2.0 *IN VITRO* MEMBRANE BARRIER TEST SYSTEMS FOR SKIN CORROSION

2.1 Background

Validation studies have been completed for an *in vitro* membrane barrier test system commercially available as Corrositex® (ICCVAM 1999; Fentem et al. 1998; Barratt et al. 1998; Gordon et al. 1994; InVitro Intl. 1995). Based on its scientific validity, this test method has been recommended for use as part of a tiered testing strategy for assessing the dermal corrosion hazard potential of chemicals, whereby any substance that qualifies for testing can be evaluated (ICCVAM 1999; ECVAM 2001). The use of an *in vitro* membrane barrier test method as part of a tiered approach reduces and refines the use of animals in testing and provides a basis for deciding on the adequacy of information for hazard classification or the need for further testing. In addition, such a test method may be used to make decisions on the corrosivity and noncorrosivity of specific classes of chemicals (e.g., organic and inorganic acids, acid derivatives[1], and bases) for certain transport testing circumstances (DOT 2002). This chapter briefly describes the principles of *in vitro* membrane barrier test systems for corrosivity followed by the recommended performance standards, which consists of essential test method components, reference chemicals, and comparison of accuracy and reliability.

2.2 Principles of *In Vitro* Membrane Barrier Test Systems for Skin Corrosion

The basis of this test system is that it detects membrane damage caused by corrosive test substances (ICCVAM 1999). The test substance is first evaluated to determine if it is compatible with the test procedure. If compatible, the substance is evaluated for category of acid or base (strong or weak) to determine the appropriate time scale used to classify the potential corrosivity of the test substance. Finally, a compatible substance is applied to the surface of the artificial membrane barrier. The time it takes for the test substance to penetrate through the membrane barrier to an underlying indicator solution determines the corrosivity classification of that test substance. Penetration of the barrier might be measured by a number of procedures, including a color change in a pH indicator dye or other properties of the solution below the barrier (e.g., electrical conductivity).

Investigators using *in vitro* membrane barrier test systems for skin corrosion must be able to demonstrate that the assay is valid for its intended use. This includes demonstrating that different preparations are consistent in barrier properties, capable of maintaining a barrier to noncorrosive substances, and able to categorize the corrosive properties of chemicals across the various subcategories of corrosivity described by the UN Packing Group classification system. For *in vitro* membrane barrier test systems, the UN Packing Group classification assigned is based on the time it takes the test substance to penetrate through the membrane barrier. For Corrositex®, the validated *in vitro* reference test method, a color change in the underlying Chemical Detection System (CDS) indicates that the membrane barrier has been penetrated. The CDS changes color when a chemical or chemical mixture changes the pH of the solution to less than 4.5 or greater than 8.5.

[1] "Acid derivative" is a non-specific class designation and is broadly defined as an acid produced from a chemical substance either directly or by modification or partial substitution. This class includes anhydrides, haloacids, salts, and other types of chemicals.

In vitro membrane barrier test systems may be used to test solids, liquids, and emulsions. The liquids can be aqueous or nonaqueous; solids can be soluble or insoluble in water. The samples may be pure chemicals, dilutions, formulations, or waste. No prior treatment of the sample is required. A limitation of the validated *in vitro* membrane barrier test method is that many noncorrosive chemicals and chemical mixtures and some corrosive chemicals and chemical mixtures do not qualify for testing. Test chemicals and chemical mixtures are considered nonqualifying if they do not cause a color change in the CDS. Aqueous substances with a pH in the range of 4.5 to 8.5 often do not qualify for testing; however, 85% of chemicals tested in this pH range were noncorrosive in animal tests (ICCVAM 1999).

2.3 Essential Test Method Components

The following is a description of the essential test method components of *in vitro* membrane barrier test systems for corrosivity. A sample protocol for the validated reference test method is available at *http://iccvam.niehs.nih.gov*.

