ESTABLISHMENT OF LD_{50} REFERENCE VALUES FOR CHEMICALS USED IN VALIDATION STUDIES OF *IN VITRO* ACUTE TOXICITY ASSAYS

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Abstract

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and NICEATM convened an international workshop in October 2000 to evaluate the validation status of *in vitro* methods for predicting acute systemic toxicity (http://iccvam.niehs.nih.gov). Workshop participants recommended further evaluation of the usefulness of *in vitro* methods for predicting rodent and human acute toxicity. NICEATM and ECVAM subsequently designed a multi-laboratory validation study to evaluate the utility of two *in vitro* cytotoxicity tests using 72 chemicals. A major aspect of the study design was the selection of the rodent LD_{50} reference value for each chemical. LD_{50} studies were located through literature searches and secondary references. Studies were reviewed to identify the most appropriate LD_{50} reference value for each chemical. Criteria used to select reference LD_{50} values included: 1) similarity of age, gender, and species to that recommended in current acute lethality testing guidelines, and 2) quality of the data, including conduct in accordance with standardized test guidelines and Good Laboratory Practices. Chemical-specific examples of the selection decisions for reference LD_{50} values will be provided. These reference data will be used to evaluate the extent that *in vitro* test methods can predict rodent LD_{50} values. Supported by NIEHS contract N01-ES-85424.

Introduction

In October, 2000, the International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity was convened to evaluate the validation status of *in vitro* methods for predicting acute systemic toxicity. Workshop participants recommended further evaluation of the usefulness of *in vitro* methods for predicting rodent and human acute toxicity (ICCVAM 2001). One Breakout Group of participants developed recommendations for selection of chemicals that could be used in validation of individual tests or prediction models. NICEATM and the European Centre for the Validation of Alternative Methods (ECVAM) subsequently designed a multi-laboratory validation study to evaluate the utility of two *in vitro* cytotoxicity tests using 72 chemicals.[1]

A major aspect of the study design was the selection of the rat LD_{50} reference value for each chemical. Primary LD_{50} studies were located through database searching, literature searches, and secondary references. To identify the most appropriate LD_{50} reference value, studies were evaluated using a weight of evidence approach. The primary selection criteria for the most appropriate LD_{50} values included the use of young adult rats of a common laboratory strain/stock (ICCVAM 2002), oral gavage administration, documentation of experimental parameters such as method of administration, doses used, number of animals and deaths at each dose, and report of a measure of variability for the LD_{50}. These reference data will be used to evaluate the extent that *in vitro* test methods can predict rodent LD_{50} values.[2]

[1]See poster entitled "Selection of Reference Chemicals for the Validation of *In Vitro* Cytotoxicity Assays for Predicting *In Vivo* Acute Systemic Toxicity" by Strickland et al. for more information on chemical selection.

[2]See poster entitled "Validation Study Design to Evaluate *In Vitro* Cytotoxicity Assays for Predicting Rodent and Human Acute Systemic Toxicity" by Stokes et al. for more information on the use of these values.

