GREEN

Safety Assessment of Hydroxypropyl Bis(N-Hydroxyethyl-P-Phenylenediamine) HCl as Used in Cosmetics

CIR EXPERT PANEL MEETING

JUNE 10-11, 2013

May 17, 2013

MEMORANDOM

To: CIR Expert Panel and Liaisons

From: Lillian C. Becker, M.S.
Scientific Analyst and Writer

Subject: Draft Report of Hydroxypropyl bis(N-Hydroxyethyl-p-Phenylenediamine) HCl As Used In Cosmetics

This is the first time the Panel is seeing this draft report. Most of the available data on this hair dye were unpublished data submitted by industry.

A scientific literature review of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was posted in February, 2013. Comments from industry have been addressed. There were no comments submitted from the public.

The Panel is to review the data on this hair dye. If the information is sufficient to reach a conclusion on safety, the Panel is to issue a tentative report. If not, then the Panel is to issue an insufficient data announcement and list the data needs.

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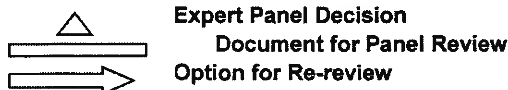
Hydroxypropyl Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl — June 2013

SAFETY ASSESSMENT FLOW CHART

Public Comment	CIR	Expert Panel	Re-Reviews	Report Color
	Draft Priority List		15 years or New Data; or request	
60 day public comment period	Draft Priority List	DRAFT PRIORITY LIST		Buff Cover
ANNOUNCE	Priority List INGREDIENT	PRIORITY LIST	Re-review to Panel	Buff Cover
		Is new data cause to reopen?		
		YES / NO — Does new data support adding new ingredients?		
Statement	Feb 2013 SLR Decision not to reopen the report*	NO / YES**		
60 day public comment period	Draft Report	DRAFT REPORT June 2013	Draft Amended Report	Green Cover 1st time
	Table	Table / ISD / TR		
	ISD Notice	ISD		
60 day public comment period	Draft TR ISD	DRAFT TENTATIVE	Draft Amended Tentative Report	Pink Cover
	Table	Table		
	Tentative Report	Issue TR	Tentative Amended Report	
60 day Public comment period	Draft FR	DRAFT FINAL REPORT	Draft Amended Final Report	Blue Cover
	Table	Table / Difft. Concl.		
PUBLISH	Final Report	Issue FR		

*The CIR Staff notifies of the public of the decision not to re-open the report and prepares a draft statement for review by the Panel. After Panel review, the statement is issued to the Public.

**If Draft Amended Report (DAR) is available, the Panel may choose to review; if not, CIR staff prepares DAR for Panel Review.



Expert Panel Decision
Document for Panel Review
Option for Re-review

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History of Hydroxypropyl Bis(N-hydroxyethyl-p-phenylenediamine)HCl

February, 2013 – SLR was posted for public comment.

June, 2013 – Panel examines Draft Report.

Hydroxypropyl bis (N-hydroxyethel-p-phenylenediamine) HCl Data Profile for June, 20113 Writer - Lillian Becker

	ADME			Acute toxicity			Repeated dose toxicity			Irritation			Sensitization					
	Dermal Penetration	Log K_{ow}	Use	Oral	Dermal	Inhale	Oral	Dermal	Inhale	Ocular Irritation	Dermal Irr. Animal	Dermal Irr Human	Sensitization Animal	Sensitization Human	Repro/Devel toxicity	Genotoxicity	Carcinogenicity	Phototoxicity
	X		X	X	X		X			X	X		X		X	X		X

Search Strategy for
Hydroxypropyl bis-(Hydroxyethyl-p-Phenylenediamine)HCl

Chemical name and CAS no. searched in SciFinder and the internet. Hits for SCCP and SCCS reports.

Safety Assessment of Hydroxypropyl Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl as Used in Cosmetics

Status: Draft Report for Panel Review
Release Date: May 17, 2013
Panel Meeting Date: June 10-11, 2013

The 2012 Cosmetic Ingredient Review Expert Panel members are: Chairman, Wilma F. Bergfeld, M.D., F.A.C.P.; Donald V. Belsito, M.D.; Curtis D. Klaassen, Ph.D.; Daniel C. Liebler, Ph.D.; Ronald A Hill, Ph.D. James G. Marks, Jr., M.D.; Ronald C. Shank, Ph.D.; Thomas J. Slaga, Ph.D.; and Paul W. Snyder, D.V.M., Ph.D. The CIR Director is F. Alan Andersen, Ph.D. This report was prepared by Lillian C. Becker, Scientific Analyst/Writer.

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INTRODUCTION

This is a safety assessment of the oxidative hair dye hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl as used in hair dye products.

CHEMISTRY

Definition and Structure

The structure of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (CAS no. 128729-28-2) is shown in Figure 1.

Physical and Chemical Properties

The physical and chemical properties are provided in Table 1. Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl is a substituted aromatic amine salt. The log Kow @ 20°C of this hair dye is -5.[1]

Ultraviolet light absorption was reported in the range of 200 – 400 nm (0.01 g/L in deionized water) with a peak at 258 nm.[2] There is a less well-defined peak at 302 nm. In the visible range of 350 – 800 nm (10 g/L in deionized water) there was a peak at 415.5 nm. There is a less well-defined, lower peak at 570 nm.

Impurities

In studies submitted to the SCCS, the purity of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine)HCl was reported to range from 94.6% - 99.8%.[3] Other impurities which were below the detection limit include: 2-phenylamino-ethanol (< 200 µg/g), 1,3-bis-[(2-hydroxy-ethyl)-(4-nitroso-phenyl)-amino]propan-2-ol (< 100µg/g), and 1,3-bis-[(2-hydroxyethyl)-phenylamino]-propan-2-ol (<100 µg/g).

USE

Cosmetic

Data on ingredient usage are provided to the Food and Drug Administration (FDA) Voluntary Cosmetic Registration Program (VCRP).[4] A survey was conducted by the Personal Care Products Council (Council) of the maximum use concentrations for ingredients in this group.[5] Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was reported to be used in 75 hair dyes and colors at a maximum concentration of 0.28%.

Because this ingredient is only used in one category, no use table was developed.

While an earlier opinion of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl stated that this hair dye may be used up to 3.0% (before mixing with hydrogen peroxide for application) so that the final concentration applied by the consumer does not exceed 1.5%, the current SCCP position is that hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine is safe at a maximum concentration on the head of 0.4%.[3,6]

TOXICOKINETICS

Absorption, Distribution, Metabolism, and Excretion

Dermal/Percutaneous

^{14}C-Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (25 mg/kg in water) administered to the clipped skin of Wistar Hans rats for 30 min was primarily recovered in the application site wash and the dressing (males, 94.2 ± 3.91%; females, 96.86 ± 2.96%).[7] Recovery in urine and feces was < 1%. Recovery in the skin (dermis and epidermis) was < 0.2%. Of the small amount that was absorbed, most of the test substance was eliminated in the feces (> 80%) within 72 h. There were no gender differences in the results.

When applied to human skin for 30 min in a diffusion cell, < 0.2% of a radio-labeled hair dye (20 mg/cm^2) containing hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (3.67 ± 0.25%) mixed with either hydrogen peroxide or water was recovered in the receptor cell.[8] The receptor fluid was sampled at 0, 0.5, and 1 h then every hour up to 24 h. Most of the dye was recovered from the skin surface (93.9 ± 2.7% and 98.2 ± 4.0%, respectively). The stratum corneum contained 1.78 ± 0.87% and 1.32 ± 0.96% of the dye and the epidermis/dermis contained 0.55 ± 0.33% and 1.85 ± 1.68%, respectively.

When applied to human skin for 30 min in a diffusion cell, ~0.01% of a radio- labeled hair dye (14.0 µL in water; ~20 mg/cm^2) containing hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (0.34%, 0.8%) mixed with either hydrogen peroxide or water was recovered in the receptor cell.[1] Samples were collected from the receptor cell at 0.5 h and 1 h, then hourly after that. Most of the dye was recovered from the skin surface (98.31 ± 2.68% and 98.72 ± 2.27%, respectively). The epidermis/dermis contained 0.90 ± 0.92% and 0.80 ± 0.77% of the applied dye, respectively.

When applied to heat separated human abdominal or breast epidermis in a Franz diffusion cell (n = 7), 0.004% (in the absence of hair) and 0.005% (in the presence of hair) of the administered dose was present in the receptor cell.[3] Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine)HCl (3.3% in a formulation containing 0.64% p-aminophenol and then mixed 1:1 with hydrogen peroxide for a final concentration of 1.65%; 40 mg) was applied to the skin for 30 min, with or without finely chopped bleached hair.

Oral

When ^{14}C-hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was orally administered to Wistar Han rats, the mean plasma total radioactivity levels increased from time 0 until the c_{max} (1558 ± 157 ng-eq/g for males and 1678 ± 540 ng-eq/g for females) at 1 h (males) or 2 h (females), and then decreased until the last quantifiable time points at 6 h (281 ± 15 ng-eq/g) or 8 h (224 ±53 ng-eq/g), respectively.[9] Blood samples (n = 3/sex/time point) were collected at 0, 1, 2, 4, 6, 8, 24, 48, and 72 h after treatment.

Other rats (n = 3/sex) were weighed and urine/feces/cage wash collected for 0 - 6 h and 6 – 24 h then every 24 h up to 168 h. Following oral gavage of the isotope mixture at 100 mg/kg, the mean total cumulative excretion of the radioactive dose in the summed excreta over the 168-h period was 98.3 ± 2.7% and 96.3 ± 3.4% for the males and females, respectively. A mean of 2.5 ± 0.3% and 95.4 ± 2.5% of the absorbed test substance was eliminated in the urine and feces, respectively, for the males and 3.7 ± 0.3% and 88.6 ± 9.3% for the females. The cage contained < 5% absorbed test substance for both sexes. Most of the radioactivity (90.7% and 72.9 %, respectively) was eliminated in the summed urine and feces within 24 h, > 95% of which was in the feces.[9]

TOXICOLOGICAL STUDIES

Acute Toxicity

Dermal – Non-Human

The dermal LD_{50} for hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was > 2000 mg/kg (the maximum dose tested) for Sprague-Dawley rats (n = 5/sex).[10] One rat had a slight decrease in spontaneous activity at 4 and 6 h after treatment.

Oral – Non-Human

The oral LD_{50} of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was reported to be 2186 mg/kg (C.I. 1797-2965) for Sprague-Dawley rats (n = 5 females, 5/sex for 2000 mg/kg group).[11] The fasted rats were administered the test substance (1100, 1600, 2000, 2600 mg/kg in water; 10 ml/kg). No deaths occurred in the 1100 and 1600 mg/kg female groups. In the 2000 mg/kg group, 2/5 females and 3/5 males died. In the 2600 mg/kg group, 4/5 females died. Except for 2 animals which died on day 3, all deaths occurred within 30 minutes of treatment. Hypoactivity, sedation, piloerection and dyspnea were observed in both sexes. Males exhibited lateral decubitus. The first signs were observed at 30 min after treatment. For those that did not die, recovery was complete on day 7 for the females and day 5 for the males.

Wistar HanIbm:WIST (SPF) rats (n = 2) exhibited a reduction of spontaneous activity eyelid closure, and apathy when orally administered 1500 mg/kg hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl.[12] At 2000 mg/kg, there was a reduction of spontaneous activity eyelid closure, apathy, abdominal position, and one death observed.

Repeated Dose Toxicity

Oral – Non-Human

In a range finding study where Sprague-Dawley rats (n not provided) were administered hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (50, 200, 800 mg/kg/d) for 2 weeks, the rats in the high dose group had a slight decrease in body weight gain, glucose level, and total proteins.[13,14] A dose of 800 mg/kg/d resulted in: ptyalism and signs of poor clinical condition in both sexes, slightly lower body weight gain in males (-11% compared to controls), lower glucose (-26%) and higher triglyceride (x 1.5) levels in males, and in the kidneys, minimal to slight brownish pigment in the tubular epithelium and slightly higher incidence and severity of tubular dilatation in both sexes. The mid dose group had a slight decrease in glucose level. There were no effects observed in the low dose group.

The NOAEL for hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was 25 mg/kg/d when administered to rats for 13 weeks.[14] The test substance (25, 100, 400 mg/kg/d in water) was administered to Sprague-Dawley rats (n = 10/sex) by gavage; the rats were then killed and necropsied. There were no clinical signs in the low dose group.

There was ptyalism, loud breathing, and/or regurgitation in the mid and high dose groups from week 4. Pink urine, brown colored tails, and brown or black feces were also observed in these groups. One male from each of the mid and high dose groups died; aspiration pneumonia due to regurgitation was considered a contributing factor. Body weights and feed consumption were similar to controls. Opacification of the lens was observed in one female in the high dose group. Females in the high dose group had higher activated partial thromboplastin time and higher urea and creatinine levels were observed in females in the mid and high dose groups. Urinalysis and macroscopic examination of tissues were unremarkable. Microscopic examination revealed tubular basophilia in the kidneys of the males in the high dose group and many of the organs and tissues had a brownish pigmentation, probably due to the color of the test material. Subacute to chronic aspiration pneumonia was observed in the mid and high dose group.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY

The NOAEL for reproductive and developmental toxicity was > 800 mg/kg/d for Crl CD (SD) BR Sprague-Dawley rats (n = 25) orally administered hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (50, 200, 800 mg/kg/d in

water on days 6 – 15 of pregnancy).[13] On day 20, the dams were killed and necropsied. Other than colored urine in one dam in the low dose group and all the dams in the mid and high dose groups, there were no clinical signs. The necropsies were unremarkable. The mean number of corpora lutea, implantation sites, post-implantation loss, number of live fetuses, sex ratio, and fetal body weights were similar to controls. There were no treatment related anomalies in the fetuses.

GENOTOXICITY

In Vitro

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine)HCl (312.5, 625, 1250, 2500, 5000 µg/plate with metabolic activation; 62.5, 125, 250, 500, 1000 µg/plate without) was not mutagenic to *Salmonella typhimurium* (strains TA98, TA100, TA1535, TA1537) and *Escherichia coli* (strain WP2uvrA) except for weak mutagenic activity observed (2.2 fold increase in revertant colonies) at 5000 µg/plate with metabolic action in the TA100 strain.[15] The test with metabolic activation was repeated (125, 250, 500, 1000, 2000 µg/plate) with the same result. The test without metabolic activation was repeated at the same concentrations with the same result.

In a mammalian cytogenetic assay using Chinese hamster ovary (CHO) cells, hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (30, 100, 300, 1000, 3000, 5000 µg/mL with metabolic activation; 12.5, 25, 50, 100, 150 µg/mL without) did not induce an increase in aberrant cell frequency with metabolic activation at any exposure except for 100 µg/mL without metabolic activation.[16]

When this assay was repeated (125, 250, 500, 750, 1000 µg/mL with metabolic activation; 12.5, 25, 50, 75, 100 µg/mL without), the test substance did not induce an increase in aberrant cell frequency with metabolic activation.[16] However, without metabolic activation, the test substance increased the instances of cells with structural chromosome aberrations at 75 µg/mL. In each case, these positive findings in the without metabolic activation group were not observed at a higher dose level, suggesting the finding was not relevant to assessing a dose-response.

In Vivo

In an unscheduled DNA synthesis (UDS) assay of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (150, 1500 mg/kg in distilled water; 10 ml/kg) using Wistar HanIbm:WIST (SPF) rats (n = 4), there was no induction of UDS in the hepatocytes of the treated rats.[12] The hepatic samples were collected at 2 h (1500 mg/kg) and 16 h (150, 1500 mg/kg) after the rats were administered a single oral dose of the test substance. The hepatocytes were cultured and the cells examined for UDS.

In a micronucleus test, hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (375, 750, 1500 mg/kg/d) orally administered for 2 days to Swiss OF1 mice (n = 5/sex) did not induce damage to the chromosomes or the mitotic apparatus of the bone marrows cells of the mice.[17]

In a micronucleus test, hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (500, 1000, 2000 mg/kg/d) orally administered for 2 days to Sprague-Dawley rats (n = 5/sex) did not induce damage to the chromosomes or the mitotic apparatus of the bone marrows cells of the mice.[18]

CARCINOGENICITY

No published carcinogenicity studies were discovered and no unpublished data were submitted.

IRRITATION AND SENSITIZATION

Irritation

Dermal – Non-Human

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (10% in purified water; 0.05 ml) was not irritating in a repeated application irritation test using Dunkin-Hartley guinea pigs (n = 3/sex).[19] The test substance was administered to the clipped skin daily for 14 days. The guinea pigs were killed and the test site examined microscopically. There were no clinical signs. There was a very slight erythema on all guinea pigs on day 9 and two on days 10 and 15. Almost all of the animals had dry skin at the test site. There was a slight black coloration of the skin starting on days 3 and 4 that could have masked very slight erythema.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (5% in distilled water; 0.05 ml) was not irritating in a patch test using New Zealand White rabbits (n = 3).[20] The test substance was administered to clipped skin under semiocclusion for 4 h and observed at 1, 24, 48, and 72 h and then daily up to day 9. No skin reactions were observed in one rabbit. Very slight or well-defined erythema was observed at 24 or 72 h after treatment in the other two rabbits. No edema was observed. There was dryness of the skin observed on days 5 - 8 in one rabbit. Mean scores over 24, 48 and 72 h for each animal were 0.0, 1.7, and 0.3 out of 4 for erythema and 0.0 for edema.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (100% dampened with water; 500 mg) was an irritant to male New Zealand White rabbits.[21] The test substance was administered to clipped skin under occlusion for 3 min (n = 1), 1 h (n = 1), and 4 h (n = 3). After 3 min, erythema (masked by a black coloration of the test site) was observed and persisted up to day 10. Slight edema was noted 1 h after removal of the dressing. After 1 h, slight to severe erythema was observed on

days 1- 11. Severe to slight edema was observed on days 1 - 6. After 4 h, erythema (masked by a black coloration of the test site) persisted up to day 15. Slight to severe edema was observed on days 1 - 5 in two rabbits. The third rabbit had slight edema 1 h after removal of the dressing. The mean scores over 24, 48 and 72 h for individual rabbits were 0.0, 2.7 and 3.3 out a possible 4 for edema. Due to the skin coloration, scores for erythema could not be calculated.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (0.5 g in 0.5 mL distilled water) had a primary irritation index of 3.4 when administered to the intact and abraded clipped skin of New Zealand White rabbits (n = 3).[22] Slight to well-defined erythema and slight to severe edema were observed.

Ocular

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (100%; 0.1 mL) caused opalescent corneal opacity, iridial inflammation, and severe conjuctival irritation as well as sloughing of the cornea, hemorrhage, and a pale appearance of the nictitating membrane when administered to the eye of one New Zealand White rabbit.[23]

In a repeat of the above experiment (100 mg; n = 1), the test material caused severe ocular reactions including severe to marked chemosis, slight to moderate conjunctival redness, iris lesions, and moderate to marked corneal opacity. Neovascularisation of the cornea was observed at 72 hours.[3]

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (5% in water; 0.1 mL) was not an ocular irritant to New Zealand White rabbits (n =3).[24] The eyes were not rinsed and were observed at 1, 24, 48, and 72 h after administration.

Sensitization

Dermal – Non-Human

In a Buehler test, hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (50% in distilled water; 0.5 mL) administered to the clipped skin of Dunkin-Hartley guinea pigs (n = 10/sex) did not induce sensitization when challenged (5% and 20%).[25] During the induction period, very slight to slight cutaneous reactions were observed in 8/20 guinea pigs.

In a guinea pig maximization assay using Dunkin-Hartley guinea pigs (n = 10), hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (0.1% in a sterile isotonic aqueous NaCl solution; 0.5 mL) administered by subcutaneous injections did not induce sensitization when challenged at 25% administered in a dermal patch.[26]

In a Magnusson-Kligman maximization test using Dunkin-Hartley guinea pigs (n = 10/sex), hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (1% in sterile isotonic saline) administered by intradermal injections and challenged at 50% in a dermal patch was classified as a sensitizer.[27] At 24 h, very slight, well-defined, and marked erythema were observed in 2/20, 11/20 and 7/20 guinea pigs, respectively. There was also slight edema observed in 11 guinea pigs and severe edema in one animal. Dryness of the skin was observed in 9/20 guinea pigs. Very slight black coloration of the skin was observed in 3 guinea pigs. At 48 h, very slight, well-defined, marked, and severe erythema were noted in 1/20, 4/20, 1/20 and 2/20 guinea pigs, respectively. Crust formation was observed in 3 guinea pigs. Dryness of the skin was observed in 14/20 guinea pigs. The dryness was severe enough to mask the evaluation of erythema in 5/20 treatment sites. Very slight to slight black coloration of the skin was observed in 5 guinea pigs. The very slight erythema which did not persist at the 48-h reading in two guinea pigs was attributed to a possible slight irritant reaction. All of the other skin lesions were attributed to a sensitization effect.

Phototoxicity

Dermal administration of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl (10% in purified water; 0.2 mL) to Dunkin Hartley guinea pigs (n = 10) did not cause phototoxicity or photosensitization when exposed to UVA or UVB lamp light.[28] For the phototoxicity assay, the test substance was gently massaged into the shaved backs of the guinea pigs and 30 min later they were irradiated by UVB (312 nm) then UVA (365 nm). For the photosensitization assay, the guinea pigs were administered the test substance and irradiated 6 more times. After a 20-day rest, the test substance was administered and the test sites were irradiated again (left flank UVA, right flank UVB). The test sites were scored for reactions at 1, 6, 24, and 48 h after application.

HAIR DYE EPIDEMIOLOGY

Hydroxypropyl bis (N-hydroxyehtyl-p-phenylenediamine) HCl is an oxidative hair dye ingredient. While the safety of individual hair dye ingredients are not addressed in epidemiology studies that seek to determine links, if any, between hair dye use and disease, such studies do provide broad information. Currently available epidemiology studies provided insufficient evidence to support a causal association between personal hair dye use and a variety of tumors and cancers. A detailed summary of the available hair dye epidemiology data is available at http://www.cir-safety.org/cir-findings.

SUMMARY

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl is an oxidative hair dye used in 75 hair dyes and colors at a maximum concentration of 0.28%.

Europe has established a 0.4% limit on hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl when used as an oxidative hair dye.

Less than 1% of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was recovered in the urine and feces when dermally applied to the skin of rats. Less than 0.2% of the dye was recovered in the receptor cells using human skin. Most of orally administered test substance was eliminated through urine and feces and cleared from the blood of rats within 6 – 8 h.

The dermal LD_{50} was > 2000 mg/kg for rats. The oral LD_{50} for rats was 2186 mg/kg.

The NOAEL for hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was 25 mg/kg/d for 13 weeks for rats.

The NOAEL for reproductive and developmental toxicity was > 800 mg/kg/d orally administered to rats on gestation days 6 – 15.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was not genotoxic to *S. typhimurium* and *E. coli* in an Ames test and a mammalian cytogenetic assay using CHO cells. The test substance was not genotoxic in an unscheduled DNA synthesis assay and two micronucleus tests.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was not irritating to guinea pigs up to 10% and rabbits up to 5%. It was severely irritating to rabbits at 100%.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was a severe ocular irritant to rabbits at 100% but was not an irritant at 5%.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was not sensitizing to guinea pigs up to 50% when applied dermally. However, when applied by intradermal injection, the test substance was sensitizing at 1%.

Hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl was not phototoxic at 10% when exposed to either UVA or UVB light.

Currently available epidemiology studies provided insufficient evidence to support a causal association between personal hair dye use and a variety of tumors and cancers.

DISCUSSION

Discussion to be developed at the June, 2013 Panel meeting.

CONCLUSION

Conclusion to be developed at the June, 2013 Panel meeting.

TABLES AND FIGURES

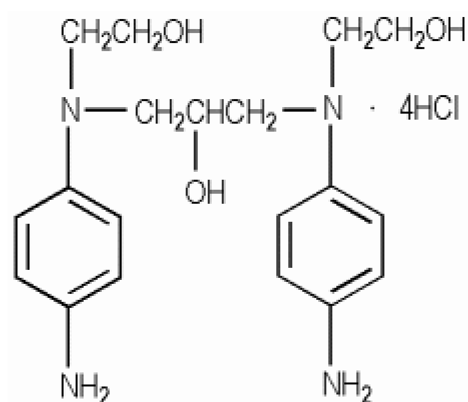

Figure 1. Chemical structure of hydroxypropyl bis(N-hydroxyethyl-p-phenylenediamine) HCl.

Table 1. Physical and chemical properties of hydroxypropyl bis(N-hydroxyethel-p-phenylenediamine) HCl.

Property	Value	Reference
Physical Form	Powder	23
Color	Blue-grey	23
	White-grey	1
	Beige	10
Odor	Strong, irritating	2,3
Molecular Weight g/mol	506.3	1
Water Solubility g/L	760	1
Other Solubility g/L @ 22°C		
Ethanol	< 1	2
DMSO	≥ 20	2
log K_{ow} @ 20 °C	-5	1

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May 17, 2013

MEMORANDOM

To: CIR Expert Panel and Liaisons

From: Lillian C. Becker, M.S.
Scientific Analyst and Writer

Subject: Unpublished Data for the Draft Report of Hydroxypropyl bis(N-Hydroxyethyl-p-Phenylenediamine) HCI As Used In Cosmetics

Most of the information in this report is unpublished data submitted by industry. The data submitted are:

A. Local Tolerance Study after repeated topical application for 2 weeks in guinea pigs;
B. 13-week oral Toxicity study in rats
C. Acute Dermal Toxicity study in rats
D. Acute Eye Irritation study in rabbits
E. Eye Irritation study in rabbits
F. Acute Oral Toxicity study in rats
G. Acute Dermal Irritation study in rabbits at 5%
H. Acute Dermal Irritation study in rabbits
I. Skin Irritation study in rabbits
J. Reverse Mutation Assay
K. Micronucleus Test by Oral Route in mice
L. Micronucleus Test by Oral Route in rats
M. Skin Sensitization Test in guinea pigs (Buehler assay)
N. In Vitro Mammalian Cytogenetic Test in CHO Chinese hamster ovary cells
O. Pharmacokinetcs Study in Plasma, Excretion Balance and Tissue Distribution by Cutaneous Route in rats
P. Sensitization in guinea pigs (Magnusson & Kligman)
Q. Skin Sensitization Test in guinea pigs (Magnusson &Kligman)
R. In Vitro Percutaneous Absorbtion using human skin
S. In Vitro Percutaneous Absorption using human skin
T. In Vivo/In Vitro Unscheduled DNA Synthesis using rat hepatocytes
U. Oral ADME study using rats
V. Dermal Phototoxic and Photoallergenic Potential using guinea pigs
W. Oral Embryotoxicity/Teratogenicity study using rats
X. Concentration of Use survey

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(Email) cirinfo@cir-safety.org (Website) www.cir-safety.org

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TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY TITLE

LOCAL TOLERANCE STUDY AFTER REPEATED TOPICAL APPLICATION FOR 2 WEEKS IN GUINEA-PIGS with the test substance diluted at 10%

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

23 July 1997

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)

Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

14933 TSG

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STATEMENT OF THE STUDY DIRECTOR

The study was performed in compliance with the Principles of Good Laboratory Practice Regulations:

. O.E.C.D. principles of Good Laboratory Practice, Decision Concerning Mutual Acceptance of Data in the Assessment of Chemicals, C(81)30(final) Annex 2. 12 May 1981.

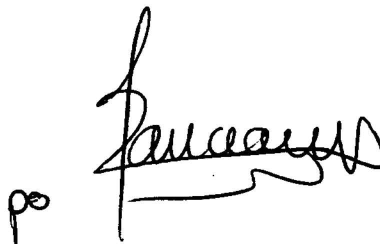
. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Journal Officiel du 9 mars 1990), Ministère de l'Industrie et de l'Aménagement du Territoire.

. US Food and Drug Administration, Good Laboratory Practice Regulations 21 CFR Part 58, December 22, 1978 (and subsequent amendments).

. Japanese Good Laboratory Practice, Notice of Pharmaceutical Affairs Bureau, Ministry of Health and Welfare, March 31, 1982 (and subsequent amendments).

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at the Centre International de Toxicologie (C.I.T.), Miserey, 27005 Evreux, France.



Toxicology

S. de Jouffrey Date: 23 July 1997
Study Director
Head of Short-term and
Environmental Toxicology

OTHER SCIENTISTS INVOLVED IN THIS STUDY

For Pharmacy: P.O. Guillaumat
Doctor of Pharmacy

For Toxicology: C. Pelcot
Study Supervisor

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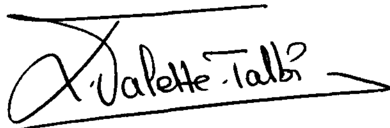
STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspections	Dates		
	Inspections	Report to Study Director	Report to Management (*)
Protocol	30 Oct. 96	30 Oct. 96	30 Oct. 96
Study	23 Jan. 97	24 Jan. 97	24 Jan. 97
Report	4 June 97	24 June 97	24 June 97

The inspections were performed in compliance with C.I.T. Quality Assurance Unit procedures and the Good Laboratory Practice Regulations.

(*) The dates mentioned correspond to the dates of signature of audit reports by Study Director / Management.



L. Valette-Talbi Date: 23 July 1997
Doctor of Biochemistry
Head of Quality Assurance Unit
and Scientific Archives

SUMMARY

At the request of the effects of repeated application of the test substance (batch No. OP 18) diluted at 10% to the skin was evaluated in guinea-pigs. The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

A volume of 0.05 ml of a 10% (w/w) solution of the test substance in purified water was applied to the left flank of six guinea-pigs (three males and three females) once daily, at approximately the same time each day, for 14 consecutive days (days 1 to 14).
The test substance was applied over the same area of clipped skin, measuring approximately 2 cm x 2 cm. The test site was not covered by a dressing.
The right flank received purified water under the same experimental conditions.
Cutaneous reactions were evaluated in each animal immediately before each application and approximately 24 hours after the last application by comparing the reactions on both flanks.
Residual test substance was removed before each scoring using a moistened gauze pad.
Photographs of the treated application sites of each animal were performed immediately before treatment on days 1, 5, 9, 12 and on day 15.

At the end of the observation period, the animals were killed and cutaneous samples were taken from the treated sites.

Results

No clinical signs and no mortality were noted during the study.

During the treatment period, only a very slight erythema was observed on the left flank of all animals on day 9 and of two animals on days 10 and 15.

Dryness of the skin was also noted on the left flank of almost all animals.

A slight black colouration of the skin was observed in all animals; it appeared on day 3 or 4 and could have masked a very slight erythema in almost all animals.

Maximum Weekly Mean Irritation Index was non-calculable due to the skin colouration.

Conclusion

Under our experimental conditions, the repeated application for 14 days of the test substance (batch No. OP 18) diluted at 10% to the skin of guinea-pigs induced a slight black colouration of the application sites but no significative irritation reaction.

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1. INTRODUCTION

The objective of this study was to evaluate the effects of repeated application of the test substance to the skin of the guinea-pigs.

Applications were made daily for 14 consecutive days.

The results of the study are of value in predicting the skin irritancy of the test substance in Man following repeated application.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance used in the study was supplied by

Documentation supplied by the Sponsor identified the test substance as follows:
. name:
  - protocol and labelling:
. batch number:
  - protocol: none
  - labelling: OP 18
. description: greyish powder
. container: two glass flasks
. date of receipt: 10 January 1997
. storage conditions: at +4°C, protected from light and stored under argon gas
. purity: 97.6%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Vehicle

The vehicle used was purified water.

2.1.3 Preparation

On each day of treatment, the test substance was prepared at a concentration of 10% (w/w) in the vehicle.
This concentration was chosen as the substance is expected to be applied to human hair and skin at a much lower concentration (approximately 1%).

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2.2. TEST SYSTEM

2.2.1 Animals

Species and strain: Dunkin-Hartley guinea-pigs.
Reason for this choice: species generally accepted by the regulatory authorities for this type of study.
Breeder: Centre d'Elevage Lebeau, 78950 Gambais, France.
Number: ten animals (five males and five females).
Age and weight: on day 1 of treatment, the animals had 1-3 months old and had a mean body weight of 402 ± 41 g for the males and 437 ± 25 g for the females.
Acclimatization: at least five days before the beginning of the study.
Identification of the animals: the animals were identified individually by an ear-tattoo.

2.2.2 Environmental conditions

During the acclimatization period and throughout the study, the conditions in the animal room were as follows:

. temperature: 21 ± 2°C
. relative humidity: 30 to 70%
. light/dark cycle: 12 h/12 h
. ventilation: about 12 cycles/hour of filtered, non-recycled air.

The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity and ventilation) were checked monthly.
During the acclimatization period and throughout the study, the animals were individually housed in polycarbonate cages (48 cm x 27 cm x 20 cm) equipped with a polypropylene bottle. Sifted and dusted sawdust was provided as litter (SICSA, 92142 Alfortville, France). An analysis of potential residues and major contaminants is performed periodically (Laboratoire Wolff, 92110 Clichy, France).

2.2.3 Food and water

During the study, the animals had free access to "Guinea-pigs sustenance reference 106 pelleted diet" (U.A.R., 91360 Villemoisson-sur-Orge, France).
Food was periodically analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was contained in bottles.
Bacteriological and chemical analysis of the diet and water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Preparation of the animals

The right and left flanks (2 cm x 2 cm) of all animals were clipped and shaved on day -1. Both flanks were then clipped and shaved on days 4, 8, 11 and 14, approximately 6 hours after treatment.

2.3.2 Administration of the test substance

The test substance was applied daily, at a volume of 0.05 ml to the left flank of each animal. The application was repeated for 14 consecutive days, at approximately the same time each day. The test site was not covered by a dressing after application of the test substance.
Purified water was applied to the right flank under the same experimental conditions.

2.4. SCORING OF CUTANEOUS REACTIONS

Cutaneous reactions were evaluated in each animal immediately before each application and approximately 24 hours after the last application, according to the following numerical scale:

Erythema and eschar formation

. No erythema 0
. Very slight erythema (barely perceptible) 1
. Well-defined erythema 2
. Moderate to severe erythema 3
. Severe erythema (beet redness) to slight eschar formation (injuries in depth) 4

Oedema formation

. No oedema 0
. Very slight oedema (barely perceptible) 1
. Slight oedema (visible swelling with well-defined edges) 2
. Moderate oedema (visible swelling raised more than 1 millimetre) 3
. Severe oedema (visible swelling raised more than 1 millimetre and extending beyond area of exposure) 4

All other lesions were noted.

2.5. CLINICAL EXAMINATIONS

The animals were observed twice a day during the study in order to record clinical signs and to check for mortality.

2.6. BODY WEIGHT

The animals were weighed individually on the day of allocation into the groups, on the first day of the study (day 1) and each week until the end of the study (day 15).

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2.7. PHOTOGRAPHS

Photographs of the treated application sites of each animal were performed immediately before treatment on day 1 then on days 5, 9, 12 and 15.

2.8. PATHOLOGY

2.8.1 Necropsy

On day 15, after the last observation period, the animals were killed by CO_2 inhalation in excess.

2.8.2 Cutaneous samples

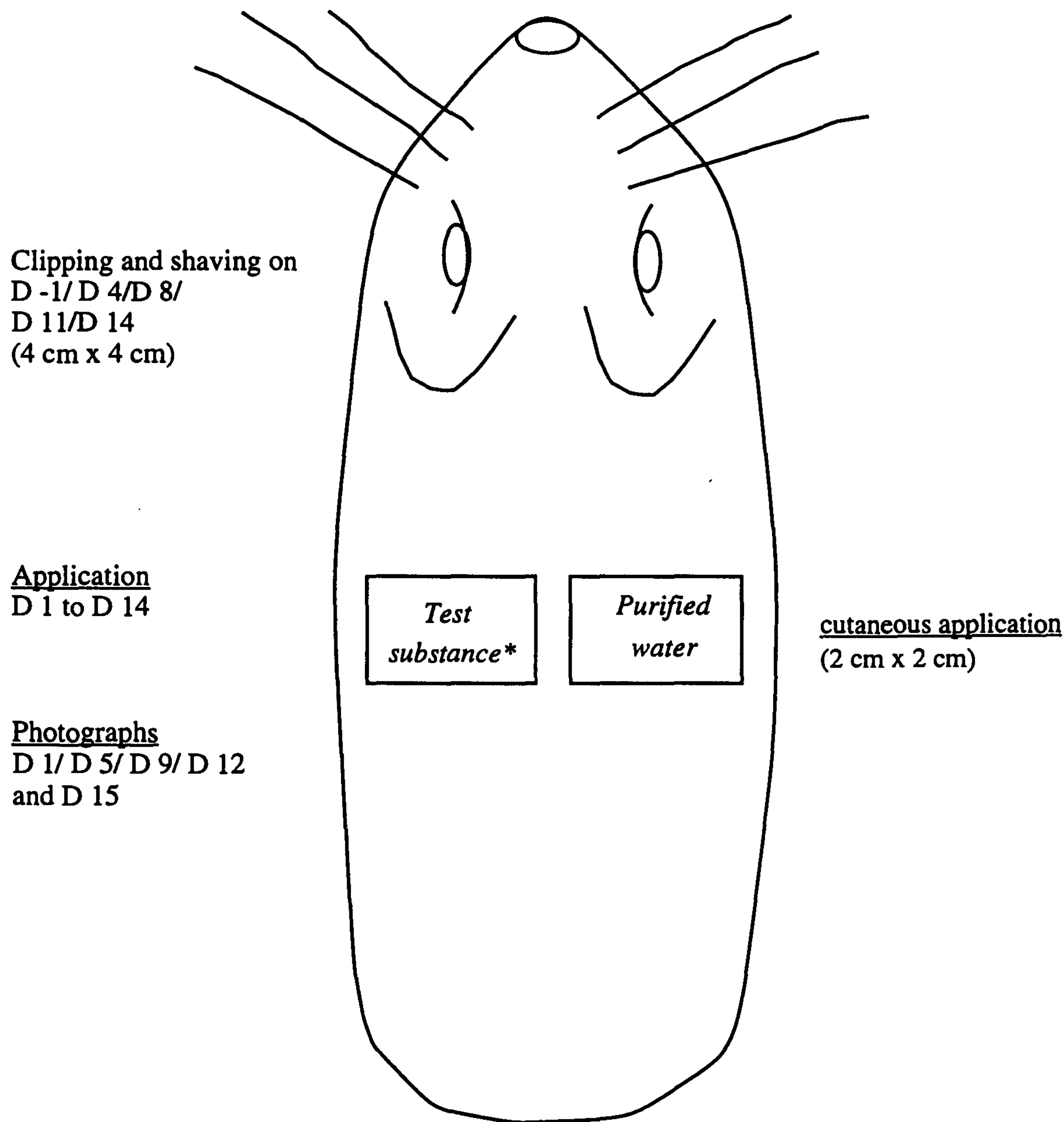
On day 15, a skin sample was taken from the treatment sites of the posterior left and right flanks of all animals. The samples were preserved in 10% buffered formalin.

2.8.3 Microscopic examination

No histological examination was performed.

Figure 1: summary diagram

Chronology



* at a concentration of 10% (w/w)

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2.10. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date	Day
Arrival of the animals	9 January 1997	-14
Weighing, allocation of the animals	22 January 1997	-1
Photographs, scoring First treatment, weighing	23 January 1997	1
Scoring, treatment	24 January 1997	2
Scoring, treatment	25 January 1997	3
Scoring, treatment Clipping and shaving approximately 6 hours after treatment	26 January 1997	4
Photographs, scoring, treatment	27 January 1997	5
Scoring, treatment	28 January 1997	6
Scoring, treatment	29 January 1997	7
Scoring, treatment, weighing Clipping and shaving approximately 6 hours after treatment	30 January 1997	8
Photographs, scoring, treatment	31 January 1997	9
Scoring, treatment	1 February 1997	10
Scoring, treatment Clipping and shaving approximately 6 hours after treatment	2 February 1997	11
Photographs, scoring, treatment	3 February 1997	12
Scoring, treatment	4 February 1997	13
Scoring, treatment Clipping and shaving approximately 6 hours after treatment	5 February 1997	14
Photographs, scoring 24 hours after treatment, weighing, sacrifice of the animals and skin samples	6 February 1997	15

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2.11. PROTOCOL ADHERENCE

The study was performed in accordance with the original protocol No. 14933 TSG.
The minor fluctuation of temperature recorded outside of the target ranges specified in the protocol were not considered to have an impact on the validity or integrity of the study.

2.12. ARCHIVES

The study archives:
- . protocol and possible amendments,
- . raw data,
- . correspondence,
- . final study report and possible amendments,
- . histological specimens:
  - tissues in preservative

are stored in the premises of C.I.T., Miserey, 27005 Evreux, France, for five years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. RESULTS

3.1. CLINICAL SIGNS

No clinical signs and no mortality were observed during the study.

The body weight gain of the animals was normal (figure 2, appendix 3).

3.2. SCORING OF CUTANEOUS REACTIONS

Observations of cutaneous reactions are presented in appendix 4.

During the treatment period, only a very slight erythema was observed on the left flank of all animals on day 9 and in two animals on days 10 and 15.

Dryness of the skin was also noted on the left flank of almost all animals.

A slight black colouration of the skin was observed in all animals; it appeared on day 3 or 4 and could have masked a very slight erythema in almost all animals.

Maximum Weekly Mean Irritation Index was non-calculable due to the skin colouration.

3.3. PATHOLOGY

3.3.1 Cutaneous samples
On day 15, a skin sample was taken from the treatment sites of the posterior left and right flanks of all animals. The samples were preserved in 10% buffered formalin

3.3.2 Microscopic examination
No microscopic examinations were performed.

3.4. PHOTOGRAPHS

Photographs of treated site are presented in appendix 5.

4. CONCLUSION

Under our experimental conditions, the repeated application for 14 days of the test substance (batch No. OP 18) diluted at 10% to the skin of guinea-pigs induced a slight black colouration of the application sites but no significative irritation reaction.

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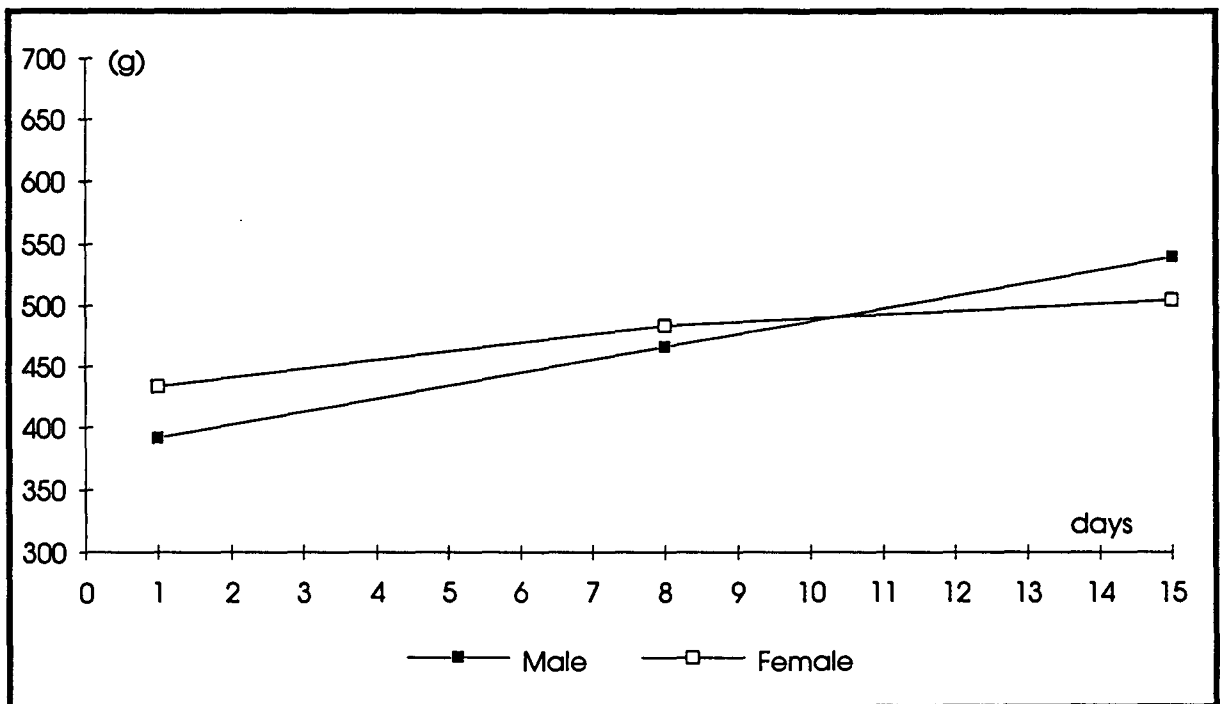
Figure 2: Body weight gain (g)



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APPENDICES

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1. Analytical certificate

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DEVELOPPEMENT ANALYTIQUE
& QUALITE

9 janvier 1997

BULLETIN D'ANALYSE
54562 lot 18

ASPECT	Poudre grise
SOLUBILITE	Soluble à 10% dans l'eau
Titre	97.6 %
Absorbance à 258± 5 nm (c= 0.01 g/l dans l'eau)	0.48
Teneur en ethylglycol	0.08%
Teneur en isopropanol	0.01%

Durée de validité : Décembre 1997

Responsable du laboratoire de
Developpement Analytique et Qualité

Le n° de code ... est synonyme
de " –

10/1/97

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2. Diet formula

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Ref: 106
COMPLETE DIET
GUINEA-PIG MAINTENANCE DIET
Appearance: 4.5 mm diameter granules
Conditioning: bags of 25 kgs

Daily portion: Guinea-pigs 35-50 g, water *ad libitum.*

FORMULA %

Cereals	42
Grain biproducts and legumes	46
Vegetable protein (soya bean meal, yeast)	9
Vitamin and mineral mixture	3

AVERAGE ANALYSIS %

Calorific value (KCal/kg)	2600
Moisture	10
Proteins	17
Lipids	3
Carbohydrates (N.F.E.)	49
Fibre	13
Minerals (ash)	8

AMINO ACID VALUES
(calculated in mg/kg)

Arginine	8500
Cystine	2500
Lysine	7200
Methionine	2100
Tryptophan	2000
Glycine	6000

FATTY ACID VALUES
(calculated in mg/kg)

Palmitic acid	3600
Palmitoleic acid	0
Stearic acid	700
Oleic acid	5900
Linoleic acid	11200
Linolenic acid	3000

MINERALS (calculated in mg/kg)

	Nat. val.	CMV val.	Total
P	7400	1400	8800
Ca	5400	5600	11000
K	12000	0	12000
Na	1300	1950	3250
Mg	3270	130	3400
Mn	60	40	100
Fe	170	150	320
Cu	10	15	25
Zn	40	45	85
Co	0.1	1.5	1.6
I	0	0	0
Cl	0	0	0

VITAMINS (calculated per kg)

	Nat. val.	CMV val.	Total
Vitamin A	3500 IU	7500 IU	11000 IU
Vitamin D3	30 IU	2000 IU	2030 IU
Vitamin B1	6 mg	6.4 mg	12.4 mg
Vitamin B2	5 mg	6.4 mg	11.4 mg
Vitamin B3	22 mg	26 mg	48 mg
Vitamin B6	0.7 mg	2.7 mg	3.4 mg
Vitamin B12	0.003 mg	0.012 mg	0.015 mg
Vitamin C	0 mg	400 mg	400 mg
Vitamin E	15 mg	60 mg	75 mg
Vitamin K3	5 mg	12.6 mg	17.6 mg
Vitamin PP	97 mg	14.5 mg	111.5 mg
Folic acid	2.2 mg	1.3 mg	3.5 mg
P.A.B. acid	0 mg	2.5 mg	2.5 mg
Biotin	0.02 mg	0.06 mg	0.08 mg
Choline	1010 mg	60 mg	1070 mg
Meso-Inositol	0 mg	62.5 mg	62.5 mg

This food is supplemented with stabilized coated vitamin C, avoiding the need of other food substances (greenery, ascorbic acid) if used within 4 months of date of manufacture.

U.A.R., 7 rue Galliéni, 91360 Villemoisson - Tel: 69.04.03.57 - Fax : 69.04.81.97
(Ref. Doc. UAR: 1992)

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3. Individual body weight values

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INDIVIDUAL BODY WEIGHT VALUES
(g)

Group	Sex	Animals	Days					
			-1	1	(1)	8	(1)	15
2	Male	82	360	309	96	405	79	484
		83	405	416	58	474	66	540
		84	442	450	69	519	76	595
		M	402	392	74	466	74	540
		SD	41	74	20	57	7	56
	Female	85	459	460	25	485	63	548
		86	410	409	56	465	34	499
		87	443	433	68	501	-32	469
		M	437	434	50	484	22	505
		SD	25	26	22	18	49	40

(1) = Body weight gain
M = Mean
SD = Standard Deviation

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4. Individual observation of cutaneous reactions

CUTANEOUS REACTIONS

Left flank

Group 2	Animals	Days 2	3	4	5	6	7	8	9	10	11	12	13	14	15
Erythema	82	0	0	0/C	0/C	0	0/C	0/C	1/C/S	1/C	0/C	0/C	0/C	0/C	0/C
	83	0	0	0/C	0/C	0	0/C	0/C	1/C/S	1/C	0/C	0/C	C1	C1	1/C
	84	0	0/C	0/C	0/C/S	0/C	0/C	0/C	1/C/S	C1/S	C1	C1	0/C	C1	1/C
	85	0	0	0/C	C1	0/C	0/C	0/C	1/C	C1	C1	C1	C1	C1	C2
	86	0	0	0/C	0/C	0/C	0/C	0/C	1/C/S	C1	0/C	0/C	C1	C1	C1
	87	0	0	0/C	C1	0	0/C	0/C	1/C/S	0/C	0/C	0/C	C1	C1	C1
Oedema	82	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	84	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	85	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	86	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	87	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	(D.I.)	0,0	0,0	0,0	(1)	0,0	0,0	0,0	1,0	(1)	(1)	(1)	(1)	(1)	(1)
	(W.I.)			W.I.1	=	(1)					W.I.2	=	(1)		

Max.I. = (1)

D.I. = Daily irritation indices
W.I. = Weekly mean irritation indices
Max.I. = Maximum weekly index
S = Dryness of the skin
C = Black colouration of the skin
C 1 = Black colouration of the skin which could mask a very slight erythema
C 2 = Black colouration of the skin which could mask a well-defined erythema
(1) = non calculable

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CUTANEOUS REACTIONS

Right flank

	Group 2 Animals	Days 2	3	4	5	6	7	8	9	10	11	12	13	14	15
Erythema	82	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	84	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	85	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	86	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	87	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Oedema	82	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	83	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	84	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	85	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	86	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	87	0	0	0	0	0	0	0	0	0	0	0	0	0	0

(D.I.)	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
(W.I.)			W.I.1	=	0,0					W.I.2	=	0,0		

Max.I. = 0,0

D.I. = Daily irritation indices
W.I. = Weekly mean irritation indices
Max.I. = Maximum weekly index

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STUDY TITLE
13-WEEK TOXICITY STUDY
BY ORAL ROUTE IN RATS

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR
Catherine Fabreguettes

STUDY COMPLETION DATE
21st July 1995

PERFORMING LABORATORY
Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER
12069 TCR (94/1/082)

Volume 1

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STATEMENT OF THE STUDY DIRECTOR

The study was performed in compliance with the principles of Good Laboratory Practice Regulations:
. O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981,
. Decret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire).

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at the Centre International de Toxicologie (C.I.T.), Miserey, 27005 Evreux, France.

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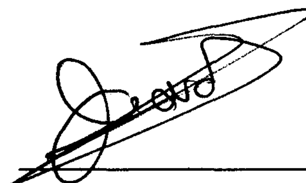

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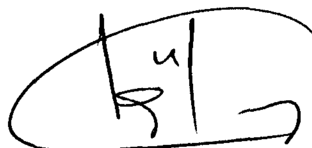

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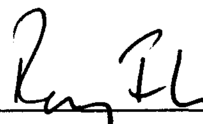

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SCIENTISTS INVOLVED IN THE STUDY (continued)

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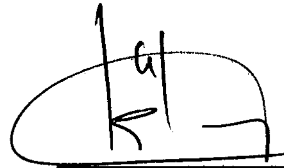

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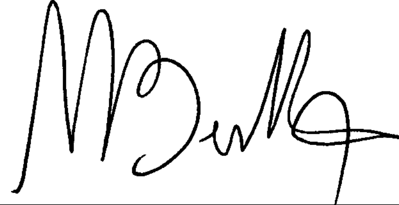

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Director of Histopathology and
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STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspections	Dates (day/month/year)		
	Inspections	Report to Study Director (*)	Report to Management (*)
Protocol	23.9.94	23.9.94	26.9.94
Protocol	12.10.94	12.10.94	12.10.94
Study	6.12.94	13.12.94	15.12.94
Study	8.12.94	13.12.94	15.12.94
Study	14.12.94	16.12.94	16.12.94
Study	3.1.95	12.1.95	13.1.95
Study	16.1.95	17.1.95	17.1.95
Study	1.2.95	2.2.95	3.2.95
Study	1.2.95	2.2.95	3.2.95
Study	28.2.95	13.3.95	13.3.95
Study	2.3.95	6.3.95	6.3.95
Study	8.3.95	13.3.95	13.3.95
Study	30.3.95	14.4.95	20.4.95
Study	8.6.95	15.6.95	15.6.95
Report	22.6.95	26.6.95	27.6.95

The inspections were performed in compliance with C.I.T. Quality Assurance Unit procedures and the principles of Good Laboratory Practice Regulations.

(*) The dates mentioned correspond to the dates of signature of audit reports by Study Director and Management.

L. Valette-Talbi Date: 21.7.95
Doctor of Biochemistry
Head of Quality Assurance Unit
and Scientific Archives

SUMMARY

The objective of this study was to evaluate the potential toxicity of the test substance, (batch No. Pil 4X), when administered daily by gavage to Sprague-Dawley rats for 13 weeks.
The test substance is a dye.

Methods

Three groups of 10 males and 10 females each received the test substance, daily by gavage at 25, 100 or 400 mg/kg/day for 13 weeks. An additional group of 10 males and 10 females received the vehicle alone and acted as control group.
The animals were checked daily for clinical signs and mortality. Food consumption and body weight were recorded once a week. Ophthalmological examinations were performed before the treatment period and on week 13. Haematological and blood biochemical investigations and urinalysis were performed on week 13.
Any animal found dead during the treatment period was subjected to a macroscopic examination and a full spectrum of tissues was preserved for microscopic examination.
At the end of the treatment period, the animals were killed and submitted to a full macroscopic examination. Selected organs were weighed and a microscopic examination was performed on all tissue specimens for animals of the control and high dose groups; macroscopic lesions and target tissues only (liver, kidneys and lungs) were examined for animals of the low and intermediate dose groups.

Results

Clinical signs
No clinical signs were observed in animals given 25 mg/kg/day.
Ptyalism was observed in some animals given 100 mg/kg/day and in almost all animals given 400 mg/kg/day, from week 4.
Loud breathing and/or regurgitation were observed in some males given 100 or 400 mg/kg/day.
Pink coloured urine, brown coloured tail, brown or black coloured faeces were observed in almost all animals given 100 or 400 mg/kg/day; these last findings were probably related to the elimination of the test substance or its metabolites.

Mortality
The mortalities occurring at 100 mg/kg/day (1 male) or 400 mg/kg/day (1 male) might be treatment-related. Moderate to marked aspiration pneumonia due to regurgitation was considered as a contributory factor.

Body weight, food consumption and efficiency of food utilization
The body weight, food consumption and efficiency of food utilization were similar in control and treated animals.

Ophthalmology
Opacification of lens (posterior-partial), slight on left and moderate on right, was observed in one female given 400 mg/kg/day. A relationship to the administration of the test substance cannot be excluded.

Haematology
The slightly higher activated partial thromboplastin time noted in females given 400 mg/kg/day was considered to be of minor toxicological importance.

Blood biochemistry
The slightly higher urea and creatinine levels observed in females given 100 or 400 mg/kg/day were considered to be of minor toxicological importance.

Urinalysis
No test substance-related changes were observed.

Organ weight
No relevant differences from controls were recorded.

Macroscopic examination
No findings of toxicological importance were observed.
Brownish coloured hair and body extremities were observed in some animals given 400 mg/kg/day (related to the staining properties of the test substance).

Microscopic examination
Slightly higher severity of tubular basophilia was noted in kidneys of the males given 400 mg/kg/day.
Brownish pigment accumulation was noted in many organs and tissues (kidneys, alimentary tract, liver and/or mesenteric lymph nodes) among the animals given 100 or 400 mg/kg/day (related to the staining properties of the test substance).
Subacute to chronic aspiration pneumonia was noted among the animals of both sexes given 100 or 400 mg/kg/day.

Conclusion

The administration of the test substance, (batch No. Pil 4X), daily by gavage for 13 weeks to rats was well-tolerated at 25 mg/kg/day. At 100 and 400 mg/kg/day, mortality of one animal was seen in each group, attributed to chronic aspiration pneumonia. This finding was probably due to an irritant effect of the test substance on the respiratory tract and was equally observed in almost all the animals from these groups at histopathological examination. At the same doses, signs of urinary elimination of the test substance or its metabolite were observed.
In addition, at 400 mg/kg/day, ptyalism was observed in almost all animals. Slightly higher activated partial thromboplastin time and slightly higher urea and creatinine levels were observed in females. Bilateral opacification of lens was observed in one female. Microscopically in kidney, slightly higher severity of tubular basophilia was noted for the males. The other treatment-related findings were due to the staining properties of the test substance.
Consequently, under our experimental conditions, the No Observable Adverse Effect Level is 25 mg/kg/day.

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1. INTRODUCTION

This study was performed at the request of Société
The objective of this study was to evaluate the potential toxicity of the test substance, when administered daily by oral route (gavage) to Sprague-Dawley rats for 13 weeks.
The rat was chosen because it is a rodent species commonly requested by regulatory authorities and the Sprague-Dawley strain was selected due to the background data available from previous studies performed at our laboratory.
The test substance is a dye.
The oral route was used since it is expected to ensure an absorption of the test substance at least equal to that following administration by dermal route which is the route of exposure in humans.
Dose-levels were based on the results of a previous 2-week toxicity study by gavage in rats (C.I.T/Study No. 12068 TSR).
This protocol complies with:
. O.E.C.D. guideline No. 408, 12th May 1981,
. E.E.C. Directive No. 87/302/E.E.C., 18th November 1987.

2. MATERIALS AND METHODS

2.1. TEST SUBSTANCE

2.1.1 Identification

2.1.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol and labelling:
. batch number:
  - protocol and labelling: Pil 4X
. description: beige powder
. quantity and container: 1 plastic flask of 4 kg; this quantity was dispatched at C.I.T. into 7 flasks (flasks 2, 4 and 5 were used for the present study)
. date of receipt: 26.7.94
. storage conditions: at room temperature, away from light, and in addition away from humidity (from 1.8.94 onwards); the content of each flask was kept under nitrogen until use.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.1.2 Vehicle

The vehicle was water for injections, batch Nos. 0419, 0675, 1112 and 1519, provided by Fresenius (92316 Sèvres, France).

2.1.2 Preparation

The test substance was given in the vehicle which was previously degassed by sonication for at least 10 minutes, and in which nitrogen bubbled for 2 to 3 minutes; this vehicle was then kept under argon until use.
The test substance was dissolved in vehicle in order to achieve a concentration of 80 mg/ml, and then homogenized using a magnetic stirrer. The 5 and 20 mg/ml preparations were prepared by direct dilution of the 80 mg/ml preparation. The preparations were then conditioned in sealed bottles, under argon atmosphere and away from light.
The test substance preparations were made for up to 7 days of treatment, according to known stability and were stored at +4°C, away from light pending utilization. They were delivered each day to the animal room, protected from light and maintained under continuous stirring during the dosing procedure (except for vehicle).

2.1.3 Chemical analysis of the test substance and preparations

2.1.3.1 Chemical analysis of the test substance

A sample of the test substance was analysed by C.I.T. by a potentiometric method provided by the Sponsor, on day 2 and after the *in vivo* study, to assess the stability of the test substance.
In addition, due to the low sensitivity of the measurement performed at C.I.T., a control was also performed by the Sponsor before the beginning of the treatment period and during the *in vivo* study (week 10).

2.1.3.2 Chemical analysis of the preparations

Stability

Stability has been checked for preparations at 10 mg/ml and 160 mg/ml in the embryotoxicity/teratogenicity study by oral route (gavage) (CIT/Study No. 12357 RSR) previously performed on the batch Pil 4X.
For the present study, the stability of a solution of the test substance at 5 mg/ml was checked before the second day of treatment of the study. The solution was sampled just after preparation, after 2 hours in sealed bottle followed by 1 hour in opened bottle at room temperature, and after 4 and 9 days storage at +4°C (4 sampling times in total). Each sample was diluted with degassed solvent containing a stabilizer (ascorbic acid at 0.01% w/v) and kept frozen at -20°C until analysis on day 9 (except samples taken after 3 hours storage at room temperature and after 9 days storage at +4°C).
Each sample was analysed in duplicate.

Concentration

For each group, including control, one preparation intended for use during weeks 1, 4, 8 and 12 was sampled and analysed.

The analytical procedure and results are presented in appendix 2.

2.2. TEST SYSTEM

2.2.1 Animals

Eighty-eight Sprague-Dawley rats (44 males and 44 females) of the Crl CD (SD) BR strain were supplied by Charles River France (76410 Saint-Aubin-lès-Elbeuf, France) and received at C.I.T. on 29.11.94.
Upon their arrival the animals were given a clinical examination to ensure that they were in good clinical condition.
A 8-day acclimatization period to the conditions of the study preceded the beginning of the treatment period.

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Before the beginning of the treatment period, the required number of animals (40 males and 40 females) was selected according to the body weight and clinical condition and allocated, by sex to the groups, according to a computerized stratified procedure so that the average body weight of each group was similar.
The remaining animals were killed later.
Each animal was then identified by ear tattoo.
At the beginning of the treatment period, the animals were approximately 6 weeks old and had a mean body weight of 219 g for the males (217 g to 221 g) and 182 g for the females (181 g to 184 g).

2.2.2 Environmental conditions

Upon their arrival at C.I.T., the animals were housed in a protected zone.
The animal room was disinfected before the arrival of the animals and cleaned regularly thereafter. Microbiological analyses of the air and the surfaces of the walls and floor of the animal room are performed regularly (Laboratoire Départemental d'Analyses d'Evreux, 27000 Evreux, France).
The environmental conditions in the animal room were set as follows:
. temperature: 21 ± 2°C
. relative humidity: 50 ± 20%
. light/dark cycle: 12h/12h (07:00 - 19:00)
. ventilation: about 12 cycles/hour of filtered, non-recycled air.
The temperature and relative humidity were recorded continuously and records retained in the study archives.
The housing conditions (temperature, relative humidity and ventilation) were checked monthly.
The animals were housed in suspended wire-mesh cages (43.0 x 21.5 x 18.0 cm) and each cage contained 2 rats of the same sex and group. A metallic tray was placed under each cage and contained autoclaved sawdust (SICSA, 94142 Alfortville, France). Bacteriological analysis of the sawdust and detection of possible contaminants (pesticides, heavy metals) are performed periodically (Laboratoire Départemental d'Analyses d'Evreux, 27000 Evreux, France; Laboratoire Municipal et Régional de Rouen, 76000 Rouen, France).
Bottles and sawdust were changed once a week, feeders, trays, cages and racks once every 4 weeks.
The cages were not randomized in the room but placed in numerical order, vertically on the racks. Fortnightly, all the racks were moved around clockwise, rack by rack.

There were no deviations in temperature and relative humidity.

2.2.3 Food and water

The animals had free access to A04 C pelleted diet, batch Nos. 40927 and 41114 (U.A.R., 91360 Villemoisson-sur-Orge, France) distributed weekly except as noted in "Laboratory investigations". The diet formula is presented in appendix 3. Each batch of food was analysed (composition, contaminants) by the supplier.
The animals had free access to bottles containing tap water filtered using a 0.22 micron filter (Millipore S.A., 78140 Vélizy, France).
Bacteriological and chemical analyses of water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are made periodically (Laboratoire Départemental d'Analyses d'Evreux, 27000 Evreux, France; Laboratoire Municipal et Régional de Rouen, 76000 Rouen, France; Centre de Nutrition Humaine, 54000 Nancy, France). Results are archived at C.I.T.

There were no known contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

Rationale for dose-level selection

The dose-levels were determined by the Sponsor following the results of the preliminary 2-week toxicity study by oral route (gavage) in rats performed at 50, 200 and 800 mg/kg/day (CIT/Study No. 12068 TSR).
Under the experimental conditions of this study, except slightly lower glucose level (-15% in males), no signs of toxicity were noted at 50 mg/kg/day.
At 200 mg/kg/day, only lower glucose level (-22%) and higher triglyceride level (x 1.5) were noted in males.
The principal signs of toxicity were observed at 800 mg/kg/day; they were, when compared to the control group: ptyalism and signs of poor clinical condition in both sexes, slightly lower body weight gain in males (-11%), lower glucose level (-26%) and higher triglyceride level (x 1.5) in males and in the kidneys, minimal to slight brownish pigment in the tubular epithelium and slightly higher incidence and severity of tubular dilatation in both sexes.
On the basis of these results, the dose-levels of 0, 25, 100 and 400 mg/kg/day were chosen for this study.

2.3.1 Dose-levels and groups

The groups, dose-levels and animal numbers are detailed in the following table:

Group	Animals per group	Dose-level mg/kg/day	Animal numbers
Males			
1	10	0	L22351 to L22360
2	10	25	L22361 to L22370
3	10	100	L22371 to L22380
4	10	400	L22381 to L22390
Females			
1	10	0	L22421 to L22430
2	10	25	L22431 to L22440
3	10	100	L22441 to L22450
4	10	400	L22451 to L22460

Between day 9 and day 22, an error was made in the preparation procedure of the high dose-level group. The weight of test substance was not substracted from the total weight of the preparation at the time of addition of vehicle, leading to an error in the final preparation of approximately -7%.
The same difference from theoretical concentration (-7%) was obtained for the low and intermediate dose-level groups as these preparations are made by appropriate dilution in the vehicle of the high dose-level preparation.

In addition, in the 100 mg/kg/day group, following an inversion of 2 animals at the weighing of day 15, the animal L22445 was given 1.12 ml of preparation instead of 1.22 ml (-8%) and the animal L22446 was given 1.22 ml of preparation instead of 1.12 ml (+9%), between day 17 (23.12.94) and day 23 (29.12.94).

As none of these changes reached more than + or -8% of error, they were not considered to alter the value of the study.

2.3.2 Administration

The test substance was administered by gavage using a glass syringe fitted with a metal gavage tube. Each animal was given the test substance once a day, at the same approximate daily time, 7 days a week over a period of 92 or 93 days.
During the administration, the test substance preparations (except vehicle) were maintained under constant magnetic stirring.
The quantity of the test substance administered to each animal was adjusted according to the most recently recorded body weight.
Control animals received the vehicle alone under the same conditions.
A constant dosage volume of 5 ml/kg/day was used.

2.4. CLINICAL EXAMINATIONS

2.4.1 Clinical signs

Clinical signs were observed for each animal at least once a day, at the same approximate daily time.

2.4.2 Mortality

All animals were checked at least twice a day for mortality and signs of morbidity, including weekends and public holidays.
The males found dead were subjected to a macroscopic examination and a full spectrum of tissues was preserved for a microscopic examination (see § 2.6. PATHOLOGY).

2.4.3 Food consumption

The quantity of food consumed by the animals of each cage was recorded once a week (over a 7-day period) until the end of the study.
Food intake per animal and per day was calculated using the amount of food given and left in each cage, divided by 2. In the summary tables, n corresponds to the number of cages.
When one of the two animals from the same cage died, the number of days for which that animal was present in the cage was taken into consideration for the calculation of the food consumption.

2.4.4 Body weight

Body weight was recorded for each animal, once before allocation of the animals into groups, on the first day of treatment, then once a week until the end of the study.

2.4.5 Efficiency of food utilization

Efficiency of food utilization was estimated by calculation of food conversion ratios.
The food conversion ratio was calculated on a weekly basis for each sex and each group, using body weight and food consumption means.

$$FCR = \frac{FC}{BWG}$$

FCR = food conversion ratio
FC = mean food consumption (g/animal/week)
BWG = mean body weight gain(g/animal/week).

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2.4.6 Ophthalmological examinations

Ophthalmological examinations were performed on all animals of each sex from the control and high dose-level groups once before the beginning of the treatment period and in all animals on week 13.
These examinations included corneal reflexes and the examination of the appendages, optic media and fundus by indirect ophthalmoscopy (All Pupil, Keeler, Windsor Berks, England). Prior to examination, the pupils of the animals were dilated using Mydriaticum® (Merck Sharp & Dohme-Chibret, 75008 Paris, France).
In some animals, in order to confirm the observations made by indirect ophthalmoscopy, an examination using a slit-lamp (biomicroscope Zeiss, 92153 Suresnes, France) was performed.

2.5. LABORATORY INVESTIGATIONS

Approximately 24 hours after treatment, blood samples were taken from the orbital sinus of the animals under light ether anaesthesia. The samples were collected in tubes containing the appropriate anticoagulant (see below).
For urine and blood collection, the animals were deprived of food and placed in metabolism cages over approximately 18 hours. The urine was collected into a tube containing thymol crystals.

2.5.1 Haematology

The following parameters were determined in all surviving animals on week 13.

Parameter	Apparatus/Method	Unit
Blood collected on EDTA		
Erythrocytes (RBC)	Bayer Diagnostics H1 (1) Haematology Analyzer/laser	T/l
Haemoglobin (HB)	Bayer Diagnostics H1 Haematology Analyzer/Drabkin	g/dl
Mean Cell Volume (MCV)	Bayer Diagnostics H1 Haematology Analyzer/laser	fl
Packed Cell Volume (PCV)	Bayer Diagnostics H1 Haematology Analyzer/calculated	l/l
Mean Cell Haemoglobin Concentration (MCHC)	Bayer Diagnostics H1 Haematology Analyzer/calculated/laser	g/dl
Mean Cell Haemoglobin (MCH)	Bayer Diagnostics H1 Haematology Analyzer/calculated	pg
Thrombocytes (PLAT)	Bayer Diagnostics H1 Haematology Analyzer/laser	G/l
Leucocytes (WBC)	Bayer Diagnostics H1 Haematology Analyzer/ peroxidase cytochemistry/laser morphometry	G/l

(1) Bayer Diagnostics (95331 Domont, France)

Parameter	Apparatus/Method	Units
Differential White Cell Count with cell morphology	Bayer Diagnostics H1 Haematology Analyzer/ peroxidase cytochemistry/laser morphometry (if reject, a microscopic control was determined after May Grünwald staining) (2) (a)	
. neutrophils (N)		% and G/l
. eosinophils (E)		% and G/l
. basophils (B)		% and G/l
. lymphocytes (L)		% and G/l
. monocytes (M)		% and G/l
Reticulocytes (RETIC) (b)	Microscopic/ Bright cresyl blue staining (3)	p.1000
Blood collected on sodium citrate		
Prothrombin Time (PT)	ACL 300 Thromboplastin (IL France) (4)	s
Activated Partial Thromboplastin Time (APTT)	ACL 300 Ellagic acid (IL France)	s
Fibrinogen (FIB)	ACL 300 Thromboplastin (IL France)	g/l

(a) Blood smears were prepared for all sampled animals.
(b) Blood smears were prepared for all sampled animals. In the absence of anaemia, the reticulocyte count was not determined.
(2) Merck Clévenot (77500 Chelles, France)
(3) Prolabo (75011 Paris, France)
(4) IL France (75562 Paris, France)

2.5.2 Blood biochemistry

The following parameters were determined in all surviving animals on week 13.

Parameter	Apparatus/Method	Unit
Blood collected on lithium heparinate		
Sodium (Na^+)	Hitachi 717 Selective electrode (Boehringer) (1)	mmol/l
Potassium (K^+)	Hitachi 717 Selective electrode (Boehringer)	mmol/l
Chloride (Cl^-)	Hitachi 717 Selective electrode (Boehringer)	mmol/l

(1) Boehringer (38242 Meylan, France)

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Parameter	Apparatus/Method	Unit
Calcium (Ca^{++})	Hitachi 717 Ortho cresol-phthalein (Boehringer)	mmol/l
Inorganic phosphorus (I.PHOS)	Hitachi 717 Phosphomolybdic reaction (Boehringer)	mmol/l
Glucose (GLUC)	Hitachi 717 GOD-PAP (Boehringer)	mmol/l
Urea (UREA)	Hitachi 717 Urease UV (Boehringer)	mmol/l
Creatinine (CREAT)	Hitachi 717 Jaffe without deproteinisation (Boehringer)	µmol/l
Total Bilirubin (TOT.BIL)	Hitachi 717 Jendrassik (Boehringer)	µmol/l
Total Proteins (PROT)	Hitachi 717 Biuret (Boehringer)	g/l
Albumin (ALB)	Hitachi 717 Bromocresol green (Boehringer)	g/l
Albumin/globulin ratio (A/G)	Hitachi 717 Calculated	1
Cholesterol (CHOL)	Hitachi 717 CHOD-PAP (Boehringer)	mmol/l
Triglycerides (TRIG)	Hitachi 717 GPO-PAP (Boehringer)	mmol/l
Alkaline phosphatase (ALP)	Hitachi 717 DGKC Standard/30°C (Boehringer)	IU/l
Aspartate aminotransferase (ASAT)	Hitachi 717 IFCC Standard/30°C (Boehringer)	IU/l
Alanine aminotransferase (ALAT)	Hitachi 717 IFCC Standard/30°C(Boehringer)	IU/l

2.5.3 Urinalysis

The following parameters were determined in all surviving animals on week 13.

Parameter	Apparatus/Method	Unit
Quantitative parameters		
Volume (VOLUME)		ml
pH (pH)	10-Multistix SG test strips (Ames) (1)	
Specific gravity (SP.GRAV)	10-Multistix SG test strips (Ames) Refractometer (2) when superior to 1025	x1000
Semi-quantitative parameters		
Proteins (PROT)	10-Multistix SG test strips (Ames)	(a)
Glucose (GLUC)	10-Multistix SG test strips (Ames)	(a)
Ketones (CETO)	10-Multistix SG test strips (Ames)	(a)
Bilirubin (BILI)	10-Multistix SG test strips (Ames)	(a)
Nitrites (NITR)	10-Multistix SG test strips (Ames)	(a)
Blood (BLOOD)	10-Multistix SG test strips (Ames)	(a)
Urobilinogen (UROB)	10-Multistix SG test strips (Ames)	U%(=Ehrlich unit/100ml)
Cytology	microscopic	
. leucocytes (WBC)		(a)
. erythrocytes (RBC)		(a)
. cylinders (CYLIN)		(a)
. magnesium ammonium phosphate crystals (AMM.PH.)		(a)
. calcium phosphate crystals (CAL.PH)		(a)
. calcium oxalate crystals (CAL.OX.)		(a)
. cells (CELLS)		(a)
Qualitative parameters		
Appearance (APP)		(a)
Colour (COLOUR)		(a)

(a) see key or grading of cell frequency to appendix with individual values.
(1) Ames, Miles (75755 Paris, France)
(2) Aventec (92170 Vanves, France)

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2.6. PATHOLOGY

2.6.1 Sacrifice

On completion of the treatment period, after about 18 hours fasting, all surviving animals were asphyxiated using carbon dioxide and killed by exsanguination.

2.6.2 Organ weights

For all animals killed at the end of the treatment period, the body weight was recorded before necropsy and the following organs were weighed wet as soon as possible after dissection:

adrenals, kidneys, ovaries, testes
heart, liver, spleen, thymus

Paired organs were weighed separately.

2.6.3 Macroscopic examination

A complete macroscopic examination was performed on all animals including those found dead during the study.
All gross observations were recorded individually.

2.6.4 Preservation of tissues

For all animals including those found dead during the study, all the macroscopic lesions and the following tissues were preserved in 10% buffered formalin (except for the eyes and pituitary gland which were fixed in formol-sublimate for the animals killed at the end of the treatment period) and except for the testes and the epididymides which were fixed in Bouin's fluid:

adrenals
aorta
brain including medulla/pons, cerebellar and cerebral cortex
caecum
colon
duodenum
eyes with Harderian glands*
femoral bone with articulation*
heart
ileum
jejunum
kidneys
liver
lungs with bronchi
lymph nodes (mandibular and mesenteric)
mammary glands*
oesophagus
ovaries
pancreas
pituitary gland
prostate*
rectum
salivary glands* (sublingual and submaxillary)
sciatic nerve
seminal vesicles*
skeletal muscle*
skin*
spinal cord* (cervical, thoracic and lumbar)
spleen
sternum with bone marrow
stomach with forestomach
testes and epididymides
thymus
thyroids with parathyroids
tongue*
trachea
urinary bladder
uterus (horns and cervix)
vagina*

The tissues marked by * were preserved in fixative for possible future microscopic examination.

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2.6.5 Microscopic examination

All tissues required for microscopic examination were embedded in paraffin wax, sectioned at approximately 4 microns in thickness and stained with hematoxylin-eosin.
A P.A.S. staining was also performed for testes and epididymides.
Microscopic examination was performed on:

. all macroscopic lesions and tissues listed above (except those marked by *) in animals of the control and high dose groups killed at the end of the treatment period and in animals found dead during the study.
. all macroscopic lesions, liver, kidneys and lungs of all animals of the low and intermediate dose groups.

2.7. STATISTICAL ANALYSIS

The following sequence was used for the statistical analysis of body weight, food consumption, haematology, blood biochemistry, urinalysis and organ weight data:
The normality of the distribution of the values in each group was checked by Kolmogorov-Smirnov's test (1948).
If the distribution was normal, the homogeneity of variances between the groups was assessed by Bartlett's test (1937) (more than 2 groups) or Fisher's test (1934) (2 groups).
If no significant heterogeneity of the variances was established, the comparison between treated and control groups was performed by Dunnett's test (1955).
If the variances were heterogeneous, the comparison between treated and control groups was performed by Dunn's test (1964) (more than 2 groups) or by Mann Whitney's test (1947) (2 groups).
If the distribution of values in the groups was not normal, the analysis was repeated after logarithmic transformation of the values (except for organ weights).
If this logarithmic transformation failed to normalise the distribution of the values, comparison of treated and control groups was performed by Dunn's test using original values.

2.8. ARCHIVES

Study documentation and materials:

. protocol and amendments,
. raw data,
. correspondence,
. final report and possible amendments,
. tissues in preservative, blocks, histological slides,
. haematological slides,
. samples of the control and test substances,

are stored on the premises of C.I.T., 27005 Miserey, Evreux, France for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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2.9. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Dates	Days
Protocol approved by:		
. Study Director	12.10.94	
. Study Monitor	21.10.94	
Arrival of the animals	29.11.94	-8
Preidentification and weighing	30.11.94	-7
Randomization and identification of the animals	2.12.94	-5
Ophthalmological examinations	5.12.94	-2
First day of treatment	7.12.94	1
Week 13		
Ophthalmological examinations	3.3.95	87
Haematology, blood biochemistry, and urinalysis	6.3.95	90
Last day of treatment	8 or 9.3.94	92 or 93
Days of necropsy	9 or 10.3.94	93 or 94

3. RESULTS

3.1. CHEMICAL ANALYSIS OF THE TEST SUBSTANCE AND PREPARATIONS

3.1.1 Chemical analysis of the test substance

Controls performed before and after the *in vivo* study by C.I.T. to assess the stability of the test substance were insufficiently sensitive to confirm acceptable stability of the test substance. Therefore, analyses were also performed by the Sponsor which have confirmed the stability of the test substance.

3.1.2 Chemical analysis of the preparations

Stability

Satisfactory stability was previously demonstrated for the embryotoxicity/teratogenicity study by oral route (gavage) (CIT/Study No. 12357 RSR). For this study, preparations at 10 mg/ml and 160 mg/ml were uped until a maximum of 9 days followed by 2 hours in a sealed bottle and then 1 hour in an open bottle at room temperature, storage at +4°C.
For the present study, the satisfactory stability of a solution of the test substance at 5 mg/ml was also demonstrated under the same storage conditions.

Concentration

Throughout the study, a satisfactory concordance between obtained and nominal concentrations was found.

Detailed results are presented in appendix 2.

3.2. CLINICAL EXAMINATIONS

3.2.1 Clinical signs (tables 1 and 2, appendix 4)

No clinical signs were observed in animals given 25 mg/kg/day.
Ptyalism was observed in 2/10 males and 1/10 females given 100 mg/kg/day and in 10/10 males and 7/10 females given 400 mg/kg/day, from day 22 (week 4).
Loud breathing was observed in 1/10 males given 100 mg/kg/day, from week 6 and during 13 days and in 3/10 males given 400 mg/kg/day, from week 6.
Regurgitation was noted in 1/10 males given 100 mg/kg/day and 3/10 males given 400 mg/kg/day, on day 90 (week 13).
These clinical signs were attributed to the treatment with the test substance.

The following observations were noted and were probably related to the elimination of the test substance or its metabolites:

. pink coloured urine in all females given 100 mg/kg/day and in all animals given 400 mg/kg/day (from day 1),
. brown coloured tail in 9/10 males and 5/10 females given 400 mg/kg/day (from week 11),
. brown coloured faeces in 6/10 males and 10/10 females given 100 mg/kg/day (from week 14),
. black coloured faeces in all animals given 400 mg/kg/day (from week 13).

These signs were considered to be an indirect proof of the absorption of the test substance.

As the other clinical signs (including: chromorhinorrhea, chromodacryorrhea, scabs on head, area of hair loss on head, back or on right and/or left forelimbs) appeared with a low frequency and were noted with a similar incidence in control and treated groups they were not attributed to the administration of the test substance.

3.2.2 Mortality (appendix 5)

The mortality occurring during the course of the study was as follows:

25 mg/kg/day group:
Male L22369 was found dead on day 78 (week 12).
There were no ante mortem clinical signs.
Dilated and reddish lungs were noted at necropsy which were correlated at microscopy to moderate acute aspiration pneumonia together with post mortem stasis.

100 mg/kg/day group:
Male L22376 was found dead on day 90 (week 13).
There were no ante mortem clinical signs.
No macroscopic change was noted at necropsy and moderate acute aspiration pneumonia and post mortem stasis were noted at microscopy.

400 mg/kg/day group:
Male L22388 was found dead on day 91 (week 13).
Regurgitation was observed in this male on day 90.
Multiple raised greyish/whitish foci were noted in the lungs which were correlated at microscopy to a marked acute aspiration pneumonia.

For all these males, moderate to marked acute aspiration pneumonia was considered to be the cause of death and due to misdosing or regurgitation.
Taking into consideration the absence of regurgitation in the animals given 25 mg/kg/day, it can be deduced that the death of this animal is accidental, due to misdosing and not treatment-related. On the contrary, due to the relatively frequent occurence of regurgitation at 100 or 400 mg/kg/day, the mortalities in these two groups might be treatment-related.

3.2.3 Food consumption (tables 3 and 4, appendix 6)

No relevant differences from controls were noted in any group.
The slightly lower mean food consumption recorded only on week 9 in males given 100 or 400 mg/kg/day (-8% and -9% respectively) was considered not to be toxicological significance.

3.2.4 Body weight (figures 1 and 2, tables 5 and 6, appendix 7)

The mean body weight gain of all treated males and females was similar to that of respective controls.

3.2.5 Efficiency of food utilization (table 7)

The mean efficiency of food utilization of all treated males and females was similar to that of respective controls.

3.2.6 Ophthalmological examinations (appendix 8)

The relevant findings noted at the end of the treatment period were:

. opacification of lens (posterior-partial), slight on left and moderate on right in 1/10 females (L22460) given 400 mg/kg/day. Although this finding was noted with a low incidence in this group, as this was not recorded in control or other treated animals and not observed in our background data for rats of this age, a relationship to administration of the test substance cannot be excluded.
. bilateral partial corneal opacification in 1/10 males given 25 mg/kg/day (L22366). This finding was not recorded in higher dose groups and can be seen spontaneously in animals of that strain and that age. Accordingly, this was considered not to be related to test substance administration.

