OECD Guideline for the Testing of Chemicals -

Draft Documents

D R A F T

DRAFT PROPOSAL
(February 1995)

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

Acute Dermal Photoirritation Screening Test

INTRODUCTION

1. Information derived from the dermal photoirritation test serves to indicate the existence of possible hazards likely to arise from topical application to skin of the test substance in association with exposure to light. Compounds administrated systemically - and which may then be distributed in the body, including the skin - may also induce photoirritant reactions when the skin is irradiated, but are not the subject of this guideline.

2. This guideline can be used to determine the potential of a substance to induce skin irritation following single percutaneous treatment of the animals used in the test.

3. A definition of dermal photoirritation is given in Annex 1

INITIAL CONSIDERATIONS

4. In the assessment and evaluation of the toxic characteristics of a chemical the determination of photoirritation may be relevant. However, photoirritation testing in animal should only be carried out after initial information on dermal irritation/corrosion has been obtained by *in vitro* tests or acute testing (1, 2).

5. In the interest of animal welfare, it is important that the unnecessary use of animals is avoided, and that any testing which is likely to produce severe responses in animals is minimized. Consequently, test substances meeting any of the following criteria should not be tested in animals for acute dermal photoirritation:

i) test substances that have predictable corrosive potential based on structure-activity relationships and/or physicochemical properties such as strong acidity or alkalinity, e.g., when the material to be applied has a pH of 2 or less or 11.5 or greater (alkaline or acidic reserve (3) should also be taken into account);

ii) test substances which have been shown to be highly toxic by the dermal route; and,

iii) test substances which have not shown absorption of light in the range of 310-420 nm.

6. In addition, it is strongly recommended that photoirritation testing should start with *in vitro* test(s). Subsequently, it may not be necessary to test *in vivo* test substances for which photoirritation has been demonstrated *in vitro*. However, this animal screening test could be used to confirm negative results from *in vitro* photoirritation test(s). (See Annex 2 : Sequential Approach to Photoirritation Testing)

7. The sensitivity of the screening test must be checked by means of a positive control study with a selection of known photoirritants of different compound classes and with different photoirritation

mechanisms, such as naturally occurring psoralens (8-methoxypsoralen (8-MOP), CAS Number 298-81-7); dyes (acridine); coal-tar derivatives (anthracene); and, drugs (phenothiazine). Other positive controls suitable for the category of compound being evaluated may also be used. Considering the stability of the compound, it may be useful to prepare the positive control preparation on the day of use.

8. When photoirritation testing is being conducted on a routine basis and no changes are made in the experimental design or selection of animals, it should be sufficient to include the positive control study only at a certain interval, e.g. once a year.

PRINCIPLE OF THE TEST

9. Information derived from this acute dermal photoirritation screening test serves to indicate possible hazards likely to arise from topical application of a test substance to the skin in combination with exposure to ultraviolet (UV)-light.

10. The differences in reactions between the irradiated and the non-irradiated skin site of the test animals are evaluated in order to establish whether or not the test substance possesses photoirritation potential under the conditions of this test.

DESCRIPTION OF THE METHOD

Selection of animal species

11. Although several mammalian species including guinea pigs or rats can be used, the albino rabbit is the preferred species, unless it can be expected that the test substance is also likely to be tested for potential photosensitization. In that situation, use of the guinea pig for this test would be preferred. The rationale for the choice of preferred species involves some of the following considerations:

- Albino rabbits are considered as the most suitable species for assessing acute dermal irritation properties of compounds;

- In this manner results of the acute dermal irritation/corrosion test (2) can be of use for comparative purposes in order to establish the concentration to be applied in the acute dermal photoirritation screening test; in a similar way, if additional study of the test substance for photosensitization is likely, the use of the guinea pig as the species of choice would allow comparison of the results of the photoirritation test with those studies;

- On the large skin surfaces on the back and flanks of rabbits and, to a lesser extent, guinea pigs, several substances can be tested simultaneously limiting the number of animals used for screening tests; in addition, each animal can serve as its own control;

- Rabbits that have already been used for acute eye irritation testing, without a severe response, can also be employed; and,

- Also alternative species for which background data exist, such as rats or mice, can also be used with this test. Due to their smaller body surface area, the experimental design will need to be modified and would probably require an increase in the number of animal used.

D R A F T

Housing and feeding conditions

12. The temperature in the experimental animal room should be 20 ± 3 °C for rabbits (or 22 ± 3 °C for rodents including the guinea pig). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The animals should be housed individually.

Preparation of the animals

13. Health young adult animals, with a body weight range of 2000-3500 g (albino rabbit) or 300-500 g (guinea pig), are randomly assigned to the test. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatization to the laboratory conditions.

The fur on the back and flanks of the animals selected for the study should be removed by clipping or shaving within 24 hours before testing. Care should be taken to avoid abrading of the skin. Some strains of rabbit have dense patches of hair that are more prominent at certain times of the year. Such areas of dense hair growth should not be used as test or control sites.

14. Only animals with healthy intact skin should be used. Animals with cutaneous irritation or skin lesions must not be used.

15. The entire trunk of each animal is wrapped with a non-irritating, self-adhesive gauze tape (20 cm width). Two square areas of circa 2.5 cm x 2.5 cm are cut from the adhesive gauze tape on the left flank of the animal. In this way, the surrounding area of the test sites, and the right flank are protected against irradiation. One of the two test sites on the left flank is not treated with the test substance so that it can be used as an irradiation irritation control site. (IICS; untreated but irradiated)

Preparation of doses

16. The test materials are to be applied neat, or as solutions, emulsion or suspensions in water or a suitable vehicle that reproduces the proposed formulation and use. Otherwise, the choice of vehicle should be limited to water, acetone, or ethanol.

Preparation of UV-irradiation

17. The UV source is a major factor in achieving reproducibility of the method. Since the majority of known photoirritant materials absorb light in the UVA wavelengths, it is customary to carry out photoirritation test to using sources which emit UVA. The recommended UVA-dose to be given is ca 10 J/cm^2, if UVB is also used, the recommended dose is 0.1 J/cm^2.

18. Where reliable predictions of the action spectrum based on the absorption spectrum of the test compound cannot be made, spectral coverage extended to include UVB should be considered. Certain chemicals such as benzoyl peroxide are activated by UVB and some such as doxycycline are augmented by UVB. However relevant, sub-erythematous doses of UVB-light are very difficult to apply. Moreover, sub-erythematous doses of UVB-light may damage healthy skin without showing clinical effects.

19. The irradiation dose should not cause erythema to the untreated skin or marked temperature increase leading to possible pain.

20. The wavelength of UVA and UVB are mentioned in the definition, given in Annex 1.

PROCEDURE

Number and sex of animals

21. At least 3 animals should be used. Additional animals may be required to clarify equivocal responses.

22. Either males or females can be used. If females are used, they should be nulliparous and not pregnant.

Dosage

23. To select the appropriate concentration of the test substance to be used in this screening test information concerning acute dermal irritating/corrosive properties of the test substance is considered essential, in addition to results from *in vitro* testing. Such information could be derived from the acute dermal irritation/corrosion test (2). If an undiluted liquid test substance was found to be non-irritating in such a test it should be used undiluted in this photoirritation test as well. Otherwise, the highest concentration of the test liquid that is non- or only slightly irritating should be used. When testing solids found to be non-irritating in acute dermal irritation/corrosion studies (2), the highest concentration possible in water, or where necessary a suitable vehicle that reproduces the proposed formulation and use should be used. Otherwise, the highest non- or slightly irritating concentrations should be used.

Application of the test substance

24. A non-irritating or only slightly irritating concentration of the test substance is to be tested in a single dose applied to two test sites on the intact skin of several experimental animals, each animal serving as his own control. One test site is irradiated with UVA(B)-light at a high, but sub-erythematogenic dose (usually 10 Joules/cm^2 for UVA and 0.1 Joules/cm^2 for UVB), the other test site is not irradiated.

25. An volume of 0.025 ml of the selected concentration of the test substance is brought into contact with the separate area of intact skin, left of the midline of the back, whereas the IICS is left untreated or treated with an identical volume of the vehicle used for the test substance.

Experimental schedule

26. Just prior to the cutaneous application of the test substance, it is recommended that animals are placed in a suitable restrainer to avoid ingestion of the test substance. During the irradiation period, the animals should continue to be restrained in appropriate holders. Prior to irradiation, the head of the animals must be protected to avoid ocular effects.

27. The exposed test site and IICS are irradiated with UVA(B)-light by means of an irradiation device containing parallel placed UVA fluorescent lamps or tubes (wavelength 310-420 nm, wavelength peak at 365 nm) until a dose of ca 10 Joules/cm^2 is received. If UVB is added the UVB-dose should be approximately 0.1 Joule/cm^2. To consider possible photoaugmenting effects, simultaneous irradiation by UVB is preferred to irradiation by UVA followed by UVB. This may require the addition of other UVB tubes in the lamp or the use of filter to achieve the desired exposures. Because of the lower dose of UVB, less time of exposure than that for UVA is usually necessary because of the energy characteristics of available lamp and irradiation systems.

28. The time of exposure can be calculated by means of the following formula:

$$t\,(\text{min}) = \frac{\text{irradiation dose } (J/cm^2) \times 1000}{\text{irradiance } (mW/cm^2) \times 60} \quad (1\,J/sec = 1\ \text{Watt})$$

29. The time of exposure need not to exceed two hours and the use of fluorescent lamps enables the simultaneous exposure of at least three animals. In addition, they do not produce excessive heat; therefore, irradiation from a short distance (ca 10 cm) is possible without adverse effects on skin or body temperature of the animals.

30. Immediately after irradiation, the test sites should be wiped clean using water, saline or an appropriate solvent without altering the existing response or the integrity of the epidermis. Next, the adhesive tape is removed and subsequently the same amount of the test substance is applied as a thin layer onto a similar shaven skin area right of the midline of the back, in the same sequence as, and opposite to, the irradiated treatment and control (IICS) sites on the left flank. A second site on the right side remains untreated as was done on the left side. The right side treatment and control sites are not irradiatated so that each animal serves as its on control for irritation due to either irradiation or the test substance alone. The animal is kept restrained for the same period of time that was used for irradiation of the left side. After the exposure period, residual test substance are removed in a similar way as was done for the irradiated test sites. Thereafter the animal is returned to its cage.

Observations

31. The duration of the observation period should not be fixed rigidly, but should be sufficiently long to fully evaluate possible photoirritation potential of the test compound. Normally, it need not exceed 72 hours after treatment.

32. The resulting skin reaction should be scored and evaluated by the method of Draize *et al.*(4). Since the exposure period for the UV-irradiated test and control sites ends prior to the exposure period for the non-UV-irradiated test and control sites, the observation periods for the irradiated and non-irradiated sites will not be at the same time. The time difference between the observation periods equals the time needed for the UV irradiation. The observation of the irradiated sites should be made approximately 30-60 minutes, and at 4, 24, 48, and 72 hours after the termination of the irradiation exposure period. Similarly, the same time pattern for observations should be used for the non-irradiated sites beginning at the end of the exposure to the test substance at the non-irradiated test sites. Observations, after those at the 4 hour post-exposure times (i.e., at 24, 48 and 72 hours) can be made at the same time for all sites.

33. A summary of the grading system by Draize *et al.*(4) is given in the table.

N.B. A skin thickness caliper may be used for a quantitative estimate of edema.

DRAFT

TABLE: GRADING OF SKIN REACTION

Evaluation of skin reactions

A. 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); eschar formation (injuries in depth: ischemia, haemorrhages, and incrustation)	4

Maximum possible: 4

B. 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)	
Severe oedema (raised more than 1 millimetre, extending beyond the area of exposure)	4

Maximum possible: 4

Histopathological examination may be carried out to clarify doubtful reactions.

DATA AND REPORTING

Data

34. Data must be summarized in tabular form, showing for each animal, and for each of the four control and treatment sites, the individual irritation scores for erythema (including eschar formation) and for oedema at 30-60 minutes, and at 4, 24, 48, and 72 hours after treatment. In addition, the mean scores for these parameters at each observation time point should be calculated.

Evaluation of results

35. Care should be taken in the evaluation of agents belonging to certain pharmacologic categories which could interfere with the evaluation of erythema and oedema (e.g. vasoconstrictors, vasodilators, antiinflammatory drugs).

36. Results are evaluated, taking into consideration the scores obtained primarily at the time of maximum skin response at each control and treatment site. The HCS should not show any skin reaction. However, if a reaction is observed, its severity and type should be taken into account in the evaluation of observed skin reactions at the other test and control sites.

37. In absence of skin reaction on the treated, non-irradiated test site and on the IICS, the observed skin reactions on the irradiated control and treated test site should be considered as signs that the test substance has the potential to cause photoirritation. If skin reactions are observed both on the non-irradiated and irradiated sites treated with the test substance, the difference in type, severity, and persistence of these reactions should be evaluated in order to establish whether or not the reaction of the irradiated test site are signs of photoirritation.

Test report

38. The test report must include the following information:

Test substance:

- Physical nature, purity and physicochemical properties;
- photostability (if possible);
- identification data.

Vehicle (if appropriate):

- justification for choice of vehicle.

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.

Test conditions:

- rationale for the selected dose level;
- details of test substance formulation/diet preparation,achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the substance and cleansing of the test sites;
- details of food and water quality;
- details of the irradiation (UV source and emission spectra, radiometer used, UV dose (J/cm^2), irradiance of the UV source (W/cm^2), duration of exposure, distance from UV source to test site, etc.)

Results:

- body weight/body weight changes;
- food and water consumption;
- toxic response data including for each individual animal: erythema and eschar formation; oedema; tabulation of the individual and mean values obtained by grading the skin reactions for each control and treatment site with and without UVA(B)-irradiation; and, any other signs of toxicity or unusual findings; and,
- if no positive control has been included in the conducted test, historical data for such controls should be given, indicating the time period elapsed since the reference compound was last tested.

Discussion of results.

Conclusions.

LITERATURE

(1) OECD Guidelines for Testing of Chemicals, nr. 402 adopted 24 Feb 1987.

(2) OECD Guidelines for Testing of Chemicals, nr. 404, adopted 17 July 1992.

(3) Young J.R., How M.J., Walker A.P. and Worth W.M.H. (1988). Classification as corrosive or irritant to skin of preparations contain acidic or alkaline substances without testing on animals. Toxicology *In Vitro*, 2, 19-26.

(4) Draize J.H., Woodard G. and Calvery H.O. (1944). Methods for the Study of Irritation and Toxicity of Substances Applies Topically to the Skin and Mucous Membranes. J. Pharmacol. Exp. Ther. 82, 377-390.

