A Prevalidation Study on *In Vitro* Skin Corrosivity Testing

The Report and Recommendations of ECVAM Workshop 6[1,2]

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Preface

This is the report of the sixth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on *In Vitro* Skin Corrosivity Testing was held in Angera, Italy on 12–14 January 1994, under the chairmanship of Philip Botham. The workshop was concerned primarily with the discussion of a prevalid-

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[1]European Centre for the Validation of Alternative Methods. [2]This document represents the agreed report of the participants as individual scientists.

ation study on alternative methods for skin corrosivity testing. The results and conclusions of the prevalidation study are given in this report, in addition to some specific recommendations which relate to future requirements in skin corrosivity testing. A follow-up meeting was held at ECVAM in February 1995 to finalise the workshop report, to review further test optimisation conducted since January 1994, and to discuss the design of a validation study on alternative methods for corrosivity testing, which is to be sponsored by ECVAM.

Introduction

The potential for chemical-induced skin corrosion is an important consideration in establishing procedures for the safe handling, packing and transport of chemicals. The standard approach for assessing skin corrosion and irritation has been to apply the test chemical to the shaved skin of albino rabbits (2). The production of irreversible full-thickness necrosis of the skin (which is the endpoint of corrosion) is determined by visual inspection of the skin for up to 21 days following exposures to the test material of up to four hours' duration. This test is included in international regulatory requirements for the testing of new chemicals, for example, the Code of Federal Regulations (3) and OECD testing guidelines (4).

Regulations originating from the United Nations (UN; 5) require the labelling of packaged chemicals for international transport purposes. The UN guidelines recommend that corrosives be classifed into potency categories, termed "packing groups". Packing groups I, II and III are assigned on the basis of the capacity of a chemical, when tested on the intact skin of albino rabbits, to produce skin corrosion within three minutes, one hour or four hours, respectively. In October 1993, these UN guidelines were accepted by the US Department of Transportation (DoT). European regulations (6) require classification according to risk phrases, determined according to whether a chemical causes corrosion within three minutes (R35; analogous to packing group I) or four hours (R34; analogous to packing groups II and III).

Testing for skin corrosion/irritation in laboratory animals can cause them discomfort or pain. For this reason, alternative methods for trying to identify corrosive substances have been developed. The severity of the skin lesion (i.e. tissue destruction) lends itself to using non-animal methods that can detect severe tissue damage. Thus, the feasibility of using non-animal tests for assessing skin corrosion is probably greater than for trying to detect toxic effects which are exerted by subtle multifactorial mechanisms.

As in several other areas of toxicity testing, the evolution of alternative tests for detecting chemicals which are corrosive to the skin has resulted in the production of model test systems which have been designed for a particular market and/or to fulfil a specific need. The *in vitro* test systems investigated in this prevalidation study cover a range of biological complexity: excised rat skin (the transcutaneous electrical resistance [TER] assay), a three-dimensional human skin model (Skin^{2TM}; Advanced Tissue Sciences, La Jolla, CA, USA), and a biochemical matrix (CORROSITEX^{TM}; InVitro International, Irvine, CA, USA).

The most mature alternative method for corrosivity testing, the TER assay (7), has been used successfully as a routine in-house prescreen for several years. When used in screening mode, the TER test is employed to predict corrosivity potential rather than the degree of corrosive effect (i.e. potency), and it is used primarily to guide humane *in vivo* skin testing. The TER method has undergone intralaboratory and interlaboratory validation (7, 8), although these studies would not meet current standards for the conduct of validation studies, such as those defined by Balls *et al.* (9).

Recently, two commercially available test systems, CORROSITEX (10) and a protocol using Skin^2 (11), have been developed to predict the skin corrosivity potentials of chemicals and to rank them with respect to their degree of corrosive effect (typically to assign chemicals to UN packing groups). CORROSITEX is an assay system based on the penetration of a test material through a non-living barrier. It has been granted regulatory approval by the US DoT, in the form of an exemption (limited to defined chemical classes) for the detection of corrosives, such that a positive result in this non-animal test enables the chemical to be classified as a corrosive without this being confirmed in the standard animal procedure. However, a negative response does not obviate the need for an animal test for subsequent classification. The Skin^2 Model ZK1350 *in vitro* skin corr-

osion test, which involves the topical application of test materials to the stratum corneum of three-dimensional human skin cultures, has now also been granted similar DoT approval, despite the fact that neither CORROSITEX nor the Skin^2 protocol have undergone formal interlaboratory validation.

The TER, CORROSITEX and Skin^2 tests are therefore at different stages of optimisation, evaluation and validation. The need for a prevalidation assessment of the tests, prior to the possible undertaking of a formal validation study, was recognised early in 1993 by scientists from the Central Toxicology Laboratory (CTL) at ZENECA and from the Environmental Safety Laboratory (ESL) at Unilever. Subsequently, representatives from laboratories which were familiar with the *in vitro* tests were canvassed regarding the possibility of them actively participating in such a prevalidation study.

The aims of the prevalidation study were:

1. to evaluate the relative performances of the TER, CORROSITEX and Skin^2 methods in correctly predicting defined corrosive and non-corrosive test chemicals;
2. to undertake an initial assessment of the interlaboratory variabilities in the methods, by conducting each test in at least two laboratories; and
3. to assess the relative states of optimisation, evaluation and validation of the tests.

The test chemicals (Table I) were selected on the basis of their availability and the confidence with which they could be classified unambiguously as "corrosive" or "non-corrosive". *In vivo* data were obtained from in-house studies or from the scientific literature (12), or by reference to the 1991 Comité Européen des agents de Surface et leurs Intermédiaires Organiques (CESIO) classifications or to manufacturers' data. All animal tests were reported to have been conducted in accordance with OECD Test Guideline 404. Corrosivity classifications were from three sources: animal data, hazard data sheets, or Annex 1 of the Dangerous Substances Directive (13). The selection of the test chemicals was based primarily on the availability of the test material and associated data. Thus, the criteria for selection were not as stringent as those employed by other groups (for example, the European Centre for the Ecotoxicology and Toxicology of Chemicals [ECETOC]) when choosing the chemicals to be tested in formal validation studies. However, the test chemicals chosen were considered to be adequate for the primary objective of the study, i.e. for an assessment of the relative performances of the three tests.

In this prevalidation study, each *in vitro* test was conducted to the same agreed protocol in at least two different laboratories, in accordance with the principles of Good Laboratory Practice (GLP). The laboratories involved encompassed industrial, contract and government establishments located either in the USA or in Europe (Table II).

Materials and Methods

Test chemicals

Most of the test chemicals (25 corrosives, 25 non-corrosives; Table I) were commercially available, and were obtained from Aldrich (Gillingham, Kent, UK), Akzo (Hersham, Surrey, UK), Monsanto (Basingstoke, Hants., UK), Unichema (Gouda, The Netherlands), Hoechst (Hounslow, Middlesex, UK), K&K Greeff (Croydon, Surrey, UK), Albright and Wilson (Oldbury, W. Midlands, UK), ZENECA (Macclesfield, Cheshire, UK) or ICI (Leatherhead, Surrey, UK). The sources of the corrosive/non-corrosive classifications are indicated in Table I. All test samples were independently coded by Unilever ESL. Each chemical was assigned a single code number and complete sets of test materials were dispatched to the participating laboratories. The identities of the coded chemicals were unknown to the participants. To ensure the integrity of the raw data, test results were lodged with ECVAM prior to the code being broken. Subsequently, the identities of the test chemicals were revealed and the participating laboratories analysed their own data.

TER assay

In the *in vitro* skin corrosivity test developed by Oliver *et al.* (7, 14, 15), substances are applied for up to 24 hours to the epidermal surfaces of skin discs obtained from humanely killed young rats. Corrosive substances produce an irreversible loss of normal stratum corneum integrity and function, which is measured as a reduction in the inherent TER below a predetermined (corrosive) threshold level; irritant substances do not reduce the TER below the threshold level. The original protocol (14) has been refined by the use of

Table I: Test chemicals

Trade name	Chemical name (if different)	Chemical class	Appearance
Corrosives			
Acetic acid (glacial)[a]		Organic acid	Clear liquid
Acrylic acid (99%)[a]		Organic acid	Clear liquid
Armeen CD[b]	Cocoamine	Organic base	Clear liquid
Armeen TD[b]	Tallowamine	Organic base	Opaque gel
Arquad 16-50[b]	Hexadecyltrimethyl-ammonium chloride, 50% in isopropanol	Cationic surfactant	Clear liquid
Arquad DMMCB-50[c]	Coco(C12)dimethylbenzyl-ammonium chloride, 50% in aqueous ethylene glycol	Cationic surfactant	Clear viscous liquid
Bromoacetic acid (8%)[a]		Organic acid	Clear liquid
Bromoacetic acid (55.6%)[a]		Organic acid	Clear liquid
Butylamine (40%)[a]		Organic base	Clear liquid
Capric/caprylic (45:55) acid[b]		Organic acid	Clear liquid
Caprylic acid[b]		Organic acid	Clear liquid
Cyclohexylamine (11.9%)[a]		Organic base	Clear liquid
1,4-Diaminobutane (30%)[a]		Organic base	Clear liquid
Dichloroacetic acid (36.1%)[a]		Organic acid	Clear liquid
Diethylamine (35%)[a]		Organic base	Yellow liquid
Duoquad T-50[b]	Pentamethyl-*N*-tallow-1,3-propanediammonium chloride, 50% in isopropanol	Cationic surfactant	Yellow liquid
Formic acid (33.9%)[a]		Organic acid	Clear liquid
Hexanoic acid[a]		Organic acid	Clear yellow liquid
Mercaptoacetic acid (15.1%)[a]		Organic acid	Clear liquid
Proxel BD[b] (biocide A)	1,2-Benzisothiazolin-3-one (33%) in aqueous propylene glycol	Neutral organic	Tan opaque liquid
Pyrrolidine (34.5%)[a]		Organic base	Yellow liquid
Sodium hydroxide (4.88%)[a]		Inorganic	Clear liquid
Sodium metasilicate[b]		Inorganic	Granular powder[c]
Sodium silicate A140[b]		Inorganic	Clear gel
Synprolam 35X2[b]	C13-15Alkyl-di(2-hydroxyethyl)amine	Organic base	Clear viscous liquid

[a] *Jacobs & Martens (12) classification from animal data.*

[b] *Original animal data.*

[c] *Prepared in distilled water at 1g/ml.*

Table I: continued

Trade name	Chemical name (if different)	Chemical class	Appearance
Non-corrosives			
Armeen 2C[d]	Dicocoamine	Organic base	Crystalline powder[c]
Aromox DMMCD-W[b]	Coco(C12)dimethylamine oxide (30%)	Amine oxide	Clear liquid
Arquad C-33-W[d]	Coco(C12)trimethyl-ammonium chloride, 33% in water	Cationic surfactant	Clear gel
Butylbenzene[a]		Neutral organic	Clear liquid
Dequest 2000[e]	Aminotris(methylphosphonic acid), 50% in water	Organic acid	Clear liquid
Dowanol PNB[f]	Propylene glycol *n*-butyl ether	Neutral organic	Clear liquid
Elfan OS 46[d]	C12-14α-Olefin sulphonate, sodium salt	Anionic surfactant	Yellow viscous liquid
Empicol LZPV/C[d]	Sodium dodecyl sulphate	Anionic surfactant	Dry pellets[c]
Empigen OB[d]	Coco(C12)dimethylamine oxide (30%)	Amine oxide	Clear liquid
Empilan CME[d]	Fatty acid monoethanolamide coco	Neutral organic	Dry chips[c]
Empilan KB2[d]	Fatty alkylethoxylate 2EO	Neutral organic	White opaque cream
Ethomeen T/25[b]	Polyoxyethylene(15)tallowamine	Organic base	Yellow viscous liquid
Genamin KDM-F[d]	Behenyl(C20-22)trimethyl-ammonium chloride, 80% in isopropanol	Cationic surfactant	Powdered flakes[c]
Genapol LRO[d]	Coco(C12)2EO sulphate, sodium salt (70%)	Anionic surfactant	Clear gel
n-Hexanol[a]		Neutral organic	Clear liquid

[a] *Jacobs & Martens (12) classification from animal data.*

[b] *Original animal data.*

[c] *Prepared in distilled water at 1g/ml.*

[d] *CESIO classification from animal data.*

[e] *Harmonised Electronic Dataset (HEDSET) data.*

[f] *Manufacturers' data sheet and summary of test data.*

Table I: continued

Trade name	Chemical name (if different)	Chemical class	Appearance
Hostaphat KLD[d]	Alkyl(4EO)phosphate ester	Neutral organic	Clear viscous liquid
Lauric acid[b]		Organic acid	Fine powder[c]
n-Nonanol[a]		Neutral organic	Clear liquid
Oleic/caprylic (80:20) acid[b]		Organic acid	Yellow liquid
Proxel AB[b] (biocide B)	1,2-Benzisothiazolin-3-one (33%), aqueous	Neutral organic	Opaque tan liquid
Sodium perborate[e]		Inorganic	Crystalline powder[c]
Sodium percarbonate[e]		Inorganic	Granular powder[c]
Sodium silicate H100[b]		Inorganic	Clear viscous liquid
Triethanolamine[a]		Organic base	Clear viscous liquid
n-Undecanol[a]		Neutral organic	Clear liquid

[a] *Jacobs & Martens (12) classification from animal data.*

[b] *Original animal data.*

[c] *Prepared in distilled water at 1g/ml.*

[d] *CESIO classification from animal data.*

[e] *Harmonised Electronic Dataset (HEDSET) data.*

magnesium sulphate rather than sodium chloride as the electrolyte solution. This has been found to reduce the incidence of false positive results obtained with solvents and surfactants (16). The protocol used in this prevalidation study has been evaluated previously with 88 industrial substances (16), and with 20 test materials in a blind interlaboratory trial (8). The assay produces very few false negative results, but some false positive results are obtained with test materials containing surfactants and solvents.

Animals

Male Wistar albino rats, aged between 23 and 30 days, were used. These were supplied by Charles River (Manston, Kent, UK) or Iffa-Credo (69210 L'Arbresle, France).

Methodology

The TER assay was performed as described previously (8, 16). Rats (23–25 days old) were shaved to remove hair from the dorsal surface without abrading the skin, and were then washed in an antibiotic bath. Another antibiotic wash was performed three days later. At 28–30 days old, the rats were humanely killed. During this period, rats are in the telogen phase of hair growth. Thus, during the preparation stages, there is no hair growth, and the stratum corneum recovers from the effects of shaving and is not damaged by bacterial growth. The quality of the stratum corneum is critical to the success of the assay, and so the stage of hair growth must be controlled by using animals of exactly the correct age.

Table II: Tests and laboratories involved in the prevalidation study

TER assay	CORROSITEX™	Skin^{2TM} assay
Rhône-Poulenc, France	Microbiological Associates, USA	Procter & Gamble, USA
ZENECA CTL, UK	InVitro International, USA	ZEBET, Germany
		Huntingdon Research Centre, UK

TER assay = transcutaneous electrical resistance assay.

