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7.0 RELIABILITY OF THE 3T3 AND NHK NRU TEST METHODS

The reliability of the 3T3 and NHK NRU test methods was assessed by determining intra- and inter-laboratory reproducibility. Intralaboratory reproducibility is the agreement of results produced when people in the same laboratory perform the method using the same test protocol at different times (ICCVAM 2003). Interlaboratory reproducibility is the agreement of results among different laboratories using the same protocol and reference substances. Interlaboratory reproducibility indicates the extent to which a method can be successfully transferred among laboratories. Repeatability, usually applied to results within a laboratory, is the closeness of agreement between test results obtained when the procedure is performed on the same substance under identical conditions within a given time. This study was not designed to assess intralaboratory repeatability.

The interlaboratory reproducibility of the test results was assessed by comparing the laboratory-specific IC_{50} - LD_{50} regressions for the 3T3 and NHK NRU test methods to the mean (i.e., across-laboratory mean) laboratory regressions (see **Section 7.2.1**). This comparison is relevant because the 3T3 and NHK NRU test methods are intended for use with IC_{50} - LD_{50} regressions to determine starting doses for acute oral toxicity tests. Interlaboratory reproducibility of the 3T3 and NHK NRU test methods was also determined using ANOVA, CV analysis, and comparison of maximum:minimum IC_{50} ratios calculated using laboratory mean values (see **Sections 7.2.2, 7.2.3, and 7.2.4**, respectively), as discussed in **Section 5.5.2.2**. Inter- and intra-laboratory reproducibility of the PC (SLS) was determined using ANOVA, CV analysis, and/or linear regression over time (see **Section 7.3**). The extent of laboratory concordance in selecting the solvent to be used for each test substance (described in **Section 2.10**) is provided in **Section 7.4**.

7.1 Reference Substances Used to Determine the Reliability of the 3T3 and NHK NRU Test Methods

The validation study was designed for the purpose of using the IC_{50} results of 72 reference substances (see **Table 3-2**) to determine the reliability of the IC_{50} values from the 3T3 and NHK NRU test methods. The number of reference substances used for the reproducibility analysis was not the same as the number of reference substances used for the accuracy analyses in **Section 6.4**. In the former case, only reference substances for which all three laboratories reported replicate IC_{50} values were used, while in the latter case, substances with rat acute oral LD_{50} data only and at least one laboratory reporting replicate IC_{50} values were used. **Table 7-1** lists the reference substances that failed to yield sufficient toxicity for the calculation of an IC_{50} in each laboratory, and the number of remaining reference substances with replicate IC_{50} values. The laboratories obtained acceptable IC_{50} values for 66 to 68 reference substances using the 3T3 NRU test method, and for 69 to 70 substances using the NHK NRU test method. When only reference substances with IC_{50} values from all three laboratories are considered, 64 and 68 substances were available to evaluate the reliability of the 3T3 and NHK NRU test methods, respectively. The substances that were excluded from the 3T3 reliability analysis were carbon tetrachloride, disulfoton, gibberellic acid, lithium carbonate, methanol, 1,1,1-trichloroethane, valproic acid, and xylene. The substances that were excluded from the NHK reliability analysis were carbon tetrachloride, methanol, 1,1,1-trichloroethane, and xylene.

Table 7-1 Reference Substances Excluded from Reproducibility Analyses Because of Insufficient Cytotoxicity

Laboratory	3T3 NRU Test Method		NHK NRU Test Method	
	Reference Substances Lacking IC ₅₀ Results	N ¹	Reference Substances Lacking IC ₅₀ Results	N ¹
ECBC	Carbon tetrachloride Methanol 1,1,1-Trichloroethane Xylene	68	Carbon tetrachloride Methanol Xylene	69
FAL	Carbon tetrachloride Disulfoton Gibberellic acid Lithium carbonate Methanol Xylene	66	1,1,1-Trichloroethane Carbon tetrachloride Xylene	69
IIVS	Carbon tetrachloride Lithium carbonate Methanol Valproic acid	68	Carbon tetrachloride 1,1,1-Trichloroethane	70

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU= Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; N=Number of substances. ²Number of substances with replicate IC₅₀ values.

Despite the fact that IC₅₀ values were not obtained by all the laboratories for all reference substances, **Table 7-2** shows that the complete range of LD₅₀ responses, as defined by the GHS classification for acute oral toxicity in **Table 3-1**, was covered by the reference substances for which replicate IC₅₀ values were obtained. The 3T3 NRU IC₅₀ values ranged from 0.005 to 38,878 µg/mL, while the NHK values covered a larger range, from 0.00005 to 49,800 µg/mL (see **Tables 5-4** and **5-5**).

Table 7-2 Number of Reference Substances Tested vs Number of Reference Substances Yielding IC₅₀ Values from Each Laboratory, by GHS Acute Oral Toxicity Category

GHS Category ¹ (mg/kg)	Reference Oral LD ₅₀ ²	3T3 NRU Test Method ³	NHK NRU Test Method ³
LD ₅₀ ≤ 5	7	6	7
5 < LD ₅₀ ≤ 50	12	12	12
50 < LD ₅₀ ≤ 300	12	12	12
300 < LD ₅₀ ≤ 2000	16	14	16
2000 < LD ₅₀ ≤ 5000	11	9	9
LD ₅₀ > 5000	14	11	12

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU= Neutral red uptake; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005).

¹GHS category for acute oral toxicity.

²Number of reference substances tested in each category. Reference acute oral LD₅₀ values from rats and mice were generated after evaluating LD₅₀ values located through literature searches and references from toxicity databases such as RTECS® (from **Table 4-2**).

³Number of reference substances with IC₅₀ values from all three laboratories.

7.2 Reproducibility Analyses for the 3T3 and NHK NRU Test Methods

The interlaboratory reproducibility of the 3T3 and NHK NRU IC₅₀ values was assessed by comparing the laboratory-specific IC₅₀-LD₅₀ linear regressions for each method to a regression calculated using the mean IC₅₀ values of the laboratories. The interlaboratory reproducibility of the 3T3 and NHK NRU test methods was also assessed using ANOVA, CV analysis, and analysis of the laboratory mean maximum:minimum IC₅₀ ratios, as described in **Section 5.5.2.2**. Intralaboratory reproducibility was assessed using a CV analysis.

7.2.1 Comparison of Laboratory-Specific IC₅₀-LD₅₀ Linear Regression Analyses to the Mean Laboratory Regression

The comparisons of laboratory-specific IC₅₀-LD₅₀ linear regressions to the mean laboratory regression for each method were made because the 3T3 and NHK NRU test methods are intended for use with IC₅₀-LD₅₀ regressions to determine starting doses for acute oral toxicity tests. Laboratory-specific IC₅₀-LD₅₀ linear regressions were generated and displayed graphically for each method using the 64 and 68 reference substances for the 3T3 and NHK NRU test methods, respectively, as indicated in **Section 7.1**. The regressions used the geometric mean IC₅₀ values for each substance with the rodent acute oral LD₅₀ reference value (**Table 4-2**). To determine whether the laboratory-specific regressions were significantly different from one another, they were compared against the mean laboratory regression for each NRU test method that was calculated using the geometric mean of the laboratory mean IC₅₀ values and the rodent acute oral LD₅₀ reference values. The mean laboratory regression for each NRU test method is in **Figure 7-1** with 95% confidence limits, and shows that the laboratory-specific regressions were all within the 95% confidence limits of the mean laboratory regression.

7.2.2 ANOVA Results for the 3T3 and NHK NRU Test Methods

The ANOVA was performed as discussed in **Section 5.5.2.2**. Because the sample sizes from this study were small, usually three observations per laboratory, there may be differences that were statistically significant only because there were too few observations within the laboratories to adequately characterize variability or because the within-laboratory variability was small.

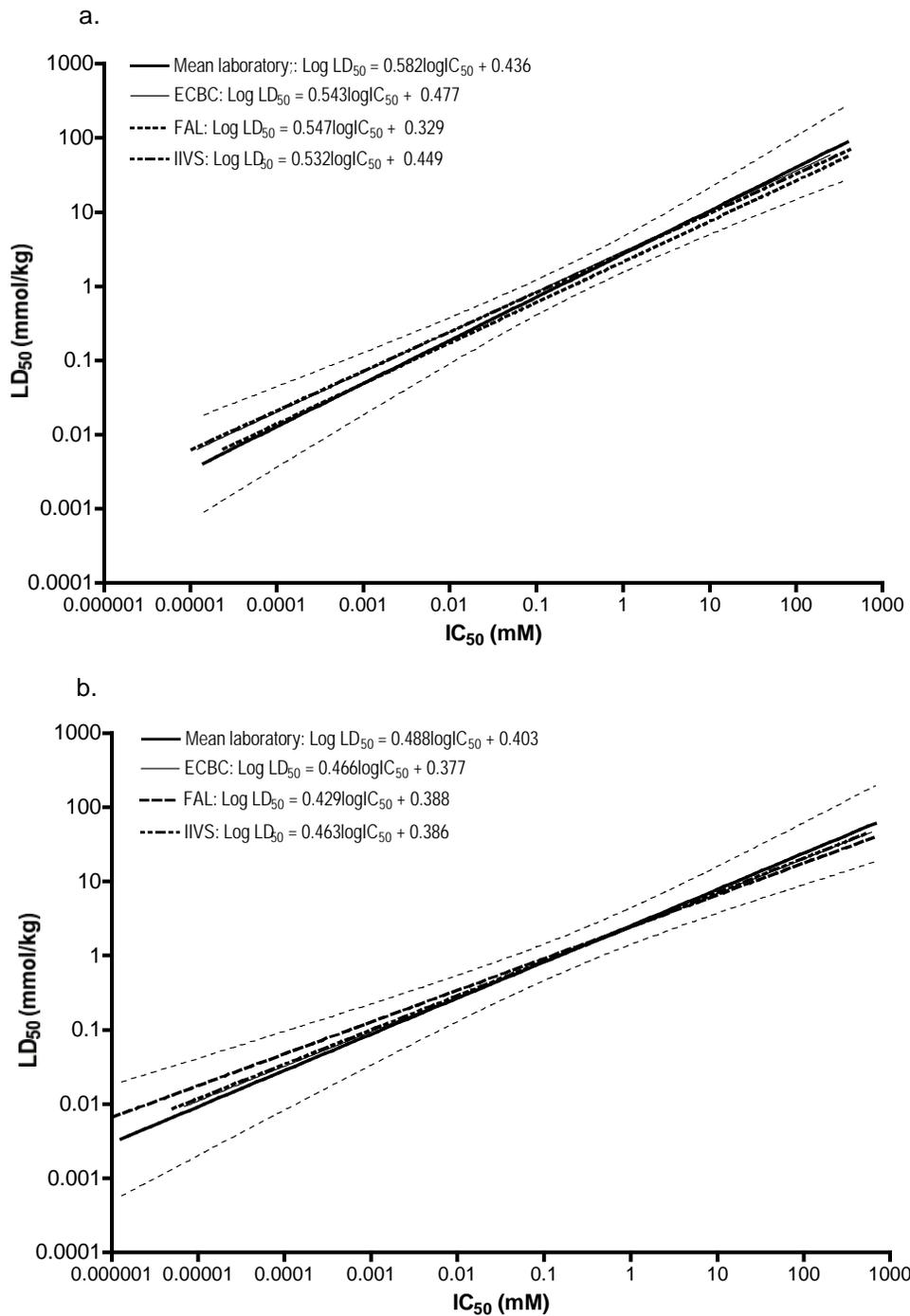
7.2.2.1 *Differences Among the IC₅₀ Values in Laboratories Using the 3T3 NRU Test Method*

The ANOVA results in **Table 7-3** show that there were statistically significant ($p < 0.01$) differences among the laboratories for 23 of the 64 (36%) reference substances evaluated. The p values from the contrast analyses, post-hoc tests to determine which laboratory was significantly different from the others at $p < 0.01$ (see **Section 5.5.2.2**), are also provided in **Table 7-3**. The substances for which statistically significant ANOVA and contrast results were obtained are listed in **Table 7-4** along with columns showing the laboratory with significantly differing values from the other two laboratories. Because significant laboratory differences may have resulted from the insolubility or volatility of the test substance, **Table 7-4** also indicates whether any laboratory reported insolubility or volatility during conduct of the test. Insolubility was suggested by the presence of precipitates in either the stock solutions or in cell culture. Volatility was identified by the need for plate sealers to contain volatile contamination of lower concentration wells by higher concentrations. Insolubility and volatility were reported for only six of the 23 chemicals showing significant

interlaboratory variability. In contrast, 22 of the 41 substances that were classified as generating interlaboratory reproducible data exhibited precipitates and/or volatility.

For the 23 substances that yielded significantly different results among laboratories, contrast analyses indicated that the IC₅₀ values produced by ECBC and FAL were frequently different from the other laboratories. ECBC tended to report the lowest IC₅₀ values (i.e., highest toxicity) among the laboratories while FAL tended to report the highest values of the three laboratories. ECBC reported significantly different results from the other two laboratories for 15 of the 23 substances; for 13 of the 15, ECBC's mean value IC₅₀ was the lowest among the laboratories. FAL reported significantly different results from the other two laboratories for 20 of the 23 substances; for 18 of the 20, FAL's IC₅₀ value was the highest among the laboratories. IIVS reported significantly different values for 11 of the 26 substances, with no tendency toward highest or lowest IC₅₀ values.