DRAFT

ANNEX 1

DEFINITIONS

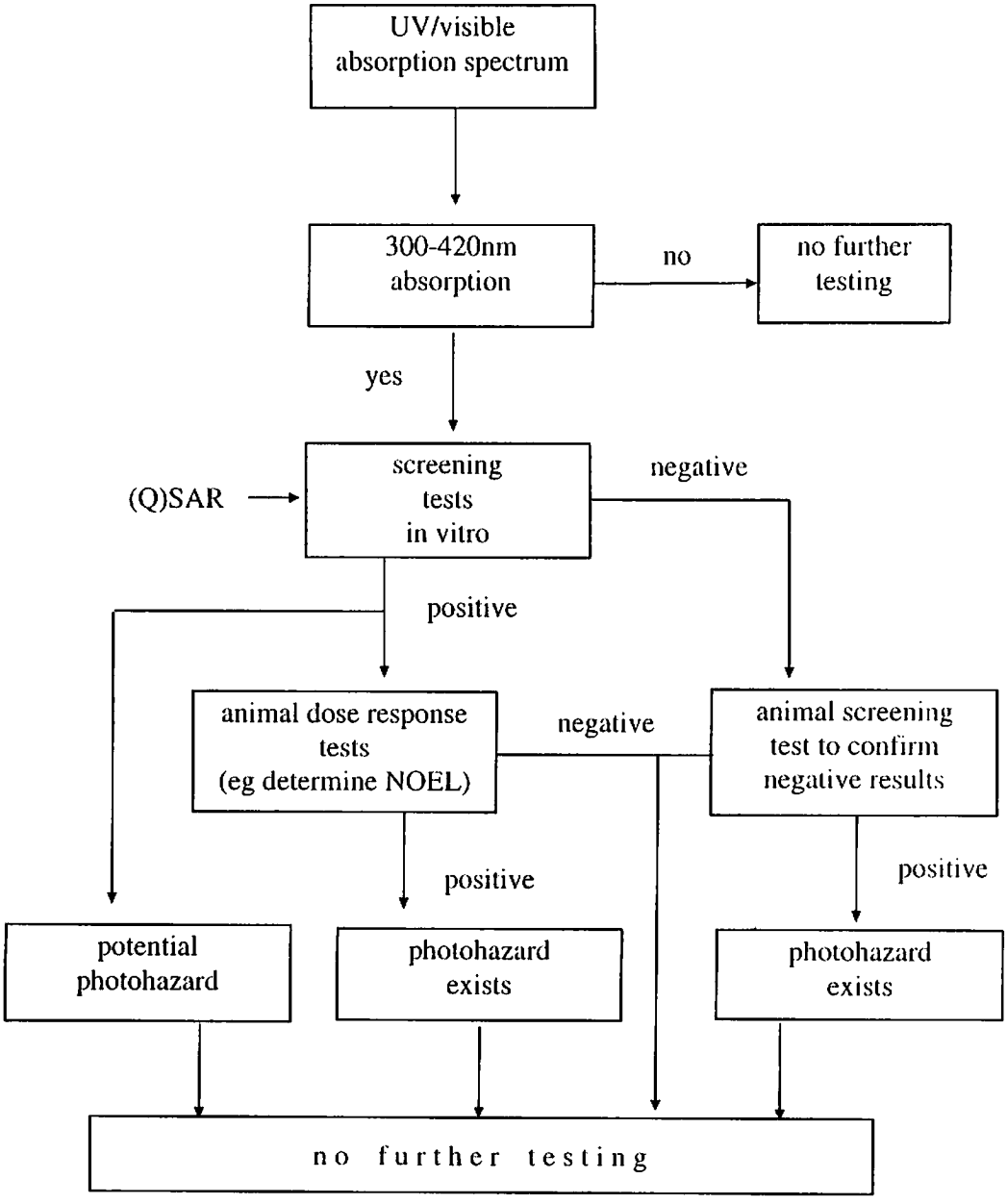
Dermal photoirritation is an inflammatory reaction of the skin that is elicited after a single exposure of the skin to a chemical which only occurs after activation by subsequent exposure to light (ultraviolet radiation). The skin reaction is characterized by erythema, possible eschar formation, and oedema.

The "Commission Internationale de L'eclairage" (CIE) defined UVA as ultraviolet irradiation (UVR) in the spectral range between 315 - 380 nm ; UBV as 280 - 315 nm.(C.I.E. publication No.17, 1970).

DRAFT

ANNEX 2

Sequential Approach to Photoirritation



DRAFT PROPOSAL
(February 1995)

OECD GUIDELINE FOR TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE

Acute Dermal Photoirritation Dose-Response Test

INTRODUCTION

1. Information derived from dermal photoirritation tests serve to indicate the existence of possible hazards likely to arise from topical application to skin of the test substance in association with exposure to light. Compounds administered systemically - and which may then be distributed in the body, including the skin - may also induce photoirritant reactions when the skin is irradiated.

2. This guideline can be used to determine the potential of a substance to induce skin irritation and provide information on the dose response characteristics of the photoirrititation following the percutaneous treatment of the animals used in the test.

3. A definition of dermal photoirritation is given in Annex 1.

INITIAL CONSIDERATIONS

4. In the assessment and evaluation of the toxic characteristics of a chemical the determination of photoirritation may be relevant. However, photoirritation testing in animals should only be carried out after initial information on dermal irritation/corrosion has been obtained by *in vitro* tests or acute testing (1, 2).

5. In the interest of animal welfare, it is important that the unnecessary use of animals is avoided, and that any testing which is likely to produce severe responses in animals is minimized. Consequently, test substances meeting any of the following criteria should not be tested in animals for acute dermal photoirritation:

i) test substances that have predictable corrosive potential based on structure-activity relationships and/or physicochemical properties such as strong acidity or alkalinity, e.g., when the material to be applied has a pH of 2 or less or 11.5 or greater (alkaline or acidic reserve (3) should also be taken into account);

ii) test substances which have been shown to be highly toxic by the dermal route; and,

iii) test substances which have not shown absorption of light in the range of 310-420 nm.

6. In addition, it is strongly recommended that photoirritation testing should start with *in vitro* test(s). Next, negative results from *in vitro* test(s) could be confirmed in the Acute Dermal Photoirritation Screening Test (4), as appropriate. It may not be necessary to test *in vivo* materials for which photoirritation has been demonstrated *in vitro*. However, this test can be used to study dose-response characteristics and to definitive a no-observed-adverse-effect-level (NOAEL) for photoirritation.
(See Annex 2 : Sequential Approach to Photoirrititation Testing)

7. The sensitivity of this test must be checked by means of a positive control study with a selection of known photoirritants of different compound classes and with different photoirritation mechanisms, such as naturally occurring psoralens (8-methoxypsoralen (8-MOP), CAS Number 298-81-7); dyes (acridine); coal-tar derivatives (anthracene); and, drugs (phenothiazine). Other positive controls suitable for the category of compound being evaluated may also be used. Considering the stability of the compound, it may be useful to prepare the positive control preparation on the day of use.

8. When photoirritation testing is being conducted on a routine basis and no changes are made in the experimental design or selection of animals, it should be sufficient to include the positive control study only at a certain interval, e.g. once a year.

PRINCIPLE OF THE TEST

9. Information derived from this acute dermal photoirritation dose-response test serves to indicate and characterise the dose-response characteristics of possible hazards likely to arise from topical application of a test substance to the skin in combination with exposure to ultraviolet (UV)-light.

10. The differences in reactions between the irradiated and the non-irradiated test animals are evaluated in order to establish whether or not the test substance possesses photoirritation potential under the conditions of this test.

DESCRIPTION OF THE METHOD

Selection of animal species

11. Although several mammalian species including albino rabbit or rat can be used, the guinea pig is the preferred species unless it can be expected that the test substance is unlikely to be tested for potential photosensitization. In that situation, use of the rabbit for this study would be preferred. The rationale for the choice of species involves some of the following considerations:

- Albino rabbits are considered as the most suitable species for assessing acute dermal irritation properties of compounds;

- In this manner results of the acute dermal irritation/corrosion test (2) can be of use for comparative purposes in order to establish the concentration range to be applied in this acute dermal photoirritation dose-response test ; in a similar way, if additional study of the test substance for photosensitization is likely, the use of the guinea pig as the species of choice would allow comparison of the results of the photoirritation test with those studies;

- The large skin surfaces on the back and flanks of rabbits and, to a lesser extent guinea pigs permit the use of different concentrations of the same substance to be examined simultaneously; in addition, each animal can serve as its own control thus limiting the number of animals used for obtaining information on the dose-response characteristic of the test substance and; and,

- Although alternate species for which background data exist, such as rats or mice, can also be used with this test. Due to their smaller body surface area, the experimental design will need to be modified and would probably require an increase in the number of animals used.

Housing and feeding conditions

12. The temperature in the experimental animal room should be 22°C(±3°C) for guinea pigs and other rodents, and 20 ± 3 °C for rabbits. Although the relative humidity should be at least 30% and preferably not

exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

13. The animals should be housed individually.

Preparation of the animals

14. Health young adult animals, with a body weight range of 300-500 g (guinea pig) or 2000-3500 g (rabbit), are randomly assigned to the test. The animals are identified uniquely and kept in their cages for at least five days prior to the start of the study to allow for acclimatization to the laboratory conditions.

15. The fur on the back and flanks of the animals selected for the study should be removed by clipping or shaving within 24 hours before testing. Care should be taken to avoid abrading of the skin.
Some strains of rabbit have dense patches of hair that are more prominent at certain times of the year. Such areas of dense hair growth should not be used as test or control sites.

16. Only animals with healthy intact skin should be used. Animals with cutaneous irritation or skin lesions must not be used.

Preparation of doses

17. The test materials are to be applied neat, or as solutions, emulsion or suspensions in water or a suitable vehicle that reproduces the proposed formulation and use. Otherwise, the choice of vehicle should be limited to water, acetone, or ethanol.

Preparation of UV-irradiation

18. The UV source is a major factor in achieving reproducibility of the method. Since the majority of known photoirritant materials absorb light in the UVA wavelengths, it is customary to carry out photoirritation tests using sources which emit UVA. The recommended UVA-dose to be given is ca 10 J/cm^2, if UVB is also used, the recommended dose is 0.1 J/cm^2.

19. Where reliable predictions of the action spectrum based on the absorption spectrum of the test compound cannot be made, spectral coverage extended to include UVB should be considered. Certain chemicals such as benzoyl peroxide are activated by UVB and some such as doxycycline are augmented by UVB. However relevant, sub-erythematous doses of UVB-light are very difficult to apply. Moreover, sub-erythematous doses of UVB-light may damage healthy skin without showing clinical effects.

20. The irradiation dose should not cause erythema to the untreated skin or marked temperature increase leading to possible pain.

21. The wavelength of UVA and UVB are mentioned in the definition, given in Annex 1.

PROCEDURE

Number and sex of animals

22. 10 animals are assigned to the test. Additional animals may be required to clarify equivocal responses.

23. Either males or females can be used. If females are used, they should be nulliparous and not pregnant.

Dosage

24. The concentrations at which the compound is used in this test should produce no or only slight skin irritation at 1, 4, 24, 48 and 72 hours after application.

25. To select the appropriate concentration of the test substance to be used in this dose-response test information concerning acute dermal irritating/corrosive properties of the test substance is considered essential, in addition to results from *in vitro* testing. Such information could be derived from the acute dermal irritation/corrosion test (2). If an undiluted liquid test substance was found to be non-irritating in such a test, it should be used undiluted as the highest concentration applied in this photoirritation test as well. Otherwise, the highest concentration of the test liquid that is expected to cause no or only slight irritation should be used. When testing solids found to be non-irritating in acute dermal irritation/corrosion studies (2), the highest concentration possible in water, or where necessary a suitable vehicle that reproduces the proposed formulation and use should be used. Otherwise, the highest non- or slightly irritating concentrations should be used.

26. The low dose selected should be sufficiently high to ensure an adequate margin for human safety taking into account conditions of use and exposure.

Application of the test substance

27. Each animal has four application sites, measuring 1.5 cm by 1.5 cm, laid out in a square on the animal's back. No substance is applied to one site, the untreated or vehicle control. The other sites are treated with different concentrations of the same substance. It is recommended that the lowest dose selected be applied to the site adjoining or adjacent to the untreated control site. The highest concentration used, or the positive control, should be applied to the treatment site opposite the control site.

28. A volume of 0.025 ml/cm^2 of the selected concentrations of the test substance and the positive control substance or the vehicle, as appropriate, should be applied with a micropipette to its designated site. The various sites should be placed in such a way as to prevent mixing the different concentrations of test substance, positive and vehicle controls.

Experimental schedule

29. Just prior to the cutaneous application of the test concentrations, it is recommended that animals are placed in a suitable restrainer to avoid ingestion of the test compound. During the irradiation period, the animals should continue to be restrained in appropriate holders. Prior to irradiation, the head of the animals must be protected to avoid ocular effects.

30. Approximately thirty minutes following the percutaneous application of the different concentrations of the test substance and/or the positive and vehicle controls to the designated sites on all ten animals, six animals are exposed to a non-erythemogenic dose of UV radiation. There should be no or only slight skin reaction in untreated, control site of the animals exposed to UV radiation. Depending on the nature of the

vehicle used or the absorption rate of the compound, the time interval between the completion of the percutaneous application of the test substance and the start of irradiation may be longer than 30 minutes.

31. Irradiated with UVA(B)-light is carried out by means of an irradiation device containing parallel placed UVA fluorescent lamps or tubes (wavelength 310-420 nm, wavelength peak at 365 nm) until a dose of ca 10 Joules/cm^2 is received. If UVB is added the UVB-dose should be approximately 0.1 Joule/cm^2. To consider possible photoaugmenting effects, simultaneous irradiation by UVB is preferred to irradiation by UVA followed by UVB. This may require the addition of other UVB tubes in the lamp or the use of filter to achieve the desired exposures. Because of the lower dose of UVB, less time of exposure than that for UVA is usually necessary because of the energy characteristics of available lamp and irradiation systems. The remaining four animals are treated in the same way as UV-irradiation group except that this group is not irradiated. The purpose is to ascertain the absence of irritation due to the compound. If there is reliable data concerning acute dermal irritating/corrosive properties of the test substance under the experimental conditions being used for this test, a reduction in the size of group of animals that are not irradiated can be considered.

32. The dose of UVA should be approximately 10 Joule/cm^2; if UVB is add the UVB-dose should be approximately 0.1 Joule/cm^2. To consider possible photoaugmenting effects, simultaneous irradiation by UVB is preferred to irradiation by UVA followed by UVB. This may require the addition of other UVB tubes in the lamp or the use of filter to achieve the desired exposures. Because of the lower dose of UVB, less time of exposure than that for UVA is usually necessary because of the energy characteristics of available lamp and irradiation systems.

33. The time of exposure can be calculated by means of following formula:

$$t\,(\text{min}) = \frac{\text{irradiation dose (J/cm}^2\text{)} \times 1000}{\text{irradiance (mW/cm}^2\text{)} \times 60} \qquad (1\text{J/sec} = 1\text{ Watt})$$

34. The time of exposure need not to exceed two hours and the use of fluorescent lamps enables the simultaneous exposure of at least three animals. In addition, they do not produce excessive heat, therefore, irradiation from a short distance (ca 10 cm) is possible without adverse effects on skin or body temperature of the animals.

35. Immediately after irradiation, the test sites should be wiped clean using sterile gauze moistened with water, saline, or an appropriate solvent without altering the existing response of the integrity of the epidermis. The non-irradiation group should also be wiped.

Observations

36. The duration of the observation period should not be fixed rigidly, but should be sufficiently long to fully evaluate possible photoirritation potential of the test compound. Normally, it need not exceed 72 hours after treatment and irradiation.

37. Each test animal, irradiated or not, is examined 1, 4, 24, 48 and 72 hours after the irradiation period is completed. When appropriate, additional examinations can be included.

38. A summary of the grading system by Draize *et al.* (5) is given in the table.

N.B. A skin thickness caliper may be used for a quantitative estimate of edema.

TABLE: GRADING OF SKIN REACTION

A. 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); eschar formation (injuries in depth: ischemia, haemorrhages, and incrustation)	4

Maximum possible: 4

B. 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)	
Severe oedema (raised more than 1 millimetre, extending beyond the area of exposure)	4

Maximum possible: 4

Histopathological examination may be carried out to clarify doubtful reactions.

DATA AND REPORTING

Data

39. Data must be summarized in tabular form, showing for each animal and each of the four control and treatment sites, the individual irritation scores for erythema (including eschar formation) and for oedema at 1, 4, 24, 48, and 72 hours after treatment. In addition, the mean scores for these parameters at each observation time point should be calculated.

Evaluation of results

40. Care should be taken in the evaluation of agents belonging to certain pharmacologic categories which could interfere with the evaluation of erythema and oedema (e.g. vasoconstrictors, vasodilators, antiinflammatory drugs).

41. Results are evaluated, taking into consideration the scores obtained primarily at the time of maximum skin response at each control and treatment site. The control sites should not show any skin reaction. However, if a reaction is observed, its severity and type should be taken into account in the evaluation of observed skin reactions at the other test and control sites.

42. In absence of skin reaction on the non-irradiated animal's control and treatment sites, the observed skin reactions on the irradiated control and treated test sites should be considered as signs that the test substance has the potential to cause photoirritation, and the dose-response relationship of each treatment site

should be carefully analysed. If skin reactions are observed both on the non-irradiated and irradiated sites treated with the test substance, the difference in type, severity, and persistence of these reactions should be evaluated in order to establish whether or not the reaction of the irradiated test sites are signs of photoirritation. The dose-response relationships among the different concentrations of the test substance and between irradiated and non-irradited animals is important in this regard.