The dorsal skin was removed from the rat as a single pelt. The excess fat was removed and the pelt was then mounted, epidermal side uppermost, onto polytetrafluoroethylene (PTFE) tubes (International Market Supply, Macclesfield, Cheshire, UK) and secured with a rubber 'O' ring. Excess tissue was trimmed away and the 'O' ring/PTFE tube interface sealed with soft paraffin wax. The tube was supported by a spring clip inside a plastic tube containing electrolyte solution (154mM $MgSO_4$ in deionised or distilled water). Three discs were taken from each pelt and the TER was measured (as described below) as a quality control procedure: only pelts showing a TER of greater than 10kΩ/skin disc were used in the assay. The quality control discs were discarded, and new discs from the acceptable pelts (up to nine discs per pelt) were mounted onto PTFE tubes, which were then randomised to avoid bias from individual pelts.

The test materials were applied to the epidermal surfaces of at least three skin discs per chemical, at room temperature, for 24 hours. For liquids, 150µl of test material was applied; for solids, 100mg (or sufficient to cover the skin disc) of test material was applied, along with 150µl of water to ensure good contact with the skin. At the end of the exposure period, the chemicals were removed with a jet of tap water. The stratum corneum was rinsed with aqueous ethanol (70%), to reduce the surface tension, prior to the addition of electrolyte solution (3ml). The TER was then measured using a resistance meter in alternating current mode (AIM Databridge, AIM Instruments, Huntingdon, Cambs., UK).

Data evaluation and analysis

The mean TER for the skin discs was calculated for each substance. Test materials giving mean TER values below 5kΩ/skin disc (the corrosive threshold; 8) are classified as skin corrosives.

CORROSITEX

In the CORROSITEX system, a test material is applied directly to a biobarrier. If it alters the biobarrier sufficiently to be able to pass through into a second compartment, then the chemical is detected by a colour or physical change in a liquid (the "chemical detection system" [CDS]) which is located directly below the biobarrier. The time required for this change to occur (the "breakthrough time") is reported to be inversely proportional to the degree of corrosivity of the test material, i.e. the longer it takes to detect a change in the CDS, the less corrosive is the substance.

Chemicals

The coded chemicals were dispatched only to one of the laboratories conducting CORROSITEX (Microbiological Associates [MA], Rockville, MD, USA). Following the completion of testing by MA, the remainder of the test materials were forwarded to InVitro International (IVI; Irvine, CA, USA). IVI also received samples of the coded test materials from The Procter & Gamble Company (P&G; Cincinnati, OH, USA), which had been excess to their requirements when undertaking the Skin^2 assay. However, there were two test materials (later identified as sodium silicate A140 and Synprolam 35X2, following breaking of the code) which could not be tested by IVI, because there were insufficient amounts remaining.

Reagents
CORROSITEX kits were supplied by IVI. The kits included liquid CDS, lyophilised biobarrier matrix, biobarrier diluent, membrane discs and glass scintillation vials (used to hold all the components during the assay).

Methodology
Firstly, the compatibilities of the coded chemicals with the test kit were determined, i.e. whether they possessed the chemical or physical properties which enabled them to be detected by the CDS. Test material (50µl) was mixed with CDS (500µl). If a noticeable colour or physical change occurred in the CDS within a 5-minute observation period, the sample was considered to qualify for subsequent testing in the CORROSITEX system.

The biobarrier was prepared according to the instructions provided with the kits. In outline, CORROSITEX diluent (50ml) was slowly mixed (with stirring for about 20 minutes) with biobarrier matrix (5g) in a beaker kept in a water bath at 60–68°C. After solubilisation of the matrix, the hot solution was pipetted into discs placed in 24-well plates. Each biobarrier was inspected carefully and discarded if any air bubbles were present. The plates were sealed with plastic film and refrigerated (2–8°C) for at least two hours before use.

Triplicate vials were set up for each test material. CDS (22ml) was pipetted into the vials, and a biobarrier disc was then placed into each vial. Test materials (liquids: 500µl; solids: 500mg) were placed onto the discs. All vials were left uncapped during the test. The time of the first physical or colour change of the CDS was recorded, either to the nearest minute (Laboratory A) or to the nearest hundredth of a minute (Laboratory B). A single pellet of sodium hydroxide, placed on the biobarrier, served as the positive control for all experiments. The assay acceptance criterion (Laboratory A) was that the positive control breakthrough time was within two standard deviations of the historical mean value (11.6 ± 1.1 minutes; n = 37).

Data evaluation and analysis
A chemical was considered to be corrosive if it penetrated the biobarrier and was detected by the CDS in less than 4 hours. Suggested cut-off times of 3 minutes and 1 hour can be used to assign chemicals to UN packing groups I and II, respectively. However, in this study, only corrosive/non-corrosive classifications were determined.

The mean values from triplicate measurements were used to determine whether the test chemicals were corrosives or non-corrosives. Data are presented as means ± SD, to give some indication of intralaboratory and interlaboratory reproducibility. Interlaboratory reproducibility was also assessed by performing a regression analysis on the paired data from both laboratories.

Skin^2 Model ZK1350 in vitro skin corrosion test

The Skin^2 Model ZK1350 test is based on the topical application of test materials to the stratum corneum of three-dimensional human skin cultures (11, 17, 18). Following a 10-second exposure period, the extent of cell damage is determined using the MTT (3[4,5-dimethylthiazol-2-yl],2,5-diphenyltetrazolium) reduction assay, to assess the degree of corrosivity of the test chemical. The Skin^2 cultures are grown from neonatal human skin cells and contain dermal and epidermal components. Neonatal fibroblasts are seeded onto inert nylon mesh and are grown into a dermal tissue containing fibroblasts and naturally secreted extracellular matrix and growth factors. Keratinocytes are seeded on top of this dermal tissue, and they then differentiate into a functional epidermis. Basal, spinous and granular layers of keratinocytes are present, as well as a multilayered stratum corneum (11). Biochemical and ultrastructural characterisation of the human skin cultures have demonstrated the presence of differentiation markers and metabolising enzyme activities comparable to those of intact human skin (11, 19, 20).

The Skin^2 Model ZK1350 test was performed in three independent laboratories, designated A, B and C. The laboratories were familiar with the basic test procedure, but no specialised training on this test protocol was conducted. Test kits were purchased from Advanced Tissue Sciences, La Jolla, CA, USA. Each test kit contained 24 human skin cultures (9mm × 9mm), cell culture medium and various other items required to conduct the test. One kit was used to test five chemicals (each in a single experiment using quadruplicate cultures) using a 10-second exposure period. Four control cultures (treated with distilled water) per kit were evaluated.

Methodology
The test was performed as described in the directions for use included with each kit. On the day before the test was performed, each

Skin^2 cell culture was removed from the surface of the agarose used for shipping purposes and was placed onto a Millicell culture insert above serum-free Dulbecco's minimum essential medium (DMEM)-based assay medium (1ml). The cultures were incubated (5% CO_2, 90% humidity) overnight at 37°C. In those test kits which were to be used over a three-day period (Laboratory B), the Skin^2 cell cultures were placed onto a Millicell culture insert above maintenance medium (1ml) and incubated for 24 hours. The medium was then changed to serum-free DMEM-based assay medium, and the cultures were incubated overnight prior to use on the following day.

Liquid and semi-solid test materials were evaluated undiluted. Test materials (15µl — a volume sufficient to just cover the surface of each cell culture) were dispensed using positive displacement micropipettes onto small (18mm diameter) glass cover slips. Powdered and granular materials were prepared by grinding them with a mortar and pestle. The ground material (1g) was mixed with distilled water (1ml) to obtain a "100% solution". Aliquots (15µl) were then dispensed onto the glass cover slips. Ten of the 50 test materials were difficult to pipette accurately; with these, approximately 15µl was spread on the cover slip over an area of about 9mm × 9mm.

The epidermal side of the skin cultures was placed onto the test material on the glass cover slip for exactly 10 seconds. The cultures were then washed with copious amounts of calcium-containing phosphate-buffered saline (PBS) to remove residual test material.

The effects of the test materials on cell viability were determined using the MTT reduction assay (21, 22), employing either MTT Topical Cytotoxicity Assay test kits (Advanced Tissue Sciences) or reagents obtained from Sigma (St. Louis, MI, USA). Skin cultures were incubated on a shaker plate at 37°C for two hours with medium containing MTT (2mg/ml; 2ml per 35mm well). The cultures were then washed with PBS and the insoluble blue formazan product extracted with isopropanol (4ml/culture) for 60 minutes at room temperature. Aliquots (200µl) of the isopropanol extracts were transferred to 96-well plates and the intensity of colour determined using a microplate reader set at a wavelength of 540nm.

Data evaluation and analysis

For each test material, the average viability of the treated skin cultures (conducted in quadruplicate) was calculated as a percentage of the untreated control values using the equation: (mean A_{540} of the chemical-treated cultures ÷ mean A_{540} of the untreated control cultures) × 100. The percentage viability values were then used to classify the material as corrosive (≤ 80% viability) or non-corrosive (> 80% viability). Although data from the Skin^2 assay can be used to classify degrees of corrosivity (for example, to assign chemicals to UN packing groups), only corrosive/non-corrosive classifications were determined in this study.

Results and Discussion

TER assay

The data obtained with the TER assay are given in Tables III and IV. All the substances were compatible with the test system. The only technical difficulty encountered was in the rinsing of the skin discs with certain adherent materials (specifically, this was noted for the two formulations containing caprylic acid by Laboratory B).

The interlaboratory comparison of the TER assay results is shown in Figure 1. For the corrosive materials, 23 of the 25 were classified similarly by both laboratories; the two exceptions were capric/caprylic acid and caprylic acid (Table IV). Subsequently, it was shown that the false negative results in Laboratory B were caused by insufficient rinsing of the skin discs before measurement of the TER. The remaining layer of the test material acted as a barrier, thereby increasing the TER value. For the non-corrosive materials, again 23 of the 25 were classified similarly by both laboratories. The two substances which gave different results were Dequest 2000 and Empilan KB2 (Table IV).

Of the 25 corrosive materials, Laboratory A classified 24 as corrosive and one (Proxel BD) as non-corrosive; Laboratory B classified 22 as corrosive and three (Proxel BD, capric/caprylic acid and caprylic acid) as non-corrosive (Table III). The false negative results from the two materials containing caprylic acid were the result of a technical problem, as described earlier. Following interlaboratory comparison of the results, these two materials were re-tested and gave positive results in Laboratory B. Of the 25 non-corrosives, Laboratory A classified 11 as corrosive and Laboratory B classified 13 as corrosive (Table III). The false positives found

Table III: *In vitro–in vivo* comparisons

	TER assay		CORROSITEX™ assay		Skin²™ assay		
Laboratory	**A**	**B**	**A**	**B**	**A**	**B**	**C**
Samples tested	50	50	50	48	50	50	50
Qualified samples	50[a]	50[a]	38	35	50[a]	50[a]	50[a]
Corrosives identified correctly	24	22	17[b]	16[b]	16	24	21
Non-corrosives identified correctly	14	12	11[b]	12[b]	19	22	19
Concordance (%)	76	68	74[b]	80[b]	70	92	80
False positives	11	13	6[b]	5[b]	6	3	6
False negatives	1	3	4[b]	2[b]	9	1	4
Sensitivity (%)	96	88	81[b]	89[b]	64	96	84
Specificity (%)	56	48	65[b]	71[b]	76	88	76

All test substances were judged to "qualify", since they were all compatible with the test system.

Values have been calculated on the basis of the samples which could be tested.

TER assay = transcutaneous electrical resistance assay.

only in Laboratory B were Dequest 2000 and Empilan KB2.

The TER assay has been in regular use in several laboratories for over five years. The results do not allow prediction of the potential severity of corrosive effects (i.e. assignment into packing classes, etc.), but they provide a means of distinguishing between potential corrosives and non-corrosives. The results of this prevalidation study confirm previous experiences with the assay and the published data. False negative results are rare, but false positive results are relatively common for materials containing certain solvents or surfactants (16). This is because they tend to solubilise the stratum corneum, thus allowing the passage of ions and reducing the electrical resistance. Users of the assay need to be aware of this problem and should either avoid testing certain products and/or take into account the chemical class of the test material when interpreting the results. Of the false positive results obtained in this study, sodium percarbonate was the only material which was not a solvent or a surfactant.

Thus, the TER assay was able to classify correctly 24 out of 25 corrosive materials. Although about half of the 25 non-corrosives were classified incorrectly, all but one of these would have been regarded as possible false positives on the basis of their chemical class. Therefore, with a knowledge of the chemical nature and physicochemical properties of the test materials, the TER assay is able to provide a good indication of the potential corrosivities of most test materials.

CORROSITEX

The results obtained with the CORROSITEX system for the 50 test chemicals are shown in Tables III and V. Laboratory B was not supplied with sufficient amounts of samples to be able to complete the testing of sodium silicate A140 and Synprolam 35X2.

In the initial compatibility determination, some of the test chemicals did not cause a visible change in the CDS, and thus did not qualify for testing (i.e. they were incompatible with the CORROSITEX test system). In Laboratory A, 38 out of the 50 chemicals qualified (76%); in Laboratory B, 35 out of the 48 chemicals tested were judged to qualify (73%). The two samples which could not be

Table IV: Corrosivity of the test chemicals as determined in the transcutaneous electrical resistance assay

Chemical	Laboratory A		Laboratory B	
	kΩ/disc Mean ± SD[a]	C/NC	kΩ/disc Mean ± SD[b]	C/NC
Corrosives				
Acetic acid (glacial)	1.3 ± 0.4	C	1.5 ± 0.3	C
Acrylic acid	1.3 ± 0.3	C	1.3 ± 0.1	C
Armeen CD	1.1 ± 0.1	C	1.5 ± 0.1	C
Armeen TD	4.3 ± 1.1	C	2.5 ± 0.9	C
Arquad 16-50	1.1 ± 0.4	C	0.9 ± 0.2	C
Arquad DMMCB-50	0.8 ± 0.1	C	0.5 ± 0.0	C
Bromoacetic acid (8%)	3.1 ± 0.5	C	3.6 ± 2.2	C
Bromoacetic acid (55.6%)	1.6 ± 0.3	C	2.2 ± 0.2	C
Butylamine	0.8 ± 0.1	C	1.8 ± 0.0	C
Capric/caprylic acid	1.6 ± 0.8	C	10.4 ± 6.8	NC
Caprylic acid	3.8 ± 1.8	C	13.4 ± 7.4	NC
Cyclohexylamine	1.1 ± 0.2	C	1.5 ± 0.0	C
1,4-Diaminobutane	0.8 ± 0.1	C	0.8 ± 0.1	C
Dichloroacetic acid	1.7 ± 0.4	C	3.0 ± 0.7	C
Diethylamine	0.9 ± 0.2	C	0.9 ± 0.1	C
Duoquad T50	2.6 ± 1.1	C	1.6 ± 0.1	C
Formic acid	1.8 ± 1.4	C	2.7 ± 0.2	C
Hexanoic acid	1.0 ± 0.4	C	1.4 ± 0.6	C
Mercaptoacetic acid	1.2 ± 0.2	C	1.6 ± 0.3	C
Proxel BD	10.5 ± 3.0	NC	9.4 ± 3.4	NC
Pyrrolidine	0.6 ± 0.1	C	0.8 ± 0.0	C
Sodium hydroxide	1.3 ± 0.2	C	1.2 ± 0.2	C
Sodium metasilicate	1.1 ± 0.2	C	0.9 ± 0.1	C
Sodium silicate A140	1.9 ± 1.2	C	2.9 ± 0.4	C
Synprolam 35X2	2.0 ± 0.5	C	1.5 ± 0.3	C

[a] $n = 6$.

