Episkin™, EpiDerm™, and Rat Skin Transcutaneous Electrical Resistance (TER)

In Vitro Test Methods for Assessing the Dermal Corrosivity Potential of Chemicals

Background Review Document

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Executive Summary

Corrosive substances are defined as chemicals causing visible destruction of, or irreversible alterations in living tissue by chemical action at the site of contact (29 CFR 1917.28). Dermal corrosivity testing is conducted to identify chemicals that potentially pose this hazard to humans upon contact. U.S. Federal regulations and guidelines include test methods for assessing dermal corrosivity, appropriate chemical packaging and labeling, appropriate transport and/or storage methods, and awareness education programs for workers in industrial settings. For regulatory purposes, corrosive chemicals and chemical mixtures are typically identified using an *in vivo* test method involving the application of chemicals or chemical mixtures to the intact skin of a rabbit. The skin is visually evaluated for corrosion within three minutes, and at one or four hours after application. Animal welfare considerations have led to efforts to develop *in vitro* alternative test methods.

Three alternative *in vitro* test methods – EpiDerm™, EPISKIN™, and the Rat Skin Transcutaneous Electrical Resistance (TER) assay – were developed and have subsequently been accepted as replacement assays for traditional *in vivo* corrosivity testing in the European Union (EU) (Anon., 2000). ICCVAM has implemented an expedited test method review process to consider methods which have been validated and adopted by other countries (ICCVAM, 2001). This process will accelerate interagency consideration of these test methods, thereby avoiding duplication of effort and unnecessary delays in recommending useful test methods to Federal agencies in accordance with Public Law 106-545.

Validation and Regulatory Acceptance Status of EPISKIN™, EpiDerm™, and Rat Skin TER

Independent validation studies on these three *in vitro* assays were conducted by the European Center for the Validation of Alternative Methods (ECVAM) (Barratt et al., 1998; Fentem et al.,

1998; Liebsch et al., 2000). The ECVAM Management Team concluded from these studies that the EpiDerm™, Rat Skin TER, and EPISKIN™ methods were considered scientifically validated for use as replacements for the animal test for distinguishing between corrosive and non-corrosive chemicals for all of the chemical types studied. Further, EPISKIN™ was able to distinguish between chemicals in the EU skin corrosion hazard classes (R35 and R34) and United Nations (UN) packing group classifications (I and II/III), for all of the chemical types tested (Fentem et al., 1998).[1]

A review of these validation studies and the analyses conducted by reviewers for NICEATM are presented in the background review materials as follows: Summary Report of the EPISKIN™ *In Vitro* Assay for Assessing Dermal Corrosivity (Tice and Haneke (1), drafted May 13, 1999, revised July 24, 2001; Tab 3.2); Summary Report of the EpiDerm™ *In Vitro* Assay for Assessing Dermal Corrosivity (Tice, drafted May 31, 2001, July 24, 2001; Tab 4.2); and Summary Report of the Rat Skin Transcutaneous Electrical Resistance (TER) *In Vitro* Assay for Assessing Dermal Corrosivity (Tice and Haneke (2), drafted May 13, 1999, revised July 24, 2001; Tab 5.2).

Subsequent to the ECVAM studies, the validation status of these three methods was evaluated by the ECVAM Scientific Advisory Committee (ESAC) (Balls and Corcelle, 1998; Balls and Hellsten, 2000). EPISKIN™ and Rat Skin TER were also evaluated by the European Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (Anon., 1999). EpiDerm™, EPISKIN™, and Rat Skin TER were adopted by the European Commission (Anon., 2000). The ESAC concluded that the Rat Skin TER and the EPISKIN™ tests were scientifically validated for use as replacements for the animal test for distinguishing between corrosive and non-corrosive

[1] UN packing group classifications I, II, and III are assigned based on the capacity of a chemical, when tested on the intact skin of rabbits, to produce skin corrosion following exposure intervals of 3 minutes, 1 hour, or 4 hours, respectively (Fentem et al., 1998). EU regulations require classification of chemicals according to certain risk phases, such as those assigned based on whether the chemical causes corrosion following a 3-minute application (R35 – "causes severe burns"; analogous to packing group I) or 4 hours (R34 – "causes burns"; analogous to packing groups II and III) (Barratt et al., 1998; Fentem et al., 1998).

chemicals and that these *in vitro* tests were ready to be considered for regulatory acceptance (Balls and Corcelle, 1998). Based on a review of the results of the ECVAM-funded independent pre-validation/validation study on EpiDerm™, coordinated by ZEBET (Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments, Berlin, Germany), the ESAC concluded that EpiDerm™ can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the draft EU and Organisation for Economic Co-operation and Development (OECD) test guidelines on skin corrosion (Balls and Hellsten, 2000). The SCCNFP concluded that the validated Rat Skin TER and EPISKIN™ methods were considered to be applicable to the safety evaluation of cosmetic ingredients or mixtures of ingredients (Anon., 1999).

The OECD has developed a draft test guideline (TG) on *in vitro* skin corrosion tests detailing the procedures of the Rat Skin TER assay and a generic Human Skin Model Assay (*In Vitro* Skin Corrosion Tests, Draft New Guideline, November 1999). A generic skin model assay was proposed based on OECD's policy not to recommend TGs for tests requiring equipment or material from a unique source. Review and comment on the OECD draft TG was requested from member countries in early 2000. Based on the comments submitted by the national coordinators to OECD on the proposed TG, a number of scientific/technical issues exist that require further consideration. The primary concerns identified are the lack of guidance on interpreting borderline results; the lack of sufficient detail on the generic *in vitro* human skin model assay, and the lack of consistency with the Globally Harmonised Classification System (GHS) with respect to the treatment of negative results in the *in vitro* skin corrosion tests (OECD ENV/JM/TG(2001)7).

Due to the nature of the concerns identified, it was concluded at the OECD's 13th Working Group of National Coordinators of the Test Guidelines Programme (May 30 – June 1, 2001 in Paris, France) that an Extended Expert Consultation Meeting should be convened. This workshop is to be held on November 1-2, 2001 in Berlin, Germany. The objectives of this meeting will include: i) the explanation, clarification, discussion, and agreement on all technical issues raised during the TG comment period, and ii) identification of additional work, if any, to be considered in order to approve the Guideline proposal.

EPISKIN™

EPISKIN™ is a three-dimensional human skin model composed a human collagen (Types III and I) matrix, representing the dermis, covered with a film of Type IV human collagen, upon which stratified differentiated epidermis derived from human keratinocytes has been laid. Test materials can be applied directly to the stratum corneum. The model utilizes cell viability as the measured endpoint. The mode of application (topical) of the test material mimics the route of human exposure. For use in corrosivity testing, the test material (liquids: 50 µL; solids: 20 mg) is applied to an epidermis unit for 3, 60, and 240 minutes. Cell viability is assessed by measuring mitochondrial activity using the MTT (a tetrazolium salt) assay as compared to concurrent negative controls. A 35% decrease in cell viability is used to indicate a potential for human corrosivity.

ECVAM conducted an independent validation study on the EPISKIN™ method as an *in vitro* replacement assay for *in vivo* corrosivity testing (Fentem et al., 1998). Sixty chemicals were evaluated in duplicate in three different laboratories; chemical selection and *in vivo* reference data were described by Barratt et al. (1998). The ECVAM validation chemical test set included organic acids, organic bases, neutral organics, phenols, inorganic acids, inorganic bases, inorganic salts, electrophiles, and soaps/surfactants. ECVAM concluded the set of test chemicals represented the best possible group for evaluating the performance characteristics of the *in vitro* assays, given the limited availability of unequivocal animal data (Barratt et al., 1998).

An analysis was conducted, by reviewers supporting NICEATM, to evaluate the performance characteristics of the EPISKIN™ assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the EPISKIN™ evaluation consisted of data from the ECVAM validation study only; other data were not located. For ease of comparison, chemicals evaluated in the EPISKIN™ assay were classified into the same chemical and product class designations used in the ICCVAM Peer Review of Corrositex® (ICCVAM, 1999). A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative

calls), the chemical was eliminated from inclusion in the performance calculations. Based on the database of 60 chemicals and chemical mixtures used in the validation study, EPISKIN™ had an accuracy of 83% (50/60 chemicals or chemical mixtures), a sensitivity of 82% (23/28 chemicals or chemical mixtures), and a specificity of 84% (27/32 chemicals or chemical mixtures). Furthermore, EPISKIN™ was able to distinguish between known R35/I and R34/II & III chemicals.

Inter- and intra-laboratory reproducibility of EPISKIN™ was also evaluated by Fentem et al. (1998). In each laboratory, each chemical was tested three times using three different batches of EPISKIN™. Intra- and inter-laboratory reliability was evaluated using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Of the 60 chemicals tested, 42 gave the same corrosivity classification in all three experiments in all three laboratories. For six chemicals, one test resulted in a classification differing from the other eight test results. For seven chemicals, the number of discordant results among the nine tests varied from two to three. For the remaining five chemicals, the number of discordant results among the nine tests varied from four to five. Although there were differences for some chemicals in trials between experiments within and between laboratories, ECVAM concluded that EPISKIN™ met the criteria agreed by the Management Team concerning acceptable intra- and inter-laboratory reproducibility (Fentem et al., 1998).

EpiDerm™

The EpiDerm™ skin model is mechanistically and functionally related to EPISKIN™. The assay consists of normal, human epidermal keratinocytes which have been cultured in chemically defined medium to produce a stratified, highly differentiated, organotypic tissue model of the human epidermis. The EpiDerm™ tissue consists of metabolically and mitotically active cells which are organized into a basal, spinous, and granular layer along with a multi-layered stratum corneum (MatTek Corporation, 2000). Like EPISKIN™, the EpiDerm™ tissue approximates the barrier of normal human skin, and further, the topical mode of application of the test material in

EpiDerm™ mimics the route of human exposure. For use in corrosivity testing, the test material (liquids and semi-solids: 50 µL; solids: 25 mg plus 25 µl of H_2O) is applied to a tissue for three and 60 minutes. Per test compound, replicate plates are used for each test period. As with EPISKIN™, cell viability is assessed by measuring mitochondrial activity using the MTT assay. A test chemical is classified as corrosive if it induces ≥50% decrease in relative cell viability at 3 minutes or ≥85% decrease in relative cell viability at 60 minutes.

ECVAM conducted an independent validation study on EpiDerm™ as an *in vitro* replacement assay for *in vivo* corrosivity testing (Liebsch et al., 2000). Twenty-four chemicals representative of the 60 chemicals tested in the Fentem et al. (1998) ECVAM validation study for the EPISKIN™ assay were tested. In selecting the 24 chemicals, care was taken to ensure a balanced representation of the chemical classes in this subset. The 24 chemicals selected included 12 corrosive and 12 non-corrosive chemicals -- organic acids and bases, neutral organic bases, phenols, inorganic acids and bases, electrophiles, and surfactants.

An analysis was conducted, by reviewers supporting NICEATM, to evaluate the performance characteristics of the EpiDerm™ assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the evaluation of EpiDerm™ consisted of data from the ECVAM pre-validation/validation study only (Liebsch et al., 2000); other data were not located. For ease of comparison, chemicals were classified into the same chemical and product class designations used in the ICCVAM Peer Review of Corrositex® (ICCVAM, 1999) and a weight-of-evidence approach was used for classifying any discordant results. Based on the database of 24 chemicals and chemical mixtures used in the validation study, EpiDerm™ had an accuracy of 92% (22/24 chemicals or chemical mixtures), a sensitivity of 92% (11/12 chemicals or chemical mixtures), and a specificity of 83% (10/12 chemicals or chemical mixtures). Unlike EPISKIN™, EpiDerm™ was not able to distinguish between known R35/I and R34/II & III chemicals.

In the validation study, each chemical was tested twice using different tissue lots in each of three laboratories. Intra- and inter-laboratory reliability was evaluated using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors),

scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Of the 24 chemicals tested, 19 gave the same corrosivity classification in the two replicates in all three laboratories (6 tests). For three chemicals, one test resulted in a classification differing from the other five test results. Two discordant results in six tests were found for the two remaining chemicals. Based on the results obtained, ECVAM concluded that EpiDerm™ provided excellent reliability (Liebsch et al., 2000).

Rat Skin TER

In the Rat Skin TER assay, test materials (liquids: 150 µL; solids 100 mg plus 150 µL of water) are applied for two and 24 hours to the epidermal surfaces of skin discs obtained from the skin of humanely killed young rats. Nine to 15 discs can be prepared from one rat pelt. Corrosive materials are identified by the ability of the chemical to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction of the inherent transcutaneous electrical resistance below a predetermined threshold level of 5 kΩ.

A prevalidation study of the Rat Skin TER assay was conducted during 1993 and 1994 (Botham et al., 1995) to evaluate the relative performance, to assess interlaboratory variability, and to evaluate the validation status of the method. Subsequently, in 1997, the Rat Skin TER method was also validated in an independent ECVAM study as an *in vitro* replacement test method for traditional *in vivo* testing and was tested using the same 60 chemicals and chemical mixtures as EPISKIN™ (Fentem et al., 1998).

An analysis was conducted, by reviewers supporting NICEATM, to evaluate the performance characteristics of the Rat Skin TER assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the TER evaluation consisted of data from three published sources (Botham et al., 1992; Botham et al., 1995; Fentem et al., 1998). For ease of comparison, chemicals evaluated in the TER assay were classified into the same chemical and product class designations used in the ICCVAM Peer Review of Corrositex® evaluation (ICCVAM, 1999). A weight-of-evidence approach was used for classifying discordant results as previously described.

Based on a database of 122 chemical and chemical mixtures, TER had an accuracy of 81% (99/122 chemicals or chemical mixtures), a sensitivity of 94% (51/54 chemicals or chemical mixtures), and a specificity of 71% (48/68 chemicals or chemical mixtures). These performance characteristics were not different when the Botham et al. (1992) and (1995) studies were evaluated independently of the ECVAM validation study (Fentem et al., 1998). Rat Skin TER was not capable of classifying chemicals or chemical mixtures by UN corrosivity packing group.

In the ECVAM validation study (Fentem et al., 1998), the intra- and inter- laboratory reliability was evaluated using a relative mean square diagram, scatter diagrams, and range diagrams as previously described. In this study, the inter- and intra-laboratory reproducibility were approximately equivalent, with no evidence of systematic differences between experiments within a laboratory. Of the 60 chemicals tested, 37 gave the same corrosivity classification in both experiments in all three laboratories (6 tests). For 11 chemicals, one test resulted in a classification differing from the other five test results. For the remaining 12 chemicals, the number of discordant results among the six tests varied from two to three. ECVAM concluded the TER assay was both reliable and reproducible.

Summary Conclusions

The existing database of information, along with conclusions and recommendations of review bodies and regulatory authorities, was used to develop responses for two questions for each test:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?

2. Does the assay adequately consider and incorporate, where scientifically feasible and applicable, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

In response to the first question, the performance characteristics of all three *in vitro* methods indicate, in specific testing circumstances, that these tests may be considered useful as part of an integrated testing strategy for assessing the dermal corrosion potential of chemicals. Only the EPISKIN™ skin model was adequate for assigning packing groups according to the EU skin corrosion hazard classes (R34/R35) and the UN packing group classifications (I and II/III). However, since the performance of EPISKIN™ was not assessed for distinguishing between UN packing group II and packing group III, all R34 classifications would be conservatively classified as packing group II.

Each of the three *in vitro* corrosivity methods sufficiently considers and incorporates, where scientifically feasible and applicable, the 3Rs of animal use (refinement, reduction, and replacement alternatives). When incorporated into an integrated testing approach, the use of EpiDerm™ and EPISKIN™ offer advantages with respect to animal use refinement, reduction, and replacement. The Rat Skin TER method offers animal welfare advantages, including animal use refinement and reduction; this method reduces the number of animals used as skin from one humanely killed rat may be used to test up to five chemicals. Similar to EpiDerm™ and EPISKIN™, the use of the Rat Skin TER assay as part of the integrated testing strategy for corrosivity/irritation reduces and refines the use of animals.

ICCVAM Recommendations

Based on an evaluation of the ECVAM validation studies and all other available data, ICCVAM concludes that there are sufficient data to substantiate the use of these assays for assessing the dermal corrosion potential of chemicals in a weight-of-evidence approach in an integrated testing scheme (e.g., OECD GHS and/or the OECD Revised Proposals for Updated Test Guidelines 404 and 405: Dermal and Eye Corrosion/Irritation Studies (ENV/JM/TG(2001)2). The integrated testing schemes for dermal irritation/corrosion allow for the use of validated and accepted *in vitro* methods. In this approach, positive *in vitro* corrosivity responses do not generally require further testing and can be used for classification and labeling. Negative *in vitro* corrosivity responses would be followed by *in vivo* dermal corrosion/irritation testing. (Note: The first animal used in the irritation/corrosivity assessment would be expected to identify any chemical

corrosives that were false negatives in the *in vitro* test). Furthermore, as is appropriate for any *in vitro* assay, there is the opportunity for confirmatory testing if false positive results are indicated based on a weight-of-evidence evaluation of supplemental information, such as pH, structure-activity relationships (SAR), and other chemical and testing information.

ICCVAM concludes also that each of the three *in vitro* corrosivity methods sufficiently consider and incorporate, where scientifically feasible and applicable, the 3Rs of animal use (refinement, reduction, and replacement alternatives). When EpiDerm™ and EPISKIN™ are used as part of the integrated testing strategy for corrosivity/irritation, there is a reduction in the number of animals required because positive results usually eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal could be required to identify a corrosive chemical (one animal is used if the *in vitro* test is negative). Compared to the rabbit corrosivity test, the Rat Skin TER method reduces the number of animals used because skin from only one rat may be used to test up to five chemicals. Similar to EpiDerm™ and EPISKINä , the use of the Rat Skin TER assay as part of the integrated testing strategy for corrosivity/irritation reduces and refines the use of animals by providing a basis for decisions on further animal testing.

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Tice R, Haneke K (2). Summary Report of the Rat Skin Transcutaneous Electrical Resistance (TER) *In Vitro* Assay for Assessing Dermal Corrosivity. Drafted May 13, 1999, Revised July 24, 2001. Included in the Background Review Document, Tab 5.2.

ICCVAM Draft Proposed Recommendations on Three *In Vitro* Methods for Assessing the Dermal Corrosivity Potential of Chemicals: EPISKIN™, EpiDerm™, and Rat Skin Transcutaneous Electrical Resistance (TER)

[**NOTE**: The following statements represent the draft proposed test recommendations from the ICCVAM Corrosivity Working Group (CWG) for consideration and endorsement by the ICCVAM at their August 27, 2001 meeting. Following endorsement by ICCVAM, public comment on the proposed recommendations will then be solicited by NICEATM via a Federal Register notice. Following receipt and consideration of any public comments, the CWG will prepare final test recommendations for ICCVAM approval. These will be forwarded to agencies for their consideration and adoption where appropriate.]

I. Introduction

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has developed proposed recommendations for the use of three *in vitro* test methods – EpiDerm™, EPISKIN™, and Rat Skin Transcutaneous Electrical Resistance (TER) – to assess the dermal corrosivity potential of chemicals and chemical mixtures. Validation studies for these methods were conducted by the European Centre for the Validation of Alternative Methods (ECVAM) (Baratt et al., 1998; Fentem et al., 1998; Liebsch et al., 2000). The validation status of these three methods has been evaluated by the ECVAM Scientific Advisory Committee (ESAC) (Balls and Corcelle, 1998; Balls and Hellsten, 2000), and EPISKIN™ and Rat Skin TER have also been evaluated by the European Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (Anon., 1999). These three methods have been adopted for regulatory use within the European Union (EU) by the European Commission (Anon., 2000).

ICCVAM has implemented an expedited test method review process to consider methods which have been validated and adopted by other countries (ICCVAM, 2001). This process will accelerate interagency consideration of these test methods, thereby avoiding duplication of effort and unnecessary delays in recommending useful test methods to Federal agencies. ICCVAM and the ICCVAM Corrosivity Working Group (CWG) considered background review documents

prepared by the NTP Interagency Center for the Evaluation of Alternative Methods (NICEATM) on all three corrosivity methods. Based on the information provided, it was not considered necessary to organize a formal, independent scientific peer review panel evaluation. ICCVAM proposed recommendations on the test methods were subsequently developed and are provided below. These recommendations and the background review documents will be made available for public comment. Following receipt and consideration of public comments, ICCVAM will develop and forward final recommendations on these methods to U.S. agencies for their consideration and adoption where appropriate.

II. **Background**

A. ECVAM Evaluation

Validation studies on these three *in vitro* assays were conducted by ECVAM (Barratt et al., 1998; Fentem et al., 1998; Liebsch et al., 2000). Based on the results, the ECVAM Management Team concluded that EpiDerm™, Rat Skin TER, and EPISKIN™ were scientifically valid for use as replacements for the animal test currently used to distinguish between corrosive and non-corrosive chemicals and for all chemical classes (Fentem et al., 1998; Liebsch et al., 2000). Of the three test methods, only EPISKIN™ was able to distinguish between chemicals in the EU skin corrosion hazard classes (R35 and R34) and United Nations (UN) packing group classifications (I and II/III) (Fentem et al., 1998).[1] A review of these validation studies and the analyses conducted by reviewers for NICEATM are presented in the background review materials as follows: Summary Report of the EPISKIN™ *In Vitro* Assay for Assessing Dermal Corrosivity (Tice and Haneke (1), drafted May 13, 1999, revised July 24, 2001; Tab 3.2); Summary Report of the EpiDerm™ *In Vitro* Assay for Assessing Dermal Corrosivity (Tice, drafted May 31, 2001, July 24, 2001; Tab 4.2); and Summary Report of the

[1] UN packing group classifications I, II, and III are assigned based on the capacity of a chemical, when tested on the intact skin of rabbits, to produce skin corrosion following exposure intervals of 3 minutes, 1 hour, or 4 hours, respectively (Fentem et al., 1998). EU regulations require classification of chemicals according to certain risk phases, such as those assigned based on whether the chemical causes corrosion following a 3-minute application (R35 – "causes severe burns"; analogous to packing group I) or 4 hours (R34 – "causes burns"; analogous to packing groups II and III) (Barratt et al., 1998; Fentem et al., 1998).

Rat Skin Transcutaneous Electrical Resistance (TER) *In Vitro* Assay for Assessing Dermal Corrosivity (Tice and Haneke (2), drafted May 13, 1999, revised July 24, 2001; Tab 5.2).

B. Status in Organisation for Economic Co-operation and Development (OECD) Test Guidelines Program

The OECD has developed a draft test guideline (TG) on *in vitro* skin corrosion tests detailing the procedures of the Rat Skin TER assay and a generic Human Skin Model Assay (*In Vitro* Skin Corrosion Tests, Draft New Guideline, November 1999). A generic skin model assay was proposed based on OECD's policy not to recommend TGs for tests requiring equipment or material from a unique source. Review and comment on the OECD draft TG was requested from member countries in early 2000. Based on the comments submitted by the national coordinators to OECD on the proposed TG, a number of scientific/technical issues exist that require further consideration. The primary concerns identified are the lack of guidance on interpreting borderline results; the lack of sufficient detail on the generic *in vitro* human skin model assay, and the lack of consistency with the Globally Harmonised Classification System (GHS) with respect to the treatment of negative results in the *in vitro* skin corrosion tests (OECD ENV/JM/TG(2001)7).

Due to the nature of the concerns identified, it was concluded at the OECD's 13th Working Group of National Coordinators of the Test Guidelines Programme (May 30 – June 1, 2001 in Paris, France) that an Extended Expert Consultation Meeting should be convened. This workshop is to be held on November 1-2, 2001 in Berlin, Germany. The objectives of this meeting will include: i) the explanation, clarification, discussion, and agreement on all technical issues raised during the TG comment period, and ii) identification of additional work, if any, to be considered in order to approve the Guideline proposal.

III. **Proposed ICCVAM Test Method Recommendations**

Based on evaluation of the ECVAM validation studies and all other available data, ICCVAM concludes that there are sufficient data to substantiate the use of these assays for assessing the dermal corrosion potential of chemicals in a weight-of-evidence approach in an integrated testing scheme (e.g., OECD GHS and/or the OECD Revised Proposals for Updated Test Guidelines 404

and 405: Dermal and Eye Corrosion/Irritation Studies (ENV/JM/TG(2001)2). The integrated testing schemes for dermal irritation/corrosion allow for the use of validated and accepted *in vitro* methods. In this approach, positive *in vitro* corrosivity responses do not generally require further testing and can be used for classification and labeling. Negative *in vitro* corrosivity responses shall be followed by *in vivo* dermal corrosion/irritation testing. (Note: The first animal used in the irritation/corrosivity assessment would be expected to identify any chemical corrosives that were false negatives in the *in vitro* test). Furthermore, as is appropriate for any *in vitro* assay, there is the opportunity for confirmatory testing if false positive results are indicated based on a weight-of-evidence evaluation of supplemental information, such as pH, structure-activity relationships (SAR), and other chemical and testing information.

ICCVAM concludes also that each of the three *in vitro* corrosivity methods sufficiently consider and incorporate, where scientifically feasible and applicable, the 3Rs of animal use (refinement, reduction, and replacement alternatives). When EpiDerm™ and EPISKIN™ are used as part of the integrated testing strategy for corrosivity/irritation, there is a reduction in the number of animals required because positive results usually eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal could be required to identify a corrosive chemical (one animal is used if the *in vitro* test is negative). Compared to the rabbit corrosivity test, the Rat Skin TER method reduces the number of animals used because skin from only one rat may be used to test up to five chemicals. Similar to EpiDerm™ and EPISKIN™, the use of the Rat Skin TER assay as part of the integrated testing strategy for corrosivity/irritation reduces and refines the use of animals by providing a basis for decisions on further animal testing.

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ECVAM Protocol for EPISKIN™: an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: March 1997
Confirmed: October 2000

NOTE: This protocol presents the standard operating procedure used in the ECVAM Skin Corrosivity Validation Study (1996/1997). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

EPISKIN™ Test

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the EPISKIN™ reconstituted human epidermis.

Objectives and Application

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency, hazard identification
PURPOSE OF TESTING	:	classification and labelling

Proposed replacement for the *in vivo* Draize rabbit skin corrosivity test to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.

Basis of the Method

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin. The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. The determination of skin corrosion potential is therefore included in international regulatory requirements for the testing of chemicals, for example, in OECD testing guideline 404 (Anon., 1992); Annex V of Directive 67/548/EEC (Anon., 1992) and in the U.S. Code of Federal Regulations (Anon., 1991). Corrosivity is usually determined *in vivo* using the Draize rabbit skin test (Draize *et al*., 1944).

The present test is based on the experience that corrosive chemicals show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis. The test is designed to predict and classify the skin corrosivity potential of a chemical by assessment of its effect on a reconstituted human epidermis.

EPISKIN Standard Model™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin corrosivity testing involves topical application of test materials to the surface of the skin, and the subsequent assessment of their effects on cell viability. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT. (Fentem *et al*., 1998)

Experimental Description

Endpoint and Endpoint Detection	:	cell viability as determined by reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT
Test System	:	EPISKIN™ reconstructed human epidermis system *

Test materials are applied to the stratum corneum of the epidermal model (one epidermis unit per test material) for three different exposure periods: 3 minutes, 1 hour, and 4 hours. Exposure to the test chemical was terminated by rinsing with PBS (phosphate buffered saline). EPISKIN cultures exposed to the control compounds for 240 min serve as the controls for all three exposure periods. For each test material, three independent tests with three different batches of EPISKIN are to be undertaken.