3.3. LABORATORY INVESTIGATIONS

3.3.1 Haematology (tables 8 and 9, appendix 9)

Slightly higher activated partial thromboplastin time was noted in females given 400 mg/kg/day (+21%). Almost all individual values were higher than the upper limit of controls (16.9 s) but close of the upper limit of our background data (21.7 s) for rats of this age. Consequently, this difference from controls was considered to be of minor toxicological importance.

The slight differences from controls noted in the other haematological parameters (including: mean cell haemoglobin concentration in females given 400 mg/kg/day) were considered to be of no toxicological importance, although statistically significant, since the individual values were within the normal range of our background data.

3.3.2 Blood biochemistry (tables 10 and 11, appendix 10)

Slightly higher urea and creatinine levels were observed in females given 100 or 400 mg/kg/day when compared to controls (urea: +25% and +30%, creatinine: +16% and +22% at 100 and 400 mg/kg/day respectively). In both groups, 5/10 females were slightly higher to the upper value of the control animals. Nevertheless, as these differences were not associated with any relevant microscopic finding, this was considered to be of minor toxicological significance.

Slightly lower glucose level was recorded in animals given 100 or 400 mg/kg/day when compared to control groups (males: -8% and -11%, females: -10% and -9% at 100 and 400 mg/kg/day respectively). As these differences from controls were slight, sometimes not dose-related, and as all individual values were close or within the normal range of background data, this change was considered to be of no toxicological importance.

The other differences from controls (including: lower potassium or cholesterol level, lower aspartate aminotransferase activity) were considered to be of no toxicological importance, as they were not dose-related and the individual values were within the range of our background data.

3.3.3 Urinalysis (tables 12 and 13, appendix 11)

No test substance-related changes were observed.

3.4. PATHOLOGY

3.4.1 Organ weights (table 14, appendix 12)

Statistically significant lower mean absolute kidney weight (-13%) was noted for the males given 100 mg/kg/day when compared to controls. As this was not recorded for the high dose males, this minor difference was considered to bear no relationship to treatment with the test substance.
No other relevant differences in organ weights were noted between the animals of the control and treated groups.

3.4.2 Macroscopic examination (table 15, appendix 13)

Brownish colour of hair and extremities was noted for 5/10 males and 9/10 females given 400 mg/kg/day. This was considered to be related to the staining properties of the test substance.

A brownish/blackish focus (approx. 0.2 cm in diameter) was noted in the glandular mucosa of stomach of 1/10 males given 400 mg/kg/day. This was not associated with any microscopic change.

Several raised greyish/whitish foci were noted in the mucosa of forestomach of another male of the same group.
As these findings can be seen spontaneously in untreated laboratory rats of this strain, their reported low incidence in the high dose group was considered to be of no toxicological importance.

The other macroscopic findings noted were those which are commonly recorded in untreated laboratory rats of this strain.

3.4.3 Microscopic examination (table 16, appendix 13)

Minimal to slight brownish pigment accumulation was noted in many organs and tissues and was noted as follows:

kidneys

. minimal to slight brownish pigment accumulation in cortical cells of 7/10 males and 1/10 females given 100 mg/kg/day and 10/10 males and 8/10 females given 400 mg/kg/day.

alimentary tract

. minimal to slight brownish pigment (either intracellular or extracellular) accumulation in the lamina propria of the mucosa of the small (duodenum, jejunum, ileum) and/or large (caecum, colon, rectum) intestine for all males and females given 400 mg/kg/day. A few brownish pigment deposits were also noted in the glandular lumens of colon or rectum for some of these animals.

liver

. minimal presence of brownish pigment laden macrophages for 2/10 females given 100 mg/kg/day and brownish pigment laden Kupffer cells, hepatocytes and macrophages for 1/10 females given 400 mg/kg/day.

mesenteric lymph nodes

. minimal to slight brownish pigment accumulation in macrophages for 8/10 males and 2/10 females given 400 mg/kg/day.

These changes were considered to be related to the staining properties of the test substance. Consequently, no further investigation was performed on the alimentary tract, mesenteric lymph nodes for the low and intermediate dose animals.

Minimal or slight tubular basophilia, often unilateral, was noted in the kidneys of 4/10 control males, 3/10 males given 25 mg/kg/day, and 4/10 males and 3/10 females given 100 mg/kg/day.
Minimal to moderate tubular basophilia, often bilateral was noted for 6/10 males given 400 mg/kg/day.
The slightly higher severity of tubular basophilia noted for the males given 400 mg/kg/day was considered to be related to treatment with the test substance.
The minimal unilateral tubular basophilia noted for the females given 100 mg/kg/day was considered to bear no relationship to treatment with the test substance, as this was not recorded for the females given 400 mg/kg/day.

Minimal ulceration associated with brownish pigment deposition was noted in mucosa of rectum for 1/10 males given 400 mg/kg/day. The low incidence of this finding was considered to be of no toxicological importance.

Lungs

Minimal to moderate subacute to chronic aspiration pneumonia was noted for 1/10 females given 25 mg/kg/day, 5/10 males and 6/10 females given 100 mg/kg/day and 5/10 males and 5/10 females given 400 mg/kg/day. This change was characterized by the presence of foamy macrophages in bronchioles and peribronchiolar alveoli, sometimes centred on few cholesterol clefts. The peribronchiolar alveoli showed cuboidal epithelial metaplasia and/or septal fibrosis together with atelectasia. In addition, some brownish pigment laden macrophages were also noted within the lesion for the animals given 100 or 400 mg/kg/day, but not for the low dose female.

Mild chronic aspiration pneumonia can be seen in studies where the test substance is administered either by gavage, intravascularly, or by admixture in the diet (Gopinath et Al., 1987).
This type of pneumonia might have been developed by accidental aspiration of the test substance, either by deposition at the laringeal orifice (even, in cases of correct oral dosing) or as a result of ptyalism and/or regurgitation.
Taking into consideration the relatively frequent occurence of these findings in animals given 100 or 400 mg/kg/day, the relationship of aspiration pneumonia to the treatment cannot be ruled out.

The few other microscopic findings noted were those which are commonly recorded in untreated laboratory rats of this strain and age. Furthermore, their incidence, severity and morphological characteristics were similar between the control and treated groups. Consequently, they were considered to bear no relationship to treatment with the test substance.

4. CONCLUSION

The administration of the test substance, (batch No. Pil 4X), daily by gavage for 13 weeks to rats was well-tolerated at 25 mg/kg/day. At 100 and 400 mg/kg/day, mortality of one animal was seen in each group, attributed to chronic aspiration pneumonia. This finding was probably due to an irritant effect of the test substance on the respiratory tract and was equally observed in almost all the animals from these groups at histopathological examination. At the same doses, signs of urinary elimination of the test substance or its metabolite were observed.
In addition, at 400 mg/kg/day, ptyalism was observed in almost all animals. Slightly higher activated partial thromboplastin time and slightly higher urea and creatinine levels were observed in females. Bilateral opacification of lens was observed in one female. Microscopically in kidney, slightly higher severity of tubular basophilia was noted for the males. The other treatment-related findings were due to the staining properties of the test substance.
Consequently, under our experimental conditions, the No Observable Adverse Effect Level is 25 mg/kg/day.

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DEVIATION FROM THE PROTOCOL

STUDY No.: **12069 TCR - 94/1/082**

SPONSOR: **Société**

STUDY MONITOR:

TEST SUBSTANCE:

Justification: punctual errors in the preparation procedure and weighing of the animals

3. TREATMENT

Between day 9 and day 15 and between day 16 and day 22 (between 15.12.94 and 28.12.94), an error in the preparation procedure of the high dose group was done. The weight of test substance was not deduced of the total weight of the preparation at the time of addition of vehicle.
The error on the final preparation was approximately -7%.
The same difference from theoretical concentration (-7%) was obtained for the low and intermediate dose groups as these preparations are obtained by appropriate dilution in the vehicle of the high dose preparation.

In addition, in the 100 mg/kg/day group, following an inversion of 2 animals at the weighing of day 15, the animal L22445 was given 1.12 ml of preparation instead of 1.22 ml (-8%) and the animal L22446 was given 1.22 ml of preparation instead of 1.12 ml (+9%), between day 17 (23.12.94) and day 23 (29.12.94).

Comments of the Study Director:

These minor deviations did not compromise the validity or integrity of the study.

Scientific Management
J.F. Le Bigot or A. Simonnard
Date: 16.1.95
Signature:

Study Director
C. Fabreguettes
Date: 16.1.95
Signature:

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STUDY TITLE

ACUTE DERMAL TOXICITY IN RATS

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

3rd November 1994

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12042 TAR

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3. Body weight of control animals
   (C.I.T. Historical data of animals dosed by the dermal route) 24 to 26

SUMMARY

At the request of , the acute toxicity of the test substance, (batch No. Pil 4x), by dermal route was evaluated in rats according to O.E.C.D. (No. 402, 24th February 1987) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice.

Methods

The test substance was administered by dermal route to a group of 10 Sprague-Dawley rats (5 males and 5 females).

The test substance in its original form was prepared on a moistened compress at a dose of 2000 mg/kg and then applied to the skin of the animals.

After 24 hours under a semi-occlusive dressing, any residual test substance was removed using a dry compress.

The animals were checked for clinical signs, mortality and body weight gain for a period of 14 days following the single application of the test substance.

A necropsy was performed on each animal killed at the end of the study.

The interpretation of results was carried out according to the classification criteria laid down in Council Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C.

Results

A slight decrease in spontaneous activity was observed in 1 out of 10 animals, 4 and 6 hours after treatment. No other clinical signs were noted during the study.

No cutaneous reactions were observed.

The body weight gain of the animals was not affected by treatment with the test substance.

No deaths occurred at 2000 mg/kg.

A macroscopic examination revealed no abnormalities in the animals killed at the end of the study.

Conclusion

Under our experimental conditions, the LD_{50} of the test substance, (batch No. Pil 4x), when administered by dermal route in rats was higher than **2000 mg/kg**.

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Labelling

Chimie
Commission Directive 93/21/E.E.C.

Labelling not indicated for the test substance.

1. INTRODUCTION

The objective of this study was to evaluate the toxicity of the test substance, following a single dermal application in rats.

In the assessment of the toxic characteristics of a test substance, determination of acute dermal toxicity is usually an initial step. It provides information on health hazards to man likely to arise from a short-term exposure on the skin.

The test substance is applied at a single dose to the skin of a group of 10 rats (5 males and 5 females).

This study was conducted in compliance with:
. O.E.C.D. guideline No. 402, 24th February 1987,
. E.C. Directive No. 92/69/E.E.C., B_3, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4x
  - labelling: Pil 4x
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: . at room temperature, protected from light,
  . protected from humidity from 1.8.94
. purity: see analytical certificate

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Other substance

Water for injections, batch No. 9784 (Biosédra, 92240 Malakoff, France) was used to disperse the test substance and ensure a good contact with the skin.

2.1.3 Preparation

The test substance was applied in its original form.

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2.2. TEST SYSTEM

2.2.1 Animals

Species, strain: rat, Sprague-Dawley ICO: OFA-SD (IOPS Caw).
Reason for this choice: rodent species commonly requested by the international regulations for this type of study.
Breeder: Iffa Crédo, 69210 L'Arbresle, France.
Number and sex: one group of 10 animals (5 males and 5 females).
Age/weight: on the day of treatment, the animals were approximately 8 weeks old, and had a mean body weight of 268 ± 10 g for the males and 214 ± 7 g for the females.
Acclimatization: at least 5 days before the beginning of the study.
Identification of the animals: the animals were identified individually by earmarks or ear-notches.

2.2.2 Environmental conditions

During the acclimatization period and during the main test, the conditions in the animal room were as follows:
temperature: 21 ± 2°C
relative humidity: 30 to 70%
light/dark cycle: 12 h/12 h
ventilation: about 12 cycles/hour of filtered, non-recycled air.
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity, light/dark cycle and ventilation) were checked monthly.
The animals were housed in polycarbonate cages covered with a stainless steel lid.
During the acclimatization period each cage (48 x 27 x 20 cm) contained 4 to 7 animals of the same sex.
During the treatment period each cage (35.5 x 23.5 x 19.3 cm) contained 1 rat. Each cage contained graded, dust-free sawdust (SICSA, 94142 Alfortville, France).
Bacteriological analysis of the sawdust and detection of possible contaminants (pesticides, heavy metals) are performed periodically.

2.2.3 Food and water

All the animals had free access to A04 C pelleted diet (U.A.R., 91360 Villemoisson-sur-Orge, France).
Each batch of food was analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was contained in bottles.
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

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2.3. TREATMENT

2.3.1 Preparation of the animals

On the day before treatment, the dorsal area (6 cm x 8 cm) of each animal was clipped of hair using electric clippers. Only animals with healthy intact skin were used for the study.

2.3.2 Mode of treatment

As the test substance was anticipated to be non-toxic at 2000 mg/kg, a limit test was performed by application of 2000 mg/kg of the test substance to a group of 10 animals (5 males and 5 females).

A single dose of 2000 mg/kg of the test substance in its original form was prepared on a hydrophilic gauze patch (Semes France, 54183 Heillecourt, France) pre-moistened with 2 ml of water for injections and then applied to an area of the skin representing approximately 10% (5 x 6 cm for the females and 5 x 7 cm for the males) of the body surface of the animals. This was calculated according to Meeh's formula (1). The test substance and the patch were held in contact with the skin for 24 hours by means of an adhesive hypoallergenic aerated semi-occlusive dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and a restraining bandage (Laboratoires 3M Santé, 92245 Malakoff, France). This dressing prevented ingestion of the test substance by the animal. Any residual test substance was removed with a dry gauze patch.

The dose applied to each animal was adjusted according to body weight determined on the day of treatment.

2.3.3 Date of treatment

The single administration was performed on 18.8.94 in the morning (day 1) and was followed by a 14-day observation period until 1.9.94 (day 15).

2.4. CLINICAL EXAMINATIONS

2.4.1 Clinical signs

The animals were observed frequently during the hours following application of the test substance, for detection of possible treatment-related clinical signs. Observation of the animals was made at least once a day for a period of 14 days, to determine whether any of the clinical signs were reversible or not. Clinical signs were recorded for each animal individually. Local cutaneous reactions were also noted when necessary.

(1) Meeh's formula (AFNOR-T03-23 Standard, August 1980, France)

$S = k\sqrt[3]{p^2}$

S = Total surface of the animal in dm^2

p = Weight of the animal in grams

k = 0.09 for the rat

2.4.2 Mortality

The animals were checked frequently during the hours following application of the test substance for mortality or signs of morbidity, then at least twice a day thereafter.

2.4.3 Body weight

The animals were weighed individually just before administration of the test substance then on days 8 and 15.

The body weight gain of the treated animals was compared to a reference curve of C.I.T. control animals with the same initial weight.

2.5. PATHOLOGY

2.5.1 Necropsy

On day 15, the animals were killed by CO_2 inhalation in excess and a macroscopic examination was performed.

2.5.2 Macroscopic examination

After opening the thoracic and abdominal cavities, a macroscopic examination of the main organs (digestive tract, heart, kidneys, liver, lungs, pancreas, spleen and any other organs with obvious abnormalities) was performed.
In case of macroscopic lesions, organ samples were taken and preserved in 10% buffered formalin.

2.5.3 Microscopic examination

No microscopic examination was performed.

2.6. DATA EVALUATION

Evaluation of the innoxiousness or toxicity of the test substance following a single dermal application in rats should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all the abnormalities including behavioural and clinical abnormalities, macroscopic lesions, body weight changes, mortality and any other toxic effects.
Classification of the test substance is based on the following criteria:

Commission Directive 93/21/E.E.C.

LD_{50} dermal route (mg/kg)	Labelling sentence	Indication of danger	Symbol
≤ 50	R27 Very toxic in contact with skin	Very toxic	T^+
50 < and ≤ 400	R24 Toxic in contact with skin	Toxic	T
400 < and ≤ 2000	R21 Harmful in contact with skin	Harmful	X_n
> 2000	None	None	None

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2.7. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,

are stored on the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. RESULTS

3.1. CLINICAL EXAMINATIONS

3.1.1 Clinical signs (tables 1 and 2)
Hypoactivity was observed in 1 animal (female No. 02) 4 and 6 hours after treatment.

No other clinical signs were noted during the study.

No cutaneous reactions were observed during the study.

3.1.2 Mortality (table 1)
No deaths occurred during the observation period.

3.1.3 Body weight (treated animals: figure 1, table 3)
(control animals: appendix 3)
The body weight gain of the animals was normal.

3.2. PATHOLOGY (table 4)

Macroscopic examination of the main organs of the animals killed at the end of the study revealed no apparent abnormalities.

4. CONCLUSION

Under our experimental conditions, the LD_{50} of the test substance, (batch No. Pil 4x), when administered by dermal route in rats was higher than **2000 mg/kg**.

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, le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par $AgNO_3$	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

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TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY TITLE

ACUTE EYE IRRITATION IN RABBITS

with the test substance diluted at 5%

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

26th July 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12909 TAL (95/1/042)

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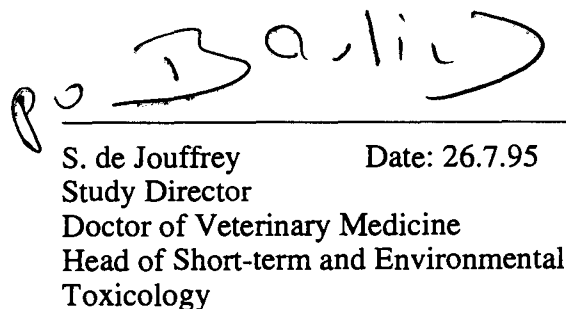
STATEMENT OF THE STUDY DIRECTOR

The study was performed in compliance with the principles of Good Laboratory Practice Regulations:

. O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981,
. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire).

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at the Centre International de Toxicologie (C.I.T.), Miserey, 27005 Evreux, France.



Toxicology

S. de Jouffrey Date: 26.7.95
Study Director
Doctor of Veterinary Medicine
Head of Short-term and Environmental Toxicology

OTHER SCIENTISTS INVOLVED IN THIS STUDY

For Pharmacy: J. Richard
Doctor of Pharmacy

For Toxicology: C. Pelcot
Study Supervisor

STATEMENT OF QUALITY ASSURANCE UNIT

1. Specific study inspections

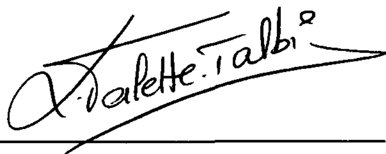
Type of inspections	Dates (day/month/year)	
	Inspections	Report to Study Director / Management (*)
Protocol	5.5.95	10.5.95
Study	5.7.95	7.7.95

2. Routine inspections performed on other studies of the same type according to a frequency defined in Q.A.U. procedures

Inspected phase	Dates (day/month/year)	
	Inspections	Report to Study Director / Management (*)
Treatment/test substance	7.2.95	10.2.95
Test substance/preparation	9.3.95	10.3.95

The inspections were performed in compliance with C.I.T. Quality Assurance Unit procedures and the Good Laboratory Practice Regulations.

(*) The dates mentioned correspond to the dates of signature of audit reports by Study Director / Management.



L. Valette-Talbi Date: 26.7.95
Doctor of Biochemistry
Head of Quality Assurance Unit
and Scientific Archives

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SUMMARY

At the request of , the ocular irritation that could be induced by the test substance, (batch No. Pil 4X) diluted at a concentration of 5%, was evaluated in rabbits according to O.E.C.D. (No. 405, 24th February 1987) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

Having confirmed that the test substance diluted at a concentration of 5% was not irritant when administered by cutaneous route, a single dose of 0.1 ml of a preparation containing the test substance diluted at a concentration of 5% (w/w) in distilled water was instilled into the conjunctival sac of the left eye of 3 male New Zealand White rabbits.

The eyes were not rinsed after administration of the test substance. Ocular reactions were observed approximately 1, 24, 48 and 72 hours after the administration.

The mean score of the values recorded for each animal after 24, 48 and 72 hours was calculated.

The interpretation of results was carried out according to the classification criteria laid down in Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C.

Results

No ocular reactions were observed during the study.

Conclusion

Under our experimental conditions, the test substance, (batch No. Pil 4X) diluted at a concentration of 5% (w/w) in water, was considered non-irritant when administered by ocular route in rabbits.

1. INTRODUCTION

The objective of this study was to evaluate the potential of the test substance,
diluted at a concentration of 5% (w/w), to induce ocular irritation following a single administration in rabbits.

In the assessment of the toxic characteristics of a test substance, determination of the irritant and/or corrosive effects on the eyes of mammals is an important initial step.
. When the acute dermal irritation study in the rabbit demonstrates that the test substance is not corrosive or severely irritant, one may presume that the test substance is unlikely to produce severe effects on the eye. In this case, the acute eye irritation study may be performed directly on 3 rabbits.
. When the acute dermal irritation study in the rabbit demonstrates that the test substance is severely irritant, one can presume that the test substance is likely to produce severe effects on the eye. In this case, the acute eye irritation study must be performed with caution.

Information derived from this test serves to indicate the possible existence of hazards to Man likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

The test substance is administered by ocular route as a single dose into one of the eyes of each experimental animal.

The untreated eye serves as a control. The degree of irritation is evaluated and numerically scored at specific intervals.

This study was conducted in compliance with:
. O.E.C.D. guideline No. 405, 24th February 1987,
. E.C. Directive No. 92/69/E.E.C., B_5, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société
Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4X
  - labelling: Pil 4X
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: at room temperature, protected from light and from humidity from 1.8.94. The test substance, deconditioned in flasks numbered from 1 to 7, was stored under nitrogen gas from 2.1.95 until use of each flask
. purity: 99.8%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Vehicle

Distilled water, batch No. 1811 (Biosédra, 92240 Malakoff, France).

2.1.3 Preparation

On the day of treatment, the test substance was diluted at a concentration of 5% (w/w) in the vehicle. The pH of the preparation was approximately 1.

2.2. TEST SYSTEM

2.2.1 Animals

Sex, species, strain: Male New Zealand White rabbits.
Reason for this choice: species commonly requested by the international regulations for this type of study.
Breeder: Elevage Cunicole de Val de Selle, 80160 Prouzel, France.
Number of animals and identification: 3 animals were used, as recommended by the international regulations and taking into account that a good correlation of results can be obtained with either 3 or 6 animals (1). The animals were identified individually with a metal tag in the ear.
Weight: on the day of treatment, the animals had a mean body weight of 2.6 ± 0.2 kg.
Acclimatization: at least 5 days before the beginning of the study.
Selection of the animals: the day before treatment, the eyes of each animal were examined in order to use only animals without any signs of ocular irritation. Animals showing signs of ocular irritation, ocular defects or pre-existing corneal injury were not used.

2.2.2 Environmental conditions

During the acclimatization period and during the main test, the environmental conditions in the animal room were as follows:
temperature: 18 ± 3°C
relative humidity: 30 to 70%
light/dark cycle: 12 h/12 h
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity and light/dark cycle) were checked regularly.
The animals were housed individually in polystyrene cages (35 x 55 x 32 cm or 48.2 x 58 x 36.5 cm). Each cage was equipped with a food container and a water bottle.

(1) Talsma, D. M.; Leach, C. L.; Hatoum, N. S.; Gibbons, R. D.; Roger, J. C.; Garvin, J. P.: Reducing the number of rabbits in the Draize eye irritancy test: A statistical analysis of 155 studies conducted over 6 years. Fundamental and Applied Toxicology. 10: 1, 146-153 (1988).

2.2.3 Food and water

All the animals had free access to 112 C pelleted diet (U.A.R., 91360 Villemoisson-sur-Orge, France).
Each batch of food was analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) and contained in bottles was provided *ad libitum.*
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet or water at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Preparation of the animals

The day before treatment, the animals' eyes were checked for abnormalities. Only animals without ocular defects were used for the study.

2.3.2 Administration of the test substance

As the cutaneous study performed at C.I.T. (CIT/Study No. 12908 TAL) revealed no irritant effects, the test substance was then administered by ocular route.

A single dose of 0.1 ml of a preparation containing the test substance diluted at a concentration of 5% (w/w) in the vehicle was instilled into the conjunctival sac of the left eye of 3 animals after gently pulling the lower lid away from the eyeball.

The lower and upper eyelids were held together for about one second to avoid any loss of test substance. The right eye, which remained untreated, served as a control.
The eyes were not rinsed after administration of the test substance.

2.3.3 Date of treatment

Animal number	Date of treatment (day 1)	End of the observation period
01	18.5.95	21.5.95
02	18.5.95	21.5.95
03	18.5.95	21.5.95

2.4. OCULAR EXAMINATIONS

The eyes were examined approximately 1, 24, 48 and 72 hours after administration of the test substance.

When there is no evidence of irritation after 72 hours, the study is ended.

2.5. DESCRIPTION AND EVALUATION OF OCULAR REACTIONS

Ocular reactions were evaluated for each animal according to the following numerical scale:

2.5.1 Conjunctival lesions and discharge

Chemosis (lids and/or nictitating membranes)

. no swelling 0
. any swelling above normal (includes nictitating membranes) 1
. obvious swelling with partial eversion of lids 2*
. swelling with lids about half-closed 3*
. swelling with lids more than half-closed 4*

Redness (refers to palpebral and bulbar conjunctivae, cornea and iris)

. blood vessels normal 0
. a number of blood vessels definitely hyperaemic (injected) 1
. diffuse, crimson colour, individual vessels not easily discernible 2*
. diffuse, beefy red 3*

Discharge

. absence of discharge 0
. slight discharge (does not include small amounts normally found in inner canthus) 1
. discharge with moistening of lids and hairs adjacent to lids 2
. discharge with moistening of lids and hairs on wide area around the eye 3

2.5.2 Iris lesions

. normal 0
. markedly deepened rugae, congestion, swelling, moderate circum-corneal hyperaemia, or injection, any of these or combination of any thereof, iris still reacting to light (sluggish reaction is positive) 1*
. no reaction to light, haemorrhage, gross destruction (any or all of these) 2*

2.5.3 Corneal lesions

Cornea (direct examination or, if necessary, with an Ultra Violet lamp)
To determine the presence or absence of corneal opacification and to evaluate the affected area, 1 or 2 drops of 0.5% sodium fluorescein solution can be instilled into the eye (however, this must be performed after a period of 24 hours).
If corneal opacification is difficult to determine, the eye can be examined under a U.V. lamp (a clear fluorescence is visible in the areas of opacification).

Opacity (degree of intensity: area most dense taken for reading)

. no ulceration or opacity 0
. scattered or diffuse areas of opacity (other than slight dulling or normal lustre), details of iris clearly visible 1*
. easily discernible translucent area, details of iris slightly obscured 2*
. nacrous areas, no details of iris visible, size of pupil barely discernible 3*
. opaque cornea, iris not discernible through the opacity 4*

* indicates positive effect

Area of opacity
. one quarter (or less) but not zero 1
. greater than one quarter but less than a half 2
. greater than one half but less than three quarters 3
. greater than three quarters up to whole area 4

Any other lesions observed were noted.

2.6. INTERPRETATION OF RESULTS AND CLASSIFICATION OF SUBSTANCES

The results obtained were evaluated in conjunction with the nature and the reversibility or irreversibility of the scores observed, whilst taking into account all the reactions of the treated animals. Classification of the test substance is based on the criteria laid down in Council Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C. on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances.

2.6.1 Interpretation of the results

Criteria of irritation
A substance or a preparation is considered as irritant if it induce ocular lesions appearing within 72 hours and lasting at least 24 hours after administration of the test substance.
In both cases, all the scores obtained for each reading (24, 48 and 72 hours) of a reaction must be used to calculate the respective mean values.

2.6.2 Classification of the substances

Irritant substances and preparations
- Xi symbol, indication of danger "irritant",
- sentences indicating particular hazards:

R 36: "Irritant for eyes"
An ocular lesion must be considered as marked if the mean score reaches one of the following values:
. opacity of the cornea ≥ 2, but < 3,
. lesion of the iris ≥ 1, but ≤ 1.5,
. redness of the conjunctivae ≥ 2.5,
. oedema of the conjunctivae (chemosis) ≥ 2.

Or else, if the test is performed on 3 animals, if at least 2 of them show lesions equal to 1 of the above-mentioned values, except the iris for which the value will have to be equal or higher than 1, but lower than 2 and the redness of the conjunctivae for which the value will have to be equal to or higher than 2.5.

R 41: "Risk of serious damage to eyes"
Ocular lesions must be considered as severe if the mean score reaches one of the following values:
. opacity of the cornea ≥ 3,
. iridian lesion > 1.5.

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Or else, when the test is performed on 3 animals, if at least 2 of them show lesions equal to one of the following values:

. opacity of the cornea ≥ 3,
. iridian lesion = 2.

Ocular lesions were considered as severe when they persisted at the end of the observation period.

2.7. ARCHIVES

The study archives:

. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,

are stored on the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. **RESULTS** (table 1)

No ocular reactions were noted during the study.

4. CONCLUSION

Under our experimental conditions, the test substance, diluted at a concentration of 5% (w/w) in water, was considered non-irritant when administered by ocular route in rabbits.

Table 1: Individual ocular examinations and mean score of the values recorded after 24, 48 and 72 hours for each animal

Rabbit number	Region of eye	Description of ocular reactions	Scores 1h D1	24h D2	48h D3	72h D4	Mean irritation score (1)	Interpretation (+) (-)
01	Conjunctivae	Chemosis	0	0	0	0	0,0	(-)
		Redness	0	0	0	0	0,0	(-)
		Discharge	0	0	0	0	0,0	
	Iris		0	0	0	0	0,0	(-)
	Corneal opacity	Intensity	0	0	0	0	0,0	(-)
		Area	0	0	0	0	0,0	
	Other		*	*	*	*		
	Fluorescein		/	U	/	/		
02	Conjunctivae	Chemosis	0	0	0	0	0,0	(-)
		Redness	0	0	0	0	0,0	(-)
		Discharge	0	0	0	0	0,0	
	Iris		0	0	0	0	0,0	(-)
	Corneal opacity	Intensity	0	0	0	0	0,0	(-)
		Area	0	0	0	0	0,0	
	Other		*	*	*	*		
	Fluorescein		/	U	/	/		
03	Conjunctivae	Chemosis	0	0	0	0	0,0	(-)
		Redness	0	0	0	0	0,0	(-)
		Discharge	0	0	0	0	0,0	
	Iris		0	0	0	0	0,0	(-)
	Corneal opacity	Intensity	0	0	0	0	0,0	(-)
		Area	0	0	0	0	0,0	
	Other		*	*	*	*		
	Fluorescein		/	U	/	/		

(1) mean of scores on days 2, 3 and 4
h = hour
D = day
(+) = irritant according to E.E.C. criteria
(-) = non-irritant according to E.E.C. criteria
* = None
U = Fluorescein batch No. 3461
/ = Fluorescein not used

APPENDICES

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1. Analytical certificate

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le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par $AgNO_3$	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

Lot utilisable jusqu'au 25/11/95

10/11/94

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2. Diet formula

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Ref: 112

COMPLETE DIET

RABBIT MAINTENANCE DIET

Appearance: 4.5 mm diameter granules

Conditioning: bags of 25 kgs

Daily portion: in accordance with race and body weight, Rabbits 100-150 g, water *ad libitum.*

FORMULA %

Cereals	43.8
Grain biproducts and legumes	49
Vegetable proteins (soya bean meal, yeast)	4.2
Vitamin and mineral mixture	3

AVERAGE ANALYSIS %

Calorific value (KCal/kg)	2200
Moisture	10
Proteins	13
Lipids	2.7
Carbohydrates (N.F.E.)	49.3
Fibre	17
Minerals (ash)	8

AMINO ACID VALUES (calculated in mg/kg)

Arginine	6800
Cystine	2100
Lysine	4600
Methionine	1600
Tryptophan	1400
Glycine	5200

FATTY ACID VALUES (calculated in mg/kg)

Palmitic acid	6400
Palmitoleic acid	0
Stearic acid	600
Oleic acid	6400
Linoleic acid	12100
Linolenic acid	2400

MINERALS (calculated in mg/kg)

	Nat. val.	CMV val.	Total
P	3500	3500	7000
Ca	4500	4500	9000
K	11600	0	11600
Na	400	1600	2000
Mg	2100	100	2200
Mn	40	40	80
Fe	160	140	300
Cu	12	15	27
Zn	30	45	75
Co	0.1	1.5	1.6
I	0	0	0
Cl	500	3000	3500

VITAMINS (calculated per kg)

	Nat. val.	CMV val.	Total
Vitamin A	2850 IU	6500 IU	9350 IU
Vitamin D3	30 IU	1000 IU	1030 IU
Vitamin B1	4.3 mg	0 mg	4.3 mg
Vitamin B2	3.8 mg	0 mg	3.8 mg
Vitamin B3	16 mg	0 mg	16 mg
Vitamin B6	1 mg	1 mg	2 mg
Vitamin B12	0 mg	0 mg	0 mg
Vitamin E	16 mg	10 mg	26 mg
Vitamin K3	6 mg	1 mg	7 mg
Vitamin PP	55 mg	5 mg	60 mg
Folic acid	0 mg	0 mg	0 mg
Biotin	0 mg	0 mg	0 mg
Choline	850 mg	200 mg	1050 mg
Meso-Inositol	0 mg	0 mg	0 mg

Available under quality "Control Ref.: 112 C"

U.A.R., 7 rue Galliéni, 91360 Villemoisson - Tel: 69.04.03.57 - Fax : 69.04.81.97
(Ref. Doc. UAR: 1992)

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HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

SPAUL 90010 - PIL1:

JOURNAL OFFICIEL EYE IRRITATION

TEST IN THE RABBIT

PROJECT NUMBER 109/408

AUTHOR: R.L. Guest

ISSUED BY:

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PROJECT NUMBER: 109/408

SAFEPHARM LABORATORIES LIMITED

QUALITY ASSURANCE UNIT REPORT

The routine inspection of short term toxicity studies at Safepharm Laboratories is carried out as a continuous process designed to ensure that where possible all critical phases of a particular study type are inspected at least once per month. Dates of inspection for this study type are given below:

STUDY TYPE	DATE(S) OF INSPECTION
Eye Irritation:	02/08/90, 06/08/90, 07/08/90, 16/08/90, 22/08/90, 23/08/90
General Facilities Audit conducted:	31/08/90

This report has been audited by Safepharm Laboratories Quality Assurance Unit and is an accurate account of the procedures followed and accurately records the original raw laboratory data generated in this study.

Date of report audit: 14/09/90

J.R. Pateman M.I. Biol., C. Biol.
QUALITY ASSURANCE MANAGER

..........J R Pateman..........

DATE: ....18/09/90..........

GOOD LABORATORY PRACTICE STATEMENT

I, the undersigned, hereby declare that this study was performed in compliance with the following principles of Good Laboratory Practice:

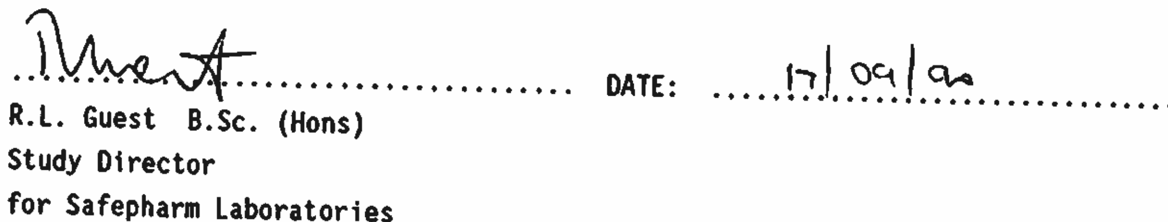
Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.

*United States Environmental Protection Agency, Title 40, Code of Federal Regulation, Part 160 and Part 792.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health 1989.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 NohSan Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

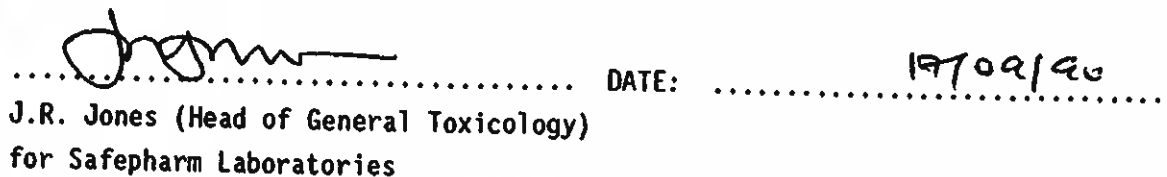
Japan Ministry of Health and Welfare, Notification No. Yakuhatsu 313, Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatsu 870, Pharmaceutical Affairs Bureau, 5 October 1988.



R.L. Guest B.Sc. (Hons)
Study Director
for Safepharm Laboratories

*Under the provisions of a Memorandum of Understanding between the U.K. Department of Health and the United States Environmental Protection Agency, effective for five years from March 1988.

Approved for Issue by:



J.R. Jones (Head of General Toxicology)
for Safepharm Laboratories

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SUMMARY OF RESULTS

STUDY SPONSOR	:	
PROJECT NUMBER	:	109/408
REFERENCE	:	SPAUL 90010
TEST MATERIAL	:	· PIL1

1. A study was performed to assess the irritancy potential of the test material to the eye of the New Zealand White rabbit. The method used followed that described in the Journal Officiel de la Republique Francaise 24 October 1984 "Official Method for Evaluation of Eye Irritation".

2. A single application of the test material to the non-irrigated eye of one rabbit produced opalescent corneal opacity, iridial inflammation and severe conjunctival irritation. Other adverse ocular reactions noted were sloughing of the cornea, haemorrhage and pale appearance of the nictitating membrane.

   In accordance with current U.K. Government Home Office guidelines relating to eye irritation testing, and for humane reasons, the animal was killed after the day one observation. No other animals were treated.

3. The test material produced a maximum total score of 63 and was considered to be at least very irritant to the rabbit eye (based on one rabbit only).

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IMEXINE OAX - PIL1:

JOURNAL OFFICIEL EYE IRRITATION

TEST IN THE RABBIT

1. INTRODUCTION

The study was performed according to Safepharm Standard Protocol Number JORF 2A and was carried out in order to assess the irritancy potential of a test material following a single application to the rabbit eye. The study was designed to comply with the method described in the Journal Officiel de la Republique Francaise 24 October 1984 "Official Method for Evaluation of Eye Irritation".

The test system was chosen because the rabbit has been shown to be a suitable model for this type of study and is recommended in the test method. The results of the study are believed to be of value in predicting the likely eye irritancy potential of the test material to man.

The study was conducted in accordance with the internationally accepted general principles of Good Laboratory Practice and Safepharm Standard Operating Procedures.

The study was performed between 6 August 1990 and 7 August 1990.

2. TEST MATERIAL

2.1 Description, Identification and Storage Conditions

The test material was supplied by the study sponsor as follows:

Description	:	blue/grey-coloured powder
Container	:	glass screw-top bottle
Sponsor's identification	:	PIL1
Date of arrival	:	21 March 1990
Storage conditions	:	room temperature

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2. TEST MATERIAL (contd)

2.2 Method of Preparation

For the purpose of this study the test material was used as supplied.

The composition and stability of the test material were not determined.

3. TEST SYSTEM

3.1 Specification

One New Zealand White rabbit was supplied by Rosemead Rabbits, Waltham Abbey, Essex, U.K. At the start of the study the animal weighed 2.33 kg and was approximately twelve to sixteen weeks old. After a minimum acclimatisation period of five days the animal was given a number unique within the study which was written with a black indelible marker-pen on the inner surface of the ear and on a cage label.

3.2 Husbandry

The animal was individually housed in a suspended metal cage. Free access to mains drinking water and food (Rabbit Diet, Preston Farmers Limited, New Leake, Boston, Lincolnshire, U.K.) was allowed throughout the study.

The animal room was maintained at a temperature of 20 - 23°C and relative humidity of 60 - 63%. On one occasion the temperature was outside the upper limit specified in the protocol (21°C). This did not affect the purpose or integrity of the study. The rate of air exchange was approximately 15 changes per hour and the lighting was controlled by a time switch to give 12 hours light and 12 hours darkness.

4. PROCEDURE

Immediately before the start of the test, both eyes of the provisionally selected test rabbit were examined for evidence of ocular irritation or defect with the aid of a light source from a standard ophthalmoscope.

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4. PROCEDURE (contd)

On the day of the test the animal was held firmly but gently until quiet. A volume of 0.1 ml of the test material, weighing approximately 54 mg (as measured by gently compacting the required volume into an adapted syringe), was placed into the right eye of the rabbit by gently pulling the lower lid away from the eyeball to form a cup into which the test material was dropped. The upper and lower eyelids were held together for about one second immediately after application, to prevent loss of the test material, and then released. The left eye remained untreated and was used for control purposes.

Assessment of ocular damage/irritation was made approximately 1 and 24 hours following treatment, according to the numerical evaluation given in Appendix I, (ie. Draize J.H. 1959, Association of Food and Drug Officials of the United States, Austin, Texas, "The Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics"). Any other adverse ocular effects were also noted. Examination of the eye was facilitated by use of the light source from a standard ophthalmoscope.

In accordance with current U.K. Government Home Office guidelines relating to eye irritation testing, and for humane reasons, the animal was killed after the day one observation. No further animals were treated.

5. INTERPRETATION OF RESULTS

The numerical values corresponding to the animal, tissue and observation time were recorded. The data relating to the conjunctivae were designated by the letters A (redness), B (chemosis) and C (discharge), those relating to the iris designated by the letter D and those relating to the cornea by the letters E (degree of opacity) and F (area of opacity). For each tissue the total score was calculated as follows:

Total score for conjunctivae = $(A + B + C) \times 2$

Total score for iris = $D \times 5$

Total score for cornea = $(E \times F) \times 5$

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5. INTERPRETATION OF RESULTS (contd)

The numerical data obtained was used to assess the ocular irritation potential of the test material. This was achieved by adding together the total scores for the cornea, iris and conjunctivae for each of the observations. Each value obtained was designated the Individual Eye Irritation Rating (I.E.I.) and the highest of these values was used to give an approximation by placing the test material into one of six categories as Appendix II.

6. ARCHIVES

On completion of the study all raw laboratory data and a copy of the final report were transferred to Safepharm Laboratories Central Archives, London Road, Shardlow, Derbyshire, U.K., where they will be retained for a period of ten years.

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7. RESULTS

Individual and total scores for ocular irritation are given in Table 1.

A dulling of the normal lustre of the corneal surface was noted one hour after treatment. Opalescent corneal opacity was noted at the one day observation.

Iridial inflammation was noted one hour and one day after treatment.

Moderate conjunctival irritation was noted one hour after treatment developing into severe conjunctival irritation at the one day observation.

Other adverse ocular effects noted in the treated eye were sloughing of the cornea and haemorrhage and pale appearance of the nictitating membrane,

In accordance with current U.K. Government Home Office guidelines relating to eye irritation testing, and for humane reasons, the animal was killed after the day one observation. No other animals were treated.

The test material produced a maximum total score of 63 and was regarded as at least VERY IRRITANT to the rabbit eye (based on one rabbit only).

8. CONCLUSION

The test material, - PIL1, was regarded as at least very irritant to the rabbit eye (based on one rabbit only).

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PROJECT NUMBER: 109/408

- PILI : ACUTE EYE IRRITATION TEST IN THE RABBIT

TABLE 1 INDIVIDUAL SCORES AND TOTAL SCORES FOR OCULAR IRRITATION

Rabbit Number and Sex (Bodyweight Kg)	241 Male (2.33)	
Time After Treatment	1 hour	1 day
CORNEA		
E = Degree of Opacity	d	2 Sl
F = Area of Opacity	4	4
Score (E x F) x 5	0	40
IRIS		
D	1	1
Score (D x 5)	5	5
CONJUNCTIVAE		
A = Redness	2	2 HPa
B = Chemosis	2	4
C = Discharge	3	3
Score (A + B + C) x 2	14	18
Total Score	19	63

d = dulling of the normal lustre of the corneal surface
H = haemorrhage of the nictitating membrane and conjunctival membrane
Pa = pale appearance of nictitating membrane
Sl = sloughing of the cornea

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A P P E N D I C E S

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APPENDIX I

DRAIZE SCALE FOR SCORING OCULAR IRRITATION

1. CONJUNCTIVAE

(A) Redness (refers to palpebral and bulbar conjunctivae excluding cornea and iris)

Vessels normal	0
Vessels definitely injected above normal	1
More diffuse, deeper crimson red, individual vessels not easily discernible	2
Diffuse beefy red	3

(B) Chemosis

No swelling	0
Any swelling above normal (includes nictitating membrane)	1
Obvious swelling with partial eversion of lids	2
Swelling with lids about half closed	3
Swelling with lids half closed to completely closed	4

(C) Discharge

No discharge	0
Any amount different from normal (does not include small amounts observed in inner canthus of normal animals)	1
Discharge with moistening of the lids and hairs just adjacent to lids	2
Discharge with moistening of the lids and hairs a considerable area around the eye	3

THE TOTAL SCORE = (A + B + C) x 2 MAXIMUM TOTAL = 20

2. IRIS

(D) Values

Normal	0
Folds above normal, congestion, swelling, circumcorneal injection (any or all of these or combination of any thereof) iris still reacting to light (sluggish reaction is positive)	1
No reaction to light, haemorrhage, gross destruction (any or all of these)	2

THE TOTAL SCORE = D x 5 MAXIMUM TOTAL = 10

3. CORNEA

(E) Degree of Opacity (most dense area used)

No opacity	0
Scattered or diffuse areas, details of iris clearly visible	1
Easily discernible translucent areas, details of iris slightly obscured	2
Opalescent areas, no details of iris visible, size of pupil barely discernible	3
Opaque, iris invisible	4

(F) Area of Cornea involved

One quarter (or less) but not zero	1
Greater than one quarter but less than half	2
Greater than half but less than three quarters	3
Greater than three quarters, up to whole area	4

THE TOTAL SCORE = (E x F) x 5 MAXIMUM TOTAL = 80

MAXIMUM TOTAL SCORE POSSIBLE = 110

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APPENDIX II

OFFICIAL METHOD OF EVALUATION OF EYE IRRITATION

Maximum Mean Eye Irritation Rating (M.E.I.)	Mean Eye Irritation Rating (E.I.)	Individual Eye Irritation Rating (I.E.I.)	Classification
< 5	At 1 day = 0		Non-irritant
> 5 and < 15	At 2 days < 2		Very slightly irritant
> 15 and < 25	At 4 days < 2		Slightly irritant
> 25 and < 50	At 7 days < 20	< 30 in 6 rabbits and < 15 in at least 4 rabbits at 7 days	Irritant
> 50 and < 80	At 7 days < 40	< 60 in 6 rabbits and < 30 in at least 4 rabbits at 7 days	Very irritant
> 80			Extremely irritant

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STUDY TITLE

ACUTE ORAL TOXICITY
IN RATS

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

14th November 1994

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12041 TAR

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SUMMARY

At the request of Société , the acute toxicity of the test substance, (batch No. Pil 4x), by oral route was evaluated in rats according to O.E.C.D. (No. 401, 24th February 1987) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice.

Methods

In the limit test, the test substance was administered by oral route to 1 group of 10 fasted Sprague-Dawley rats (5 males and 5 females). The test substance was prepared in water for injections and then administered at a dose of 2000 mg/kg using a dose volume of 10 ml/kg. As the mortality was 50%, thereafter the test substance was administered in the same conditions to 3 groups of 5 females in each at dose levels of 1100, 1600 and 2600 mg/kg.

The animals were checked for clinical signs, mortality and body weight gain for a period of 14 days following the single administration of the test substance.

A necropsy was performed on each animal found dead during the study or killed at the end of the study.

The interpretation of results was carried out according to the classification criteria laid down in Council Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C.

The LD_{50} was calculated according to Probit's method.

Results

Main clinical signs were hypoactivity, sedation, piloerection and dyspnoea in both sexes. In males, lateral decubitus was also observed. First signs were noted 30 minutes after treatment. Recovery was complete on day 7 for the females and day 5 for the males.

No death was noted in the 1100 and 1600 mg/kg dose groups for females. At 2000 mg/kg, 40% (2/5 rats) and 60% (3/5) mortality was observed for females and males respectively. Eighty percent mortality was noted in the 2600 mg/kg female group. Except for 2 animals which died on day 3, all mortality occured within 30 minutes after treatment.

Body weight gain of the surviving animals was normal.

A macroscopic examination revealed no abnormalities in the animals found dead during the study or killed at the end of the study.

Conclusion

The acute oral LD_{50} value of the test substance (batch No. Pil 4x) was:
. females: 2186 (1797-2965) mg/kg with 95% confidence interval limits.

The toxicity of the test substance was comparable in males.

Labelling

Council Directive 93/21/E.E.C.

Labelling not indicated for the test substance.

1. INTRODUCTION

The objective of this study was to evaluate the toxicity of the test substance, following a single oral administration in rats.

In the assessment of the toxic characteristics of a test substance, determination of acute oral toxicity is usually an initial step. It provides information on health hazards to man likely to arise from a short-term exposure by the oral route.

The test substance is administered by gavage at a single dose to several groups of rats (5 males and/or 5 females each).

The LD_{50} (Median Lethal Dose) is the statistically derived single dose of the test substance that can be expected to cause death in 50% of the treated animals.

The study was conducted in compliance with:
. O.E.C.D. guideline No. 401, 24th February 1987.
. E.C. Directive No. 92/69/E.E.C., B_1, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4x
  - labelling: Pil 4x
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: . at room temperature, protected from light,
  . protected from humidity from 1.8.94
. purity: see analytical certificate

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Vehicle

The vehicle used was water for injections, batch Nos. 9784 and 9799 (Biosédra, 92240 Malakoff, France).

2.1.3 Preparation

On the day of the treatment, the test substance was prepared in the vehicle

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2.2. TEST SYSTEM

2.2.1 Animals

Species, strain: rat, Sprague-Dawley ICO: OFA-SD (IOPS Caw).
Reason for this choice: rodent species commonly requested by the international regulations for this type of study.
Breeder: Iffa Crédo, 69210 L'Arbresle, France.
Number and sex: . a variable number of males and females for the preliminary test,
. 1 group of 10 animals (5 males and 5 females) and 3 groups of 5 females each for the main test.
Age/weight: on the day of treatment, the animals were approximately 6 weeks old, and had a mean body weight of 175 ± 3 g for the males and 148 ± 4 g for the females.
Acclimatization: at least 5 days before the beginning of the study.
Allocation/identification of the animals: the animals of each sex were randomly assigned to the treatment groups according to body weight within a range not exceeding ± 20% of the mean body weight. Animals were identified individually by earmarks or earnotches.

2.2.2 Environmental conditions

During the acclimatization period and during the main test, the conditions in the animal room were as follows:
temperature: 21 ± 2°C
relative humidity: 30 to 70%
light/dark cycle: 12 h/12 h
ventilation: about 12 cycles/hour of filtered, non-recycled air.
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity, light/dark cycle and ventilation) were checked monthly.
The animals were housed in polycarbonate cages (48 x 27 x 20 cm) covered with a stainless steel lid. Each cage contained 4 to 7 animals of the same sex during the acclimatization period and 5 rats of the same sex during the treatment period. Each cage contained graded, dust-free sawdust (SICSA, 94142 Alfortville, France).
Bacteriological analysis of the sawdust and detection of possible contaminants (pesticides, heavy metals) are performed periodically.

2.2.3 Food and water

All the animals had free access to AO4 C pelleted diet (U.A.R., 91360 Villemoisson-sur-Orge, France), except as noted in "2.3.1 Treatment".
Each batch of food was analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was contained in bottles.
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Fasting of the animals

The animals were fasted for an overnight period of approximately 18 hours before dosing, but had free access to water. Food was given approximately 4 hours after administration of the test substance.

2.3.2 Doses and treatment

As the test substance was anticipated to be non-toxic at 2000 mg/kg, a limit test was performed by administering 2000 mg/kg of the test substance to 1 group of 10 animals (5 males and 5 females).
The test substance was prepared in the vehicle and administered under a volume of 10 ml/kg.
As mortality of the animals was 50% in the limit test, a preliminary test was performed on a reduced number of animals in order to define the range of doses to be administered for evaluation of the LD_{50}. The results of this preliminary test, not included in the report, enabled us to constitute 3 groups of 5 females each.

Dose (mg/kg)	Volume (ml/kg)	Date of treatment	End of observation period
1100	10	6.9.94	20.9.94
1600	10	6.9.94	20.9.94
2000	10	11.8.94	25.8.94
2600	10	6.9.94	20.9.94

The administration was performed in a single dose by oral route using a stainless steel round-tipped probe (diameter: 18 G.2", Perfektum: Poffer & Sons Inc., New Hyde Park, New York 11040, U.S.A.) fitted to a 2 ml glass syringe (0.02 ml graduations, Record: Carrieri, 75005 Paris, France).

The volume administered to each animal was adjusted according to body weight determined on the day of treatment.

2.4. CLINICAL EXAMINATIONS

2.4.1 Clinical signs

The animals were observed frequently during the hours following administration of the test substance, for detection of possible treatment-related clinical signs. Observation of the animals was made at least once a day for a period of 14 days, to determine whether any of the clinical signs were reversible or not. Clinical signs were recorded for each animal individually.

2.4.2 Mortality

The animals were checked frequently during the hours following administration of the test substance for mortality or signs of morbidity, then at least twice a day thereafter. The time of any deaths was recorded individually, in terms of the number of hours or days after dosing.

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2.4.3 Body weight

The animals were weighed individually just before administration of the test substance then on days 8 and 15.

Individual weights of animals found dead during the study were measured at necropsy if no signs of "cannibalism" appeared and when survival exceeds 24 hours.

The body weight gain of the treated animals was compared to a reference curve of C.I.T. control animals with the same initial weight.

2.5. PATHOLOGY

2.5.1 Necropsy

The animals found dead during the study were subjected to a macroscopic examination as soon as possible.
On day 15, the surviving animals were killed by CO_2 inhalation in excess and a macroscopic examination was performed.

2.5.2 Macroscopic examination

After opening the thoracic and abdominal cavities, a macroscopic examination of the main organs (digestive tract, heart, kidneys, liver, lungs, pancreas, spleen and any other organs with obvious abnormalities) was performed.
In case of macroscopic lesions, organ samples were taken and preserved in 10% buffered formalin.

2.5.3 Microscopic examination

No microscopic examination was performed.

2.6. DETERMINATION OF THE LD_{50}

The LD_{50} value, expressed in milligrams of test substance per kilogram of animal (mg/kg), was calculated according to Probit-Analysis, i.e. Finney's method, published by E. Weber and combined with Bliss's method.
The 70 to 95% confidence interval limits were calculated statistically according to Fieller's method.

References

Weber, E.: Grunden der biologischen Statistik, Gustav Fischer Verlag, Stuttgart, 1972.

Bliss, C.I.: The determination of dosage-mortality curves from small number. Quart. J. Pharm. 11, 192-216 (1938).

Fieller: A fundamental formula in the statistics of biological assay and some applications. Quarterly Journal of Pharmacy and Pharmacology, 117-123 (1944).

2.7. DATA EVALUATION

Evaluation of the toxicity of the test substance following a single oral administration in rats should include the relationship, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities including behavioural and clinical abnormalities, macroscopic lesions, body weight changes, mortality and any other toxic effects.
Classification of the test substance is based on the following criteria:

Commission Directive 93/21/E.E.C.

LD_{50} oral route (mg/kg)		Labelling sentence	Indication of danger	Symbol
≤ 25	R28	Very toxic if swallowed	Very toxic	T^+
25 < and ≤ 200	R25	Toxic if swallowed	Toxic	T
200 < and ≤ 2000	R22	Harmful if swallowed	Harmful	X_n
> 2000		None	None	None

2.8. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,

are stored on the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. RESULTS

3.1. CLINICAL EXAMINATIONS

3.1.1 Clinical signs (tables 1 and 2, appendix 3)

In females, sedation, hypoactivity, dyspnoea and piloerection were observed. The first signs appeared 30 minutes after treatment (hypoactivity or sedation). In the 1100, 1600 and 2600 mg/kg dose groups, the surviving females appeared normal 24 hours after treatment. In the 2000 mg/kg dose group, signs persisted for 2 days (dyspnoea), 3 days (sedation and piloerection) or 6 days (hypoactivity).

All signs had subsided on day 7.

The same clinical signs were observed in males treated at 2000 mg/kg; in addition, lateral decubitus was noted in 1 animal 24 hours after treatment. Hypoactivity was observed 30 minutes after treatment, all the surviving males appeared normal on day 5.

3.1.2 Mortality (tables 1 and 2, appendix 3)

In females, 80% mortality was noted in the 2600 mg/kg dose group, all deaths were observed 30 minutes after treatment. Forty percent mortality was noted in the 2000 mg/kg dose group, 1 female died 30 minutes after treatment and another female died on day 3. No mortality was observed in the other 2 dose groups.

In males, 60% mortality was noted in the 2000 mg/kg dose group , 2/5 animals died 30 minutes after treatment and another male died on day 3.

3.1.3 Body weight (treated animals: figures 1 and 2, table 3, appendix 4)
(control animals: appendix 5)

The body weight gain of the surviving animals was comparable to historical control data for animals of the same strain, age and initial body weight.

3.2. PATHOLOGY (appendix 6)

Macroscopic examination of the main organs of the animals found dead during the study or killed at the end of the study revealed no apparent abnormalities.

3.3. DETERMINATION OF THE LD_{50} (appendix 7)

The acute oral LD_{50} value of the test substance was:
. females: 2186 (1797-2965) mg/kg with 95% confidence interval limits.

The toxicity of the test substance was comparable in males.

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CLASSIFICATION OF THE TEST SUBSTANCE

According to Council Directive 93/21/E.E.C. (27th April 1993) adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C. on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances,

and concerning the toxicity by oral route, the test substance should not be classified.

le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par $AgNO_3$	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

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TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY TITLE

ACUTE DERMAL IRRITATION
IN RABBITS
with the test substance diluted at 5%

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

26th July 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12908 TAL (95/1/041)

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SUMMARY

At the request of Société , potential of the test substance, (batch No. Pil 4X) diluted at a concentration of 5%, to induce dermal irritation was evaluated in rabbits according to O.E.C.D. (No. 404, 17th July 1992) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

A single dose of 0.5 ml of a preparation containing the test substance diluted at a concentration of 5% (w/w) in distilled water was prepared on a dry gauze pad, and then applied to a 6 cm^2 clipped area of the skin of 3 male New Zealand White rabbits.

The test substance was held in contact with the skin by means of a semi-occlusive dressing for 4 hours then any residual test substance was removed by means of a gauze pad moistened with water. Cutaneous reactions were observed approximately 1, 24, 48 and 72 hours after removal of the dressing and then daily until day 9 at the latest in order to observe their reversibility.

The mean score of the values for erythema and oedema recorded for each animal after 24, 48 and 72 hours was calculated.

The interpretation of results was carried out according to the classification criteria laid down in Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C.

Results

No skin reactions were noted in one rabbit.
In the two other animals, very slight or well-defined erythema was observed for 24 or 72 hours after treatment. No oedema was observed. Dryness of the skin was noted from day 5 to day 8 in one animal.

Mean scores over 24, 48 and 72 hours for each animal were 0.0, 1.7 and 0.3 out of 4 for erythema and 0.0 for oedema.

Conclusion

Under our experimental conditions, the test substance, (batch No. Pil 4X) diluted at a concentration of 5% (w/w) in water, was considered non-irritant when administered by cutaneous route in rabbits.

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1. INTRODUCTION

The objective of this study was to evaluate the potential of the test substance, diluted at a concentration of 5% (w/w), to induce dermal irritation following a single administration in rabbits.

In the assessment of the toxic characteristics of a test substance, determination of the irritant and/or corrosive effects on the skin of mammals is an important initial step.

. When the test substance is not strongly acidic or alkaline, one may presume that the test substance is unlikely to produce severe irritant effects on the skin. In this case, the acute skin irritation study may be performed directly on 3 rabbits.

. When the test substance is acidic or alkaline, one can presume that the test substance is likely to produce severe effects on the skin. In this case, the acute skin irritation study must be performed with caution.

Information derived from this test serves to indicate the possible existence of hazards to Man likely to arise from exposure of the skin to the test substance.

The test substance is applied as a single dose to the skin of each experimental animal.

An area of untreated skin on each animal serves as a control. The degree of irritation is evaluated and scored numerically at specific intervals.

This study was conducted in compliance with:

. O.E.C.D. guideline No. 404, 17th July 1992,

. E.C. Directive No. 92/69/E.E.C., B_4, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société
Documentation supplied by the Sponsor identified the test substance as follows:

. denomination:
  - protocol:
  - labelling:

. batch number:
  - protocol: Pil 4X
  - labelling: Pil 4X

. description: beige powder

. quantity and container: 4 kg in a plastic pot

. date of receipt: 26.7.94

. storage conditions: at room temperature, protected from light and from humidity from 1.8.94. The test substance, deconditioned in flasks numbered from 1 to 7, was stored under nitrogen gas from 2.1.95 until use of each flask

. purity: 99.8%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

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2.1.2 Vehicle

Distilled water, batch No. 1811 (Biosédra, 92240 Malakoff, France).

2.1.3 Preparation

On the day of treatment, the test substance was diluted at a concentration of 5% (w/w) in the vehicle. The pH of the preparation was approximately 1.

2.2. TEST SYSTEM

2.2.1 Animals

Sex, species, strain: Male New Zealand White rabbits.
Reason for this choice: species commonly requested by the international regulations for this type of study.
Breeder: Elevage Cunicole de Val de Selle, 80160 Prouzel, France.
Number of animals and identification: 3 animals were used, as recommended by the international regulations and taking into account that a good correlation of results can be obtained with either 3 or 6 animals (1). The animals were identified individually with a metal tag in the ear.
Weight: on the day of treatment, the animals had a mean body weight of 2.3 ± 0.1 kg.
Acclimatization: at least 5 days before the beginning of the study.
Selection of the animals: the day before treatment, the skin of each animal was examined in order to use only animals without any signs of cutaneous irritation. Animals showing signs of cutaneous irritation, cutaneous defects or pre-existing dermal injury were not used.

2.2.2 Environmental conditions

During the acclimatization period and during the main test, the environmental conditions in the animal room were set as follows:
temperature: 18 ± 3°C
relative humidity: 30 to 70%
light/dark cycle: 12 h/12 h
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity and light/dark cycle) were checked regularly.
The animals were housed individually in polystyrene cages (35 x 55 x 32 cm or 48.2 x 58 x 36.5 cm). Each cage was equipped with a food container and a water bottle.

2.2.3 Food and water

All the animals had free access to 112 C pelleted diet (U.A.R., 91360 Villemoisson-sur-Orge, France).
Each batch of food was analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

(1) Hatoum, N.S.; Leach, C.L.; Talsma, D.M.; Gibbons, R.D.; Garvin, P.J.: A statistical basis for using fewer Rabbits in dermal irritation testing. Journal of the American College of Toxicology. 9: 49-60 (1990).

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) and contained in bottles was provided *ad libitum.*
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet or water at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Preparation of the animals

The day before treatment, the flanks of each animal were clipped using electric clippers. Only animals showing no obvious signs of irritancy were used for the study.

2.3.2 Application of the test substance

As no irritant effects were anticipated, 3 animals were used.

A dose of 0.5 ml of a preparation containing the test substance diluted at a concentration of 5% (w/w) in the vehicle was applied to a 6 cm^2 dry hydrophilic gauze pad (Semes France, 54183 Heillecourt, France) and this was then applied to the right flank of the animals for 4 hours.

The left flank did not receive any test substance and served as a control. The test substance and the gauze pads were held in contact with the skin by means of an adhesive hypoallergenic aerated semi-occlusive dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and a restraining bandage (Laboratoires 3M Santé, 92245 Malakoff, France).

Subsequently, the dressings were removed and any residual test substance was wiped off with a gauze pad moistened with water.

The animals were then replaced into their individual cages.

2.3.3 Date of treatment

Animal number	Date of treatment (day 1)	End of the observation period
01	10.5.95	13.5.95
02	10.5.95	18.5.95
03	10.5.95	13.5.95

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2.4. CUTANEOUS EXAMINATIONS

The skin was examined approximately 1, 24, 48 and 72 hours after removal of the dressing.

When there is persistent cutaneous irritation after 72 hours, the observation period is extended to a maximum of 14 days (until day 15) in order to determine the progress of the lesions and their reversibility or irreversibility.

2.5. DESCRIPTION AND EVALUATION OF CUTANEOUS REACTIONS

Dermal irritation was evaluated for each animal according to the following scoring scale:

Erythema and eschar formation:

. no erythema 0
. very slight erythema (barely perceptible) 1
. well-defined erythema 2
. moderate to severe erythema 3
. severe erythema (beet redness) to slight eschar formation (injuries in depth) 4

Oedema formation

. no oedema 0
. very slight oedema (barely perceptible) 1
. slight oedema (edges of area well-defined by definite raising) 2
. moderate oedema (raised approximately 1 millimetre) 3
. severe oedema (raised more than 1 millimetre and extending beyond area of exposure) 4

Any other lesions were noted.

2.6. INTERPRETATION OF RESULTS AND CLASSIFICATION OF SUBSTANCES

The results obtained were evaluated taking into consideration the nature and the reversibility or irreversibility of the findings observed. Classification of the test substance is based on the criteria laid down Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C. on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances.

The substances and preparations are classified as corrosive or irritant, according to the following criteria:

2.6.1 Criteria of corrosion

A substance or a preparation is considered as corrosive if when applied to the healthy and intact skin it induces, in at least one animal during the dermal irritation tests, tissular destruction on the whole depth of the skin, or if the result can be predicted, for example: strong acid or alkaline reactions.

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Corrosive substances and preparations

- C symbol, indication of danger: "corrosive",
- sentences indicating particular hazards:

R 34: "Causes burns"
If, when applied to the healthy and intact animal skin, it induces tissular lesions on the whole depth of the skin after an exposure period not exceeding 4 hours, or if the results can be predicted.

R 35: "Causes severe burns"
If, when applied to the healthy and intact animal skin, it induces tissular lesions on the whole depth of the skin after an exposure period not exceeding 3 minutes, or if the result can be predicted.

2.6.2 Criteria of irritation

A substance or a preparation tested on 3 animals is considered as irritant when it induces skin inflammation lasting at least 24 hours after a 4-hour exposure period and if at least 2 of them show an erythema and eschar formation or an oedema formation with a scoring equal to or higher than 2.

All the scores obtained from each reading (24, 48 and 72 hours) of a reaction are used to calculate the respective mean values.

Irritant substances and preparations

- Xi symbol, indication of danger: "irritant",
- sentence indicating particular hazards:

R 38: "Irritating to skin"

2.7. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,

are stored on the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. RESULTS (table 1)

No skin reactions were noted in one rabbit.
In the two other animals, very slight or well-defined erythema (grade 1 or 2) was observed for 24 or 72 hours after treatment. No oedema was observed. Dryness of the skin was noted from day 5 to day 8 in one animal.

Mean scores over 24, 48 and 72 hours for each animal were 0.0, 1.7 and 0.3 for erythema and 0.0 for oedema.

4. CONCLUSION

Under our experimental conditions, the test substance, diluted at a concentration of 5% (w/w) in water, was considered non-irritant when administered by cutaneous route in rabbits.

le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par $AgNO_3$	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

Lot utilisable jusqu'au 29/11/95

10/11/94

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STUDY TITLE

ACUTE DERMAL IRRITATION IN RABBITS

TEST SUBSTANCE

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

13th January 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12043 TAL

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Table 3: Individual cutaneous examinations and mean score of the values recorded after 24, 48 and 72 hours for each animal (application 4 hours) 19 and 20

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SUMMARY

At the request of Société , potential of the test substance, (batch No. Pil 4x), to induce dermal irritation was evaluated in rabbits according to O.E.C.D. (No. 404, 17th July 1992) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

Following the recommendations of the Sponsor, the application of the test substance was performed in a first assay for a period of 3 minutes in 1 animal. As the test substance was non-corrosive, the test substance was applied in a second assay during 4 hours to the same animal. The results were then confirmed on 2 other animals.

A single dose of 500 mg of the test substance in its original form was prepared on a moistened gauze pad, and then applied to a 6 cm^2 clipped area of the skin of 3 male New Zealand White rabbits.

The test substance was held in contact with the skin for 3 minutes, 1 hour or 4 hours by means of a semi-occlusive dressing. Cutaneous reactions were observed approximately 1, 24, 48 and 72 hours after removal of the dressing and then daily until day 15 in order to observe their reversibility or irreversibility.

Any residual test substance was removed by means of a gauze pad moistened with water for injections.

The mean score of the values for erythema and oedema recorded for each animal after 24, 48 and 72 hours was calculated.

The interpretation of results was carried out according to the classification criteria laid down in Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C.

Results

After exposure of 3 minutes (1 animal)
Erythema was masked by a black colouration of the test site which was present up to day 10. Slight oedema was noted 1 hour after removal of the dressing only.

After exposure of 1 hour (1 animal)
Slight to severe erythema was observed from day 1 to day 11. Severe to slight oedema was noted from days 1 to 6.

After exposure of 4 hours (3 animals)
Erythema was masked by a black colouration of the test site which was present up to day 15. Slight to severe oedema was noted from day 1 to day 5 in 2 animals. In the third animal, slight oedema was observed 1 hour after removal of the dressing only.

Mean scores over 24, 48 and 72 hours for individual animals were 0.0, 2.7 and 3.3 for oedema.

Due to the skin colouration, scores for erythema could not be calculated.

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Conclusion

As the mean scores for oedema for 2 out of the 3 animals reached the criteria values for irritation, under our experimental conditions, the test substance, (batch No. Pil 4x), was considered as **irritant** when administered by cutaneous route in rabbits.

Labelling

Commission Directive 93/21/E.E.C.

Symbol : X_i

Indication of danger : Irritant

Sentence R38 : "Irritating to skin".

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1. INTRODUCTION

The objective of this study was to evaluate the potential of the test substance,
to induce dermal irritation following a single administration in rabbits.

In the assessment of the toxic characteristics of a test substance, determination of the irritant and/or corrosive effects on the skin of mammals is an important initial step.
. When the test substance is not strongly acidic or alkaline, one may presume that the test substance is unlikely to produce severe irritant effects on the skin. In this case, the acute skin irritation study may be performed directly on 3 rabbits.
. When the test substance is acidic or alkaline, one can presume that the test substance is likely to produce severe effects on the skin. In this case, the acute skin irritation study must be performed with caution.

Information derived from this test serves to indicate the possible existence of hazards to Man likely to arise from exposure of the skin to the test substance.

The test substance is applied as a single dose to the skin of each experimental animal.

An area of untreated skin on each animal serves as a control. The degree of irritation is evaluated and scored numerically at specific intervals.

This study was conducted in compliance with:
. O.E.C.D. guideline No. 404, 17th July 1992.
. E.C. Directive No. 92/69/E.E.C., B_4, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4x
  - labelling: Pil 4x
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: . at room temperature, protected from light,
  . protected from humidity from 1.8.94
. purity: see analytical certificate.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

The pH of the test substance at a concentration of 5% in water, as mentioned in the test substance data sheet, was 1.

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2.1.2 Other substances

Water for injections, batch Nos. 9784 and 9186 (Biosédra, 92240 Malakoff, France) was used in order to disperse the test substance and ensure a good contact with the skin and remove residual test substance after removal of the dressing.

2.1.3 Preparation

The test substance was applied in its original form.

2.2. TEST SYSTEM

2.2.1 Animals

Sex, species, strain: Male New Zealand White rabbits.
Reason for this choice: species commonly requested by the international regulations for this type of study.
Breeder: Elevage Cunicole de Val de Selle, 80160 Prouzel, France.
Number of animals and identification: 3 animals were used, as recommended by the international regulations and taking into account that a good correlation of results can be obtained with either 3 or 6 animals (1). The animals were identified individually with a metal tag in the ear.
onal regulations. The animals were identified individually with a metal tag on the ear.
Weight: on the day of treatment, the animals had a mean body weight of 2.7 ± 0.2 kg.
Acclimatization: at least 5 days before the beginning of the study.
Selection of the animals: the day before treatment, the skin of each animal was examined in order to use only animals without any signs of cutaneous irritation. Animals showing signs of cutaneous irritation, cutaneous defects or pre-existing dermal injury were not used.

2.2.2 Environmental conditions

During the acclimatization period and during the main test, the environmental conditions in the animal room were set as follows:
temperature: 18 ± 3°C
relative humidity: 30 to 70%
light/dark cycle: 12 h/12 h
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity and light/dark cycle) were checked monthly.
The animals were housed individually in polystyrene cages (35 x 55 x 32 cm or 48.2 x 58 x 36.5 cm). Each cage was equipped with a food container and a water bottle.

2.2.3 Food and water

All the animals had free access to 112 C pelleted diet (U.A.R., 91360 Villemoisson-sur-Orge, France).
Each batch of food was analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

(1) Hatoum, N.S.; Leach, C.L.; Talsma, D.M.; Gibbons, R.D.; Garvin, P.J.: A statistical basis for using fewer Rabbits in dermal irritation testing. Journal of the American College of Toxicology. 9: 49-60 (1990).

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Drinking water filtered by a F.G. Millipore membrane (0.22 micron) and contained in bottles was provided *ad libitum*.
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet or water at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Preparation of the animals

The day before treatment, the flanks of each animal were clipped using electric clippers. Only animals showing no obvious signs of irritancy were used for the study.

2.3.2 Application of the test substance

As the test substance was anticipated to be irritant, a preliminary assay was conducted on 1 animal (No. 01). The duration of exposure was 3 minutes on one flank and 1 hour and 4 hours on the other flank. As the test substance was not very irritant, the results were then confirmed using 2 additional animals (Nos. 02 and 03) for a period of 4 hours.

A dose of 500 mg of the test substance in its original form was applied to a 6 cm^2 hydrophilic gauze pad (Semes France, 54183 Heillecourt, France) moistened with 0.5 ml of distilled water and this was then applied to the right flank of the animals (treatment for 1 hour, animal No. 01 or treatment for 4 hours, animal Nos. 01, 02 and 03) or to the left flank of the animal No. 01 (treatment for 3 minutes).

The anterior left flank (animal No. 01) or the left flank (animal Nos. 02 and 03) did not receive any test substance and served as a control. The test substance and the gauze pad were held in contact with the skin by means of an adhesive hypoallergenic aerated semi-occlusive dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and a restraining bandage (Laboratoires 3M Santé, 92245 Malakoff, France).

Subsequently, the dressings were removed and any residual test substance was wiped off with a gauze pad moistened with water for injections.

The animals were then replaced into their individual cages.

2.3.3 Date of treatment

Animal number	Date of treatment (day 1)	End of the observation period
01	18.8.94	1.9.94
02	23.8.94	6.9.94
03	23.8.94	4.9.94

2.4. CUTANEOUS EXAMINATIONS

The skin was examined approximately 1, 24, 48 and 72 hours after removal of the dressing.

When there is persistent cutaneous irritation after 72 hours, the observation period is extended to a maximum of 14 days (until day 15) in order to determine the progress of the lesions and their reversibility or irreversibility.

2.5. DESCRIPTION AND EVALUATION OF CUTANEOUS REACTIONS

Dermal irritation was evaluated for each animal according to the following scoring scale:

Erythema and eschar formation:

. no erythema 0
. very slight erythema (barely perceptible) 1
. well-defined erythema 2
. moderate to severe erythema 3
. severe erythema (beet redness) to slight eschar formation (injuries in depth) 4

Oedema formation

. no oedema 0
. very slight oedema (barely perceptible) 1
. slight oedema (edges of area well-defined by definite raising) 2
. moderate oedema (raised approximately 1 millimetre) 3
. severe oedema (raised more than 1 millimetre and extending beyond area of exposure) 4

Any other lesions were noted.

2.6. INTERPRETATION OF RESULTS AND CLASSIFICATION OF SUBSTANCES

The results obtained were evaluated taking into consideration the nature and the reversibility or irreversibility of the findings observed. Classification of the test substance is based on the criteria laid down Directive 93/21/E.E.C. Commission Directive of 27th April 1993 adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C. on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances.

The substances and preparations are classified as corrosive or irritant, according to the following criteria:

2.6.1 Criteria of corrosion

A substance or a preparation is considered as corrosive if when applied to the sound and intact skin it induces, in at least one animal during the dermal irritation tests, tissular destruction on the whole depth of the skin, or if the result can be predicted, for example: strong acid or alkaline reactions.

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Corrosive substances and preparations

- C symbol, indication of danger: "corrosive",
- sentences indicating particular hazards:

R 34: "Causes burns"
If, when applied to the sound and intact animal skin, it induces tissular lesions on the whole depth of the skin after an exposure period not exceeding 4 hours, or if the results can be predicted.

R 35: "Causes severe burns"
If, when applied to the sound and intact animal skin, it induces tissular lesions on the whole depth of the skin after an exposure period not exceeding 3 minutes, or if the result can be predicted.

2.6.2 Criteria of irritation
A substance or a preparation tested on 3 animals is considered as irritant when it induces skin inflammation lasting at least 24 hours after a 4-hour exposure period and if at least 2 of them show an erythema and eschar formation or an oedema formation with a scoring equal to or higher than 2.

All the scores obtained from each reading (24, 48 and 72 hours) of a reaction are used to calculate the respective mean values.

Irritant substances and preparations

- Xi symbol, indication of danger: "irritant",
- sentence indicating particular hazards:

R 38: "Irritating to skin"

2.7. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,

are stored on the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. RESULTS (tables 1 to 3)

After exposure of 3 minutes (1 animal)
Black colouration of the test site that could mask an erythema of grade 4 or grade 2 was noted from days 1 to 10. No erythema was observed thereafter. Slight oedema (grade 2) was noted 1 hour after removal of the dressing only. Dryness of the skin was noted at the test site from days 7 to 11.

After exposure of 1 hour (1 animal)
Black colouration of the test site did not mask the erythema. Severe erythema (grade 4) was observed from days 1 to 9. It was moderate on day 10 (grade 2) and slight on day 11 (grade 1). Severe or slight oedema (grade 4 or 2) was noted from days 1 to 6. Dryness of the skin (from day 7 to day 12) and crusts (from day 11 to day 15) were noted at the test site.

After exposure of 4 hours (3 animals)
Black colouration of the test site that could mask an erythema of grade 4 or grade 2 was noted from day 1 on all animals. It persisted up to days 6, 11 or 15. No erythema was noted thereafter. Slight or severe oedema (grade 2 or 4) was noted from days 1 to 5 in 2 animals. In the third animal, slight oedema (grade 2) was observed 1 hour after removal of the dressing only.

Dryness of the skin was noted between days 4, 6 or 7 to days 10 or 11 on all animals.

Mean scores over 24, 48 and 72 hours for individual animals were 0.0, 2.7 and 3.3 for oedema.

Due to the skin colouration, scores for erythema could not be calculated.

4. CONCLUSION

As the mean scores for oedema for 2 out of the 3 animals reached the criteria values for irritation, under our experimental conditions, the test substance, was considered as **irritant** when administered by cutaneous route in rabbits.

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CLASSIFICATION OF THE TEST SUBSTANCE

According to Council Directive 93/21/E.E.C. (27th April 1993) adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C. on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances,

concerning the potential skin irritant effects, the test substance should be classified as follows:

Symbol : X_i

Indication of danger : Irritant

Sentence indicating particular hazards : R38: "Irritating to skin"

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1,3-Bis-[(4-AMINO-PHENYL)-(2-HYDROXY-ETHYL)-AMINO]-PROPAN-2-OL, TETRAHYDROCHLORIDE -
Bulletin d'analyse établi sur le lot pil.4X

* IDENTIFICATION

A) Poudre ivoire, plus ou moins agglomérée, irritante, à odeur forte.

B) Solubilité à 25 °C

1 g + 10 ml d'eau = soluble

0,10 g + 10 ml d'éthanol à 95 % = soluble

C) Spectres UV (solution aqueuse à 0,010 g/l)

λ_{max} = 258 nm Absorbance = 0,472

D) Spectre visible (solution aqueuse à 10 g/l)

λ_{max} = 415 nm Absorbance = 0,602

E) Spectre infra-rouge (IR)

Principaux nombres d'ondes : 3371 - 2844 - 2572 - 1635 - 1611 - 1585 - 1516 - 1492 - 824 cm^{-1}

* PURETE

- Eau (Karl Fischer) = 1,75 % (p/p)
- Impuretés (HPLC)
  - 2-phénylamino-éthanol < 200 ppm (non détecté)
  - 1,3-bis-[(2-hydroxy-éthyl)-phénylamino]-propan-2-ol < 100 ppm (non détecté)
  - 1,3-bis[(2-hydroxy-éthyl)-(4-nitroso-phényl)-amino]-propan-2-ol < 100 ppm (non détecté)

* TITRE

- Le titre [déterminé par potentiométrie - NaOH] du lot pil.4X est de 98,8 %
- Chlorures (potentiométrie - nitrate d'argent) = 7,57 (mEq/g) - (Théorie : 7,9 mEq/g)

Bulletin d'analyses établi le 15/11/1994 à partir du dossier analytique émis le 2/11/1994

Directeur du Département de Chimie Analytique

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le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par $AgNO_3$	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

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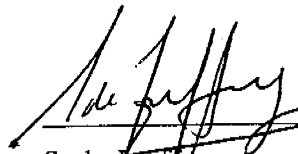
STUDY TITLE: ACUTE DERMAL IRRITATION IN RABBITS

MOTIVE: Error in table 2

SCIENTIST INVOLVED IN THIS AMENDMENT:

Toxicology



S. de Jouffrey Date: 24.4.95
Study Director
Doctor of Veterinary Medicine
Head of Short-term and
Environmental Toxicology

QUALITY ASSURANCE UNIT:

L. Valette-Talbi Date: 24.4.95
Doctor of Biochemistry
Head of Quality Assurance Unit and
Scientific Archives

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HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

SPAUL 90011

PIL 1:

SKIN IRRITATION

TEST IN THE RABBIT

PROJECT NUMBER 109/409

Date Started: 12 July 1990

Experimental Procedures:

Date Completed: 15 July 1990

AUTHOR: R.L. Guest

STUDY SPONSOR:

ISSUED BY:

Safepharm Laboratories Limited
P.O. Box No. 45
DERBY
DE1 2BT
U.K.

Telephone: DERBY (0332) 792896

Facsimile: (0332) 799018

Telex: 377079 SAFPHM G

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PROJECT NUMBER: 109/409

SAFEPHARM LABORATORIES LIMITED

QUALITY ASSURANCE UNIT REPORT

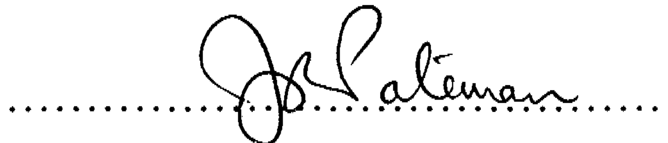
The routine inspection of short term toxicity studies at Safepharm Laboratories is carried out as a continuous process designed to ensure that where possible all critical phases of a particular study type are inspected at least once per month. Dates of inspection for this study type are given below:

STUDY TYPE	DATE(S) OF INSPECTION
Skin Irritation:	13/07/90, 18/07/90, 31/07/90, 31/07/90
General Facilities Audit conducted:	06/07/90

This report has been audited by Safepharm Laboratories Quality Assurance Unit and is an accurate account of the procedures followed and accurately records the original raw laboratory data generated in this study.