[b] $n = 3$.

C = corrosive; NC = non-corrosive.

tested by Laboratory B were both qualified by Laboratory A. Only one chemical (caprylic acid) was qualified by one laboratory and not by the other. Thus, 12 (Arquad 16-50, Arquad DDMMCB, Duoquad T50, Proxel BD, butylbenzene, Dowanol PNB, Elfan OS 46, *n*-hexanol, Hostaphat KLD, lauric acid, *n*-nonanol and *n*-undecanol) of the set of 50 chemicals were found to be incompatible with CORROSITEX in both laboratories. The 12 materials which were not qualified by both laboratories comprised seven neutral organics, three cationic surfactants, an organic acid and an anionic surfactant. The one additional material not qualified by Laboratory B was an organic acid. It has been reported previously that many organic solvents are not detected by the CORROSITEX CDS (10).

Table IV: continued

Chemical	Laboratory A kΩ/disc Mean ± SD[a]	Laboratory A C/NC	Laboratory B kΩ/disc Mean ± SD[b]	Laboratory B C/NC
Non-corrosives				
Armeen 2C	13.3 ± 2.0	NC	13.2 ± 5.8	NC
Aromox DMMCD-W	1.2 ± 0.4	C	1.6 ± 0.0	C
Arquad C-33-W	2.3 ± 1.1	C	2.0 ± 0.4	C
Butylbenzene	3.9 ± 3.2	C	3.8 ± 2.2	C
Dequest 2000	7.9 ± 4.0	NC	3.2 ± 0.5	C
Dowanol PNB	6.0 ± 1.9	NC	7.1 ± 1.2	NC
Elfan OS 46	1.1 ± 0.3	C	0.8 ± 0.2	C
Empicol LZPV/C	1.5 ± 0.3	C	1.4 ± 0.3	C
Empigen OB	1.5 ± 0.6	C	1.4 ± 0.3	C
Empilan CME	13.8 ± 4.9	NC	12.7 ± 2.2	NC
Empilan KB2	7.6 ± 1.1	NC	2.7 ± 0.5	C
Ethomeen T/25	12.3 ± 3.5	NC	13.6 ± 1.2	NC
Genamin KDM-F	6.7 ± 2.8	NC	6.9 ± 3.2	NC
Genapol LRO	1.8 ± 0.8	C	4.8 ± 2.0	C
n-Hexanol	3.6 ± 1.1	C	2.3 ± 0.3	C
Hostaphat KLD	2.9 ± 0.9	C	3.3 ± 0.3	C
Lauric acid	6.9 ± 2.8	NC	14.6 ± 4.1	NC
n-Nonanol	2.5 ± 0.7	C	1.5 ± 1.0	C
Oleic/caprylic acid	10.6 ± 4.1	NC	18.5 ± 6.5	NC
Proxel AB	11.0 ± 1.8	NC	9.4 ± 3.4	NC
Sodium perborate	18.1 ± 5.3	NC	10.2 ± 2.1	NC
Sodium percarbonate	2.2 ± 2.0	C	1.6 ± 0.4	C
Sodium silicate H100	6.2 ± 2.9	NC	6.2 ± 0.9	NC
Triethanolamine	11.2 ± 1.9	NC	11.6 ± 5.3	NC
n-Undecanol	14.2 ± 3.0	NC	9.2 ± 3.0	NC

[a] $n = 6$.

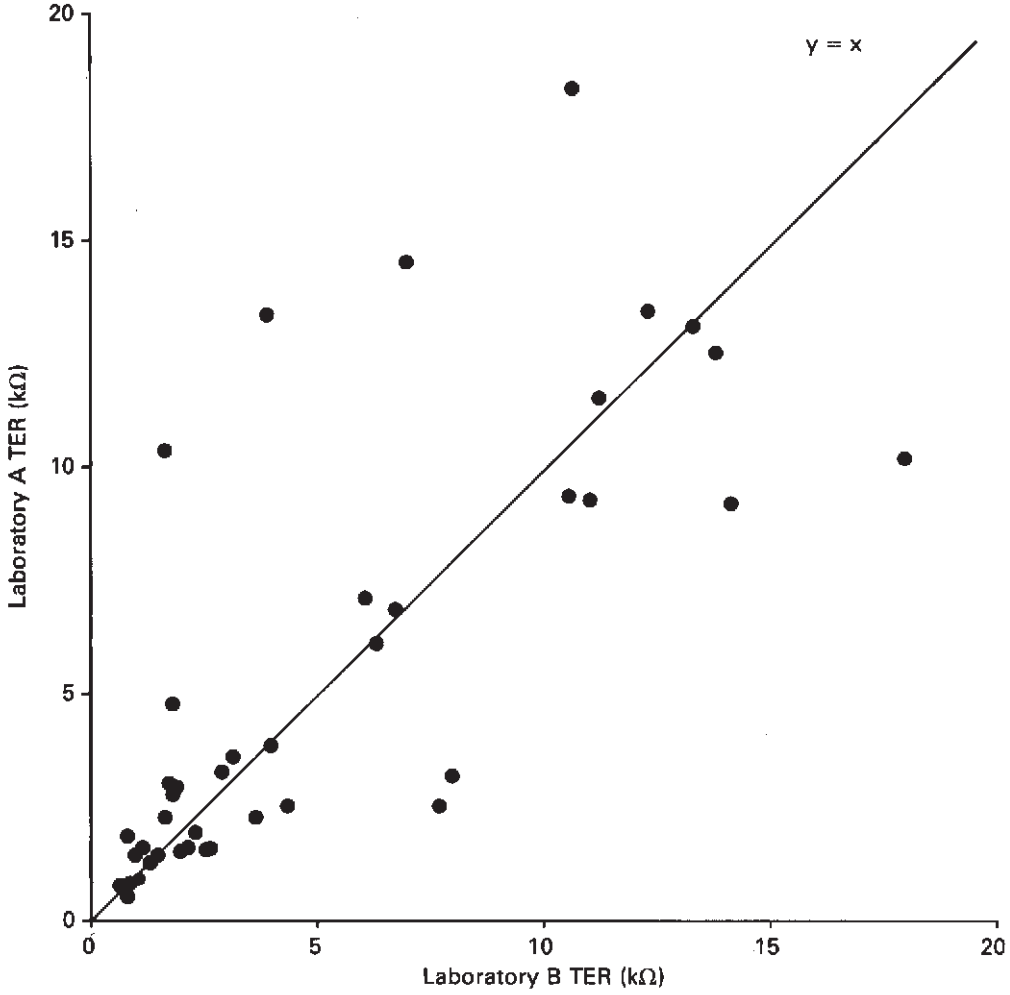
[b] $n = 3$.

C = corrosive; NC = non-corrosive.

The intralaboratory reproducibility (triplicate determinations) of CORROSITEX was very good. The coefficients of variation (CV) for Laboratory A, which recorded breakthrough times only to the nearest minute, ranged from 0–9.1% (mean: 3.2%; n = 23). The results obtained in Laboratory B, which actually recorded data to the nearest hundredth of a minute, were even more reproducible (CV: 0–0.5%; mean: 0.13%; n = 22).

Laboratory A classified 23 materials as corrosive and 15 as non-corrosive; Laboratory B identified 21 materials as corrosive and 14 as non-corrosive (Table V). Empicol LZPV/C was classified differently in the two laboratories, and caprylic acid was identified as non-

Figure 1: Interlaboratory comparison of data from the transcutaneous electrical resistance (TER) assay



corrosive by Laboratory A but did not qualify ("NQ") according to Laboratory B. Similar breakthrough times were reported for the 21 chemicals which were classified as corrosive in both laboratories. Thirteen test materials were identified as non-corrosive by both laboratories.

The interlaboratory comparison of CORROSITEX breakthrough times is shown in Figure 2. For breakthrough times below about 40 minutes, the values obtained in both laboratories were very similar. However, for times greater than 40 minutes, faster breakthrough times were observed in Laboratory A than in Laboratory B. Thus, there could be some systematic difference in the determination of the endpoint between the two laboratories. Further investigation of the effects of environmental conditions on the data generated, such as possible differences due to

Table V: Corrosivity of the test chemicals as determined using the CORROSITEX™ assay

Chemical	Laboratory A		Laboratory B	
	Time (minutes)[a] Mean ± SD	C/NC	Time (minutes)[a] Mean ± SD	C/NC
Corrosives				
Acetic acid (glacial)	21.7 ± 0.6	C	28.5 ± 0.0	C
Acrylic acid	28.0 ± 0.0	C	29.0 ± 0.0	C
Armeen CD	159 ± 5.3	C	212 ± 0.6	C
Armeen TD	> 240	NC	> 240	NC
Arquad 16-50	NQ	?	NQ	?
Arquad DMMCB-50	NQ	?	NQ	?
Bromoacetic acid (8%)	28.7 ± 1.2	C	34.6 ± 0.2	C
Bromoacetic acid (55.6%)	6.3 ± 0.6	C	5.1 ± 0.0	C
Butylamine	28.3 ± 0.6	C	36.8 ± 0.1	C
Capric/caprylic acid	> 240	NC	> 240	NC
Caprylic acid	> 240	NC	NQ	?
Cyclohexylamine	43.3 ± 1.2	C	48.7 ± 0.1	C
1,4-Diaminobutane	26.7 ± 0.6	C	30.9 ± 0.0	C
Dichloroacetic acid	12.7 ± 0.6	C	20.5 ± 0.0	C
Diethylamine	34.0 ± 0.0	C	33.0 ± 0.0	C
Duoquad T50	NQ	?	NQ	?
Formic acid	15.7 ± 0.6	C	18.5 ± 0.0	C
Hexanoic acid	95.3 ± 1.5	C	165 ± 0.4	C
Mercaptoacetic acid	37.7 ± 2.1	C	35.6 ± 0.1	C
Proxel BD	NQ	?	NQ	?
Pyrrolidine	27.7 ± 0.6	C	27.0 ± 0.0	C
Sodium hydroxide	17.0 ± 1.0	C	22.0 ± 0.1	C
Sodium metasilicate	18.0 ± 1.0	C	22.4 ± 0.0	C
Sodium silicate A140	20.0 ± 0.0	C	NT	?
Synprolam 35X2	> 240	NC	NT	?

[a] *Biobarrier breakthrough time, as described in Materials and Methods.*

NQ = not qualified (incompatible with the test system); C = corrosive; NC = non-corrosive; NT = not tested (insufficient sample); ? = cannot be classified.

the ambient temperature at which the assay is conducted, may help explain this discrepancy.

Comparisons of the *in vitro* corrosivity classifications with those assigned to the test chemicals prior to undertaking the prevalidation study (Table I), which are based mainly on *in vivo* corrosivity data, are summarised in Table III. The classifications based on the *in vitro* data obtained by Laboratory A agreed with the assigned (*in vivo*) classifications for 28 of the 38 qualified test chemicals (74%). The corrosivities of six materials (Dequest 2000, Empicol LZPV/C, sodium perborate, sodium percarbonate, sodium silicate H100 and triethanolamine) were overestimated, and those of four materials (Armeen TD, capric/caprylic acid, caprylic acid and Syn-

Table V: continued

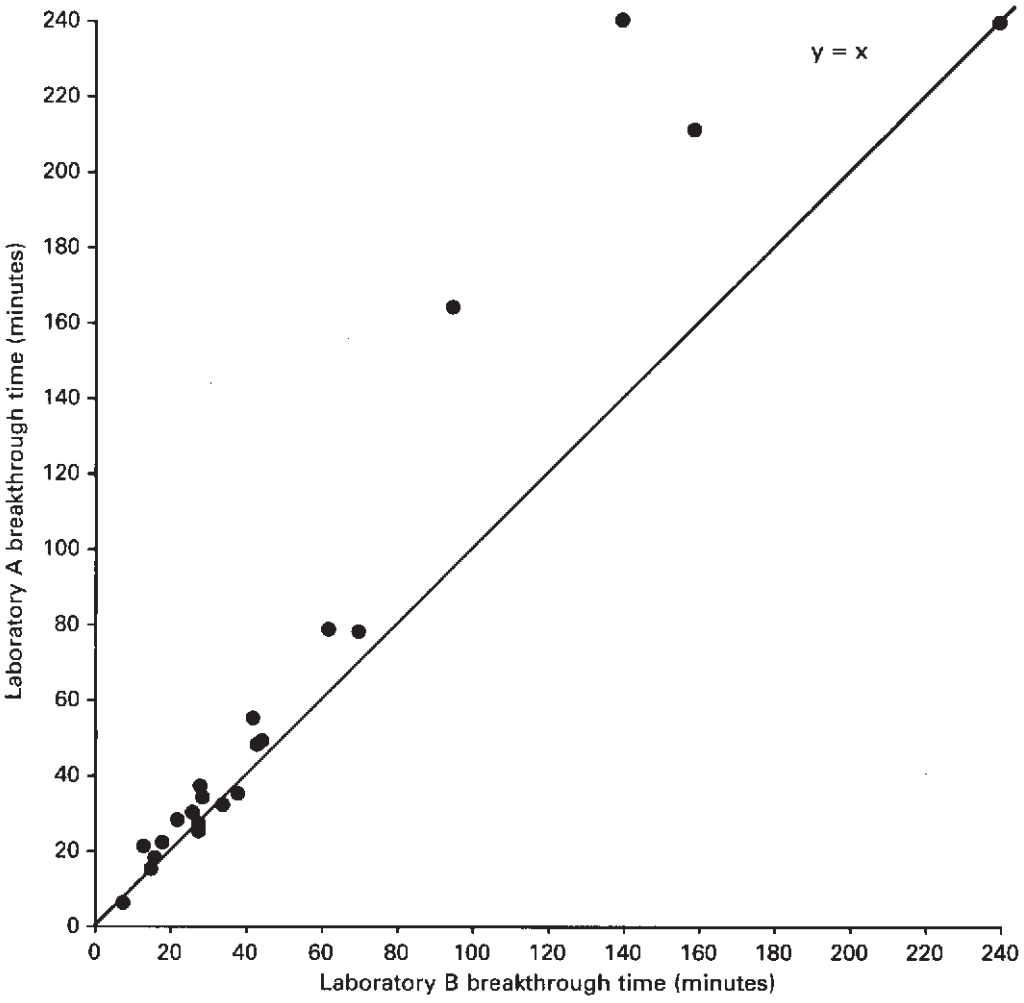
Chemical	Laboratory A Time (minutes)[a] Mean ± SD	Laboratory A C/NC	Laboratory B Time (minutes)[a] Mean ± SD	Laboratory B C/NC
Non-corrosives				
Armeen 2C	>240	NC	> 240	NC
Aromox DMMCD-W	> 240	NC	> 240	NC
Arquad C-33-W	> 240	NC	> 240	NC
Butylbenzene	NQ	?	NQ	?
Dequest 2000	14.3 ± 0.6	C	15.1 ± 0.0	C
Dowanol PNB	NQ	?	NQ	?
Elfan OS 46	NQ	?	NQ	?
Empicol LZPV/C	140 ± 4.0	C	> 240	NC
Empigen OB	> 240	NC	> 240	NC
Empilan CME	> 240	NC	> 240	NC
Empilan KB2	> 240	NC	> 240	NC
Ethomeen T/25	> 240	NC	> 240	NC
Genamin KDM-F	> 240	NC	> 240	NC
Genapol LRO	> 240	NC	> 240	NC
n-Hexanol	NQ	?	NQ	?
Hostaphat KLD	NQ	?	NQ	?
Lauric acid	NQ	?	NQ	?
n-Nonanol	NQ	?	NQ	?
Oleic/caprylic acid	> 240	NC	> 240	NC
Proxel AB	> 240	NC	> 240	NC
Sodium perborate	63.3 ± 0.6	C	78.3 ± 0.1	C
Sodium percarbonate	69.7 ± 4.2	C	77.6 ± 0.0	C
Sodium silicate H100	45.0 ± 0.0	C	49.9 ± 0.0	C
Triethanolamine	42.7 ± 1.5	C	55.3 ± 0.1	C
n-Undecanol	NQ	?	NQ	?