The viability of the epidermis is assessed by measuring the mitochondrial activity. The tissues are incubated for 3 hours with MTT solution (0.3 mg/l; 2.2 ml per well). MTT, a yellow-coloured tetrazolium salt, is reduced by succinate dehydrogenase into a blue formazan precipitate in the mitochondria of living cells. The precipitated formazan is extracted overnight by using acidified isopropanol (0.85 ml), and is then quantified spectrophotometrically at a wavelength between 545nm and 595nm.

All experimental procedures have to be conducted at room temperature (18-28°C); if the temperature is below 20°C, the 3-hour MTT incubation should be carried out in a warmer environment of 20-28°C. NaCl (50 µl) and glacial acetic acid (50 µl) are used as negative and positive controls, respectively.

Some highly reactive chemicals can produce fumes, which may affect adjacent units in the same plate. It is recommended that if there is any suspicion that a material could cause fumes, it should be tested alone in a single plate. It is particularly important that the negative control units are not exposed to fumes from other units, hence it is recommended to routinely incubate positive and negative controls in a separate plate.

NOTE: The commercial availability of EPISKIN (SADUC-Biomatériaux Imedex, Chaponost, France) was restricted following the completion of the validation study to enable new production facilities to be completed. It is likely to be available again during 2000. In a subsequent small catch up study, the EPIDERM nodel has been tested and accepted for the assessment of the corrosive potential of chemical substances (INVITTOX No. 119).

Test Compounds

A total of 60 test compounds, consisting of 11 organic acids, 10 organic bases, 9 neutral organics, 5 phenols, 7 inorganic acids, 4 inorganic bases, 3 inorganic salts, 8 electrophiles, 3 soaps/surfactants have been tested in the ECVAM validation study.

Details of the test compounds and test results are available in **dbVas** of ECVAM SIS.

Prediction Model

The test results are interpreted on the basis of the exposure time needed to cause cell viability to decrease below 35%. The determination of the UN packing groups and EU classifications is summarized in the table reported in the section 4.1. **"Interpretation of test results"** of the present standard operating procedure.

Status

This method has been evaluated in the **ECVAM Skin Corrosivity Validation Study** conducted under the auspices of ECVAM during 1996 and 1997 (Fentem *et al*., 1998). The ECVAM Scientific Advisory Committee (ESAC) agreed that the results obtained with the EPISKIN™ test in the ECVAM international validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The test proved applicable to testing of all the above reported chemical classes of different physical forms. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good.

The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied; it was also able to distinguish between known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. Based on the outcome of the study, the ESAC unanimously endorsed the statement that the EPISKIN test was scientifically validated for use as a replacement for the animal test and that this test was ready to be considered for regulatory acceptance (10th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 1998). (Anon., 1998b).

· The 27th meeting of the Committee for Adaptation to Technical Progress of "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" agreed that the human skin model assays, which meet certain criteria, would form part of "Annex V method B.40. Skin Corrosion", February 2000 (Commission Directive 2000/33/EC). Furthermore, these models are now under consideration for inclusion in the OECD Guidelines. Further details on the ECVAM Validation Study are available in **dbVas** of the ECVAM SIS.

Last update: May 2000

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Procedure Details, March 1997*

EPISKIN™ TEST

NOTE: This protocol presents the standard operating procedure used in ECVAM Skin Corrosivity Validation Study (1996/1997).

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* The accuracy of the SOP has been confirmed in October 2000.

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1. Introduction

Product Description
The EPISKIN-SM™ (Standard Model) kit contains 12 reconstructed epidermis units. Each reconstructed epidermis unit consists of a human collagen (Types III and I) matrix, representing the dermis, covered with a film of Type IV human collagen, upon which stratified differentiated epidermis derived from human keratinocytes has been laid. Test materials can be applied directly to the stratum corneum.

Precautions
The epidermal cells are taken from healthy volunteer donors negative to anti-HIV 1 and 2, and to hepatitis C, antibodies, and to hepatitis B antigens. Nevertheless, normal handling procedures for biological materials should be followed:

(a) it is recommended that gloves are worn during handling; and
(b) after use, the epidermis, the material in contact with it, and the culture medium, should be decontaminated (for example, by using a 10% solution of bleach or a 1% solution of pyosynthene), prior to disposal.

Quality Control
EPISKIN-SM kits are manufactured according to defined quality assurance procedures (certified ISO 9001). All biological components of the epidermis and the kit culture medium have been tested for the presence of viruses, bacteria and mycoplasma. The quality of the final product is assessed by undertaking an MTT cell viability test and a cytotoxicity test with sodium dodecylsulphate (SDS). For reasons connected with the nature of the product, it is shipped before all of the necessary checks have been completed. A release form certifying the conformity (or otherwise) of the batch is sent to the user, by fax, on the day of delivery of the kit.

2. Materials

2.1. KIT CONTENTS

DESCRIPTION	USE
1 EPISKIN-SM plate containing 12 reconstructed epidermis units (area: 0.38cm^2)	each reconstructed epidermis is attached to the base of a tissue culture vessel with an O-ring set and maintained on nutritive agar for transport
1 12-well assay plate	for assays
1 flask of sterile assay medium	basic medium for use in assays
1 EPISKIN-SM biopsy punch	for easy sampling of epidermis

1 lot of "MTT reagents":

1 flask MTT reagent	to reconstitute
1 flask PBS 10x wash solution	to dilute
1 flask 4N NaOH	to adjust pH of wash solution
1 flask extraction solution of isopropanol acid (ready to use)	

1 flask negative control (NaCl, 9g/l) 1 flask positive control (glacial acetic acid)	specific controls for the corrosivity test

2.2. MATERIALS NOT PROVIDED WITH THE KIT

- 500ml wash bottle
- 5ml glass tubes with corks
- 200µl micropipette
- Multidispenser micropipette (2.2ml)
- 50µl or 100µl positive displacement micropipette (for applying thick or viscous samples)
- Vacuum source and Pasteur pipettes
- Small forceps
- Timers
- Microplate reader with filter of 545-595nm and 96-well microplates; or spectrophotometer and 1ml microcells
- Vortex mixer
- Non-sterile ventilated cabinet

3. Experimental Procedures and Timing

Details of the kit and assay procedures should be registered on the reporting form (Annex 1).

3.1. RECEIPT OF TEST KIT

Check the date of dispatch written on the package. Before opening the EPISKIN-SM kit:
(a) inspect the colour of the agar medium used for transport and check that its pH is acceptable: orange colour = good; yellow or violet colour = not acceptable;
and
(b) inspect the colour of the temperature indicator to verify that the kit has not been exposed to a temperature above 40°C: the indicator changes from white to grey at 40°C.
In the event of any anomaly, immediately contact the Sales Administration Department at SADUC (Tel: +33 78 56 72 72; Fax: +33 78 56 00 48).

Place the assay medium supplied with the kits at 2-8°C. Leave the EPISKIN-SM kits in their packaging at room temperature until the assays are to be undertaken.

3.2. APPLICATION AND RINSING

Safety precautions: MTT and corrosive materials are dangerous. Work in a non-sterile, ventilated, cabinet, wear protective gloves, and a mask and safety glasses, as necessary.
Pre-warm the assay medium to 37°C. An approximate timing for conducting the test procedure is given below as a guide.

9.30: proceed with the application of test material for the 4-hour samples

(a) Fill the appropriate number of wells of an assay plate with pre-warmed culture medium (2.2ml per well). Mark the plate lids with the application time (4 hours) and the code numbers of the chemicals to be tested (1 well per chemical), or negative control (3 wells) or positive control (3 wells).
(b) Open the EPISKIN-SM kits and place an epidermis unit into each prepared well. Mark each epidermis unit with the appropriate code number.

9.45: application of the products during **4 hours:**

(c) Add 50µl of test material to each well by using the positive displacement pipette.
(d) In the case of solids, the material should be crushed to a powder, if necessary, and 20mg applied evenly to the epidermal surface (with difficult materials, use sufficient to cover the epidermal surface); add 100µl NaCl (9g/l saline) to ensure good contact with the epidermis.
(e) Add 50µl NaCl (9g/l saline) to each of the three negative control wells.
(f) Add 50µl glacial acetic acid to each of the three positive control wells.
(g) Replace the lid on the plate and incubate for 4 hours (± 5 minutes) in a ventilated cabinet at room temperature (18-28°C).

Note: The negative and positive controls incubated for 4 hours will act as controls for all of the incubation times.

10.00: proceed with the application of test material for the 1-hour samples

(a) Fill the appropriate number of wells of an assay plate with pre-warmed culture medium (2.2ml per well). Mark the plate lids with the application time (1 hour) and the code numbers of the chemicals to be tested (1 well per chemical).
(b) Open the EPISKIN-SM kits and place an epidermis unit into each prepared well. Mark each epidermis unit with the appropriate code number.

10.15: application of the products during **1 hour**

(c) Add 50µl of test material to each well by using the positive displacement pipette.
(d) In the case of solids, apply 20mg and add 100µl of NaCl (9g/l), as described previously for the 4-hour samples.
(e) Replace the lid on the plate and incubate for 1 hour (± 5 minutes) in a ventilated cabinet at room temperature (18-28°C).

10.30: proceed with the application of test material for the 3-minute samples

(a) Prepare the MTT solution (0.3mg/ml; enough for 2.2ml per well for the entire assay) and the PBS 1x wash solution, as indicated in the "MTT reagents" leaflet accompanying the test kit.
(b) Fill the appropriate number of wells of an assay plate with pre-warmed culture medium (2.2ml per well). Mark the plate lids with the application time (3 minutes) and the code numbers of the chemicals to be tested (1 well per chemical).
(c) Open the EPISKIN-SM kits and place an epidermis unit into each prepared well. Mark each epidermis unit with the appropriate code number.

10.45: application of the products during **3 minutes**

(d) Add 50µl of test material to each well by using the positive displacement pipette. Proceed well by well at 20-second intervals, with the aid of multiple timers (test a maximum of 5 or 6 materials at a time). Ensure that the exposure period is exactly 3 minutes for each well
(e) In the case of solids, apply 20mg and add 100µl of NaCl (9g/l), as described previously for the 4-hour samples.
(f) Remove the EPISKIN-SM unit and rinse thoroughly with PBS 1x solution, to remove all of the test material from the epidermal surface.
(g) Replace the EPISKIN-SM unit in the culture medium.
(h) When all of the units have been rinsed:
- remove the culture medium
- place the units on absorbent paper, or remove the rest of the PBS from the epidermal surface with a Pasteur pipette linked to a vacuum source (be careful not to touch the epidermis)
- add 2.2ml of the MTT solution (0.3mg/ml) to each well
- replace the lid on the plate. If the ambient temperature is 20-28°C, leave to incubate for 3 hours (± 5 minutes) in a ventilated cabinet at room temperature, **protected from light**. If the ambient temperature is below 20°C, then leave to incubate for 3 hours (± 5 minutes) at temperature of 20-28°C, **protected from light**. An incubator (with or without CO_2), or a warm location within the laboratory, may be used. It is important that all the samples from each exposure time are treated identically.

11.15: rinse the 1-hour samples and replace the culture medium with 2.2ml of MTT solution (0.3mg/ml), as described above.

11.45: place 0.85ml of acidified isopropanol into labelled glass tubes (one tube corresponding to one well of the tissue culture plate). Label each tube with the name of the test material and the incubation time.

13.45: rinse the 4-hour samples and replace the culture medium with 2.2ml of MTT solution

(0.3mg/ml), as described above.

3.3. FORMAZAN EXTRACTION

At the end of each incubation with MTT (*14.15*, *14.45* and *17.00*), the formazan extraction should be undertaken:

(a) place the units on absorbent paper
(b) remove the MTT solution from each well
(c) take a biopsy of the epidermis by using the biopsy punch, by placing the epidermis unit on the plate lid
(d) separate the epidermis from the collagen matrix with the aid of forceps, and place both parts (epidermis and collagen matrix) into the acidified isopropanol
(e) cork each tube and mix thoroughly by using a vortex mixer
(f) ensure that the acidified isopropanol is in good contact with all of the material
(g) store at room temperature overnight, protected from light.

3.4. ABSORBANCE/OPTICAL DENSITY MEASUREMENTS

Following the formazan extraction (left overnight):

(a) mix each tube by using a vortex mixer
(b) let the solution settle for 1-2 minutes, so that any cell fragments do not interfere with the absorbance readings
(c) place a 200µl sample from each tube into the wells of a 96-well plate (labelled appropriately)
(d) read the optical densities (OD) of the samples at a wavelength between 545nm and 595nm using acidified isopropanol solution as the blank.
(e) record the results on the template given in Annex 2.

Note: if a spectrophotometer is used rather than a plate reader, place a 500µl sample from each tube and 500µl isopropanol (not acidified) in a 1ml microcell and read the OD at 545-595nm using the acidified isopropanol solution as the blank.

4. Calculations of viability percentages and acceptability criteria

Record all calculations on the Data Report Form (Annex 3).

Viability (%) = 100 x (OD test material/mean OD negative control at 4 hours)

(a) calculate the mean OD of the 3 negative control values: this corresponds to 100% viability. Based on historical data the minimum acceptable mean OD for negative controls is 0.115 (mean ± 2SD). The maximum acceptable mean OD for the negative control is 0.4 (to allow for incubations at 28°C).
(b) calculate the mean OD of the 3 positive control values: the % viability of the positive control is calculated relative to the mean negative control. Based on historical data (mean ±2SD), the acceptable mean percentage viability range for positive controls is 0-20%.
(c) calculate the % viability following exposure to the test material at each incubation time as the OD expressed as a percentage of the mean negative control value.
(d) assay acceptability criteria*:* for an assay to be acceptable, the mean positive and negative control values should fall within the ranges given above.
In those cases where the mean values fall outside the range, the assay should be repeated, except in cases where the same chemical has been tested on at least two other occasions (with acceptable control values) and the results of all of the tests give the same corrosivity classification.

4.1 INTERPRETATION OF TEST RESULTS

The test results are interpreted on the basis of the exposure time needed to cause cell viability to decrease below 35%. The determination of the packing group is summarized in the following table:

Classification	**Packing group**	**Criteria for *In Vitro* interpretation**
UN	Corrosive class I	If viability < 35% after 3 min exposure
	Corrosive class II	If viability ≥ 35% after 3 min exposure and < 35% after 1 hour exposure
	Corrosive class III	If viability ≥ 35% after 1 hour exposure and < 35% after 4 hours exposure
	Non corrosive	If viability ≥ 35% after 4 hours exposure
EU	Corrosive class R35	If viability < 35% after 3 min exposure
	Corrosive class R34	If viability ≥ 35% after 3 min exposure and < 35% after 4 hours exposure
	Non-corrosive	If viability ≥ 35% after 4 hours exposure

In cases where the viability values from individual skin units are highly variable, causing different corrosivity classifications, the chemical should normally be re-tested. If one or more sets of data are considered to be incorrect (or inconsistent with data from other runs), the results should be replaced by those generated in a repeat run.

In cases where the viability values fall below 35%, but longer exposure times give values of >35% (or values higher than the earlier time point), the results should be considered to be doubtful. The run should normally be repeated.

Annex 1

ECVAM SKIN CORROSIVITY VALIDATION STUDY

EPISKIN ™

Assay report form

Experimental center: .....

Kit Reception

Lot number

Date of Receipt

Observations.

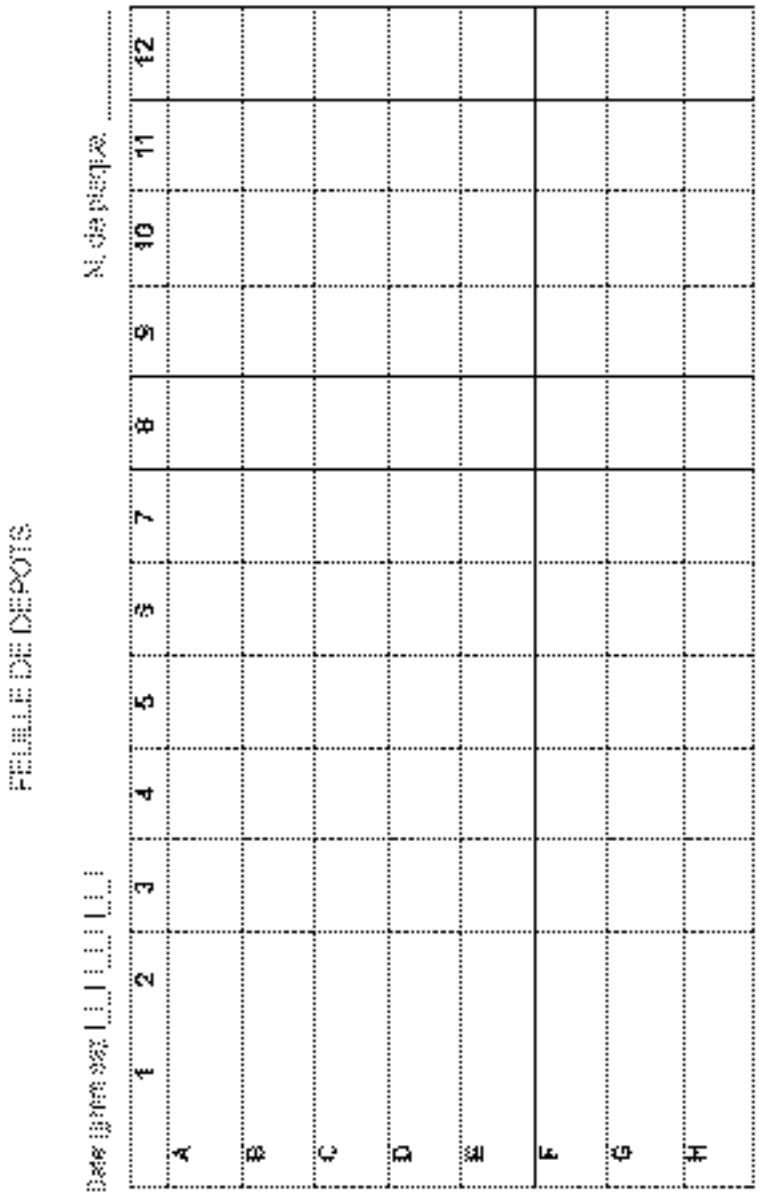
Assay

Date of Assay.

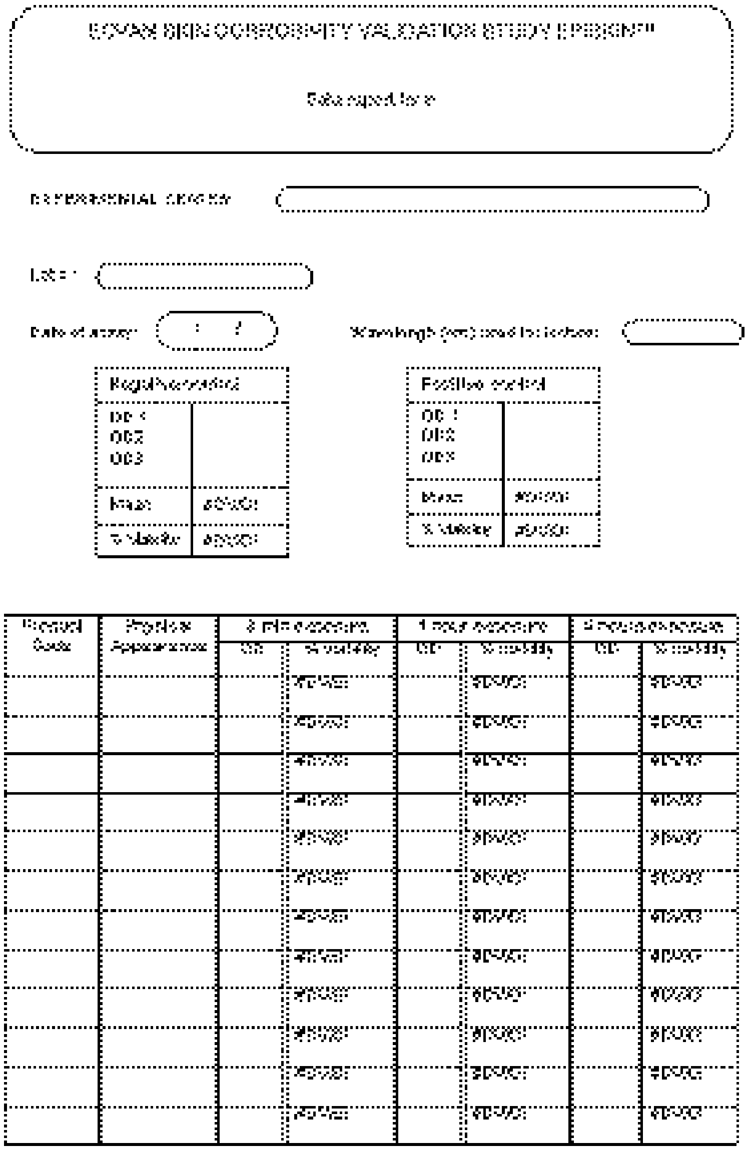
Code of tested product

Observations

Annex 2



Annex 3



ECVAM SKIN CORROSIVITY VALIDATION STUDY EPISKIN™

Data report form

EXPERIMENTAL CENTRE

Lot #:

Date of assay: / /

Wavelength (nm) used for lecture:

Negative control	
OD 1 OD2 OD3	
Mean	#DIV/0!
% Viability	#DIV/0!

Positive control	
OD 1 OD2 OD3	
Mean	#DIV/0!
% Viability	#DIV/0!

Product Code	Physical Appearance	3 min exposure		1 hour exposure		4 hours exposure	
		OD	% viability	OD	% viability	OD	% viability
			#DIV/0!		#DIV/0!		#DIV/0!
			#DIV/0!		#DIV/0!		#DIV/0!
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			#DIV/0!		#DIV/0!		#DIV/0!

Annex 4

ECVAM SKIN CORROSIVITY VALIDATION STUDY EPISKIN™

Data compilation form

EXPERIMENTAL CENTER:

Product Code	Physical Appearance	Lot N. Episkin	% viability 3 min	% viability 1hr	% viability 4 hrs

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Summary Report of the EPISKIN™ *In Vitro* Assay for Assessing Dermal Corrosivity

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PURPOSE

This report focuses on the performance of EPISKIN™ to determine the usefulness and limitations of the assay for the identification of potential human corrosive chemicals. This report also discusses how the EPISKIN™ assay compares to the *in vivo* rabbit skin corrosivity test and to other *in vitro* corrosivity tests (Rat Skin Transcutaneous Electrical Resistance [TER], EpiDerm™, and Corrositex®). The data and assessments in the European Centre for the Validation of Alternative Methods (ECVAM) formal validation study on EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998) were reviewed. Additionally, an independent analysis of the performance data, based on the information provided in Fentem et al. (1998), was conducted.

EVALUATION OF REGULATORY AND SCIENTIFIC RATIONALE

EPISKIN™ is one of several *in vitro* corrosivity assays evaluated as alternatives to the *in vivo* rabbit corrosivity test by ECVAM in a formal validation study (Fentem et al., 1998). EPISKIN™ is a three-dimensional human skin model that measures cell viability. Because it is a human skin model, it may be more relevant to assessing human skin corrosivity potential than a test based on skin from another species. Also, the mode of application (topical) of the test material mimics the route of human exposure.

EPISKIN™ has been approved by the ECVAM Scientific Advisory Committee for use in corrosivity testing in Europe (Balls and Corcelle, 1998) and EPISKIN™ has also been evaluated and approved for its intended use by the European Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (Anon., 1999). This method has been adopted for regulatory use within the European Union (EU) by the European Commission (Anon., 2000).

EVALUATION OF THE TEST METHOD

A standard kit contains media, reagents, and 12 epidermis units. The epidermis units provided in the test kit are comprised of a reconstructed epidermis and a functional stratum corneum. For use in corrosivity testing, the test material (liquids: 50 µL; solids: 20 mg) is topically applied to an epidermis unit for 3, 60, and 240 minutes. Per test compound, one epidermis unit is needed for each of the three test periods. Cell viability is assessed by measuring mitochondrial activity using the MTT (a tetrazolium salt) assay. A 35% decrease in cell viability is used to indicate a potential for human corrosivity. The scientific and mechanistic basis of the test and the rationale for using a 35% decrease in cell viability as the criterion for identifying potential human corrosivity were not discussed by Fentem et al. (1998). However, mechanistically, corrosivity is associated with cell death.

EVALUATION OF TEST METHOD DATA QUALITY

Only limited validation test data are available on EPISKIN™. In the single published validation study by Fentem et al. (1998), ECVAM evaluated 60 chemicals. The chemical selection procedure was described in sufficient detail by Barratt et al. (1998). The main criterion for including chemicals in the study was that their corrosivity classification (C= corrosive; NC = noncorrosive) was based on unequivocal animal data (Barratt et al., 1998). The ECVAM validation chemical test set included organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1 NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC), and soaps/surfactants (3NC). Despite the small numbers of chemicals in some categories, ECVAM concluded that the set of test chemicals represented the best possible group for evaluating the performance characteristics of the *in vitro* assays, given the limited availability of unequivocal animal data (Barratt et al., 1998).

Each chemical was tested three times by each of three different laboratories. The tests were stated to have been conducted in the "spirit" of GLP (Fentem et al., 1998). A formal audit of the ECVAM data by a Quality Assurance Unit was not conducted; however, it was stated that all data submitted by the participating laboratories were verified against the original data sheets by ECVAM staff on at least three separate occasions.

EVALUATION OF TEST METHOD PERFORMANCE

For this summary report, an analysis was conducted, similar to the performance analysis conducted for the ICCVAM Peer Review of Corrositex®; the current analysis evaluated the performance characteristics of the EPISKIN™ assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the EPISKIN™ evaluation consisted of data from the ECVAM validation study only; other data were not located. For ease of comparison, chemicals evaluated in the EPISKIN™ assay were classified into the same chemical and product class designations used in the Corrositex® evaluation. A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls), the chemical was eliminated from inclusion in the performance calculations.

Based on the database of 60 chemicals and chemical mixtures used in the validation study (Table 1), EPISKIN™ had an accuracy of 83% (50/60 chemicals or chemical mixtures), a sensitivity of 82% (23/28 chemicals or chemical mixtures), and a specificity of 84% (27/32 chemicals or chemical mixtures). Furthermore, EPISKIN™ was able to distinguish between known R35/I and R34/II & III chemicals[1]. Based on these data, ECVAM concluded that EPISKIN™ was valid for

[1] UN packing group classifications I, II, and III are assigned based on the capacity of a chemical, when tested on the intact skin of rabbits, to produce skin corrosion following exposure intervals of 3 minutes, 1 hour, or 4 hours, respectively (Fentem et al., 1998). EU regulations require classification of chemicals according to certain risk phases, such as those assigned based on whether the chemical causes corrosion following a 3-minute application (R35 – "causes severe burns"; analogous to packing group I) or 4 hours (R34 – "causes burns"; analogous to packing groups II and III) (Barratt et al., 1998; Fentem et al., 1998).

use as a replacement for the *in vivo* rabbit skin test for distinguishing between corrosive and noncorrosive chemicals for all of the chemical classes studied (Fentem et al., 1998; Balls and Corcelle, 1998). Because of the relatively small numbers of chemicals evaluated in some chemical classes (i.e., cleaners and detergents), definitive conclusions as to the adequacy of EPISKIN™ for some classes of chemicals were difficult to make with a high degree of confidence. Additionally, no assessment could be made with respect to mixtures. However, it was stated that taking into account the relative simplicity of the mechanism of action of corrosives, this method would be generally applicable across all chemical classes (Fentem et al., 1998).