2.3.1 Test Method Components (Membrane Barrier, Categorization Solutions, Indicator Solution)

Membrane Barrier: The membrane barrier consists of two components -- a proteinaceous macromolecular aqueous gel and an underlying, permeable supporting membrane. The proteinaceous gel, composed of protein (e.g., keratin, collagen, or mixtures of proteins) forming a gel matrix, serves as the target for the test substance. It should be impervious to liquids and solids but able to be corroded and made permeable, presumably by the same mechanism(s) of corrosion that operates on living skin. The permeable supporting membrane provides mechanical support to the proteinaceous gel during the gelling process and exposure to the test substance, preventing sagging or shifting of the gel. The supporting membrane should be readily permeable to test substances so as not to interfere with its passage through to the indicator solution. The proteinaceous material is placed on the surface of the supporting membrane and allowed to gel prior to placing the membrane barrier over the indicator solution. The proteinaceous gel should be of equal thickness and density throughout, and with no air bubbles or defects that could affect its permeability or response to a corrosive test substance. The fully constructed membrane barrier should be stored under predetermined conditions shown to preclude deterioration of the gel (drying, microbial growth, etc) or loss of uniformity (shifting or cracking), which would degrade its performance. The acceptable storage period should be determined and membrane barrier preparations not used after that period.

Test Substance Categorization System: Experience with the validated reference system has shown that “strong” acids or bases and “weak” acids or bases behave somewhat differently in the time required to breakthrough the barrier membrane relative to their corrosive potential *in vivo*. Scoring of all test substances on a scale appropriate for strong acids and bases led to an over prediction of corrosivity for weak acids and bases. Thus, two scoring scales of breakthrough times are used to determine corrosivity (and UN Packing Group classification) or noncorrosivity for strong acids and bases and one for weak acids and bases. If a categorization system is used, objective criteria must be developed to place test substances into the appropriate categories for scoring. Changes in the pH of calibrated buffer solutions (one for acids and one for bases) could be used for this purpose. Specific ranges for strong and weak acids or bases should be defined.

Indicator Solution: An indicator solution responds to the presence of a test substance. This response can be assessed as an observable color change in a pH indicator dye, or by other types of chemical or electrochemical reactions. A pH-specific indicator dye or combination of dyes (e.g., cresol red and methyl orange) that will show a color change in response to the presence of the test substance can be used. The measurement system could be visual or electronic. Test substances must be determined to be capable of causing a measurable response in the indicator solution before they are considered qualified for evaluation in the test system.

2.3.2 Test Procedure

Test Substance Compatibility: Prior to testing, a qualification or compatibility test is performed to determine if the test substance can be detected by the indicator solution. The indicator system and the conditions of exposure used for the compatibility test must reflect the exposure in the subsequent corrosivity test. If the test substance is not detectable by the indicator solution, then the test system cannot be used to evaluate the corrosivity of that test substance.

Test Substance Categorization: If appropriate for the assay, a test substance that has been qualified by the compatibility test should be subjected to a categorization test (i.e., a screening test to distinguish between weak and strong acids or bases) to determine the appropriate breakthrough timescale to use for determining corrosivity and GHS skin corrosivity subcategory.

Assembly of the Test Method Components: The membrane barrier is positioned in a vial (or tube) containing the indicator solution so that the supporting membrane is in full contact with the indicator solution and with no air bubbles present. Care should be taken to ensure that barrier integrity is maintained.

Application of Test Substances: The assay is performed at room temperature (17-25°C), and a test substance is at room temperature when applied. A suitable amount of the test substance (e.g., 500 µL of liquid or 500 mg finely powdered solid) for the validated reference test method (InVitro Intl. 1995) is carefully layered onto the upper surface of the membrane barrier and distributed evenly. An appropriate number of replicates (e.g., four, as is used in the validated reference method) are prepared for each test substance and the concurrent controls. The time of addition of the test substance is recorded. To ensure that short corrosion times can be accurately recorded, the application times of the test substance to the replicate vials are staggered.

2.3.3 Control Substances

Solvent Controls: In tests that involve the use of a vehicle or solvent with the test substance, the vehicle or solvent must be compatible with the barrier system (i.e., not alter the integrity of the membrane barrier system) and should not alter the corrosivity of the test substance. When applicable, solvent (or vehicle) controls should be tested concurrently with the test substance to demonstrate the compatibility of the solvent with the barrier system.

