The Dermal Up-and-Down Procedure: An Alternative Method for Acute Dermal Systemic Toxicity Testing

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Abstract

U.S. agencies that regulate chemicals and chemical products require acute dermal systemic toxicity testing to estimate the potential for life-threatening or fatal toxicity from dermal exposures. The proposed dermal up-and-down procedure (UDP) is a sequential sampling design that can potentially reduce the use of animals for acute dermal systemic toxicity testing of nontoxic compounds by 85%. Sequential testing is a powerful statistical sampling technique that allows fewer animals to be used than simultaneous testing of multiple groups of animals with multiple doses as specified in current regulatory test guidelines. In the proposed dermal UDP, individual animals are dosed sequentially, with 48 hours between doses. The response of each animal is used to determine the dose applied to the next animal. If an animal dies, the next animal is tested at a lower dose. If an animal lives, the next animal is tested at a higher dose (with the exception of testing at the default starting doses). The proposed dermal UDP merges the main and limit tests in the current test guidelines into a single test by starting at traditional limit test doses, 2000 mg/kg or 5000 mg/kg, based on regulatory needs. The dose-spacing factor for test substance doses is 4.2. The default doses based on a starting dose of 5000 mg/kg are 5000, 1200, 300, 70, 15, and 4 mg/kg. If test results are to be based on 2000 mg/kg as a starting dose, then the default doses are 2000, 500, 100, 25, and 5 mg/kg. If an investigator, prior to testing animals, expects that the dose expected to produce lethality in 50% of the animals tested (LD_{50}) is less than the default starting dose, testing should start one step below the estimated LD_{50}. The proposed dermal UDP can reduce animal use while providing regulatory agencies with a dermal LD_{50} estimate for dermal hazard classification. (ILS staff supported by NIEHS Contract N01-ES-35504; SRA staff supported by NIEHS Contract GS-23F-8806H.)

Introduction

Acute poisonings from chemicals, pharmaceuticals, and other products are a significant public health problem.

- In 2010, 2.4 million human poisoning cases were reported to U.S. poison control centers (Bronstein et al. 2011). The dermal route of exposure contributed to 7.2% (172,318) of these cases.
- Acute dermal systemic toxicity tests are performed to determine the potential of chemicals and products to cause life-threatening or fatal acute toxicity in humans. Regulatory agencies use the dermal estimated LD_{50}, the dose expected to produce lethality in 50% of the animals tested, to classify dermal hazards for chemicals and products (see **Figure 1**).
  - Hazard classifications are used as the basis for required hazard labels (see **Table 1**) to warn consumers and workers about the potential hazards of a chemical or product, to provide appropriate precautions necessary to avoid or limit chemical exposures that could lead to adverse health effects, and as the basis for packaging requirements for the transport of hazardous substances.

Acute toxicity testing accounts for approximately 50% of the animals used for toxicity testing and accounts for the majority of animals used in testing that experience unrelieved pain and distress. Acute dermal systemic toxicity testing is one of the four most commonly conducted toxicity tests worldwide.

- The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), which is composed of 15 Federal regulatory and research agencies, is charged by law (42 U.S.C. 285l-3) with promoting the regulatory acceptance of scientifically valid tests that can replace, reduce, or refine (lessen or avoid pain and distress) the use of animals in testing.
- ICCVAM previously reviewed and recommended a revised acute oral toxicity up-and-down procedure (oral UDP) as a valid test method to assess acute oral systemic toxicity (ICCVAM 2001). The oral UDP was subsequently adopted by U.S. and international agencies. The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) developed an up-and-down procedure for acute dermal systemic toxicity testing (proposed dermal UDP) as an alternative method to reduce and refine animal use for required regulatory testing.

Figure 1. Hazard Classification Systems for Acute Dermal Systemic Toxicity

Dermal LD_{50} (mg/kg)	GHS	EPA	CPSC	OSHA	DOT
50	1	I	Highly Toxic	1	I
200	2			2	II
1000	3	II	Toxic	3	III
2000	4			4	
5000	5	III			
	Unclassified	IV			

Abbreviations: CPSC = Consumer Product Safety Commission; DOT = Department of Transportation; EPA = Environmental Protection Agency; GHS = U.N. Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); LD_{50} = dose expected to produce lethality in 50% of the animals tested; OSHA = Occupational Safety and Health Administration.