EVALUATION OF TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

The inter- and intra-laboratory reliability of EPISKIN™ was evaluated in the ECVAM validation study (Fentem et al., 1998). In each laboratory, each chemical was tested three times using three different batches of EPISKIN™. Intra- and inter-laboratory reliability was evaluated using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Of the 60 chemicals tested, 42 gave the same corrosivity classification in all three experiments in all three laboratories. In seven cases, the median results for the three laboratories gave identical predictions. In only three cases did one laboratory give results that were consistently in a different classification category than those from the other laboratories. In an additional three cases, the median result from one laboratory was in a different category than those from the other laboratories, and in five cases, chemicals gave results that crossed the classification boundaries in more than one laboratory. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that EPISKIN™ met the criteria agreed by the Management Team concerning acceptable intra- and inter-laboratory reproducibility (Fentem et al., 1998). Due to the lack of quantitative data, by experiment and laboratory, for individual chemicals in the published studies, no independent evaluation of repeatability or reproducibility for EPISKIN™ could be conducted. However, after reviewing the intra- and inter-laboratory evaluations conducted by ECVAM, it was concluded that the analyses were appropriate and that the conclusions were accurate.

Table 1. Performance of the EPISKIN™ Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Fentem et al., 1998)

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	**60**	**83**	**(50/60)**	**82**	**(23/28)**	**84**	**(27/32)**
Organic and Inorganic Acids and Bases[1]	**41**	**78**	**(32/41)**	**81**	**(21/26)**	**73**	**(11/15)**
Organic and Inorganic Bases and Base Mixtures[2]	**14**	**64**	**(9/14)**	**60**	**(6/10)**	**75**	**(3/4)**
Organic and Inorganic Acids and Acid Mixtures	**20**	**85**	**(17/20)**	**100**	**(11/11)**	**67**	**(6/9)**
Amines	**10**	**60**	**(6/10)**	**57**	**(4/7)**	**67**	**(2/3)**
Inorganic Bases and Base Mixtures	**4**	**75**	**(3/4)**	**67**	**(2/3)**	**100**	**(1/1)**
Acid Derivatives	**7**	**86**	**(6/7)**	**80**	**(4/5)**	**100**	**(2/2)**
Surfactants	**5**	**80**	**(4/5)**	**NA**	**(0/0)**	**80**	**(4/5)**
Industrial Chemicals	**10**	**100**	**(10/10)**	**100**	**(1/1)**	**100**	**(9/9)**
Cleaners and Detergents	**1**	**100**	**(1/1)**	**NA**	**(0/0)**	**100**	**(1/1)**

[1] This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives

[2] This chemical class includes amines, inorganic bases, and base mixtures.

OTHER SCIENTIFIC REVIEWS

In March 1999, a search of the open literature was conducted to locate additional EPISKIN™ studies. Six databases (Medline, Toxline, Embase, Biosis, Caba, and LifeSci) were searched using the key terms "Episkin", and "Epi" within one word of "skin". The search found no additional relevant studies conducted with EPISKIN™. In May 2001, another search was conducted to locate additional EPISKIN™ studies. Four databases (PubMed, Web of Science, Toxline, and Current Contents Connect) were searched using the same search strategy and no additional relevant studies were found.

OTHER CONSIDERATIONS

The EPISKIN™ kit contains all of the necessary materials to conduct the test and does not require additional preparation. No animals are used in this test. ECVAM concluded that, compared to the *in vivo* test method, EPISKIN™ costs less to perform (Fentem et al., 1998). The cost for conducting EPISKIN™ is reported by L'OrÈal Recherche (e-mail communication from Odile de Silva, L'OrÈal Recherche) to be approximately $450 per kit (Table 2). When compared to other *in vitro* corrosivity test methods, the cost of EPISKIN™ is stated to be greater than that of the Corrositexâ and EpiDerm™ assays and somewhat less than the Rat Skin TER (Fentem et al., 1998). However, currently, the EPISKIN™ kit is not commercially available. The time needed to conduct the EPISKIN™ assay is greater than the Corrositex® assay, comparable to the EpiDerm™ assay, and less than the Rat Skin TER assay.

RELATED ISSUES

Refinement, Reduction, and Replacement

Since the method is designed as a replacement for animals, EPISKIN™ would clearly reduce the requirement for animal testing for corrosivity. Therefore, it has the potential to eliminate the use of animals for the determination of corrosivity. If used in an integrated approach, EPISKIN™ provides for reduction and refinement of animal use.

Comparison to Other *In Vitro* Assays

General comparative information on the TER, EPISKIN™, and Corrositexâ assays is provided in Tables 2 through 5.

Table 2. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays

	Rat Skin TER	EPISKIN™ (prediction model B)	EpiDerm™ (prediction model 2)	Corrositex®
Test Method Description	Acceptable	Acceptable	Acceptable	Acceptable
Adequacy/Completeness of Protocol	Acceptable	Acceptable	Acceptable	Acceptable
Usefulness for Assessing Corrosivity/Non-corrosivity	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Usefulness for Determining Packing Groups	Not Acceptable (Fentem et al., 1998)	Can group as UN packing group II/III or I (Fentem et al., 1998)[a]	Not Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Repeatability and Reproducibility	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (Fentem et al., 1998; ICCVAM, 1999)
Animal Use Refinement, Reduction, and Replacement Considerations	Refines and reduces animal use when used as a stand-alone test or in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.	Refines and reduces animal use when used in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.
Cost	~$500-850/test	~$450/test kit[b]	~$200/test chemical	~$300/test chemical
Study duration	2 work-days	1 work-day	1 work-day	≤ 4 hr/chemical

a Since the performance of EPISKINä was not assessed for distinguishing between UN packing groups II and III, all R34 classifications would be conservatively classified as UN packing group II.

b One to three chemicals may be tested per test kit; however, it is recommended by the supplier that each test chemical be assayed using 3 different skin batches/kits which equates to a total cost of ~$430/ test chemical.

Table 3. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays Based on an ICCVAM Weight-of-Evidence Approach by Chemical using Data from the ECVAM and other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™	EpiDerm™ (prediction model 2)	Corrositex®
Number of Chemicals	122	60	24	163
Overall Sensitivity[a]	94% (51/54)	82% (23/28)	92% (11/12)	85% (76/89)
Overall Specificity[a]	71% (48/68)	84% (27/32)	83% (10/12)	72% (52/74)
Overall Accuracy[a]	81% (99/122)	83% (50/60)	92% (22/24)	79% (128/163)
Test Chemical Interlaboratory Coefficient of Variation	Median = 34.7 Range = 3.8-322 n^b = 120	Median = 11.3 Range = 3.9-148.8 n^b = 20	Median = 12.3 Range = 0.9-51.2 n^b = 144	Median = 30.3 Range = 7.7-252.5 n^b = 180

a Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method.

b The total number of independent values, which is calculated as the number of chemicals tested multiplied by the number of sample times for each chemical.

Table 4. General comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® assays from independent test results in the ECVAM validation studies (Fentem et al., 1998; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™ (prediction model B)	EpiDerm™ (prediction model 2)
Number of Chemicals Tested in ECVAM Validation Study	60 (Fentem et al., 1998)	60/24[a] (Fentem et al., 1998)	24 (Liebsch et al., 2000)
Sensitivity[b]	88% (140/159)	83% (201/243) / 88% (87/99)	88% (63/72)
Specificity[b]	72% (142/196)	80% (237/297) / 79% (92/117)	86% (62/72)
Accuracy[b]	79% (282/355)[c]	81% (438/540) / 83% (179/216)	87% (125/144)
False Positive Rate[b]	28% (54/196)	20% (60/297) / 21% (25/117)	14% (10/72)
False Negative Rate[b]	12% (19/159)	17% (42/243) / 12% (12/99)	13% (9/72)
Number of Trials[d]	355	540 / 216	144
Test Chemical Inter-laboratory Coefficient of Variation	Median = 34.7 Range = 10-322 n^d = 360	Median = 30.2 Range = 7.7-252.5 n^d = 540	Median = 12.3 Range = 0.9-51.2 n^d = 144

a The first numbers for accuracy, sensitivity, specificity, false positive rate, and false negative rate correspond to the 60 chemicals tested in the ECVAM Skin Corrosivity Test using EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998); the latter values correspond to a direct comparison of EpiDerm™ and EPISKIN™ for the same 24 materials tested in both systems (Liebsch et al., 2000).

b Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative.

c The percentages are based on the number of correct trials among the total number of trials (i.e., independent tests) provided in parenthesis.

d The total number of trials conducted in the validation study minus the non-qualified (NQ) results. This number is equal to the number of chemicals multiplied by the number of participating laboratories multiplied by the number of replicate tests.

Table 5. Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000)

No.	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN-B [a]	EpiDerm
1	Hexanoic acid	ORGAC	R34/II&III	R35	R35	N/A
29	65/35 Octanoic/decanoic acid	ORGAC	R34/II&III	R34	R35	N/A
36	2-Methylbutyric acid	ORGAC	R34/II&III	R35	R34	N/A
40	Octanoic acid (caprylic acid)	ORGAC	R34/II&III	R35	R34/C	C
47	60/40 Octanoic/decanoic acids	ORGAC	R34/II&III	R34	R34/C	C
50	55/45 Octanoic/decanoic acids	ORGAC	R34/II&III	R35	R34	N/A
7	3,3'-Dithiodipropionic acid	ORGAC	NC	NC	NC	N/A
12	Dodecanoic acid (lauric acid)	ORGAC	NC	NC	NC	NC
26	Isotearic acid	ORGAC	NC	NC	NC	NC
34	70/30 Oleine/octanoic acid	ORGAC	NC	NC	NC	N/A
58	10-Undecenoic acid	ORGAC	NC	NC	R34	N/A
2	1,2-Diaminopropane	ORGBA	R35/I	R35	R34/C	C
15	Dimethyldipropylenetriamine	ORGBA	R35/I	R35	R34/C	C
38	Tallow amine	ORGBA	R35/II	2R34/2NC/2NQ	NC	N/A
55	1-(2-Aminoethyl)piperazine	ORGBA	R34/II	R35	NC	N/A
13	3-Methoxypropylamine	ORGBA	R34/II&III	R35	R34	N/A
17	Dimethylisopropylamine	ORGBA	R34/II&III	R35	R34/C	C
45	n-Heptylamine	ORGBA	R34/II&III	R35	NC	C
10	2,4-Xylidine (2,4-Dimethylaniline)	ORGBA	NC	R34	R34	N/A
35	Hydrogenated tallow amine	ORGBA	NC	NC	NC	NC
59	4-Amino-1,2,4-triazole	ORGBA	NC	NC	NC	NC
8	Isopropanol	NORG	NC	NC	NC	N/A
11	2-Phenylethanol	NORG	NC	NC	NC	N/A
16	Methyl trimethylacetate (referred to as Methyl 2,2-dimethylpropanoate in EpiDerm™)	NORG	NC	NC	NC	C
19	Tetrachloroethylene	NORG	NC	NC	NC	NC
22	n-Butyl propionate	NORG	NC	NC	NC	N/A
27	Methyl palmitate	NORG	NC	NC	NC	N/A
44	Benzyl acetone	NORG	NC	NC	NC	NC
51	Methyl laurate	NORG	NC	NC	NC	N/A
56	1,9-Decadiene	NORG	NC	NC	NC	NC
3	Carvacrol	PHEN	R34/II&III	R34	R34	N/A
23	2-tert-Butylphenol	PHEN	R34/II&III	R35	R34/C	C
9	*o*-Methoxyphenol (Guaiacol)	PHEN	NC	NC	R34	N/A
30	4,4-Methylene-bis-(2,6-di-tert-butylphenol)	PHEN	NC	NC	NC	N/A
49	Eugenol	PHEN	NC	NC	NC	NC
4	Boron trifluoride dihydrate	INORGAC	R35/I	R35	R35/C	C
28	Phosphorus tribromide	INORGAC	R35/I	R35	R35/C	C

32	Phosphorus pentachloride	INORGAC	R35/I	R35	R34	N/A
25	Sulfuric acid (10% wt.)	INORGAC	R34/II&III	R34	R34	N/A
57	Phosphoric acid	INORGAC	R34/II	R35	R34	N/A
43	Hydrochloric acid (14.4% wt)	INORGAC	R34/II&III	R35	R34	N/A
53	Sulfamic acid	INORGAC	NC	R34	R34/C	C
18	Potassium hydroxide (10% aq.)	INORGBA	R34/II&III	R35	R34/C	C
42	2-Mercaptoethanol, Na salt (45% aq.)	INORGBA	R34/II&III	R35	NC	N/A
21	Potassium hydroxide (5% aq.)	INORGBA	NC	R35	R34	N/A
24	Sodium carbonate (50% aq.)	INORGBA	NC	R34	NC	NC
20	Ferric [iron (III)] chloride	INORGSAL	R34/II	R35	R34	N/A
52	Sodium bicarbonate	INORGSAL	NC	R34	NC	N/A
54	Sodium bisulfite	INORGSAL	NC	3R34/3NC	NC	N/A
5	Methacrolein	ELECTRO	R34/II&III	NC	R34/C	NC
14	Allyl bromide	ELECTRO	R34/II&III	R35	R34	N/A
48	Glycol bromoacetate (85%)	ELECTRO	R34/II&III	NC	R34/C	C
6	Phenethyl bromide	ELECTRO	NC	NC	NC	N/A
31	2-Bromobutane	ELECTRO	NC	3R34/3R35	NC	N/A
33	4-(Methylthio)-benzaldehyde	ELECTRO	NC	NC	NC	N/A
39	2-Ethoxyethyl methacrylate	ELECTRO	NC	NC	NC	N/A
46	Cinnamaldehyde	ELECTRO	NC	NC	NC	N/A
37	Sodium undecylenate (33% aq.)	SOAP	NC	R35	R34	N/A
41	20/80 Coconut/palm soap	SOAP	NC	NC	NC	N/A
60	Sodium lauryl sulfate (20% aq.)	SOAP	NC	R35	NC	NC

Overall corrosivity classifications were determined by the majority of the reported results obtained from each assay. If results do not show a majority, a definitive classification could not be determined.

Definitions are as follows: C = Corrosive; NC = Non-corrosive; R34 is equivalent to packing groups II and/or III; R35 is equivalent of packing group I, except for tallow amine (R35/II); NQ = Non-qualified; N/A = Not applicable because not tested; ORGAC = Organic acid; ORGBA = Organic base; NORG = Neutral organics; PHEN = phenol; INORGAC = Inorganic acid; INORGBA = Inorganic base; INORGSAL = Inorganic salt; ELECTRO = Electrophile; SOAP = Soap surfactant

a For EPISKIN™, prediction model B was the more complex prediction model and was the only model considered in detail by the ECVAM Management Team (Fentem et al., 1998).

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

ECVAM concluded that EPISKIN™ was an *in vitro* replacement assay for *in vivo* corrosivity testing. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that EPISKIN™ was both reliable and reproducible. For some chemical or product classes (e.g., industrial chemicals, cleaners and detergents), the small number of chemicals and/or the unbalanced distribution of corrosive and

noncorrosive chemicals does not allow accurate conclusions to be made on the performance of EPISKIN™ for those chemical classes.

The two major questions to be addressed for *in vitro* corrosivity assays are:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?

2. Does the assay adequately consider and incorporate, where scientifically feasible, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

In response to the first question, the performance characteristics of the EPISKIN™ method indicates, in specific testing circumstances, that this test may be considered useful as part of an integrated testing strategy for assessing the dermal corrosion potential of chemicals. Only the EPISKIN™ skin model was adequate for assigning packing groups according to the EU skin corrosion hazard classes (R34/R35) and the UN packing group classifications (I and II/III). However, since the performance of EPISKIN™ was not assessed for distinguishing between UN packing group II and packing group III, all R34 classifications would be conservatively classified as packing group II.

In response to the second question, EPISKIN™ sufficiently considers and incorporates the 3Rs. Specifically, the use of EPISKIN™ offers advantages with respect to animal welfare considerations, including animal use refinement, reduction, and replacement. Similarly, the use of this assay as part of an integrated approach reduces and refines the use of animals by providing a basis for decisions on further testing. When this method is used as part of the integrated testing strategy for corrosivity/irritation, there is a reduction in the number of animals required because positive results usually eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal could be required to identify a corrosive chemical (one animal is used if the *in vitro* test is negative).

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ECVAM Protocol for EPIDERM™: an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: October 24, 1997
Confirmed: October 2000

NOTE: This protocol presents the standard operating procedure used in the ECVAM Prevalidation of Epiderm™ Skin Corrosivity test (1997-1998). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

EPIDERM™ Skin Corrosivity Test

The corrosivity potential of a chemical may be predicted by measurement of its cytotoxic effect, as reflected in the MTT assay, on the Epiderm™ human epidermal model.

Objectives

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency, hazard identification
PURPOSE OF TESTING	:	classification and labelling

Proposed replacement for the *in vivo* Draize rabbit skin corrosivity test (OECD testing guideline 404, Anon., 1992b; and Annex V of Directive 67/548/EEC, Anon., 1992a) to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.

Basis of the Method

The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. The two major mechanisms of skin corrosion are the destruction (erosion or solubilisation) of the skin penetration barrier (stratum corneum) including the viable skin cells underneath, and the rapid penetration of highly cytotoxic chemicals through the skin barrier without involving its destruction.

The present test is based on the experience that corrosive chemicals show cytotoxic effects following short-term exposure of the stratum corneum of the epidermis. The test is designed to predict and classify the skin corrosivity potential of a chemical by assessment of its effect on a reconstituted three-dimensional human epidermis model. Cytotoxicity is expressed as the reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT.

Experimental Description

Endpoint and Endpoint Detection	:	Cell viability as determined by reduction of mitochondrial dehydrogenase activity measured by formazan production from MTT
Test Parameter	:	50% viability
Test System	:	EpiDerm™ human epidermal model system

On day of receipt EpiDerm™ tissues are placed in the refrigerator. Next day, at least one hour before starting the assay, the tissues are transferred to 6-well plates with assay medium, which is immediately replaced before the test is started. The test is performed on a total of 4 tissues per test material, together with a negative control and a positive control.

Two tissues are used for a three-minute exposure to the test chemical and two for a one-hour exposure. 50 µl of the undiluted test material (liquids, semi-solids) or ~ 25 mg solid +25 µl H_2O

are added into the MILLICELL® insert on top of the Epi-200 tissues. The remaining tissues are concurrently treated with 50µl distilled water (negative control) and with 50µl 8N-KOH (positive control). After the exposure period, the tissues are washed with phosphate buffered saline (PBS) to remove residual test material. Rinsed tissues are kept in 24-well plates (holding plates) in 300 µl serum free assay medium until 12 tissues (=one application time) have been dosed and rinsed. The assay medium is then replaced with 300 µl MTT-medium and tissues are incubated for three hrs (37°C, 5% CO_2). After incubation, tissues are washed with PBS and formazan is extracted with 2 ml isopropanol (either for 2 hrs or overnight). The optical density of extracted formazan is determined spectrophotometrically at 570 nm (or 540 nm) and cell viability is calculated for each tissue as a % of the mean of the negative control tissues. The skin corrosivity potential of the test materials is classified according to the remaining cell viability following exposure to the test material for either of the two exposure times.

Test Compounds

A total of 24 test compounds were chosen from the 60 chemicals tested in the ECVAM Skin Corrosivity Validation Study (1996/1997). These compounds included 4 organic acids, 6 organic bases, 4 neutral organics, 2 phenols, 3 inorganic acids, 2 inorganic bases, 2 electrophiles and 1 soap/surfactant.

Prediction Model

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after exposure compared to the negative control tissues concurrently treated with H_20. A chemical is classified “corrosive”, if the relative tissue viability after 3 min exposure to a test material is decreased below 50% (PM1). In addition, those materials classified "non corrosive" after 3 min (viability ≥ 50%) are classified "corrosive" if the relative tissue viability after 1 hr treatment with a test material is decreased below 15 % (PM2). For details see the section 4. **"Evaluation, Prediction Models (PM1 and PM2)"** reported in the present standard operating procedure.

Status

Following presentation of the outcome to the Management Team of the ECVAM Skin Corrosivity Validation Study on 22 April 1998, it was recommended to carry out a small catch up study of the Epiderm test rather than a formal validation study. This "Prevalidation Study of the Epiderm™ Skin Corrosivity Test" (March 1997-April 1998) has successfully been concluded (Liebsch *et al.*, 2000). Based on the outcome of the study (Botham & Fentem, 1999), ESAC unanimously endorsed the statement that the Epiderm human skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the EU and draft OECD test guidelines on skin corrosion (14th meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 2000; Anon., 2000b).

The 27th meeting of the Committee for Adaptation to Technical Progress of “Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances” agreed that the human skin model assays, which meet certain criteria, would form part of “Annex V method B.40. Skin Corrosion”, February 2000 (Commission Directive 2000/33/EC). Furthermore, these models are now under consideration for inclusion in the OECD Guidelines. Further details may be obtained from the contact person.

Remarks

After in 1993/94 two *in vitro* assays (Corrositex and Skin^2 ZK 1350) had achieved limited regulatory acceptance (exemptions for the use with specified chemical classes) by the US DOT, an international prevalidation study on three *in vitro* tests for skin corrosivity was performed in 1996. As a follow-up to this study, a formal validation study, initiated and sponsored by ECVAM, has been conducted (1996/97). Tests being evaluated were the rat skin Transcutaneous Electrical Resistance (TER) assay; CORROSITEX™ test; Skin^2 ZK 1350 and EPISKIN™ test (protocol numbers: 115, 116, 117, 118 respectively). As an outcome of this Validation Study, two tests (TER assay and EPISKIN™) have scientifically been validated as a replacement to the animal test. Following the successfully conclusion of this study, the production of the two *in vitro* 3-D models of reconstructed human skin/epidermis (Skin^2 and EPISKIN) was interrupted by the manufactors. Skin^2 is no longer produced, while EPISKIN will be available again shortly.

The present EpiDerm™ assay is, therefore, used as a substitute for the two models. The need for a substitute test is supported by experience of a similar performance of different models in skin corrosivity testing (ECETOC, 1995) and by the OECD tier strategy for the classification of skin irritancy/corrosivity, developed by the US EPA and the German BgVV which includes the use of validated *in vitro* tests (OECD, 1996) for positive classification.

Details on the validation study are available in **dbVas** of the ECVAM SIS.

Last update: October 2000

Procedure Details, 24 October 1997

EPIDERM™ SKIN CORROSIVITY TEST

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Note: The protocol presents the standard operation procedure used in the Prevalidation of Epiderm™ Skin Corrosivity test (1997-1998).

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1. Introduction and remarks

The SOP is based on a method developed at Procter & Gamble in 1996. The SOP was drafted at ZEBET in Phase I of the prevalidation study and a database comprising 96 tests with 50 chemicals was produced using the first Draft SOP. The SOP was then refined according to discussions with P&G and with the partner laboratories participating in phases II and III (Huntingdon Life Sciences, UK and BASF AG, D) which lead to the attached final SOP.

2. Materials

2.1 MATERIALS, NOT PROVIDED WITH THE KITS

Sterile, blunt-edged forceps	For transferring tissues from agarose
500 ml wash bottle	For rinsing tissue after test material exposure
200 ml beaker	For collecting PBS washes
Sterile disposable pipettes, pipette tips and pipetters	For diluting, adding, and removing media and test materials. For topically applying test materials to tissues
37°C incubator 5% CO_2	For incubating tissues prior to and during assays
Vacuum source/trap (optional)	For aspirating solutions
Laminar flow hood (optional)	For transferring tissues under sterile conditions
37°C water bath	For warming Media and MTT solution
Mortar and Pestle	For grinding granulars

Adjustable Pipet 1 ml	For pipetting assay medium under inserts (0.9 ml)
Pipet 300 µl	For pipetting MTT medium into 24-well plates
Pipet 2 ml	For pipetting MTT extraction solution into 24-well plate
Pipet 200 µl	For pipetting extracted formazan from 24-well plate into 96 well plate to be used in a plate photometer
Pipet 50 µl	For application of liquid test materials
Positive displacement pipet 50 µL	For application of semi-solid test materials
Sharp spoon (NaCl weight: 25±1 mg) Aesculap, Purchase No.: FK623	For application of solids
(bulb headed) sound	To aid levelling the spoon (spoonful)
Laboratory balance	For pipette verification and checking spoonful weight
96-well plate photometer 570 or 540 nm	For reading OD
Shaker for microtiter/MILLICELL® plates	For extraction of formazan
Stop-watches	To be used during application of test materials
Potassium Hydroxyde, 8 N (Sigma # 17-8)	To be used as positive control with each kit
Dulbeccos PBS (ICN # 196 0054) or (ICN # 196 1054) or (ICN # 176 0020) or (ICN # 176 0022)	Use for rinsing tissues Use as ready solution or dilute from 10x concentrate or prepare from PBS powder
HCl	For pH adjustment of PBS
NaOH	For pH adjustment of PBS
H_2O, pure (distilled or aqua pur)	To be used as negative control with each kit
Two additional 24-well plates	Use for preparing the "holding plates"

2.2. EPI-200 KIT COMPONENTS

Examine all kit components for integrity. If there is a concern call MatTek Corporation immediately (Mitch Klausner, ☎ +1-508-881-6771, Fax +1-508-879-1532).

1	Sealed 24-well plate	Contains 24 inserts with tissues on agarose
2	24-well plates	Use for MTT viability assay
4	6-well plates	Use for storing inserts, or for topically applying test agents
1 bottle	Maintenance Medium	Do not use in present assay

1 bottle	Serum-Free Assay Medium	DMEM-based medium
1 bottle	PBS Rinse Solution (100 ml)	Use for rinsing the inserts in MTT assay
1 vial	1% Triton X-100 Solution (10 ml)	Skin irritant reference chemical **Do not use in present assay**
1	MTT Assay Protocol	MatTek Corporation: steps are included in the present protocol

2.3. MTT-100 ASSAY KIT COMPONENTS

1 vial, 2 ml	MTT concentrate	
1 vial, 8 ml	MTT diluent (supplemented DMEM)	For diluting MTT concentrate prior to use in the MTT assay
1 bottle, 60 ml	Extractant Solution (Isopropanol)	For extraction of formazan crystals

3. Methods

3.1. EXPIRATION AND KIT STORAGE

Epi-200 kits are shipped from Boston on Monday. If possible, make sure that they are arriving in the laboratory on Tuesday. Upon receipt of the EpiDerm tissues, place the sealed 24 well plates and the assay medium into the refrigerator (4°C). Place the MTT concentrate containing vial in the freezer (-20°C) and the MTT diluent in the refrigerator (4°C).

Part #	**description**	**conditions**	**shelf life**
Epi-200	EpiDerm cultures	refrigerator (4°C)	until Friday, of the week of delivery
Epi-100	assay medium	refrigerator (4°C)	7 days
MTT-099	MTT diluent	refrigerator (4°C)	7 days
MTT-100	MTT concentrate	freezer (-20°C)	2 months

Record lot numbers of all components shown on the lot/production label on sealed tray in the Methods Documentation Sheet (see ANNEX)

3.2. QUALITY CONTROLS

3.2.1. Assay Acceptance Criterion 1: Negative Controls

The absolute OD_{570} or OD_{540} of the negative control tissues in the MTT-test is an indicator of tissue viability obtained in the testing laboratory after the shipping and storing procedure and under specific conditions of the assay.
Tissue viability is meeting the acceptance criterion if the mean OD of the two tissues is $OD \geq 0.8$.