Positive (Corrosive) Controls: A positive control chemical should be tested concurrently with the test substance to demonstrate that the *in vitro* membrane barrier test system is functioning properly. The positive control should be well characterized for its corrosive activity and should generate a response that is low to intermediate within the range of corrosive responses for the assay. Thus, extremely corrosive (UN Packing Group I) or noncorrosive chemicals are of limited utility, while

a Packing Group II substance would allow detection of a too rapid or too slow breakthrough time. To measure performance of the test method close to the cut off time between corrosive and noncorrosive, a weak Packing Group III substance might be employed. An acceptable positive control response range must be developed based on the historical range of breakthrough times for the positive control(s) employed. In each study, the positive control should be evaluated to determine if the breakthrough time is within the acceptable positive control range. For the validated reference test method, the acceptable breakthrough time for sodium hydroxide pellets, a Packing Group II positive control, ranges from 10.6 to 15.9 minutes.

Negative (Noncorrosive) Controls: A noncorrosive substance should also be tested concurrently with the test substance as another quality control measure to demonstrate the functional integrity of the membrane barrier. Examples of noncorrosive substances used as negative controls in the validated reference test method include 10% citric acid or 6% propionic acid.

Benchmark Controls: Benchmark controls may be useful to demonstrate that the test method is functioning properly for detecting the dermal corrosivity potential of chemicals of a specific chemical class or a specific range of responses, or for evaluating the relative corrosivity potential of a corrosive test substance. Appropriate benchmark controls should have the following properties:

- consistent and reliable source(s) for the chemical
- structural and functional similarity to the class of the substance being tested
- known physical/chemical characteristics
- supporting data on known effects in animal models
- known potency in the range of response (including moderate response)

2.3.4 Measurement of Membrane Barrier Penetration

Each vial is appropriately monitored and the time of the first change in the indicator solution (i.e., barrier penetration) is recorded. The difference in time between application of the test substance and penetration of the membrane barrier is determined.

2.3.5 Interpretation of Results

According to the established time parameters for each UN Packing Group, the time (in minutes) elapsed between application of the test substance and barrier penetration is used to predict the corrosivity of a test substance. For a test to be considered acceptable, the concurrent positive control must give the expected penetration response time, and, when included, the concurrent solvent control must not be corrosive.

2.3.6 Classification of Test Substances

The time (in minutes) elapsed between application and appearance of a color change in the CDS is used to classify the test substance in terms of corrosivity and, if applicable, UN Packing Group.

2.3.7 Test Report

The test report should include the following information, if relevant to the conduct of the study:

Test and Control Substances

- Chemical name(s) such as Chemical Abstract Services (CAS) preferred name and Registry Number (RN), followed by other names, if known
- Purity and composition of the substance or preparation (in percentage[s] by weight)
- Physicochemical properties such as physical state, volatility, pH, stability, chemical class, water solubility relevant to the conduct of the study
- Treatment of the test/control substances prior to testing, if applicable (e.g., warming, grinding)
- Stability, if known

Justification of the Test Method and Protocol Used

Test Method Integrity

- The procedure used to ensure the integrity (i.e., accuracy and reliability) of the test method over time
- If the test method employs proprietary components, the procedure used to ensure their integrity from "lot-to-lot" and over time
- The procedures that the user may employ to verify the integrity of the proprietary components

Criteria for an Acceptable Test

- Acceptable concurrent negative control ranges based on historical data
- Acceptable concurrent positive control ranges based on historical data

Test Conditions

- Apparatus and preparation procedures used
- Source and composition of the biological membrane barrier
- Composition and properties of the qualification and detection solutions
- Method of measurement of effect
- Details of test procedure used (e.g., test substance amounts, number of replicates, method of application, observation times)
- Description of any modifications of the test procedure
- Reference to historical data of the model
- Description of the evaluation and classification criteria used