Table 1. Internet-Accessible Databases with LD_{50} Information

Database	Sponsor
• Registry of Toxic Effects of Chemical Substances (RTECS®) • NIOSH Pocket Guide to Chemical Hazards	National Institute for Occupational Safety and Health (NIOSH)
• Integrated Risk Information System (IRIS)	U.S. Environmental Protection Agency (U.S. EPA) Office of Research and Development (ORD)
• Toxic Chemical Release Inventory (TRI) • GENE-TOX	The National Library of Medicine (NLM); U.S. EPA
• Developmental and Reproductive Toxicology/Environmental Teratology Information Center (DART®/ETIC)	U.S. EPA; NLM; The National Institute of Environmental Health Sciences (NIEHS); National Center for Toxicological Research (NCTR)
• Oil and Hazardous Materials/Technical Assistance Data System (OHM/TADS)	U.S. EPA Office of Waste and Water Management
• ChemRTK High Production Volume (HPV) Challenge Program • OPPT Chemical Fact Sheets • Chemical Information Collection and Data Development	U.S. EPA Office of Pollution Prevention and Toxics (OPPT)
• Pesticide Product Information System (PPIS)	U.S. EPA Office of Pesticide Programs (OPP)
• Toxic Substances Control Act Test Submissions (TSCATS)	U.S. EPA OPPT
• Chemical Ingredients Database	U.S. EPA Office of Pesticide Programs (OPP); California EPA Department of Pesticide Regulation
• TOXLINE® • Hazardous Substances Data Bank (HSDB) • ChemIDplus	NLM [TOXNET]
• Chemical Carcinogensis Research Information System (CCRIS) • National Cancer Institute Website	National Cancer Institute (NCI); National Institutes of Health (NIH); U.S. Department of Health and Human Services (U.S. DHHS)
• Chemical Hazard Response (CHRIS)	U.S. Coast Guard
• Emergency Response Guidebook (ERG 2000)	Transport Canada; U.S. Department of Transportation (U.S. DOT); Secretariat of Communications and Transportation of Mexico
• Agency for Toxic Substances and Disease Registry (ATSDR)	U.S. Department of Health and Human Services (U.S. HHS)
• National Toxicology Program (NTP) Chemical Health and Safety Database	NIEHS
• Center for Drug Evaluation and Research (CDER)	U.S. Food and Drug Administration (U.S. FDA)
• National Transportation Library	U.S. DOT
• Consumer Product Safety Commission Website	U.S. Consumer Product Safety Commission (U.S. CPSC)
• The EXtension TOXicology NETwork (EXTOXNET)	University of California, Davis, Oregon State University, Michigan State University, Cornell University, and the University of Idaho
• The Right-to-Know Network (RTK NET)	Office of Management and Budget Watch; Center for Public Data access
• CHEMINDEX • CHEMINFO	Canadian Centre for Occupational Health and Safety (CCOHS) CHEMpendium™
• Chemical Evaluation Search and Retrieval System (CESARS)	Michigan Department of Natural Resources; Ontario Ministry of the Environment; (CCOHS) CHEMpendium™
• CIS Chemical Information (ILO/CIS)	The International Programme on Chemical Safety (IPCS); CCOHS; Labour Organisation (ILO) Occupational Safety and Health Information Centre (CIS)
• Concise International Chemical Assessment Documents (CICADS)	IPCS; CCOHS; World Health Organization (WHO), the International Labour Organisation (ILO), and the United Nations Environment Programme (UNEP)
• Environmental Health Criteria (EHC) monographs • Health and Safety Guides (HSG) • International Agency for Research on Cancer (IARC)	IPCS; CCOHS; WHO
• International Chemical Safety Cards (ICSC) • IPCS/EC Evaluation of Antidotes Series	IPCS; CCOHS; Commission of the European Union
• Joint Expert Committee on Food Additives (JECFA) • Joint Meeting on Pesticide Residues (JMPR) • Pesticide Data Sheets (PDSs)	IPCS; CCOHS; WHO; Food and Agriculture Organization (FAO) of the United Nations
• Poisons Information Monographs (PIMs)	IPCS; CCOHS
• Organisation for Economic Co-operation and Development (OECD) Screening Information Data Sets (SIDS)	IPCS; CCOHS; International Register of Potentially Toxic Chemicals (IRPTC); United Nations Environmental Programme (UNEP)
• Deutsches Institut fur Medizinische Dokumentation und Information (DIMDI) [The German Institute for Medical Documentation and Information] • Registry of Cytotoxicity (RC)	Zentralstelle zur Erfassung und Bewertungvon Ersatz- und Erganzungsmethoden zum Tiervers uch (ZEBET) [German Centre for the Documentation and Validation of Alternative Methods]
• International Uniform ChemicaL Information Database (IUCLID)	European Chemicals Bureau
• European Centre for the Validation of Alternative Methods Scientific Information Service (ECVAM SIS)	European Commission Joint Research Centre
• Multicentre Evaluation of In Vitro Cytotoxicity (MEIC)	Scandinavian Society for Cell Toxicology
• New Jersey Hazardous Substance Fact Sheets	New Jersey Department of Health and Senior Services
• HAZARDTEXT®; MEDITEXT®; INFOTEXT®; SARATEXT®; REPROTEXT®; REPROTOX®	TOMES Plus®, MICROMEDEX, Greenwood Village, CO
• CHEMFINDER	CambridgeSoft Corporation
• Pesticide Action Network Pesticide Database	Pesticide Action Network North America
• SCORECARD	Environmental Defense
• Material Safety Data Sheets (MSDS)	Interactive Living Paradigms Incorporated