43. A positive response should be defined, taking into account the incidence, type and severity of reactions induced by positive and negative controls under the particular experimental conditions chosen. When the results are judged to be positive, a dose (concentration)-response relationship should be established and a no-observed-adverse-effect-level (NOAEL) determined.

Test report

44. The test report must include the following information:

Test and reference substance:

- physical nature,purity and physicochemical properties;
- photostability (if possible);
- identification data.

Vehicle(if appropriate):

- justification for choice of vehicle.

Test animals:

- species/strain used;
- number,age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.

Test conditions:

- rationale for selection of the dose (concentration) levels;
- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the substance and cleansing of the test sites;
- details of food and water quality;
- details of the irradiation (UV source and emission spectra, radiometer used, UV-dose (J/cm^2), irradiance of the UV-source (W/cm^2), duration of exposure, distance from UV source to test site, etc.).

Results:

- body weight/body weight changes;
- food and water consumption;
- toxic response data including for each individual animal (irradiated and not): erythema and eschar formation; oedema; tabulation of the individual and mean values obtained by grading the skin reactions for each control and treatment sites; and, any other signs of toxicity or unusual findings; and,
- if no positive control has been included in the conducted test, historical data for such controls should be given, indicating the time period elapsed since the reference compound was last tested.

Discussion of results.

- dose-response relationships
- no-observed-adverse-effect level

Conclusions.

LITERATURE

(1) OECD Guidelines for Testing of Chemicals, nr. 402 adopted 24 Feb 1987.

(2) OECD Guidelines for Testing of Chemicals, nr. 404, adopted 17 July 1992.

(3) Young J.R., How M.J., Walker A.P. and Worth W.M.H. (1988). Classification as corrosive or irritant to skin of preparations contain acidic or alkaline substances without testing on animals. Toxicology *In Vitro*, 2, 19-26.

(4) OECD Guidelines for Testing of Chemicals, Draft Proposal for a New Guideline: Acute Dermal Photoirritation Screening Test, February 1995.

(5) Draize J.H., Woodard G. and Calvery H.O. (1944). Methods for the Study of Irritation and Toxicity of Substances Applies Topically to the Skin and Mucous Membranes. J. Pharmacol. Exp. Ther. 82, 377-390.

ANNEX 1

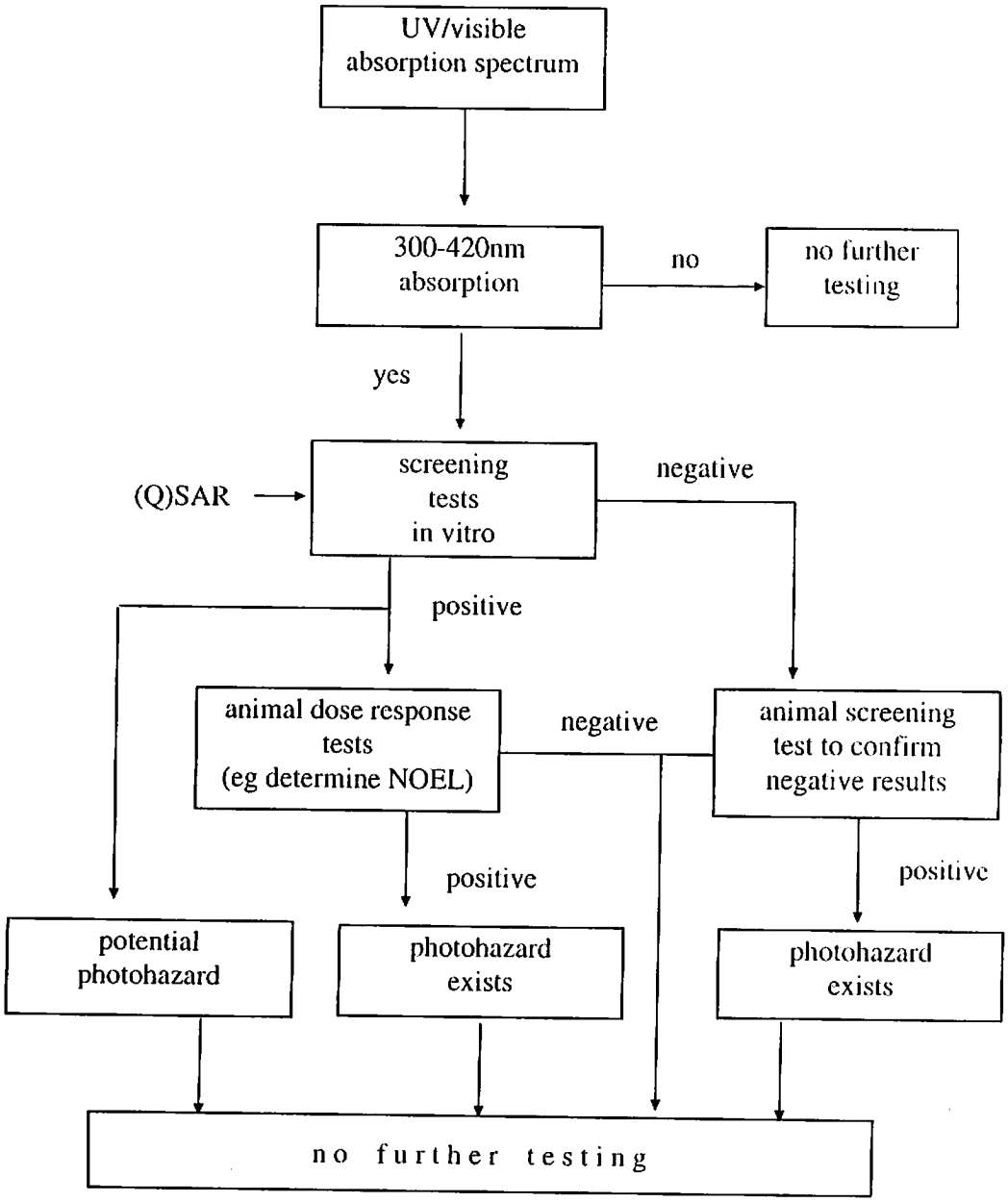
DEFINITIONS

Dermal photoirritation is an inflammatory reaction of the skin that is elicited after a single exposure of the skin to a chemical which only occurs after activation by subsequent exposure to light (ultraviolet radiation). The skin reaction is characterized by erythema, possible eschar formation, and oedema.

The "Commission Internationale de L'eclairage" (CIE) defined UVA as ultraviolet irradiation (UVR) in the spectral range between 315 - 380 nm ; UBV as 280 - 315 nm.(C.I.E. publication No.17, 1970).

ANNEX 2

Sequential Approach to Photoirritation Testing



D R A F T

DRAFT DOCUMENT
(June 1996)

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE

Percutaneous Absorption: *in vivo* Method

INTRODUCTION

1. Exposure to many chemicals occurs mainly via the skin whilst the majority of toxicological studies performed in laboratory animals use the oral route of administration. The use of oral route of administration can present difficulties in making safety assessments following dermal exposure. The *in vivo* percutaneous absorption study set out in this guideline provides the linkage necessary to extrapolate from oral studies when making safety assessments following dermal exposure. In cases, where an *in vivo* percutaneous study has been done, the determination of the acute dermal toxicity (Test Guideline 402) is usually not necessary. However, orally administered compounds, but not those absorbed through the skin, are often metabolically changed in liver before reaching general circulation. This may complicate the extrapolation from oral to dermal toxicity.

2. A chemical must cross a large number of cell layers of the skin before it can reach circulation. The rate determining layer is the stratum corneum consisting of dead cells. Permeability through the skin depends both on the lipophilicity of the chemical and the thickness of the outer layer of epidermis. In general, the skin of rats and rabbits appear to be more permeable that that of human, whereas the skin permeability of guinea pig, pigs and monkeys often are similar than that of human.

3. The methods for measuring percutaneous absorption can be divided into two categories; *in vivo* and *in vitro*. The in vivo method is capable of providing good information, in various laboratory species, on skin absorption. More recently *in vitro* methods have been developed. These utilise transport across full or partial thickness animal or human skin to a fluid reservoir. It is possible to estimate *in vivo* absorption by extrapolating from *in vitro* data (1)(2).

INITIAL CONSIDERATIONS

4. The *in vivo* method, described in this Guideline, allows the determination of the rate of penetration of the test substance through the skin and its subsequent uptake into blood and body distribution. The technique has been extensively used (3)(4)(5). Although *in vitro* percutaneous absorption studies may in many cases be appropriate, and should be preferred whenever scientifically justified/adequate, there may be situations where only an *in vivo* study can provide the necessary data for risk evaluation.

5. The advantages of the *in vivo* method are that it is well documented, uses a physiologically and metabolically intact system, uses a species common to many toxicity studies and can be modified for use with other species. The disadvantages are the use of live animals, the need for radiolabelled material to

ensure reliable results, difficulties in determining the early absorption phase and the differences in permeability of the preferred species (rat) and human skin. Animal skin is generally more permeable and therefore may overestimate human percutaneous absorption (6)(7)(8).

PRINCIPLE OF THE TEST

6. The test substance, preferably radiolabelled, is applied to the clipped skin of several groups of animals at one or more appropriate dose levels. The compound is allowed to remain in contact with the skin for a fixed period of time under a suitable dressing. Ingestion of the test substance must be prevented by applying protective devices (e.g. collars). At the end of the exposure time the dressing is removed and the skin is cleaned with an appropriate cleansing agent, the dressing and the cleansing materials are retained for analysis and a fresh dressing applied. The animals are housed prior to, during and after the exposure period in individual metabolism cages and the excreta and expired air over these periods are collected for analysis. The collection of expired air can be omitted when there is sufficient information that no volatile radioactive metabolites are formed. At the end of the sampling time the animals are killed, blood is collected for analysis, the application site removed for analysis and the carcass is analyzed for any unexcreted material. The samples are assayed by appropriate means and the degree of percutaneous absorption is estimated(6)(7)(8).

DESCRIPTION OF THE METHOD

Selection of animal species

7. The rat is the recommended species, but hairless animals and species having skin absorption rates more similar to those of human, can also be used (6)(7)(8). Young adults healthy animals of commonly used laboratory strains should be employed. At the commencement of the study, the weight variation of animals used should not exceed ± 20% of the mean weight.

Housing and feeding conditions

8. The temperature in the experimental animal room should be 22° C (± 3° C). Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used and should be freely available together with an unlimited supply of drinking water. During the study, and preferably also during the acclimatisation, the animals are individually housed in metabolism cages. Since food and water spillage would compromise the results, the probability of such events should be minimised.

Preparation of animals

9. The animals are marked to permit individual identification and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

10. Following the acclimatisation period and at least sixteen hours prior to dosing, each animal will have an area of skin in the region of the shoulders and the back clipped. The permeation properties of damaged skin are different from intact skin and care should be taken to avoid abrading the skin. The area must be large enough to allow reliable calculation of the absorbed amount of test substance per square cm skin, preferably at least 10 square cm. The skin may be washed several hours after clipping on the day

prior to dosing. However, careful consideration must be given to the possibility of modifying the skin or its barrier properties. The reasons for washing and the choice of the cleansing agent should be justified in the report. After preparation, the animals are returned to metabolism cages.

Test substance

11. The test substance should, if possible, be radiolabelled in a metabolically stable position, preferably with ^{14}C and of a suitable radiochemical purity (>98 %). The radiolabelled compound should be diluted, when appropriate, with non-radiolabelled substance. The specific activity and radiochemical purity of the test substance must be known. A non-radiolabelled compound may be utilized provided that suitable validated assay procedures for the compound in the relevant samples exist and the metabolism of the compound is well characterised.

Preparation of the dose

12. The test substance should be prepared where appropriate in a suitable vehicle (e.g. acetone) to allow good contact with the skin. Liquids should normally be tested undiluted. Typical use dilutions could be tested in addition. The stability of the dose material under the proposed conditions of administration should be known. Since the permeation properties of skin can be remarkably modified by the vehicle, careful consideration should be given to the use of solvents in these studies. Normally, the material used in commercial formulations should be considered first when a vehicle is necessary.

PROCEDURE

Number and sex of animals

13. At least four animals of one sex should be used for each sampling time. If there are data available that demonstrate substantial differences in toxicity between males and females, the more sensitive sex should be chosen. If there are no such data, then either gender could be used.

Administration of the dose

14. Immediately prior to dosing, the animal is removed from its metabolism cage and an 'O'-ring of a material with which neither the test substance nor vehicle interacts and of known internal diameter between 3-4 cm is glued to the skin over the clipped area using cyanoacrylate adhesive. When the ring is securely attached to the skin the appropriate volume of the test substance should be applied as a single dose evenly over the clipped skin within the 'O'-ring to at least 4 animals per time-point. It is recommended that the animals are sedated or anaesthetised when the 'O'-ring is adhered and the compound is administered. It is recommended that a volume of 10 µl/cm² should be applied although larger volumes can be used where appropriate, provided there is no run-off to non-application sites. A suitable cover is then glued over the 'O'-ring. The 'O'-ring should be thick enough to account for non-occlusive conditions and to prevent rubbing off the cover on the treated skin. The animals are then returned to their individual metabolism cages.

Exposure period and observations

15. An exposure period of 6 or 24 hours should be used, the choice being based on the expected exposure duration and on the expected penetration/absorption kinetics of the test compound. The animals

should be observed for signs of toxicity/abnormal reactions at regular intervals during the exposure period. At the end of the exposure period the treated skin should be observed for possible reactions of irritation.

Removal of unabsorbed dose

16. At the end of the exposure period the animals should be anaesthetised and the cover removed from each animal and retained separately for analysis. The treated skin of all animals should be washed at least 3 times, with an cleansing agent using cotton swabs. Care must be taken to avoid contaminating other parts of the body. The cleansing agent should have minimal effects on the uptake of the test substance whilst effectively removing it from the skin. The skin should be dried with cotton swabs. All swabs and washings should be retained for analysis as one set per animal. A fresh cover should be applied to the 'O'-rings of those animals forming the later time point groups prior to their return to individual metabolism cages.

Sampling times

17. Samples are collected 6 and 24 hours after the initial skin contact, and then daily until the end of the experiment. Whilst three time points will normally be sufficient, the envisaged purpose of the compound or existing kinetic data may suggest more appropriate or additional time points for study.

Sampling

18. The metabolism cages should permit separate collection of urine and faeces throughout the study. They should also allow collection of ^{14}C-carbon dioxide and volatile ^{14}C-compounds, which should be analysed when produced in quantity (>5%). The urine, faeces and trap fluids (e.g. ^{14}C-carbon dioxide and volatile ^{14}C-compounds) should be individually collected from each group at each sampling time. If there is sufficient information that no volatile radioactive metabolites are formed, open cages can be used.

19. At the appropriate times of kill for each group, the individual animals should be humanely killed and blood collected for analysis of whole blood and plasma. The cover and 'O'-ring should be removed for analysis. The dose site skin and a similar area of non-dosed, clipped skin should be removed from each animal for separate analysis. The carcasses of the individual animals should be retained for analysis. Usually analysis of the total carcass content will be sufficient. Target organs should be removed for separate analysis (if indicated by other studies). After collection of the samples from the metabolism cages at the time of kill, the cages and their traps should be washed with an appropriate solvent. The resulting washes can be pooled and a representative sample retained for analysis. Similar washes should be obtained from equipment used to remove the dose site, etc.

Analysis

20. The amount of compound in each sample should be analysed by suitably validated procedures for either parent compound and metabolites or radiolabelled material in the appropriate milieu. The recovery of material should be 100 ± 10%. Where radiolabelled material is concerned total compound and metabolites will be assessed. A specific assay for metabolites may be used to give a better assessment of percutaneous absorption than the assay for the parent compound alone.

DRAFT

DATA AND REPORTING

Data

21. The following measurements should be made for each group of animals, at each time/point.

- quantity of compound associated with the protective appliances (cover and 'O'-ring);
- quantity of compound that can be washed from the skin;
- quantity of compound in/on skin that cannot be washed from the skin;
- blood concentration of compound;
- quantity of compound in the excreta;
- quantity of compound remaining in the carcass and any organs removed for separate analysis.

22. The quantity of compound in the excreta and in the carcass will allow an estimate of the total amount absorbed at each time point and the percentage applied dose absorbed. An estimate of the amount of compound absorbed per square cm of skin over the exposure period can also be obtained. Permeability constant should be calculated to enable comparison of different results, as constants are independent of the concentration of test substance, and the contact time. Usually it can be considered, that the dose remained in the skin is potentially absorbed systemically.

