CENTRE DE RECHERCHES BIOLOGIQUES

Baugy, June 17, 1993

STUDY NO 920086 E

[redacted]

STUDY OF CUTANEOUS SENSITIZATION USING THE MAGNUSSON AND KLIGMAN MAXIMIZATION TEST IN GUINEA PIG

Sponsor :

ELF ATOCHEM SA
La Défense 10, cedex 42
92091 PARIS LA DEFENSE
FRANCE

STUDY : Study of cutaneous sensitization using the MAGNUSSON and KLIGMAN test in Guinea Pig.

COMPOUND : [redacted]

SPONSOR : ELF ATOCHEM SA

SPONSOR CONTACT : Mr REGNIER

ADDRESS : La Défense 10, Cedex 42
92091 PARIS LA DEFENSE - FRANCE.

LOCATION OF STUDY : CENTRE DE RECHERCHES BIOLOGIQUES
Chemin de Montifault
18800 BAUGY - FRANCE

RESPONSIBLE STAFF FOR CERB :

Scientific and Technical Director :

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30/06/93 (Date) — Signature

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C. BESSON
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30/06/93 (Date) — Signature

Quality Assurance :

C. VIGIER

28/06/93 (Date) — Signature

The authentic results of the experiment form the basis of the present report.

This report consists of 42 pages numbered from 1 to 42 including 5 appendices.

CONTENTS

I. SUMMARY

Study of the sensitizing capacity of compound [redacted] was undertaken in the Guinea Pig using the technique of MAGNUSSON and KLIGMAN in comparison with a negative control group receiving water for injection and a positive control group receiving dinitrochlorobenzene (D.N.C.B., 1 %).

The maximum slightly irritant concentration determined by intradermal administration and used during the primary induction phase was compound [redacted] diluted 3/4 in water for injection.

The maximum non-irritant concentration (M.N.I.C.) determined by epicutaneous administration and used during the sensitization and challenge phases was compound [redacted] diluted 1/2 in water for injection.

Under the experimental conditions adopted, 1 % D.N.C.B. showed a degree of allergenicity class V at 24 hours. Its sensitizing capacity was very strong at 24 hours in the Guinea Pig.

Water for injection showed a degree of allergenicity of class I at 24 hours. It may be considered as being free of any sensitizing capacity in the Guinea Pig.

Compound [redacted] showed a degree of allergenicity of class I at 24 hours.

Under the experimental conditions adopted, compound [redacted] may be considered as being free of any sensitizing capacity in the Guinea Pig.

II. EXPERIMENTAL PROTOCOL ADOPTED

II.1 AIM :

The aim of this study was to evaluate the possible delayed sensitization capacity of compound [redacted]. The experimental method was inspired of MAGNUSSON - KLIGMAN (1969) and GUILLOT (1983).

II.2 METHOD :

The method described can be applied to all compounds in liquid, or semi-liquid. The protocol complied with the requirements of O.E.C.D. Guideline No. 406 (May 12, 1981).

The protocol n° 92.04.10.05 signed by the legal responsible for the sponsor of the study and by the experimenters may be found in appendix 1.

The study was carried out according to Good Laboratory Practices as published by :

- **the French Ministry of Social Affairs and National Solidarity : State Secretariat for Health.**
  Guideline concerning Good Laboratory Practices (G.L.P.) in the area of experimental toxicology.
  Guideline dated May 31, 1983, official text No. 1065 ; reference SN-S83/25.
- **E.E.C. Directive 87/18. This directive refers to recommendation CC81/30 Appendix 2 of the O.E.C.D.**
- **Food and Drug Administration : G.L.P. 21 C.F.R. Part 58 dated December 22, 1978 and amendments of April 11, 1980 and September 4, 1987.**

II.3 COMPOUND :

The analytical certificate concerning the compound is supplied with the study report in Appendix 3.

- Name : [redacted]
- Batch No. : [redacted]
- Record number (CAL) : [redacted]
- Origin : ELF ATOCHEM SA
- Date received : Saturday April 18 1992
- Amount : 1 bottle of 107 g
- Appearance : [redacted]
- pH : [redacted]

II.4 GENERAL CHARACTERISTICS OF STUDIES :

II.4.1 ANIMALS :

- SPECIES : Hartley strain albino Guinea Pigs.
- ORIGIN : INTERFAUNA FRANCE S.A.R.L. -les Sillons" - 37600 SAINT-JEAN-SAINT- GERMAIN specialized breeding station.
- WEIGHT : Male animals had a mean weight of 586 g ± 32 g, and females a mean weight of 513 g ± 21 g on the day of randomization (D-1).
- SEX : Males and females.
  Females were nulliparous and non-gravid.
- NUMBER :

  Preliminary studies : 8

  Intradermal administration : 4 (2 males + 2 females)
  Epicutaneous administration : 4 (2 males + 2 females).

  Final study : 40 animals divided into three groups :

  - 10 negative controls (5 males + 5 females)
  - 10 positive controls (5 males + 5 females)
  - 20 treated with the study compound (10 males + 10 females)
- DATE RECEIVED : On 22.04.1992.
- ACCLIMATIZATION : for 13 days before the study in the area where the experiment was to take place.
- IDENTIFICATION : They were identified per cage, individually by an aqueous solution of picric acid tagging.

- HOUSING : Five male or female animals from each treatment group were kept per cage of standard size, on dust-free white wood shavings as bedding.