Selection Criteria for Rodent Oral LD_{50} Reference Values

A weight of evidence approach was used to determine the most appropriate rat oral LD_{50} value to use as a reference value. The weight of evidence judgment requires the consideration of relevant study parameters and an assessment of the quality and quantity of data reported in each LD_{50} experiment to make an informed selection of the most appropriate study(ies). Since this judgment involves considerations of the quality and adequacy of data, the level of detail reported by the individual studies regarding the experimental design and results plays a major role in determining their adequacy. The following table lists the experimental design parameters used as factors.

Table 2. Weight of Evidence Factors

Increased Weighting	Decreased Weighting
Commonly used laboratory rat strain/stock	Uncommon or undefined rat strain/stock
Young adult rats (8-12 weeks preferred)	No details on sex, age Rats older or younger than 8-12 weeks
LD_{50} confidence limits	No LD_{50} confidence limits
Relatively large number of animals/dose group	Relatively small number of animals/dose group
Number and spacing of dose groups	Limited/missing number and spacing of dose groups
Relatively small confidence limits	Relatively large confidence limits
Gavage administration	Other method/route
GLP or GLP-like lab documentation	Non-GLP
Individual animal data	Only animal group data
Other studies of less quality have similar values	Values quite different from the majority of similar studies

Example: Selection of Rat Oral LD_{50} Value from Primary References

Arsenic (III) Trioxide

- Eight primary LD_{50} references, reporting nine values, were identified (see Tables 3 and 4).
- LD_{50} values ranged from 13 to 385 mg/kg.
- Harrison et al. (1958), LD_{50} = **24.2 + 2.9 mg/kg,** was selected as providing the most reliable reference value.

Positive Weighting Factors include:

- Rat gender and strain/stock: Male Sprague-Dawley
- Rat age: Estimated as 5-6 weeks, from reported weights, was closest to the desired age of 8 - 12 weeks without being greater. Weights of 125 – 200 g correspond to 5 - 6 weeks of age according to charts from Taconic Farms.
- Measure of variability: Reported as ± 2.9 mg As_2O_3/kg.
- Documentation was most complete: Included sex, strain/stock, and weight of the animals, and details of chemical administration.
- Thirty (30) animals were used for each of five doses (150 animals total). Deaths at each dose were reported:

Dose (mg As/kg)	Percent Mortality (at 96 hr post dosing)
10	30.0
20	66.7
30	90.0
40	93.3
50	100

Other studies (and reasons for exclusion):

- Kitagawa et al. (1982). LD_{50} = 81.5 mg/kg. Fewer animals used per dose (10 animals for each of four doses and control). Fewer doses used (four vs. five).
- Harrison et al. (1958). LD_{50} = 232.7 mg/kg. Chemical was administered in food rather than oral gavage.
- Done and Peart (1971). LD_{50} = 385 mg/kg. Rats were older than 12 weeks (13 – 41 weeks) and compound was administered in gel capsules to anesthetized rats.
- Dieke and Richter (1946). LD_{50} = 138 mg/kg. Used wild Norway rats. The level of reporting was not as complete as other studies. Did not report age of rats or doses used.
- Pryor et al. (1983). LD_{50} = 32.6 mg/kg. The animal strain/stock and age were unreported, doses used were unspecified, and the number of animals and animals/dose were reported as ranges.
- Peardon et al. (1972). LD_{50} = 140 mg/kg. Used wild Norway rats. The level of reporting was not as complete as other studies. Did not report sex, weight, or age of the animals.
- Lehman et al. (1951). LD_{50} = 13 mg/kg. The following items were not reported: measure of variability, rat strain, age and sex, doses, and number of animals/dose.
- Tulakino and Novikov (1987). LD_{50} = 14.6 mg/kg. No experimental details, other than the sex of the animals, are known.