Test report

23. The test report must include the following information:

Test substance:

- physical nature, purity and physicochemical properties;
- identification data;
- details of radiolabelling, if appropriate;
- concentration as mg/ml and dose as $\mu l/cm^2$ or as mg/cm^2.

Vehicle (if appropriate):

- justification for choice of vehicle; if other than water;
- pH of vehicle

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet etc.;
- individual weights of animals at the start of the test.

Test conditions:

- details of test substance preparation, amount applied, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test substance (site of application, assay methods, occlusion/no occlusion, extraction, detection, validation);
- details of food and water quality.

Results:

- toxic response data, including signs of toxicity;
- absorption data at each time point;
- statistical treatment of results, where appropriate.

Interpretation of the results, including comparison with any available data on percutaneous absorption of the test compound.

Discussion of results.

LITERATURE

(1) Scott RC, Batten PL, Clowes HM, Jones BK and Ramsay JD (1992). Further Validation of an *in vitro* method to reduce the need for *in vivo* studies for measuring the absorption of chemicals through rat skin. *Fundam. Appl. Toxicol.* 19, 484-492.

(2) ECETOC (1993) Percutaneous Absorption. European Centre for Ecotoxicology and Toxicology of Chemicals, Monograph No. 20.

(3) Zendzian RP (1989) Skin Penetration Method suggested for Environmental Protection Agency Requirements. *J. Am. Coll. Toxicol.* 8 (5), 829-835.

(4) Kemppainen BW, Reifenrath WG (1990) Methods for skin absorption. CRC Press Boca Raton, FL, USA.

(5) EPA (1992) Dermal Exposure Assessment: Principles and Applications. Exposure Assessment Group, Office of Health and Environmental Assessment.

(6) Bronaugh RL, Wester RC, Bucks D, Maibach HI and Sarason R (1990) *In vivo* percutaneous absorption of fragrance ingredients in rhesus monkeys and humans. Fd. *Chem. Toxic.* 28, 369-373.

(7) Feldman RJ and Maibach HI (1970) Absorption of some organic compounds through the skin in man. *J. Invest. Dermatol.* 54, 399-404.

(8) Scott RC, Dugard PH (1989) The properties of skin as a diffusion barrier and route for absorption. Handbook Exp. Pharmacol. 87/II, 93-114.

(9) Wester RC, Maibach HI (1991) *In vivo* percutaneous absorption. In: Dermatotoxicology, 4th ed., Marzulli FM, Maibach HI, Hemisphere Publishing Company, New York, pp. 75-96.

(10) Zendzian RP (1991) US Environmental Protection Agency. Dermal absorption studies of pesticides. Subdivision F: Hazard Evaluation: Humans and domestic animals. Series 85-3.

DRAFT

NEW PROPOSAL
(May 1996)

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A NEW GUIDELINE

Dermal Delivery and Percutaneous Absorption: *in vitro* Method

INTRODUCTION

1. The methods for measuring dermal delivery and percutaneous absorption can be divided into two categories: *in vivo* and *in vitro*. The *in vivo* method is well established and provides pharmacokinetic information, in a range of animal species but particularly the rat, on skin absorption. This method is separately described in another guideline. *In vitro* methods have been developed which, when conducted to best experimental principles and correctly interpreted, can give data which adequately reflects those from *in vivo* experiments. These *in vitro* methods measure the diffusion of chemicals into and across skin to a fluid reservoir and can utilise 'dead' skin to measure diffusion only, or fresh, metabolically active skin to simultaneously measure diffusion and skin metabolism. They have found particular use as a screen for comparing delivery of chemicals to skin from different formulations and can also provide useful models for assessing risk due to percutaneous absorption in man.

2. This guideline presents general principles for measuring the deposition and permeation of a chemical through excised skin. Skin from many mammalian species, including humans can be used. The permeability properties of skin are usually maintained after excision from the body and active transport of chemicals through the skin has not been identified. The principal diffusion barrier has been identified as the stratum corneum. The skin has been shown to have the capability to metabolise some chemicals during percutaneous absorption, but this process is not thought to be rate limiting for most chemicals in terms of actual absorbed dose, although it may affect the nature of the material entering the blood stream.

INITIAL CONSIDERATIONS

3. The principles of the *in vitro* methods described in this guideline are applicable in all cases where the stratum corneum is believed to be the rate limiting barrier to percutaneous absorption. There is sufficient experience to enable the *in vitro* methods to be used to predict percutaneous absorption *in vivo*.

4. The advantages of the *in vitro* method are that it can be used with skin from humans and other species; many replicate measurements can be made from the same subject; live animals are not used; diverse exposure conditions can be studied; and it can be used for compounds which are extensively metabolised either in the skin or systemically. The disadvantages include; limited supply of human skin; lack of pharmacokinetic data; species differences in skin permeability; and that sink conditions of the peripheral blood flow cannot be fully reproduced. The limited supply of human skin samples will mean that skin from animals will be used in many studies. There has been concern shown about the extrapolation of *in vitro* data to the *in vivo* situation, but, provided appropriate experimental conditions are used, acceptable *in vivo* predictions can be made.

PRINCIPLE OF THE TEST

5. The test substance, which can be radiolabelled, is applied to the surface of a skin sample separating the two chambers of a diffusion cell. The substance remains on the skin for a specified time under specified conditions, before removal by rinsing with an appropriate solvent or cleaning agent. The receptor cell fluid is sampled at time points throughout the experiment and analyzed for the test substance and, in metabolically active systems, for its metabolites, by appropriate methods. At the end of the experiment, the distribution of the test substance and its metabolites in the test system are quantified. The integrity of the membrane used and, where appropriate, its metabolic activity should be demonstrated.

6. Dermal delivery is defined as the sum of the absorbed test compound in skin and in the receptor fluid at the end of the experiment. Percutaneous absorption is the proportion of that delivered amount which may become systemically available in a given time. For first estimates of percutaneous absorption, the skin levels should be included in the percutaneous absorption determinations for all finite dose experiments. Where skin levels after terminal rinsing are much greater than receptor fluid levels, which when added together may lead to over estimation of percutaneous absorption, the distribution and interaction of the test material in the skin can be studied by measuring the distribution in skin with time or using autoradiographic techniques to aid in understanding the potential fate of the skin reservoir. For infinite dose experiments, used to measure permeability constants, if the skin levels at the end of the experiment are equal to or greater than the receptor fluid levels, the study should be repeated with skin and receptor fluid levels determined at each sample time point by using separate diffusion cells.

DESCRIPTION OF THE METHOD

Diffusion cell design

7. There are several types of glass, teflon, or stainless steel diffusion cells which consist of a donor chamber and a receptor chamber between which the skin is positioned. The cell should be designed so that it provides a good seal around the skin, enables easy sampling, allows good mixing of the receptor solution in contact with the underside of the skin, and good temperature control of the cell and its contents. Static and flow-through diffusion cells are both acceptable. Flow-through cells can offer advantages by enhancing sink conditions. Furthermore, if metabolically active skin is required, flow-through cells with a suitable tissue culture medium would be needed.

Receptor fluid

8. Physiological buffer solutions, such as tissue culture medium, or a balanced salt solution containing serum albumin, to help solubilise lipophilic compounds, should normally be used as the receptor fluid. This system will maintain the barrier properties, pH and viability of the skin membrane. When preliminary or screening studies are performed the precise composition of the receptor fluid used should be justified, adequate solubility of the test compound in it should be demonstrated and its effects on membrane integrity should be demonstrated by using chemicals with relevant physical properties and well documented permeability characteristics. When metabolism is being studied, the receptor fluid must maintain skin viability throughout the experiment. In the flow-through system the rate of flow should provide at least five receptor compartment volume changes per hour. In a static cell system, the fluid should be stirred and sampled regularly.

DRAFT

Skin membranes

9. Skin from human or animal sources can be used, but the choice should be justified. Either epidermal membranes (enzymically, heat or chemically separated), or split thickness skin (200 - 400 um thick) prepared with a dermatome are recommended. Full thickness skin, in general, is not recommended, unless specifically required for determination of dermal levels of test compound. Membrane preparation may result in damage to the skin and the integrity of the prepared membrane must be checked. When skin metabolism is being investigated, freshly excised skin should be used as soon as possible after excision and under conditions known to maintain metabolic activity. When membranes have been stored prior to use, evidence should be presented to show that barrier function is maintained. Any treatment of the skin with antimicrobials and/or cleansing preparations should be reported.

Membrane integrity

10. It is essential to ensure that the skin membranes used are undamaged. This may be achieved by measurement of the penetration of a standard molecule whose penetration characteristics are known (e.g. tritiated water), either before treatment with the test compound or, when donor compartment conditions allow, concurrently. Transepidermal water loss can be used as an indicator of membrane integrity. The criteria for the rejection of data should be stated.

Skin metabolism

11. The viability of human and animal skin can be maintained for at least 24 hours in flow-through diffusion cells with a balanced salt solution or tissue culture media. The influence of metabolism on percutaneous absorption of a test chemical can thereby be directly assessed. The potential importance of skin metabolism may be examined in separate experiments that determine if metabolites are formed (in appreciable amounts) in dermatomed slices or skin homogenates. Alternatively, useful information on the potential for human skin metabolism can be obtained from percutaneous absorption/metabolism studies involving viable animal skin.

Test substance

12. The test substance should be of known composition, purity and stability, and, ideally radiolabelled at a metabolically suitable site. Its solubility properties in the receptor phase should be known.

Test preparation

13. The test substance can be applied to the skin directly without the use of a solvent or delivery system. In most cases, the material will be applied in an appropriate vehicle which may range from a simple solution to a complex phased mixture. Solvent or formulation may affect release of the test compound to the stratum corneum or affect the barrier properties of the skin. The delivery system chosen must be justified in the protocol.

Test preparation application

14. Applications to the skin surface, i.e. the stratum corneum, may be infinite, where large volumes per unit area are applied, or finite, where limited quantities per unit are applied. Under normal human conditions of exposure to chemicals, finite doses are usually encountered and these may reach a maximum of up to 10 mg/cm^2 of skin. In the infinite dose application situation, which is required to determine the

permeability constant, the membrane is often fully hydrated, which may lead to increased penetration. Application rates or volumes should thus be justified in the protocol. Skin exposure may be for duration of the experiment or for shorter times (e.g. to mimic a specific type of human exposure). The skin rinsing technique used for short-term exposures should not damage the membrane. The vehicle and dose applied should mimic the intended "in use" conditions.

Temperature and humidity

15. The diffusion of chemicals (and therefore their absorption) is affected by temperature. A constant temperature of the diffusion chamber and skin should be close to normal skin temperature of 32°C. It is important that this is thermostatically controlled. Different cell designs will require different waterbath or heated block temperatures to ensure that the receptor/skin is at its physiological norm. With regard to humidity, it is appropriate to ensure adequate hydration of the skin, particularly if the preparation is to be maintained for more than just a few hours. Donor chambers may be occluded or left unoccluded during exposure to the test chemical, depending on the objective of the experiment and the volatility of the test substance.

Study time

16. A period of sampling of 24 hours is normally required, to allow for adequate characterisation of the absorption profile. For chemicals which penetrate the skin rapidly, this may not be necessary, but for chemicals which penetrate slowly longer times may be required. The exposure time and sample period should be defined in the protocol.

Terminal procedures

17. At the end of the exposure period, which in many cases will also be the end of the experiment, the skin should be rinsed of excess test preparation and the rinsing collected for analysis. The receptor compartment should be emptied, rinsed and the total collected for analysis. The cell should be dismantled and the skin membrane should be analyzed, whenever possible, for parent compound and metabolites by suitable extraction and analytical techniques. Extraction efficiency should also be demonstrated. In some cases, the skin may be fractionated into the exposed area of skin and area of skin under the cell flange, and into stratum corneum, epidermis and dermis fractions, for separate analysis. The cells should be rinsed for residual test preparation determination. The skin analysis procedure should be defined in the protocol.

Sampling

18. When appropriate, the sampling rate of the receptor fluid in static cells should enable the absorption profile to be shown in graphic form and the rate of absorption to be determined. In flow-through cells the rate of perfusion and method of analysis will determine the sampling rates. More frequent sample collections may be needed for measurements of steady-state absorption rates.

Analysis

19. The amount of test material in the receptor solution, rinsings and in the treated area of skin should be analyzed, whenever posible, using a suitably validated chemical or radiochemical technique. When metabolically maintained skin is used, the receptor solution should be analyzed for the presence of metabolites and parent compound. Whenever feasible, a mass balance of the test chemical following topical application should be undertaken.

D R A F T

DATA AND REPORTING

Data

20. The percutaneous absorption profile from the analysis of receptor solution with time and the mass balance of each cell, should be presented. When infinite dose conditions of exposure are used, the data may also permit the calculation of lag time and permeability constant. When finite dose conditions of exposure are used, the total penetration expressed as a percentage of the dose can be presented. Data to demonstrate the integrity of the membrane used and, where appropriate, evidence of metabolic activity, should also be presented.

Test report

21. The test report must include the requirements stipulated in the protocol of the test and should comprise the following data as appropriate:

Test substance:

- physical nature, physicochemical properties, purity (radiochemical purity);
- identification data;
- solubility in receptor solution.

Test preparation:

- formulation and justification of use.

Test conditions:

- source and site of skin membrane, method of preparation, storage conditions prior to use, any pretreatment (cleaning, antibiotic treatments etc), integrity measurements, metabolic status, justification of use;
- cell design, receptor formulation;
- details of application of test preparation and quantification of dose applied;
- details of removal of test preparation from the skin e.g. skin rinsing;
- details of analysis of skin and any fractionation techniques employed to demonstrate skin distribution;
- assay methods, extraction techniques, limits of detection and analytical method validation.

Results:

- overall recoveries of the experiment (Application = Skin rinsing + Skin + Receptor + Cell washings);
- tabulation of individual cell recoveries;
- penetration quantification (receptor recoveries only and/or plus skin residues).

Discussion of results:

- Including comparison with relevant published data.

Conclusions:

- Including an expert appraisal of the data and the limitations of the use of the data for extrapolation to human risk assessment.

LITERATURE

1. ECETOC Monograph No. 20. Percutaneous Absorption ECETOC, Brussels(1993).
2. Bronaugh RL, Stewart RF and Congdon ER. Methods for *in vitro* percutaneous absorption studies II. Animal models for human skin. Toxicol App Pharmacol 62 474 (1982).
3. COLIPA Cosmetic ingredients: Guidelines for Percutaneous Absorption/Penetration
4. COLIPA, Brussels (1995)
5. Collier SW, Sheik NM, Sakr A,Lichtin JL, Stewart RF and Bronaugh RL. Maintenance of skin viability during in vitro percutaneous absorption/metabolism studies. Toxicol. App. Pharmacol. 99 522 (1989).
6. Howes D, Guy R, Hadgraft J, et al. Methods for assessing percutaneous absorption. The report and recommendations of ECVAM workshop 13 . ATLA 24, 81 (1996)
7. Grisson RE, Brownie C and Guthrie FE. *In vivo* and *in vitro* dermal penetration of lipophilic and hydrophillic pesticides in mice. Bull Environ Contam Toxicol 38 917 (1987).
8. Scott RC and Ramsey JR. Comparison of the *in vivo* and *in vitro* percutaneous absorption of a lipophilic molecule (Cypemethrin, a pyrethroid insecticide). J Invest Dermatol 89 (1987).

9. Scott RC, Batten PL, Clowes HM et al. Further validation of an *in vitro* method to reduce the need for *in vivo* studies for measuring the absorption of chemicals through the skin. Fund Appl Toxicol 19 484 (1992).

402

Adopted:
24 Feb 1987

OECD GUIDELINE FOR TESTING OF CHEMICALS

"Acute Dermal Toxicity"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Solid or liquid test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- Melting point/boiling point
- pH (where appropriate)

• Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

In the assessment and evaluation of the toxic characteristics of a substance, determination of acute dermal toxicity is useful where exposure by the dermal route is likely. It provides information on health hazards likely to arise from a short-term exposure by the dermal route. Data from an acute dermal toxicity study may serve as a basis for classification and labelling. It is an initial step in establishing a dosage regimen in subchronic and other studies and may provide information on dermal absorption and the mode of toxic action of a substance by this route.