Table 1. Labeling Requirements for Acute Dermal Systemic Toxicity Hazards

Acute Dermal GHS Category	1	2	3	4	5	Unclassified
LD_{50} (mg/kg)[a]	≤50	>50 to ≤200	>200 to ≤1000	>1000 to ≤2000	>2000 to ≤5000	>5000
Signal Word for Label[b]	DANGER	DANGER	DANGER	WARNING	WARNING	NR
Hazard Statement for Label[b]	Fatal in contact with skin	Fatal in contact with skin	Toxic in contact with skin	Harmful in contact with skin	May be harmful in contact with skin	NR

Abbreviations: GHS = U.N. Globally Harmonized System of Classification and Labelling of Chemicals; LD_{50} = dose expected to produce lethality in 50% of the animals tested; NR = none required.

[a] Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011).

[b] Label Review Manual (EPA 2011).

Current Acute Dermal Systemic Toxicity Testing Guidelines

Regulatory agencies require and accept acute dermal systemic toxicity data produced using the current guidelines:

- Organisation for Economic Co-operation and Development (OECD) Test Guideline 402 (OECD 1987)
- EPA Health Effects Test Guidelines: OPPTS 870.1200 (EPA 1998)

The OECD and EPA test guidelines are nearly identical (**Figure 2**), with each containing main test and limit test options.

- The main test is used to estimate LD_{50} values and classify substances expected to have LD_{50} values that would require hazard classification (i.e., substances that are acutely toxic by the dermal route of exposure).
  - Requires a minimum of 20 animals: three doses of test substance, five animals of the same sex per dose group, plus one dose for five animals of the opposite sex
- The limit test is used for substances expected to have LD_{50} values that would not require hazard classification (i.e., substances that are relatively nontoxic by the dermal route of exposure).
  - Requires a single dose of either 2000 mg/kg or 5000 mg/kg, based on regulatory need, applied to five animals of each sex. If the test substance is more toxic than the limit test dose, then the main test is used to determine the LD_{50} value.

Proposed Dermal UDP

The proposed dermal UDP is based on the revised oral UDP (ICCVAM 2001). Computer simulation modeling was used to develop and evaluate different dermal UDP protocols that were based on existing acute dermal systemic toxicity test protocols.

- Computer simulations of animal outcomes save animals while permitting an evaluation of the performance of multiple test designs in estimating the dermal LD_{50} in thousands of simulated tests.
- Computer simulations were appropriate for this evaluation because, compared with the current acute dermal systemic toxicity protocol, the proposed dermal UDP designs involved changes in only the sampling technique and calculation of LD_{50} values.
- Under certain assumptions, computer simulations provide a confidence interval for the true LD_{50} of the population.

The proposed dermal UDP uses the same test substance application techniques as the current dermal test methods and the sequential testing design of the oral UDP. Substances known to be corrosive to the skin should not be tested using the dermal UDP, but all other substances with regulatory requirements for testing are amenable to testing (**Figure 3**).

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Figure 2. Acute Dermal Systemic Toxicity Testing Procedures Using Current Guidelines

- **ANIMAL SELECTION**
  Young adult rats, guinea pigs, or rabbits
- **DOSE LEVELS/DOSE SELECTION**
  •Limit Test: 2000 mg/kg or 5000 mg/kg
  •Main Test: At least 3 doses spaced appropriately to produce test groups with a range of toxic effects and mortality rates; need sufficient data to produce a dose-response curve and, where possible, permit determination of the LD_{50}.
- **NUMBER OF ANIMALS**
  •Limit Test: Requires 5 females and 5 males
  •Main Test: Requires at least 5 animals per dose of one sex and at least one group of 5 animals of the other sex to establish that animals of this sex are not markedly more sensitive to the test substance.
- **PREPARATION OF SKIN**
  Clip/shave fur from the dorsal area of the trunk of the test animals. Do not abrade skin. Clipped area should be approximately 10% of body surface area.
- **APPLICATION OF TEST SUBSTANCE**
  Uniformly apply substance to clipped/shaved area. Hold substance in place with occlusive/semi-occlusive dressing for 24 hours. Remove residual.
- **OBSERVATION**
  Perform clinical observations at least once per day (14-day period). Take appropriate actions to minimize loss of animals to the study. Record time of death.
- **PATHOLOGY**
  Weigh/sacrifice surviving animals at end of test. Perform gross necropsy on all animals.
- **EVALUATION OF RESULTS**
  Evaluate the relationship between exposure of the animals to the test substance and the incidence/severity of all abnormalities, including behavioral and clinical abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects. Calculate the LD_{50} using probit or moving averages techniques.