Results

- Tabulation of test results from individual test samples; (i.e., the time in minutes elapsed between application and barrier penetration for the test substance and the positive, negative, solvent, and benchmark controls reported as individual replicate data, as well as means ± the standard deviation for each trial)

Description of Other Effects Observed

Discussion of the Results

Conclusion

2.4 Reference Chemicals

To ensure that a proposed *in vitro* membrane barrier test method possesses reliability and accuracy characteristics that are comparable to the validated reference test method, the 40 reference chemicals listed in **Table 2-1** must be used. However, to demonstrate technical proficiency, users of the validated reference test method or other similar validated test method that adhere to these

performance standards may want to evaluate their ability to correctly identify the dermal corrosivity classification of a subset of twelve of the chemicals (e.g., 3 noncorrosives, 3 from each Packing Group subcategory) that were correctly identified by the reference test method (see **Table 2-1**). The 40 reference chemicals represent relevant chemical classes and the range of corrosivity responses (i.e., noncorrosives; Packing Group I, II, and III corrosives) and were selected from the 163 chemicals used for the validation of the *in vitro* reference test method. These 40 chemicals consist of eight acid derivatives, eight inorganic acids, eight organic acids, seven organic bases, two acid esters, four inorganic bases, one electrophile, one quaternary ammonium, and one surfactant. They represent the minimum number of reference chemicals that should be used to evaluate the performance of a mechanistically and functionally similar, proposed test method. These chemicals should not be used to develop the prediction model for the proposed test method. If any of the recommended chemicals are unavailable, other chemicals for which adequate *in vivo* reference data are available could be substituted. To the extent possible, the substituted chemical(s) should be of the same chemical class as the original chemical(s). If desired, additional chemicals representing other chemical or product classes and for which adequate reference data are available can be used to more comprehensively evaluate the accuracy of the proposed test method. However, these additional chemicals should not include any that had been used to develop the prediction model for the proposed test method.

The distribution of chemicals in this list by corrosivity and UN Packing Group classification are:

- 12 Noncorrosive Chemicals
- 28 Corrosive Chemicals
  - 9 UN Packing Group I
  - 9 UN Packing Group II
  - 10 UN Packing Group III

2.5 Accuracy and Reliability

When evaluated using the minimum list of recommended reference chemicals in **Table 2-1**, the reliability and accuracy (i.e., sensitivity, specificity, false positive rates, and false negative rates) of the proposed *in vitro* membrane test method should be at least comparable to that of the validated *in vitro* membrane barrier test method (ICCVAM 1999). Noncorrosive and corrosive chemicals, ranging in activity from strong to weak, and representing relevant chemical classes are included so that the performance of the proposed test method can be determined and compared to that of the validated reference test method. For purposes of transportation hazard classification, the list of corrosive chemicals also covers the range of UN Packing Group classifications (ICCVAM 1999; ECVAM 2001). Including these substances will allow for the determination of whether the breakthrough times used to assign test substances to different UN Packing Groups are appropriate.

The penetration times associated with the assignment of each UN Packing Group (or other classification) must be determined for each composition of barrier, indicator, and categorization system. The reliability of the proposed *in vitro* test system, as well as its ability to over- and under-predict known corrosive substances, should be determined prior to testing new chemicals. Based on experience with the validation of different *in vitro* test methods, one effective approach used to establish intra- and inter-laboratory reproducibility for a test method not previously validated is

Table 2-1 Recommended Chemicals for Validation of New *In Vitro* Membrane Corrosivity Test Methods