[a] *Biobarrier breakthrough time, as described in Materials and Methods.*

NQ = not qualified (incompatible with the test system); C = corrosive; NC = non-corrosive; NT = not tested (insufficient sample); ? = cannot be classified.

prolam 35X2) were underestimated. The sensitivity and specificity were 81% and 65%, respectively. The classifications based on the *in vitro* data recorded by Laboratory B agreed with the assigned classifications for 28 of the 35 qualified test chemicals (80%). The corrosive effects of five materials (Dequest 2000, sodium perborate, sodium percarbonate, sodium silicate H100 and triethanolamine) were overestimated, while those of two materials (Armeen TD and capric/caprylic acid) were underestimated. The sensitivity and specificity were 89% and 71%, respectively. The materials whose corrosive effects were overestimated by both laboratories were three inorganics, an organic base and an

Figure 2: Interlaboratory comparison of data from the CORROSITEX™ assay



organic acid. Those which were underestimated were organic acids (two) and organic bases (two).

Following the completion of the blind part of this study, IVI determined that the corrosive effects of materials with low acid/alkaline reserve capacities were often overpredicted by CORROSITEX. A screening test was therefore introduced to assign materials to one of four categories: A_1 — high acid content; B_1 — high base content; A_2 — low acid content; and B_2 — low base content. The cut-off breakthrough time for distinguishing between corrosives and non-corrosives was adjusted (from 240 minutes to 45 minutes) for materials designated A_2 or B_2. Thus, if these materials resulted in a change in the CDS within 45 minutes, they were classified as corrosive; if the breakthrough time was greater than 45 minutes, the material was considered to be non-corrosive. Subsequent analysis of the data for the chemicals tested

during this prevalidation study, to incorporate this new procedure for handling materials with low acid/alkaline reserve capacities, resulted in the re-classification of four (Laboratory A) or five (Laboratory B) chemicals (data not shown), and slightly increased the *in vitro–in vivo* concordance values to 79% (Laboratory A) and 83% (Laboratory B).

Skin^2 Model ZK1350 in vitro skin corrosion test

The results obtained using the Skin^2 test are summarised in Tables III and VI. All substances were compatible with the test system. Certain highly viscous test materials were difficult to pipette accurately, and the skin cultures were applied to these test materials with light pressure, to ensure even contact.

Comparison of the percentage viabilities from the three independent laboratories indicates reasonably good reproducibility, although there are several outliers (Figure 3). An analysis of variance (ANOVA) showed no significant evidence of systematic interlaboratory differences based on the percentage viability data ($p > 0.1$). Comparison of the corrosive/non-corrosive classifications (Table VI) indicates agreement between all three laboratories for 30 of the 50 chemicals (i.e. 60%). Pairwise comparisons indicate better agreement between Laboratories B and C (80%) than between Laboratory A and either of the other two Laboratories (A/B, 70%; A/C, 60%). Although the laboratories were familiar with the basic assay procedures, this study was the first time that this specific Skin^2 protocol had been conducted in each of the three laboratories. It is probable that additional experience and training will improve the interlaboratory reproducibility of the results obtained.

The ability of the Skin^2 test to predict the corrosive/non-corrosive classifications of the test chemicals is summarised in Table III. The concordance between the *in vitro* and *in vivo* classifications ranged from 70–92% for the three Laboratories. There were four false negative results in either two or all three of the laboratories: caprylic acid, mercaptoacetic acid, Proxel BD and sodium silicate. There were three false positive results in either two or all of the laboratories: Arquad C-33-W, Empigen OB and Empilan KB2. Thus, the Skin^2 test is promising in terms of its ability to identify corrosives and non-corrosives.

The interlaboratory comparisons were acceptable, but formal training of the participants is recommended prior to a formal validation study, to ensure uniform conduct of the test across laboratories. To determine intralaboratory versus interlaboratory sources of variability, it would be useful to include more repeat experiments (for example, three independent experiments) with each chemical in each laboratory, rather than undertaking a single experiment with quadruplicate cell cultures.

General Discussion

As described in the Introduction, the purpose of this study was to evaluate three *in vitro* tests for their abilities to detect substances which are corrosive to skin. This was not a validation study in terms of any of the currently accepted criteria, such as those described by Balls *et al.* (9). The principal objective was a relative comparison of the three assays, to determine which, if any, had been sufficiently well-developed to be considered for inclusion in a formal validation study.

No attempt was made to select the test materials from a wide range of chemical classes as, ideally, would be the case for a formal validation study. Test substances were selected on the basis of their skin corrosivity potentials and not their irritancy potentials, and so no conclusions can be drawn regarding the irritant effects of the test materials.

The relative performances of the tests have been assessed by comparing their interlaboratory reproducibilities and the values for their sensitivity, specificity and concordance (Table III).

Interlaboratory reproducibility

TER assay

For the corrosive substances, only two were identified differently in the two laboratories; similarly, for the non-corrosives, only two were classified differently. Thus, for the 50 substances tested, both laboratories agreed in their assessment for 46 materials (i.e. 92%).

CORROSITEX

The assessment of CORROSITEX has to take into account those test substances which do not qualify (NQ) in the assay. In some cases, a substance may not qualify in one laboratory whereas another laboratory can obtain a result (for example, caprylic acid; Table V). Therefore,

Table VI: Corrosivity of the test chemicals as determined using the Skin^{2TM} assay

Chemical	Laboratory A Viability (%)[a] Mean ± SD[b]	C/NC	Laboratory B Viability (%)[a] Mean ± SD[b]	C/NC	Laboratory C Viability (%)[a] Mean ± SD[b]	C/NC
Corrosives						
Acetic acid (glacial)	4.1 ± 14.7	C	5.9 ± 7.8	C	24.3 ± 13.8	C
Acrylic acid	0 ± 1.7	C	1.9 ± 1.7	C	4.2 ± 2.4	C
Armeen CD	18.4 ± 8.8	C	7.4 ± 2.1	C	7.2 ± 1.5	C
Armeen TD	48.8 ± 13.0	C	15.5 ± 3.7	C	17.9 ± 2.9	C
Arquad 16-50	98.0 ± 7.7	NC	61.0 ± 14.8	C	50.3 ± 12.6	C
Arquad DMMCB-50	31.8 ± 20.4	C	44.3 ± 2.8	C	44.6 ± 20.7	C
Bromoacetic acid (8%)	47.6 ± 7.8	C	33.5 ± 12.2	C	40.2 ± 4.6	C
Bromoacetic acid (55.6%)	2.7 ± 8.2	C	7.3 ± 4.2	C	15.6 ± 7.9	C
Butylamine	0 ± 1.4	C	1.4 ± 0.9	C	2.3 ± 0.9	C
Capric/caprylic acid	72.8 ± 32.6	C	54.5 ± 4.4	C	69.9 ± 18.2	C
Caprylic acid	88.8 ± 22.3	NC	98.8 ± 43.2	NC	78.0 ± 25.4	C
Cyclohexylamine	1.3 ± 3.1	C	12.6 ± 3.5	C	15.2 ± 15.4	C
1,4-Diaminobutane	101 ± 24.3	NC	64.6 ± 31.7	C	61.6 ± 27.8	C
Dichloroacetic acid	22.5 ± 14.3	C	34.5 ± 29.4	C	14.6 ± 7.9	C
Diethylamine	0.7 ± 0.9	C	26.0 ± 13.7	C	10.0 ± 7.8	C
Duoquad T50	85.1 ± 9.1	NC	68.5 ± 22.4	C	79.9 ± 15.3	C
Formic acid	80.6 ± 27.9	NC	13.4 ± 20.6	C	24.2 ± 18.8	C
Hexanoic acid	85.8 ± 29.2	NC	63.0 ± 25.8	C	42.1 ± 7.7	C
Mercaptoacetic acid	95.0 ± 36.2	NC	59.0 ± 17.3	C	84.2 ± 12.0	NC
Proxel BD	95.4 ± 23.5	NC	66.1 ± 4.3	C	102 ± 11.7	NC
Pyrrolidine	8.9 ± 17.9	C	30.2 ± 29.6	C	9.1 ± 4.0	C
Sodium hydroxide	61.2 ± 40.0	C	55.7 ± 33.2	C	30.1 ± 10.9	C
Sodium metasilicate	65.7 ± 10.4	C	35.3 ± 28.6	C	106 ± 9.1	NC
Sodium silicate A140	82.4 ± 12.0	NC	73.7 ± 13.0	C	102 ± 6.5	NC
Synprolam 35X2	47.4 ± 22.3	C	29.2 ± 3.9	C	56.0 ± 12.6	C

[a] *Cell viability, as a percentage of the control, was determined following topical treatment of human skin cultures with test chemical for 10 seconds.*

[b] *n = 4; standard deviations (SD) for four skin samples per test material, conducted in parallel, are given. Since these values have been calculated with respect to four independent controls (mean = 100% viability), which themselves showed variation (SD =± 10%), the SD values quoted should not be used for further statistical calculations.*

C = corrosive (≤ 80% viability); NC = non-corrosive (> 80% viability).

Table VI: continued

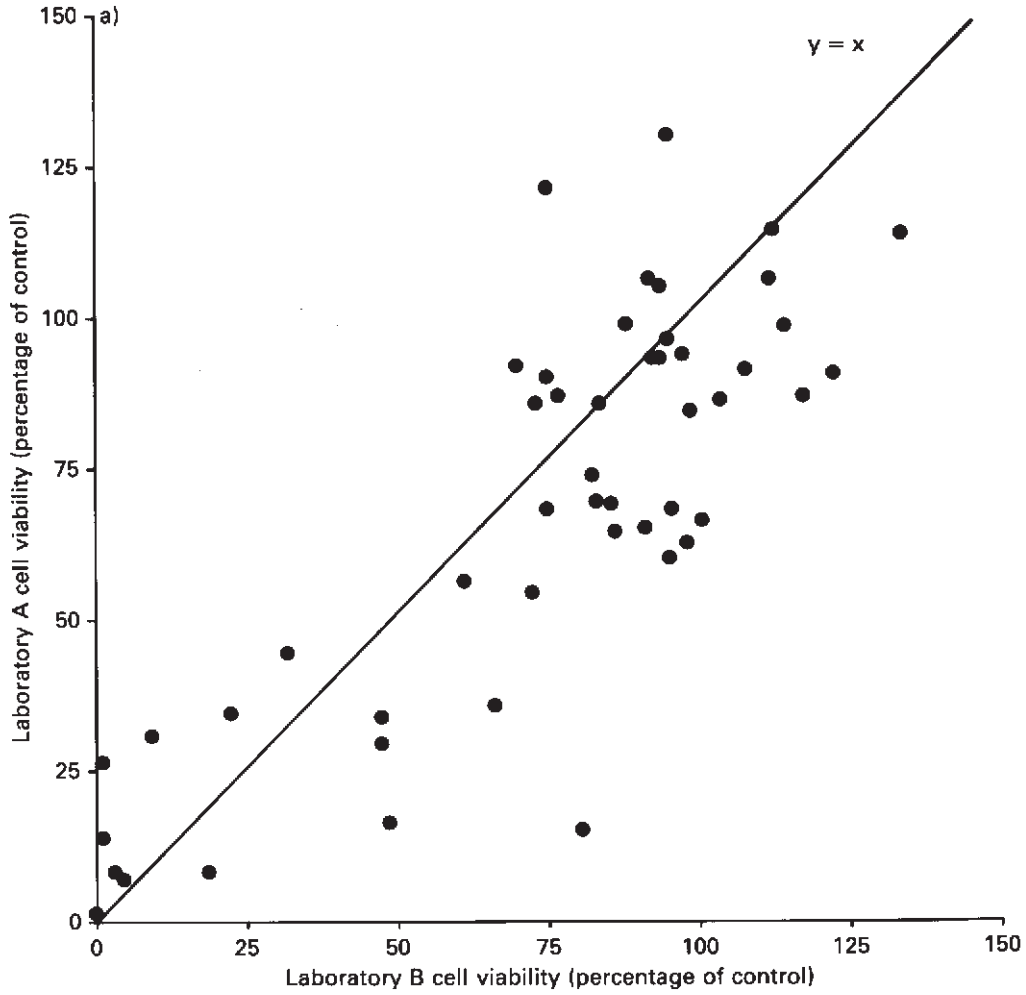
Chemical	Laboratory A Viability (%)[a]		Laboratory B Viability (%)[a]		Laboratory C Viability (%)[a]	
	Mean ± SD[b]	C/NC	Mean ± SD[b]	C/NC	Mean ± SD[b]	C/NC
Non-corrosives						
Armeen 2C	108 ± 26.9	NC	90.0 ± 26.0	NC	96.1 ± 5.7	NC
Aromox DMMCD-W	123 ± 7.4	NC	89.4 ± 10.5	NC	62.0 ± 10.1	C
Arquad C-33-W	83.0 ± 12.7	NC	68.7 ± 30.3	C	63.5 ± 22.9	C
Butylbenzene	134 ± 17.4	NC	112 ± 37.3	NC	79.9 ± 19.3	C
Dequest 2000	95.0 ± 28.3	NC	95.4 ± 17.0	NC	85.4 ± 14.8	NC
Dowanol PNB	75.0 ± 7.8	C	121 ± 24.4	NC	113 ± 32.4	NC
Elfan OS 46	93.5 ± 7.4	NC	92.3 ± 7.3	NC	103 ± 5.4	NC
Empicol LZPV/C	90.7 ± 25.5	NC	64.1 ± 6.8	C	108 ± 12.3	NC
Empigen OB	75.5 ± 5.1	C	88.8 ± 4.3	NC	77.8 ± 13.1	C
Empilan CME	73.3 ± 15.7	C	84.5 ± 20.0	NC	102 ± 7.9	NC
Empilan KB2	75.0 ± 32.1	C	66.3 ± 6.1	C	73.3 ± 8.9	C
Ethomeen T/25	93.5 ± 21.0	NC	105 ± 25.3	NC	73.5 ± 7.9	C
Genamin KDM-F	77.3 ± 9.3	C	85.6 ± 17.3	NC	110 ± 3.4	NC
Genapol LRO	69.9 ± 24.8	C	90.5 ± 19.4	NC	95.0 ± 17.9	NC
n-Hexanol	103 ± 6.5	NC	85.2 ± 17.7	NC	87.4 ± 4.6	NC
Hostaphat KLD	83.7 ± 17.3	NC	84.5 ± 8.7	NC	94.2 ± 3.8	NC
Lauric acid	92.4 ± 7.8	NC	92.2 ± 21.5	NC	99.7 ± 14.9	NC
n-Nonanol	115 ± 15.0	NC	98.3 ± 15.7	NC	92.4 ± 12.7	NC
Oleic/caprylic acid	113 ± 5.7	NC	113 ± 25.6	NC	120 ± 14.3	NC
Proxel AB	98.6 ± 5.1	NC	82.9 ± 6.1	NC	104 ± 10.6	NC
Sodium perborate	95.3 ± 24.6	NC	130 ± 38.0	NC	108 ± 7.3	NC
Sodium percarbonate	112 ± 19.0	NC	106 ± 16.9	NC	93.7 ± 5.6	NC
Sodium silicate H100	91.7 ± 10.9	NC	106 ± 18.6	NC	88.4 ± 16.7	NC
Triethanolamine	117 ± 13.7	NC	85.6 ± 11.1	NC	102 ± 9.6	NC
n-Undecanol	97.5 ± 11.2	NC	93.5 ± 5.5	NC	103 ± 7.5	NC