• Definitions

Acute dermal toxicity is the adverse effects occurring within a short time of dermal application of a single dose of a test substance.

Dose is the amount of test substance applied. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g. mg/kg).

The LD50 (median lethal dose), dermal, is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of treated animals when applied to the skin. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.

402

"Acute Dermal Toxicity"

Dosage is a general term comprising the dose, its frequency and the duration of dosing.

Dose-response is the relationship between the dose and the proportion of a population sample showing a defined effect.

Dose-effect is the relationship between the dose and the magnitude of a defined biological effect either in an individual or in a population sample.

- Principle of the test method

The test substance is applied to the skin in graduated doses to several groups of experimental animals, one dose being used per group. Subsequently, observations of effects and deaths are made. Animals which die during the test are necropsied, and at the conclusion of the test the surviving animals are sacrificed and necropsied. Animals showing severe and enduring signs of distress and pain may need to be humanely killed. Dosing test substances in a way known to cause marked pain and distress due to corrosive or irritating properties need not be carried out.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Healthy young adult animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Approximately 24 hours before the test, fur should be removed from the dorsal area of the trunk of the test animals by clipping or shaving. Care must be taken to avoid abrading the skin, which could alter its permeability.

Not less than 10 per cent of the body surface area should be clear for the application of the test substance. The weight of the animal should be taken into account when deciding on the area to be cleared and on the dimensions of the covering.

When testing solids, which may be pulverised if appropriate, the test substance should be moistened sufficiently with water or, where necessary, a suitable vehicle to ensure good contact with the skin. When a vehicle is used, the influence of the vehicle on penetration of skin by the test substance should be taken into account. Liquid test substances are generally used undiluted.

402

"Acute Dermal Toxicity"

- Experimental animals

Selection of species

The adult rat, rabbit or guinea pig may be used. Other species may be used but their use would require justification. The following weight ranges are suggested to provide animals of a size which facilitates the conduct of the test: rats, 200 to 300 g; rabbits, 2.0 to 3.0 kg; guinea pigs, 350 to 450 g. Animals with healthy, intact skin are required.

Note: In acute toxicity tests with animals of a higher order than rodents, the use of smaller numbers should be considered. Doses should be carefully selected, and every effort should be made not to exceed moderately toxic doses. In such tests, administration of lethal doses of the test substance should be avoided.

Number and sex

At least 5 animals are used at each dose level. They should all be of the same sex. If females are used, they should be nulliparous and non-pregnant. The use of a smaller number of animals may be justified in some cases. Where information is available demonstrating that a sex is markedly more sensitive, animals of this sex should be dosed.

Housing and feeding conditions

Animals should be caged individually. The temperature of the experimental animal room should be 22°C (± 3°) for rodents, 20° (± 3°) for rabbits, and the relative humidity 30-70 per cent. Where the lightings is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

- Test conditions

Dose levels

These should be sufficient in number, at least three, and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data should be sufficient to produce a dose-response curve and, where possible, permit an acceptable determination of the LD50.

Limit test

A limit test at one dose level of at least 2000 mg/kg body weight may be carried out in a group of 5 male and 5 female animals, using the procedures described above. If compound-related mortality is produced, a full study may need to be considered.

402

"Acute Dermal Toxicity"

Observation period

The observation period should be at least 14 days. However, the duration of observation should not be fixed rigidly. It should be determined by the toxic reactions, rate of onset and length of recovery period, and may thus be extended when considered necessary. The time at which signs of toxicity appear and disappear, their duration and the time of death are important, especially if there is a tendency for deaths to be delayed.

- Performance of the test

The test substance should be applied uniformly over an area which is approximately 10 per cent of the total body surface area. With highly toxic substances the surface area covered may be less, but as much of the area should be covered with as thin and uniform a film as possible.

Test substances should be held in contact with the skin with a porous gauze dressing and non-irritating tape throughout a 24-hour exposure period. The test site should be further covered in a suitable manner to retain the gauze dressing and test substance and ensure that the animals cannot ingest the test substance. Restrainers may be used to prevent the ingestion of the test substance, but complete immobilisation is not a recommended method.

At the end of the exposure period, residual test substance should be removed, where practicable using water or an appropriate solvent.

- Clinical examinations

Observations should be recorded systematically as they are made. Individual records should be maintained for each animal. Following application of the test substance, the animals should be observed frequently during the first day and then a careful clinical examination should be made at least once each day. Additional observations should be made daily with appropriate actions taken to minimise loss of animals to the study, e.g. necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals. Cageside observations should include changes in fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behaviour

pattern. Particular attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. The time of death must be recorded as precisely as possible.

Individual weights of animals should be determined shortly before the test substance is applied, weekly thereafter, and at death; changes in weight should be calculated and recorded when survival exceeds one day. At the end of the test surviving animals are weighed and then sacrificed.

- Pathology

Necropsy of all animals should be carried out and all gross pathological changes should be recorded. Microscopic examination of organs showing evidence of gross pathology in animals surviving 24 or more hours should also be considered because it may yield useful information.

- Assessment of toxicity in the other sex

After completion of the study in one sex, at least one group of 5 animals of the other sex is dosed to establish that animals of this sex are not markedly more sensitive to the test substance. The use of fewer animals may be justified in individual circumstances. Where adequate information is available to demonstrate that animals of the sex tested are markedly more sensitive, testing in animals of the other sex may be dispensed with.

3. DATA AND REPORTING

- Treatment of results

Data may be summarised in tabular form, showing for each test group the number of animals at the start of the test, time of death of individual animals at different dose levels, number of animals displaying other signs of toxicity, description of toxic effects and necropsy findings.

Animals which are humanely killed due to compound-related distress and pain are recorded as compound-related deaths.

The LD50 may be determined by any accepted method, e.g. Bliss (4), Litchfield and Wilcoxon (3), Finney (5), Weil (6), Thompson (7), Miller and Tainter (8).

402

"Acute Dermal Toxicity"

- Evaluation of results

The dermal LD50 value should always be considered in conjunction with the observed toxic effects and the necropsy findings. The LD50 value is a relatively coarse measurement, useful only as a reference value for classification and labelling purposes, and an expression of the lethal potential of the test substance following dermal exposure.

Reference should always be made to the experimental animal species in which the LD50 value was obtained. An evaluation should include an evaluation of relationships, if any, between the animals' exposure to the test substance and the incidence and severity of all abnormalities, including behavioural and clinical abnormalities, gross lesions, body weight changes, effect on mortality, and any other toxic effects.

- Test report

The test report should include the following information:

- species/strain/source used; environmental conditions;
- sex of animals dosed;
- tabulation of response data by dose level (i.e. number of animals that died or were killed during the test, number of animals showing signs of toxicity, number of animals exposed);
- time of dosing and time of death after dosing;
- LD50 value for the sex dosed (intact skin) determined at 14 days with the method of determination specified;
- 95 per cent confidence interval for the LD50 (where this can be provided);
- dose-mortality curve and slope (where permitted by the method of determination);
- pathology findings; and
- results of any test on the other sex.

- Interpretation of the results

A study of acute toxicity by the dermal (percutaneous) route and determination of a dermal LD50 provides an estimate of the relative toxicity of a substance by the dermal route of exposure.

"Acute Dermal Toxicity"

Extrapolation of the results of acute dermal toxicity studies and dermal LD50 values in animals to man is valid only to a limited degree. The results of an acute dermal toxicity study should be considered in conjunction with data from acute toxicity studies by other routes.

4. LITERATURE

1. WHO Publication: Environmental Health Criteria 6, *Principles and Methods for Evaluating the Toxicity of Chemicals*. Part I, Geneva, 1978.

2. National Academy of Sciences, Committee for the Revision of NAS Publication 1138, *Principles and Procedures for Evaluating the Toxicity of Household Substances*, Washington, 1977.

3. Litchfield, J.T. and Wilcoxon, F., *J. Pharmacol., Exp. Ther.*, 96, 99-113, 1949.

4. Bliss, C.I., *Quart. J. Pharm. Pharmacol., 11*, 192-216, 1938.

5. Finney, D.G., *Probit Analysis*. (3rd Ed.) London, Cambridge University Press, 1971.

6. Weil, C.S., *Biometrics, 8*, 249-263, 1952.

7. Thompson, W., *Bact. Rev., 11*, 115-141, 1947.

8. Miller, L.C. and Tainter, M.L., *Proc. Soc. Exp. Biol. Med. NY, 57*, 261-264, 1944.

404
Adopted:
17.07.92

OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Acute Dermal Irritation/Corrosion

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed in light of scientific progress. In the review, special attention is given to possible improvements in relation to animal welfare. This updated version of the original guideline 404 (adopted in 1981) is the outcome of a meeting of OECD experts held in Paris in May 1991.

2. The main differences between this and the original version of the guideline are: a) the inclusion of data from *in vitro* tests in the information on which a decision not to proceed to an *in vivo* test can be based; and b) the possibility to use one animal in a first step of the *in vivo* procedure allowing certain chemicals to be exempted from further testing.

3. Definitions used are set out in the Annex.

INITIAL CONSIDERATIONS

4. In the interests of animal welfare, it is important that the unnecessary use of animals is avoided, and that any testing which is likely to produce severe responses in animals is minimised. Consequently, test materials meeting any of the following criteria should not be tested in animals for dermal irritation/corrosion:

i) materials that have predictable corrosive potential based on structure-activity relationships and/or physicochemical properties such as strong acidity or alkalinity, e.g., when the material to be applied has a pH of 2 or less or 11.5 or greater (alkaline or acidic reserve (1) should also be taken into account);

ii) materials which have been shown to be highly toxic by the dermal route;

iii) materials which, in an acute dermal toxicity test (2), have been shown not to produce irritation of the skin at the limit test dose level of 2000 mg/kg body weight.

In addition, it may not be necessary to test *in vivo* materials for which corrosive properties are predicted on the basis of results from *in vitro* tests (3).

PRINCIPLE OF THE *IN VIVO* TEST

5. The substance to be tested is applied in a single dose to the skin of one or more experimental animals, untreated skin areas of the test animal(s) serving as control. The degree of irritation is read

and scored at specified intervals and is further described in order to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility of the effects observed. Animals showing severe distress and/or pain at any stage of the test must be humanely killed.

DESCRIPTION OF THE *IN VIVO* METHOD

Selection of animal species

6. Several mammalian species may be used. The albino rabbit is the preferred species.

Number and sex of animals

7. Three healthy adult animals are required for the complete test. Male and/or female animals can be used. Additional animals may be used to clarify equivocal responses. Sometimes the test can be performed with one animal only.

Housing and feeding conditions

8. Animals should be individually housed. The temperature of the experimental animal room should be 20°C (± 3°C) for rabbits, 22°C (± 3°C) for rodents and the relative humidity 30 to 70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Conventional laboratory diets are suitable for feeding and an unrestricted supply of drinking water should be available.

Preparation of the animals

9. Approximately 24 hours before the test, fur should be removed by close-clipping the dorsal area of the trunk of the animals. Care should be taken to avoid abrading the skin and only animals with healthy intact skin should be used.

10. Some strains of rabbit have dense patches of hair which are more prominent at certain times of the year. Such areas of dense hair growth should not be used as patch sites.

PROCEDURE

Application of the test substance

11. The test substance should be applied to a small area (approximately 6 cm^2) of skin and covered with a gauze patch, which is held in place with non-irritating tape. In the case of liquids or some pastes, it may be necessary to apply the test substance to the gauze patch and then apply that to the skin. The patch should be loosely held in contact with the skin by means of a suitable semi-occlusive dressing for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test substance should be prevented.

12. Liquid test substances are generally used undiluted. When testing solids (which may be pulverised if considered necessary), the test substance should be moistened with the smallest amount of water, or where necessary a suitable vehicle, needed to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of the skin by the test substance should be taken into account.

13. At the end of the exposure period, normally 4 hours, residual test substance should be

removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Dose level

14. A dose of 0.5 ml of liquid or 0.5 g of solid or semi-solid is applied to the test site.

Exposure of one animal

15. If it is suspected that the test substance might produce severe irritancy/corrosion, a single animal test should be employed. When it is suspected that the substance may cause corrosion, three test patches are applied simultaneously to the animal. The first patch is removed after three minutes. If no serious skin reaction is observed, the second patch is removed after one hour. If the observations at this stage indicate that exposure can humanely be allowed to extend to four hours, the third patch is removed after four hours and the responses are graded. If a corrosive effect is observed after either three minutes or one hour exposure, the test is immediately terminated by removal of the remaining patches. Alternatively, the three patches may be applied sequentially. When it is suspected that the substance may cause severe irritancy, a single patch should be applied to the animal for four hours.

Exposure of a further two animals

16. If neither a corrosive effect nor a severe irritant effect is observed after a four hour exposure, the test should be completed using two additional animals, each with one patch only, for an exposure period of four hours.

Exposure of three animals

17. If it is expected that the test substance will not produce severe irritancy or corrosion, the test may be started using three animals, each receiving one patch for an exposure period of four hours.

Observation period

18. The duration of the observation period should not be fixed rigidly but should be sufficient to evaluate fully the reversibility of the effects observed.

Clinical observations and grading of skin reactions

19. Animals should be examined for signs of erythema and oedema and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal. Dermal irritation is scored and recorded according to the grades in the table below. Further observations may be needed to establish reversibility. In addition to the observation of irritation, all lesions and other toxic effects should be recorded and fully described.

TABLE: GRADING OF SKIN REACTION

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 eschar formation preventing grading of erythema	4

Maximum possible: 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

Maximum possible: 4

Histopathological examination may be carried out to clarify doubtful reactions.

DATA AND REPORTING

Data

20. Data should be summarised in tabular form, showing for each individual animal the irritation scores for erythema and oedema at 60 minutes, 24, 48 and 72 hours after patch removal, all lesions, a description of the degree and nature of irritation, corrosion or reversibility, and any other toxic effects observed.

Test report

21. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Vehicle:

- justification for choice of vehicle.

Test animals:

- species/strain used;
- number, age and sex of animals;

- source, housing conditions, diet, etc.;
- individual weights of animals at the start and at the conclusion of the test.

Test conditions:

- technique of patch site preparation;
- details of patch materials used and patching technique;
- details of test substance preparation, application and removal.

Results:

- tabulation of irritation response data for each individual animal for each observation time period (e.g. 60 minutes, 24, 48 and 72 hours after patch removal);
- description of all lesions observed;
- narrative description of the degree and nature of irritation observed, and any histopathological findings;
- description of any other toxic effects in addition to dermal irritation/corrosion.

Discussion of the results.

If an *in vitro* test is performed before the *in vivo* test, the description or reference of the test, including details of the procedure, must be given together with results obtained with the test and reference substances.

LITERATURE

(1) Young J.R., How M.J., Walker A.P. and Worth W.M.H. (1988). Classification as corrosive or irritant to skin of preparations containing acidic or alkaline substances without testing on animals. Toxicology *In Vitro*, 2, 19-26.

(2) OECD, Paris (1987). Guideline 402.

(3) ECETOC Monograph No. 15, "Skin Irritation", European Chemical Industry, Ecology and Toxicology Centre, Brussels, July, 1990.

ANNEX

DEFINITIONS

Dermal irritation is the production of reversible inflammatory changes in the skin following the application of a test substance.

Dermal corrosion is the production of irreversible tissue damage in the skin following the application of a test substance.

405

Adopted:
24 Feb 1987

OECD GUIDELINE FOR TESTING OF CHEMICALS

"Acute Eye Irritation/Corrosion"

1. INTRODUCTORY INFORMATION

• Prerequisites

- Solid or liquid test substance
- Chemical identification of test substance
- Purity (impurities) of test substance
- Solubility characteristics
- pH and buffer capacity (where appropriate)
- Melting point/boiling point

• Standard documents

There are no relevant international standards.

2. METHOD

A. INTRODUCTION, PURPOSE, SCOPE, RELEVANCE, APPLICATION AND LIMITS OF TEST

In the assessment and evaluation of the toxic characteristics of a substance, determination of the irritant and/or corrosive effects on eyes of mammals is an important initial step. Information derived from this test serves to indicate the possible existence of hazards likely to arise from exposure of the eyes and associated mucous membranes to the test substance.

