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International Journal of Pharmaceutics 317 (2006) 54–60
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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High throughput microsomal stability assay for insoluble compounds

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Received 15 January 2006; received in revised form 3 March 2006; accepted 3 March 2006
Available online 17 March 2006

Abstract

High throughput metabolic stability assays are widely implemented in drug discovery to guide structural modification, predict in vivo performance, develop structure-metabolic stability relationships, and triage compounds for in vivo animal studies. However, these methods are often developed and validated using commercial drugs. Many drug discovery compounds differ from commercial drugs, with many having high lipophilicity, high molecular weight and low solubility. The impact of very low solubility on metabolic stability assay results was explored. Two metabolic stability assays, the 'aqueous dilution method' and the 'cosolvent method, were compared. For commercial drugs and most discovery compounds having reasonable drug-like properties, the two methods gave comparable results. For highly lipophilic, insoluble drug discovery compounds, the 'aqueous dilution method' gave artificially higher stability results. The cosolvent method performs compound dilutions in solutions with higher organic solvent content and adds solutions directly to microsomes to assist with solubilization, minimize precipitation and reduce non-specific binding to plastics. This method is more applicable in drug discovery where compounds of a wide range of solubility are studied.

Keywords: Metabolism; stability; Solubility; High throughput; LC–MS; Automation; Robotics

1. Introduction

Metabolic stability is an important property of drug candidates (Thompson, 2001; Di and Kerns, 2005; Saunders, 2004; Eddershaw et al., 2000; Ansede and Thakker, 2004). Metabolism affects clearance, half-life and oral bioavailability, and it governs how much and how often the compound should be dosed (Dickins, 2004; Chaturvedi et al., 2001). Traditionally, the metabolic stability assay was used mainly for development candidates during late-stage drug discovery. At this stage, if compounds have any metabolic liabilities, it is often to late to fix the molecules. Consequently, poor metabolism/PK was one of the major causes for drugs to fail in development Kennedy (1997). Nowadays, compounds are evaluated much earlier in drug discovery for ADME/TOX properties in order to reduce the attrition rate due to poor PK/bioavailability (Kerns, 2001; Kola and Landis, 2004; Herbst and Dickinson, 2005; Di and Kerns, 2003; Kerns and Di, 2005). High throughput metabolic stability assays have been widely implemented in many pharmaceutical companies to support early drug discovery (Masimirembwa et al., 2001; Jenkins et al., 2004; Linget, 1999; Bertrand et al., 2000; Eddershaw and Dickins, 1999; Di et al., 2003; Caldwell et al., 1999; Korfmacher et al., 1999; Xu et al., 2002). They are being used to predict in vivo performance Obach (1999), triage compounds for in vivo animal studies Di and Kerns (2005), guide structural modification MacKenzie et al. (2002), and develop structure–metabolism relationships Nassar et al. (2004). Several strategies have been developed to increase throughput and turnaround time of metabolic stability assays in order to provide quality and timely data to project teams, including the single time point approach to reduce sample preparation and analysis (Di et al., 2004; Zhao et al., 2005), cassette dosing or "N-in-1" approach to pool samples and reduce the number of samples to be analyzed (Zhao et al., 2005; Rajanikanth et al., 2003), application of parallel technologies to analyze samples simultaneously (Jenkins et al., 2004; Lindqvist et al., 2004; Ong et al., 2004), using a combined ESI-APCI ion source (ESCi) to increase sample coverage Gallagher et al. (2003), and online extraction with high throughput LC–MS analysis (Laven et al., 2004; Kerns et al., 2004; Janiszewski et al., 2001). Assay conditions have been

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0378-5173/$ – see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.ijpharm.2006.03.007

optimized to achieve high speed and high quality information. The successful implementation of high throughput ADME/TOX assays reduced the attrition rate due to poor PK/bioavailability from more than 40% in 1991 to less than 10% in 2000 Drews (2000).

Solubility is a major issue in drug discovery, owing to the increased lipophilicity and molecular weight of drug candidates produced through combinatorial synthesis (Lipinski et al., 1997; Lipinski, 2004a). It has been estimated that more than 30% of drug discovery compounds have an aqueous solubility of less than 10 μM, which is a concentration typically used for primary screening in HTS and bioassays Lipinski (2001). These poorly soluble compounds tend to give erratic assay results, have artificially low potency and inaccurate SAR (Lipinski et al., 1997; Lipinski, 2004b; Di and Kerns, in press). While the industry is starting to address solubility issues in bioassays, the impact of compound insolubility on metabolic stability has not yet been explored.