Date of report audit: 03/08/90

J.R. Pateman M.I. Biol., C. Biol.
QUALITY ASSURANCE MANAGER



DATE: 16/08/90

PROJECT NUMBER: 109/409

GOOD LABORATORY PRACTICE STATEMENT

I, the undersigned, hereby declare that this study was performed in compliance with the following principles of Good Laboratory Practice:

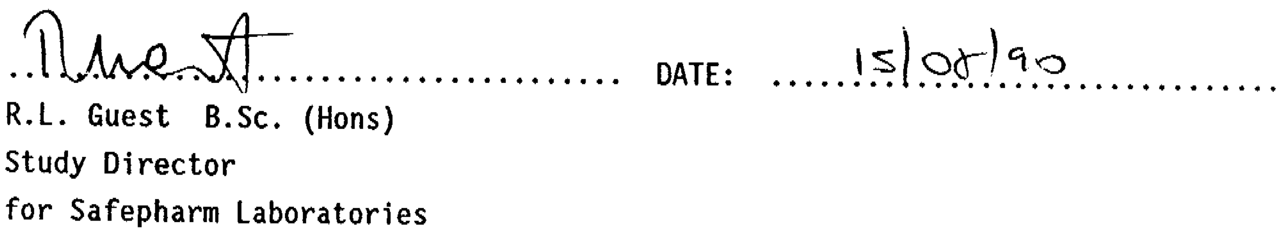
Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.

*United States Environmental Protection Agency, Title 40, Code of Federal Regulation, Part 160 and Part 792.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health 1989.

Japan Ministry of Agriculture, Forestry and Fisheries, 59 Nohsan Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

Japan Ministry of Health and Welfare, Notification No. Yakuhatsu 313, Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatsu 870, Pharmaceutical Affairs Bureau, 5 October 1988.

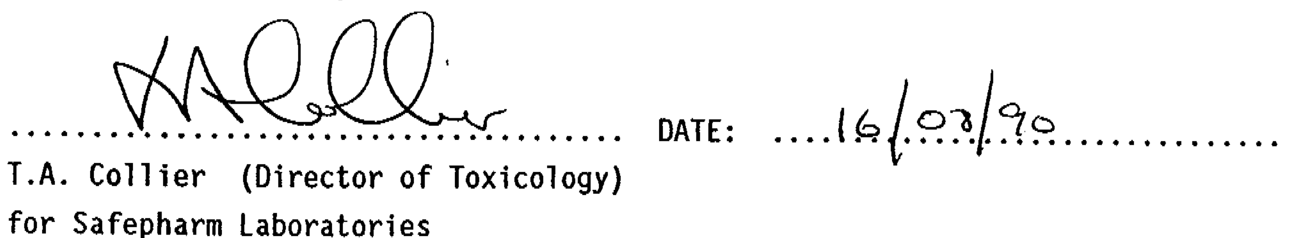


.... DATE: ....15/08/90....

R.L. Guest B.Sc. (Hons)
Study Director
for Safepharm Laboratories

* Under the provisions of a Memorandum of Understanding between the UK Department of Health and the United States Environmental Protection Agency, effective for five years from March 1988.

Approved for Issue by:



.... DATE: ....16/08/90....

T.A. Collier (Director of Toxicology)
for Safepharm Laboratories

SUMMARY OF RESULTS

STUDY SPONSOR :

PROJECT NUMBER : 109/409

L'OREAL REFERENCE : SPAUL 90011

TEST MATERIAL : PIL 1

1. A study was performed to assess the irritancy potential of the test material to the skin of the New Zealand White rabbit. The method used was based on that described in the Journal Officiel de la Republique Francaise, 21 February 1982.

2. A single, 24-hour, occluded application of the test material to the intact and abraded skin of three rabbits produced slight to well-defined erythema and slight to severe oedema.

3. The test material produced a primary irritation index of 3.4 and was classified as irritant to rabbit skin.

PIL 1:

JOURNAL OFFICIEL SKIN IRRITATION TEST IN THE RABBIT

INTRODUCTION

The study was performed according to Safepharm Standard Protocol Number JORF 1A and was carried out in order to assess the irritancy potential of a test material following a single, 24-hour, occluded application to intact and abraded rabbit skin. The study was based on the method described in the Journal Officiel de la Republique Francaise, 21 February 1982. The test system was chosen because the rabbit has been shown to be a suitable model for this type of study and is recommended in the test method.

The results of the study are believed to be of value in predicting the likely skin irritation potential of the test material to man.

The study was conducted in accordance with the internationally accepted general principles of good laboratory practice and Safepharm Standard Operating Procedures.

METHODS

1. Animals and Animal Husbandry

Three New Zealand White rabbits were supplied by David Percival Ltd., Moston, Sandbach, Cheshire, U.K. At the start of the study the animals weighed 2.21 - 2.41 kg and were approximately twelve to sixteen weeks old. After a minimum acclimatisation period of five days, each animal was given a number unique within the study which was written with a black indelible marker-pen on the inner surface of the ear and on the cage label.

The animals were individually housed in suspended metal cages. Free access to mains drinking water and food (Rabbit Diet, Preston Farmers Limited, New Leake, Boston, Lincolnshire, U.K.) was allowed throughout the study.

The animal room was maintained at a temperature of 18 - 23°C and relative humidity of 65 - 68%. The rate of air exchange was approximately 15 changes per hour and the lighting was controlled by a time switch to give 12 hours light and 12 hours darkness.

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2. Test Material and Experimental Preparation

The test material was supplied , as follows:

Description	:	blue/grey-coloured powder
Container	:	glass screw-top bottle
Sponsor's identification	:	PIL 1
Batch number	:	PIL 1
Date of arrival	:	21 March 1990
Storage conditions	:	room temperature

For the purpose of this study the test material was moistened with distilled water immediately before application.

The identification and stability of the test material were not determined.

3. Procedure

Approximately twenty-four hours prior to the commencement of the test, each of a group of three rabbits was clipped free of fur from the dorsal/ flank area using veterinary clippers. Only animals with a healthy intact epidermis by gross observation were selected for the study. Immediately before treatment on the day of the test, the right side of each rabbit was abraded using the tip of a scalpel blade. Abrasion consisted of superficial skin injury sufficient to disturb the stratum corneum but not deep enough to cause bleeding. The left side of each rabbit remained intact.

On the day of the test suitable test sites were selected on the back of each rabbit. A quantity of 0.5g of the test material moistened with 0.5 ml of distilled water was introduced under a composite patch which consisted of a 2.5 cm x 2.5 cm square of surgical gauze BPC (two layers thick) backed by a 3.5 cm x 3.5 cm square of thin latex rubber sheeting. The loaded patches were applied to the abraded and intact skin sites of each animal and secured in position by a 5.0 cm x 5.0 cm square of surgical adhesive tape (BLENDERM). To prevent the animals from interfering with the patches, the trunk of each rabbit was wrapped in an elasticated corset (TUBIGRIP); the animals were then returned to their cages for the duration of the exposure period.

3. <u>Procedure</u> (contd)

Twenty-four hours after application the corset and patches were removed from each animal and any residual test material removed by gentle swabbing with cotton wool soaked in distilled water.

Approximately one hour following the removal of the patches, and 48 hours later, (i.e. 24 and 72 hours after the test material was applied), the test sites were examined for evidence of primary irritation and scored according to the following scale i.e. Draize J.H. (1959) Association of Food and Drug Officials of the United States, Austin, Texas, "The Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics":-

EVALUATION OF SKIN REACTIONS

Erythema and Eschar Formation	*Value*
No erythema	*0*
Very slight erythema (barely perceptible)	*1*
Well-defined erythema	*2*
Moderate to severe erythema	*3*
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	*4*

Oedema Formation	
No oedema	*0*
Very slight oedema (barely perceptible)	*1*
Slight oedema (edges of area well-defined by definite raising)	*2*
Moderate oedema (raised approximately 1 millimetre)	*3*
Severe oedema (raised more than 1 millimetre and extending beyond the area of exposure)	*4*

In addition, a full description of any serious lesions, a description of the degree and nature of irritation, corrosion or reversibility, and any other toxic effects were made.

4. Interpretation of Results

The scores for erythema and oedema at the 24 and 72-hour readings were totalled for the intact and abraded sites of all three test rabbits (24 values) and this total was divided by 12 to give the primary irritation index of the test material. The test material was classified according to the following scheme as described in the Journal Officiel de la Republique Francaise, 21 February 1982:

Primary Irritation Index	Classification of Irritancy
0 - 0.5	Non-irritant
> 0.5 - 2.0	Slight irritant
> 2.0 - 5.0	Irritant
> 5.0 - 8.0	Very irritant

ARCHIVES

On completion of the study, all raw laboratory data and a copy of the final report were transferred to Safepharm Laboratories Central Archives, London Road, Shardlow, Derbyshire, U.K., where they will be retained for a period of ten years.

RESULTS

The individual scores for erythema/eschar and oedema, are given in Table 1.

Staining of the skin was noted at all intact and abraded skin sites during the study. This did not affect evaluation of the skin responses.

Well-defined erythema was noted at all intact and abraded skin sites at the 24-hour observation and at one intact and one abraded skin site at the 72-hour observation. The remaining intact and abraded skin sites showed very slight erythema at this time.

Severe oedema, extending ventrally below the treatment site was noted at one intact and one abraded skin site at the 24-hour observation. The two remaining intact and abraded skin sites showed slight oedema at this time. Slight oedema continued to be noted at one intact and one abraded skin site at the 72-hour observation. An incident of very slight oedema was also noted at one abraded skin site at the 72-hour observation,.

No other adverse reactions were noted during the study.

The test material produced a primary irritation index of 3.4 and was classified as IRRITANT to rabbit skin.

CONCLUSION

The test material, PIL 1, was found to be irritant to rabbit skin under the conditions of the study.

TABLE 1

INDIVIDUAL SKIN REACTIONS

TEST MATERIAL: PIL 1

SKIN REACTION	SKIN SITE	READING (HOURS)	INDIVIDUAL SCORES - RABBIT NUMBER & SEX 156 Female	163 Male	166 Male	TOTAL
ERYTHEMA & ESCHAR FORMATION	INTACT	24	2 STA	2 STA	2 STA	6
		72	1 STA	1 STA	2 STA	4
	ABRADED	24	2 STA	2 STA	2 STA	6
		72	1 STA	1 STA	2 STA	4
OEDEMA FORMATION	INTACT	24	2	2	4 Oe	8
		72	0	0	2	2
	ABRADED	24	2	2	4 Oe	8
		72	1	0	2	3

SUM OF 24 & 72 HOUR READINGS (S) : 41

PRIMARY IRRITATION INDEX (S/12) : 41/12 = 3.4

CLASSIFICATION : IRRITANT

STA = staining
Oe = oedema extending ventrally below treatment site

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STUDY TITLE

REVERSE MUTATION ASSAY
ON BACTERIA *SALMONELLA TYPHIMURIUM*
AND *ESCHERICHIA COLI*

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Brigitte Molinier

STUDY COMPLETION DATE

27th February 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12089 MMJ

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SUMMARY

The objective of this study was to evaluate the potential of the test substance (batch No. Pil 4X) to induce a reverse mutation in bacteria *Salmonella typhimurium* and *Escherichia coli* (Ames test). This test enables the detection of base-pair substitution or frameshift mutagens.

The test substance is a dyeing substance.

Methods

A preliminary toxicity test was performed to define the doses to be used for the mutagenicity study. The test substance was then tested in two independent tests, with or without a metabolic activation system, the S9 mix, prepared from a liver microsomal fraction (S9) of rats induced with Aroclor 1254.

The tests were performed according to the direct plate incorporation method except the second test with S9 mix, according to the preincubation method (1 hour, 37°C).

Four strains of bacteria *Salmonella typhimurium*: TA 1535, TA 1537, TA 98 and TA 100 and one strain of *Escherichia coli*: WP2uvrA were used. Each strain was exposed to 5 doses of the test substance (3 plates/dose). After 48 to 72 hours of incubation at 37°C, the revertant colonies were scored.

The test substance was dissolved in distilled water.

The doses of were:

. without S9 mix: for the 4 *Salmonella typhimurium* strains:
 - 1st test: 62.5, 125, 250, 500, 1000 µg/plate,
 - 2nd test: 125, 250, 500, 1000, 2000 µg/plate.

. with S9 mix for the 4 *Salmonella typhimurium* strains and both with or without S9 mix for the *Escherichia coli* strain:
 - 312.5, 625, 1250, 2500, 5000 µg/plate,

5000 µg/plate being the top dose recommended by international regulations.

The doses of the positive controls were as follows:

without S9 mix:

. 1 µg/plate of sodium azide (NaN_3): TA 1535 and TA 100 strains,
. 50 µg/plate of 9-Aminoacridine (9AA): TA 1537 strain,
. 0.5 µg/plate of 2-Nitrofluorene (2NF): TA 98 strain,
. 2 µg/plate of N-ethyl-N-nitro-nitrosoguanidine (ENNG):WP2uvrA strain.

with S9 mix:

. 2 µg/plate of 2-Anthramine (2AM): Salmonella typhimurium strains,
. 10 µg/plate of 2-Anthramine (2AM): WP2uvrA strain.

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Results

For both tests, the control results were equivalent to those usually obtained in our Laboratory. The number of revertants induced by the positive controls was higher than the controls, indicating the sensitivity of the test system.

The test substance did not induce any significant increase in the number of revertants, without S9 mix, in any of the 5 strains and with S9 mix in the TA1535, TA1537, TA98 and WP2uvrA strains. In the TA100 strain, a weak positive response (a 2.2 fold increase) was noted in the second test at 5000 µg/plate, but no significant increase was noted in the first or the third tests.

Conclusion

Under our experimental conditions, the test substance (batch No. Pil 4X) did not show mutagenic activity in the reverse mutation assay on *Salmonella typhimurium* and *Escherichia coli*. Equivocal results were obtained with S9 mix in one test out of 3 in one strain of *Salmonella typhimurium*: TA100 and at the highest concentration only.

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1. INTRODUCTION

This study was performed at the request of

The objective of the study was to evaluate the potential of the test substance (batch No. Pil 4X) to induce a reverse mutation in bacteria *Salmonella typhimurium* and *Escherichia coli* (Ames test).

This test (Ames et al., 1975; Maron and Ames, 1983) enables the detection of base-pair substitution or frameshift mutagens. Mutagenic substances can induce reversion in histidine-deficient strains of *Salmonella typhimurium* or in tryptophan-deficient strains of *Escherichia coli* which are then able to grow and form colonies in a histidine(tryptophan)-limited medium, while non-reverted strains cannot.

The test substance is a dyeing substance.

This study was designed in accordance with the following guidelines:
. E.C. Directive No. 92/69/E.E.C., Annex V, B_{14}, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Identification

2.1.1.1 Test substance

The test substance, used in the study was supplied

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pilot 4X
  - labelling: Pil 4 X
. description: beige powder
. quantity and container: 4 kg in a plastic tube, this quantity was dispatched at C.I.T. into 7 flasks
. date of receipt: 26.7.94
. storage conditions: at room temperature, away from light and in addition away from humidity (from 1.8.94 onwards); the content of each flask was also kept under nitrogen until use.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in paragraph 6) provided by the Sponsor.

2.1.1.2 Vehicle

The vehicle was distilled water.

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2.1.2 Preparation

The test substance was dissolved in the vehicle at a concentration of 50 mg/ml for both tests.
The preparations were made immediately before use.

2.1.3 Positive controls

Five known mutagens, dissolved in dimethylsulfoxide, were used to check the sensitivity of the test system:

	Dose (µg/plate)	Strains
Without S9 mix		
. Sodium azide (NaN_3)	1	TA 1535 - TA 100
9-Aminoacridine (9AA)	50	TA 1537
2-Nitrofluorene (2NF)	0.5	TA 98
N-ethyl-N-nitro-nitrosoguanidine (ENNG)	2	WP2uvrA
With S9 mix		
. 2-Anthramine (2AM)	2	TA 1535 - TA 1537 - TA 98 - TA 100
	10	WP2uvrA

2.2. TEST SYSTEM

2.2.1 Bacterial strains

The 4 strains of *Salmonella typhimurium* (Ames et al., 1975; Maron and Ames, 1983): TA 1535, TA 1537, TA 98 and TA 100, are supplied by B.N. Ames' Laboratory (University of California, Berkeley, U.S.A.). The strain of *Escherichia coli*: WP2uvrA is supplied by S. Venitt's Laboratory (I.C.R., Sutton, England). They are stored in a cryoprotective medium containing 1 ml nutrient broth and 0.09 ml dimethylsulfoxide in a liquid nitrogen container.

Each strain derived from *Salmonella typhimurium* LT 2 contains one mutation in the histidine operon, resulting in a requirement for histidine. The strain of *Escherichia coli* contains one mutation in the tryptophan operon, resulting in a requirement for tryptophan. In addition, to increase their sensitivity to mutagenic substances, additional mutations have been added:

. the rfa mutation causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules that do not penetrate the normal bacteria cell wall,

. the uvr (uvrB for *Salmonella typhimurium* and uvrA for *Escherichia coli*) mutation is a deletion of a gene code for the DNA excision repair system, which renders the bacteria unable to use this repair mechanism to remove the damaged DNA,

. the addition of the pKM 101 ampicillin resistant plasmidic R-factor in the strains TA 98 and TA 100 enhances their detection sensitivity to some mutagens.

Genotype of the bacterial strains

Strains	Histidine mutation	Additional mutations		
TA 1535	His G 46	rfa	uvrB	
TA 100	His G 46	rfa	uvrB	Factor R
TA 1537	His C 3076	rfa	uvrB	
TA 98	His D 3052	rfa	uvrB	Factor R
WP2uvrA	Trp E	-	uvrA	

The TA 1535, TA 100 and WP2uvrA strains are reverted by base-pair substitution mutagens and the TA 1537 and TA 98 strains by frameshift mutagens.

2.2.2 Metabolic activation system: S9 mix

The S9 mix consists of induced enzymatic systems contained in rat liver microsomal fractions (S9) and the cofactors necessary for their function. S9 was performed at C.I.T. according to Ames et al. The S9 fraction comes from liver homogenates from rats induced with Aroclor 1254 (500 mg/kg) by intraperitoneal route.
The S9 fraction was preserved in sterile tubes within a liquid nitrogen container.
The S9 mix was used at 10% S9 in the S9 mix. The S9 mix was prepared immediately before use at +4°C and maintained at this temperature throughout the experiment.

The S9 mix contained per ml:
. 5 µmoles Glucose-6-Phosphate,
. 4 µmoles NADP,
. 33 µmoles KCl,
. 8 µmoles $MgCl_2$,
. 100 µmoles sodium phosphate pH 7.4,
. 100 µl S9 (batchs No. 37 and 38, protein concentration: 30 and 27 g/l respectively),
. sterile distilled water q.s.p. 1 ml.

2.3. EXPERIMENTAL DESIGN

The day before treatment, cultures were inoculated from frozen permanents: a crystal was sampled under sterile conditions and put into approximately 6 ml of nutrient broth. The nutrient broth was then placed under agitation in an incubator at 37°C for about 14 hours.
The tests were performed according to:
. direct plate incorporation method (both tests without S9 mix, first test with S9 mix): the test substance solution, 0.5 ml of S9 mix (when required) and 0.1 ml of the strain were added to 2 ml molten agar containing traces of either histidine for the *Salmonella typhimurium* strains or tryptophan for the *Escherichia coli* strain and biotin and maintained at 45°C. After rapid homogenization, the mixture was spread out on a Petri plate containing minimum medium.
. preincubation method (second and third tests with S9 mix): the test substance solution, 0.5 ml of S9 mix and 0.1 ml of the strain were incubated for 60 minutes at 37°C prior adding the overlay agar and pouring onto the surface of a minimum agar plate.
After 48 to 72 hours of incubation at 37°C, revertants were scored with an automatic counter (Artek counter, model 880, O.S.I., 75015 Paris, France).

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2.3.1 Preliminary toxicity test

To assess the toxicity of the test substance to the bacteria, 6 doses (1 plate/dose) were tested in the WP2uvrA, TA 98 and TA 100 strains, with or without S9 mix. The top dose was determined according to the international regulations, i.e. not exceeding 5000 µg/plate.

In the event of toxicity, the bacterial lawn is sparse compared to control plates and/or the number of colonies is decreased.

The sterility of the test substance was checked during this test and was found to be satisfactory.

2.3.2 Mutagenicity tests

Rationale for dose selection

The top dose was selected according to the results of the preliminary toxicity test and to the following criteria:

. For non-toxic, freely soluble test substances, the top dose is 5000 µg/plate, according to international regulations.
. For non-toxic, poorly soluble test substances, the top dose is the lowest precipitating dose.
. For toxic test substances, irrespective of solubility, the top dose is based on the level of toxicity: moderately to markedly sparse bacterial lawn and/or decrease by approximately 50% of the number of revertants when compared to the controls. However, precipitation should not interfere with the scoring of the test.

Five doses of the test substance (3 plates/dose) were tested on each strain, with or without S9 mix.

During each test, the following controls were made using triplicate plates:

. negative control: untreated strain, without S9 mix,
. vehicle control: strain treated with the vehicle,
. positive control: strain treated with the known mutagens mentioned in § 2.1.3.

The sterility of the S9 mix was checked during each test (before the beginning and at the end of the experiment) and was found to be satisfactory.

2.4. ASSESSMENT OF DATA

Treatment of results

During each test, for each strain and for each experimental point, the number of revertants per plate was scored. The individual results and the mean number of revertants, with the corresponding standard deviation and ratio (mutants obtained in the presence of the test substance/mutants obtained in the presence of the vehicle), are presented in a table.

Acceptance criteria

This study was considered valid because the following criteria were fully met:

. the number of revertants of the controls was within the range of our historical data,
. the number of revertants of the positive controls was higher than that of the controls and was within the range of our historical data.

Evaluation criteria

The following criteria were used as an aid for determining a positive response:

. a reproducible and significant dose relationship.

and/or

. a reproducible and significant increase (i.e. a doubling in the number of revertants for at least one of the tested strains when compared to that of the controls) for at least one of the doses.

A test substance is considered as non-mutagenic in this test system if the above two criteria are not fully met.

Biological significances were considered during the evaluation.

2.5. ARCHIVES

The study archives:

. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments

are stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

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2.6. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date	
Protocol approved by:		
. Study Director	7.7.94	
. Sponsor	21.7.94	
Preliminary test		
. treatment	26.8.94	
First mutagenicity test		
. treatment	30.8.94	
Second mutagenicity test		
. treatment	20.9.94	22.11.94*

* strain TA100 with S9 mix.

3. RESULTS

3.1. PRELIMINARY TOXICITY TEST (table 1)

The test substance was freely soluble in the vehicle (distilled water) at 50 mg/ml.

Consequently, the doses were: 10, 100, 500, 1000, 2500, 5000 µg/plate.

When 0.1 ml of this solution was added to 2 ml molten agar, corresponding to the top dose of 5000 µg/plate, no precipitate was observed in the Petri plate when scoring the revertants, but a strong yellow colouration.

The test substance was not toxic at the doses used with S9 mix for the 3 strains and without S9 mix for WP2uvrA.

The test substance was toxic at doses higher than 1000 µg/plate in the TA98 and TA 100 strains, without S9 mix: the bacterial lawn was very sparse compared to the control and the number of revertants had decreased. At lower doses, no toxicity was observed.

3.2. MUTAGENIC ACTIVITY (tables 2 to 6)

The control results were equivalent to those usually obtained in our Laboratory. The number of revertants induced by the positive controls was higher than the controls, indicating the sensitivity of the test system.

The selected doses were without S9 mix: for the 4 *Salmonella typhimurium* strains:
- 1st test: 62.5, 125, 250, 500, 1000 µg/plate,
- 2nd test: 125, 250, 500, 1000, 2000 µg/plate.

. with S9 mix for the 4 *Salmonella typhimurium* strains and both with or without S9 mix for the *Escherichia coli* strain:
- 312.5, 625, 1250, 2500, 5000 µg/plate,

5000 µg/plate being the top dose recommended by international regulations.

The test substance did not induce any significant increase in the number of revertants, without S9 mix, in any of the 5 strains, and with S9 mix in the TA1535, TA1537, TA98 and WP2uvrA strains. In the TA100 strain, a weak positive response (a 2.2 fold increase) was noted in the second test at the highest concentration only, but no significant increase was noted in the first or the third tests.

4. CONCLUSION

Under our experimental conditions, the test substance (batch No. Pil 4X) did not show mutagenic activity in the reverse mutation assay on *Salmonella typhimurium* and *Escherichia coli*. Equivocal results were obtained with S9 mix in one test out of 3 in one strain of *Salmonella typhimurium*: TA100 and at the highest concentration only.

5. REFERENCES

Ames, B. N.; Durston, W. E.; Yamasaki, E. and Lee, F. D.: Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Nat. Acad. Sci. U.S.A., 70, 2281-2285 (1973).

Ames, B. N.; Mc Cann, D. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the *Salmonella* Mammalian-microsome mutagenicity test. Mutation Research, 31, 347-364 (1975).

Maron, D.M. and Ames B. N.: Revised methods for the *Salmonella* mutagenicity test. Mutation Research, 113, 173-215 (1983).

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le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par AgNO3	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

Lot utilisable jusqu'au 25/11/95

10/11/94

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8. PROTOCOL AND AMENDMENTS

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CIT

centre international de toxicologie

MISEREY BP 563 27005 ÉVREUX CEDEX FRANCE TÉL. 32 29 26 26 TÉLÉCOPIE 32 67 87 05

Miserey, 7th July 1994

REVERSE MUTATION ASSAY ON BACTERIA *SALMONELLA TYPHIMURIUM* AND *ESCHERICHIA COLI*

Protocol from	:	Centre International de Toxicologie Miserey BP 563 - 27005 Evreux Cédex France
Sponsor	:	Société
Address	:	
Study Monitor	:	
Study Director	:	B. Molinier
Study Number	:	12089 MMJ

INTRODUCTION

The objective of this study is to evaluate the potential of the test substance to induce a reverse mutation in bacteria *Salmonella typhimurium* and *Escherichia coli* (Ames test).

This test enables the detection of base-pair substitution or frameshift mutagens. Mutagenic substances can induce reversion in histidine-deficient strains of *Salmonella typhimurium* or tryptophan-deficient strains of *Escherichia coli* which are then able to grow and form colonies in a histidine(tryptophan)-limited medium, while non-reverted strains cannot.

Two independent tests, with or without a metabolic activation system, the S9 mix, will be performed. This allows the detection of mutagenic activity of a test substance and/or its metabolites.

The tests will be performed according to the direct plate incorporation method, except the second test with S9 mix, according to the preincubation method (1 hour, 37°C).

Four strains of *Salmonella typhimurium*: TA 1535, TA 1537, TA 98 and TA 100 and one strain of *Escherichia coli*: WP2uvrA will be used. Each strain will be exposed to 5 doses of the test substance (3 plates/dose). After 48 to 72 hours of incubation at 37°C, the revertant colonies will be scored.

This protocol complies with:

. E.C. Directive No. 92/69/E.E.C., Annex V, B_{14}, 31st July 1992.

The study will be conducted in compliance with the following Good Laboratory Practice Regulations:

. Council Directive 87/18/E.E.C. of 18 December 1986 on the harmonization of laws, regulations or administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (O.J. n° L 15 of 17.1.87).

. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire).

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MATERIALS AND METHODS

1. TEST AND CONTROL SUBSTANCES

1.1 Identification

1.1.1 Test substance

. **Denomination** :
. **Batch No.** : Pilot 4X
. **Description** : powder
. **Storage conditions** : ...(1)...
. **Physico-chemical properties:** i.e. purity, composition, stability and expiry date which refer to the batch to be used and handling conditions will be indicated in the test substance data sheet (to be completed by the Sponsor). An analytical certificate will also be provided by the Sponsor.
. **Required amount** : 2 g

1.1.2 Vehicle

The vehicle will be selected according to the results of solubility tests performed before the preliminary toxicity test (2).

1.2 Preparation

The test substance will be dissolved in the vehicle to provide approximately 10 to 20 fold the final dose. The preparations will be made immediately before use.

1.3 Positive controls

Five known mutagens, dissolved in dimethylsulfoxide, will be used to check the sensitivity of the test system:

	Doses (µg/plate)	Strains
Without S9 mix		
. sodium azide	1	TA 1535 - TA 100
. 9-Aminoacridine	50	TA 1537
. 2-Nitrofluorene	0.5	TA 98
. N-ethyl-N-nitro-nitrosoguanidine	2	WP2uvrA
With S9 mix		
. 2-Anthramine	2	TA 1535 - TA 1537 - TA 98 - TA 100
. 2-Anthramine	10	WP2uvrA

(1) To be specified, dated and signed by the Sponsor
(2) Will be documented in the raw data and specified in the final report

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2. TEST SYSTEM

2.1 Bacterial strains

The 4 strains of *Salmonella typhimurium*: TA 1535, TA 1537, TA 98 and TA 100, are supplied by B.N. Ames' Laboratory (University of California, Berkeley, U.S.A.). The strain of *Escherichia coli*: WP2uvrA is supplied by S. Venitt's Laboratory (I.C.R., Sutton, England). They are stored in a cryoprotective medium containing 1 ml nutrient broth and 0.09 ml dimethylsulfoxide in a liquid nitrogen container.

Each strain derived from *Salmonella typhimurium* LT 2 contains one mutation in the histidine operon, resulting in a requirement for histidine. The strain of *Escherichia coli* contains one mutation in the tryptophan operon, resulting in a requirement for tryptophan. In addition, to increase their sensitivity to mutagenic substances, additional mutations have been added:

. the rfa mutation causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules that do not penetrate the normal bacteria cell wall,

. the uvr (uvrB for *Salmonella typhimurium* and uvrA for *Escherichia coli*) mutation is a deletion of a gene code for the DNA excision repair system, which renders the bacteria unable to use this repair mechanism to remove the damaged DNA,

. the addition of the pKM 101 ampicillin resistant plasmidic R-factor in the strains TA 98 and TA 100 enhances their detection sensitivity to some mutagens.

Genotype of the bacterial strains

Strains	Histidine mutation	Additional mutations
TA 1535	His G 46	rfa uvrB
TA 100	His G 46	rfa uvrB Factor R
TA 1537	His C 3076	rfa uvrB
TA 98	His D 3052	rfa uvrB Factor R
WP2uvrA	Trp E	- uvrA

The TA 1535, TA 100 and WP2uvrA strains are reverted by base-pair substitution mutagens and the TA 1537 and TA 98 strains by frameshift mutagens.

2.2 Metabolic activation system: S9 mix

The S9 mix consists of induced enzymatic systems contained in rat liver microsomal fractions (S9) and the cofactors necessary for their function. S9 will be performed at C.I.T. The S9 fraction comes from liver homogenates from rats induced with Aroclor 1254 (500 mg/kg) by the intraperitoneal route.
The S9 fraction is preserved in sterile tubes within a liquid nitrogen container.

The S9 mix will be used at 10% S9 in the S9 mix. The S9 mix will be prepared immediately before use at +4°C and maintained at this temperature throughout the experiment.

The S9 mix will contain per ml:
. 5 µmoles Glucose-6-Phosphate,
. 4 µmoles NADP,
. 33 µmoles KCl,
. 8 µmoles $MgCl_2$,
. 100 µmoles sodium phosphate pH 7.4,
. 100 µl S9,
. sterile distilled water q.s.p. 1 ml.

3. EXPERIMENTAL DESIGN

The day before treatment, cultures will be inoculated from frozen permanents: a crystal will be sampled under sterile conditions and put into approximately 6 ml of nutrient broth. The nutrient broth will then be placed under agitation in an incubator at 37°C for about 14 hours.
The tests will be performed according to:
. direct plate incorporation method (both tests without S9 mix; first test with S9 mix): the test substance solution, 0.5 ml of S9 mix when required and 0.1 ml of the strain, will be added to 2 ml molten agar containing traces of either histidine for the *Salmonella typhimurium* strains or tryptophan for the *Escherichia coli* strain and biotin and maintained at 45°C. After rapid homogenization, the mixture will be spread out on a Petri plate containing minimum medium.
. preincubation method (second test with S9 mix): the test substance solution, 0.5 ml of S9 mix and 0.1 ml of the strain will be incubated for 60 minutes at 37°C prior adding the overlay agar and pouring onto the surface of a minimum agar plate.
After 48 to 72 hours of incubation at 37°C, revertants will be scored with an automatic counter (Artek counter, model 880, O.S.I., 75015 Paris, France).

3.1 Preliminary toxicity test

To assess the toxicity of the test substance to the bacteria, at least 6 doses (1 plate/dose) will be tested in the WP2uvrA, TA 98 and TA 100 strains, with or without S9 mix. The top dose will be determined according to the solubility of the test substance in the vehicle, but will not exceed 5000 µg/plate according to the international regulations.

In the event of toxicity, the bacterial lawn is sparse compared to control plates and/or the number of colonies is decreased.

The sterility of the test substance will be checked during this test.

3.2 Mutagenicity tests

Rationale for dose selection
The choice of the doses will be performed by the Study Director and will be documented in the raw data throughout the study.
The top dose will be selected according to the results of the preliminary toxicity test and to the following criteria:
. For non-toxic, freely soluble test substances, the top dose is 5000 µg/plate, according to international regulations.
. For non-toxic, poorly soluble test substances, the top dose is the lowest precipitating dose.

. For toxic test substances, irrespective of solubility, the top dose is based on the level of toxicity: moderately to markedly sparse bacterial lawn and/or decrease by approximately 50% of the number of revertants when compared to the controls. However, precipitation should not interfere with the scoring of the test.

In two independent tests, 5 doses of the test substance (3 plates/dose) will be tested on each strain, with or without metabolic activation.

During each test, the following controls will be made using triplicate plates:

. vehicle control: strain treated with the vehicle,

. positive control: strain treated with the known mutagens mentioned in § 1.3.

The sterility of the S9 mix will be checked during each test: before the beginning and at the end of the experiment.

4. ASSESSMENT OF DATA

Treatment of results

During each test, for each strain and for each experimental point, the number of revertants per plate will be scored. The individual results and the mean number of revertants, with the corresponding standard deviation and ratio (mutants obtained in the presence of the test substance/mutants obtained in the presence of the vehicle), will be presented in a table.

Acceptance criteria

This study will be considered valid if the following criteria are fully met:

. the number of revertants of the controls is within the range of our historical data,

. the number of revertants of the positive controls is higher than that of the controls and within the range of our historical data.

Evaluation criteria

The following criteria will be used as an aid for determining a positive response:

. a reproducible and significant dose relationship,

and/or

. a reproducible and significant increase (i.e. a doubling in the number of revertants for at least one of the tested strains when compared to that of the controls) for at least one of the doses.

A test substance will be considered as non-mutagenic in this test system if the above two criteria are not fully met.

The results will be considered as inconclusive if there are dramatic differences between the first and the second tests. In this case, a third test will be performed.

Both biological and statistical significance will be considered together in the evaluation.

5. PROCEDURES

The procedures used during the study will be those documented in the relevant C.I.T. procedures manual.

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6. AMENDMENTS TO THE PROTOCOL

If necessary, amendments to the protocol will be made after agreement between the Study Director and the Study Monitor.

7. REPORTING

The Study Director will contact the Study Monitor when necessary.
The final report in English **with a French summary**, will contain all data collected throughout the study.
Number of copies of the final report: 3 (1 + 2 unbound)
Proposed issue of the draft report: one month after the end of the study.

8. QUALITY ASSURANCE UNIT

The Quality Assurance Unit will conduct inspections according to Good Laboratory Practice Regulations (as specified on page 2).
The dates on which the findings of these inspections are reported to the Study Director and C.I.T. Management will be specified in the final report.

9. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments

will be stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

10. TIME SCHEDULE

Beginning of the study: 2nd fortnight of August, 1994
End of the study: 2nd fortnight of September, 1994

AMENDMENT TO PROTOCOL

STUDY No.: **12089 MMJ**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 01

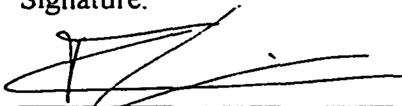
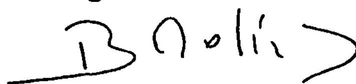
Page 1 / 1

Justification: complementary information concerning the Good Laboratory Practice Regulations.
Date of application: 26.8.94

INTRODUCTION

The study will be conducted in compliance with the following Good Laboratory Practice Regulations:
O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or A. Simonnard	B. Molinier	6/10/94
Date: 27.9.94	Date: 27.9.94	Date:
Signature:	Signature:	Signature:

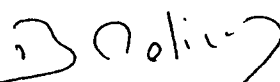
AMENDMENT TO PROTOCOL

STUDY No.: **12089 MMJ**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 02

Page 1 / 1

Justification: perfomance of supplementary test.
Date of application: 21.11.94

At the request of the sponsor, a supplementary test will be performed with the test substance " on the TA 100 strain with S9 mix (using the preincubation method).

Scientific management	Study Director	Study Monitor
J.F Le Bigot or A. Simonnard	B. Molinier	
Date: 21.11.94	Date: 21.11.94	Date:
Signature:	Signature: 	Signature: 23/12/94

AMENDMENT TO PROTOCOL

STUDY No.: **12089 MMJ**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 03

Page 1 / 1

Justification: complementary of information
Date of application: 30.8.94

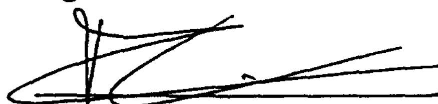
MATERIALS AND METHODS

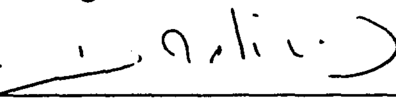
3. EXPERIMENTAL DESIGN

3.2 Mutagenicity tests

During each test, negative control: untreated strain, without S9 mix, will be made.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or A. Simonnard	B. Molinier	
Date: 26.12.94	Date: 26.12.94	Date:
Signature:	Signature:	Signature:





23/1/94

AMENDMENT TO PROTOCOL

STUDY No.: **12089 MMJ**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 04

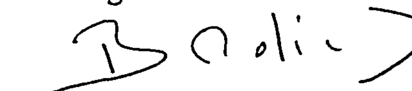
Justification: complementary information concerning the Good Laboratory Practice Regulations.
Date of application: 26.8.94

INTRODUCTION

The study will not be conducted in compliance with the following Good Laboratory Practice Regulations:

Council Directive 87/18/E.E.C. of 18 December 1986 on the harmonization of laws, regulations or administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (O.J. n° L 15 of 17.1.87).

Scientific management	Study Director	Study Monitor
J.F Le Bigot or A. Simonnard	B. Molinier	6.2.95
Date: 27.1.95	Date: 27.1.95	Date:
Signature:	Signature:	Signature:



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SPONSOR

STUDY TITLE
MICRONUCLEUS TEST
BY ORAL ROUTE IN MICE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

TEST SUBSTANCE

STUDY DIRECTOR
Brigitte Molinier

STUDY COMPLETION DATE
9th August 1995

PERFORMING LABORATORY
Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER
12287 MAS (94/1/081)

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SUMMARY

The objective of this study was to evaluate the potential of the test substance, (batch No. Pil 4X), to induce damage to the chromosomes or the mitotic apparatus in bone marrow cells of mice.

Methods

A preliminary toxicity test was performed to define the doses to be used for the cytogenetic study.
In the main study, three groups of 5 male and 5 female Swiss OF1 mice received the test substance by oral route, at doses of 375, 750 and 1500 mg/kg/day for 2 consecutive days.
One group of 5 males and 5 females received the vehicle alone (distilled water) by oral route, and acted as control group.
One group of 5 males and 5 females received cyclophosphamide once at 50 mg/kg, by oral route.

All the animals were killed 24 hours after the last administration and bone marrow smears were prepared.

For each animal, the micronuclei were counted in 2000 polychromatic erythrocytes. The polychromatic (PE) and normochromatic (NE) erythrocyte ratio was established by scoring a total of 1000 erythrocytes (PE + NE).

Results

In the vehicle control group, the mean values of micronucleated polychromatic erythrocytes (MPE) were within the range of our historical data.

Cyclophosphamide induced a highly significant increase ($p < 0.001$) in the number of MPE, indicating the sensitivity of the test system under our experimental conditions. In addition, the PE/NE ratio decreased significantly ($p < 0.001$) showing the toxic effect of this substance to bone marrow cells.

In all groups treated with the mean values of MPE were similar to those of the vehicle group, and no statistically significant differences were observed. At 1500 mg/kg/day, the PE/NE ratio was lower ($p < 0.05$) than that of the vehicle control group.

Conclusion

Under our experimental conditions, the test substance, (batch No. Pil 4X), did not induce damage to the chromosomes or the mitotic apparatus of bone marrow cells of mice treated by oral route at 375, 750 and 1500 mg/kg/day for 2 consecutive days in the micronucleus test.

1. INTRODUCTION

This study was performed at the request of Société

The objective of this study was to evaluate the potential of the test substance, to induce damage to the chromosomes or the mitotic apparatus in bone marrow cells of mice.

Apart from detecting chromosome breakage events (clastogenesis), the micronucleus test is capable of detecting chemicals which induce whole chromosome loss (aneuploidy) in the absence of clastogenic activity.

The basis of this test is the increase in the number of micronucleated polychromatic erythrocytes in the bone marrow of mice exposed to a chemical which induces cytogenetic damage. Chromosomal fragments or entire chromosomes which are left behind at cell division are not incorporated into the nuclei of daughter cells. Most of these fragments condense and form one or more micronuclei in the cytoplasm. The visualization of micronuclei is facilitated in erythrocytes because their nucleus is extruded during erythropoiesis.

Substances which inhibit either proliferation or maturation of erythroblasts and those which kill nucleated cells, decrease the proportion of polychromatic erythrocytes (PE) when compared to normochromatic erythrocytes (NE). Thus, the cytotoxicity of a substance can be determined by a decrease in the PE/NE ratio.

This test was performed according to the method described by Schmid (1975) and modified by Salamone and coll. (1980).

This study was designed in accordance with the following guidelines:

. O.E.C.D. guideline No. 474, 26th May 1983 and revised Draft document of December 1994,

. E.E.C. Directive No. 92/69/E.E.C., Annex V, B_{12}, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, , used in the study was supplied by Société
Documentation supplied by the Sponsor identified the test substance as follows:

. denomination:
  - protocol:
  - labelling:

. batch number:
  - protocol: Pil 4X
  - labelling: Pil 4X

. description: beige powder

. quantity and container: 4 kg in a plastic pot

. date of receipt: 26.7.94

. storage conditions: at room temperature, protected from light and from humidity from 1.8.94. The test substance, reconditioned in flasks numerated from 1 to 7, was stored under nitrogen gas until use of each flask

. purity: 99.8%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in paragraph 6) provided by the Sponsor.

2.1.2 Vehicle

The vehicle was distilled water (C.I.T., Millipore).

2.1.3 Preparation

The test substance was given in the vehicle. The test substance was ground to a fine powder using a mortar and pestle, dissolved in the vehicle in order to achieve the concentrations of 37.5, 75 and 150 mg/ml and then homogenized using a magnetic stirrer. The preparations were made immediately before use.

2.1.4 Positive control

The positive control was cyclophosphamide (CPA Endoxan-Asta).
It was dissolved in distilled water at a concentration of 5 mg/ml.
The preparation was made immediately before use.

2.2. TEST SYSTEM

2.2.1 Animals

Number: . 10 male and 10 female mice for the preliminary toxicity test
. 56 mice: 28 males and 28 females for the cytogenetic study.
Strain: Swiss OF1/ICO:OF1 (IOPS Caw).
Reason for this choice: rodent species commonly requested by the international regulations for this type of study.
Breeder: Iffa Crédo, L'Arbresle, France.
Age: on the day of treatment the animals were approximately 7 weeks old.
Veterinary care at C.I.T.: upon their arrival at C.I.T., the animals were given a complete examination to ensure that the animals are in good clinical condition.
Acclimatization: at least 5 days before the day of treatment.
Constitution of groups: upon arrival, the animals were randomly allocated to the groups by sex. Subsequently, each group was assigned to a different treatment group.
Identification: individually on the tail.

2.2.2 Environmental conditions

Upon their arrival at C.I.T., the animals were housed in an animal room. The animal room conditions were set as follows:
. temperature: 21 ± 2°C
. relative humidity: 50 ± 20%
. light/dark cycle: 12 h/12 h (07:00 - 19:00)
. ventilation: filtered and non-recycled fresh air.

The housing conditions (temperature, relative humidity) are checked regularly.
The animals were housed in polycarbonate cages and each cage contained 5 animals of the same sex and group (3 for the supplementary animals, see § 2.3.2.1). Each cage contained autoclaved sawdust (SICSA, Alfortville, France).
Bacteriological analysis and detection of possible contaminants (pesticides, heavy metals) of the sawdust are conducted periodically by approved laboratories. Results are archived at C.I.T.

2.2.3 Food and water

All animals had free access to AO4 C pelleted sustenance diet (U.A.R., Villemoisson-sur-Orge, France) and tap water (filtered using a 0.22 micron filter).
Each batch of food was analysed (composition and contaminants) by the supplier.
Bacteriological and chemical analyses of diet and water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed regularly by approved laboratory.
Results are archived at C.I.T.
There were no known contaminants in the diet, water or sawdust at levels likely to influence the outcome of the study.

2.3. EXPERIMENTAL DESIGN

Rationale for dose selection

The choice of doses was performed according to the following criteria.
A limit test using one dose should be performed, if no observable toxic effects are produced at 2000 mg/kg/day and if genotoxicity would not be expected based upon data from structurally related substances.
In all other cases, three doses are used for the first sampling time. For the latest sampling time, only the top dose is used.

. top dose:
- if no observable toxic effects are produced at 2000 mg/kg/day, it is chosen as the top dose,
- if observable toxic effects are produced, the top dose is defined as the dose producing signs of toxicity, such that a higher dose would be expected to produce lethality.

. middle and low doses:
- the 2 other doses are separated by no more than a factor between 2 and $\sqrt{10}$.

2.3.1 Preliminary toxicity test

In order to determine the top dose, several preliminary assays were performed on groups of 6 animals (3 males and 3 females). Clinical signs and mortality were recorded for a period of 48 hours. At the end of this period, the animals were killed after CO_2 inhalation in excess.

2.3.2 Cytogenetic study

2.3.2.1 Groups and times of sacrifice

Group	Dose (mg/kg/day)	Animals per group Male	Animals per group Female	Time of sacrifice (1)
vehicle	-	5	5	
treated	375	5	5	
	750	5	5	24 h
	1500	5	5	
treated supplementary (2)	1500	3	3	
CPA	50	5	5	

(1) Sacrifice after the last administration.

(2) Since mortality occurs during the study, these supplementary animals replaced those which die.

2.3.2.2 Administration

The test substance was administered by gavage using a dose volume of 10 ml/kg.
Each animal was given the test substance twice.
The quantity of the test substance administered to each animal was adjusted according to the body weight recorded at the time of dosing.
The vehicle control animals received the vehicle alone, under the same conditions.
The positive control animals received cyclophosphamide once, by oral route.

2.3.2.3 Preparation of smears

At the time of sacrifice, all the animals were killed by CO_2 inhalation in excess. The femurs of the mice were removed and the bone marrow eluted out using fetal calf serum. After centrifugation, the supernatant was removed and the cells in the sediment were suspended by shaking. A drop of this cell suspension was placed and spread on a slide. The slides were air-dried and stained with Giemsa. All the slides were coded for scoring.

2.4. EVALUATION OF THE RESULTS

Analysis

For each animal, the micronuclei were counted in 2000 polychromatic erythrocytes; the polychromatic (PE) and normochromatic (NE) erythrocyte ratio was established by scoring a total of 1000 erythrocytes (PE + NE).

Treatment of results

All the individual data are presented in tabular form. The number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio are given for each animal.
The means and the standard deviations of the number of MPE and the PE/NE ratio are given for each experimental group.

Statistical analysis

The mean number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio from the treated groups were compared to the simultaneous vehicle groups. The intergroup comparison was performed using:
. for MPE, the χ^2 test,
. for the PE/NE ratio, the Student's "t" test,
in which p = 0.05 was used as the lowest level of significance.

Evaluation criteria

Biological relevance of the results was considered first. In addition, the following criteria were used as an aid for determining a positive response:
. a statistically significant increase in the number of MPE when compared to the vehicle group,
. this increase should double the number of MPE of our historical data.

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2.5. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,
. slides,

are stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

2.6. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date
Protocol approved by:	
. Study Director	26.9.94
. Sponsor	21.10.94
Preliminary toxicity test	
. Beginning of treatment	6.12.94
Cytogenetic test	
. Arrival of the animals and randomization	13.4.95
. Identification of the animals and first day of treatment	18.4.95

3. RESULTS

3.1. PRELIMINARY TOXICITY TEST (table 1)

In order to select the top dose, 1000 and 2000 mg/kg/day were administered on 2 consecutive days by oral route to groups of 3 male and 3 female mice and 1500 mg/kg/day to one group of 4 male and 4 female mice.

At 2000 mg/kg/day, one female died 6 hours after the second treatment.

At 1500 mg/kg/day, one male died 24 hours after the second treatment.

No clinical signs were observed after the administration of 1000 mg/kg/day.

Consequently, 1500 mg/kg/day was chosen as the top dose. The 2 other doses were: 750 or 375 mg/kg/day.

3.2. CYTOGENETIC TEST (tables 2 and 3)

After treatment with the test substance, one female per group died just after the first administration.

In the vehicle control group, the mean values of micronucleated polychromatic erythrocytes (MPE) were within the range of our historical data presented in paragraph 7.

Cyclophosphamide induced a highly significant increase ($p < 0.001$) in the number of MPE, indicating the sensitivity of the test system under our experimental conditions. In addition, the PE/NE ratio decreased significantly ($p < 0.001$) showing the toxic effect of this substance to bone marrow cells.

In all groups treated with the mean values of MPE were similar to those of the vehicle group, and no statistically significant differences were observed. At 1500 mg/kg/day, the PE/NE ratio was lower ($p < 0.05$) than that of the vehicle control group.

4. CONCLUSION

Under our experimental conditions, the test substance, (batch No. Pil 4X), did not induce damage to the chromosomes or the mitotic apparatus of bone marrow cells of mice treated by oral route at 375, 750 and 1500 mg/kg/day for 2 consecutive days in the micronucleus test.

5. REFERENCES

Schmid, W.: The micronucleus test. Mutation Research, 31, 1975, 9-15.

Salamone, M.; Heddle, J.; Stuart, E. and Katz, M.: Toward an improved micronucleus test. Studies on 3 model agents, mitomycin C, CPA and dimethylbenzanthracene. Mutation Research, 74, 1980, 347-356.

Heddle, J. and Salamone, M.: The micronucleus assay. I. in vivo. In: short-term test for chemical carcinogens. Springer Verlag New-York (Stich and San eds.), 1981, 243-249.

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', le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par $AgNO_3$	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

Lot utilisable jusqu'au 25/11/95

10/11/94

C I T

centre international de toxicologie

MISEREY BP 563 27005 ÉVREUX CEDEX FRANCE TÉL. 32 29 26 26 TÉLÉCOPIE 32 67 87 05

Miserey, 26th September 1994

MICRONUCLEUS TEST BY ORAL ROUTE IN MICE

Protocol from : Centre International de Toxicologie
Miserey
BP 563 - 27005 Evreux Cédex
France

Sponsor : Société

Address :

Study Monitor :

Study Director : B. Molinier

Study Number : 12287 MAS

L'Oréal reference : ...(1)...

(1) To be specified by the Sponsor

GROUPEMENT D'INTERET ECONOMIQUE REGI PAR L'ORDONNANCE DU 23 SEPTEMBRE 1967 / R.C. EVREUX C 788 060 465 00010
AU CAPITAL DE 5 550 000 FRANCS

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INTRODUCTION

The objective of this study is to evaluate the potential of the test substance to induce cytogenetic damage in the bone marrow cells of mice (micronucleus test).

Apart from detecting chromosome breakage events (clastogenesis), the micronucleus test is capable of detecting chemicals which induce whole chromosome loss (aneuploidy) in the absence of clastogenic activity.

The basis of this test is the increase in the number of micronuclei in immature polychromatic erythrocytes in the bone marrow of mice exposed to a chemical which induces cytogenetic damage. Chromosomal fragments or entire chromosomes which are left behind at cell division will not be incorporated into the nuclei of daughter cells. Most of these fragments condense and form one or more micronuclei in the cytoplasm. The visualization of micronuclei is facilitated in erythrocytes because their nucleus is extruded during erythropoiesis.

Substances which inhibit either proliferation or maturation of erythroblasts and those which kill nucleated cells, decrease the proportion of polychromatic erythrocytes (PE) when compared to normochromatic erythrocytes (NE). Thus, the cytotoxicity of a substance can be determined by a decrease in the PE/NE ratio.

This protocol complies with:

. O.E.C.D. guideline No. 474, 26th May 1983 and revised Draft document of May 1994,
. E.E.C. Directive No. 92/69/E.E.C., Annex V, B_{12}, 31st July 1992.

The study will be conducted in compliance with the following Good Laboratory Practice Regulations:

. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire),
. O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

MATERIALS AND METHODS

1. TEST AND CONTROL SUBSTANCES

1.1 Identification

1.1.1 Test substance

. **Denomination** :
. **Batch No.** : Pil 4X
. **Description** : beige powder
. **Storage conditions** : room temperature, protected from light
. **Physico-chemical properties:** i.e. purity, composition, stability and expiry date which refer to the batch to be used and handling conditions will be indicated in the test substance data sheet (to be completed by the Sponsor). An analytical certificate will also be provided by the Sponsor.
. **Required amount** : 15 g

1.1.2 Vehicle

The vehicle will be selected according to the results of solubility tests performed before the preliminary toxicity test (1).

1.2 Preparation

The test substance will be given in the vehicle. The preparations will be performed immediately before use.

1.3 Positive controls

One known clastogen, dissolved in distilled water, will be used to check the sensitivity of the test system: cyclophosphamide (CPA, Endoxan-Asta) used at 50 mg/kg.

(1) Will be documented in the raw data and specified in the final report

2. TEST SYSTEM

2.1 Animals

Number: . some male or female mice for the preliminary toxicity test (the number used will be documented in the raw data)
. 56 mice: 28 males and 28 females for the cytogenetic study.

Strain: Swiss OF1/ICO:OF1 (IOPS Caw).

Reason for this choice: rodent species commonly requested by the international regulations for this type of study.

Breeder: Iffa Crédo, L'Arbresle, France.

Age: on the day of treatment the animals will be approximately 6 weeks old.

Veterinary care at C.I.T.: upon their arrival at C.I.T., the animals will be given a complete examination to ensure that the animals are in good clinical condition.

Acclimatization: at least 5 days before the day of treatment.

Constitution of groups: upon arrival, the animals will be allocated randomly by sex to the groups. Subsequently, each group will be assigned to a different treatment group.

Identification: individual tail identification upon treatment.

2.2 Environmental conditions

Upon their arrival at C.I.T., the animals will be housed in an animal room. The animal room conditions will be set as follows:

. temperature: 21 ± 2°C
. relative humidity: 50 ± 20%
. light/dark cycle: 12 h/12 h (07:00 - 19:00)
. ventilation: filtered and non-recycled fresh air.

The temperature and relative humidity are under continuous control and recording. The records are checked daily and retained.

The animals will be housed in polycarbonate cages and each cage will contain 5 mice of the same sex and group (3 for the supplementary animals, see § 3. TREATMENT). The floor of each cage will contain autoclaved sawdust (SICSA, Alfortville, France).

Bacteriological analysis and detection of possible contaminants (pesticides, heavy metals) of the sawdust will be made periodically.

2.3 Food and water

All animals will have free access to AO4 C pelleted diet (U.A.R., Villemoisson-sur-Orge, France) and tap water (filtered using a 0.22 micron filter) contained in bottles.

Bacteriological and chemical analysis of diet and water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed regularly.

Results are archived at C.I.T.

There are no known contaminants in the diet, water or sawdust at levels likely to influence the outcome of the study.

3. TREATMENT

3.1 Preliminary toxicity test

In order to determine the top dose and depending upon the amount of information supplied by the Sponsor, several preliminary assays might be necessary on groups of 6 animals (3 males and 3 females). Clinical signs and any mortality will be recorded for a period of 48 hours. At the end of this period, the animals will be sacrificed after CO_2 inhalation in excess.

3.2 Cytogenetic study

Rationale for dose selection

The choice of doses will be performed by the Study Director and will be documented in the raw data throughout the study, using the following criteria:

. if no observable toxic effects are produced at 2000 mg/kg/day then a limit test using one dose, i.e.: the top dose recommended by international regulations: 2000 mg/kg/day will be performed.

. the top dose is defined as the dose producing signs of toxicity, such that higher doses would be expected to produce lethality. The 2 other doses will be separately by no more than a factor between 2 and $\sqrt{10}$.

3.2.1 Groups and times of sacrifice

Group	Dose (mg/kg)	Animals per group Male	Female	Time of sacrifice (1)
vehicle	-	5	5	
treated	Low dose	5	5	
	Middle dose	5	5	24 h
	Top dose	5	5	
treated supplementary (2)	Top dose	3	3	
CPA	50	5	5	

(1) Sacrifice after the last administration.

(2) If mortality occurs during the study, these supplementary animals will replace those which die. If no mortality occurs, these animals will not be used for the study, i.e. bone marrows will not be taken.

3.2.2 Administration

. Route: oral, since it is a possible route of exposure in humans

. Frequency: two treatments at 24-hour interval

. Volume: ≤ 25 ml/kg

. CPA: oral route

3.2.3 Preparation of the smears

At the time of sacrifice, all the animals will be killed after CO_2 inhalation in excess. The femurs of the mice will be removed and the bone marrow will be eluted out with fetal calf serum. After centrifugation, the supernatant will be removed and the cells in the sediment will be suspended by shaking. A drop of this cell suspension will be placed and spread on a slide. The slides will be air-dried and stained with May-Grünwald-Giemsa. All the slides will be coded for scoring.

4. EVALUATION OF THE RESULTS

Analysis

For each animal, the micronuclei will be counted in 2000 polychromatic erythrocytes; the polychromatic (PE) and normochromatic (NE) erythrocyte ratio will be established by scoring a total of 1000 erythrocytes (PE + NE).

Treatment of results

All the individual data will be presented in tabular form. The number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio will be given for each animal.
The means and the standard deviations of the number of MPE and of the PE/NE ratio will be given for each experimental group.

Statistical analysis

The mean number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio from the treated groups will be compared to the simultaneous vehicle groups. The intergroup comparison will be performed using:

. for MPE, the X^2 test,

. for the PE/NE ratio, the Student's "t" test,

in which p = 0.05 will be used as the lowest level of significance.

Evaluation criteria

The following criteria will be used as an aid for determining a positive response:

. a statistically significant increase in the number of MPE when compared to the vehicle group,

. this increase should double the number of MPE of our historical data.

The results will be considered negative if the above criteria are not fully met.

Both biological and statistical significance will be considered together in the evaluation.

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5. PROCEDURES

The procedures used during the study will be those documented in the relevant C.I.T. procedures manual.

6. AMENDMENTS TO THE PROTOCOL

If necessary, amendments to the protocol will be made after agreement between the Study Director and the Study Monitor.

7. REPORTING

The Study Director will contact the Study Monitor when necessary.
The final report, in English, will contain all data collected throughout the study.
Number of copies of the final report: 3 (1 + 2 unbound)
Proposed issue of the draft report: 1 month after the end of the study.

8. QUALITY ASSURANCE UNIT

The Quality Assurance Unit will conduct inspections according to Good Laboratory Practice Regulations (as specified on page 2).
The dates on which the findings of these inspections are reported to the Study Director and C.I.T. Management will be specified in the final report.

9. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments
. slides

will be stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

10. TIME SCHEDULE

Beginning of the study: 1st fortnight of November 1994
End of the study: 2nd fortnight of December 1994

AMENDMENT TO PROTOCOL

STUDY No.: **12287 MAS**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 01

Justification: complementary information to the protocol.
Date of application: 6.12.94

MATERIALS AND METHOD

1. TEST AND CONTROL SUBSTANCES

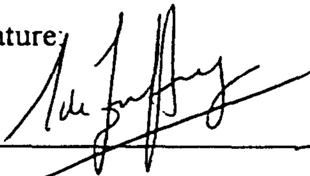
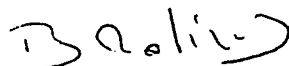
1.1 Identification

.Storage conditions: room temperature, away from light, and in addition away from humidity (from 1.8.94 onwards); the content of each flask will also be kept under nitrogen until use.

10. TIME SCHEDULE

Date of the beginning of the study: 6.12.94

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	
Date: 13.2.95	Date: 13.2.95	Date: 3/3/95
Signature:	Signature:	Signature:

AMENDMENT TO PROTOCOL

STUDY No.: **12287 MAS**

SPONSOR: **Société**

TEST SUBSTANCE:

AMENDMENT No.: 02 Page 1 / 1

Justification: modification to the protocol.

Date of application: 18.4.95

MATERIALS AND METHODS

2. TEST SYSTEM

2.1 Animals

Age: on the day of treatment the animals will be approximately 7 weeks old for the third cytogenetic study.

3. TREATMENT

A third cytogenetic study will be performed since the EP/EN ratio of the first and second studies was slightly lower than usually obtained in our Laboratory.
The conditions will be as follows:

Group	dose (mg/kg/day)	Animals per group Male	Animals per group Female	Time of sacrifice (1)
vehicle	-	5	5	
treated	375	5	5	
treated	750	5	5	24 h
treated	1500	5	5	
CPA	50	5	5	

(1): Sacrifice after the last administration

The results of the first and second cytogenetic studies will not be included in the final report, slides corresponding to those studies will be archived.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	
Date: 10.5.95	Date: 10.5.95	Date: 8/6/95
Signature:	Signature:	Signature:

AMENDMENT TO PROTOCOL

STUDY No.: **12287 MAS**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 03

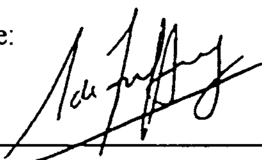
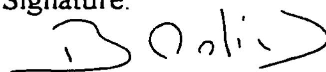
Justification: modification to the protocol.
Date of application: 24.2.95

MATERIALS AND METHOD

3. TREATMENT

3.2.3 Preparation of the smears

The slides will be air-dried and stained with Giemsa.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	
Date: 2.6.95	Date: 2.6.95	Date: 19/6/95
Signature: 	Signature: 	Signature:

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SPONSOR

STUDY TITLE
MICRONUCLEUS TEST
BY ORAL ROUTE IN RATS

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR
Brigitte Molinier

STUDY COMPLETION DATE
25th October 1995

PERFORMING LABORATORY
Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER
12931 MAR (95/1/044)

SUMMARY

The objective of this study was to evaluate the potential of the test substance (batch No. pil 4X) to induce damage to the chromosomes or the mitotic apparatus in bone marrow cells of rats.

Methods

A preliminary toxicity test was performed to define the doses to be used for the cytogenetic study.
For the main study, three groups of five male and five female Sprague-Dawley rats received the test substance twice separated by 24 hours by oral route, at doses of 500, 1000 and 2000 mg/kg/day.
One group of five males and five females received the vehicle alone (distilled water) by oral route, and acted as control group.
One group of five males and five females received cyclophosphamide once at 15 mg/kg, by oral route.

All the animals were killed 24 hours after the last administration. Bone marrow smears were then prepared.

For each animal, the micronuclei were counted in 2000 polychromatic erythrocytes. The polychromatic (PE) and normochromatic (NE) erythrocyte ratio was established by scoring a total of 1000 erythrocytes (PE + NE).

Results

In the two vehicle control groups, the mean values of micronucleated polychromatic erythrocytes (MPE) were within the range of published data.

Cyclophosphamide induced a highly significant increase ($p < 0.001$) in the number of MPE, indicating the sensitivity of the test system under our experimental conditions.

In all groups treated with the mean values of MPE were similar to those of their respective vehicle groups, and no statistically significant differences were observed. The PE/NE ratio was not statistically significantly different from that of the respective vehicle control groups.

Conclusion

Under our experimental conditions, the test substance (batch No. pil 4X) did not induce damage to the chromosomes or the mitotic apparatus in bone marrow cells of rats when treated by oral route at 500, 1000 and 2000 mg/kg/day in the micronucleus test.

1. INTRODUCTION

This study was performed at the request of Société

The objective of this study was to evaluate the potential of the test substance, to induce damage to the chromosomes or the mitotic apparatus in bone marrow cells of rats.

Apart from detecting chromosome breakage events (clastogenesis), the micronucleus test is capable of detecting chemicals which induce whole chromosome loss (aneuploidy) in the absence of clastogenic activity.

The basis of this test is the increase in the number of micronucleated polychromatic erythrocytes in the bone marrow of rats exposed to a chemical which induces cytogenetic damage. Chromosomal fragments or entire chromosomes which are left behind at cell division are not incorporated into the nuclei of daughter cells. Most of these fragments condense and form one or more micronuclei in the cytoplasm. The visualization of micronuclei is facilitated in erythrocytes because their nucleus is extruded during erythropoiesis.

Substances which inhibit either proliferation or maturation of erythroblasts and those which kill nucleated cells, decrease the proportion of polychromatic erythrocytes (PE) when compared to normochromatic erythrocytes (NE). Thus, the cytotoxicity of a substance can be determined by a decrease in the PE/NE ratio.

This test was performed according to the method described by Schmid (1975) and modified by Salamone and coll. (1980).

This study was designed in accordance with the following guidelines:

. O.E.C.D. guideline No. 474, 26th May 1983 and revised Draft document of December 1994.
. Commission Directive No. 92/69/E.E.C., Annex V, B_{12}, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by

Documentation supplied by the Sponsor identified the test substance as follows:

. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4X
  - labelling: Pil 4X
. description: beige powder
. quantity and container: 4 kg contained in 1 plastic pot, this quantity was dispatched at C.I.T. into 7 flasks
. date of receipt: 26.7.94
. storage conditions: room temperature, protected from light, from humidity from 1.8.94 and conserved under nitrogen until use from 2.1.95
. purity: 99.8%.

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Data relating to the characterization of the test substance are documented in an analytical certificate (presented in paragraph 6) provided by the Sponsor.

2.1.2 Vehicle

The vehicle was distilled water (C.I.T., millipore).

2.1.3 Preparation

The test substance was given in the vehicle. The test substance was dissolved in the vehicle in order to achieve the concentrations of 50, 100 and 200 mg/ml and then homogenized using a magnetic stirrer.
The preparations were made immediately before use.

2.1.4 Positive control

The positive control was cyclophosphamide (CPA Endoxan-Asta).
It was dissolved in distilled water at a concentration of 1.5 mg/ml.
The preparation was made immediately before use.

2.2. TEST SYSTEM

2.2.1 Animals

Number: . three male and three female rats for the preliminary toxicity test
. 56 rats: 28 males and 28 females for the cytogenetic study.
Strain: Sprague-Dawley ICO: OFA-SD (IOPS Caw).
Reason for this choice: rodent species commonly requested by the international regulations for this type of study.
Breeder: Iffa Crédo, L'Arbresle, France.
Age: on the day of treatment the animals were approximately seven weeks old.
Veterinary care at C.I.T.: upon their arrival at C.I.T., the animals were given a complete examination to ensure that the animals are in good clinical condition.
Acclimatization: at least five days before the day of treatment.
Constitution of groups: upon arrival, the animals were randomly allocated to the groups by sex. Subsequently, each group was assigned to a different treatment group.
Identification: individual tail identification upon treatment.

2.2.2 Environmental conditions

Upon their arrival at C.I.T., the animals were housed in an animal room. The animal room conditions were set as follows:
. temperature: 21 ± 2°C
. relative humidity: 50 ± 20%
. light/dark cycle: 12 h/12 h (07:00 - 19:00)
. ventilation: about 12 cycles/hour of filtered non-recycled fresh air.

The housing conditions (temperature, relative humidity and ventilation) are checked regularly.
The animals were housed in polycarbonate cages and each cage contained five animals of the same sex and group (three for the supplementary animals, see § 2.3.2.1). Each cage contained autoclaved sawdust (SICSA, Alfortville, France).
Bacteriological analysis and detection of possible contaminants (pesticides, heavy metals) of the sawdust are conducted periodically by approved laboratories. Results are archived at C.I.T.

2.2.3 Food and water

All animals had free access to AO4 C pelleted sustenance diet (U.A.R., Villemoisson-sur-Orge, France) and tap water (filtered using a 0.22 micron filter).
Each batch of food was analysed (composition and contaminants) by the suplier.
Bacteriological and chemical analyses of diet and water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed regularly by approved laboratory.
Results are archived at C.I.T.

There are no known contaminants in the diet, water or sawdust at levels likely to influence the outcome of the study.

2.3. EXPERIMENTAL DESIGN

Rationale for dose selection

The choice of doses will be performed according to the following criteria specified in international regulations.

. top dose:
- if no observable toxic effects are produced at 2000 mg/kg/day, this will be the top dose, as recommended by international regulations
- if observable toxic effects are produced, the top dose is defined as the dose producing signs of toxicity, such that a higher dose would be expected to produce lethality.

The two other doses will be separated by no more than a factor between 2 and $\sqrt{10}$.

2.3.1 Preliminary toxicity test

In order to determine the top dose and depending upon the amount of information supplied by the Sponsor, one preliminary assay was performed on group of six animals (three males and three females). Clinical signs and any mortality were recorded for a period of 48 hours. At the end of this period, the animals were sacrificed after CO_2 inhalation in excess.

2.3.2 Cytogenetic study

2.3.2.1 Groups and times of sacrifice

Group	Dose (mg/kg/day)	Animals per group Male	Animals per group Female	Time of sacrifice (1)
vehicle	-	5	5	
treated	500	5	5	
	1000	5	5	24 h
	2000	5	5	
treated supplementary (2)	2000	3	3	
CPA	15	5	5	

(1) Sacrifice after the last administration.
(2) Since no mortality occurs, these animals were not used for the study, i.e. bone marrows were not taken.

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2.3.2.2 Administration

The test substance was administered by the oral route using a dose volume of 10 ml/kg. Each animal was given the test substance twice separated by 24 hours.
The quantity of the test substance administered to each animal was adjusted according to the body weight recorded at the time of dosing.
The vehicle control animals received the vehicle alone, under the same conditions.
The positive control animals received cyclophosphamide alone, by oral route.

2.3.2.3 Preparation of smears

At the time of sacrifice, all the animals were killed after CO_2 inhalation in excess. The femurs of the rats were removed and the bone marrow eluted out using fetal calf serum. After centrifugation, the supernatant was removed and the cells in the sediment were suspended by shaking. A drop of this cell suspension was placed and spread on a slide. The slides were air-dried and stained with Giemsa. All the slides were coded for scoring.

2.4. EVALUATION OF THE RESULTS

Analysis

For each animal, the micronuclei were counted in 2000 polychromatic erythrocytes; the polychromatic (PE) and normochromatic (NE) erythrocyte ratio was established by scoring a total of 1000 erythrocytes (PE + NE).

Treatment of results

All the individual data are presented in tabular form. The number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio are given for each animal.
The means and the standard deviations of the number of MPE and the PE/NE ratio are given for each experimental group.

Statistical analysis

The mean number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio from the treated groups were compared to the simultaneous vehicle group. The intergroup comparison was performed using:

. for MPE, the χ^2 test,
. for the PE/NE ratio, the Student's "t" test,
in which p = 0.05 was used as the lowest level of significance.

Evaluation criteria

Biological relevance of the results was considered first. In addition, the following criteria may be used as an aid for determining a positive response:

. a statistically significant increase in the number of MPE when compared to the vehicle group.

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2.5. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments
. slides

are stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for five years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

2.6. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date
Protocol approved by:	
. Study Director	10.4.95
. Sponsor	2.5.95
Preliminary toxicity test	
. Beginning of treatment	20.6.95
Cytogenetic test	
. Arrival of the animals and randomization	17.8.95
. Identification of the animals and first day of treatment	22.8.95
Second day of treatment	23.8.95

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3. RESULTS

3.1. PRELIMINARY TOXICITY TEST (table 1)

The top dose for the cytogenetic study, 2000 mg/kg/day was administered to three males and three females.

Since no observable toxic effects were noted, the top dose chosen for the main study was 2000 mg/kg/day. The two other doses were 1000 and 500 mg/kg/day.

3.2. CYTOGENETIC TEST (tables 2 and 3)

No clinical signs and no mortality were observed in all the animals of both sexes.

The mean values of MPE, as well as the PE/NE ratio for the vehicle and positive controls, were as specified in the acceptance criteria and within the range of our historical data.

The mean values of MPE, as well as the PE/NE ratio in the groups treated with the test substance, were equivalent to those of the vehicle group.

4. CONCLUSION

Under our experimental conditions, the test substance (batch No. pil 4X) did not induce damage to the chromosomes or the mitotic apparatus in bone marrow cells of rats when treated by oral route at 500, 1000 and 2000 mg/kg/day in the micronucleus test.

5. REFERENCES

Schmid, W.: The micronucleus test. Mutation Research, 31, 1975, 9-15.

Salamone, M.; Heddle, J.; Stuart, E. and Katz, M.: Toward an improved micronucleus test. Studies on 3 model agents, mitomycin C, CPA and dimethylbenzanthracene. Mutation Research, 74, 1980, 347-356.

Heddle, J. and Salamone, M.: The micronucleus assay. I. in vivo. In: short-term test for chemical carcinogens. Springer Verlag New-York (Stich and San eds.), 1981, 243-249.

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le 1er Juillet 1994

Bulletin d'analyse certifié
Pil 4 X

Aspect	Poudre beige
Titre par AgNO3	97,2 %
Titre par NaOH	96.1 %
HPLC	% relatif à 258 nm

Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
Impureté non identifiée (λ_{max} = 222 nm)	0.01 %
	99.8 %
Impureté non identifiée(λ_{max} = 258 nm)	0.18 %

Responsable Développement
Analytique et Qualité

Lot utilisable jusqu'au 25/11/95

10/11/94

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INTRODUCTION

The objective of this study is to evaluate the potential of the test substance to induce cytogenetic damage in the bone marrow cells of rats (micronucleus test).

Apart from detecting chromosome breakage events (clastogenesis), the micronucleus test is capable of detecting chemicals which induce whole chromosome loss (aneuploidy) in the absence of clastogenic activity.

The basis of this test is the increase in the number of micronuclei in immature polychromatic erythrocytes in the bone marrow of rats exposed to a chemical which induces cytogenetic damage. Chromosomal fragments or entire chromosomes which are left behind at cell division will not be incorporated into the nuclei of daughter cells. Most of these fragments condense and form one or more micronuclei in the cytoplasm. The visualization of micronuclei is facilitated in erythrocytes because their nucleus is extruded during erythropoiesis.

Substances which inhibit either proliferation or maturation of erythroblasts and those which kill nucleated cells, decrease the proportion of polychromatic erythrocytes (PE) when compared to normochromatic erythrocytes (NE). Thus, the cytotoxicity of a substance can be determined by a decrease in the PE/NE ratio.

This protocol complies with:

- O.E.C.D. guideline No. 474, 26th May 1983 and revised Draft document of December 1994,
- E.E.C. Directive No. 92/69/E.E.C., Annex V, B_{12}, 31st July 1992.

The study will be conducted in compliance with the following Good Laboratory Practice Regulations:

- Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire),
- O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

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MATERIALS AND METHODS

1. TEST AND CONTROL SUBSTANCES

1.1 Identification

1.1.1 Test substance

. **Denomination** :
. **Batch No.** : Pil 4X
. **Description** : beige powder
. **Storage conditions:** at room temperature, protected from light and humidity and kept under nitrogen
. **Physico-chemical properties:** i.e. purity, composition, stability and expiry date which refer to the batch to be used and handling conditions will be indicated in the test substance data sheet (to be completed by the Sponsor). An analytical certificate will also be provided by the Sponsor.
. **Required amount:** 20 g

1.1.2 Vehicle

. **Denomination:** distilled water

1.2 Preparation

The test substance will be given in the vehicle. The preparations will be performed immediately before use.

1.2.1 Positive controls

One known clastogen, dissolved in distilled water, will be used to check the sensitivity of the test system: cyclophosphamide (CPA, Endoxan-Asta) used at 15 mg/kg.

2. TEST SYSTEM

2.1 Animals

Number: . some male or female rats for the preliminary toxicity test (the number used will be documented in the raw data)
. 56 rats: 28 males and 28 females for the cytogenetic study.

Strain: Sprague-Dawley rats.

Reason for this choice: rodent species commonly requested by the international regulations for this type of study.

Breeder: Iffa Crédo, L'Arbresle, France.

Age: on the day of treatment the animals will be approximately 7 weeks old.

Veterinary care at C.I.T.: upon their arrival at C.I.T., the animals will be given a complete examination to ensure that the animals are in good clinical condition.

Acclimatization: at least 5 days before the day of treatment.

Constitution of groups: upon arrival, the animals will be allocated randomly by sex to the groups. Subsequently, each group will be assigned to a different treatment group.

Identification: individual tail identification upon treatment.

2.2 Environmental conditions

Upon their arrival at C.I.T., the animals will be housed in an animal room. The animal room conditions will be set as follows:
. temperature: 21 ± 2°C
. relative humidity: 50 ± 20%
. light/dark cycle: 12 h/12 h (07:00 - 19:00)
. ventilation: filtered and non-recycled fresh air.

The temperature and relative humidity are under continuous control and recording. The records are checked daily and retained.

The animals will be housed in polycarbonate cages and each cage will contain 5 rats of the same sex and group (3 for the supplementary animals, see § 3. TREATMENT). The floor of each cage will contain autoclaved sawdust (SICSA, Alfortville, France).

Bacteriological analysis and detection of possible contaminants (pesticides, heavy metals) of the sawdust will be made periodically.

2.3 Food and water

All animals will have free access to AO4 C pelleted diet (U.A.R., Villemoisson-sur-Orge, France) and tap water (filtered using a 0.22 micron filter) contained in bottles.

Bacteriological and chemical analysis of diet and water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed regularly.

Results are archived at C.I.T.

There are no known contaminants in the diet, water or sawdust at levels likely to influence the outcome of the study.

3. TREATMENT

3.1 Preliminary toxicity test

In order to determine the top dose and depending upon the amount of information supplied by the Sponsor, several preliminary assays might be necessary on groups of 6 animals (3 males and 3 females). Clinical signs and any mortality will be recorded for a period of 48 hours. At the end of this period, the animals will be sacrificed after CO_2 inhalation in excess.

3.2 Cytogenetic study

Rationale for dose selection

The choice of doses will be performed by the Study Director and will be documented in the raw data throughout the study, using the following criteria:

. if no observable toxic effects are produced at 2000 mg/kg then a limit test using one dose, i.e.: the top dose recommended by international regulations: 2000 mg/kg will be performed.

. the top dose is defined as the dose producing signs of toxicity, such that higher doses would be expected to produce lethality. The 2 other doses will be separately by no more than a factor between 2 and $\sqrt{10}$.

3.2.1 Groups and times of sacrifice

Group	Dose (mg/kg/day)	Animals per group Male	Female	Sampling time
vehicle	-	5	5	
treated	Low dose	5	5	
	Middle dose	5	5	24 h
	Top dose	5	5	
treated supplementary (1)	Top dose	3	3	
CPA	15	5	5	

(1) If mortality occurs during the study, these supplementary animals will replace those which die. If no mortality occurs, these animals will not be used for the study, i.e. bone marrow will not be taken.

3.2.2 Administration

. Route: oral, since it is expected to assure an absorption at least equal to the dermal route, which is the route of exposure in human.
. Frequency: 2 treatments at 24-hour interval
. Volume: ≤ 20 ml/kg
. CPA: oral route, only one administration

3.2.3 Preparation of the smears

At the sampling time, all the animals will be killed after CO_2 inhalation in excess. The femurs of the rats will be removed and the bone marrow will be eluted out with fetal calf serum. After centrifugation, the supernatant will be removed and the cells in the sediment will be suspended by shaking. A drop of this cell suspension will be placed and spread on a slide. The slides will be air-dried and stained with May-Grünwald-Giemsa. All the slides will be coded for scoring.

4. EVALUATION OF THE RESULTS

Analysis

For each animal, the micronuclei will be counted in 2000 polychromatic erythrocytes; the polychromatic (PE) and normochromatic (NE) erythrocyte ratio will be established by scoring a total of 1000 erythrocytes (PE + NE).

Treatment of results

All the individual data will be presented in tabular form. The number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio will be given for each animal.
The means and the standard deviations of the number of MPE and of the PE/NE ratio will be given for each experimental group.

Statistical analysis

The mean number of micronucleated polychromatic erythrocytes (MPE) and the PE/NE ratio from the treated groups will be compared to the simultaneous vehicle groups. The intergroup comparison will be performed using:
. for MPE, the X^2 test,
. for the PE/NE ratio, the Student's "t" test,
in which p = 0.05 will be used as the lowest level of significance.

Evaluation criteria

The following criteria will be used as an aid for determining a positive response:
. a statistically significant increase in the number of MPE when compared to the vehicle group,
. this increase should double the number of MPE of our historical data.

The results will be considered negative if the above criteria are not fully met.

Both biological and statistical significance will be considered together in the evaluation.

5. PROCEDURES

The procedures used during the study will be those documented in the relevant C.I.T. procedures manual.

6. AMENDMENTS TO THE PROTOCOL

If necessary, amendments to the protocol will be made after agreement between the Study Director and the Study Monitor.

7. REPORTING

The Study Director will contact the Study Monitor when necessary.
The final report, in English, will contain all data collected throughout the study.
Number of copies of the final report: 3 (1 + 2 unbound)
Proposed issue of the draft report: 1 month after the end of the study.

8. QUALITY ASSURANCE UNIT

The Quality Assurance Unit will conduct inspections according to Good Laboratory Practice Regulations (as specified on page 2).
The dates on which the findings of these inspections are reported to the Study Director and C.I.T. Management will be specified in the final report.

9. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments
. slides

will be stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

10. TIME SCHEDULE

Beginning of the study: 1st fortnight of July, 1995
End of the study: 2nd fortnight of August, 1995

AMENDMENT TO PROTOCOL

STUDY No.: **12931 MAR**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 01

Justification: information concerning the doses perfomed, the coloration of the slides and the time schedule.
Date of application: 20.6.95

MATERIALS AND METHODS

3. TREATMENT

3.2 Cytogenetic study

Rationale for dose selection

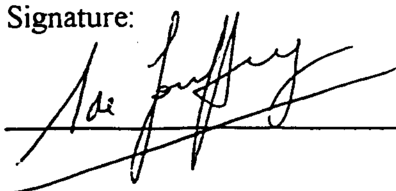
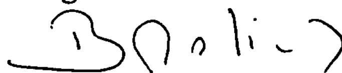
. if no observable toxic effects are produced at 2000 mg/kg, then this will be the top dose used. Two other doses will be also perfomed separated by no more than a factor between 2 and $\sqrt{10}$.

3.2.3 Preparation of the smears

The slides will be air-dried and stained with Giemsa.

10. TIME SCHEDULE

Date of the beginning of the study: 20.6.95.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	
Date: 24.7.95	Date: 24.7.95	Date:
Signature: 	Signature: 	Signature:

AMENDMENT TO PROTOCOL

STUDY No.: **12931 MAR**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 02

Page 1 / 1

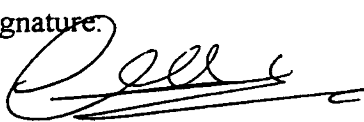
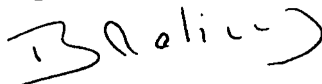
Date of application: 7.8.95

MATERIALS AND METHODS

3. TREATMENT

A second cytogenetic study will be performed since the EP/EN ratio of the first study was slightly lower than usually obtained in our Laboratory. Indeed after scoring of five slides of the vehicle control group, the value obtained was between 0.39 and 0.66.

The results of the first cytogenetic study will not be included in the final report, the slides corresponding to the first study will be archived, but not scored except as mentioned above.

Scientific management	Study Director	Study Monitor
P.o J.F Le Bigot or S. de Jouffrey	B. Molinier	
Date: 7.8.95 M. Attia	Date: 7.8.95	Date:
Signature: 	Signature: 	Signature: 10/8/95

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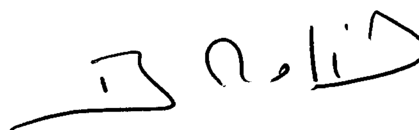
STUDY TITLE: **MICRONUCLEUS TEST BY ORAL ROUTE IN RATS**

MOTIVE: Correction on page 5, § Results, first line:
"In the vehicle control group" instead of "In the two vehicle control groups".

