Evaluation of Potential False Negative Corrosive Chemicals in Proposed *In Vitro* Dermal Irritation Assays

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Introduction

An estimated 12% to 21% of dermal corrosives (i.e., substances that produce irreversible tissue damage in the skin) are not identified as corrosives by at least one of the four currently adopted *in vitro* corrosivity tests (i.e., EPISKIN™, EpiDerm™, rat skin TER, and Corrositex™) (ICCVAM 1999, 2002; Fentem et al. 1998). Although classified as complete replacements for dermal corrosivity by the European Centre for the Validation of Alternative Methods (ECVAM), these *in vitro* corrosivity test methods are recommended in the United States for use in a weight-of-evidence tiered testing strategy. In such a strategy, substances testing negative for corrosivity would be tested *in vivo* for dermal irritation in up to three rabbits using a sequential testing approach. Therefore, corrosive substances incorrectly identified as false negatives in an *in vitro* corrosivity test would be identified correctly during the *in vivo* dermal irritation test.

Recently, ECVAM completed a validation study to evaluate the ability of EpiDerm™ and EPISKIN™, using modified protocols, to also detect chemicals that induce dermal irritation (i.e., those that produce reversible inflammatory changes in the skin). Corrosive or irritating compounds are detected by their ability to kill cells, as measured by the ability of live cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT).

Based on the results obtained in the dermal irritation validation study, the ECVAM Scientific Advisory Committee (ESAC) concluded that EPISKIN™ could be used as a stand-alone replacement for the Draize skin irritation test. However, if *in vitro* dermal corrosion and irritation methods are to be used as complete replacements for animals, then *in vitro* testing strategies must be capable of identifying the approximately 12% to 21% false negative corrosive substances that are currently identified using the 1-3 animal dermal irritation/corrosivity protocol.

Accordingly, it is important to evaluate how the *in vitro* dermal irritation test methods will respond to corrosive substances, especially those that have produced false negative results in *in vitro* corrosivity tests. This resulting information will be needed for regulatory authorities to consider the usefulness and limitations of *in vitro* dermal irritation assays for regulatory hazard classification purposes.

The following study plan, developed in conjunction with ICCVAM and with input from the ECVAM Validation Study Management Team, will generate the critical information needed by regulatory authorities. This plan focuses specifically on EPISKIN™, based on the recent ESAC recommendations that it may be used as a replacement for the Draize skin irritation test (ESAC recommended that EpiDerm™ be used only as a screen for dermal irritants). For reference, **Table 1** provides a general comparison of the corrosivity and irritation protocols for EPISKIN™.

Table 1 Comparison of Corrosive and Irritation Protocols for EPISKIN™

	EPISKIN™	
	Corrosivity Protocol	**Irritation Protocol**
Endpoint measured	MTT reduction	MTT reduction + IL-1α release
Pre-exposure incubation	None	18-20 hr in maintenance medium at 37°C/5% CO2
Quantity of test substance applied	Liquids: 50 µL Solids: 20 mg (w/ 10 µL NaCl)	Liquids: 10 µL Solids: 10 mg
Duration of exposure	3 min, 1 hr, 4 hr at room temperature	15 min at room temperature
Rinsing of test substance	PBS	PBS
Post-exposure incubation	None	42 hr at 37°C/5% CO2
Acceptance criteria for control data	Negative control (saline; 4 hr): OD545-OD595 = 0.113 to 0.309 Positive control (glacial acetic acid; 4 hr): 0-20% cell viability	Negative control (saline): OD545-OD595 = 0.6 to 0.9 Positive control (5% SDS): 0-20% cell viability
Decision criteria for a positive response	Relative cell viability <35% at any exposure duration	Relative cell viability ≤50%

MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; PBS = phosphate buffered saline

Study Objectives

The primary objectives of this study are to evaluate:

- How corrosive substances classified as negative in any of the four accepted *in vitro* corrosivity test methods will act in the EPISKIN™ dermal irritation test method. Such data might allow for criteria to be established for identifying such substances as corrosives.
- The extent to which various combinations of *in vitro* dermal corrosivity and irritation test methods offer the greatest accuracy.
- The extent to which controlling for substances that directly reduce MTT (i.e., act as false negatives) would correct some of the false negative corrosive results.

Database Of Substances To Be Tested

At least 15 *in vivo* rabbit dermal corrosive substances have produced false negative results in one or more of the four accepted *in vitro* corrosivity test methods (**Table 2**). This list includes any substances that produced a false negative result in any laboratory in the ECVAM *in vitro* corrosivity validation studies (Fentem et al. 1998) or in any studies reviewed by ICCVAM (ICCVAM 2002). Corrosive compounds that were reported as negative in an *in vitro* human skin model recently developed in Japan (i.e., VitroLife-Skin™) for the detection of skin corrosives are also included.