Figure 7-1 Mean Laboratory and Laboratory-Specific 3T3 and NHK NRU Regressions



Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake; NHK=Normal human epidermal keratinocytes. Solid lines show the mean laboratory linear regressions for the 3T3 NRU (a) and the NHK NRU (b) test methods with dashed curved lines to show the 95% confidence limits of the regression. The regressions were calculated using 64 and 68 reference substances for the 3T3 and NHK NRU test methods, respectively, as described in **Section 7.1**. Regressions used geometric mean IC_{50} values and reference acute oral LD_{50} values from **Table 4-2**.

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
Acetaminophen	50.1	1.6		28	1.7	0.171	
ECBC	40.8		22		1.61		NA
FAL	66.2		35		1.82		NA
IIVS	43.4		26		1.64		NA
Acetonitrile	8484	1.5		21	3.93	0.553	
ECBC	6433		2		3.81		NA
FAL	9690		58		3.99		NA
IIVS	9330		13		3.97		NA
Acetylsalicylic acid	760	3.1		56	2.88	<0.001	
ECBC	646		10		2.81		0.581
FAL	1234		24		3.09		<0.001
IIVS	401		16		2.6		<0.001
5-Aminosalicylic acid	1698	1.4		19	3.23	0.054	
ECBC	1467		14		3.17		NA
FAL	2070		16		3.32		NA
IIVS	1557		12		3.19		NA
Aminopterin	0.007	2.4		54	-2.14	0.036	
ECBC	0.005		20		-2.28		NA
FAL	0.012		46		-1.93		NA
IIVS	0.005		23		-2.33		NA
Amitriptyline HCl	7.23	1.3		14	0.86	0.348	
ECBC	6.03		23		0.78		0.163
FAL	7.86		28		0.9		0.469
IIVS	7.81		18		0.89		0.445
Arsenic trioxide	2.51	3.9		61	0.4	0.004	

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
ECBC	2.41		33		0.38		0.527
FAL	1.04		7		0.02		0.002
IIVS	4.09		52		0.61		0.006
Atropine sulfate	85.6	2.5		49	1.93	0.049	
ECBC	54.1		55		1.73		NA
FAL	133		31		2.12		NA
IIVS	70		8		1.85		NA
Boric acid	2228	3.3		69	3.35	0.01	
ECBC	1497		32		3.18		NA
FAL	3987		17		3.6		NA
IIVS	1202		48		3.08		NA
Busulfan	135	8.0		119	2.13	0.002	
ECBC	40		48		1.6		0.012
FAL	321		56		2.51		<0.001
IIVS	43.7		4		1.64		0.033
Cadmium chloride	0.565	1.4		39	-0.25	0.124	
ECBC	0.48		14		-0.32		NA
FAL	0.4		32		-0.4		NA
IIVS	0.817		53		-0.09		NA
Caffeine	161	1.4		18	2.21	0.481	
ECBC	133		10		2.12		NA
FAL	157		52		2.2		NA
IIVS	191		7.5		2.28		NA
Carbamazepine	109	1.8		35	2.04	0.049	
ECBC	83		14		1.92		NA

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
FAL	152		37		2.18		NA
IIVS	91.8		12		1.96		NA
Carbon tetrachloride	NA	NA		NA	NA	NA	
ECBC	NA		NA		NA		NA
FAL	NA		NA		NA		NA
IIVS	NA		NA		NA		NA
Chloral hydrate	187	1.6		25	2.27	0.004	
ECBC	151		10		2.18		0.008
FAL	241		10		2.38		0.002
IIVS	170		12		2.23		0.181
Chloramphenicol	161	4.9		67	2.21	<0.001	
ECBC	55.3		22		1.74		<0.001
FAL	273		30		2.44		0.001
IIVS	156		18		2.19		0.165
Citric acid	829	2.4		41	2.92	0.002	
ECBC	473		29		2.68		0.001
FAL	1148		13		3.06		0.003
IIVS	865		19		2.94		0.298
Colchicine	0.047	4.7		85	-1.33	0.001	
ECBC	0.02		11		-1.70		0.0028
FAL	0.093		45		-1.03		0.0005
IIVS	0.028		1		-1.55		0.0914
Cupric sulfate pentahydrate	70.6	21.6		85	1.85	<0.001	
ECBC	82.7		4		1.92		0.001
FAL	123		44		2.09		<0.001

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
IIVS	5.7		31		0.76		<0.001
Cycloheximide	0.293	5.9		104	-0.53	0.021	
ECBC	0.125		45		-0.9		NA
FAL	0.647		70		-0.19		NA
IIVS	0.109		23		-0.96		NA
Dibutyl phthalate	78.3	9.2		124	1.89	<0.001	
ECBC	23.5		17		1.37		0.012
FAL	191		50		2.28		<0.001
IIVS	20.7		7		1.32		0.005
Dichlorvos	20.3	3.3		57	1.31	0.002	
ECBC	9.8		35		0.99		0.001
FAL	32.8		6		1.52		0.002
IIVS	18.3		11		1.26		0.823
Diethyl phthalate	113	1.7		28	2.05	0.127	
ECBC	85.5		34		1.93		0.092
FAL	147		26		2.17		0.07
IIVS	106		24		2.03		0.846
Digoxin	520	2.8		62	2.72	0.043	
ECBC	351		39		2.54		NA
FAL	892		36		2.95		NA
IIVS	317		21		2.5		NA
Dimethylformamide	5242	1.1		6	3.72	0.296	
ECBC	5343		10		3.73		NA
FAL	5483		9		3.74		NA
IIVS	4900		4		3.69		NA

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
Diquat dibromide monohydrate	15.1	9.3		120	1.18	0.017	
ECBC	3.9		23		0.59		NA
FAL	36.1		98		1.56		NA
IIVS	5.4		25		0.73		NA
Disulfoton	98.6	2.3		55	1.99	0.003	
ECBC	137		55		2.14		NA
FAL	NA		NA		NA		NA
IIVS	60.4		87		1.78		NA
Endosulfan	8.02	4.2		78	0.9	0.046	
ECBC	5.3		57		0.72		NA
FAL	15.2		78		1.18		NA
IIVS	3.6		42		0.56		NA
Epinephrine bitartrate	59.4	1.2		12	1.77	0.048	
ECBC	51.5		12		1.71		NA
FAL	63.4		11		1.8		NA
IIVS	63.4		3		1.8		NA
Ethanol	6731	1.6		23	3.83	0.075	
ECBC	5360		33		3.73		NA
FAL	8420		14		3.93		NA
IIVS	6413		5		3.81		NA
Ethylene glycol	25292	1.7		26	4.4	0.007	
ECBC	18325		9		4.26		0.004
FAL	31650		24		4.50		0.01
IIVS	25900		12		4.41		0.505
Fenpropathrin	27.2	2.5		49	1.43	0.301	

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
ECBC	22.6		11		1.35		NA
FAL	42.4		63		1.63		NA
IIVS	16.7		12		1.22		NA
Gibberellic Acid	7842	1.0		3	3.89	0.621	
ECBC	8027		11		3.9		NA
FAL	NA		NA		NA		NA
IIVS	7657		10		3.88		NA
Glutethimide	192	2.3		43	2.28	<0.001	
ECBC	167		4		2.22		0.029
FAL	284.3		7		2.45		<0.001
IIVS	125.3		7		2.1		<0.001
Glycerol	28904	1.9		33	4.46	0.846	
ECBC	20000		15		4.3		NA
FAL	38878		73		4.59		NA
IIVS	27833		39		4.44		NA
Haloperidol	6.26	1.5		24	0.8	0.006	
ECBC	5.3		12		0.72		0.03
FAL	8		8		0.9		0.002
IIVS	5.5		12		0.74		0.061
Hexachlorophene	4.48	1.7		27	0.65	0.174	
ECBC	5		48		0.7		NA
FAL	5.3		33		0.72		NA
IIVS	3.1		9		0.49		NA
Lactic acid	3073	1.2		12	3.49	0.16	
ECBC	2943		11		3.47		NA

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
FAL	3487		16		3.54		NA
IIVS	2790		9		3.45		NA
Lindane	161	2.9		58	2.21	0.066	
ECBC	125		95		2.1		NA
FAL	266		36		2.43		NA
IIVS	90.4		122		1.96		NA
Lithium carbonate	NA	NA		NA	NA	NA	NA
ECBC	564		12		2.75		NA
FAL	NA		NA		NA		NA
IIVS	NA		NA		NA		NA
Meprobamate	539	2.5		54	2.73	<0.001	
ECBC	353		14		2.55		NA
FAL	877		15		2.94		NA
IIVS	386		2		2.59		NA
Mercury chloride	4.32	1.7		33	0.64	0.021	
ECBC	3.5		5		0.54		NA
FAL	6		31		0.78		NA
IIVS	3.5		3		0.54		NA
Methanol	NA	NA		NA	NA	NA	NA
ECBC	NA		NA		NA		NA
FAL	NA				NA		NA
IIVS	NA				NA		NA
Nicotine	378	1.7		25	2.58	0.128	
ECBC	272		24		2.43		NA
FAL	412		33		2.61		NA

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
IIVS	450		12		2.65		NA
Paraquat	23.3	1.2		8	1.37	1	
ECBC	21.3		34		1.33		NA
FAL	24.9		67		1.4		NA
IIVS	23.7		64		1.37		NA
Parathion	61.8	6.4		111	1.79	0.014	
ECBC	22.7		53		1.36		NA
FAL	141		70		2.15		NA
IIVS	22		22		1.34		NA
Phenobarbital	612	1.5		21	2.79	0.232	
ECBC	634		21		2.8		NA
FAL	726		35		2.86		NA
IIVS	476		23		2.68		NA
Phenol	70.9	2.1		41		0.011	
ECBC	50.2		22		1.7		NA
FAL	104		24		2.02		NA
IIVS	58.1		12		1.76		NA
Phenylthiourea	119	7.9		90	2.08	0.007	
ECBC	30.1		66		1.48		0.004
FAL	239		28		2.38		0.006
IIVS	89		25		1.95		0.718
Physostigmine	28.8	1.9		30	1.46	0.149	
ECBC	28.2		53		1.45		NA
FAL	37.8		5		1.58		NA
IIVS	20.4		33		1.31		NA

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
Potassium chloride	3635	1.1		7	3.56	0.846	
ECBC	3352		14		3.53		NA
FAL	3842		31		3.58		NA
IIVS	3710		11		3.57		NA
Potassium cyanide	64.3	10.4		127	1.81	<0.001	
ECBC	15.3		25		1.18		0.001
FAL	159		52		2.2		<0.001
IIVS	18.9		5		1.28		0.006
Procainamide HCl	443	1.2		11	2.65	0.007	
ECBC	400		4		2.6		0.008
FAL	431		1		2.63		0.396
IIVS	497		8		2.7		0.003
2-Propanol	3563	1.6		23	3.55	0.001	
ECBC	2610		9		3.42		<0.001
FAL	3970		4		3.6		0.004
IIVS	4110		4		3.61		0.002
Propranolol HCl	14.9	1.3		16	1.17	0.488	
ECBC	13.6		32		1.13		NA
FAL	13.5		51		1.13		NA
IIVS	17.6		21		1.25		NA
Propylparaben	29.9	3.0		64	1.48	0.001	
ECBC	20.9		16		1.32		0.045
FAL	51.8		29		1.71		<0.001
IIVS	17.1		12		1.23		0.003
Sodium arsenite	0.873	2.8		55	-0.06	0.028	

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
ECBC	0.5		6		-0.3		NA
FAL	1.4		57		0.15		NA
IIVS	0.7		17		-0.15		NA
Sodium chloride	4764	1.1		3	3.68	0.759	
ECBC	4790		5		3.68		NA
FAL	4625		13		3.67		NA
IIVS	4877		9		3.69		NA
Sodium dichromate dihydrate	0.602	1.2		9	-0.22	0.822	
ECBC	0.603		14		-0.22		NA
FAL	0.657		37		-0.18		NA
IIVS	0.547		17		-0.26		NA
Sodium fluoride	79.8	1.6		22	1.9	0.016	
ECBC	61.3		9		1.79		NA
FAL	96.1		18		1.98		NA
IIVS	82		7		1.91		NA
Sodium hypochlorite	1211	2.5		57	3.08	0.04	
ECBC	823		13		2.92		NA
FAL	805		46		2.91		NA
IIVS	2005		44		3.3		NA
Sodium oxalate	40.8	1.6		23	1.61	0.643	
ECBC	42		41		1.62		NA
FAL	31		28		1.49		NA
IIVS	49.5		53		1.69		NA
Sodium selenate	34.5	4.3		60	1.54	<0.001	
ECBC	12.7		13		1.1		<0.001

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
FAL	54.2		19		1.73		<0.001
IIVS	36.5		14		1.56		0.026
Strychnine	199	4.7		83	2.3	<0.001	
ECBC	389		21		2.59		<0.001
FAL	124		16		2.09		0.018
IIVS	83.5		6		1.92		<0.001
Thallium Sulfate	7.5	4.9		72	0.88	0.165	
ECBC	2.8		24		0.45		NA
FAL	13.4		78		1.13		NA
IIVS	6.3		28		0.8		NA
Trichloroacetic acid	928	1.6		27	2.97	0.005	
ECBC	762		13		2.88		0.022
FAL	1220		6		3.09		0.002
IIVS	801		14		2.9		0.069
1,1,1-Trichloroethane	15538	2.2		52	4.19	<0.001	
ECBC	NA		NA		NA		NA
FAL	21250		11		4.33		NA
IIVS	9827		2		3.99		NA
Triethylenemelamine	0.568	16.9		135	-0.25	<0.001	
ECBC	0.086		11		-1.07		<0.001
FAL	1.45		18		0.16		<0.001
IIVS	0.169		29		-0.77		0.002
Triphenyltin hydroxide	0.022	1.7		29	-1.66	0.688	
ECBC	0.026		17		-1.59		NA
FAL	0.026		81		-1.59		NA

Table 7-3 Interlaboratory Reproducibility of the IC₅₀ Values from the 3T3 NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
IIVS	0.015		55		-1.83		NA
Valproic acid	1177	3.3		76	3.07	<0.001	
ECBC	547		12		2.74		NA
FAL	1807		10		3.26		NA
IIVS	NA		NA		NA		NA
Verapamil HCl	35.2	1.2		10	1.55	0.23	
ECBC	32		18		1.51		NA
FAL	34.6		5		1.54		NA
IIVS	38.9		11		1.59		NA
Xylene	NA	NA		NA	NA	NA	NA
ECBC	NA		NA		NA		NA
FAL	NA		NA		NA		NA
IIVS	724		12		2.86		NA

Abbreviations: 3T3=3T3 fibroblasts; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL= Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; NA=No acceptable IC₅₀ results reported or calculation was not performed (e.g., for contrast results); CV=Coefficient of variation.