Chemical[1]	CASRN	Chemical Class[2]	Conc[3] (%)	UN *In Vivo* PG[4]	Validated Test Method PG	pH[3]
Fluorosulfonic acid	7789-21-1	inorganic acid	neat	I	I	0
Nitric acid	7697-37-2	inorganic acid	90	I	I	0
Phosphorus pentachloride	10026-13-8	inorganic acid	98	I	I	0
Selenic acid	7783-08-6	inorganic acid	95	I	I	0
Boron trifluoride dehydrate	13319-75-0	inorganic acid	96	I	I	0.4
Phosphorus tribromide	7789-60-8	inorganic acid	97	I	I	1.0
Sulfuric acid, 10% wt.	7664-93-9	inorganic acid	10	I	I	1.2
Benzyl chloroformate	501-53-1	acid derivative	95	I	NC	2.5
1,2-Diaminopropane	78-90-0	organic base	NA	I	II	8.3
Phosphoric acid	7664-38-2	inorganic acid	85	II	II	0.4
Valeryl chloride	638-29-9	acid derivative	98	II	II	0.5
Acetic acid	64-19-7	organic acid	99+	II	II	1.9
Caprylic acid	124-07-2	organic acid	95	II	NC	2.7
Capric:caprylic acid (45:55)	68937-75-7	organic acid	95	II	NC	3.0
Ammonium hydrogen difluoride	1341-49-7	acid derivative	98	II	II	5.2
1-(2-Aminoethyl) piperazine	140-31-8	organic base	99	II	II	11.8
Ethanolamine	141-43-5	organic base	99+	II	II	11.8
Sodium hydroxide	1310-73-2	inorganic base	100	II	II	13.8
Cyanuric chloride	108-77-0	acid derivative	99	III	III	1.7
Benzenesulfonyl chloride	98-09-9	acid derivative	Neat	III	III	1.8
Crotonic acid	107-93-7	organic acid	99+	III	III	2.3
Butyric anhydride	106-31-0	acid derivative	99	III	III	3.1
Hydroxylamine sulfate	10039-54-0	organic acid	97+	III	III	3.6
2-Methylbutyric acid	600-07-7	organic acid	NA	III	III	3.6
Dicyclohexylamine	101-83-7	organic base	99	III	III	9.6
N,N-Dimethyl benzylamine	103-83-3	organic base	99	III	III	10.7
Tetraethylenepent-amine	112-57-2	organic base	neat	III	III	11.9
2-Ethylhexylamine	104-75-6	organic base	98	III	III	12.0
Maleic acid	110-16-7	organic acid	99	NC	II	1.3

Chemical[1]	CASRN	Chemical Class[2]	Conc[3] (%)	UN *In Vivo* PG[4]	Validated Test Method PG	pH[3]
Copper(II) chloride	7447-39-4	acid derivative	97	NC	II	3.0
Eugenol	97-53-0	organic acid	NA	NC	NC	3.7
Chromium(III) fluoride	7788-97-8	acid derivative	97	NC	NC	3.9
Cinnamaldehyde	14371-10-9	electrophile	100	NC	NC	3.9
Ethyl triglycol methacrylate	39670-09-2	acid ester	neat	NC	NC	4.5
Nonyl acrylate	2664-55-3	acid ester	neat	NC	NC	6.9
Benzalkonium chloride	8001-54-5	quaternary ammonium	100	NC	NC	7.6
Sodium acid carbonate	144-55-8	inorganic base	100	NC	NC	8.3
Sodium undecylenate	3398-33-2	surfactant	33	NC	NC	8.3
Sodium carbonate, 50% aqueous	497-19-8	inorganic base	100	NC	II	11.7
Calcium carbonate	471-34-1	inorganic base	neat	NC	NC	12.6

Abbreviations: CASRN = Chemical Abstracts Service Registry Number; Conc = concentration; NA = not available; NC = noncorrosive; PG = Packing Group; UN = United Nations.

[1]These chemicals, sorted first by *in vivo* rabbit skin corrosivity response and then by pH, represent the range of chemical classes and corrosivity responses [e.g., noncorrosives; UN Packing Groups I, II, and III corrosives] used to validate Corrositex® (ICCVAM 1999). The goal of the selection process was to include, to the extent possible, chemicals that: were representative of the range of corrosivity responses (e.g., noncorrosives; UN Packing Groups I, II, and III corrosives) that the validated reference test method is capable of measuring or predicting; were representative of the chemical classes used during the validation process; reflected the overall performance characteristics of the validated reference test method; have chemical structures that were well-defined; induced reproducible results in the validated reference test method; induced definitive results in the *in vivo* reference test; were commercially available; and were not associated with prohibitive disposal costs.

