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Evaluation of a Solubility Protocol for In Vitro Cytotoxicity Testing

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Introduction

Solubility is an important determinant of toxicity in an *in vitro* assay system since it affects the availability of potential toxins to the cells. Solubility in any particular solvent is a specific property of the involved chemical structures while solubility determination, like any measurement, can vary from lab to lab with certain precision and accuracy. To eliminate solubility and solvent selection as potential sources of variation in multi-laboratory validation studies, the management teams often recommend an appropriate solvent for each test article and may also specify the concentrations to be tested (Knox et al. 1986; Spielmann et al. 1998; Clothier et al. 1999).

During a validation study to evaluate two neutral red uptake (NRU) *in vitro* cytotoxicity assays for estimating acute *in vivo* systemic toxicity, we evaluated a solubility protocol (NICEATM 2003) designed to identify the solvent that would provide the highest soluble concentration of a test chemical for *in vitro* testing. To avoid the use of different solvents by the labs when testing the same chemical, the Study Management Team (SMT) assigned the solvents used for *in vitro* testing. The objectives of this part of the validation study were to:

- Evaluate the utility and appropriateness of the solubility protocol
- Evaluate the concordance among labs in the solvent selected for each of the 72 chemicals tested in the validation study

Methods

The study design was for three labs to test 72 coded chemicals in two *in vitro* basal cytotoxicity assays (for more information, see Poster 1628). While the three *in vitro* test labs used the protocol presented here, a fourth lab purchased and coded the chemicals, performed solubility testing using a different protocol, and distributed aliquots to the *in vitro* test labs.

Solubility Protocol

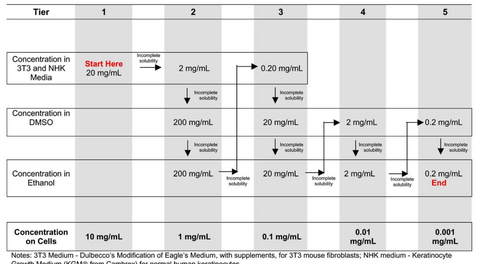
The order of preference for solvents was culture media, dimethyl sulfoxide (DMSO), and ethanol (ETOH). The protocol was based on a US Environmental Protection Agency guideline (US EPA 1998) and involved testing for solubility in a particular solvent, beginning at a relatively high concentration and proceeding to successively lower concentrations by adding more solvent as necessary for dissolution. At each concentration, mixing procedures (i.e., vortexing, sonicating, and heating to 37ºC) were employed as necessary. Testing stopped when, upon visual observation, the procedure produced a clear solution with no cloudiness or precipitate. Chemicals considered insoluble in media were tested using DMSO and chemicals considered insoluble in DMSO were tested using ETOH. Practical limitations were the large volumes of solvent required for testing the more insoluble chemicals since approximately 50 mg test material would require >> 250 mL solvent.

The chemical purchaser/distributor was the first to evaluate the solubility of the test chemicals, first in media, then in DMSO, and then in ETOH at 400 and 200 mg/mL. Based on this experience, a solubility protocol for the *in vitro* labs was developed to test at lower test article concentrations and to test with the various solvents at concentrations that would be equivalent when applied to the cultures. The solubility flow chart in Figure 1 shows, for example, that 2 mg/mL medium and 200 mg/mL DMSO or ETOH are equivalent concentrations since they yield 1 mg/mL in cell culture. (Note: When applied to cultures, medium was diluted by one-half. DMSO and ETOH were diluted by 200 to achieve 0.5% [v/v] final concentrations.) At each concentration, the following mixing procedures were employed, as necessary to completely dissolve the test chemical, in this order:

- Gently mix. Vortex (1 –2 min).
- Sonicate for up to 5 min.
- Warm to 37°C for 5 - 60 min.

If test chemical was still undissolved, the next concentration/solvent was tested.

Figure 1.