[a] *Cell viability, as a percentage of the control, was determined following topical treatment of human skin cultures with test chemical for 10 seconds.*

[b] *n = 4; standard deviations (SD) for four skin samples per test material, conducted in parallel, are given. Since these values have been calculated with respect to four independent controls (mean = 100% viability), which themselves showed variation (SD =± 10%), the SD values quoted should not be used for further statistical calculations.*

C = corrosive (≤ 80% viability); NC = non-corrosive (> 80% viability).

Figure 3: Interlaboratory comparison of data from the Skin^{2TM} assay

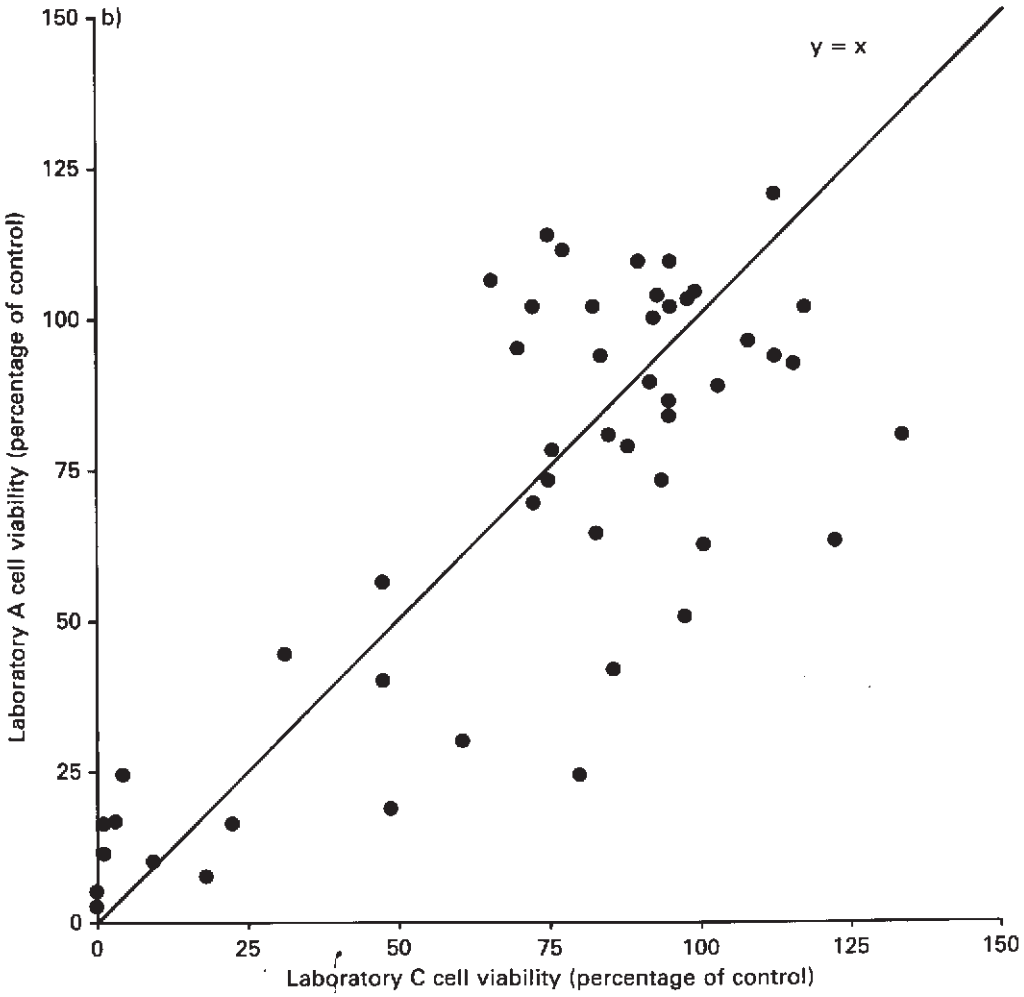


for this analysis NQ is taken to be an outcome of the assay, and is included in the interlaboratory comparison. Of the 25 corrosives tested, only one was classified differently by the two laboratories. However, two of the corrosive substances were not tested in one of the laboratories because there was insufficient sample available. For the non-corrosives, again only one substance was classified differently. Thus, for the 48 substances tested in both laboratories, the assessment agreed for 46 (i.e. 96%).

Skin^2 Model ZK1350 in vitro skin corrosion test

Three laboratories carried out the Skin^2 assay, whereas only two laboratories conducted both of the other tests. Agreement on the corrosive/non-corrosive classification across all three laboratories was obtained for 30 (i.e. 60%) of the test substances. Pairwise comparisons between the three laboratories indicated higher degrees of agreement of the results obtained (60%, 70% and 80%). Whilst this is better than the comparison across the three laboratories,

Figure 3: continued

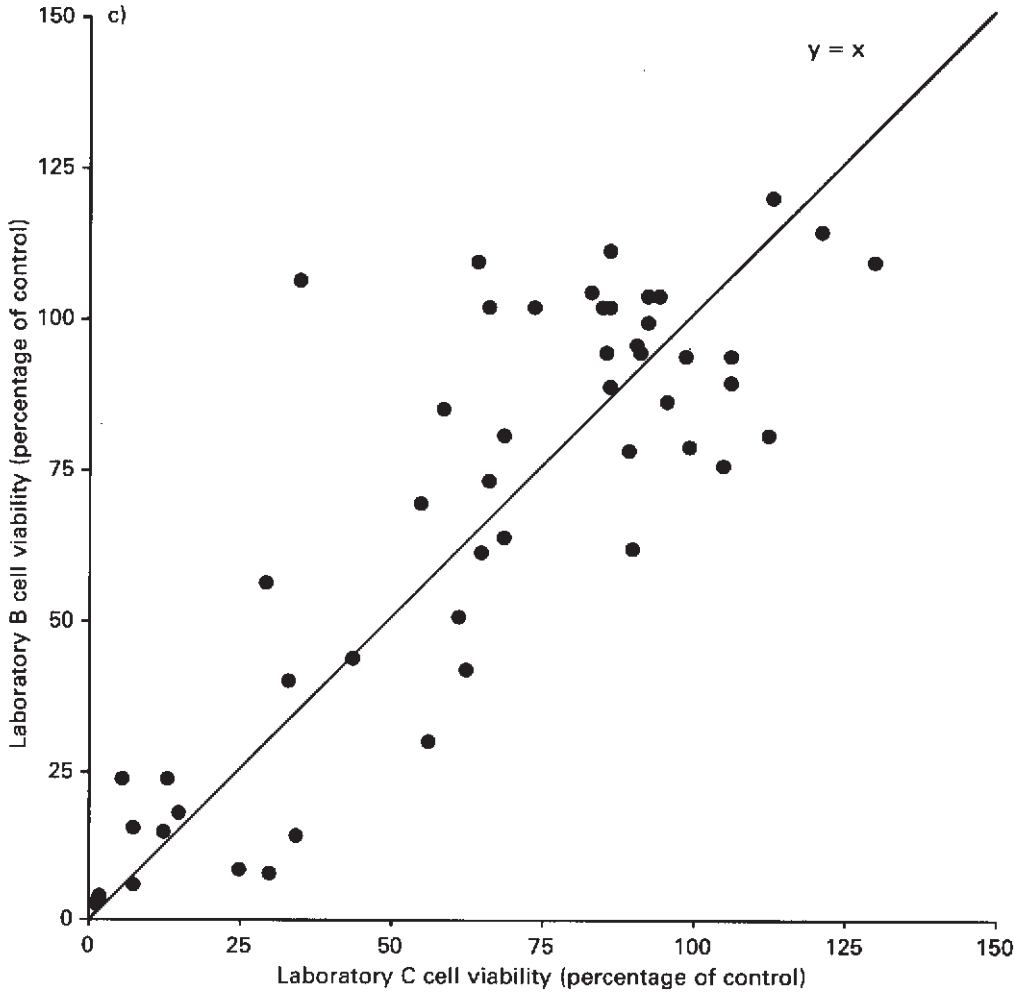


the agreement is still markedly lower than that for the TER and CORROSITEX assays. The reasons for the relatively low reproducibility of the Skin[2] assay were not investigated as part of this study, but may relate to different degrees of training and experience across the participating laboratories. It is recommended that possible sources of interlaboratory variation be identified and resolved prior to the inclusion of the Skin[2] assay in a formal validation study.

Sensitivity/specificity/concordance

For assays which provide results that can be assigned to one of two categories, the analysis proposed by Cooper *et al.* (23) may be used. The analysis provides several indices which can be used to assess the overall performance of assays in identifying correctly the hazard of test substances. Although this study was designed to assess the relative performances of

Figure 3: continued



the three *in vitro* tests, the indices derived are also useful in this context. The *in vitro–in vivo* comparisons for all the tests in each laboratory are given in Table III.

Concordance is a measure of the ability of an assay to assign a test substance to its true category. Sensitivity is a measure of the ability of an assay to identify correctly the "active" test substances. For these indices, there were no appreciable differences between the three *in vitro* tests. Specificity is a measure of the ability of an assay to discriminate between "active" and "inactive" test substances. In this particular case, it is assessed by scoring the number of non-corrosives identified correctly. The highest specificity (76–88%) was found with the Skin[2] assay. CORROSITEX performed moderately well, giving specificities of

65% and 71%, whilst the TER assay showed relatively low specificity (values of 48% and 56%) because it gave too many false positives.

Conclusions and Recommendations

On the basis of the results of this study, it is impossible to conclude that any one test performed better than the other two. Each method has both strengths and weaknesses. The TER assay showed high sensitivity, but was characterised by relatively low specificity (i.e. too many false positives). As with all *ex vivo* tests, the TER assay has both the advantage of close relevance to the *in vivo* animal model and the disadvantage of requiring animal tissue. CORROSITEX gave high concordance and specificity values, but its overall utility is reduced by the significant proportion of non-qualifying test materials. The overall performance of the Skin^2 assay was quite creditable, but one laboratory reported significantly different results from the other two, suggesting that there are technical issues which should be addressed.

There are well-established Standard Operating Procedures for all of the tests, and it has been demonstrated that they can all be transferred from one laboratory to another. A clear, unambiguous endpoint is defined for each *in vitro* test, in order to distinguish between corrosive and non-corrosive substances. Although two of the methods, CORROSITEX and Skin^2, are marketed as having the capability to distinguish between degrees of corrosive effects (for example, to assign chemicals to the UN packing groups I–III), this was not addressed within this prevalidation study. To further clarify the state of optimisation and evaluation of the three tests, it is recommended that the following additional work is carried out:

1. investigations are undertaken to try to reduce the false positive rate of the TER assay;
2. the TER assay is assessed for its ability to distinguish between chemicals with different degrees of corrosivity (for example, the UN packing groups or EU classification groups R34 and R35);
3. investigations are undertaken to try to modify the CORROSITEX CDS, specifically to reduce the number of non-qualifying test materials, and to reduce the subjectivity of the assay;
4. investigations are conducted with the Skin^2 assay with the aim of reducing the interlaboratory variability (in terms of predicting corrosive/non-corrosive classifications); and
5. the laboratories conducting the Skin^2 assay should consider re-evaluating the threshold value for distinguishing between corrosives and non-corrosives.

Depending on the outcome of this additional work, it is recommended that a formal validation study is then conducted involving the TER, CORROSITEX and Skin^2 assays. For such a study, a suitable set of test materials should be selected, probably from the reference chemical set which is currently being prepared by an ECETOC Task Force (A.P. Walker, personal communication). It is further recommended that such a study be undertaken in the light of recent recommendations concerning the conduct of validation studies (9), and taking into account the lessons learned during previous validation studies (24).

Furthermore, future validation studies should, wherever possible, incorporate knowledge from structure-activity relationships (qualitative and quantitative). Such information may be useful in guiding the selection of appropriate test chemicals, so that they cover different mechanistic classes and represent an even spread of *in vivo* potencies (25, 26). Appendix A provides an example of the application of quantitative structure-activity relationships (QSAR) to the prediction of skin corrosivity.