3.2.2. Assay Acceptance Criterion 2: Positive Control

Potassium Hydroxyde as 8.0 normal ready made solution (Sigma # 17-8) is used as positive reference and has to be tested with each kit according to section 3.4. A 3 minutes application of 8.0 n KOH will reveal a mean relative tissue viability of ~20%.
An assay is meeting the acceptance criterion if mean relative tissue viability of the 3 min Positive Control is ≤ 30%.

3.2.3. Maximum inter tissue viability difference

In the present test protocol each chemical is tested on 2 tissues per application time (3 min and 1 hr). Thus, in contrast to the first test version (which used only 3 min application on 4 tissues) statistically outlying tissues cannot be identified any more. According to the historical data base existing at ZEBET the mean difference between untreated tissue duplicates is 9% ± 7% (S.D.).
A difference > 30% between two tissues treated identically should be regarded as a rejection criterion, and re-testing of the chemical is recommended if the resulting viability is near to a classification cut-off.
Note: If necessary, calculate % difference between the mean of the 2 tissues (= 100%) and one of the two tissues. If this difference is > 15% then rejection should be considered.

3.3. PREPARATIONS

3.3.1. MTT solution (prepare freshly on day of testing)

Thaw the MTT concentrate (MTT-100) and dilute with the MTT diluent (MTT-099). Store the remaining MTT solution in the dark at 4°C for later use on the same day (do not store until next day).
Note: Some test chemicals may reduce MTT, which will result in a blue colour without any involvement of cellular mitochondrial dehydrogenase. Although in the present assay the test chemicals are rinsed off and the DMEM medium beneath the tissues is changed before contact with MTT medium, some amount of a test chemical may be released by the tissues into the MTT medium and directly reduce the MTT, which would be interpreted as "tissue viability".

To check MTT reducing capability a solution of MTT in DMEM (1.0 mg/ml) can be prepared and ~100 µL (liquid test material) or 30 mg (solid test material) added to 1 ml MTT medium. If the mixture turns blue/purple after about 1 hr at room temperature, the test material is presumed to have reduced the MTT. This check can only be used to explain unexpected results, but it can not be used for quantitative correction of results.

3.3.2. Dulbecco's PBS

Using ICN FLOW 10× DPBS (Cat. no. see section 2.1 **"Materials, not provided with the kits"**) dilute 1 in 10 with distilled water and adjust to pH 7.0 with either NaOH or HCl.
Record the pH adjustment in the MDS. If PBS powder is used: prepare PBS according to supplier instructions.
Note: 1 Litre is sufficient for all rinsing performed with one kit. If PBS is prepared from 10x concentrates or powder and not sterilised after preparation do not use PBS for more than one week.

3.3.3. Test materials

Safety Instruction

1. For handling of non-coded test chemicals follow instructions given in the Material Safety Data Sheet.
2. If coded chemicals are supplied from BIBRA, no information regarding the safe handling will be provided. Therefore, all test materials must be treated as if they were corrosives and work must be performed in accordance with chemical safety guidelines (use ventilated cabinet, wear gloves, protect eyes and face).

Except solids all test materials are applied neat (undiluted):

Liquids	**:**	Dispense 50 µl directly atop the Epi-200 tissue. If necessary spread to match size of tissue. Record the use of spreading in the MDS.
Semi-solids	**:**	Dispense 50 µl using a positive displacement pipet directly atop the Epi-200 tissue. If necessary spread to match size of tissue. Record the use of spreading in the MDS.
Solids	**:**	Crush and grind test material in a mortar with pestle wherever this improves the consistency. Fill 25 mg application spoon (see section 2.1. **"Materials not provided with the kits"**) with fine ground test material. Level the "spoonful" by gently scratching the excess material away with an appropriate aid, avoiding compression ("packing") of the test material [#]. Add 25 µl H_20 for wetting of the test material (increase volume of H_20 in case of materials where this is not enough for wetting). If necessary spread to match size of tissue. Record in the MDS if grinding was not used and if spreading or increasing H_20 volume was necessary.

[#]**Note**: "Packing" can be avoided by using a rod shaped sound instead of a flat spatula. If a bulb headed sound is used the bulb can be used to empty the spoon completely.

3.4 EXPERIMENTAL PROCEDURE

Note: Since the present test is a short term test which makes use of the epidermis model over a period of only 5 hours, sterility is not as important as is in other applications of EpiDerm™. Nevertheless, it is important to keep assay media sterile and to keep risk of contamination at a low level.

Day prior to testing

1. Upon receipt of the EpiDerm kit(s), place the sealed 24 well plates containing the tissues and the assay medium into the refrigerator (4° C). Place the vial containing the MTT concentrate in the freezer (-20°C).
2. Preparation of PBS according to section 3.3.2 **"Dulbecco's PBS"**.

Day of testing

Introductory note: One kit is used for testing 4 test chemicals, negative control and positive control, each of them applied both for 3 min and 1 hr to two tissue replicates. Thus, the experimental design can be either that the 3 min applications are completed first and subsequently the 1 hr experiment is performed, or, alternatively, that the 3 min applications are performed during the exposure period of the 1 hr experiment. The following steps are describing the latter option.

1). Before treatment pre-warm the assay medium in a 37°C waterbath.

2). Pipet 0.9 ml of the assay medium into each well of four sterile 6-well plates.

3). At least 1 hour before dosing, remove the EpiDerm tissues from the refrigerator. Under sterile conditions using sterile forceps, transfer the inserts into four 6-well plates containing the pre-warmed assay medium.

Note: Care should be taken to remove all adherent agarose sticking to the outside of the inserts. Any air bubbles trapped underneath the insert should be released. Label the 6 well plates (lid and bottom) indicating the test material.

4). Place the four 6-well plates containing the tissues into a humidified (37°C, 5% CO_2) incubator for at least 1 hour prior to dosing
(pre-incubation).

5). Prepare MTT solution according to section 3.3.1 **"MTT solution"**.
6). Before pre-incubation is complete, prepare two 24-well plates to be used as "holding plates", one for the 3 min experiment, the other for the 1 hr experiment. In addition, prepare two 24-well plates for the MTT assay: Use the plate design shown below. Pipette 300 µL of either pre-warmed assay medium or MTT medium in each well. Place the 4 plates in the incubator.

24-well plate design (used as "holding plates" and for MTT assay)

NC	C1	C2	C3	C4	PC
NC	C1	C2	C3	C4	PC

3 min

NC	C1	C2	C3	C4	PC
NC	C1	C2	C3	C4	PC

1 hr

NC = Negative Control
C1-C4 = Test Chemical 1,2,3,4
PC = Positive Control
7). After pre-incubation is completed (at least 1 hr) replace medium by 0.9 ml fresh assay medium in all four 6-well plates. Place two 6-well plates (3 min experiment) back into the incubator, the other two 6-well plates are used for the 1 hour experiment. Use the following plate design:
6-well plate design (chemical treatment and incubation)

Negative control	test material 1	test material 2
Negative control	test material 1	test material 2

plate A (3 min)

negative control	test material 1	test material 2
negative control	test material 1	test material 2

plate C (1 hour)

Test material 3	test material 4	positive control
Test material 3	test material 4	positive control

plate B (3 min)

test material 3	test material 4	positive control
test material 3	test material 4	positive control

plate D (1 hour)

Note: To avoid experimental errors it is recommended to use NC and PC at **identical** positions in all experiments. In contrast, test chemicals should be positioned differently in the two independent experiments.

8). **1 hour experiment**: Add 50 µL H_2O (negative control) into the first insert atop the EpiDerm tissue. Set the timer to 1 hr and start it, repeat the procedure with the second tissue. Proceed with test material 1 - 4 and the positive control in the same manner until all 12 tissues are dosed and rinsed. Place both 6-well plates into the incubator (37°C, 5 % CO_2). Record start time in the

MDS.
9) **3 minutes experiment**: Add 50 µL H_2O (negative control) into the first insert atop the EpiDerm tissue. Set the timer to 3 min and start it. Repeat the procedure with the second tissue. Important: keep a constant time interval between dosing (e.g. 40 sec.). After 3 min of application, with forceps, remove the first insert immediately from the 6-well plate. Using a wash bottle gently rinse the tissue with PBS (20 times) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all 12 tissues are dosed and rinsed.
10) **3 minutes:** once all tissues have been dosed and rinsed and are in the holding plate, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for the plate for 3 hours (37°C, 5% CO_2).
11) **1 hour:** after the 1 hour period of test material exposure (in the incubator) is completed with forceps remove the first insert from the 6-well plate. Using a wash bottle gently rinse the tissue with PBS (20 times) to remove any residual test material. Remove excess PBS by gently shaking the insert and blot bottom with blotting paper. Place insert in the prepared holding plate. Proceed with test materials 1 - 4 and the positive control in the same manner until all 12 tissues are rinsed.
12) **1 hour:** once all tissues have been rinsed and are in the holding plate, remove inserts from the holding plate, blot bottom and transfer into the 24-well plate, prepared for the MTT assay. Place plate in the incubator, record start time of MTT incubation in the MDS and incubate for the plate for 3 hours (37°C, 5% CO_2).
13) **3 minutes:** After the 3 hour MTT incubation period is complete, aspirate MTT medium from all 12 wells (e.g. gently using a suction pump), refill wells with PBS and aspirate PBS. Repeat the rinsing twice and make sure tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.
14) **3 minutes:** Immerse the inserts by gently pipetting 2 ml extractant solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.
15) **3 minutes:** Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. Record start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.
16) **1 hour:** After the 3 hour MTT incubation period is complete, aspirate MTT medium from all 12 wells (e.g. gently using a suction pump), refill wells with PBS and aspirate PBS. Repeat the rinsing twice and make sure tissues are dry after the last aspiration. Transfer inserts to new 24 well plates.
17) **1 hour:** Immerse the inserts by gently pipetting 2 ml extractant solution (isopropanol) into each insert. The level will rise above the upper edge of the insert, thus completely covering the tissue from both sides.
18) **1 hour:** Seal the 24 well plate (e.g. with a zip bag) to inhibit isopropanol evaporation. Record start time of extraction in the MDS. Extract either over night without shaking at room temperature or, alternatively, 2 hours with shaking (~120 rpm) at room temperature.

Second day of testing (only if formazan has been extracted over night!)
19) After the extraction period is complete for both, the **3 min** and the **1 hr** experiment, pierce the inserts with an injection needle (~ gauge 20, ~0.9 mm diameter) and allow the extract to run into the well from which the insert was taken. Afterwards the insert can be discarded. Place the 24-well plates on a shaker for 15 minutes until solution is homogeneous in colour.
20) Per each tissue transfer 3 × 200µL aliquots * of the blue formazan solution into a 96-well flat bottom microtiter plate, both from the **3 min** exposure and from the **1 hr** exposure. For the 96

well plate, use exactly the plate design given next page, as this configuration is used in the data spreadsheet. Read OD in a plate spectrophotometer at 570 nm, without reference filter. Alternatively, ODs can be read at 540 nm.

* **Note**: In contrast to normal photometers, in plate readers pipetting errors influence the OD. Therefore, 3 formazan aliquots shall be taken from each tissue extract. In the data sheet these 3 aliquots will be automatically reduced to one value by calculating the mean of the three aliquots. Thus, for calculations from each single tissue only one single mean OD-value is used.

Note: Readings are performed without reference filter, since the "classical" reference filter often used in the MTT test (630 nm) is still within the absorption curve of formazan. Since filters may have a ± tolerance in some cases the reference filter reduces the dynamics of the signal (OD) up to 40%.

Fixed 96 well-plate design (for OD reading in plate photometer, 3 aliquots per tissue)

NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	3 min
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	1 hour
NC	NC	C1	C1	C2	C2	C3	C3	C4	C4	PC	PC	
tissue 1	tissue 2	tissue 1	tissue 2	tissue 1	tissue 2	tissue 1	tissue 2	tissue 1	tissue 2	Tissue 1	Tissue 2	

3.5. DOCUMENTATION

3.5.1. Method Documentation Sheet, MDS (see ANNEX)

The MDS allows to check the correct set up, calibration and function of the equipment as well as correct weights, applications etc. The MDS is designed as a paper document "in the spirit of GLP". For each kit, make a hardcopy of the MDS, fill in and sign the requested information, starting the day prior to testing and ending after the test has been conducted.

Note (1): If several tests are performed per week, pipette verification (weighing H_20 on a balance) is only necessary once at the beginning of each week. Nevertheless, if adjustable pipettes are used the correct adjustment shall be checked and recorded in the MDS before each test.

Note (2): If solids cannot be sufficiently ground to a fine powder, it is recommended to check the weight of the levelled application spoon and record this weight in the MDS.

3.5.2. Data Spreadsheet

The MS EXCEL spreadsheet "C-SPREAD.XLS" is provided by ZEBET. Data files of optical densities (ODs) generated by the microplate reader are copied from the reader software to the Windows Clipboard and then pasted into the first map of the EXCEL spreadsheet in the 96-well format given above (Note: Only 72 wells of the 96 wells are used!).

The spreadsheet consists of three maps, named Import, MDS_Information and Spread**.** The first map (Import) is used for pasting the OD values (cursor position: A20!). Use the second map (MDS information) for the entry of the requested information (tissue lot-no., test material codes, date...), they will be copied from there to the other maps. The third map (Spread) does the calculations and provides a column graph of the results.

File names to be used in prevalidation phase III:
Since each single XLS file contains the data of 4 test chemicals, each of them coded by BIBRA with a four digit number there is no way to use "intelligent" file names which would allow to recognise the test chemicals from the file names. Therefore, file names should first give the testing laboratory name (3 digits), then a dash (1 digit) and then the test number (2 digits):
BAS-01.XLS, BAS-02.XLS, ...BAS-12.XLS
HLS-01.XLS, HLS-02.XLS, ...HLS-12.XLS
ZEB-01.XLS, ZEB-02.XLS, ...ZEB-12.XLS

4. Evaluation, Prediction Models (PM 1 and PM 2)

Note: The mathematical rule for the prediction or classification of *in vivo* skin corrosivity potential from the *in vitro* data is called Prediction Model (PM). For the present test two prediction models are defined, one definitive model (PM 1), based on published data (Perkins et al., 1996) which have been confirmed by extensive testing at ZEBET during Phase I of the present prevalidation study.

Nevertheless, the data base obtained in Phase I indicated that sensitivity was a bit too low (71%) to be used as a full animal replacement test, whereas the specificity of the test was very high (89%). Since a shift of the cut-off for classification would not have sufficiently increased the sensitivity, the test design was changed by including a second, longer application time of 1 hr for the test chemicals. This changed test design was experimentally tested at ZEBET when the prevalidation study had already proceeded to Phase II. Therefore, ZEBET was able to test only those chemicals again, which were classified negative with the 3 min EpiDerm™ protocol. The data indicated that the sensitivity was increased (some false negatives were predicted now correct as corrosives) but the influence of this change on the total predictive capacity of the assay could not be sufficiently investigated. Therefore, a second, tentative prediction model (PM 2) was defined, which has to be verified / falsified by the data obtained in Phase III of the present prevalidation study.

4.1. PREDICTION MODEL 1

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 min treatment compared to the negative control tissues concurrently treated with H_20. A chemical is classified "corrosive", if the relative tissue viability after 3 min treatment with a test material is decreased below 50 %:

mean tissue viability (% negative control)	**Prediction C / NC**
< 50	Corrosive
≥ 50	Non-corrosive

4.2. PREDICTION MODEL 2

Corrosivity potential of the test materials is predicted from the relative mean tissue viabilities obtained after 3 min treatment compared to the negative control tissues concurrently treated with H_20. A chemical is classified "corrosive" in any case, if the relative tissue viability after 3 min treatment with a test material is decreased below 50 %. In addition, those materials classified "non corrosive" after 3 min (viability ≥ 50%) are classified "corrosive" if the relative tissue viability after 1 hr treatment with a test material is decreased below 15 %.

mean tissue viability (% negative ctrl.)	**Prediction C / NC**
3 min: < 50	Corrosive
3 min:. ≥ 50 and 1 hour: < 15	Corrosive
3 min:. ≥ 50 and 1 hour: ≥ 15	Non-corrosive

ANNEX: METHODS DOCUMENTATION SHEET (MDS)

ASSAY No:.....DATE:.....
XLS file name:.....

Kit receipt

EpiDerm kit received (day/date):	Day used:
EpiDerm Lot no.:	Production date:
Epi-100 Assay medium Lot no.:	Expiration date:
MTT concentrate Lot no.:	Date:
MTT diluent Lot no.:	Date:
MTT extractant Lot no.:	Date:
Booked in by (ID):	

PBS preparation

DPBS Lot no.:	Expiration date:	
Vol 10x DPBS:	Vol water:	Initial pH:
NaOH used to adjust pH:		Final pH:
HCl used to adjust pH:		Final pH:
Prepared by (ID):		

Incubator verification

Incubator #	CO_2 (%)	Temperature (°C)	Check water in reservoir (✓)

ID / date:

Pipette verification (triplicate weightings)
Note: Perform pipette verification only once per week and refer to it in all assays of this week.
But: If adjustable pipettes are used, check correct adjustment daily and mark with (✓).

Verification	0.9 ml	300 µL	200 µL	25 µL	50 µL
	H_20 weight (mg)				
1. 2. 3.					

ID / date:

Dosing procedure
Please mark (✓) the type of application. Also, mark (✓) wetting with H_20. If significantly more than 25 µL of H_20 had to be used for wetting solids record ~ volume. REMARKS: record, if spreading was necessary or if crushing and grinding was not used (because it did not improve consistence of test material).

TEST MATERIAL CODE	LIQUID 50 µl (✓)	SEMI-SOLID	SOLID spoon (✓) + x µl H_2O		Material Characterisation §	REMARKS
Neg. Control						
Pos. Control						

§ use your own wording, like: "highly viscous"

Record experimental design of the 6-well plates

plate A (3 min)

Negative control		
Negative Control		

plate C (1 hour)

negative control		
negative control		

plate B (3 min)

		positive control
		positive control

plate D (1 hour)

		Positive Control
		Positive Control

(record code numbers of test materials) ID / date:

Time protocols:

Procedure	**Start**	**Stop**
1 hr pre-incubation of tissues		
1 hr chemical application (incubator)		
3 hrs MTT incubation (1 hr experiment)		
3 hrs MTT incubation (3 min experiment)		
Formazan extraction		

ID / Date:

Check plate photometer filter (_)

reading filter: 570 nm	
reading filter: 540 nm	

ID / Date:

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Summary Report of the EpiDerm™ *In Vitro* Assay for Assessing Dermal Corrosivity

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PURPOSE

This report focuses on the performance of EpiDerm™ to determine the usefulness and limitations of the assay for the identification of potential human corrosive chemicals. This report discusses also how EpiDerm™ compares to EPISKIN™, a mechanistically related *in vitro* human skin model system, and to other validated *in vitro* corrosivity tests (Rat Skin Transcutaneous Electrical Resistance [TER] and Corrositex®). The data and assessments reviewed for this report included the European Centre for the Validation of Alternative Methods (ECVAM) formal pre-validation/validation study on EpiDerm™ (Liebsch et al., 2000) and additional information formally submitted by MatTek, the commercial source of the assay, to ICCVAM for consideration (see MatTek Submission to ICCVAM; September 13, 2000).

EVALUATION OF REGULATORY AND SCIENTIFIC RATIONALE

EpiDerm™ is one of several *in vitro* corrosivity assays formally evaluated by ECVAM as alternatives to the *in vivo* rabbit corrosivity test (Fentem et al., 1998; Liebsch et al., 2000). The assay is a three-dimensional human skin model that uses cell viability as a measure of toxicity (i.e., corrosivity). Because EpiDerm™ is a human skin model, it may be more relevant to assessing human skin corrosivity potential than a test based on skin from another species. Also, the mode of application (topical) of the test material mimics the route of human exposure.

EpiDerm™ has been approved by the ECVAM Scientific Advisory Committee for use in corrosivity testing in Europe (Balls and Hellsten, 2000). This method has also been adopted for regulatory use within the European Union (EU) by the European Commission (Anon., 2000).

EVALUATION OF THE TEST METHOD

A standard kit contains media, reagents, and 24 tissues. The tissues provided in the test kit consist of normal, human epidermal keratinocytes cultured in a chemically defined medium to produce a stratified, highly differentiated, organotypic tissue model of the human epidermis. An EpiDerm™ kit is equipped with sufficient amounts of medium, washing solutions, and sterile, disposable tissue culture plasticware to test four test materials and concurrent negative and positive controls. For use in corrosivity testing, the test material (liquids: 50 µL; solids: 25 mg) is topically applied to a tissue for 3 and 60 minutes. Per test compound, replicate plates are used for each test period. Cell viability is assessed by measuring mitochondrial activity using the MTT (a tetrazolium salt) assay. A test chemical is classified as corrosive if it induces a 50% or greater decrease in relative cell viability at 3 minutes or an 85% or greater decrease in relative cell viability at 60 minutes. The scientific rationale for these decision criteria are based on a correlative analysis of the ability of a number of corrosive (C) and non-corrosive (NC) chemicals to induce histopathological necrosis and an associated reduction in cell viability (Perkins et al., 1996). EpiDerm™ will complement EPISKIN™, an ECVAM-validated *in vitro* corrosivity method, by providing an alternative and commercially available method.

Information on differences and similarities between EpiDerm™ and EPISKIN™ are detailed in Table 1.

EVALUATION OF TEST METHOD DATA QUALITY

The performance of EpiDerm™ was evaluated in three phases (Liebsch et al., 2000). Phase I was conducted by ZEBET (Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments, Berlin, Germany), and involved protocol and prediction model refinement using 50 chemicals. Phase II involved the transfer of the protocol to a second laboratory (Huntington Life Sciences) and the reproducibility of the assay was assessed by the repeat testing of 11 chemicals. In addition, in Phase II, ZEBET tested those chemicals classified as false negative in Phase I, aiming to refine the protocol and prediction model by increasing test sensitivity. Phase III was a formal evaluation of the reliability and performance of the assay using three laboratories (ZEBET, Huntington Life Sciences, and BASF AG), in which a blind trial conducted with 24 test chemicals was performed using the refined final protocol. In designing the Phase III study, ECVAM based its validation process on experimental results demonstrating that the EpiDerm™ and EPISKIN™ assays were mechanistically identical (Roguet et al., 1999). For Phase III, ECVAM selected a subset of 24 chemicals from the 60 chemicals tested in the EPISKIN™ ECVAM validation study (Fentem et al., 1998). The selection of the 60 chemicals in the original validation study was based on unequivocal animal data (Barratt et al., 1998). Care was taken to ensure a balanced representation of the chemical classes in this subset, as well as to minimize the number of chemicals previously in Phase I (there was an overlap of 5 chemicals). The 24 chemicals selected included 12 corrosive tested and 12 non-corrosive chemicals -- four organic acids (2 C; 2 NC), six organic bases (4 C, 2 NC), four neutral organic bases (4 NC), two phenols (1 C, 1 NC), three inorganic acids (2 C; 1 NC), two inorganic bases (1 C; 1 NC), two electrophiles (2 C), and one surfactant (1 NC).

Table 1. General Protocol Comparison between EPISKIN™ and EpiDerm™

	EPISKIN™	EpiDermä
Assay	Reconstructed human epidermis and a functional stratum corneum (not an animal model). Tissue approximates the barrier of normal human skin.	
Known limits of use	No known restrictions except for chemicals that reduce MTT. Although a relatively small numbers of chemicals have been evaluated in some chemical classes (i.e., cleaners and detergents), classified by ECVAM as otherwise without limits.	
Tissue construct acceptability	QC measures are based on historical laboratory control data.	
Materials, equipment, and supplies needed	Similar	
Replicates	Single tissue (culture)/experiment (ECVAM) or 3 replicates/ experiments (OECD)	Duplicate tissues/experiment, experiment replication if needed
Dosing procedures	Liquids: 50 µL applied neat Solids: 20 mg + saline	Liquids: 50 µL applied neat Solids: 25 mg + 25 µL H2O
Exposure duration	3 minutes, 1 hour, 4 hours	3 minutes, 1 hour
Endpoint	Relative cell viability compared to concurrent negative control, based on MTT assay (measure of mitochondrial function); assay based on optical density.	
Negative and positive controls	no vehicle control (undiluted test material used) Negative control: saline Positive control: glacial acetic acid	no vehicle control (undiluted test material used) Negative control: water Positive control: 8.0 N KOH
Acceptable range of control responses	Negative control: 4-hour optical density at 545-595 nm = 0.113-0.309 for MTT incubations at 20-28°C. Positive control: viability at 4 hours must be 0-20%.	Negative control: 3-min and 1-hour optical density at 570 or 540 nm = ≥0.8. Positive control: viability at 3 min must be ≤30%.
Data analysis	Determination of relative viability at each exposure duration. No statistical analysis.	
Positive Response	Relative cell viability <35% at any exposure duration (=packing group).	Relative cell viability <50% after 3 minutes and/or <15% after 60 minutes.
Criteria for accepting or rejecting a test	Acceptable control values Test repeated if inconsistent toxicity response pattern across exposure durations (i.e., less toxicity at a longer exposure duration) or if corrosivity classification is variable	Acceptable control values Test repeated if difference in viability between duplicate tissues >30% and the corrosivity classification is variable, or (recommended) if the resulting viability is near to a classification cut-off.

The tests were conducted in the "spirit" of GLP. Each chemical was tested twice using independent lots of tissue by each of three different laboratories. A formal audit of the ECVAM data by a Quality Assurance Unit was not conducted; however, it was stated that all data submitted by the participating laboratories were verified against the original data sheets by ECVAM staff.

EVALUATION OF TEST METHOD PERFORMANCE

For this summary report, an analysis was conducted, similar to the performance analysis conducted for the ICCVAM Peer Review of Corrositex®; the current analysis evaluated the performance characteristics of the EpiDerm™ assay against the corresponding *in vivo* rabbit corrosivity data and the corresponding *in vitro* corrosivity data generated by EPISKIN™. The database used in the evaluation of the performance characteristics of EpiDerm™ consisted of data from the ECVAM pre-validation/validation study only (Liebsch et al., 2000); other data were not located.

For ease of comparison, chemicals evaluated in the EpiDerm™ assay were classified into the same chemical and product class designations used in the Corrositex® evaluation. A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls), the chemical was eliminated from inclusion in the performance calculations.

Based on the database of 24 chemicals and chemical mixtures used in the validation study (Tables 2 and 4), EpiDerm™ had an accuracy of 92% (22/24 chemicals or chemical mixtures), a sensitivity of 92% (11/12 chemicals or chemical mixtures), and a specificity of 83% (10/12 chemicals or chemical mixtures). From these data, ECVAM concluded that EpiDerm™ was valid for use as a replacement for the *in vivo* rabbit skin test for distinguishing between corrosive and non-corrosive chemicals for all of the chemical classes studied (Liebsch et al., 2000). As for EPISKIN™, due to the relatively small numbers of chemicals evaluated in some chemical classes, definitive conclusions as to the adequacy of EPISKIN™ or EpiDermä for some classes of chemicals were difficult to make with a high degree of confidence. However, taking into account the relative simplicity of the mechanism of action of corrosives, ECVAM concluded that the EpiDerm™ method would be generally applicable across all chemical classes (Fentem et al., 1998; Liebsch et al., 2000). A comparison of the ability of EpiDerm™ and EPISKIN™ to correctly identify corrosive and non-corrosive chemicals among the 24 chemicals tested in Phase III is provided in Table 2. Both assays are nearly identical in their performance (see also Table 4).