  These cages were placed in an air-conditioned animal house (17°C-21°C) kept at a constant relative humidity of between 45 % and 65 %, except during cleaning periods in which non-recycled filtered air was changed approximately ten times per hour. The artificial daylight cycle was 12 hours light and 12 hours darkness.

- FOOD : UAR 106 special Guinea Pig foodstuff. An analytical certificate concerning foodstuff may be found in Appendix 4.

- DRINKING WATER : Tap water distributed "ad libitum" in polycarbonate feed bottles with a stainless steel teat. A water sample was obtained every three months and sent to the Direction des Services Vétérinaires, 216, rue Louis Mallet, 18014 BOURGES Cedex, FRANCE for analysis. The water analytical certificate in Appendix 5 corresponds to the sample obtained on the date closest of the start of the study.

II.4.2 PRINCIPLE :

Animals were treated three times (Figure 1).

The test consisted of three phases :

- INDUCTION : first contact between the body and compound [redacted]
- SENSITIZATION : second contact with compound [redacted] Recognition of allergen and multiplication of cellular and/or humoral transformations.
- CHALLENGE : third contact with compound [redacted] leading or not to the finding of a clinical manifestation.

II.5 EXPERIMENTAL PROTOCOL :

II.5.1 PRE-EXPERIMENTAL PROCEDURE :

- PREPARATION OF ANIMALS : the day prior to each application Guinea Pigs used were shaved using AESCULAP FAVORITA II GT 104 clipper. An area of approximately 24 cm^2 was exposed on the back, behind the head, at the level of the shoulders and flanks.
- SELECTION OF ANIMALS : only healthy animals, with a skin free of any trace of cutaneous lesions, were selected.

II.5.2 PRELIMINARY STUDIES :

II.5.2.1 Intradermal administration :

Determination of the maximum concentration causing slight irritation (with neither scab nor necrosis) in all animals by intradermal injection.

- Animals : 2 males and 2 females.

- Treatment :

Intradermal injections were administered in the dorsal region using 0.1 ml of compound [redacted] or of its dilutions. Injections were administered at 4 sites using one concentration per site, i.e. 4 concentrations per animal. Water for injection was used as a non-irritant and non-sensitizing vehicle.

- Concentration : concentrations selected were compound [redacted] undiluted and diluted 3/4, 1/2 and 1/4.

- Grading of results : Lesions were evaluated for each concentration 24 hours after injections using the scale published by MAGNUSSON and KLIGMAN :

No reaction	0
Slight erythema (scarcely visible)	1
Moderate erythema (clearly visible)	2
Intense erythema with edema	3

II.5.2.2 Epicutaneous application :

a. Determination of M.N.I.C. (Maximum Non-Irritant Concentration) by epicutaneous application under an ELASTOPLASTE® non occlusive dressing.

- Animals : 2 males and 2 females.

- Treatment :

Epicutaneous application of 0.5 ml of compound [redacted] or of its dilution over an area of 8 cm² using one concentration on each flank, i.e. two concentrations per animal.

- Concentrations :

Compound [redacted] was applied undiluted or diluted 1/2 in water for injection to the skin prepared for this purpose. The piece of gauze on which the compound or its dilution was deposited was held in place for 48 hours using an ELASTOPLASTE® non-occlusive dressing.

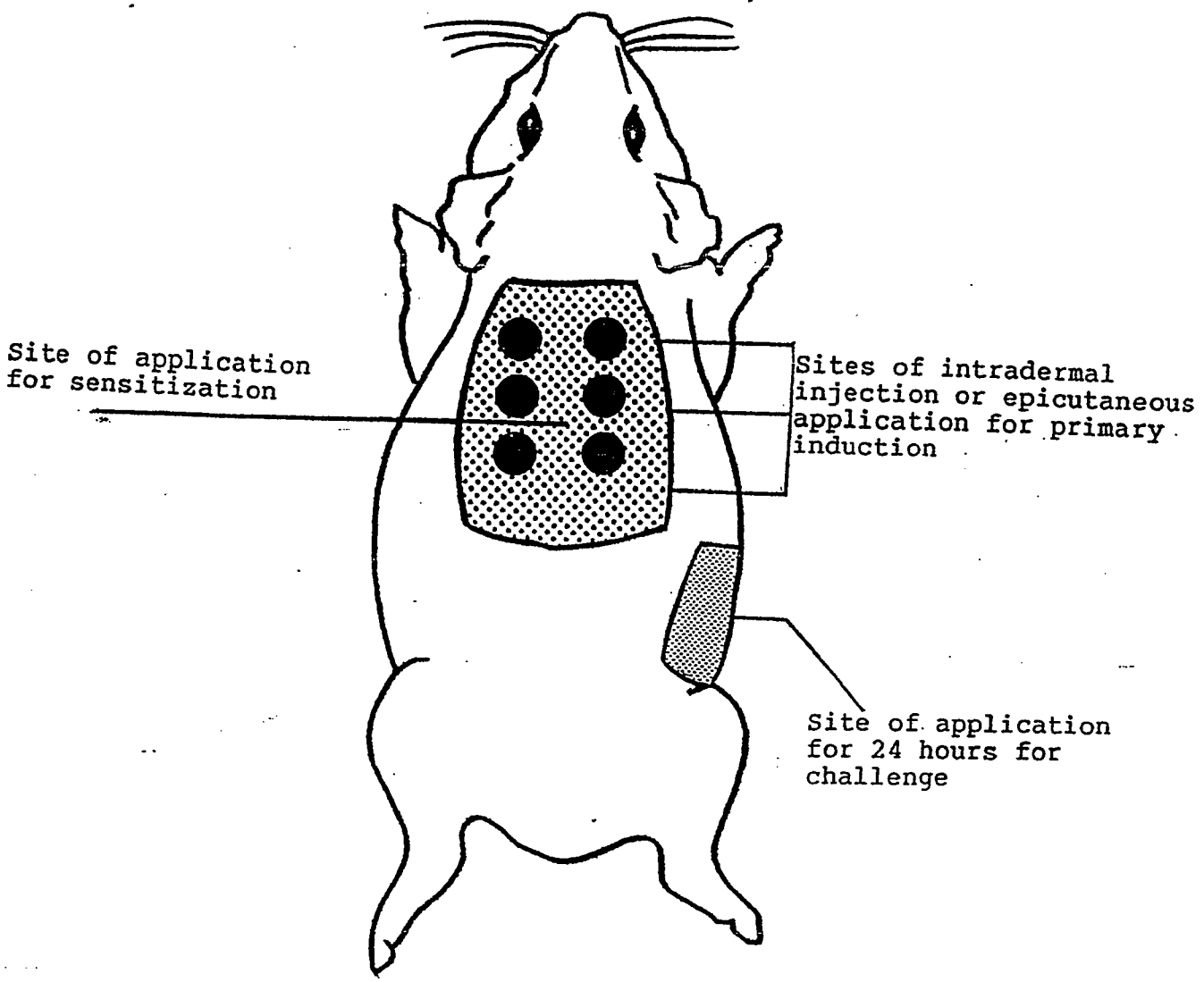
- Grading of results : lesions were evaluated for each concentration one hour after removal of dressings using the scale already described in II.5.2.1.

