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3.0 REFERENCE SUBSTANCES USED FOR VALIDATION OF THE 3T3 AND NHK NRU TEST METHODS

This section discusses the rationale for the selection of the 72 reference substances tested to validate the 3T3 and NHK NRU test methods for determining starting doses for rodent acute oral systemic toxicity testing. Information regarding chemical class and physical/chemical characteristics is provided, as is the available information on toxicological characteristics, such as target, organ, extent of metabolism, and mechanism of action, for the 72 reference substances. Such information may be useful for characterizing the performance of the 3T3 and NHK NRU test methods for various chemical types. Chemical supplier and purity information are provided as well as the methods for purchasing, coding, and distributing the substances to the testing laboratories.

3.1 Rationale for the Reference Substances Selected for Testing

This section describes the procedure used to select the 72 reference substances tested in the NICEATM/ECVAM validation study.

3.1.1 Reference Substance Selection Criteria

The SMT selected reference substances for testing in 2001 and 2002 using a process based primarily on general recommendations made by Workshop 2000 participants (ICCVAM 2001b). The following criteria were used:

- the toxicities of the reference substances should be evenly distributed across the expected range of LD_{50} values (i.e., the GHS classification for acute oral toxicity [UN 2005])
- the reference substances should cover a wide range of structural and use classes, according to the needs of various user communities
- substances with human toxicity data and/or human exposure potential (i.e., substances of interest to society) should be included

Table 3-1 shows the GHS classification scheme which classifies chemicals into five toxicity categories or an unclassified group based on acute oral LD_{50} values (UN 2005).

Table 3-1 UN GHS[1] Classification Scheme for Acute Oral Toxicity

Category	LD_{50} (mg/kg)
1	$LD_{50} \leq 5$
2	$5 < LD_{50} \leq 50$
3	$50 < LD_{50} \leq 300$
4	$300 < LD_{50} \leq 2000$
5	$2000 < LD_{50} \leq 5000$
Unclassified	$LD_{50} > 5000$

[1]Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005)

For the purposes of toxicity classification, the rodent oral LD_{50} values for individual reference substances were obtained from readily available toxicological databases. Rat LD_{50} values were preferred, but mouse LD_{50} values were used (three reference substances) when rat data were unavailable. However, mouse data were not used in the regression analyses (See **Section 6**). The Registry of Cytotoxicity (RC) is a database of acute oral LD_{50} values for rats and mice obtained from RTECS® and IC_{50} values from *in vitro* cytotoxicity assays using multiple cell lines and cytotoxicity endpoints for chemicals with known molecular weights (Halle 1998). The toxicological databases, in order of preference, were:

- the RC, which contains LD_{50} values that came largely from the 1983/84 RTECS® (Halle et al. 1998)
- the current RTECS® (MDL Information Systems 2001, 2002)
- the current Hazardous Substances Data Bank (HSDB; U.S. National Library of Medicine [NLM] 2001, 2002).

To assure that a wide range of structural and use classes were selected, reference substances of interest to the various U.S. regulatory agencies, as determined from chemical lists (personal communication) from the agencies, were included. Chemicals with human toxicity data and/or human exposure potential (i.e., chemicals of interest to society) were chosen by mining publicly available databases (e.g., NTP test database) for potential candidates.

3.1.2 Candidate Reference Substances

Sources of Candidate Chemicals

The process of identifying the 72 reference substances started with the compilation of a database that ultimately contained 116 candidate chemicals. The intent of the SMT was to compile a database with more than 12 chemicals in each toxicity category that also met the other criteria, and then to prioritize the chemicals in each category to select the 72 reference substances to be tested. As recommended by the Workshop 2000 participants (ICCVAM 2001a), the following publicly available databases and other indicated sources were used to identify candidate chemicals:

- the MEIC program, which collected human toxicity data and *in vitro* toxicity data from 61 test methods for the first 50 chemicals (Ekwall et al. 1998)
- the RC (Halle 1998), which contains a compilation of *in vitro* cytotoxicity and *in vivo* rodent LD_{50} data for 347 chemicals
- the Toxic Exposure Surveillance System (TESS) (Litovitz et al. 2000), which compiles reports of toxic human exposures from poison control centers throughout the United States
- pesticides recommended for consideration by the EPA Office of Pesticide Programs (OPP)
- the *Guidance Document* (ICCVAM 2001b), which reported the NRU results for 11 RC chemicals using protocols similar to those used in the NICEATM/ECVAM validation study
- the U.S. NTP test database, which contains information on the toxicity of chemicals relevant to human exposure (NTP 2002)
- the EPA High Production Volume (HPV) Challenge Program, which is a voluntary testing program to provide the public with a complete set of baseline health and environmental effects data for each chemical that is manufactured within or imported into the United States at amounts > 1 million pounds/year (EPA 2000)

Selection of Candidate Chemicals

The complete list of candidate chemicals is provided in **Table 3-2.** The left side of **Table 3-2** presents selected chemicals and the right side presents the alternate chemicals. The candidate chemicals are grouped by GHS acute oral toxicity classification. For each candidate chemical, the table provides the corresponding rat or mouse oral LD_{50} value, the database(s) or other source(s) used to identify the chemical as a potential candidate, and the type of product and/or use for the chemical. Product/use categories were identified from HSDB (NLM 2001, 2002) or RTECS® (MDL Information Systems 2001, 2002).

The final list of candidate chemicals compiled by the SMT included:

- 65 MEIC chemicals. These include the first 50 chemicals evaluated by MEIC as well as another 15 chemicals that were identified for future evaluation (C. Clemedson, personal communication 2001). Twenty of these chemicals were identified for the EDIT program, a follow-on project to the MEIC study to develop supplementary toxicity and kinetic tests (to determine distribution of chemicals in the body and biotransformation of chemicals to more toxic metabolites) to improve the prediction of human toxicity by the battery of tests identified as the best predictors in the MEIC program (Clemedson et al. 2002). The EDIT chemicals were selected by excluding MEIC chemicals that were volatile, those that precipitated at the IC_{50} dose level, and those with sparse or insufficient data on human toxicity or mechanism of acute toxicity
- 16 pesticides with extensive human exposure nominated by the EPA OPP. These included fenpropathrin, endosulfan, bromoxynil (phenol), fipronil, carbaryl, rotenone, metaldehyde, molinate, 1,3-dichloropropene, dichlorvos, chlorpyrifos, sodium arsenite, triphenyltin hydroxide, cycloheximide, acrolein, and boric acid. Pentachlorophenol was also nominated, but was already on the candidate list since it was a MEIC chemical

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Table 3-2 Candidate Chemicals for the 3T3 and NHK NRU Test Methods Validation Study