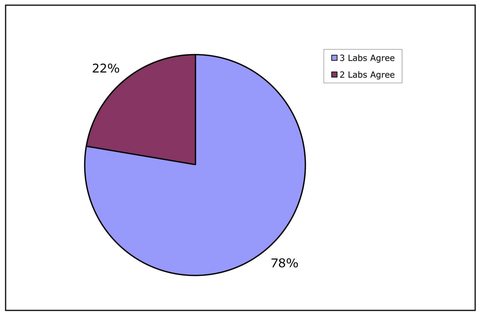


Notes: 3T3 Medium - Dulbecco's Modification of Eagle's Medium, with supplements, for 3T3 mouse fibroblasts; NHK medium - Keratinocyte Growth Medium (KGM® from Cambrex) for normal human keratinocytes.

Flow Chart for Determination of Test Chemical Solubility in Medium, DMSO, or Ethanol

Results

Figure 2.



Lab Agreement on Solvent

U.S. Department of Health and Human Services
National Institutes of Health
National Institute of Environmental Health Sciences

Table 1. Solubility Results in mg/mL

Chemical	Chemical Distributor[a]				SMT[b] Selection	Lab 1[c]				Lab 2[c]				Lab 3[c]			
	3T3[d] Medium	NHK[d] Medium	DMSO	ETOH		3T3[d] Medium	NHK[d] Medium	DMSO	ETOH	3T3[d] Medium	NHK[d] Medium	DMSO	ETOH	3T3[d] Medium	NHK[d] Medium	DMSO	ETOH
Phase I																	
Arsenic III trioxide	0.25	0.05	< 2	< 2	Medium	0.025[e]	0.025[e]	< 0.2	< 0.2	0.135[e]	0.135[e]	< 0.2	< 0.2	< 0.02[e]	< 0.02[e]	< 0.2	< 0.2
Ethylene glycol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Propranolol HCl	< 2	10	200	20	DMSO	0.2	2	200	NT	20	20	200	NT	20	2	NT	NT
Phase II																	
Aminopterin	2	2	NT	NT	DMSO	2.0	< 2	200	NT	< 2	2	200	NT	0.2	0.2	200	NT
Cadmium II chloride	< 2	< 2	200	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	20	< 20
Chloramphenicol	2	2	400	< 200	DMSO	2.0	< 2	200	NT	< 2	< 2	200	NT	0.2	0.2	20	20
Colchicine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lithium carbonate	0.25	10	< 2	NT	Medium	0.2	2.0	< 20	< 20	0.2	2	< 200	< 200	0.2	2	< 2	< 2
Potassium chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
2-Propanol	400	400	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium fluoride	20	20	< 200	< 200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium selenate	200	200	< 200	< 200	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phase III																	
Acetaminophen	10	10	400	< 200	DMSO	2	2	NT	NT	2	2	NT	NT	< 2	< 2	200	NT
Acetylsalicylic acid	10	10	400	200	DMSO	2	2	NT	NT	< 2	< 2	200	NT	2	2	NT	NT
5-Aminosalicylic acid	2	2	< 200	< 200	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Amitriptyline HCl	200	200	NT	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	0.2	0.2	200	NT
Atropine sulfate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Boric acid	40	40	200	< 200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Busulfan	< 2	< 2	40	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	50[f]	< 200	< 0.2	< 0.2	20	< 200
Caffeine	10	10	20	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Carbamazepine	< 2	< 2	40	< 200	DMSO	0.2	0.2	20	20	< 2	< 2	200	NT	< 0.2	< 0.2	2	< 20
Carbon tetrachloride	2	10	NT	NT	DMSO	20	20	NT	NT	< 0.2	< 0.2	2	NT	20	20	NT	NT
Chloral hydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Citric acid	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Cupric sulfate pentahydrate	1	0.5	< 2	2	Medium	2	0.2	< 200	< 200	2	2	NT	NT	0.2	0.2	< 200	NT
Cycloheximide	20	20	400	< 200	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Dibutylphthalate	< 2	< 2	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Dichlorvos	10	10	NT	NT	DMSO	2	2	NT	NT	< 2	< 2	200	NT	2	2	NT	NT
Diethylphthalate	< 2	< 2	400	400	DMSO	< 2	< 2	200	NT	0.2	< 0.2	200	NT	< 2	< 2	200	NT
Digoxin	0.05	0.05	200	< 200	DMSO	< 2	< 2	200	NT	< 0.2	< 0.2	200	NT	< 2	< 2	200	NT