The maximum slightly irritant concentration determined by intradermal administration was used during the primary induction phase.

The maximum non-irritant concentration (M.N.I.C.) determined by epicutaneous administration was used during the sensitization and challenge phases.

FIGURE N° 1

SITES OF ADMINISTRATION



II.5.3 FINAL STUDY :

II.5.3.1 Study design :

The study design called for the use of 40 animals (20 of each sex) randomized into 3 groups on the basis of the criterion of body weight and selected according to their general condition.

The 3 groups of animals involved in the study consisted of :

1. A negative control group of 5 animals of each sex, used to confirm that under the experimental conditions adopted, the appearance of skin lesions in the treated group in the absence of any lesion in the negative control group was indeed evidence of an allergic reaction.

2. A positive control group of 5 animals of each sex, serving to validate the sensitivity of the method used. This confirmed that D.N.C.B., a compound recognized as being allergenic in the Guinea Pig, fully exerted its properties under the experimental conditions adopted here.

3. A group treated with compound [redacted] of 10 animals of each sex, used to determine whether compound [redacted] had allergenic properties under the experimental conditions adopted here.

II.5.3.2 Primary induction (time D1) :

The induction phase took place on D1, the first day of the trial. Each Guinea Pig received 6 injections in the retroscapular region on either side of the vertebral column, using a previously shaved area of approximately 24 cm^2 (4 cm x 6 cm).

Two intradermal injections of 0.1 ml were administered with three different preparations.

TREATMENT OF NEGATIVE CONTROLS :

- Sites 1 : 0.1 ml of complete FREUND's adjuvant diluted 50 % in sterile and pyrogen-free isotonic sodium chloride solution, per site.
- Sites 2 : 0.1 ml of water for injection, per site.
- Sites 3 : 0.1 ml of an emulsion of equal volume of water for injection and of complete FREUND's adjuvant diluted 50 % in sterile and pyrogen-free isotonic sodium chloride solution, per site.

TREATMENT OF POSITIVE CONTROLS :

- Sites 1 : 0.1 ml of complete FREUND's adjuvant diluted 50 % in sterile and pyrogen-free isotonic sodium chloride solution, per site.
- Sites 2 : 0.1 ml of 1 % D.N.C.B., per site.
- Sites 3 : 0.1 ml of an emulsion of equal volume of 1 % D.N.C.B. and of complete FREUND's adjuvant diluted 50 % in sterile and pyrogen-free isotonic sodium chloride solution, per site.

TREATMENT OF TREATED ANIMALS :

- Sites 1 : 0.1 ml of complete FREUND's adjuvant diluted 50 % in sterile and pyrogen-free isotonic sodium chloride solution, per site.
- Sites 2 : 0.1 ml of compound [redacted] at the maximum slightly irritant concentration determined during the preliminary study, per site.
- Sites 3 : 0.1 ml of an emulsion of equal volume of complete FREUND's adjuvant diluted 50 % in sterile and pyrogen-free isotonic sodium chloride solution and of compound [redacted] at the maximum slightly irritant concentration determined during the preliminary study, per site.

II.5.3.3 Sensitization (time D9) :

- Preparatory phase (time D8) : creation of local irritation.

On D8, topical application of 0.5 ml of a 10% suspension of sodium lauryl sulfate in paraffin oil was performed to previously shaved skin, at 6 injection sites of D1.

- **Actual sensitization** (time D9):

Sensitization involved cutaneous application at injection sites of D1.

Negative controls received 0.5 ml of water for injection by cutaneous application on a piece of absorbant gauze measuring 8 cm^2. The application site was protected by a piece of absorbant gauze measuring approximately 24 cm^2 held in place for 48 hours by an ELASTOPLASTE® non-occlusive dressing.

Positive controls received 0.5 ml of 1 % D.N.C.B. solution under the same conditions.

Treated animals received under the same conditions 0.5 ml of compound [redacted] at the maximun non-irritant concentration determined during the preliminary study.