Table 3. Factors Evaluated for Weight of Evidence Judgment

Reference	LD50 (mg/kg)	Rat Information				Dosing				Measure of Variability
		Strain/ Stock	Age	Gender	Total No.	Method	Doses	Animals/ dose	Deaths/ dose	
Harrison et al. 1958	**24.2**	**X**	**5-6 wk**	**X**	**150**	**X**	**5**	**30**	**X**	**X**
Kitagawa et al. 1982	81.5	X	5 wk	X	50	X	5	10	X	X
Harrison et al. 1958	232.7	X	5-6 wk	X	140	LD	7	20	X	X
Done & Peart 1971	385	X	14 wk	X	~70	LD	7			X
Dieke & Richter 1946	138	LD	LD	X	41	X				X
Pryor et al. 1983	32.6			X	40-60	X	5-6	8-10		X
Peardon et al. 1972	140	LD				X				
Lehman 1951	13					X				
Tulakino & Novikov 1987[a]	14.6			X						

Notes: X -- Information that meets criteria was reported; LD -- Information provided, but attributes were less desirable than other studies; Blank cells -- No information provided.
[a]Reference not translated from Russian

Table 4. Rat OralLD_{50} Reference Values for Arsenic Trioxide

LD50 (mg/kg) As_2O_3	LD50 Measure of Variability (mg/kg) As_2O_3	LD50 Calculation Method	Animal Information Provided (stock, weight, age)	Gender	Method of Oral Administration	Doses	Observations	Notes	Primary Reference
13			Rats		Stomach tube		Violent gastroenteritis, diarrhea, rice water stools.	From US FDA Division of Pharmacology; fasted animals.	Lehman 1951.
14.6			Rats	Male			No clinical signs given.	Russian; not translated.	Tulakino & Novikov 1987. RTECS 2002 REFERENCE
24.2 (reported as 15.1 mg As/kg)	**+/- 2.9 (reported as +/- 1.8 mg As/kg)**	**de Beer EJ. 1945. JPET 85:1.**	**Sprague-Dawley; 125 - 200 g**	**Male**	**Intra-esophageal via feeding needles**	**In 0.03 mL distilled water/g body weight; max volume 8 mL; 10 - 50 mg As/kg.**	**LD50 calculated at 96 hours; rats observed for 2 weeks; no difference between males & females; 95 dead at 24 hr; Deaths/dose at 96 hr (male): 10 mg As/kg -- 9/30; 20 mg As/kg -- 20/30; 30 mg As/kg -- 27/30; 40 mg As/kg -- 28/30; 50 mg As/kg -- 30/30.**	**5 groups of 30 rats (150 total); fasted 24 hr; results given only for males.**	**Harrison et al. 1958.**
32.6	28.4 - 36.7 95% confidence interval	Probit Analysis. Finney DJ. 1971. Statistical Methods in Biological Assay, 2nd ed. London:Griffin Press.	Rats	Male	Intubated	In 2 mL/kg distilled water; 5 - 6 doses.	Deaths recorded daily for 7 days.	Animals acclimated to environment for 2 weeks before testing; used only healthy rats; 5 - 6 groups of 8 - 10 rats.	Pryor et al. 1983.