Selected Chemicals				Alternate Chemicals				
GHS Category[1]/Chemical	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Product/Use[4]**	**GHS Category[1]/Chemical**	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Notes[5]**	**Product/Use[4]**
$LD_{50} \leq 5$ mg/kg								
Mercury II chloride	1	MEIC, EDIT, RC (outlier), TESS, NTP	Preservative/ manufacturing/ insecticide	Aflatoxin B1	5.0	RC (outlier)	Prohibitively expensive	Food contaminant
Triethylenemelamine	1	RC (outlier), NTP	Manufacturing/ insect sterilant					
Sodium selenate	2[a]	TESS, NTP	Feed additive					
Busulfan	2	RC (outlier), NTP	Pharmaceutical (antineoplastic)					
Cycloheximide	2	RC (outlier), NTP	Antibiotic/fungicide					
Disulfoton	2	RC (outlier), EPA, NTP	Pesticide (insecticide)					
Parathion	2	RC (outlier), EPA, NTP	Pesticide (insecticide)					
Strychnine	2[a]	MEIC, TESS, EPA	Pesticide (rodenticide)					
Aminopterin	3[b]	RC	Pharmaceutical (antineoplastic); Rodenticide					
Phenylthiourea	3	RC (outlier), NTP	Pesticide (rodenticide)					
Epinephrine bitartrate	4[b]	RC (outlier), NTP (HCl salt)	Pharmaceutical (adrenergic)					
Physostigmine	5[a]	EHS	Pharmaceutical (anticholinesterase)					
$5 < LD_{50} \leq 50$ mg/kg								
Colchicine	6[b]	MEIC, RC, TESS, NTP	Pharmaceutical (gout suppressant)	2,4-Dinitrophenol	30	RC (outlier), NTP, HPV		Pesticide (fungicide/ insecticide) manufacturing
Potassium cyanide	10	MEIC, EDIT, RC (outlier), TESS	Electroplating	t-Butylamine	44[a]	EPA, NTP, HPV		Manufacturing

Table 3-2 Candidate Chemicals for the 3T3 and NHK NRU Test Methods Validation Study

Selected Chemicals				Alternate Chemicals				
GHS Category[1]/Chemical	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Product/Use[4]**	**GHS Category[1]/Chemical**	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Notes[5]**	**Product/Use[4]**
Dichlorvos	17[a]	TESS, EPA, NTP, HPV	Pesticide (insecticide)	Acrolein	46	RC, TESS, EPA, NTP, HPV	Volatile (BP=52°C)	Pesticide (herbicide/ rodenticide/ algicide), manufacturing
Digoxin	18[b]	MEIC, EDIT, RC (outlier), TESS	Pharmaceutical (antiarrhythmic)					
Fenpropathrin	18[a]	EPA	Pesticide (insecticide)					
Endosulfan	18[a]	TESS, EPA, NTP	Pesticide (insecticide)					
Arsenic III trioxide	20	MEIC, EDIT, RC, TESS, EPA, NTP	Pesticide (insecticide)					
Thallium I sulfate	29[b]	MEIC, EDIT, RC (outlier), TESS	Pesticide (rodenticide/ insecticide)					
Sodium arsenite	41[a]	TESS, NTP	Pesticide (herbicide, insecticide, fungicide)					
Triphenyltin hydroxide	44	RC, EPA, NTP, HPV	Pesticide (fungicide/ insecticide)					
Sodium dichromate dihydrate	50	RC, EPA, GD, NTP	Oxidizing agent					
Nicotine	50	MEIC, EDIT, RC (outlier), TESS, EPA, NTP	Pharmaceutical (stimulant)					
$50 < LD_{50} \leq 300$ mg/kg								
Paraquat	58	MEIC, EDIT, RC (outlier), TESS, EPA	Pesticide (herbicide)	Pentachlorophenol	51	MEIC, RC (outlier), NTP		Disinfectant
Hexachlorophene	61	MEIC, RC, TESS, NTP	Disinfectant	Amphetamine sulfate	55	MEIC, EDIT, RC (outlier), TESS, NTP	DEA	Pharmaceutical (stimulant)
Lindane	76	MEIC, EDIT, RC (outlier), EPA, NTP	Pesticide (insecticide)	Rotenone	60	RC, TESS, EPA, NTP		Pesticide (insecticide/ piscicide)

Table 3-2 Candidate Chemicals for the 3T3 and NHK NRU Test Methods Validation Study

Selected Chemicals				Alternate Chemicals				
GHS Category[1]/Chemical	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Product/Use[4]**	**GHS Category[1]/Chemical**	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Notes[5]**	**Product/Use[4]**
Cadmium II chloride	88	RC, TESS, GD, NTP	Consumer/ industrial products	Furfural	65[a]	NTP, HPV		Solvent, food additive
Verapamil HCl	108	MEIC, EDIT, RC (outlier), TESS. NTP	Pharmaceutical (antiarrhythmic)	p-Phenylenediamine	80	RC, GD, NTP, HPV		Dyeing
Haloperidol	128[a]	MEIC, TESS	Pharmaceutical (antipsychotic)	Chlorpyrifos	82[a]	TESS, EPA, NTP		Pesticide (insecticide)
Sodium oxalate	155	MEIC, EDIT, RC, TESS, NTP	Paints, cleaners	Dextropropoxyphene HCl	83	MEIC, RC (outlier), TESS		Pharmaceutical (analgesic)
Phenobarbital	163	MEIC, RC (outlier), TESS, NTP	Pharmaceutical (anticonvulsant)	Methadone	86[a]	MEIC,TESS, NTP	DEA	Pharmaceutical (analgesic)
Sodium I fluoride	180	MEIC, RC, TESS, EPA, NTP	Electroplating, fluoridation	Fipronil	92[a]	EPA		Pesticide (insecticide)
Caffeine	192	MEIC, RC (outlier), TESS, NTP, HPV	Pharmaceutical (stimulant), food additive	Pentobarbital	125	MEIC, RC TESS	DEA	Pharmaceutical (sedative)
Diquat dibromide	231	MEIC, RC, TESS	Pesticide (herbicide)	Bromoxynil (phenol)	190[a]	EPA		Pesticide (herbicide)
Cupric sulfate * 5 H2O	300	MEIC, RC, TESS, EPA, NTP	Pesticide (insecticide/ fungicide)	Diphenylhydantoin	199	MEIC, RC, TESS, NTP		Pharmaceutical (anticonvulsant)
				Metaldehyde	227[a]	TESS, EPA		Pesticide (molluscicide)
				Carbaryl	230	RC, EPA. NTP		Pesticide (insecticide)
300 < LD_{50} ≤ 2000 mg/kg								
Amitriptyline HCl	319	MEIC, EDIT, RC, TESS	Pharmaceutical (antidepressant)	Ferrous sulfate	319	MEIC, RC, TESS		Food additive
Phenol	414	MEIC, RC, TESS, EPA, NTP, HPV	Disinfectant	Warfarin	324	MEIC, RC, TESS, EPA		Pharmaceutical (anticoagulant), pesticide
Propranolol HCl	470[b]	MEIC, RC, TESS, GD	Pharmaceutical (antiarrhythmic)	Disopyramide	333[a]	MEIC, TESS		Pharmaceutical (antiarrythmic)
Chloral hydrate	479	MEIC, RC, TESS, NTP	Pharmaceutical (sedative)	Barium II nitrate	355	MEIC, RC, TESS, NTP		Pyrotechnic
Glutethimide	600	MEIC, RC,	Pharmaceutical	Thioridazine HCl	358	MEIC, RC, TESS		Pharmaceutical

Table 3-2 Candidate Chemicals for the 3T3 and NHK NRU Test Methods Validation Study