- **Rest phase** : D11 to D21.

Animals were left at rest from D11 to D21, i.e. for 11 days.

II.5.3.4 Challenge (time D22) :

On D22, 0.5 ml of compound [redacted] at the maximun non-irritant concentration determined during the preliminary study were applied topically to the right lateral abdominal region, in an area never previously in contact with the compound.

Application was as described at time D9 to previously shaved skin.

The piece of gauze was held in place for 24 hours using an ELASTOPLASTE® non-occlusive dressing.

Under the same conditions as above, negative control animals received water for injection. Positive control animals received 1 % D.N.C.B. solution.

II.6 CLINICAL MONITORING OF ANIMALS :

Animals were monitored daily throughout the study period. This clinical examination was designed to seek any possible abnormalities due to treatment.

In case of mortality, the cause was defined whenever possible.

II.7 READING OF SKIN REACTIONS (times D23, D24 and D25) :

Determination of degree of allergenicity :

Possible skin reactions were studied 6 hours, 24 hours and 48 hours after removal of the dressing.

Results were graded for each animal and by group using the following scale:

No reaction	0
Slight erythema (scarcely visible)	1
Moderate erythema (clearly visible)	2
Intense erythema with edema.	3

Any other cutaneous abnormality (thickening, vesicles, dryness or other lesions) or any general behavioral changes were recorded daily for each animal using a clinical case report form.

Determination of the degree of allergenicity at time 24 hours was based upon the percentage of animals in the group showing a reaction rather than the severity of the latter.

Classification involving 5 degrees, used the following scale :

Percentage of animals sensitized	Degree of allergenicity (classification)	Sensitizing capacity
0 - 8	I	Very slight
9 - 28	II	Slight
29 - 64	III	Moderate
65 - 80	IV	Strong
81 - 100	V	Very strong

II.8 DATA ENTRY OF RESULTS :

All results were recorded as and when obtained using forms identified by the study number.

II.9 REPORT :

The experimental report included all findings noted during the course of the study, and in particular information concerning :

- observation of animals
- recording of mortality and of its causes
- skin reactions
- classification of the compound [redacted] .

II.10 QUALITY ASSURANCE :

The Quality Assurance Unit ensured that working procedures relative to this type of study were strictly complied with by periodic inspections at random over the course of the year.

Data in the experimental report were audited by the Quality Assurance Unit, in accordance with standard operating procedures at the Centre.

II.11 RECORDS :

The protocol, raw data, the correspondence, the report and a sample of the compound have been stored for five years at CERB - 18800 BAUGY, FRANCE, starting from the date of the final report.

At the end of this period, CERB will contact the sponsor in order to jointly determine to either :

- continue storage of records,
- return the data to the sponsor,
- destroy the data.

III. RESULTS

III.1 DEVIATION FROM PROTOCOL N° 92.04.10.05 :

The weight of males was more than 550 g on the day of randomization (586 g ± 32 g)

III.2 PRELIMINARY STUDIES :

III.2.1 INTRADERMAL ADMINISTRATION :

- Dates of study :

Start: 28.04.1992.
End : 29.04.1992.

The maximum slightly irritant concentration determined by intradermal administration and used during the primary induction phase was compound {[redacted]} diluted 3/4 in water for injection.

III.2.2 EPICUTANEOUS ADMINISTRATION :

- Dates of study :

Start: 28.04.1992.
End : 30.04.1992.

The maximum non-irritant concentration (M.N.I.C.) determined by epicutaneous administration and used during the sensitization and challenge phases was compound {[redacted]} diluted 1/2 in water for injection.

III.3 FINAL STUDY :

III.3.1 DATES :

Induction	:	05.05.1992 (D1)
Preparatory phase	:	12.05.1992 (D8)
Sensitization	:	13.05.1992 (D9)
Challenge	:	26.05.1992 (D22)
Readings	:	27.05.1992 (D23)
		28.05.1992 (D24)
		29.05.1992 (D25)

III.3.2 MONITORING OF ANIMALS :

Animals were subjected to daily clinical monitoring throughout the study period.

On D17, a weight loss was noted in the male No 920341 of the negative control group.
On D18, this animal was found dead in its cage. At necropsy, a great weight loss was noted, organs were in an advanced autolysis stage. The stomach and intestines were empty.

From D18 to D22, the female No 920373 of the group treated with compound [redacted] showed a decrease of motricity due to a paralysis and an edema of hindlegs.

On D23, no symptomatology was recorded.

III.3.3 READING OF SKIN REACTIONS :

Results of findings are summarized in the table overleaf (Table 1).

TABLE 1

DETERMINATION OF DEGREE OF ALLERGENICITY

ALLERGEN	TIME	No. of animals/grade				% of animals sensitized	CLASS
		0	1	2	3		
NEGATIVE CONTROLS (1)	6 H	9	-	-	-	0	-
	24 H	9	-	-	-	0	I
	48 H	9	-	-	-	0	-
POSITIVE CONTROLS (2)	6 H	-	1	9	-	100	-
	24 H	1	6	3	-	90	V
	48 H	6	2	2	-	40	-
FORAPERLE 321	6 H	20	-	-	-	0	-
	24 H	20	-	-	-	0	I
	48 H	20	-	-	-	0	-

(1) water for injection.
(2) 1 % dinitrochlorobenzene.

III.3.4 CLASSIFICATION OF COMPOUND :

Compound [redacted] showed a degree of allergen of class I at 24 hours.

Under the experimental conditions adopted, compound [redacted] may be considered as being free of any sensitizing capacity in the Guinea Pig.