SCIENTIST INVOLVED IN THIS AMENDMENT:

Toxicology



B. Molinier Date: 20.2.96
Study Director
Doctor of Toxicology
Toxicologist
Head of Genetic Toxicology

QUALITY ASSURANCE UNIT:

L. Valette-Talbi Date: 20.2.96
Doctor of Biochemistry
Head of Quality Assurance Unit and
Scientific Archives

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SPONSOR

STUDY TITLE

SKIN SENSITIZATION TEST
IN GUINEA-PIGS
(Buehler test: 3 applications)

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

6th October 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12910 TSG (95/1/043)

SUMMARY

At the request of Société , the potential of the test substance, (batch No. Pil 4X), to induce delayed contact hypersensitivity following cutaneous application was evaluated in guinea-pigs according to the method established by Buehler and O.E.C.D. (No. 406, 17th July 1992) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

Thirty guinea-pigs (15 males and 15 females) were allocated to 2 groups: a control group 1 (5 males and 5 females) and a treated group 2 (10 males and 10 females).
During a 2-week induction period, animals of the treated group 2 received on days 1, 8 and 15 an application of 0.5 ml of the test substance at a concentration of 50% (w/w) in the vehicle. The test substance was prepared on a dry gauze pad and then applied on an area of the skin of approximately 4 cm^2 on the anterior left flank. After each application, the test substance was held in place for 6 hours by means of an occlusive dressing. After a 14-day rest period (from day 15 to day 28), a cutaneous challenge application of 0.5 ml of the test substance at a concentration of 5% (w/w) in the vehicle (right flank) and the application of 0.5 ml of the test substance at a concentration of 20% (w/w) in the vehicle (left flank) were performed on a non-treated area of the posterior region of the animals. Cutaneous reactions were evaluated 24 and 48 hours after the removal of the patches of the challenge application by comparing the reactions on both flanks.

At the end of the observation period, the animals were killed. In the absence of macroscopic cutaneous reactions in all the animals, no cutaneous samples were taken from the challenge application sites.

The sensitivity of the guinea-pigs in C.I.T. experimental conditions were checked in a recent study with a positive sensitizer: Dinitro 2.4 Chlorobenzene. During induction period, the test substance was applied at 1% (days 1 and 8) and 0.1% (day 15) concentrations. At cutaneous challenge application, 0.5% were tested on right flank and paraffin oil on the left flank.

Results

No clinical signs and no deaths were noted during the study.

During the induction period, very slight to slight cutaneous reactions were observed in 8/20 animals.

After 24 and 48 hours following the removal of the patches of the cutaneous challenge application of the test substance, no cutaneous reactions were recorded.

In a recent study, the species and strain which were used showed a satisfactory sensitization response in 50% animals using a positive sensitizer (appendix 5).

Conclusion

Under our experimental conditions and according to the method established by Buehler, the application of the test substance, (batch No. Pil 4X), at a concentration of 50% (w/w) (induction) and 5 or 20% (w/w) (challenge), did not induce any sensitization reaction in guinea-pigs.

Labelling

Commission Directive 93/21/E.E.C.

Labelling not indicated for the test substance.

1. INTRODUCTION

The objective of this study, performed according to the Buehler method (1), was to evaluate the potential of the test substance, to induce delayed contact hypersensitivity in guinea-pigs.

The results of the study are of value in predicting the contact sensitization potential of the test substance in Man.

During an induction period, 3 cutaneous applications of the test substance were performed, covered by an occlusive dressing for 6 hours on each occasion. After a rest period of 14 days, a challenge application with the test substance was performed in order to provoke a cutaneous sensitization reaction.

The study was conducted in compliance with:
. O.E.C.D. guideline No. 406, 17th July 1992.
. E.C. Directive No. 92/69/E.E.C., B_6, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4X
  - labelling: Pil 4X
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: at room temperature, protected from light and from humidity from 1.8.94. The test substance, deconditioned in flasks numbered from 1 to 7, was stored under nitrogen gas from 2.1.95 until use of each flask
. purity: 99.8%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Vehicle

The vehicle used was distilled water, batch No. 1811 (Biosédra, 92240 Malakoff, France).

(1) Buehler, E. V.: Toxicol. Appl. Pharmacol. 6, 341 (1964), also Arch. Dermatol. 91, 171 (1965).

2.1.3 Preparation

The test substance was prepared in the vehicle.

2.2. TEST SYSTEM

2.2.1 Animals

Species and strain: Dunkin-Hartley guinea-pigs.
Reason for this choice: species recommended by the international regulations for sensitization studies. The strain used has been shown to produce a satisfactory sensitization response using known positive sensitizers.
Breeder: Centre d'Elevage Lebeau, 78950 Gambais, France.
Number: 30 animals (15 males and 15 females).
Allocation of the animals to the groups: on day -1, the animals were weighed and randomly allocated to 2 groups: a control group 1 consisting of 10 animals (5 males and 5 females) and a treated group 2 consisting of 20 animals (10 males and 10 females).
Age and weight: on day 1 of treatment, the animals had 1-3 months old and had a mean body weight of 334 ± 19 g for the males and 329 ± 17 g for the females.
Acclimatization: at least 5 days before the beginning of the study.
Identification of the animals: the animals were identified individually by an ear-tattoo.

2.2.2 Environmental conditions

During the acclimatization period and throughout the study, the conditions in the animal room were as follows:
. temperature: 21 ± 2°C
. relative humidity: 30 to 70%
. light/dark cycle: 12 h/12 h
. ventilation: about 12 cycles/hour of filtered, non-recycled air.
During the acclimatization period and throughout the study, the animals were individually housed in polycarbonate cages (48 x 27 x 20 cm) equipped with a polypropylene bottle. Sifted and dusted sawdust was provided as litter (SICSA, 92142 Alfortville, France). An analysis of potential residues and major contaminants is performed periodically (Laboratoire Wolff, 92110 Clichy, France).

2.2.3 Food and water

During the study, the animals had free access to "Guinea-pigs sustenance reference 106 diet" (U.A.R., 91360 Villemoisson-sur-Orge, France).
Food was periodically analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was contained in bottles.
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Preliminary test

A preliminary test was performed to define the Maximum Non-Irritant Concentration to be tested.

0.5 ml of the test substance prepared in the vehicle was applied to a clipped area of the skin of approximately 4 cm^2. The test substance was prepared on a dry gauze pad and then held in place by means of an occlusive dressing for 6 hours.
Twenty-four and 48 hours followed removal of the patches, scoring of cutaneous reactions was performed.
No residual test substance was observed upon removal of the dressings.

2.3.2 Main test

2.3.2.1 Preparation of the animals

The application sites of all animals were clipped and shaved 24 hours before each application, as follows:

- the anterior flank (4 cm x 4 cm) during the induction period,
- the posterior right and left flanks (4 cm x 4 cm) on day 28, day before the challenge application.

2.3.2.2 Induction period

The animals of the treated group 2 were treated by cutaneous application once a week for 2 consecutive weeks. On days 1, 8 and 15, 0.5 ml of the test substance at a concentration of 50% (w/w) in the vehicle was prepared on a 4 cm^2 dry gauze pad (Semes France, 54183 Heillecourt, France) then applied to the anterior left flank. The test substance was prepared using a 1 ml polypropylene single use syringe (0.01 ml graduations, Térumo: C.M.L., 77140 Nemours, France). An adhesive anallergenic waterproof plaster (Laboratoires des Professions Médicales, 92240 Malakoff, France) was placed around the trunk of the animals and held in place for 6 hours.
On removal of the dressing, any residual test substance was removed by means of a gauze pad moistened using water.

The animals of the control group 1 were given in the same experimental conditions vehicle on the anterior left flank.

2.3.2.3 Rest period

Following the induction period, the animals received no treatment for 14 days, from day 15 to day 28 inclusive.

2.3.2.4 Challenge application

On day 29, all animals received an application of 0.5 ml of the test substance at a concentration of 5% (w/w) in the vehicle on an area of 4 cm^2 (2 cm x 2 cm) of the posterior right flank (not treated before) and 0.5 ml of the test substance at a concentration of 20% (w/w) in the vehicle on the posterior left flank.
The test substance was held in contact with the skin for 6 hours by means of an adhesive hypoallergenic dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and an adhesive anallergenic waterproof plaster (Laboratoires des Professions Médicales, 92240 Malakoff, France).
On removal of the dressing, any residual test substance was removed by means of a gauze pad moistened using water.

2.4. SCORING OF CUTANEOUS REACTIONS

Twenty-four hours after each removal dressing during the induction period, then 24 and 48 hours after removal of the dressing following the challenge application, the application sites were observed in order to evaluate cutaneous reactions according to the following scale:

Erythema and eschar formation

. No erythema 0
. Very slight erythema (barely perceptible) 1
. Well-defined erythema 2
. Moderate to severe erythema 3
. Severe erythema (beet redness) to slight eschar formation (injuries in depth) 4

Oedema formation

. No oedema 0
. Very slight oedema (barely perceptible) 1
. Slight oedema (visible swelling with well-defined edges) 2
. Moderate oedema (visible swelling raised more than 1 millimetre) 3
. Severe oedema (visible swelling raised more than 1 millimetre and extending beyond area of exposure) 4

Any other lesions were noted.

2.5. CLINICAL EXAMINATIONS

The animals were observed twice a day during the study in order to record clinical signs and to check for mortality.

2.6. BODY WEIGHT

The animals were weighed individually on the day of allocation into the groups, on the first day of the study (day 1) then on days 8, 15, 22 and 31.

2.7. PATHOLOGY

2.7.1 Necropsy

On day 31, after the 48-hour observation period, the animals were killed by CO_2 inhalation in excess.

2.7.2 Skin samples

No skin samples were taken.

2.7.3 Microscopic examination

No histological examination was performed.

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2.8. DETERMINATION OF THE ALLERGENICITY LEVEL

The animals show a positive reaction if reactions are different from those noted in control animals and if the macroscopic cutaneous reactions are clearly visible (erythema and/or oedema score ≥ 2) or, if the "doubtful" macroscopic reactions are confirmed by the microscopic examination as being due to the sensitization process. Sensitization reactions are characterized at microscopic examination by basal spongiosis, reactional acanthosis of the epidermis and infiltration of mononucleated cells into the dermis (1).

According to the E.E.C. directive 93/21/E.E.C. published in the Journal Officiel des Communautés Européennes, when the reactions are positive in at least 15% of the treated animals, the test substance has sensitization properties and the sentence "R 43: Induces sensitization by contact with the skin" must apply.

(1) Duprat, P. ; Delsaut. L. ; Gradiski, D. ; Lepage, M. : Investigations histopathologiques et cytologiques lors de la mise en évidence, chez le cobaye, d'une allergie cutanée de type retardé. Revue Méd. Vét. 127: 7, 1083-1101 (1976).

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2.9. SUMMARY DIAGRAMS

Figure 1: control group

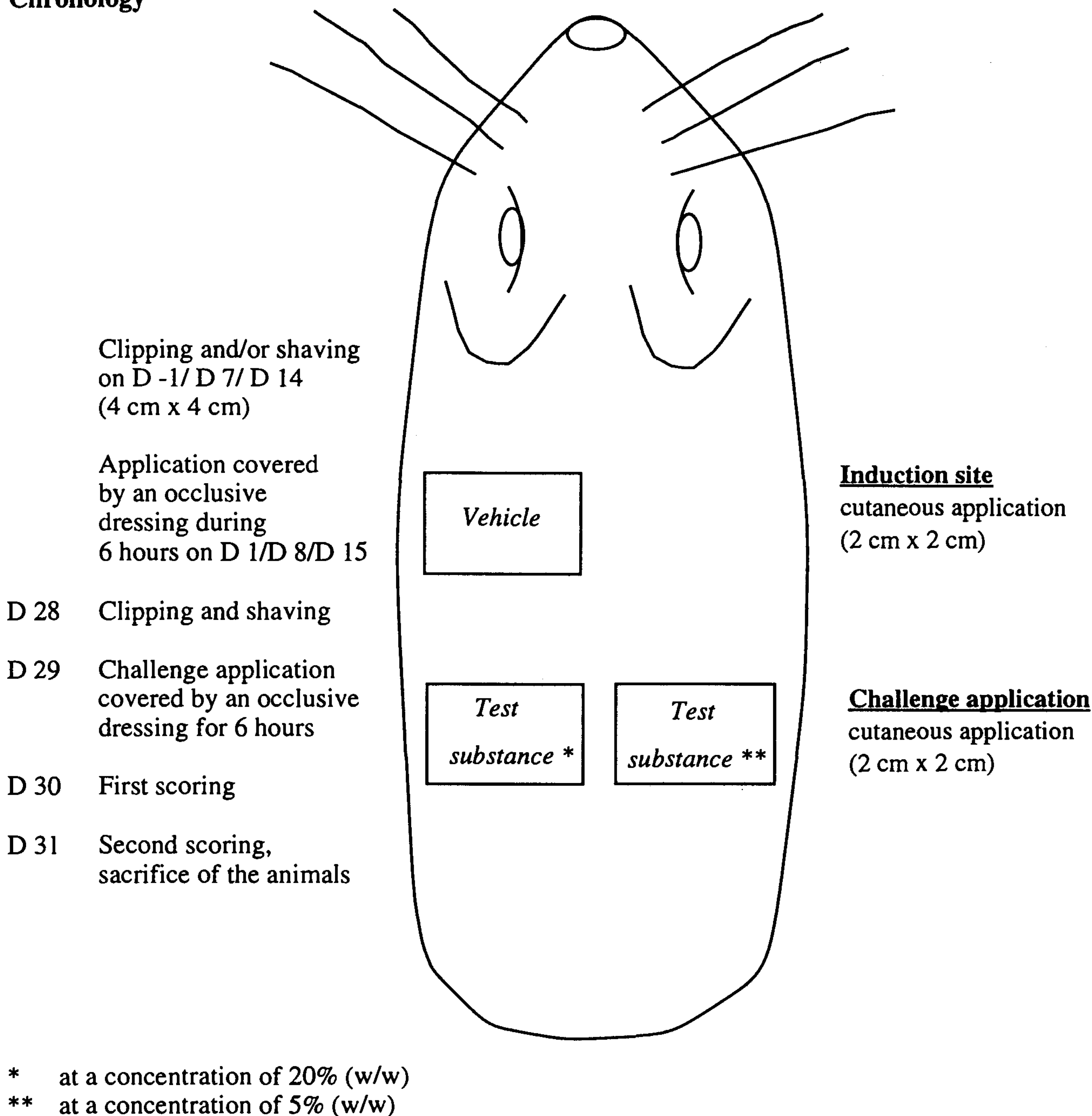

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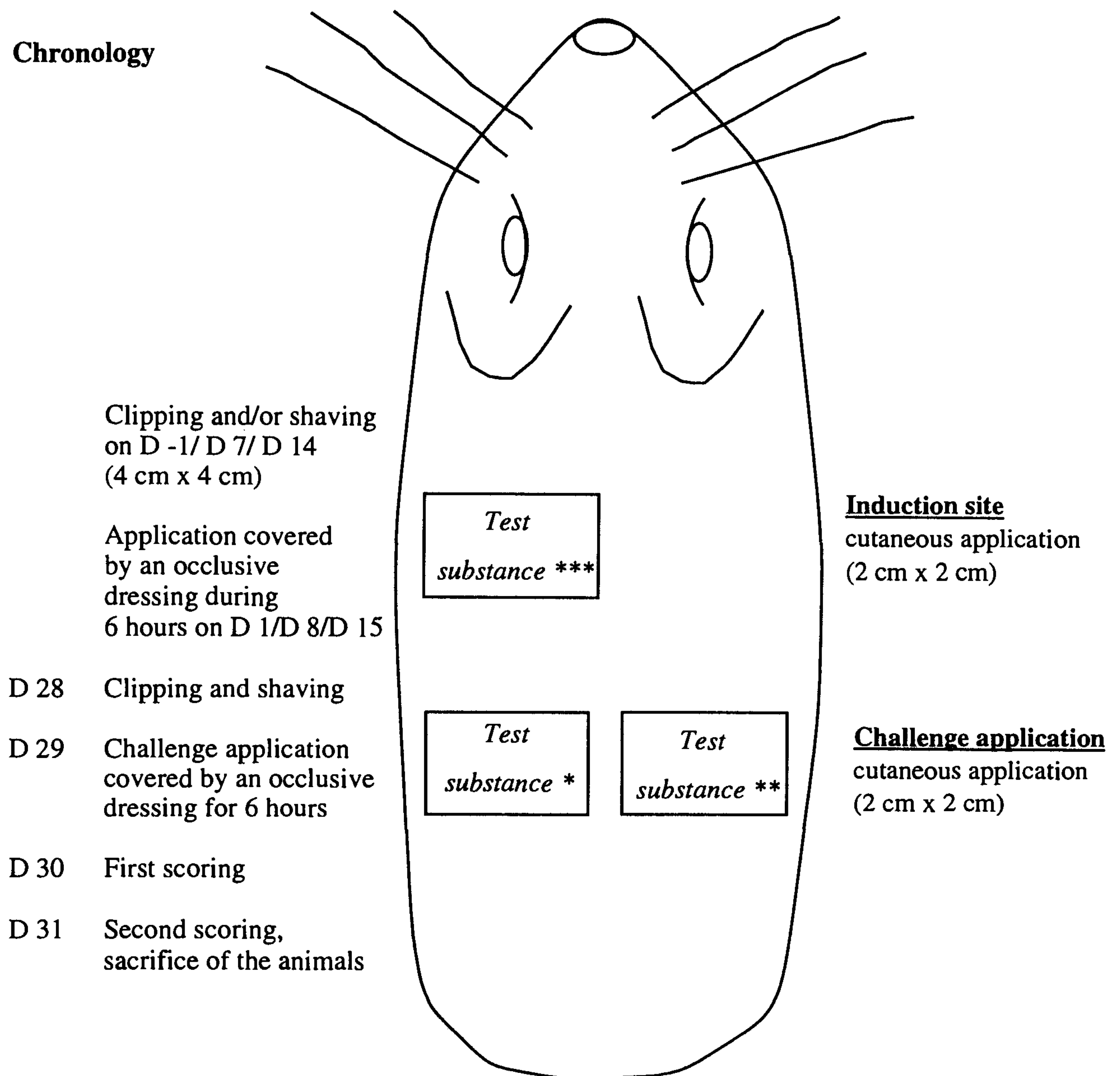
Figure 2: treated group



* at a concentration of 20% (w/w)
** at a concentration of 5% (w/w)
*** at a concentration of 50% w/w)

2.10. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date	Day
Arrival of the animals	11.5.95	-5
Weighing, allocation of the animals into groups	15.5.95	-1
Induction period		
. first application	16.5.95	1
. second application	23.5.95	8
. third application	30.5.95	15
Challenge application	13.6.95	29
Scoring of cutaneous reactions		
. after 24 hours	14.6.95	30
. after 48 hours	15.6.95	31
Weighing, sacrifice of the animals	15.6.95	31

2.11. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final study report and possible amendments,

are stored in the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

3. RESULTS

3.1. PRELIMINARY STUDY

The maximal concentration which could be obtained in the vehicle was 50% (w/w). One test was performed if this concentration was irritant.

Animal number	Concentration of the test substance % (w/w)		Scoring after removal of the dressing (1) 24 hours		48 hours	
			E	O	E	O
Male No. 01	50	RF	0	0	0	0
		LF	0	0	0	0
Female No. 01	50	RF	0	0	0	0
		LF	0	0	0	0

E : erythema
O : oedema
RF: right flank
LF: left flank

(1) No residual test substance was observed at removal of the dressings.

Concentrations chosen for the main study were 50% (w/w) for the induction phase and 20% (w/w) and 5% (w/w) for the challenge application. These latest concentrations were chosen on the request of the Study Monitor.

3.2. MAIN STUDY

3.2.1 Clinical signs

No clinical signs or mortalities were observed during the study.

The body weight gain of the animals was normal (figures 3 and 4, appendix 3).

3.2.2 Scoring of cutaneous reactions

Observations of cutaneous reactions are presented in appendix 4.

3.2.2.1 Induction period

Very slight or slight cutaneous reactions were observed in 8/10 animals during the induction period.

3.2.2.2 Challenge application

No cutaneous reactions were observed 24 and 48 hours after removal of the dressing.

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4. CONCLUSION

Under our experimental conditions and according to the method established by Buehler, the application of the test substance, (batch No. Pil 4X), at a concentration of 50% (w/w) (induction) and 5 or 20% (w/w) (challenge), did not induce any sensitization reaction in guinea-pigs.

SUMMARY

At the request of Société the potential of the test substance, (batch No. Pil 4X), to induced delayed contact hypersensitivity following cutaneous application was evaluated in guinea-pigs according to the method established by Buehler and O.E.C.D. (No. 406, 17th July 1992) and E.C. (92/69/E.E.C.) guidelines. The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

Thirty guinea-pigs (15 males and 15 females) were allocated to 2 groups: a control group 1 (5 males and 5 females) and a treated group 2 (10 males and 10 females).
During a 2-week induction period, animals of the treated group 2 received on days 1, 8 and 15 an application of 0.5 ml of the test substance at a concentration of 50% (w/w) in the vehicle. The test substance was prepared on a dry gauze pad and then applied to an area of the skin of approximately 4 cm^2 on the anterior left flank. After each application, the test substance was held in place for 6 hours by means of an occlusive dressing. After a 14-day rest period (from day 15 to day 28), a cutaneous challenge application of 0.5 ml of the test substance at a concentration of 5% (w/w) in the vehicle (right flank) and the application of 0.5 ml of the test substance at a concentration of 20% (w/w) in the vehicle (left flank) were performed on a non-treated area of the posterior region of the animals. Cutaneous reactions were evaluated 24 and 48 hours after the removal of the pads of the challenge application by comparing the reactions on both flanks.

At the end of the observation period, the animals were killed. In the absence of macroscopic cutaneous reactions in all the animals, no cutaneous samples were taken from the challenge application sites.

The sensitivity of the guinea-pigs in C.I.T. experimental conditions were checked in a recent study with a positive sensitizer: Dinitro 2.4 Chlorobenzene. During induction period, the test substance was applied at 1% (days 1 and 8) and 0.1% (day 15) concentrations. At cutaneous challenge application, 0.5% were tested on right flank and paraffin oil on the left flank.

Results

No clinical signs and no deaths were noted during the study.

During the induction period, very slight to slight cutaneous reactions were observed in 11/20 animals.

After 24 and 48 hours following the removal of the pads of the cutaneous challenge application of the test substance, no cutaneous reactions were recorded.

In a recent study, the species and strain which were used showed a satisfactory sensitization response in 50% animals using a positive sensitizer (appendix 5).

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3. RESULTS

3.1. PRELIMINARY STUDY

The maximal concentration which could be obtained in the vehicle was 50% (w/w). One test was performed to check if this concentration was irritant.

Animal number	Concentration of the test substance % (w/w)		Scoring after removal of the dressing (1)			
			24 hours		48 hours	
			E	O	E	O
Male No. 01	50	RF	0	0	0	0
		LF	0	0	0	0
Female No. 01	50	RF	0	0	0	0
		LF	0	0	0	0

E : erythema
O : oedema
RF : right flank
LF : left flank

(1) No residual test substance was observed at removal of the dressings.

Concentrations chosen for the main study were 50% (w/w) for the induction phase and 20% (w/w) and 5% (w/w) for the challenge application. These latest concentrations were chosen on the request of the Study Monitor.

3.2. MAIN STUDY

3.2.1. Clinical signs

No clinical signs or mortalities were observed during the study.

The body weight gain of the animals was normal (figures 3 and 4, appendix 3).

3.2.2. Scoring of cutaneous reactions

Observations of cutaneous reactions are presented in appendix 4.

3.2.2.1 Induction period

Very slight or slight cutaneous reactions were observed in 11/20 animals during the induction period.

3.2.2.2 Challenge application

No cutaneous reactions were observed 24 and 48 hours after removal of the dressing.

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SPONSOR

STUDY TITLE

***IN VITRO* MAMMALIAN CYTOGENETIC TEST**
IN CHO CHINESE HAMSTER OVARY CELLS

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR
Brigitte Molinier

STUDY COMPLETION DATE
30th November 1995

PERFORMING LABORATORY
Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER
12090 MIC

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SUMMARY

The objective of this study was to evaluate the potential of the test substance (batch No. Pil 4X) to induce chromosome breakage (clastogenesis) in Chinese hamster ovary (CHO) cells.

Methods

The test substance was tested, with or without a metabolic activation system, the S9 mix, prepared from a liver microsomal fraction (S9) of rats induced with Aroclor 1254.

For each culture, the cells were seeded in the culture medium and incubated at 37°C.

After 24 hours, the conditions of treatment were as follows, using 2 cultures/experimental point:

. without S9 mix: the test or control substances remained in the culture medium until harvest.
. with S9 mix: the test or control substances remained in a culture medium containing 1.5% S9 for 3 hours. The cells were then rinsed and fresh culture medium was added. The cultures were then incubated until harvest.

For the first test, one harvest time was used: 20 hours. For the second, two harvest times were used: 20 hours and 44 hours, i.e. approximately 1.5 times cell cycle and 24 hours later.
Before harvest, each culture was treated for 1.5 hours with a colcemid solution to block cells at the metaphase-stage of mitosis. The cell preparations were stained and screened microscopically for mitotic index and for aberrations: 200 well-spread metaphases per dose were read, whenever possible.

The test substance was dissolved in distilled water.

The doses of were as follows:
First test:
. 12.5, 25, 50, 100, 150 µg/ml without S9 mix,
. 30, 100, 300, 1000, 3000, 5000 µg/ml with S9 mix.
Second test:
. 12.5, 25, 50, 75, 100 µg/ml without S9 mix,
. 125, 250, 500, 750, 1000 µg/ml with S9 mix.

The chromosomal aberrations scoring was performed on cells treated at the doses of:
First test:
. 25, 50, 100 µg/ml without S9 mix, 100 µg/ml being the dose which showed moderate to marked toxicity (decreased of the mitotic index by 63% as compared to the controls),
. 100, 300, 1000 µg/ml with S9 mix, 1000 µg/ml being the lowest precipitating dose.
Second test:
. 75 µg/ml without S9 mix, for which moderate to marked toxicity was seen: a reduction by 53% at the 20-hour harvest and by 71% at the 44-hour harvest,
. 750 µg/ml with S9 mix.

The doses of the positive control substances were as follows:
. without S9 mix: 25 µg/ml of methylmethane sulfonate,
. with S9 mix: 30 µg/ml of cyclophosphamide.

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Results

The aberrant cell frequency in the vehicle controls was within the range of our historical data (presented in paragraph 7).

The aberrant cell frequency in the positive controls was significantly higher ($p < 0.001$) than that of the vehicle controls, indicating the sensitivity of the test system.

In the first test, the test substance did not induce any significant increase in the aberrant cell frequency, with S9 mix at the 3 doses or without S9 mix at 25 or 50 µg/ml. At 100 µg/ml, the incidence of cells with structural chromosomal aberrations was significantly higher than that of the controls (17.9% vs. 3.5%, gap excluded).

In the second test, the positive aspect of the response without S9 mix was confirmed: the frequency of cells with structural chromosome aberrations was high at 75 µg/ml, 30% or 22% at the 20-hour or 44-hour harvest respectively.
With S9 mix, the results were negative at 750 µg/ml. It was judged not necessary to read all the remaining slides.

Conclusion

Under our experimental conditions, the test substance (batch No. Pil 4X) showed direct clastogenic activity without S9 mix in this chromosomal aberration test performed in Chinese hamster ovary (CHO) cells, at the highest dose level which induced a reduction greater than 50% in the mitotic index.

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1. INTRODUCTION

This study was performed at the request of Société

The objective of this study was to evaluate the potential of the test substance to induce chromosome breakage (clastogenesis) in Chinese hamster ovary (CHO) cells.

This study was designed in accordance with the following guidelines:
. E.C. Directive No. 92/69/E.E.C., Annex V, B_{10}, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Identification

2.1.1.1 Test substance

The test substance, used in the study was supplied by

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
- protocol:
- labelling:

. batch number:
- protocol: Pilot 4X
- labelling: Pil 4X

. description: beige powder
. quantity and container: 4 kg contained in 1 plastic pot, this quantity was dispatched at C.I.T. into 7 flasks
. date of receipt: 26.7.94
. storage conditions: room temperature, protected from light, from humidity and conserved under nitrogen until use
. purity: 99.8%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in paragraph 6) provided by the Sponsor.

2.1.1.2 Vehicle

The vehicle was distilled water.

2.1.2 Preparation

The test substance was dissolved in the vehicle at a concentration of 750 mg/ml for the first test with S9 mix, and of 22.5 mg/ml for the first test without S9 mix, and of 30 mg/ml for the second test.
The preparations were made immediately before use.

2.1.3 Positive controls

Two known mutagens, dissolved in distilled water, were used to check the sensitivity of the test system:
. without S9 mix: methylmethane sulfonate (MMS), used at 25 µg/ml,

. with S9 mix: cyclophosphamide (CPA), used at 30 µg/ml.

2.2. TEST SYSTEM

2.2.1 Cells

CHO cells are an established cell line chosen for cytogenetic assays because the aberrations can be easily scored; they have a karyotype with 18-22 chromosomes and the assays are reproducible. They are characterized by their rapid growth (average cell cycle time of 12-14 hours) and their low spontaneous aberrant cell frequency.
They are stored in a cryoprotective medium containing 20% fetal calf serum and 10% dimethylsulfoxide (DMSO) within a liquid nitrogen container.

2.2.2 Metabolic activation system: S9 mix

The S9 mix consists of induced enzymatic systems contained in rat liver microsomal fractions (S9) and the cofactors necessary for their function. S9 was performed at C.I.T. according to Ames et al. (1975). The S9 fraction comes from liver homogenates from rats induced with Aroclor 1254 (500 mg/kg) by the intraperitoneal route.
The S9 fraction was preserved in sterile tubes within a liquid nitrogen container, until use.
The S9 mix was used at 10% S9 in the S9 mix. The S9 mix was prepared immediately before use at +4°C and maintained at this temperature throughout the experiment.

The S9 mix contained per ml:
. 5 µmoles Glucose-6-Phosphate,
. 4 µmoles NADP,
. 33 µmoles KCl,
. 8 µmoles $MgCl_2$,
. 100 µmoles sodium phosphate pH 7.4,
. 100 µl S9 (batch Nos. 37 and 40, protein concentration: 30 and 33 g/l respectively),
. sterile distilled water q.s.p. 1 ml.

2.3. EXPERIMENTAL DESIGN

Rationale for dose selection

The top dose was selected according to the following criteria:
. for non-toxic, freely soluble test substances, the top dose is 5000 µg/ml, according to international regulations
. for non-toxic, poorly soluble test substances, the top dose is the lowest precipitating dose.
. for toxic test substances, irrespective of solubility, the top dose is based on the level of toxicity: decrease by 50-75% of the mitotic index. However, precipitation should not interfere with the scoring of the test.

Consequently the doses were as follows:

First test:

For treatment:
12.5, 25, 50, 100, 150 µg/ml, without S9 mix,
30, 100, 300, 1000, 3000, 5000 µg/ml with S9 mix.

For scoring:
25, 50, 100 µg/ml without S9 mix,
100, 300, 1000 µg/ml with S9 mix.

Second test:

For treatment:
12.5, 25, 50, 75, 100 µg/ml, without S9 mix,
125, 250, 500, 750, 1000 µg/ml with S9 mix.

For scoring:
75 µg/ml without S9 mix,
750 µg/ml with S9 mix.

The following controls were used as a reference using duplicate cultures (except for the first test with S9 mix, vehicle and positive control using quadriplicate cultures).
. vehicle control: culture treated with the vehicle (20 or 100 µl distilled water/3 ml culture medium),
. positive controls: culture treated (only for the first harvest time) with:
- MMS, without S9 mix,
- CPA, with S9 mix.

Treatment
For each culture, approximately 5.10^5 cells were seeded in 3 ml of Eagles minimal essential medium modified by Dulbecco (DMEM) containing 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and fungison (0.25 µg/ml) in a 25 cm^2 flask. The flasks were then placed at 37°C in a humidified atmosphere of 5% CO_2/95% air.
After 24 hours, the conditions of treatment were as follows, using 2 cultures/experimental point:

. without S9 mix: the test or control substances remained in the culture medium until harvest.
. with S9 mix: the test or control substances remained in a culture medium containing 1.5% S9 for 3 hours. The cells were then rinsed and fresh culture medium was added. The cultures were then incubated until harvest, i.e. approximately 1.5 times cell cycle.

For the first test, one harvest time was used: 20 hours. For the second, two harvest times were used: 20 hours and 44 hours, i.e. approximately 1.5 times cell cycle and 24 hours later.

Before harvest (1.5 hours), each culture was treated with a colcemid solution to block cells at the metaphase-stage of mitosis and harvested. After hypotonic treatment (KCl 0.075 M), the cells were fixed in a methanol/acetic acid mixture (3/1; v/v), spread on glass slides and stained with Giemsa. At least 2 slides/culture were prepared. All the slides were coded for scoring.

2.4. ASSESSMENT OF DATA

Analysis
The cytotoxicity of the test substance was evaluated using the mitotic index (number of cells in mitosis/number of cells examined), which determines whether a substance induces mitotic inhibition. The number of cells in mitosis was evaluated on a total of 1000 cells.
In the event where the cells were exposed to more than 3 doses of the test substance, then the mitotic index was evaluated for all the doses, but chromosomal abnormalities were scored only on the slides corresponding to 3 doses, selected according to the criteria explained previously in paragraph "Rationale for dose selection". All the controls were examined for mitotic index and chromosomal aberrations.

200 metaphases/dose (with 18 to 22 chromosomes) were analysed whenever possible. The following structural aberrations (Savage, 1975; International Nomenclature, 1985): gaps, chromatid and chromosomal breaks and exchanges, and others (multiple aberrations and pulverizations) and the following numerical aberrations: polyploidy, hyperdiploidy and endoreduplication were recorded for each metaphase.

Treatment of results
All the data are presented in tabular form in which the total number of aberrations and the aberrant cell frequency, including and excluding gaps, are shown. A cell having any of the above mentioned structural chromosomal aberrations was recorded as one aberrant cell. If the cell has 2 or more aberrations, the aberrant cell frequency was scored accordingly, so that the total aberrant cell frequency is not necessarily equivalent to the total number of aberrations.

Acceptance criteria
This study was considered valid because the following criteria were fully met:
. the aberrant cell frequency in the controls was within the range of our historical data,
. the aberrant cell frequency of the positive controls was significantly higher than that of the controls and was within the range of our historical data.

Statistical analysis
The aberrant cell frequency (excluding gaps) in treated cultures was compared to that of the control cultures. The comparison was performed using the X^2 test, in which $p = 0.05$ was used as the lowest level of significance.

Evaluation criteria
The following criteria were used as an aid for determining a positive response:
. a reproducible and/or statistically significant increase in the aberrant cell frequency for at least one of the doses.

A test substance was considered as non-clastogenic in this test system if there is no significant increase in aberrant cell frequency at any dose above concurrent control frequencies.

Both biological and statistical significance was considered together in the evaluation.

2.5. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments
. slides

are stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

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2.6. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date
Protocol approved by:	
. Study Director	7.7.94
. Sponsor	21.7.94
First test:	
. Treatment	
- without S9 mix	31.8.94
- with S9 mix	24.8.94
Second test:	
. Treatment	
- without S9 mix	26.7.95
- with S9 mix	8.8.95

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3. RESULTS (tables 1 to 6)

Rationale for dose selection

The test substance was freely soluble in the vehicle (distilled water) at 750 mg/ml.

When 20 µl of this solution were added to 3 ml of culture medium, corresponding to the top dose of 5000 µg/ml, a marked precipitate was observed before harvest. At 3000 µg/ml, the precipitation was moderate and at 1000 µg/ml a slight precipitation occurred.

The cells were exposed to the following doses of

- 30, 100, 300, 1000, 3000, 5000 µg/ml, both with and without S9 mix.

Without S9 mix, because of the presence of round and refringent cells and the poor quality of the slides at 300 and 1000 µg/ml, the treatment was repeated using the following doses:

- 12.5, 25, 50, 100, 150 µg/ml.

The results of mitotic index were as follows:
Without S9 mix, at 150 µg/ml, the mitotic index was reduced by approximately 90% to the controls; at 100 µg/ml, by 63% to the controls; at 50, 25 or 12.5 µg/ml, by 35-45% to that of the controls.

With S9 mix, at 30, 100, 300 or 1000 µg/ml, the mitotic index was similar to that of the controls for all treatment levels.

Consequently, the chromosomal abnormalities were scored on the slides corresponding to the following doses:
. 25, 50, 100 µg/ml without S9 mix,
. 100, 300, 1000 µg/ml with S9 mix.

For the second test, the doses were as follows:
. without S9 mix: 12.5, 25, 50, 75, 100 µg/ml,
. with S9 mix: 125, 250, 500, 750, 1000 µg/ml.
Without S9 mix, at 100 µg/ml the mitotic index was reduced by approximately 90% for both harvest times; at 75 µg/ml, it was reduced by 53% at the 20-hour harvest and by 71% at the 44 hours.
Chromosome aberrations were scored at 75 µg/ml without S9 mix, and at 750 µg/ml with S9 mix.

Clastogenic activity

The aberrant cell frequency in the vehicle controls was within the range of our historical data (presented in paragraph 7).

The aberrant cell frequency in the positive controls was significantly higher ($p < 0.001$) than that of the vehicle controls, indicating the sensitivity of the test system.

In the first test, the test substance did not induce any significant increase in the aberrant cell frequency, with S9 mix at the 3 doses or without S9 mix at 25 or 50 µg/ml. However, at 100 µg/ml, the incidence of cells with structural chromosomal aberrations was significantly higher than that of the controls (17.9% vs. 3.5%, gap excluded).
In the second test, the positive aspect of the response without S9 mix was confirmed: the frequency of cells with structural chromosome aberrations was high at 75 µg/ml, 30% or 22% at the 20-hour or 44-hour harvest respectively.

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With S9 mix, the results were negative at 750 µg/ml. It was judged not necessary to read all the remaining slides.

4. CONCLUSION

Under our experimental conditions, the test substance (batch No. Pil 4X) showed direct clastogenic activity without S9 mix in this chromosomal aberration test performed in Chinese hamster ovary (CHO) cells, at the highest dose level which induced a reduction greater than 50% in the mitotic index.

5. REFERENCES

Ames, B. N.; Mc Cann, D. and Yamasaki, E.: Methods for detecting carcinogens and mutagens with the *Salmonella* Mammalian-microsome mutagenicity test. Mutation Res., 31, 347-364 (1975).

Savage, J.R.K.: Classification and relationships of induced chromosomal structural changes. Journal of Medical Genetics (1975), 12, 103-122.

An International System for Human Cytogenetic Nomenclature (edited by D.G. Harnden et al.) S. Karger, Switzerland (1985).

C I T

centre international de toxicologie

MISEREY BP 563 27005 ÉVREUX CEDEX FRANCE TÉL. 32 29 26 26 TÉLÉCOPIE 32 67 87 05

Miserey, 7th July 1994

IN VITRO MAMMALIAN CYTOGENETIC TEST IN CHO CHINESE HAMSTER OVARY CELLS

Protocol from	:	Centre International de Toxicologie Miserey BP 563 - 27005 Evreux Cédex France
Sponsor	:	
Address	:	
Study Monitor	:	
Study Director	:	B. Molinier
Study Number	:	12090 MIC

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INTRODUCTION

The objective of this study is to evaluate the potential of the test substance to induce chromosome breakage (clastogenesis) in Chinese hamster ovary cells (CHO).

Two independent tests with or without a metabolic activation system, the S9 mix, will be performed. This allows the detection of clastogenic activity of a test substance and/or its metabolites. The first test will use a single harvest time, at about 1.5 cell cycle times after the beginning of treatment. The second test will include an additional harvest time approximately 24 hours later in the event of negative or equivocal results in the first test. In case of positive results, then only one test will be performed.

The cells will be exposed to at least 3 doses of the test substance (2 cultures/dose). 1.5 hour before harvesting, the cells will be treated with a colcemid solution to block them at the metaphase-stage of mitosis. The chromosomal preparations will be stained and screened microscopically for mitotic index and for aberrations: 200 well-spread metaphases per dose will be read, whenever possible.

This protocol complies with:

. E.C. Directive No. 92/69/E.E.C., Annex V, B_{10}, 31st July 1992.

The study will be conducted in compliance with the following Good Laboratory Practice Regulations:

. Council Directive 87/18/E.E.C. of 18 December 1986 on the harmonization of laws, regulations or administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (O.J. n° L 15 of 17.1.87).

. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire).

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MATERIALS AND METHODS

1. TEST AND CONTROL SUBSTANCES

1.1 Identification

1.1.1 Test substance

. **Denomination** :
. **Batch No.** : Pilot 4X
. **Description** : powder
. **Physico-chemical properties:** i.e. purity, composition, stability and expiry date which refer to the batch to be used and handling conditions will be indicated in the test substance data sheet (to be completed by the Sponsor). An analytical certificate will also be provided by the Sponsor
. **Required amount:** 5 g

1.1.2 Vehicle

The vehicle will be selected according to the results of solubility tests performed before the first assay (2).

1.2 Preparation

The test substance will be dissolved in the vehicle to provide approximately 30 to 300 fold the final dose, depending on the vehicle. The preparations will be made immediately before use.

1.3 Positive controls

Two known mutagens, dissolved in distilled water, will be used to check the sensitivity of the test system:
. without S9 mix: methylmethane sulfonate (MMS), used at 25 µg/ml
. with S9 mix: cyclophosphamide (CPA), used at 30 µg/ml

2. TEST SYSTEM

2.1 Cells

CHO cells are an established cell line chosen for cytogenetic assays because the aberrations can be easily scored; they have a karyotype with 18-22 chromosomes and the assays are reproducible. They are characterized by their rapid growth (average cell cycle time of 12-14 hours) and their low spontaneous aberrant cell frequency.
They are stored in a cryoprotective medium containing 20% fetal calf serum and 10% dimethylsulfoxide (DMSO) within a liquid nitrogen container.

2.2 Metabolic activation system: S9 mix

The S9 mix consists of induced enzymatic systems contained in rat liver microsomal fractions (S9) and the cofactors necessary for their function. S9 will be performed at C.I.T. The S9 fraction comes from liver homogenates from rats induced with Aroclor 1254 (500 mg/kg) by the intraperitoneal route.
The S9 fraction is preserved in sterile tubes within a liquid nitrogen container, until use.

The S9 mix will be used at 10% S9 in the S9 mix. The S9 mix will be prepared immediately before use at +4°C and maintained at this temperature throughout the experiment.

The S9 mix will contain per ml:
. 5 µmoles Glucose-6-Phosphate,
. 4 µmoles NADP,
. 33 µmoles KCl,
. 8 µmoles $MgCl_2$,
. 100 µmoles sodium phosphate pH 7.4,
. 100 µl S9,
. sterile distilled water q.s.p. 1 ml.

3. EXPERIMENTAL DESIGN

Rationale for dose selection

The choice of doses will be performed by the Study Director and will be documented in the raw data throughout the study.
The top dose will be selected according to the following criteria:
. for non-toxic, freely soluble test substances, the top dose is 5000 µg/ml, according to international regulations.
. for non-toxic, poorly soluble test substances, the top dose is the lowest precipitating dose.
. for toxic test substances, irrespective of solubility, the top dose is based on the level of toxicity: decrease by 50-75% of the mitotic index. However, precipitation should not interfere with the scoring of the test.

At least 3 doses of the test substance (2 cultures/dose) will be tested, with or without metabolic activation, the cells being harvested at about 1.5 times normal cell cycle after the beginning of treatment. If negative or equivocal results are obtained, the test will be repeated and will include an additional harvest approximately 24 hours later. If positive results are obtained, the test will not be repeated.

During each test, the following controls will be made using duplicate cultures:
. vehicle control: culture treated with the vehicle, if other than water or culture medium,
. positive control: culture treated (only for the first harvest time) with:
  - MMS, without S9 mix,
  - CPA, with S9 mix.

Treatment

For each culture, approximately 5.10^5 cells will be seeded in 3 ml of Eagles minimal essential medium modified by Dulbecco (DMEM) containing 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml) and fungison (0.25 µg/ml) in a 25 cm^2 flask. The flasks will then be placed at 37°C in a humidified atmosphere of 5% CO_2/95% air.

After 24 hours, the conditions of treatment will be as follows, using 2 cultures/experimental point:

First test:

. without S9 mix: the test or control substances remained in the culture medium for 20 hours until harvest, i.e. approximately 1.5 times cell cycle.

. with S9 mix: the test or control substances remained in a culture medium containing 1.5% S9 for 3 hours. The cells will then be rinsed and fresh culture medium will be added. The cultures will then be incubated for 20 hours after the beginning of treatment, until harvest, i.e. approximately 1.5 times cell cycle.

Second test:

. without S9 mix, the test or control substances remained in the culture medium either for 20 hours or for 44 hours, until harvest, i.e. approximately 1.5 times cell cycle and 24 hours after.

. with S9 mix, the test or control substances remained in the culture medium for 3 hours. The cells were then rinsed and fresh culture medium was added. The cultures were then incubated either for 20 hours or for 44 hours after the beginning of treatment, until harvest, i.e. approximately 1.5 times cell cycle and 24 hours after.

Each culture will then be treated for 1.5 hours with a colcemid solution to block cells at the metaphase-stage of mitosis and harvested. After hypotonic treatment (KCl 0.075 M), the cells will be fixed in a methanol/acetic acid mixture (3/1; v/v), spread on glass slides and stained with Giemsa. At least 2 slides/culture will be prepared. All the slides will be coded for scoring.

4. ASSESSMENT OF DATA

Analysis

The cytotoxicity of the test substance will be evaluated using the mitotic index (number of cells in mitosis/number of cells examined), which determines whether a substance induces mitotic inhibition. The number of cells in mitosis will be evaluated on a total of 1000 cells.

In the event where the cells are exposed to more than 3 doses of the test substance, then the mitotic index will be evaluated for all the doses, but chromosomal abnormalities will be scored only on the slides corresponding to 3 doses, selected according to the criteria explained previously in paragraph "Rationale for dose selection". All the controls will be examined for mitotic index and chromosomal aberrations.

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200 metaphases/dose (with 18 to 22 chromosomes) will be analysed whenever possible. The following structural aberrations: gaps, chromatid and chromosomal breaks and exchanges, and others (multiple aberrations and pulverizations) and the following numerical aberrations: polyploidy, hyperdiploidy and endoreduplication will be recorded for each metaphase.

Treatment of results

All the data will be presented in tabular form in which the total number of aberrations and the aberrant cell frequency, including and excluding gaps, will be shown. A cell having any of the above mentioned structural chromosomal aberrations will be recorded as one aberrant cell. If the cell has 2 or more aberrations, the aberrant cell frequency will be scored accordingly, so that the total aberrant cell frequency will not necessarily be equivalent to the total number of aberrations.

Acceptance criteria

This study will be considered valid if the following criteria are fully met:

. the aberrant cell frequency in the controls is within the range of our historical data,

. the aberrant cell frequency of the positive controls is significantly higher than that of the controls and is within the range of our historical data.

Statistical analysis

For each test and for each harvest time, the aberrant cell frequency (excluding gaps) in treated cultures will be compared to that of the control cultures. The comparison will be performed using the X^2 test, in which $p = 0.05$ will be used as the lowest level of significance.

Evaluation criteria

The following criteria will be used as an aid for determining a positive response:

. a reproducible and statistically significant increase in the aberrant cell frequency for at least one of the doses.

A test substance will be considered as non-clastogenic in this test system if there is no significant increase in aberrant cell frequency at any dose above concurrent control frequencies and in both of the 2 tests and the 2 harvest times.

Both biological and statistical significance will be considered together in the evaluation.

5. PROCEDURES

The procedures used during the study will be those documented in the relevant C.I.T. procedures manual.

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6. AMENDMENTS TO THE PROTOCOL

If necessary, amendments to the protocol will be made after agreement between the Study Director and the Study Monitor.

7. REPORTING

The Study Director will contact the Study Monitor when necessary.
The final report, **in English with a French summary**, will contain all data collected throughout the study.
Number of copies of the final report: 3 (1 + 2 unbound)
Proposed issue of the draft report: one month after the end of the study.

8. QUALITY ASSURANCE UNIT

The Quality Assurance Unit will conduct inspections according to Good Laboratory Practice Regulations (as specified on page 2).
The dates on which the findings of these inspections are reported to the Study Director and C.I.T. Management will be specified in the final report.

9. ARCHIVES

The study archives:
. protocol and possible amendments
. raw data
. correspondence
. final report and possible amendments
. slides

will be stored on the premises of C.I.T., 27005 Miserey, Evreux, France, for 5 years after the end of the experimental study. At the end of this period, the study archives will be returned to the Sponsor.

10. TIME SCHEDULE

Beginning of the study: will be specified later
End of the study: will be specified later

AMENDMENT TO PROTOCOL

STUDY No.: **12090 MIC**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 01

Page 1 / 1

Justification: complementary information .
Date of application: 24.8.94

INTRODUCTION

The study will not be conducted in compliance with the following Good Laboratory Practice Regulations:

Council Directive 87/18/E.E.C. of 18 December 1986 on the harmonization of laws, regulations or administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (O.J. n° L 15 of 17.1.87).

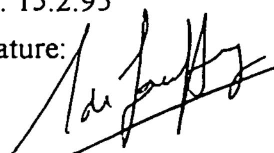
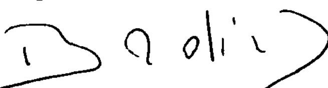
but with the following Good Laboratory Practice Regulations:

O.E.C.D. Principle of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

3.EXPERIMENTAL DESIGN

During the test , the following controls will made using duplicate cultures, except with S9 mix (quadriplicate cultures).

. vehicle control: culture treated with distilled water.

Scientific management	Study Director	Study Monitor
J.F. Le Bigot ou S. de Jouffrey	B. Molinier	9/3/95
Date: 15.2.95	Date: 15.2.95	Date:
Signature: 	Signature: 	Signature:

AMENDMENT TO PROTOCOL

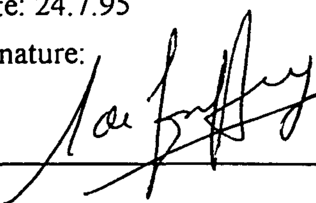
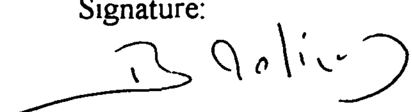
STUDY No.: **12090 MIC**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 02

Justification: information concerning the repeat test.
Date of application: 24.7.95

MATERIALS AND METHODS

3. EXPERIMENTAL DESIGN

At the request of the sponsor, the test will be repeated and will include an additional harvest approximately 24 hours later.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	
Date: 24.7.95	Date: 24.7.95	Date:
Signature: 	Signature: 	Signature:

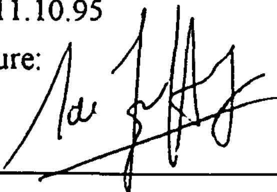
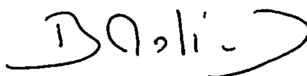
AMENDMENT TO PROTOCOL

STUDY No.: **12090 MIC**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 03

Page 1 / 1

Justification: information concerning the repeat test.
Date of application: 11.10.95

In agreement with the Sponsor, only the slides of the second test corresponding to the top doses (without S9 mix: 75µg/ml, with S9 mix: 750µg/ml) were scored for both harvests.

Scientific management	Study Director	Study Monitor
J.F Le Bigot or S. de Jouffrey	B. Molinier	12/10/95
Date: 11.10.95	Date: 11.10.95	Date:
Signature: 	Signature: 	Signature:

AMENDMENT TO PROTOCOL

STUDY No.: **12090 MIC**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 04

Page 1 / 1

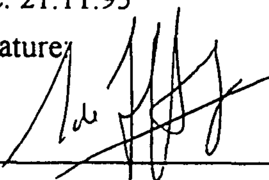
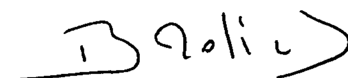
Justification: information concerning scoring of mitotic index of the repeat test.
Date of application: 26.7.95

MATERIALS AND METHODS

4. ASSESSMENT OF DATA

Analysis
- without S9 mix, the mitotic index was scored on 500 cells.
- with S9 mix, it was not scored.

This minor deviation has no impact on quality of this study since mitotic index is only an indirect measurement of cytotoxicity (OECD Guideline Draft Sept 95).

Scientific management	Study Director	Study Monitor
J.F. Le Bigot ou S. de Jouffrey	B. Molinier	24/11/95
Date: 21.11.95	Date: 21.11.95	Date:
Signature: 	Signature: 	Signature:

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TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

ADME STUDY

PHARMACOKINETICS STUDY IN PLASMA, EXCRETION BALANCE AND TISSUE DISTRIBUTION AFTER SINGLE ADMINISTRATION BY CUTANEOUS ROUTE TO RATS

STUDY DIRECTOR

Terence Appelqvist

STUDY COMPLETION DATE

27 September 1999

PERFORMING LABORATORY

CIT
Centre International de Toxicologie
BP 563 - 27005 Evreux - France

LABORATORY STUDY NUMBER

16432 PAR

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Figure 3: mean (n=6) cumulative excretion of radioactivity (as % of dose) following a single cutaneous application (25mg/kg) of ^{14}C to Wistar Han rats: males and females 27

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STATEMENT OF THE STUDY DIRECTOR

The study was performed in compliance with the principles of Good Laboratory Practice as described in:

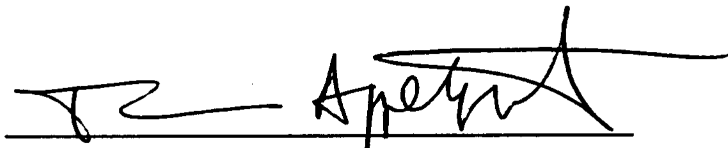
. O.E.C.D. principles of Good Laboratory Practice, Decision Concerning Mutual Acceptance of Data in the Assessment of Chemicals, C(81)30(final) Annex 2. 12 May 1981,
. Council Directive 87/18/E.E.C. of 18 December 1986 on the harmonization of laws, regulations or administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (O.J. n° L 15 of 17.1.87),
. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Journal Officiel du 9 mars 1990), Ministère de l'Industrie et de l'Aménagement du Territoire,
. US Food and Drug Administration, Good Laboratory Practice Regulations 21 CFR Part 58, December 22, 1978 (and subsequent amendments),
. Japanese Ministry of International Trade and Industry, Good Laboratory Practice Directive, March 31, 1984; Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85.

The study was conducted in compliance with the following Animal Health regulation:

. Council Directive No. 86/609/E.E.C. of 24th November 1986 on the harmonization of laws, regulations or administrative provisions relating to the protection of animals used for experimental or other scientific purposes.

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at CIT, Centre International de Toxicologie, B.P. 563, Miserey, 27005 Evreux, France.



T. Appelqvist Date: 27 September 1999
Study Director
Bachelor of Pharmacy, Ph.D.
Head of Pharmacokinetics

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SCIENTISTS INVOLVED IN THE STUDY

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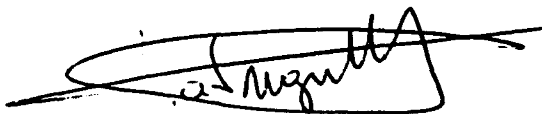
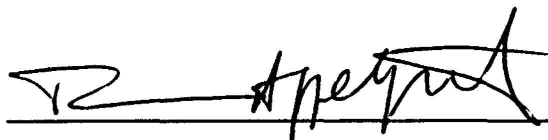
P.O. Guillaumat Date: 27 September 1999
Doctor of Pharmacy
Head of Pharmacy

Analytical Chemistry



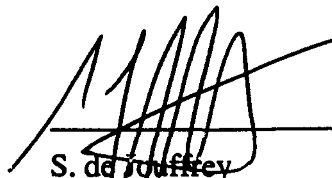
G. Fabreguettes Date: 27 September 1999
D.E.S.S. (Analytical Chemistry)

Pharmacokinetics



T. Appelqvist Date: 27 September 1999
Bachelor of Pharmacy, Ph.D.
Head of Pharmacokinetics

CIT Management



S. de Jouffrey Date: 27 September 1999
Doctor of Veterinary Medicine
Director of Operations

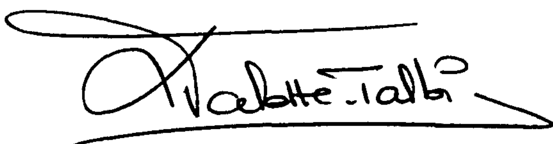
STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspection	Dates		
	Inspection	Reported to Study Director (*)	Reported to Management (*)
Protocol	2 March 1998	4 March 1998	9 March 1998
Study	27 October 1998	3 November 1998	3 November 1998
Study	3 November 1998	4 November 1998	5 November 1998
Report	9 August 1999	9 September 1999	23 September 1999

In addition to the above-mentioned inspections, at about the same time as this study, described in the present report, process-based and routine facility inspections of critical procedures relevant to this study were also made by the Quality Assurance Unit.
The findings of these inspections were reported to the Study Director and to CIT Management.

The inspections were performed in compliance with CIT Quality Assurance Unit procedures and the Good Laboratory Practice.

The reported methods and procedures were found to describe those used and the results to constitute an accurate and complete reflection of the study raw data.



L. Valette-Talbi Date: 27 September 1999
Doctor of Biochemistry, Ph.D.
Head of Quality Assurance Unit
and Scientific Archives

(*) The dates indicated correspond to the dates of signature of audit reports by Study Director and Management.

SUMMARY

Objective

The objective of this ADME study, performed at the request of the Sponsor, was to evaluate the plasma pharmacokinetics and excretion balance in urine and faeces of the test substance, ^{14}C after single administration by the cutaneous route to rats.

Methods

Twenty-four Wistar Han rats (12 males and 12 females), weighing an average 180 g and 142 g for the males and females respectively (seven weeks old), were divided into two groups (numbered, 1 and 2) and each received a single administration of the radiolabelled test substance, ^{14}C (in water for injections) by the cutaneous route (30 minute exposure over 10% of the body surface area) at the nominal dose-level of 25 mg/kg. The animals of group 1 (nine males and nine females) were used for plasma pharmacokinetic investigations, whilst group 2 (three males and three females) were placed individually in metabolism cages and used for excretion balance. A constant radioactive dose of 2.2 MBq/kg was used. During the exposure period, the site was protected with light bandages; thereafter, the bandages were removed and the site washed and dried.

For group 1; blood samples were collected (from three rats/sex/time point), under isoflurane anaesthesia, predose, 1, 2, 4, 6, 8, 24, 48 and 72 hours post-application of test substance. After the final blood sampling, all animals were killed and stored frozen.

For group 2; weighed urine, faeces and cage-wash were collected predose, and then every 24 hours (except initial urine fractions, collected 0-6 and 6-24 hours) until 168 hours post-application of test substance. Thereafter, the animals were killed, weighed and the test and a control application sites were "stripped" and skin biopsies taken. Application sites and various organs were removed, weighed (including remaining carcass) and stored frozen until analyzed for total radioactivity (application site) or discarded (remaining samples) in view of the very low absorption found.

Blood samples were centrifuged to obtain plasma. Each plasma sample, selected tissues/organs, strippings and excreta samples were analyzed for total radioactivity after the appropriate preparation. Mass balance calculations were performed.
During the study, the animals were observed for clinical signs, morbidity and mortality. Body weight was recorded pre-dosing and on the day of treatment.

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Results

Plasma pharmacokinetics (group 1)
Following topical application (30 minutes exposure) of the isotopic mixture at a nominal 25 mg/kg, the radioactivity in all plasma samples was below quantifiable limits (< 18.2 ng-eq/g, <1.6 Bq/g).

Excretion balance (group 2)
Following topical application of the isotopic mixture at a nominal 25 mg/kg, the mean (± standard deviation) total cumulative excretion of the radioactive dose in the excreta (during 30 minute exposure period, and a 168 hour period following washing of the site), was 0.51 ± 0.24% and 0.52 ± 0.03% for males and females, respectively. A mean 0.014 ± 0.007% and 0.48 ± 0.23% of the administered dose was eliminated respectively in the urine and faeces for the males; for the females the values were respectively 0.014 ± 0.002% and 0.50 ± 0.03%. For both sexes, less than 0.1% of the dose was in the cage-wash. Further, a total mean 94.2 ± 3.91% (males) and 96.85 ± 2.96% (females) of the applied dose was recovered in the site dressings/wash, and 0.14 ± 0.02% (males) and 0.09 ± 0.02% (females) remained in the skin application site. Most (>85%) of the total radioactivity recovered in the excreta was eliminated (mainly in faeces) within 72 hours of dosing. Virtually no radioactivity (nearly all values were below quantifiable limits) was found in the strippings. The results were homogenous for all animals/sexes.

Absorption of radioactivity following topical application
The minimal fraction of the topical dose absorbed systemically was a mean 0.49% for the males and 0.51% for the females, as estimated from the individual summed cumulative urine and faecal excretion data. The maximum fraction of the dose available for eventual systemic absorption was estimated to be 0.65 and 0.61% for the males and females, respectively, and was considered to be the sum of the recovered radioactivity in the excreta and skin site.

Mortality and clinical signs
No mortality or morbidity were observed. One animal developed a small blister at the application site.

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Conclusion

The plasma pharmacokinetics and elimination of radioactivity in excreta was investigated following topical administration of ^{14}C at 25 mg/kg, to male and female rats. This study showed that;

- following topical exposure for 30 minutes, the mean plasma radioactivity levels remained below quantifiable levels,
- the absolute minimal and maximal fractions of the dose absorbed were extremely low at, respectively, 0.49 and 0.67% for males and 0.51 and 0.63% for females (estimated from summed urine plus faecal elimination data and summed excreta plus skin site and stripping data, respectively),
- the radioactivity recovered in the skin site (dermis and epidermis) was extremely low at, respectively, 0.14% for the males and 0.09% for the females; radioactivity in the strippings (stratum corneum) was below quantifiable limits,
- the recovery of radioactivity in the site dressing/wash was very high at 94.2% of the dose for the males and 96.9% for females,
- the absorbed radioactivity was >80% eliminated (mainly in the faeces) within 72 hours; specifically, the mean urine/faeces ratios over whole collection period were 0.029 and 0.028 for males and females, respectively,
- the pharmacokinetic and excretion results were homogenous for the individual animals per sex, and no gender differences were apparent.

No metabolism investigations were performed as the levels of radioactivity present in the plasma and urine samples were too low for the analytical techniques available.

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1. INTRODUCTION

1.1. OBJECTIVE

This study was performed at the request of the Sponsor.
The objective of this study was to evaluate the pharmacokinetics in plasma, excretion balance in urine and faeces, and possible tissue distribution of the test substance, ^{14}C after single administration by the cutaneous route to rats.

is a dye used in hair products.

The rat was chosen because it is a rodent species commonly requested by the international regulations for this type of study and the same species was used in toxicity studies.

The dose-level was chosen by the Sponsor.

The cutaneous route was used since it is the normal route of exposure in humans.

2. EXPERIMENTAL CONDITIONS

2.1. TEST SUBSTANCES AND VEHICLE

2.1.1 Identification

2.1.1.1 Radiolabelled test substance

The radiolabelled test substance, ^{14}C was supplied by Isotopchim, France.

Documentation supplied by the Sponsor identified the radiolabelled test substance as follows:

. denomination:
  - protocol: ^{14}C [U-Ring-14C] as synonym
  - labelling: [U-Ring-14C]

. batch number:
  - protocol and labelling: 98218A

. description: black powder
. quantity and container: two glass vials each containing 74 MBq
. date of receipt: 21 August 1998
. specific activity: 23.4 mCi/mmol corresponding to 1.708 MBq/mg
. storage conditions: at -20 °C and under nitrogen.

An analytical certificate provided by Isotopchim is presented in Appendix 1.

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2.1.1.2 Non radiolabelled test substance

The non radiolabelled test substance, was supplied by the Sponsor.

Documentation supplied by the Sponsor identified the non radiolabelled test substance as follows:

. denomination:
  - protocol and labelling:
. batch number:
. - protocol and labelling: 0500591
. description: grey powder
. quantity and container: one smoked glass vial containing 10 g
. date of receipt: 19 October 1998
. storage conditions: at +4 °C, away from light and under argon atmosphere
. expiry date: October 1999.

An analytical certificate provided by the Sponsor is presented in Appendix 1.

2.1.2 Vehicle

The vehicle was water for injection, batch No. 9562, supplied by Fresenius (Sèvres, France).

2.1.3 Formulation procedure

The day before dosing, the contents of the vial containing 2 mCi of isotopic powder (equivalent to a total of 43.33 mg of test substance) were transferred stepwise, using approximately 2 ml ethanol (batch No. VRG080228G, supplied by Carlo Erba, Rueil-Malmaison, France), to a gauged (5 ml) preparation vial, and then made to volume with more ethanol to obtain a solution of 0.4 mCi/ml (8.666 mg/ml).
After mixing, 2.027 ml (0.811 mCi, 17.56 mg) of the solution was transferred to a gauged flask (34.1 ml) and the solvent was evaporated under a nitrogen gas stream. The dried residue was stored at -20 °C, protected from light and humidity until the day of treatment.

On the day of dosing, the non-radiollabelled test substance (323 mg) was added to the dried residue followed by the vehicle (to 34.1 ml) to achieve the target concentration of 10 mg/ml.
The formulation was kept under magnetic stirring and protected from light until delivery.

2.1.4 Chemical analysis of the radiolabelled test substance and the administered formulation

2.1.4.1 Determination of the total radioactivity and radiochemical purity of the radiolabelled test substance

Before the treatment, the radiolabelled test substance solution was analyzed in triplicate by liquid scintillation counting to determine the total radioactivity (results expressed as MBq/ml).
In addition, the radiolabelled test substance solution was analyzed (in duplicate) by HPLC/UV/on line radioactivity detection to determine the radiochemical purity (results expressed as %) using a basic method provided by the supplier of the radiolabelled compound.

2.1.4.2 Total radioactivity of the administered formulation

On the day of treatment, three samples (weighed accurately) were taken from the formulation and analyzed by liquid scintillation counting to determine the total radioactivity.
After treatment, the same analysis of radioactivity was undertaken (in triplicate) on an aliquot of the formulation which had passed through the same administration device as that used for the treatment of the animals.
The results are expressed as Bq/g of formulation.

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2.1.4.3 Radiochemical purity of the administered formulation

On the day of treatment, the formulation was analyzed (in duplicate) by HPLC/UV/on line radioactivity detection to determine the radiochemical purity (results expressed as %).

Full details of the analytical procedures used are presented in Appendix 2.

2.2. MATERIALS AND METHODS

2.2.1 Animals

A total of 28 Wistar Han rats (14 males and 14 females) of the Ico: WI (IOPS AF/Han) strain were supplied by Iffa Crédo (l'Arbresle, France) and received at CIT on 20 October 1998.
On arrival, the animals were given a clinical examination to ensure they were in good condition. A higher number of animals than necessary was acclimatized in order to permit the selection and/or replacement of individuals.
A seven-day acclimatization period to the conditions of the study preceded the day of treatment. The required number of animals (12 males and 12 females) was selected according to body weight and clinical condition and allocated by sex, to groups, according to a computerized randomization procedure.
Each animal was identified by an individual ear tattoo. At the beginning of the study, each animal received a unique CIT identity number.
On the day of treatment, the animals were approximately seven weeks old and had a mean body weight of 180 g for the males (range: 169 g to 190 g) and 142 g for the females (range: 132 g to 151 g).

2.2.2 Environmental conditions

From arrival at CIT, the animals were housed in a barriered rodent unit.
The animal room conditions were set as follows:

- . temperature : 21 ± 2 °C
- . relative humidity: 50 ± 20%
- . light/dark cycle : 12h/12h (07:00 - 19:00)
- . ventilation : approximately 12 cycles/hour of filtered, non-recycled air.

The corresponding instrumentation and equipment are checked and calibrated at regular intervals. The temperature and relative humidity were recorded continuously and the records were checked daily and filed.
The animal room was disinfected before the arrival of the animals and cleaned regularly thereafter. Microbiological analyses of the air and the surfaces of the walls and floor of the animal room are performed regularly by an external laboratory and the results archived at CIT.

2.2.3 Housing

The animals for plasma pharmacokinetics (group 1) were housed in threes in suspended wire-mesh cages (43.0 x 21.5 x 18.0 cm). A metal tray containing autoclaved sawdust (SICSA, Alfortville, France) was placed under each cage.
The animals for the excretion balance (group 2) were housed individually in metabolism cages.
Cages were not randomized in the room but placed in numerical order, vertically (normal cages) or horizontally (metabolism cages) on the racks.

2.2.4 Food and water

All animals had free access to A04C P2.5 powdered maintenance diet, batch No. 80609 (UAR, Villemoisson-sur-Orge, France), distributed daily and tap water (filtered using a 0.22 micron filter) contained in bottles. The rats were fasted overnight (food only) before dosing; the food was given about four hours after dosing on the day of treatment.
Each batch of diet was analyzed by the supplier for composition and contaminant levels. The diet formula is presented in Appendix 9.

2.2.5 Contaminants analyses

Bacterial and chemical analyses of the sawdust, diet and water are performed periodically by external laboratories. These analyzes include the detection of possible contaminants (sawdust: pesticides and heavy metals; diet and water: pesticides, heavy metals and nitrosamines).
No contaminants were known to have been present in the diet, drinking water or sawdust at levels which may be expected to have interfered with or prejudiced the outcome of the study.

2.3. TREATMENT

2.3.1 Dose-levels and groups

The dose-levels used were based on the intended concentration of use in hair dye preparations (0.25% w/w when applied on hair), multiplied by a factor of 4 to increase the radioactive dose possible. The test substance was applied for a period of half an hour to the skin, as this corresponds to the exposure period during the normal use of the test substance by humans. The animals were divided into two groups and treated cutaneously with the test substance as indicated below:

Group	Animals per group	Purpose	Nominal dose-level (mg/kg)	Radioactive dose (MBq/kg)	Animal numbers
1	9 males	Plasma pharmacokinetics	25	2.2	U20451 to U20459
	9 females				U20481 to U20489
2	3 males	Excretion balance and distribution	25	2.2	U20460 to U20462
	3 females				U20490 to U20492

2.3.2 Administration

The cutaneous route was used since it is the normal mode of exposure in humans.
Before dosing, the animals were deprived of food for an overnight period. The food was reoffered about four hours after application of the test formulation.
The test formulation was administered by cutaneous application using a plastic syringe fitted with a metal probe. The same metal probe was used for all animals.
For cutaneous application, the day before dosing, the dorsum of the animals was clipped free of hair (an area equivalent to approximately 10% of the total body surface area over the interscapular/upper back region; 20 cm² for a 150 g rat, 25 cm² for a 200 g rat), as close to the skin as possible, using electric clippers. Then, on the day of dosing, the test substance formulation was spread evenly and thinly over the exposed skin. The animals wore a gauze pad (Stérilux ES, 8x10 cm, Hartmann, Châtenois, France) and light non-occlusive bandages (Urgo band, 3 m x 7 cm, Fournier, Chenove, France) to protect the site for 30 minutes post administration. After 30 minutes, the treated area was washed to remove all traces of the test substance using cotton swabs dampened with water.

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The bandages and swabs were kept at room temperature pending radioactivity analysis by liquid scintillation counting (as triplicate samples after extraction) at CIT to determined the recovered applied dose.

For each animal, the syringe with metal probe was weighed before and after administration in order to determine precisely the quantity of isotopic mixture administered.
Before use, the syringe plus probe was pre-saturated with an aliquot of the formulation which was not administered to the animals.
The formulation was stirred continuously and kept on ice pending filling of the administration device.
A constant dosage-volume of 20 µl/cm² was used.

2.4. BLOOD SAMPLINGS (group 1)

Blood samples were taken as follows:
. predose and 1, 2, 4, 6, 8, 24, 48 and 72 hours after the beginning of the application period.

Three animals/sex were sampled at each time point, and each animal was sampled three times in total, using the following scheme:
. first three animals/sex: predose, 2 hours and 24 hours post-application,
. second three animals/sex: 1 hour, 6 hours and 48 hours post-application,
. remaining three animals/sex: 4 hours, 8 hours and 72 hours post-application.

Blood samples (1 ml at the first and second sampling per animal, and maximal volume at the third sampling) were taken into tubes containing lithium heparinate, from the orbital sinus of the animals. For the blood sampling, the animals were lightly anaesthetized by isoflurane. The blood samples was kept on ice (+4 °C).
Plasma was obtained by centrifugation of the blood (4000 rpm for 10 min at +4 °C).
The plasma was stored at -20 °C until analyzed for radioactivity at CIT.
All surviving animals were killed after the last collection (see 2.9. Pathology: sacrifice). Thereafter, the carcasses were stored frozen at -20 °C at CIT until destroyed with the agreement of the Study Monitor.

2.5. EXCRETION BALANCE (group 2)

Urine, faeces and cage wash were collected at the following times:
. during a 24 hours period before dosing (urine and faeces) and then during the period 0-6h, 6-24h (urine) or 0-24h (faeces), 24-48h, 48-72h, 72-96h, 96-120h, 120-144h and 144-168h (urine and faeces) after beginning of the application period,
. after each collection of faeces the cages and cones were carefully rinsed with approximately 20 ml of water (except at 168 h when 200 ml was used).
The urine and faeces were collected in tubes at room temperature.
All the samples collected were weighed in tared bottles and then frozen at -20 °C (except cage wash water, which was kept at room temperature).

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All animals were killed after the last collection (see § 2.9. Pathology: sacrifice) and their carcasses weighed.
Thereafter, skin from the application site and a sample (same size) from elsewhere (back, hindquarters) were fully stripped (13 times, using MIRAGE invisible tape, 19 mm, GUILBERT) and excised and weighed (after biopsies were taken). After stripping, three skin biopsies (6 mm diameter, using a biopsy punch) from both the excised application and non-application site were taken (and were separated into the dermis and epidermis, using a cryomicrotome, by making a 40 µm slice), and the brain, heart, kidneys, liver, lungs, spleen, stomach, small intestine, large intestine, pancreas, yellow fat sample, skeletal muscle sample and bone sample were dissected out, weighed and stored frozen at -20 °C at CIT. The remaining carcasses were reweighed and then stored frozen (along with the strippings and biopsies) at -20 °C at CIT.
The carcasses and tissue/organ samples or homogenates were stored pending analysis and/or until destroyed with the agreement of the Study Monitor.

2.6. PHARMACOKINETIC ANALYSIS

The minimal fraction of the topical dose absorbed was estimated using urine and faeces data and was considered to be equal to the total summed urinary and faecal cumulative excretion of radioactivity during the study period after topical application. The maximum fraction of the dose available for eventual systemic absorption was considered to be the sum of the total recovered radioactivity in the urine, faeces and skin application site, as a previous study (*CIT/Study No. 16434 PAR*), where the same test compound was given by the oral route, had shown that any systemically absorbed radioactivity was quickly eliminated in the excreta (i.e., not-retained in the tissues/organs).

2.7. RADIOACTIVITY DETERMINATION (LIQUID SCINTILLATION COUNTING)

2.7.1 Sample preparation

Test formulations and extract from bandages/swabs
A small aliquot was weighed into a counting vial and counted after the addition of Ready-Safe scintillation solution. Three samples per test formulation and two samples per extract were counted.
For the bandages/swabs, the remaining fibrous material after analysis and removal of solvent was digested with sulphuric acid and then oxidized (in duplicate) and analyzed as for faeces samples below.

Plasma (group 1)
An aliquot of each sample was weighed into a counting vial and counted after the addition of Ready-Safe Scintillation solution.
The sample preparation was performed in duplicate.

Urine, cage wash (group 2)
An aliquot of each urine or cage wash sample was weighed into a counting vial and counted after the addition of Ready-Safe or Ready-Gel scintillation solution, respectively.
The sample preparation was performed in duplicate.

Faeces (group 2)
Each sample was placed in a suitable tared container and ethanol added. The contents of the container were weighed. The sample was then homogenized using an ultra turrax.
About 0.3 g of the resultant tissue homogenate was oxidized using a Model 307 oxidizer (Packard). The radioactive gases produced were collected in a mixture of Carbosorb and Permafluor, and analyzed by LSC.

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The sample oxidation was performed in duplicate, starting from a single homogenate for each faeces sample.

Skin (group 2) (application site only)
The skin was solubilized in alcoholic potassium hydroxide solution followed by homogenization. Thereafter, two aliquots per sample were analyzed for radioactivity after addition of Hionic Fluor scintillant.

Strippings (group 2)
The strippings (application site and non-application site separately) for each animal were pooled as follows:
. strip 1,
. strips 2 to 5,
. strips 6 to 9,
. strips 10 to 13.
Thereafter, the pooled strips were solubilized with Soluene 350/Isopropanol mixture and counted for radioactivity after addition of Hionic-Fluor scintillation solution.

Carcasses, biopsy samples, general tissues and organs (group 2)
No analysis was performed as no radioactivity was retained in the tissues/carcasses.

2.7.2 Analytical apparatus and counting method

A Beckman LS 6000 TA counter was used.
An external standard source (Cesium 137) was used to monitor sample quenching; the dpm values of each sample were obtained from the corresponding cpm values using a quench standard curve.
All biological samples were counted for 10 minutes.
Each biological sample was counted in duplicate and the mean dpm value was used to calculate the corresponding radioactivity concentration (as Bq/g or Bq/sample).
Full details of the analytical procedures used are shown in Appendices 2 and 3.

2.8. CLINICAL EXAMINATIONS

2.8.1 Clinical signs

Each animal was observed at least once a day, at approximately the same time each day, for the recording of clinical signs.

2.8.2 Morbidity and mortality

Each animal was checked at least twice a day (including during weekends and public holidays) for mortality or signs of morbidity.

2.8.3 Body weight

Body weight was recorded for each animal once before allocation of the animals to groups, on the day of dosing and after killing (group 2 only).

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2.9. PATHOLOGY: sacrifice

After the last excreta collection or blood sampling, the surviving animals were killed by cervical dislocation under excess isoflurane anesthesia and weighed (group 2, only).
A macroscopic examination was not performed on any of the study animals.
The carcasses of the animals (after removal of the specified organs where relevant; see § 2.5. Excretion balance), were stored frozen at -20 °C (with the organs, where relevant), until destruction without analysis in view of the complete mass balance.

2.10. ARCHIVING

The study documentation and specimens generated during the course of the study are archived at CIT, 27005 Miserey, Evreux, France for five years after the end of the *in vivo* phase of the study.
The archived study materials include:
. protocol and amendment,
. raw data,
. correspondence,
. final report and possible amendments.

On completion of this period, the archived study materials will be returned to the Sponsor, or may be archived at CIT for a further period (at additional cost).
In addition raw data witch is not specific to the study including, but not limited to, certificates of analyses for food, water and bedding (if applicable) and records of environmental data and equipment calibration will also be archived at CIT for at least 30 years.

2.11. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedures	Dates	Study days
Protocol approved by:		
. Study Director	4 March 1998	
. Study Monitor	9 March 1998	
Arrival of the animals	20 October 1998	-7
. Preidentification, weighing	21 October 1998	-6
. Randomization and identification	22 October 1998	-5
. Pre-treatment urine, faeces and cage wash samples (group 2)	23 October 1998	-4
Day of dosing	27 October 1998	1
. Blood sampling (group 1)	27 to 30 October 1998	1 to 4
. Urine, faeces and cage wash samples (group 2)	27 October to 3 November 1998	1 to 8
Sacrifice: **group 1**	28 to 30 October 1998	2 to 4
group 2	3 November 1998	8

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2.12. PROTOCOL ADHERENCE

The study was performed in accordance with Study Protocol No. 16432 PAR and subsequent amendment with the following deviations from the agreed Study Protocol:

. the mean body weight of the males was about 180 g (instead of 250 g) and the mean body weight of females was about 142 g (instead of 200 g),
. the animals had free access to A04C P2.5 powdered maintenance diet (instead of A04C pelleted diet) distributed daily,
. the administration of the test formulation was performed using a plastic syringe (instead of a glass syringe),
. Ready-Gel scintillation fluid (instead of Ready-Safe) was used to analyze the cage-wash samples,
. for analytical purposes, various parameters (i.e., number of aliquots and size of aliquots) were modified from those stated in the protocol,
. Guilbert tape (instead of Scotch Magic) was used for skin stripping,
. metabolism cages were placed horizontally (and not vertically) in the racks,
. due to analytical difficulties, the bandages were solubilized in acid and oxidized for radiochemical analysis,
. skin samples (application site only) were analyzed without prior oxidation using Hionic-Fluor scintillant (instead of Carbosorb/Permafluor),
. test formulations were protected from light after manufacture.

These minor deviations were not considered to have compromised the validity or integrity of the study.

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3. RESULTS

3.1. CHEMICAL ANALYSIS OF THE THE RADIOLABELLED TEST SUBSTANCE AND THE ADMINISTERED FORMULATION

3.1.1 Determination of total radioactivity and radiochemical purity of the radiolabelled test substance

The total radioactivity of the radiolabelled test substance solution deviated by -12% from the expected value. However, this was not considered to have had an impact on the integrity of the study.
The results of the analyses demonstrated a satisfactory radiochemical purity of the radiolabelled test substance i.e. 99.1% before administration to the animals.

3.1.2 Total radioactivity of the administered formulation

The total radioactivity of the formulation administered by cutaneous application was found to be somewhat low; deviations from the nominal radioactivity were -15.8% (before the treatment) and -15.3% (after passing through the same administration device as that used for the treatment of the animals). This finding is not surprising in view of the test substance total radioactivity results above, and, in a similar manner, was not considered to have had an impact on the study integrity.

3.1.3 Radiochemical purity of the administered formulation

The results of the analyses demonstrated a satisfactory radiochemical purity of the formulation for cutaneous application since the mean value obtained was 98.4%.

Detailed results are presented in Appendix 2.

3.2. ADMINISTRATION SYSTEM WEIGHTS AND RADIOACTIVE DOSES

The weights of the test substance preparations, and the calculated doses both as total radioactivity (Bq) and mg/kg (using the theoretical concentration), given to the animals of each group are shown in Appendix 2. There was generally a good agreement between the theoretical and actual dose-levels achieved, as the maximum deviation observed was +30.8% for animal U20487 of group 1 (pharmacokinetics).

3.3. PLASMA PHARMACOKINETICS (Table 1, Appendix 3)

There were no relevant deviations to the scheduled blood sampling times.

Following topical application of the isotopic mixture at a nominal 25 mg/kg for an exposure period of 30 minutes, the mean plasma total radioactivity levels were below quantifiable limits at all time points for all animals. The limit of quantitation for the test substance in plasma was >18.2 ng-eq/g (>1.6 Bq/g).

3.4. EXCRETION BALANCE (Table 2, Figures 1 to 3, Appendices 3, 4, 5 and 6)

Following topical application of the isotopic mixture of the test substance at a nominal 25 mg/kg, the mean (± standard deviation) total cumulative excretion of the radioactive dose in the urine, faeces and cage wash during the 30 minute exposure period and over a 168 hour period following washing of the site, was 0.51 ± 0.24% for the males, 0.52 ± 0.03% for the females and 0.52 ± 0.15% for both sexes. The radioactivity was eliminated principally (≥94% of the total recovered in the excreta) in the faeces; specifically, a mean 0.48 ± 0.23%, 0.50 ± 0.03% and 0.49 ± 0.15% of the dose was found in the faeces over the study period for the males, females and both sexes, respectively, whereas for urine the values were, respectively, 0.014 ± 0.007%, 0.014 ± 0.002% and 0.014 ± 0.005%. For cage wash, the elimination values were 0.021 ± 0.002% (males), 0.007 ± 0.011% (females) and 0.016 ± 0.006% (all animals) of the dose over the study period. The radioactivity was eliminated moderately fast, as an average 85.9% (males), 94.1% (females) and 88.5% (both sexes) of the total recovered dose in the excreta was eliminated within 72 hours following dosing; 95.7% of this recovered radioactivity was in the faeces for the males, females and both sexes. Further, a total mean 94.2 ± 3.9% (males), 96.8 ± 3.0% (females) and 95.5 ± 3.4% (both sexes) of the applied dose was recovered in the site dressings and associated wash fluid and materials, and 0.14 ± 0.02% (males), 0.09 ± 0.02% (females) and 0.11 ± 0.03% (both sexes) remained in the "stripped" skin application site (dermis and epidermis). Virtually no radioactivity (except for one male, all values were non quantifiable, i.e., < 0.66 Bq/g of extraction solvent) was found in the skin strippings (stratum corneum).