Selected Chemicals				Alternate Chemicals				
GHS Category[1]/Chemical	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Product/Use[4]**	**GHS Category[1]/Chemical**	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Notes[5]**	**Product/Use[4]**
		TESS	(sedative)					(antipsychotic)
Atropine sulfate	623	MEIC, EDIT, RC, TESS	Pharmaceutical (antimuscarinic)	Methylphenidate	367[a]	NTP	DEA	Pharmaceutical (stimulant)
Valproic acid	1695 [b]	RC, MEIC, TESS, NTP	Pharmaceutical (anticonvulsant)	Molinate	369[a]	EPA, NTP		Pesticide (herbicide)
Meprobamate	794[a]	MEIC, TESS	Pharmaceutical (antidepressant)	2,4-Dichlorophenoxy-acetic acid	369	MEIC, RC, TESS, EPA, NTP, HPV		Pesticide (herbicide)
Acetylsalicylic acid	1000	MEIC, EDIT, RC, TESS, NTP	Pharmaceutical (analgesic)	Orphenadrine HCl	425	MEIC, RC, NTP		Pharmaceutical (analgesic)
Lithium I sulfate	1187[b]	MEIC, RC, TESS, NTP (Cl salt)	Pharmaceutical (mood stabilizer)	Trichlorfon	451	RC, EPA, GD, NTP		Pesticide (insecticide)
Procainamide	1950[a]	MEIC, TESS	Pharmaceutical (antiarrythmic)	Quinidine sulfate	456	MEIC, RC, NTP (base)		Pharmaceutical (antiarrhythmic)
Carbamazepine	1957[a]	MEIC, TESS	Pharmaceutical (antiepileptic)	1,3-Dichloropropene	470[a]	TESS, EPA, NTP		Pesticide (nematocide)
				Theophylline	600 [b]	MEIC, RC, TESS, NTP		Pharmaceutical (antiasthmatic)
				Isoniazid	650	MEIC, RC, TESS, NTP		Pharmaceutical (antibiotic)
				Diazepam	709	MEIC, EDIT, RC, TESS, NTP	DEA	Pharmaceutical (anxiolytic)
				Maprotiline	760[a]	MEIC, TESS		Pharmaceutical (antidepressant)
				Methyleugenol	810[a]	NTP		Food additive
				Diphenhydramine HCl	855	MEIC, RC, TESS, NTP		Pharmaceutical (antihistamine)
				Malathion	885	MEIC, EDIT, RC, TESS, EPA, NTP		Pesticide (insecticide)
				Salicylic acid	891	RC, TESS, GD, NTP, HPV		Pharmaceutical (analgesic)
				Chloroform	908	MEIC, RC, NTP, HPV	Volatile (BP=61°C)	Solvent
				Chloroquine diphosphate	970	MEIC, RC		Pharmaceutical (antimalarial))

Table 3-2 Candidate Chemicals for the 3T3 and NHK NRU Test Methods Validation Study

Selected Chemicals				Alternate Chemicals				
GHS Category[1]/Chemical	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Product/Use[4]**	**GHS Category[1]/Chemical**	**Rodent Oral LD_{50}[2] (mg/kg)**	**Source[3]**	**Notes[5]**	**Product/Use[4]**
				Ibuprofen	1009	RC, TESS, GD		Pharmaceutical (analgesic)
				Nalidixic acid	1349	RC, GD, NTP		Pharmaceutical (antibiotic)
				Dichloromethane	1597	MEIC, RC, TESS, NTP, HPV	Volatile (BP=40°C)	Solvent
				Antipyrene	1800	RC, GD		Pharmaceutical (analgesic)
2000 < LD_{50} ≤ 5000 mg/kg								
Acetaminophen	2404	MEIC, EDIT, RC, TESS, NTP	Pharmaceutical (analgesic)					
Potassium I chloride	2602	MEIC, RC, TESS, NTP	Pharmaceutical (electrolyte), manufacturing					
Boric aid	2660[a]	TESS, EPA, NTP	Pesticide (insecticide)					
Carbon tetrachloride	2799	MEIC, RC, TESS, NTP, HPV	Solvent					
Dimethylformamide	2800	RC, GD, NTP, HPV	Solvent					
Sodium chloride	2998	MEIC, EDIT, RC, TESS, EPA, NTP	Pharmaceutical (electrolyte), food additive					
Citric Acid	3000[a]	EPA, NTP, HPV	Food additive					
Chloramphenicol	3393	MEIC, RC, NTP	Pharmaceutical (antibiotic)					
Lactic acid	3730	RC, NTP, HPV	Food additive					
Acetonitrile	3798	RC, NTP, HPV	Solvent					
Xylene	4300	MEIC, RC, TESS, NTP, HPV	Solvent					
Trichloroacetic acid	4999	RC, NTP	Fixative					
LD_{50} > 5000 mg/kg								

Table 3-2 Candidate Chemicals for the 3T3 and NHK NRU Test Methods Validation Study

Selected Chemicals				Alternate Chemicals				
GHS Category[1]/Chemical	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Product/Use[4]	GHS Category[1]/Chemical	Rodent Oral LD_{50}[2] (mg/kg)	Source[3]	Notes[5]	Product/Use[4]
2-Propanol	5843	MEIC, RC, TESS, EPA, NTP, HPV	Disinfectant					
Gibberellic acid	6305	RC, EPA, NTP	Plant growth regulator					
Propylparaben	6326[b]	RC (outlier), NTP	Food additive					
5-Aminosalicylic acid	7749[b]	RC (outlier), NTP	Pharmaceutical (antibiotic)					
Ethylene glycol	8567	MEIC, EDIT, RC, TESS, NTP, HPV	Antifreeze					
Diethyl phthalate	8602	RC (outlier), NTP, HPV	Plasticizer					
Sodium hypochlorite	8910[d]	TESS, NTP	Disinfectant					
1,1,1-Trichloroethane	10298	MEIC, RC, NTP, HPV	Solvent					
Dibutyl phthalate	11998	RC (outlier), NTP, HPV	Plasticizer					
Glycerol	12691	RC, GD, NTP, HPV	Solvent					
Methanol	13012	MEIC, EDIT, RC, TESS, NTP, HPV	Solvent					
Ethanol	14008	MEIC, RC (outlier), TESS, EPA, NTP, HPV	Solvent					

[1]GHS-Globally Harmonized System of Classification and Labelling of Chemicals for acute oral toxicity (UN 2005).

[2]LD_{50} data are from the Registry of Cytotoxicity (Halle 1998) and are for rats, the preferred species for oral acute toxicity studies, unless otherwise noted. Data with decimal places are rounded to the nearest one.

[3]Sources used to identify candidate chemicals: EDIT = Evaluation-guided Development of New *In vitro* Test Batteries; EPA = pesticides registered with the Environmental Protection Agency; EHS = EPA's Extremely Hazardous Substance list; HPV = High Production Volume chemicals (i.e., those that are imported into or produced in the United States in amounts $\geq$ 1,000,000 lbs/year; GD = *Guidance Document* (ICCVAM 2001b); MEIC = Multicentre Evaluation of In Vitro Cytotoxicity; NTP = National Toxicology Program; RC = Registry of Cytotoxicity with chemicals classified as regression outliers shown in parentheses; TESS = Toxic Exposure Surveillance System (Litovitz et al. 2000).

[4]Product/use categories from Hazardous Substances Data Bank (NLM 2002) or Registry of Toxic Effects of Chemical Substances ([RTECS®], MDL Information Systems 2002). Pharmaceutical uses from Gilman et al. (1985) or Thomson PDR® (2004).