While the presence of organic solvent in aqueous buffer can enhance the solubility of compounds, the assay can only tolerate a very small amount of organic solvent (e.g., DMSO ≤ 0.2%, acetonitrile ≤ 1%). This can limit the solubility of many drug discovery compounds in the metabolic stability assay. A high amount of organic solvent can inhibit CYP450 enzyme activity (Busby et al., 1999; Chauret et al., 1998; Easterbrook et al., 2001; Hickman et al., 1998). Consequently, many high throughput microsomal stability assays include an aqueous dilution step to reduce the percentage of organic solvent before adding the test compounds to the microsomes for incubation, as well as to increase pipetting accuracy. Insoluble compounds can potentially precipitate during aqueous dilution, non-specifically bind to plastic-ware and lead to unreliable data.

In this study, we compared two microsomal stability assays. One method uses an aqueous dilution step before addition of compounds to the microsomal proteins (aqueous dilution method). The other method uses an organic cosolvent without aqueous dilution, with the test compound being added directly to the microsomal protein from an organic media (cosolvent method). The objective was to investigate the impact of different sample preparation schemes on insoluble compounds for the metabolic stability assay as a precaution for achieving optimal assay results with insoluble compounds.

2. Experimental

2.1. Materials

All reagents used were of the highest grade commercially available. The test compounds were obtained from Aldrich and Sigma Chemical Co. (St. Louis, MO), Fluka (Ronkonkoma, NY), and Wyeth compounds were obtained from Wyeth Research (Princeton, NJ). NADPH regenerating agent Solutions A and B and rat liver microsomes (SD, male) were obtained from BD Gentest (Woburn, MA). Mouse liver microsomes (C56BL6, male) were purchased from XenoTech (Lenexa, KS). Ninety-six well plates were obtained from Corning Incorporated (Acton, MA).

2.2. Instrumentation and software

A Packard Multiprobe™ II EX HT (eight probes) robot with WinPrep™ software was used for sample preparation (Perkin-Elmer, Downers Grove, IL). Model 1100 HPLC pumps (Agilent Technology, Piscataway, NJ) were used. A CTC Twin Pal autosampler (LEAP Technologies, Carrboro, NC) equipped with six cooled well plate holder drawers (12 tray capacity), a syringe injection valve, and a 10-port valve to which 2 trapping cartridges (Keystone Aquasil C18 10 mm × 2.1 mm, 5 μm, Bellefonte, PA) were attached, was used. A column was used after the trapping cartridges (Keystone Aquasil C18 50 mm × 2.1 mm, 5 μm, Bellefonte, PA). A triple quadrupole Micromass Quattro Micro™ mass spectrometer (Waters, Milford, MA), with electrospray ionization (ESI) were used for sample analysis. Instruments were controlled by Masslynx software (Version 4.0, Waters, Milford, MA). plog *P* was calculated using ProLogP software from CompuDrug.

2.3. HPLC conditions

Column	Aquasil C18, 50 mm × 2.1 mm, 5 μm (Thermo, Bellefonte, PA)		
Loading	0.1% formic acid in water, flow rate 3 mL/min		
Mobile phase	A = 0.1% formic acid in water		
	B = 0.1% formic acid in acetonitrile		
	Gradient		
	Time	A (%)	B (%)
	0	70	30
	1	5	95
	1.6	5	95
	1.7	70	30
	2.5	70	30
Flow rate	0.9 mL/min, split 0.2 mL/min to MS		
Detection	ESI, MRM		
Injection	20 μL		
Column temperature	Ambient		

2.4. Sample preparation for microsomal stability

The *aqueous dilution method* was described previously (Di et al., 2003, 2004). An incubation time of 15 min was used.