81.5	70.5 - 94.3	Bliss-Probit method	Sprague-Dawley; 5 weeks	Male	Gavage	In saline at 51.2, 66.5, 86.5, 112.5, 146.2 mg/kg.	Rats observed 6 hours after dosing and once a day for 1 - 2 weeks; vomiting and diarrhea; 27/50 died, most within 3 days.	Animals acclimated to environment for 1 week before testing; 5 groups of 10 rats; fasted 16 hours.	Kitagawa et al. 1982.
138	+/- 13 (standard error)	Litchfield JT Jr, Fetig JW. 1941. On a graphic solution of the dosage-effect curve. Bull Johns Hopkins Hosp 69: 276 - 286.	Wild Norway (trapped in Baltimore, MD); 148 - 493 g (ave. = 253 g); adult	Male and female	Gavage via metal needle	In 10% acacia solution; 1mL/100 g body weight.	Rats survived from 6 - 72 hours.	41 rats used (~ equal number of male and female); overnight fasting; assays performed in winter and repeated in summer; LD50 values from combined information; final LD50 was higher than winter LD50; attributed to not having enough rats in winter.	Dieke & Richter 1946.
140		Statistical formula based on mortality rates	Wild Norway		Stomach tube	Used a number of doses at different concentrations.	Enteritis and neuritis.	Equal number of rats/dose.	Peardon et al. 1972.
232.7 (reported as 145.2 mg As/kg)	+/- 14.0 (reported as +/- 8.7 mg As/kg)	de Beer EJ. 1945. JPET 85:1.	Sprague-Dawley; 125 - 200 g	Male	In 3 g Purina rat chow consumed in 1 hour	Pure arsenic trioxide mixed with food; 30.1 - 338 mg As/kg.	LD50 calculated at 96 hours; rats observed for 2 weeks; no difference between males and females; 76 dead at 24 hr; Deaths/dose at 96 hr: 30.1 mg As/kg -- 0/20; 91 mg As/kg -- 2/20; 128.1 mg As/kg -- 6/20; 180.9 mg As/kg -- 12/20; 207.8 mg As/kg -- 18/20; 269.0 mg As/kg -- 20/20; 338.0 mg As/kg -- 20/20.	7 groups of 20 rats each (140 total); fasted 24 hr; results given only for males.	Harrison et al. 1958.
385	350 - 424 95% confidence limits	Litchfield and Wilcoxon method	Holtsman; 300 - 500 g; 100 - 300 days old (13 - 41 weeks)	Male and female	Gelatin capsules administered under light anesthesia	20, 50, 100, 250, 500, 750, 1000 mg/kg.	Death occurred within 4 days.	~ 70 rats used; 24 hour fasting.	Done & Peart 1971.