[5]Only chemicals expected to be too volatile for the cytotoxicity assay system have "volatile" notations. BP = Boiling point. DEA (U.S. Drug Enforcement Agency) refers to Schedule II controlled substances. Chemicals with no "DEA" notation are expected to be under less strict control.

[a]RTECS® (MDL Information Systems 2002).

[b]Mouse.

- five chemicals associated with the highest incidence of toxic exposures reported by U.S. poison control centers participating in the TESS (Litovitz et al. 2000): hypochlorite, acetaminophen, ethanol, diphenhydramine, and isopropanol. The five chemicals with the greatest incidence of toxic exposures among children were the same, except that oxalate replaced ethanol. Most of these chemicals were already identified as candidate chemicals due to their inclusion in the MEIC study. Since hypochlorite (sodium salt) and diphenhydramine, were not already included, they were added to the list of candidates
- 11 chemicals recommended in the *Guidance Document* (ICCVAM 2001b) for qualifying *in vitro* cytotoxicity assays for the prediction of starting doses using the RC regression. These chemicals were recommended because the IC_{50} and LD_{50} data for these chemicals fit the RC regression line extremely well. These chemicals were sodium dichromate dihydrate, cadmium chloride, p-phenylenediamine, DL-propranolol HCl, trichlorfon, ibuprofen, nalidixic acid, salicylic acid, antipyrene, dimethylformamide, and glycerol
- 16 chemicals from the NTP
  - furfural, methyleugenol, and methylphenidate, scheduled for testing by the NTP National Center for Toxicogenomics (NCT) (G. Boorman, personal communication 2001), were added. Acetaminophen, another hepatotoxin to be tested by the NCT, was already a candidate chemical because it was included in the MEIC study. Chromium (VI), recommended by the NTP for consideration due to the potential for human exposure via drinking water (NTP 2002) was represented in the list of candidate chemicals by sodium dichromate dihydrate, which was also recommended in the *Guidance Document* (ICCVAM 2001b)
  - dibutyl phthalate, 5-aminosalicylic acid, propylparaben, gibberellic acid, and diethyl phthalate were added to increase the number of chemicals with LD_{50} values > 5000 mg/kg
  - trichloroacetic acid was added to increase the number of chemicals in the $2000 < LD_{50} \leq 5000$ mg/kg category

- sodium selenate was added to increase the number of chemicals in the $LD_{50} \leq 5$ mg/kg category to 12
- six chemicals that were also on the HPV list were added. Lactic acid, citric acid, and acetonitrile were added to increase the number of chemicals in the $2000 < LD_{50} \leq 5000$ mg/kg category. Tert-butylamine, 2,4-dinitrophenol, and acrolein were added to increase the number of chemicals in the $5 < LD_{50} \leq 50$ mg/kg category

- eight additional RC chemicals in the $LD_{50} \leq 5$ mg/kg category. These were: triethylenemelamine, busulfan, disulfoton, parathion, aminopterin, phenylthiourea, epinephrine bitartrate, and aflatoxin B1

The goal to identify more than 12 candidate chemicals for each toxicity category was unrealized for three toxicity categories. The most toxic category ($LD_{50} \leq 5$ mg/kg), and least toxic categories ($2000 < LD_{50} \leq 5000$ mg/kg, $LD_{50} > 5000$ mg/kg), contained only 12 candidate chemicals each. The intermediate toxicity categories ($50 < LD_{50} \leq 300$ mg/kg, > $300 < LD_{50} \leq 2000$ mg/kg), however, contained two to three times the minimum number of candidate chemicals.

3.1.3 Selection of Reference Substances for Testing

Using the candidate chemical database, 72 reference substances (12 unclassified chemicals and 12 chemicals from each the five GHS acute oral toxicity hazard categories) were selected for use in the NICEATM/ECVAM validation study. The criteria for prioritizing the candidate chemicals were:

- the availability of rodent acute oral toxicity data (e.g., RC, RTECS®)
- the availability of human acute oral toxicity data and/or relevance for human exposure (e.g., MEIC, EDIT, TESS, NTP)
- the lack of excessive volatility as estimated by SMT chemical consultants. Since the cells are exposed for 48 hours while incubated at 37°C in 96-well plates, volatilization from wells with high reference substance concentrations would reduce the extent of cytotoxicity and potentially contaminate other wells in close proximity

- the lack of U.S. Drug Enforcement Agency (DEA) controls. Excluding chemicals that are listed in DEA Schedules I and II from consideration obviates the requirement for U.S. laboratories to obtain a DEA license and adhere to strict chemical storage and control procedures
- practical considerations such as cost and disposal issues

If more than twelve candidate chemicals in a GHS category met the above criteria, then selection was based on two further considerations. One consideration was the distribution of chemical toxicities within each toxicity category (i.e., the goal was to select chemicals that represented the entire range of toxicity within each category). Another consideration, which applied only to candidate chemicals selected from the RC database, was the fit of the chemical to the RC regression. Chemicals with the best fit to the RC regression were preferentially selected to prevent the entire set of reference substances from having proportionally more "outlier" substances (i.e., greater than one-half log from the RC regression) than the entire RC database.

The final list of selected reference substances is provided by GHS acute oral toxicity category on the left side of **Table 3-2.**

3.2 Rationale for the Number of Reference Substances Selected

Seventy-two reference substances were used to evaluate the ability of the 3T3 and NHK NRU test methods to estimate the acute oral LD_{50} and thus the starting dose for *in vivo* acute oral toxicity tests. The SMT determined the number of reference substances for testing by first using the GHS classification scheme for acute oral toxicity (UN 2005) to assure that the candidate chemicals covered the complete range of toxicity, (see **Table 3-1**) then deciding how many chemicals would be tested per category. To adequately cover the range of toxicity within each of the six toxicity groups, the SMT decided to test 12 chemicals per group. Seventy-two reference substances (12 substances/group with six groups) were deemed adequate by the SMT, the ICCVAM Acute Toxicity Working Group (ATWG), ICCVAM, and ECVAM.

The total number of reference substances was comparable to the number used in other contemporary *in vitro* test method multilaboratory validation studies. The European Cosmetic, Toiletry, and Perfumery Association evaluation of multiple alternatives to the Draize eye irritation test used 55 reference substances (Brantom et al. 1997). ECVAM's evaluations of *in vitro* dermal corrosivity test methods (Fentem et al. 1998) and *in vitro* dermal irritation assays (Botham 2004) used 60 reference substances.

3.3 Characteristics of the Selected Reference Substances

The physical/chemical and toxicological information in **Appendix F** may be useful for characterizing the performance of the *in vitro* NRU assays for various chemical types. **Appendix F-1** lists the selected reference substances in alphabetical order with information on the CASRN, purity, supplier, pH, and concentrations tested in the *in vitro* NRU cytotoxicity assays. **Appendix F-2** also provides the reference substances in alphabetical order, but with the available information on molecular weight, chemical class, water solubility, acid/base dissociation constant (pK), boiling point, lipid solubility (log K_{ow}), major toxic effects, ability to pass the blood:brain barrier, metabolic activation/inactivation, and mechanism of lethality. The remainder of **Section 3.3** summarizes selected characteristics of the reference substances.