The *cosolvent method* was performed as follows. DMSO stock solutions of test compounds were prepared at 0.5 mM concentration. Diluted solutions of test compounds were prepared by adding 50 μL of each DMSO stock solution to 200 μL of acetonitrile to make 0.1 mM solutions in 20% DMSO/80% acetonitrile. Rat liver microsomal solution was prepared by adding 1.582 mL of concentrated rat liver microsomes (20 mg/mL protein concentration) to 48.291 mL of pre-warmed (to 37 °C) 0.1 M potassium phosphate buffer (pH 7.4) containing 127 μL of 0.5 M EDTA to make a 0.6329 mg/mL (protein) microsomal solution. 11.2 μL of each test compound diluted solution was each added directly to 885 μL of rat liver microsomal solution (allowing direct binding of drugs to microsomal proteins and lipids to minimize precipitation and non-specific binding to the plasticware). This solution was mixed and 180 μL was transferred to

Time 0 and Time 15 min plates (each in duplicate wells). For the Time 15 min plate, NADPH regenerating agent (45 μL) was added to each well to initiate the reaction, the plate was incubated at 37° C for 15 min, followed by quenching of the reaction by adding 450 μL of cold acetonitrile to each well. For the Time 0 plate, 450 μL if cold acetonitrile was added to each well, followed by addition of NADPH regenerating agent (45 μL) and no incubation. All of the plates were centrifuged at 3000 rpm for 15 min and the supernatants were transferred to other well plates for analysis by LC–MS. The final assay conditions for the cosolvent method are shown in Table 1.

2.5. Solubility determination

Solubility was determined at pH 7.4 using a pION PSR4s instrument and software Avdeef (2003). Compounds were initially dissolved in DMSO at 8 mg/mL. Thirteen microliters of this stock solution was added to 1.0 mL of pH 7.4 pION buffer. The solution is mixed and allowed to settle for 18 h at room temperature, then filtered through a 0.2 μm filter plate (Corning, Acton, MA). The concentration of the filtrate was quantitated using a UV plate reader (Molecular Devices, Sunnyvale, CA). The solubility was derived using a single point standard.

Table 1
Final assay conditions for the cosolvent microsomal stability assay

Cosolvent assay	Conditions
Substrate concentration	1 μM
Microsomal protein	0.5 mg/mL
Organic solvents	0.2% DMSO, 0.8% acetonitrile[a]
Incubation time	15 min

[a] In the aqueous dilution method, organic solvents are 0.2% DMSO only.