IV. CONCLUSION :

The sensitizing capacity of compound [redacted] was studied in the male and female Guinea Pig using the technique of MAGNUSSON and KLIGMAN, in comparison with a negative control group receiving water for injection and a positive control group receiving 1 % D.N.C.B.

The maximum slightly irritant concentration determined by intradermal administration and used during the primary induction phase was compound [redacted] diluted 3/4 in water for injection.

The maximum non-irritant concentration (M.N.I.C.) determined by epicutaneous administration and used during the sensitization and challenge phases was compound [redacted] diluted 1/2 in water for injection.

Under the experimental conditions adopted, 1 % D.N.C.B. showed a degree of allergenicity of class V at 24 hours. Its sensitizing capacity was very strong at 24 hours in the Guinea Pig.

Water for injection showed a degree of allergenicity of class I at 24 hours. It may be considered as being free of any sensitizing capacity in the Guinea Pig.

Compound [redacted] showed a degree of allergenicity of class I at 24 hours.

Under the experimental conditions adopted, compound [redacted] may be considered as being free of any sensitizing capacity in the Guinea Pig.

APPENDIX 1

Protocol n° 92.04.10.05

CERB - PROTOCOL N° 92.04.10.05

TRIAL : [redacted]

Study of cutaneous sensitization using the MAGNUSSON and KLIGMAN test in Guinea Pig.

SPONSOR : ELF ATOCHEM SA
La Défense 10, Cedex 42
92091 PARIS LA DEFENSE

SITE OF TRIAL : CENTRE DE RECHERCHES BIOLOGIQUES
Chemin de Montifault
18800 BAUGY, FRANCE

APPROBATION :

SPONSOR CONTACT :

Mr REGNIER

22/04/92 ---------- Date ---------- Signed

RESPONSIBLE STAFF FOR CERB :

Scientific and Technical Director :

S. RICHARD

16/04/92 ---------- Date ---------- Signed

Head of Toxicology Department :

C. AUDEVAL-GERARD

16/04/92 ---------- Date ---------- Signed

Study Director :

C. BESSON

14/04/92 ---------- Date ---------- Signed

Quality Assurance :

C. VIGIER

16/04/92 ---------- Date ---------- Signed

[redacted]

STUDY OF CUTANEOUS SENSITIZATION USING THE MAGNUSSON AND KLIGMAN MAXIMIZATION TEST IN GUINEA PIG

I - AIM :

The aim of this study is to evaluate the possible delayed sensitization capacity of compound [redacted]. The experimental method is inspired of MAGNUSSON - KLIGMAN (1969) and GUILLOT (1983).

II - METHOD :

The method described can be applied to all compounds in liquid, or semi-liquid. The protocol complies with the requirements of OECD Guideline No. 406 (May 12, 1981).

The study is carried out according to Good Laboratory Practices as published by :

- **the French Ministry of Social Affairs and National Solidarity : State Secretariat for Health.**
  Guideline concerning Good Laboratory Practices (G.L.P.) in the area of experimental toxicology.
  Guideline dated May 31, 1983, official text No. 1065 ; reference SN-S83/25.
- **E.E.C. Directive 87/18. This directive refers to recommendation CC81/30 Appendix 2 of the O.E.C.D.**
- **Food and Drug Administration : G.L.P. 21 C.F.R. Part 58 dated December 22, 1978 and amendments of April 11, 1980 and September 4, 1987.**

III - COMPOUND : [redacted]

DATA SUPLIED BY THE SPONSOR :

The substance information sheet and the analytical certificate were received on April 10 1992.

DATA TO BE SUPPLIED BY THE SPONSOR :

Please fill in the attached substance information form.

AMOUNT OF SUBSTANCE TO BE SUPPLIED :

50 ml of substance [redacted] will be necessary for carrying out this study.

IV GENERAL CHARACTERISTICS OF STUDIES :

IV.1 ANIMALS :

- SPECIES : Hartley strain albino Guinea Pigs.
- ORIGIN : From a specialized breeding station.
- WEIGHT : Between 350 g and 550 g on the day of randomization (D-1).
- SEX : Males and females.
  Females were nulliparous and non-gravid.
- NUMBER :

Preliminary studies : 4 males, 4 females.

Final study : 20 males, 20 females.

- ACCLIMATIZATION : for at least 5 days before the study in the area where the experiment is to take place.
- IDENTIFICATION : the animals are identified by an aqueous solution of picric acid tagging.
- HOUSING : Five male or female animals from each treatment group are kept per cage of standard size, on dust-free white wood shavings as bedding.

  These cages are placed in an air-conditioned animal house (17°C-21°C) kept at a constant relative humidity of between 45 % and 65 %, except during cleaning periods in which no recycled filtered air is changed approximately ten times per hour. The artificial daylight cycle is 12 hours light and 12 hours darkness.
- FOOD : UAR 106 foodstuff. An analytical certificate concerning foodstuff is included in the study report.

- DRINKING WATER : Tap water distributed "ad libitum" in polycarbonate feed bottles with a stainless steel teat. A water sample is obtained every three months and sent to the Direction des Services Vétérinaire, 216, rue Louis Mallet, 18014 BOURGES Cedex, FRANCE for analysis. A water analytical certificate is included the study report.

IV-2 PRINCIPLE :

The test consists of 3 phases.

- INDUCTION : first contact between the body and compound [redacted]
- SENSITIZATION : second contact with compound [redacted] Recognition of allergen and multiplication of cellular and/or humoral transformations.
- CHALLENGE : third contact with compound [redacted] leading or not to the finding of a clinical manifestation.