Table 2. Summary of Results for EpiDerm™ and EPISKIN™ Compared to *In Vivo* Rabbit Results

Material	EPISKIN™	EpiDerm™
Corrosive	11/12	11/12
Non-corrosive	11/12	10/12

EVALUATION OF TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

The inter- and intra-laboratory reliability of EpiDerm™ was evaluated in the ECVAM pre-validation/validation study (Liebsch et al., 2000). In Phase III, each chemical was tested twice using different tissue lots in each of three laboratories (i.e., 144 tests were conducted). Of 72 replicate tests, 5 (6.9%) did not replicate. Regarding inter-laboratory reproducibility, three of the 24 chemicals (12.5%) were not predicted by all three laboratories (i.e., the performance characteristics of the three laboratories were nearly identical). Intra- and inter-laboratory reliability was evaluated formally using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Based on the results obtained, ECVAM concluded that EpiDerm™ provided excellent reliability (Liebsch et al., 2000). After reviewing the intra- and inter-laboratory evaluations conducted by ECVAM, it was concluded that the analyses were appropriate and that the conclusions were accurate.

OTHER SCIENTIFIC REVIEWS

In May 2001, a search of the open literature was conducted to locate additional EpiDerm™ studies. Four databases (PubMed, Web of Science, Toxline, and Current Contents Connect) were searched using the key terms "EpiDerm", and "Epi" within one word of "derm". Additional references were obtained from the MatTek technical references section at www.mattek.com. The search found no additional relevant studies conducted with EpiDerm™.

OTHER CONSIDERATIONS

Like EPISKIN™, the EpiDerm™ kit contains all of the necessary materials to conduct the test and does not require additional preparation. No animals are used in this test. The cost for conducting EpiDerm™ is reported by MatTek (e-mail communication from Mitch Klausner, MatTek Corporation) to be approximately $800 per kit or $200 per test chemical (Table 3). This cost is less than the *in vivo* rabbit skin test and similar to that for the other validated *in vitro* corrosivity assays (Fentem et al., 1998). The time needed to conduct the EpiDerm™ is similar to EPISKIN™.

RELATED ISSUES

Refinement, Reduction, and Replacement

Since the method is designed as a replacement for animals, EpiDerm™ would clearly reduce the requirement for animal testing for corrosivity. Therefore, it has the potential to eliminate the use of animals for the determination of corrosivity. If used in an integrated testing approach, EpiDerm™ provides for reduction and refinement of animal use.

Comparison to Other *In Vitro* Assays

General comparative information on EpiDerm™ compared to Rat Skin TER, EPISKIN™, and Corrositex® is provided in Tables 3 through 6. In contrast to Corrositex® and EPISKIN™, EpiDerm™, like Rat Skin TER, cannot be used to identify packing group classifications.

Table 3. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays

	Rat Skin TER	EPISKIN™ (prediction model B)	EpiDerm™ (prediction model 2)	Corrositex®
Test Method Description	Acceptable	Acceptable	Acceptable	Acceptable
Adequacy/Completeness of Protocol	Acceptable	Acceptable	Acceptable	Acceptable
Usefulness for Assessing Corrosivity/Non-corrosivity	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Usefulness for Determining Packing Groups	Not Acceptable (Fentem et al., 1998)	Can group as UN packing group II/III or I (Fentem et al., 1998)[a]	Not Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Repeatability and Reproducibility	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (Fentem et al., 1998; ICCVAM, 1999)
Animal Use Refinement, Reduction, and Replacement Considerations	Refines and reduces animal use when used as a stand-alone test or in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.	Refines and reduces animal use when used in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.
Cost	~$500-850/test	~$450/test kit[b]	~$200/test chemical	~$300/test chemical
Study duration	2 work-days	1 work-day	1 work-day	≤ 4 hr/chemical

a Since the performance of EPISKINä was not assessed for distinguishing between UN packing groups II and III, all R34 classifications would be conservatively classified as UN packing group II.

b One to three chemicals may be tested per test kit; however, it is recommended by the supplier that each test chemical be assayed using 3 different skin batches/kits which equates to a total cost of ~$430/ test chemical.

Table 4. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays Based on an ICCVAM Weight-of-Evidence Approach by Chemical using Data from the ECVAM and other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™	EpiDerm™ (prediction model 2)	Corrositex®
Number of Chemicals	122	60	24	163
Overall Sensitivity[a]	94% (51/54)	82% (23/28)	92% (11/12)	85% (76/89)
Overall Specificity[a]	71% (48/68)	84% (27/32)	83% (10/12)	72% (52/74)
Overall Accuracy[a]	81% (99/122)	83% (50/60)	92% (22/24)	79% (128/163)
Test Chemical Interlaboratory Coefficient of Variation	Median = 34.7 Range = 3.8-322 n^b = 120	Median = 11.3 Range = 3.9-148.8 n^b = 20	Median = 12.3 Range = 0.9-51.2 n^b = 144	Median = 30.3 Range = 7.7-252.5 n^b = 180

a Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method.

b The total number of independent values, which is calculated as the number of chemicals tested multiplied by the number of sample times for each chemical.

Table 5. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays from Independent Test Results in the ECVAM Validation Studies (Fentem et al., 1998; Liebsch et al., 2000)

	TER	EPISKIN™ (prediction model B)	EpiDerm™ (prediction model 2)
Number of Chemicals Tested in ECVAM Validation Study	60 (Fentem et al., 1998)	60/24[a] (Fentem et al., 1998)	24 (Liebsch et al., 2000)
Sensitivity[b]	88% (140/159)	83% (201/243) / 88% (87/99)	88% (63/72)
Specificity[b]	72% (142/196)	80% (237/297) / 79% (92/117)	86% (62/72)
Accuracy[b]	79% (282/355)[c]	81% (438/540) / 83% (179/216)	87% (125/144)
False Positive Rate[b]	28% (54/196)	20% (60/297) / 21% (25/117)	14% (10/72)
False Negative Rate[b]	12% (19/159)	17% (42/243) / 12% (12/99)	13% (9/72)
Number of Trials[d]	355	540 / 216	144
Test Chemical Inter-laboratory Coefficient of Variation	Median = 34.7 Range = 10-322 n^d = 360	Median = 30.2 Range = 7.7-252.5 n^d = 540	Median = 12.3 Range = 0.9-51.2 n^d = 144

a The first numbers for accuracy, sensitivity, specificity, false positive rate, and false negative rate correspond to the 60 chemicals tested in the ECVAM Skin Corrosivity Test using EPISKINä (Barratt et al., 1998; Fentem et al., 1998); the latter values correspond to a direct comparison of EpiDermä and EPISKINä for the same 24 materials tested in both systems (Liebsch et al., 2000).

b Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative.

c The percentages are based on the number of correct trials among the total number of trials (i.e., independent tests) provided in parenthesis.

d The total number of trials conducted in the validation study minus the non-qualified (NQ) results. This number is usually equal to the number of chemicals multiplied by the number of participating laboratories multiplied by the number of replicate tests.

Table 6. Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000)

No.	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN-B [a]	EpiDerm
1	Hexanoic acid	ORGAC	R34/II&III	R35	R35	N/A
29	65/35 Octanoic/decanoic acid	ORGAC	R34/II&III	R34	R35	N/A
36	2-Methylbutyric acid	ORGAC	R34/II&III	R35	R34	N/A
40	Octanoic acid (caprylic acid)	ORGAC	R34/II&III	R35	R34/C	C
47	60/40 Octanoic/decanoic acids	ORGAC	R34/II&III	R34	R34/C	C
50	55/45 Octanoic/decanoic acids	ORGAC	R34/II&III	R35	R34	N/A
7	3,3'-Dithiodipropionic acid	ORGAC	NC	NC	NC	N/A
12	Dodecanoic acid (lauric acid)	ORGAC	NC	NC	NC	NC
26	Isotearic acid	ORGAC	NC	NC	NC	NC
34	70/30 Oleine/octanoic acid	ORGAC	NC	NC	NC	N/A
58	10-Undecenoic acid	ORGAC	NC	NC	R34	N/A
2	1,2-Diaminopropane	ORGBA	R35/I	R35	R34/C	C
15	Dimethyldipropylenetriamine	ORGBA	R35/I	R35	R34/C	C
38	Tallow amine	ORGBA	R35/II	2R34/2NC/2NQ	NC	N/A
55	1-(2-Aminoethyl)piperazine	ORGBA	R34/II	R35	NC	N/A
13	3-Methoxypropylamine	ORGBA	R34/II&III	R35	R34	N/A
17	Dimethylisopropylamine	ORGBA	R34/II&III	R35	R34/C	C
45	n-Heptylamine	ORGBA	R34/II&III	R35	NC	C
10	2,4-Xylidine (2,4-Dimethylaniline)	ORGBA	NC	R34	R34	N/A
35	Hydrogenated tallow amine	ORGBA	NC	NC	NC	NC
59	4-Amino-1,2,4-triazole	ORGBA	NC	NC	NC	NC
8	Isopropanol	NORG	NC	NC	NC	N/A
11	2-Phenylethanol	NORG	NC	NC	NC	N/A
16	Methyl trimethylacetate (referred to as Methyl 2,2-dimethylpropanoate in EpiDerm™)	NORG	NC	NC	NC	C
19	Tetrachloroethylene	NORG	NC	NC	NC	NC
22	n-Butyl propionate	NORG	NC	NC	NC	N/A
27	Methyl palmitate	NORG	NC	NC	NC	N/A
44	Benzyl acetone	NORG	NC	NC	NC	NC
51	Methyl laurate	NORG	NC	NC	NC	N/A
56	1,9-Decadiene	NORG	NC	NC	NC	NC
3	Carvacrol	PHEN	R34/II&III	R34	R34	N/A
23	2-tert-Butylphenol	PHEN	R34/II&III	R35	R34/C	C
9	*o*-Methoxyphenol (Guaiacol)	PHEN	NC	NC	R34	N/A
30	4,4-Methylene-bis-(2,6-di-tert-butylphenol)	PHEN	NC	NC	NC	N/A
49	Eugenol	PHEN	NC	NC	NC	NC
4	Boron trifluoride dihydrate	INORGAC	R35/I	R35	R35/C	C
28	Phosphorus tribromide	INORGAC	R35/I	R35	R35/C	C

32	Phosphorus pentachloride	INORGAC	R35/I	R35	R34	N/A
25	Sulfuric acid (10% wt.)	INORGAC	R34/II&III	R34	R34	N/A
57	Phosphoric acid	INORGAC	R34/II	R35	R34	N/A
43	Hydrochloric acid (14.4% wt)	INORGAC	R34/II&III	R35	R34	N/A
53	Sulfamic acid	INORGAC	NC	R34	R34/C	C
18	Potassium hydroxide (10% aq.)	INORGBA	R34/II&III	R35	R34/C	C
42	2-Mercaptoethanol, Na salt (45% aq.)	INORGBA	R34/II&III	R35	NC	N/A
21	Potassium hydroxide (5% aq.)	INORGBA	NC	R35	R34	N/A
24	Sodium carbonate (50% aq.)	INORGBA	NC	R34	NC	NC
20	Ferric [iron (III)] chloride	INORGSAL	R34/II	R35	R34	N/A
52	Sodium bicarbonate	INORGSAL	NC	R34	NC	N/A
54	Sodium bisulfite	INORGSAL	NC	3R34/3NC	NC	N/A
5	Methacrolein	ELECTRO	R34/II&III	NC	R34/C	NC
14	Allyl bromide	ELECTRO	R34/II&III	R35	R34	N/A
48	Glycol bromoacetate (85%)	ELECTRO	R34/II&III	NC	R34/C	C
6	Phenethyl bromide	ELECTRO	NC	NC	NC	N/A
31	2-Bromobutane	ELECTRO	NC	3R34/3R35	NC	N/A
33	4-(Methylthio)-benzaldehyde	ELECTRO	NC	NC	NC	N/A
39	2-Ethoxyethyl methacrylate	ELECTRO	NC	NC	NC	N/A
46	Cinnamaldehyde	ELECTRO	NC	NC	NC	N/A
37	Sodium undecylenate (33% aq.)	SOAP	NC	R35	R34	N/A
41	20/80 Coconut/palm soap	SOAP	NC	NC	NC	N/A
60	Sodium lauryl sulfate (20% aq.)	SOAP	NC	R35	NC	NC

Overall corrosivity classifications were determined by the majority of the reported results obtained from each assay. If results do not show a majority, a definitive classification could not be determined.

Definitions are as follows: C = Corrosive; NC = Non-corrosive; R34 is equivalent to packing groups II and/or III; R35 is equivalent of packing group I, except for tallow amine (R35/II); NQ = Non-qualified; N/A = Not applicable because not tested; ORGAC = Organic acid; ORGBA = Organic base; NORG = Neutral organics; PHEN = phenol; INORGAC = Inorganic acid; INORGBA = Inorganic base; INORGSAL = Inorganic salt; ELECTRO = Electrophile; SOAP = Soap surfactant

a For EPISKIN™, prediction model B was the more complex prediction model and was the only model considered in detail by the ECVAM Management Team (Fentem et al., 1998).

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

ECVAM concluded that EpiDerm™ was an *in vitro* replacement assay for *in vivo* corrosivity testing. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that EpiDerm™ was both reliable and reproducible; the author of this report concurs with that conclusion.

The two major questions to be addressed for *in vitro* corrosivity assays are:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?

2. Does the assay adequately consider and incorporate, where scientifically feasible, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

In response to the first question, the performance characteristics of the EpiDerm™ method indicates, in specific testing circumstances, that this test may be considered useful as part of an integrated testing strategy for assessing the dermal corrosion potential of chemicals.

In response to the second question, EpiDerm™ sufficiently considers and incorporates the 3Rs. Specifically, the use of EpiDerm™ offers advantages with respect to animal welfare considerations, including animal use refinement, reduction, and replacement. Similarly, the use of the EpiDerm™ assay as part of an integrated approach reduces and refines the use of animals by providing a basis for decisions on further testing. When these methods are used as part of an integrated testing strategy for corrosivity, there is a reduction in the number of animals required because positive results typically eliminate the need for animal testing, and when further testing in animals is determined to be necessary, only one animal is required to confirm a corrosive chemical. Follow-up testing using *in vivo* methods, when deemed necessary, could also employ test agent dilution schemes to minimize possible pain in any individual animal.

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ECVAM Protocol for Rat Skin Transcutaneous Electrical Resistance: an *In Vitro* Assay for Assessing Dermal Corrosivity

Original Draft: July 1996
Confirmed: October 2000

NOTE: This protocol presents the standard operating procedure used in the ECVAM Skin Corrosivity Validation Study (1996/1997). ECVAM confirmed the accuracy of the SOP in October 2000, and this protocol was supplied by Dr. Andrew Worth of ECVAM via email on May 22, 2001.

Rat Skin Transcutaneous Electrical Resistance (TER) Test

The corrosivity potential of a chemical may be predicted from its effects on the transcutaneous electrical resistance of rat skin and from its effects on the penetration of sulforhodamine B dye through the skin.

Objectives and Applications

TYPE OF TESTING	:	screening, replacement
LEVEL OF ASSESSMENT	:	toxic potential, toxic potency
PURPOSE OF TESTING	:	hazard identification, classification and labelling

Proposed replacement for the *in vivo* method, the Draize rabbit skin corrosivity test, to be used for hazard identification and classification of corrosive potential to fulfil international regulatory requirements pertaining to the handling, packing and transport of chemicals.
When used in screening mode, the TER test is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency) (Fentem *et al.*, 1998).

Basis of the Method

Most international regulatory classification schemes define chemically induced dermal corrosion as full thickness destruction (necrosis) of the skin tissue, while some extend the definition of corrosion to include any irreversible alterations caused to the skin. The potential to induce skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals.

The determination of skin corrosion potential is therefore included in international regulatory requirements for the testing of chemicals, for example, in OECD testing guideline 404 (Anon., 1992); Annex V of Directive 67/548/EEC (Anon., 2000) and in the U.S. Code of Federal Regulations (Anon., 1991).

Corrosivity is usually determined *in vivo* using the Draize rabbit skin test (Draize *et al.*, 1944). The present test is based on the experience that transcutaneous electrical resistance (TER) measurements are believed to be of value in predicting severe cutaneous effects *in vivo*. The TER assay developed and evaluated by Oliver and coworkers (Barlow *et al.*, 1991; Oliver *et al.*, 1986; 1988; Oliver, 1990) has been used successfully as a routine in-house test for several years (Fentem *et al.*, 1998).

As an outcome of the ECVAM prevalidation study for protocol optimization, a second endpoint, dye binding (sulforhodamine B) has been added to reduce the number of false positive predictions encountered previously with surfactants and neutral organics.

Experimental Description

Endpoint and Endpoint Detection	:	- changes in transcutaneous electrical resistance (kΩ); - dye binding (sulforhodamine B) determined by optical density measurements;
Test System	:	isolated rat skin.

Liquid or solid test material is applied to the inner epidermal surface of discs of freshly isolated rat dorsal skin. After the exposure periods of 2 and 24 hours, the skin is washed and transcutaneous electrical resistance is measured. If the electrical resistance values are <5kΩ and the substance is a surfactant or neutral organic, then the sulforhodamine B dye is applied to the epidermal surface of each skin disc. The discs are washed and then subjected to a dye extraction procedure. The amount of dye extracted is determined from optical density measurements. The changes in the endpoints are then compared to HCl and H_2O, the positive and negative controls.

Test Compounds

A total of 60 test compounds, consisting of 11 organic acids, 10 organic bases, 9 neutral organics, 5 phenols, 7 inorganic acids, 4 inorganic bases, 3 inorganic salts, 8 electrophiles and 3 soaps/surfactants were tested in the ECVAM validation study. Details on the test compounds and test results are available from dbVas of the ECVAM SIS.

Prediction Model

Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a predetermined threshold level.

If the transcutaneous electrical resistance readings are ≤5kΩ at either of the contact periods, and the substance is a surfactant or neutral organic, then the dye penetration results are considered.

For detailed information see section 11, “Interpretation of results” of the present standard operating procedure.

Discussion

The TER assay is robust, requires inexpensive and readily available equipment, and can be performed by most laboratory personnel provided that care is taken during the critical steps of disc preparation and washing. The assay is inexpensive to perform in comparison with the three-dimensional tissue culture models and the CORROSITEX assay, and the technology is not protected by patent. These factors support the overall applicability of the TER assay in routine testing. The validation study has demonstrated the accuracy of the TER assay in identifying C and NC chemicals (Fentem *et al.*, 1998).

Status

The TER assay has been evaluated in intralaboratory and interlaboratory studies (Botham *et al.*,

1992; Oliver *et al.*, 1986, 1988), and it performed creditably in the prevalidation study conducted during 1993 and 1994 (Botham *et al.*, 1995). This method has been evaluated in the ECVAM Skin Corrosivity Validation Study conducted in 1996 and 1997 (Fentem *et al.*, 1998). Based on the outcome of the study, the ECVAM Scientific Advisory Committee (ESAC) concluded that the results obtained with the rat skin TER test in the "ECVAM Skin Corrosivity Validation Study" were reproducible. The test proved applicable to testing all the above reported chemical classes of different physical forms. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good.

ESAC unanimously endorsed the statement that the rat skin TER test was scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and non-corrosive chemicals, and that this test was ready to be considered for regulatory acceptance (10^{th} meeting at ECVAM of the ECVAM Scientific Advisory Committee, European Commission, March 1998; Anon., 1998). The 27^{th} meeting of the Committee for Adaptation to Technical Progress of "Directive 67/548/EEC on the Classification, Packaging and Labelling of Dangerous Substances" agreed that the TER Test would form part of "Annex V method B.40. Skin Corrosion", February 2000 (Directive 2000/33/EC). Furthermore, this test is now under consideration for inclusion in the OECD Guidelines. Further details on the ECVAM Validation Study are available from dbVas of the ECVAM SIS.

Last update: May 2000

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Procedure Details, July 1996*

RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST

NOTE: This protocol presents the standard operating procedure evaluated in the ECVAM Skin Corrosivity Validation Study (1996/1997).

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* The accuracy of the SOP has been confirmed in October 2000.

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1. INTRODUCTION AND OBJECTIVES

The purpose of this technique is to assess the degree of the skin corrosive potential of a test chemical *in vitro*. The results obtained from the transcutaneous electrical resistance (TER) measurements are believed to be of value in predicting severe cutaneous effects (degree of skin corrosive potential) *in vivo*. As a prelude to formal validation, the TER assay was evaluated in a prevalidation study (Botham *et al.*, 1995). Preliminary evaluation of the results indicated that the TER test required optimisation, to enable differentiation between different classes of corrosive materials, and to reduce the number of over-predictions (false positives). The results of this optimisation (Hadfield & Lewis, 1996; unpublished data), indicated that the modified electrical resistance test was able to differentiate between classes of corrosive materials (R35/R34) and, by the addition of a second endpoint, dye binding, was able to reduce the number of false positive predictions. The following protocol was therefore devised for use in the ECVAM international validation study on *in vitro* tests for skin corrosivity (Barratt *et al.*, 1998; Fentem *et al.*, 1998).

2. SAFETY PRECAUTIONS

Standard local safety precautions should be adopted. All materials should be handled in accordance with their potential hazards.

3. ANIMALS AND HUSBANDRY

20-23 day old Wistar rats are purchased for use in the test. Animals are acclimatised for a minimum of one night, depending on the day of delivery. On the day after arrival they are shaved and washed: animals are held securely and the dorsal flank hair is carefully removed with small animal clippers. The animals are then washed by careful wiping, whilst submerging the area in a one-litre volume of antibiotic solution (see following section 4). Animals are washed again on the third or fourth day following the first wash, and they are then used within 3 days (animals must

not be older than 31 days for pelt preparation).

4. PREPARATION OF ANTIBIOTIC SOLUTION

An antibiotic solution is prepared by adding streptomycin, penicillin, chloramphenicol and amphotericin B to 1 litre of luke-warm deionised water. The resulting antibiotic solution should contain the following concentrations: 8mgml^{-1} streptomycin; 800µgml^{-1} penicillin; 10µgml^{-1} chloramphenicol; and 10µgml^{-1} amphotericin B. Streptomycin, penicillin, chloramphenicol and amphotericin B are available from standard laboratory suppliers. It is also acceptable to use mixtures of antibiotics containing glutamine which are commercially available. Appropriate inhalation safety procedures should be followed when handling antibiotics.

5. PREPARATION OF SKIN AND MOUNTING ON *IN VITRO* APPARATUS

Animals are humanely killed by inhalation of a rising concentration of CO_2 followed by cervical dislocation. The dorsal skin of each animal is then removed and stripped of excess fat by carefully peeling it away from the skin by using the thumb and forefinger covered with paper towel. The pelt is placed over the end of a 10 mm diameter polytetrafluoroethylene (PTFE) tube ensuring that the epidermal surface is in contact with the tube. A rubber 'O' ring is press-fitted over the end of the tube to hold the skin in place, and excess tissue is trimmed away with a scalpel blade. Tube and 'O ' ring dimensions are shown in Figure 3. The rubber 'O' ring is then carefully sealed to the end of the PTFE tube with petroleum jelly (or soft paraffin wax), applied with a scalpel blade. The tube is supported by a spring ("Terry") clip inside a plastic receptor chamber containing 10ml of magnesium sulphate solution (154mM; see Figure 1). The PTFE tube is uniquely numbered with a label prior to test substance application.

Skin discs of approximately 0.79cm^2 can be obtained from any number of animals. However, the viability of each pelt must be assessed prior to use in the test by using the following method: two discs are taken from each pelt and prepared as described above. Electrical resistance measurements are then taken for each disc (see section 7). Both discs must produce resistance values of ≥10kΩ. The two discs are then discarded and the remainder of the pelt is used in the test. If both discs fall below the 10kΩ threshold, the pelt is discarded. If one disc falls below this threshold, another is tested; if this also falls below the threshold, the pelt is discarded. If the disc produces a TER measurement of ≥10kΩ, the pelt can be used in the test.

PTFE tubes and rubber 'O' rings are available from IMS, Dane Mill, Broadhurst Lane, Congleton, Cheshire CW12 1LA, UK.

6. TEST CHEMICAL APPLICATION AND REMOVAL

A measured volume of liquid test material (0.15ml) is applied to the inner epidermal surface (see Figure 1). When using solid test materials, a sufficient amount of solid material is applied to the surface of the disc ensuring that the whole surface of the epidermis is covered. Deionised or distilled water (0.15ml) is then added on top of the solid material and the tubes are shaken. Three skin discs are used for each time point per chemical. Test chemicals are applied for contact periods of 2 and 24 hours. After the required contact time, the test chemical is removed by washing with a jet of tap water, at room temperature, for approximately 10 seconds or until no further test material can be removed.

Control substances for the TER test and the dye binding assay:

Positive	-	36% HCI
Negative	-	DH_2O

All to be tested at the 24-hour contact period only.

Test substances should have maximum contact with the skin. For some solids this may be achieved by warming up to 30°C to melt the test substance, or by grinding to produce a granular material or powder.

Where measured test substance TER values are higher than the negative (water) control values (for example, waxy solids which may become liquids at approximately room temperature), the skin surface can be washed with water at up to 37°C. The skin should be visually inspected to determine if the skin is coated with test substance. The TER value should then be re-measured. If the value is less than or equal to the upper limit of the negative (water) control range, and if the skin disc appears to be free of residue, it can be accepted. If the TER value does not reduce to the upper limit of the negative control range after washing with the warm water, the disc should be rejected.

7. TRANSCUTANEOUS ELECTRICAL RESISTANCE MEASUREMENTS

The transcutaneous electrical resistance is measured using an AIM electronic databridge 401 or 6401 (available from H. Tinsley and Co., 275 King Henry's Drive, New Addington, Croydon, Surrey CR0 0AE, UK).

Prior to measuring the electrical resistance, the surface tension of the skin is reduced by adding a small volume of 70% ethanol sufficient to cover the epidermis. After approximately 3 seconds, the ethanol is removed by inverting the tube. The PTFE tube is then replaced in the receptor chamber and the tissue is hydrated by the addition of 3ml of magnesium sulphate solution (154mM) to the inside of the PTFE tube; any air bubbles are dislodged by slight tapping.

The stainless steel electrodes of the databridge are then placed on either side of the skin disc to take the resistance measurement in kΩ/skin disc (see Figure 2). Electrode dimensions and the length of the electrode exposed below the crocodile clips are shown in Figure 3. The inner (thick) electrode clip is rested on the top of the PTFE tube during resistance measurement, to ensure that a consistent length of electrode is submerged in the $MgSO_4$ solution. The outer (thin) electrode is positioned inside the receptor chamber, so that it rests on the bottom of the chamber. The distance between the bottom of the Terry clip and the bottom of the PTFE tube is set at 7.0 cm, to reduce the variability of resistance measurements between individual skin discs, which is influenced by the distance between the electrodes. The electrical resistance is then recorded from the databridge display.

If the reading falls above 20kΩ this may be due to the test material coating the epidermal surface of the skin disc. Removal of this coating can be performed by holding a gloved thumb over the end of the tube and shaking it for approximately 10 seconds; the $MgSO_4$ solution is then poured away. If any test material is present it may be seen as a residue in the $MgSO_4$ solution. The transcutaneous electrical resistance of the skin is then measured as described previously.

8. SULFORHODAMINE B DYE APPLICATION AND REMOVAL

If the electrical resistance values are ≤5kΩ at the 2- and/or 24-hour contact periods, an assessment of dye penetration is carried out on the 24-hour contact period tissues. If the skin disc was punctured during the jet washing procedure to remove the test chemical, then that particular tube is excluded from further testing.