Dimethylformamide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Diquat dibromide monohydrate	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Disulfoton	< 2	< 2	500	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Endosulfan	< 0.05	< 0.05	40	NT	DMSO	< 0.2	< 0.2	20	< 200	< 0.2	< 0.2	2	< 200	< 0.2	< 0.2	20	< 200
Epinephrine bitartrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	2	2	NT	NT
Ethanol	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Fenpropathrin	< 20	< 20	500	NT	DMSO	< 2	< 2	200	NT	< 0.2	< 0.2	200	NT	< 2	< 2	200	NT
Gibberellic acid	10	10	NT	NT	Medium	2	2	NT	NT	2	2	NT	NT	2	2	NT	NT
Glutethimide	< 2	< 2	500	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Glycerol	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Haloperidol	< 20	< 20	40	NT	DMSO	< 0.2	< 0.2	20	< 20	< 0.2	< 0.2	20	< 20	< 2	< 2	20	< 20
Hexachlorophene	0.05	< 0.05	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Lactic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Lindane	< 0.05	< 0.05	400	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	20	< 200
Meprobamate	1	1	200	NT	DMSO	2	2	200	NT	2	2	200	NT	< 0.2	< 0.2	200	NT
Mercury II chloride	0.125	0.125	400	< 200	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	200	NT
Methanol	40	40	400	400	DMSO	20	20	NT	NT	20	20	NT	NT	< 2	< 2	200	NT
Nicotine	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Paraquat	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Parathion	0.05	< 0.05	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Phenobarbital	2	2	200	< 200	DMSO	2	2	NT	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Phenol	40	40	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Phenylthiourea	2	2	400	< 200	DMSO	2	< 2	200	NT	20	20	NT	NT	< 2	< 2	200	NT
Physostigmine	2	2	400	200	DMSO	2	2	NT	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Potassium cyanide	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Procainamide HCl	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Propylparaben	0.25	0.25	400	400	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 2	< 2	200	NT
Sodium arsenite	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium chloride	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium dichromate dihydrate	400	400	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium hypochlorite	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Sodium oxalate	< 0.05	20	0.125	< 0.05	Medium	< 0.2	20	0.2	< 2	20	20	NT	NT	< 0.2	< 0.2	< 0.2	< 0.2
Strychnine	< 2	< 2	2	2	Medium	0.2	< 0.2	2	2	0.2	0.2	< 200	< 200	< 0.2	< 0.2	< 0.2	< 0.2
Thallium I sulfate	1	0.5	< 2	< 2	Medium	0.2	0.2	< 200	< 200	< 0.2	< 0.2	< 0.2	< 0.2	0.2	0.2	< 20	< 200
Trichloroacetic acid	200	200	NT	NT	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
1,1,1-Trichloroethane	10	10	400	400	Medium	20	20	NT	NT	20	20	NT	NT	20	20	NT	NT
Triethylene-melamine	< 2	< 2	2	< 20	DMSO	0.2	0.2	< 200	< 200	< 0.2	< 0.2	2	< 2	< 0.2	< 0.2	< 0.2	< 0.2
Triphenyltin hydroxide	< 0.05	< 0.05	10	< 20	DMSO	< 0.2	< 0.2	2	< 20	< 0.2	< 0.2	2	< 200	< 2	< 2	2	< 20
Valproic acid	10	2	NT	NT	DMSO	2	2	NT	NT	< 2	< 2	200	NT	2	< 2	200	NT
Verapamil HCl	< 0.05	0.25	200	NT	DMSO	< 2	< 2	200	NT	< 2	< 2	200	NT	< 0.2	< 0.2	20	NT
Xylene	1	1	500	NT	DMSO	< 2	< 2	200	NT	2	< 2	200	NT	< 2	< 2	200	NT