The excretion profiles were homogeneous for the individual animals and both sexes. In view of the complete mass balance obtained for the excretion experiment, no organs or carcasses were analyzed for radioactivity.
The summarised total cumulated excretion (CE) and recovered radioactivity (RA) data (over the exposure and 168 hour period) (means ± standard deviation), expressed as percentage of the administered dose (%), are shown in the table below;

Route and dose	Animal number and sex	CE in urine (%)	CE in faeces (%)	CE in cage wash (%)	RA in dressing (%)	RA in skin (%)	Total RA (%)
Topical, 25 mg/kg	3, males	0.014 ± 0.007	0.48 ± 0.23	0.021 ± 0.002	94.2 ± 3.9	0.14 ± 0.02	94.8 ± 3.8
	3, females	0.014 ± 0.002	0.50 ± 0.03	0.011 ± 0.005	96.8 ± 3.0	0.09 ± 0.02	97.5 ± 2.9
	6, both	0.014 ± 0.005	0.49 ± 0.15	0.016 ± 0.006	95.5 ± 3.4	0.11 ± 0.03	96.2 ± 3.4

<u>Minimal and maximal fraction of topical dose absorbed</u>
The minimal fraction of radioactivity absorbed following topical application was estimated from the summed urine and faeces total cumulative excretion data to be 0.49% for males, 0.51% for females and 0.50% for both sexes.

The maximum fraction of the dose available for eventual systemic absorption was estimated to be 0.65, 0.61 and 0.63% for males, females both sexes, respectively, and was considered to be the sum of the recovered radioactivity in the excreta and skin site (as the test substance and/or metabolites are known to be eliminated rapidly in the excreta following oral absorption, and are not retained in the carcass).

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3.5. CLINICAL SIGNS, MORBIDITY AND MORTALITY (Appendix 7)

During the study, no morbidity or mortality were observed. One female (U20484) from the pharmacokinetics group 1, had a small blister in the neck region (administration site) after the exposure period; this was considered to be test treatment related and was indicative of a slight local irritant effect.

3.6. BODY WEIGHT (Appendix 8)

On the day of treatment, the males and females of the plasma pharmacokinetics group 1 weighed a mean (± standard deviation) 179 ± 7.8 g and 141 ± 5.6 g, respectively. For group 2, excretion balance, the mean weights were 182 ± 4.5 g for the males and 145 ± 3.5 g for the females.

3.7. PATHOLOGY

In the absence of observed morbidity, at the end of the scheduled biological sampling periods, all surviving study animals were killed. The carcasses from the plasma pharmacokinetic animals (group 1) were stored frozen until discarded without radiochemical analysis. The excretion balance animals (group 2) were dissected/processed as described in the protocol, and the various samples and carcasses were stored frozen. In the presence of a complete elimination of radioactivity during the study period, all the latter samples were discarded without radioactivity analysis.

4. CONCLUSION

The plasma pharmacokinetics and elimination of radioactivity in excreta was investigated following topical administration of ^{14}C at 25 mg/kg, to male and female rats. This study showed that;

- . following topical exposure for 30 minutes, the mean plasma radioactivity levels remained below quantifiable levels,
- . the absolute minimal and maximal fractions of the dose absorbed were extremely low at, respectively, 0.49 and 0.67% for males and 0.51 and 0.63% for females (estimated from summed urine plus faecal elimination data and summed excreta plus skin site and stripping data, respectively),
- . the radioactivity recovered in the skin site (dermis and epidermis) was extremely low at, respectively, 0.14% for the males and 0.09% for the females; radioactivity in the strippings (stratum corneum) was below quantifiable limits,
- . the recovery of radioactivity in the site dressing/wash was very high at 94.2% of the dose for the males and 96.9% for females,
- . the absorbed radioactivity was >80% eliminated (mainly in the faeces) within 72 hours; specifically, the mean urine/faeces ratios over whole collection period were 0.029 and 0.028 for males and females, respectively,
- . the pharmacokinetic and excretion results were homogenous for the individual animals per sex, and no gender differences were apparent.

No metabolism investigations were performed as the levels of radioactivity present in the plasma and urine samples were too low for the analytical techniques available.

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10. Protocol and amendment

AMENDMENT TO PROTOCOL

STUDY No.: **16432 PAR**

SPONSOR:

TITLE: ^{14}C **; Pharmacokinetics study in plasma, excretion balance and tissue distribution after single administration by cutaneous route to rats.**

AMENDMENT No.: 1 Page 1/2

Justification: To amend protocol, add expiry date and synonym for test substance and supply information not known at protocol finalisation.

Date of application: 26 October 1998.

2. TEST AND CONTROL SUBSTANCES

2.1.1 Radiolabelled test substance

To add synonym and supply information:

. Synonym: [U-Ring-14C]
. Batch No.: 98218A
. Description: grey powder
. Specific activity: 23.4 mCi/mmol
. Storage conditions: -20°C, away from light and under nitrogen.

2.1.2 Non radiolabelled test substance

To add expiry date and supply information:

. Batch No.: 0500591
. Description: grey powder
. Expiry date.: October 1999
. Storage conditions: +4°C, away from light and under argon atmosphere

3. TEST SYSTEM

3.2 Environmental conditions

Amend to read:

light/dark cycle: 12 h/12 h (07:00-19:00)

AMENDMENT TO PROTOCOL

STUDY No.: **16432 PAR**
SPONSOR: I.
TITLE: ^{14}C **Pharmacokinetics study in plasma, excretion balance and tissue distribution after single administration by cutaneous route to rats.**
AMENDMENT No.: 1 Page 2/2

6. EXCRETION BALANCE AND DISTRIBUTION (group 2)

Amend to read:
Urine, faeces ... beginning of the application period,
. after each collection of faeces the cages and trays will be carefully rinsed with not more than 20 ml of water (except at 168 h when 200 ml will be used).

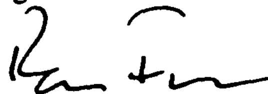
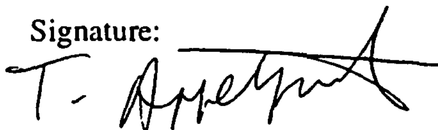
Remove the following phrase;
Other collections may be made if excretion of the compound is not complete 168 h after treatment.

11. REPORTING

Amend to read:
The Study Director ... Bq/animal, Bq/kg and mg/kg as appropriate.
For the excretion balance study ... also be cumulated) and the overall mass balance will be calculated.

14. TIME SCHEDULE

To specify:
Day of treatment: 27 October 1998
End of study: 3 November 1998

Scientific management	Study Director	Study Monitor
S. de Jouffrey or R. Forster	T. Appelqvist	28/10/98
Date: 26 October 1998	Date: 26 October 1998	Date:
Signature: 	Signature: 	Signature:

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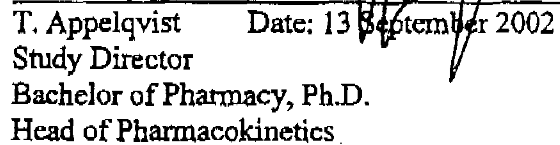
STUDY TITLE: PHARMACOKINETICS STUDY IN PLASMA, EXCRETION BALANCE AND TISSUE DISTRIBUTION AFTER SINGLE ADMINISTRATION BY CUTANEOUS ROUTE TO RATS

MOTIVE: to correct minor "rounding" errors in the report at the request of the Sponsor.

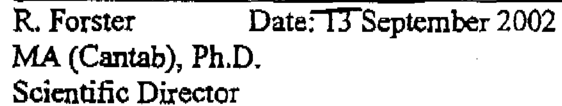
SCIENTISTS INVOLVED IN THIS AMENDMENT:

Pharmacokinetics



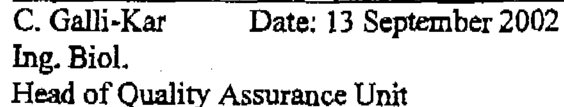
T. Appelqvist Date: 13 September 2002
Study Director
Bachelor of Pharmacy, Ph.D.
Head of Pharmacokinetics

CIT Management



R. Forster Date: 13 September 2002
MA (Cantab), Ph.D.
Scientific Director

QUALITY ASSURANCE UNIT:



C. Galli-Kar Date: 13 September 2002
Ing. Biol.
Head of Quality Assurance Unit

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Results

Plasma pharmacokinetics (group 1)
Following topical application (30 minutes exposure) of the isotopic mixture at a nominal 25 mg/kg, the radioactivity in all plasma samples was below quantifiable limits (< 18.2 ng-eq/g, <1.6 Bq/g).

Excretion balance (group 2)
Following topical application of the isotopic mixture at a nominal 25 mg/kg, the mean (± standard deviation) total cumulative excretion of the radioactive dose in the excreta (during 30 minute exposure period, and a 168 hour period following washing of the site), was 0.51 ± 0.24% and 0.52 ± 0.03% for males and females, respectively. A mean 0.014 ± 0.007% and 0.48 ± 0.23% of the administered dose was eliminated respectively in the urine and faeces for the males; for the females the values were respectively 0.014 ± 0.002% and 0.50 ± 0.03%.
For both sexes, less than 0.1% of the dose was in the cage-wash. Further, a total mean 94.2 ± 3.91% (males) and 96.8 ± 2.96% (females) of the applied dose was recovered in the site dressings/wash, and 0.14 ± 0.02% (males) and 0.09 ± 0.02% (females) remained in the skin application site. Most (>85%) of the total radioactivity recovered in the excreta was eliminated (mainly in faeces) within 72 hours of dosing. Virtually no radioactivity (nearly all values were below quantifiable limits) was found in the strippings. The results were homogenous for all animals/sexes.

Absorption of radioactivity following topical application
The minimal fraction of the topical dose absorbed systemically was a mean 0.49% for the males and 0.51% for the females, as estimated from the individual summed cumulative urine and faecal excretion data. The maximum fraction of the dose available for eventual systemic absorption was estimated to be 0.65 and 0.61% for the males and females, respectively, and was considered to be the sum of the recovered radioactivity in the excreta and skin site.

Mortality and clinical signs
No mortality or morbidity were observed. One animal developed a small blister at the application site.

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Conclusion

The plasma pharmacokinetics and elimination of radioactivity in excreta was investigated following topical administration of ^{14}C at 25 mg/kg, to male and female rats. This study showed that;

- following topical exposure for 30 minutes, the mean plasma radioactivity levels remained below quantifiable levels,
- the absolute minimal and maximal fractions of the dose absorbed were extremely low at, respectively, 0.49 and 0.65% for males and 0.51 and 0.61% for females (estimated from summed urine plus faecal elimination data and summed excreta plus skin site, respectively),
- the radioactivity recovered in the skin site (dermis and epidermis) was extremely low at, respectively, 0.14% for the males and 0.09% for the females; radioactivity in the strippings (stratum corneum) was below quantifiable limits,
- the recovery of radioactivity in the site dressing/wash was very high at 94.2% of the dose for the males and 96.8% for females,
- the absorbed radioactivity was >80% eliminated (mainly in the faeces) within 72 hours; specifically, the mean urine/faeces ratios over whole collection period were 0.029 and 0.028 for males and females, respectively,
- the pharmacokinetic and excretion results were homogenous for the individual animals per sex, and no gender differences were apparent.

No metabolism investigations were performed as the levels of radioactivity present in the plasma and urine samples were too low for the analytical techniques available.

3.4. EXCRETION BALANCE (Table 2, Figures 1 to 3, Appendices 3, 4, 5 and 6)

Following topical application of the isotopic mixture of the test substance at a nominal 25 mg/kg, the mean (± standard deviation) total cumulative excretion of the radioactive dose in the urine, faeces and cage wash during the 30 minute exposure period and over a 168 hour period following washing of the site, was 0.51 ± 0.24% for the males, 0.52 ± 0.03% for the females and 0.52 ± 0.15% for both sexes. The radioactivity was eliminated principally (≥94% of the total recovered in the excreta) in the faeces; specifically, a mean 0.48 ± 0.23%, 0.50 ± 0.03% and 0.49 ± 0.15% of the dose was found in the faeces over the study period for the males, females and both sexes, respectively, whereas for urine the values were, respectively, 0.014 ± 0.007%, 0.014 ± 0.002% and 0.014 ± 0.005%. For cage wash, the elimination values were 0.021 ± 0.002% (males), 0.011 ± 0.005% (females) and 0.016 ± 0.006% (all animals) of the dose over the study period. The radioactivity was eliminated moderately fast, as an average 85.9% (males), 94.1% (females) and 88.5% (both sexes) of the total recovered dose in the excreta was eliminated within 72 hours following dosing; 95.7% of this recovered radioactivity was in the faeces for the males, females and both sexes. Further, a total mean 94.2 ± 3.9% (males), 96.8 ± 3.0% (females) and 95.5 ± 3.4% (both sexes) of the applied dose was recovered in the site dressings and associated wash fluid and materials, and 0.14 ± 0.02% (males), 0.09 ± 0.02% (females) and 0.11 ± 0.03% (both sexes) remained in the "stripped" skin application site (dermis and epidermis). Virtually no radioactivity (except for one male, all values were non quantifiable, i.e., < 0.66 Bq/g of extraction solvent) was found in the skin strippings (stratum corneum).
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The summarised total cumulated excretion (CE) and recovered radioactivity (RA) data (over the exposure and 168 hour period) (means ± standard deviation), expressed as percentage of the administered dose (%), are shown in the table below;

Route and dose	Animal number and sex	CE in urine (%)	CE in faeces (%)	CE in cage wash (%)	RA in dressing (%)	RA in ski (%)	Total RA (%)
Topical, 25 mg/kg	3, males	0.014 ± 0.007	0.48 ± 0.23	0.021 ± 0.002	94.2 ± 3.9	0.14 ± 0.02	94.8 ± 3.8
	3, females	0.014 ± 0.002	0.50 ± 0.03	0.011 ± 0.005	96.8 ± 3.0	0.09 ± 0.02	97.5 ± 2.9
	6, both	0.014 ± 0.005	0.49 ± 0.15	0.016 ± 0.006	95.5 ± 3.4	0.11 ± 0.03	96.2 ± 3.4

Minimal and maximal fraction of topical dose absorbed

The minimal fraction of radioactivity absorbed following topical application was estimated from the summed urine and faeces total cumulative excretion data to be 0.49% for males, 0.51% for females and 0.50% for both sexes.

The maximum fraction of the dose available for eventual systemic absorption was estimated to be 0.65, 0.61 and 0.63% for males, females both sexes, respectively, and was considered to be the sum of the recovered radioactivity in the excreta and skin site (as the test substance and/or metabolites are known to be eliminated rapidly in the excreta following oral absorption, and are not retained in the carcass).

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3.5. CLINICAL SIGNS, MORBIDITY AND MORTALITY (Appendix 7)

During the study, no morbidity or mortality were observed. One female (U20484) from the pharmacokinetics group 1, had a small blister in the neck region (administration site) after the exposure period; this was considered to be test treatment related and was indicative of a slight local irritant effect.

3.6. BODY WEIGHT (Appendix 8)

On the day of treatment, the males and females of the plasma pharmacokinetics group 1 weighed a mean (± standard deviation) 179 ± 7.8 g and 141 ± 5.6 g, respectively. For group 2, excretion balance, the mean weights were 182 ± 4.5 g for the males and 145 ± 3.5 g for the females.

3.7. PATHOLOGY

In the absence of observed morbidity, at the end of the scheduled biological sampling periods, all surviving study animals were killed. The carcasses from the plasma pharmacokinetic animals (group 1) were stored frozen until discarded without radiochemical analysis. The excretion balance animals (group 2) were dissected/processed as described in the protocol, and the various samples and carcasses were stored frozen. In the presence of a complete elimination of radioactivity during the study period, all the latter samples were discarded without radioactivity analysis.

4. CONCLUSION

The plasma pharmacokinetics and elimination of radioactivity in excreta was investigated following topical administration of ^{14}C , at 25 mg/kg, to male and female rats. This study showed that;

- following topical exposure for 30 minutes, the mean plasma radioactivity levels remained below quantifiable levels,
- the absolute minimal and maximal fractions of the dose absorbed were extremely low at, respectively, 0.49 and 0.65% for males and 0.51 and 0.61% for females (estimated from summed urine plus faecal elimination data and summed excreta plus skin site, respectively),
- the radioactivity recovered in the skin site (dermis and epidermis) was extremely low at, respectively, 0.14% for the males and 0.09% for the females; radioactivity in the strippings (stratum corneum) was below quantifiable limits,
- the recovery of radioactivity in the site dressing/wash was very high at 94.2% of the dose for the males and 96.8% for females,
- the absorbed radioactivity was >80% eliminated (mainly in the faeces) within 72 hours; specifically, the mean urine/faeces ratios over whole collection period were 0.029 and 0.028 for males and females, respectively,
- the pharmacokinetic and excretion results were homogenous for the individual animals per sex, and no gender differences were apparent.

No metabolism investigations were performed as the levels of radioactivity present in the plasma and urine samples were too low for the analytical techniques available.

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SPONSOR

STUDY TITLE

SKIN SENSITIZATION TEST
IN GUINEA-PIGS
(Maximization method of
Magnusson, B. and Kligman, A.M.)

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

15th February 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12045 TSG

SUMMARY

At the request of Société , the potential of the test substance, (batch No. Pil 4 X), to induce delayed contact hypersensitivity following intradermal injection and cutaneous application was evaluated in guinea-pigs according to the maximization method of Magnusson and Kligman. The study was conducted in compliance with the principles of Good Laboratory Practice Regulations.

Methods

Fifteen guinea-pigs (females) were allocated to 2 groups: a control group 1 (5 females) and a treated group 2 (10 females).
The sensitization potential of the test substance was evaluated after a 10-day induction period during which time the animals were treated with isotonic aqueous NaCl solution (control group) or the test substance (treated group). On day 1, in presence of Freund's complete adjuvant, 0.1 ml of the test substance at a concentration of 0.1% (w/w) in the vehicle was administered by intradermal route. On day 8, 0.5 ml of the test substance at a concentration of 50% (w/w) in the vehicle was applied by cutaneous route during 48 hours by means of an occlusive dressing. After a period of 12 days without treatment, a challenge cutaneous application of 0.5 ml of the vehicle (left flank) and 0.5 ml of the test substance at a concentration of 25% (w/w) in the vehicle (right flank) were administered to all animals. The test substance and the vehicle were prepared on a dry gauze pad then were applied to the skin and held in place for 24 hours by means of an occlusive dressing. Cutaneous reactions on the challenge application sites were then evaluated 24 and 48 hours after removal of the dressing.

After the final scoring period, the animals were killed and no cutaneous samples were taken.

The sensitivity of the guinea-pigs in C.I.T. experimental conditions were checked in a recent study with a positive sensitizer: Dinitro 2.4 Chlorobenzene. During induction period the test substance was applied at 0.05% (day 1) and 0.5% (day 8) concentrations. At cutaneous challenge application, 0.1% and 0.5% were tested on both flanks.

Results

No clinical signs and no deaths were noted during the study.

Slight erythema was observed in 6/10 animals 24 hours after removal of the dressing and in 5/10 animals 48 hours after removal of the dressing. No cutaneous reaction was observed in the control group.

The guinea-pigs which were used in a recent study showed a satisfactory sensitization response in 100% animals using a positive sensitizer (appendix 5).

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Conclusion

Under our experimental conditions and according to the maximization method established by Magnusson and Kligman, cutaneous reactions attributable to the sensitization potential of the test substance, (batch No. Pil 4 X), at the concentration of 25% (w/w) were observed in 50% of the guinea-pigs.

The sensitization potential of the test substance was not confirmed on a second challenge phase.

Labelling

Commission Directive 93/21/E.E.C.

Symbol : X_i

Indication of danger : Irritant

Sentence R43 : "May cause sensitization by skin contact"

1. INTRODUCTION

The objective of this study, performed according to maximization method established by Magnusson and Kligman (1), was to evaluate the potential of the test substance, to induce delayed contact hypersensitivity in guinea-pigs.

The results of the study are of value in predicting the contact sensitization potential of the test material in Man.

During the induction period, the test substance was administered by intradermal route (together with an adjuvant to maximise potential reactions) and cutaneous route. After a rest period of 12 days, a challenge application with the test substance was performed in order to provoke a cutaneous sensitization reaction.

The study was conducted in compliance with:
. O.E.C.D. guideline No. 406, 17th July 1992,
. E.C. Directive No. 92/69/E.E.C., B_6, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4x
  - labelling: Pil 4x
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: . at room temperature, protected from light,
  . protected from humidity from 1.8.94.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Vehicle

The vehicle used was isotonic aqueous NaCl solution, batch No. 3067 (Biosédra, 92240 Malakoff, France).

(1) Magnusson, B.; Kligman, A.M.: The identification of contact allergens by animal assay.
Société The guinea-pig maximization test. J. Invest. Derm. 52: 268-276 (1969).

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2.1.3 Preparation

The test substance was prepared in the vehicle.

2.1.4 Other substance

The other substance used was Freund's complete adjuvant, batch No. 063H8800 (Sigma, 38297 Saint-Quentin-Fallavier, France).

2.2. TEST SYSTEM

2.2.1 Animals

Species and strain: Dunkin-Hartley guinea-pigs.
Reason for this choice: species recommended by the international regulations for sensitization studies. The strain used has been shown to produce a satisfactory sensitization response using known positive sensitizers.
Breeder: Centre d'Elevage Lebeau, 78950 Gambais, France.
Number: 15 nulliparous and non-pregnant females.
Allocation of the animals to the groups: on day -1, the animals were weighed and randomly allocated to 2 groups: a control group 1 consisting of 5 females and a treated group 2 consisting of 10 females.
Weight: on day 1, the animals had a mean body weight of 342 ± 28 g.
Acclimatization: at least 5 days before the beginning of the study.
Identification of the animals: the animals were identified individually by an ear-tattoo.

2.2.2 Environmental conditions

During the acclimatization period and throughout the study, the conditions in the animal room were as follows:
. temperature: 21 ± 2°C
. relative humidity: 30 to 70%
. light/dark cycle: 12 h/12 h
. ventilation: about 12 cycles/hour of filtered, non-recycled air.
The air was non-recycled and filtered.
During the acclimatization period and throughout the study, the animals were housed individually in polycarbonate cages (48 x 27 x 20 cm) equipped with a polypropylene bottle. Calibrated and dust-free sawdust was provided as litter (SICSA, 92142 Alfortville, France). An analysis of potential residues and major contaminants is performed periodically (Laboratoire Wolff, 92110 Clichy, France).

2.2.3 Food and water

During the study, the animals had free access to "Guinea-pigs sustenance reference 106 diet" (U.A.R., 91360 Villemoisson-sur-Orge, France).
Food was periodically analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was contained in bottles.
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

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2.3. TREATMENT

2.3.1 Preliminary test

A preliminary test was performed to define the concentration to be tested in the main study.

By intradermal route
Determination of the Minimum Irritant Concentration (M.I.C.):
. 24 hours before treatment, the dorsal region of the animals was clipped,
. the test substance was prepared in an appropriate vehicle,
. intradermal administration of the test substance (volume 0.1 ml) at increasing concentrations was performed in order to determine the maximum concentration which does not cause necrosis or ulceration, but does cause an irritation,
. evaluation of the potential cutaneous reactions, 24 hours after injection.

By cutaneous route
Determination of the Minimum Irritant Concentration (M.I.C.) and Maximum Non-Irritant Concentration (M.N.I.C.):
. 24 hours before treatment, the dorsal region of the animals was clipped,
. the test substance was prepared in an appropriate vehicle,
. 0.5 ml of each concentration was applied to a gauze pad of approximately 4 cm^2 and then held in place by an occlusive dressing for 24 hours,
. potential cutaneous reactions were evaluated 24 and 48 hours after removal of the gauze pads.

2.3.2 Main study

2.3.2.1 Preparation of the animals

For all animals and before each treatment, the application sites were:
. clipped on days -1 and 7 (scapular area 4 cm x 2 cm),
. clipped again and shaved on day 21 (each flank 2 cm x 2 cm).

2.3.3 Induction phase by intradermal and cutaneous routes

2.3.3.1 Intradermal route

On day 1, 6 intradermal injections were made into a clipped area (4 cm x 2 cm) in the scapular region, using a needle (diameter: 0.50 x 16 mm, Térumo: C.M.L., 77140 Nemours, France) mounted on a 1 ml glass syringe (0.01 ml graduations, Record: Carrieri, 75005 Paris, France). Three injections of 0.1 ml were injected into each side of the animal, as follows:

Control group (figure 1)
. Freund's complete adjuvant diluted to 50% (v/v) with an injectable isotonic solution (NaCl at 0.9%),
. vehicle,
. a mixture of 50/50 (w/v) Freund's complete adjuvant diluted to 50% (v/v) with a sterile isotonic aqueous NaCl solution and the vehicle.

Treated group (figure 2)
. Freund's complete adjuvant diluted to 50% (v/v) with a sterile isotonic aqueous NaCl solution,
. test substance at a concentration of 0.1% (w/w) in the vehicle,
. a mixture 50/50 (w/v) of Freund's complete adjuvant diluted to 50% (v/v) with a sterile isotonic aqueous NaCl solution, and, the test substance at a concentration of 0.1% (w/w) in the vehicle.

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2.3.3.2 Cutaneous route

As the test substance was shown to be irritant after occlusive cutaneous treatment during preliminary test, a local irritation by sodium laurylsulphate was not necessary on day 7.

On day 8, a cutaneous application on the 6 injection areas (4 cm x 2 cm) of the scapular region was performed.

Control group

. application of 0.5 ml of the vehicle.

Treated group

. application of 0.5 ml of a slightly irritant concentration of the test substance i.e. 50% (w/w) in the vehicle.

The test substance and the vehicle were prepared on a dry gauze pad (Semes France, 54183 Heillecourt, France), which was then applied to the scapular region and held in place for 48 hours by means of an adhesive hypoallergenic dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and an adhesive anallergenic waterproof plaster (Laboratoire des Professions Médicales, 92240 Malakoff, France).
No residual test substance was observed at removal of the dressing.
One hour after removal of the occlusive dressing, cutaneous reactions were recorded.

2.3.3.3 Challenge phase

At the end of the rest period on day 22, the test substance was applied at the Maximum Non-Irritant Concentration (M.N.I.C.) i.e. 25% (w/w) in the vehicle.

On day 22, the animals from both groups received an application of 0.5 ml of the M.N.I.C. of the test substance on the posterior right flank, and 0.5 ml of the vehicle on the posterior left flank. This application was performed using a 1 ml plastic syringe (0.01 ml graduations, Térumo: C.M.L., 77140 Nemours, France). The test substance and vehicle were prepared on a dry gauze pad (Semes France, 54183 Heillecourt, France), then applied to a 4 cm^2 (2 cm x 2 cm) clipped area of the skin. The gauze pad was held in contact with the skin for 24 hours by means of an occlusive, hypoallergenic dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and an adhesive anallergenic waterproof plaster (Laboratoire des Professions Médicales, 92240 Malakoff, France).
No residual test substance was observed at removal of the dressing.

2.4. SCORING OF CUTANEOUS REACTIONS

Twenty-four and 48 hours after removal of the dressing from the challenge application site, the both flanks of the treated and control animals were observed in order to evaluate cutaneous reactions, according to the following scale:

Erythema and eschar formation

. No erythema 0
. Very slight erythema (barely perceptible) 1
. Well-defined erythema 2
. Moderate to severe erythema 3
. Severe erythema (beet redness) to slight eschar formation (injuries in depth) 4

Oedema formation

. No oedema 0
. Very slight oedema (barely perceptible) 1
. Slight oedema (visible swelling with well-defined edges) 2
. Moderate oedema (visible swelling raised more than 1 millimetre) 3
. Severe oedema (visible swelling raised more than 1 millimetre and extending beyond the area of exposure) 4

Any other lesions were noted.

2.5. CLINICAL EXAMINATIONS

The animals were observed twice a day during the study in order to record clinical signs and to check for mortality.

2.6. BODY WEIGHT

The animals were weighed individually on the day of allocation into the groups, on the first day of the study (day 1), then on days 8, 15 and 25.

2.7. PATHOLOGY

2.7.1 Necropsy

On day 25, after the 48-hour observation period, the animals of group 1 were killed by CO_2 inhalation in excess. At the request of the Sponsor, animals of group 2 were killed at a later date.

2.7.2 Cutaneous samples

On day 25, no skin samples were taken.

2.7.3 Microscopic examination

No histological examinations were performed.

2.8. DETERMINATION OF THE ALLERGENICITY LEVEL

The treated animals show a positive reaction if macroscopic cutaneous reactions are clearly visible (erythema and/or oedema ≥ 2) and more marked than the most severe reactions of the control animals, or, if "doubtful" macroscopic reactions are confirmed at microscopic examination as being due to the sensitization process. Sensitization reactions are characterized at microscopic examination by basal spongiosis, reactional acanthosis of the epidermis and infiltration of mononucleated cells into the dermis (1).

Determination of the allergenicity level
The allergenicity level of the test substance is calculated by comparing the number of animals showing positive reactions with the number of surviving treated animals at the end of the study.

% of animals showing a reaction	Allergenicity level	Classification
0 - 8	I	very weak
9 - 28	II	weak
29 - 64	III	moderate
65 - 80	IV	strong
81 - 100	V	very strong

According to the E.E.C. directive 93/21/E.E.C., when the reactions are positive in at least 30% of the treated animals, the test substance has sensitization properties and the sentence "R 43: May cause sensitization by skin contact" must be applied.

(1) Duprat, P. ; Delsaut. L. ; Gradiski, D. ; Lepage, M. : Investigations histo–pathologiques et cytologiques lors de la mise en évidence, chez le cobaye, d'une allergie cutanée de type retardé. Revue Méd. Vét. 127: 7, 1083-1101 (1976).

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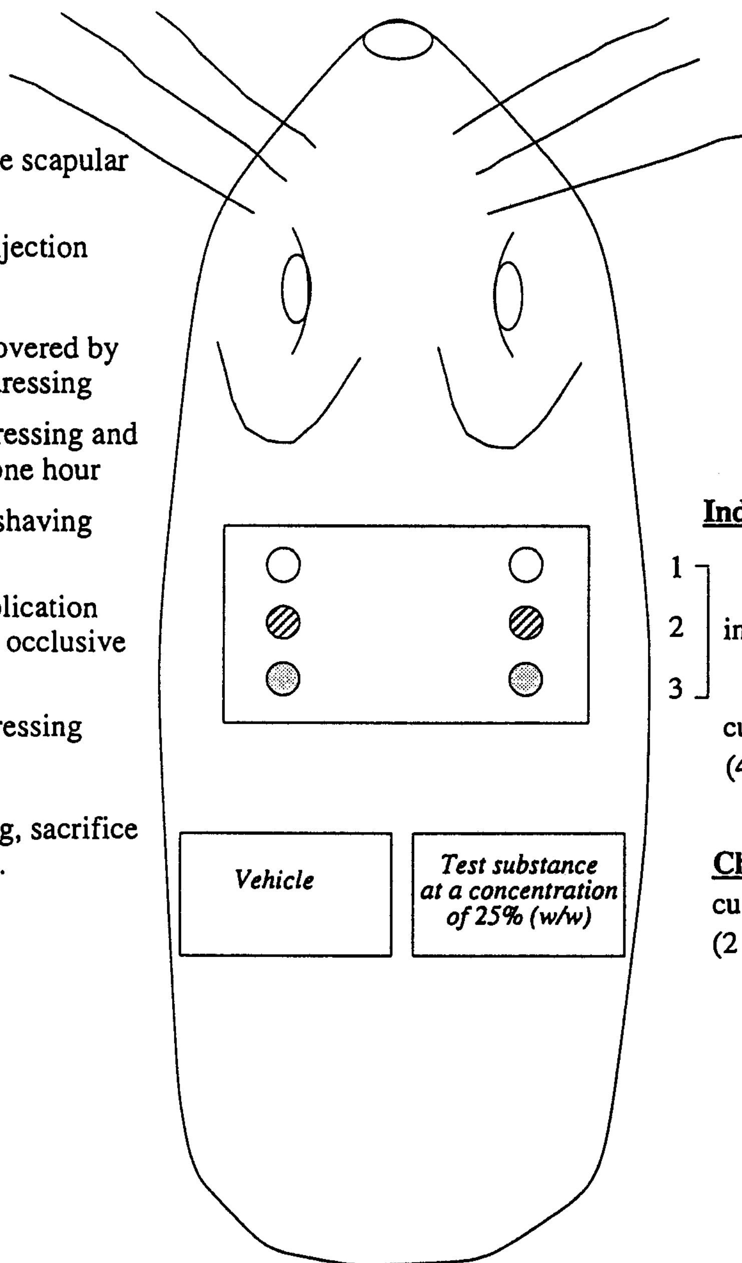
2.9. SUMMARY DIAGRAMS

Figure 1: control group

Chronology

Day -1	Clipping of the scapular region
Day 1	Intradermal injection
Day 7	Clipping
Day 8	Application covered by an occlusive dressing
Day 10	Removal of dressing and scoring after one hour
Day 21	Clipping and shaving of the flanks
Day 22	Challenge application covered by an occlusive dressing
Day 23	Removal of dressing
Day 24	First scoring
Day 25	Second scoring, sacrifice of the animals.



Intradermal injections

1	50% Freund's complete adjuvant and NaCl at 0.9% solution
2	vehicle
3	1 + 2, 50/50 (w/v)

Figure 2: treated group

Chronology

Day -1 Clipping of the scapular region

Day 1 Intradermal injection

Day 7 Clipping

Day 8 Application covered by an occlusive dressing

Day 10 Removal of dressing and scoring after one hour

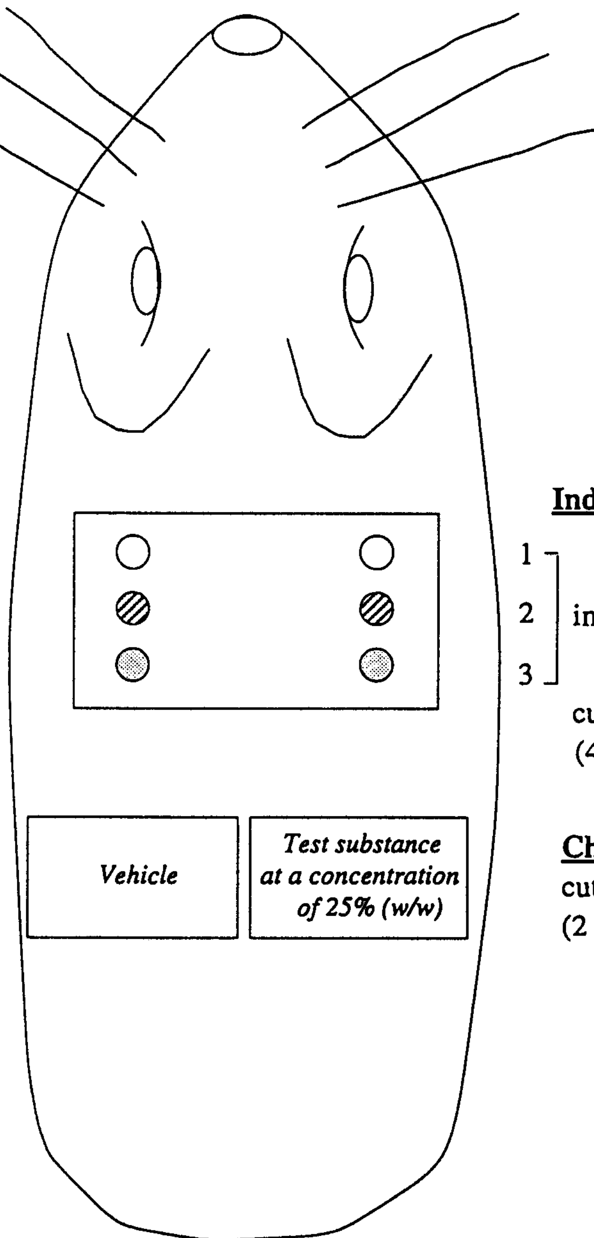
Day 21 Clipping and shaving of the flanks

Day 22 Challenge application covered by an occlusive dressing

Day 23 Removal of dressing

Day 24 First scoring

Day 25 Second scoring, sacrifice of the animals.



Induction site

1, 2, 3 intradermal injections

cutaneous application (4 cm x 2 cm)

Challenge application

cutaneous application (2 cm x 2 cm)

Intradermal injections

1. 50% Freund's complete adjuvant and NaCl at 0.9% solution
2. test substance and vehicle (w/w)
3. 1 + 2, 50/50 (w/v)

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2.10. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date	Day
Arrival of the animals	18.8.94	-5
Allocation of the animals into groups	22.8.94	-1
Weighing, induction by intradermal injection	23.8.94	1
Clipping	29.8.94	7
Weighing, induction by cutaneous route	30.8.94	8
Removal of occlusive dressings and scoring of local reactions after 1 hour	1.9.94	10
Weighing	6.9.94	15
Challenge cutaneous application	13.9.94	22
Removal of occlusive dressings	14.9.94	23
Scoring of cutaneous reactions after		
. 24 hours	15.9.94	24
. 48 hours	16.9.94	25
Weighing	16.9.94	25
Sacrifice of the animals	21.9.94	

2.11. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final study report and possible amendments,

are stored on the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

3. RESULTS

3.1. PRELIMINARY STUDY

3.1.1 Administration by intradermal route

Several tests were performed to determine the minimal irritant concentration which did not provoke necrosis or ulceration.

Animal number	Concentration of the test substance % (w/w)	Scoring after treatment 24 hours
First assay		
female 01	5	necrosis
female 02	5	necrosis
female 01	10	necrosis
female 02	10	necrosis
female 01	25	necrosis
female 02	25	necrosis
Second assay		
male 03	0.1	C
male 04	0.1	C
male 03	0.5	C
male 04	0.5	C
male 03	1	C
male 04	1	C

C = black colouration of skin which could mask an eventual erythema.
Concentration used in the main study was 0.1% (w/w) of the test substance.

3.1.2 Application by cutaneous route

The maximal test substance concentration which could be obtained in the vehicle was 100%. Several assays were conducted to determine the Maximum Non-Irritant Concentration.

Animal number	Concentration of the test substance %(w/w)		Scoring after removal of the dressing (1) (2) 24 hours E	24 hours O	48 hours E	48 hours O
First assay						
female 01	50	RF	0	0	0	0
	50	LF	0	0	0	0
female 02	50	RF	0	0	0	0
	50	LF	1	0	0	0
Second assay						
female 01	25	RF	0	0	0	0
	25	LF	0	0	0	0
female 02	25	RF	0	0	0	0
	25	LF	0	0	0	0

M.N.I.C. is 25% of the test substance.
E: erythema
O: oedema

(1) No residual test substance was observed.
(2) Any residual were wiped off water for injections, batch No. 9186 (Bioséadra, 92240 Malakoff, France).

3.2. MAIN STUDY

3.2.1 Clinical examinations

No clinical signs or mortalities were observed during the study.

The body weight gain of the treated animals was normal when compared to that of the control animals (figure 3, appendix 3).

3.2.2 Scoring of cutaneous reactions (appendix 4)

3.2.2.1 End of the induction period

On day 10, after removal of the dressing, irritation in control and treated groups were observed at the intradermal injection sites.

3.2.2.2 Challenge application

After the challenge application, a very slight (score of 1), erythema was observed at the following frequency:

Erythema

Groups	Sex	Erythema score	Scoring of the cutaneous parameters			
			24 hours		48 hours	
			LF	RF	LF	RF
Control 1	Female	0	5/5	5/5	5/5	5/5
Treated 2	Female	0	10/10	4/10	10/10	5/10
		1	-	6/10	-	5/10

LF: left flank (control)
RF: right flank (treated)

No oedemas were observed 24 and 48 hours after removal of the dressing of the challenge cutaneous application of the test substance.
No cutaneous reaction was observed in the control group.

Slight erythema was observed on 60% of the animals 24 hours after removal of the dressing and on 50% of the animals 48 hours after removal of the dressing. Dryness of the skin was observed on 5/10 animals at the 48-hour reading. These reactions were attributed to a sensitization potential of the test substance.

4. CONCLUSION

Under our experimental conditions and according to the maximization method established by Magnusson and Kligman, cutaneous reactions attributable to the sensitization potential of the test substance, (batch No. Pil 4 X), at the concentration of 25% (w/w) were observed in 50% of the guinea-pigs.

CLASSIFICATION OF THE TEST SUBSTANCE

Cutaneous sensitization induced by the test substance,

According to Council Directive 93/21/E.E.C. (27th April 1993) adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C. on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances,

Concerning the potential sensitizing effect by skin contact, the test substance should be classified as follows:

Symbol : X_i

Indication of danger : Irritant

Sentence indicating particular hazards : R43: "May cause sensitization by skin contact".

MACROSCOPIC EXAMINATION OF CUTANEOUS REACTIONS

Challenge application

Group	Sex	Animal No.	Day 24 scoring period (after 24 hours) Erythema LF	Erythema RF	Oedema LF	Oedema RF	Day 25 scoring period (after 48 hours) Erythema LF	Erythema RF	Oedema LF	Oedema RF
Control	Female	76	0	0	0	0	0	0	0	0
1		77	0	0	0	0	0	0	0	0
		78	0	0	0	0	0	0	0	0
		79	0	0	0	0	0	0	0	0
		80	0	0	0	0	0	0	0	0
Treated	Female	81	0	1	0	0	0	1/S	0	0
2		82	0	0	0	0	0	0	0	0
		83	0	0	0	0	0	0	0	0
		84	0	1	0	0	0	0	0	0
		85	0	1	0	0	0	1/S	0	0
		86	0	1	0	0	0	1/S	0	0
		87	0	1	0	0	0	1/S	0	0
		88	0	1	0	0	0	1/S	0	0
		89	0	0	0	0	0	0	0	0
		90	0	0	0	0	0	0	0	0

LF : left flank (control)
RF : right flank (treated)
S : dryness of the skin

Purpose: check the sensitivity of Dunkin-Hartley guinea-pigs to a positive control test article

Method : Magnusson and Kligman

Test substance : DINITRO 2.4 CHLOROBENZENE

C.I.T. Study - Date : January 1994 (CIT/Study No. 11284 TSG)

Number of animals : 5 females

Induction : 0.05% intradermal route day 1
0.5% cutaneous route day 8

Challenge application : 0.1% right flank
0.5% left flank

Conclusion

Under our experimental conditions and according to the Magnusson and Kligman method, DINITRO 2.4 CHLOROBENZENE at a concentration of 0.5% induced positive skin sensitization reactions in 100% of the guinea-pigs.

INDIVIDUAL REACTIONS: CHALLENGE PHASE MACROSCOPIC FINDINGS

Group	Sex	Animals	24-hour scoring period Erythema LF		Oedema LF		48-hour scoring period Erythema LF		Oedema LF		Conclusion LF	
			Erythema LF	Erythema RF	Oedema LF	Oedema RF	Erythema LF	Erythema RF	Oedema LF	Oedema RF	LF	RF
Treated	Female	76	2	1	0	0	2/S	1/S	0	0	+	+/-
		77	2/S	1/S	0	0	2/S	2/S	0	0	+	+
		78	3	2	2	2	3/S	2/S	0	0	+	+
		79	4/S	2/S	2	0	4/A	2/S	0	0	+	+
		80	2/S	1	0	0	2/S	1/S	0	0	+	+/-

+/- : borderline
\+ : hypersensitizing reaction
S : dryness of the skin
A : crust
LF : left flank
RF : right flank

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SPONSOR

STUDY TITLE

SKIN SENSITIZATION TEST
IN GUINEA-PIGS
(Maximization method of
Magnusson, B. and Kligman, A.M.)

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

5th February 1996

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

13354 TSG (96/1/013)

SUMMARY

At the request of Société , the potential of the test substance (batch No. Pil 4X) to induce delayed contact hypersensitivity was evaluated in guinea-pigs according to the maximization method of Magnusson and Kligman and to O.E.C.D. (No. 406, 17th July 1992) and E.C. (92/69/E.E.C., B_6) guidelines. The study was conducted in compliance with the principles of Good Laboratory Practice Regulations.

Methods

Thirty guinea-pigs were allocated to two groups: a control group 1 (five males and five females) and a treated group 2 (ten males and ten females).

On day 1, in the dorsal region between the shoulders, intradermal injections of Freund's complete adjuvant mixed with the test substance (treated group) or the vehicle (control group) were prepared.

On day 7, the same region received a topical application of sodium laurylsulfate in vaseline (10% w/w) in order to induce local irritation.

On day 8, this same test site was treated by topical application of the test substance (treated group) or the vehicle (control group) and was covered by an occlusive dressing for 48 hours.

After a rest period of 12 days, all animals of the treated and control groups were challenged by a topical application of the test substance to the right flank. The left flank served as control and received the vehicle only.

Test substance and vehicle were maintained under an occlusive dressing for 24 hours. Skin reactions were evaluated approximately 24 and 48 hours later.

Test substance concentrations were as follows:

Induction (treated group)
. intradermal injections: at 1% (w/w) in sterile isotonic saline solution (0.9% NaCl),
. topical application: at 50% (w/w) in sterile isotonic saline solution (0.9% NaCl).

First challenge (all groups)
. topical application: at 50% (w/w) in sterile isotonic saline solution (0.9% NaCl).

At the end of the study, animals were killed and cutaneous samples were taken from the challenge application sites from all the animals. No histological examinations were performed on the cutaneous reactions.

The sensitivity of the guinea-pigs in C.I.T. experimental conditions were checked in a recent study with a positive sensitizer: 2,4-dinitro chlorobenzene. During induction period, the test substance was applied at 0.1% (day 1) and 1% (day 8) concentrations. At cutaneous challenge application, 1% (w/w) was tested on the right flank.

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The interpretation of results was carried out according to the classification criteria laid down in Council Directive 93/21/E.E.C. (27th April 1993) adapting to technical progress for the eighteenth time Council Directive 67/548/E.E.C.

Results

No clinical signs and no deaths were noted during the study.

After the challenge application, no cutaneous reactions were observed in the control group.

In the treated group, at the 24-hour reading, very slight, well-defined and marked erythema were observed in 2/20, 11/20 and 7/20 animals, respectively. The erythema was accompanied by slight oedema in 11 animals and by severe oedema in one animal. Dryness of the skin was noted in 9/20 animals. Very slight black colouration of the skin was observed in 3 animals.

At the 48-hour reading, very slight, well-defined, marked and severe erythema were noted in 1/20, 4/20, 1/20 and 2/20 animals, respectively. Crust were observed in 3 animals. Dryness of the skin was observed in 14/20 animals. It was severe enough to mask the evaluation of the erythema in 5/20 animals. Very slight to slight black colouration of the skin was observed in 5 animals.

The very slight erythema which did not persit at the 48-hour reading in two animals, were attributed to a possible slight irritant reaction. All the other skin lesions were attributed to a sensitisation effect.

The guinea-pigs which were used in a recent study, showed a satisfactory sensitization response in 100% animals using a positive sensitizer (appendix 5).

Conclusion

Under our experimental conditions and according to the maximization method of Magnusson and Kligman, cutaneous reactions attributable to the sensitization potential of the test substance (batch No. Pil 4X) at a concentration of 50% (w/w) were observed in 18/20 (90%) guinea-pigs.

Classification

Concerning the potential sensitizing effect by skin contact, according to Commission Directive 93/21/E.E.C., the test substance shoud be classified as follows:

Symbol : X_i

Indication of danger: Irritant

Sentence R 43 : "May cause sensitization by skin contact"

1. INTRODUCTION

The objective of this study, performed according to the maximization method of Magnusson and Kligman (1), was to evaluate the potential of the test substance to induce delayed contact hypersensitivity in guinea-pigs.

The results of the study are of value in predicting the contact sensitization potential of the test material in Man.

The study was conducted in compliance with:
. O.E.C.D. guideline No. 406, 17th July 1992.
. E.C. Directive No. 92/69/E.E.C., B_6, 31st July 1992.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance, used in the study was supplied by Société

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination:
  - protocol:
  - labelling:
. batch number:
  - protocol: Pil 4X
  - labelling: Pil 4X
. description: beige powder
. quantity and container: 4 kg in a plastic pot
. date of receipt: 26.7.94
. storage conditions: at room temperature, protected from light and from humidity from 1.8.94. The test substance, deconditioned in flasks numbered from 1 to 7, was stored under nitrogen gas from 2.1.95 until use of each flask
. purity: 99.8%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.2 Vehicle

The choice of the vehicle was based on tests to check the homogeneity of the preparation (for topical and intradermal injections) and its free passage through a needle (for intradermal injections). The highest concentration which satisfied these criteria was called the maximal practicable concentration.

(1) Magnusson, B.; Kligman, A.M.: The identification of contact allergens by animal assay. The guinea-pig maximization test. J. Invest. Derm. 52: 268-276 (1969).

The vehicle used was sterile isotonic saline solution (0.9% NaCl), batch No. 4040 (Laboratoire Fresenius, 92316 Sèvres, France).

2.1.3 Preparation

The test substance was prepared at appropriate concentrations in the vehicle or Freund's complete adjuvant.
All preparations were made freshly on the morning of administration and any unused material was discarded that same day.

2.1.4 Other substances

The other substances used were Freund's complete adjuvant, batch No. 084H8800 (Sigma, 38297 Saint-Quentin-Fallavier, France); sodium laurylsulphate, batch No. 83H0841 (Sigma, 38297 Saint-Quentin-Fallavier, France) and vaseline, batch No. 4036 (Coopérative Pharmaceutique Française, 77000 Melun, France).

2.2. TEST SYSTEM

2.2.1 Animals

Species and strain: Dunkin-Hartley guinea-pigs.
Reason for this choice: species recommended by the international regulations for sensitization studies. The strain used has been shown to produce a satisfactory sensitization response using known positive sensitizers.
Breeder: Centre d'Elevage Lebeau, 78950 Gambais, France.
Number: 30 animals (15 males and 15 nulliparous and non-pregnant females).
Allocation of the animals to the groups: on day -1, the animals were weighed and randomly allocated to two groups: a control group 1 consisting of ten animals (five males and five females) and a treated group 2 consisting of 20 animals (ten males and ten females).
Weight: on day 1, the animals were approximately three months old and had a mean body weight ± standard deviation of 375 ± 33 g for the males and 311 ± 14 g for the females.
Acclimatization: at least five days before the beginning of the study.
Identification of the animals: ear-tattoo.

2.2.2 Environmental conditions

During the acclimatization period and throughout the study, the conditions in the animal room were set as follows:
. temperature: 21 ± 2°C
. relative humidity: 30 to 70%
. light/dark cycle: 12 h/12 h
. ventilation: about 12 cycles/hour of filtered, non-recycled air.
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity, light/dark cycle and ventilation) were checked regularly.
During the acclimatization period and throughout the study, the animals were housed individually in polycarbonate cages (48 cm x 27 cm x 20 cm) equipped with a polypropylene bottle.
Dust-free sawdust was provided as litter (SICSA, 92142 Alfortville, France).
Bacteriological analysis of the sawdust and detection of possible contaminants (pesticides, heavy metals) are performed periodically.

2.2.3 Food and water

During the study, the animals had free access to "106 diet" (U.A.R., 91360 Villemoisson-sur-Orge, France).
Each batch of food was analysed (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was provided *ad libitum*.
Bacteriological and chemical analysis of the water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

It was verified that no contaminants in the diet or water at levels likely to influence the outcome of the study were present.

2.3. TREATMENT

2.3.1 Preliminary test

A preliminary test was conducted in order to determine the concentrations to be tested in the main study.

By intradermal route:
. 24 hours before treatment, the dorsal region of the animals was clipped,
. the test substance was prepared in an appropriate vehicle,
. intradermal administrations of the test substance (0.1 ml) at different concentrations were performed in the dorsal region between the shoulders,
. cutaneous reactions were evaluated approximately 24, 48 hours and six days after injection.

By cutaneous route:
. 24 hours before treatment, both flank regions of the animals were clipped,
. if necessary, the test substance was prepared in an appropriate vehicle,
. the test substance (0.5 ml for each concentration) was applied to a dry gauze pad of approximately 4 cm^2 which was held in place by an occlusive dressing for 24 hours,
. cutaneous reactions were evaluated approximately 24 and 48 hours after removal of the dressings.

Criteria for selection of concentrations
The following criteria were used:
. the concentrations should be well-tolerated systemically and locally,
. intradermal injections should cause moderate irritant effect (no necrosis or ulceration of the skin),
. topical application for the induction should cause at most weak or moderate skin reactions or be the maximal practicable concentration,
. topical application for the challenge should be the highest concentration which does not cause irritant effect.

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2.3.2 Main study

2.3.2.1 Preparation of the animals

For all animals and before each treatment, the application sites were:
. clipped on days -1 and 7 (scapular area 4 cm x 2 cm),
. clipped and shaved on day 21 (each flank 2 cm x 2 cm),
. clipped again on day 25 (each flank 2 cm x 2 cm).

2.3.3 Induction phase by intradermal and cutaneous routes

2.3.3.1 Intradermal route

On day 1, six injections were made deep into the dermis of a clipped area (4 cm x 2 cm) in the dorsal region between the shoulders, using a needle (diameter: 0.50 x 16 mm, Térumo: C.M.L., 77140 Nemours, France) mounted on a 1 ml glass syringe (0.01 ml graduations, Record: Carrieri, 75005 Paris, France).
Three injections of 0.1 ml were made into each side of this shoulder region, as follows:

Injection sites*	Treated group	Control group
Anterior	1: FCA diluted at 50% (v/v) with 0.9% NaCl	1: FCA diluted at 50% (v/v) with 0.9% NaCl
Middle	2: test substance at 0.1% (w/w) in vehicle	2: vehicle
Posterior	mixture of 50/50 (w/v) of 1 and 2	mixture of 50/50 (w/v) of 1 and 2

* : three pairs of sites
FCA: Freund's complete adjuvant

2.3.3.2 Cutaneous route

On day 7, the scapular area was clipped. As the test substance was shown to be non-irritant during the preliminary tests, the animals were treated with 0.5 ml of sodium laurylsulphate (10% w/w) in vaseline in order to induce local irritation.
On day 8, a topical application to the region of the intradermal injections (4 cm x 2 cm) was performed.

<u>Control group</u>
. application of 0.5 ml of the vehicle.

<u>Treated group</u>
. application of 0.5 ml of the test substance at the chosen concentration.

The test substance and the vehicle were prepared on a dry gauze pad (Semes France, 54183 Heillecourt, France), which was then applied to the dorsal region between the shoulders and held in place for 48 hours by means of an adhesive hypoallergenic dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and an adhesive anallergenic waterproof plaster (Laboratoire des Professions Médicales, 92240 Malakoff, France).
On removal of the dressing, if present, any residual test substance was removed by means of a dry or a moistened gauze pad.
Cutaneous reactions were recorded one hour after removal of the occlusive dressing.

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2.3.3.3 Challenge phase

On day 22, the animals from both groups received an application of 0.5 ml of the test substance at the chosen concentration to the posterior right flank, and 0.5 ml of the vehicle to the posterior left flank. This application was performed using a 1 ml plastic syringe (0.01 ml graduations, Térumo: C.M.L., 77140 Nemours, France). The test substance and vehicle were prepared on a dry gauze pad (Semes France, 54183 Heillecourt, France), then applied to a 4 cm^2 (2 cm x 2 cm) clipped area of the skin. The gauze pad was held in contact with the skin for 24 hours by means of an occlusive, hypoallergenic dressing (Laboratoires de Pansements et d'Hygiène, 21300 Chenove, France) and an adhesive anallergenic waterproof plaster (Laboratoire des Professions Médicales, 92240 Malakoff, France).

On removal of the dressing, if present, any residual test substance was removed by means of a dry or a moistened gauze pad.

2.4. SUMMARY DIAGRAMS

Figure 1: control group

Chronology

Day -1 Clipping of the scapular region

Day 1 Intradermal injection

Day 7 Clipping + Sodium laurylsulphate

Day 8 Application covered by an occlusive dressing

Day 10 Removal of dressing and scoring after one hour

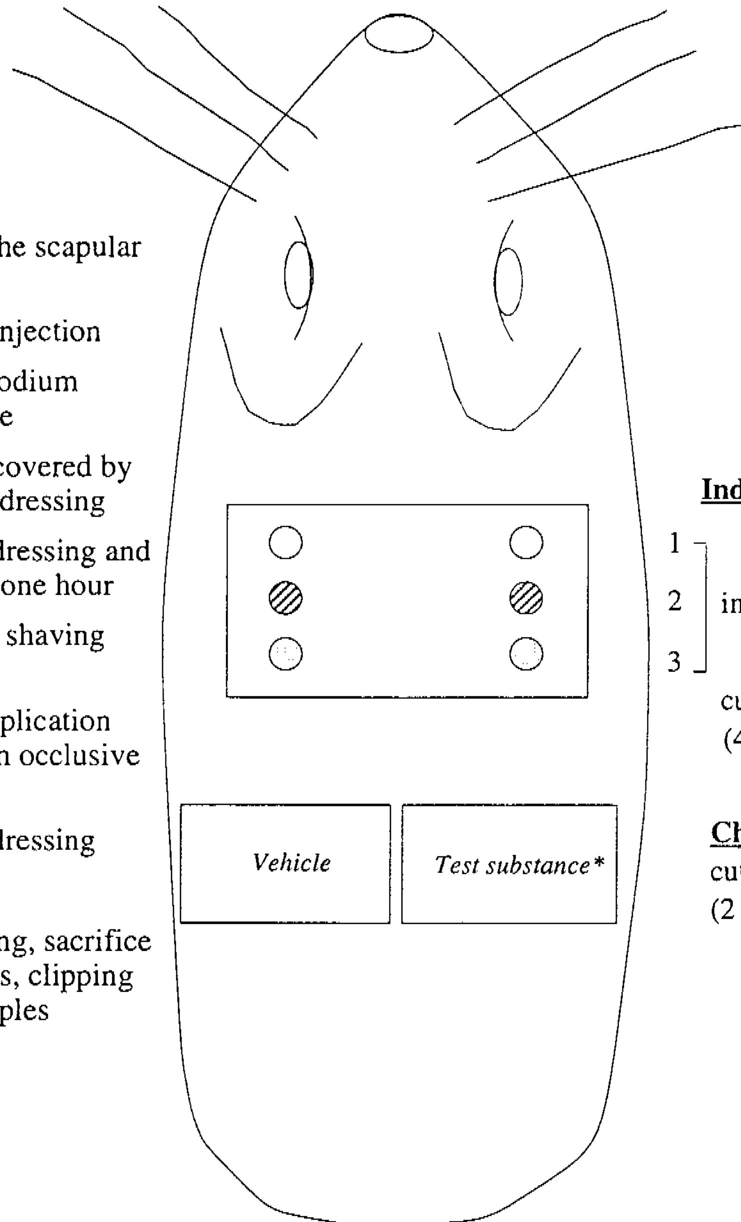
Day 21 Clipping and shaving of the flanks

Day 22 Challenge application covered by an occlusive dressing

Day 23 Removal of dressing

Day 24 First scoring

Day 25 Second scoring, sacrifice of the animals, clipping and skin samples



Induction site

1, 2, 3 intradermal injections

cutaneous application (4 cm x 2 cm)

Challenge application

cutaneous application (2 cm x 2 cm)

Intradermal injections

1 50% Freund's complete adjuvant and sterile isotonic solution (0.9% NaCl)

2 vehicle

3 1 + 2, 50/50 (w/v)

* at a concentration of 50% (w/w)

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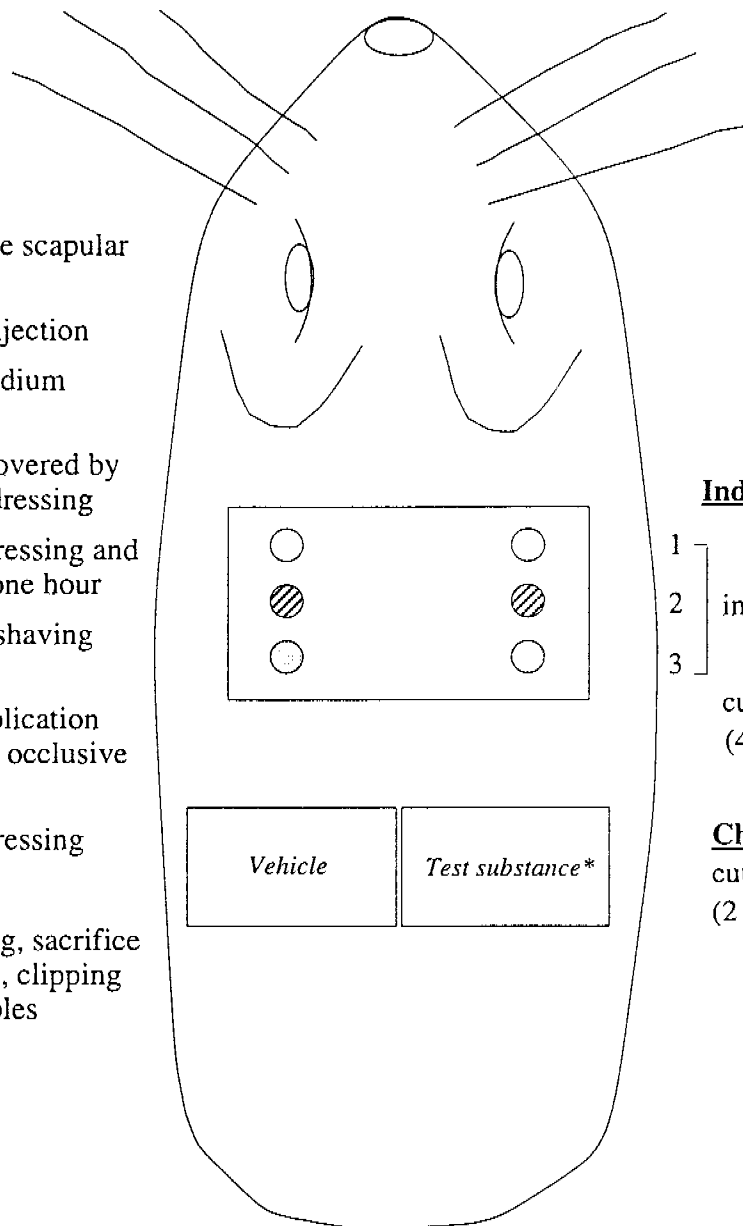
Figure 2: treated group

Chronology

Day -1	Clipping of the scapular region
Day 1	Intradermal injection
Day 7	Clipping + Sodium laurylsulphate
Day 8	Application covered by an occlusive dressing
Day 10	Removal of dressing and scoring after one hour
Day 21	Clipping and shaving of the flanks
Day 22	Challenge application covered by an occlusive dressing
Day 23	Removal of dressing
Day 24	First scoring
Day 25	Second scoring, sacrifice of the animals, clipping and skin samples



Induction site

1, 2, 3 — intradermal injections

cutaneous application (4 cm x 2 cm)

Challenge application

cutaneous application (2 cm x 2 cm)

Intradermal injections

1 — 50% Freund's complete adjuvant and sterile isotonic solution (0.9% NaCl)

2 — test substance at the chosen concentration

3 — 1 + 2, 50/50 (w/v)

* at a concentration of 50% (w/w)

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2.5. SCORING OF CUTANEOUS REACTIONS

Twenty-four and 48 hours after the challenge application, both flanks of the treated and control animals were observed in order to evaluate cutaneous reactions, according to the following scale:

Erythema and eschar formation

. No erythema 0
. Very slight erythema (barely perceptible) 1
. Well-defined erythema 2
. Moderate to severe erythema 3
. Severe erythema (beet redness) to slight eschar formation (injuries in depth) 4

Oedema formation

. No oedema 0
. Very slight oedema (barely perceptible) 1
. Slight oedema (visible swelling with well-defined edges) 2
. Moderate oedema (visible swelling raised more than 1 millimetre) 3
. Severe oedema (visible swelling raised more than 1 millimetre and extending beyond the area of exposure) 4

Any other lesions were noted.

2.6. CLINICAL EXAMINATIONS

The animals were observed twice a day during the study in order to check for clinical signs and mortality.

2.7. BODY WEIGHT

The animals were weighed individually on the day of allocation into the groups, on the first day of the study (day 1), on days 8 and 15 and on the last day of the study.

2.8. PATHOLOGY

2.8.1 Necropsy

At the end of the study, all the animals were killed by CO_2 inhalation in excess. No necropsy was performed.

2.8.2 Cutaneous samples

At the end of the study, skin samples were taken from the posterior left and right flanks of all the animals. The samples were preserved in 10% buffered formalin.

2.8.3 Microscopic examination

No histological examinations were performed.

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2.9. DETERMINATION OF THE ALLERGENICITY LEVEL

The treated animals show a positive reaction if macroscopic cutaneous reactions are clearly visible (erythema ≥ 2) and if the treated animals have a greater intensity or duration of response than the maximum reaction seen in control animals, or, if macroscopic reactions are confirmed at microscopic examination as being due to the sensitization process. Sensitization reactions are characterized at microscopic examination by basal spongiosis, reactional acanthosis of the epidermis and infiltration of mononucleated cells into the dermis (1).

Determination of the allergenicity level
The allergenicity level of the test substance is calculated by comparing the number of animals showing positive reactions with the number of surviving treated animals at the end of the study.

% of animals showing a reaction	Allergenicity level	Classification
0 - 8	I	very weak
9 - 28	II	weak
29 - 64	III	moderate
65 - 80	IV	strong
81 - 100	V	very strong

According to the Commission Directive 93/21/E.E.C., when the reactions are positive in at least 30% of the treated animals, the test substance has sensitization properties and the sentence "R 43: May cause sensitization by skin contact" must be applied.

(1) Duprat, P. ; Delsaut. L. ; Gradiski, D. ; Lepage, M. : Investigations histo-pathologiques et cytologiques lors de la mise en évidence, chez le cobaye, d'une allergie cutanée de type retardé. Revue Méd. Vét. 127: 7, 1083-1101 (1976).

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2.10. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date	Day
Arrival of the animals	31.8.95	-8
Weighing and allocation of the animals into groups	7.9.95	-1
Weighing, induction by intradermal injection	8.9.95	1
Laurylsulfate application	14.9.95	7
Weighing, induction by cutaneous route	15.9.95	8
Removal of occlusive dressings and scoring of local reactions after one hour	17.9.95	10
Weighing	22.9.95	15
Challenge cutaneous application	29.9.95	22
Removal of occlusive dressings	30.9.95	23
Scoring of cutaneous reactions after		
. 24 hours	1.10.95	24
. 48 hours	2.10.95	25
Weighing, sacrifice of the animals and skin samples	2.10.95	25

2.11. ARCHIVES

The study documentation and materials, namely:
. protocol and possible amendments,
. raw data,
. correspondence,
. final report and possible amendments,
. histological specimens:
  - tissues in preservative
  - possible blocks and slides

are stored in the archives of C.I.T., Miserey, 27005 Evreux, France, for five years after the end of the *in vivo* phase of the study. At the end of this period, the study documentation will be returned to the Sponsor.

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3. RESULTS

3.1. PRELIMINARY STUDY

3.1.1 Administration by intradermal route

Several tests were performed in order to determine the concentration to be used in the main study.

Animal number	Concentration of the test substance % (w/w)	Scoring after treatment 24 hours	48 hours	6 days
Male No. 01	0.1	C*	C*	irritation
	1	C*	crust	crust
	5	C*	crust	necrosis
Female No. 01	0.1	C*	C*	irritation
	1	C*	crust	crust
	5	C*	crust	necrosis

*: black colouration which could mask an eventual necrosis

Concentration chosen for the main study was 0.1% (w/w).

3.1.2 Application by cutaneous route

The maximal practicable concentration was 50% (w/w). One test was performed in order to check if this concentration was irritant.

Animal number	Concentration of the test substance % (w/w)		Scoring after removal of the dressing (1) 24 hours E	O	48 hours E	O
Male No. 01	50	RF	0	0	0	0
		LF	0	0	0	0
		RF	0	0	0	0
Female No. 01	50	LF	0	0	0	0

E : erythema
O : oedema
RF: right flank
LF: left flank
(1): Residual test substance was wiped off with a moistened gauze pad.

Concentration chosen for the topical application of the induction phase (day 8) and for the challenge application was 50% (w/w).

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3.2. MAIN STUDY

3.2.1 Clinical examinations

No clinical signs and no mortalities were observed during the study.

The body weight gain of the treated animals was normal when compared to that of the control animals (figures 3 and 4, appendix 3).

3.2.2 Scoring of cutaneous reactions

3.2.2.1 End of the induction period

On day 10, after topical application of the induction period, signs of irritation were observed at the test site (dorsal region between shoulders) in the control and treated groups.

3.2.2.2 Challenge application

Skin reactions were as follows:

Sex	Animal number	Control group							
		24 hours				48 hours			
		Erythema		Oedema		Erythema		Oedema	
		LF	RF	LF	RF	LF	RF	LF	RF
Male	151	0	0	0	0	0	0	0	0
	152	0	0	0	0	0	0	0	0
	153	0	0	0	0	0	0	0	0
	154	0	0	0	0	0	0	0	0
	155	0	0	0	0	0	0	0	0
Female	166	0	0	0	0	0	0	0	0
	167	0	0	0	0	0	0	0	0
	168	0	0	0	0	0	0	0	0
	169	0	0	0	0	0	0	0	0
	170	0	0	0	0	0	0	0	0

LF : left flank (control)
RF: right flank (treated)

Sex	Animal number	Treated group							
		24 hours				48 hours			
		Erythema		Oedema		Erythema		Oedema	
		LF	RF	LF	RF	LF	RF	LF	RF
Male	156	0	3/C	0	0	0	C2/S	0	0
	157	0	2/S	0	0	0	2/S	0	2
	158	0	1/S	0	0	0	0	0	0
	159	0	2/S	0	0	0	LS/C	0	0
	160	0	2/S	0	2	0	LS/C	0	0
	161	0	3/S	0	0	0	2/S	0	0
	162	0	2/S	0	2	0	4/A	0	4
	163	0	2	0	2	0	C2	0	2
	164	0	3/S/C	0	2	0	2/S	0	0
	165	0	3/S/C	0	2	0	4/A	0	4
Female	171	0	3/S	0	4	0	3/S	0	4
	172	0	3	0	2	0	LS	0	2
	173	0	2	0	0	0	1/S	0	0
	174	0	3	0	2	0	LS/C	0	2
	175	0	2	0	0	0	0/S	0	0
	176	0	1	0	0	0	LS	0	0
	177	0	2	0	2	0	LA	0	3
	178	0	2	0	2	0	2/S	0	2
	179	0	2	0	2	0	0/S	0	0
	180	0	2	0	2	0	0	0	0

LF : left flank (control)
RF: right flank (treated)
C : black colouration of the skin
C2 : black colouration of the skin which could mask an eventual erythema at grade 1 or 2
S : dryness of the skin
LS : scoring mask by dryness
LA: scoring mask by crusts
A : crusts

Residual test substance was removed by means of a gauze pad moistened with water.

After the challenge application, no cutaneous réactions were observed in the control group.

In the treated group, at the 24-hour reading, very slight, well-defined and marked erythema (grades 1 to 3) were observed in 2/20, 11/20 and 7/20 animals, respectively. The erythema was accompanied by slight oedema (grade 2) in 11 animals and by severe oedema (grade 4) in one animal. Dryness of the skin was noted in 9/20 animals. Very slight black colouration of the skin was observed in 3 animals.

At the 48-hour reading, very slight, well-defined, marked and severe erythema (grades 1 to 4) were noted in 1/20, 4/20, 1/20 and 2/20 animals, respectively. Slight, marked or severe oedema (grades 2 to 4) were noted in 5, 1 and 3 animals respectively. Crust were observed in 3 animals. Dryness of the skin was observed in 14/20 animals. It was severe enough to mask the evaluation of the erythema in 5/20 animals. Very slight to slight black colouration of the skin was observed in 5 animals.
The very slight erythema which did not persit at the 48-hour reading in two animals, were attributed to a possible slight irritant reaction. All the other skin lesions were attributed to a sensitisation effect.

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3.2.3 Pathology

3.2.3.1 Microscopic examination

No microscopic examinations were performed.

4. CONCLUSION

Under our experimental conditions and according to the maximization method of Magnusson and Kligman, cutaneous reactions attributable to the sensitization potential of the test substance (batch No. Pil 4X) at a concentration of 50% (w/w) were observed in 18/20 (90%) guinea-pigs.

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IN VITRO
PERCUTANEOUS ABSORPTION
OF
HYDROXYPROPYL-BIS-(N-HYDROXYETHYL-P-PHENYLENE DIAMINE) HCL
(COLIPA A121,

Study director :

Laboratory technician :

Study completion date : 04/03/04

Performing laboratory :

Study number : 16 056

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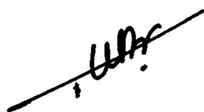
STATEMENT OF THE STUDY DIRECTOR

The study was conducted in the spirit of GLP as described in :

Revised OECD principles of Good Laboratory Practice,
[C(97) 186 / Final]. 26th November, 1997.

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at :



date : 04/03/04

Study Director
Groupe Biodisponibilité Cutanée et Métabolisme

STUDY DATES

Dates of protocol approval by the study director : 14/09/01

Date of experimental penetration studies :

Date (dd/mm/yy)	**Experiment**	**Hair dye**
18/09/01	1	175339 + H_2O_2
03/10/01	2	175339 + H_2O_2
16/10/01	3	175338 + H_2O
22/10/01	4	175339 + H_2O_2
05/11/01	5	175338 + H_2O

Completion of experimental work : 16/11/01

SCIENTISTS INVOLVED IN THE STUDY

date signature

MANAGING DIRECTOR : 01/03/04

STUDY DIRECTOR : 04/03/04

LABORATORY TECHNICIAN : 08/03/04

STATEMENT OF QUALITY ASSURANCE UNIT

The protocol, experiments, raw data and report were inspected by the Quality Assurance Unit, on the following dates :

TYPE OF INSPECTION	Dates of inspection	Reported to Study Director
Protocol	19/09/01	19/09/01
Penetration studies : Control of the formulations	16/10/01	17/10/01
Report	02/03/04	04/03/04

The inspections were performed in the spirit of GLP and documented in report sent to Study Director.

date : 12/03/04

QA Manager

SUMMARY

The aim of this study was to evaluate under in-use conditions the *in vitro* human percutaneous absorption and cutaneous distribution of the primary intermediate **HYDROXYPROPYL-BIS-(N-HYDROXYETHYL-P-PHENYLENE DIAMINE) HCL (COLIPA A 121,** incorporated at 3.67 ± 0.25 % (w/w) into two typical hair dye formulations containing or not a coupler (m-AMINOPHENOL). The formulations were applied on the skin surface after mixing (1/1, w/w) with H_2O_2 or H_2O respectively.

This study was conducted according to the COLIPA, SCCNFP and OECD Guidelines.

This study was performed on human dermatomed skin samples mounted in flow-through diffusion cells. Skin integrity was checked by TEWL before the hair dye application.

The following hair dye mixtures (prepared immediately before the application) were studied :

- the hair dye formulation **175339** containing the primary intermediate (unlabelled + [^{14}C] = 3.53 ± 0.09 %, w/w) associated to a coupler (m-AMINOPHENOL 1.29 % (w/w)) applied in a 1/1 (w/w) mixture with the developer **hydrogen peroxide** (final concentration = 1.76 ± 0.04 %, w/w), in order to study the penetration of the primary intermediate under usual dyeing conditions.
- the hair dye formulation **175338** containing the primary intermediate alone (unlabelled + [^{14}C] = 3.87 ± 0.32 %, w/w) applied in a 1/1 (w/w) mixture with **water** (final concentration = 1.93 ± 0.12 %, w/w), in order to study the penetration of the primary intermediate itself.

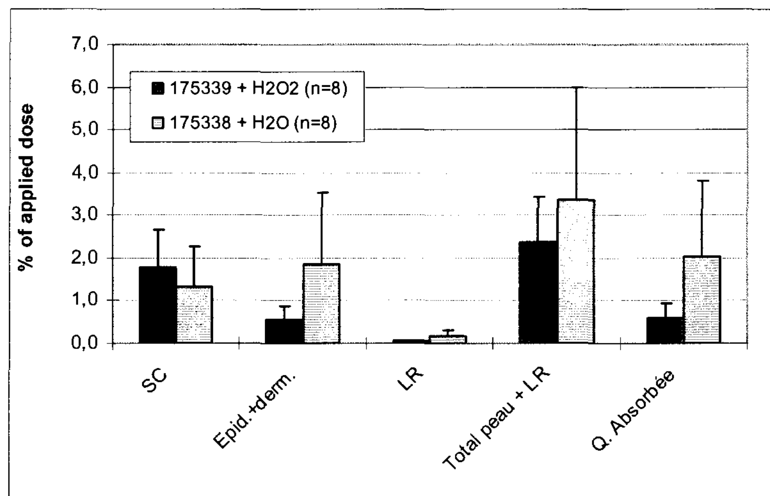
The hair dye mixtures were applied once at the dose of 20 mg/cm² (20.6 ± 1.6 mg/cm², corresponding to 378.4 ± 36.0 µg/cm² of the primary intermediate) on the skin surface.

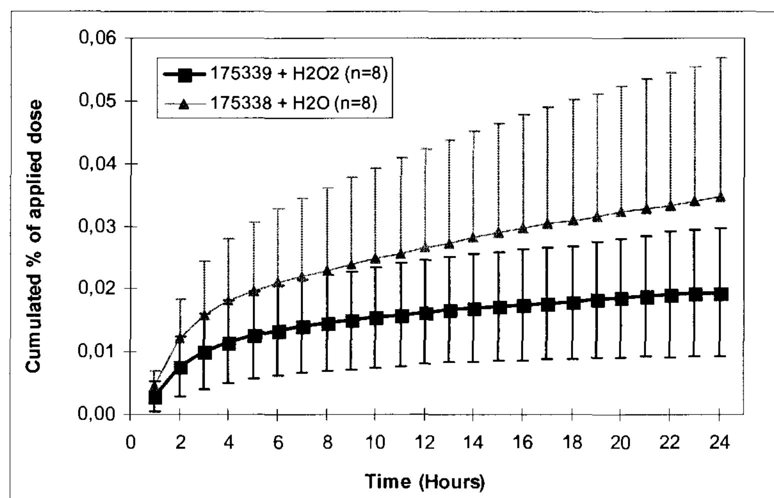
Thirty minutes after application, the hair dye mixture remaining on the skin surface was removed following a well-standardized washing procedure.

Twenty four hours after application, percutaneous absorption of **[^{14}C]** and/or [^{14}C] by-products was determined by measuring the concentration of **the [^{14}C] primary intermediate** and/or [^{14}C] by-product by liquid scintillation counting in the following compartments : Skin excess, Stratum corneum (tape stripping), Epidermis + dermis and Receptor fluid.

The kinetics of **[^{14}C]** and/or [^{14}C] by-products diffusion in the receptor fluid was also evaluated.

Hair dye mixture	175339 + H_2O_2 (n = 8)	175338 + H_2O (n = 8)
Skin excess		
$\mu g_{eq}/cm^2$ (CV%)	345.1 ± 38.3 (11%)	385.3 ± 35.2 (9%)
% of the applied dose (CV%)	**93.9 ± 2.7** (3%)	**98.2 ± 4.0** (4%)
Stratum corneum (SC)		
$\mu g_{eq}/cm^2$ (CV%)	6.29 ± 2.29 (36%)	5.17 ± 3.55 (69%)
% of the applied dose (CV%)	**1.78 ± 0.87** (49%)	**1.32 ± 0.96** (72%)
Epidermis + dermis		
$\mu g_{eq}/cm^2$ (CV%)	1.97 ± 1.12 (57%)	7.11 ± 6.24 (88%)
% of the applied dose (CV%)	**0.55 ± 0.33** (60%)	**1.85 ± 1.68** (91%)
Receptor fluid (RF)		
$\mu g_{eq}/cm^2$ (CV%)	0.19 ± 0.10 (55%)	0.75 ± 0.56 (75%)
% of the applied dose (CV%)	**0.05 ± 0.03** (57%)	**0.19 ± 0.14** (74%)
Total recovery		
% of the applied dose (CV%)	**96.3 ± 3.0** (3%)	**101.5 ± 3.5** (3%)





Under the present experimental conditions, this study showed that :

- For both hair dye mixtures, most of the hair dye applied on the skin surface was removed with the washing procedure (i.e., 93.9 ± 2.7 % of the applied dose for **175339 [^{14}C] + H_2O_2** and 98.2 ± 4.0 % of the applied dose for **175338 [^{14}C] + H_2O**).

- Concerning the skin distribution of **[^{14}C]** and/or [^{14}C] by-products, some differences were observed between the two hair dye mixtures :

  - **In the stratum corneum** : similar amounts were found for **175339 [^{14}C] + H_2O_2** and **175338 [^{14}C] + H_2O**, with respectively 1.78 ± 0.87 % of the applied dose (6.29 ± 2.29 $\mu g_{eq}/cm^2$) and 1.32 ± 0.96 % of the applied dose (5.17 ± 3.55 $\mu g_{eq}/cm^2$). Those amounts retained in the stratum corneum 24 hours post-application were not considered to be percutaneously absorbed and thus did not contribute to the systemic dose at this time.
  - **In the epidermis + dermis** : significantly lower amounts of **[^{14}C]** and/or [^{14}C] by-products were found for **175339 [^{14}C] + H_2O_2** in comparison to **175338 [^{14}C] + H_2O**, with respectively 0.55 ± 0.33 % (1.97 ± 1.12 $\mu g_{eq}/cm^2$) and 1.85 ± 1.68 % of the applied dose (7.11 ± 6.24 $\mu g_{eq}/cm^2$).
  - **In the receptor fluid** : the amounts of **[^{14}C]** and/or [^{14}C] by -products were significantly lower for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O**, respectively 0.05 ± 0.03 % (0.19 ± 0.10 $\mu g_{eq}/cm^2$) and 0.19 ± 0.14 % of the applied dose (0.75 ± 0.56 μg_{eq}/cm.

  The skin distribution clearly shows that, in the presence of developer (H_2O_2), the production of high molecular weight products (formed by oxidation and coupling reactions between the primary intermediate and the coupler) decreases the diffusion of the primary intermediate through the deeper skin layers into the receptor fluid and therefore its absorption.

- **"Total skin + receptor fluid" amounts** of **[^{14}C]** and/or [^{14}C] by-products (SC + epidermis + dermis + receptor fluid) did not differ significantly for both hair dye mixtures tested : 2.38 ± 1.06 % of the applied dose for **175339 [^{14}C] + H_2O_2**, and 3.36 ± 2.63 % of the applied dose for **175338 [^{14}C] + H_2O**, corresponding to 8.44 ± 2.65 $\mu g_{eq}/cm^2$ and 13.02 ± 9.63 $\mu g_{eq}/cm^2$ respectively.

- **Absorbed amounts [^{14}C]** and/or [^{14}C] by-products (epidermis + dermis + receptor fluid) were significantly lower for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O** with :

  - **0.60 ± 0.33 %** of the applied dose (**2.16 ± 1.10 $\mu g_{eq}/cm^2$**) for **175339 [^{14}C] + H_2O_2**, which represents the amount to be taken into account for the calculation of the safety factor,
  - **2.04 ± 1.76 %** of the applied dose (**7.86 ± 6.49 $\mu g_{eq}/cm^2$**) for **175338 [^{14}C] + H_2O**.