Figure 3. Up-and-Down Procedure for Acute Dermal Systemic Toxicity Testing

- **ANIMAL SELECTION**
  Young adult rats or rabbits. Females are preferred, but males can be used if demonstrated to be the more susceptible sex.
- **DOSE LEVELS/DOSE SELECTION**
  •Starting Dose: 2000 mg/kg or 5000 mg/kg depending on regulatory needs (Limit Test is incorporated into Main Test)
  •Dose-Spacing Factor: 4.2 for test substance concentrations
  •Default Doses: 2000, 500, 100, 25, and 5 mg/kg or 5000, 1200, 300, 70, 15, and 4 mg/kg
- **NUMBER OF ANIMALS**
  •Continue sequential dosing of individual animals depending on outcomes of all animals tested up to that time (3 to 15 animals).
  •Dose single animals in sequence (48 hours between doses) with a dose-spacing factor of 4.2.
  •If animal dies, test the next animal at a lower dose. If animal lives, next animal is tested at a higher dose.
  •Stop test when one stopping rule is met.
- **STOPPING RULES**
  •Upper testing bound (i.e., 2000 or 5000 mg/kg) is reached, and 3 consecutive animals survive at that bound.
  •Five outcome reversals (i.e., one animal dies, next one lives; or one animal lives and next one dies) occur in any 6 animals tested.
  •At least 4 animals follow the first reversal, and specified likelihood ratios exceed 2.5.
  •A maximum of 15 animals has been tested.
- **PREPARATION OF SKIN**
  Clip/shave fur from the dorsal area of the trunk of the test animals. Do not abrade skin. Clipped area should be approximately 10% of body surface area.
- **APPLICATION OF TEST SUBSTANCE**
  Uniformly apply substance to clipped/shaved area. Hold substance in place with occlusive/semi-occlusive dressing for 24 hours. Remove residual.
- **OBSERVATION**
  Perform clinical observations at least once per day (14-day period). Take appropriate actions to minimize loss of animals to the study. Record time of death.
- **PATHOLOGY**
  Weigh/sacrifice surviving animals at end of test. Perform gross necropsy on all animals.
- **EVALUATION OF RESULTS**
  Evaluate the relationship between exposure of the animals to the test substance and the incidence/severity of all abnormalities, including behavioral and clinical abnormalities; gross lesions, body weight changes, effects on mortality, and any other toxic effects. Calculate the LD_{50} by applying maximum likelihood methods to the mortality data.

Accuracy of the Dermal UDP for Hazard Classification

The extent to which the proposed dermal UDP would classify chemicals into the same GHS dermal hazard categories as the current dermal test was calculated across true LD_{50} values above or below the limit dose of 5000 mg/kg. Each LD_{50} was tested using 1000 computer simulations of each method. For these simulations, the starting dose for the dermal UDP was 5000 mg/kg. A dose-spacing factor of 4.2 was used. Following are five hypothetical substances corresponding to the midpoints of the GHS categories and one substance above the limit dose.

