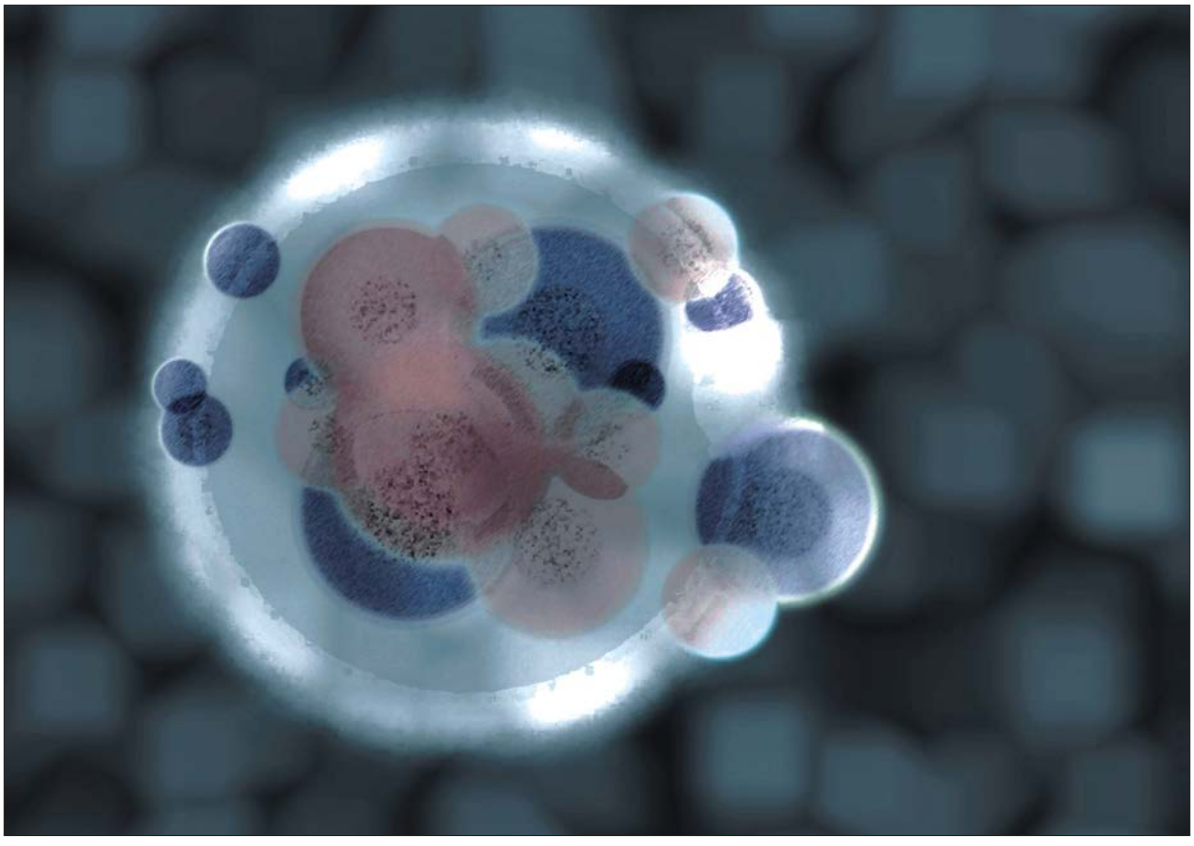

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Optimization of Rat Liver Microsomal Stability Assay Usimg HPLC

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Abstract: Microsomal stability of atenolol, propranolol HCl, verapamil HCl, imipramine HCl, midazolam HCl andrographolide and 14-Deoxyandrographolide was performed and quantitation was done by HPLC. The results with a single batch microsome showed good day-to-day reproducibility and variation in percent stability was within ±5.71% of the average for all the compounds for the entire study period of 2 years. Microsome was found to be stable at -80°C store for at least upto 2 years. Two batches of microsomes from each of 3 vendors were also used for stability study where similar type of animal, microsome preparation method and assay protocol were used. The findings demonstrated variations for both batch to batch and vendor to vendor were within 10%. Results indicated that variability in stability data can be minimized by selecting a fixed type of animal, keeping similar method of microsome preparation and following same assay protocol.