1. STUDY OBJECTIVE

The aim of this study was to evaluate under in-use conditions the *in vitro* human percutaneous absorption and cutaneous distribution of the primary intermediate **HYDROXYPROPYL-BIS-(N-HYDROXYETHYL-P-PHENYLENE DIAMINE) HCL (COLIPA A 121,** incorporated at 3.67 ± 0.25 % (w/w) into two typical hair dye formulations containing or not a coupler (m-AMINOPHENOL). The formulations were applied on the skin surface after mixing (1/1, w/w) with H_2O_2 or H_2O respectively.

This study was conducted according to the COLIPA [1], SCCNFP [2] and OECD [3] Guidelines.

[1] Diembeck W, Beck H, Benech-Kieffer F, Courtellemont P, Dupuis J, Lovell W, Paye M, Spengler J, Steiling W. Test Guidelines for *in vitro* Assessment of Dermal Absorption and Percutaneous Penetration of Cosmetic Ingredients. *Food and Chemical Toxicology, 1999,* ***37*** *: 191-205.*

[2] Basic criteria for the *in vitro* assessment of percutaneous absorption of cosmetic ingredients. SCCNFP/0167/99 Final, 23/06/99.

[3] OECD Environmental Health and Safety Publications, Series on Testing and Assessment n°28. Draft guidance document for the conduct of skin absorption studies, Paris, December 2000.

This study was performed on human dermatomed skin. This skin model was shown to be predictive [4] for *in vivo* results in Humans. Moreover, results obtained with the same model and method showed a good reproducibility [5].

[4] Bronaugh RL. Methods for *in vitro* percutaneous absorption. Toxicology Methods, 1995, **5** (4), 265-273.

[5] Benech-Kieffer F., Wegrich P., Schwarzenbach R., Klecak G., Weber T., Leclaire J. and Schaefer H. Percutaneous absorption of sunscreens *in vitro* : Interspecies comparison, skin models and reproducibility aspects. Skin Parmacol Appl. Skin Physiol. 2000 ; 13 : 324-335.

2. STUDY DESIGN

This study was performed on human dermatomed skin samples mounted in flow-through diffusion cells according to the experimental protocol P94 (presented in Appendix 1 ; see also the protocol deviations in Appendix 2).

The following hair dye mixtures (prepared immediately before the application) were studied :

- the hair dye formulation **175339** containing the primary intermediate (unlabelled + [^{14}C] = 3.53 ± 0.09 %, w/w) associated to a coupler (m-AMINOPHENOL 1.29 % (w/w)) applied in a 1/1 (w/w) mixture with the developer **hydrogen peroxide** (final concentration = 1.76 ± 0.04 %, w/w), in order to study the penetration of the primary intermediate under usual dyeing conditions.
- the hair dye formulation **175338** containing the primary intermediate alone (unlabelled + [^{14}C] = 3.87 ± 0.32 %, w/w) applied in a 1/1 (w/w) mixture with **water**

(final concentration = 1.93 ± 0.12 %, w/w), in order to study the penetration of the primary intermediate itself.

The hair dye mixtures were applied once at the dose of 20 mg/cm² (20.6 ± 1.6 mg/cm², corresponding to 378.4 ± 36.0 µg/cm² of the primary intermediate) on the skin surface.
Thirty minutes after application, the hair dye mixture remaining on the skin surface was removed following a well-standardized washing procedure.

Twenty four hours after application, percutaneous absorption of **[^{14}C]**
and/or [^{14}C] by-products was determined by measuring the concentration of **the [^{14}C] primary intermediate** and/or [^{14}C] by-product by liquid scintillation counting in the following compartments :

- Skin excess
- Stratum corneum
- Epidermis + dermis
- Receptor fluid

The kinetics of **[^{14}C]** and/or [^{14}C] by-products penetration in the receptor fluid was evaluated by measuring the concentration of **[^{14}C]**
and/or [^{14}C] by-products by liquid scintillation counting in the receptor fluid fractions up to 24 hours.

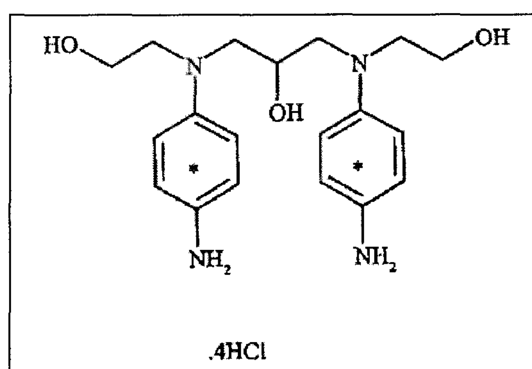
3. MATERIAL AND METHODS

3.1. Test substance and formulations

3.1.1. Test substance

3.1.1.1. Radiolabelled test substance :

Chemical name	:	**[RING-U-^{14}C]** called **[^{14}C]**
Supplier	:	AMERSHAM
Batch number	:	CFQ12295
Specific activity	:	2.52 GBq/mmol corresponding to 68 mCi/mmole
Molecular weight	:	508.5 g/mol
Date of receipt	:	29/11/00
Storage conditions	:	at -20°C, in the absence of light, moisture and air.
Radiochemical purity	:	95.1 % (analyzed by HPLC on 24/11/00 by AMERSHAM, Appendix 3). The purity of each utilized aliquot was checked by HPLC (Appendix 5).



3.1.1.2. Unlabelled test substance :

INCI name	:	**HYDROXYPROPYL-BIS-(N-HYDROXYETHYL-p-PHENYLENE DIAMINE) HCL**
Trade name	:	
COLIPA Code	:	**A121**
Supplier	:	
Supplier code	:	
Batch number	:	05046551
Date of receipt	:	11/09/2001
Storage conditions	:	at room temperature, protected from light and moisture
MW	:	506.3 g/mol
Log P ($_{oct/H_2O}$)	:	-1.2 (Software CLOGP 3.64)

Product specifications for the test substance (labelled and unlabelled) are presented in Appendix 3.

3.1.2. Tested formulations

Unlabelled formulations n° **175339** (primary intermediate + coupler), **175338** (primary intermediate alone) and **175337** (placebo) were supplied by (').

Placebo formulation **175337** has not been applied on the skin. It was used as control during the analytical controls of the tested formulations.

Detailed composition of the formulations is described in the appendix 1 of the experimental protocol presented in Appendix 1.

Stability of the formulations ***:***

The stability of the unlabelled formulations was checked at room temperature (RT) and 66°C before the study (Ref ANA20010906A).

The results are shown on Table 1.

Table 1 : Stability of the hair dye formulations (mean ± SD, CV %)

Formulation N°	Storage	Theoretical concentration	Concentration	CV	Deviation from theory
175339	1 WE / RT	2.8 %	**2.703 %**	0.5 %	**- 3.5 %**
	1 WE / 66°C	2.8 %	**2.664 %**	0.6 %	**- 4.6 %**
175338	1 WE / RT	2.8 %	**2.675 %**	0.8 %	**- 4.5 %**
	1 WE / 66°C	2.8 %	**2.678 %**	0.4 %	**- 4.4 %**

The radiolabelled hair dye formulations and mixtures were prepared, before each experiment, as follows :

- Preparation of the radiolabelled hair dye formulations :
  600 µL (corresponding to 596.3 ± 1.0 mg) of the corresponding non radiolabelled formulation (containing *ca* 2.8 % (w/w) of were added to the vial containing 400 µCi of **[14C]** (corresponding to *ca* 0.5 %) and mixed with a spatula.
- Preparation of the radiolabelled hair dye mixture :
  ca 150 mg (exactly weighed) of the corresponding radiolabelled formulation (containing *ca* 3.3 % (w/w) of unlabelled + [14C] were mixed to *ca* 150 mg (exactly weighed) of H_2O_2 or H_2O with a spatula.

Analytical control of the unlabelled and the radiolabelled hair dye formulations and mixtures :

During each experiment, the non radiolabelled and radiolabelled hair dye formulations and the radiolabelled hair dye mixtures were controlled for :

- Radiochemical purity of **[14C]** in the radiolabelled hair dye formulations (HPLC),
- Concentration of in the hair dye formulations before the addition of **[14C]** (HPLC) : *ca* 2.8 %,
- Total concentration of (unlabelled + [14C]) in the radiolabelled hair dye formulations (HPLC) : *ca* 3.3 % and in the corresponding hair dye mixture after mixing with H_2O_2 or H_2O : *ca* 1.7% (calculation),
- Radioactive concentration (expressed in mCi/g) of the hair dye formulations and mixtures (liquid scintillation counting) : *ca* 0.67 mCi/g and 0.33 mCi/g respectively.

The HPLC method is presented in Appendix 4.

The results are summarized in Table 2.
Individual results are presented in Appendix 5.

Table 2 : Control of the unlabelled and the radiolabelled hair dye formulations and mixtures (mean ± SD, CV %)

Hair dye formulations	**175339** (n = 3)	**175338** (n = 2)
Purity (%)	95.2 ± 1.2 (1.3 %)	95.0 ± 1.1 (1.2 %)
Concentration (%) (unlabelled)	3.08± 0.16 (5.0 %)	3.30 ± 0.28 (8.6 %)
Total concentration (%) (unlabelled + [^{14}C])	3.53 ± 0.09 (2.4 %)	3.87 ± 0.32 (8.3 %)
Radioactive concentration (mCi/g)	0.71 ± 0.02 (3.4 %)	0.72 ± 0.03 (4.1 %)

Hair dye mixtures	**175339 + H_2O_2** (n = 6)	**175338 + H_2O** (n = 4)
Total concentration (%) (unlabelled + [^{14}C])	1.76 ± 0.04 (2.4 %)	1.93 ± 0.12 (6.3 %)
Radioactive concentration (mCi/g)	0.34 ± 0.02 (4.9 %)	0.33 ± 0.01 (1.5 %)

3.2. Test system

3.2.1. Origin, nature and preservation of skin samples

The human skin samples were obtained from abdominal plastic surgery ; they were transported at 4°C and kept frozen (-20°C) until their utilization.
The characteristics of the skin donors (identification, date of removal, age, gender and anatomical site) are described in Appendix 6.

3.2.2. Number of skin donors and samples

The study was carried out according to protocol P94 (four diffusion cells per experiment) (Appendix 1).

Table 3 describes the number of skin donors and samples used in the study.

Table 3 : Number of skin donors and samples

	175339 + H_2O_2	**175338 + H_2O**	**Total**
Number of donors	4	4	4
Skin samples per donor	2	2	4
Number of experiments during the study	3	2	5
Number of diffusion cells performed	12	8	20
Number of diffusion cells interpreted	8	8	16

According to the protocol P94, the following experiments were performed :

Experiment 1 :

Cell A	Cell B	Cell C	Cell D
Donor 1	Donor 2	Donor 1	Donor 2
175339 + H_2O_2		**175339 + H_2O_2**	

The results of this experiment were rejected as the individual total recoveries were out of the range 85-115 % : they were ranging from 73.9 % to 79.8 % due to bad recoveries of in the washing solutions (where the presence of black grains was observed).

Experiment 2 :

Cell A	Cell B	Cell C	Cell D
Donor 2	Donor 1	Donor 2	Donor 1
175339 + H_2O_2		**175339 + H_2O_2**	

Experiment 3 :

Cell A	Cell B	Cell C	Cell D
Donor 2	Donor 3	Donor 2	Donor 3
175338 + H_2O		**175338 + H_2O**	

Experiment 4 :

Cell A	Cell B	Cell C	Cell D
Donor 3	Donor 4	Donor 3	Donor 4
175339 + H_2O_2		**175339 + H_2O_2**	

Experiment 5 :

Cell A	Cell B	Cell C	Cell D
Donor 1	Donor 4	Donor 1	Donor 4
175338 + H_2O		**175338 + H_2O**	

3.2.3. Preparation of skin samples

All skin biopsies were visually checked to ensure they were unaltered after clinical removal.

After thawing, skin samples were dermatomed and cut into pieces of 2 cm X 2 cm ; their thickness was 466 ± 117 µm.

Skin samples thickness are presented in Appendix 6.

3.3. Percutaneous absorption study

3.3.1. Evaluation of HYDROXYPROPYL-BIS-(N-HYDROXYETHYL-p-PHENYLENE DIAMINE) HCL *solubility in receptor fluid*

The receptor fluid chosen for this study was **PBS Buffer w/o Ca^{++}, Mg^{++} Instamed 9.55 g/l**.

211.2 mg of were introduced in 100 ml of receptor fluid (solution corresponding to the maximal amount of substance (200 µg/ml) which could be found in the receptor fluid, assuming a 100 % absorption in 24 hours). The solution was continuously stirred for 5 minutes at 32°C.

Under these conditions, was completely soluble (yield 99.6 ± 0.4 %) in the receptor fluid, its solubility was equal to **210 µg/ml.**

3.3.2. Cell preparation

Each skin sample was mounted in a diffusion cell, with an area of 2 cm^2 and a receiver compartment of 3 ml volume.
Skin samples were kept between two silicone membranes in order to maintain the cohesion with the cell compartments.

The epidermal side of skin samples was exposed to the ambient conditions of the laboratory environment while the dermal side was in contact with the receptor fluid.

The receptor fluid was continuously stirred by a small Teflon-covered stirring bar.
Skin surface temperature was 32.4 ± 0.2°C ; it was maintained by thermostating the receptor solution.

3.3.3. Skin integrity test

Approximately one hour after the skin was mounted in the diffusion cell, and just before the application of the hair dye formulation, the integrity of each skin sample was checked by measuring TransEpidermal Water Loss (TEWL).

Individual results are presented in Appendix 6.

Table 4 : TEWL values (mean ± SD, CV %)

	175339 + H_2O_2 (n = 8)	175338 + H_2O (n = 8)
TEWL value ($g/m^2/h$)	0.7 ± 0.9 (121 %)	2.8 ± 3.4 (124%)

According to POS 16/008, 14 samples of the 16 samples had a lower TEWL value than the limit fixed at 5.4 $g/m^2/h$ (see Appendix 2). Those samples were kept as the cutaneous distribution of the test substance was relevant.
These lower values were attributed to measuring conditions. In case of skin integrity problems, TEWL values would have been increased.

3.3.4. Application of formulations

After mixing the hair dye formulation (1/1, w/w) with H_2O_2 or H_2O, the hair dye mixture was applied once at a dose of 20 mg/cm^2 on the skin surface with a positive displacement pipette.
The exact amount applied was determined by double weighing of the pipette.
Individual applied doses are presented in tables in Appendix 7.
Mean results are presented in table 5.

Table 5 : Applied doses (mean ± SD, CV %)

	175339 + H_2O_2 (n = 8)	**175338 + H_2O** (n = 8)
Quantity of hair dye mixture (mg/cm²)	20.76 ± 2.13 (10.3%)	20.37 ± 0.68 (3.4%)
Quantity of (µg/cm²)	367.8 ± 40.2 (10.9%)	392.1 ± 25.4 (6.5 %)

3.3.5. Start and end of the diffusion experiment

The experiment was immediately started after the application of the hair dye mixture. 30 minutes after application, the hair dye mixture remaining on the skin surface was removed following a well-standardized washing procedure :

- 10 x 1 ml of water,
- 1 ml of a 2 % sodium dodecyl sulfate aqueous solution,
- 10 x 1 ml of water,
- 3 cotton swabs to dry the skin.

Twenty four hours after application, the cells were dismantled and each skin sample was removed and analyzed in order to perform the cutaneous distribution of **[^{14}C]** and/or [^{14}C] by-products.

3.3.6. Analysis of the cutaneous distribution

The following parameters were evaluated :

↳ **Analysis** of **[^{14}C]** and/or [^{14}C] by-products quantities in the following compartments :

- **Skin excess**
- **Stratum corneum** (isolated by tape strippings)
- **Epidermis + dermis**
- **Receptor fluid**

↳ **"Total skin + Receptor fluid" amounts** of **[^{14}C]** and/or [^{14}C] by-products : stratum corneum + epidermis + dermis + receptor fluid.

↳ **Absorbed amounts** of **[^{14}C]** and/or [^{14}C] by-products : epidermis + dermis + receptor fluid.

↳ **Kinetics** of **[^{14}C]** and/or [^{14}C] by-products diffusion in the receptor fluid.

Results are expressed as :

- **μg_{eq}/cm²** (µg equivalent of / cm²)
- **%** of / applied dose

3.3.7. *Statistical analysis*

Statistical analysis of the results were subcontracted to EFFI-STAT under the responsibility of (Modélisation et Statistique, Direction des report DCZ/01-24).

The following **statistical methods** with their respective software were used :

- **Descriptive statistics** (means, standard deviations,...) ⇨ Software = **SAS** (Version 6.12),
- **Mixed model variance analysis** using skin donor as random factor (repeated measures on this factor) and the hair dye mixtures as fixed factor ⇨ Software = **SAS** (Version 6.12, proc Mixed),
- For the kinetics comparison, **mixed model variance analysis** using skin donor as random factor (repeated measures on this factor) and the hair dye mixtures and the time as fixed factor, taking into account the hair dye mixture*time interaction ⇨ Software = **SAS** (Version 6.12, proc Mixed).

The significance between the means is evaluated at the risk α = 5 %.

4. PROTOCOL ADHERENCE

The study was performed in accordance with Protocol P94, and with the following deviations (see details in appendix 2) from the agreed protocol :

- The concentration of the test substance in the unlabelled and radiolabelled formulations and in the radiolabelled hair dye mixtures were higher than those mentioned in Protocol P94 and their respective radioactive concentrations were lower,
- For the measurements of radioactive concentration in the [^{14}C] Formulations, aliquots of [^{14}C] Formulations were mixed with 10 ml of water containing 10 µg/ml of ascorbic acid.
- NaOH 1N was added to the washing solutions before liquid scintillation counting in order to dissolve the black grains of hair dye mixture that were observed after the first experiment.
- Skin samples having a TEWL below the lower limit were kept because it was attributed to measuring conditions and not to skin integrity problems (for which values would have been increased).

These deviations were not considered to have compromised the validity or the integrity of the study.

5. RESULTS

Results of cutaneous distribution of **[^{14}C]** and/or [^{14}C] by-products are presented in tables 6 and 7 and in figures 1 (% of the applied dose) and 3 ($\mu g_{eq}/cm^2$).

Kinetics of **[^{14}C]** and/or [^{14}C] by-products diffusion in the receptor fluid are presented in figures 2 (% of the applied dose) and 4 ($\mu g_{eq}/cm^2$).

Individual results are presented in Appendix 7.

*Table 6 : Cutaneous distribution of **[^{14}C]** and/or [^{14}C] by-products after application on human dermatomed skin. Results (mean ± SD) are expressed as $\mu g_{eq}/cm^2$ and % of the applied dose.*

Hair dye mixture	175339 + H_2O_2 (n = 8)	175338 + H_2O (n = 8)
Skin excess		
$\mu g_{eq}/cm^2$ (CV%)	345.1 ± 38.3 (11%)	385.3 ± 35.2 (9%)
% of the applied dose (CV%)	**93.9 ± 2.7** (3%)	**98.2 ± 4.0** (4%)
Stratum corneum (SC)		
$\mu g_{eq}/cm^2$ (CV%)	6.29 ± 2.29 (36%)	5.17 ± 3.55 (69%)
% of the applied dose (CV%)	**1.78 ± 0.87** (49%)	**1.32 ± 0.96** (72%)
Epidermis + dermis		
$\mu g_{eq}/cm^2$ (CV%)	1.97 ± 1.12 (57%)	7.11 ± 6.24 (88%)
% of the applied dose (CV%)	**0.55 ± 0.33** (60%)	**1.85 ± 1.68** (91%)
Receptor fluid (RF)		
$\mu g_{eq}/cm^2$ (CV%)	0.19 ± 0.10 (55%)	0.75 ± 0.56 (75%)
% of the applied dose (CV%)	**0.05 ± 0.03** (57%)	**0.19 ± 0.14** (74%)
Total recovery		
% of the applied dose (CV%)	**96.3 ± 3.0** (3%)	**101.5 ± 3.5** (3%)

*Table 7 : "Total skin + Receptor fluid" and absorbed amounts of **[^{14}C]** and/or [^{14}C] by-products after application on human dermatomed skin. Results (mean ± SD) are expressed as $\mu g_{eq}/cm^2$ and % of the applied dose.*

Hair dye mixture	175339 + H_2O_2 (n = 8)	175338 + H_2O (n = 8)
"Total skin + RF" amount [1]		
$\mu g_{eq}/cm^2$ (CV%)	8.44 ± 2.65 (31%)	13.02 ± 9.63 (74%)
% of the applied dose (CV%)	**2.38 ± 1.06** (44%)	**3.36 ± 2.63** (78%)
Absorbed amount [2]		
$\mu g_{eq}/cm^2$ (CV%)	2.16 ± 1.10 (51%)	7.86 ± 6.49 (83%)
% of the applied dose (CV%)	**0.60 ± 0.33** (55%)	**2.04 ± 1.76** (86%)

[1] "Total skin + RF" amount = SC + epidermis + dermis + receptor fluid
[2] Absorbed amount = Epidermis + dermis + receptor fluid

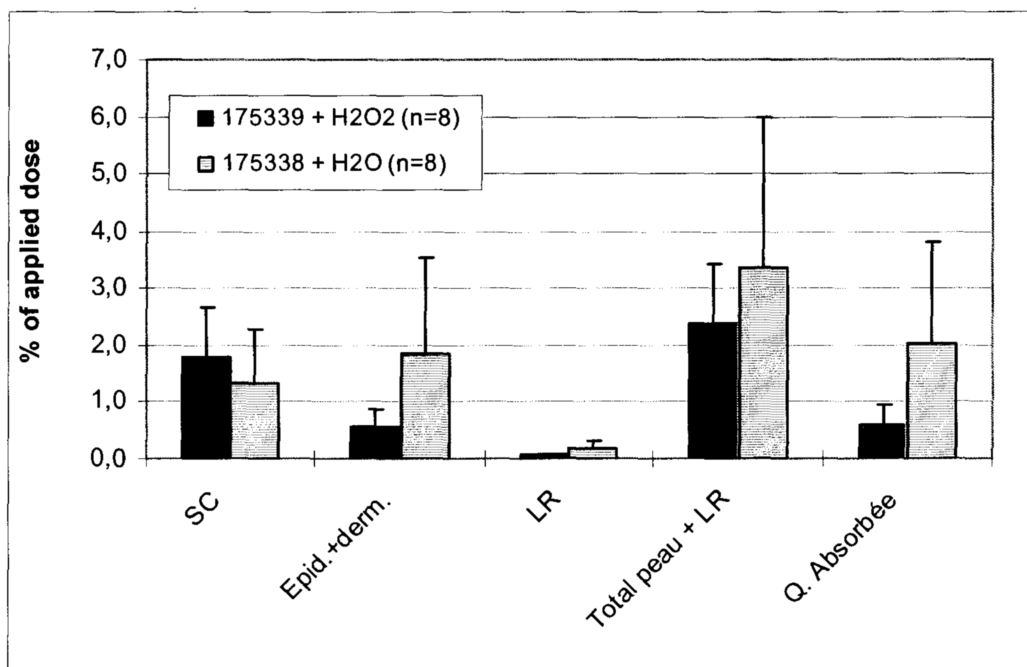


Fig. 1 : Cutaneous distribution of $[^{14}C]$ and/or $[^{14}C]$ by-products after application on human dermatomed skin. Results (mean ± SD) are expressed as % of the applied dose.

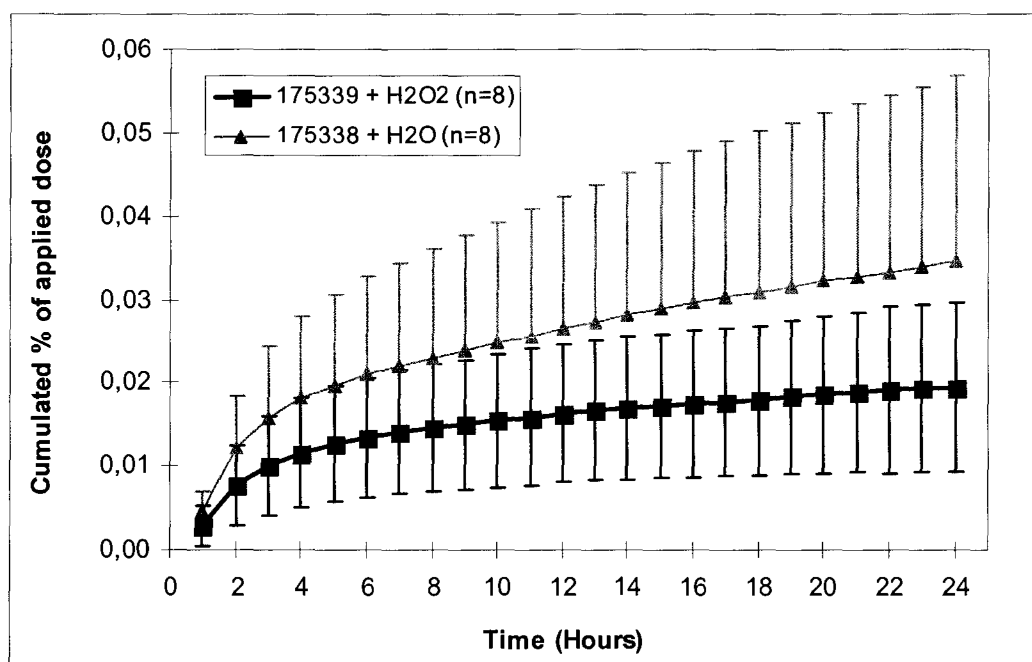


Fig. 2 : Kinetics of $[^{14}C]$ and/or $[^{14}C]$ by-products diffusion in the receptor fluid. Results (mean ± SD) are expressed as % of the applied dose.

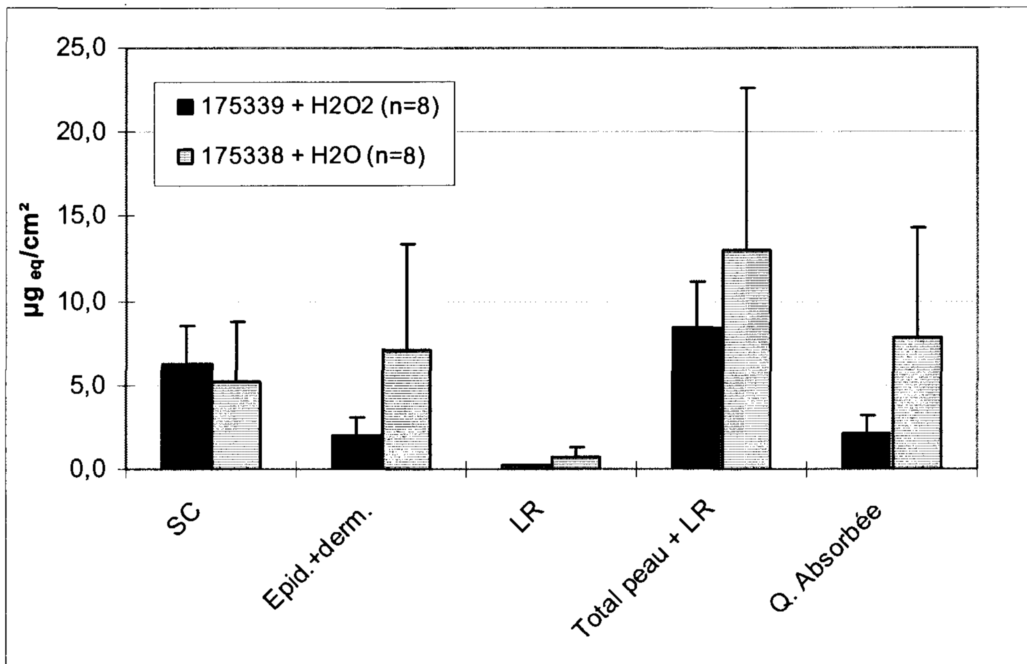


Fig. 3 : Cutaneous distribution of ***[14C]*** *and/or [14C] by-products after application on human dermatomed skin. Results (mean ± SD) are expressed as µg$_{eq}$/cm².*

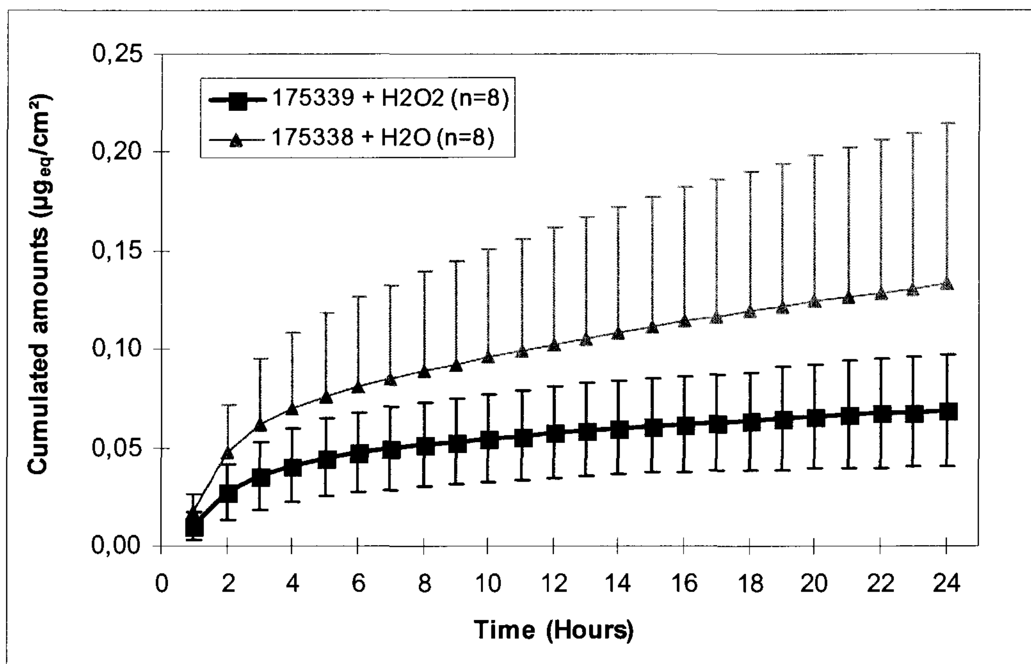


Fig. 4 : Kinetics of ***[14C]*** *and/or [14C] by-products diffusion in the receptor fluid. Results (mean ± SD) are expressed as µg$_{eq}$/cm².*

6. DISCUSSION

↳ Skin distribution

Skin excess : The recoveries of the washing protocol performed 30 minutes after application were complete (*ca* 100%) for both hair dye mixtures. The addition of NaOH 1N allowed to dissolve the black grains of hair dye mixture observed in the washing and cotton swab solutions. Significantly lower amounts of **[^{14}C]** and/or [^{14}C] by-products ($p < 0.05$) were removed with the washing protocol for **175339 [^{14}C] + H_2O_2** in comparison to **175338 [^{14}C] + H_2O** (i.e., 93.9 ± 2.7 % and 98.2 ± 4.0 % of the applied dose respectively).

In the stratum corneum, similar amounts of **[^{14}C]** and/or [^{14}C] by-products were found for the two hair dye mixtures : 1.78 ± 0.87 % of the applied dose for **175339 [^{14}C] + H_2O_2** and 1.32 ± 0.96 % of the applied dose for **175338 [^{14}C] + H_2O**.

In the epidermis + dermis, significantly lower amounts of **[^{14}C]** and/or [^{14}C] by-products ($p < 0.05$) were found for **175339 [^{14}C] + H_2O_2** in comparison to **175338 [^{14}C] + H_2O**, 0.55 ± 0.33 % and 1.85 ± 1.68 % of the applied dose respectively.

The ratio "epidermis + dermis / absorbed amounts" was similar for both hair dye mixtures : *ca* 92 % for **175339 [^{14}C] + H_2O_2** and 91 % for **175338 [^{14}C] + H_2O**. Similarly, 95-98 % of the "Total skin + Receptor fluid" amounts were located in the skin for both hair dye mixtures, the remaining 2-5 % being located in the receptor fluid, traducing a skin storage of **[^{14}C]** and/or [^{14}C] by-products.

In the receptor fluid (RF), the amounts of **[^{14}C]** and/or [^{14}C] by-products were significantly lower ($p < 0.05$) for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O**, with 0.05 ± 0.03 % and 0.19 ± 0.14 % of the applied dose respectively.

The **"Total skin + RF" amounts** (SC + epidermis + dermis + RF) of **[^{14}C]** and/or [^{14}C] by-products were not significantly different for the two hair dye mixtures. They represented 2.38 ± 1.06 % of the applied dose for **175339 [^{14}C] + H_2O_2** and 3.36 ± 2.63 % of the applied dose for **175338 [^{14}C] + H_2O** respectively.

On the other hand, the **absorbed amounts** (epidermis + dermis + RF) of **[^{14}C]** and/or [^{14}C] by-products were significantly lower ($p < 0.05$) for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O**, 0.60 ± 0.33 % and 2.04 ± 1.76 % of the applied dose respectively.

Total recoveries of **[^{14}C]** and/or [^{14}C] by-products were also significantly lower ($p < 0.05$) for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O**, 96.3 ± 3.0 % and 101.5 ± 3.5 % of the applied dose respectively.

Therefore, for each hair dye mixture and each parameter, normalizing data by the total recovery (Normalized data = data * 100 / total recovery) gave similar results on the statistical point of view, except for the skin excess where no significant difference was observed between the 2 hair dye mixtures as summarized below :

- Skin excess : NS
- Stratum corneum : NS
- Epidermis + dermis : $p < 0.05$, 175339 [^{14}C] + H_2O_2 < 175338 [^{14}C] + H_2O
- Receptor fluid : $p < 0.05$, 175339 [^{14}C] + H_2O_2 < 175338 [^{14}C] + H_2O
- "Total skin + RF" : NS
- Absorbed amount : $p < 0.05$, 175339 [^{14}C] + H_2O_2 < 175338 [^{14}C] + H_2O

Normalized data (mean ± SD) are presented in Appendix 8.

↳ Cutaneous penetration kinetics

The shape of the kinetics was similar for the two hair dye mixtures.

But, the kinetics of **[^{14}C]** and/or [^{14}C] by-products diffusion in the receptor fluid were significantly different after 2 hours post-application, with **175339 [^{14}C] + H_2O_2** being lower than **175338 [^{14}C] + H_2O**.

Finally, the major part of the transcutaneous penetration of **^{14}C]** and/or [^{14}C] by-products took place during the first 3 hours after application, clearly showing the absence of a reservoir effect in the stratum corneum.

↳ Comparison of the tested hair dye mixtures

The statistical analysis clearly demonstrated that the diffusion of the primary intermediate through the epidermis + dermis into the receptor fluid and therefore its absorption are lower for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O**. In fact, in the case of **175339 [^{14}C] + H_2O_2**, the primary intermediate and the coupler underwent oxidation and coupling reactions in the presence of developer (hydrogen peroxide), leading to higher molecular weight reaction products than in the case of **175338 [^{14}C] + H_2O** where the primary intermediate is alone (without coupler) and no oxidation and coupling reactions occurred. Moreover, for this hair dye mixture, considering that is a rather big (MW = 506) and hydrophilic (Log P = -1.2), it is interesting to notice that higher amounts of **[^{14}C]** and/or [^{14}C] by-products were found in the epidermis + dermis (more hydrophilic skin layers) than in the stratum corneum (more lipophilic skin layer). On the other hand, the diffusion of **[^{14}C]** and/or [^{14}C] by-products in the receptor fluid was low due to its high molecular weight.

Finally, no explanation was found concerning the higher variability (higher CV) of the results obtained for **175338 [^{14}C] + H_2O** than for **175339 [^{14}C] + H_2O_2**.

7. CONCLUSION

According to our experimental conditions, this study showed that :

↳ For both hair dye mixtures, most of the hair dye applied on the skin surface was removed with the washing procedure (i.e., 93.9 ± 2.7 % of the applied dose for **175339 [^{14}C] + H_2O_2** and 98.2 ± 4.0 % of the applied dose for **175338 [^{14}C] + H_2O**).

↳ Concerning the skin distribution of **[^{14}C] I** and/or [^{14}C] by-products, some differences were observed between the two hair dye mixtures :

- **In the stratum corneum**, similar amounts were found for **175339 [^{14}C] + H_2O_2** and **175338 [^{14}C] + H_2O**, with respectively **1.78 ± 0.87 %** of the applied dose (**6.29 ± 2.29 $\mu g_{eq}/cm^2$**) and **1.32 ± 0.96 %** of the applied dose (**5.17 ± 3.55 $\mu g_{eq}/cm^2$**). Those amounts retained in the stratum corneum 24 hours post-application were not considered to be percutaneously absorbed and thus did not contribute to the systemic dose at this time.
- **In the epidermis + dermis**, significantly lower amounts of **[^{14}C]** and/or [^{14}C] by-products were found for **175339 [^{14}C] + H_2O_2** in comparison to **175338 [^{14}C] + H_2O**, with respectively 0.55 ± 0.33 % (**1.97 ± 1.12 $\mu g_{eq}/cm^2$**) and 1.85 ± 1.68 % of the applied dose (**7.11 ± 6.24 $\mu g_{eq}/cm^2$**).
- **In the receptor fluid**, the amounts of **[^{14}C]** and/or [^{14}C] by-products were significantly lower for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O**, respectively 0.05 ± 0.03 % (**0.19 ± 0.10 $\mu g_{eq}/cm^2$**) and 0.19 ± 0.14 % of the applied dose (**0.75 ± 0.56 $\mu g_{eq}/cm^2$**).

The skin distribution clearly showed that, in the presence of developer, the production of high molecular weight products (formed by oxidation and coupling reactions between the primary intermediate and the coupler) decreased the diffusion of the primary intermediate through the deeper skin layers to the receptor fluid and therefore its absorption.

↳ **"Total skin + receptor fluid" amounts** of **[^{14}C]** and/or [^{14}C] by-products (SC + epidermis + dermis + receptor fluid) did not differ significantly for both hair dye mixtures : **2.38 ± 1.06 %** of the applied dose for **175339 [^{14}C] + H_2O_2**, and **3.36 ± 2.63 %** of the applied dose for **175338 [^{14}C] + H_2O**, corresponding to **8.44 ± 2.65 $\mu g_{eq}/cm^2$** and **13.02 ± 9.63 $\mu g_{eq}/cm^2$** respectively.

↳ **Absorbed amounts [^{14}C] IMEXINE OAX** and/or [^{14}C] by-products (epidermis + dermis + receptor fluid) were significantly lower for **175339 [^{14}C] + H_2O_2** than for **175338 [^{14}C] + H_2O** with :

- **0.60 ± 0.33 %** of the applied dose (**2.16 ± 1.10 $\mu g_{eq}/cm^2$**) for **175339 [^{14}C] + H_2O_2**, which represents the amount to be taken into account for the calculation of the safety factor,

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- **2.04 ± 1.76 %** of the applied dose (**7.86 ± 6.49 $\mu g_{eq}/cm^2$**) for **175338 [^{14}C] + H_2O**.

8. QUALITY ASSURANCE

The study was audited in compliance with the requirements of Good Laboratory Practice by the Quality Assurance Unit. The corresponding quality assurance statement is presented at the beginning of the report (p. 6/89).

9. ARCHIVING PROCEDURE

All the documentation relative to this study will be kept under the study number : « **16056** » in .

APPENDIX 2

PROTOCOL DEVIATIONS

PROTOCOL DEVIATIONS

Some deviations with regard to protocol P94 were made for different reasons explained below :

1. Concentration of test substance in the formulations and hair dye mixtures

The concentration of the test substance in the unlabelled and radiolabelled formulations and in the radiolabelled hair dye mixtures were higher than those mentioned in Protocol P94 and their respective radioactive concentrations were lower :

Results are expressed as mean measured values :

Formulations	**Theoretical concentration**	**175339**	**175338**
Unlabelled test substance	2.8 %	3.08 %	3.30 %
Radiolabelled test substance	0.2 %	0.53 %	0.54 %
Total concentration	3 %	3.53 %	3.87 %
Radioactive concentration	1 mCi/g	0.71 mCi/g	0.72 mCi/g

Hair dye mixtures	**Theoretical concentration**	**175339 + H_2O_2**	**175338 + H_2O**
Total concentration	1.5 %	1.76 %	1.93 %
Radioactive concentration	0.5 mCi/g	0.34 mCi/g	0.33 mCi/g

For the measurement of the radioactive concentration in the [^{14}C] Formulations, aliquots of [^{14}C] Formulations were mixed with 10 ml of water containing 10 µg/ml of ascorbic acid instead of 5 ml of mobile phase.

2. Samples treatment for the washing solutions and cotton swabs

Due to the black grains observed in the washing solutions and in the cotton swabs solutions in experiment 1 leading to the bad total recoveries obtained, the following treatment was followed for the other experiments :

- Washing solution : the washing solution was divided in 2 aliquots. 8ml of NaOH 1N was added to each aliquot which was then put at 50°C during 96 hours. Each aliquot was then divided in 6 parts and Ultima Gold was added for the liquid scintillation counting.

- Cotton swabs : 5 ml of NaOH 1N was added to the cotton swabs. The solution was then put at 50°C during 96 hours. 3 aliquots of 250 µl were counted by liquid scintillation after addition of Ultima Gold.

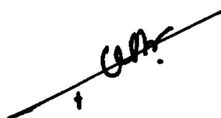
3. Skin integrity test

According to POS 16/008, 14 samples of the 16 samples had a lower TEWL value than the limit fixed at 5.4 g/m²/h (see Appendix 2). Those samples were kept as the cutaneous distribution of the test substance was relevant.
These lower values were attributed to measuring conditions. In case of skin integrity problems TEWL values would have been increased and higher than the upper limit of 13.0 g/m²/h.

Table 1 : Sample with TEWL value below the limit

Skin donor	TEWL (g/m²/h)	Experiment	Hair dye mixture
1010620B0445	0.1 0.4	2	175339 + H_2O_2
	1.1 0.4	5	175338 + H_2O
LLA065	2.8 0.1	2	175339 + H_2O_2
	1.4 1.2	3	175338 + H_2O
1010717B0462	0.7 0.3	4	175339 + H_2O_2
	0.9	3	175338 + H_2O
1010717B0457	0.7 0.7	4	175339 + H_2O_2
	0.7	5	175338 + H_2O

These deviations were not considered to have compromised the validity or integrity of the study.



Study director Date : 04/03/04

APPENDIX 3

PRODUCT SPECIFICATIONS

(Batch 05046551)

[RING-U-^{14}C]
(Code CFQ12295)

Département de Chimie analytique

(batch 05046551)
Analytical certificate

1. **NOMENCLATURE**

1.1 Chemical name

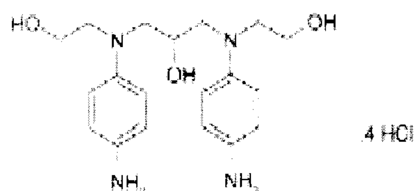
1,3-Bis-[(4-Amino-phenyl)-(2-hydroxy-ethyl)-amino]-propan-2-ol,tetrahydrochloride

1.2 INCI Name

Hydroxypropyl bis (N-hydroxyethyl-p-phenylenediamine) HCl

2. **IDENTIFICATION**

2.1 Structural formula



- Empirical formula : C_{19} H_{28} N_4 O_3, H_4 Cl_4
- Molecular weight : [(360.46 + 145.84)] = 506.30 g/mol

2.2 Description

An ivory powder

2.3 Infra-red spectrophotometry

The infra-red spectrum of batch 05046551 complies with the structural formula

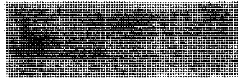
3. **ASSAY :**

Determination of titer by potentiometry

Neutralisation of the tetrahydrochloride functions arranged in pairs.

The titer of batch 05046551 is 95.3 g/100g

Le 29 Janvier 2002 **Head of Analytical Chemistry Department**



CAUTION - RADIOACTIVE MATERIAL

Product Specification

Before using this product, please read the instructions overleaf for safe handling, storage and disposal.
This product has been manufactured to the quality assurance standard ISO9002

Nycomed Amersham plc
Amersham Place
Little Chalfont
Buckinghamshire HP7 9NA
England

[ring-U-[14]
Code CFQ12295
Pack size 14.8 MBq, 400µCi

Technical data

Specific activity
determined by mass spectrometry : 2.52 GBq/mmol 68 mCi/mmol

Molecular weight : 508.5 (at this specific activity)

Radiochemical purity

by high performance liquid chromatography : 95.1%

Column	:	Supelco Supiex PKB-100 (150 x 4.6mm)
Solvent	:	20mM ammonium acetate (aqueous) pH 7.5:methanol (775:225)
Gradient	:	isocratic
Flow rate	:	1.0 ml/min
UV detection	:	326 nm

Analysed on 24th November 2000

Chemical identity

The material co-chromatographs with customer supplied material in the above chromatographic system.

The mass spectrum is consistent with the proposed structure and a non-labelled reference.

All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Pharmacia Biotech group which supplies them. A copy of these terms and conditions is available on request.
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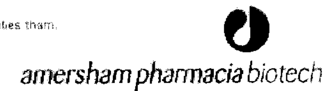


Packaging and storage

[ring-U-^{14}C]... is supplied as a solid in a polypropylene insert contained within a borosilicate multidose vial with additional screw cap.

Storage at -20°C in the absence of moisture light and air is recommended

---

Preparation

[ring-U-^{14}C] was prepared from 2-[ring-U-^{14}C]anilinoethanol by the method supplied by the customer

The product was purified by crystallisation.

Safety warnings and precautions

Warning: For research use only.
Not recommended or intended for diagnosis of disease in humans or animals.
Do not use internally or externally in humans.

Caution: Radioactive material **For professional users only**

Instructions relating to the handling, use, storage and disposal of radioactive materials

1 Upon receipt, vials or ampoules containing radioactive material should be checked for contamination. All radioactive materials should be stored in specially designated areas and suitable shielding should be used where appropriate. Access to these areas should be restricted to authorized personnel only.

2 Radioactive material should be used by responsible persons only in authorized areas. Care should be taken to prevent ingestion or contact with skin or clothing. Protective clothing, such as laboratory overalls, safety glasses and gloves should be worn whenever radioactive materials are handled. Where this is appropriate, the operator should wear personal dosimeters to measure radiation dose to the body and fingers

3 No smoking, drinking or eating should be allowed in areas where radioactive materials are used. Avoid actions that could lead to the ingestion of radioactive materials, such as the pipetting of radioactive solutions by mouth.

4 Vials containing radioactive materials should not be touched by hand, wear suitable protective gloves as normal practice. Use forceps when handling vials containing 'hard' beta emitters such as phosphorus-32 or gamma emitting labelled compounds. Ampoules likely to contain volatile radioactive compounds should be opened only in a well ventilated fume cabinet.

5 Work should be carried out on a surface covered with absorbent material or in enamel trays of sufficient capacity to contain any spillage. Working areas should be monitored regularly.

6 Any spills of radioactive material should be cleaned immediately and all contaminated materials should be decontaminated or disposed of as radioactive waste via an authorized route. Contaminated surfaces should be washed with a suitable detergent to remove traces of radioactivity.

7 After use, all unused radioactive materials should be stored in specifically designated areas. Any radioactive product not required or any materials that have come into contact with radioactivity should be disposed of as radioactive waste via an authorized route.

8 Hands should be washed after using radioactive materials. Hands and clothing should be monitored before leaving the designated area, using appropriate instruments to ensure that no contamination has occurred. If radioactive contamination is detected, hands should be washed again and rechecked. Any contamination persisting on hands and clothing should be reported to the responsible person so that suitable remedial actions can be taken.

9 Certain national/international organisations and agencies consider it appropriate to have additional controls during pregnancy. Users should check local regulations.

Most countries have legislation governing the handling, use, storage, disposal and transportation of radioactive materials. The instructions set out above complement local regulations or codes of practice. Such regulations may require that a person be nominated to oversee radiological protection. Users of radioactive products must make themselves aware of and observe local regulations or codes of practice which relate to such matters.

CAUTION - Substance not yet fully tested.
The full chemical and toxicological properties of this compound are unknown to Nycomed Amersham plc.
The safety precautions given above will generally provide adequate protection from any non-radioactive hazards associated with this material in the form and quantity supplied.

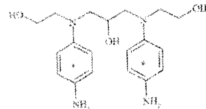
The users of this product should also refer to any information they have available on the properties and hazards of this product.

In Confidence

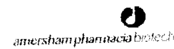
PREPARATIVE DETAILS OF

[ring-U-^{14}C]

CFQ12295



The preparation was carried out to Amersham Pharmacia Biotech's Custom Preparation procedures, which are certified to ISO9002, between 1st November 2000 and 28th November 2000 and is described in Preparation File CFQ12295.



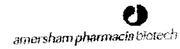
In Confidence

Prepared by:-

Alan Simmonds
C-14 Custom Preparations Group
Amersham Pharmacia Biotech
Cardiff Laboratories
Forest Farm Estate
Whitchurch
Cardiff
CF14 7YT

Reviewed and approved by:-

_______ Team Leader, C-14 Custom Preparations



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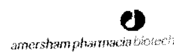

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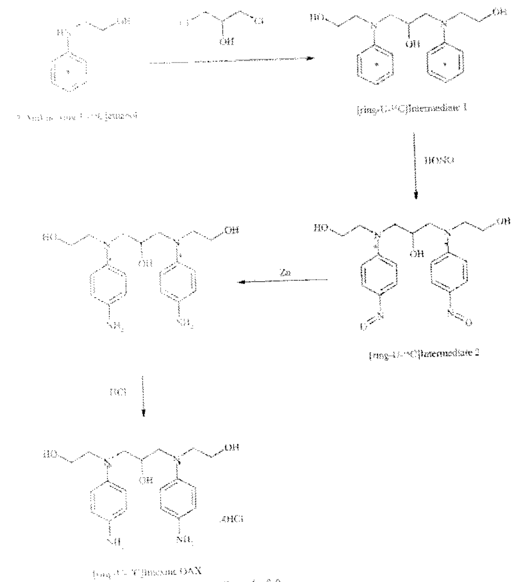
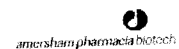
1. REACTION SCHEME





2. LIST OF REAGENTS

Material	Supplier/Cat No	Lot No
Ammonia solution	BDH 100126T	B399307923
Ammonium chloride	BDH 100173D	A244378 038
Calcium carbonate	Aldrich 23,921-6	04522TS
1,3-Dichloropropan-2-ol	Aldrich 15,687-6	8261977
Ethanol	Hayman	00/680A3
Hydrochloric acid	BDH 101254H	K27010652942
Hydrogen chloride	Aldrich 29,542-6	91020016
Sodium nitrite	Aldrich 23,721-3	CR10514AR
Zinc	Riedel-de-Haan 14409	82780

3. EXPERIMENTAL

3.1 Preparation of [ring-U-^{14}C]Intermediate 1

Material	Supplier/Cat No	Lot No
2-Anilino[ring-U-^{14}C]ethanol	Amersham Pharmacia Biotech	AJS12295/17/2
Calcium carbonate	Aldrich 23,921-6	04522TS
1,3-Dichloropropan-2-ol	Aldrich 15,687-6	8261977

Method

Calcium carbonate (435mg, 4.35mmol), water (5ml) and 1,3-dichloropropan-2-ol (356µl, 3.73mmol) were added to 2-[ring-U-^{14}C]anilinoethanol (165mCi, 6.22mmol). This mixture was stirred at reflux for 18 hours under an atmosphere of nitrogen. A further aliquot of 1,3-dichloropropan-2-ol (70µl, 0.73mmol) was added and reflux continued for 2 hours.

The reaction mixture was cooled to room temperature, ethyl acetate (20ml) was added and filtered to remove the solids. The filtrate was partitioned and the aqueous phase extracted twice with ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulphate. This was filtered and the filtrate concentrated to a brown oil by rotary evaporation.

This material was purified by flash silica chromatography eluting with ethyl acetate. The fractions containing [ring-U-^{14}C]Intermediate 1 were combined and concentrated to a white solid by rotary evaporation and dried *in vacuo* over phosphorus pentoxide, labelled AJS12295/25/2.

Yield 133.6mCi, 691.4mg, 81%.

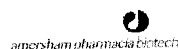
3.2 Preparation of [ring-U-^{14}C]Intermediate 2

Material	Supplier/Cat No	Lot No
[ring-U-^{14}C]Intermediate 1	Amersham Pharmacia Biotech	AJS12295/25/2
Sodium nitrite	Aldrich 23,721-3	CR10514AR
Hydrochloric acid	BDH 101254H	K27010652942
Ammonia solution	BDH 100126T	B399307923

Method

[ring-U-^{14}C]Intermediate 1 (130mCi, 671mg, 2.02mmol) was dissolved in water (4.12ml) and concentrated hydrochloric acid (1.17ml) at 0°C under an atmosphere of nitrogen. A solution of sodium nitrite (289mg, 4.18mmol) in water (610µl) was added dropwise to give a deep red solution. Stirring was continued for 2½ hours. 20% Ammonia solution (3ml) was added and the brown precipitate isolated by filtration. The solid was dried *in vacuo* over phosphorus pentoxide to give a brown solid, labelled AJS12295/37/1.

Yield 123mCi, 749.9mg, 94.5%.



3.3 Preparation of [ring-U-^{14}C]Imexine OAX

Material	Supplier/Cat No	Lot No
[ring-U-^{14}C]Intermediate 2	Amersham Pharmacia Biotech	AJS12295/37/1
Ammonium chloride	BDH 100173D	A244378 038
Zinc	Riedel-de-Haan 14409	82780
Ethanol	Hayman	00/680A3
Hydrogen chloride	Aldrich 29,542-6	91020016

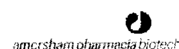
Method

[ring-U-^{14}C]Intermediate 2 (123mCi, 2mmol) and ammonium chloride (41mg, 0.766mmol) were dissolved in water (465µl) and ethanol (3.2ml). Zinc dust (1.327g, 20.6mmol) was added and the mixture heated at reflux for 2 hours under an atmosphere of nitrogen.

The reaction mixture was cooled to room temperature and filtered into a flask containing 1.3M ethanolic hydrogen chloride (6.8ml) under a blanket of nitrogen. This gave a deep blue solution from which a gum separated, and treatment with diethyl ether gave a light blue solid, labelled AJS12295/39/1.

Yield 73mCi, 578.5mg, 57%.

This product was analysed by high performance liquid chromatography and mass spectrometry, the results of which are presented in the Product Specification Sheet.



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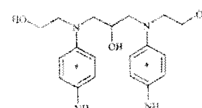
ANALYTICAL PACKAGE

[ring-U-14C]

CFQ12295



4HCl

APPENDIX A

prepared by

Alan Simmonds
Carbon-14 Custom Preparations Group
Amersham Pharmacia Biotech
Cardiff Laboratories
Whitchurch
Cardiff
CF14 7YT

The preparation was carried out to Amersham Pharmacia Biotech's Custom Preparation procedures, which are certified to ISO9002, between 31st October 2000 and 28th November 2000 and is described in Preparation File CFQ12295.

Reviewed by

amersham pharmacia biotech

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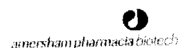

amersham pharmacia biotech

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Compound    : CFQ12295 [ring-U-14C]IMEXINE OAX
Sample Info        : APB MATERIAL
Injected           : 24/11/00
Injection Time     : 7:09:00 PM              Injected by          :   PH
Initial Flow Rate:             1.0ml/min     Vial Number          :    1
Initial Temp.      :                         Injection Number     :    1
Initial Pressure   :           106.4   bar   Injection Volume     :  1 µl
Solvent A :20mM AMM ACETATE(aq) pH7.5:MeOH 775:225
Solvent B :N/A
Column Used        :  SUPLEX PKB-100                Length:     150mm
Code               :  58932                       Diameter:     4.6mm
Manufacturer       :  SUPELCO                Particle Size:       5µm
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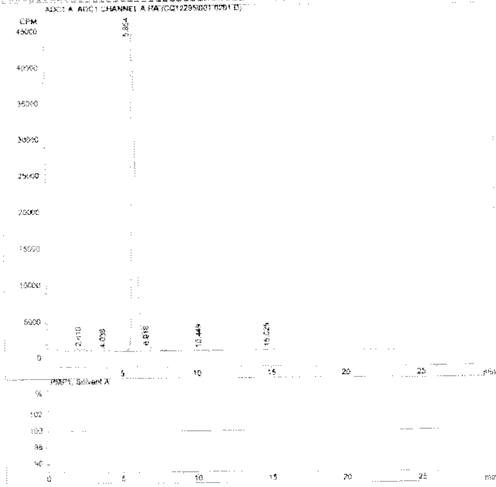