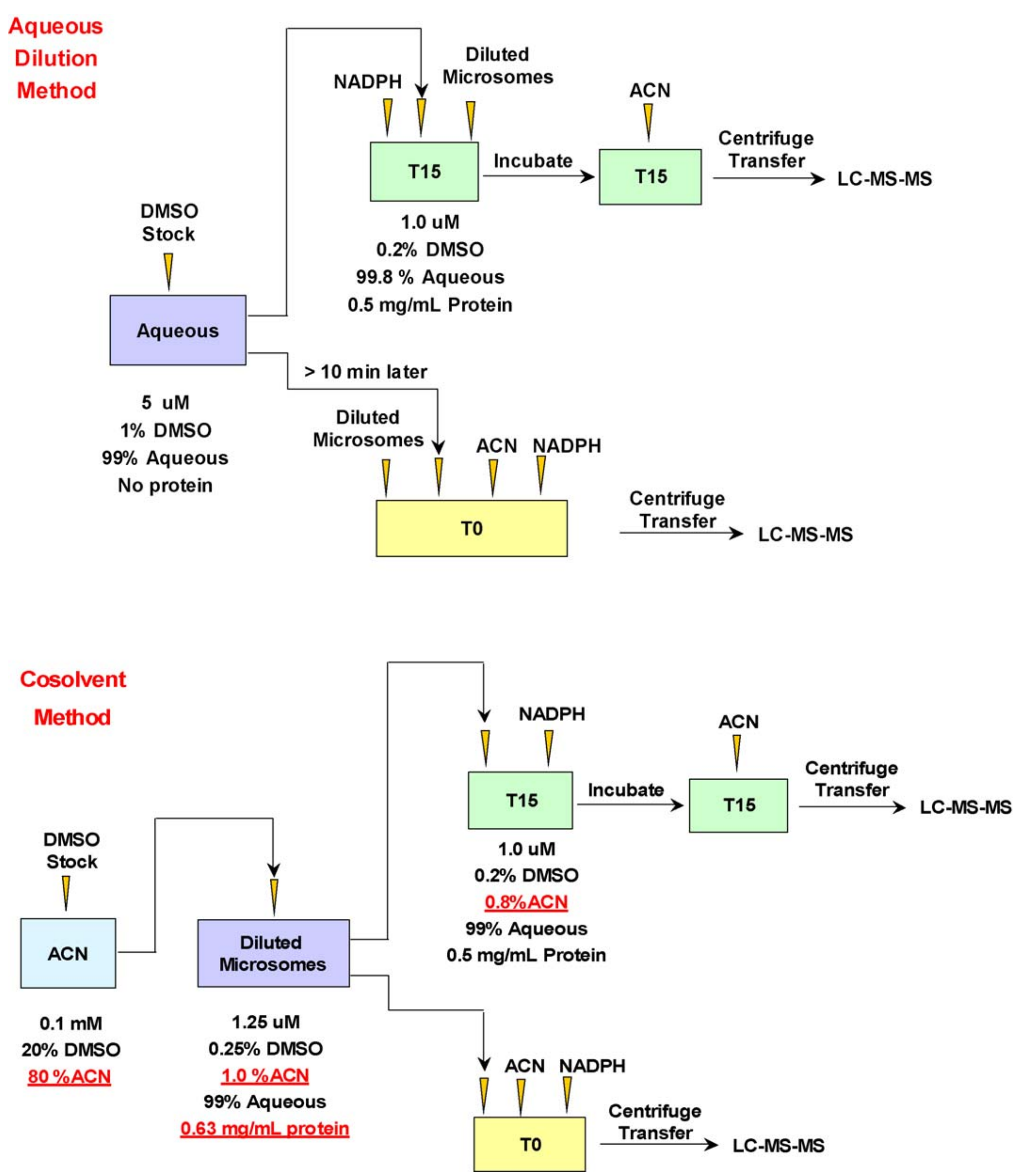

Fig. 1. The aqueous dilution and cosolvent microsomal stability assays.

3. Results and discussions

3.1. Comparison of results from the aqueous dilution and cosolvent methods

3.1.1. Commercial drugs

Thirty-three commercial drugs, including some very insoluble compounds, such as miconazole, ketoconazole, probenecid, terfenadine and phenytoin, were used to validate the assay (Avdeef, 2003; Glomme, 2005). The compounds were assayed in rat liver microsomes using both methods. A schematic representation of the methods is shown in Fig. 1. The results are shown in Table 2 and Fig. 2. The half-lives obtained from both the aqueous dilution and the cosolvent methods were very comparable, with a correlation coefficient (R^2) of 0.97 for the 33 commercial drugs. This suggests that even for the poorly soluble commercial drugs, the aqueous dilution step in the assay did not significantly affect the metabolic stability. The MW of the compounds ranged from 175 to 531, and log P of the compounds ranged from −1.55 to 6.88. Even though some of the compounds violate one of Lipinski's rule of 5, most compounds have drug-like properties in general. The low substrate concentration of 1 μM used in microsomal incubation is advantageous for insoluble compounds, which is less demanding on solubility as compared to bioassays that are typically performed at a higher concentration (e.g., 10 μM). If microsomal stability assays are performed at higher concentration (e.g., 5–10 μM), solubility will be a problem for low solubility compounds. The aqueous dilution method is suitable for metabolic stability determination of most commercial drugs that have reasonable drug-like properties.

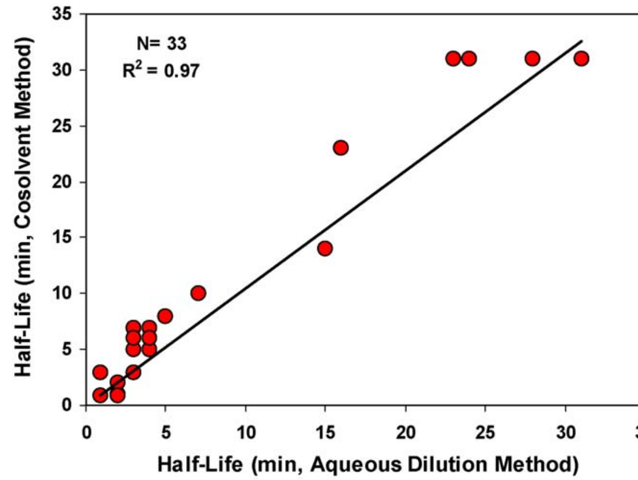

Fig. 2. Comparison of rat microsomal stability using the aqueous dilution and cosolvent methods for 33 commercial drugs.