150µl of a 10% (w/v) dilution of sulforhodamine B dye in DH_2O is applied to the epidermal surface of each skin disc for 2 hours. To remove any excess/unbound dye, the skin discs are then jet-washed with tap water at room temperature for approximately 10 seconds (or until the water runs clear). Each skin disc is carefully removed from the PTFE tube and placed in a 20ml scintillation vial containing 8ml of deionised water. The vials are agitated gently for 5 minutes to remove any further excess/unbound dye. This rinsing procedure is then repeated. Each skin disc is removed and placed into another 20ml scintillation vial containing 5ml of 30% (w/v) sodium dodecyl sulphate (SDS) in DH_2O and is incubated overnight at 60°C.

After incubation, each skin disc is removed and discarded and the remaining solution is centrifuged in a 15ml centrifuge tube at 1000rpm for 8 minutes at 21°C (relative centrifugal force ≈ 175g). A 1ml sample of the supernatant is then placed into another 15ml centrifuge tube and diluted 1 in 5 (v/v) (i.e. 1ml + 4ml) with 30% (w/v) SDS in DH_2O. The optical density of the solution is determined at 565.5nm and the results are recorded.

Sulforhodamine B (90% dye content) and SDS are available from Sigma Chemical Company, Poole, UK.

9. FURTHER INFORMATION

Experience with the TER assay has shown that there are two critical stages. Experienced users pay particular attention to: a) skin disc preparation, ensuring removal of all fatty tissues and a complete seal of the skin on the PTFE tube; b) washing of the disc to remove as much of the test substance as possible. Residues of test substance remaining on the skin may affect the resistance values (for example, waxy substances, which solidify on the skin's surface). The positive controls TER values can drift with time (within days) if the samples are not fresh aliquoted from the stock acid maintained according to the storage recommendations on the label.

10. CALCULATION OF DYE CONTENT/DISC

The dye content, in µg/disc, is calculated from the optical density values as follows:

Sulforhodamine B dye molar extinction coefficient = 8.7×10^4,
Molecular Weight = 580,
No correction for the purity of the dye is made,
Optical density = 0.973,

$$\frac{0.973 \leftrightarrow 10^{-4}}{8.7} = 0.112 \leftrightarrow 10^{-4} = 11.2 \leftrightarrow 10^{-6} = 11.2 \mu M = 11.2 \mu\text{mol/l}$$

$$11.2 \leftrightarrow 580 \leftrightarrow 10^{-6} = 6496 \leftrightarrow 10^{-6}\ \textbf{\textit{g/l}} = 6.496 \leftrightarrow 10^{-3}\ \textbf{\textit{g/l}}$$

Dye was extracted into 5ml of solvent:

$$\frac{6.496 \times 10^{-3}}{200} = 0.325 \times 10^{-4}\ \textbf{\textit{g/l}} = 32.5 \times 10^{-6}\ \textbf{\textit{g/l}}$$

Solution was diluted 1 in 5 (v/v):

$$32.5 \times 10^{-6} \times 5 = 162.5 \times 10^{-6} = 162.5\mu\textbf{\textit{g/disc}}$$

The sulforhodamine B dye content is determined for each skin disc. A mean dye content is then calculated for the three skin discs at 24 hours. If a skin disc is punctured during the washing procedure used to remove the dye, then the individual dye content is recorded but it is excluded from the calculation of the mean.

All results are recorded on the data sheet shown in Appendix 1.

11. INTERPRETATION OF RESULTS

a) Results are accepted on condition of adherence to the ranges given below. If the positive and negative control results for the experiment do not fall within the accepted ranges, the data on the test substance cannot be interpreted and the experiment must be repeated.

Dye binding assay		TER assay	
36% HCl positive control range (µg/disc)	Distilled water negative control range (µg/disc)	36% HCl positive control range (kΩ)	Distilled water negative control range (kΩ)
40 - 100	15 – 35	0.5 - 1.0	10 - 25

b) If the transcutaneous electrical resistance readings obtained for all test substance contact periods are >5kΩ, then the substance is classified as non-corrosive.

c) If the transcutaneous electrical resistance readings are ≤5kΩ after a 2-hour contact period, and the substance is not a surfactant or neutral organic, then the substance is classified as corrosive (R35).

d) If the transcutaneous electrical resistance readings are ≤5kΩ after a 24-hour contact period (but >5kΩ after 2 hours contact), and the substance is not a surfactant or neutral organic, then the substance is classified as corrosive (R34).

e) If the transcutaneous electrical resistance readings are ≤5kΩ at either of the contact periods, and the substance is a surfactant or neutral organic, then the dye penetration results are considered.

f) If the mean disc dye content is ≥mean disc dye content of the 36% HCI positive control obtained concurrently in the experiment at the 24-hour contact period, then the substance is a true positive and is therefore classified as corrosive (R34).

g) If the mean disc dye content is < mean disc dye content of the 36% HCI positive control obtained concurrently in the experiment at the 24-hour contact period, then the substance is a false positive and is therefore classified as non-corrosive.

A flow diagram for interpretation of the results is attached.

Figure 1

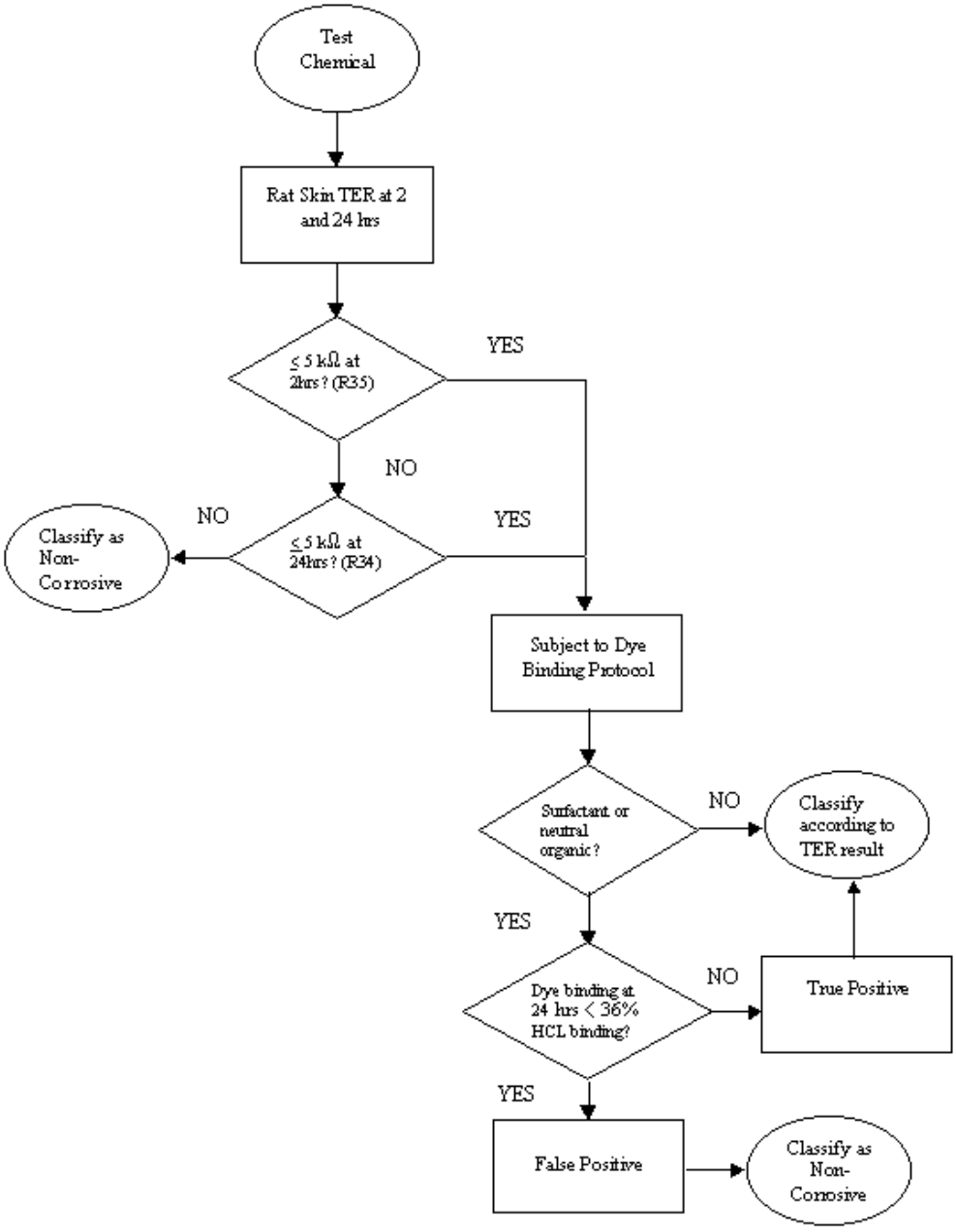

Test Chemical
Rat Skin TER at 2 and 24 hrs
≤ 5 kΩ at 2hrs ? (R35)
YES
NO
≤ 5 kΩ at 24hrs ? (R34)
NO
YES
Classify as Non-Corrosive
Subject to Dye Binding Protocol
Surfactant or neutral organic ?
NO
Classify according to TER result
YES
Dye binding at 24 hrs < 36% HCL binding?
NO
True Positive
YES
False Positive
Classify as Non-Corrosive

Figure 2

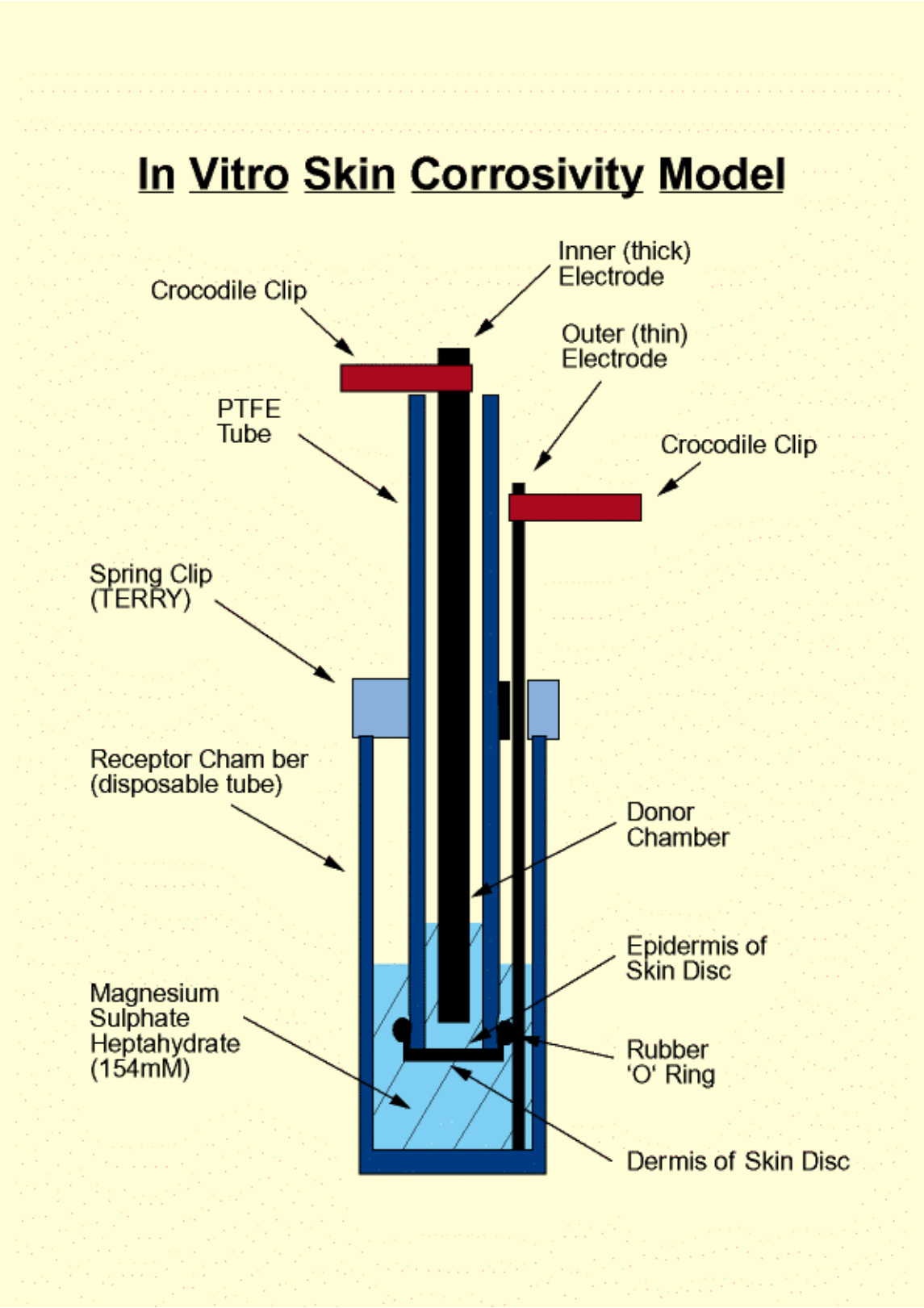
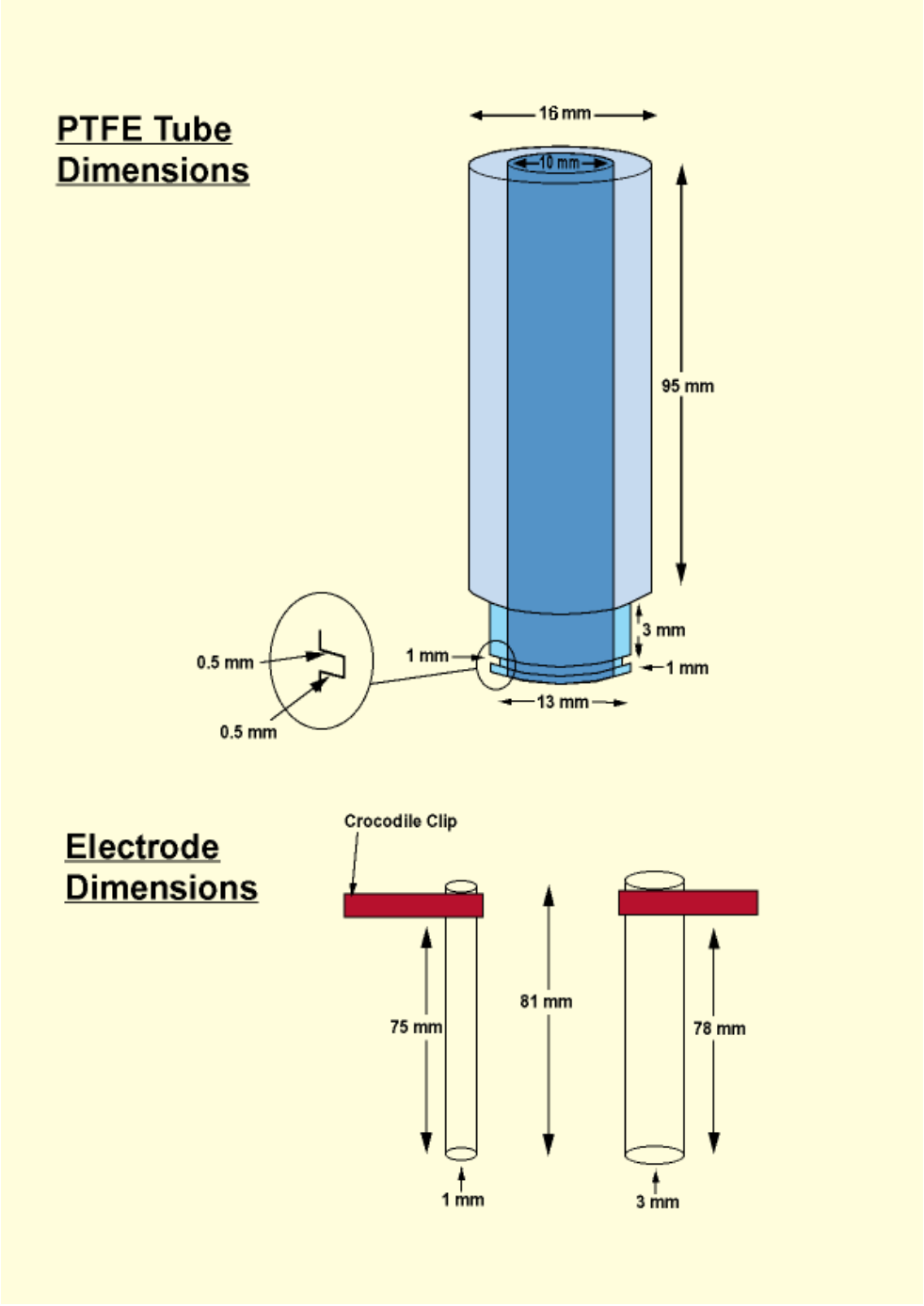

In Vitro Skin Corrosivity Model
Inner (thick) Electrode
Crocodile Clip
Outer (thin) Electrode
PTFE Tube
Crocodile Clip
Spring Clip (TERRY)
Receptor Cham ber (disposable tube)
Donor Chamber
Epidermis of Skin Disc
Magnesium Sulphate Heptahydrate (154mM)
Rubber 'O' Ring
Dermis of Skin Disc

Figure 3


PTFE Tube Dimensions
16 mm
10 mm
95 mm
3 mm
1 mm
13 mm
0.5 mm
1 mm
0.5 mm
Electrode Dimensions
Crocodile Clip
75 mm
81 mm
78 mm
1 mm
3 mm

APPENDIX 1: ECVAM Validation Study

Transcutaneous Electrical Resistance Test/Dye Binding - Results Summary Sheet

Laboratory Study Number: ____________ Laboratory Name: ____________ Experiment Number: ____________

Test Chemical and appearance	Contact Time (hours)	Tissue number	Electrical Resistance (kohms)	Mean TER ± S.D. (kohms)
	2			
	24			

Dye Contact Time (hours)	Optical Density at 565.5 nm (diluted 1 in 5)	Dye Concentration (µg/disc)	Mean Dye Concentration ± S.D. (µg/disc)	10% SDS Positive Control Mean Dye Concentration ± S.D. (µg/disc)	Classification	
2					R35	PG I
					R34	PG II/III
					NC	NC

Signature: ____________ Date: ____________

Checked by: ____________ Date: ____________

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Summary Report of the Rat Skin Transcutaneous Electrical Resistance *In Vitro* Assay for Assessing Dermal Corrosivity

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PURPOSE

This report focuses on the performance of the Rat Skin Transcutaneous Electrical Resistance (TER) assay to determine the usefulness and limitations of the assay for the identification of potential human corrosive chemicals. This report also discusses how Rat Skin TER compares to the *in vivo* rabbit skin corrosivity test and to other *in vitro* corrosivity tests (EPISKIN™, EpiDerm™, and Corrositex®). The data and assessments reviewed included an interlaboratory trial (Botham et al., 1992), a prevalidation study (Botham et al., 1995), and a validation study (Barratt et al., 1998; Fentem et al., 1998). Additionally, an independent analysis of the Rat Skin TER performance data, taking into account the totality of the database, was conducted.

EVALUATION OF REGULATORY AND SCIENTIFIC RATIONALE

The Rat Skin TER assay has been in use for over five years (Botham et al., 1995). This assay is one of several *in vitro* corrosivity assays evaluated as alternatives to the *in vivo* rabbit corrosivity test by the European Centre for the Validation of Alternative Methods (ECVAM) in a formal validation study (Fentem et al., 1998).

The assay has been approved by the ECVAM Scientific Advisory Committee for use in corrosivity testing in Europe (Balls and Corcelle, 1998) and has also been evaluated and accepted for its intended use by the European Scientific Committee for Cosmetic Products and Non-food Products (SCCNFP) (Anon., 1999). This method has been adopted for regulatory use within the European Union (EU) by the European Commission (Anon., 2000).

EVALUATION OF THE TEST METHOD

In the Rat Skin TER assay, test materials (liquids: 150 µL; solids 100 mg plus 150 µL of water) are applied for 2 and 24 hours to the epidermal surfaces of skin discs obtained from the skin of humanely killed young rats. Nine to 15 discs can be prepared from one rat pelt. Pelts must give a resistance value greater than 10 kΩ to be acceptable for use in the test. To test each chemical, three skin discs are used per time period, in addition to a concurrent positive and negative control. Corrosive materials are identified by the ability of the chemical to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction of the inherent transcutaneous electrical resistance below a predetermined threshold level of 5 kΩ. The validation protocol developed by ECVAM included a dye-binding assay, which is used to reduce the number of false positives encountered in the prevalidation study for surfactants and solvents. The scientific and mechanistic basis of the test and the rationale for using a 5 kΩ criterion for identifying potential human corrosivity were not discussed by Botham et al. (1995) or Fentem et al. (1998).

EVALUATION OF TEST METHOD DATA QUALITY

The Rat Skin TER assay was evaluated in three studies: an interlaboratory trial (Botham et al., 1992), a prevalidation study (Botham et al., 1995), and an ECVAM validation study (Fentem et al., 1998). The interlaboratory trial was based on an evaluation of 20 chemicals (6 corrosives/14 noncorrosives), while the prevalidation and ECVAM validation studies evaluated 50 chemicals (25C/25NC) and 60 chemicals (27C/33NC), respectively. The main criterion for including chemicals in the study was that their corrosivity classification was based on unequivocal animal data (Barratt et al., 1998). The ECVAM validation chemical test set included organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1 NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC), and soaps/surfactants (3NC). Despite the small numbers of chemicals in some categories, ECVAM concluded that the set of test chemicals used in the validation study represented the best possible group for evaluating the performance characteristics of the *in vitro* assays, given the limited availability of unequivocal animal data (Barratt et al., 1998).

In the validation study, each chemical was tested twice in each of three different laboratories. The tests were stated to have been conducted in the "spirit" of GLP (Fentem et al., 1998). A formal audit of the ECVAM data by a Quality Assurance Unit was not conducted; however, it was stated that all data submitted by the participating laboratories were verified against the original data sheets by ECVAM staff on at least three separate occasions.

EVALUATION OF TEST METHOD PERFORMANCE

For this summary report, an analysis was conducted, similar to the performance analysis conducted for the ICCVAM Peer Review of Corrositex®; the current analysis evaluated the performance characteristics of the Rat Skin TER assay against the corresponding *in vivo* rabbit corrosivity data. The database used in the Rat Skin TER evaluation consisted of data from three published sources (Botham et al., 1992; Botham et al., 1995; Fentem et al., 1998). For ease of comparison, chemicals evaluated in the Rat Skin TER assay were classified into the same chemical and product class designations used in the Corrositex® evaluation. A weight-of-evidence approach was used for classifying discordant results within or between laboratories; in instances where discordant results could not be resolved (i.e., there was an equal number of positive and negative calls), the chemical was eliminated from inclusion in the performance calculations.

The results of the overall performance analysis for the Rat Skin TER assay are presented in Table 1. Based on a database of 122 chemical and chemical mixtures, this assay had an accuracy of 81% (99/122 chemicals or chemical mixtures), a sensitivity of 94% (51/54 chemicals or chemical mixtures), and a specificity of 71% (48/68 chemicals or chemical mixtures). These performance characteristics were not different when the Botham et al. (1992 and 1995) studies were evaluated independently of the ECVAM validation study (Fentem et al., 1998) (Tables 2 and 3, respectively). The performance characteristics for the Rat Skin TER assay remained consistent when evaluated against various chemicals classes, including organic and inorganic acids and bases, organic and inorganic bases and base mixtures, organic and inorganic acids and

acid mixtures. Based on the validation study results only, ECVAM concluded that the Rat Skin TER assay was valid for use as a replacement for the *in vivo* rabbit skin test for distinguishing between corrosive and noncorrosive chemicals for all of the chemical types studied (Fentem et al., 1998; Balls and Corcelle, 1998). ECVAM concluded also that the Rat Skin TER assay was not capable of classifying chemicals or chemical mixtures by packing group (i.e., it could not distinguish between known R35/I and R34/II & III chemicals). However, it was stated that taking into account the relative simplicity of the mechanism of action of corrosives, this method would be generally applicable across all chemical classes (Fentem et al. 1998).

EVALUATION OF TEST METHOD RELIABILITY (REPEATABILITY/ REPRODUCIBILITY)

The Rat Skin TER assay has been evaluated for repeatability and/or reproducibility in three different studies. In the Botham et al. (1992) interlaboratory trial, no statistically significant level of interlaboratory variability was found for corrosives (6 compounds), noncorrosives (14 compounds), or for all test materials (20 compounds); variability among the three independent laboratories was assessed using ANOVA. An intralaboratory analysis was not possible. In the prevalidation study (Botham et al., 1995), the agreement for the classifications obtained by both participating laboratories was 92% (23 of 25 C and 23 of 25 NC chemicals).

In the ECVAM validation study (Fentem et al., 1998), the 60 chemicals were each tested twice by each of three laboratories. Intra- and inter- laboratory reliability was evaluated using a relative mean square diagram (determined using a two-way ANOVA with laboratory and experiments as factors), scatter diagrams to assess the possibility of divergence between results obtained in different laboratories, and range diagrams to summarize the overall performance of the tests. Based on their analyses, ECVAM concluded that inter- and intra-laboratory variability was approximately equivalent, with no evidence of systematic differences between experiments within a laboratory. Of the 60 chemicals tested, 37 gave the same corrosivity classification in both experiments in all three laboratories. For ten of the remaining 23 chemicals, only one experiment resulted in a classification differing from the other 5 predictions. Although there were differences for some chemicals in calls between experiments within and between laboratories, ECVAM concluded that the Rat Skin TER assay was reliable and reproducible. Due to the lack of quantitative data for individual chemicals in the published studies, no independent evaluation of repeatability or reproducibility for the Rat Skin TER assay could be conducted. However, after reviewing the intra- and inter-laboratory evaluations conducted by ECVAM, it was concluded that the analyses were appropriate and that the conclusions were accurate.

Table 1. Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Overall)[1]

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	**122**	**81**	**(99/122)**	**94**	**(51/54)**	**71**	**(48/68)**
Organic and Inorganic Acids and Bases[2]	**64**	**91**	**(58/64)**	**98**	**(44/45)**	**74**	**(14/19)**
Organic and Inorganic Bases and Base Mixtures[3]	**27**	**93**	**(25/27)**	**100**	**(20/20)**	**71**	**(5/7)**
Organic and Inorganic Acids and Acid Mixtures	**31**	**94**	**(29/31)**	**100**	**(20/20)**	**82**	**(9/11)**
Amines	**21**	**95**	**(20/21)**	**100**	**(15/15)**	**83**	**(5/6)**
Inorganic Bases and Base Mixtures	**6**	**83**	**(5/6)**	**100**	**(5/5)**	**0**	**(0/1)**
Acid Derivatives	**6**	**67**	**(4/6)**	**80**	**(4/5)**	**0**	**(0/1)**
Surfactants	**21**	**62**	**(13/21)**	**100**	**(4/4)**	**53**	**(9/17)**
Industrial Chemicals	**26**	**73**	**(19/26)**	**50**	**(1/2)**	**75**	**(18/24)**
Cleaners and Detergents	**7**	**86**	**(6/7)**	**100**	**(2/2)**	**80**	**(4/5)**

[1]This analysis contains data from Fentem et al. (1998), Botham et al. (1995), and Botham et al. (1992).

[2]This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives.

[3]This chemical class includes amines, inorganic bases, and base mixtures.