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Data file   : P:\HP1100\DATA\CQ12295\001-0201.D                    CQ12295.M
Sample Name: B08413A1
Compound    : CFQ12295 [ring-U-14C]IMEXINE OAX
```

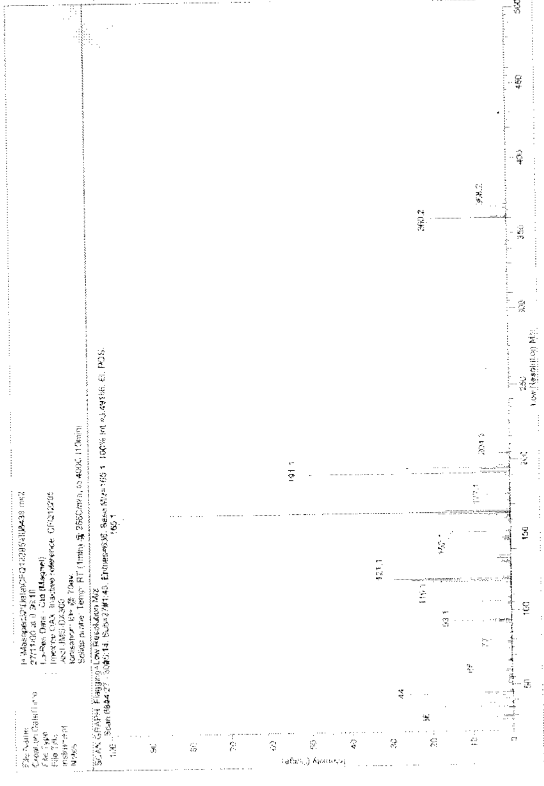
Integration Report

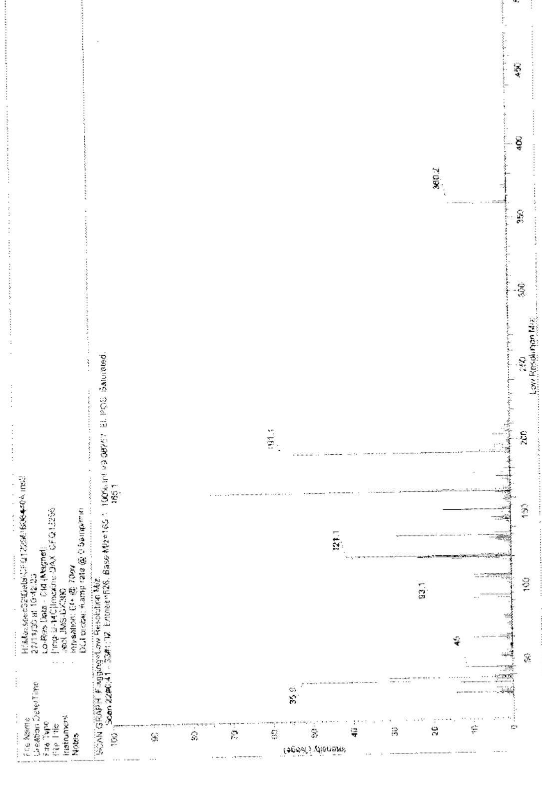
ADC1 A, ADC1 CHANNEL A RA

#	RT.	PkType	Compound	Ht	Ht%	Width	Area	Area%
1	2.410	MM		410.2	0.9	0.829	26408.2	2.2
2	4.036	MM		153.3	0.3	0.248	2279.9	0.2
3	5.854	MF		43823.7	97.3	0.341	896555.8	95.1
4	6.918	FM		467.2	1.0	0.621	17418.8	1.8
5	10.469	MM		62.6	0.1	0.327	1229.4	0.1
6	15.035	MM		126.2	0.3	0.651	5008.9	0.5

Beta   Sat, 25 Nov 2000 07:59:43 am   Page 1 of 2
Nycomed Amersham plc

Beta   Sat, 25 Nov 2000 07:59:43 am   Page 2 of 2
Nycomed Amersham plc


165.1
191.1
121.1
360.2
Low Resolution M/z


165.1
191.1
121.1
93.1
360.2
Low Resolution M/z

APPENDIX 8

NORMALIZED DATA

For each parameter and each hair dye mixture, individual **normalized data** (expressed as % of the applied dose) were calculated by multiplying the data by 100 and dividing by the total recovery (Normalized data = data * 100 / total recovery). Mean and SD were then calculated.

***Normalized data** for cutaneous distribution of*
[^{14}C] and/or [^{14}C] by-products
after application on human dermatomed skin.
Results (mean ± SD) are expressed as % of the applied dose, (CV%).

Hair dye mixture	175339 + H_2O_2 (n = 8)	175338 + H_2O (n = 8)
Skin excess	**97.5 ± 1.0** (1%)	**96.7 ± 2.6** (3%)
Stratum corneum (SC)	**1.84 ± 0.86** (47%)	**1.30 ± 0.94** (72%)
Epidermis + dermis	**0.56 ± 0.33** (59%)	**1.82 ± 1.66** (91%)
Receptor fluid (RF)	**0.05 ± 0.03** (57%)	**0.19 ± 0.14** (74%)
"Total skin + RF" amount [1]	**2.46 ± 1.03** (42%)	**3.30 ± 2.59** (78%)
Absorbed amount [2]	**0.62 ± 0.33** (53%)	**2.00 ± 1.73** (86%)

[1] "Total skin + RF" amount = SC + epidermis + dermis + receptor fluid
[2] Absorbed amount = Epidermis + dermis + receptor fluid

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FINAL REPORT

Test Facility Study No. 783975, Report No. 29007

The *In Vitro* Percutaneous Absorption of Radiolabelled (A121) Through Human Skin

TEST FACILITY:

Charles River Laboratories
Tranent
Edinburgh
EH33 2NE
UK

SPONSOR:

4 SUMMARY

(Colipa code A121) is a hair dye developed by Recherche. is a primary intermediate used in oxidative hair dye formulations.

As part of the safety evaluation of the product, an *in vitro* study was required to assess the rate and extent of absorption of following the topical application of the hair dye to human skin under in-use conditions. For this purpose, the hair dye was tested under oxidative and non-oxidative conditions. [^{14}C]- was incorporated into a typical oxidative hair dye formulation at *ca* 0.8% (w/w) associated to the coupler m-aminophenol (at *ca* 0.34%, w/w) before mixing with developer (1:1, w/w), to give a final concentration of of *ca* 0.4% (w/w). This will be referred to as the oxidative hair dye test preparation. was incorporated into the same typical oxidative hair dye formulation (without coupler) at *ca* 0.8% (w/w) before being mixed with water (1:1, w/w). This will be referred to as the non-oxidative hair dye test preparation.

The study was conducted according to the OECD principles of Good Laboratory Practice and was performed following the SCCP, COLIPA and OECD test guideline for skin penetration studies and the accompanying OECD guidance document.

The integrity of the skin was checked by determination of the permeability coefficient for tritiated water which was < 3.5×10^{-3} cm/h for all selected membranes. [^{14}C]- was applied in two test preparations (oxidative and non-oxidative) to human split-thickness skin membranes mounted in flow-through diffusion cells *in vitro*. Both oxidative and non-oxidative hair dye test preparations were applied at a target application rate for the formulation of *ca* 20 mg/cm^2.

Absorption was assessed by collecting receptor fluid (PBS) samples from 0 to 0.5, 0.5 to 1 h and then hourly from 1 to 24 h post dose (flow rate 1.5 mL/h). At 30 min post dose, the skin was washed with water, sodium dodecyl sulphate (SDS) solution (*ca* 2% w/v) and water again. The skin was then dried with tissue paper swabs. At 24 h post dose the underside of the skin was rinsed with receptor fluid. The skin was then removed from the flow-through cells, dried and the stratum corneum removed by tape stripping. The remaining skin was divided into exposed and unexposed skin. All liquid samples were analysed by liquid scintillation counting and the remaining samples were analysed by combustion/ liquid scintillation counting.

A summary of the mean results is provided in the table below.

Formulation / Test Preparation		Oxidative	Non-Oxidative
Target	Concentration in Formulation (%, w/w)	0.80	0.80
Actual	Concentration in Formulation (%, w/w)	0.87	0.85
Target	Concentration in Test Preparation (%, w/w)	0.40	0.40
Actual	Concentration in Test Preparation (%, w/w)	0.41	0.41
Target Application Rate of Test Preparation (mg/cm^2)		20.00	20.00
Actual Application Rate of Test Preparation (mg/cm^2)		21.22	20.18
(% Applied Dose)		(Mean ± SD)	
Dislodgeable Dose		94.64 ± 3.13	96.43 ± 2.64
Unabsorbed Dose *		98.31 ± 2.68	98.72 ± 2.27
Absorbed Dose **		0.01 ± 0.00	0.01 ± 0.01
Dermal Delivery ***		0.90 ± 0.92	0.80 ± 0.77
Mass Balance		99.21 ± 2.41	99.52 ± 2.17
(µg equiv./cm^2)		(Mean ± SD)	
Dislodgeable Dose		82.92 ± 2.54	80.28 ± 2.13
Unabsorbed Dose *		86.14 ± 2.09	82.19 ± 1.90
Absorbed Dose **		0.01 ± 0.00	0.01 ± 0.01
Dermal Delivery ***		0.78 ± 0.80	0.66 ± 0.64
Mass Balance		86.92 ± 1.70	82.85 ± 1.76

* Unabsorbed dose = dislodgeable dose + stratum corneum + unexposed skin
** Absorbed dose = receptor fluid + receptor rinse
*** Dermal Delivery = epidermis + dermis + absorbed dose

In conclusion, [^{14}C]- in oxidative and non-oxidative test preparations was applied topically to human skin *in vitro*. Under the present experimental conditions, for [^{14}C]- in the oxidative test preparation, most of the applied dose was removed at 30 min post dose (94.19% of the applied dose). At 24 h post dose, a further 0.45% was removed. Therefore, the total dislodgeable dose was 94.64% of the applied dose. At 24 h post dose, the absorbed dose and dermal delivery were 0.01% (0.01 µg equiv/cm^2) and 0.90% (0.78 µg equiv/cm^2) of the applied dose, respectively. Under the present experimental conditions, for [^{14}C]- in the non-oxidative test preparation, most of the applied dose was removed at 30 min post dose (95.66% of the applied dose). At 24 h post dose, a further 0.77% was removed. Therefore, the total dislodgeable dose was 96.43% of the applied dose. At 24 h post dose, the absorbed dose and dermal delivery were 0.01% (0.01 µg equiv/cm^2) and 0.80% (0.66 µg equiv/cm^2) of the applied dose, respectively. The amount of test item found in the epidermis and dermis accounted for > 98% of the dermal delivery.

The dermal absorption figure to be taken into consideration for the calculation of the margin of safety are the mean values of 0.78 µg equiv./cm^2 for the oxidative test preparation and 0.66 µg equiv./cm^2 for the non-oxidative test preparation, taking into account the very low total absorbed and dermal delivery values. Additionally, samples with the highest dermal delivery values were calculated using some receptor fluid samples that were below the limit of reliable measurement, therefore, this supports the use of mean values for the risk assessment purposes.

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5 INTRODUCTION.

(Colipa code A121) is a hair dye developed by Recherche. is a primary intermediate used in oxidative hair dye formulations.

A study was required to assess the rate and extent of absorption of the primary intermediate following the topical application of the hair dye to human skin under in-use conditions. For this purpose, the hair dye was tested under oxidative and non-oxidative conditions. [^{14}C]- was incorporated into a typical oxidative hair dye formulation at *ca* 0.8% (w/w) associated to the coupler m-aminophenol (at *ca* 0.34%, w/w) before mixing with developer (1:1, w/w), to give a final concentration of of *ca* 0.4% (w/w). This will be referred to as the oxidative hair dye test preparation. was incorporated into the same typical oxidative hair dye formulation (without coupler) at *ca* 0.8% (w/w) before being mixed with water (1:1, w/w). This will be referred to as the non-oxidative hair dye test preparation.

The study was conducted at:

Charles River Laboratories
Tranent
Edinburgh
EH33 2NE
UK

Key dates in the conduct of the study were as follows:

Study Initiation:	10 December 2007
Experimental Start Date:	11 December 2007
Experimental Completion Date:	22 February 2008
Study Completion Date:	See Compliance Statement page for date of Study Director's Signature

The study was conducted according to Charles River Laboratories Study No. 783975. A copy of the protocol is provided in Appendix 1.

All data generated and recorded during this study, including a copy of the final report, will be stored in the Scientific Archives of Charles River Laboratories, Preclinical Services Edinburgh for 2 years after the issue of the final report. After the 2 year period the Sponsor will be consulted regarding the disposal, transfer or continued storage of the raw data.

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6 TEST GUIDELINES AND REFERENCES.

This study was performed in accordance with Good Laboratory Practice regulations. This study was also performed in accordance with the following documents:

OECD Guideline for Testing of Chemicals, Guideline 428: Skin Absorption: *In Vitro* Method (2004).

OECD Environmental Health and Safety Publications Series on Testing and Assessment No. 28. Guidance Document for the Conduct of Skin Absorption Studies (2004).

Basic Criteria for the *In Vitro* Assessment of Percutaneous Absorption of Cosmetic Ingredients. SCCP/0970/06 Updated March 2006.

COLIPA (1995). Cosmetic Ingredients: Guidelines for Percutaneous Absorption/ Penetration. The European Cosmetic and Perfumery Association.

Diembeck W, Beck H, Benech-Kieffer F, Courtellemont P, Dupuis J, Lovell W, Paye M, Spengler J, Steiling W. Test Guidelines for *In Vitro* Assessment of Dermal Absorption and Percutaneous Penetration of Cosmetic Ingredients. *Food and Chemical Toxicology* (1999) **37**: 191-205.

In Vitro percutaneous absorption of Hydroxypropyl-bis (N-hydroxyethyl-p-phenylenediamine) HCl. (Colipa A121, Study No. 16056, 2004.

7 EXPERIMENTAL PROCEDURES

7.1 Materials

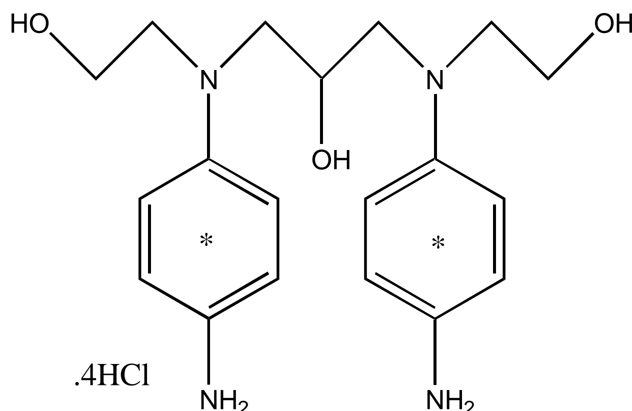
7.1.1 Radiolabelled Test Item

Carbon-14 labelled [ring-U-^{14}C]- also referred to as [^{14}C]- code no. CFQ40063 Batch 1, 74 MBq (2 mCi) was supplied in 2 containers by GE Healthcare UK Limited (Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK). A Certificate of Analysis was supplied with the radiolabelled test item stating the following:

Radiochemical purity: 99.0% (by HPLC, on 21 November 2007)
Specific activity: 2.48 GBq/mmol, 67 mCi/mmol
Molecular weight: 508.4 (at this specific activity)

A copy of the Certificate of Analysis is provided in Appendix 2. The test item was stored at *ca* -80°C in the dark.

The structure and site of labelling (*) of as [^{14}C]- are shown below:



7.1.2 Non-Radiolabelled Test Item

batch no. 0133411 was supplied by . The test item was stored at *ca* 4°C temperature in the dark under nitrogen gas. A copy of the Certificate of Analysis is provided in Appendix 3. The name and properties of the test item are summarised below:

INCI name:	Hydroxypropyl-bis-(N-hydroxyethyl-p-phenylenediamine), HCl
Colipa Code:	A121
Chemical name:	1, 3-bis-[(4-Aminophenyl)-(2-hydroxyethyl)-amino]-propan-2-ol, tetrahydrochloride
Trade Name:	
CAS Registry Number:	128729-28-2 (4HCl)
Description:	White to grey powder
Purity:	99.6% (HPLC)
Molecular Weight:	506.3 g/mol
Log $K_{o/w}$:	-5 at 20°C (dir. 92/69/EEC, A8 method and OECD 107)
Solubility:	Water 760 g/L (Dir. 92/69/EEC, A6 method and OECD 105)
Expiry Date:	April 2008

7.1.3 Formulations

The following formulations were prepared by Laboratoires de Dévéloppement and were stored at ambient temperature in the dark. The formulations were dispatched from Recherche, . A copy of

the composition of ingredients is provided in Appendix 4. This composition of ingredients was supplied by the Sponsor.

Formulation 1: Formulation containing the coupler m-aminophenol (at *ca* 0.34% (w/w)) to which [^{14}C]- will be added.

Formulation 2: Developer (hydrogen peroxide, *ca* 6%)

Formulation 3: Formulation containing no test item and no coupler (Placebo formulation) to which [^{14}C]- will be added.

7.1.4 Other Materials

Phosphate buffered saline tablets and sodium dodecyl sulphate were supplied by Sigma, Fancy Road, Poole, Dorset, UK.

Solvable® was supplied by PerkinElmer LAS (UK) Ltd, Chalfont Road, Bucks, UK.

Aquasafe 500® liquid scintillation fluid was obtained from Zinsser Analytic Maidenhead, UK.

Gilson Microman pipettes were supplied by Gilson S.A. B.P. 45-95400 Villiers-le-Bel, France.

Peristaltic Pump (Watson-Marlow U205) was supplied by Watson-Marlow Bredel pumps Limited, Falmouth, UK.

Saline (0.9%, w/v) was supplied by Baxter Healthcare Limited, Thetford, Norfolk, UK.

Variomag magnetic stirring plate was supplied by H+P Labortechnik AG, Bruckmannring 15-19, Oberschleißheim, Germany.

Zimmer ® electric dermatome (Ref 88 21 06) was supplied by Zimmer Orthopaedic Products, Dover, Ohio 44622, USA.

All other materials were obtained by Charles River Laboratories and were of analytical grade where possible.

7.2 HPLC Method Transfer

The HPLC method transfer for analysis of was performed using the following equipment and conditions:

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<u>Equipment</u>

Agilent 1100 Series HPLC Equipment

<u>Conditions</u>

Column: SUPELCO Suplex PKB100 (150 mm x 4.6 mm, 5 µm)
Mobile Phase A: 0.02 M Ammonium Acetate (pH 7.5):Methanol (77.5:22.5, v/v)
Injection Solution: Ascorbic acid (0.01%, w/v) in water
Run Time: 20 min
Mobile Phase Conditions: Isocratic
Flow Rate: 1.0 mL/min
Column Temperature: Ambient
Auto-Sampler Temperature: 4°C
UV Wavelength: 326 nm

Data was captured by Labsystems Atlas 2002 (Thermosystems) product version 6.18 (hereafter referred to as Altas) and quantified by integration of peak areas.

The acceptance criteria for this method was a correlation coefficient ($r^2 \geq 0.995$) for linearity, within 15% of calculated concentration compared with the weighed concentration for accuracy, a response factor within 5% for reproducibility and coefficient of variation (CV) for peak area within 1% for repeatability. This method was accepted and the results are provided in the table below and in greater detail in Appendix 5.

Parameter	Result
Linearity (r^2)	≥ 0.9997
Reproducibility (%)	≤ 5.52
Repeatability (CV, %)	≤ 0.57
Accuracy (%)	≤ 2.47

A typical HPLC chromatogram for one of the standards and one of the QCs are presented in Appendices 6 and 7, respectively.

7.3 Human Skin Samples

Six samples of full-thickness human skin (5 breast and 1 abdomen) were obtained from donors aged 38 to 84 years old. The skin samples were supplied by two Tissue Banks. The skin was transferred to Charles River Laboratories on dry ice where, when required, it was cleaned of subcutaneous fat and connective tissue using a scalpel blade. The skin samples were washed in cold running water and dried using tissue paper. The skin samples were then cut into smaller pieces (where appropriate) and stored at *ca* -20°C until they were used in the

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study. The age and sex of the donor and site from which the skin was taken were recorded centrally and in the study records. The sample details are shown in Appendix 8.

7.4 Preparation of Split-Thickness Skin Membranes

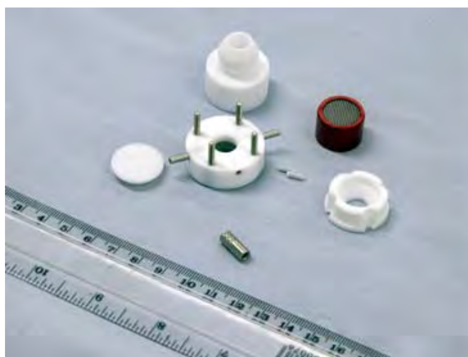
Human skin samples were removed from storage and allowed to thaw at ambient temperature. The thickness of the uncut skin membranes was measured using a micrometer.
Split-thickness membranes were prepared by pinning the full-thickness skin, stratum corneum uppermost, onto a raised cork board and cutting at a setting equivalent to 200-500 µm depth using a Zimmer® electric dermatome. The membranes were then laid out onto aluminium foil and the thickness of the membranes measured using a micrometer. The split-thickness membranes were stored at *ca* -20°C. The thickness of the full-thickness and split-thickness skin membranes is provided in Appendix 9.

7.5 Flow-Through Diffusion Cell Apparatus

An automated flow-through diffusion cell apparatus (Scott/Dick, University of Newcastle-upon-Tyne, UK) was used (see photograph below). The flow-through diffusion cells were placed in a steel manifold heated *via* a circulating water bath to maintain the skin surface temperature at *ca* 32°C (Appendix 10). The cells were connected to multi-channel peristaltic pumps from their afferent ports with the receptor fluid effluent dropping *via* fine bore tubing into scintillation vials on a fraction collector.

The surface area of exposed skin within the cells was 0.64 cm^2. The receptor chamber volume was 0.25 mL. The peristaltic pumps were adjusted to maintain a flow-rate of *ca* 1.5 mL/h (Appendix 10).

<u>A Photograph of the Flow-Through Diffusion Cell</u>



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7.6 Receptor Fluid

Calcium and magnesium free phosphate buffered saline (PBS) was used as the receptor fluid. The receptor fluid was degassed in a sonic bath prior to use.

The receptor fluid was deemed to be acceptable and not rate limiting for absorption as the test item is readily soluble in water (> 760g/L).

The stability of the test item in the receptor fluid was not assessed. This was not deemed necessary as the radiolabelled test item, which will have been absorbed through the skin into the receptor fluid would be analysed by liquid scintillation counting.

7.7 Flow-Through Diffusion Cell Preparation

When required, split-thickness skin membrane samples were removed from storage and allowed to thaw at ambient temperature. Sections of *ca* 1.5 x 1.5 cm were cut out, positioned on the receptor chamber of the diffusion cell containing a magnetic flea and the donor chamber was tightened into place with screws. The cells were then placed in the heated manifold and connected to the peristaltic pump. A Variomag magnetic stirrer was switched on to mix the contents of the receptor chamber. An equilibration period of *ca* 15 min was allowed while receptor fluid was pumped through the receptor chambers at *ca* 1.5 mL/h. The effluent was then collected for *ca* 30 min and retained as blank samples for use in the barrier integrity assessment.

7.8 Barrier Integrity Assessment

Tritiated water (250 µL, *ca* 100,000 disintegrations per minute [d.p.m.]) was applied to the surface of each skin sample and the donor chamber occluded. Penetration of tritiated water was assessed by collecting hourly fractions for 2 h and analysing the fractions by liquid scintillation counting. Permeability coefficients (k_p) were calculated for each skin sample. Any human skin sample exhibiting a k_p greater than 3.5×10^{-3} cm/h was excluded from subsequent absorption measurements. A cross reference of skin cell number, donor number and tritiated water permeability coefficient (k_p) is presented in Appendix 11. At the end of the 2 h period, residual tritiated water was removed from the skin surface by rinsing with water (*ca* 2 mL). The skin was then dried with tissue paper. An equilibration period of *ca* 2.5 h was allowed prior to collection of the pre-dose sample which was collected at ca 1 h.

7.9 Skin Temperature Confirmation

The skin temperature of cells that had not been dosed was also recorded at the time of dosing and then periodically over the next 24 h (Appendix 12).

7.10 Formulation of Test Preparations

7.10.1 Oxidative Test Preparation

7.10.1.1 Preparation of Oxidative Formulation

All preparation procedures stated below, up until the addition of the Developer (Section 7.10.1.2), were carried out in a nitrogen atmosphere. Three dose vials were required to be prepared due to dosing constraints and the nature of the formulation.

One vial containing *ca* 1000 µCi of [^{14}C]- was removed from *ca* -80°C storage and allowed to reach ambient temperature. Formulation 1 (952.93 mg) was then added to the vial and the formulation was mixed by sonication and vortexing.

To determine the radioactive homogeneity and concentration, six weighed 10 µL aliquots were taken into individual vials and 10 mL water added. The samples were mixed by inversion and duplicate 1 mL aliquots removed. Scintillation fluid (*ca* 10 mL) was then added and the samples analysed by liquid scintillation counting.

By radioactivity, the concentration of in the formulation was calculated to be 0.87% (w/w). The concentration was 108.24% of the target concentration of 0.8% (w/w). The formulation was homogeneous with a CV of 2.23%.

7.10.1.2 Preparation of Oxidative Test Preparation

Three aliquots of formulation (170.57 mg, 169.40 mg and 172.79 mg) were transferred into new vials. Immediately prior to dosing, Developer (171.31 mg, 169.45 mg and 173.48 mg) was added to the aliquots, respectively. The contents of each vial were mixed using a pipette tip for *ca* 1-2 min.

7.10.2 Non-Oxidative Test Preparation

7.10.2.1 Preparation of Non-Oxidative Formulation

All preparation procedures stated below, up until the preparation of the three vials for dosing were carried out in a nitrogen atmosphere. Three dose vials were required to be prepared due to dosing constraints and the nature of the formulation. Each vial was used for separate dosing occasions.

One vial containing *ca* 1000 µCi of [^{14}C]- was removed from *ca* -80°C storage and allowed to reach ambient temperature. Formulation 3 (944.30 mg) was added to the vial. The vial was then mixed by sonication and vortexing.

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To determine the radioactive homogeneity and concentration, six weighed 10 µL aliquots were taken into individual vials and 10 mL water added. The samples were mixed by inversion and duplicate 1 mL aliquots removed. Scintillation fluid (*ca* 10 mL) was then added and the samples analysed by liquid scintillation counting.

By radioactivity, the concentration of in the formulation was calculated to be 0.85% (w/w). The concentration was 106.59% of the target concentration of 0.8% (w/w). The formulation was homogeneous with a CV of 2.65%.

7.10.2.2 Preparation of Non-Oxidative Test Preparation

Three aliquots of formulation (77.00 mg, 189.11 mg and 189.62 mg) were transferred into new vials for dosing. Immediately prior to dosing, water (77.08 mg, 188.35 mg and 189.23 mg) was added to the aliquots, respectively. The contents of each vial were mixed using a pipette prior to dosing.

7.11 Confirmation of Concentration of Oxidative and Non-Oxidative Formulations

Prior to dosing before the addition of developer or water, three *ca* 25 µL weighed aliquots of both oxidative and non-oxidative formulations (Sections 7.10.1.1 and 7.10.2.1) were taken and mixed with *ca* 3.975 mL of ascorbic acid. The concentration of was then confirmed using the HPLC method described in Section 6.2.

By HPLC, the concentration of in the oxidative formulation was calculated to be 0.83% (w/w) (CV, 5.57%). An example HPLC chromatogram is presented in Appendix 13. The concentration of in the non-oxidative formulation was calculated to be 0.80% (w/w) (CV, 2.57%). An example HPLC chromatogram is presented in Appendix 14. For the absorption study, all calculations are based on the concentration obtained by liquid scintillation counting.

7.12 Confirmation of Radiochemical Purity of [^{14}C]-

The solutions prepared for the concentration confirmation (Section 7.11) were used to determine the radiochemical purity of [^{14}C]- in the two formulations. This was performed using the same equipment and conditions as used for the method transfer except that the following equipment was also used:

Canberra Packard Radiomatic™Flo-one® Scintillation Analyser (Model 150TR).

Data was captured by Atlas quantified by integration of peak areas.

The chemical authenticity of the [^{14}C]- was confirmed by co-chromatography with authentic non-radiolabelled The radiochemical purity of [^{14}C]-

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in the oxidative and non-oxidative formulations was determined to be 98.3% and 98.2%, respectively. Representative HPLC traces are provided in Appendices 15 and 16.

7.13 Application of Test Preparation

7.13.1 Oxidative Test Preparation

Dosing was divided into three due to the short exposure time (30 min) and the time taken to wash each sample. A separate vial of test preparation, as prepared in Section 7.10.1.2, was used for each separate dosing period.

The test preparation (vial 1) was applied over the surface of the stratum corneum of the exposed skin using an M25 Gilson Microman positive displacement pipette set to deliver 14.0 µL corresponding to *ca* 12.8 mg (*ca* 20 mg/cm^2) for cells 2, 3, 4 and 5. The test preparation (vial 2) was applied over the surface of the stratum corneum of the exposed skin for cells 11, 12, 13 and 14 and the test preparation (vial 3) was applied over the surface of the stratum corneum of the exposed skin for cells 15, 16, 18 and 21. The donor chambers were left open to the atmosphere. To accurately quantify the radioactivity applied to the skin samples, seven 14.0 µL aliquots of test preparation were taken throughout. These were analysed by liquid scintillation counting.

By radioactivity, the concentration of in the test preparation was calculated to be 0.41% (w/w). The mean application rate of the oxidative test preparation and was calculated to be 21.22 mg/cm^2 (target 20 mg/cm^2).

7.13.2 Non-Oxidative Test Preparation

Dosing was divided into three due to the short exposure time (30 min) and the time taken to wash each sample. A separate vial of test preparation, as prepared in Section 7.10.2.2, was used for each separate dosing period.

The test preparation (vial 4) was applied over the surface of the stratum corneum of the exposed skin using an M25 Gilson Microman positive displacement pipette set to deliver 13.4 µL corresponding to *ca* 12.8 mg (*ca* 20 mg/cm^2) for cells 23, 24, 25 and 26. The test preparation (vial 2) was applied over the surface of the stratum corneum of the exposed skin for cells 31, 32, 33 and 35 and the test preparation (vial 3) was applied over the surface of the stratum corneum of the exposed skin for cells 36, 37, 40 and 42. The donor chambers were left open to the atmosphere. To accurately quantify the radioactivity applied to the skin samples, seven 13.4 µL aliquots of test preparation were taken throughout. These were analysed by liquid scintillation counting.

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By radioactivity, the concentration of in the test preparation was calculated to be 0.41% (w/w). The mean application rate of the non-oxidative test preparation and was calculated to be 20.18 mg/cm^2 (target 20 mg/cm^2).

7.14 Formulation Stability

Approximately 24 h after dosing, three 25 µL weighed aliquots of both formulations (Sections 7.10.1.1 and 7.10.2.1) were mixed with injection solution. The concentration and radiochemical purity of were then determined using the HPLC methods described in Section 7.2, respectively.

The concentration of in the oxidative formulation was calculated to be 0.79% (w/w) (CV, 3.37%). The concentration of in the non-oxidative formulation was calculated to be 0.79% (w/w) (CV, 5.37%). The radiochemical purity of [^{14}C]- in the oxidative and non-oxidative formulations was determined to be 98.3% and 98.2%, respectively. This along with the information from Sections 7.11 and 7.12 is summarised in the table below:

Formulation	Time (h)	Concentration (%, w/w)	Radiochemical Purity (%)
Oxidative	0	0.83	98.3
	24	0.79	98.3
Non-Oxidative	0	0.80	98.2
	24	0.79	98.2

Over the 24 h monitoring period, concentration and purity of in both the oxidative and non-oxidative formulations were within 5% of those calculated at 0 h. This was within the criteria for the limit of acceptance of the HPLC method (section 7.2) and therefore the stability of in the oxidative formulation over the live phase of the study was considered to be acceptable.

7.15 Sampling Information

7.15.1 Receptor Fluid

Receptor fluid was collected from 0 to 0.5 h, 0.5 to 1 h and in hourly fractions from 1 to 24 h. All receptor fluid samples were mixed with scintillation fluid (*ca* 10 mL) and then analysed by liquid scintillation counting.

7.15.2 Terminal Exposure Procedures (30 min Post Dose)

At 30 min post exposure, each skin was washed with ten successive *ca* 320 µL (500 µL/cm^2) aliquots of water using an automatic pipette set to deliver 320 µL. Each aliquot was aspirated three times onto the skin surface prior to removal. The pipette tip was removed and replaced by a fresh pipette tip. A single *ca* 320 µL aliquot of sodium dodecyl sulphate (SDS) solution

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in water (2% w/v) was applied to each skin. This was aspirated 3 times. Each skin was washed with a further ten successive *ca* 320 µL aliquots of water with three aspirations between each aliquot. The pipette tips were retained for subsequent analysis. The water and SDS solution were pooled in one skin wash vial per skin sample and water (*ca* 10 mL) was added. Duplicate weighed aliquots (*ca* 0.75 mL) were removed from each skin wash vial, mixed with scintillation fluid (*ca* 10 mL) and analysed by liquid scintillation counting. The pipette tips were mixed with 1ml of methanol and (ca 10 mL) of scintillation fluid and analysed directly by liquid scintillation counting. The skin surface was then dried with 3 tissue swabs which were then placed into a single vial. Water (*ca* 10 mL) was added to each tissue swab vial and aliquots were removed. These were mixed with scintillation fluid (*ca* 10 mL) and analysed by liquid scintillation counting.

7.15.3 Terminal Exposure Procedures (24 h Post Dose)

At 24 h post dose, *i.e.* after a 23.5 h monitoring period, each diffusion cell was disconnected from the receptor fluid pump lines. The underside of the skin was rinsed (receptor rinse) with receptor fluid (*ca* 1-2 mL), which was mixed with scintillation fluid (*ca* 10 mL) and analysed by liquid scintillation counting. The receptor rinse represented the absorbed test item, which was in the receptor chamber, but had not been collected into the 23-24 h receptor fluid fraction.

The donor and receptor chambers were dismantled and the skin removed. The donor and receptor chambers were transferred into a pre-weighed pot (cell wash) containing water (*ca* 40 mL) which had previously been weighed. The water was mixed and left to extract the test item for *ca* 30 min. The pot was placed in a sonic bath for *ca* 10 min during this time. The donor and receptor chambers were removed from the cell wash pot and then duplicate weighed *ca* 1 mL were removed, mixed with scintillation fluid (*ca* 10 mL) and analysed by liquid scintillation counting.

The skin was dried as previously described and then the 24 h tissue swab was analysed as described for the 30 min tissue swab samples. The stratum corneum was removed with *ca* 20 successive tape strips leaving the epidermis visibly intact. Where a small piece of epidermis was removed, the tape strip number was recorded and this is presented in Appendix 17. The tape strips were placed in individual vials and mixed with 1 mL of methanol and 10 mL scintillation fluid.

The skin under the cell flange (unexposed skin) was cut away from the exposed skin with scissors. The epidermis of exposed skin was then separated by heat separation under a layer of cling film. Each skin sample was placed in an appropriately labelled vial containing *ca* 1 mL Solvable®. Vials were heated to *ca* 65°C for ca 2 hours until fully dissolved, then *ca* 10 mL scintillation fluid and 50 µL stannous chloride was added prior to liquid

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scintillation counting analysis. The cling film was placed in individual vials and mixed with 1 mL of water and 10 mL scintillation fluid and analysed by liquid scintillation counting.

7.16 Storage of Samples

All bulk samples not immediately analysed were stored at *ca* -20°C. After analysis, samples were returned to storage at *ca* -20°C.

7.17 Quantification of Total Radioactivity

All samples, except for the tritiated water samples, were counted for 5 min together with representative blanks using a liquid scintillation analyser (Packard 2100-TR) with automatic quench correction by external standard. Representative blank sample values were subtracted from sample count rates to give net d.p.m. per sample. Prior to analysis, samples were allowed to stabilise with regard to light and temperature. The tritiated water samples were treated as above, except that they were subject to liquid scintillation counting for 1 min only.

7.18 Limit of Reliable Measurement

A limit of reliable measurement of 30 d.p.m. above background has been instituted in these laboratories. Any occasions where results arose from data below the limit of reliable measurement have been noted in the Results section of the report. Counts that are below 30 d.p.m. above background represent a true value. This means that data are recorded with values that are less than the limit of reliable measurement. For the lowest application in this study, [^{14}C]- at a concentration of 0.4% (w/w), the limit of reliable measurement is calculated to be 160.2 pg equiv./cm^2 or 0.1602 ng equiv./cm^2. Results calculated from data less than 30 d.p.m above background have been highlighted with an asterisk (*) in the results tables.

The instrumental equipment used to quantify radioactivity records data to a fraction of a d.p.m, and reports these values as a mean rounded value over the counting period (5 min). For this study, a lowest detection value of 1 d.p.m has been set. Therefore, for the lowest application in this study the detection of 1 d.p.m is calculated to be 5.3 pg equiv./cm^2.

Test Preparation	dpm above background	ng equiv./cm^2	pg equiv./cm^2
Oxidative	30	0.1602	160.2
	1	0.0053	5.3
Non Oxidative	30	0.1602	160.2
	1	0.0053	5.3

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8 CALCULATIONS

The following calculations were performed:

8.1 Permeability Coefficient (k_p) of Water

Cumulative absorption of tritiated water was calculated for each skin sample by summing the net d.p.m. for each hourly fraction from 0 to 2 h. The slope of the absorption *versus* time curve from 0 to 2 h (*ie* 3 data points) was calculated by linear regression to give an absorption rate (d.p.m/cm^2/h).

$$\text{Absorption rate (d.p.m./cm}^2\text{/h)} = \frac{\text{slope (d.p.m./h)}}{\text{exposed area (cm}^2\text{)}}$$

This was then converted to the permeability coefficient (k_p) from the dose application rate of tritiated water as follows:

$$K_p = \frac{\text{absorption rate (d.p.m./cm}^2\text{/h)}}{\text{Specific activity (d.p.m./cm}^3\text{)}}$$

8.2 Absorption of [^{14}C]- (Flux and Percentage Absorbed)

The absorbed dose was calculated from each individual sample (receptor fluid samples were given as cumulative absorbed dose) radioactivity (d.p.m), specific activity (SA) and dose area as follows:

$$\text{Absorbed dose (µg equiv./cm}^2\text{)} = \frac{\text{sample radioactivity (d.p.m.)}}{\text{SA (d.p.m./µg equiv.) x exposure area (cm}^2\text{)}}$$

In addition, the percentage absorbed dose was also calculated for each sample as follows:

$$\text{Absorbed dose (\%)} = \frac{\text{sample radioactivity (d.p.m.)}}{\text{applied dose (d.p.m.)}} \text{ x } 100\%$$

8.3 Data Presentation

Data presented in results, tables, figures and appendices are computer generated and rounded appropriately for inclusion in the report. As a consequence, calculation of values from data presented will, in some instances, yield minor variations.

9 DEFINITIONS

The definitions used in this report were taken directly from the OECD guidance document as follows:

Absorbed Dose (*in vitro*)

The mass of test item reaching the receptor fluid or systemic circulation within a specified period of time.

Absorbable Dose

Represents that present on or in the skin following washing.

Absorption (Dermal, Percutaneous and Skin Absorption)

Diffusion of chemicals from the outer surface of the skin to the receptor fluid or systemic circulation.

Absorption Profile

A graphical representation of cumulative absorption as a function of time.

Absorption Rate

Mass of test item passing through a unit area of skin into the receptor fluid or systemic circulation, per unit time ($\mu g/cm^2/h$).

Adsorption

Reversible binding or adherence of the test item to any component of the test system.

Applied Dose

The mass of test preparation containing a specified mass of test item applied per cm^2 of skin.

Dermal Delivery

The sum of the applied dose found in the treated skin and the absorbed dose at the end of the experiment.

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Dislodgeable Dose

The mass of test item that is removable from the application site.

Exposure Period

The time from application of test preparation to removal at skin washing.

Finite Dose

The amount of test preparation applied to the skin where a maximum absorption rate of the test item may be achieved for a certain time interval but is not maintained.

Flux

The mass of the test item passing through a unit area of skin per unit of time under steady-state conditions (in $\mu g/cm^2/h$).

In-Use Preparation

The preparation of test item which relates directly to potential human exposure (eg cosmetic or agrochemical formulations and dilutions thereof, a mixture of industrial chemicals in a solvent, *etc*).

Infinite Dose

The amount of test preparation applied to the skin where a maximum absorption rate of the test item is achieved and maintained.

Lag Time

Derived from a graph of cumulative absorbed dose and time. Intercept of the tangent of the linear part of the absorption profile with the x-axis.

Penetration Enhancer

An adjuvant, which facilitates penetration of the test item through skin.

Percentage Absorption

The mass of test item absorbed (over a given time period) divided by the mass of test item applied multiplied by 100.

Permeability Coefficient (K_p)

A value, in units of cm/h, that represents the rate at which a chemical penetrates the skin. This is calculated from the flux divided by the applied concentration.

Steady-State

The part of the absorption profile where the absorption rate remains constant.

Test Substance (Test Item)

A single chemical entity whose penetration characteristics are under investigation.

Test Preparation

The actual material which is applied to the skin. Usually the test preparation will be the "in-use" preparation that reflects actual use conditions; alternatively it may be a mixture of the test item in a carrier or solvent to facilitate application to the skin.

Unabsorbed Dose

Represents that washed from the skin surface after exposure and any present on the non-occlusive cover, including any dose shown to volatilise from the skin during exposure.

This is also defined as the sum of the mass of test item in the dislodgeable dose, the unexposed skin and stratum corneum.

10 PROTOCOL ADHERENCE

The study was performed in accordance with the protocol for Charles River Laboratories Study No. 783975 with the following deviations from the protocol:

Protocol Sections 13.4 & 13.5

Aliquots of 25 µL of the formulation were taken for HPLC analysis. The smaller aliquot volume was used due to the limited amount of the formulation prepared.

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11 RESULTS AND DISCUSSION

11.1 Absorption Study – Oxidative Test Preparation

A total of 12 samples of human skin obtained from 6 different donors were dosed topically with [^{14}C]- in the Oxidative Test Preparation (0.4%, w/w) at an application rate of *ca* 20 mg/cm . All samples had similar absorption profiles (Figure 1) except for cell 11 which was rejected. Cell 11 was rejected as an outlier for absorbed dose, therefore, the following results are provided as mean values (n = 11).

The distribution of radioactivity at 24 h post dose is provided in Table 1. The mean mass balance was 99.21% of the applied dose at 24 h post dose. At the end of the 30 min exposure period, 94.19% of the applied dose was washed off (88.55%, 4.61% and 1.03% recovered in the skin wash, tissue swab and pipette tips, respectively). A further 0.45% of the applied dose was removed (cell wash and 24 h tissue swabs contained 0.34% and 0.10% of the applied dose, respectively). The material recovered in the cell wash was almost certainly material that had been dislodged from the skin at 30 min post dose during the washing procedure. Therefore, the total dislodgeable dose was 94.64% of the applied dose. The mean total unabsorbed dose was 98.31% of the applied dose. This consisted of the dislodgeable dose, unexposed skin (<0.01%) and the radioactivity associated with the stratum corneum (3.67%). The first five tape strips contained 2.42% of the applied dose. These initial tape strips may be considered to be on the stratum corneum surface and not associated with the stratum corneum. There was a steady decrease in the recovery of radioactivity associated with the stratum corneum. Tapes 6-10, 11-15 and 16-20 contained a further 0.78%, 0.29% and 0.18%, respectively. The results are presented in Table 2 and graphically in Figure 2. Those amounts retained by the stratum corneum at 24 h are not considered to be dermally absorbed and thus do not contribute to the systemic dose. The absorbed dose (0.01%) was the sum of the receptor fluid (0.01%) and the receptor rinse (<0.01%). Dermal delivery (0.90%) was the sum of the absorbed dose and the epidermis (0.73%), dermis (0.16%). The absorption profile is provided in Table 3 and Figure 3.

The distribution by mass, of [^{14}C]- at 24 h post dose is shown in Table 4. The mass balance, dislodgeable dose, unabsorbed dose, absorbed dose and dermal delivery were 86.92, 82.92, 86.14, 0.01 and 0.78 µg equiv./cm^2, respectively. The distribution of radioactivity through the stratum corneum is provided in Table 5 and graphically in Figure 4.

The absorption profile, by mass, is provided in Table 6 and Figure 5. There was a lag time of < 1 h. Any steady state flux reached was of too short a time period to be quantified.

The raw data results expressed as d.p.m. are provided in Appendix 18.

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11.2 Absorption Study – Non-Oxidative Test Preparation

A total of 12 samples of human skin obtained from 6 different donors were dosed topically with [^{14}C]- in the Non-Oxidative Test Preparation (0.4%, w/w) at an application rate of *ca* 20 mg/cm^2. All samples had similar absorption profiles (Figure 6). No cells were rejected. The following results are provided as mean values (n = 12).

The distribution of radioactivity at 24 h post dose is provided in Table 7. The mean mass balance was 99.52% of the applied dose at 24 h post dose. At the end of the 30 min exposure period, 95.66% of the applied dose was washed off (93.09%, 2.27% and 0.29% recovered in the skin wash, tissue swab and pipette tips, respectively). A further 0.77% of the applied dose was removed (cell wash and 24 h tissue swabs contained 0.64% and 0.14% of the applied dose, respectively). The material recovered in the cell wash was almost certainly material that had been dislodged from the skin at 30 min post dose during the washing procedure. Therefore, the total dislodgeable dose was 96.43% of the applied dose. The mean total unabsorbed dose was 98.72% of the applied dose. This consisted of the dislodgeable dose, unexposed skin (0.02%) and the radioactivity associated with the stratum corneum (2.26%). The first five tape strips contained 1.42% of the applied dose. These initial tape strips may be considered to be on the stratum corneum surface and not associated with the stratum corneum. There was a steady decrease in the recovery of radioactivity associated with the stratum corneum. Tapes 6-10, 11-15 and 16-20 contained a further 0.48%, 0.21% and 0.16%, respectively. The results are presented in Table 8 and graphically in Figure 7. Those amounts retained by the stratum corneum at 24 h are not considered to be dermally absorbed and thus do not contribute to the systemic dose. The absorbed dose (0.01%) was the sum of the receptor fluid (0.01%) and the receptor rinse (<0.01%). Dermal delivery (0.80%) was the sum of the absorbed dose and the epidermis (0.70%), dermis (0.08%). The absorption profile is provided in Table 9 and Figure 8.

The distribution by mass, of [^{14}C]- at 24 h post dose is shown in Table 10. The mass balance, dislodgeable dose, unabsorbed dose, absorbed dose and dermal delivery were 82.85, 80.28, 82.19, 0.01 and 0.66 µg equiv./cm^2, respectively. The distribution of radioactivity through the stratum corneum is provided in Table 11 and graphically in Figure 9.

The absorption profile, by mass, is provided in Table 12 and Figure 10. There was a lag time of < 1 h. Any steady state flux reached was of too short a time period to be quantified.

The raw data results expressed as d.p.m. are provided in Appendix 19.

11.3 Comparison of [^{14}C]- in Oxidative and Non-Oxidative Test Preparations

A statistical comparison of [^{14}C]- in both the oxidative and non oxidative test preparations was carried out using an unpaired T-Test (Table 13).

When comparing the results as either % applied dose or µg equiv./cm^2, there were no significant differences for dermal delivery of [^{14}C]- for the two test preparations. Dislodgeable dose and total absorbed dose were significantly higher for the non-oxidative test preparation compared to the oxidative test preparation when comparing µg equiv./cm^2 results. However, only total absorbed dose was significantly higher for the non-oxidative test preparation compared to the oxidative test preparation when comparing % applied dose.

11.4 Discussion of Variability – Total Absorbed Dose and Dermal Delivery

When reviewing the variability in the total absorbed dose and dermal delivery of [^{14}C]- for the oxidative and non-oxidative test preparations, three major factors were further considered; (a) the low total absorbed dose and dermal delivery values, (b) the effectiveness of the washing procedure at 30 min and (c) inter-donor variability in skin barrier function.

(a) The mean total absorbed dose [^{14}C]- over 24 hours was very low (0.01% corresponding to 0.01 µg equiv./cm^2) for both test preparations. Similarly, dermal delivery of [^{14}C]- was lower than 1% of the applied dose (0.90 ± 0.92% and 0.80 ± 0.77% for the oxidative and the non-oxidative test preparations, respectively).

Due to these low values, any fluctuation between samples (*i.e.* 1% of applied dose) would result in a large increase in the relative variability of the data set. This is particularly true given that the variability observed in dermal absorption studies has been recognised to increase inversely to a decreasing skin penetration rate[1]. In addition, the physico-chemical properties of the test items are known to contribute to the overall level of variability of the data[2]. For hydrophilic molecules such as (water solubility 760 g/L and log $K_{o/w}$ = -5) a larger impact is often seen.

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[1] SCCP Opinion on Basic Criteria for the *In Vitro* Assessment of Dermal Absorption of Cosmetic Ingredients, updated March 2006. SCCP/0970/06, adopted by the SCCP during the 7th plenary of 28 March 2006.

[2] Akomeah FK, Martin GP, Brown MB. Variability in Human Skin Permeability *in vitro*: Comparing Penetrants with Different Physicochemical Properties. J. Pharm. Sci., 2007, 96 (4): 824-834.

The vast majority of the material considered to be dermally delivered was associated with the epidermis (>80%). Additionally, amounts found in the receptor fluid were very low, with the material found in the epidermis and dermis accounting for >98% of the material considered to be dermally delivered. Therefore, any variability in these compartments will have a significant bearing on the overall level of variability.

(b) It should be noted that review of variability for total absorbed dose or dermal delivery by CV alone should be assessed with caution. Review of CV alone for each sample type does not take into consideration the influence that one sample type may have over another (*i.e.* washing procedure influencing total absorbed dose and dermal delivery). However, by reviewing variability as the SD of % applied dose, these differences and influences were highlighted.

The washing procedure at 30 min post dose had an influence on total absorbed dose and dermal delivery of [^{14}C]- The 30 min washing procedure for the oxidative and non-oxidative test preparations was both efficient and consistent for all samples (94.19 ± 3.14% and 95.66 ± 3.39%, respectively).

However, when comparing the test item removed from the skin at 30 min post dose (SD: ± 3.14% or ± 3.39%) with the dermal delivery (SD: ± 0.92% or ± 0.77%), the variability in the washing (SD) was three to four times greater than the variability for either the total absorbed dose or dermal delivery of [^{14}C]-

(c) When reviewing the variability in total absorbed dose and dermal delivery, differences in the barrier function of the different skin donors was considered. The results were relatively consistent for total absorbed dose and dermal delivery for each individual skin donor. For example, donor no. 0211 had the highest epidermis and dermal delivery amount of [^{14}C]- for both the oxidative and non-oxidative test preparations.

Therefore, taking into consideration the very low mean total absorbed dose (≤ 0.01% of applied dose) and the relatively low mean dermal delivery (≤ 0.90% of applied dose) of [^{14}C]- the variability in the washing procedure (SD: *ca* 3% of applied dose) and inter-donor variability in skin barrier function, the data was considered to be very consistent for total absorbed dose and dermal delivery of [^{14}C]- for both the oxidative and non-oxidative test preparations. Also, given that the criteria of barrier integrity and mass balance were achieved, this study was considered to be valid.

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12 CONCLUSIONS

In conclusion, [^{14}C]- in oxidative and non-oxidative test preparations was applied topically to human skin *in vitro*. Under the present experimental conditions, for [^{14}C]- in the oxidative test preparation, most of the applied dose was removed at 30 min post dose (94.19% of the applied dose). At 24 h post dose, a further 0.45% was removed. Therefore, the total dislodgeable dose was 94.64% of the applied dose. At 24 h post dose, the absorbed dose and dermal delivery were 0.01% (0.01 µg equiv/cm^2) and 0.90% (0.78 µg equiv/cm^2) of the applied dose, respectively. Under the present experimental conditions, for [^{14}C]- in the non-oxidative test preparation, most of the applied dose was removed at 30 min post dose (95.66% of the applied dose). At 24 h post dose, a further 0.77% was removed. Therefore, the total dislodgeable dose was 96.43% of the applied dose. At 24 h post dose, the absorbed dose and dermal delivery were 0.01% (0.01 µg equiv/cm^2) and 0.80% (0.66 µg equiv/cm^2) of the applied dose, respectively. The amount of test item found in the epidermis and dermis accounted for > 98% of the dermal delivery.

The dermal absorption figure to be taken into consideration for the calculation of the margin of safety are the mean values of 0.78 µg equiv./cm^2 for the oxidative test preparation and 0.66 µg equiv./cm^2 for the non-oxidative test preparation, taking into account the very low total absorbed and dermal delivery values. Additionally, samples with the highest dermal delivery values were calculated using some receptor fluid samples that were below the limit of reliable measurement, therefore, this supports the use of mean values for the risk assessment purposes.

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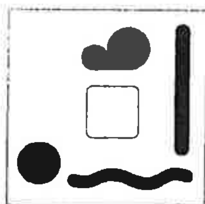

CCR PROJECT 508800

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

Project No.:
95/1/006

IN VIVO / IN VITRO UNSCHEDULED DNA SYNTHESIS IN RAT HEPATOCYTES

WITH

REPORT

Study Completion Date:
August 10, 1995

RCC
Group

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Deviation to the Protocol

1) Historical Controls (1993 - 1994)

Negative controls:

Nuclear grains:		Net grains:	
Range:	0.02 to 9.92	Range:	-0.01 to -12.29
Mean:	5.63*± 6.38	Mean:	-4.45 ± 2.45

Positive controls:

Range:	18.01 to 107.84	Range:	8.38 to 92.43
Mean:	52.91#± 19.44	Mean:	37.72 ± 17.33

*** = mean of 83 male rats (calculated from the means of 100 scored cells per animal)**
 # = mean of 70 male rats (calculated from the means of 100 scored cells per animal)

No historical data from female rats are available since male rates are prefered for the performance of UDS studies.

2) Guidelines

OECD-Draft guideline: "DNA Damage and Repair/Unscheduled DNA Synthesis in Mammalian liver cells *in vivo*", Paris, **1981**

Reasons for the alterations: updating

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STATEMENT OF COMPLIANCE

Project Number: 508800

Test Material :

Study Director: Dr. Wolfgang Völkner

Title: *In vivo/in vitro* Unscheduled DNA Synthesis in Rat Hepatocytes with

This study performed in the testing facility of **C C R** was conducted in compliance with Good Laboratory Practice Regulations.

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Anlage 1 ("Annex 1"), dated July 25, 1994 (BGBL. I 1994 S. 1703).

"The OECD Principles of Good Laboratory Practice", Paris 1981."

There were no circumstances that may have affected the quality or integrity of the study.

Study Director

C C R
Dr. Wolfgang Völkner

Date: August 21, 1995

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QUALITY ASSURANCE UNIT

C C R, Cytotest Cell Research GmbH & Co. KG,
In den Leppsteinswiesen 19
D-64380 Roßdorf

Statement

Project Number: 508800

Test Material :

Study Director: Dr. Wolfgang Völkner

Title: *In vivo/in vitro* Unscheduled DNA Synthesis in Rat Hepatocytes with

This report was audited by the Quality Assurance Unit and the conduct of this study was inspected on the following dates.

Phases and Dates of QAU Inspections/ Audits	Dates of Reports to the Study Director and to Management
Protocol Audit: March 27, 1995	March 27, 1995
Process Inspection: July 07, 1995	July 07, 1995
Draft Audit: August 03, 1995	August 03, 1995

Head of Quality Assurance Unit Frauke Hermann

i. V. U. Wener

Date: August 18, 1995

SUMMARY

The test article (Batch No. Pil 4 X) was assessed in the in *vivo/in vitro* UDS assay for its potential to induce DNA repair (UDS) in the hepatocytes of rats.

The test article was formulated in aqua dest. This suspending agent was used as vehicle control. The volume administered orally was 10 ml/kg body weight (b.w.). After a treatment period of 2 and 15 hours, respectively, the livers of narcotised animals were perfused to achieve hepatocytes suspensions. Primary hepatocyte cultures were established and exposed for 4 hours to ^{3}HTdR (methyl-^{3}H-thymidine) which is incorporated if UDS occurs (2).

The test article was tested at the following dose levels:

2 hour preparation interval:	1500 mg/kg b.w.
16 hour preparation interval:	150 and 1500 mg/kg b.w.

The highest dose was estimated by a pre-experiment to be suitable. The animals expressed toxic reactions. After administration of a higher dose level one out of two animals died.

For each dose level, including the vehicle and positive control, hepatocytes from three treated animals were assessed for the occurrence of UDS.

No behavioural toxic reactions of the animals occurred at any of the treatment periods or dose groups. In addition, the viability of the hepatocytes was not substantially affected due to the *in vivo* pre-treatment with the test article.

Animals treated with the highest dose (1500 mg/kg b.w.) showed black coloured fluid of the peritoneal cavity.

No dose level of the test article revealed UDS induction in the hepatocytes of the treated animals as compared to the current vehicle controls.

An appropriate reference mutagen (2-AAF, 100 mg/kg b.w.) was used as positive control. Treatment with 2-AAF revealed distinct increases in the number of nuclear and net grain counts.

Conclusion

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test article did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats.

Therefore, is considered to be non-effective in this *in vivo/in vitro* UDS test system.

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OBJECTIVE

Aims of the Study

This in *vivo/in vitro* experiment was performed to assess the potential of the test article to induce repairable DNA-damage. Repair was measured as unscheduled DNA-synthesis (UDS) by the uptake of radiolabelled ^{3}HTdR into hepatocytes of rats (6,7).

Reasons for the Study

The UDS test is an assay for the detection of chemically induced effects on DNA. Lesions in DNA produced by chemicals may lead to genetic changes (mutations) through mis-repair.

It is generally accepted that somatic mutations contribute to the formation of cancer. Chemicals which damage DNA and induce DNA repair synthesis therefore have the potential of being carcinogenic. Consequently, the aim of the UDS assay is to detect potential carcinogens (7,9).

Rats are exposed to the test article by an appropriate route. After a treatment period of 2 hours or 16 hours, respectively, the animals are narcotised and the liver perfusion is initiated. Primary hepatocyte cultures are established and exposed for 4 hours to ^{3}HTdR which is incorporated if UDS occurs (2).

The UDS test measures the DNA repair synthesis after excision and removal of a stretch of DNA containing the region of damage induced by chemical or physical agents. The test is based commonly on the incorporation of tritium labeled thymidine (^{3}HTdR) into the DNA of mammalian cells which are not in the S-phase of the cell cycle. The uptake of ^{3}HTdR in cells isolated from rats exposed *in vivo* is determined by autoradiography.

The autoradiography approach involves a short *in vitro* culturing period of hepatocytes from treated rats on cover slips and exposing them to medium containing ^{3}HTdR. At the end of that period the cultures are processed for autoradiography and the amount of incorporated radioactivity is determined by silver grain counting. The few cells undergoing replicative DNA synthesis can be recognised by their heavy labeling in the autoradiographs and they are excluded from counting.

To investigate a potential peak response of the test article the highest dose level is tested at two different exposure times.

To validate the test a reference mutagen is tested parallel to the test article.

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MATERIALS AND METHODS

Test Article

The test article and the information concerning the test article were provided by the sponsor.

Name:	
Batch No.:	Pil 4 X
Aggregate State at RT:	solid
Colour:	beige
Analysis:	July 01,1994
Purity:	99.8 %
Stability Pure:	not indicated by the sponsor
Stability in solvent:	oxydation
Storage:	room temperature, light and humid protected
Expiration Date:	not indicated by the sponsor

On the day of the experiment (immediately before treatment), the test article was formulated in aqua dest. The vehicle was chosen according to its relative nontoxicity for the animals. All animals were challenged orally once. The volume administered was 10 ml/kg b.w. (2).

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Controls

Vehicle Control

The vehicle of the test article was used as vehicle control.

Name:	Aqua dest
Route and frequency of administration:	singly, orally
Volume administered:	10 ml/kg b.w.

Positive Control Substance

Name:	2-AAF; 2-Acetylaminofluorene
Supplier:	FLUKA FEINCHEMIKALIEN GmbH D-89231 Neu-Ulm
Catalogue no.:	00300
CAS no.:	53-96-3
Purity:	> 99 %
Dissolved in:	dimethyl sulfoxide/polyethylene glycol 400 (1 + 9)
Dosing:	100 mg/kg b.w.
Route and frequency of administration:	singly, orally
Volume administered:	10 ml/kg b.w.

Solution prepared on day of administration.

The stability of the positive control substance in vehicle was unknown, but a DNA repair response in the expected range (3) means biological stability demonstration.

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Test System

Reasons for the Choice of the Experimental Animal Species

The rat is an animal which has been used for many years as suitable experimental animal in genotoxicity investigations. There are many data available from such investigations which may be helpful in the interpretation of results from the UDS test. In addition, the rat is an experimental animal in many physiological, pharmacological, and toxicological studies. Data from such experiments may also be useful for the design and the performance of the UDS test.

Strain:	Wistar HanIbm: WIST (SPF)
Source:	BRL, CH-4414 Füllinsdorf
Number of animals:	20 (males)
Acclimatisation:	minimum 5 days
Age of the animals:	6 - 10 weeks
Initial body weight at start of treatment:	Mean value 203.6 g (SD ± 10.17 g)

According to the suppliers assurance the animals were in healthy condition. The animals underwent quarantine in the animal house of CCR for at least five days after their arrival. During this period the animals did not show signs of illness or altered behaviour.

The animals were distributed into the test groups at random and identified by cage number.

Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions.

Housing:	single
Cage Type:	Makrolon Type II, with wire mesh top (Ehret, D-79312 Emmendingen)
Bedding:	granulated soft wood bedding (Altromin, D-32791 Lage/Lippe)
Feed:	pelleted standard diet (Altromin 1324, D-32791 Lage/Lippe)
Drinking Water:	tap water, ad libitum (Gemeindewerke Roßdorf, D-64380 Roßdorf)
Environment:	temperature 21 ± 3°C relative humidity 30 - 85 %* artificial light 6.00 a.m. - 6.00 p.m.

* Sometimes the humidity was higher than given in the protocol. This deviation has no influence on the study.

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Experimental Performance

Pre-Experiment for Toxicity

A preliminary study on acute toxicity was performed with a small group of animals using the same conditions as in the UDS study concerning: animal strain; starvation period; vehicle; route, frequency, and volume of administration.

The animals were challenged orally (gavage) and examined for acute toxic symptoms at intervals of 1 h and 24 h after administration of the test article.

Main-Experiment

Dose Selection

The maximum tolerated dose was determined to be the dose that caused toxic reactions (reduced spontaneous activity, eyelid closure, apathy) without having major effects on survival within 24 hours. For the low dose one tenth of the high dose level was tested.

Study Procedure

Test groups:

Four male rats were assigned to each test group. The animals were identified by their cage numbers as shown below in the table.

Test group	hours post-treatment 2 h	hours post-treatment 16 h
Vehicle control		5 - 8
Low dose		9 - 12
High dose	1 - 4	13 - 16
Positive control		17 - 20

Treatment

The animals were starved overnight (2 hours treatment) or approximately 6 hours (16 hours treatment) before receiving the test article, water was available continuously. At the beginning of the treatment the animals were weighed and the individual volume to be administered was adjusted to the body weight of the animals. The animals received the test article once.

Four animals (males) were treated per dose group.

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Isolation of the Primary Hepatocytes

After anaesthetising the rats with Na-pentobarbital (Narcoren) the liver was perfused through the vena portae with Hanks' balanced salt solution (HBSS, Gibco/BRL, D-76344 Eggenstein) supplemented with collagenase (0.05 % w/v, Boehringer Mannheim, D-68305 Mannheim) adjusted to pH 7.4 and maintained at 37° C (8).

The hepatocytes were isolated from the liver and washed twice with HBSS. The crude cell suspension was filtered through a stainless steel mesh to yield a single cell suspension. The quality of the actual performed perfusion was determined by the trypan blue dye exclusion method. In addition, the number of the isolated cells was determined.

Culture Conditions

The washed hepatocytes were centrifuged and transfered into Williams medium E (WME, Gibco/BRL, D-76344 Eggenstein) supplemented (1) with:

Hepes	2,38 mg/ml	Glutamin	0,29 mg/ml
Penicillin	100 units/ml	Insulin	0,50 µg/ml
Streptomycin	0,10 mg/ml	Fetal Calf Seum (FCS)	100 µl/ml

The medium without the cells was adjusted to pH 7.6.

At least three cultures were established for each animal. Aliquotes of 2.5 ml with freshly isolated hepatocytes in complete culture medium (2.0×10^5 living cells/ml) were added to 35 mm six-well cluster dishes (Greiner, D-72603 Nürtingen) containing one gelatinised 25 mm round plastic coverslip (Thermanox, Nunc, D-65203 Wiesbaden) per well.

After an attachment period of approximately 1.5 h in a 95 % air/ 5 % CO_2 humidified incubator at 37° C the culture medium was discarded. Then the cell layer was rinsed once with PBS to remove non-adherent cells (9). Subsequently ^{3}HTdR (5 µCi/ml, specific activity 20 Ci/mmol; New England Nuclear, D-63033 Dreieich) in 2.0 ml culture medium (WME, 1% FCS) was added to the cultures. After a labeling time of 4 h the cells were washed twice with WME supplemented with 1 % FCS and 0.25 mM unlabeled thymidine. Cultures were incubated overnight using the same medium (2). To prepare for autoradiography the medium was - replaced by a hypotonic solution of 1 % sodium citrate for 10 minutes to swell the nuclei for better grain quantification (9). The cells on the coverslips were then fixed by three changes of methanol:acetic acid (3+1 v/v) for 20 minutes each, rinsed with 96 % ethanol, and air dried.

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Autoradiographic Processing

The cover slips were mounted the side carrying the cells up on glass slides and coated with KODAK NTB2 (Tecnomara, D-35463 Fernwald) photographic emulsion in the dark. The coated slides were stored in light proofed boxes in the presence of a drying agent for 12 - 14 days at 4° C. The photographic emulsion is then developed with KODAK Dektol Developer (Tecnomara, D-35463 Fernwald) at room temperature, fixed in TETENAL (Tetenal, D-22844 Norderstedt) and stained with hematoxylin/eosin.

Quantification of UDS

Evaluation was performed microscopically on coded slides using NIKON microscopes with oil immersion objectives. The cells for scoring were randomly selected according to a fixed scheme. The number of silver grains above the nucleus was counted automatically using the ARTEK 880 or 982 counter. In addition, the number of grains of one nuclear-sized cytoplasm adjacent to the nucleus was counted (2). At least two slides per animal and 50 cells per slide were evaluated. Heavily labeled S-phase cells were excluded from counting.

Three animals per group were evaluated as described above. The remaining animal per test group would be evaluated if an animal dies spontaneously or in case of technical problems concerning the isolation of the hepatocytes.

Data Recording

The data generated were recorded in the raw data. The results were presented in tabular form, including experimental groups with the test article, vehicle and positive controls.

The nuclear and cytoplasm grain counts, as well as the net grain counts (nuclear - cytoplasmic grains) were reported separately (5). The mean counts with standard deviation were used to describe the distribution of ^{3}HTdR incorporation in the nucleus, the cytoplasm, and for the net grains, respectively.

Evaluation of Results

Nuclear and net grain counts are estimated together. Increased net grains should be based on enhanced nuclear grain counts rather than on decreased cytoplasmic grain counts.

A test article is classified as positive if the mean number of net grains is higher than five per nucleus at one of the test points.

A group average between 0 and 5 net grains is considered as a marginal response. A dose-related increase in nuclear and net grains and/or a substantial shift of the percentage distribution of the nuclear grain counts to higher values provide additional informations to confirm a positive response with less than 5 net grains.

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Statistical significance may give further evidence for a positive evaluation.

Statistical significance can be evaluated by means of the non-parametric Mann-Whitney test (4).

A test article producing net grains not greater than 0 at anyone of the test points is considered non-effective in this system.

Historical Controls (1993 - 1994)

Negative controls:

Nuclear grains:		Net grains:	
Range:	0.02 to 9.92	Range:	-0.01 to -12.29
Mean:	5.63*± 6.38	Mean:	-4.45 ± 2.45

Positive controls:

Range:	18.01 to 107.84	Range:	8.38 to 92.43
Mean:	52.91#± 19.44	Mean:	37.72 ± 17.33

*** = mean of 83 male rats (calculated from the means of 100 scored cells per animal)**
 # = mean of 70 male rats (calculated from the means of 100 scored cells per animal)

No historical data from female rats are available since male rates are prefered for the performance of UDS studies.

BIOMETRY

A statistical evaluation of the results was not necessary to perform as the number of nuclear and net grain counts of the groups treated with the test article were in the range of the corresponding controls.A statistical evaluation of the results was not necessary to perform, since the number of nuclear grain counts of the groups treated with the test article were in the range of the controls and net grain values obtained were consistently negative.

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RESULTS

Pre-Experiment

In 2 consecutive pre-experiments 2 male rats received orally a single dose of 1500, and 2000 mg/kg b.w., respectively, of formulated in aqua dest. The volume administered was 10 ml/kg b.w..

The treated animals expressed toxic reactions as shown below in the tables:

Dose: 1500 mg/kg b.w.

toxic reactions	hours post-treatment 1 h	24 h
reduction of spontaneous activity	2	2
eyelid closure	1	-
apathy	2	2

Dose: 2000 mg/kg b.w.

toxic reactions	hours post-treatment 1 h	24 h
reduction of spontaneous activity	1	1
abdominal position	1	-
eyelid closure	1	1
apathy	1	1
death	1	-

On the basis of these data 1500 mg/kg b.w. was estimated to be close to the maximum tolerated dose.

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TABLES OF RESULTS

Table 1: Vability and number of the hepatocytes

Test Article:

dose [mg / kg b.w.]	treatment period	Animal No.	Viability* [%]	Number of isolated cells [x 10 ^{6}]
1500	2h	1	71	854
	2h	2	78	642
	2h	3	69	762
Vehicle (Aqua dest)	16h	5	71	882
	16h	6	77	748
	16h	7	78	592
150	16h	9	74	723
	16h	10	91	652
	16h	11	72	782
1500	16h	13	77	487
	16h	14	67	1027
	16h	15	70	755
100 (2AAF)	16h	17	75	707
	16h	18	85	571
	16h	19	71	604

* = Viability determined by means of trypan blue dye exclusion assay

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Summary of the Results

Table 2: Individual Animals

Test article:

Treatment, Period	(Animal no.)	mg /kg b.w.	Grains per nucleus Mean*	Grains per nucleus Standard deviation	Grains per Cytoplasma area Mean*	Grains per Cytoplasma area Standard deviation	Net grains per nucleus Mean*	Net grains per nucleus Standard deviation
Vehicle control, 16 h	(5)	Aqua dest	7.58	± 2.56	15.08	± 4.76	-7.50	± 4.17
	(6)		12.39	± 4.85	15.48	± 4.65	-3.09	± 4.21
	(7)		14.19	± 5.26	17.06	± 5.03	-2.87	± 4.75
Pos. control**, 16 h	(17)	100.00	37.25	± 10.97	14.86	± 5.45	22.39	± 9.43
	(18)		97.51	± 31.39	24.41	±10.98	73.10	± 26.64
	(19)		103.57	± 23.14	23.39	±10.83	80.18	± 19.77
1st Dose level, 16 h	(9)	150.00	8.98	± 3.22	14.71	± 3.88	-5.73	± 4.64
	(10)		11.97	± 4.29	16.14	± 4.89	-4.17	± 4.71
	(11)		11.21	± 5.36	20.86	± 6.63	-9.65	± 5.44
2nd Dose level, 2h	(1)	1500.00	9.63	± 3.64	16.88	± 4.70	-7.25	± 4.71
	(2)		10.76	± 4.13	20.02	± 5.05	-9.26	± 5.52
	(3)		8.60	± 2.78	15.28	± 3.87	-6.68	± 4.03
2nd Dose level, 16 h	(13)	1500.00	12.18	± 4.67	22.48	± 6.65	-10.30	± 5.81
	(14)		11.18	± 4.03	20.82	± 4.85	-9.64	± 4.73
	(15)		11.06	± 4.47	16.67	± 4.86	-5.61	± 4.68

* Mean of 100 cells
** Positive control: 2-AAF

Percentage Distribution of the Nuclear Grain Counts

Table 3: Percentage Distribution of the Nuclear Grain Counts

Test article:

Treatment, Period (Animal no.)	Mean*	>0	>1	>5	>10	>20	>30	>40	>50	>60	>70	>80	>90	>100
Vehicle control, 16 h Aqua dest														
(5)	7.58	100	100	83.0	11.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(6)	12.39	100	100	91.0	64.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(7)	14.19	100	100	98.0	72.0	12.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pos. control**, 16 h 100.00 mg/kg														
(17)	37.25	100	100	100	100	97.0	69.0	34.0	13.0	3.0	1.0	0.0	0.0	0.0
(18)	97.51	100	100	100	100	100	100	100	100	91.0	79.0	59.0	47.0	42.0
(19)	103.57	100	100	100	100	100	100	100	100	100	96.0	84.0	66.0	47.0
1st Dose level, 16 h 150.00 mg/kg														
(9)	8.98	100	100	85.0	33.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(10)	11.97	100	100	96.0	63.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(11)	11.21	100	100	93.0	44.0	4.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd Dose level, 2h 1500.00 mg/kg														
(1)	9.63	100	100	88.0	30.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(2)	10.76	100	100	88.0	49.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(3)	8.60	100	100	83.0	28.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd Dose level, 16 h 1500.00 mg/kg														
(13)	12.18	100	100	95.0	67.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(14)	11.18	100	100	95.0	52.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(15)	11.06	100	100	85.0	59.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* Mean of 100 cells, absolute value of nuclear grains
** Vehicle Control: Aqua dest
*** Positive control: 2-AAF

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Table 4: Dose Groups

Test article:

Treatment	Period	Grains per nucleus		Grains per Cytoplasma area		Net grains per nucleus	
		Mean*	Standard deviation	Mean*	Standard deviation	Mean*	Standard deviation
Vehicle control Aqua dest	(16 h)	11.39	± 5.19	15.87	± 4.88	-4.49	± 4.87
Pos. control** 100.00 mg/kg	(16 h)	79.44	± 37.98	20.89	± 10.34	58.56	± 32.53
1st Dose level 150.00 mg/kg	(16 h)	10.72	± 4.54	17.24	± 5.86	-6.52	± 5.44
2nd Dose level 1500.00 mg/kg	(2 h)	9.66	± 3.66	17.39	± 4.96	-7.73	± 4.90
2nd Dose level 1500.00 mg/kg	(16h)	11.47	± 4.41	19.99	± 6.02	-8.52	± 5.49

Table 5: Percentage Distribution of the Nuclear Grain Counts

Test article:

Treatment/ Period	Mean*	>0	>1	>5	>10	>20	>30	>40	>50	>60	>70	>80	>90	>100
Vehicle control Aqua dest / 16 h	11.39	100	100	90.7	49.0	5.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pos. control** 100.00 mg/kg / 16 h	79.44	100	100	100	100	99.0	89.7	78.0	71.0	64.7	58.7	47.7	37.7	29.7
1st Dose level 150.00 mg/kg / 16 h	10.72	100	100	91.3	46.7	2.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd Dose level 1500.00 mg/kg / 2 h	9.66	100	100	86.3	35.7	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2nd Dose level 1500.00 mg/kg / 16 h	11.47	100	100	91.7	59.3	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* Mean of 3 animals, 100 cells each

** Positive control: 2-AAF

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RESULTS AND DISCUSSION

The test article was assessed in the in *vivo*/*in vitro* UDS assay for its potential to induce DNA repair (UDS) in the hepatocytes of rats with doses of 150 mg/kg b.w. (16h preparation interval) and 1500 mg/kg b.w. (2 h and 16h preparation interval).

The highest dose was estimated by a pre-experiment to be suitable. The animals expressed toxic reactions. After administration of a higher dose (2000 mg/kg b.w) one out of two animals died.

The viability of the hepatocytes was not substantially affected due to the *in vivo* pre-treatment with the test article at any of the treatment periods or dose groups. The interindividual variations obtained for the numbers and the viabilities of the isolated hepatocytes are in the range of our historical laboratory control (3).

No dose level of the test article revealed UDS induction in the hepatocytes of the treated animals as compared to the current vehicle controls. Neither the nuclear grains nor the resulting net grains were distinctly enhanced due to the *in vivo* treatment of the animals with the test article for 2 hours or 16 hours, respectively. Therefore, the net grain values obtained after treatment with the test article were consistently negative.

In addition, no substantial shift to higher values was obtained in the percentage distribution of the nuclear grain counts.

An appropriate reference mutagen (2-AAF, 100 mg/kg b.w.) was used as positive control. In vivo treatment with 2-AAF revealed distinct increases in the number of nuclear and net grain counts.

Conclusions

In conclusion, it can be stated that during the described study and under the experimental conditions reported, the test article did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats.

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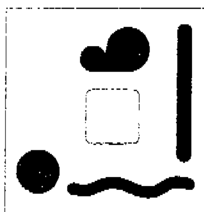

CCR PROJECT 508800

Project No.: 95/1/006

FIRST AMENDMENT TO REPORT

(3 pages)

TITLE: IN VIVO / IN VITRO UNSCHEDULED DNA SYNTHESIS IN RAT HEPATOCYTES WITH

SPONSOR:

STUDY MONITOR:

DATE OF AMENDMENT: NOVEMBER 06, 1997

RCC

Group

CONCERNING: **SUMMARY (page 9)**

PRESENT:

The test article Batch No. Pil 4 X) was assessed in the in *vivo/in vitro* UDS assay for its potential to induce DNA repair (UDS) in the hepatocytes of rats.

The test article was formulated in aqua dest. This suspending agent was used as vehicle control. The volume administered orally was 10 ml/kg body weight (b.w.). After a treatment period of 2 and 15 hours, respectively, the livers of narcotised animals were perfused to achieve hepatocytes suspensions. Primary hepatocyte cultures were established and exposed for 4 hours to ^{3}HTdR (methyl-^{3}H-thymidine) which is incorporated if UDS occurs (2).

...

N E W:

The test article (Batch No. Pil 4 X) was assessed in the in *vivo/in vitro* UDS assay for its potential to induce DNA repair (UDS) in the hepatocytes of rats.

The test article was formulated in aqua dest. This suspending agent was used as vehicle control. The volume administered orally was 10 ml/kg body weight (b.w.). After a treatment period of 2 and **16** hours, respectively, the livers of narcotised animals were perfused to achieve hepatocytes suspensions. Primary hepatocyte cultures were established and exposed for 4 hours to ^{3}HTdR (methyl-^{3}H-thymidine) which is incorporated if UDS occurs (2).

...

REASON FOR THE ALTERATION:

Typing error

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SPONSOR

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

ADME STUDY

PHARMACOKINETICS STUDY IN PLASMA, EXCRETION BALANCE AND TISSUE DISTRIBUTION AFTER SINGLE ADMINISTRATION BY ORAL ROUTE (GAVAGE) TO RATS

STUDY DIRECTOR
Terence Appelqvist

STUDY COMPLETION DATE
28 July 1999

PERFORMING LABORATORY
CIT
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LABORATORY STUDY NUMBER
16434 PAR

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STATEMENT OF THE STUDY DIRECTOR

The study was performed in compliance with the principles of Good Laboratory Practice as described in:

. O.E.C.D. principles of Good Laboratory Practice, Decision Concerning Mutual Acceptance of Data in the Assessment of Chemicals, C(81)30(final) Annex 2. 12 May 1981,

. Council Directive 87/18/E.E.C. of 18 December 1986 on the harmonization of laws, regulations or administrative provisions relating to the application of the Principles of Good Laboratory Practice and the verification of their applications for tests on chemical substances (O.J. n° L 15 of 17.1.87),

. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Journal Officiel du 9 mars 1990), Ministère de l'Industrie et de l'Aménagement du Territoire,

. US Food and Drug Administration, Good Laboratory Practice Regulations 21 CFR Part 58, December 22, 1978 (and subsequent amendments),

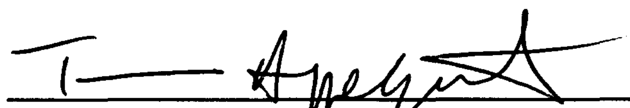
. Japanese Ministry of International Trade and Industry, Good Laboratory Practice Directive, March 31, 1984; Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85.

The study was conducted in compliance with the following Animal Health regulation:

. Council Directive No. 86/609/E.E.C. of 24th November 1986 on the harmonization of laws, regulations or administrative provisions relating to the protection of animals used for experimental or other scientific purposes.

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained during the performance of the study.

This study was performed at CIT, Centre International de Toxicologie, B.P. 563, Miserey, 27005 Evreux, France.



T. Appelqvist Date: 28 July 1999
Study Director
Bachelor of Pharmacy, Ph.D.
Head of Pharmacokinetics

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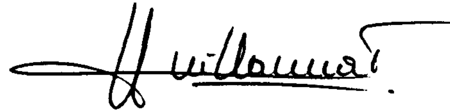
SCIENTISTS INVOLVED IN THE STUDY

Pharmacy



P.O. Guillaumat Date: 28 July 1999
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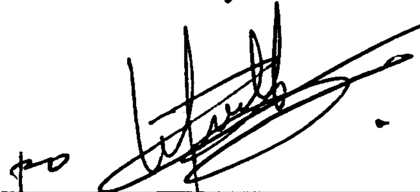
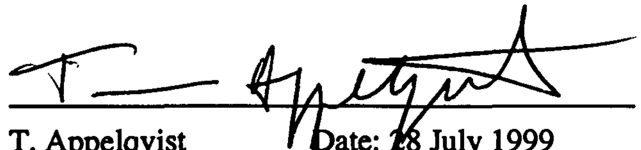


G. Fabreguettes Date: 28 July 1999
D.E.S.S. (Analytical Chemistry)

Pharmacokinetics



T. Appelqvist Date: 28 July 1999
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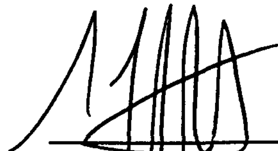
CIT Management



S. de Jouffrey Date: 28 July 1999
Doctor of Veterinary Medicine
Director of Operations

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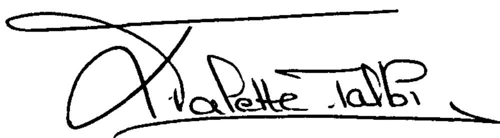
STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspection	Dates		
	Inspection	Reported to Study Director (*)	Reported to Management (*)
Protocol	3 March 1998	4 March 1998	9 March 1998
Study	5 November 1998	10 November 1998	11 November 1998
	12 November 1998	12 November 1998	13 November 1998
Report	21 April 1999	21 May 1999	4 June 1999

At about the same time as this study, described in the present report, process-based and routine facility inspections of critical procedures relevant to this study were made by the Quality Assurance Unit.
The findings of these inspections were reported to the Study Director and to CIT Management.

The inspections were performed in compliance with CIT Quality Assurance Unit procedures and the principles of Good Laboratory Practice.

The reported methods and procedures were found to describe those used and the results to constitute an accurate and complete reflection of the study raw data.



L. Valette-Talbi Date: 28 July 1999
Doctor of Biochemistry, Ph.D.
Head of Quality Assurance Unit
and Scientific Archives

(*) The dates indicated correspond to the dates of signature of audit reports by Study Director and Management.

SUMMARY

Objective

The objective of this study, performed at the request of the Sponsor, was to evaluate the plasma pharmacokinetics and excretion balance in urine and faeces of the test substance, ^{14}C after single administration by the oral route to rats.

Methods

Twenty-four Wistar Han rats (12 males and 12 females), weighing an average 177 g and 148 g for the males and females respectively (seven weeks old), were divided into two groups (numbered, 1 and 2) and each received a single administration of the radiolabelled test substance, ^{14}C (in water for injections) by oral gavage at the nominal dose-level of 100 mg/kg. The animals of group 1 (9 males and 9 females) were used for plasma pharmacokinetic investigations, whilst group 2 (3 males and 3 females) were placed individually in metabolism cages and used for excretion balance. A constant radioactive dose of 1.85 MBq/kg was used.

For group 1; blood samples were collected (from 3 rats/sex/time point), under isoflurane anaesthesia, predose, 1, 2, 4, 6, 8, 24, 48 and 72 hours post-gavage. After the final blood sampling, all animals were killed and stored frozen until discarded.

For group 2; weighed urine, faeces and cage-wash were collected predose, and then every 24 hours (except initial urine fractions, collected 0-6 and 6-24 hours) until 168 hours post-gavage. Thereafter, the animals were killed, weighed and various organs/tissues removed, weighed (including remaining carcass) and stored frozen, until discarded without analysis as the radioactivity was completely eliminated within the 168 hour period.

Blood samples were centrifuged to obtain plasma. Each plasma and excreta sample were analyzed for total radioactivity after the appropriate preparation. Pharmacokinetic modelling and mass balance calculations were performed.
During the study, the animals were observed for clinical signs, morbidity and mortality. Body weight was recorded pre-dosing and on the day of treatment.

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Results

Plasma pharmacokinetics (group 1)
Following oral administration of the isotopic mixture at a nominal 100 mg/kg, the mean (± standard deviation) plasma total radioactivity levels increased from time zero until the C_{max} (1558 ± 157 ng-eq/g for males and 1678 ± 540 ng-eq/g for females) at 1 (males) or 2 hours (females) post-dosing, and then decreased quickly until the last quantifiable time points at 6 (for males; 281 ± 15 ng-eq/g) or 8 hours (for females; 224 ± 53 ng-eq/g), post-gavage. The limit of quantification was >138 ng-eq/g.
The principal derived pharmacokinetic parameters (using non-compartmental methods) are shown below;

Sex	$t_{1/2z}$ (h)	AUC_{0-h} (ng-eq*h/g)	$AUC_{0-\infty}$ (ng-eq*h/g)
Males	1.964	4559	5355
Females	2.297	6674	7416

The results showed a low inter-animal variability; in addition, the calculated pharmacokinetic parameters were similar for both sexes.

Excretion balance (group 2)
Following oral gavage of the isotopic mixture at a nominal 100 mg/kg, the mean (± standard deviation) total cumulative excretion of the radioactive dose in the summed excreta over a 168 hour period was 98.3 ± 2.7 and 96.3 ± 3.4 % for the males and females, respectively. A mean 2.5 ± 0.3 % and 95.4 ± 2.5 % of the administered dose was eliminated respectively in the urine and faeces for the males; for the females the values were, respectively, 3.7 ± 0.3 % and 88.6 ± 9.3 %. For both sexes, less than 5 % of the dose was in the cage-wash. Most radioactivity (90.7 and 72.9 % of the dose for males and females, respectively) was eliminated rapidly in the summed urine and faeces within 24 hours following gavage; of this radioactivity, >95 % was in the faeces. The results were generally homogenous for all animals/sexes.

Absorption of radioactivity following oral dosing
The minimal fraction of the oral dose absorbed was in the ranges 2.2 to 2.7 % (males) and 3.3 to 4.0 % (females), as estimated from the individual cumulative urinary excretion data after gavage dosing.

Mortality and clinical signs
No test substance related mortality, morbidity or clinical signs were observed.

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Conclusion

The plasma pharmacokinetics and elimination of radioactivity in excreta was investigated following oral administration of ^{14}C at 100 mg/kg, to male and female rats. This study showed that;

- following oral gavage, the plasma radioactivity levels increased quickly to the C_{max} (1558 ng-eq/g for males and 1678 ng-eq/g for females, at 1 or 2 hours, respectively) and then fell gradually to last quantifiable time points at 6 (males; 281 ng-eq/g) or 8 hours (females; 224 ng-eq/g),
- the calculated $t_{1/2z}$, AUC_{0-h} and $AUC_{0-\infty}$ were 1.964 h, 4559 ng-eq*h/g and 5355 ng-eq*h/g, respectively, for the males, and 2.297 h, 6674 ng-eq*h/g and 7416 ng-eq*h/g, respectively, for the females,
- following oral gavage, the radioactive dose was quickly and completely eliminated (mainly in the faeces); specifically, the mean cumulative excretion of the dose in a 168 hour period was 98.3 % for the males and 96.3 % for the females; of this recovered dose, >70.0 % was eliminated within 24 hours in faeces; the total excretion values for urine and faeces were 2.5 and 95.4 %, respectively, for males (urine/faeces ratio of 0.03) and 3.7 and 88.6 %, respectively, for females (urine/faeces ratio of 0.04),
- minimal fraction of oral dose absorbed was low (estimated from the urine elimination data to be in the range 2.2 to 4.0 %),
- the pharmacokinetic and excretion results were homogenous for the individual animals per sex, and no gender differences were apparent.

No metabolism assays were performed as the techniques for determination of metabolites were not available.

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1. INTRODUCTION