Table 2
Comparison of rat microsomal stability using the aqueous dilution and cosolvent methods

#	Commercial name	MW	log P	Half-life (min) aqueous dilution method	Half-life (min) cosolvent method
1	Astemizole	459	6.88	3	7
2	Atenolol	266	0.71	>30	>30
3	Bifonazole	310	5.44	3	5
4	Bupivacaine	288	4.85	0.9	3
5	Chlorpromazine	319	4.71	2	2
6	Cimetidine	252	−0.3	>30	>30
7	Debrisoquine	175	−0.31	15	14
8	Isoxicam	335	−0.39	>30	>30
9	Labetalol	328	2.06	3	3
10	Miconazole	416	5.91	4	5
11	Norfloxacin	319	0.86	>30	>30
12	Nortriptyline	263	5.63	2	1
13	Ofloxacin	361	1.51	>30	>30
14	Promethazine	284	4.55	0.9	0.9
15	Propranolol	259	3.62	0.9	0.9
16	Quinidine	324	3.15	3	6
17	Sufamethoxazole	253	0.05	>30	>30
18	Terbutaline	225	0.71	>30	>30
19	Thymidine	242	−1.55	>30	>30
20	Triflupromazine	352	5.12	3	3
21	Danazol	337	5.41	2	2
22	Felodipine	384	4.92	2	0.9
23	Albendazole	265	2.83	7	10
24	Ketoconazole	531	3.75	>30	>30
25	Piroxicam	331	−0.19	28	>30
26	Probenecid	285	2.59	23	>30
27	Terfenadine	472	6.46	4	7
28	Phenytoin	252	1.3	24	>30
29	Indomethacin	358	2.92	>30	>30
30	Dipyridamole	505	4.77	4	6
31	Verapamil	455	5.92	4	6
32	Loperamide	477	3.63	5	8
33	Zolpidem	307	2.50	16	23

3.1.2. Wyeth research compounds from a nuclear receptor project

The comparison of metabolic stability data from the two methods for eight Wyeth research compounds from a nuclear receptor project is shown in Table 3. The compounds all have poor properties, with high molecular weight (∼500–600), very high log P (∼9–12) and low solubility (0–8 μg/mL). Several compounds have much lower metabolic stability half-lives using the cosolvent method than using the aqueous dilution method. This suggests that precipitation or non-specific binding to the plastic-ware caused artificially higher stability results using the

Table 3
Comparison of mouse microsomal stability using the aqueous dilution and cosolvent methods for eight Wyeth research compounds from a nuclear receptor program

Compounds	Half-life (min) aqueous dilution method	Half-life (min) cosolvent method	MW	plog P	Solubility (μg/mL)
1	>30	22	613	10	1.0
2	>30	13	615	10	1.0
3	>30	5	523	9	1.0
4	>30	7	522	9	0.0
5	>30	9	597	10	0.0
6	>30	>30	626	12	2.0
7	>30	>30	582	9	8.0
8	>30	>30	481	6	6.0

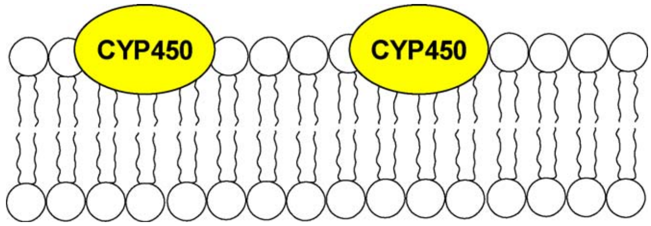

Fig. 3. Membrane bound cyp450 enzymes.

aqueous dilution method. In the cosolvent method, the compounds are added directly to the liver microsomes, allowing lipophilic insoluble compounds to bind to the proteins and lipids in the microsomes and minimize precipitation and non-specific binding to the plastic-ware. This enables maximum metabolism by the metabolizing enzymes. CYP450 is a membrane bound protein (Fig. 3). Both phospholipids and proteins are good solublizers of lipophilic compounds. Adding compounds directly to microsomes can minimize solubility issues. Drug discovery research compounds typically have less desirable properties than commercial drugs. They have higher MW, higher log P and lower solubility. Even though the two microsomal stability assays gave comparable results for commercial drugs, for highly lipophilic insoluble research compounds the aqueous dilution method tends to give artificially higher stability results.