¹Results reported on the same row with chemical names are the means of all the laboratories. Results reported on the same row as laboratories are the laboratory means.

²Maximum laboratory mean IC₅₀ divided by minimum laboratory mean IC₅₀.

³p <0.01 indicated statistical significance.

⁴Contrasts were performed if ANOVA was significant (p <0.01) to determine which laboratory was different from the other two laboratories. Significant contrasts were denoted by p <0.01. No contrast tests were performed if only two laboratories reported IC₅₀ values.

Table 7-4 Reference Substances with Significant ANOVA Differences Among Laboratories for the 3T3 NRU Test Method

Reference Substance	Significant Contrast Results ¹			Insoluble/ Volatile ²
	ECBC	FAL	IIVS	
Acetylsalicylic acid		H	L	
Arsenic trioxide		L	H	Precipitate
Busulfan		H		
Chloral hydrate	L	H		
Chloramphenicol	L	H		
Citric acid	L	H		
Colchicine	L	H		
Cupric sulfate pentahydrate	M	H	L	
Dibutyl phthalate		H	L	Precipitate
Dichlorvos	L	H		Precipitate
Ethylene glycol	L			
Glutethimide		H	L	
Haloperidol		H		
Meprobamate	L	H	M	
Phenylthiourea	L	H		
Potassium cyanide	L	H	M	Precipitate /Volatile
Procainamide HCl	L		H	
2-Propanol	L	M	H	Volatile
Propylparaben		H	L	
Sodium selenate	L	H		
Strychnine	H		L	Precipitate
Trichloroacetic acid		H		
Triethylenemelamine	L	H		

Abbreviations: ANOVA=Analysis of variance; 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake; H=Laboratory reported the highest mean IC₅₀; L=Laboratory reported the lowest mean IC₅₀; M=Laboratory reported a mean IC₅₀ between the values of the other two laboratories; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

¹Laboratories significantly different from the other two at p <0.01.

²From **Table 5-11**. Precipitate reported by at least one laboratory is indicated by “Precipitate”. Use of plate sealers by at least one laboratory to prevent volatile contamination of control wells indicated by “Volatility”.

7.2.2.2 *Differences Among the IC₅₀ Values in Laboratories Using the NHK NRU Test Method*

The ANOVA results in **Table 7-5** indicate that there were statistically significant ($p < 0.01$) laboratory differences for six of the 68 (9%) reference substances evaluated. These substances are listed in **Table 7-6** along with columns showing which laboratory's IC₅₀ values were statistically significantly different from the other two (as indicated by the contrast results), and indications of insolubility or volatility during conduct of the assay. Insolubility was reported for three of the six substances, but none of the six substances were volatile.

For the six substances that yielded significantly different IC₅₀ values among the laboratories, ECBC reported the highest IC₅₀ value for four substances and the lowest for one, FAL reported the lowest values for three substances and the highest for two, and IIVS reported the highest IC₅₀ value for one substance and the lowest for two.

7.2.3 CV Results for the 3T3 and NHK NRU Test Methods

CV values were calculated as described in Section 5.5.2.2. **Tables 7-3** and **7-5** provide the intra- and inter-laboratory CV values for the individual reference substances. **Table 7-7** summarizes the CV values for each method and shows that median and mean values were often similar. Median CV values were frequently lower than the corresponding means, which indicated that large individual CV values skewed the CV distributions.

7.2.3.1 *Reproducibility of Intralaboratory CV Values*

Table 7-7 shows that the intralaboratory CV values and mean intralaboratory CV values were the same, 26%, for both NRU test methods. The median intralaboratory CV values were also similar: 23% and 24% for the 3T3 and the NHK NRU test method, respectively. Of the three laboratories, FAL had the highest mean and median CV values and IIVS had the lowest for both methods.

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
Acetaminophen	526	1.3		13	2.72	0.181	
ECBC	558		15		2.75		NA
FAL	447		19		2.65		NA
IIVS	571		14		2.76		NA
Acetonitrile	10104	1.2		8	4	0.964	
ECBC	10868		72		4.04		NA
FAL	10153		19		4.01		NA
IIVS	9290		4		3.97		NA
Acetylsalicylic acid	613	1.4		15	2.79	0.060	
ECBC	631		3		2.8		NA
FAL	694		14		2.84		NA
IIVS	514		15		2.71		NA
5-Aminosalicylic acid	52.3	2.6		47	1.72	0.044	
ECBC	29.9		22		1.48		NA
FAL	78.2		54		1.89		NA
IIVS	48.8		16		1.69		NA
Aminopterin	682	1.6		27	2.83	0.025	
ECBC	889		20		2.95		NA
FAL	545		8		2.74		NA
IIVS	611		12		2.79		NA
Amitriptyline HCl	9.76	1.4		19	0.99	0.365	
ECBC	10.8		31		1.03		NA
FAL	7.57		72		0.88		NA
IIVS	10.9		10		1.04		NA
Arsenic trioxide	10.4	8.2		91	1.02	<0.001	

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
ECBC	7.77		33		0.89		0.694
FAL	2.55		75		0.41		<0.001
IIVS	20.9		31		1.32		0.0006
Atropine sulfate	91.9	1.3		13	1.96	0.988	
ECBC	85.4		12		1.93		0.8903
FAL	104		85		2.02		0.9069
IIVS	83.2		25		1.92		0.9832
Boric acid	473	1.2		8	2.67	0.931	
ECBC	440		31		2.64		0.9692
FAL	517		73		2.71		0.7391
IIVS	464		2		2.67		0.768
Busulfan	278	1.2		11	2.44	0.659	
ECBC	253		27		2.4		NA
FAL	268		72		2.43		NA
IIVS	313		12		2.5		NA
Cadmium chloride	1.98	1.2		10	0.3	0.733	
ECBC	2.2		37		0.34		NA
FAL	1.88		65		0.27		NA
IIVS	1.86		8		0.27		NA
Caffeine	661	1.4		21	2.82	0.296	
ECBC	817		31		2.91		NA
FAL	591		32		2.77		NA
IIVS	574		1		2.76		NA
Carbamazepine	128	4.0		85	2.11	0.432	
ECBC	66.1		13		1.82		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
FAL	253		129		2.4		NA
IIVS	63.9		8		1.81		NA
Carbon tetrachloride	NA	NA		NA	NA	NA	
ECBC	NA		NA		NA		NA
FAL	NA		NA		NA		NA
IIVS	NA		NA		NA		NA
Chloral hydrate	137	1.4		17	2.14	0.302	
ECBC	140		24		2.15		NA
FAL	159		32		2.2		NA
IIVS	112		2		2.05		NA
Chloramphenicol	366	1.3		13	2.56	0.750	
ECBC	318		45		2.5		NA
FAL	414		44		2.62		NA
IIVS	367		22		2.56		NA
Citric acid	424	1.7		25	2.63	0.006	
ECBC	526		16		2.72		0.009
FAL	312		17		2.49		0.002
IIVS	433		5		2.64		0.483
Colchicine	0.007	1.6		22	-2.16	0.174	
ECBC	0.005		46		-2.28		NA
FAL	0.008		10		-2.12		NA
IIVS	0.008		21		-2.09		NA
Cupric sulfate pentahydrate	197	1.1		4	2.29	0.374	
ECBC	190		10		2.28		NA
FAL	195		6		2.29		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
IIVS	207		3		2.32		NA
Cycloheximide	0.082	2.3		43	-1.09	0.302	
ECBC	0.053		22		-1.28		NA
FAL	0.12		78		-0.92		NA
IIVS	0.071		19		-1.15		NA
Dibutyl phthalate	32.6	2.2		41	1.51	0.408	
ECBC	28.3		27		1.45		NA
FAL	47.4		73		1.68		NA
IIVS	22		6		1.34		NA
Dichlorvos	11.1	1.4		20	1.05	0.181	
ECBC	8.56		27		0.93		NA
FAL	12.4		30		1.09		NA
IIVS	12.2		3		1.09		NA
Diethyl phthalate	145	2.6		44	2.16	0.049	
ECBC	174		8		2.24		NA
FAL	71.5		94		1.85		NA
IIVS	189		18		2.28		NA
Digoxin	0.00314	107.6		88	-2.5	<0.001	
ECBC	0.00538		13		-2.27		<0.001
FAL	0.00005		36		-4.29		<0.001
IIVS	0.00398		7		-2.4		<0.001
Dimethylformamide	7856	1.5		19	3.9	<0.001	
ECBC	9353		2		3.97		<0.001
FAL	7817		1		3.89		0.508
IIVS	6397		3		3.81		<0.001

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
Diquat dibromide monohydrate	4.73	1.9		37	0.67	0.217	
ECBC	3.59		23		0.56		NA
FAL	6.77		55		0.83		NA
IIVS	3.84		8		0.58		NA
Disulfoton	378	5.8		99	2.58	<0.001	
ECBC	140		19		2.15		0.002
FAL	808		26		2.91		<0.001
IIVS	186		32		2.27		0.018
Endosulfan	2.35	2.4		43	0.37	0.029	
ECBC	3.44		17		0.54		NA
FAL	1.42		50		0.15		NA
IIVS	2.19		20		0.34		NA
Epinephrine bitartrate	90.6	1.5		24	1.96	0.119	
ECBC	115		9		2.06		NA
FAL	81.7		35		1.91		NA
IIVS	75		16		1.88		NA
Ethanol	10184	1.4		18	4.01	0.035	
ECBC	8290		5		3.92		NA
FAL	12013		19		4.08		NA
IIVS	10250		9		4.01		NA
Ethylene glycol	42600	1.3		15	4.63	0.063	
ECBC	38000		12		4.58		NA
FAL	49800		9		4.7		NA
IIVS	40000		13		4.6		NA
Fenpropathrin	2.6	2.0		39	0.41	0.031	