[2]Chemical class assigned by Barratt et al. (1998) and InVitro International, as provided to ICCVAM (1999).

[3]The concentration tested and the pH values were obtained from the original sources as indicated in ICCVAM (1999).

[4] Within the UN Globally Harmonized System of Classification and Labeling of Chemicals (GHS), the PG classifications correspond as follows: PG I = 1A, PG II = 1B, PG III = 1C (UNECE 2003).

to test each of the reference chemicals three times in each of three independent laboratories. The accuracy of the validated *in vitro* membrane barrier test method for the 40 reference chemicals, and the corresponding values obtained for the complete database considered by ICCVAM in its evaluation of this test method are summarized in **Table 2-2**. The accuracy of the validated *in vitro* membrane barrier test method for the reference chemicals and the corresponding values obtained for the total database compiled during the ICCAM evaluation process are not identical due to constraints associated with the chemical selection process.

The reliability of the proposed test method should also be comparable to that of the validated reference method. However, an assessment of inter-laboratory reproducibility is not essential if the test method is to be used in one laboratory only. The overall inter-laboratory reproducibility of the proposed *in vitro* membrane barrier test method for correctly classifying the UN Packing

group of a test substance detected as corrosive should be at least 93% (ICCVAM 1999; Fentem et al. 1998). In terms of membrane breakthrough times, the overall median coefficient of variation (CV) should not exceed 30% for studies conducted in different laboratories and should not exceed 5% for replicate measurements within an experiment (ICCVAM 1999; Fentem et al. 1998).

Table 2-2 Accuracy of the Validated *In Vitro* Membrane Barrier Test System (Corrositex®) for Skin Corrosion[1]

Source	# of Chemicals	Sensitivity[2]	Specificity[2]	False Negative Rate[2]	False Positive Rate[2]	UN Packing Group Accuracy[2]
Reference Chemicals	40	89% (25/28)	75% (9/12)	11% (3/28)	25% (3/12)	96% (24/25)
ICCVAM (1999)	163	85% (76/89)	70% (52/74)	15% (13/89)	30% (22/74)	Not Determined

Definitions: Sensitivity is defined as the proportion of all positive chemicals or chemical mixtures that are correctly classified as positive in a test. Specificity is defined as the proportion of all negative chemicals or chemical mixtures that are correctly classified as negative in a test. False positive rate is defined as the proportion of all negative chemicals or chemical mixtures that are falsely identified as positive. False negative rate is defined as the proportion of all positive chemicals or chemical mixtures that are falsely identified as negative. UN Packing Group Accuracy reflects the frequency with which Corrositex® correctly assigned the UN Packing Group classification to a substance the *in vitro* test method correctly classified as corrosive.

[1]The validation database is limited to those chemicals that qualified for testing in Corrositex®. The ability of the validated *in vitro* membrane barrier test system to correctly identify the corrosivity potential of the reference chemicals and the corresponding performance characteristics obtained for the complete database evaluated during the ICCVAM evaluation process are not identical due to the constraints associated with the reference chemical selection process. The goal of the selection process was to include chemicals that were representative of the range of corrosivity responses (e.g., noncorrosives; UN Packing Groups I, II, and III corrosives) that the validated reference test method is capable of measuring or predicting; were representative of the chemical classes used during the validation process; reflected the overall performance characteristics of the validated reference test method; have a chemical structure that was well-defined; induced reproducible results in the validated reference test method; induced definitive results in the *in vivo* reference test; were commercially available; and were not associated with prohibitive disposal costs.

[2]In this analysis (see ICCVAM [1999]), a substance is first classified as positive or negative for corrosivity within each laboratory based on the majority of test results obtained (when replicate testing was conducted). Next, the substance is classified as positive or negative for corrosivity based on the majority of test results obtained in multiple laboratories (when multiple laboratory studies were conducted). This approach was used due to the considerable variability in the database in the number of times a substance was tested.