- Substance 1: LD_{50} = 25 mg/kg --- GHS Category 1 (<50 mg/kg)
- Substance 2: LD_{50} = 125 mg/kg --- GHS Category 2 (>50 and ≤200 mg/kg)
- Substance 3: LD_{50} = 600 mg/kg --- GHS Category 3 (>200 and ≤1000 mg/kg)
- Substance 4: LD_{50} = 1500 mg/kg --- GHS Category 4 (>1000 and ≤2000 mg/kg)
- Substance 5: LD_{50} = 3500 mg/kg --- GHS Category 5 (>2000 and ≤5000 mg/kg)
- Substance 6: LD_{50} = 10,000 mg/kg --- GHS unclassified (>5000 mg/kg)

The percentage of simulations correctly classified by the proposed dermal UDP was slightly less than that of the current dermal test for three of the four substances (**Table 2**). The percentage of simulations correctly classified for the substance with LD_{50} = 3500 mg/kg was slightly higher for the dermal UDP, 61% (612/1000), versus 58% (576/1000) for the current dermal test.

Table 2. Classification Rates for GHS Dermal Hazard Categories

True LD_{50} (mg/kg)	Current Traditional Dermal Test			Dermal UDP		
	Correct Hazard Category	Under-classified Category	Over-classified Category	Correct Hazard Category	Under-classified Category	Over-classified Category
25	85% (GHS 1)	15% (11% GHS 2)	NA	76% (GHS 1)	24% (GHS 2)	NA
125	72% (GHS 2)	22% (16% GHS 3)	7% (7% GHS 1)	62% (GHS 2)	32% (31% GHS 3)	7% (7% GHS 1)
600	76% (GHS 3)	18% (11% GHS 4)	6% (4% GHS 2)	65% (GHS 3)	32% (26% GHS 4)	3% (3% GHS 2)
1500	55% (GHS 4)	27% (20% GHS 5)	18% (16% GHS 3)	48% (GHS 4)	33% (30% GHS 5)	19% (19% GHS 3)
3500	58% (GHS 5)	31% (31% GHS Unc)	12% (7% GHS 4)	61% (61% GHS 5)	24% (24% GHS Unc)	15% (14% GHS 4)
10,000	95% (GHS Unc)	NA	5% (3% GHS 5)	79% (GHS Unc)	NA	21% (20% GHS 5)

Abbreviations: GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2011); LD_{50} = dose expected to produce lethality in 50% of the animals tested; NA = not applicable; UDP = up-and-down procedure; Unc = unclassified.

Underclassified means a determination that results in a higher (i.e., less toxic) GHS category than the true one. *Overclassified* means a determination that results in a lower (i.e., more toxic) GHS category than the true one. The percentages in parentheses show which under- or overclassified category was most represented.

Table 3 compares the underclassification (false negative) rates for the proposed dermal UDP and the current dermal tests for correctly categorizing substances into a toxic category (LD_{50} < 5000 mg/kg) versus a nontoxic category (LD_{50} > 5000 mg/kg). False negative results are those in which a substance was categorized as Unclassified when the true LD_{50} was less than 5000 mg/kg. The proposed dermal UDP underclassified fewer tests than the current dermal test for the five simulated LD_{50} values that were <5000 mg/kg.

Table 3. Underclassification (False Negative) Rate for the Current Dermal Test Compared to the Proposed Dermal UDP: Hazard vs. Nonhazard

True LD_{50} (mg/kg)	Underclassification (False Negative) Rate[a]	
	Current Dermal Test	Dermal UDP
25	3% (30/1000)	0% (0/1000)
125	4% (43/1000)	0% (0/1000)
600	6% (56/1000)	0% (1/1000)
1500	7% (70/1000)	3% (30/1000)
3500	31% (305/1000)	24% (235/1000)

Abbreviations: LD_{50} = dose expected to produce lethality in 50% of the animals tested; UDP = up-and-down procedure.

Rates were calculated from the results of 1000 simulations for each test protocol. Both tests used a dose-spacing factor of 4.2. The starting dose for the dermal UDP was 5000 mg/kg, and the mid dose for the current dermal test was the LD_{50}.

For LD_{50} = 10,000 mg/kg, the dermal UDP incorrectly identified more tests as positive (i.e., LD_{50} > 5000 mg/kg) than the current dermal test. The false positive rate of the dermal UDP was 21% (208/1000), and the false positive rate of the current dermal test was 5% (50/1000).