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
ECBC	3.73		27		0.57		NA
FAL	2.23		28		0.35		NA
IIVS	1.82		17		0.26		NA
Gibberellic Acid	2866	1.0		2	3.46	0.862	
ECBC	2850		14		3.45		NA
FAL	2940		9		3.47		NA
IIVS	2807		4		3.45		NA
Glutethimide	177	1.1		5	2.25	0.968	
ECBC	187		34		2.27		NA
FAL	170		14		2.23		NA
IIVS	176		16		2.24		NA
Glycerol	27108	1.9		31	4.43	0.200	
ECBC	34267		45		4.53		NA
FAL	18023		46		4.26		NA
IIVS	29033		16		4.46		NA
Haloperidol	3.57	1.1		7	0.55	0.935	
ECBC	3.69		27		0.57		NA
FAL	3.72		49		0.57		NA
IIVS	3.29		35		0.52		NA
Hexachlorophene	0.031	2.2		41	-1.5	0.097	
ECBC	0.027		16		-1.57		NA
FAL	0.046		44		-1.34		NA
IIVS	0.021		11		-1.67		NA
Lactic acid	1308	1.0		1	3.12	0.904	
ECBC	1290		4		3.11		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
FAL	1320		5		3.12		NA
IIVS	1313		11		3.12		NA
Lindane	19.3	1.5		20	1.29	0.203	
ECBC	19.1		17		1.28		NA
FAL	23.2		31		1.37		NA
IIVS	15.6		15		1.19		NA
Lithium carbonate	477	1.3		13	2.68	0.295	
ECBC	411		29		2.61		NA
FAL	486		20		2.69		NA
IIVS	535		6		2.73		NA
Meprobamate	516	4.7		61	2.71	0.027	
ECBC	761		15		2.88		NA
FAL	163		116		2.21		NA
IIVS	624		14		2.8		NA
Mercury chloride	5.87	1.3		15	0.77	0.120	
ECBC	6.87		15		0.84		NA
FAL	5.4		19		0.73		NA
IIVS	5.35		2		0.73		NA
Methanol	1616	1.9		42	3.21	0.007	
ECBC	NA		NA		NA		NA
FAL	1133		19		3.05		NA
IIVS	2100		11		3.32		NA
Nicotine	113	1.4		17	2.05	0.700	
ECBC	94.3		26		1.97		NA
FAL	134		59		2.13		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
IIVS	112		25		2.05		NA
Paraquat	66.1	2.0		40	1.82	0.047	
ECBC	48.3		13		1.68		NA
FAL	96.6		39		1.98		NA
IIVS	53.4		10		1.73		NA
Parathion	31.4	1.2		8	1.5	0.845	
ECBC	34		30		1.53		NA
FAL	31.2		38		1.49		NA
IIVS	29		29		1.46		NA
Phenobarbital	478	1.9		39	2.68	0.027	
ECBC	693		26		2.84		NA
FAL	360		27		2.56		NA
IIVS	381		18		2.58		NA
Phenol	77.7	1.6		22	1.89	0.094	
ECBC	59.1		36		1.77		NA
FAL	93.2		6		1.97		NA
IIVS	80.8		6		1.91		NA
Phenylthiourea	346	1.5		19	2.54	0.133	
ECBC	363		16		2.56		NA
FAL	401		21		2.6		NA
IIVS	272		26		2.44		NA
Physostigmine	172	1.5		22	2.24	0.623	
ECBC	164		3		2.21		NA
FAL	213		112		2.33		NA
IIVS	139		6		2.14		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
Potassium chloride	2279	1.3		13	3.36	0.396	
ECBC	2560		17		3.41		NA
FAL	2287		28		3.36		NA
IIVS	1990		8		3.3		NA
Potassium cyanide	45.1	5.3		86	1.65	0.340	
ECBC	29.3		24		1.47		NA
FAL	89		112		1.95		NA
IIVS	16.9		13		1.23		NA
Procainamide HCl	1764	1.4		16	3.25	0.053	
ECBC	1480		14		3.17		NA
FAL	1787		12		3.25		NA
IIVS	2027		11		3.31		NA
2-Propanol	5541	1.7		26	3.74	0.033	
ECBC	5263		11		3.72		NA
FAL	4273		27		3.63		NA
IIVS	7087		7		3.85		NA
Propranolol HCl	36.9	1.5		21	1.57	0.003	
ECBC	38.27		12		1.58		0.325
FAL	43.8		6		1.64		0.006
IIVS	28.6		11		1.46		0.001
Propylparaben	16.8	1.3		16	1.23	0.066	
ECBC	18.1		13		1.26		NA
FAL	18.6		15		1.27		NA
IIVS	13.8		9		1.14		NA
Sodium arsenite	0.532	2.4		44	-0.27	0.061	

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
ECBC	0.79		32		-0.1		NA
FAL	0.336		56		-0.47		NA
IIVS	0.47		14		-0.33		NA
Sodium chloride	2724	3.2		51	3.44	0.045	
ECBC	3583		7		3.55		NA
FAL	1118		124		3.05		NA
IIVS	3470		9		3.54		NA
Sodium dichromate dihydrate	0.737	1.5		19	-0.13	0.258	
ECBC	0.784		14		-0.11		NA
FAL	0.851		36		-0.07		NA
IIVS	0.576		17		-0.24		NA
Sodium fluoride	47.4	1.4		15	1.68	0.313	
ECBC	48.7		14		1.69		NA
FAL	39.7		24		1.6		NA
IIVS	53.7		13		1.73		NA
Sodium hypochlorite	1580	1.5		20	3.2	0.313	
ECBC	1863		31		3.27		NA
FAL	1243		46		3.09		NA
IIVS	1633		11		3.21		NA
Sodium oxalate	355	1.0		1	2.55	0.926	
ECBC	355		15		2.55		NA
FAL	350		42		2.54		NA
IIVS	360		26		2.56		NA
Sodium selenate	11.2	2.2		40	1.05	0.134	
ECBC	7.47		12		0.87		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
FAL	16.1		59		1.21		NA
IIVS	10		13		1		NA
Strychnine	69.3	1.9		39	1.84	0.364	
ECBC	100		76		2		NA
FAL	52.5		53		1.72		NA
IIVS	55.1		6		1.74		NA
Thallium Sulfate	0.16	1.6		23	-0.8	0.405	
ECBC	0.198		51		-0.7		NA
FAL	0.153		20		-0.82		NA
IIVS	0.127		16		-0.9		NA
Trichloroacetic acid	427	1.6		24	2.63	0.134	
ECBC	348		18		2.54		NA
FAL	541		28		2.73		NA
IIVS	394		13		2.6		NA
1,1,1-Trichloroethane	NA	NA		NA	NA	NA	
ECBC	8137		7		3.91		NA
FAL	NA		NA		NA		NA
IIVS	NA		NA		NA		NA
Triethylenemelamine	1.95	1.3		12	0.29	0.562	
ECBC	1.69		57		0.23		NA
FAL	2.03		23		0.31		NA
IIVS	2.13		23		0.33		NA
Triphenyltin hydroxide	0.013	3.0		55	-1.89	0.088	
ECBC	0.021		32		-1.68		NA
FAL	0.007		106		-2.15		NA

Table 7-5 Reproducibility of the IC₅₀ Values from the NHK NRU Test Method

Reference Substance/Laboratory	Arithmetic Mean IC ₅₀ (mg/mL) ¹	Maximum: Minimum Mean IC ₅₀ Ratio ²	Arithmetic IntraLab %CV	Arithmetic InterLab %CV	Log Arithmetic Mean IC ₅₀ (mg/mL) ¹	ANOVA P ³	Contrast P ⁴
IIVS	0.011		32		-1.96		NA
Valproic acid	533	1.6		28	2.73	0.081	
ECBC	468		25		2.67		0.331
FAL	702		23		2.85		0.032
IIVS	430		17		2.63		0.135
Verapamil HCl	68.7	1.3		14	1.84	0.624	
ECBC	60.5		22		1.78		NA
FAL	79.4		42		1.9		NA
IIVS	66.2		8		1.82		NA
Xylene	NA	NA		NA	NA	NA	
ECBC	NA		NA		NA		NA
FAL	NA		NA		NA		NA
IIVS	486		38		2.69		NA

Abbreviations: NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; NA=No acceptable IC₅₀ results reported or calculation was not performed (e.g., for contrast results); CV=Coefficient of variation.

¹Results reported on the same row with chemical names are the means of all the laboratories. Results reported on the same row as laboratories are the laboratory means.

²Maximum laboratory mean IC₅₀ divided by minimum laboratory mean IC₅₀.

³p <0.01 indicated statistical significance.

⁴Contrasts were performed if ANOVA was significant (p <0.01) to determine which laboratory was different from the other two laboratories. Significant contrasts were denoted by p <0.01. No contrast tests were performed if only two laboratories reported IC₅₀ values.

Table 7-6 Reference Substances with Significant ANOVA Differences Among Laboratories for the NHK NRU Test Method

Reference Substance	Significant Contrast Results ¹			Solubility/ Volatility ²
	ECBC	FAL	IIVS	
Arsenic trioxide		L	H	Precipitate
Citric acid	H	L		Precipitate
Digoxin	H	L		
Dimethylformamide	H		L	
Disulfoton	L	H		Precipitate
Propranolol HCl		H	L	

Abbreviations: ANOVA=Analysis of variance; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; H=Laboratory reported the highest mean IC₅₀; L=Laboratory reported the lowest mean IC₅₀; ECBC=Edgewood Chemical Biological Center; FAL= Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

¹Laboratories significantly different from the other two at p <0.01

²From **Table 5-11**. Precipitate reported by at least one laboratory.

7.2.3.2 Reproducibility of Interlaboratory CV Values

The mean and median interlaboratory CV for the reference substances were lower in the NHK NRU test method (mean=28%; median=21% vs. mean=47%; median=37% for 3T3 (see **Table 7-7**).

Table 7-7 Summary of CV Results for the 3T3 and NHK NRU Test Methods

CV	3T3 NRU Test Method				NHK NRU Test Method			
	N	Mean	Median	Range	N	Mean	Median	Range
Intralaboratory CV	198	26%	23%	1-122%	204	26%	24%	1-129%
ECBC	64	23%	17%	2-95%	68	23%	20%	2-76%
FAL	64	33%	31%	1-98%	68	43%	34%	1-129%
IIVS	64	21%	14%	1-122%	68	13%	13%	1-35%
Interlaboratory CV	64	47%	37%	3-135%	68	28%	21%	1-91%

Abbreviations: CV=Coefficient of variation; 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; N=number of values; ECBC=Edgewood Chemical Biological Center; FAL= Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

Note: For the 3T3 method, the following substances were excluded because all laboratories did not obtain sufficient IC₅₀ data: carbon tetrachloride; disulfoton; gibberellic acid; lithium carbonate; methanol; 1,1,1-trichloroethane; valproic acid; and xylene. For the NHK method, the following substances were excluded because all laboratories did not obtain sufficient IC₅₀ data: carbon tetrachloride; methanol; 1,1,1-trichloroethane; and xylene.

7.2.3.3 Variation of CV with Chemical Property

To identify chemical characteristics that may be associated with high or low CV values, their associations were assessed for chemical class along with the following chemical attributes: physical state (i.e., solid or liquid), solubility, volatility, molecular weight, log K_{ow}, IC₅₀, and boiling point. The CVs were also examined with respect to their association with the GHS acute oral toxicity class (UN 2005). For categorical characteristics such as physical form, solubility (i.e., precipitate/no precipitate), volatile/not volatile, and chemical class, the mean

CV values and ranges for the groups were compared to one another and to the overall mean CV and CV range for each method. No statistical analyses were performed for these comparisons. Spearman correlation analyses were performed for chemical characteristics measured by continuous variables, such as molecular weight, log K_{ow} , and IC_{50} , and boiling point.

7.2.3.4 Results of Intralaboratory CV Analysis

The intralaboratory CV analysis (see **Table 7-8**) uses one mean intralaboratory CV for each reference substance that was calculated from the intralaboratory CV values from each laboratory. There seemed to be little difference in CV values among the categorical physical/chemical/toxicological attributes. The mean intralaboratory CV values for solids and liquids were similar (26 vs. 23% for 3T3; 27 vs. 24% for NHK). The mean intralaboratory CV values for reference substances for which precipitates were observed were similar to values for substances with no precipitates were observed (32 vs. 23% for 3T3; 24 vs. 27% for NHK). The mean intralaboratory CV values for substances that exhibited volatility were similar to those that did not (31 vs. 25% for 3T3; 27 vs. 26% for NHK). Similarly, the substances grouped by GHS acute oral toxicity category (UN 2005) had mean intralaboratory CV values that were similar (20-33% for 3T3; 19-31% for NHK) to the overall mean CV values (26% for both test methods). However, the mean intralaboratory CV values for both NRU test methods tended to increase with decreasing LD_{50} .

Mean intralaboratory CV values were calculated for the chemical classes that contained at least three of the reference substances included in the reproducibility analyses (i.e., 64 substances for 3T3 and 68 substances for NHK). Reference substances in the amide chemical class had unusually low mean intralaboratory CV values for both the 3T3 (13%) and the NHK (10%) NRU test method compared with the overall mean CV (26% for both test methods), but there were only three substances in this chemical class (acetaminophen, dimethylformamide, procainamide HCl). Organic sulfur compounds had a high mean intralaboratory CV for the 3T3 test method (46%), but not for the NHK NRU test method (29%) compared with the overall mean intralaboratory CV for both test methods (26%). The intralaboratory CV values for the remaining chemical classes were unremarkable compared with the overall mean intralaboratory CV values.

For the characteristics amenable to correlation analysis, none of the Spearman correlation coefficients were large (absolute value of $r_s < 0.6$), but several were statistically significantly different from zero ($p < 0.05$). Molecular weight ($p=0.016$), IC_{50} ($p=0.002$), and boiling point ($p=0.009$) exhibited statistically significant correlations to intralaboratory CV for the 3T3 test NRU method. The higher molecular weight substances had higher intralaboratory CV values and the substances with lower IC_{50} values had higher intralaboratory CV values. The finding that substances with higher boiling points had higher CV values was consistent with the categorical analysis of volatility. The substances that exhibited volatile characteristics (i.e., cross contamination of VC wells) in the 3T3 NRU test method had slightly higher mean intralaboratory CV values (31%) than the substances that did not exhibit volatile characteristics (25%).