1.1. OBJECTIVE

This study was performed at the request of the Sponsor.
The objective of this study was to evaluate the pharmacokinetics in plasma, excretion balance in urine and faeces, and possible tissue distribution of the test substance, ^{14}C
after single administration by the oral route (gavage) to rats.

is a dye used in hair products.

The rat was chosen because it is a rodent species commonly requested by international regulations for this type of study and the same species was used in toxicity studies.

The dose-level was chosen by the Sponsor.

The oral route was used since it is expected to ensure an absorption at least equal to the cutaneous route which is the route of exposure in humans.

2. EXPERIMENTAL CONDITIONS

2.1. TEST SUBSTANCES AND VEHICLE

2.1.1 Identification

2.1.1.1 Radiolabelled test substance

The radiolabelled test substance, ^{14}C was supplied by Isotopchim, France.

The documentation supplied identified the radiolabelled test substance as follows:
. denomination:
. - protocol: 14C [U-Ring-14C], as synonym
. - labelling: [U-Ring-14C]
. batch number:
. - protocol and labelling: 98218A
. description: grey powder
. quantity and container: two glass vials, each containing 74 MBq
. date of receipt: 21 August 1998
. specific activity: 23.4 mCi/mmol corresponding to 1.708 MBq/mg
. storage conditions: at -20 °C, away from light and under nitrogen.

An analytical certificate provided by Isotopchim is presented in Appendix 1.

2.1.1.2 Non radiolabelled test substance

The non radiolabelled test substance, was supplied by the Sponsor.

The documentation supplied identified the non radiolabelled test substance as follows:
. denomination:
  - protocol and labelling:
. batch number:
  - protocol and labelling: 0500591
. description: grey powder
. quantity and container: one smoked glass vial containing 10 g
. date of receipt: 19 October 1998
. storage conditions: at +4 °C, away from light and under argon atmosphere
. expiry date: October 1999.

An analytical certificate provided by the Sponsor is presented in Appendix 1.

2.1.2 Vehicle

The vehicle was water for injections, batch No. 9171, supplied by Fresenius (Sèvres, France).

2.1.3 Formulation procedure

Before the day of dosing, the contents of one vial containing 2 mCi of isotopic powder (equivalent to a total of 43.33 mg of test substance) were transferred stepwise, using appoximately 5 ml ethanol (batch No. VRG080228G, supplied by Carlo Erba, Rueil-Malmaison, France), to a gauged (5 ml) preparation flask, to obtain an ethanolic solution of 0.4 mCi/ml (8.665 mg/ml).
After mixing, 1.216 ml (0.486 mCi, 10.54 mg) of the obtained solution was transferred to a gauged flask (48.65 ml) and the solvent was evaporated under a nitrogen gas stream. The dried residue was stored at -20 °C, protected from light and humidity, until the day of treatment.

On the day of dosing, the non-radiollabelled test substance (962 mg) was added to the dried residue. Subsequently, the vehicle was added to 48.65 ml to achieve the target concentration of 20 mg/ml.
The formulation was kept under magnetic stirring and protected from light until delivery.

2.1.4 Chemical analysis of the radiolabelled test substance and the administered formulation

2.1.4.1 Determination of total radioactivity and radiochemical purity of the radiolabelled test substance

Before the treatment, the radiolabelled test substance solution was analyzed in triplicate by liquid scintillation counting to determine the total radioactivity (results expressed as MBq/ml).
In addition, the radiolabelled test substance solution was analyzed (in duplicate) by HPLC/UV/on line radioactivity detection to determine the radiochemical purity (results expressed as %) using a basic method provided by the supplier of the radiolabelled compound.

2.1.4.2 Total radioactivity of the administered formulation

On the day of treatment, three samples (weighed accurately) were taken from the formulation and analyzed by liquid scintillation counting to determine the total radioactivity.
After treatment, the same analysis of radioactivity was undertaken (in triplicate) on an aliquot of the formulation which had passed through the same administration device as that used for the treatment of the animals.
The results are expressed as Bq/g of preparation.

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2.1.4.3 Radiochemical purity of the administered formulation

On the day of treatment, the formulation was analyzed (in duplicate) by HPLC/UV/on line radioactivity detection to determine the radiochemical purity of the isotopic mixture (results expressed as %).

Full details of the analytical procedures used are presented in Appendix 2.

2.2. MATERIALS AND METHODS

2.2.1 Animals

A total of 28 Wistar Han rats (14 males and 14 females) of the Ico: WI (IOPS AF/Han) strain were supplied by Iffa Crédo, l'Arbresle, France and received at CIT on 29 October 1998.
On arrival, the animals were given a clinical examination to ensure they were in good condition. A higher number of animals than necessary was acclimatized in order to permit the selection and/or replacement of individuals.
A seven day acclimatization period to the conditions of the study preceded the day of treatment. The required number of animals (12 males and 12 females) was selected according to body weight and clinical condition and allocated by sex, to groups, according to a computerized randomization procedure.
Each animal was identified by an individual ear tattoo. At the beginning of the study, each animal received a unique CIT identity number.
On the day of treatment, the animals were approximately seven weeks old and had a mean body weight of 177 g for the males (range: 161 g to 184 g) and 148 g for the females (range: 132 g to 158 g).

2.2.2 Environmental conditions

From arrival at CIT, the animals were housed in a barriered rodent unit.
The animal room conditions were set as follows:

. temperature : 21± 2 °C
. relative humidity : 50 ± 20%
. light/dark cycle : 12h/12h (07:00 - 19:00)
. ventilation : approximately 12 cycles/hour of filtered, non-recycled air.

The corresponding instrumentation and equipment are checked and calibrated at regular intervals. The temperature and relative humidity were recorded continuously and the records were checked daily and filed.
The animal room was disinfected before the arrival of the animals and cleaned regularly thereafter. Microbiological analyses of the air and the surfaces of the walls and floor of the rodent unit are performed regularly by an external laboratory and the results archived at CIT.

2.2.3 Housing

The animals for plasma pharmacokinetics (group 1) were housed in threes in suspended wire-mesh cages (43.0 x 21.5 x 18.0 cm). A metal tray containing autoclaved sawdust (SICSA, Alfortville, France) was placed under each cage.
The animals for the excretion balance (group 2) were housed individually in metabolism cages.

Cages were not randomized in the room but placed in numerical order, vertically (normal cages) or horizontally (metabolism cages) on the racks.

2.2.4 Food and water

All animals had free access to A04 C powdered maintenance diet, batch Nos. 80609 and 80729 (UAR, Villemoisson-sur-Orge, France), distributed daily and tap water (filtered using a 0.22 micron filter) contained in bottles. The rats were fasted overnight (food only) before dosing; the food was given about four hours after dosing on the day of treatment.
Each batch of diet was analyzed by the supplier for composition and contaminant levels. The diet formula is presented in Appendix 10.

2.2.5 Contaminants analyses

Bacterial and chemical analyses of the sawdust, diet and water are performed periodically by external laboratories. These analyses include the detection of possible contaminants (sawdust: pesticides and heavy metals; diet and water: pesticides, heavy metals and nitrosamines).
No contaminants were known to have been present in the diet, drinking water or sawdust at levels which may be expected to have interfered with or prejudiced the outcome of the study.

2.3. TREATMENT

2.3.1 Dose-levels and groups

The dose-level was selected on the basis of acute oral toxicity studies performed at CIT in which no mortality was observed at 100 mg/kg.
The animals were divided into two groups and treated orally with the test substance as indicated below:

Group	Animals per group	Purpose	Dose-level mg/kg	Radioactive dose MBq/kg	Animal numbers
1	9 males 9 females	Plasma pharmacokinetics	100	1.85	U20511 to U20519 U20541 to U20549
2	3 males 3 females	Excretion balance	100	1.85	U20520 to U20522 U20550 to U20552

2.3.2 Administration

The oral route was used since it is expected to ensure an absorption at least equal to the cutaneous route which is the route of exposure in humans.
Before dosing, the animals were deprived of food for an overnight period. The food was reoffered about four hours after dosing.
The test formulation was administered by gavage using a plastic syringe fitted with a metal probe. The same metal probe was used for all animals.
For each animal, the syringe with metal probe was weighed before and after administration in order to determine precisely the quantity of isotopic mixture administered.
Before use, the syringe plus probe was pre-saturated with an aliquot of the formulation which was not administered to the animals.
The formulation was stirred continuously pending filling of the administration device.
A constant dosage-volume of 5 ml/kg was used.

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2.4. BLOOD SAMPLINGS (group 1)

Blood samples were taken as follows:
. predose and 1, 2, 4, 6, 8, 24, 48 and 72 hours after administration.
Three animals/sex were sampled at each time point, and each animal was sampled three times in total, using the following scheme:
. first 3 animals/sex: predose, 2 hours and 24 hours post-dosing,
. second 3 animals/sex: 1 hour, 6 hours and 48 hours post-dosing,
. remaining 3 animals/sex: 4 hours, 8 hours and 72 hours post-dosing.
Blood samples (1 ml at the first and second sampling per animal, and maximal volume at the third sampling) were taken into tubes containing lithium heparinate, from the orbital sinus of the animals. For the blood sampling, the animals were lightly anaesthetized by isoflurane. The blood samples were kept on ice (+4 °C).
Specifically for female U20541 (assigned to sampling set 1), which was found dead at anaesthesia for the two hour sampling, the second blood sample was taken from the aorta. Subsequently, this rat was replaced by U20547 (from sampling set 3; therefore, four blood samples were taken from this animal) for the scheduled 24 hour blood sampling.
Plasma was obtained by centrifugation of the blood (4000 rpm for 10 min at +4 °C).
The plasma was stored at -20 °C until analyzed for radioactivity at CIT.
All surviving animals were killed after the last collection (see § 2.9. Pathology: sacrifice). Thereafter, the carcasses were stored frozen at -20 °C at CIT until discarded with the agreement of the Sponsor.

2.5. EXCRETION BALANCE (group 2)

Urine, faeces and cage wash were collected at the following times:
. during the 24 hour period before dosing (urine and faeces) and then during the period 0-6h, 6-24h (urine) or 0-24h (faeces), 24-48h, 48-72h, 72-96h, 96-120h, 120-144h and 144-168h (urine and faeces) after dosing,
. after each collection of faeces the cages and cones and trays were carefully rinsed with approximately 20 ml of water (except at 168h when 200 ml were used).
The urine and faeces were collected in tubes at room temperature.
All the samples collected were weighed in tared bottles and then frozen at -20 °C (except cage wash water, which were kept at room temperature).
All animals were killed after the last collection (see § 2.9. Pathology: sacrifice) and their carcasses weighed.
Thereafter, a skin sample, the brain, heart, kidneys, liver, lungs, spleen, stomach, small intestine, large intestine, pancreas, yellow fat sample, skeletal muscle sample and bone sample were dissected out, weighed and stored frozen at -20 °C at CIT pending possible radioactivity analysis. The remaining carcasses were reweighed and then stored frozen at -20 °C at CIT pending possible radioactivity analysis. In view of the excretion balance results, all tissue/organ/carcass samples were discarded without analysis after the agreement of the Sponsor.

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2.6. RADIOACTIVITY DETERMINATION (LIQUID SCINTILLATION COUNTING)

2.6.1 Sample preparation

Test formulations

A small aliquot was weighed into a counting vial and counted after addition of Ready-safe scintillation solution.
Three samples per test preparation were counted.

Plasma (group 1)

An aliquot of each sample was weighed into a counting vial and counted after addition of Ready-safe Scintillation solution.
The sample preparation was performed in duplicate.

Urine, cage wash (group 2)

An aliquot of each urine and cage-wash sample was weighed into a counting vial and counted after addition of Ready-Safe or Ready-Gel scintillation solution, respectively.
The sample preparation was performed in duplicate.

Faeces (group 2)

Each sample was placed in a suitable tared container and ethanol added. The contents of the container was weighed. The sample was then homogenized using an ultra turrax.
About 0.3 g of the resultant tissue homogenate was oxidized using an Model 307 oxidizer (Packard). The radioactive gases produced were collected in a mixture of nine volumes Carbosorb and nine volumes Permafluor, and analyzed by LSC.
The sample oxidation was performed in duplicate, starting from a single homogenate for each faeces sample.

Carcasses, tissues and organs (group 2)

No analysis was performed as all radioactivity was eliminated in the excreta.

2.6.2 Analytical apparatus and counting method

A Beckman LS 6000 TA counter was used.
An external standard source (Cesium 137) was used to monitor sample quenching; the dpm values of each sample were obtained from the corresponding cpm values using a quench standard curve.
All biological samples were counted for 10 minutes.
Each biological sample was counted in duplicate and the mean dpm value was used to calculate the corresponding radioactivity concentration (as Bq/g, ng-eq/g or Bq/sample).

Full details of the analytical procedures used are shown in Appendices 2 and 3.

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2.7. PHARMACOKINETIC ANALYSIS

Pharmacokinetic analysis was performed via a non-compartmental approach, using Topfit computer software (version 2.0, Dr. Karl Thomae GmbH, Schering AG and Gödecke AG, 1993);

. terminal rate constant (λ_z) and half-life ($t_{1/2z}$) were determined by log-linear regression of the terminal slope of the plasma concentration versus time profiles,
. maximum plasma concentration (C_{max}) and corresponding time point (t_{max}) were determined directly from the data,
. area under the data curve (AUC_{0-h}) was calculated using the linear trapezoidal rule and was extrapolated to infinity ($AUC_{0-\infty}$) using the calculated $t_{1/2z}$.

The minimal fraction of oral dose absorbed was estimated using urine data and was considered to be equal to the total urinary cumulative excretion of radioactivity during the study period after gavage.

2.8. CLINICAL EXAMINATIONS

2.8.1 Clinical signs

Each animal was observed at least once a day, at approximately the same time, for the recording of clinical signs.

2.8.2 Morbidity and mortality

Each animal was checked at least twice a day (including during weekends) for mortality or signs of morbidity.

2.8.3 Body weight

Body weight was recorded for each animal once before allocation of the animals to groups, on the day of dosing and after killing.

2.9. PATHOLOGY: sacrifice

After the last excreta collection or blood sampling, the surviving animals were killed by cervical dislocation under excess isoflurane anesthesia and weighed (group 2, only).
A macroscopic *post-mortem* examination was not performed on any of the study animals.
The carcasses of the animals (after removal of the specified organs where relevant; see § 2.5. Excretion balance and distribution), were stored frozen at -20 °C (with the organs, where relevant), until disposal without radioactivity analysis.

2.10. ARCHIVING

The study documentation and specimens generated during the course of the study are archived at CIT (Evreux, France) for five years after the end of the *in vivo* phase of the study.
The archived study materials include:
. protocol and amendment,
. raw data,
. correspondence,
. final report and possible amendments,

On completion of this period, the archived study materials will be returned to the Sponsor, or may be archived at CIT for a further period.

2.11. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedures		Dates	Study days
Protocol approved by:			
. Study Director		4 March 1998	
. Study Monitor		9 March 1998	
Arrival of the animals		29 October 1998	-7
. Weighing		29 October 1998	-7
. Preidentification		30 October 1998	-6
. Randomization and identification		2 November 1998	-3
. Pre-treatment urine, faeces and cage wash samples (group 2)		3 November 1998	-2
Day of dosing		5 November 1998	1
. Blood sampling (group 1)		5 to 8 November 1998	1 to 4
. Urine, faeces and cage wash samples (group 2)		5 to 12 November 1998	1 to 8
Sacrifice:	**group 1**	6 to 8 November 1998	2 to 4
	group 2	12 November 1998	8

2.12. PROTOCOL ADHERENCE

The study was performed in accordance with Study Protocol No. 16434 PAR and subsequent amendment, with the following deviations from the agreed Study Protocol:

. plastic (and not glass) syringes were used for the treatment,
. powdered (and not pelleted diet) was given to the study animals,
. as female U20541 died at anaesthesia for the two hour sampling, the blood sample was taken from the aorta; for the 24 hour blood sampling, the dead female was replaced by U20547 (which was effectively sampled four times),
. the study start and end days were both delayed by one day from those specified in amendment 1,
. body weights of carcasses from group 1 were not retained in the report,
. purity of the radiolabelled test substance was analysed (in common with Study 16432 PAR) on 26 October 1998 and not the end of treatment,
. volumes of urine and cage-wash analysed were in the range 0.2 to 5 ml (and not 0.5 to 1 ml), and cage-wash was analysed using Ready-Gel (and not Ready-Safe) scintillant,
. the radiolabelled test substance was dissolved in ethanol for the purposes of quantifying and transfer to the preparation flask, and then evaporated to dryness under nitrogen gas stream,
. metabolism cages were placed horizontally (and not vertically) in the racks,
. food was distributed daily (and not weekly).

These minor deviations were not considered to have compromised the validity or integrity of the study.

3. RESULTS

3.1. CHEMICAL ANALYSIS OF THE THE RADIOLABELLED TEST SUBSTANCE AND THE ADMINISTERED FORMULATION

3.1.1 Determination of total radioactivity and radiochemical purity of the radiolabelled test substance

The total radioactivity of the radiolabelled test substance solution was 12 % less than the expected value. This was not considered to have compromised the integrity of the study.
The results of the analyses demonstrated a satisfactory radiochemical purity of the radiolabelled test substance i.e. 98.1 %.

3.1.2 Total radioactivity of the administered formulation

The total radioactivity of the formulation administered was found to be lower than expected; the deviations from the nominal radioactivity were -19.4 % (before the treatment) and -19.2 % (after passing through the same administration device as that used for the treatment of the animals). This was not considered to have compromised the integrity of the study.

3.1.3 Radiochemical purity of the administered formulation

The results of the analyses demonstrated a satisfactory radiochemical purity of formulation administered since the mean value obtained was 99.0 %.

Detailed results are presented in Appendix 2.

3.2. ADMINISTRATION SYSTEM WEIGHTS AND RADIOACTIVE DOSES

The weights of the test substance preparations, and the calculated doses both as total radioactivity (Bq) and mg/kg (using theoretical concentration), given to the animals of each group are shown in Appendix 2. There was a good agreement between the theoretical and actual dose-levels achieved, as the maximum deviation observed was +3 % for animals U20511 (male) and U20549 (female) of group 1 (pharmacokinetics).

3.3. PLASMA PHARMACOKINETICS (Tables 1, Figure 1, Appendices 4 and 5)

Following oral gavage of the isotopic mixture at a nominal 100 mg/kg, the mean (± standard deviation) plasma total radioactivity levels increased quickly from time zero to the C_{max} (1558 ± 157 ng-eq/g for males, 1678 ± 540 ng-eq/g for females and 1569 ± 104 ng-eq/g for both sexes) at 1 (males and both sexes) or 2 hours (females), post-dosing. Subsequently, the plasma radioactivity levels decreased quickly until the last quantifiable time points at 6 (281 ± 15 ng-eq for males) or 8 hours (224 ± 53 and 205 ± 58 ng-eq/g for females and both sexes, respectively), post-gavage. The limit of quantification for the test substance in plasma was >138 ng-eq/g.

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The main pharmacokinetic parameters (calculated using non-compartmental methods, where relevant) for the animals are summarized below;

Sex	$t_{1/2z.}$ (h)	$\lambda_{z.}$ (1/h)	$C_{max.}$ (ng-eq/g)	$t_{max.}$ (h)	$AUC_{0-h.}$ (ng-eq*h/g)	$AUC_{0-\infty.}$ (ng-eq*h/g)
Males	1.964	0.3529	1558	1	4559	5355
Females	2.297	0.3018	1678	2	6674	7416
Both sexes	2.498	0.2775	1569	1	5851	6589

The respective plasma concentration versus time profiles and calculated pharmacokinetic parameters were similar for both sexes. The results showed a low inter-animal variability.

3.4. EXCRETION BALANCE (Table 2, Figures 2 to 4, Appendices 6 and 7)

Following oral gavage of the isotopic mixture of the test substance at the nominal dose-level of 100 mg/kg, the mean (± standard deviation) total cumulative excretion of the radioactive dose over a 168 hour period in urine, faeces and cage wash was 98.3 ± 2.7 % for the males, 96.3 ± 3.4 % in the females and 97.3 ± 3.0 % for all animals. The radioactivity was eliminated principally in the faeces; specifically, a mean 95.4 ± 2.5 %, 88.6 ± 9.3 % and 92.0 ± 7.2 % of the dose was found in the faeces over the study period for the males, females and both sexes, respectively, whereas for urine the values were, respectively, 2.5 ± 0.3 %, 3.7 ± 0.3 % and 3.1 ± 0.7 %. The orally given radioactivity was eliminated rapidly, as an average 90.7 % (males), 95.2 % (females) and 81.7 % (both sexes) of the dose (in summed urine and faeces) was eliminated within 24 hours following dosing; 97.3 %, 86.3 % and 96.4 % of this recovered radioactivity was, respectively, in the faeces of the males, females and both sexes. Over the 168 hour period, 0.35 ± 0.10 % (males), 4.09 ± 5.94 % (females) and 2.22 ± 4.28 % (all animals) of the dose was found in the cage wash. For female U20550, 10.2 % of the dose was found in the final (large volume) cage wash fraction; in view of the results obtained for the other study animals, this radioactivity was considered to have originated from slight regurgitation of stomach contents following dosing, resulting in contamination of the higher levels of the cage which were only washed extensively during the last wash fraction.

The excretion profiles were homogeneous for the individual animals and both sexes. In view of the complete mass balance obtained for the excretion data, no carcasses and/or tissues were analyzed for radioactivity.

The summarised total cumulated excretion (CE) data (over the 168 hour period) (means ± standard deviation), expressed as percentage of the administered dose (%), are shown in the table below;

Route and dose	Animal number and sex	CE in urine (%)	CE in faeces (%)	CE in cage wash (%)	Total CE (%)
Oral gavage, 100 mg/kg	3, males	2.5 ± 0.3	95.4 ± 2.5	0.35 ± 0.10	98.3 ± 2.7
	3, females	3.7 ± 0.3	88.6 ± 9.3	4.09 ± 5.94	96.3 ± 3.4
	6, all animals	3.1 ± 0.7	92.0 ± 7.2	2.22 ± 4.28	97.3 ± 3.0

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Minimal fraction of oral dose absorbed

The minimal fraction of radioactivity absorbed following oral dosing was estimated from urine total cumulative excretion data to be in the range 2.2 to 2.7 % (mean, 2.5 %) for males and 3.3 to 4.0 % (mean, 3.7 %) for females.

3.5. CLINICAL SIGNS, MORBIDITY AND MORTALITY (Appendix 8)

During the study, one animal (female, U20541) in the plasma pharmacokinetics group (group 1) died after anaesthesia (for blood sampling purposes) without any prior clinical signs. Hence, as this death was an isolated incident, it was considered to be procedure (and not test substance) related (though no macroscopic *post-mortem* examination was performed). Overall, no test substance related mortality, morbidity or clinical signs were observed.

3.6. BODY WEIGHT (Appendix 9)

On the day of treatment, the males and females of the plasma pharmacokinetics group 1 weighed a mean (± standard deviation) 175 ± 7.3 g and 147 ± 9.8 g, respectively. For group 2, excretion balance, the mean weights were 181 ± 2.1 g for the males and 150 ± 5.5 g for the females.

3.7. PATHOLOGY

In the absence of observed morbidity, at the end of the scheduled biological sampling periods, all surviving study animals were killed. The carcasses from the plasma pharmacokinetic animals (group 1) were stored frozen until discarded without radiochemical analysis. The excretion balance animals (group 2) were dissected/processed as described in the protocol, and the various samples and carcasses were stored frozen. In the presence of a complete elimination of radioactivity during the study period, all the latter samples were discarded without radioactivity analysis.

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4. CONCLUSION

The plasma pharmacokinetics and elimination of radioactivity in excreta was investigated following oral administration of ^{14}C at 100 mg/kg, to male and female rats. This study showed that;

. following oral gavage, the plasma radioactivity levels increased quickly to the C_{max} (1558 ng-eq/g for males and 1678 ng-eq/g for females, at 1 or 2 hours, respectively) and then fell gradually to last quantifiable time points at 6 (males; 281 ng-eq/g) or 8 hours (females; 224 ng-eq/g),

. the calculated $t_{1/2}$, AUC_{0-h} and $AUC_{0-\infty}$ were 1.964 h, 4559 ng-eq*h/g and 5355 ng-eq*h/g, respectively, for the males, and 2.297 h, 6674 ng-eq*h/g and 7416 ng-eq*h/g, respectively, for the females,

. following oral gavage, the radioactive dose was quickly and completely eliminated (mainly in the faeces); specifically, the mean cumulative excretion of the dose in a 168 hour period was 98.3 % for the males and 96.3 % for the females; of this recovered dose, >70.0 % was eliminated within 24 hours in faeces; the total excretion values for urine and faeces were 2.5 and 95.4 %, respectively, for males (urine/faeces ratio of 0.03) and 3.7 and 88.6 %, respectively, for females (urine/faeces ratio of 0.04),

. minimal fraction of oral dose absorbed was low (estimated from the urine elimination data to be in the range 2.2 to 4.0 %),

. the pharmacokinetic and excretion results were homogenous for the individual animals per sex, and no gender differences were apparent.

Table 1

DETERMINATION OF THE TOTAL RADIOACTIVITY IN PLASMA (ng-eq/g) FOLLOWING A SINGLE ORAL ADMINISTRATION (100 mg/kg) OF ^{14}C TO WISTAR HAN RATS

Animal	Sampling times (h)								
	0	1	2	4	6	8	24	48	72
U20511	BLQ		1296				BLQ		
U20512	BLQ		1113				BLQ		
U20513	BLQ		1050				BLQ		
U20514		1628			291			BLQ	
U20515		1379			289			BLQ	
U20516		1668			263			BLQ	
U20517				359		149			BLQ
U20518				575		BLQ			BLQ
U20519				550		BLQ			BLQ
Male mean	**BLQ**	**1558**	**1153**	**495**	**281**	**BLQ**	**BLQ**	**BLQ**	**BLQ**
SD	[1]	157	127	118	15	[1]	[1]	[1]	[1]
U20541	BLQ		2239				[-]		
U20542	BLQ		1162				BLQ		
U20543	BLQ		1632				BLQ		
U20544		1553			386			BLQ	
U20545		1634			488			BLQ	
U20546		1550			411			BLQ	
U20547				491		175	BLQ		BLQ
U20548				711		217			BLQ
U20549				1046		281			BLQ
Female mean	**BLQ**	**1579**	**1678**	**749**	**428**	**224**	**BLQ**	**BLQ**	**BLQ**
SD	[1]	47	540	280	53	53	[1]	[1]	[1]
Overall mean	**BLQ**	**1569**	**1415**	**622**	**355**	**205**	**BLQ**	**BLQ**	**BLQ**
SD	[1]	104	454	237	88	58	[1]	[1]	[1]

BLQ: Below limit of quantification (< 138 ng-eq/g)
[1] Not calculated
[-]: animal dead after sampling 2 hours post-dosing and replaced by female U20547

Table 2

TOTAL CUMULATIVE EXCRETION OF RADIOACTIVITY OVER A 168-HOUR PERIOD (as % of dose) FOLLOWING A SINGLE ORAL ADMINISTRATION (100 mg/kg) OF ^{14}C TO WISTAR HAN RATS

Animal Sex	Animal Identification	Urine %	Faeces %	Cage wash liquid %	Cumulative excretion %
Male	U20520	2.2	92.7	0.46	95.3
	U20521	2.7	97.7	0.27	100.7
	U20522	2.6	96.0	0.31	98.8
Female	U20550	3.7	77.8	10.9	92.5
	U20551	4.0	94.8	0.23	99.0
	U20552	3.3	93.1	1.11	97.5
Male mean		2.5	95.4	0.35	98.3
SD		0.3	2.5	0.10	2.7
Female mean		3.7	88.6	4.09	96.3
SD		0.3	9.3	5.94	3.4
Overall mean		3.1	92.0	2.22	97.3
SD		0.7	7.2	4.28	3.0

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Figure 1

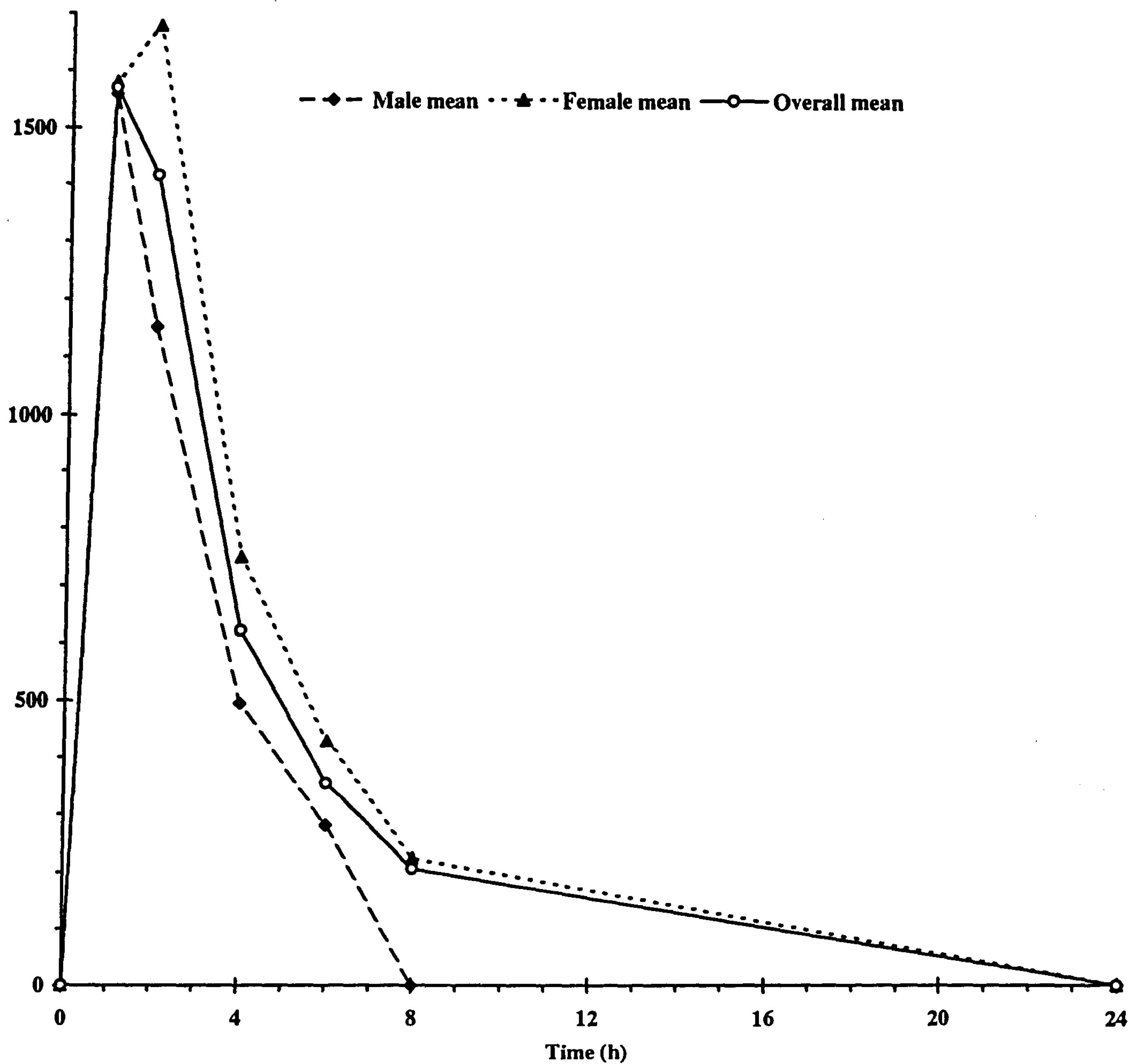

Figure 2

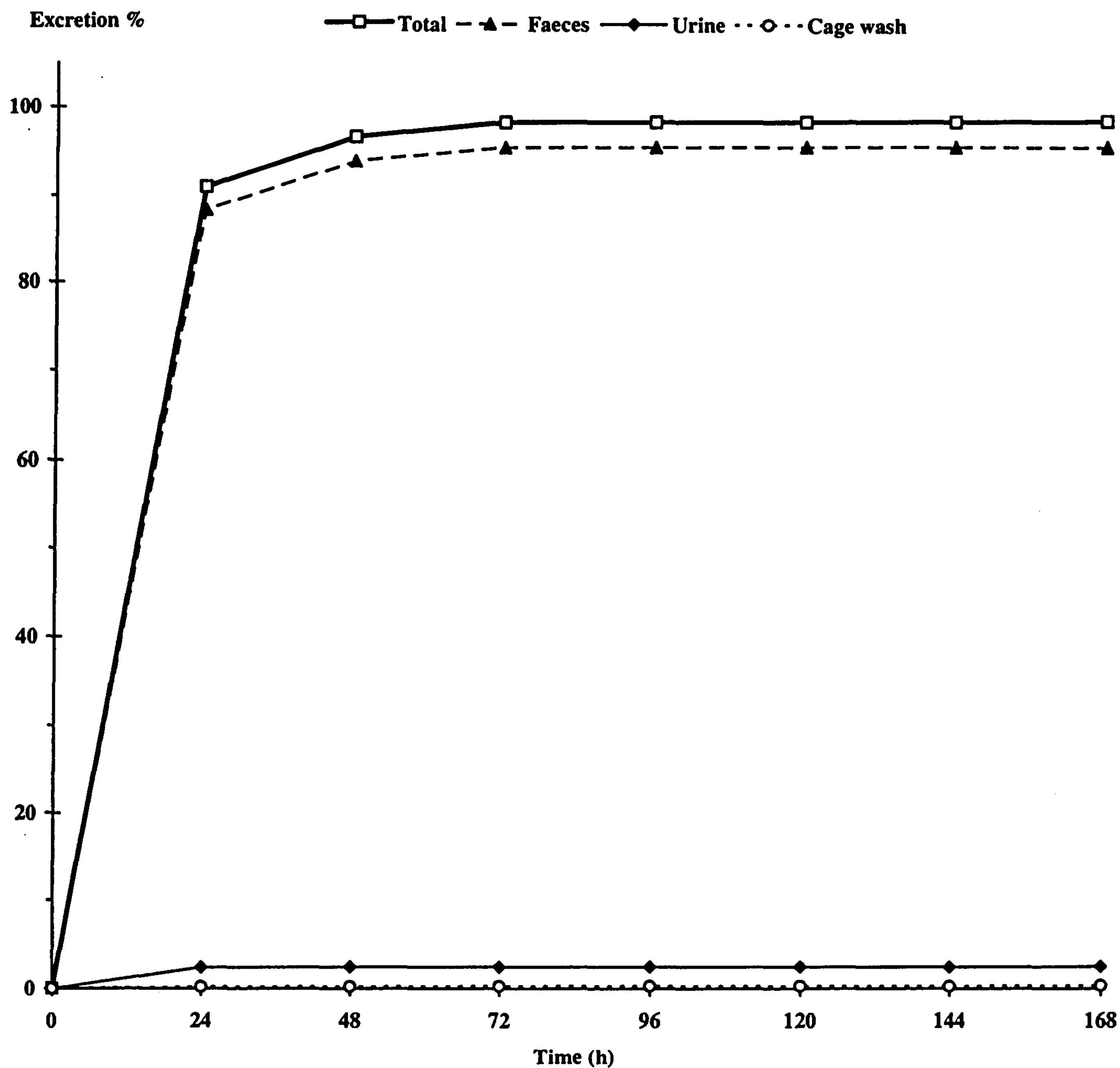

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Figure 3

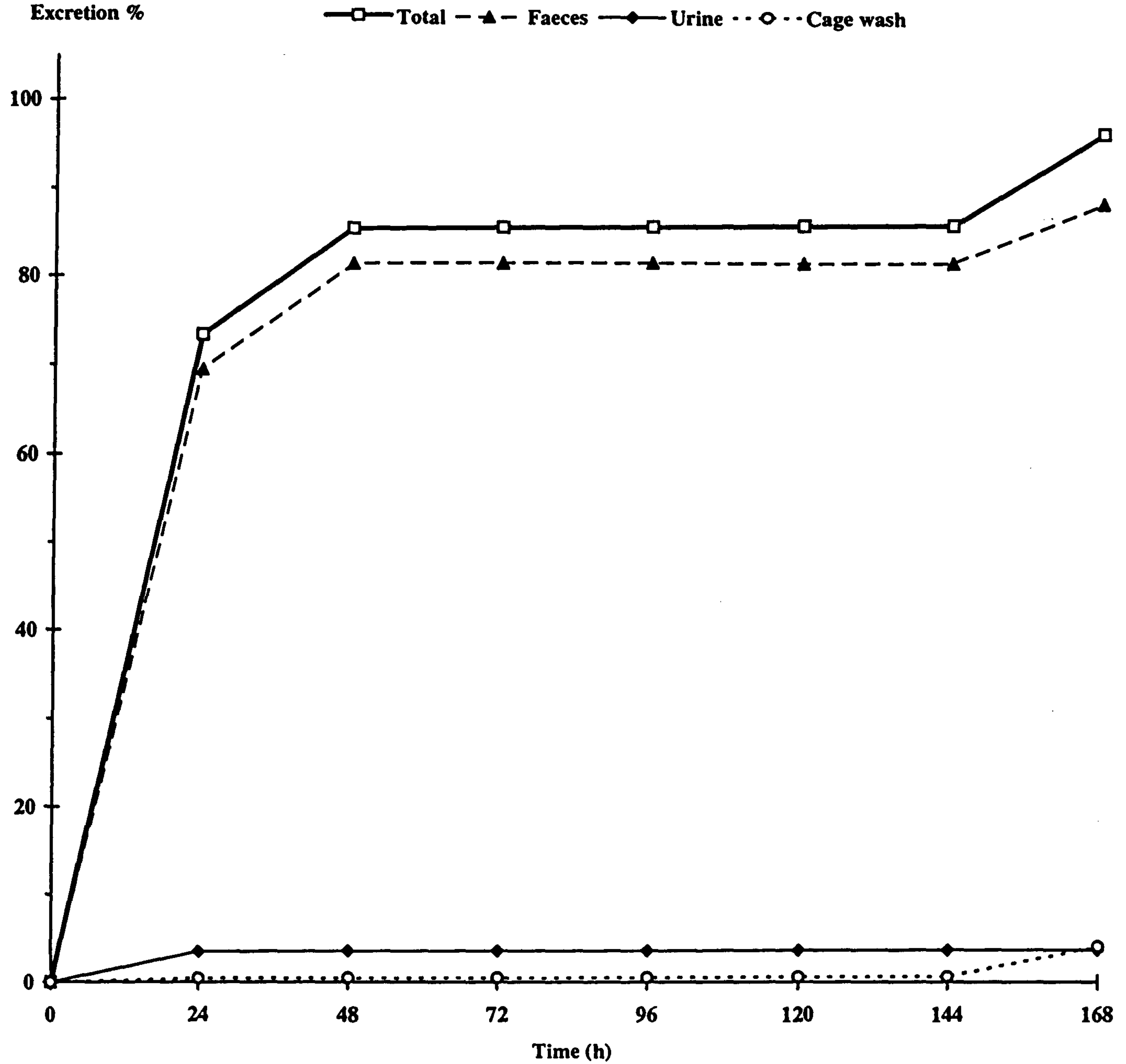

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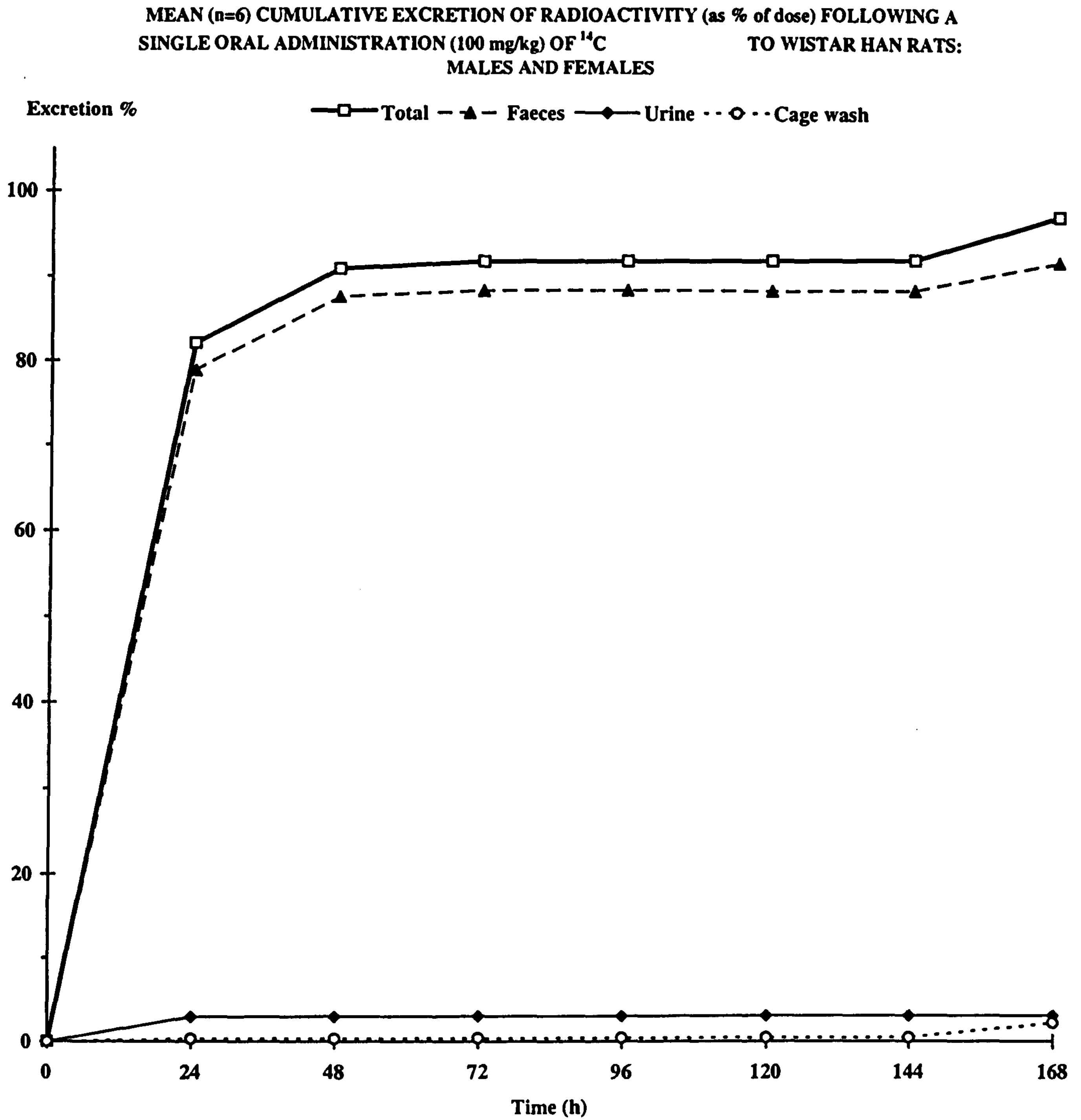
Figure 4



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AMENDMENT TO PROTOCOL

STUDY No.: **16434 PAR**

SPONSOR:

TITLE: ^{14}C **Pharmacokinetics study in plasma, excretion balance and tissue distribution after single administration by oral route (gavage) to rats.**

AMENDMENT No.: 1 Page 1/2

Justification: To amend protocol, add expiry date and synonym for test substance and supply information not known at protocol finalisation.

Date of application: 2 November 1998.

2. TEST AND CONTROL SUBSTANCES

2.1.1 Radiolabelled test substance

To add synonym and supply information:

- **Synonym:** [U-Ring-14C]
- **Batch No.:** 98218A
- **Description:** grey powder
- **Specific activity:** 23.4 mCi/mmol
- **Storage conditions:** -20°C, away from light and under nitrogen.

2.1.2 Non radiolabelled test substance

To add expiry date and supply information:

- **Batch No.:** 0500591
- **Description:** grey powder
- **Expiry date.:** October 1999
- **Storage conditions:** +4°C, away from light and under argon atmosphere

3. TEST SYSTEM

3.2 Environmental conditions

Amend to read:

light/dark cycle: 12 h/12 h (07:00-19:00)

AMENDMENT TO PROTOCOL

STUDY No.: **16434 PAR**

SPONSOR:

TITLE: ^{14}C **Pharmacokinetics study in plasma, excretion balance and tissue distribution after single administration by oral route (gavage) to rats.**

AMENDMENT No.: 1 Page 2/2

6. EXCRETION BALANCE AND DISTRIBUTION (group 2)

Amend to read:

Urine, faeces ... beginning of the application period,

. after each collection of faeces the cages and trays will be carefully rinsed with not more than 20 ml of water (except at 168 h when 200 ml will be used).

Remove the following phrase;

Other collections may be made if excretion of the compound is not complete 168 h after treatment.

11. REPORTING

Amend to read:

The Study Director ... Bq/animal, Bq/kg and mg/kg as appropriate.

For the excretion balance study ... also be cumulated) and the overall mass balance will be calculated.

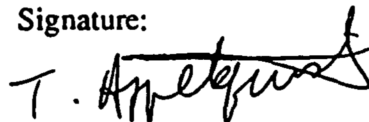
14. TIME SCHEDULE

To specify:

Day of treatment: 4 November 1998

End of study: 11 November 1998

Scientific management	Study Director	Study Monitor 3/11/98
S. de Jouffrey or R. Forster	T. Appelqvist	
Date: 2 November 1998	Date: 2 November 1998	Date:
Signature:	Signature:	Signature:



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STUDY TITLE

PHOTOTOXIC AND PHOTOALLERGENIC POTENTIAL BY DERMAL ROUTE IN GUINEA-PIGS

(according to the method of Unkovic, J., 1983)

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Stéphane de Jouffrey

STUDY COMPLETION DATE

21 July 1997

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)

Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

14932 TSG

SUMMARY

At the request of , the phototoxic and photoallergenic potential of the test substance (batch No. OP 18) was evaluated by dermal route in Guinea-pigs according to the method established by Unkovic, J. (1983). The study was conducted in compliance with the Principles of Good Laboratory Practice Regulations.

Methods

Twenty-five male Dunkin Hartley guinea-pigs were allocated to four groups: an irradiated control group (group 1, five animals), a group treated with the test substance (group 2, five animals), a group treated with the test substance and irradiated (group 3, ten animals) and a vehicle control group with irradiation (group 4, five animals).
The phototoxic potential of the test substance was evaluated 1, 6 and 24 hours after a single treatment. The photoallergenic potential of the test substance was assessed after several treatments during an induction period of 8 days with topical applications of the substance on the anterior scapular area followed by a period of 20 days without treatment and/or irradiation, then a challenge application on the posterior area of the right (U.V.A.) and left (U.V.B.) flanks of the animals.
At each treatment, 0.2 ml of the test substance at a concentration of 10% (w/w) in purified water was applied by dermal route. The irradiation dose of U.V.A. and U.V.B. was infra-erythematogenic. The cutaneous reactions were evaluated at the treatment sites during the induction and after the challenge application.
After the final scoring, the animals were sacrificed and cutaneous samples were taken from the challenge application sites of all the animals. No histological examination was performed on the cutaneous samples.

The sensitivity of Dunkin-Hartley guinea pigs under C.I.T. experimental conditions was checked in recent studies: 5-METHOXYPSORALENE was used as positive phototoxic substance and CHLORPROMAZINE as positive photosensitizer.

Results

No clinical signs and no deaths related to treatment with the test substance were noted during the study.

The body weight gain of the treated animals was normal when compared to that of the control animals.

Phototoxic potential

The very slight cutaneous reactions observed in 4/10 animals of the treated group 3 were similar to that noted in the control groups 1 and 4 and remained within the range of a local reaction at an infra-erythematogenic dose of UV radiations (erythema, grade 0.5).

Photoallergenic potential

After the challenge application of the test substance and irradiation of the treatment site, the only cutaneous reaction observed in animals of group 3 was a very slight or weak erythema (grade 0.5 or 1) which was similar to that noted in the control groups 1, 2 and 4 and remained within the range of a local reaction at an infra-erythematogenic dose of UV irradiation.

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In the recent studies performed using positive substances, the guinea-pigs showed phototoxic reactions in 100% of the animals (appendix 5) and macroscopic photoallergenic reactions in 40% of the animals (appendix 6).

Conclusion

Under our experimental conditions and according to the method established by Unkovic, J., (1983) the application of the test substance, (batch No. OP 18), diluted at 10% (w/w) in water did not induce any phototoxic or photoallergenic reactions in guinea-pigs.

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1. INTRODUCTION

The objective of this study, performed according to the method published by Unkovic, J. (1983) (1) serves to indicate the potential of the test substance to induce phototoxicity and photoallergenicity in guinea-pigs.

The results of this study are considered to be of value in predicting potential hazards in humans likely to arise from topical application of a test substance in association with exposure to light.

The study comprised 3 phases:
. a single treatment by cutaneous application of the test substance with ultra-violet irradiation to assess phototoxic potential,
. an induction phase, performed by repeated cutaneous application of the test substance with ultra-violet irradiation,
. a challenge phase, performed by cutaneous route with ultra-violet irradiation, to assess photo-allergenic potential.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Test substance

The test substance used in the study was supplied by

Documentation supplied by the Sponsor identified the test substance as follows:
. name:
  - protocol: none
  - labelling:
. batch number:
  - protocol: none
  - labelling: OP 18
. description: greyish powder
. container: two glass flasks
. date of receipt: 10 January 1997
. storage conditions: at +4°C, protected from light and stored under argon gas
. purity: 97.6%.

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

(1) Unkovic, J.; Mazue, G. and Girard, J.: Prévisions des accidents de produits photo-dynamisants chez l'homme à l'aide d'un modèle expérimental animal. Communication présentée aux XVIIIèmes journées "Peau et Toxiques" des Centres anti-poisons, Paris, 20 et 21 septembre 1979, Sci. Tech. Ani. Lab. 8, N° 3 : 149-160 (1983)

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2.1.2 Vehicle

The choice of the vehicle was based on tests to check the homogeneity of the preparation. The highest concentration which satisfied the criteria was called the maximal practicable concentration.
The vehicle used was purified water.

2.1.3 Preparation

On each day of treatment, the test substance was prepared at the concentration of 10% (w/w) in the vehicle.

2.2. TEST SYSTEM

2.2.1 Animals

Sex, species and strain: male Dunkin-Hartley guinea-pigs.
Reason for this choice: species recommended by the international regulations for sensitization studies. The strain used has been shown to produce a satisfactory response using known phototoxic substances.
Breeder: Centre d'Elevage Lebeau, 78950 Gambais, France.
Number and allocation of the animals to the groups: on day -1, the animals were weighed and randomly allocated to four groups:
. preliminary test: 8 animals,
. group 1 (irradiation control group): five animals irradiated but not treated,
. group 2 (test substance control group): five animals treated but not irradiated,
. group 3 (test group): ten animals treated and irradiated,
. group 4 (vehicle group): five animals treated with the vehicle and irradiated.
Weight: on day 1 of treatment, the animals had a mean body weight of 331 ± 21 g.
Acclimatization: at least five days before the beginning of the study.
Identification of the animals: the animals were identified individually with an ear-tattoo.

2.2.2 Environmental conditions

During the acclimatization period and throughout the study, the conditions in the animal room were as follows:
. temperature: 21 ± 2°C
. relative humidity: 30 to 70%
. light/dark cycle: 12 h/12 h
. ventilation: approximately 12 cycles/hour of filtered, non-recycled air.
The temperature and relative humidity were recorded continuously and records retained.
The housing conditions (temperature, relative humidity and ventilation) were checked monthly.
During the acclimatization period and throughout the study, the animals were housed individually in suspended wire-mesh cages (42.0 cm x 21.5 cmx 18.0 cm) equipped with a poly-propylene bottle. Sifted and dust-free sawdust was provided as litter (SICSA, 92142 Alfortville, France). An analysis of potential residues and major contaminants is performed periodically (Laboratoire Wolff, 92110 Clichy, France).

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2.2.3 Food and water

During the study, the animals had free access to "Guinea-pigs sustenance reference 106 pelleted diet" (U.A.R., 91360 Villemoisson-sur-Orge, France).
Food was analysed periodically (composition and contaminants) by the supplier.
The diet formula is presented in appendix 2.

Drinking water filtered by a F.G. Millipore membrane (0.22 micron) was contained in bottles. Bacteriological and chemical analysis of the water and diet and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are performed periodically.
Results are archived at C.I.T.

There were no contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

2.3. TREATMENT

2.3.1 Preliminary test

A preliminary test was performed to define the concentration to be tested in the main study and to determine the Maximum Non-Irritant Concentration (M.N.I.C.):
. the day before treatment, the anterior scapular area or the flanks of two animals was clipped and shaved,
. 0.2 ml of the test substance was applied diluted at appropriate concentrations in an appropriate vehicle,
. cutaneous reactions were evaluated 1, 6 and 24 hours after application of the test substance.

2.3.2 Main study

The main study comprised four groups according to the results of the preliminary test.

2.3.2.1 Preparation of animals (table 1)

The application site of the animals was clipped and shaved whenever necessary.

2.3.2.2 Irradiation

Only the animals from groups 1, 3 and 4 were irradiated.
Animals were immobilized and irradiated using an ultra-violet lamp "Toxicotronic" 312/365 nm (Vilbert/Lourmat, 77202 Marne-la-Vallée, France). The lamp consists of two groups of three fluorescent tubes producing either U.V.A. (365 nm) or U.V.B. (312 nm).
The irradiation was performed in two stages, first irradiation with U.V.B. and then irradiation with U.V.A. at the infra-erythematogenic dose (score of the erythema inferior than or equal to 0.5). The irradiation doses were 9 joules/cm^2 for U.V.A. and 0.1 joule/cm^2 for U.V.B.
The non-irradiated part of the back and flanks were protected from the ultra-violet rays.

2.3.2.3 Phototoxic potential

The phototoxic potential of the test substance was determined on day 1 (1 and 6 hours) and day 2 (24 hours) after a single application and irradiation on day 1 in the animals from group 3.
An application of 0.2 ml of the test substance at a concentration of 10% (w/w) in the vehicle was applied to the anterior scapular region on an area of 9 cm^2 in the animals from groups 2 and 3.
A gentle massage was given to facilitate penetration of the test substance into the epidermis.
As the test substance was administered in a vehicle, 0.2 ml of the vehicle was applied under the same experimental conditions to the animals from group 4.
The U.V.B. and U.V.A. irradiation of the animals from group 3 and 4 started approximately 30 minutes after application of the test substance.

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The animals from group 1 were irradiated without application of the test substance.

2.3.2.4 Photoallergenic potential

The photoallergenic potential of the test substance was determined in the animals from group 3 which were already used for the phototoxic evaluation and when the latter has shown to be negative.
The test was performed in three stages (induction, rest period and challenge application).

2.3.2.4.1 Induction period (from day 1 to day 8)

On each day of treatment (from day 2 to day 8), another dose of 0.2 ml of the test substance at the concentration of 10% (w/w) in the vehicle was applied to the anterior scapular region on an area of 9 cm^2 (3 cm x 3 cm) of each animal from groups 2 and 3 (table 1).
The animals from group 4 were treated with 0.2 ml of the vehicle.
Only the animals from group 3 and 4 were irradiated after each application of the test substance.
The non-irradiated region (back and flanks) was protected from the ultra-violet irradiation.
Irradiation started after a minimum of 30 minutes after application of the test substance.
The animals from group 1 were irradiated only.
During the induction period (including day 1), the animals were treated and/or irradiated on 6 out of 8 days (table 1).
Cutaneous reactions were scored 24 hours after each application of the test substance and/or irradiation (see § 2.3.3).

2.3.2.4.2 Rest period (day 9 to day 28)

After the 6th application, the animals from groups 1, 2, 3 and 4 received no further applications of the test substance and/or irradiation for a period of 20 days.

2.3.2.4.3 Challenge phase

On day 28, the animals from groups 1, 2, 3 and 4 were clipped and shaved. The following day (day 29), the challenge application sites showed no cutaneous reactions.
On day 29, 0.2 ml of the test substance at a concentration of 10% (w/w) in the vehicle was applied to two areas of 4 cm^2 (2 cm x 2 cm) of the distal part of the back (areas which had not previously been treated with the test substance or irradiated) of the animals from groups 2 and 3.
The animals from group 4 were treated with 0.2 ml of the vehicle.

After a minimum of 30 minutes after application of the test substance (animals from groups 2 and 3), the animals from groups 1 and 3 were irradiated: the left flank was U.V.B.-irradiated only, the right flank was U.V.A.-irradiated only, at a level which did not cause erythema.
Animals from group 1 and 4 were irradiated under the same conditions as the animals from group 3.
Cutaneous reactions were scored 1, 6, 24 and 48 hours after the challenge application.

2.3.3 Evaluation of the cutaneous reactions

The erythema and oedema observations were evaluated (table 1) according to the following scoring system:

. no erythema 0
. questionable erythema (infra-erythematogenic dose) 0.5
. weak erythema 1
. well-defined erythema 2
. severe erythema 3
. severe erythema with oedema (beet redness and trauma) 4

Any other lesions were noted.

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2.4. CLINICAL EXAMINATIONS

2.4.1 Morbidity and mortality

The animals were checked at least twice a day for mortality or signs of morbidity

2.4.2 Clinical signs

The animals were observed at least twice a day for clinical signs.

2.4.3 Body weight

Body weight was recorded for each animal once before allocation of the animals into groups, at the start of the study (on day 1) and then once a week until the end of the study (day 31).

2.5. PATHOLOGY

2.5.1 Necropsy

A macroscopic examination of the main organs was performed on the animals found dead during the study.
On day 31, after the 48-hour observation period, the surviving animals were killed by CO_2 inhalation in excess.

2.5.2 Cutaneous samples

On day 31, due to lesions and/or colouration of the skin, a skin sample was taken from the treatment sites of the posterior left and right flanks of all surviving animals. The samples were preserved in 10% buffered formalin.

2.5.3 Microscopic examination

No histological examinations were performed.

2.6. INTERPRETATION OF THE RESULTS

2.6.1 Phototoxic potential

Animals from group 3 were considered to show macroscopic phototoxic reactions if the cutaneous reactions noted 1 hour, 6 and 24 hours after the 1st treatment were clearly different to those of the animals from the control groups 1, 2 and 4 (well-defined erythema, having a score of 2 or more). Following microscopic examination, only animals with "burn"-type reactions were scored as positive.

2.6.2 Photoallergenic potential

Animals from group 3 were considered as showing macroscopic photoallergenic reactions if the cutaneous reactions noted 1 hour, 6, 24 and 48 hours after the challenge application were clearly different to those of the animals from control groups 1, 2 and 4 (well-defined erythema, having a score of 2 or more). Following microscopic examination, only guinea-pigs showing reaction due to a sensitization process were scored as positive.

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2.7. CONCLUSION

The test was considered positive if most of the treated and irradiated animals from group 3 showed obvious positive cutaneous reactions when compared to the animals from control groups 1, 2 and 4.
The test was considered negative if none of the animals from group 3 showed obvious positive macroscopic reactions or if the histopathological examination did not confirm evidence of a sensitization process, if they reveal reactions similar to those from control groups 1, 2 and possibly 4.
The test was considered "doubtful" if 1 or 2 animals from group 3 showed positive cutaneous reactions or if, on the other hand, obvious positive macroscopic reactions were found whose origins cannot be determined histologically.

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Table 1

Arrival of the animals: 23 January 1997
Allocation of the animals into groups: 27 January 1997

Phase	Time (day)	Group 1	Group 2	Groups 3 and 4	Date
	-1	Clipping and shaving of dorsal surface	Clipping and shaving of dorsal surface	Clipping and shaving of dorsal surface	27 Jan. 97
INDUCTION / PHOTOTOXICITY	1	Scoring/shaving/ irradiation Scoring (1-6 h)	Scoring/shaving/ application Scoring (1-6 h)	Scoring/shaving/ application/irradiation Scoring (1-6 h)	28 Jan. 97
INDUCTION / PHOTOTOXICITY	2	Scoring (24 h)/shaving ---------- Irradiation	Scoring (24 h)/shaving ---------- Application	Scoring (24 h)/shaving ---------- Application/irradiation	29 Jan. 97
INDUCTION	3	Scoring/shaving irradiation	Scoring/shaving application	Scoring/shaving application/irradiation	30 Jan. 97
INDUCTION	4	Scoring/shaving irradiation	Scoring/shaving application	Scoring/shaving application/irradiation	31 Jan. 97
INDUCTION	5	Scoring/shaving	Scoring/shaving	Scoring/shaving	1 Feb. 97
INDUCTION	6	Rest	Rest	Rest	2 Feb. 97
INDUCTION	7	Shaving/irradiation	Shaving/application	Shaving/application/ irradiation	3 Feb. 97
INDUCTION	8	Scoring/shaving /irradiation	Scoring/shaving /application	Scoring/shaving/ application/irradiation	4 Feb. 97
REST	9	Scoring	Scoring	Scoring	5 Feb. 97
REST	28	Clipping and shaving of lower back	Clipping and shaving of lower back	Clipping and shaving of lower back	24 Feb. 97
PHOTOALLERGENICITY	29	Scoring/shaving/ irradiation Scoring (1-6 h)	Scoring/shaving/ application/irradiation Scoring (1-6 h)	Scoring/shaving/ application/irradiation Scoring (1-6 h)	25 Feb. 97
PHOTOALLERGENICITY	30	Scoring of reactions 24 hours	Scoring of reactions 24 hours	Scoring of reactions 24 hours	26 Feb. 97
PHOTOALLERGENICITY	31	Scoring of reactions 48 hours/weighing/ sacrifice/shaving/ skin samples	Scoring of reactions 48 hours/weighing/ sacrifice/shaving/ skin samples	Scoring of reactions 48 hours/weighing/ sacrifice/shaving/ skin samples	27 Feb. 97

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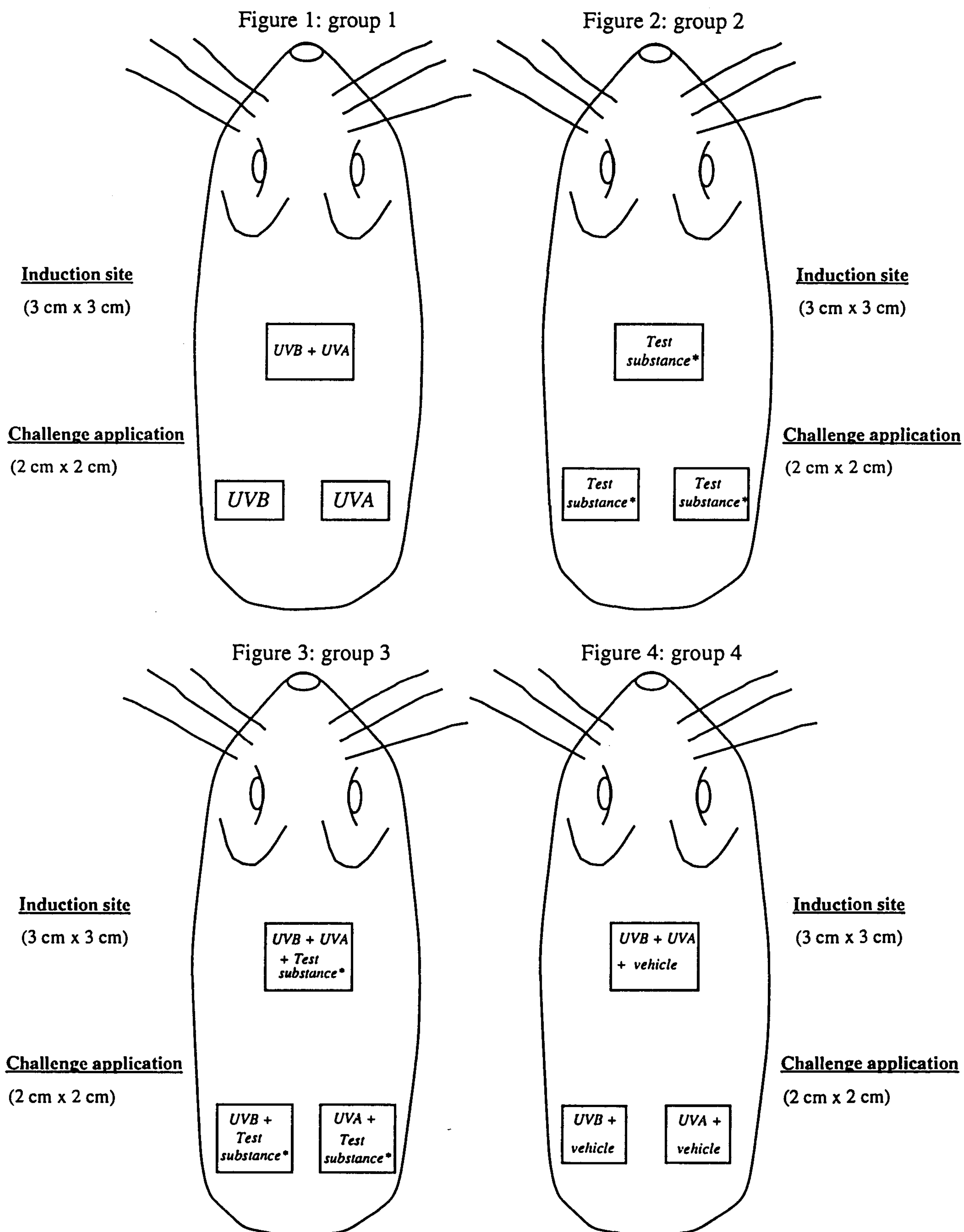
2.8. SUMMARY DIAGRAM



* at the concentration of 10% (w/w)

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2.9. ARCHIVES

The study archives:
. protocol and possible amendments,
. raw data,
. correspondence,
. final study report and possible amendments,
. possible histological specimens:
  - tissues in preservative

are stored in the premises of C.I.T., Miserey, 27005 Evreux, France, for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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3. RESULTS

3.1. PRELIMINARY STUDY

The maximal administrable concentration which could be obtained in the vehicle was 50% (w/w) of the test substance in the vehicle. Several tests were performed to determine the Maximal Non-Irritant Concentration (M.N.I.C.) after application of the test substance without irradiation.

Concentration of the test substance % (w/w)		Animal number	Scoring 1 hour	Scoring 6 hours	Scoring 24 hours
First assay					
50	DR	01	1	1	0
25	LF	01	0	0	0
10	RF	01	0	0	0
50	DR	02	1	1	0
25	LF	02	0	0	0
10	RF	02	0	0	0
Second assay					
25	DR	03	1	2	1/C
50	RF	03	1	1	0/C
25	DR	04	1	2/C	0/C
50	RF	04	0	1	0/C
Third assay					
10	DR	05	0	1	1
10	DR	06	0	1	0
Forth assay					
5	DR	07	0	1	0/C
5	DR	08	0	1	0/C

LF : left flank
RF : right flank
DR: dorsal region
C : black colouration of the skin

Score of 0: no erythema
Score of 1: weak erythema
Score of 2: well-defined erythema

Consequently the concentration of 10% (w/w) was chosen for the main study (induction period and challenge phase).

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3.2. MAIN STUDY

3.2.1 Clinical examinations

Sedation and piloerection were noted in one animal (No. 146) from group 2 on day 4, it was found dead on day 5. Two other animals (No. 140 from group 1 and No. 152 from group 3) were also found dead on day 5; no clinical signs were observed prior to death.
No macroscopic abnormalities were observed at necropsy (see paragraph 3.2.4.1).
This species is quite fragile and spontaneous clinical signs and mortality, without macroscopic abnormalities are commonly observed in untreated control animals, as was observed in animal No. 140 of group 1.
None of these clinical signs and mortality were therefore attributable to treatment.

3.2.2 Scoring of cutaneous reactions (appendix 4)

3.2.2.1 Evaluation of phototoxic reactions on days 1 and 2

After the first treatment, an infra-erythematogenic erythema (grade 0.5) was observed as follows:

Group	Score	Day 1 1 hour	Day 1 6 hours	Day 2
1 Irradiated Control	0 0.5	2/5 3/5	2/5 3/5	2/5 3/5
2 Treated with the test substance	0	5/5	5/5	5/5
3 Treated with the test substance then irradiated	0 0.5	7/10 3/10	6/10 4/10	7/10 3/10
4 Vehicle control then irradiated	0 0.5	3/5 2/5	2/5 3/5	2/5 3/5

The very slight cutaneous reactions observed in 4/10 animals of the treated group 3 were similar to that noted in the control groups 1 (3/5 animals) and 4 (3/5 animals) and remained within the range of a local reaction at an infra-erythematogenic dose of UV irradiation (erythema, grade 0.5).
A slight black colouration of the skin was observed in 3/10 animals from group 3 and in 4/5 animals from group 2. This colouration was attributed to the test substance.

3.2.2.2 Evaluation of cutaneous reactions from day 3 to day 9

A black colouration of the skin, which could mask a weak to severe erythema was observed in all animals from groups 2 and 3. Dryness of the skin and crusts were also noted in a few animals of these groups.

3.2.3 Evaluation of photoallergenic reactions from day 29 to day 31

After the challenge cutaneous application, an infra-erythematogenic (grade 0.5) or weak (grade 1) erythema was observed as follows:

Group	Scores	Day 29 1 hour LF	Day 29 1 hour RF	Day 29 6 hours LF	Day 29 6 hours RF	Day 30 24 hours LF	Day 30 24 hours RF	Day 31 48 hours LF	Day 31 48 hours RF
1 Irradiated Control	0	2/4	4/4	2/4	4/4	4/4	4/4	4/4	4/4
	0.5	2/4	-	2/4	-	-	-	-	-
2 Treated with the test substance	0	4/4	4/4	4/4	4/4	-	1/4	-	1/4
	0.5	-	-	-	-	-	-	-	-
	1	-	-	-	-	4/4	3/4	4/4	3/4
3 Treated with the test substance then irradiated	0	6/9	5/9	6/9	4/9	6/9	5/9	8/9	6/9
	0.5	3/9	4/9	3/9	5/9	3/9	3/9	1/9	3/9
	1	-	-	-	-	-	1/9	-	-
4 Vehicle control then irradiated	0	4/5	4/5	4/5	4/5	5/5	5/5	5/5	5/5
	0.5	1/5	1/5	1/5	1/5	-	-	-	-

LF : left flank (U.V.B.)
RF: right flank (U.V.A.)

After the challenge application of the test substance then irradiation of the treatment site, the only cutaneous reactions observed in animals of group 3 were very slight or weak erythema (grade 0.5 or 1) which were similar to that noted in the control groups 1, 2 and 4 and remained within the range of a local reaction at an infra-erythematogenic dose of UV radiations.

3.2.4 Pathology

3.2.4.1 Necropsy

Examination of the main organs of the thoracic and abdominal cavities (digestive tract, heart, kidneys, liver, lungs, pancreas, spleen) of animal Nos. 140, 146 and 152 did not reveal any macroscopic abnormalities.

3.2.4.2 Microscopic examination

No microscopic examination was performed.

4. CONCLUSION

Under our experimental conditions and according to the method established by Unkovic, J., (1983), the application of the test substance (batch No. OP 18) diluted at 10%, did not induce any phototoxic or photoallergenic reactions in guinea-pigs.

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SCORING OF CUTANEOUS REACTIONS

Phototoxicity and induction for photoallergy

Groups	Animal No.	Days: before	1: 1 h	1: 6 h	2	3	4	5	8	9
1M Irradiated Control	140	0	0	0	0	0	0.5	-	-	-
	141	0	0.5	0.5	0.5	0	0	0	0.5	0.5
	142	0	0.5	0.5	0.5	0.5	0.5	0	0	0.5
	143	0	0	0	0	0	0	0	0	0
	144	0	0.5	0.5	0.5	0	0	0	0/S	0/S
2M Treated with the test substance	145	0	0/C	0/C	0/C	C2	C2	C2/S	C2/S	1/C/S
	146	0	0/C	0/C	0/C	C1	C1	-	-	-
	147	0	0/C	0/C	0/C	C2	C2	C2/S	C2/S	C1/S
	148	0	0	0	0	0/C	C1	C1	C1/S	C1/S/A
	149	0	0/C	0/C	0/C	C3	C3	C3	C3	C1/S/A
3M Treated with the test substance and irradiated	150	0	0	0	0	0/C	C1	C1	C2/S	C2/S
	151	0	0	0	0	0	0/C	0/C	C2/S	C2/S
	152	0	0/C	0/C	0/C	C1	C1	-	-	-
	153	0	0	0	0	0/C	0/C	0/C	C2/S	C1
	154	0	0.5/C	0.5/C	0.5/C	0/C	C1	C1	C2/S	C2
	155	0	0.5	0.5/C	0.5/C	C1	C1	0/C	C1/S	C2/S
	156	0	0	0.5	0	0/C	0/C	0/C	C1	C1
	157	0	0.5	0.5	0.5	C1	C1	0/C	C1	C1
	158	0	0	0	0	C1	C1	0/C	C1	0/C/S
	159	0	0	0	0	0/C	C1	C1	C2	C2/S
4M Vehicle control and irradiated	160	0	0	0	0	0	0	0	0.5	0
	161	0	0.5	0.5	0.5	0.5	0.5	0	0	0
	162	0	0	0.5	0.5	0	0	0	0	0
	163	0	0	0	0	0.5	0.5	0.5	0.5/S	0/S
	164	0	0.5	0.5	0.5	0	0	0	0	0

S : dryness of the skin
C: black colouration of the skin
- : dead animal
h : hours

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SCORING OF CUTANEOUS REACTIONS

Photoallergy

Groups	Animal No.	Before treatment		Days 29, 1 h		Days 29, 6 h		30		31	
		LF	RF	LF	RF	LF	RF	LF	RF	LF	RF
1M Irradiated Control	140	-	-	-	-	-	-	-	-	-	-
	141	0	0	0.5	0	0.5	0	0	0	0	0
	142	0	0	0	0	0	0	0	0	0	0
	143	0	0	0.5	0	0.5	0	0	0	0	0
	144	0	0	0	0	0	0	0	0	0	0
2M Treated with the test substance	145	0	0	0/C	0/C	0/C	0/C	1/C	1/C	1/C	1/C
	146	-	-	-	-	-	-	-	-	-	-
	147	0	0	0/C	0/C	0/C	0/C	1/C	1/C	1/C	1/C
	148	0	0	0/C	0/C	0/C	0/C	1/C	0/C	1/C	0/C
	149	0	0	0/C	0/C	0/C	0/C	1/C	1/C	1/C	1/C
3M Treated with the test substance and irradiated	150	0	0	0.5/C	0/C	0.5/C	0/C	0.5/C	0/C	0	0/C
	151	0	0	0.5/C	0.5/C	0.5/C	0.5/C	0.5/C	1/C	0.5/C	0.5/C
	152	-	-	-	-	-	-	-	-	-	-
	153	0	0	0.5/C	0.5/C	0.5/C	0.5/C	0.5/C	0.5/C	0/C	0/C
	154	0	0	0/C	0.5/C	0/C	0.5/C	0/C	0.5/C	0	0.5/C
	155	0	0	0/C	0/C	0	0	0	0	0	0
	156	0	0	0	0	0	0.5	0	0	0	0
	157	0	0	0/C	0.5/C	0/C	0.5/C	0/C	0.5/C	0/C	0.5/C
	158	0	0	0	0	0	0	0	0	0	0
	159	0	0	0	0/C	0	0/C	0	0/C	0	0
4M Vehicle control and irradiated	160	0	0	0	0	0	0	0	0	0	0
	161	0	0	0	0	0	0	0	0	0	0
	162	0	0	0.5	0.5	0.5	0.5	0	0	0	0
	163	0	0	0	0	0	0	0	0	0	0
	164	0	0	0	0	0	0	0	0	0	0

LF: left flank (U.V.B.)
RF: right flank (U.V.A.)
C : black colouration of the skin
h : hours
- : dead animal

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SPONSOR

STUDY TITLE

EMBRYOTOXICITY/TERATOGENICITY STUDY BY ORAL ROUTE (GAVAGE) IN RATS

TEST SUBSTANCE

HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL

STUDY DIRECTOR

Marie-Hélène Savary

STUDY COMPLETION DATE

17th May 1995

PERFORMING LABORATORY

Centre International de Toxicologie (C.I.T.)
Miserey - 27005 Evreux - France

LABORATORY STUDY NUMBER

12357 RSR

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STATEMENT OF THE STUDY DIRECTOR

The study was conducted in compliance with the principles of Good Laboratory Practice Regulations:

. Décret N° 90-206 du 7 mars 1990 concernant les Bonnes Pratiques de Laboratoire (Ministère de l'Industrie et de l'Aménagement du Territoire),

. O.E.C.D. Principles of Good Laboratory Practice, C(81)30(final) Annex 2. May 12, 1981.

I declare that this report constitutes a true and faithful record of the procedures undertaken and the results obtained in the performance of the study.

The study was performed at the Centre International de Toxicologie (C.I.T.), Miserey, 27005 Evreux, France.

M.H. Savary Date: 17.5.95
Study Director
Doctor of Sciences
Toxicologist
Head of Reproductive Toxicology

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SCIENTISTS INVOLVED IN THE STUDY

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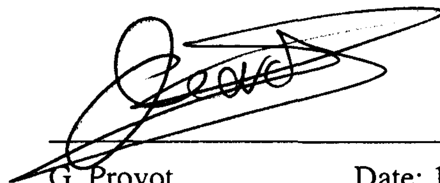

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J.F. Le Bigot Date: 17.5.95
Doctor of Sciences
General Director

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STATEMENT OF QUALITY ASSURANCE UNIT

Type of inspections	Dates (day/month/year)		
	Inspections	Report to Study Director (*)	Report to Management (*)
Protocol	12.10.94	12.10.94	13.10.94
Study	18.11.94	21.11.94	24.11.94
Study	24.11.94	28.11.94	28.11.94
Study	5.12.94	6.12.94	7.12.94
Report	27.3.95	9.5.95	7.5.95

The inspections were performed in compliance with C.I.T. Quality Assurance Unit procedures and the principles of Good Laboratory Practice Regulations.

(*) The dates mentioned correspond to the dates of signature of audit reports by Study Director and Management.

L. Valette-Talbi Date: 17.5.95
Doctor of Biochemistry
Head of Quality Assurance Unit
and Scientific Archives

SUMMARY

The objective of the study was to evaluate the potential toxic effects of the test substance, (batch No. Pil 4X), on the embryonic and fetal development when administered daily by oral route(gavage) to Sprague-Dawley female rats during organogenesis (day 6 to day 15 of pregnancy inclusive).
The test substance is a dye.

Methods

Three groups of 25 mated female rats received daily by gavage the test substance, at the dose levels of 50, 200 or 800 mg/kg/day, from day 6 to day 15 of pregnancy inclusive. Simultaneously, a group of 25 mated females was given the vehicle only (water for injections) and acted as a control group. Day 0 was designated as the day of mating.
Clinical signs including signs of abortion and mortality were checked daily. Food consumption and body weight were recorded at designated intervals during pregnancy.
On day 20 of pregnancy, females were killed, examined macroscopically and fetuses removed by Caesarean section. Litter parameters were recorded: number of corpora lutea, implantation sites, resorptions, dead and live fetuses. Fetuses were weighed, sexed and submitted to external, soft tissue and skeletal examinations.

Results

Maternal data

Reddish coloured urine was observed in 1 out of 25 females given 50 mg/kg/day and all females given 200 or 800 mg/kg/day. These signs were the proof of the absorption of the test substance and were not considered to be signs of toxicity.
The mean food consumption and body weight gain of females with completed pregnancy were similar in the control and treated groups. No macroscopic changes atrributed to treatment were noted at necropsy of females.

Litter data

The mean number of corpora lutea, implantation sites, post-implantation loss, mean number of live fetuses, sex-ratio and mean fetal body weight were similar in the control and treated groups.

Fetal observations

No treatment-related external anomalies or malformations, soft tissue anomalies or malformations or skeletal variations, anomalies or malformations were observed in fetuses of any group.

Conclusion

The test substance, (batch No. Pil 4X), administered daily by oral route to pregnant Sprague-Dawley female rats during organogenesis at the dose levels of 50, 200 or 800 mg/kg/day was well-tolerated by the pregnant female at all dose levels and was not embryotoxic nor teratogenic. Only reddish coloured urine was noted, proof of absorption of the test substance.
Under our experimental conditions, the No Adverse Effect Level was defined as 800 mg/kg/day in terms of maternotoxicity and embryo/fetal development.

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1. INTRODUCTION

This study was performed at the request of Société .
The objective of this study was to evaluate the potential toxic effects of the test substance, on the embryonic and fetal development when administered daily by oral route (gavage) to Sprague-Dawley female rats during organogenesis (day 6 to day 15 of pregnancy inclusive).
The rat was chosen because it is a rodent species commonly requested by regulatory authorities and the Sprague-Dawley strain was selected due to the background data available from previous studies performed at our laboratory.
The test substance is a dye.
The oral route was used since it is expected to ensure an absorption of the test substance at least equal to the dermal route which is the route of exposure in humans.
Doses were based on the results of a previous study (2-week toxicity study by oral route in rats (CIT/Study No. 12068 TSR).

This study was designed in accordance with the following guidelines:
. O.E.C.D. guideline No. 414, 12th May 1981,
. E.E.C. Recommendation No. 87/302/E.E.C., 18th November 1987.

2. MATERIALS AND METHODS

2.1. TEST AND CONTROL SUBSTANCES

2.1.1 Identification

2.1.1.1 Test substance

The test substance, used in the study was supplied by .

Documentation supplied by the Sponsor identified the test substance as follows:
. denomination
  - protocol and labelling:
. batch number
  - protocol and labelling: Pil 4X
. description: beige powder
. quantity and container: 4 kg in a plastic tub; this quantity was dispatched at C.I.T. into 7 flasks (flasks 1 and 2 were used for the present study)
. date of receipt: 26.7.94
. storage conditions: at room temperature, away from light, and in addition away from humidity (from 1.8.94 onwards).

Data relating to the characterization of the test substance are documented in an analytical certificate (presented in appendix 1) provided by the Sponsor.

2.1.1.2 Vehicle

The vehicle was water for injections, batch No. 0419, provided by Biosédra (92240 Malakoff, France).

2.1.2 Preparation

The test substance was given in the vehicle which was previously degased by sonication for at least 10 minutes, and in which nitrogen bubbled for 2 to 3 minutes; this vehicle was then kept under argon until use.
The test substance was dissolved in the vehicle in order to achieve the concentration of 160 mg/ml, and then homogenized using a magnetic stirrer. The 10 mg/l and 40 mg/l preparations were prepared by direct dilution of the 160 mg/l preparation. The obtained preparations were then kept under argon until use.
The test substance preparations were made for up to 4 days of treatment, according to known stability. They were delivered to the animal room, protected fom light and maintained under conditions stirring during the dosing procedure (except for vehicle).

2.1.3 Chemical analysis of preparations

. Stability
Before the beginning of the treatment period, the stability of solutions at 10 mg/ml and 160 mg/ml was checked. Each preparation was sampled just after preparation, after 2 hours in sealed bottles followed by 1 hour in opened bottles at room temperature and after 4 and 9 days of storage at +4°C.
Each sample was diluted with degazed solvent containing a stabilizer (ascorbic acid at 0.01% w/v) and kept frozen at -20°C until analysis on day 9 (except samples taken on the day of preparation, after 3 hours storage at room temperature and after 9 days storage at +4°C).
Each sample was analysed in duplicate.

. Concentration
Each preparation (control group included) was checked for achieved concentration of the test substance on the first day of treatment of the first mated females and on the last day of treatment of the last mated females.

The analytical procedure and detailed results are presented in appendix 2.

2.2. TEST SYSTEM

2.2.1 Animals

One hundred Sprague-Dawley mated female rats of the Crl CD (SD) BR strain were supplied by Charles River France (76410 Saint-Aubin-lès-Elbeuf, France) and received at C.I.T. on 16.11.94 (36 females), 17.11.94 (32 females) and 18.11.94 (32 females) corresponding to day 1 of pregnancy. Upon their arrival the animals were given a clinical examination to ensure that they were in good clinical condition.
A 5-day acclimatization period to the conditions of the study preceded the beginning of the treatment period.
The females were allocated to the groups before the beginning of the treatment period, according to the body weight recorded on day 2 of pregnancy and according to a stratified procedure so that the average body weight of each group was similar.
Each animal was then identified by ear tattoo.
At the beginning of the treatment period, the animals were approximately 10 weeks old and had a mean body weight of 260 g (208 to 321 g).

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2.2.2 Environmental conditions

Upon their arrival at C.I.T., the animals were housed in a protected zone.
The animal room was disinfected before the arrival of the animals and cleaned regularly thereafter. Microbiological analyses of the air and the surfaces of the walls and floor of the animal room are performed regularly (Laboratoire Départemental d'Analyses d'Evreux, 27000 Evreux, France).
The environmental conditions in the animal room were set as follows:
. temperature: 21 ± 2°C
. relative humidity: 50 ± 20%
. light/dark cycle: 12h/12h (07:00 - 19:00)
. ventilation: about 12 cycles/hour of filtered, non-recycled air
The temperature and relative humidity were recorded continuously and records retained in C.I.T.'s archives.
The housing conditions (temperature, relative humidity, light/dark cycle and ventilation) were checked monthly.
The animals were housed individually in polycarbonate cages (43.0 x 21.5 x 20.0 cm) and each cage contained autoclaved sawdust (SICSA, 94142 Alfortville, France). Bacteriological analysis of the sawdust and detection of possible contaminants (pesticides, heavy metals) are performed periodically (Laboratoire Départemental d'Analyses d'Evreux, 27000 Evreux, France; Laboratoire Municipal et Régional de Rouen, 76000 Rouen, France).
Bottles, sawdust and cages were changed at least once a week.

2.2.3 Food and water

The animals had free access to A04 C pelleted diet, batch No. 40728 (U.A.R., 91360 Villemoisson-sur-Orge, France) distributed weekly. The diet formula is presented in appendix 3.
Each batch of food was analysed (composition, contaminants) by the supplier. Results are archived at C.I.T.
The animals had free access to bottles containing tap water filtered using a 0.22 micron filter (Millipore S.A., 78140 Vélizy, France).
Bacteriological and chemical analyses of water and detection of possible contaminants (pesticides, heavy metals and nitrosamines) are made periodically (Laboratoire Départemental d'Analyses d'Evreux, 27000 Evreux, France; Laboratoire Municipal et Régional de Rouen, 76000 Rouen, France; Centre de Nutrition Humaine, 54000 Nancy, France). Results are archived at C.I.T.

There were no known contaminants in the diet, water or sawdust at levels likely to have influenced the outcome of the study.

2.3. MATING

Females were mated at the breeder's facilities.
The day of the positive mating was designated day 0 of pregnancy.

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2.4. TREATMENT

Rationale for dose selection

The dose levels were determined in agreement with the Sponsor following the results of a previously 2-week toxicity study by oral route in rats (CIT/Study No. 12068 TSR) performed at the dose levels of 50, 200 and 800 mg/kg/day. The results showed that the dose level of 800 mg/kg/day induced a slight decrease in body weight gain, glucose level and total proteins. The 200 mg/kg/day dose level induced only a slight decrease in glucose level. No effects were noted at 50 mg/kg/day. Therefore these same dose levels of 50, 200 and 800 mg/kg/day were retained for the present study.

2.4.1 Doses and groups

The groups, doses and animal numbers are detailed in the following table:

Group	Animals per group	Dose (mg/kg/day)	Animal numbers
1	25	0	L21441 to L21465
2	25	50	L21466 to L21490
3	25	200	L21491 to L21515
4	25	800	L21516 to L21540

2.4.2 Administration

The test substance was administered by gavage using a glass syringe fitted with a metal probe. Each animal was given the test substance once a day, at the same approximate daily time, 7 days a week from day 6 to day 15 of pregnancy inclusive.
During the administration, the test substance preparations (except vehicle) were maintained under constant magnetic stirring.
The quantity of the test substance was adjusted according to the most recently recorded body weight.
Control animals received the vehicle alone under the same conditions.
A constant dose volume of 5 ml/kg/day was used.

2.5. CLINICAL EXAMINATIONS

2.5.1 Clinical signs

Clinical signs were observed for each animal at least once a day, at the same approximate daily time.

2.5.2 Mortality

All animals were checked at least twice a day for mortality and signs of morbidity, including weekends and Public Holidays during the treatment period.
Any female killed prematurely was subjected to a macroscopic examination. The number of corpora lutea and implantation sites was recorded.

2.5.3 Food consumption

The quantity of food consumed by each animal was recorded at the following intervals: days 2-6, 6-9, 9-12, 12-15 and 15-20 of pregnancy.
Food intake per animal and per day was calculated using the amount of food given and left in each feeder.

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2.5.4 Body weight

Body weight was recorded for each animal on days 2, 6, 9, 12, 15 and 20 of pregnancy.

2.6. HYSTERECTOMIES

On day 20 of pregnancy, the females were killed by asphyxiation using carbon dioxide. The fetuses were removed by Caesarean section.
The ovaries and uterus of females were examined to determine:
- number of corpora lutea,
- number and distribution of live and dead fetuses,
- number and distribution of early and late resorptions,
- number of implantation sites.

In apparently non-pregnant females, presence of implantation sites was checked using the Salewski staining technique.
After hysterectomies, the dams were subjected to a macroscopic examination.

2.7. EXAMINATION OF FETUSES

2.7.1 Body weight

Each live fetus was weighed.

2.7.2 External examination

Each fetus was submitted to an external examination (including palate) to check for the presence of malformations. Dead fetuses were discarded therafter.

2.7.3 Soft tissue examination

Approximately half of the live fetuses were fixed in Bouin's fluid. Soft tissue examination was performed according to free-hand sectioning technique (Wilson technique).
The soft tissue findings were classified into malformations and anomalies. Malformations referred as severe changes preventing normal development and/or capable of provoking death. Anomalies referred as slight, non-permanent structural and not obviously detrimental changes.

2.7.4 Skeletal examination

The remaining fetuses per litter were placed in alcohol and the skeleton was stained with alizarin red S, in order to be examined (modified Dawson technique).
The skeletal findings were classified into skeletal variations, anomalies and malformations. Malformations referred as severe permanent structural changes which could be detrimental. Anomalies referred as slight, non-permanent structural and not obviously detrimental changes. Variations referred as slight, non-permanent structural changes observed in more than 10% of the historical population.

2.7.5 Sex of fetuses

The sex of live fetuses was determined at the time of the evisceration after fixation in alcohol or at the time of the Wilson's sections. The sex of dead fetuses was determined at the time of hysterectomies.

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2.8. ASSESSMENT OF DATA/STATISTICAL ANALYSIS

Expression of data

Data are expressed as group mean values ± standard deviation: body weight, food consumption, number of implantation sites, corpora lutea, fetuses, resorptions or as percentage values: pre and post-implantation loss, fetal observations.

The following calculations were performed for each group:

Pre-implantation loss:

$$\frac{\text{Number of corpora lutea - Number of implantation sites}}{\text{Number of corpora lutea}} \times 100$$

Post-implantation loss:

$$\frac{\text{Number of implantation sites - Number of live fetuses}}{\text{Number of implantation sites}} \times 100$$

Observations in fetuses:

$$\frac{\text{Number of fetuses with a particular observation}}{\text{Total number of fetuses examined}} \times 100$$

In addition, the total number of litters within each group containing fetuses with a particular observation was calculated.

Statistical analysis

Mean values were compared by one-way analysis of variance and Dunnett's test.
Percentage values were compared by Fisher's exact probability test.

2.9. ARCHIVES

The study archives:
. protocol and amendments,
. raw data,
. correspondence,
. final report and possible amendments,
. stained skeletons,
. Wilson' sections,
. samples of test substance and vehicle,

are stored on the premises of C.I.T., 27005 Miserey, Evreux, France for 5 years after the end of the *in vivo* study. At the end of this period, the study archives will be returned to the Sponsor.

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2.10. CHRONOLOGY OF THE STUDY

The chronology of the study is summarized as follows:

Procedure	Date
Protocol approved by:	
. Study Director	12.10.94
. Study Monitor	21.10.94
Mating of females (day 0 of pregnancy)	
. First female	15.11.94
. Last female	17.11.94
First day of treatment (day 6 of pregnancy)	
. First female	21.11.94
. Last female	23.11.94
Last day of treatment (day 15 of pregnancy)	
. First female	30.11.94
. Last female	2.12.94
Hysterectomies (day 20 of pregnancy)	
. First female	5.12.94
. Last female	7.12.94

3. RESULTS

3.1. CHEMICAL ANALYSIS OF PREPARATION

. Stability
The results of analyses revealed a good stability 9 days at +4°C for investigated solutions (concentrations of 10 mg/ml and 160 mg/ml) under the conditions of preparation previously mentioned (§ 2.1.3).

. Concentration
A satisfactory concordance between obtained and nominal concentrations was found for the preparations performed on the first day of treatment of the first mated females and on the last day of treatment of the last mated females.

Detailed results are presented in appendix 2.

3.2. MATERNAL DATA

3.2.1 Pregnancy status

The pregnancy status is summarized in the table below:

Dose (mg/kg/day)	0	50	200	800
Mated females	25	25	25	25
Non-pregnant females	6	4	3	2
Pregnant females	19	21	22	23
. killed prematurely	0	0	0	1
. aborted	0	0	0	0
. alive at term	19	21	22	22
. total resorption	0	0	0	0
. completed pregnancy	19	21	22	22

3.2.2 Clinical signs (table 1, appendix 4)

Reddish coloured urine was observed in 1 out of 25 females given 50 mg/kg/day and all females given 200 or 800 mg/kg/day. These signs were the proof of the absorption of the test substance and were not considered to be signs of toxicity.
One female of the 800 mg/kg/day (L21520) group presented signs of poor clinical conditions on day 9 and/or day 10 (locomotor difficulties and bent head). This sign which was observed only in one female was considered to bear no relationship to treatment.

3.2.3 Mortality/sacrifice (table 1, appendix 4)

No deaths were noted in females of the control, 50 and 200 mg/kg/day groups.
In the 800 mg/kg/day group, the female that presented clinical signs (L21520) was killed on day 10 of pregnancy.

3.2.4 Abortions (table 1, appendix 4)

No abortions occurred during the study.

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3.2.5 Food consumption (figure 1, table 2, appendix 5)

The mean food consumption of females with completed pregnancy was similar in the control and treated groups.

3.2.6 Body weight (figures 2 and 3, tables 3 and 4, appendix 6)

The mean body weight gain of females with completed pregnancy was similar in the control and treated groups when considering the overall treatment period from day 6 to day 15 of pregnancy.

3.2.7 Maternal necropsy observations (table 5, appendix 7)

No macroscopic changes attributed to treatment were noted.

3.3. LITTER DATA (table 6, appendices 8 to 10)

3.3.1 Corpora lutea and implantation sites

The mean number of corpora lutea and implantation sites was similar in the control and 200 mg/kg/day groups. They were higher in the 50 and 800 mg/kg/day groups. As the treatment began after implantation of concepti, this increase was not attributed to treatment.

3.3.2 Post-implantation loss

3.3.2.1 Resorptions

The rate of resorptions was similar in the control and treated groups.

3.3.2.2 Dead fetuses

No dead fetuses were noted in the control, 200 and 800 mg/kg/day groups.
In the 50 mg/kg/day group, 1 out of 300 fetuses was dead. As this death occurred only at the low dose level and as it concerned only 1 fetus, it was not attributed to treatment.

3.3.2.3 Total post-implantation loss

The post-implantation loss was similar in the control and treated groups.

3.3.3 Live fetuses

3.3.3.1 Mean number

The mean number of live fetuses was similar in the control and 200 mg/kg/day groups. It was higher in the 50 and 800 mg/kg/day groups as a consequence of the higher number of implantation sites which was not attributed to treatment.

3.3.3.2 Body weight

The mean fetal body weight was similar in the control and treated groups.

3.3.3.3 Sex-ratio

The sex-ratio was similar in the control and treated groups.

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3.4. FETAL EXAMINATIONS (tables 7 to 9, appendix 11 to 13)

3.4.1 Fetal external examinations

No external anomalies or malformations were observed in fetuses of any group.

3.4.2 Fetal soft tissue examinations

3.4.2.1 Fetal soft tissue anomalies

Dilated renal pelvis was noted in 1 fetus of each of the control, 50 and 200 mg/kg/day groups vs. none in the 800 mg/kg/day group.
Ureteral dilatation was noted in 1 fetus of the control group and 2 fetuses of the 200 mg/kg/day group.
These anomalies for which the incidence was very low and not dose-related were considered to be spontaneous in nature.

3.4.2.2 Fetal soft tissue malformations

No fetal soft tissue malformations were noted in fetuses of the control, 200 and 800 mg/kg/day groups.
In the 50 mg/kg/day group, one out of 145 fetuses had a ventricular cerebral dilatation. Considering that this malformation occurred only in one fetus of the low dose group, it was considered to be spontaneous in nature.

3.4.3 Fetal skeletal examinations

3.4.3.1 Fetal skeletal variations

The incidence of fetal skeletal variations was similar in the control and treated groups.

3.4.3.2 Fetal skeletal anomalies

The incidence of skeletal anomalies was similar in the control and treated groups.

3.4.3.3 Fetal skeletal malformations

No fetal skeletal malformations were noted in the control and 200 mg/kg/day groups.
In the 50 and 800 mg/kg/day groups, one fetus of each group had a wavy rib. This malformation which occurred only in one fetus and whose incidence was not dose-related was considered to be spontaneous in nature.

4. CONCLUSION

The test substance, (batch No. Pil 4X), administered daily by oral route to pregnant Sprague-Dawley female rats during organogenesis at the dose levels of 50, 200 or 800 mg/kg/day was well-tolerated by the pregnant female at all dose levels and was not embryotoxic nor teratogenic. Only reddish coloured urine was noted, proof of absorption of the test substance.
Under our experimental conditions, the No Adverse Effect Level was defined as 800 mg/kg/day in terms of maternotoxicity and embryo/fetal development.

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5. REFERENCES

Dawson, A.B.: A note of the staining of the skeleton of cleared specimens with alizarin red S. Stain Technol., 1926: 1; 123-124.

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Fisher, R.A.: Statistical methods for research workers (5th ed). Edinburgh: Oliver and Boyd (1934).

Salewski (E.): Färbemethode zum makroskopischen Nachweis von Implantationstellen am Uterus der Ratte. Arch. Exp. Path. Pharmak., 247, 367 (1964).

Wilson, J.G.: Methods for administering agents and detecting malformations in experimental animals. In Teratology, Principles and Techniques; J.G. Wilson and J. Warkany eds, the University of Chicago Press, Chicago and London, 1965, pp 262-277.

AMENDMENT TO PROTOCOL

STUDY No.: **12357 RSR**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 01 **Page 1 / 1**

Justification: data unavailable in the protocol
Date of application: 21.11.94

Add: Reference Number: 94/1/083

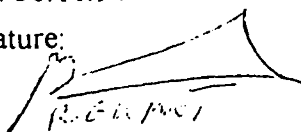
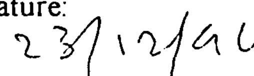
1. TEST AND CONTROL SUBSTANCES

1.2 Preparation

The test substance ... light.
The test substance preparations ... CIT Pharmacy for up to 9 days of use.

14. TIME SCHEDULE

Arrival of the first animals: 16.11.94
First day of treatment: 21.11.94
First day of hysterectomy: 5.12.94

Scientific management	Study Director	Study Monitor
J.F Le Bigot or A. Simonnard	M.H. Savary	
Date: 30.11.94	Date: 30.11.94	Date:
Signature: 	Signature:	Signature: 

AMENDMENT TO PROTOCOL

STUDY No.: **12357 RSR**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 02

Justification: changes in protocol
Date of application: 10.2.95

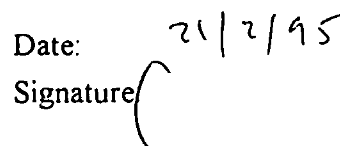
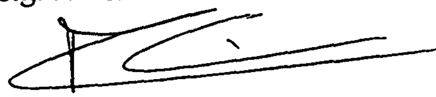
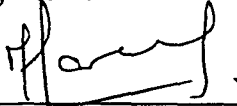
INTRODUCTION

Suppress:
. Council Directive 87/18/E.E.C. ... chemical substances (O.J. n° L 15 of 17.1.87)

10. REPORTING

Suppress:
with a summary in French

Scientific management	Study Director	Study Monitor
J.F. Le Bigot or A. Simonnard	M.H. Savary	
Date: 10.2.95	Date: 10.2.95	Date: 21/2/95
Signature:	Signature:	Signature

AMENDMENT TO PROTOCOL

STUDY No.: **12357 RSR**
SPONSOR: **Société**
TEST SUBSTANCE:
AMENDMENT No.: 03

Justification: precision on randomization of animals
Date of application: 17.11.94

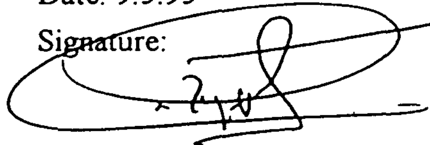
2. TEST SYSTEM

2.1 Animals

...

- **Constitution of groups:** the females ... groups, before the beginning of the treatment period according to the body weight recorded on day 2 of pregnancy and according to a stratified body weight procedure ... similar.

...

Scientific management	Study Director	Study Monitor
J.F. Le Bigot or R. Glomot	M.H. Savary	
Date: 9.5.95	Date: 9.5.95	Date:
Signature: 	Signature:	Signature:

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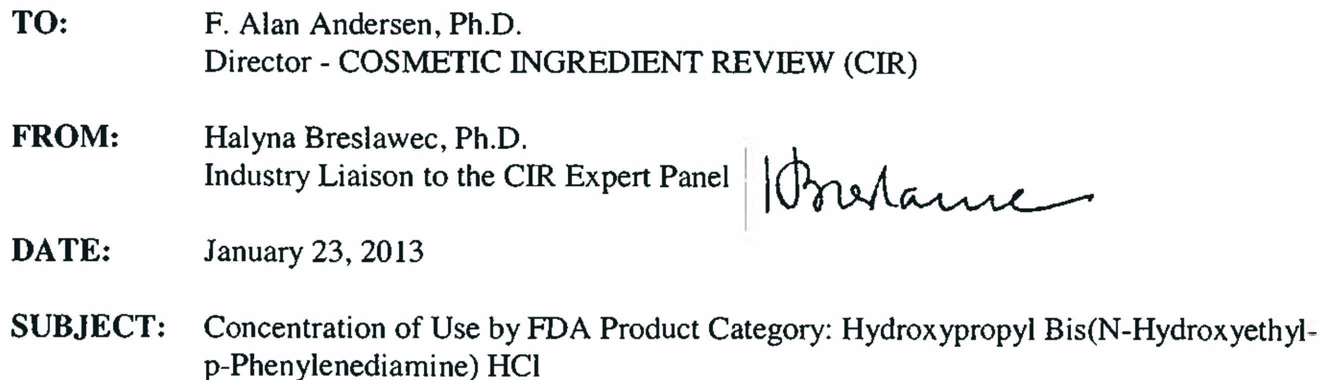
Memorandum



TO: F. Alan Andersen, Ph.D.
Director - COSMETIC INGREDIENT REVIEW (CIR)

FROM: Halyna Breslawec, Ph.D.
Industry Liaison to the CIR Expert Panel

DATE: January 23, 2013

SUBJECT: Concentration of Use by FDA Product Category: Hydroxypropyl Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl

1101 17th Street, N.W., Suite 300 | Washington, D.C. 20036-4702 | 202.331.1770 | 202.331.1969 (fax) | www.personalcarecouncil.org

Concentration of Use by FDA Product Category
Hydroxypropy Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl

FDA Code*	Product Category	Maximum Concentration of Use
06A	Hair dyes and colors (all types requiring caution statement and patch tests)	0.28%

*Product category codes used by FDA

Information collected in 2012
Table prepared January 23, 2013

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FDA VCRP Use DATA 2012

06A - Hair Dyes and Colors (all types requiring caution statements and patch tests)	128729282	HYDROXYPROPYL BIS(N-HYDROXYETHYL-P-PHENYLENEDIAMINE) HCL	75

Memorandum

TO: F. Alan Andersen, Ph.D.
Director - COSMETIC INGREDIENT REVIEW (CIR)

FROM: Hair Coloring Technical Committee (HCTC) of the Personal Care Products Council

DATE: March 25, 2013

SUBJECT: Comments on Scientific Literature Review (SLR): Safety Assessment of Hydroxypropyl Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl as Used in Cosmetics

We appreciate the opportunity to comment on the Scientific Literature Review (SLR) on Hydroxypropyl Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl.

The (SLR) on Hydroxypropyl Bis(N-Hydroxyethyl-p-Phenylenediamine) HCl includes the following request for data: "CIR is asking for submissions of characterization, toxicity, dermal penetration data on the reaction products of this hair dye with hydrogen peroxide."

This request shows a lack of understanding of the chemistry of oxidative hair dyes and should not have been included in the SLR.

At the December 2011 CIR Expert Panel meeting, Dr. Julie Skare, representing our committee, gave a presentation on oxidative hair dye chemistry. She indicated that the intermediate compounds formed during reaction with hydrogen peroxide are very short-lived, and concluded that "Safety assessment of oxidative hair dyes is driven by the toxicological evaluation of the ingredients (i.e., precursors and couplers) rather than by the reaction products formed during use."

Based on Dr. Skare's presentation, the CIR Expert Panel drafted the following paragraph (copied from the 6-Hydroxyindole draft report) to be included in the discussion sections of CIR reports on oxidative hair dyes (underlining added for emphasis): "The Panel noted that the use of oxidative hair dye formulations involves exposure to precursors and coupling agents as well as to their reaction products. While reaction intermediates may be formed, <u>human exposure is to the precursors and coupling agents and to reaction products, not to reaction intermediates. The exposures to the precursors and couplers are low (they are consumed in the color forming reaction), and the exposures to reaction products are even lower (they are adsorbed into the hair shaft itself and physically retained there). Therefore, safety assessments of oxidative hair dyes are driven by the toxicological evaluation of the ingredients (i.e. precursors and coupling agents).</u>"

Data requests for oxidative hair dye ingredients should focus on the ingredient (precursor or coupling agent) under review, not reaction products, and especially not the short-lived intermediates that result from reaction with hydrogen peroxide.