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References

1. Anon. (1994). ECVAM News & Views. *ATLA* **22**, 7–11.
2. Draize, J.H., Woodand, G. & Calvery, H.O. (1944). Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics* **82**, 377–390.
3. Anon. (1991). Code of Federal Regulations. Method of Testing Corrosion to the Skin. *Transportation Title* **49**, Part 173.136, Appendix A. Washington, DC: Office of the Federal Register, National Archives and Records Administration.
4. Anon. (1992). *OECD Guideline for Testing of Chemicals*, Number 404. (Adopted by the Council on 17 July 1992). 6pp. Paris: OECD.
5. Anon. (1977). United Nations Economic and Social Council. Joint meeting of the RID safety committee and the group of experts on the transportation of dangerous goods. *Trans/GE 15/R* **274**, 2.
6. Anon. (1983). Guide to the classification and labelling of dangerous substances and preparations. Criteria for the choice of chemicals indicating special risks (R phrases) and safety advice (S phrases). *Official Journal of the European Communities* **L257**, 13–20.
7. Oliver, G.J.A., Pemberton, M.A. & Rhodes, C. (1988). An *in vitro* model for identifying skin corrosive chemicals. I. Initial validation. *Toxicology in Vitro* **2**, 7–17.
8. Botham, P.A., Hall, T.J., Dennett, R., McCall, J.C., Basketter, D.A., Whittle, E., Cheesman, M., Esdaile, D.J. & Gardner, J. (1992). The skin corrosivity test *in vitro*: results of an interlaboratory trial. *Toxicology in Vitro* **6**, 191–194.
9. Balls, M., Blaauboer, B.J., Fentem, J.H., Bruner, L., Combes, R.D., Ekwall, B., Fielder, R.J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., 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.
10. Gordon, V.C., Harvell, J.D. & Maibach, H.I. (1994). Dermal corrosion, the CORROSITEX system: a DOT accepted method to predict corrosivity potential of test materials. In *Alternative Methods in Toxicology, Volume 10, In Vitro Skin Toxicology: Irritation, Phototoxicity, Sensitization* (ed. A. Rougier, A.M. Goldberg & H.I. Maibach), pp. 37–45. New York: Mary Ann Liebert.
11. de Wever, B. & Rheins, L.A. (1994). Skin^{2TM}: an *in vitro* human skin analogue. In *Alternative Methods in Toxicology, Volume 10, In Vitro Skin Toxicology: Irritation, Phototoxicity, Sensitization* (ed. A. Rougier, A.M. Goldberg & H.I. Maibach), pp. 121–131. New York: Mary Ann Liebert.
12. Jacobs, G. & Martens, M. (1987). Evaluation of the test method for skin irritation as prescribed by OECD and EEC. *Journal of Toxicology — Cutaneous and Ocular Toxicology* **6**, 215–226.
13. Anon. (1984). Commission Directive 84/449/EEC of 25 April 1984 adapting to technical progress for the sixth time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances: skin irritation. *Official Journal of the European Communities* **L251**, 106–108.
14. Oliver, G.J.A., Pemberton, M.A. & Rhodes, C. (1986). An *in vitro* skin corrosivity test: modifications and validation. *Food and Chemical Toxicology* **24**, 507–512.
15. Oliver, G.J.A. (1990). The evaluation of cutaneous toxicity: past and future. In *Skin Pharmacology and Toxicology: Recent Advances* (ed. C.L. Galli, C.N. Hensby & M. Marinovich), pp. 147-163. New York: Plenum Press.
16. Barlow, A., Hirst, R.A., Pemberton, M.A., Rigden, A., Hall, T.J., Botham, P.A. & Oliver, G.J.A. (1991). Refinement of an *in vitro* test for the identification of skin corrosive chemicals. *Toxicology Methods* **1**, 106–115.
17. Naughton, G.K., Jacobs, L. & Naughton, B.A. (1989). A physiologic skin model for *in vitro* toxicity studies. In *Alternative Methods in Toxicology, Volume 7, In Vitro Toxicology: New Directions* (ed. A.M. Goldberg), pp. 183–189. New York: Mary Ann Liebert.
18. Perkins, M.A. & Osborne, R. (1993). Development of an *in vitro* method for skin corrosion testing. *Journal of Investigative Dermatology* **100**, 535.
19. Slivka, S.R., Landeen, L., Zimber, M.P. & Bartel, R.L. (1993). Biochemical characterization, barrier function, and drug metabolism of an *in vitro* skin model. *Journal of Investigative Dermatology* **100**, 39–45.
20. Contard, P., Bartel, R.L., Jacobs, L. 2nd, Perlish, J.S., MacDonald, E.D. 2nd, Handler, L., Cone, D. & Fleischmajer, R. (1993). Culturing keratinocytes and fibroblasts in a three-dimensional mesh results in epidermal differentiation and formation of a basal lamina-anchoring zone. *Journal of Investigative Dermatology* **100**, 34–38.
21. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55–63.
22. Triglia, D., Sherard Braa, S., Yonan, C. & Naughton, G.K. (1991). Cytotoxicity testing using neutral red and MTT assays on a three-dimensional human skin substrate. *Toxicology in Vitro* **5**, 573–578.
23. Cooper, J.A., Saracci, R. & Cole, P. (1979). Describing the validity of carcinogen screening tests. *British Journal of Cancer* **39**, 87–89.
24. Fentem, J.H., Prinsen, M.K., Spielmann, H., Walum, E. & Botham, P.A. Validation: lessons learned from practical experience. *Toxicology in Vitro*, in press.
25. Chamberlain, M. & Barratt, M.D. (1995). Practical applications of QSAR to *in vitro* toxicology illustrated by consideration of eye irritation. *Toxicology in Vitro*, in press.
26. Barratt, M.D., Castell, J.V., Chamberlain, M., Combes, R.D., Dearden, J.C., Fentem, J.H., Gerner, I., Giuliani, A., Gray, T.J.B., Livingstone, D.J., Provan, W.McL., Rutten, A.A.J.J.L., Verhaar, H.J.M. & Zbinden, P. (1995). The integrated use of alternative approaches for predicting toxic hazard. The report and recommendations of an ECVAM workshop. *ATLA*, in press.

Appendix A: Quantitative Structure-activity Relationships for Skin Corrosivity

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Summary

Quantitative structure-activity relationships (QSARs) have been derived which relate skin corrosivity data on organic chemicals (acids, bases, phenols and neutral chemicals) to their log P (log [octanol/water partition coefficient]) values, molecular volumes, melting points and pKa/pKb values. Data sets were analysed using principal components analysis. For each group of chemicals, plots of the first two principal components of the above parameters, which broadly model skin permeability and cytotoxicity, showed that the analysis was able to discriminate well between corrosive and non-corrosive chemicals. The QSARs derived should be useful for predicting the skin corrosivity potentials of new or untested chemicals within these categories.

Introduction

The principles of quantitative structure-activity relationships (QSAR) are based on the premise that the properties of a chemical are implicit in its molecular structure. It therefore follows that, if the mechanism for the activity of a group of chemicals can be elucidated and relevant parameters can be measured or calculated, then, in principle, a structure-activity relationship can be established.

A substance which is classified as a skin "corrosive" is one which causes irreversible destruction of the tissue when applied to rabbit skin for a period of up to four hours, as defined in the EU Annex V method (1). Within the set of 50 chemicals used for the prevalidation trial of *in vitro* tests for skin corrosivity, there are a number of different classes of chemicals working via different mechanisms.

1. Inorganic acids, bases and oxidising agents: these are expected to have low skin permeabilities by virtue of their high polarities. They are possibly corrosive because they are able to erode the stratum corneum to get to the tissue beneath.
2. Anionic and cationic surfactants: these also have low skin permeabilities due to the presence of charged headgroups and to their large molecular volumes. As a category, anionic surfactants do not appear to be corrosive. Cationics are stronger surfactants than anionics, and they are also more cytotoxic. Corrosivity may result from solubilisation of the stratum corneum by the surfactant.
3. Organics: their skin permeabilities are generally greater than those for inorganics and surfactants because of their greater hydrophobicities. It is postulated that corrosivity results from the chemical first penetrating the skin; if it is sufficiently cytotoxic, then the underlying cells are killed.

Corrosivity appears to be a property of some acids, bases and phenols; neutral molecules do not appear to be corrosive. Of the chemicals used in the prevalidation study, six are inorganics, eight are surfactants, two are mixtures and 30 are organics (of which three are duplicated). The QSARs described below are based on these 30 organic chemicals (Table I), and on additional sets of organic acids (Table II), bases (Table III) and phenols (Table IV). The parameters required to model skin corrosivity are the same as those which are used to model percutaneous absorption, together with a measure of cytotoxicity (pKa, i.e. $-\log_{10}$ [dissociation constant]).

The relationship between the physicochemical properties of chemicals and their skin permeabilities has recently been explored by Flynn (2), who was able to demonstrate that, for groups of molecules of similar sizes, algorithms based on log P values can be used to obtain a first estimate of skin permeability coefficients. This work was refined further by Potts & Guy (3), who showed that the human *in vitro* permeability coefficients for over 90 chemicals could be reasonably represented by

Table I: Skin corrosivity QSARs: descriptors for data set of 30 chemicals

	Chemical	C/NC	Log P	Mol. vol. (Å^3)	m.p. (°C)	pKa/pKb[a]
1.	Armeen CD[b]	C	5.238	179.0	37	3.37
2.	Armeen TD[c]	C	7.382	229.5	38	3.40
3.	Ethomeen T/25[d]	NC	5.144	759.1	37	5.50
4.	Aromox DMMCDW	NC	7.377	216.4	37	14.0
5.	*n*-Hexanol	NC	1.952	94.67	37	14.0
6.	Butylbenzene	NC	4.212	122.5	37	14.0
7.	*n*-Nonanol	NC	3.560	130.4	37	14.0
8.	*n*-Undecanol	NC	4.632	162.5	37	14.0
9.	Acetic acid	C	− 0.319	46.16	37	4.75
10.	Hexanoic acid	C	1.773	97.06	37	4.88
11.	Triethanolamine	NC	− 2.677	122.4	37	6.23
12.	Dichloroacetic acid	C	0.178	80.97	37	1.48
13.	Bromoacetic acid	C	− 0.131	69.39	37	2.69
14.	Mercaptoacetic acid	C	− 0.038	66.24	37	3.33
15.	Formic acid	C	− 0.641	34.57	37	3.75
16.	Acrylic acid	C	0.103	55.84	37	4.25
17.	1,4-Diaminobutane	C	− 0.70	82.55	37	2.85
18.	Pyrrolidine	C	0.253	62.37	37	2.73
19.	Cyclohexylamine	C	1.324	89.72	37	3.34
20.	Diethylamine	C	0.709	69.72	37	3.51
21.	Butylamine	C	0.950	72.11	37	3.23
22.	Caprylic acid	C	2.845	122.0	37	4.89
23.	Lauric acid	NC	4.989	180.5	46	4.90
24.	Armeen 2C	NC	11.43	335.4	47	3.50
25.	Dowanol PNB	NC	1.119	119.4	37	14.0
26.	Empilan KB2	NC	5.002	245.5	37	14.0
27.	Empilan CME	NC	3.657	219.7	68	14.0
28.	Dequest 2000	C[e]	− 3.133	185.7	37	1.85
29.	Proxel AB	NC	1.532	102.2	60	14.0
30.	Synprolam 35X2	C	5.151	275.5	37	5.50

[a] *pKa values are for acids; pKb values are for bases (pKb = 14.0 − pKa); neutral chemicals, i.e. those without acidic or basic groups, are given a value of 14.0.*

[b] *Cocoamine, modelled as n-dodecylamine.*

[c] *Tallowamine, modelled as n-hexadecylamine.*

[d] *Polyoxyethylene(15)tallowamine, modelled as n-polyoxyethylene(15)-n-hexylamine.*

[e] *Classification is based on results from a 24-hour rabbit covered patch test.*

C = corrosive; NC = non-corrosive; Mol. vol. = molecular volume; m.p. = melting point.

Table II: Skin corrosivity QSARs: descriptors for data set of 20 organic acids

	Chemical	C/NC	Log P	Mol. vol. (Å^3)	m.p. (°C)	pKa
1.	Acetic acid	C[a]	− 0.319	46.16	37	4.75
2.	Hexanoic acid	C	1.773	97.06	37	4.88
3.	Dichloroacetic acid	C[a]	0.178	80.97	37	1.48
4.	Bromoacetic acid	C[a]	− 0.131	69.39	37	2.69
5.	Mercaptoacetic acid	C	− 0.038	66.24	37	3.75
6.	Formic acid	C[a]	− 0.641	34.57	37	3.75
7.	Acrylic acid	C[a]	0.103	55.84	37	4.25
8.	Caprylic acid	C	2.845	122.0	37	4.89
9.	Lauric acid	NC	4.989	180.5	46	4.90[b]
10.	Myristic acid	NC	6.061	210.5	51	4.90[b]
11.	Benzoic acid	NC	1.780	90.16	123	4.19
12.	2,4,6-Trichlorophenol	NC[a]	3.694	123.2	66	6.00
13.	2-Bromobenzoic acid	NC	2.099	101.6	150	2.84
14.	4-Nitrophenylacetic acid	NC	1.134	119.9	155	3.85
15.	Citric acid	NC	− 3.086	132.1	154	3.14
16.	Decanoic acid	NC	3.917	151.1	37	4.90[b]
17.	Lactic acid	C	− 0.621	67.39	37	3.08
18.	Oxalic acid	C[a]	0.452	59.91	106	1.23
19.	Salicylic acid	NC	1.997	92.64	160	2.97
20.	*trans*-Cinnamic acid	NC	2.066	106.8	134	4.44

[a] *Classifications are taken from Annex 1 of the EU Dangerous Substances Directive (1); all other classifications are taken from suppliers' data.*

[b] *Extrapolated values.*

C = corrosive; NC = non-corrosive; Mol. vol. = molecular volume; m.p. = melting point.

a multiple regression relationship involving log P values and molecular weights ($r^2 = 0.67$). Recent work at Unilever ESL (4) has shown that the permeability coefficients for 60 of the chemicals investigated by Potts & Guy (3) can be modelled by multiple regression analysis using log P values, molecular volumes and melting points ($r^2 = 0.904$). The melting point can be used to compute aqueous solubility when linked with the log P value (5).

In the work described below, the three parameters used in the previous study (4) are used to model the skin permeability part of the mechanism of corrosivity, while pKa/pKb values (pKb = 14 − pKa) are used as a measure of the cytotoxicity of a particular chemical. The relevance of the pKa value to the mechanism of cytotoxicity is discussed later.