Notes: [a]Used a different solubility protocol from the *in vitro* cytotoxicity labs. [b]Solvents selected by the SMT for cytotoxicity testing. Chemical distributor results were used to determine solvents for Phases I and II. Results from all labs were used to determine solvents for Phase III. Media were treated as one result. If insoluble in one medium and soluble in DMSO, DMSO was selected. [c]Used protocol in Figure 1. [d]Dulbecco's Modification of Eagle's Medium. [e]Keratinocyte Growth Medium (KGM® from Cambrex). [f]Protocol deviation.
In vitro labs agreed on solvent. In vitro labs did not agree on solvent. Protocol did not provide enough information to select a solvent.
NT– not tested.

Table 2. Differences in Media Solubility

Chemical	Lab 1	Lab 2	Lab 3
Lithium carbonate	3T3	3T3	3T3
Sodium oxalate	3T3		
Propranolol HCl	3T3		NHK
Aminopterin	NHK	3T3	
Copper sulfate pentahydrate	NHK		
Valproic acid			NHK
Chloramphenicol	NHK		
Diethylphthalate		NHK	
Phenylthiourea	NHK		
Strychnine	NHK		
Xylene		NHK	
Total	**8**	**4**	**3**

Notes: [†]3T3 - solubility lower in 3T3 medium than in NHK medium, NHK – solubility lower in NHK medium than in 3T3 medium. No entry indicates no difference in solubility for the NHK and 3T3 media.

Although solubility in 3T3 medium was the same as that for NHK medium for 85% (61/72) of the chemicals, solubility was different for 11 chemicals in at least one lab. Two chemicals exhibited lower solubility in the 3T3 medium while seven chemicals had lower solubility in NHK medium in at least one lab. Two chemicals, propranolol HCl and aminopterin, had lower solubility in 3T3 medium in one lab and lower solubility in NHK medium in another lab. Although the differences for most chemicals were reported by one of three labs, medium solubility differences for lithium carbonate, propranolol HCl, and aminopterin were reported by at least two labs.

Conclusions

- For *in vitro* cytotoxicity testing, culture medium was used for 38 chemicals, DMSO was used for 34 chemicals, and ETOH was not used.
- Lab agreement was good for identifying the appropriate solvent. The *in vitro* labs selected the same solvent for 78% of the chemicals tested. Agreement was poor only with chemicals that exhibited low solubility, ~ 2 mg/mL, in medium. This was a limit concentration for determining whether to use medium or DMSO. If a chemical was insoluble in medium at 2 mg/mL, but soluble in DMSO at 200 mg/mL, then DMSO was selected as the solvent.
- Due to the 0.2 mg/mL lower limit for solubility testing, at least one *in vitro* lab failed to determine solubility for five chemicals (arsenic trioxide, sodium oxalate, strychnine, thallium sulfate, and triethylenemelamine). To determine the level of solubility for such compounds, future protocols should include testing at concentrations below 0.2 mg/mL. The chemical distributor used a protocol that required testing at concentrations as low as 0.05 mg/mL and never reported insolubility.
- Sixty-one of 72 chemicals were soluble in the 3T3 and NHK media to the same extent, despite differences in media composition.
- Solubility may be in the eye of the beholder. Differences in solubility among the labs may be attributed, in part, to the subjectivity of visual observations in judging whether the chemicals had fully dissolved.

References

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