3.3.1 Source Databases Represented by the Selected Reference Substances

The primary sources of chemicals, which reflect the level of societal interest, were well represented in the final list of reference substances. **Table 3-3** shows the distribution of reference substances by GHS category from the MEIC, EDIT, TESS, NTP, and HPV lists. Forty-two (58%) of the 72 selected chemicals were MEIC chemicals (17 of the 42 MEIC chemicals [40%] were EDIT chemicals), 46 (64%) chemicals were involved in human poisonings report by TESS, 51 (71%) chemicals have been evaluated by the NTP, and 18 (25%) chemicals are listed in the EPA's HPV Challenge Program. Some chemicals were found in more than one source.

The other major source of chemicals was the RC. As shown in **Table 3-4**, 58 (81%) of the selected chemicals were included in the RC. Since one of the regression formulas evaluated in the NICEATM/ECVAM validation study was the RC regression, the fit of the RC chemicals to the regression was relevant (Halle 1998). Halle (1998) defined outliers as those chemicals with log IC_{50}-log LD_{50} points that were > 0.699 (i.e., log 5) from the RC regression. For each toxicity category, **Table 3-4** shows the number of RC outliers selected for testing and the corresponding number of outliers in the RC. Although the percentage of outliers for the selected chemicals in several GHS categories is similar to the RC, the total percentage of RC outliers in the set of reference substances (i.e., 38% [22/58]) is greater than the total percentage of outliers in the RC (i.e., 27% [95/347]). For the reference substances, the RC prediction model underpredicted toxicity (i.e., actual LD_{50} is lower than predicted) for 17 outliers and overpredicted toxicity (i.e., actual LD_{50} is higher than predicted) for five outliers. **Figure 3-1** shows the 58 RC chemicals selected for testing with the remaining 289 RC data points and the RC regression. In the figure, the outliers are those points outside the RC prediction interval. The 17 outlier chemicals for which toxicity is underpredicted are below the lower prediction interval and the five outliers for which toxicity is overpredicted are above the upper prediction interval.

Table 3-3 Distribution of Candidate Chemicals and Reference Substances by Source[1] and Toxicity Category

GHS[2] Category (LD_{50} in mg/kg)	Reference Substances/ Candidate Chemicals	MEIC Reference/ MEIC Candidates	EDIT Reference/ EDIT Candidates	TESS Reference/ TESS Candidates	NTP Reference/ NTP Candidates	HPV Reference/ HPV Candidates
$LD_{50} \leq 5$	12/13	2/2	1/1	3/3	5/5	0/0
$5 < LD_{50} \leq 50$	12/15	6/6	5/5	9/10	8/11	2/5
$50 < LD_{50} \leq 300$	12/26	11/17	4/5	11/19	9/18	1/3
$300 < LD_{50} \leq 2000$	12/38	12/29	3/5	12/27	5/23	1/5
$2000 < LD_{50} \leq 5000$	12/12	6/6	2/2	6/6	12/12	6/6
$LD_{50} > 5000$	12/12	5/5	2/2	5/5	12/12	8/8
Total	72/116	42/65	17/20	46/70	51/81	18/27

[1]Substances may be represented in more than one source (see **Table 3-2**).

[2]GHS = Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

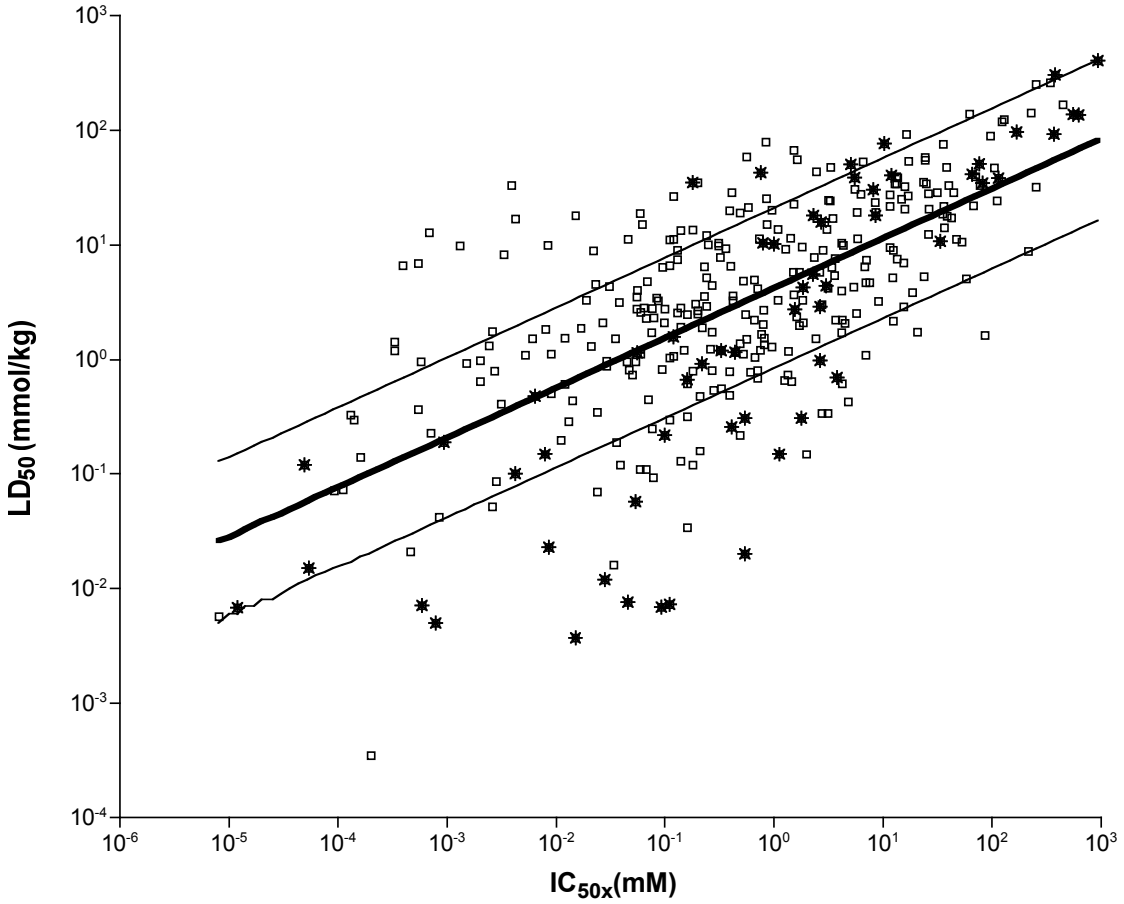
MEIC = Multicentre Evaluation of In Vitro Cytotoxicity**;** EDIT= Evaluation-Guided Development of *In vitro* Tests; TESS =Toxic Exposure Surveillance System; NTP = U.S. National Toxicology Program; HPV = EPA High Production Volume program.