Table 7-8 Intralaboratory CV Values by Chemical Characteristics for the 3T3 and NHK NRU Test Methods

Class/Attribute	3T3 NRU Test Method			NHK NRU Test Method		
	N ¹	Range	Mean	N ¹	Range	Mean
All chemicals	64	1-122%	26%	68	1-129%	26%
Chemical form						
Solid	51	4-84	26	53	6-57	27
Liquid	13	6-48	23	15	2-40	24
Solubility						
Precipitate ²	18	11-84	32	19	2-47	24
No precipitate	46	4-55	23	49	7-57	27
Volatility³						
Volatile	10	6-84	31	9	11-50	27
Nonvolatile	54	4-55	25	59 ²	2-57	26
Chemical Class						
Alcohol	9	6-42	22	9	10-37	22
Amide	3	4-28	13	3	2-16	10
Amine	3	9-35	18	3	10-24	18
Carboxylic acid	13	4-41	18	14	2-48	23
Heterocyclic	14	6-59	31	14	13-50	32
Organophosphorous	2	NA	NA	3	20-32	26
Organic sulfur	4	36-59	46	5	21-27	29
Phenol	5	14-30	20	5	11-31	19
Polycyclic	4	19-35	27	5	9-38	20
Inorganic	14	9-43	25	15	6-50	29
Inorganic chlorine	5	9-33	19	5	12-50	32
Inorganic sodium	6	9-34	20	6	17-47	30
GHS Acute Oral Toxicity Class						
LD ₅₀ ≤ 5 mg/kg	6	9-46	27	7	20-40	30
5 < LD ₅₀ ≤ 50	12	13-59	32	12	12-50	31
50 < LD ₅₀ ≤ 300	12	11-84	33	12	17-37	25
300 < LD ₅₀ ≤ 2000	14	4-51	22	16	6-57	25
2000 < LD ₅₀ ≤ 5000	9	9-32	20	9	7-50	30
LD ₅₀ > 5000	11	6-42	20	12	2-40	19
Correlations	N	r_s	P value	N	r_s	P value
Molecular weight	64	0.301	0.016	68	0.181	0.140
Log K _{ow}	45 ⁴	0.121	0.430	48 ⁴	0.310	0.032
IC ₅₀	64	-0.382	0.002	68	-0.346	0.004
Boiling point	24 ⁵	0.520	0.009	24 ⁵	0.226	0.289

Abbreviations: CV=Coefficient of variation; 3T3=BALB/c 3T3 fibroblasts; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); NA=Not applicable because class had less than three observations; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; N=Number of values; r_s=Spearman correlation coefficient; K_{ow}=Octanol:water partition coefficient.

¹One intralaboratory CV for each chemical was calculated by averaging the CV values for each reference substance.

²Identified by laboratory reports of precipitate in the stock reference substance solutions or in cell culture (see **Table 5-11**).

³Identified by laboratory reports of using plate sealers to avoid contamination of the VC wells (see **Table 5-11**).

⁴Number of reference substances with CV values and log K_{ow} data.

⁵Number of reference substances with CV values and boiling point data.

Among the IC₅₀ values obtained using the NHK NRU test method, two of the characteristics amenable to correlation analysis were statistically significantly different from zero, although the correlation coefficients did not have large magnitudes (absolute value of r_s < 0.4). The log

K_{ow} ($p=0.032$) and IC_{50} ($p=0.004$) exhibited statistically significant correlations ($p < 0.05$) to the intralaboratory CV. Log K_{ow} was positively correlated (i.e., higher log K_{ow} values were associated with higher mean intralaboratory CV), but the IC_{50} was negatively correlated (i.e., higher log IC_{50} values were associated with lower mean intralaboratory CV) to mean intralaboratory CV.

7.2.3.5 Results of the Interlaboratory CV Analysis

Table 7-9 shows the analysis of the interlaboratory CV values. There seemed to be little difference in interlaboratory CV values for most of the categorical physical/chemical characteristics. The mean interlaboratory CV values for solids and liquids were similar (48% for solids vs. 42% for liquids for 3T3, and 28% for solids vs. 21% for liquids for NHK), as were the values for substances for which precipitates were observed versus no precipitates (58% vs. 43% for 3T3, and 24% vs. 28% for NHK), and the values for substances that exhibited volatile characteristics (51% for volatile substances vs. 46% for nonvolatile substances for 3T3, and 32% for volatile substances vs. 26% for nonvolatile substances for NHK).

Mean interlaboratory CV values were calculated for the chemical classes that contained at least three of the reference substances included in the reproducibility analyses (i.e., 64 substances for 3T3 and 68 substances for NHK). Reference substances in the amide chemical class had low mean interlaboratory CV values for both the 3T3 (15%) and the NHK (16%) NRU test methods compared with the overall mean interlaboratory CV (47% and 28%, respectively). Substances in the amine class also had low mean interlaboratory CV values for the 3T3 NRU (13%), but not for the NHK NRU (20%). Organic sulfur compounds had unusually high mean interlaboratory CV values for the 3T3 test method (100%), but not for the NHK NRU (36%) compared with the overall mean interlaboratory CV (47% and 28%, respectively). Because of the low number of reference substances in these classes, these results were deemed to not be significant.

Mean interlaboratory CV values tended to be large for chemicals in the most toxic GHS acute oral toxicity categories, especially with the 3T3 NRU test method. The mean interlaboratory CV for reference substances in the $LD_{50} \leq 5$ mg/kg (72%) and $5 < LD_{50} \leq 50$ mg/kg (78%) classes were larger than the mean overall interlaboratory CV (47%). For the NHK NRU test method, the mean interlaboratory CV for chemicals in the $5 < LD_{50} \leq 50$ mg/kg (37%) and $5 < LD_{50} \leq 50$ mg/kg (41%) classes were larger than the mean overall interlaboratory CV (28%).

For the characteristics amenable to correlation analysis, none of the correlation coefficients were large (absolute value of $r_s < 0.6$), but IC_{50} ($p=0.015$) and boiling point ($p=0.007$) exhibited statistically significant correlations ($p < 0.05$) to interlaboratory CV in the 3T3 test NRU method. There was a negative correlation between interlaboratory CV and IC_{50} , but the correlation between boiling point and interlaboratory CV was positive. The positive correlation of CV with boiling point was largely consistent with the categorical analysis of volatility. The substances that exhibited volatile characteristics in the 3T3 NRU test method had slightly higher mean CV values than substances that did not exhibit volatile characteristics (51% vs. 46%). Only the IC_{50} was significantly correlated ($p=0.014$) to

interlaboratory CV with a negative correlation ($r_s=-0.271$) when the NHK NRU test method was used.

Table 7-9 Interlaboratory 3T3 and NHK NRU Test Method CV Values Sorted by Chemical Characteristics

Class/Attribute	3T3 NRU Test Method			NHK NRU Test Method		
	N	Range	Mean	N	Range	Mean
All chemicals	64 ¹	3-135%	47%	68 ¹	1-91%	28%
Chemical Form						
Solids	51	3-135	48	53	1-91	28
Liquids	13	6-124	42	15	1-44	21
Solubility						
Precipitate ²	18	7-127	58	19	1-91	24
No precipitate	46	3-135	43	49	1-88	28
Volatility						
Volatile ³	10	21-127	51	9	8-86	32
Nonvolatile	54	3-135	46	59	1-91	26
Chemical Class						
Alcohol	9	12-119	38	9	11-31	20
Amide	3	6-28	15	3	13-19	16
Amine	3	10-16	13	3	14-24	20
Carboxylic acid	13	6-124	38	14	1-61	26
Heterocyclic	14	8-135	57	14	5-85	32
Organic sulfur	4	78-119	100	5	8-99	36
Organophosphorous	2	NA	NA	3	8-99	42
Phenol	5	19-64	41	5	15-47	28
Polycyclic	4	14-85	44	5	2-88	30
Inorganic	14	3-127	50	15	4-91	30
Inorganic chlorine	5	3-127	45	5	10-86	35
Inorganic sodium	6	3-60	34	6	15-51	32
GHS Acute Oral Toxicity Class						
LD ₅₀ ≤5 mg/kg	6	12-135	72	7	12-99	37
5 < LD ₅₀ ≤50	12	33-127	78	12	8-91	41
50 < LD ₅₀ ≤300	12	8-120	37	12	10-41	26
300 < LD ₅₀ ≤2000	14	11-85	35	16	1-61	20
2000 < LD ₅₀ ≤5000	9	3-69	29	9	1-85	27
LD ₅₀ >5000	11	6-124	41	12	2-44	23
Correlations	N	r_s	P value	N	r_s	P value
Molecular weight	64	0.245	0.051	68	0.169	0.168
Log K _{ow}	45 ⁴	0.151	0.324	48 ⁴	0.210	0.151
IC ₅₀	64	-0.304	0.015	68	-0.297	0.014
Boiling point	22 ⁵	0.563	0.007	25 ⁵	-0.051	0.809

Abbreviations: CV=Coefficient of variation; 3T3=BALB/c 3T3 fibroblasts; GHS=Globally Harmonized System of Classification and Labelling of Chemicals (UN 2005); NA=Not applicable because class had less than three observations; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; N=Number of values; r_s =Spearman correlation coefficient; K_{ow}=Octanol:water partition coefficient.

¹One intralaboratory CV for each chemical was calculated by averaging the CV values for each reference substance.

²Identified by laboratory reports of precipitate in the stock reference substance solutions or in cell culture (see **Table 5-11**).

³Identified by laboratory reports of using plate sealers to avoid contamination of the VC wells (see **Table 5-11**).

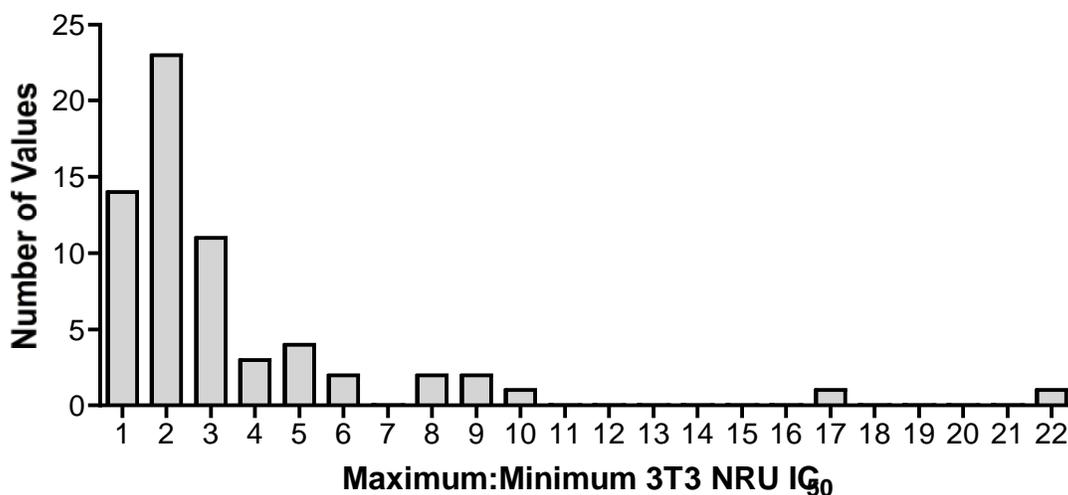
⁴Number of reference substances with CV values and log K_{ow} data.

⁵Number of reference substances with CV values and boiling point data.

7.2.4 Comparison of Maximum to Minimum IC₅₀ Values Using Laboratory Means

Interlaboratory reproducibility was also compared by calculating maximum to minimum mean IC₅₀ values using the laboratory means from each method, so that the reproducibility of the IC₅₀ values could be compared with the reproducibility of the reference LD₅₀ values derived in **Section 4.2**. The **Figure 7-2** frequency histogram for the 3T3 NRU test method maximum:minimum mean IC₅₀ values shows that approximately half (37) of the 64 reference substances produced ratios less than 2.5-fold of each other, and only nine chemicals had ratios greater than 5.5-fold, including one substance (cupric sulfate pentahydrate) that had a ratio of 22.

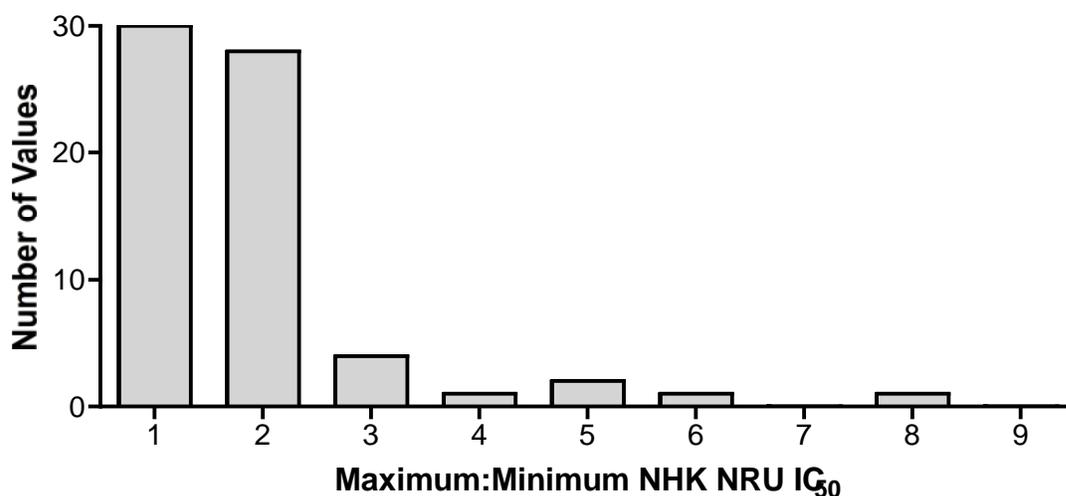
Figure 7-2 Frequency of Maximum:Minimum 3T3 NRU IC₅₀ Ratios



Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake.

Bars show the number of substances with maximum:minimum 3T3 NRU IC₅₀ ratios within ± 0.5 units of the bar label (e.g., the first bar indicates that there were 14 reference substances for which the laboratory mean maximum:minimum 3T3 NRU IC₅₀ ratios were 0.5 to 1.4). The analysis includes 64 reference substances. Carbon tetrachloride, disulfoton, gibberellic acid, lithium carbonate, methanol, 1,1,1-trichloroethane, valproic acid, and xylene were excluded because not all laboratories obtained IC₅₀ values.