Materials and Methods

Molecules were constructed using Sybyl 6.0 software (Tripos Associates, Bracknell, Berks., UK). After energy minimisation and calculation of log P values, using the CHEMICALC system (6), the molecular structures were imported into the TSAR spreadsheet (Oxford Molecular Ltd, Sandford-on-Thames, Oxon, UK), where molecular volumes were calcul-

Table III: Skin corrosivity QSARs: descriptors for data set of 21 organic bases

	Chemical	C/NC	Log P	Mol. vol. (Å^3)	m.p. (°C)	pKb
1.	Armeen CD[a]	C	5.238	179.0	37	3.37
2.	Armeen TD[b]	C	7.382	229.5	38	3.40
3.	Ethomeen T/25[c]	NC	5.144	759.1	37	5.50[d]
4.	Triethanolamine	NC	− 2.677	122.4	37	6.23[e]
5.	1,4-Diaminobutane	C	− 0.70	82.55	37	2.85
6.	Pyrrolidine	C	0.253	62.37	37	2.73
7.	Cyclohexylamine	C[f]	1.324	89.72	37	3.34
8.	Diethylamine	C	0.709	69.72	37	3.51
9.	Butylamine	C	0.950	72.11	37	3.23
10.	Dicocoamine	NC	11.43	335.4	47	3.50[d]
11.	Pyridine	NC	0.653	57.03	37	8.75
12.	3-Picoline	NC	1.072	69.99	37	8.32
13.	4-Picoline	NC[f]	1.072	70.06	56	7.98
14.	Acridine	NC	3.539	118.5	110	8.42
15.	Benzylamine	C[f]	1.087	83.36	37	4.67
16.	Imidazole	NC	− 0.079	45.71	91	7.05
17.	Isoquinoline	NC	1.847	87.67	37	8.58
18.	3-Toluidine	NC	1.327	82.60	37	9.27
19.	Morpholine	C[f]	− 0.602	70.64	37	5.67
20.	Quinoline	NC	2.096	87.70	37	9.10
21.	2,3-Lutidine	NC	1.561	82.94	37	7.43

[a] *Cocoamine, modelled as n-dodecylamine.*

[b] *Tallowamine, modelled as n-hexadecylamine.*

[c] *Polyoxyethylene(15)tallowamine, modelled as n-polyoxyethylene(15)-n-hexylamine.*

[d] *Extrapolated values.*

[e] *Value is taken from Fasman (8).*

[f] *Classifications are taken from Annex 1 of the EU Dangerous Substances Directive (1); all other classifications are taken from suppliers' data.*

C = corrosive; NC = non-corrosive; Mol. vol. = molecular volume; m.p. = melting point.

ated and QSARs were subsequently determined (using principal components analysis).

pKa and pKb values were obtained from the *Handbook of Chemistry and Physics* (7), except where indicated. Melting points were obtained from chemicals catalogues. All chemicals with melting points of 37°C or lower were assigned a default value of 37°C, on the basis that they would be in the liquid state when applied to rabbit skin.

Four data sets (30 of the chemicals used in the prevalidation study, Table I; 20 organic acids, Table II; 21 organic bases, Table III; and 15 phenols, Table IV), each consisting of four independent variables, were analysed by principal components analysis. In principal components analysis, the original variables are transformed into a new orthogonal set of linear combinants called principal components. The variance from the original descriptors is

Table IV: Skin corrosivity QSARs: descriptors for data set of 15 phenols

	Chemical	C/NC	Log P	Mol. vol. (Å^3)	m.p. (°C)	pKa
1.	Phenol	C[a]	1.391	65.84	42	9.89
2.	1-Naphthol	NC	2.585	96.58	96	9.34
3.	2-Naphthol	NC	2.585	96.48	123	9.51
4.	2,4-Dinitrophenol	NC	1.588	104.9	108	3.69
5.	2,5-Dinitrophenol	NC	1.588	105.0	109	5.15
6.	2-Nitrophenol	NC	1.862	85.16	45	7.15
7.	3-Nitrophenol	NC	1.117	85.65	98	8.28
8.	4-Nitrophenol	NC[a]	1.117	85.81	115	7.17
9.	Catechol	NC[a]	0.806	72.79	106	9.85
10.	Hydroquinone	NC	0.580	72.78	175	10.35
11.	Resorcinol	NC[a]	0.580	72.68	113	9.81
12.	*p*-Cresol	C[a]	1.810	78.85	37	10.17
13.	*m*-Cresol	C[a]	1.810	78.77	37	10.01
14.	*o*-Cresol	C[a]	1.810	78.76	37	10.17
15.	2,4,6-Trichlorophenol	NC[a]	3.964	123.2	66	6.00

[a] *Classifications are taken from Annex 1 of the EU Dangerous Substances Directive (1); all other classifications are taken from suppliers' data.*

C = corrosive; NC = non-corrosive; Mol. vol. = molecular volume; m.p. = melting point

greatest in the first principal component, less in the second component, and so on, allowing multi-component data sets to be reduced to two-dimensional or three-dimensional plots without significant loss of information.

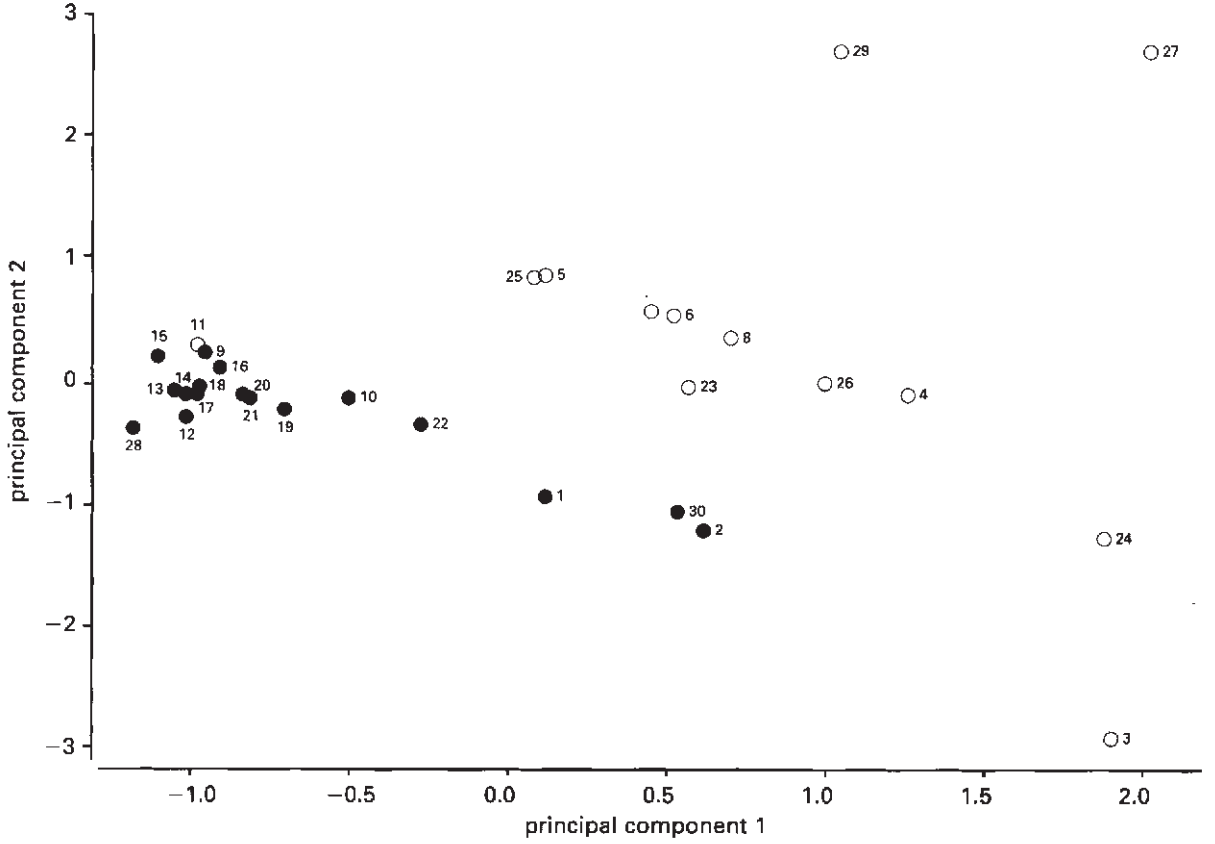
Results and Discussion

The plot of the first two principal components from the data set of 30 chemicals (Table I) is shown in Figure 1, and the principal components analysis is presented in Table V. The chemicals classified as corrosive are clearly separated from those classified as non-corrosive, with the neutral chemicals forming a separate group from the acids and bases. Subsequently, the analysis was repeated with the nine neutral chemicals omitted (Figure 2; Table VI). Again, corrosive chemicals are found in one group, while the four non-corrosive acids and bases are scattered around the edges of this group.

Since this data set is poorly balanced in terms of the numbers of corrosive and non-corrosive acids and bases, larger data sets of acids (Table II) and bases (Table III), having approximately equal numbers of corrosives and non-corrosives, were compiled from Annex 1 of the EU Dangerous Substances Directive (1) and suppliers' data. The analysis was also applied to a group of phenols (Table IV). Plots of the first two principal components of these data sets (Figures 3, 4 and 5; Table VII) again show that chemicals which are classified as corrosive are clearly separated from those classified as non-corrosive.

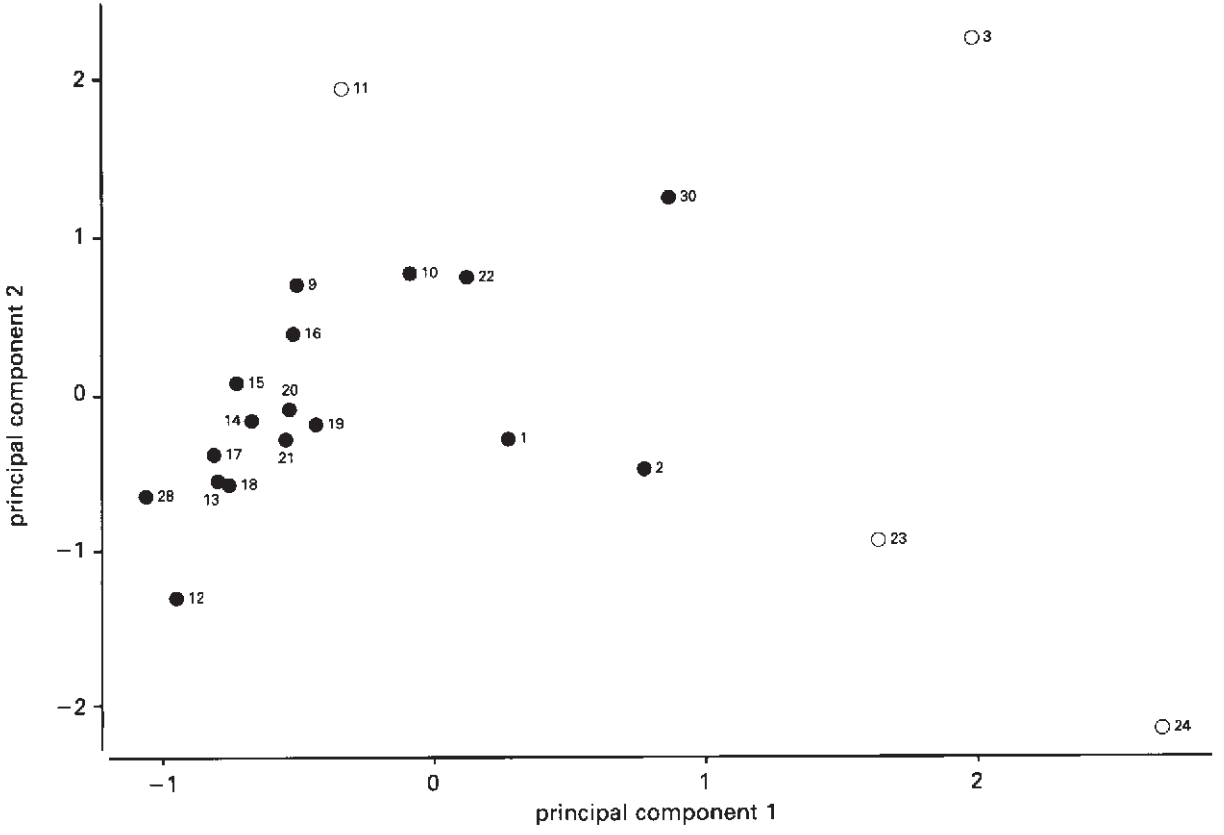
The counterbalancing effects of the different variables on corrosivity can be seen in all of the figures. Chemicals with lower log P values, larger molecular volumes or higher melting points (features associated with lower skin permeability and lower solubility) are less likely to be found in "corrosive areas" of the plots, unless they are particularly acidic or basic, for example oxalic acid, which is very polar but has a very low pKa value. On the other hand, short-chain aliphatic carboxylic acids (for example, hexanoic acid), which are regarded as relatively weak acids, are class-

Figure 1: Figure 1: Plot of the first two principal components of log P, molecular volume, melting point and pKa/pKb for 30 organic chemicals



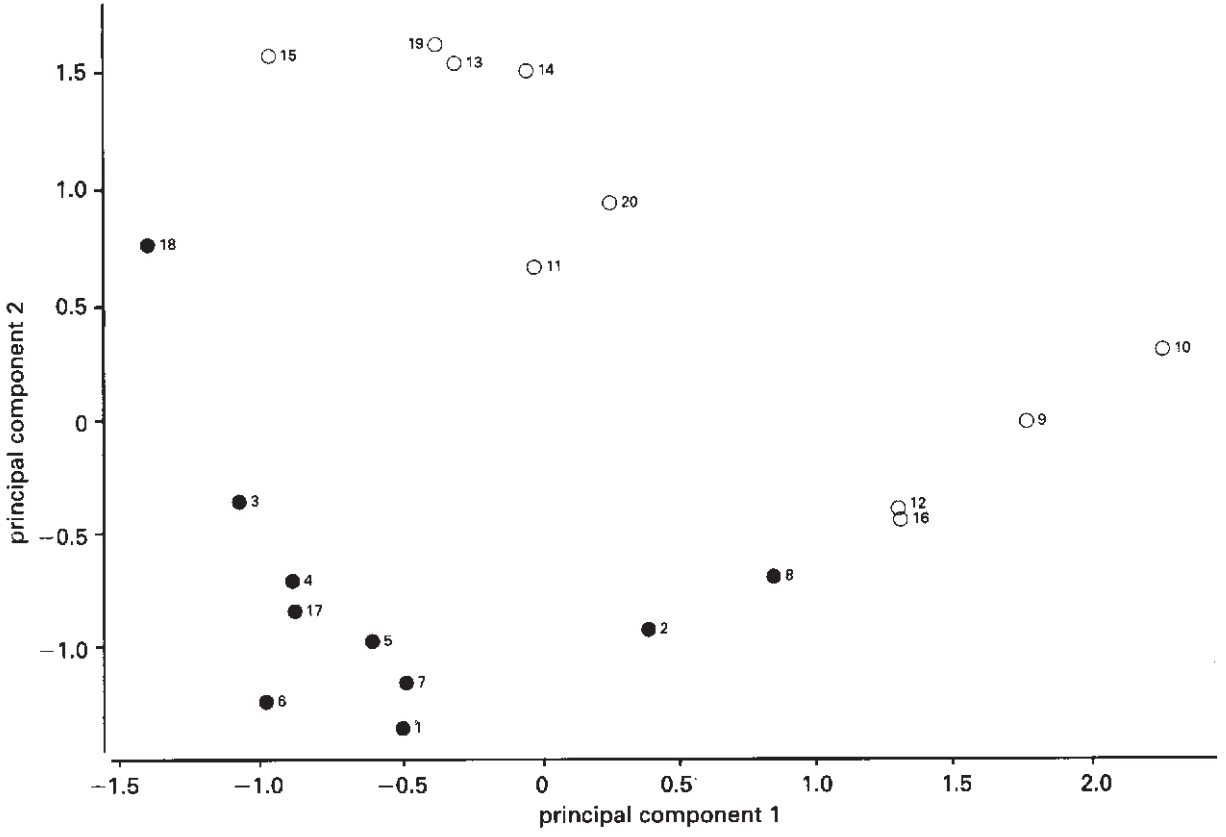
The identities of the chemicals are as given in Table I. The principal components analysis is shown in Table V. Corrosive (●), non-corrosive (○).