For the purposes of this list, a ***false negative*** corrosive substance includes those classified as:

- ***NEGATIVE***, where all test outcomes yielded a noncorrosive response
- ***QUESTIONABLE***, where a majority of the test outcomes yielded a corrosive response, but one or more responses were noncorrosive
- ***DISCORDANT***, where a majority of the test outcomes yielded a noncorrosive response, but some responses were classified as corrosive.

A phased study design (**Figure 1**) will be followed such that subsets of the complete list of substances (see **Table 3**) will be tested in initial phases of the study to provide support or information for further testing of the entire list of substances in a subsequent phase. Information obtained in each phase will provide useful information towards addressing the study objectives described above.

Some of the discordant results relative to the *in vivo* rabbit skin test may be explained by the inherent physiochemical properties of certain substances or by inaccurate reports of hazard classification. For example:

- Carvacrol, n-heptylamine, 2-mercaptoethanol (45%) sodium salt, methacrolein, tallow amine, and 2-tert-butylphenol reportedly reduce MTT directly.
- Methacrolein polymerizes over time, which potentially results in reduced toxicity each time its container is opened.
- Sulfuric acid 10% was incorrectly identified as CORROSIVE in the list of ICCVAM performance standards chemicals (the POISINDEX® database indicates that sulfuric acid is corrosive only at concentrations greater than 10%).
- Sulfamic acid may have been incorrectly identified as non-corrosive. Sulfamic acid has been reported to be a corrosive substance at high concentrations or as a moist paste.
- Tallow amine does not wash off of the surface; waxy/non-removable substances are an established limitation of the *in vitro* skin irritation test methods.

However, including these substances in the proposed study will expand the database, and thus potentially strengthen the justification for the exclusion of such substances in these types of test methods. An updated corrosivity protocol, which includes a test for direct MTT reduction, will be conducted. This approach allows for a quantitative assessment and correction for direct MTT reduction, thereby reducing the potential for false negatives.

The proposed study design also includes testing the reference substances for *in vitro* skin equivalent corrosive test methods included in the ICCVAM document, "Recommended Performance Standards for *In Vitro* Test Methods for Skin Corrosion" (ICCVAM 2004). Use of these reference substances will provide responses of EPISKIN™ dermal irritation protocol to known corrosives and non-corrosives that were correctly identified using the dermal corrosivity protocols, and to corrosive substances that were incorrectly identified as non-corrosives in one or more test results. The total database of chemicals includes 12 non-corrosive substances and 20 corrosive substances, for a total of 32 substances (**Table 3**).

Table 2 False Negative Substances from *In Vitro* Corrosivity Tests

Chemical	UN *In Vivo* PG1	*In Vivo* Study			*In Vitro* Result2				
		Exp time	Result (day observed)	n	Corrositex™	EpiDerm™	EPISKIN™	TER	VitroLife-Skin
Allyl bromide	II/III	3 min 4 hr	NC Cr, TD (≤7)	1/1 2/2	Not corrosive (4NC/2NQ)	NT	AC	AC	NT
1-(2-Aminoethyl) piperazine	II	NA			AC	NT	Not corrosive (9NC)	AC	NT
Caprylic acid*	II/III	4 hr	Ne (2)	3/3	Not corrosive (6NC)	AC	AC	Corrosive (4C/2NC)	Corrosive (10C/1NC)
Carvacrol1	II/III	4 hr	E,H (≤2)	4/4	AC	NT	AC	Corrosive (5C/1NC)	NT
Ferric chloride	II	NA			AC	NT	Corrosive (5C/4NC)	AC	NT
Glycol bromoacetate (85%)*	II/III	4 hr	Ne (1 hr)	1/1	AC	AC	NT	Not corrosive (6NC)	NT
n-Heptylamine*1	II/III	4 hr	Bu (1 hr)	6/6	AC	Corrosive (4C/2NC)	NT	AC	Not corrosive (NA)
2-Mercaptoethanol, sodium salt (45% aq.)*1	II/III	3 min 4 hr	NC TD (1 hr)	3/3 1/1	AC	NT	NT	AC	NT
Methacrolein1	II/III	4 hr	Ne (9)	3/3	Not corrosive (4NC/2NQ)	Not corrosive (5NC/1C)	NT	Not corrosive (6NC)	Corrosive (NA)
Octanoic/decanoic acids (65:35) 2	II/III	NA			Not corrosive (6NC)	NT	AC	AC	NT
Octanoic/decanoic acids (60:40) 2	II/III	4 hr	E (1, 7)	2/3, 1/3	Not corrosive (6NC)	AC	NT	AC	NT
Octanoic/decanoic acids (55:45) 2	II/III	NA			Not corrosive (6NC)	NT	AC	AC	NT
Sulfuric acid, 10%*	II/III	NA			AC	Corrosive (8C/4NC)3	AC	Corrosive (5C/1NC)	Corrosive (10C/1NC)
Tallow amine1	II	3 min	Ne (7)	2/3	Not corrosive (6NC)	NT	Not corrosive (9NC)	Corrosive (2C/2NC/2NQ)	NT
2-tert-Butylphenol*1	II/III	4 hr	Ne (1 hr, 1 day)	5/6, 1/6	AC	Corrosive (5C/1NC)	AC	Corrosive (4C/2NC)	Corrosive (NA)