The **Figure 7-3** frequency histogram for the maximum:minimum mean IC₅₀ values for the NHK NRU test method shows that ratios of 58 of the 68 chemicals were less than 2.5-fold of one another. The highest ratio of 108 for digoxin is not shown in the figure. Comparison of **Figures 7-2** and **7-3** shows that the interlaboratory reproducibility of the NHK NRU test method was better than that for the 3T3 NRU test method based on the distribution of the low maximum:minimum IC₅₀ ratios.

Figure 7-3 Frequency of Maximum:Minimum NHK NRU IC₅₀ Ratios

Abbreviations: NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake.

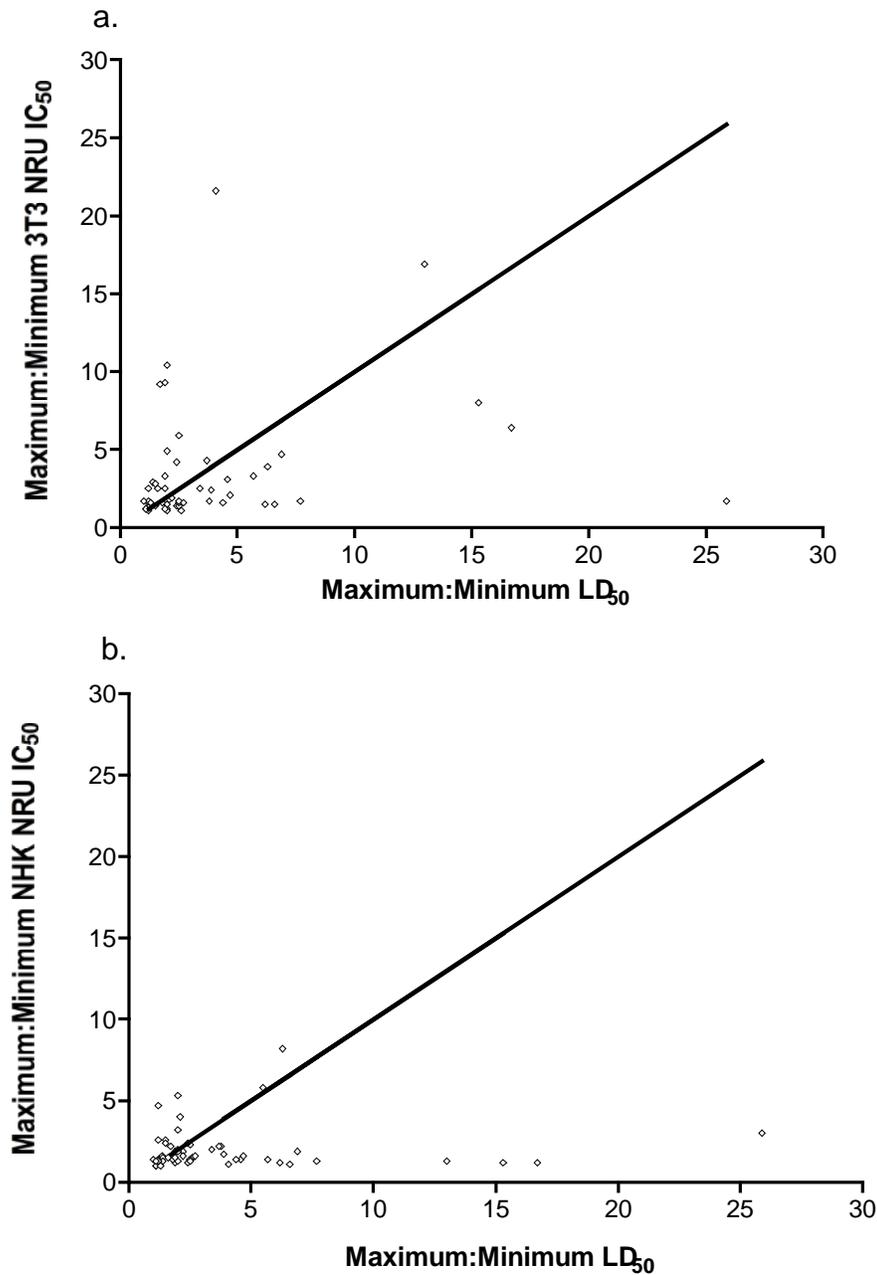
Bars show the number of substances with maximum:minimum NHK NRU IC₅₀ ratios within ± 0.5 units of the bar label (e.g., the first bar indicates that there were 30 reference substances for which the laboratory mean maximum:minimum NHK NRU IC₅₀ ratios were 0.5 to 1.4). The analysis includes 68 reference substances. Carbon tetrachloride, methanol, 1,1,1-trichloroethane, and xylene were excluded because not all laboratories obtained IC₅₀ values. The maximum:minimum IC₅₀ for digoxin of 108 was excluded from this figure.

7.2.5 Comparison of the Maximum:Minimum IC₅₀ Ratios with the Maximum:Minimum LD₅₀ Ratios

To compare the reproducibility of the NRU IC₅₀ values with that of the LD₅₀ values, the maximum:minimum IC₅₀ ratios for each method (shown in **Tables 7-3** and **7-5**) were compared with the maximum:minimum LD₅₀ ratios reported in **Table 4-2**. This analysis excluded reference substances for which fewer than three laboratories reported IC₅₀ values, and reference substances for which fewer than two acceptable acute oral LD₅₀ values were identified. As a result, there were 53 substances analysed for the 3T3 NRU test method and 57 for the NHK NRU test method. The following substances were excluded from both analyses because fewer than two acceptable LD₅₀ values could be identified: aminopterin; colchicine; digoxin; epinephrine bitartrate; glutethimide; phenylthiourea; physostigmine; procainamide HCl, propranolol HCl; propylparaben; and thallium sulfate. Carbon tetrachloride, disulfoton, gibberellic acid, lithium carbonate, methanol, 1,1,1-trichloroethane, valproic acid, and xylene, were excluded from the 3T3 analysis, and carbon tetrachloride, methanol, 1,1,1-trichloroethane, and xylene, were excluded from the NHK analysis, because fewer than three laboratories reported IC₅₀ values.

Figure 7-4 shows that the maximum:minimum LD₅₀ ratios tend to be larger than either the 3T3 NRU IC₅₀ or NHK NRU IC₅₀ ratios because there are more points below the theoretical one-to-one correspondence line than above the line. The difference between the LD₅₀ maximum:minimum values and the NRU IC₅₀ maximum:minimum values is more striking for the NHK since there are fewer points above the line for the NHK graph (**Figure 7-4b**) than for the 3T3 graph (**Figure 7-4a**).

Figure 7-4 Comparison of Maximum:Minimum NRU IC₅₀ Ratios to Maximum:Minimum LD₅₀ Ratios



Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake; NHK=Normal human epidermal keratinocytes. Comparison of maximum:minimum ratios of IC₅₀ and LD₅₀ for 53 reference substances for the 3T3 NRU test method (a) and 57 reference substances for the NHK NRU test method (b). Solid lines show the theoretical one to one correspondence of maximum:minimum IC₅₀ to maximum:minimum LD₅₀.

7.2.6 Normalization of Reference Substance IC₅₀ Values Using SLS IC₅₀ Values

As an alternate analysis for reproducibility, IC₅₀ values for reference substances were normalized to those of the corresponding SLS IC₅₀ values. This approach was tested using five reference substances for each test method to determine whether such normalization would reduce the variability, measured using CV values, of the results. The reference substances selected for this evaluation were those for which the ANOVA indicated statistically significant differences among the laboratories. Because there were a number of reference substances that met this criterion for the 3T3 NRU test method, the substances were selected so as to cover a wide range of rodent acute oral toxicity. One reference substance was selected from each GHS category with the exception of the $50 \leq LD_{50} < 300$ mg/kg category. There was no substance represented by this category because there were six acute oral toxicity categories and only five substances were used for this assessment. The reference substances, shown in **Table 7-10**, were busulfan, chloramphenicol, meprobamate, propylparaben, and triethylenemelamine. Because there were only six reference substances with significant ANOVAs in the NHK NRU test method, the last five reference substances in **Table 7-5** (citric acid, digoxin, dimethylformamide, disulfoton, and propranolol HCl) were selected for this analysis.

Millimolar units were used for the IC₅₀ values in this analysis since the mole is the most appropriate unit for measuring and comparing biological activity. The IC₅₀ value (in mM) for each reference substance was normalized to the corresponding SLS IC₅₀ value (in mM) by dividing the SLS IC₅₀ by the reference substance IC₅₀. Intra- and inter-laboratory CV values were calculated for both the IC₅₀ values and for the SLS IC₅₀:reference substance IC₅₀ ratios to determine whether this type of normalization would reduce the interlaboratory CV values.

Table 7-10 shows that the mean intralaboratory CV of the IC₅₀ values for the five substances used in the 3T3 evaluation was 22% and the interlaboratory CV was 88%. Normalizing the reference substance IC₅₀ values to the SLS IC₅₀ yielded a slightly higher intralaboratory CV of 25% and a lower interlaboratory CV of 65%. The mean intralaboratory CV of the IC₅₀ values for the five substances used in the NHK evaluation was 14% and the interlaboratory CV was 50%. Normalizing the reference substance IC₅₀ values to the SLS IC₅₀ yielded a slightly higher intralaboratory CV of 16% and a higher interlaboratory CV of 61%. When the normalization ratios are examined for each chemical-by-laboratory combination (**Table 7-10**), nine CVs increased, five decreased, and one remained the same for the 3T3 NRU test method, and eight increased, six decreased, and one remained the same for the NHK NRU test method. Thus, for the reference substances used in this analysis, normalizing the reference substance IC₅₀ to the concurrent SLS IC₅₀ did not reduce the overall variability of the measurements, as measured by the CV values.

Table 7-10 CV Values for 3T3 and NHK NRU Test Method IC₅₀ Values and Normalized IC₅₀ Values

Reference Substance	IC ₅₀ (mM) ¹	IntraLab CV ² (%)	InterLab CV ³ (%)	SLS IC ₅₀ : Substance IC ₅₀ ⁴	IntraLab CV SLS IC ₅₀ : Substance IC ₅₀ ⁵ (%)	InterLab CV SLS IC ₅₀ : Substance IC ₅₀ ⁶ (%)
3T3 NRU Test Method						
Busulfan	0.548		119	0.677		74
ECBC	0.163	48		1.05	70	
FAL	1.30	56		0.109	53	
IIVS	0.177	4		0.877	9	
Chloramphenicol	0.498		67	0.725		29
ECBC	0.171	22		0.847	30	
FAL	0.845	30		0.844	22	
IIVS	0.483	18		0.483	21	
Meprobamate	2.47		54	0.071		39
ECBC	1.62	14		0.085	23	
FAL	4.02	15		0.039	29	
IIVS	1.77	2		0.088	3	
Propylparaben	0.166		64	1.16		49
ECBC	0.116	16		1.29	20	
FAL	0.287	29		0.535	22	
IIVS	0.0949	12		1.65	9	
Triethylene-melamine	0.00278		135	191		87
ECBC	0.000421	11		354	11	
FAL	0.00710	18		21.4	24	
IIVS	0.000827	29		197	23	
Mean		22	88		25	65
NHK NRU Test Method						
Citric Acid	2.21		25	0.00587		26
ECBC	2.74	16		0.0053	14	
FAL	1.62	17		0.0076	28	
IIVS	2.25	5		0.0047	16	
Digoxin	4.02E-06		88	62378		168
ECBC	6.89E-06	13		1264	10	
FAL	6.53E-08	36		183479	44	
IIVS	5.10E-06	7		2389	26	
Dimethylformamide	107		19	0.00011		31
ECBC	128	2		0.00007	7	
FAL	107	1		0.00013	1	
IIVS	87.5	3		0.00013	19	
Disulfoton	1.38		99	0.0140		61
ECBC	0.509	19		0.022	6	
FAL	2.94	26		0.005	5	
IIVS	0.679	32		0.015	20	
Propranolol HCl	0.125		21	0.0947		20
ECBC	0.129	12		0.081	15	

Table 7-10 CV Values for 3T3 and NHK NRU Test Method IC₅₀ Values and Normalized IC₅₀ Values

Reference Substance	IC ₅₀ (mM) ¹	IntraLab CV ² (%)	InterLab CV ³ (%)	SLS IC ₅₀ : Substance IC ₅₀ ⁴	IntraLab CV SLS IC ₅₀ : Substance IC ₅₀ ⁵ (%)	InterLab CV SLS IC ₅₀ : Substance IC ₅₀ ⁶ (%)
FAL	0.148	6		0.087	25	
IIVS	0.0967	11		0.116	9	
Mean		14	50		16	61

Abbreviations: 3T3=BALB/c 3T3 fibroblasts; ECBC=Edgewood Chemical Biological Center; FAL= Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; NA=No acceptable IC₅₀ results reported or calculation was not performed (e.g., for contrast results); CV=Coefficient of variation.
¹Results reported on the same row with reference substance names are the arithmetic means of all the laboratories. Results reported on the same row as laboratories are the arithmetic laboratory means.
²CV for IC₅₀ values from the acceptable tests within each laboratory.
³CV calculated using the arithmetic mean IC₅₀ values from each laboratory.
⁴Concurrent SLS IC₅₀ in mM divided by the reference substance IC₅₀. Results reported on the same row with reference substance names are the arithmetic means of all the laboratories. Results reported on the same row as laboratories are the arithmetic laboratory means.
⁵CV for SLS IC₅₀:reference substance IC₅₀ values within each laboratory.
⁶CV calculated using the mean SLS IC₅₀:reference substance IC₅₀ values from each laboratory.