3.1.3. Structurally diverse Wyeth research compounds from 15 projects

A set of 43 structurally diverse Wyeth Research compounds from 15 different projects were used to compare microsomal stability results using both the aqueous dilution method and the cosolvent method. The results are shown in Fig. 4. For most compounds, the two methods gave comparable data. However, there was a set of the compounds that had higher half-lives in the aqueous dilution method (>30 min), but were unstable using the cosolvent method (half-life ∼5–15 min). All the outliers have high MW (>450) and high log P (>600). Three of the outliers were from the nuclear receptor project discussed previously. The two methods in general are quite comparable, with the cosolvent method giving more reliable data for highly lipophilic insoluble compounds.

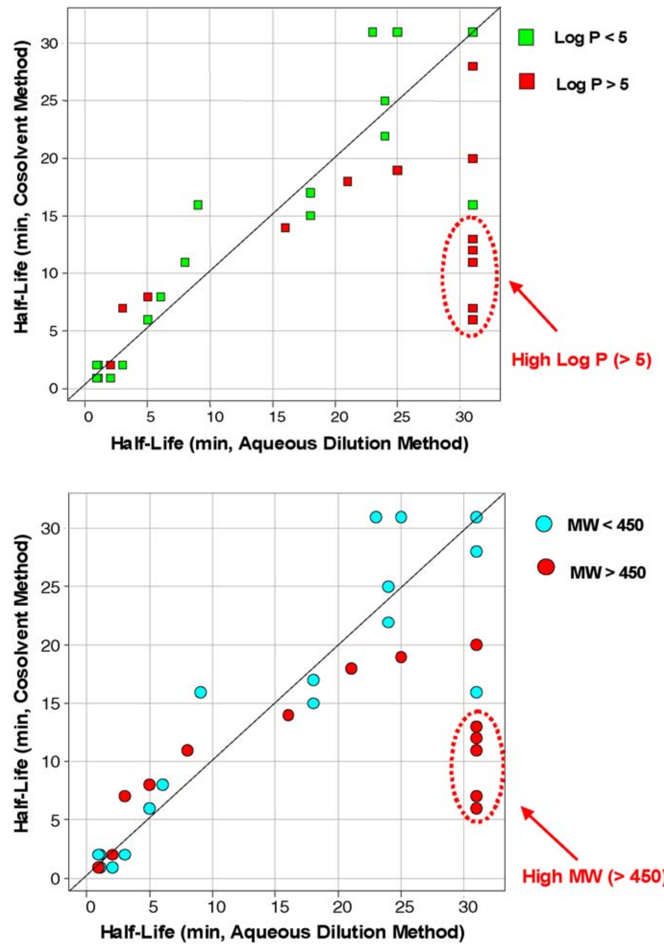

Fig. 4. Comparison of rat microsomal stability using the aqueous dilution and cosolvent methods for 43 structurally diverse Wyeth research compounds. Compounds with artificially high stability results in the aqueous dilution method tend to have high MW and high log P.

3.2. Discussion of mechanisms that effect insoluble compounds in metabolic stability assays

When compounds are added to an aqueous buffer from a DMSO stock solution, four kinds of solutions/suspensions can develop as discussed below, depending on the physico-chemical properties of the compounds (Fig. 5). For a specific compound, it can exist as a mixture of the different states.

A homogeneous solution is formed when the compound is completely dissolved in solution. The compound concentration in the assay equals the target substrate concentration of 1 μM. Molecules can interact with CYP450 enzymes freely and the rate of metabolism reflects the true stability of the compound. Half-life determined by both the aqueous dilution method and the cosolvent method would be the same when compound is completely dissolved.

A homogeneous suspension can be produced when the compound is not completely soluble in the aqueous buffer. It

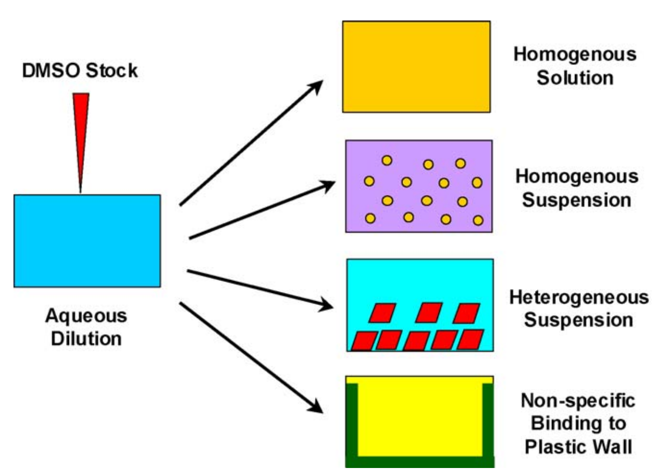

Fig. 5. Formation of various solutions after aqueous dilution of DMSO stock solution.