AC = correctly classified as corrosive in all tests; Bu =burn; C = corrosive; Cr = crust; E = eschar; H = hardening; n = number of animals with effect(s)/total number of animals tested; NA = not available; NC = noncorrosive; Ne = necrosis; NQ = not qualifying; NT = not tested; PG = packing group; TD = tissue destruction; * = included in the 12 corrosive reference chemicals in the ICCVAM performance standards (ICCVAM 2004); *italics* = not proposed for inclusion in the study; [1]II/III indicated for UN *In Vivo* PG where a test with a 1 hour exposure was not available to delineate between a PG II vs PG III; [2]Results provided are the overall *in vitro* classification (individual experimental classification); [3]Results from Japanese Society for Alternatives to Animal Experiments (JSAAE) Validation Study with EpiDerm™ and VitroLife-Skin.

[1]Chemicals that directly reduce MTT and thus may have produced erroneous results under the conditions of the original corrosivity protocols. An updated protocol that accounts for this property will be used in this study.

[2]Only one of the three different mixtures of octanoic/decanoic acids would need testing (i.e., the 60:40 mixture) since test results were the same in each test method for all three similar mixtures (i.e., either all correct, or all incorrect)

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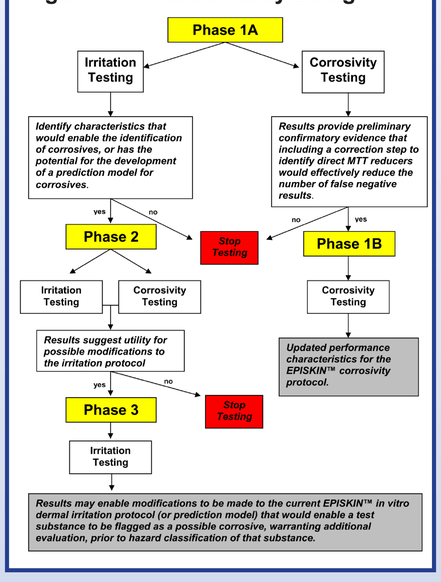
Table 3 List of Chemicals Recommended for Evaluation in the *In Vitro* Dermal Irritation Methods