• Definitions

Eye irritation is the production of reversible changes in the eye following the application of a test substance to the anterior surface of the eye.

Eye corrosion is the production of irreversible tissue damage in the eye following application of a test substance to the anterior surface of the eye.

• Principle of the test method

The substance to be tested is applied in a single dose to one of the eyes in each of several experimental animals; the untreated eye is used to provide control information.

Users of this Test Guideline should consult the Preface, in particular paragraphs 3, 4, 7 and 8.

405

"Acute Eye Irritation/Corrosion"

The degree of irritation/corrosion is evaluated and scored at specific intervals and is further described to provide a complete evaluation of the effects. The duration of the study should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed.

Animals showing severe and enduring signs of distress and pain may need to be humanely killed.

- Initial Considerations

Careful consideration needs to be given to all the available information on a substance to minimise the testing of substances under conditions that are likely to produce severe reactions. The following information may be useful in this regard:

i) Physical chemical properties and chemical reactivity. Strongly acidic or alkaline substances which, for example, can be expected to result in a pH in the eye of 2 or less, or 11.5 or greater, need not be tested owing to their probable corrosive properties. Buffer capacity should also be taken into account.

ii) Results from skin irritation studies. Materials which have demonstrated definite corrosive or severe skin irritancy in a dermal study need not be further tested for eye irritancy, it being presumed that such substances will produce similarly severe effects on the eyes.

iii) Results from well-validated alternative studies. Materials which have demonstrated potential corrosive or severe irritancy need not be further tested for eye irritation, it being presumed that such substances will produce similarly severe effects on the eyes in a test using this guideline.

B. DESCRIPTION OF THE TEST PROCEDURE

- Preparations

Both eyes of each experimental animal provisionally selected for testing should be examined within 24 hours before testing starts. Animals showing eye irritation, ocular defects or pre-existing corneal injury should not be used.

405

"Acute Eye Irritation/Corrosion"

- Experimental animals

Selection of species

A variety of experimental animals have been used, but it is recommended that testing should be performed using healthy adult albino rabbits.

A single animal test

A single animal test should be considered if marked effects are anticipated. If the results of this test in one rabbit suggest the substance to be severely irritating (reversible effect) or corrosive (irreversible effect) to the eye using the procedure described, further testing for ocular irritancy in subsequent animals may not need to be carried out. Occasionally, further testing in additional animals may be appropriate to investigate specific aspects.

Number of animals

In cases other than a single animal test, at least 3 animals should be used. Additional animals may be required to clarify equivocal responses.

Housing and feeding conditions

Animals should be individually housed. The temperature of the experimental animal room should be 22°C (± 3°) for rodents, 20°C (± 3°) for rabbits, and the relative humidity 30 to 70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Conventional laboratory diets are suitable for feeding, and an unrestricted supply of drinking water should be available.

- Test conditions

Dose level

Testing of solids and liquids

For testing liquids, a dose of 0.1 ml is used. Pump sprays should not be used, but instead the liquid should be expelled and 0.1 ml collected and instilled into the eye as described for liquids. In testing solids, pastes, and particulate substances, the amount used should have a volume of 0.1 ml, or a weight of not more than 100 mg (the weight must always be recorded). If the test material is solid or granular it should be ground to a fine dust. The volume of particulates should be measured after gently compacting them, e.g. by tapping the measuring container.

Testing of aerosols

To test a substance contained in a pressurised aerosol container, the eye should be held open and the test substance administered in a single burst of about one second from a distance of 10 cm directly in front of the eye. Care should be taken not to damage the eye. In appropriate cases, aerosols may be tested in the manner already described for pump sprays.

An estimate of the dose may be made by simulating the test as follows: the substance is sprayed through a window, the size of a rabbit eye, placed directly before a weighing paper. The weight increase of the weighing paper is considered to approximate the amount sprayed into a rabbit eye. For volatile substances the dose may be estimated by weighing the container before and after use.

Observation period

The duration of the observation period should not be fixed rigidly but should be sufficient to evaluate fully the reversibility or irreversibility of the effects observed. It normally need not exceed 21 days after instillation.

- Procedure

Application

The test substance should be placed in the conjunctival sac of one eye of each animal after gently pulling the lower lid away from the eyeball. The lids are then gently held together for about one second in order to prevent loss of the material. The other eye, which remains untreated, serves as a control.

Local anaesthetics

If it is thought that the substance could cause unreasonable pain, a local anaesthetic may be used prior to instillation of the test substance. The type and concentration of the local anaesthetic should be carefully selected to ensure that no significant differences in reaction to the test substance will result from its use. The control eye should be similarly anaesthetised.

Irrigation

The eyes of the test animals should not be washed out for 24 hours following instillation of the test substance. At 24 hours a washout may be used if considered appropriate.

405

"Acute Eye Irritation/Corrosion"

For some substances shown to be irritating by this test, additional tests using rabbits with eyes washed soon after instillation of the substance may be indicated. In these cases it is recommended that 3 rabbits be used. Half a minute after instillation, the eyes of the rabbits are washed for half a minute using a volume and velocity of flow which will not cause injury.

- Clinical observations and scoring

The eyes should be examined at 1, 24, 48 and 72 hours. If there is no evidence of irritation at 72 hours the study may be ended. Extended observation may be necessary if there is persistent corneal involvement or other ocular irritation in order to determine the progress of the lesions and their reversibility or irreversibility. In addition to the observations of the conjunctivae, cornea and iris, any other lesions which are noted should be recorded and reported. The grades of ocular reaction (Table 1) should be recorded at each examination.

Examination of reactions can be facilitated by use of a binocular loupe, hand slit-lamp, biomicroscope, or other suitable devices. After recording the observations at 24 hours, the eyes of any or all rabbits may be further examined with the aid of fluoroscein.

The grading of ocular responses is subject to various interpretations. To promote harmonization and to assist testing laboratories and those involved in making and interpreting the observations, an illustrated guide in grading eye irritation should be used.

405

"Acute Eye Irritation/Corrosion"

Table 1: GRADES FOR OCULAR LESIONS

CORNEA*	
Opacity: degree of density (area most dense taken for reading)	
No ulceration or opacity	0
Scattered or diffuse areas of opacity (other than slight dulling of normal lustre), details of iris clearly visible	1
Easily discernible translucent area, details of iris slightly obscured	2
Nacrous area, no details of iris visible, size of pupil barely discernible	3
Opaque cornea, iris not discernible through the opacity	4
IRIS	
Normal	0
Markedly deepened rugae, congestion, swelling, moderate circumcorneal 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

* The area of corneal opacity should be noted.

Table 1: GRADES FOR OCULAR LESIONS (continued)

CONJUNCTIVAE	
Redness (refers to palpebral and bulbar conjunctivae, cornea and iris)	
Blood vessels normal	0
Some blood vessels definitely hyperaemic (injected)	1
Diffuse, crimson colour, individual vessels not easily discernible	2
Diffuse beefy red	3
Chemosis: lids and/or nictating membranes	
No swelling	0
Any swelling above normal (includes nictating 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

3. DATA AND REPORTING

- Treatment of results

Data may be summarised in tabular form, showing for each individual animal the irritation scores at the designated observation time; a description of the degree and nature of irritation; the presence of serious lesions and any effects other than ocular which were observed.

- Evaluation of the results

The ocular irritation scores should be evaluated in conjunction with the nature and reversibility or otherwise of the responses observed. The individual scores do not represent an absolute standard for the irritant properties of a material. They should be viewed as reference values and are only meaningful when supported by a full description and evaluation of the observations.

- Test report

The test report should include the following information:

- species/strain used;
- physical nature and, where applicable, concentration and pH value for the test substance;
- tabulation of irritant/corrosive response data for each individual animal at each observation time (e.g. 1, 24, 48 and 72 hours);
- description of any serious lesions observed;
- narrative description of the degree and nature of irritation or corrosion observed;
- description of the method used to score the irritation at 1, 24, 48 and 72 hours (e.g. hand slit-lamp, biomicroscope, fluorescein); and
- description of any non-ocular topical effects noted.

- Interpretation of the results

Extrapolation of the results of eye irritation studies in animals to man is valid only to a limited degree. The albino rabbit is more sensitive than man to ocular irritants or corrosives in most cases. Similar results in tests on other animal species can give more weight to extrapolation from animal studies to man.

Care should be taken in the interpretation of data to exclude irritation resulting from secondary infection.

4. LITERATURE

1. WHO Publication: Environmental Health Criteria 6, *Principles and Methods for Evaluating the Toxicity of Chemicals*, Part II (in preparation).

2. United States National Academy of Sciences, Committee for the Revision of NAS Publication 1138, *Principles and Procedures for Evaluating the Toxicity of Household Substances*, Washington, 1977.

3. Draize, J.H., Woodward, G. and Calvery, H.O., *J. Pharmacol. Exp. Ther.* 82: 377-390, 1944.

4. Draize, J.H. *Appraisal of the Safety of Chemicals in Foods, Drugs, and Cosmetics - Dermal Toxicity*, pp. 49-52. Assoc. of Food and Drug Officials of the United States, Topeka, Kansas, 1965.

5. Draize, J.H. *The Appraisal of Chemicals in Foods, Drugs and Cosmetics*, pp. 36-45. Association of Food and Drug Officials of the United States, Austin, Texas, 1965.

6. United States Federal Hazardous Substances Act Regulations. Title 16, Code of Federal Regulations, 38 FR 27012, Sept. 27, 1973; 38 FR 30105, Nov. 1, 1973.

7. Loomis, T.A. *Essentials of Toxicology*. 2nd Ed. pp. 207-213. Lea & Febiger, Philadelphia, 1974.

OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Skin Sensitisation

INTRODUCTION

1. OECD Guidelines for Testing of Chemicals are periodically reviewed in light of scientific progress. In such reviews, special attention is given to possible improvements in relation to animal welfare. This updated version of the original guideline 406, adopted in 1981, is the outcome of a meeting of OECD experts held in Paris in May 1991.

2. Currently, quantitative structure-activity relationships and *in vitro* models are not yet sufficiently developed to play a significant role in the assessment of the skin-sensitisation potential of substances which therefore must continue to be based on *in vivo* models.

3. The guinea pig has been the animal of choice for predictive sensitisation tests for several decades. Two types of tests have been developed: adjuvant tests in which sensitisation is potentiated by the injection of Freunds Complete Adjuvant (FCA), and non-adjuvant tests. In the original guideline 406, four adjuvant tests and three non-adjuvant tests were considered to be acceptable. In this updated version, the Guinea Pig Maximisation Test (GPMT) of Magnusson and Kligman which uses adjuvant (1)(2)(3)(4) and the non-adjuvant Buehler Test (5)(6) are given preference over other methods and the procedures are presented in detail. It is recognised, however, that there may be circumstances where other methods may be used to provide the necessary information on sensitisation potential.

4. The immune system of the mouse has been investigated more extensively than that of the guinea pig. Recently, mouse models for assessing sensitisation potential have been developed that offer the advantages of an endpoint which is measured objectively, short duration and minimal animal treatment. The mouse ear swelling test (MEST) and the local lymph node assay (LLNA) appear to be promising. Both assays have undergone validation in several laboratories (7)(8)(9)(10)(11) and it has been shown that they are able to detect reliably moderate to strong sensitisers. The LLNA or the MEST can be used as a first stage in the assessment of skin sensitisation potential. If a positive result is seen in either assay, a test substance may be designated as a potential sensitiser, and it may not be necessary to conduct a further guinea pig test. However, if a negative result is seen in the LLNA or MEST, a guinea pig test (preferably a GPMT or Buehler Test) must be conducted using the procedure described in this guideline.

5. Definitions used are set out in the Annex.

GENERAL PRINCIPLE OF SENSITISATION TESTS IN GUINEA PIGS

6. The test animals are initially exposed to the test substance by intradermal injection and/or epidermal application (induction exposure). Following a rest period of 10 to 14 days (induction

period), during which an immune response may develop, the animals are exposed to a challenge dose. The extent and degree of skin reaction to the challenge exposure in the test animals is compared with that demonstrated by control animals which undergo sham treatment during induction and receive the challenge exposure.

ELEMENTS COMMON TO SENSITISATION TESTS IN GUINEA PIGS

Sex of animals

7. Male and/or female healthy young adult animals can be used. If females are used they should be nulliparous and non-pregnant.

Housing and feeding conditions

8. The temperature of the experimental animal room should be 20°C (± 3°C) and the relative humidity 30-70 per cent. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. It is essential that guinea pigs receive an adequate amount of ascorbic acid.

Preparation of the animals

9. Animals are acclimatised to the laboratory conditions for at least 5 days prior to the test. Before the test, animals are randomised and assigned to the treatment groups. Removal of hair is by clipping, shaving or possibly by chemical depilation, depending on the test method used. Care should be taken to avoid abrading the skin. The animals are weighed before the test commences and at the end of the test.

Reliability check

10. The sensitivity and reliability of the experimental technique used should be assessed every six months by use of substances which are known to have mild-to-moderate skin sensitisation properties.

11. In a properly conducted test, a response of at least 30% in an adjuvant test and at least 15% in a non-adjuvant test should be expected for mild/moderate sensitisers. Preferred substances are hexyl cinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4) and benzocaine (CAS No. 94-09-7). There may be circumstances where, given adequate justification, other control substances meeting the above criteria may be used.

Removal of the test substance

12. If removal of the test substance is considered necessary, this should be achieved using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

DESCRIPTION OF THE GUINEA-PIG MAXIMISATION TEST METHOD

Number of animals

13. A minimum of 10 animals is used in the treatment group and at least 5 animals in the control group. When fewer than 20 test and 10 control guinea pigs have been used, and it is not possible to conclude that the test substance is a sensitiser, testing in additional animals to give a total of at least 20 test and 10 control animals is strongly recommended.

Dose levels

14. The concentration of test substance used for each induction exposure should be well-tolerated systemically and should be the highest to cause mild-to-moderate skin irritation. The concentration used for the challenge exposure should be the highest nonirritant dose. The appropriate concentrations can be determined from a pilot study using two or three animals. Consideration should be given to the use of FCA-treated animals for this purpose.

Induction: Intradermal Injections

Day 0 - treated group

15. Three pairs of intradermal injections of 0.1 ml volume are given in the shoulder region which is cleared of hair so that one of each pair lies on each side of the midline.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the test substance in an appropriate vehicle at the selected concentration

Injection 3: the test substance at the selected concentration formulated in a 1:1 mixture (v/v) FCA/water or physiological saline.

16. In injection 3, water soluble substances are dissolved in the aqueous phase prior to mixing with FCA. Liposoluble or insoluble substances are suspended in FCA prior to combining with the aqueous phase. The concentration of test substance shall be equal to that used in injection 2.

17. Injections 1 and 2 are given close to each other and nearest the head, while 3 is given towards the caudal part of the test area.

Day 0 - control group

18. Three pairs of intradermal injections of 0.1 ml volume are given in the same sites as in the treated animals.

Injection 1: a 1:1 mixture (v/v) FCA/water or physiological saline

Injection 2: the undiluted vehicle

Injection 3: a 50% w/v formulation of the vehicle in a 1:1 mixture (v/v) FCA/water or physiological saline.

Induction: Topical Application

Day 5-7 - treated and control groups

19. Approximately twenty-four hours before the topical induction application, if the substance is not a skin irritant, the test area, after close-clipping and/or shaving is painted with 0.5 ml of 10% sodium lauryl sulphate in vaseline, in order to create a local irritation.

Day 6-8 - treated group

20. The test area is again cleared of hair. A filter paper (2 x 4 cm) is fully-loaded with test substance in a suitable vehicle and applied to the test area and held in contact by an occlusive dressing for 48 hours. The choice of the vehicle should be justified. Solids are finely pulverised and

incorporated in a suitable vehicle. Liquids can be applied undiluted, if appropriate.

Day 6-8 - control group

21. The test area is again cleared of hair. The vehicle only is applied in a similar manner to the test area and held in contact by an occlusive dressing for 48 hours.

Challenge: Topical Application

Day 20-22 - treated and control groups

22. The flanks of treated and control animals are cleared of hair. A patch or chamber loaded with the test substance is applied to one flank of the animals and, when relevant, a patch or chamber loaded with the vehicle only may also be applied to the other flank. The patches are held in contact by an occlusive dressing for 24 hours.