7.3 Historical Positive Control (PC) Data

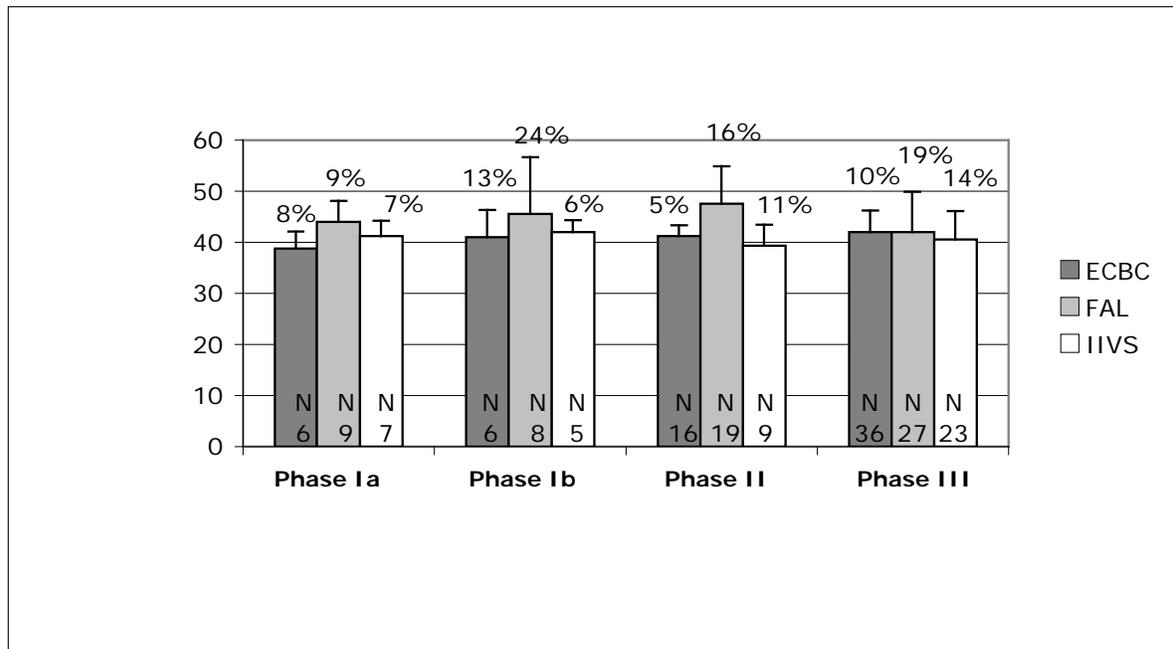
The reproducibility of the PC (SLS) data was assessed by CV analysis, ANOVA, and linear regression over time, as described in **Section 5.5.4.2**. To obtain an assessment of the true variation of SLS IC₅₀ values, the reproducibility analyses also included IC₅₀ values from SLS tests that failed the test acceptance criterion for the IC₅₀ acceptance limits determined for each study phase. Therefore, the values used for this analysis included some that were not included in **Table 5-3**. These additional SLS tests, however, passed all other test acceptance criteria. If more than one SLS test was performed in a single day (for each method and laboratory), the IC₅₀ values were averaged to determine a single IC₅₀ for the day so that the multiple results from that day would not overly influence the average.

Figure 7-5 shows the average SLS IC₅₀ values for each method, laboratory, and study phase. The SLS IC₅₀ for the 3T3 test method (**Figure 7-5a**) was relatively consistent over the entire period of the study (approximately 2.5 years). The intralaboratory CV values for the individual study phases ranged from 5% to 24% (**Figure 7-5a**). With the exception of the Phase Ib CV at FAL, the CV values for each laboratory and phase were less than 20%. The interlaboratory CV values were even smaller, 6% in Phases Ia and Ib, 10% in Phase II, and 2% in Phase III.

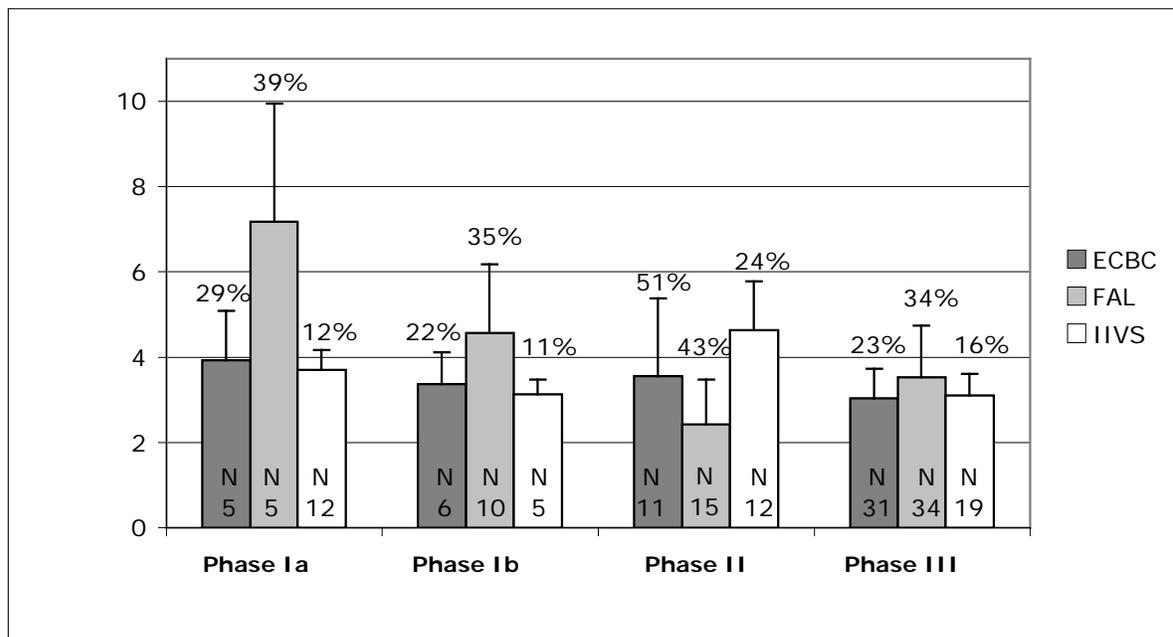
Figure 7-5b shows that the SLS IC₅₀ for the NHK NRU test method tended to vary with time, but, with the exception of the values from FAL, there appeared to be no consistent trend. The IC₅₀ values from FAL, which changed their cell culture methods after Phase Ib (see **Section 5.3.3.1**), tended to decrease over time. Although the change in cell culture methods reduced the magnitude of the IC₅₀, the variability (as evidenced by the intralaboratory CV values shown in **Figure 7-5b**) remained relatively high (CV ≥34% for all FAL study phases).

Figure 7-5 SLS IC₅₀ for Each Laboratory and Study Phase

a 3T3 NRU Test Method



b NHK NRU Test Method



Abbreviations: 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; ECBC=Edgewood Chemical Biological Center; FAL= Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; N=Number of values.

Note: Bars show mean SLS IC₅₀ values. Error bars show standard deviation. Percent values above error bars are intralaboratory CVs.

The CV values for all the laboratories and study phases show that the SLS IC₅₀ values in the NHK NRU test method are more variable within laboratories than the corresponding 3T3 SLS IC₅₀ values. The CV values for the SLS IC₅₀ for the NHK NRU test method ranged from 11 to 51%, with nine of the 12 values greater than 20%. The interlaboratory CV values, which were also greater than those for the 3T3 NRU test method, were 39% in Phase Ia, 21% in Phase Ib, 31% in Phase II, and 8% in Phase III.

7.3.1 ANOVA and Linear Regression Results for the 3T3 NRU Test Method

7.3.1.1 *Variation of SLS IC₅₀ Values with Time*

Table 7-11 shows the SLS ANOVA results from the 3T3 test method. When the IC₅₀ values in each laboratory were compared, there were no statistically significant differences ($p < 0.01$) among study phases for any laboratory. **Table 7-12** shows that the slopes of the linear regressions of the IC₅₀ values over time (expressed as index values) were significantly different from zero for ECBC and FAL ($p=0.001$ and 0.012 , respectively), but, because the slopes were so small (0.000204 and -0.000324), and in different directions, these differences were considered to be unimportant, regardless of the statistical conclusions. The slope of the IIVS regression of SLS IC₅₀ over time was not significantly different from zero ($p=0.651$; **Table 7-12**), which was consistent with the ANOVA analysis (**Table 7-11**), and showed that SLS IC₅₀ from IIVS did not vary with study phase ($p=0.854$). The ANOVA analysis, with study phase as the factor (with laboratories combined), showed that the 3T3 NRU IC₅₀ values from all the laboratories were consistent over time ($p=0.304$).

7.3.1.2 *Comparison of SLS IC₅₀ Values Among the Laboratories*

When all study phases from each laboratory were combined, ANOVA, with laboratory as the factor, showed that the SLS IC₅₀ values in the 3T3 NRU test method differed significantly among the laboratories ($p < 0.006$) (**Table 7-11**). However, as can be seen in **Figure 7-5a**, the individual laboratory SDs overlap one another.

Table 7-11 ANOVA Results for the SLS IC₅₀ Values in the 3T3 NRU Test Method

Study Phase/ Laboratory	ECBC				FAL				IIVS			
	Log Mean IC ₅₀ (mM)	SD	N	P ¹	Log Mean IC ₅₀ (mM)	SD	N	P ¹	Log Mean IC ₅₀ (mM)	SD	N	P ¹
<i>Test for differences between phases within each laboratory</i>												
Phase Ia	-0.876	0.042	6	0.031	-0.811	0.046	9	0.015	-0.850	0.034	7	0.854
Phase Ib	-0.864	0.066	6		-0.846	0.065	8		-0.838	0.025	5	
Phase II	-0.848	0.027	16		-0.796	0.057	19		-0.854	0.025	8	
Phase III	-0.842	0.036	36		-0.851	0.066	27		-0.844	0.041	23	
<i>Test for differences between laboratories (phases combined)</i>												
All Phases	-0.849	0.039	64	0.006	-0.826	0.062	63		-0.847	0.035	44	
<i>Test for differences between phases (laboratories combined)</i>												
Phase Ia	-0.839	0.049	22	0.304								
Phase Ib	-0.850	0.056	19									
Phase II	-0.831	0.047	34									
Phase III	0.845	0.045	86									

Abbreviations: ANOVA=Analysis of variance; SLS=Sodium lauryl sulfate; 3T3=BALB/c 3T3 fibroblasts; NRU=Neutral red uptake; N=Number of values; SD=Standard deviation; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

¹Statistically significant at p <0.01.

Table 7-12 Linear Regression Analysis of SLS IC₅₀ Values Over Time¹

Laboratory	Slope	P-value (Slope) ²	Intercept
3T3 NRU Test Method			
ECBC	0.000204	0.001	-0.874
FAL	-0.000324	0.012	-0.796
IIVS	0.0000304	0.651	-0.850
NHK NRU Test Method			
ECBC	-0.000559	0.002	-1.901
FAL	-0.00112	<0.001	-1.737
IIVS	-0.000445	0.002	-1.885

Abbreviations: SLS=Sodium lauryl sulfate; 3T3=BALB/c 3T3 fibroblasts; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; N=Number of values; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

¹Time was expressed as index values. The index value of each test reflected the order of testing without respect to the time lapsing between tests.

²Statistically significant from zero at p <0.05.

7.3.2 ANOVA and Linear Regression Results for the NHK NRU Test Method

7.3.2.1 *Variation of SLS IC₅₀ Values with Time*

Table 7-13 shows the ANOVA results for the NHK NRU test method. When the IC₅₀ values within each laboratory were compared by study phase, the values were statistically different (p <0.01) at each laboratory. The IC₅₀ values from the various study phases were also significantly different from one another when the laboratory data were combined (p <0.001). The change in cell culture methods at FAL after Phase Ib (see **Section 5.3.3.1**) contributed to this difference. **Table 7-13** shows that FAL had clearly the lowest log mean SLS IC₅₀ for Phases Ia and Ib. Linear regression analyses showed that the IC₅₀ slopes over time (expressed as an index values) were statistically significantly less than zero for each laboratory (see **Table 7-12**). Because the slopes were so small (-0.000559, -0.00112, and -0.000445), and negative, their statistical significance was considered to be irrelevant.

7.3.2.2 *Comparison of SLS IC₅₀ Values Among the Laboratories*

The ANOVA results, with laboratory as a factor (**Table 7-13**), showed that the SLS IC₅₀ was statistically significantly different among the laboratories when the data from the study phases were pooled (p <0.001). **Figure 7-5b** shows that the SLS data from ECBC and IIVS were rather similar to one another for Phases Ia, Ib, and III. The SLS IC₅₀ data from FAL are different from the other two laboratories for Phases Ia, Ib, and II, but the SDs for Phase III show that the data from all laboratories produced similar values.