Table 2. Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Fentem et al., 1998)

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	**58**	**81**	**(47/58)**	**93**	**(25/27)**	**71**	**(22/31)**
Organic and Inorganic Acids and Bases[1]	**39**	**85**	**(33/39)**	**96**	**(24/25)**	**64**	**(9/14)**
Organic and Inorganic Bases and Base Mixtures[2]	**13**	**85**	**(11/13)**	**100**	**(9/9)**	**50**	**(2/4)**
Organic and Inorganic Acids and Acid Mixtures	**20**	**90**	**(18/20)**	**100**	**(11/11)**	**78**	**(7/9)**
Amines	**9**	**89**	**(8/9)**	**100**	**(6/6)**	**67**	**(2/3)**
Inorganic Bases and Base Mixtures	**4**	**75**	**(3/4)**	**100**	**(3/3)**	**0**	**(0/1)**
Acid Derivatives	**6**	**67**	**(4/6)**	**80**	**(4/5)**	**0**	**(0/1)**
Surfactants	**5**	**60**	**(3/5)**	**NA**	**(0/0)**	**60**	**(3/5)**
Industrial Chemicals	**10**	**80**	**(8/10)**	**100**	**(1/1)**	**78**	**(7/9)**
Cleaners and Detergents	**1**	**100**	**(1/1)**	**NA**	**(0/0)**	**100**	**(1/1)**

NA = Not applicable

[1]This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives.

[2]This chemical class includes amines, inorganic bases, and base mixtures.

Table 3. Performance of the Rat Skin TER Assay in Predicting Corrosivity/Noncorrosivity Compared to *In Vivo* Findings (Botham et al., 1992; 1995)

Chemical or Product Class	Number of Chemicals	Accuracy		Sensitivity		Specificity	
		%	Number	%	Number	%	Number
Overall	**65**	**82**	**(53/65)**	**96**	**(27/28)**	**70**	**(26/37)**
Organic and Inorganic Acids and Bases[1]	**26**	**100**	**(26/26)**	**100**	**(21/21)**	**100**	**(5/5)**
Organic and Inorganic Bases and Base Mixtures[2]	**14**	**100**	**(14/14)**	**100**	**(11/11)**	**100**	**(3/3)**
Organic and Inorganic Acids and Acid Mixtures	**12**	**100**	**(12/12)**	**100**	**(10/10)**	**100**	**(2/2)**
Amines	**12**	**100**	**(12/12)**	**100**	**(9/9)**	**100**	**(3/3)**
Inorganic Bases and Base Mixtures	**2**	**100**	**(2/2)**	**100**	**(2/2)**	**NA**	**(0/0)**
Acid Derivatives	**0**	**NA**	**(0/0)**	**NA**	**(0/0)**	**NA**	**(0/0)**
Surfactants	**16**	**63**	**(10/16)**	**100**	**(4/4)**	**50**	**(6/12)**
Industrial Chemicals	**16**	**69**	**(11/16)**	**0**	**(0/1)**	**73**	**(11/15)**
Cleaners and Detergents	**6**	**83**	**(5/6)**	**100**	**(2/2)**	**75**	**(3/4)**

NA = Not applicable

[1]This chemical class includes chemicals from the following chemical classes: organic and inorganic bases and base mixtures, organic and inorganic acids and acid mixture, and acid derivatives.

[2]This chemical class includes amines, inorganic bases, and base mixtures.

OTHER SCIENTIFIC REVIEWS

In March 1999, a search of the open literature was conducted to locate additional Rat Skin TER studies. Six databases (Medline, Toxline, Embase, Biosis, Caba, and LifeSci) were searched using the key terms "Transcutaneous" within one word of "electrical" within one word of "resistance"; and "TER" and "rat" or "rats". The search found no additional relevant studies conducted with this assay. In May 2001, another search was performed to locate additional TER studies. Four databases (PubMed, Web of Science, Toxline, and Current Contents Connect) were searched using the same search strategy and no additional relevant studies were found.

OTHER CONSIDERATIONS

The cost for conducting the Rat Skin TER assay is reported by Syngenta Corporation (e-mail communication from Phil Botham, Syngenta CTL) to be approximately $500-800 per test. When compared to other *in vitro* methods (EPISKIN™, EpiDerm™, and Corrositex®), the cost and the time necessary to conduct the Rat Skin TER assay are greater (Table 4). Additionally, TER requires the use of animals, whereas EPISKIN™ and Corrositexâ do not.

RELATED ISSUES

Refinement, Reduction, and Replacement

The Rat Skin TER assay does not eliminate the use of animals. However, if used in an integrated approach, TER provides for the reduction and refinement of animal use.

Comparison to Other *In Vitro* Assays

General comparative information on the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositexâ assays is provided in Tables 4 through 7.

Table 4. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays

	Rat Skin TER Assay	EPISKIN™ (prediction model B)	EpiDerm™ (prediction model 2)	Corrositex®
Test Method Description	Acceptable	Acceptable	Acceptable	Acceptable
Adequacy/Completeness of Protocol	Acceptable	Acceptable	Acceptable	Acceptable
Usefulness for Assessing Corrosivity/Non-corrosivity	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Usefulness for Determining Packing Groups	Not Acceptable (Fentem et al., 1998)	Can group as UN packing group II/III or I (Fentem et al., 1998)[a]	Not Acceptable (Liebsch et al., 2000)	Acceptable (ICCVAM, 1999)
Repeatability and Reproducibility	Acceptable (Botham et al., 1992; 1995; Fentem et al., 1998)	Acceptable (Fentem et al., 1998)	Acceptable (Liebsch et al., 2000)	Acceptable (Fentem et al., 1998; ICCVAM, 1999)
Animal Use Refinement, Reduction, and Replacement Considerations	Refines and reduces animal use when used as a stand-alone test or in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.	Refines and reduces animal use when used in an integrated testing strategy.	Replaces animal use when used as a stand-alone test. Refines and reduces animal use when used in an integrated testing strategy.
Cost	~$500-850/test	~$450/test kit[b]	~$200/test chemical	~$300/test chemical
Study duration	2 work-days	1 work-day	1 work-day	≤ 4 hr/chemical

a Since the performance of EPISKINä was not assessed for distinguishing between UN packing groups II and III, all R34 classifications would be conservatively classified as UN packing group II.

b One to three chemicals may be tested per test kit; however, it is recommended by the supplier that each test chemical be assayed using 3 different skin batches/kits which equates to a total cost of ~$430/ test chemical.

Table 5. General Comparison of the Rat Skin TER Assay, EPISKIN™, EpiDerm™, and Corrositex® Assays Based on an ICCVAM Weight-of-Evidence Approach by Chemical using Data from the ECVAM and Other Validation Studies (Fentem et al., 1998; ICCVAM, 1999; Liebsch et al., 2000)

	Rat Skin TER	EPISKIN™	EpiDerm™ (prediction model 2)	Corrositex®
Number of Chemicals	122	60	24	163
Overall Sensitivity[a]	94% (51/54)	82% (23/28)	92% (11/12)	85% (76/89)
Overall Specificity[a]	71% (48/68)	84% (27/32)	83% (10/12)	72% (52/74)
Overall Accuracy[a]	81% (99/122)	83% (50/60)	92% (22/24)	79% (128/163)
Test Chemical Interlaboratory Coefficient of Variation	Median = 34.7 Range = 3.8-322 n^b = 120	Median = 11.3 Range = 3.9-148.8 n^b = 20	Median = 12.3 Range = 0.9-51.2 n^b = 144	Median = 30.3 Range = 7.7-252.5 n^b = 180

a Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method.

b The total number of independent values, which is calculated as the number of chemicals tested multiplied by the number of sample times for each chemical.

Table 6. General Comparison of the Rat Skin TER, EPISKIN™, EpiDerm™, and Corrositex® Assays from Independent Test Results in the ECVAM Validation Studies (Fentem et al., 1998; Liebsch et al., 2000)

	Rat Skin TER Assay	EPISKIN™ (prediction model B)	EpiDerm™ (prediction model 2)
Number of Chemicals Tested in ECVAM Validation Study	60 (Fentem et al., 1998)	60/24[a] (Fentem et al., 1998)	24 (Liebsch et al., 2000)
Sensitivity[b]	88% (140/159)	83% (201/243) / 88% (87/99)	88% (63/72)
Specificity[b]	72% (142/196)	80% (237/297) / 79% (92/117)	86% (62/72)
Accuracy[b]	79% (282/355)[c]	81% (438/540) / 83% (179/216)	87% (125/144)
False Positive Rate[b]	28% (54/196)	20% (60/297) / 21% (25/117)	14% (10/72)
False Negative Rate[b]	12% (19/159)	17% (42/243) / 12% (12/99)	13% (9/72)
Number of Trials[d]	355	540 / 216	144
Test Chemical Inter-laboratory Coefficient of Variation	Median = 34.7 Range = 10-322 n^d = 360	Median = 30.2 Range = 7.7-252.5 n^d =540	Median = 12.3 Range = 0.9-51.2 n^d = 144

a The first numbers for accuracy, sensitivity, specificity, false positive rate, and false negative rate correspond to the 60 chemicals tested in the ECVAM Skin Corrosivity Test using EPISKIN™ (Barratt et al., 1998; Fentem et al., 1998); the latter values correspond to a direct comparison of EpiDerm™ and EPISKIN™ for the same 24 materials tested in both systems (Liebsch et al., 2000).

b Sensitivity is defined as the proportion of all positive chemicals that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals that are correctly classified as negative in a test. Accuracy (concordance) is defined as the proportion of correct outcomes of a method. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative.

c The percentages are based on the number of correct trials among the total number of trials (i.e., independent tests) provided in parenthesis.

d The total number of trials conducted in the validation study minus the non-qualified (NQ) results. This number is equal to the number of chemicals multiplied by the number of participating laboratories multiplied by the number of replicate tests.

Table 7. Classification Results from the ECVAM Validation Studies of Rat Skin TER, EPISKIN™, and EpiDerm™ Assays as Compared to the *In Vivo* Classification (Fentem et al., 1998; Liebsch et al., 2000)

No.	Chemical	Type	*In Vivo*	Rat Skin TER	EPISKIN-B [a]	EpiDerm
1	Hexanoic acid	ORGAC	R34/II&III	R35	R35	N/A
29	65/35 Octanoic/decanoic acid	ORGAC	R34/II&III	R34	R35	N/A
36	2-Methylbutyric acid	ORGAC	R34/II&III	R35	R34	N/A
40	Octanoic acid (caprylic acid)	ORGAC	R34/II&III	R35	R34/C	C
47	60/40 Octanoic/decanoic acids	ORGAC	R34/II&III	R34	R34/C	C
50	55/45 Octanoic/decanoic acids	ORGAC	R34/II&III	R35	R34	N/A
7	3,3'-Dithiodipropionic acid	ORGAC	NC	NC	NC	N/A
12	Dodecanoic acid (lauric acid)	ORGAC	NC	NC	NC	NC
26	Isotearic acid	ORGAC	NC	NC	NC	NC
34	70/30 Oleine/octanoic acid	ORGAC	NC	NC	NC	N/A
58	10-Undecenoic acid	ORGAC	NC	NC	R34	N/A
2	1,2-Diaminopropane	ORGBA	R35/I	R35	R34/C	C
15	Dimethyldipropylenetriamine	ORGBA	R35/I	R35	R34/C	C
38	Tallow amine	ORGBA	R35/II	2R34/2NC/2NQ	NC	N/A
55	1-(2-Aminoethyl)piperazine	ORGBA	R34/II	R35	NC	N/A
13	3-Methoxypropylamine	ORGBA	R34/II&III	R35	R34	N/A
17	Dimethylisopropylamine	ORGBA	R34/II&III	R35	R34/C	C
45	n-Heptylamine	ORGBA	R34/II&III	R35	NC	C
10	2,4-Xylidine (2,4-Dimethylaniline)	ORGBA	NC	R34	R34	N/A
35	Hydrogenated tallow amine	ORGBA	NC	NC	NC	NC
59	4-Amino-1,2,4-triazole	ORGBA	NC	NC	NC	NC
8	Isopropanol	NORG	NC	NC	NC	N/A
11	2-Phenylethanol	NORG	NC	NC	NC	N/A
16	Methyl trimethylacetate (referred to as Methyl 2,2-dimethylpropanoate in EpiDerm™)	NORG	NC	NC	NC	C
19	Tetrachloroethylene	NORG	NC	NC	NC	NC
22	n-Butyl propionate	NORG	NC	NC	NC	N/A
27	Methyl palmitate	NORG	NC	NC	NC	N/A
44	Benzyl acetone	NORG	NC	NC	NC	NC
51	Methyl laurate	NORG	NC	NC	NC	N/A
56	1,9-Decadiene	NORG	NC	NC	NC	NC
3	Carvacrol	PHEN	R34/II&III	R34	R34	N/A
23	2-tert-Butylphenol	PHEN	R34/II&III	R35	R34/C	C
9	*o*-Methoxyphenol (Guaiacol)	PHEN	NC	NC	R34	N/A
30	4,4-Methylene-bis-(2,6-di-tert-butylphenol)	PHEN	NC	NC	NC	N/A
49	Eugenol	PHEN	NC	NC	NC	NC
4	Boron trifluoride dihydrate	INORGAC	R35/I	R35	R35/C	C
28	Phosphorus tribromide	INORGAC	R35/I	R35	R35/C	C

32	Phosphorus pentachloride	INORGAC	R35/I	R35	R34	N/A
25	Sulfuric acid (10% wt.)	INORGAC	R34/II&III	R34	R34	N/A
57	Phosphoric acid	INORGAC	R34/II	R35	R34	N/A
43	Hydrochloric acid (14.4% wt)	INORGAC	R34/II&III	R35	R34	N/A
53	Sulfamic acid	INORGAC	NC	R34	R34/C	C
18	Potassium hydroxide (10% aq.)	INORGBA	R34/II&III	R35	R34/C	C
42	2-Mercaptoethanol, Na salt (45% aq.)	INORGBA	R34/II&III	R35	NC	N/A
21	Potassium hydroxide (5% aq.)	INORGBA	NC	R35	R34	N/A
24	Sodium carbonate (50% aq.)	INORGBA	NC	R34	NC	NC
20	Ferric [iron (III)] chloride	INORGSAL	R34/II	R35	R34	N/A
52	Sodium bicarbonate	INORGSAL	NC	R34	NC	N/A
54	Sodium bisulfite	INORGSAL	NC	3R34/3NC	NC	N/A
5	Methacrolein	ELECTRO	R34/II&III	NC	R34/C	NC
14	Allyl bromide	ELECTRO	R34/II&III	R35	R34	N/A
48	Glycol bromoacetate (85%)	ELECTRO	R34/II&III	NC	R34/C	C
6	Phenethyl bromide	ELECTRO	NC	NC	NC	N/A
31	2-Bromobutane	ELECTRO	NC	3R34/3R35	NC	N/A
33	4-(Methylthio)-benzaldehyde	ELECTRO	NC	NC	NC	N/A
39	2-Ethoxyethyl methacrylate	ELECTRO	NC	NC	NC	N/A
46	Cinnamaldehyde	ELECTRO	NC	NC	NC	N/A
37	Sodium undecylenate (33% aq.)	SOAP	NC	R35	R34	N/A
41	20/80 Coconut/palm soap	SOAP	NC	NC	NC	N/A
60	Sodium lauryl sulfate (20% aq.)	SOAP	NC	R35	NC	NC

Overall corrosivity classifications were determined by the majority of the reported results obtained from each assay. If results do not show a majority, a definitive classification could not be determined.

Definitions are as follows: C = Corrosive; NC = Non-corrosive; R34 is equivalent to packing groups II and/or III; R35 is equivalent of packing group I, except for tallow amine (R35/II); NQ = Non-qualified; N/A = Not applicable because not tested; ORGAC = Organic acid; ORGBA = Organic base; NORG = Neutral organics; PHEN = phenol; INORGAC = Inorganic acid; INORGBA = Inorganic base; INORGSAL = Inorganic salt; ELECTRO = Electrophile; SOAP = Soap surfactant

a For EPISKIN™, prediction model B was the more complex prediction model and was the only model considered in detail by the ECVAM Management Team (Fentem et al., 1998).

SUMMARY CONCLUSIONS AND RECOMMENDATIONS

ECVAM concluded that the Rat Skin TER assay was an *in vitro* replacement assay for *in vivo* corrosivity testing (Fentem et al., 1998). The authors of this report concur with the ECVAM conclusion that the Rat Skin TER assay is both reliable and reproducible. For some chemical or product classes (e.g., cleaners and detergents), the small number of chemicals and/or the unbalanced distribution of corrosive and noncorrosive chemicals does not allow accurate conclusions to be made on the performance of this assay for these chemical classes.

The two major questions to be addressed for *in vitro* corrosivity assays are:

1. Has the assay been evaluated sufficiently and is its performance satisfactory to support the proposed use for assessing the corrosivity potential of chemicals and chemical mixtures?

2. Does the assay adequately consider and incorporate, where scientifically feasible, the 3Rs of animal use (refinement, reduction, and replacement alternatives)? Does the assay offer advantages with respect to animal welfare considerations?

In response to the first question, the performance characteristics of the Rat Skin TER assay indicates, in specific testing circumstances, that this test may be considered useful as part of an integrated testing strategy for assessing the dermal corrosion potential of chemicals.

In response to the second question, the Rat Skin TER assay sufficiently considers and incorporates the 3Rs. The assay offers animal welfare advantages, including animal use refinement and reduction; this method reduces the number of animals used as skin from one humanely killed rat may be used to test up to five chemicals. Similarly, the use of the Rat Skin TER assay as part of an integrated approach reduces and refines the use of animals by providing a basis for decisions on further testing. Follow-up testing using *in vivo* methods, when deemed necessary, could employ fewer animals and test agent dilution schemes to minimize possible pain in any individual animal.

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STATEMENT ON THE SCIENTIFIC VALIDITY OF THE RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST (AN IN VITRO TEST FOR SKIN CORROSIVITY)

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE EPISKIN™ TEST (AN *in vitro* TEST FOR SKIN CORROSIVITY)

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE RAT SKIN TRANSCUTANEOUS ELECTRICAL RESISTANCE (TER) TEST (AN IN VITRO TEST FOR SKIN CORROSIVITY)

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> The results obtained with the rat skin transcutaneous electrical resistance (TER) test in the ECVAM International validation study on in vitro tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The rat skin TER test proved applicable to testing a diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants. The concordances between the skin corrosivity classifications derived from the in vitro data and from the in vivo data were very good. The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied. The Committee therefore agrees with the conclusion from this formal validation study that the rat skin TER test is scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and non-corrosive chemicals, and that this test is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team, which is to be published in Toxicology in Vitro.[3]

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT2/ERGATT[2] workshop held in 1990, [4] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995, [5] criteria laid down by ECVAM and the ECB, [2, 6] criteria recommended at an OECD[2] workshop held in 1996, [7] and the US ICCVAM2 report on validation and regulatory acceptance. [8] The outcome of a prevalidation study on in vitro tests for skin corrosivity was published in 1995, as ECVAM workshop report 6. [9] A separate report on the selection of the test chemicals for the validation study is to be published alongside the Management Team's report in Toxicology In Vitro. [10]

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3 April 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU

Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

Dr B Blaauboer (ERGATT)
Professor J Castell (Spain)
Dr B Garthoff (EFPIA)
Dr C Hendriksen (The Netherlands)
Professor G Papadopoulos (Greece)
Dr B Rusche (Eurogroup for Animal Welfare)
Professor H Spielmann (Germany)
Professor H. Tritthart (Austria)
Professor E Walum (Sweden)
Dr P Botham (ECETOC)
Dr D Clark (UK)
Professor A Guillouzo (France)
Dr R Lorenzini (Italy)
Professor V Rogiers (Belgium)
Dr O de Silva (COLIPA)
Dr O Svendsen (Denmark)
Dr M Viluksela (Finland)
Dr F Zucco (Eurogroup for Animal Welfare)

Professor M Balls (ECVAM)
Dr J Fentem (ECVAM)
Ms S Louhimies (DGXI)
Mr A Van Elst (DGXXIV)
Mr G Corcelle (DGXI)
Dr G Fracchia (DGXII)
Dr M Robert (DGII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau. Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations.
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General Information about the ECVAM skin corrosivity validation study:

A. The study was coordinated from ECVAM, and the Management Team (MT) was chaired by Dr Julia Fentem (ECVAM). The other four MT members acted as representatives of the "lead laboratories" and each took responsibility for one of the four tests included in the validation study: Dr Rodger Curren

(Microbiological Associates Inc., USA; CORROSITEX™). Dr Lesley Earl (Unilever, UK; rat skin TER assay), Mr David Esdaile (Rhône-Poulenc Agro, France; EPISKIN™), and Dr Manfred Liebsch (ZEBET, Germany: Skin2™ assay). The study was principally funded by ECVAM, under the terms of 14 separate contracts with the participating organisations. Professor Michael Bails (ECVAM) and Dr Philip Botham (ESAC; ZENECA CTL, UK) represented the sponsors in any contacts with the MT. In addition to ECVAM, the participating organisations were: Agence du Medicament (France), BASF Aktiengesellschaft (Germany), BIBRA International (UK), COVANCE (UK), Humboldt University (Germany), Huntingdon Life Sciences (UK), INRS (France), Microbiological Associates Inc. (USA), Microbiological Associates Ltd (UK), Rhône-Poulenc Agro (France), Sanofi Recherche (France), Unilever Research (UK), ZEBET, BgVV (Germany) and ZENECA CTL (UK).

B. This study began in 1996, as a follow-up to a prevalidation study on in vitro tests for replacing the in vivo Draize rabbit test for skin corrosivity. The main objectives were to: (a) Identify tests capable of discriminating corrosives (C) from non-corrosives (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only); and (b) determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. The tests selected for inclusion In the validation study were: (a) the rat skin TER assay; (b) CORROSITEX™; (c) the Skin2™ ZK1350 corrosivity test; and (d) EPISKIN™. Each test was conducted in three independent laboratories, according to the principles, criteria and procedures for undertaking validation studies outlined previously by ECVAM in conjunction with International experts in this area. Prediction models for the four tests were clearly defined in the test protocols.

C. A test set of 60 chemicals was selected by an independent Chemicals Selection Sub-Committee, including organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC) and soaps/surfactants (3NC). The first set of ten coded chemicals was distributed independently of the MT and participating laboratories in June 1996. Further to the satisfactory completion of the first phase of the study, the remaining 50 coded chemicals were distributed in September 1996. The results obtained were submitted to ECVAM's statistician, Dr Graeme Archer, for independent analysis in consultation with Dr Hermann-Georg Holzhütter (Humboldt University, Berlin, Germany). Data analysis and preparation of the final reports took place between May and October 1997.

D. The rat skin TER assay has been used successfully as a routine in-house test for several years. When used in screening mode, the TER method is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency), and it has been used primarily to guide humane in vivo skin testing. The TER assay has been evaluated in several intralaboratory and interlaboratory studies, and it performed creditably in the prevalidation study conducted during 1993 and 1994. The test protocol evaluated in this validation study had been refined on the basis of recommendations from the prevalidation study, to include a dye binding procedure for reducing the number of false positive predictions obtained previously with test materials containing surfactants and solvents. in outline, test materials are applied for up to 24 hr to the epidermal surfaces of skin discs taken from the pelts of humanely killed young rats. Corrosive materials are identified by their ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction in the inherent TER below a predetermined threshold level (5kΩ).

Rat Skin TER Assay Prediction Model:

TER (kΩ)	Treatment time (hours)	Mean disc dye content	C/NC	EU risk phrase	UN packing group
>5	2 & 24	Nm[a]	NC	no label	-

< or = 5	2	-	C	R35	I
	24	-	C	R34	II/III
Surfactants/neutral organics:					
< or = 5	24	> or = +ve control	C	R34	II/III
	24	< +ve control	NC	no label	-

aNM = not measured

E. The prediction model for the rat skin TER test was used to classify the corrosivity potentials of the 60 test chemicals on the basis of the in vitro data obtained in the three laboratories conducting the test Comparing these in vitro classifications with the in vivo classifications independently assigned to the chemicals before the blind trial began gave the following key statistical parameters:

Sensitivity:	C	88%
	R34/II & III	18%
	R35/I	88%
Specificity:		72%
Predictivity:	C	72%
	R34/II & III	40%
	R35/I	22%
Accuracy:	C/NC	79%
	R35/R34/NC	55%

The underprediction and overprediction rates for the TER test relative to the study objectives were :

Objective (a): C v NC	underprediction rate	12%
	Overprediction rate	28%
Objective (b): R35/I v R34/II & III v NC	underprediction rate	
	R35/I-->NC	6%
	R34/II & III --> NC	14%
	overprediction rate	
	NC --> R35/I	12%
	NC --> R34/II & III	16%
	R34/II & III --> R35/I	69%

* unacceptable according to the criteria defined by the MT before undertaking the data analysis

F. In order for the rat skin TER test to be considered for use for legislative and other purposes, measures will be taken to press for the updating of OECD Testing Guideline 404 and Annex V method B.4 of Directive 67/548/EEC.

G. A statement on the scientific validity of the EPISKIN™ assay for skin corrosivity testing was also endorsed by the ESAC on 31 March 1998. The two other methods included in the validation study, CORROSITEX and Skin[2], did not meet all of the criteria for them to be considered acceptable as replacement tests. The corrosivity potentials of about 40% of the test chemicals could not be assessed with CORROSITEX, although it may be valid for testing specific classes of chemicals (such as organic bases and inorganic acids). The Skin[2] assay, as conducted in this validation study, had an unacceptably high underprediction rate (57%), although it had a specificity of 100% it is recognised that both of these methods could be useful if they were incorporated into a tiered testing strategy for skin corrosivity.

STATEMENT ON THE SCIENTIFIC VALIDITY OF THE EPISKIN™ TEST (AN *in vitro* TEST FOR SKIN CORROSIVITY)

At its 10th meeting, held on 31 March 1998 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1], unanimously endorsed the following statement:

> The results obtained with the EPISKIN™ test (involving the use of a reconstructed human skin model) in the ECVAM international validation study on *in vitro* tests for skin corrosivity were reproducible, both within and between the three laboratories that performed the test. The EPISKIN test proved applicable to testing a diverse group of chemicals of different physical forms, including organic acids, organic bases, neutral organics, inorganic acids, inorganic bases, inorganic salts, electrophiles, phenols and soaps/surfactants. The concordances between the skin corrosivity classifications derived from the *in vitro* data and from the *in vivo* data were very good. The test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied; it was also able to distinguish between known R35 (UN2 packing group I) and R34 (UN packing groups II & III) chemicals. The Committee therefore agrees with the conclusion from this formal validation study that the EPISKIN test is scientifically validated for use as a replacement for the animal test, and that it is ready to be considered for regulatory acceptance.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular, the report on the results and evaluation of the validation study by the Management Team, which is to be published in *Toxicology in vitro*.[3]

This validation study was conducted in accordance with the general principles laid down in the report of the CAAT[2]/ERGATT[2] workshop held in 1990, [4] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[5] criteria laid down by ECVAM and the ECB, [2, 6] criteria recommended at an OECD2 workshop held in 1996, [7] and the US ICCVAM2 report on validation and regulatory acceptance.[8] The outcome of a prevalidation study on *in vitro* tests for skin corrosivity was published in 1995, as ECVAM workshop report 6. [9] A separate report on the selection of the test chemicals for the validation study is to be published alongside the Management Team's report in *Toxicology in vitro.* [10]

Michael Balls
Head of Unit
ECVAM
Ispra
Italy

Guy Corcelle
Head of Unit
DGXI/E/2
Brussels
Belgium

3 April 1998

1. The ESAC was established by the European Commission, and is composed of representatives of the EU Member States, industry, academia and animal welfare, together with representatives of the relevant Commission services. The following members of the ESAC were present at the meeting on 31 March 1998:

Dr B Blaauboer (ERGATT)
Professor J Castell (Spain)
Dr B Garthoff (EFPIA)
Dr C Hendriksen (The Netherlands)
Professor G Papadopoulos (Greece)
Dr B Rusche (Eurogroup for Animal Welfare)
Professor H Spielmann (Germany)
Professor H. Tritthart (Austria)
Professor E Walum (Sweden)

Dr P Botham (ECETOC)
Dr D Clark (UK)
Professor A Guillouzo (France)
Dr R Lorenzini (Italy)
Professor V Rogiers (Belgium)
Dr O de Silva (COLIPA)
Dr O Svendsen (Denmark)
Dr M Viluksela (Finland)
Dr F Zucco (Eurogroup for Animal Welfare)

Professor M Balls (ECVAM)
Dr J Fentem (ECVAM)
Ms S Louhimies (DGXI)
Mr A Van Elst (DGXXIV)

Mr G Corcelle (DGXI)
Dr G Fracchia (DGXII)
Dr M Robert (DGII)

2. CAAT: Center for Alternatives to Animal Testing, Baltimore, USA; ECB: European Chemicals Bureau. Ispra, Italy; ERGATT: European Research Group for Alternatives in Toxicity Testing, Utrecht, The Netherlands; ICCVAM: *ad hoc* Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, USA; OECD: Organization for Economic Cooperation and Development, Paris, France; UN: United Nations.