Key words: Microsomal stability, drug metabolism, HPLC

INTRODUCTION

It is recognized that pharmacokinetic properties like absorption, distribution, metabolism and excretion (ADME) are very crucial parameters for clinical success of a drug candidate. In drug discovery research, the compounds not only to be potent and selective but also must possess drug-like properties to increase success rate in different phases of clinical studies. It has been estimated that despite of molecular modeling and parallel synthesis approach, a large portion (nearly 40%) of lead molecules fail because of poor ADME properties (Xu *et al*., 2002; Sinko, 1999; de Waterbeemd *et al*., 2001). Thus *in vitro* screening for such properties should be initiated at an early stage in discovery process to reduce attrition rate, minimize time, cost and avoid complexities associated with *in vivo* animal experiments. Among the critical properties absorption and metabolism have gained greatest attention because oral delivery is the most convenient and preferred route of drug administration (Xu *et al*., 2002). While absorption determines rate and extent of drug transport from gastro-intestinal lumen to blood, metabolic stability plays an important role in bioavailability and overall pharmacokinetic performance. Typically liver microsomes are routinely used in *in vitro* microsomal stability assay for prediction of intrinsic clearance resulting from phase-I oxidation (Linget and Vignaud, 1999). However, reported stability results varied among laboratories due to different assay conditions like liver microsomes: 0.1-1 mg mL^{-1}; test compound: 0.5-15 μM; DMSO: 0.2-2%; acetonitrile: 0.2-2%; Incubation period 5-60 min (Xu *et al*., 2002; Di *et al*., 2003; Jenkins *et al*., 2004). It is also reported that stability studies with rat liver microsomes obtained from different vendors showed wide variation in result Even different batches of microsomes obtained from a single vendor showed wide variability indicating inherent animal to animal variation, differences in microsome preparation protocol and stability issue with microsome stock at its storage condition. In drug discovery, microsomal stability data are used to rank compounds for all discovery projects over an extended period of time (Di *et al*., 2003). Thus variation in data renders misleading conclusion.

The present study was undertaken to minimize batch to batch and vendor to vendor variation by selecting a fixed method of microsome preparation, checking microsome stability at -80 C for upto 2 years and observing microsomal stability results of some standard compounds throughout the study period of 2 years. Also the effectiveness of ice-cold acetonitrile (4:3 V/V Assay volume: Acetonitrile) in order to stop phase-I oxidation was evaluated using HPLC, as well as fluoroprobes of microsomal proteins.

MATERIALS AND METHODS

Materials: Solvents for HPLC were procured from Qualigens Fine Chemicals (Mumbai, India). Nicotinamide adenine dinucleotide phosphate

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(NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and trifluoroacetic acid (TFA) were purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). All other chemicals and standard drugs were purchased from Sigma, USA.

Andrographolide and 14-Deoxyandrographolide were isolated from *Andrographis paniculata* following the method of Matsuda *et al.* (1994) and compared the spectral data with that reported in study of Matsuda *et al.* (1994). Purity of both the compounds were more than 98% (by HPLC).

High Pressure Liquid Chromatography (HPLC): Waters HPLC (Model: Alliance 2996) with PDA detector (Model 2477) and XTerra RP18 column (250×4.6 mm, 5 μ) were used for the present study. The ratio of water and acetonitrile (ACN) both containing or without containing 0.1% TFA was varied for different standard drugs following standard protocol with a total flow rate of 1 mL min^{-1} and run time not more than 15 min. The system volume was 2.50±0.02 mL. Validation of HPLC methods was based on specificity, reproducibility (% RSD for retention time and area <2% with 10 injections of same sample) and linearity.

Solubility study: As microsomal stability was designed to perform at test concentration of 10 μM in 100 mM potassium phosphate buffer pH 7.4, the solubility of the compounds was tested prior to stability study. Twenty microliter of 25 mM stock (DMSO) of compounds (atenolol, propranolol HCl, verapamil HCl, imipramine HCl, midazolam HCl, tamoxifen andrographolide and 14-Deoxy andrographolide) was mixed with 30 μL acetonitrile to obtain 10 mM stock. Two microliter of 10 mM stock was diluted to 200 μL with 100 mM phosphate buffer pH 7.4 (organic solvent 1%) and incubated at 37°C for 30 min with constant shaking. The undissolved compound, if any, was filtered through PVDF membrane filter (Millipore, MA). To 120 μL of the filtrate, 80 μL of acetonitrile was added and mixed thoroughly. The concentration in the filtrate was determined spectrophotometrically from linearity (4-80 μM) of the compound prepared in 100 mM phosphate buffer pH 7.4 containing 40% acetonitrile. The observed solubility result was multiplied by 200/120 to account for the acetonitrile addition in the test filtrate. Compounds with solubility of 10 μM and above were taken for microsomal stability assay.

Preparation of rat liver microsomes: Two batches (Batch 1 and 2) of rat liver microsomes were prepared at Indian Institute of Chemical Biology (Vendor 1), two batches (batch 3 and 4) at Jadavpur University (Vendor 2) and two batches (batch 5 and 6) at Kalyani University (Vendor 3). Ethical clearance was obtained from the animal ethical committee of all the organizations.