Observations - treated and control groups

23.
- approximately 21 hours after removing the patch the challenge area is cleaned and closely-clipped and/or shaved or depilated if necessary;
- approximately 3 hours later (approximately 48 hours from the start of the challenge application) the skin reaction is observed and recorded according to the grades shown below;
- approximately 24 hours after this observation a second observation (72 hours) is made and once again recorded.

Blind reading of test and control animals is encouraged.

TABLE: MAGNUSSON AND KLIGMAN GRADING SCALE FOR THE EVALUATION OF CHALLENGE PATCH TEST REACTIONS

0 = no visible change

1 = discrete or patchy erythema

2 = moderate and confluent erythema

3 = intense erythema and swelling

Rechallenge

24. If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. A rechallenge may also be performed on the original control group.

Clinical observations

25. All skin reactions and any unusual findings including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g.

histopathological examination, the measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

DESCRIPTION OF THE BUEHLER TEST METHOD

Number of animals

26. A minimum of 20 animals is used in the treatment group and at least 10 animals in the control group.

Dose levels

27. The concentration of test substance used for each induction exposure should be the highest to cause mild irritation. The concentration used for the challenge exposure should be the highest non-irritating dose. The appropriate concentration can be determined from a pilot study using two or three animals.

28. For water soluble test materials, it is appropriate to use water or a dilute non-irritating solution of surfactant as the vehicle. For other test materials 80% ethanol/water is preferred for induction and acetone for challenge.

Induction: Topical application

Day 0 - treated group

29. One flank is cleared of hair (closely-clipped). The test patch system should be fully loaded with test substance in a suitable vehicle (the choice of the vehicle should be justified; liquid test substances can be applied undiluted, if appropriate). The test patch system is applied to the test area and held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours.

30. The test patch system must be occlusive. A cotton pad is appropriate and can be circular or square, but should approximate 4-6 cm^2. Restraint using an appropriate restrainer is preferred to assure occlusion. If wrapping is used, additional exposures may be required.

Day 0 - control group

31. One flank is cleared of hair (closely-clipped). The vehicle only is applied in a similar manner to that used for the treated group. The test patch system is held in contact with the skin by an occlusive patch or chamber and a suitable dressing for 6 hours. If it can be demonstrated that a sham control group is not necessary, a naive control group may be used.

Days 6-8 and 13-15 - treated and control groups

32. The same application as on day 0 is carried out on the same test area (cleared of hair if necessary) of the same flank on day 6-8, and again on day 13-15.

Challenge

Day 27-29 - treated and control groups

33. The untreated flank of treated and control animals is cleared of hair (closely-clipped). An occlusive patch or chamber containing the appropriate amount of test substance is applied, at the maximum non-irritant concentration, to the posterior untreated flank of treated and control animals.

When relevant, an occlusive patch or chamber with vehicle only is also applied to the anterior untreated flank of both treated and control animals. The patches or chambers are held in contact by a suitable dressing for 6 hours.

Observations - treated and control groups

34. - approximately 21 hours after removing the patch the challenge area is cleared of hair;

- approximately three hours later (approximately 30 hours after application of the challenge patch) the skin reactions are observed and recorded according to the grades shown in the Guinea-Pig Maximisation Test (see paragraph 23);

- approximately 24 hours after the 30 hour observation (approximately 54 hours after application of the challenge patch) skin reactions are again observed and recorded.

Blind reading of test and control animals is encouraged.

Rechallenge

35. If it is necessary to clarify the results obtained in the first challenge, a second challenge (i.e. a rechallenge), where appropriate with a new control group, should be considered approximately one week after the first one. The rechallenge may also be performed on the original control group.

Clinical observations

36. All skin reactions and any unusual findings, including systemic reactions, resulting from induction and challenge procedures should be observed and recorded. Other procedures, e.g. histopathological examination, measurement of skin fold thickness, may be carried out to clarify doubtful reactions.

DATA AND REPORTING (GPMT and Buehler Test)

Data

37. Data should be summarised in tabular form, showing for each animal the skin reactions at each observation.

Test report

38. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Vehicle:

- justification of choice of vehicle.

Test animals:

- strain of guinea-pig used;

- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start and at the conclusion of the test.

Test conditions:

- technique of patch site preparation;
- details of patch materials used and patching technique;
- result of pilot study with conclusion on induction and challenge concentrations to be used in the test;
- details of test substance preparation, application and removal;
- vehicle and test substance concentrations used for induction and challenge exposures and the total amount of substance applied for induction and challenge.

Reliability check:

- a summary of the results of the latest reliability check including information on substance, concentration and vehicle used.

Results:

- on each animal including grading system;
- narrative description of the nature and degree of effects observed;
- any histopathological findings.

Discussion of the results.

If a screening assay is performed before the guinea pig test the description or reference of the test, including details of the procedure, must be given together with results obtained with the test and reference substances.

LITERATURE

(1) Magnusson B. and Kligman A.M. (1969). The identification of contact allergens by animal assay. The guinea pig maximisation test. J. Invest. Dermatol., 52, 268.

(2) Magnusson B. and Kligman A.M. (1970). Allergic Contact Dermatitis in the Guinea Pig. Charles G. Thomas; Springfield, Illinois.

(3) Magnusson B. (1980). Identification of contact sensitizers by animal assay. Cont. Derm., 6, 46.

(4) Magnusson B., Fregert S. and Wallberg J. (1979). Determination of skin sensitization potential of chemicals. Predictive testing in guinea pigs. Arbete och Hälsa, 26(E).

(5) Buehler E.V. (1965). Delayed contact hypersensitivity in the guinea pig. Arch. Dermatol., 91, 171.

(6) Ritz H.L. and Buehler E.V. (1980). Procedure for conducting the guinea pig assay. Current Concepts in Dermatology, Drill V.A. and Lazar P. (eds), Academic Press, New York, N.Y., 25-40.

(7) Kimber I., Hilton J. and Weisenberger C. (1989). The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation Contact Dermatitis, 21, 215-220.

(8) Kimber I., Hilton J. and Botham P.A. (1990). Identification of contact allergens using the murine local lymph node assay: comparisons with the Buehler OccludedPatch Test in guinea pigs. Journal of Applied Toxicology, 10(3), 173-180.

(9) Kimber I., Hilton J., Botham P.A., Basketter D.A., Scholes E.W., Miller K., Robbins M.C., Harrison P.T.C., Gray T.J.B. and Waite S.J. (1991). The murine local lymph node assay: results of an inter-laboratory trial. Toxicology Letters, 55, 203-213.

(10) Basketter D.A., Scholes E.W., Kimber I., Botham P.A., Hilton J., Miller K., Robbins M.C., Harrison P.T.C. and Waite S.J. (1991). Interlaboratory evaluation of the local lymph node assay with 25 chemicals and comparison with guinea pig test data. Toxicology Methods, 1, 30-43.

(11) Gad S.C., Dunn B.J., Dobbs D.W., Reilly C. and Walsh R.D. (1986). Development and validation of an alternative dermal sensitisation test: the mouse ear swelling test (MEST). Toxicol. Appl. Pharmacol., 84, 93-114.

ANNEX

DEFINITIONS

Skin sensitisation (allergic contact dermatitis) is an immunologically mediated cutaneous reaction to a substance. In the human, the responses may be characterised by pruritis, erythema, oedema, papules, vesicles, bullae or a combination of these. In other species the reactions may differ and only erythema and oedema may be seen.

Induction exposure: an experimental exposure of a subject to a test substance with the intention of inducing a hypersensitive state.

Induction period: a period of at least one week following an induction exposure during which a hypersensitive state may develop.

Challenge exposure: an experimental exposure of a previously treated subject to a test substance following an induction period, to determine if the subject reacts in a hypersensitive manner.

D R A F T

DRAFT DOCUMENT

(April 1997)

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR A DRAFT NEW GUIDELINE

Acute Dermal Irritation Study in Human Volunteers

INTRODUCTION

1. To date the prediction of human cutaneous irritation in view of hazard identification relies primarily on the use of experimental animals (OECD Guideline 404, adopted in 1992). There are however problems of extrapolating from animals to humans. For the risk assessment of detergent and cosmetic products, human skin patch tests are frequently used. This Guideline allows skin irritation data to be obtained directly in humans for purposes of hazard identification. The method was developed by the United Kingdom and has been extensively evaluated (1)(2)(3)(4)(5)(6)(7)(8)(9). It is crucial when adopting this approach to observe the safety/ethical standards set out below.

SAFETY/ETHICAL STANDARDS

2. In the interests of human safety the following criteria must be met before the study is initiated:

   i) The study must meet any local legal requirements and conform fully to the "Helsinki Guidelines" (10).

   ii) The study should follow the principles of Good Clinical Practice (11)

   iii) The study is reviewed and considered acceptable by an independent ethical review committee.

INITIAL CONSIDERATIONS

3. Adequate information on the toxicity profile, including percutaneous absorption data, should be available to indicate that the study does not present any significant health risk.

4. Substances should not be tested in humans when:

   - they have shown to be corrosive in a predictive assay, either *in vitro* (12) or *in vivo*;

   - a corrosive potential for human skin can be predicted on the basis of structure-activity relationships and/or physico-chemical properties such as strong acid or alkaline reserve (13);

   - they present any likelihood of skin sensitisation;

- they present any acutely toxic hazard; and/or
- they present any genotoxic hazard.

5. Further guidance on the selection of human volunteers can be found in the literature (3)(14).

PRINCIPLE OF THE TEST

6. The substance to be tested is applied in a single dose to the skin of human volunteers under occlusion. The extent of any irritation is kept to a minimum by means of a phased increase in the duration of application ranging from 15 minutes to 4 hours. Reactions are scored at 24, 48 and 72 hours, with any reaction regarded as skin irritation being sufficient to terminate treatment in the individuals concerned.

7. The principal means of evaluation is the proportion of the test panel which develops an irritant reaction in relation to a concurrent positive control substance (1). Consequently, it is important not to confuse individual variation in the susceptibility to skin irritation with the issue of the general irritation potential of the test substance.

DESCRIPTION OF THE METHOD

Selection of human volunteers

8. This Test Guideline is designed for use with human volunteers. It is not necessary specifically to select atopic individuals (4). The selected human volunteers should be generally healthy, at least 18 years of age, not pregnant and not breast-feeding. In addition, human volunteers with a known sensitivity to the test substance or showing any signs of dermatitis should not be selected for the test.

Preparation of doses

9. Liquid test substances are generally used undiluted. When testing solids (which may be pulverised if considered necessary), the test substance should be moistened with the smallest amount of water (0.2 ml) or where necessary a suitable vehicle, needed to ensure good contact with the skin. When vehicles are used, the influence of the vehicle on irritation of the skin by the test substance should be taken into account.

DRAFT

PROCEDURE

Number of volunteers

10. At least 30 volunteers are recruited to compose the test panel, with at least one third belonging to either sex. Without being excessive this number should be sufficient to provide an adequate assessment susceptible to a statistic evaluation.

Application of the test substance

11. The test substance is applied to a suitable skin site, e.g. the upper outer arm, by means of a 25 mm diameter occlusive chamber containing a gauze pad. The patch should be held in contact with the skin by means of a suitable dressing for the duration of the exposure period. It is held in place with non-irritating tape.

Dose level

12. The patch should deliver an adequate dose per unit area: approximately 50-100 mg/cm^2 is considered optimal. When testing liquid test substances, in general 0.2 ml are added onto the gauze pad. When testing solids, in general 0.2 g of the test substance are moistened and added onto the gauze pad. Alternatively, the gauze pad could be moistened.

Duration of exposure

13. To avoid unacceptably strong reactions, a cautious approach to testing must be adopted. A sequential patch procedure permits the development of a positive but not severe irritant response. The patches are applied progressively starting with a duration of 15 and 30 minutes and up to 1, 2, 3, and 4 hours. The 15 and/or 30 minutes exposure periods may be omitted if there are sufficient indications that excessive reactions will not occur following the 1-hour exposure. Progression to longer exposures at a new skin site will depend upon the absence of skin irritation (scored at least up to 48 hours) arising from the shorter exposures, in order to ensure that any delayed irritant reaction is adequately assessed

14. The application of the substance for a longer exposure period is always made to a previously untreated site.

15. At the end of the exposure period, residual test substance should be removed, where practicable, using water or an appropriate solvent without altering the existing response or the integrity of the epidermis.

Limited exposure

16. In addition to the phased increase in duration of application as described in paragraph 13, if it is suspected that the substance might produce severe irritation, a substantially reduced exposure time should be employed, possibly in a pilot group of volunteers. The progress of the study can then be defined on the basis of the data produced. Subsequent patches are only applied after the 48/72 h readings.

Clinical observation and grading of skin reactions

17. Treatment sites are examined for signs of irritation and the responses are scored at 24, 48 and 72 hours after patch removal. Skin irritation is scored and recorded according to the grading in Table 1.

Grading	Description of response
0	No reaction
1	Weakly positive reaction (usually characterised by mild erythema and/or dryness across most of the treatment site)
2	Moderately positive reaction (usually distinct erythema or dryness, possibly spreading beyond the treatment site)
3	Strongly positive reaction (strong and often spreading erythema with oedema and/or scabbing)

18. For volunteers who have a response of 1 or greater following an exposure of less than 4 hours, it is assumed that they would present a stronger reaction if exposed for 4 hours to the substance. Once a response of 1 or greater has been obtained, there is no need to subject the reacting volunteer to further treatment with the substance. Further observations may be required for proper volunteer care. In addition to the observation of irritation, any other effects should be recorded and fully described. The volunteers should be invited to make comments related to the patch applications (e.g. sensory effects) and assessors trained to note immediate responses (i.e. urticaria) when the patches are removed. Such observations may not indicate an irritant effect, but if significant, they would be taken account of in the management of the study to ensure proper volunteer care.

19. In evaluating the results for each test substance, therefore, what is measured is the number of volunteers who had, or would have had if exposure had continued, skin irritation after a 4 hour exposure. The time required for an individual to develop a response (if any) does not form part of the results; it relates only to ensuring proper care of the volunteers.

Selection of a concurrent positive control substance

20. By including a routine positive control, there is an opportunity to use it as a benchmark. Skin irritation is not an absolute phenomenon. All substances can give rise to skin irritation; it is simply a matter of dose and the nature and extent of exposure. Thus, skin irritation tests in humans are almost always comparative.

21. As positive irritant control, sodium dodecyl sulphate (SDS) of purity ≥ 99% is the preferred choice since it is the most widely used control irritant in clinical investigations (1)(15). It is also easily and widely available and free from other adverse effects.

22. SDS calibrates the panel of human volunteers and acts as a reference point. SDS is classified as a skin irritant according to EU criteria (16). It is not clear however whether SDS is at, or close to, the threshold level of response at which chemicals should be regarded as skin irritants. Thus rather than taking the neat substance, it is more appropriate to take as a reference point the minimum level of SDS regarded by at least one regional group (the EU) as a significant acute irritant to skin, which is a 20% w/v aqueous preparation (1). This is also to be preferred in the context of care of the volunteers. Experience with this positive control indicates that the response rate in panels does vary, the mean value being about 65% (1)(2)(4)(7). Where the response rate is less than 33% the capability of the study to distinguish irritants may be questioned. This is particularly true where substances of low irritant potential are to be compared statistically with the positive control.

DATA AND REPORTING

DRAFT

Data

23. Data should be summarised in tabular form, showing for each individual the irritation scores at 24, 48 and 72 hours after patch removal and any other effects observed.

Data evaluation/interpretation

24. The aim of this test is to determine whether a substance presents a significant skin irritation hazard following acute exposure. Thus if the substance produces a frequency of skin irritation in the test panel which is similar to, or greater than, the positive control, it should be regarded as a significant skin irritant. On the other hand, if it produces a level of reaction in the test panel which is substantially and significantly less than the positive control, then it may not be regarded as a significant skin irritant. It is important that interim data generated in the context of volunteer care are not confused with the endpoint data, i.e. the proportion of the panel who gave an irritant response. It is also important not to confuse individual variation in the susceptibility to skin irritation with the issue of the general irritation potential of the test substance.

Test report

25. The test report must include the following information:

Test substance:

- physical nature and, where relevant, physicochemical properties;
- identification data.