3. Fentem JH, Archer GEB, Balls M, Botham PA, Curren RD, Earl LK, Esdaile DJ, Holzhütter H-G & Liebsch M (1998) The ECVAM International validation study on *in vitro* tests for skin corrosivity. 2. Results and evaluation by the Management Team. *Toxicology in vitro*, in press.

4. Balls M, Blaauboer BJ, Brusick D, Frazier J, Lamb D, Pemberton M, Reinhardt C, Roberfroid M, Rosenkranz H, Schmid B, Spielmann H, Stammati AL & Walum E (1990) Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. *ATLA* 18: 303-337.

5. Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A, Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H & Zucco F (1995) Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. *ATLA* 23: 129-147.

6. Balls M & Karcher W (1995) The validation of alternative test methods. *ATLA* 23: 884-886.

7. Anon. (1996) *Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods.* 60pp. Paris: OECD.

8. Anon. (1997) *Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods.* 105pp. Research Triangle Park, NC: NIEHS.

9. Botham, PA, Chamberlain M, Barratt MD, Curren RD, Esdalle DJ, Gardiner JR, Gordon VC, Hildebrand B, Lewis RW, Liebsch M, Logemann P, Osborne R, Ponec M, Régnier J-F, Steiling W, Walker AP & Balls M (1995) A prevalidation study on *in vitro* skin corrosivity testing. The report and the recommendations of ECVAM workshop 6. *ATLA* 23:219-255.

10. Barratt MD, Brantom PG, Fentem JH, Gerner I, Walker AP & Worth AP (1998) The ECVAM international validation study on *in vitro* tests for skin corrosivity. 1. Selection and distribution of the

test chemicals. *Toxicology in vitro*, in press.

General information about the ECVAM skin corrosivity validation study:

A. The study was coordinated from ECVAM, and the Management Team (MT) was chaired by Dr Julia Fentem (ECVAM). The other four MT members acted as representatives of the "lead laboratories" and each took responsibility for one of the four tests included in the validation study: Dr Rodger Curren (Microbiological Associates Inc., USA; CORROSITEX™). Dr Lesley Earl (Unilever, UK; rat skin TER assay), Mr David Esdaile (Rhône-Poulenc Agro, France; EPISKIN™), and Dr Manfred Liebsch (ZEBET, Germany: Skin2™ assay). The study was principally funded by ECVAM, under the terms of 14 separate contracts with the participating organisations. Professor Michael Bails (ECVAM) and Dr Philip Botham (ESAC; ZENECA CTL, UK) represented the sponsors in any contacts with the MT. In addition to ECVAM, the participating organisations were: Agence du Medicament (France), BASF Aktiengesellschaft (Germany), BIBRA International (UK), COVANCE (UK), Humboldt University (Germany), Huntingdon Life Sciences (UK), INRS (France), Microbiological Associates Inc. (USA), Microbiological Associates Ltd (UK), Rhône-Poulenc Agro (France), Sanofi Recherche (France), Unilever Research (UK), ZEBET, BgVV (Germany) and ZENECA CTL (UK).

B. This study began in 1996, as a follow-up to a prevalidation study on *in vitro* tests for replacing the *in vivo* Draize rabbit test for skin corrosivity. The main objectives were to: (a) Identify tests capable of discriminating corrosives (C) from non-corrosives (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only); and (b) determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals. The tests selected for inclusion In the validation study were: (a) the rat skin TER assay; (b) CORROSITEX™; (c) the Skin2™ ZK1350 corrosivity test; and (d) EPISKIN™. Each test was conducted in three independent laboratories, according to the principles, criteria and procedures for undertaking validation studies outlined previously by ECVAM in conjunction with International experts in this area. Prediction models for the four tests were clearly defined in the test protocols.

C. A test set of 60 chemicals was selected by an independent Chemicals Selection Sub-Committee, including organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC) and soaps/surfactants (3NC). The first set of ten coded chemicals was distributed independently of the MT and participating laboratories in June 1996. Further to the satisfactory completion of the first phase of the study, the remaining 50 coded chemicals were distributed in September 1996. The results obtained were submitted to ECVAM's statistician, Dr Graeme Archer, for independent analysis in consultation with Dr Hermann-Georg Holzhütter (Humboldt University, Berlin, Germany). Data analysis and preparation of the final reports took place between May and October 1997.

D. EPISKIN™ is a three-dimensional human skin model comprising a reconstructed epidermis with a functional stratum corneum. Its use for skin corrosivity testing involves topical application of test materials to the surface of the skin for 3, 60 and 240 min, and the subsequent assessment of their effects on cell viability by using the MTT assay. An in-house evaluation and prevalidation of the test was conducted during 1994-96. On the basis of these studies, the test protocol was refined prior to its inclusion in this validation study.

EPISKIN Prediction Model:

Treatment time (min)	Viability (%)	C/NC	EU risk phrase	UN packing group
3	<35	C	R35	I
3 / 60	> or = 35/>35	C	R34	II

60 / 240	> or = 35 / <35 C	R34	III	
240	>35	NC	no label	-

E. The prediction model for the EPISKIN test was used to classify the corrosivity potentials of the 60 test chemicals on the basis of the *in vitro* data obtained in the three laboratories conducting the test. Comparing these *in vitro* classifications with the *in vivo* classifications independently assigned to the chemicals before the blind trial began gave the following key statistical parameters:

Sensitivity:	C	83%
	R34/II & III	75%
	R35/I	39%
Specificity:		80%
Predictivity:	C	77%
	R34/II & III	64%
	R35/I	53%
Accuracy:	C/NC	81%
	R35/R34/NC	74%

The underprediction and overprediction rates for the EPISKIN test relative to the study objectives were:

Objective (a): C v NC	underprediction rate	17%
	overprediction rate	20%
Objective (b): R35/I v R34/II&III v NC	underprediction rate	
	R35/I --> NC	17%
	R34/II & III --> NC	18%
	overprediction rate	
	NC --> R35/I	1%
	NC --> R34/II & III	19%
	R34/II & III -->R35/I	8%

F. In order for the EPISKIN test to be considered for use for legislative and other purposes, measures will be taken to press for the updating of OECD Testing Guideline 404 and Annex V method B.4 of *Directive 67/548/EEC*.

G. A statement on the scientific validity of the rat skin transcutaneous electrical resistance (TER) assay for skin corrosivity testing was also endorsed by the ESAC on 31 March 1998. The two other methods included in the validation study, CORROSITEX and Skin^2 did not meet all of the criteria for them to be considered acceptable as replacement tests. The corrosivity potentials of about 40% of the test chemicals could not be assessed with CORROSITEX, although it may be valid for testing specific classes of chemicals (such as organic bases and inorganic acids). The Skin2 assay, as conducted in this validation study, had an unacceptably high underprediction rate (57%), although it had a specificity of 100%. It is recognised that both of these methods could be useful if they were incorporated into a tiered

testing strategy for skin corrosivity.

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ECVAM European Centre for the Validation of Alternative Methods

STATEMENT ON THE APPLICATION OF THE EPIDERM™ HUMAN SKIN MODEL FOR SKIN CORROSWITY TESTING

At its 14th meeting, held on 14-15 March 2000 at the European Centre for the Validation of Alternative Methods (ECVAM), Ispra, Italy, the ECVAM Scientific Advisory Committee (ESAC)[1] unanimously endorsed the following statement:

> Following a review of the results of the ECVAM-funded prevalidaton study on the EpiDerm™ skin corrosivity test coordinated by ZEBET, it is concluded that the EpiDerm human skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the draft EU and OECD test guidelines on skin corrosion.

The ESAC has been regularly kept informed of the progress of the study, and this endorsement was based on an assessment of various documents, including, in particular; the report on the results and evaluation of the study prepared for the ESAC,[2] and a report on the study which has been accepted for publication.[3]

The validation study was conducted in accordance with the general principles laid down in the report of the CAAT[4]/ERGATT[4] workshop held in 1990,[5] guidelines contained in the report of an ECVAM/ERGATT workshop held in 1995,[6] criteria laid down by ECVAM and the ECB,[4,7] criteria recommended at an OECD[4] workshop held in 1996,[8] and the US ICCVAM[4] report on validation and regulatory acceptance.[9]

The status of the draft guidelines referred to is as follows. The Competent Authorities of it EU Member States accepted the draft guideline on skin corrosivity testing[10] into the Annex V guidelines at the 27th Meeting on Adaptation to Technical Process in relation to *Directive 67/548/EEC,*, held in Brussels on 4 February 2000. An equivalent draft guideline[11] has been circulated by the OECD Secretariat for consideration by the OECD Member Countries.

Michael Balls
Head of Unit
ECVAM
Institute for Health & Consumer Protection
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Eva Helisten
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20 March 2000

Please Note:

The Commission Directive 2000/33/EC is a priced publication,
which can be purchased at the following link:
http://europa.eu.int/eur-lex/en/archive/2000/l_13620000608en.html

or click on the blue title or page number below to access the *Official Journal* page.

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Legislation

Contents

(1) Text with EEA relevance

Acts whose titles are printed in light type are those relating to day-to-day management of agricultural matters, and are generally valid for a limited period.
The titles of all other Acts are printed in bold type and preceded by an asterisk.

Excerpts from the *Record of the 6th Plenary Meeting of the Scientific Committee for Cosmetic Products, and Non-food Products intended for Consumers (SCCNFP) Brussels, 20 January 1999* as obtained from the Internet at http://europa.eu.int/comm/food/fs/sc/sccp/out50_en.html

3.1 Report of the Co-ordinator

This plenary meeting was in particular organised to discuss and conclude on the state of the art of the alternative methods to animal testing in the safety evaluation of cosmetic ingredients or mixtures of ingredients. The SCCNFP was asked by the Commission, in the framework of Council Directive 76/768/EEC on cosmetic products, to review the status of alternative methods and provide her with a report by the end of January 1999.

A series of meetings were organised during which matters were discussed with representatives of DG III, DG XI, DG XII, ECVAM as well as with representatives of the European Cosmetic Industry. As a result, a status report was prepared by the WP 'Alternatives' and presented to the Plenary for adoption. The co-ordinator said that the committee's work for this meeting consisted of :

* the adoption of the *in vitro* methods to assess percutaneous absorption of cosmetic ingredients;

* the adoption of the status report on alternatives;

* the revision of the Notes of Guidance, and

* the establishment of a work programme for 99.

3.3 Formal opinion of the state of the art, doc. no. SCCNFP/0103/99

With reference to what is said under point 3.1 Report of the Co-ordinator, the committee was asked to review the status of alternative methods to animal testing in the safety evaluation of cosmetic ingredients and to make their conclusions available by the end of January 1999.

Three areas were considered, namely skin corrosivity, photo-toxicity and percutaneous absorption. The applicability to the cosmetic sector of the *in vitro* methods developed in these areas was reviewed and evaluated. As a consequence, the committee adopted opinions on the following methods :

* *In vitro* methods to assess skin corrosivity in the safety evaluation of cosmetic ingredients or mixtures of ingredients;

* *In vitro* methods to assess photo-toxicity in the safety evaluation of cosmetic ingredients or mixtures of ingredients;

* *In vitro* methods to assess percutaneous absorption of cosmetic ingredients.

In the state of the art, the SCCNFP concludes that the three *in vitro* methods to assess skin corrosivity (TER test and EPISKIN test) and to assess photo-toxicity (3T3 NRU PT) have been validated and can be considered to be applicable to the safety evaluation of cosmetic ingredients or mixtures of ingredients. Moreover, the SCCNFP has considered the possible usefulness of the various existing *in vitro* methodologies to evaluate the percutaneous absorption of cosmetic ingredients and will define a set of minimal criteria needed for the acceptance of *in vitro* percutaneous absorption studies for the evaluation of the toxicological profile of cosmetic ingredients.

Scientists performing such tests are requested, besides describing the respective test in detail, to elucidate also the scientific background for their choice of the used methodology, including hints to the literature.

Excerpts from the *Opinion on in vitro methods to assess skin corrosivity in the safety evaluation of cosmetic ingredients or mixtures of ingredients adopted by the plenary session of the Scientific Committee for Cosmetic Products, and Non-food Products intended for Consumers (SCCNFP) of 25 November 1998* as obtained from the Internet at
http://europa.eu.int/comm/food/fs/sc/sccp/out47_en.html

Outcome of discussions

Terms of Reference

Two *in vitro* methods developed to assess skin corrosivity of chemicals, the "Rat skin Trancutaneous Electrical Resistance (TER) test" and the "EPISKIN test" have been validated by ESAC (ECVAM Scientific Advisory Committee). The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) has been requested by DG III to advise the Commission on the applicability of the methods to the safety assessment of chemicals used as cosmetic ingredients.

Background

The European Centre for the Validation of Alternative Methods (ECVAM) has conducted in 1996-1997 a validation study of *in vitro* tests developed to assess skin corrosivity of chemicals. This study was a follow-up to a pre-validation study of tests developed for replacing the *in vivo* Draize skin corrosivity test in rabbits. The main objectives of the validation study, as defined by the sponsors and the management team before the study began, were :

(a) to identify tests capable of discriminating corrosives (C) from non corrosive (NC) for selected groups of chemicals (e.g. organic acids, phenols) and/or all chemicals (single chemical entities only);

(b) to determine whether the tests could identify correctly known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals.

Organisation of the study

The study was coordinated from ECVAM. A Management Team (MT) was constituted by four representatives of lead laboratories, each of them being responsible for one of the four tests being evaluated. The tests selected for inclusion in the validation study were the rat transcutaneous electrical resistance (TER) test, Corrositex, the Skin^2 ZK1350 corrosivity test, and Episkin. Each test was conducted in three different laboratories, according to principles, criteria and procedures previously defined by ECVAM. Prediction models for each of the four tests were defined in the test protocols.

Coordination /MT /Laboratories

Sixty chemicals were selected by an independent Chemicals Selection Sub-Committee, and distributed coded to the participating laboratories. These included organic acids (6C/5NC), organic bases (7C/3NC), neutral organics (9NC), phenols (2C/3NC), inorganic acids (6C/1NC), inorganic bases (2C/2NC), inorganic salts (1C/2NC), electrophiles (3C/5NC), and soaps/surfactants (3NC). The selection is fully described in a publication (Ref. 1); the main criterion for including chemicals in the test set was that the corrosivity classifications were based on unequivocal animal data.

The results obtained were analysed by statistician experts. The classifications of the corrosivity potential of the test chemicals, as derived from the *in vitro* data obtained in the three laboratories conducting the test, were compared to the *in vivo* classifications independently assigned to the chemicals before the blind trial, to yield sensitivity, specificity, predictivity and accuracy of the test.

Main results

The full details of the validation study have been published (Ref. 2). Two tests, with a good reproductibility within and between test laboratories, proved applicable to the testing of a diverse group of chemicals: the TER test and Episkin.

In the TER test, test materials are applied for 2 to 24 hours to the epidermal surface of skin discs taken from the pelts of humanely killed young rats, and corrosive chemicals are identified by their ability to produce a loss of normal stratum corneum integrity, which is measured as a reduction of the inherent transcutaneous electrical resistance (below a predetermined threshold level).

Episkin is a tri-dimensional human skin model with a reconstructed epidermis and a functional stratum corneum. When utilised in corrosivity testing, application of test chemicals to the surface of the skin for 3, 60 and 240 min, is followed by an assessment of cell viability.

Sensitivity, specificity, predictivity and accuracy in distinguishing corrosive from non corrosive chemicals were very high for both tests: 88, 72, 72, 79 and 83, 80, 77, 81% respectively for the TER test and Episkin. In addition, Episkin was also able to distinguish between known R35 (UN packing group I) and R34 (UN packing groups II & III) chemicals.

Opinion of the SCCNFP

ECVAM Scientific Advisory Committee (ESAC), which had been fully informed of the progression of the validation procedure, reviewed the final results and unanimously endorsed a statement that the rat skin TER test is scientifically validated for use as a replacement for the animal test for distinguishing between corrosive and noncorrosive chemicals, and that Episkin is scientifically validated as a replacement for the animal test, and that these tests are ready for regulatory acceptance.

Sixty chemicals were used for the validation of these two methodologies; twenty of them are used as cosmetic ingredients, according to the "European inventory and common nomenclature of ingredients employed in cosmetic products" (Ref. 3).

SCCNFP reviewed publications from the validation study and ESAC statements, and propose that these two methods could be applied to the safety assessment of chemicals used as cosmetic ingredients. A cosmetic ingredient or mixture of ingredients can be corrosive per se. When corrosivity cannot be excluded, testing for irritancy on animals or humans should be preceeded by a corrosivity test using one of these two validated *in vitro* methodologies.

References

1- Barratt M.D. & al. Toxicology in Vitro (1998) 12, 471-482
2- Barratt M.D. & al. Toxicology in Vitro (1998) 12, 483-524
3- Commission Decision 96/335 EC of 8 May 1996 establishing an inventory and a common nomenclature of ingredients employed in cosmetic products J.O. L 132 of 1 June 1996

Excerpts from the *alphabetical list of the scientists appointed by the Commission as members of the Scientific Committee for Cosmetic Products, and Non-food Products intended for Consumers (SCCNFP) set up by Decision 97/579/EC of 23 July 1997* as obtained from the Internet at http://europa.eu.int/comm/food/fs/sc/sccp/members_en.html.

- Klaus E. Andersen, Professor of Dermatology, Odense University Hospital, Odense (Danmark)
- Robert Anton, Professeur de Pharmacognosie, Université Louis Pasteur Strasbourg, Faculté de Pharmacie, Illkirch (France)
- Claire Marcia Chambers, Consultant Toxicologist, Chambers Toxicological Consulting, Roundwood (Ireland)
- Alessandro di Domenico, Head of Ecotoxicology Unit, Istituto Superiore di Sanità, Roma (Italia)
- Vassilios M. Kapoulas, Professor emeritus of Biochemistry, University of Ioannina, Halandri (Ellas)
- Fritz H. Kemper, Professor emeritus of Pharmacology and Toxicology, University of Münster, Münster (Deutschland) - Vice-Chair of the Committee
- Christian Laurent, Lecturer, Scientific Director, Université de Liège, Institut de Pathologie, Liège (Belgique)
- Berend A.R. Lina, Study director, Division Quality Consultant, TNO Nutrition and Food Research, Toxicology Division, Zeist (Nederland)
- Nicola Loprieno, Professor emeritus of Genetics, University of Pisa, Pisa (Italia) - Vice-Chair of the Committee
- Jean-Paul Marty, Professor of Dermopharmacology and Cosmetology, Faculty of Pharmacy, University Paris South (Paris XI), Châtenay-Malabry (France)
- José Luis Parra, Research professor, Consejo Superior de Investigaciones Cientificas, Centro de Investigación y Desarrollo, Barcelona (España)
- Thomas Platzek, Senior scientist, Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin (Deutschland)
- Suresh Chandra Rastogi, Senior Research Scientist, National Environmental Research Institute, Roskilde (Danmark)

· Vera M. Rogiers, Professor, Vrije Universiteit Brussel, Faculty of Medicine and Pharmacy, Brussel (België)

· Tore Sanner, Professor, The Norwegian Radium Hospital, Institute for Cancer Research, Oslo (Norge)

· Hans Schaefer, Invited Lecturer for Skin Physiology and Skin Pharmacology, Department of Dermatology, Charité-Hospital, Humboldt-University, Berlin (Deutschland); Former Scientific Director, L'Oréal, Clichy (France)

· Josep Vives-Rego, Professor of Microbiology, Universitat de Barcelona, Facultat de Biologia, Barcelona (España)

· Ian R. White, Director, Consultant Dermatologist, St. Thomas' Hospital, St. John's Institute of Dermatology, London (United Kingdom) - Chair of the Committee

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DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

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May 5, 2000

Maurice Zeeman, Ph.D.
U.S. Environmental Protection Agency
Office of Prevention, Pesticides, and
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Risk Assessment Division (7403)
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Washington, D.C. 20460

Dear Dr. Zeeman:

Thank you for the opportunity to provide comments on the proposed OECD draft test guideline on in vitro Skin Corrosion Tests. The following comments were generated by the Corrosivity Working Group (CWG), a subcommittee of the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The CWG is composed of knowledgeable scientists from nine participating ICCVAM agencies. The CWG, which coordinated the independent peer review of another in vitro alternative method for dermal corrosivity (Corrositexâ), reviewed the OECD documents and the ECVAM data substantiating the validity of Episkin and the rat skin TER method.

The comments are as follows:

1. Both the Episkin and the TER assays appear to be useful methods for assessing corrosivity, when used in a weight of evidence decision process within the tiered testing strategy described in the revised OECD Test Guideline 404 (OECD, 2000), and the OECD Harmonized Integrated Hazard Classification System for Human Health and Environmental Effects of Chemical Substances (OECD, 1998). Paragraph 1 should therefore be modified accordingly to ensure that the described uses are consistent with the proposed TG 404.
2. In Paragraph 6, specific guidance should be provided as to the necessity to routinely incorporate the dye-binding step when the chemical class is not known for the chemical that is to be evaluated, or if the chemical is in a chemical/product class for which the proposed method has not been adequately evaluated.

3. The "human skin model assay" section should include the specific details within the guideline as to how to construct the skin model and conduct the assay, and include the detailed Episkin™ protocol used in the ECVAM validation study. One way this could be done would be to provide a supplemental guidance/background document that provides the detailed protocol. Regardless, there must be a specific statement that the other similar test protocols must demonstrate similar or better performance characteristics with appropriate reference chemicals.
4. Paragraph 26 must better define the criteria for the following terms: "functional stratum corneum," "…must be *adequate* to resist…," "…and be *adequate* for the chemical class (es)…", "model must be *sufficiently high* to *clearly discriminate* between…," "within *acceptable limits*," and "within *specified limits*." Examples of chemicals that could be used as a positive control should be included.
5. A control chemical to verify the functionality of the stratum corneum (i.e., one that would be toxic to the underlying cells unless the stratum corneum was functional) should be included for human skin model assays (see Paragraph 26).
6. In paragraph 27, further detail should be added to explain what is considered to be "adequate for a wide range of chemicals types."
7. In Paragraph 30, detail should be added that explains how to determine the appropriate cut-off percentage cell viability for corrosive or non-corrosive chemicals, based on the model being used. Appropriate procedures for categorizing chemicals with cell viabilities near the cut-off percentage should be added.
8. In Paragraph 31, given the variations in possible construction of an artificial skin preparation, consideration should be given as to the adequacy of testing a subset of reference chemicals from the ECVAM validation study and publishing these results in a peer-reviewed journal, as sufficient evidence to demonstrate that the method yields equivalent results to the Episkin assay tested in the ECVAM study. The number and types of reference chemicals considered sufficient to evaluate 'equivalence' and the standard for acceptable reproducibility and accuracy should be provided or referenced and an appropriate rationale included.
9. Data recording forms from the ECVAM Validation Study protocols should be appended to the guideline as a suggested standard format for recording data.
10. In general, more detail is needed in both protocols. Appending the ECVAM validation study protocols may be appropriate.

Please feel free to contact Dr. Richard Hill or me if you have any questions about these comments, or if we can be of further assistance.

Sincerely,

William S. Stokes, D.V.M.
Co-Chair, ICCVAM

cc: Dr. Angela Auletta
Dr. Richard Hill
Corrosivity Working Group
ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)
Corrosivity Working Group (CWG)

Comments on the OECD Guideline for the Testing of Chemicals Draft Proposal for a New Guideline: *In Vitro* Skin Corrosion Tests, November, 1999

The following comments on the draft OECD guideline were generated at the April 25, 2000 ICCVAM CWG meeting:

1. Both the Episkin and the TER assays appear to be useful methods for assessing corrosivity, when used as a screen to identify corrosives. Negatives require *in vivo* testing because of the high false negative rates in accordance with the revised OECD Test Guideline 404. Paragraph 1 should be modified accordingly to ensure consistency with the proposed TG 404.
2. In Paragraph 6, guidance should be given as to whether to routinely incorporate the dye-binding step when the chemical class of the chemical under consideration is not known or if the chemical is in a chemical/product class that hasn't been evaluated previously.
3. The "human skin model assay" section should be more specific within the guideline or there should be supplemental guidance/background document that provides greater detail as to its construction. The detailed Episkin protocol used in the ECVAM validation study should be provided as one method that has been shown to be useful. Other similar test protocols might be found to be adequate following demonstration of similar or better performance characteristics with appropriate reference chemicals.
4. Paragraph 26 should be more specific with regard to the following terms: "functional stratum corneum," "….must be *adequate* to resist…," "….and be *adequate* for the chemical class(es)….", "model must be *sufficiently high* to *clearly discriminate* between……," "within *acceptable limits*," and "within *specified limits*." Examples of chemicals that could be used as a positive control should be included.
5. A control chemical to verify the functionality of the stratum corneum (i.e., one that would be toxic to the underlying cells unless the stratum corneum was functional) should be included for human skin model assays (see Paragraph 26).
6. In paragraph 27, further detail should be added to explain what is considered to be "adequate for a wide range of chemicals types."

7. In Paragraph 30, detail should be added that explains how to determine the appropriate cut-off percentage cell viability for corrosive or noncorrosive chemicals, based on the model being used. Appropriate procedures for categorizing chemicals with cell viabilities near the cut-off percentage should be added.
8. In Paragraph 31, given the variations in possible construction of an artificial skin preparation, consideration should be given as to the adequacy of testing a subset of reference chemicals from the ECVAM validation study, and publishing these results in a peer-reviewed journal, is sufficient to demonstrate that the method yields equivalent results to the Episkin assay tested in the ECVAM study. The number and types of reference chemicals considered sufficient to evaluate 'equivalence' and the standard for acceptable reproducibility and accuracy should be provided or referenced and an appropriate rationale included.
9. In order to set a standard procedure, data recording forms should be appended to the guideline as a suggested standard format for recording data as was done with the ECVAM Validation Study.
10. In general, more detail is needed in both protocols. Appending the ECVAM validation study protocols may be appropriate.