Table 3-4 Selected Chemicals: Distribution of Registry of Cytotoxicity (RC) Chemicals and RC Outliers[1] by Toxicity Category

GHS[2] Category (LD_{50} in mg/kg)	RC Outliers/ Total Chemicals	Candidate and Selected Chemicals		
		Candidate Chemicals	RC Reference / RC Candidates	RC Reference Outliers/ RC Reference Chemicals
$LD_{50} \leq 5$	10/11 (91%)	13	9/10	8/9 (89%)
$5 < LD_{50} \leq 50$	15/26 (58%)	15	8/10	4/8 (50%)
$50 < LD_{50} \leq 300$	24/70 (34%)	26	11/18	5/11 (45%)
$300 < LD_{50} \leq 2000$	14/139 (10%)	38	9/29	0/9 (0%)
$2000 < LD_{50} \leq 5000$	12/57 (21%)	12	10/10	0/10 (0%)
$LD_{50} > 5000$	20/44 (45%)	12	11/11	5/11 (45%)
Total	95/347 (27%)	116	58/88	22/58 (38%)

[1]Chemicals falling outside the log 5 (i.e., > ± 0.699) prediction interval for the RC regression (Halle 1998).
[2]GHS: Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

Figure 3-1 The Fifty-Eight (58) Selected Registry of Cytotoxicity (RC) Chemicals on the RC Regression



The 58 RC chemicals tested in the NICEATM/ECVAM validation study are shown by *. The RC regression, log (LD_{50}) = 0.435 x log (IC_{50x}) + 0.625, is shown by the bold line. The lighter lines show the ± log 5 (i.e., ± 0.699) prediction interval (Halle 1998). The remaining 289 RC data points are shown by the open boxes.

3.3.2 Chemical Classes Represented by the Selected Reference Substances

Medical subject headings (MeSH) from the NLM were used to determine chemical class for the selected chemicals. Of the 72 reference substances, 55 (76%) were organic compounds and 17 (24%) were inorganic compounds. The most commonly represented classes of organic compounds were heterocyclic compounds (14/55, 26%), carboxylic acids (12/55, 22%), and alcohols (10/55, 18%). **Table 3-5** shows the distribution of the selected chemicals among the GHS toxicity categories. The 14 heterocyclic compounds were rather evenly

distributed among the first four GHS toxicity categories for $LD_{50} \leq 2000$ mg/kg with the majority of the heterocyclics (11/14) in the categories for $LD_{50} < 300$ mg/kg. The majority of the carboxylic acids (10/12) and alcohols (8/10) had $LD_{50} > 300$ mg/kg. The majority of the inorganic compounds (12/17) had $LD_{50} < 300$ mg/kg.

3.3.3 Product/Use Classes Represented by the Selected Reference Substances

Product and use information for the selected chemicals was obtained from HSDB (NLM 2002) or RTECS® (MDL Information Systems 2002). Since more than one use was reported for some chemicals, the number of assigned uses (77) is greater than the number of selected chemicals. **Table 3-6** shows the distribution of products and uses of the selected chemicals among the GHS toxicity categories. Pharmaceutical (27/77; 35%) and pesticide (17/77; 22%) uses were observed most frequently. The toxicity category for $300 < LD_{50} \leq 2000$ mg/kg had the highest number of chemicals with pharmaceutical uses. Every toxicity category except for $LD_{50} > 5000$ mg/kg had at least four chemicals with pharmaceutical uses. The majority of chemicals (16/17; 94%) with pesticide uses had $LD_{50} < 300$ mg/kg. The next most frequent uses for the selected chemicals were solvents (8/77; 10%) and food additives (5/77; 6%). The toxicity categories for $LD_{50} > 2000$ mg/kg contained most of the chemicals with solvent (8/8; 100%) and food additive (4/5; 80%) uses.

3.3.4 Toxicological Characteristics of the Selected Reference Substances

Corrosivity

During the chemical selection process, the intent of the SMT was prioritize chemicals with low corrosivity because guidelines for acute systemic toxicity testing indicate that corrosive or severely irritating chemicals need not be tested (OECD 2001a, c, d). The UN and U.S. Department of Transportation Packing Group (DOT PG) classification system was used to classify the corrosivity hazard associated with the candidate chemicals. However, after chemical selection was completed and testing had begun, the SMT discovered that the PG classification system is also based on hazards other than corrosivity (e.g., dermal and inhalation toxicity, flammability, etc.). Thus, the selected chemicals were not actually prioritized by corrosivity. Subsequent information on the corrosivity of the selected chemicals was obtained from HSDB (NLM 2004) and the Material Safety Data Sheets

374 (MSDS) provided with the purchased reference substances. Seven substances had corrosive
375 notations. The MSDSs for lactic acid, sodium hypochlorite, sodium oxalate, and
376 trichloroacetic acid indicated that these chemicals should carry a corrosive label. Chloral
377 hydrate, mercury II chloride, and potassium cyanide were noted to be corrosive to eyes or
378 skin by their HSDB files.

379

Table 3-5 Distribution of Chemical Class for the 72 Reference Substances by Toxicity Category

	GHS Acute Oral Toxicity Category[1] (mg/kg)						Total
Chemical Class[2]	**≤ 5**	**> 5 - ≤ 50**	**> 50 - ≤ 300**	**> 300 - ≤ 2000**	**> 2000 - ≤ 5000**	**> 5000**	
Organic							
Heterocyclic compound	4	3	4	3	0	0	14
Carboxylic acid	1	0	1	3	3	4	12
Alcohol	2	0	0	2	1	5	10
Amide	0	0	0	1	2	0	3
Halogenated hydrocarbon	0	0	1	0	1	1	3
Cyclic hydrocarbon	0	0	1	0	1	0	2
Hydrocarbon	0	1	0	0	0	1	2
Organophosphorous compound	2	1	0	0	0	0	3
Polycyclic compound	0	1	0	1	0	0	2
Amine	0	0	1	0	0	0	1
Nitrile	0	0	0	0	1	0	1
Organometallic compound	0	1	0	0	0	0	1
Phenol	0	0	0	1	0	0	1
Total	9	7	8	11	9	11	55
Inorganic							
Arsenical	0	2	0	0	0	0	2
Sulfur compound	1	0	1	0	0	0	2
Boron compound	0	0	0	0	1	0	1
Cadmium compound	0	0	1	0	0	0	1
Ketone	0	0	1	0	0	0	1
Lithium compound	0	0	0	1	0	0	1
Mercury compound	1	0	0	0	0	0	1
Metal	0	1	0	0	0	0	1

Table 3-5 Distribution of Chemical Class for the 72 Reference Substances by Toxicity Category

	GHS Acute Oral Toxicity Category[1] (mg/kg)						Total
Chemical Class[2]	**≤ 5**	**> 5 - ≤ 50**	**> 50 - ≤ 300**	**> 300 - ≤ 2000**	**> 2000 - ≤ 5000**	**> 5000**	
Potassium, chlorine compound	0	0	0	0	1	0	1
Potassium, nitrogen compound	0	1	0	0	0	0	1
Sodium, chlorine compound	0	0	0	0	1	0	1
Sodium, chromium compound	0	1	0	0	0	0	1
Sodium, fluorine compound	0	0	1	0	0	0	1
Sodium, oxygen, chlorine compound	0	0	0	0	0	1	1
Sodium, selenium compound	1	0	0	0	0	0	1
Total	3	5	4	1	3	1	17

[1]GHS: Globally Harmonized System of Classification and Labelling of Chemicals based on oral LD_{50} (UN 2005).

≤ 5: $LD_{50} \leq 5$ mg/kg

> 5 - ≤ 50: $5 < LD_{50} \leq 50$ mg/kg

> 50 - ≤ 300: $50 < LD_{50} \leq 300$ mg/kg

> 300 - ≤ 2000: $300 < LD_{50} \leq 2000$ mg/kg

> 2000 - ≤ 5000: $2000 < LD_{50} \leq 5000$ mg/kg

> 5000: $LD_{50} > 5000$ mg/kg

[2]Based on the Medical Subject Heading [MeSH] index (NLM 2005).