[a] Test method results estimated the LD_{50} as >5000 mg/kg when the true LD_{50} < 5000 mg/kg.

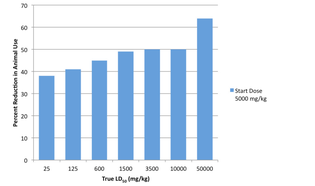
Animal Reduction Using the Dermal UDP

To determine the animal savings that could be produced by the proposed dermal UDP, the number of animals required was compared to the number of animals required by the current dermal test when testing hypothetical substances using 1000 simulations of each test protocol (**Figure 4**).

The proposed dermal UDP, similar to the oral UDP, does not require the use of both sexes of animals. This recommendation, which accounts for a savings of 5 animals per test, is based on a review of over 1351 studies that showed that female animals were more likely to have a lower LD_{50} than males when the sex-specific hazard categories were different (see SOT 2013 Poster Abstract 1573/ Poster Board 634).

- Starting dose of 5000 mg/kg (dose-spacing factor = 4.2; dose–mortality slope = 1.6)
  - Hypothetical substances with true LD_{50} values of 25, 125, 600, 1500, 3500, 10,000, and 50,000 mg/kg.
  - The mid dose for the current dermal test was chosen as the true LD_{50} and the starting dose for the proposed dermal UDP was 5000 mg/kg.
- Starting dose of 2000 mg/kg (dose-spacing factor = 4.2; dose–mortality slope = 1.6)
  - Hypothetical substances with true LD_{50} values of 25, 125, 600, 1500, 4000, and 20,000 mg/kg.
  - The mid dose for the current dermal test was chosen as the true LD_{50}, and the starting dose for the dermal UDP was 2000 mg/kg.

Figure 4. Reduction in Animal Use Using the Proposed UDP vs. the Current Dermal Toxicity Test



Abbreviations: LD_{50} = dose expected to produce lethality in 50% of the animals tested.

The number of animals used was calculated from the results of 1000 simulations for each test protocol. A dose-spacing factor of 4.2 was used. The mid dose for the current dermal test was the same as the starting dose for the dermal UDP. Simulations with a starting dose of 2000 mg/kg were performed, and the results were very similar to those with the 5000 mg/kg starting dose.

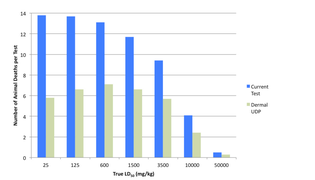
Animal Refinement Using the Dermal UDP

The reduction in animal deaths (moribund euthanasia and spontaneous deaths) through use of the proposed dermal UDP was determined by comparing deaths from using the dermal UDP to deaths using the current dermal test when testing hypothetical substances (1000 simulations; **Figure 5**). The proposed dermal UDP provides significant refinement whenever substances have an LD_{50} below 10,000 mg/kg, as fewer animals die or become moribund.

When a test substance has a dermal LD_{50} <5000 mg/kg and is initially tested using the limit test in the current dermal test, 50% to 100% of the 10 animals used are expected to die or require moribund euthanasia.

Figures 5, **6**, **7,** and **8** show examples of outcomes when using the proposed dermal UDP.

Figure 5. Reduction in Animal Deaths



Abbreviations: LD_{50} = dose expected to produce lethality in 50% of the animals tested; UDP = up-and-down procedure.

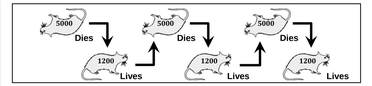
The number of animals used was calculated from the results of 1000 simulations for each test protocol. A dose-spacing factor of 4.2 was used. The mid dose for the current dermal test was the same as the starting dose for the dermal UDP. Simulations with a starting dose of 2000 mg/kg were performed, and the results were very similar to those with the 5000 mg/kg starting dose.

Examples of Dermal UDP Testing

Figure 6. Potential Test Outcome for a Nontoxic Substance (LD_{50} > 5000 mg/kg)

- 3 animals are tested sequentially. If all animals live, testing stops after 3 animals
- Stopping rule = 3 consecutive animals live at the maximum dose
- Animal savings = 85% (17/20) compared with the current dermal test

Figure 7. Potential Test Outcome for a Toxic Substance (LD_{50} = 2449 mg/kg)



- Stopping rule = 5 reversals in 6 consecutive tests (i.e., between same 2 doses).
- LD_{50} point estimate is calculated.