Table 7-13 ANOVA Results for the SLS IC₅₀ Values in the NHK NRU Test Method

Study Phase/ Laboratory	ECBC				FAL				IIVS			
	Log Mean IC ₅₀ (mM)	SD	N	P ¹	Log Mean IC ₅₀ (mM)	SD	N	P ¹	Log Mean IC ₅₀ (mM)	SD	N	P ¹
<i>Test for differences between phases within each laboratory</i>												
Phase Ia	-1.867	0.135	5	0.001	-1.656	0.125	5	<0.001	-1.904	0.060	12	<0.001
Phase Ib	-1.936	0.092	6		-1.829	0.141	10		-1.965	0.046	5	
Phase II	-2.007	0.109	11		-1.982	0.173	15		-1.863	0.058	12	
Phase III	-1.990	0.098	31		-1.941	0.113	34		-1.972	0.070	19	
<i>Test for differences between laboratories (phases combined)</i>												
All Phases	-1.971	0.113	53	<0.001	-1.879	0.175	64		-1.924	0.073	48	
<i>Test for differences between phases (laboratories combined)</i>												
Phase Ia	-1.833	0.143	22	<0.001								
Phase Ib	-1.891	0.125	21									
Phase II	-1.964	0.139	38									
Phase III	-1.971	0.100	84									

Abbreviations: ANOVA=Analysis of variance; SLS=Sodium lauryl sulfate; NHK=Normal human epidermal keratinocytes; NRU=Neutral red uptake; N=Number of values; SD=Standard deviation; ECBC=Edgewood Chemical Biological Center; FAL=Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences.

¹Statistically significant at p <0.01.

7.4 Laboratory Concordance for Solvent Selection

The solvents used for the reference substances are shown in **Table 7-14**. For Phases Ib and II, the SMT based their selection of solvents on the results provided by BioReliance (see **Table 5-9**) using the solubility protocol in **Appendix G2**. Despite the fact that the solubility of an individual substance might be different in 3T3 and NHK growth media, the SMT selected the same solvent (i.e., medium or DMSO) for both test methods, rather than having different solvents for each method.

BioReliance occasionally achieved higher solubility values for the Phase I and II substances than the three cytotoxicity laboratories (e.g., see the results for arsenic trioxide, aminopterin, and chloramphenicol in **Table 5-10**). The laboratories were using the solubility protocols in **Appendices C3** through **C6** (for Phases Ib and II), which were somewhat different from the protocol used by BioReliance. Although all the laboratories used the same protocols, they did not always obtain similar results with respect to the solvent to be used (e.g., see the results for aminopterin, cadmium chloride, and chloramphenicol in **Table 5-10**). In an attempt to avoid the selection of a solvent for which one or more laboratories could not achieve the desired solubility, the SMT used the solubility data from all the laboratories to determine the solvents to be used for each chemical tested in Phase III. **Table 7-14** shows that cell culture medium was used as the solvent for 38 substances and DMSO was used for 34 substances.

Five of the substances were insoluble in medium and DMSO in at least one testing laboratory. Arsenic trioxide was insoluble at all laboratories. IIVS also found sodium oxalate, strychnine, and triethylenemelamine insoluble in media and DMSO, and FAL found thallium sulfate insoluble in both solvents. Therefore, the SMT used the results from the laboratories that did achieve solubility to select the solvents to be used for testing these substances.

The testing laboratories selected the same solvent for 55 of the 72 reference substances (76%). Excluding the five substances that were found to be insoluble in both solvents by at least one laboratory, there were 12 substances on which the laboratories disagreed: acetaminophen, acetylsalicylic acid, carbamazepine, carbon tetrachloride, chloramphenicol, dichlorvos, meprobamate, methanol, phenobarbital, phenylthiourea, physostigmine, and valproic acid. Each laboratory reported relatively low solubility, ≤ 2 mg/mL, in medium for these substances. Because 2 mg/mL in medium is the departure point for the selection of medium or DMSO, small variations in solubility lead the laboratories to select different solvents. The solubility of acetaminophen, for example was reported as 2 mg/mL in culture media by ECBC and FAL, but < 2 mg/mL by IIVS. IIVS found it soluble in 200 mg/mL DMSO and selected DMSO as the solvent. ECBC and FAL selected culture media as the solvent. The SMT selected DMSO as the solvent for acetaminophen to be used by all laboratories so that they would all be assured of obtaining usable test results.

Table 7-14 Solvent Determinations by Laboratory

Reference Substance	Solvent Used for Testing ¹	ECBC	FAL	IIVS
Acetaminophen	DMSO	Medium	Medium	DMSO
Acetonitrile	Medium	Medium	Medium	Medium
Acetylsalicylic acid	DMSO	Medium	DMSO	Medium
Aminopterin	DMSO	DMSO	DMSO	DMSO
5-Aminosalicylic acid	Medium	Medium	Medium	Medium
Amitriptyline HCl	DMSO	DMSO	DMSO	DMSO
Arsenic III trioxide	Medium	ID	ID	ID
Atropine sulfate	Medium	Medium	Medium	Medium
Boric acid	Medium	Medium	Medium	Medium
Busulfan	DMSO	DMSO	DMSO	DMSO
Cadmium II chloride	DMSO	DMSO	DMSO	DMSO
Caffeine	Medium	Medium	Medium	Medium
Carbamazepine	DMSO	Medium	DMSO	DMSO
Carbon tetrachloride	DMSO	Medium	DMSO	Medium
Chloral hydrate	Medium	Medium	Medium	Medium
Chloramphenicol	DMSO	DMSO	DMSO	Medium
Citric acid	Medium	Medium	Medium	Medium
Colchicine	Medium	Medium	Medium	Medium
Cupric sulfate pentahydrate	Medium	Medium	Medium	Medium
Cycloheximide	Medium	Medium	Medium	Medium
Dibutyl phthalate	DMSO	DMSO	DMSO	DMSO
Dichlorvos	DMSO	Medium	DMSO	Medium
Diethyl phthalate	DMSO	DMSO	DMSO	DMSO
Digoxin	DMSO	DMSO	DMSO	DMSO
Dimethylformamide	Medium	Medium	Medium	Medium
Diquat dibromide monohydrate	Medium	Medium	Medium	Medium
Disulfoton	DMSO	DMSO	DMSO	DMSO
Endosulfan	DMSO	DMSO	DMSO	DMSO
Epinephrine bitartrate	Medium	Medium	Medium	Medium
Ethanol	Medium	Medium	Medium	Medium
Ethylene glycol	Medium	Medium	Medium	Medium
Fenpropathrin	DMSO	DMSO	DMSO	DMSO
Gibberellic acid	Medium	Medium	Medium	Medium
Glutethimide	DMSO	DMSO	DMSO	DMSO
Glycerol	Medium	Medium	Medium	Medium
Haloperidol	DMSO	DMSO	DMSO	DMSO
Hexachlorophene	DMSO	DMSO	DMSO	DMSO
Lactic acid	Medium	Medium	Medium	Medium
Lindane	DMSO	DMSO	DMSO	DMSO
Lithium I carbonate	Medium	Medium	Medium	Medium
Meprobamate	DMSO	Medium	Medium	DMSO
Mercury II chloride	DMSO	DMSO	DMSO	DMSO
Methanol	DMSO	Medium	Medium	DMSO
Nicotine	Medium	Medium	Medium	Medium
Paraquat	Medium	Medium	Medium	Medium
Parathion	DMSO	DMSO	DMSO	DMSO
Phenobarbital	DMSO	Medium	DMSO	DMSO
Phenol	Medium	Medium	Medium	Medium
Phenylthiourea	DMSO	DMSO	Medium	DMSO

Table 7-14 Solvent Determinations by Laboratory

Reference Substance	Solvent Used for Testing ¹	ECBC	FAL	IIVS
Physostigmine	DMSO	Medium	DMSO	DMSO
Potassium I chloride	Medium	Medium	Medium	Medium
Potassium cyanide	Medium	Medium	Medium	Medium
Procainamide HCl	Medium	Medium	Medium	Medium
2-Propanol	Medium	Medium	Medium	Medium
Propranolol HCl	DMSO	Medium	Medium	Medium
Propylparaben	DMSO	DMSO	DMSO	DMSO
Sodium arsenite	Medium	Medium	Medium	Medium
Sodium chloride	Medium	Medium	Medium	Medium
Sodium dichromate dihydrate	Medium	Medium	Medium	Medium
Sodium fluoride	Medium	Medium	Medium	Medium
Sodium hypochlorite	Medium	Medium	Medium	Medium
Sodium oxalate	Medium	Medium	Medium	ID
Sodium selenate	Medium	Medium	Medium	Medium
Strychnine	Medium	Medium	Medium	ID
Thallium I sulfate	Medium	Medium	ID	Medium
Trichloroacetic acid	Medium	Medium	Medium	Medium
1,1,1-Trichloroethane	Medium	Medium	Medium	Medium
Triethylenemelamine	DMSO	Medium	DMSO	ID
Triphenyltin hydroxide	DMSO	DMSO	DMSO	DMSO
Valproic acid	DMSO	Medium	DMSO	DMSO
Verapamil HCl	DMSO	DMSO	DMSO	DMSO
Xylene	DMSO	DMSO	DMSO	DMSO
DMSO Total	34	22	29	28
Medium Total	38	49	41	40

Abbreviations: DMSO=Dimethyl sulfoxide; ECBC=Edgewood Chemical Biological Center; FAL= Fund for the Replacement of Animals in Medical Experiments Alternatives Laboratory; IIVS=Institute for *In Vitro* Sciences; ID=Insufficient data to select solvent; Medium=Cell culture medium.

¹Solvents selected by the SMT for use by all laboratories.

7.5 Summary

Intra- and inter-laboratory reproducibility were assessed by comparing the laboratory-specific IC₅₀-LD₅₀ regressions to the mean, across-laboratory regression for each method, ANOVA, CV analysis, and comparison of maximum:minimum mean laboratory IC₅₀ values. ANOVA permitted statistical comparisons of laboratories and experimental averages, while controlling for other factors. CV analysis compared the relative magnitudes of variability on a standardized scale. Reproducibility was evaluated using the results from the reference substances that yielded IC₅₀ values from all three laboratories: 64 and 68 reference substances in the 3T3 and the NHK NRU test methods, respectively. The analysis of intralaboratory reproducibility, by evaluating the similarity of the laboratory specific IC₅₀-LD₅₀ regressions, showed that the laboratory regressions for both NRU test methods were within the 95% confidence limits of the laboratory mean regressions.

The ANOVA showed significant interlaboratory differences for 23 substances in the 3T3 NRU test method and six in the NHK NRU test method. Intralaboratory CV values ranged from 1-122% in the 3T3 test method and 1-129% in the NHK NRU test method. Mean interlaboratory CV values were 26% for both NRU test methods, but NHK had a lower mean

interlaboratory CV (28% vs 47% for 3T3 NRU). Interlaboratory CV values ranged from 3-135% in the 3T3 NRU test method and 1-91% in the NHK NRU test method. FAL had the highest mean intralaboratory CV in both NRU test methods (33% in 3T3, 43% in NHK).

An analysis to determine the relationship between the chemical attributes and interlaboratory CV indicated that chemical structure, physical form, solubility, and volatility had little effect on CV. The CV seemed to be related, however, to GHS acute toxicity category, IC_{50} , and boiling point. Mean interlaboratory CV values were larger for substances in the most toxic GHS categories than for substances in the other toxicity categories, especially with the 3T3 NRU test method. The mean interlaboratory CV for substances in the $LD_{50} \leq 5$ mg/kg (72%) and $5 < LD_{50} \leq 50$ mg/kg (78%) classes were larger than the mean overall interlaboratory CV (47%) with the 3T3 NRU test method. The mean interlaboratory NHK CV was 37% for substances with $LD_{50} \leq 5$ mg/kg, and 41% for substances with $5 < LD_{50} \leq 50$ mg/kg, while the mean overall interlaboratory CV was 28%. A Spearman correlation analysis showed that the IC_{50} was inversely correlated to interlaboratory CV for both the 3T3 ($p=0.015$) and NHK ($p=0.014$) test methods, and that boiling point was positively correlated to interlaboratory CV ($p=0.007$) (i.e., higher boiling points were associated with higher CV values) for the 3T3 but not the NHK NRU test method ($p=0.809$).

The ANOVA results for the PC IC_{50} in the 3T3 NRU test method showed that there were significant differences among laboratories ($p=0.006$) but not among study phases within laboratories ($p > 0.01$). However, interlaboratory CV values, which ranged from 2% to 10% for the different study phases, were small and the intralaboratory CV values ranged from 5% to 24%. The SLS IC_{50} values from the NHK NRU test method were more variable than those from the 3T3 NRU test method. The ANOVA results for SLS in the NHK NRU test method indicated that there were significant differences among laboratories ($p < 0.001$) and among study phases within laboratories ($p \leq 0.001$). A change in cell culture methods at FAL after Phase Ib decreased the SLS IC_{50} in subsequent phases, but FAL's CV values still tended to be higher than in the other laboratories. Intralaboratory CV values for the NHK SLS IC_{50} during the various study phases ranged from 11% to 51% and interlaboratory CV values for SLS in the NHK NRU test method ranged from 8% in Phase III to 39% in Phase Ia.

Cell culture medium was used as the solvent for 38 substances and DMSO was used for 34 substances. Concordance among all three laboratories in selecting the solvent for the reference substances was 76% (55/72).

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