V - EXPERIMENTAL PROTOCOL :

V.1 PRE-EXPERIMENTAL PROCEDURE :

- PREPARATION OF ANIMALS :
  The day prior to each application Guinea Pigs used are shaved using AESCULAP FAVORITA II GT 104 clipper. An area of approximately 24 cm^2 is exposed on the back, behind the head, at the level of the shoulders and flanks.
- SELECTION OF ANIMALS :
  Only healthy animals, with a skin free of any trace of cutaneous lesions, are selected.

V-2. PRELIMINARY STUDIES :

- **Intradermal administration :**

Determination of the maximum concentration causing slight irritation (with neither scab nor necrosis) in all animals by intradermal injection.

- Animals : 2 males and 2 females.

- Treatment : Intradermal injections are administered in the dorsal region using 0.1 ml of compound or of its dilutions. Injections are administered at 4 sites using one concentration per site, i.e. 4 concentrations per animal in a non-irritant and non-sensitizing vehicle.

- Concentration : concentrations used will be undiluted substance as well as 1/4, 1/2, 3/4 dilutions of the finished product.
  Dilutions will be subsequently increased in a second phase if the substance is found to be irritant at these concentrations.

- Grading of results : Lesions are evaluated for each concentration 24 hours after injections using the scale published by MAGNUSSON and KLIGMAN :

No reaction	0
Slight erythema (scarcely visible)	1
Moderate erythema (clearly visible)	2
Intense erythema with edema	3

- **Epicutaneous application :**

Determination of M.N.I.C. (Maximum Non-Irritant Concentration) by epicutaneous application under an ELASTOPLASTE® non occlusive dressing.

- Animals : 2 males and 2 females.

- Treatment : 0.5 ml of compound or of its dilution are deposited on a piece of gauze of approximately 8 cm^2 (4cm x 2 cm). The piece of gauze is applied to the skin at the level of the flanks and held in place for 48 hours using an ELASTOPLASTE® non-occlusive dressing.

- Concentrations : Compound is applied undiluted or diluted 1/2 in the chosen vehicle. Then, the dilutions are increased if the compound is irritant at these concentrations.

- Grading of results : lesions are evaluated for each concentration one hour after removal of dressings using the scale already described in V-2.

The maximum slightly irritant concentration determined by intradermal administration is used during the primary induction phase.

The maximum non-irritant concentration (M.N.I.C.) determined by epicutaneous administration is used during the sensitization and challenge phases.

V-3 FINAL STUDY :

V-3.1 Study design :

The study design calls for the use of 40 animals (20 of each sex) randomized into 3 groups on the basis of the criterion of body weight and selected according to their general condition.

The 3 groups of animals involved in the study consist of :

1. A negative control group of 5 animals of each sex, used to confirm that under the experimental conditions adopted, the appearance of skin lesions in the treated group in the absence of any lesion in the negative control group is indeed evidence of an allergic reaction.

2. A positive control group of 5 animals of each sex, serving to validate the sensitivity of the method used. This confirms that D.N.C.B.*, a compound recognized as being allergenic in the Guinea Pig, fully exerts its properties under the experimental conditions adopted here.

3. A group treated with compound [redacted] of 10 animals of each sex, used to determine whether compound [redacted] has allergenic properties under the experimental conditions adopted here.

V.3.2 Primary induction (time D1) :

The induction phase takes place on D1, the first day of the final study. Each Guinea Pig receives 6 injections in the retroscapular region on either side of the vertebral column, using a previously shaved area of approximately 24 cm^2 (4 cm x 6 cm).

* D.N.C.B. : Dinitrochlorobenzène (chloro 1-dinitro 2.4-benzene).
Use of D.N.C.B. : D.N.C.B. is used in 1 % alcoholic solution.

- Injection technique : two intradermal injections of 0.1 ml are administered for each of the three following preparations, i.e. one injection per site :
  . Complete Freund adjuvant diluted 50% in sterile and pyrogen-free isotonic sodium chloride solution.
  . Vehicle for the negative control group or D.N.C.B. for the positive control group, or substance [redacted] for the group treated with the maximum slightly irritant concentration, if this concentration has been determined in preliminary experiments,
  . 50/50 (v/v) mixture of complete FREUND's adjuvant diluted 50% in sterile and pyrogen-free isotonic sodium chloride solution, with the addition of the vehicle for the negative control group or of D.N.C.B. for the positive control group or of substance [redacted] for the group treated with the maximum slightly irritant concentration previously adopted.

V.3.3 Sensitization (time D9) :

Preparatory phase (time D8) :

- Creation of local irritation.

On D8, topical application of 0.5 ml of a 10 % suspension of sodium lauryl sulfate (dodecylhydrogenosulfate sodium salt) in paraffin oil was performed to previously shaved skin, at 6 injection sites of D1.

Actual sensitization (time D9) :

Sensitization involves cutaneous application at 6 injection sites of D1. Negative controls receive 0.5 ml of vehicle by cutaneous application. Positive controls receive 0.5 ml of 1 % D.N.C.B. solution under the same conditions. Treated animals receive under the same conditions 0.5 ml of compound [redacted] at the maximun non-irritant concentration (CMNI).

Application method :

0.5 ml of substance [redacted] are deposited on a piece of gauze of approximately 8 cm² (4 cm x 2 cm). The piece of gauze is applied to the skin and held in place for 48 hours using an ELASTOPLASTE® non-occlusive dressing.

- **Rest phase** : D11 to D21.