Figure 2: Plot of the first two principal components of log P, molecular volume, melting point and pKa/pKb for 21 organic acids and bases



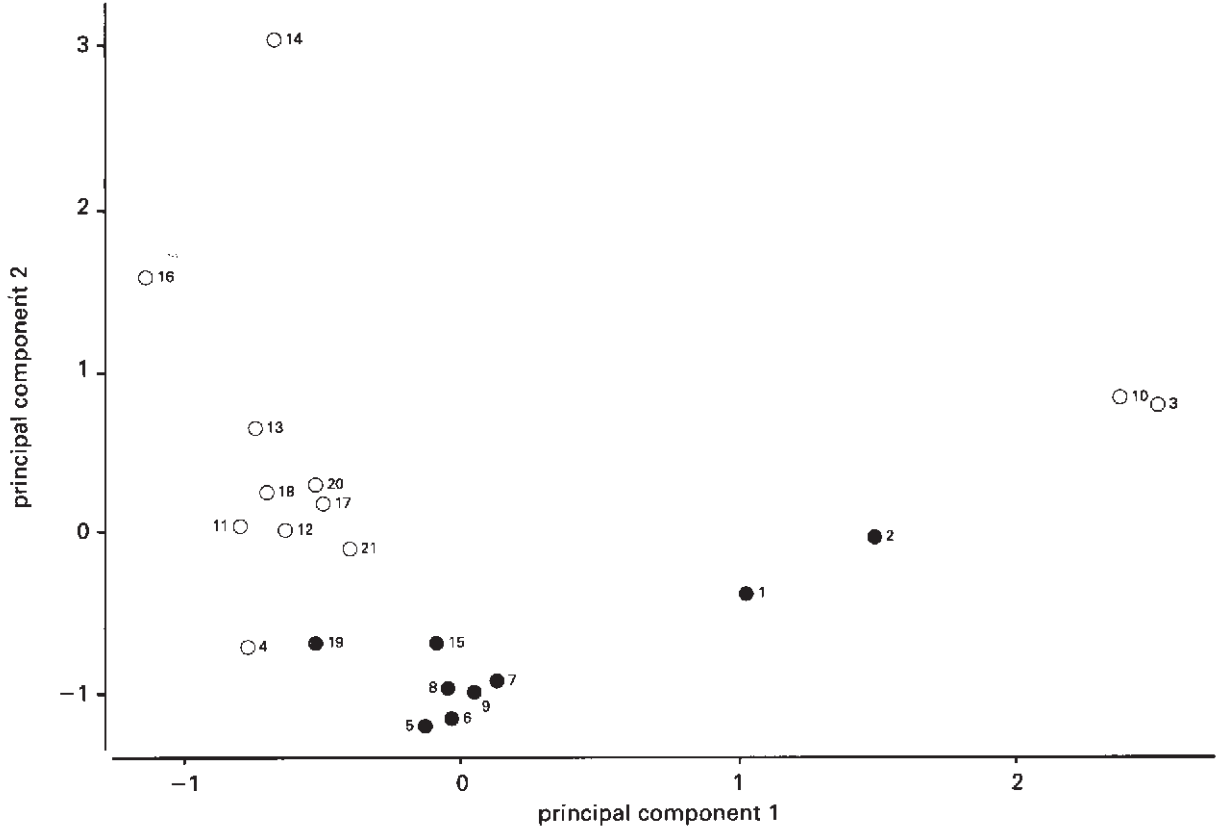
The identities of the chemicals are as given in Table I. The principal components analysis is shown in Table VI. Corrosive (●), non-corrosive (○).

Figure 3: Plot of the first two principal components of log P, molecular volume, melting point and pKa for 20 organic acids



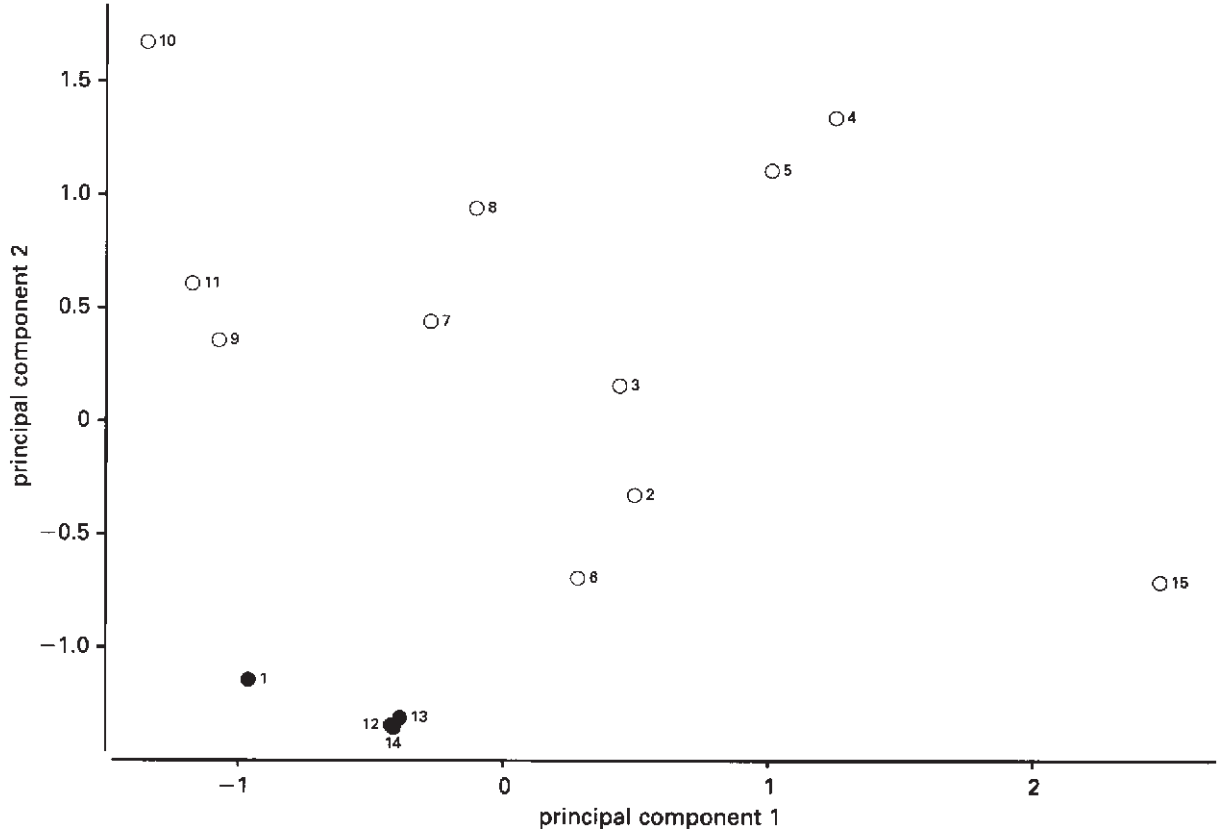
The identities of the chemicals are as given in Table II. The principal components analysis is shown in Table VII. Corrosive (●), non-corrosive (○)

Figure 4: Plot of the first two principal components of log P, molecular volume, melting point and pKb for 21 organic bases



The identities of the chemicals are as given in Table III. The principal components analysis is shown in Table VII. Corrosive (●), non-corrosive (○).

Figure 5: Plot of the first two principal components of log P, molecular volume, melting point and pKa for 15 phenols



The identities of the chemicals are as given in Table IV. The principal components analysis is shown in Table VII. Corrosive (●), non-corrosive (○).

Table V: Principal components vectors: data set of 30 chemicals

	Vectors			
	Principal Component 1	**Principal Component 2**	**Principal Component 3**	**Principal Component 4**
Log P	0.616	− 0.292	− 0.123	0.722
Molecular volume	0.521	− 0.545	0.157	− 0.638
pKa/pKb	0.437	0.539	− 0.668	− 0.269
melting point	0.399	0.572	0.717	0.013
Fraction of variance explained:				
	0.452	0.276	0.171	0.101
Total fraction of variance explained:				
	0.452	0.728	0.899	1.0

ified as corrosive apparently by virtue of their relatively high skin permeabilities (Figure 3).

Within the data set of organic bases, whilst most of the aliphatic amines are classified as corrosive, the aromatic heterocyclic bases are all classified as non-corrosive (Figure 4), since they are much weaker bases (indicated by higher pKb values). Triethanolamine, which is usually labelled as being irritant, and even as being corrosive by at least one supplier, lies in a region of the principal components plot where the distinction between corrosives and non-corrosives is unclear. Prediction of the properties of chemicals which lie in these regions may be difficult due to variabilities in the biological data.

The QSAR describing the corrosivity of phenols (Figure 5; Table VII) is best interpreted by considering the mechanism of cytotoxicity of phenols. The toxicity of phenols is associated with the uncoupling of oxidative phosphorylation from electron transport and, by studying the effects of ring substituents on the uncoupling activity (9, 10), this has

Table VI: Principal components vectors: data set of 21 acids and bases

	Vectors			
	Principal Component 1	**Principal Component 2**	**Principal Component 3**	**Principal Component 4**
Log P	0.616	− 0.238	− 0.109	0.743
Molecular volume	0.522	0.315	− 0.666	0.430
pKa/pKb	0.318	0.757	0.568	− 0.062
melting point	0.497	− 0.521	0.472	0.509
Fraction of variance explained:				
	0.525	0.255	0.158	0.063
Total fraction of variance explained:				
	0.525	0.780	0.937	1.0

Table VII: Principal components vectors: data sets of organic acids, organic bases and phenols

	Vectors			
	Principal Component 1	**Principal Component 2**	**Principal Component 3**	**Principal Component 4**
20 Organic Acids				
Log P	0.615	0.082	− 0.331	0.711
Molecular volume	0.574	0.347	− 0.302	− 0.678
melting point	− 0.114	0.891	0.399	0.182
pKa	0.528	− 0.280	0.800	− 0.053
Fraction of variance explained:				
	0.546	0.280	0.118	0.057
Total fraction of variance explained:				
	0.546	0.825	0.943	1.0
21 Organic bases				
Log P	0.622	0.346	− 0.054	0.700
Molecular volume	0.629	0.227	0.374	− 0.642
melting point	− 0.189	0.758	− 0.572	− 0.250
pKb	− 0.426	0.504	0.728	0.185
Fraction of variance explained:				
	0.430	0.298	0.175	0.097
Total fraction of variance explained:				
	0.430	0.728	0.903	1.0
15 Phenols				
Log P	0.537	− 0.429	0.497	0.530
Molecular volume	0.658	0.112	0.161	− 0.727
melting point	− 0.034	0.825	0.522	0.212
pKa	− 0.526	−0.350	0.674	− 0.382
Fraction of variance explained:				
	0.553	0.315	0.126	0.006
Total fraction of variance explained:				
	0.553	0.868	0.994	1.0

been shown to be related to both their hydrophobicities and to their proton-donating abilities. A QSAR study of the cytotoxicities of chlorophenols to V79 Chinese hamster cells (11) indicated that hydrophobicity is the dominant factor in determining their cytotoxic effects, followed by electron-withdrawing substituent effects (σ). However, hydrophobicity has to be corrected to allow for the ionisation of the phenols at physiological pH values; the correction is greater as the pKa value decreases. The pKa value is also a quantitative indicator of the proton-donating ability of a phenol, which correlates directly with σ, and thus has both positive and negative effects in determining the cytotoxicities of phenols.

When the QSARs described by Miyoshi *et al.* (10) were applied to the data set of phenols, the chemical predicted to be most cytotoxic to V79 cells was 2,4,6-trichlorophenol, followed by the nitrophenols, phenol and then the cresols. Despite their low pKa values, the dinitrophenols are less cytotoxic because their ionised state at physiological pH values lowers their effective partition into biological membranes.

In the QSAR for the skin corrosivities of phenols, parameters relating to skin permeability predominate over those which determine cytotoxicity (Table VII). The most cytotoxic chemical in the data set, 2,4,6-trichlorophenol, is a skin irritant but is not corrosive, because its relatively large molecular volume leads to lower skin permeability. The four corrosive chemicals in the data set are all small, reasonably hydrophobic, and have low melting points. Due to their relatively high pKa values, they will not be ionised at physiological pH values.

The QSARs presented here are expected to give accurate predictions of the skin corrosivity potentials of organic acids, bases and phenols. They could be used initially as a prescreen, prior to carrying out animal tests for the classification of new chemicals. Work is in progress to explore further the "borderline" areas between chemicals classified as corrosive and non-corrosive. It is also intended to extend this approach to other classes of chemicals, to discriminate between different degrees of corrosivity (for example, R34/R35) and between irritant and non-irritant substances (R38 and unclassified).

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References

1. Anon. (1984). Commission Directive 84/449/EEC of 25 April 1984 adapting to technical progress for the sixth time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances: skin irritation. *Official Journal of the European Communities* **L251**, 106–108.
2. Flynn, G.L. (1990). Physicochemical determinants of skin absorption. In *Principles of Route-to-Route Extrapolation for Risk Assessment* (ed. T.R. Gerrity & C.J. Henry), pp. 93–127. New York: Elsevier.
3. Potts, R.O. & Guy, R. (1992). Predicting skin permeability. *Pharmacological Research* **9**, 663–669.
4. Barratt, M.D. (1995). Quantitative structure-activity relationships for skin permeability. *Toxicology in Vitro* **9**, 27–37.
5. Suzuki, T. (1991). Development of an automated estimation system for both partition coefficient and aqueous solubility. *Journal of Computer-Aided Molecular Design* **5**, 149–166.
6. Suzuki, T. & Kudo, Y. (1990). Automatic log P estimation based on combined additive modelling methods. *Journal of Computer-Aided Molecular Design* **4**, 155–198.
7. Weast, R.C., ed. (1970). *Handbook of Chemistry and Physics*, 51st edn. Cleveland, OH: The Chemical Rubber Co.
8. Fasman, G.R., ed. (1976). *Handbook of Biochemistry and Molecular Biology*, 3rd edn. Cleveland, OH: The Chemical Rubber Co.
9. Stockdale, M. & Selwyn, M.J. (1971). Effects of ring substituents on the activity of phenols as inhibitors and uncouplers of mitochondrial respiration. *European Journal of Biochemistry* **21**, 565–574.
10. Miyoshi, H., Nishioka, T. & Fujita, T. (1987). Quantitative relationship between protonophoric and uncoupling activities of substituted phenols. *Biochimica et Biophysica Acta* **891**, 194–204.
11. Jansson, K. & Jansson, V. (1993). The toxicity of chlorophenols to V79 Chinese hamster cells. *Toxicology Letters* **69**, 289–294.