388 **Table 3-6 Distribution of Product/Use[1] Class for the 72 Reference Substances by Toxicity Category**

	GHS Acute Oral Toxicity Category[2] (mg/kg)						Total
Product/Use Class[1]	**≤ 5**	**> 5 - ≤ 50**	**> 50 - ≤ 300**	**> 300 - ≤ 2000**	**> 2000 - ≤ 5000**	**> 5000**	
Antibiotic/fungicide	1	0	0	0	0	0	1
Antifreeze	0	0	0	0	0	1	1
Consumer/industrial products	0	0	1	0	0	0	1
Disinfectant	0	0	1	1	0	2	4
Electroplating	0	2	0	0	0	0	2
Fluoridation	0	0	1	0	0	0	1
Feed additive	1	0	0	0	0	0	1
Fixative	0	0	0	0	1	0	1
Food additive	0	0	1	0	3	1	5
Manufacturing	1	0	0	0	1	0	2
Oxidizing agent	0	1	0	0	0	0	1
Paints, cleaners	0	0	1	0	0	0	1
Pesticide	5	7	4	0	1	0	17
Pharmaceutical	4	3	4	11	4	1	27
Plant growth regulator	0	0	0	0	0	1	1
Plasticizer	0	0	0	0	0	2	2
Preservative	1	0	0	0	0	0	1
Solvent	0	0	0	0	4	4	8

389 [1]Product/use categories from Hazardous Substances Data Bank (NLM 2002) or Registry of Toxic Effects of Chemical Substances ([RTECS®], MDL Information
390 Systems 2002). Some chemicals are counted more than once due to multiple uses.
391 ≤ 5: $LD_{50} \leq 5$ mg/kg
392 > 5 - ≤ 50: $5 < LD_{50} \leq 50$ mg/kg
393 > 50 - ≤ 300: $50 < LD_{50} \leq 300$ mg/kg
394 > 300 - ≤ 2000: $300 < LD_{50} \leq 2000$ mg/kg
395 > 2000 - ≤ 5000: $2000 < LD_{50} \leq 5000$ mg/kg
396 > 5000: $LD_{50} > 5000$ mg/kg
397 [2]GHS: Globally Harmonized System of Classification and Labelling of Chemicals based on oral LD_{50} (UN 2005).
398

Toxicity Targets

As shown in **Appendix F**, the most common toxicological effects were neurological (40 reference substances); 26 reference substances cause central nervous system (CNS) depression, seven reference substances produce CNS stimulation, four reference substances produce other CNS affects such as encephalopathy, and three reference substances attack the peripheral nervous system. Other common toxicity targets include the liver (17 reference substances), kidney (15 reference substances), and cardiovascular system (10 reference substances). No target organ information was available for gibberellic acid. Among the 72 reference substances, 27 had multiple toxicity targets.

Metabolism

Table 3-7 shows the 22 reference substances that are known or expected to produce active/toxic metabolites *in vivo*. In contrast, dichlorvos, fenpropathrin, meprobamate, phenylthiourea, and sodium dichromate are known to be rapidly inactivated by metabolism *in vivo* to less toxic compounds. Because the NHK and 3T3 cells have little (see Babich 1991) or no metabolic capability, respectively, metabolites of these compound would be unavailable *in vitro*. See **Appendix F-2** for more information on the metabolism of the selected chemicals.

Table 3-7 Reference Substances Metabolized to Active Metabolites

Known to Have Active Metabolites				Active Metabolites Expected
Acetaminophen	Carbamazepine	Digoxin	Methanol	Carbon tetrachloride
Acetonitrile	Chloral hydrate	Disulfoton	Parathion	Triethylenemelamine
Acetylsalicylic acid	Cycloheximide	Ethanol	Procainamide HCl	Valproic acid
Amitriptyline HCl	Dibutyl phthalate	Ethylene glycol	Verapamil HCl	
Busulfan	Diethyl phthalate	Glutethimide		

3.3.5 Selection of Reference Substances for Testing in Validation Study Phases Ib and II

Based on the *Guidance Document* (ICCVAM 2001b) recommendation that 10-20 chemicals be tested to qualify candidate *in vitro* cytotoxicity tests for determining starting doses for acute oral systemic toxicity assays, 12 reference substances were chosen from the 72

reference substances for testing in Phases Ib and II of the validation study (see **Table 3-8**). The criteria for choosing these reference substances, in order of importance, were:

- two reference substances must be included from each of the five GHS toxicity categories and the unclassified category
- the log LD_{50} (mmol/kg) must be within 0.699 of the RC regression (i.e., within the RC prediction interval). The *Guidance Document* (ICCVAM 2001b) recommends that reference substances for evaluating a cytotoxicity test to use with the RC regression fit the regression as closely as possible
- MEIC chemicals must be included. Cytotoxicity data from these phases (and Phase III of this study) and the available human toxicity information for the MEIC chemicals could be used to build a prediction model for estimating human lethal blood concentrations. Phase Ib reference substances arsenic trioxide and ethylene glycol are EDIT chemicals

If more than two chemicals in a GHS category met the above criteria, reference substances were selected so that the LD_{50} was as close to the RC prediction as possible and/or to represent the range of toxicity in each GHS category.

Only nine reference substances of the 72 selected reference substances fit all three criteria. One reference substance was not within the RC acceptance interval. For the most toxic category (i.e., $LD_{50} \leq 5$ mg/kg), only one RC chemical, aminopterin, was within 0.699 of the RC regression. Sodium selenate, whose fit to the RC regression was unknown and had not been tested in the MEIC study, was included in this toxicity category. In addition, neither of the two reference substances chosen for the $LD_{50} \leq 5$ mg/kg category, aminopterin and sodium selenate, were MEIC chemicals.

Table 3-8 Reference Substances Tested in Phases Ib and II

Reference Substances	CASRN	RC Reference No.	MEIC Reference No.	Rodent Oral LD_{50}[1] (mg/kg)	Observed – Predicted log LD_{50}[2]
$LD_{50} \leq 5$ mg/kg					
Aminopterin	54-62-6	3	NA	3	-0.652
Sodium selenate	13410-01-0	NA	NA	1.6[3]	NA
$5 < LD_{50} \leq 50$ mg/kg					
Colchicine	64-86-8	6	60	6[4]	-0.593
Arsenic III trioxide	1327-53-3	153	26	20	-0.591
$50 < LD_{50} \leq 300$ mg/kg					
Cadmium II chloride	10108-64-2	81	NA	88	-0.336
Sodium I fluoride	7681-49-4	106	14	180	-0.109
$300 < LD_{50} \leq 2000$ mg/kg					
DL-Propranolol HCl	350-60-90	54	23	470[4]	-0.023
Lithium I carbonate	544-13-2	327[4]	20	1187[4,5]	-0.256[4]
$2000 < LD_{50} \leq 5000$ mg/kg					
Potassium I chloride	7447-40-7	346	50	2602	0.085
Chloramphenicol	56-75-7	91	45	3393	0.441
$LD_{50} > 5000$ mg/kg					
2-Propanol	67-63-0	128	10	5843	0.396
Ethylene glycol	107-21-1	360	7	8567	0.321

[1]From the RC (Halle 1998) unless otherwise indicated. Data for rats unless otherwise indicated.
[2]Available only for chemicals included in the RC. This figure characterizes the log LD_{50} deviation from the RC regression. Outliers are > ± 0.699 from the regression line.
[3]RTECS® (MDL Information Systems 2002).
[4]Mouse data.
[5]Data for lithium sulfate.
Abbreviations: CASRN = Chemical Abstracts Service Registry Number; RC = Registry of Cytotoxicity; MEIC = Multicentre Evaluation of *In Vitro* Cytotoxicity; NA – not applicable; chemical not included in the RC and/or MEIC studies.