Animals are left at rest from D11 to D21, i.e. for 11 days.

Challenge (time D22) :

On D22, 0.5 ml of compound [redacted] at the maximun non-irritant concentration determined during the preliminary study are applied topically to the right lateral abdominal region, in an area never previously in contact with the compound.

Application is performed as described at time D9 to previously shaved skin.
The piece of gauze is held in place for 24 hours using an ELASTOPLASTE® non-occlusive dressing.

Application method :

Application is as described at time D9, to previously shaven skin.

The piece of gauze is kept in place by a non-occlusive ELASTOPLAST® tape for 24 hours.

VI CLINICAL MONITORING OF ANIMALS :

Animals are monitored daily throughout the study period. This clinical examination is designed to seek any possible abnormalities due to treatment.

In case of mortality, the cause is defined whenever possible.

VII READING OF SKIN REACTIONS (times D23, D24 and D25) :

Possible skin reactions are studied 6 hours, 24 hours and 48 hours after removal of the dressing.

Results are graded for each animal and by group using the following scale:

No reaction	0
Slight erythema (scarcely visible)	1
Moderate erythema (clearly visible)	2
Intense erythema with edema	3

Any other cutaneous abnormality (thickening, vesicles, dryness or other lesions) or any general behavioral changes are recorded daily for each animal using a clinical case report form.

Determination of the degree of allergenicity is based upon the percentage of animals in the group showing a reaction rather than the severity of the latter.

Classification involving 5 degrees, uses the following scale :

Percentage of animals sensitized	Classification	Sensitizing capacity
0 - 8	I	Very slight
9 - 28	II	Slight
29 - 64	III	Moderate
65 - 80	IV	Strong
81 - 100	V	Very strong

VIII - REFERENCES :

GUILLOT (J.P), GONNET (J.F), CLEMENT (C.) et FACCINI (J.M.)
Etude comparative de différentes méthodes choisies par l'AFNOR pour l'évalutation du pouvoir sensibilisant chez le Cobaye.
Informations Chimie, 1982, 228, 1177-192.
Comparative study of the different methods chosen by the AFNOR for the evaluation of the sensitizing potential in the guinea-pig.
Food an Chemical Toxicology, 1983, 21 (n°6), 795-805.

MAGNUSSON (B) & KLIGMAN (A.M.)
The identification of contact allergens by animal assay.
The guinea-pig maximization test.
J. Invest. Derm., 1969, 52, 268-276.

IX - RESULTS :

All results are recorded as and when obtained using forms identified by the study number.

X - REPORT :

The experimental report includes all findings noted during the course of the study, and in particular information concerning :

- observation of animals
- recording of mortality and of its causes
- skin reactions
- classification of studied compound [redacted]

XI - QUALITY ASSURANCE :

The Quality Assurance Unit ensures that working procedures relative to this type of study are strictly complied with by periodic inspections at random over the course of the year.

Data in the experimental report are audited by the Quality Assurance Unit, in accordance with standard operating procedures at the Centre.

XII RECORDS :

The protocol, any possible amendments, raw data, the correspondence, the report and a sample of the compound are stored for five years at CERB - 18800 BAUGY, FRANCE, starting from the date of the final report.

At the end of this period, CERB will contact the sponsor in order to jointly determine to either :

- continue storage of records,
- return the data to the sponsor,
- destroy the data.

XIII - PLANIFICATION :

Date of start of study : week of April 27 1992.
Results by telex : at the end of the study.
Date of submission of draft report : at the end of June 1992.
Date of submission of the English summary : at the final report submission.

APPENDIX 2

References and batch numbers of
reagents and equipment used

REFERENCES AND BATCH NUMBERS OF REAGENTS ANS EQUIPMENT USED

REAGENTS :

Complete FREUND's adjuvant : SIGMA - Reference F-4258 of batch 106 F-8860.

Sterile and pyrogen-free isotonic sodium chloride solution : MERAM of batch 56243.

D.N.C.B. : Dinitrochlorobenzene (1-chloro-2,4-dichlorobenzene).
D.N.C.B. was used in 1 % alcoholic solution.
SIGMA C-3762 of batch 10 H 05341.

90% (V/V) ethanol, Cooper of batch A 245589090152.

PEG 400 : polyoxyethylene glycol 400 : COOPER of batch A2322091130.

Sodium lauryl sulfate (sulfuric acid monododecyl ester sodium salt) : Merck - Reference 13760 of batch 029 L 765960.

Paraffin oil : COOPER reference 3108.

COMPOUND SOLVENT :

MERAM water for injection of batchs 56671 and 56663.

EQUIPMENT :

Absorbant gauze : SHV type 17 fils reference 010118RA.

ELASTOPLASTE® REF. 6 HB : of batchs 20775S and 1423S.

Syringes Terumo 1 ml of batch 91H19B5.
Terumo 5 ml of batch 91C29C5.
Terumo 10 ml of batch 91 C13S7.
Sherwood 2 ml of batch 91E022.
Henke - sass 2 ml of batch 9129451.

Needles Terumo code NN-2125R of batch 8891G10G.

APPENDIX 3

Technical data and analytical certificate
concerning compound

SERVICE DE TOXICOLOGIE
CONFIDENTIEL
Avril 1992

elf atochem sa
La Défense 10, cédex 42
92091 Paris-La Défense
France

FICHE D'INFORMATIONS

[redacted]

IDENTIFICATION

Nom commun : [redacted]
Nom chimique :

Origine; n° de lot :
N° d'archivage (CAL) :

PROPRIETES PHYSICO-CHIMIQUES

Apparence : [redacted]
Viscosité :
Température ébullition :
pH :
Densité :
Point d'éclair :
Solubilité :

INFORMATIONS TOXICOLOGIQUES ET PRECAUTION D'EMPLOI

Aucune information disponible.