precipitates as fine particles and distributes homogeneously throughout the solution. Since it is homogenous, the substrate concentration in the assay is the same as the target concentration. However, because some of the material is in the solid state, interaction with metabolizing enzymes in the liver microsomes is limited to molecules on the surface of the solid material and any molecules dissolved in the solution. This will limit the rate of metabolism. At the end of the incubation, the undissolved solid material gets extracted back into solution when acetonitrile is added to quench the reaction. The rate of metabolism is slower than if the compound is completely soluble. Under these circumstances, the apparent half-life will be longer for the aqueous dilution method than the cosolvent method.

A heterogeneous suspension is formed if a compound is not soluble in the aqueous buffer, quickly precipitates out of solution as large particles, and the particles gradually sink to the bottom of the wells. No solid material will be transferred from this aqueous solution to the microsomal incubation mixture if only the upper portion of the solution is transferred, such as when the liquid sensing function on the robot is used. Therefore, the actual concentration in the incubation will be lower than the target concentration. The transferred material is fully in solution and can interact with the CYP450 enzymes fully. Thus, if precipitation is fast, the half-life data from the aqueous dilution method will tend to be slightly higher than data from the cosolvent method, due to lower substrate concentration (Di et al., 2003). If the precipitation is slow, solid material will be transferred, and the results will be similar to the homogeneous suspension scenario, with artificially high stability and long half-life for the aqueous dilution method compared to the cosolvent method.

Lipophilic compounds tend to bind nonspecifically to the plastic walls. If this is a fast process, the actual concentration in the aqueous solution will be reduced. The transferred material will be in solution and can interact fully with enzymes. The half-life determined by the aqueous dilution method will tend to be slightly higher than that determined by the cosolvent method, due to lower substrate concentration (Di et al., 2003). If non-specific binding to the walls is a slow process, the compound will behave more like the homogenous solution.

An additional factor will lengthen the apparent half-life in the aqueous dilution method compared to the cosolvent method, if a compound has slow precipitation or slow non-specific binding to the plastics. In the aqueous dilution method, the Time 0 plate is prepared after the Time 15 min plate. Therefore the compound concentration continues to decrease owing to precipitation or adsorption, resulting in a lower compound concentration in the Time 0 sample and producing a higher % remaining and longer half-life for the compound. In the cosolvent method, compounds are added directly to microsomes, to keep them in solution, and then Time 0 and Time15 min plates are prepared. It overcomes the issue of slow precipitation and/or non-specific binding to the plastics.

For a specific compound, many of the mechanisms can happen at the same time. In general, the two methods showed comparable results for most of the compounds. For highly lipophilic insoluble compounds, the aqueous dilution method tends to give artificially higher stability. The cosolvent method is more applicable in drug discovery, where a range of compounds from drug-like to highly lipophilic insoluble compounds are regularly investigated.

4. Conclusions

Two microsomal stability assays, the aqueous dilution method and the cosolvent method, were compared using commercial drugs and discovery research compounds. For commercial drugs, both methods gave similar results. For highly lipophilic insoluble drug discovery compounds, the cosolvent method performed better than the aqueous dilution method. This method allows compounds to bind directly to microsomal lipids and proteins, to minimize precipitation and non-specific binding to plastics. For highly lipophilic insoluble compounds, the aqueous dilution method tends to give artificially high stability results. The cosolvent microsomal stability method is currently being used to screen drug discovery compounds at Wyeth Research.

While one drug discovery strategy is to eliminate insoluble compounds, new concepts are evolving to include them in the drug discovery process. Insoluble compounds can provide valuable information, such as medicinal chemistry SAR. They provide key pharmacophores, especially for highly lipophilic drug targets. Issues related to solubility can be addressed through structural modifications. Bioassays and ADME/TOX methodologies are being developed to accommodate insoluble compounds.

Acknowledgements

The authors would like to thank Guy Carter, Oliver McConnell and Magid Abou-Gharbia for their support, encouragement and leadership; Louis Leung, Irene Feingold and Jay Wrobel for useful discussions.

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