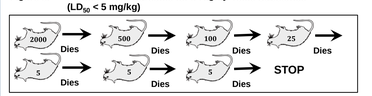
Animal Reduction

- 70% fewer animals used compared to the current dermal test (6 vs. 20)
- 80% fewer animals used compared to current limit test followed by current dermal test (6 vs. 10+20)

Animal Refinement

- 70% fewer animals (3 vs. average of 10) die/become moribund compared to the current dermal test
- 85% fewer animals (3 vs. 20 or more) die/become moribund compared with using current limit test (all 10 would die) followed by current dermal test (estimated 50% would die)

Figure 8. Potential Test Outcome for a Highly Toxic Substance (LD_{50} < 5 mg/kg)



- Three animals die at the protocol's lowest test dose (test ends)
- LD_{50} point estimate is < 5 mg/kg.

Conclusions

- The dermal UDP provides improved identification of dermal toxicity hazards compared to the current acute dermal toxicity procedure for regulatory dermal hazard classification purposes.
  - The dermal UDP identified a higher percentage of dermal hazards compared to the current dermal test (**Table 2**).
  - For some hazard categories, the dermal UDP was slightly more likely to categorize dermal hazards into a less toxic category (**Table 1**).
- The dermal UDP protocol is applicable to all testing situations. It incorporates the limit test, avoiding the need to sometimes perform the traditional limit test and sometimes perform the traditional multidose dermal toxicity test.
- The dermal UDP significantly reduces animal use in all testing situations, with animal savings up to 85% compared to the traditional acute dermal toxicity test.
- The dermal UDP provides for significant animal refinement by reducing the number of animals that die or require moribund euthanasia in all testing situations where lethality occurs, with up to 85% fewer deaths compared to the traditional test.
- The dermal UDP protocol can be readily performed by any laboratory currently performing the oral UDP, and the oral UDP software program (EPA 2001) can also be used for the dermal UDP protocol.

References

Bronstein AC, Spyker DA, Cantilena LR, Green JL, Rumack BH, Dart RC. 2011. 2010 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS): 28th Annual Report. Clin Toxicol 49: 910-941. Available: http://informahealthcare.com/doi/full/10.3109/15563650.2011.635149

EPA. 1998. Health Effects Test Guidelines: OPPTS 870.1200 - Acute Dermal Toxicity. EPA 712-C-98-192. Washington, DC:U.S. Environmental Protection Agency. Available: http://www.epa.gov.oppefrs1/publications/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-1200.pdf

EPA. 2001. The Acute Oral Toxicity Statistical Program (AOT425StatPgm). Available: http://www.epa.gov/oppfead1/harmonization/

EPA. 2011. Label Review Manual. Revised July 2011. Washington, DC:U.S. Environmental Protection Agency. Available: http://www.epa.gov/oppfead1/labeling/lrm/

ICCVAM. 2001. The Revised Up-and-Down Procedure: A Test Method for Determining the Acute Oral Toxicity of Chemicals. Volumes I and II. NIH Publication No. 02-4501. Research Triangle Park, NC:National Institute of Environmental Health Sciences. Available: http://iccvam.niehs.nih.gov/docs/acutetox_docs/udpProc/udpfn01vol_1.pdf and http://iccvam.niehs.nih.gov/docs/acutetox_docs/udpProc/udpfn01vol_2.pdf

OECD. 1987. Test No. 402. Acute Dermal Toxicity [adopted 24 February 1987]. In: OECD Guidelines for the Testing of Chemicals, Section 4: Health Effects. Paris:OECD Publishing. Available: http://dx.doi.org/10.1787/9789264070585-en

UN. 2011. GHS Implementation Web site [Internet]. Geneva, Switzerland:United Nations Economic Commission for Europe. Available: http://www.unece.org/trans/danger/publi/ghs/implementation_e.html.