Chemical	CASRN	Chemical Class[1]	UN *In Vivo* PG	PII Value	pH
***In Vivo* Corrosives**					
1-(2-Aminoethyl) piperazine	140-31-8	organic base	II	n/a	8.0
1,2-Diaminopropane	78-90-0	organic base	I	n/a	8.3
*2-Mercapoethanol, sodium salt (45% aq.)	37482-11-4	inorganic base	II/III	n/a	12.0
*2-tert-Butylphenol	88-18-6	phenol	II/III	n/a	3.9
Allyl bromide	106-95-6	electrophile	II/III	n/a	3.9
Boron trifluoride dihydrate	13319-75-0	inorganic acid	I	n/a	1.5
*Caprylic acid	124-07-02	organic acid	II/III	n/a	3.6
Carvacrol	499-75-2	phenol	II/III	n/a	3.9
Dimethyldipropylenetriamine	10563-29-8	organic base	I	n/a	8.3
Dimethylisopropylamine	996-35-0	organic base	II/III	n/a	8.3
Ferric chloride	7705-08-0	inorganic salt	II	n/a	1.0
*Glycol bromoacetate (85%)	3785-34-0	electrophile	II/III	n/a	2.0
*n-Heptylamine	111-68-2	organic base	II/III	n/a	8.4
Methacrolein	78-85-3	electrophile	II/III	n/a	3.6
Octanoic/decanoic acids (60:40)	68937-75-7	organic acid	II/III	n/a	3.9
Phosphorus tribromide	7789-60-8	inorganic acid	I	n/a	1.0
Potassium hydroxide (10% aq.)	1310-58-3	inorganic base	II	n/a	13.1
**Sulfuric acid (15%)	7664-93-9	inorganic acid	NA**	NA	NA
*Sulfuric acid (10%)	7664-93-9	inorganic acid	II/III	n/a	1.2
Tallow amine	61790-33-8	organic base	II	n/a	ND
***In Vivo* Noncorrosives**					
1,9-Decadiene	1647-16-1	neutral organic	NC	3.0	3.9
4-(Methylthio)benzaldehyde	3446-89-7	electrophile	NC	0.89	6.8
4-Amino-1,2,4-triazole	584-13-4	organic base	NC	0	5.5
Dodecanoic acid (Lauric acid)	143-07-7	organic acid	NC	0.44	ND
Eugenol	97-53-0	phenol	NC	2.92	3.7
Isostearic acid	30399-84-9	organic acid	NC	4.33	3.6
o-Methoxyphenol	90-05-1	phenol	NC	2.38	3.9
Phenethyl bromide	103-63-9	electrophile	NC	0	3.6
Sodium carbonate (50% aq.)	497-19-8	inorganic base	NC	2.33	11.7
Sodium lauryl sulfate (20% aq.)	151-21-3	surfactant	NC	6.78	3.9
Sulfamic acid	5329-14-6	inorganic acid	NC	NV	1.5
Tetrachloroethylene	127-18-4	neutral organic	NC	5.67	4.5

Abbreviations: aq = aqueous; CASRN = Chemical Abstracts Service Registry Number; NA = not available; NC = Noncorrosive; ND = not determined (or unable to measure); NV = no value given in Barratt et al. (1998), Fentem et al. (1998), or ECETOC (1995); NPC = not possible to calculate; n/a = not applicable; PII = primary irritation index; PG = Packing Group; UN = United Nations. [1]Inorganic and organic acids and bases denature proteins. Phenols denature proteins and cause dehydration. Alcohols cause dehydration. Electrophiles are highly reactive. Surfactants change surface tension and cause membranes to break apart. *Chemicals that appear in **Table 2** and that produced false negative results in one or more *in vitro* corrosivity tests. **Included as an additional known corrosive substance (POISINDEX®) due to the reported misclassification of sulfuric acid (10%) (see **Database of Substances Tested**).

Figure 1 Phased Study Design



Phase 1

Phase 1A

Objectives:

1. Determine if the irritation test protocol has characteristics that would enable the identification of corrosives, or has the potential for the development of a prediction model for corrosives.
2. Provide preliminary confirmatory evidence that including a correction step to identify direct MTT reducers would effectively reduce the number of false negative results.

Study Design:

- Eight to nine substances tested (see **Table 4**) each tested once (n=3 tissues per substance).
- Will use the optimized, validated EPISKIN™ protocols for both dermal irritation and corrosivity.
  - This "side by side" testing approach will produce data that will allow for a direct comparison of the irritation and corrosivity test method protocols with exactly the same substance lot and the same tissue lot.
  - Given the corrosivity classification (i.e., UN Packing Group) for the substances listed in **Table 4**, the longest exposure time (i.e., 4 hr for EPISKIN™) will be used in the corrosivity protocol.
  - A 3-minute exposure time will also be included to identify direct MTT reducers.
- Data will be reviewed to determine if:
  - Including the MTT correction step effectively reduces the number of false negatives (***IF SO, PROCEED TO PHASE 1B***)
  - There are any characteristics that could flag substances as potential corrosives (***IF SO, WILL PROCEED TO PHASE 2***)

Phase 1B (contingent on results from Phase 1A)

Objectives:

1. Generate a comprehensive dataset to update the performance characteristics for the corrosivity protocol when using the MTT correction step.

Study Design:

- Sixteen substances tested (see **Table 5**) each tested once (n=3 tissues per substance).
- Will use the full EPISKIN™ corrosivity protocol (i.e., which includes test substance exposure time points of 3 min, 1 hr, and 4 hr)
  - Because the focus of Phase 1B is on the potential improvement in test method sensitivity and not specificity, only corrosive substances will be tested (with the exception of the negative control substance, saline).