3.3.6 Unsuitable and Challenging Reference Substances

Several reference substances could not be adequately tested for cytotoxicity in either or both of the 3T3 or NHK NRU test methods. Under the conditions of the NRU cytotoxicity test, the following reference substances did not produce sufficient toxicity at soluble concentrations for calculation of an IC_{50} at the highest concentrations that could be tested:

- carbon tetrachloride in either the 3T3 or NHK test method in all three laboratories
- xylene in either test method in two laboratories

- methanol in the 3T3 test method in all three laboratories and in the NHK test method in two laboratories
- lithium carbonate in the 3T3 test method in two laboratories
- 1,1,1-trichloroethane in the NHK test method in two laboratories
- valproic acid in the 3T3 test method in one laboratory

Other reference substances were difficult to test, but three acceptable tests were obtained after a number of trials.

- Acetonitrile and 2-propanol were so volatile and nontoxic that, even with the use of film plate sealers, one to seven tests failed at each laboratory. Tests with these two reference substances often failed the VC and data points criteria.
- Disulfoton failed at least one test in both test methods in two laboratories due to inadequate toxicity and solubility.
- Dibutyl phthalate failed one 3T3 test at one laboratory and one NHK test at one laboratory due to inadequate toxicity and solubility.
- Lindane failed one 3T3 test due to inadequate toxicity and solubility and one 3T3 test due to volatility.
- Parathion failed one test due to inadequate toxicity and solubility in both the 3T3 and NHK test methods and one NHK test due to volatility.
- Diethyl phthalate failed one NHK test due to volatility.
- Digoxin, gibberellic acid, and strychnine failed at least one 3T3 test in more than one laboratory due to inadequate toxicity and solubility.

3.4 Reference Substance Procurement, Coding, and Distribution

Reference substances were purchased from the suppliers in the purities indicated in **Appendix F** and distributed by BioReliance Corporation (Rockville, MD). BioReliance also collected information from the suppliers on the analytical purity, composition, and stability of the reference substances. BioReliance tested the reference substances for solubility, packaged them into 4 g aliquots for shipment to the cytotoxicity testing laboratories, and archived two additional samples. All reference substances were randomly coded to conceal

the identities from the cytotoxicity testing laboratories. Each reference substance had a code unique for each testing facility. About 100 g of the positive control, SLS, was distributed to each laboratory and one additional sample was archived.

Reference substances were packaged to minimize damage during transit and shipped under appropriate storage conditions and according to proper regulatory transportation procedures. Testing facilities were notified upon shipment in order to prepare for receipt. With the exception of the positive control shipment, which was shipped directly to the Study Directors, the reference substances were shipped to the test facility Safety Officers. Reference substances shipments were accompanied by a sealed information packet containing the appropriate health and safety procedures for use (i.e., MSDS or equivalent documentation with information regarding the proper protection for handling, procedures for dealing with accidental ingestion or contact with skin or eyes, and procedures for containing and recovering spills) and a disclosure key for identifying reference substances by code. Also provided was a data sheet giving a minimum of essential information for each reference substance, including color, odor, physical state, weight or volume of sample, specific density for liquid reference substances, and storage instructions. The shipment directed the Safety Officer to:

- notify BioReliance and the SMT upon receipt of reference substances
- retain the health and safety package and provide the reference substances and chemical data sheets to the Study Director without revealing the identities of the reference substances
- notify the SMT if test facility personnel open the health and safety packet at any time during the study
- return the unopened health and safety package to BioReliance after testing is complete

Exceptions

The Safety Officer for ECBC required the information on reference substance codes before the substances were shipped to the Safety Office to satisfy the facility's environmental procedures and requirements. The reference substance codes were stored in a classified safe

located in the Safety Office, which was in a building separate from the cytotoxicity testing laboratory. Cytotoxicity testing personnel had no access to the reference substance codes. The ECBC Safety Officer opened the sealed health and safety packets for lithium carbonate and ethanol upon receipt of those substances because the code information for these substances was not included in the list originally provided. ECBC cytotoxicity testing personnel never had access to the reference substance codes.

3.5 Reference Substances Recommended by the *Guidance Document* (ICCVAM 2001b)

The *Guidance Document* method for evaluating basal cytotoxicity assays for use in predicting starting doses for acute oral toxicity assays provides the existing performance standard (ICCVAM 2001b) for the 3T3 and NHK NRU test methods. The *Guidance Document* specifically recommends testing the following 11 chemicals to qualify candidate basal cytotoxicity assays: sodium dichromate dihydrate, cadmium chloride, p-phenylenediamine, DL-propranolol HCl, trichlorfon, ibuprofen, nalidixic acid, salicylic acid, antipyrene, dimethylformamide, and glycerol (ICCVAM 2001b). Although the 11 reference chemicals recommended in the *Guidance Document* were considered as candidates for testing in the NICEATM/ECVAM validation study (see **Section 3.1.2**), only sodium dichromate dihydrate, cadmium chloride, DL-propranolol HCl, dimethylformamide, and glycerol were chosen for testing after the candidate chemicals were prioritized as described in **Section 3.1.3**. The other seven were excluded based on the criterion hierarchy used to determine the selected chemicals (e.g., were not MEIC chemicals, not identified as high exposure risk in TESS)

3.6 Summary

Seventy-two reference substances were selected for testing in the NICEATM/ECVAM validation study. The reference substances were selected to represent: (1) the complete range of *in vivo* acute oral toxicity ranges (in terms of LD_{50} values); (2) the types of substances regulated by various regulatory authorities; and (3) those with human toxicity data and/or

human exposure potential. To assure the complete range of toxicity was covered, the Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005) was used to select 12 chemicals for each acute oral toxicity category and 12 unclassified chemicals. The set of selected reference substances had the following characteristics:

- 38% (27/72) of the substances had pharmaceutical uses, 21% (15/72) had pesticide uses, 11% (8/72) had solvent uses, and 7% (5/72) had food additive uses. The remaining substances were used for a variety of manufacturing and consumer products
- relevance of the substances to human exposures was indicated by the fact that 58% (42/72) were included in the MEIC study, 24% (17/72) were included in the EDIT program, 64% (46/72) had human exposures reported by TESS, 71% (51/72) had been evaluated by NTP, and 25% (18/72) were included in EPA's HPV list
- 81% (58/72) of the substances were also included in the RC and 38% (22/58) of these were outliers with respect to the RC regression
- 76% (55/72) were organic compounds and 24% (17/72) were inorganic compounds. The most commonly represented classes of organic compounds were heterocyclic compounds (26%, 14/55), carboxylic acids (22%, 12/55), and alcohols (18%, 10/55)
- 19 substances (26%, 19/72,) were known to have active metabolites and three additional substances were expected to have active metabolites
- many of the selected chemicals had multiple target organs. The most common effects were neurological (40 chemicals), liver (17 chemicals), kidney (15 chemicals), and cardiovascular (10 chemicals). No target organ information was available for one chemical (gibberellic acid)

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