Vehicle:

- justification for the choice of vehicle used to moisten a solid test substance.

Volunteers:

- number of volunteers who are treated with the test substance;
- age/sex distribution of the volunteers.

Results:

- tabulation of irritation response data for each individual for each observation time period (e.g. 24, 48 and 72 hours after patch removal);
- description of all irritant reactions observed;
- description of any other effects in addition to irritation observed;
- statistical treatment of the results (comparison with positive control, e.g. using Fisher's exact test);
- description or reference of an *in vitro* test, if such is performed before the test in human volunteers, including details of the procedure, and results obtained with test and reference substances.

Discussion of the results.

DRAFT

LITERATURE

(1) York, M., Griffiths, H.A., Whittle, E. and Basketter, D.A. (1996). Evaluation of a human patch test for the identification and classification of skin irritation potential. Contact Dermatitis, 34, 204-212.

(2) Basketter, D.A., Griffiths, H.A., Wang, X.,M., Wilhelm, K.,P. and McFadden, J. (1996). Individual, ethnic and seasonal variability in irritant susceptibility of skin: the implications for a predictive human patch test. Contact Dermatitis, in press.

(3) Basketter, D.,A. and Reynolds, F.S. (1996). Use of human volunteers for hazard and risk assessment of skin irritation. In "Volunteers in Research and Testing", R. Combes, A. Hubbard and J. Illingworth (Eds), publ. Taylor and Francis, in press.

(4) Basketter, D., A., Blaikie, L. and Reynolds, F., S. (1996) . The impact of atopic status on a human 4 hour patch test for skin irritation. Contact Dermatitis, accepted.

(5) Dykes, P.J., Black, D.R., York, M., Dickens, A.D. and Marks, R. (1995). A stepwise procedure for evaluating irritant materials in normal volunteer subjects. Human and Experimental Toxicology, 14, 204-211.

(6) York, M., Basketter, D.A., Cuthbert, J.A. and Neilson, L. (1995). Skin irritation testing in man for hazard assessment - evaluation of four patch systems. Human and Experimental Toxicology, 14, 729-734.

(7) Basketter, D.A., Griffiths, H.,A., York, M., Patrick, E., Robinson, M. and Wilhelm, K., P. (1995). Interlaboratory evaluation of a human predictive test of skin irritation hazard. Allergology, 10, 454.

(8) Basketter, D.A., Whittle, E. and Chamberlain, M. (1994). Identification of irritation and corrosion hazards to skin: An alternative strategy to animal testing. Food and Chemical Toxicology, 32, 539-542.

(9) Basketter, D.A., Whittle, E., Griffiths, H.A. and York, M. (1994). The identification and classification of skin irritation hazard by a human patch test. Food and Chemical Toxicology, 32, 769-775.

(10) World Medical Association (1964) Declaration of Helsinki. Recommendation guiding physicians in biomedical research involving human subjects. Adopted by the 18th World Medical Assembly, Helsinki, June 1964, amended by the 29th World Medical Assembly, Tokyo, October 1975, the 35th World Medical Assembly, Venice, October 1983 and the 41st World Medical Assembly, Hong Kong, September 1989. Proceeding of the XXVIth Conference, Geneva, 1993. .

(11) ***Reference for Good Clinical Practice***

(12) Whittle, E. and Basketter, D.A. (1994) *In vitro* skin corrosivity test using human skin. Toxicology in Vitro, 8, 861-863.

(13) Young, J.R., How, M.J., Walker, A.P. and Worth, W.M.H. (1988) Classification as corrosive or irritant to skin of preparations containing acidic or alkaline substances without testing on animals. Toxicology in Vitro, 2, 19-26.

DRAFT

(14) Walker, A.,P., Basketter, D.,A., Baverel, M., Diembeck, W., Matthies, W., Mougin, D., Paye, M., Rothlisburger, R. and Dupuis, J. (1996). Test guideline for assessment of skin compatibility of cosmetic finished products in man. Food and Chemical Toxicology, in press.

(15) Lee, C.,H. and Maibach, H.I. (1995) The sodium lauryl sulfate model: an overview. Contact Dermatitis, 33, 1-7.

(16) 88/379/EEC Council Directive of 7 June 1988 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the classification, packaging and labeling of dangerous preparations. Official Journal of the European Communities L 187, 14, 1988.

ENV/MC/CHEM(97)6

DRAFT DOCUMENT
(April 1997)

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

DRAFT PROPOSAL FOR A NEW GUIDELINE 425

Acute Oral Toxicity: Up-and-Down Procedure

INTRODUCTION

1. The proposal for this guideline was submitted by the United States. The concept of the up-and-down testing approach was first described by Dixon and Mood (1)(2). In 1985, Bruce proposed to use an up-and-down procedure (UDP) for the determination of acute toxicity of chemicals (3). There exist several variations of the up-and-down experimental design for estimating an LD50. This guideline is based on the procedure of Bruce as adopted by ASTM in 1987 (4).

2. A study comparing the results obtained with the UDP, the conventional LD50 test and the Fixed Dose Procedure (FDP, Guideline 420) was published in 1995 (5). The study showed that i) the UDP yields an estimate of the LD50 which is similar to that obtained by the conventional LD50 test and hence leads to similar classification in LD50-based classification schemes, ii) classifications in the EC scheme were similar for the UDP and the FDP, and iii) of the three protocols, the UDP required the smallest number of animals: from 6 to 10 animals of one sex. Also for the Acute Toxic Class method (Guideline 423) classifications in the EC scheme were similar to the conventional LD50 test and the ATC and UDP methods require comparably small numbers of animals (6)(7).

3. Some terms used are defined in Annex 1 .

INITIAL CONSIDERATIONS

4. This test procedure is of principal value in minimising the number of animals required to estimate the acute oral toxicity of a chemical and in estimating a median lethal dose. The median lethal dose allows for comparison with historical data. In addition to the observation of mortality, it allows the observation of signs of toxicity. The latter is useful for classification purposes and in the planning of additional toxicity tests.

5. The procedure is easiest to apply to materials that produce death within one or two days. The method would not be practical to use when considerably delayed death (5 days or more) can be expected.

6. During the test, animals obviously in pain or showing signs of severe distress should be humanely killed.

PRINCIPLE OF THE TEST

7. Animals are dosed, one at a time, at 24 hour intervals. The first animal receives a dose at the level of the best estimate of the LD50. Depending on the outcome for the previous animal, the dose for the next animal is adjusted up or down. If an animal survives, the dose for the next animal is increased; if it dies, the dose for the next animal is decreased. After reaching the reversal of the initial outcome, i.e. the point where an increasing (or decreasing) dose pattern is reversed by giving a smaller (or a higher) dose, four additional animals are dosed following the same UDP. The LD50 is calculated using the method of maximum likelihood (8)(9).

DESCRIPTION OF THE METHOD

Selection of animals species

8. The preferred rodent species is the rat although other rodent species may be used. In the normal procedure female rats are used, because literature surveys of conventional LD50 tests show that, although there is little difference of sensitivity between sexes, in those cases where differences were observed, females were in general slightly more sensitive (5). When there is adequate information to infer that males are more sensitive, they should replace females in the test.

9. Healthy young adult animals should be employed. The females should be nulliparous and non-pregnant. At the commencement of the study, the weight variation of the animals should be minimal and not exceed ± 20 % of the mean weight for each sex. The test animals should be characterised as to species, strain, source, sex, weight and/or age.

Housing and feeding conditions

10. The temperature in the experimental animal room should be 22 C (± 3 C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning, the aim should be 50-60 %. Lighting should be artificial, the sequence being 12 hours light and 12 hours dark. The animals are housed individually. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

Preparation of animals

11. The animals are uniquely identified and kept in their cages for at lease five days prior to dosing for acclimatisation to the laboratory conditions. During acclimatisation the animals should be observed for ill health. Animals demonstrating signs of spontaneous disease or abnormality prior to the start of the study are eliminated from the study.

Preparation of doses

12. When necessary, the test substance is dissolved or suspended in a suitable vehicle. It is recommended that, whenever possible, the use of an aqueous solution or suspension be considered first,

followed by consideration of a solution or emulsion in oil (e.g. corn oil) and then by possible solution in other vehicles. For vehicles other than water, the toxicity of the vehicle must be known.

PROCEDURE

Full test

13. Individual animals are dosed in sequence at 24 h intervals, one at a time, and then observed for a minimum of 24 hours. However, the time intervals between dosing should not be fixed rigidly and may be adjusted as appropriate, in case of delayed mortality. The first animal is dosed at the toxicologist's best estimate of the LD50. If the animal survives, the second animal receives a higher dose, unless the limit dose was used as the starting dose. If the first animal dies or appears moribund the second animal receives a lower dose. Moribund state is characterised by symptoms such as shallow, laboured or irregular respiration, muscular weakness or tremors, absence of voluntary response to external stimuli, cyanosis and coma. Criteria for making the decision to humanely kill moribund and severely suffering animals are the subject of a separate Guidance Document. Animals killed for humane reasons are considered in the same way as animals that died on test.

14. For selecting the starting dose, all available information should be used, including information on structure-activity relationships. When the information suggests that mortality is unlikely then a limit test should be conducted (see paragraph 15). When there is no information on the substance to be tested, for animal welfare reasons it is recommended to use the starting dose of 200 or 500 mg/kg body weight.

15. The dose for each successive animal is adjusted up or down, depending on the outcome of the previous animal. If feasible, a dose progression factor of 1.3 is used. Other factors may be used, if justified. After reaching the reversal of the initial direction (the point where a decreasing dose patternrequires an increase due to a tested animal's survival or an increasing dose pattern results in a decrease due to lethality), four additional animals are dosed using the same UDP. This is the end of the normal test.

Limit test

16. Doses should not exceed 2000 mg/kg which is considered the upper limit dose. When the first animal is dosed with the upper limit dose and survives, the second animal receives the same dose. When a total of three animals have been dosed with the limit dose and no deaths have occurred, then three animals of the other sex should be tested at the limit dose level. If there is again no lethality, the test can be terminated.

Optional testing

17. Information from one sex may be adequate to assess acute toxicity. However, if found desirable, comparability of response in the other sex can be evaluated by administering to generally not more than 3 animals, doses above and below the estimated LD50. The point intermediate between doses where responses change can be taken as an approximate estimate of the lethal dose.

Administration of doses

18. The test substance is administered in a single dose by gavage, using an oral dosing needle or rubberised tubing.

19. The animals should be fasted prior to dosing by withholding food overnight. Fasted body weight of each rat is determined and the dose is calculated according to the body weight. After dosing food may be withheld for a further 3-4 hours. The volume should not exceed I ml/100g body weight, except in the case of aqueous solutions where 2 ml/100g body may be used.

Observations

20. Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter for a total of 14 days. However, the duration of the observation period should not be fixed rigidly. It should be determined by the toxic reactions, time of onset and length of recovery period, and may thus be extended when considered necessary.

21. Observations include mortality and clinical signs. These include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attentions should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Body weight

22. Individual weights of animals should be determined shortly before the test substance is administered, at least \veekly thereafter, at the time of death or at day 14 in the case of survival. Weight changes shou]d be calculated and recorded.

Pathology

23. All animals, including those which die during the test or are killed for animal welfare reasons during the test and those that survive at day 14, are subjected to gross necropsy. The necropsy should entail a macroscopic inspection of the visceral organs. As deemed appropriate, microscopic analysis of target organs and clinical chemistry may be included to gain further information on the nature of the toxicity of the test material.

DATA AND REPORTING

Data

24. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test concentration the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or killed for humane reasons, time of death of individual animals, a description and the time course of toxic

effects and reversibility, and necropsy findings.

Calculation of LD50

25. The LD50 is calculated using the maximum likelihood method (8)(9). The following statistical details may be helpful in implementing the maximum likelihood calculations suggested. All deaths, whether immediate or delayed or humane kills, are incorporated for the purpose of the maximum likelihood analysis. Following Dixon (8), the likelihood function is written as follows:

$$L = L_1 L_2 \ldots L_n,$$

where

L is likelihood of the experimental outcome, given μ and σ, and n the number of animals tested.

$L_i = 1 - F(Z_i)$ if the i^{th} animal survived, or
$L_i = F(Z_i)$ if the i^{th} animal died ,

where

F = cumulative, standard normal density,
$Z_i = [\log(d_i) - \mu] / \sigma$
d_i = dose given to the i^{th} animal
μ = log LD50, and
σ = standard deviation

An estimate of a of 0.12 is used unless a better generic or case-specific value is available.

26. The calculation can be performed using either SAS (10) or BMDP (11) computer program packages. Other computer programs may also be used. Typical instructions for these packages are given in appendices to the ASTM Standard E 1163-87 (4). The program output is an estimate of log LD50 and its standard error.

Report

27. The test report must include the following information:

Test substance:

- physical nature, purity and physicochemical properties (including isomerisation);
- identification data.

Vehicle (if appropriate):

- justification for choice of vehicle, if other than water.

Test animals:

- species/strain used;
- microbiological status of the animals, when known;
- number, age and sex of animals;
- rationale for use of males instead of females
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test, at day 7, and at day 14.

Test conditions:

- rationale for initial dose level selection and for follow-up dose levels;
- details of test substance formulation;
- details of the administration of the test substance;
- details of food and water quality (including diet type/source, water source).

Results:

- body weight/body weight changes;
- tabulation of response data by sex and dose level for each animal (i.e. animals showing signs of toxicity including nature, severity, duration of effects, and mortality);
- time course of onset of signs of toxicity and whether these were reversible for each animal;
- necropsy findings and any histopathological findings for each animal, if available.
- LD50 data;
- statistical treatment of results.

Discussion and interpretation of results.

Conclusions.

LITERATURE

(1) Dixon W.J. and Mood A.M. (1948). A Method for Obtaining and Analyzing Sensitivity Data. J. Amer. Statist. Assoc. 43, 109-126.

(2) Dixon W.J. (1991). Staircase Bioassay: The Up-and-Down Method. Neurosci. Biobehav. Rev. 15, 47-50

(3) Bruce R.D. (1985). An Up-and-Down Procedure for Acute Toxicity Testing. Fundam. Appl. Tox. 5, 151-157.

(4) ASTM (1987). E 1163-87, Standard Test Method for Estimating Acute Oral Toxicity in Rats. American Society for Testing and Materials, Philadelphia Pa, USA

(5) Lipnick R.L., Cotruvo J.A., Hill R.N., Bruce R.D., Stitzel K.A., Walker A.P., Chu I., Goddard M., Segal L., Springer J.A. and Myers R.C. (1995). Comparison of the Up-and-Down, Conventional LD50 and Fixed Dose Acute Toxicity Procedures. Fd Chem. Toxicol. 33, 223-231.

(6) Schlede E., Mischke U., Diener W. and Kayser D. (1995), The international validation study of the acute toxic class method (oral). Arch. Toxicol. 69, 659-670.

(7) Diener W., Mischke U., Schlede E. and Kayser D. (1995). The biometrical evaluation of the OECD modified version of the acute toxic class method (oral). Arch. Toxicol. 69, 729-734.

(8) Dixon W.J. (1965). The Up-and-Down Method for Small Samples. J. Amer. Statist. Assoc. 60, 967-978.

(9) Finney, D.J. (1971) Probit Analysis, 3rd ed., Cambridge University Press, Cambridge, England, 50-80.

(10) SAS User's Guide: Statistics SAS. Institute Inc., Cary, NC, USA.

(11) Dixon W.J., ed., BMDP Statistics Software. University of California Press, Berkeley, CA, USA.

ANNEX I

DEFINITIONS

Acute oral toxicity is the adverse effects occurring within a short time of oral administration of a single dose of a substance or multiple doses given within 24 hours.

Delayed death means that an animal does not die or appear moribund within 24 hours but dies later during the observation period.

Dosage is general term comprising the dose, its frequency and the duration of dosing.

Dose is the amount of test substance administered. Dose is expressed as weight (g, mg) or as weight of test substance per unit weight of test animal (e.g. mg/kg).

Moribund status of an animal is the result of the toxic properties of a test substance where death is anticipated. For making decisions as to the next step in this test, animals killed for humane reasons are considered in the same way as animals that died.

LD50 (median lethal dose), oral, is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit eight of test animal (mg/kg).