Blank Spaces: Information not provided

Table 5. Preliminary Reference LD_{50} Values for Chemicals to be Tested

GHS[1] Category/Chemical	Rodent Oral LD_{50}[2] (mg/kg)	Preliminary Selected Oral LD_{50}[3] (mg/kg)	Product/Use
LD_{50} ≤ 5 mg/kg			
Mercury II chloride	1	12	Pesticide
Triethylenemelamine	1.0	13.0	Manufacturing
Sodium selenate	1.6	6.0	Feed additive
Busulphan	2	28	Pharmaceutical
Cycloheximide	2	1	Pesticide
Disulfoton	2.0	2.3	Insecticide
Parathion	2.0	6.0	Insecticide
Strychnine	2	2.35	Pesticide
Aminopterin	3.0	3.0	Medicinal
Phenylthiourea	3.0	3.1	Pesticide
Epinephrine bitartrate	4.0	4.0	Pharmaceutical
Physostigmine	4.5	4.5	Pharmaceutical
LD_{50} > 5 - ≤ 50 mg/kg			
Colchicine	6	6	Pharmaceutical
Potassium cyanide	10	10	Electroplating
Dichlorvos (DDVP)	17	56	Pesticide
Digoxin	18	28.27	Pharmaceutical
Fenpropathrin	18	18	Pesticide
Endosulfan	18	18	Pesticide
Arsenic III trioxide	20	24	Pesticide
Thallium I sulfate	29	25	Pesticide
Sodium arsenite	41	53	Pesticide
Triphenyltin hydroxide	44	46.4	Pesticide
Sodium dichromate	50	39.02	Oxidizing agent
Nicotine	50	70	Pharmaceutical
LD_{50} > 50 - ≤ 300 mg/kg			
Paraquat	58	100	Pesticide
Hexachlorophene	61	63	Disinfectant
Lindane	76	88	Pesticide
Cadmium II chloride	88	132	Veterinary pharmaceutical
Verapamil HCl	108	114	Pharmaceutical
Haloperidol	128	850	Pharmaceutical
Sodium oxalate	155	11160	Paints, cleaners
Phenobarbital	163	220	Pharmaceutical
Sodium I fluoride	180	80	Electroplating, fluoridation
Caffeine	192	279	Pharmaceutical, food additive
Diquat	231	231	Pesticide
Cupric sulfate * 5 H2O	300	960	Pesticide
LD_{50} > 300 - ≤ 2000 mg/kg			
Amitriptyline	319	320	Pharmaceutical
Phenol	414	400	Disinfectant
Propranolol HCl	470	466	Pharmaceutical
Chloral hydrate	479	800	Pharmaceutical
Glutethimide	600	600	Pharmaceutical
Atropine sulfate	623	698.7	Pharmaceutical
Valproic acid	670	670	Pharmaceutical
Meprobamate	794	794	Pharmaceutical
Acetylsalicylic acid	1000	1430	Pharmaceutical
Lithium I sulfate	1187	613	Pharmaceutical
Procainamide	1950	1950	Pharmaceutical
Carbamazepine	1957	1957	Pharmaceutical
LD_{50} > 2000 - ≤ 5000 mg/kg			
Acetaminophen	2404	2404	Pharmaceutical
Potassium I chloride	2602	2600	Pharmaceutical, manufacturing
Sodium chloride	2998	3750	Pharmaceutical, food additive
Chloramphenicol	3393	3400	Pharmaceutical
Boric aid	2660	5140	Pesticide
Lactic acid	3730	3730	Food additive
Citric Acid	3000	3000	Food additive
Dimethylformamide	2800	7182	Solvent
Xylene	4300	8620	Solvent
Trichloroacetic acid	4999	3320	Fixative
Acetonitrile	3798	4050	Solvent
Carbon tetrachloride	2799	5453	Solvent
LD_{50} > 5000 mg/kg			
2-Propanol	5843	5338	Disinfectant
Ethylene glycol	8567	6135	Antifreeze
Ethanol	14008	11534	Solvent
1,1,1-Trichloroethane	10298	16000	Solvent
Methanol	13012	7005	Solvent
Propylparaben	6326	6332	Food additive
5-Aminosalicylic acid	7749	2800	Pharmaceutical
Sodium hypochlorite	8910	8910	Disinfectant
Dibutyl phthalate	11998	8000	Plasticizer
Glycerol	12691	27500	Solvent
Gibberellic acid	6305	6300	Plant growth regulator
Diethyl phthalate	6179	10100	Plasticizer

[1]GHS-Globally Harmonised System categories of acute oral toxicity (OECD 2001).
[2]Values used to classify chemicals into GHS toxicity categories. Data from Registry of Cytotoxicity (mostly Registry of Toxic Effects of Chemical Substances [RTECS] [1984]) or EPA Office of Pesticide Programs. The Registry of Cytotoxicity is a database of chemical specific IC_{50}s and LD_{50}s (Halle 1998).
[3]Preliminary selections of most appropriate LD_{50} data after review of the available literature.

Observations

- A number of studies reporting rat oral LD_{50} values exist for most of the validation chemicals. The highest number of values for any one chemical was 29 for acetonitrile.
- A rat oral LD_{50} value has yet to be identified for three chemicals: epinephrine bitartrate, aminopterin, and propylparaben.
- Reported rat oral LD_{50} values for individual chemicals may vary greatly.
- Some LD_{50} references are secondary references and some LD_{50} references provide totally unsupported LD_{50} values.
- The level of detail reported for lethality studies varies greatly. Some studies report only the type of animal used and other studies provide complete details on animals, administration, doses, clinical signs, and times of death.
- Very few references reported the use of GLP.

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