CONDITIONS DE CONSERVATION ET DE DESTRUCTION

Conservation	: A l'obscurité et à l'abri de la chaleur.
Stabilité	: Stable jusqu'en Avril 1993 dans ces conditions de stockage
Destruction	: Incinération.

elf atochem sa

4, cours Michelet, cedex 42
92091 Paris La Défense 10
France

ATO

BULLETIN D'ANALYSE

[redacted]

[redacted]

8 Avril 1992

Jean-François Régnier

Service de Toxicologie

APPENDIX 4

Analytical certificate concerning foodstuff

ALIMENT COMPLET

COBAYES - ENTRETIEN

Granulés : 4,5 mm

Ration journalière du Cobaye : en fonction de son âge et poids, de 35 à 50 g par jour. — Eau à v

FORMULE %

Céréales, sucre	44,50
Issues et Légumineuses	40
Protéines Végétales (Tourteaux, Levure)	9,50
Protéines Animales (Viande)	2
Composé Minéral Vitaminisé	4

ANALYSE MOYENNE %

Valeur calorifique (en Cal/kg)	2.600
Eau	10
Protides	17
Lipides	3
Glucides (E.N.A.)	49
Cellulose (Weende)	13
Minéraux	8

ACIDES AMINES

(calculés en mg/kg)

Arginine	8.500
Cystine	2.500
Lysine	7.200
Méthionine	2.100
Tryptophane	2.000
Glycine	6.000

MINERAUX

(calculés en mg/kg)

	App. Nat. (moy.)	App. per CM	TOTAUX
P	7.400	1.400	8.800
Ca	5.400	5.600	11.000
K	12.000	0	12.000
Na	1.300	1.950	3.250
Mg	3.270	130	3.400
Mn	60	40	100
Fe	170	150	320
Cu	10	15	25
Zn	40	45	85
Co	0,10	1,50	1,60
I	apporté sous forme assimilable par algues marines.		

VITAMINES

(calculées au kg)

	App. Nat. (moy.)		App. Synthét.		TOTAUX	
Vitam. A	3.400	UI	6.000	UI	9.400	UI
" D 3	30	"	2.000	"	2.030	"
" B 1	6	mg	6,40	mg	12,40	mg
" B 2	5	"	6,40	"	11,40	"
" B 3	22	"	26	"	48	"
" B 6	0,70	"	2,70	"	3,40	"
" B 12	0,003	"	0,012	"	0,015	"
" C	0	"	160	"	160	"
" E	15	"	60	"	75	"
" K 3	5	"	12,60	"	17,60	"
" PP	97	"	14,50	"	111,50	"
Ac. Folique	2,20	"	1,30	"	3,50	"
Ac. P.A.B.		"	2,50	"	2,50	"
Biotine	0,02	"	0,06	"	0,08	"
Choline	1.010	"	60	"	1.070	"
Meso-Inositol		"	62,50	"	62,50	"

La supplémentation de cet aliment avec de la vitamine C stabilisée enrobée supprime la nécessité de tout autre apport (verdure, acide ascorbique) pendant les deux premiers mois suivant fabrication.

U.A.R. 7, Rue Gallieni - 91 - VILLEMOISSON-SUR-ORGE - Tél. (1) 016.13.69 - (1) 016.21.

APPENDIX 5

Analytical certificate concerning water

Conseil Général du Cher

LABORATOIRE DÉPARTEMENTAL VÉTÉRINAIRE

agréé par le Ministère de la santé

RESULTAT D'ANALYSE D'EAU

N°d'Analyse: 920794 N° d'Ordre: 1236

*Type d'analyse: C7B3

Prélèvement d'eau parvenu le: Lundi 11 Mai 1992

Effectué par: C.E.R.B. Agent:

Origine :

Lieu: ZONE PROTEGEE LAPIN COULOIR PROPRE

Commune: BAUGY

Physicochimie	Résultats	Normes*
Turbidité en U Jackson	0,3	< 2 U
Conductivité µS/cm	652	.....
PH	7,5	6,5-9
Oxydabilité KMnO4 mg/l	1	< 5
Dureté en °Français	37	.....
T.A.C en °Français	25	.....
Ammoniaque en mg/l	0	< 0,5
Nitrites en mg/l	0	< 0,1
Nitrates en mg/l	57	< 50
Sulfates en mg/l	65	< 250
Chlorures en mg/l	20	< 200
Fer en mg/l	0	< 0,2
Manganèse en mg/l		< 0,05
Fluor en mg/l		< 1,5
		.....
		< 5

Bactériologie	Résultats	Normes*
Coliformes thermotolérants/100ml	0	0
Streptocoques fécaux/100ml	0	0
Coliformes/100ml	2	0-95%
Dénombrement des bactéries aérobies revivifiables à 37°C/ml	<1	.....
- - à 22°C/ml	2	.....
Spores de bactéries anaérobies sulfito-réductrices/20ml	0	≤1
- - /100ml		0
Salmonelles		0
Staphylocoques pathogènes		0
		0

*:Selon decret N°89-3 et suivants.

Bourges,le: Lundi 18 Mai 1992

Adj. Dir. du Laboratoire

Erick PRENGERE

Le Directeur du Laboratoire

Jean Marie GUERAUD