Table 4 Recommended Substances for Phase 1A Testing[1]

Test substance	Rationale for inclusion
1-(2-Aminoethyl) piperazine	Was 1 of 2 false negatives corrosives in EPISKIN™ corrosivity assay; was predicted corrosive in Corrositex and TER; was not tested in EpiDerm™ or VitroLife-Skin™; the other false negative corrosive was the non-removable tallow amine
Glycol bromoacetate (85%)	*In vivo* corrosive and proposed MTT reducer not tested in EPISKIN™ corrosivity assay; was corrosive in Corrositex™, EpiDerm™ and TER but not VitroLife-Skin™
2-Mercaptoethanol, sodium	*In vivo* corrosive and proposed MTT salt (45% aq) reducer not tested in EPISKIN™ corrosivity assay; was corrosive in Corrositex™ but not tested in EpiDerm™ or VitroLife-Skin™
n-Heptylamine	*In vivo* corrosive not tested in EPISKIN™ corrosivity assay or in VitroLife-Skin™; was corrosive in Corrositex™ and EpiDerm™ but not in TER
Dimethylisopropylamine	Other corrosive apparently correctly predicted during validation study (pH 8.3)
Phosphorus tribromide	Other corrosive apparently correctly predicted during validation study (pH 1.0)
Glacial acetic acid[2]	Positive control for the corrosivity protocol
Controls	
Positive Control (corrosivity protocol) Glacial acetic acid	Routine positive control
Negative Control (corrosivity protocol) Saline	Routine negative control
Positive Control (irritation protocol) 5% Sodium dodecyl sulfate	Routine positive control
Negative Controls (irritation protocol) Saline	Routine negative control

[1]For corrosivity testing, substances will be applied for 3 min and 4 hr and MTT measured immediately following each time point. For irritation testing, substances will be applied for 15 minutes, followed by a 42 hr post-incubation period prior to measuring MTT.

[2]Glacial acetic acid will tested in the corrosivity protocol as a positive control, and in the irritation protocol as a known corrosive substance. Therefore, a total of nine substances will be tested in the irritation protocol and a total of eight substances will be tested in the corrosivity protocol.

Table 5 Recommended Substances for Phase 1B Testing[1]

Chemical	UN *In Vivo* PG
1,2-Diaminopropane	I
*2-tert-Butylphenol	II/III
Allyl bromide	II/III
Boron trifluoride dihydrate	I
*Caprylic acid	II/III
Carvacrol	II/III
Dimethyldipropylenetriamine	I
Ferric chloride	II
Methacrolein	II/III
Octanoic/decanoic acids (60:40)	II/III
Potassium hydroxide (10% aq.)	II
**Sulfuric acid (15%)	NA**
*Sulfuric acid (10%)	II/III
Tallow amine	II
Glacial acetic acid (positive control)	II
Saline (negative control)	Not labeled

NA = Not available

[1]The full corrosivity protocol will be used, in which substances will be applied for 3 min, 1 hr, and 4 hr and MTT measured immediately following each time point.

*Chemicals that produced false negative results in one or more *in vitro* corrosivity tests.

**Included as an additional known corrosive substance (POISINDEX®) due to the reported misclassification of sulfuric acid (10%).

Phase 2 (contingent on results from Phase 1A)

If there are trends identified in Phase 1A that might suggest a corrosive response, and/or if a direct MTT reduction correction step would improve the performance statistics for the corrosivity protocol(s), Phase 2 will be conducted.

Objectives:

1. Conduct a more definitive analysis of any improvement in the performance of the corrosivity protocols.
2. Provide further evidence for possible modifications to the irritation protocol.

Study Design:

- The remaining substances in **Table 3** (i.e., those not included in **Table 4**) each tested once (n=3 tissues per substance).
- Will use the EPISKIN™ protocols for both dermal irritation (including any useful modifications identified from Phase 1A) and corrosivity.

Phase 3 (contingent on results from Phase 2)

- If the results of Phase 2 point to possible improvements to the current EPISKIN™ protocol (or prediction model), these modifications will be tested in Phase 3.
- Each substance, or a subset of the substances tested in Phase 2 (see **Table 3** for the complete list), will be tested once (n=3 tissues per substance) with the updated EPISKIN™ protocol (or prediction model).

Study Outcome

- These results from this study may enable modifications to be made to the current EPISKIN™ *in vitro* dermal irritation protocol (or prediction model) that would enable a test substance to be flagged as a possible corrosive, warranting additional evaluation prior to hazard classification of that substance.

NIEHS National Institute of Environmental Health Sciences

ICCVAM The Interagency Coordinating Committee on the Validation of Alternative Methods

NICEATM The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods

More information on ICCVAM and NICEATM can be accessed at http://iccvam.niehs.nih.gov/