Male Wistar rats having body weight 250-270 g were kept in standard quarantine area for two weeks followed by routine hematological tests. Animals having hematological parameters away from normal range were excluded from the study.

Ten rats were fasted overnight and sacrificed by cervical dislocation under ether anesthesia. Immediately liver was collected and perfused repeatedly with ice-cold saline. Liver was chopped into pieces and homogenized using Phosphate buffered saline (PBS) pH 7.4 (mg liver mL^{-1} PBS = 1/2). The homogenate was centrifuged at 9000 x g for 20 min at 4°C. The supernatant was further centrifuged at 100, 400 x g for 1 h at 4°C and the resulting pellets were resuspended in PBS pH 7.4 (Hasegawa *et al.*, 2002). Protein concentration was determined (Lowry *et al.*, 1951), adjusted to 20 mg mL^{-1} and stored at -80°C until use. Protein concentration of batch 1 was measured at an interval of 6 months for upto 2 years.

Microsomal stability assay: Ten microliter of 25 mM Stock (DMSO) was mixed with 240 μL acetonitrile to obtain 1 mM sub-stock. Two microliter of sub-stock was added, in triplicate, to a 96-well incubation plate (1 mL, Greiner, USA) containing 98 μL of 100 mM phosphate buffer pH 7.4. The plate was kept at 37°C for 10 min before microsome addition.

Rat liver microsome and NADPH regeneration chemicals (NADP, magnesium chloride, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were added to 100 mM phosphate buffer pH 7.4 to a concentration of 2 mg mL^{-1} and 2.6 , 6.6, 6.6 mM and 0.8 U mL^{-1}, respectively. The mixture was pre-warmed at 37°C for 20 min. One hundred microliter of this was added to each well of incubation plate. The final reaction conditions are given in Table 1. The microsomal reaction was terminated after 30 min by adding 150 μL ice-cold acetonitrile followed by vigorous shaking at 650 rpm (Thermomixer, Eppendorf, India). For 0 min samples 2 μL of compound sub-stock was added after reaction termination. The plate was centrifuged at 3500 rpm for 20 min at 20°C. Supernatant was collected and quantitated

Table 1: Reagents for microsomal stability assay

Reagent	Final concentration
NADP	1.3 mM
Glucose-6-phosphate	3.3 mM
Magnesium chloride	3.3 mM
Glucose-6-phosphate dehydrogenase	0.4 U mL^{-1}
Phosphate buffer pH 7.4	100 mM
Microsomal protein	1.0 mg mL^{-1}
Test compound	10 μM
DMSO	0.04%
Acetonitrile	0.96%

by HPLC. Percent stability (percent compound remaining after metabolism) was calculated as following:

$$\text{Stability (\%)} = \frac{100 \times \text{Peak area at 30 min}}{\text{Peak area at 0 min}}$$

For all batches such stability study was performed on the next day of microsome preparation and for batch 1 it was continued for upto 2 years at an interval of 6 months.

Reaction termination efficiency: To check if addition of 150 μL ice-cold acetonitrile (ACN) and vigorous shaking was adequate to terminate the microsomal oxidation of a reaction mixture of 200 μL the following experiments were performed, in triplicate.

HPLC study: Imipramine, having high turn over in microsomal stability, was taken as test substance. The samples were prepared as done for 0 min sample for metabolic stability. The supernatant was loaded into HPLC immediately and after 4 h. Effectiveness of reaction termination was calculated as following:

$$\text{Effectiveness (\%)} = \frac{100 \times \text{Peak area at 4h}}{\text{Peak area at 0 min}}$$

Fluorometric study: The experiment was similar to the stability study except replacement of test compound with fluoroprobes 7-Ethoxy-4-trifluoromethylcoumarine (EFC) and 3-Cyano-7-methoxycoumarine (CMC). Two hundred microliter reaction volume containing buffer with or without fluoroprobes and microsome (0.2 mg mL^{-1}) was incubated at 37°C for 30 min. Reaction was stopped by adding 150 μL ice cold acetonitrile. Set 1 contained fluoroprobe, set 2 contained fluoroprobe and microsome and set 3 contained microsome only. Buffer was present in all sets. After acetonitrile addition fluoroprobe was added to set 3 and all sets were further incubated at 37°C for 20 min, centrifuged, 200 μL supernatant was taken for fluorescence measurement at excitation 410 nm and emission 510 nm for EFC and 410/470 nm for CMC.

RESULTS AND DISCUSSION

The solubility of tamoxifen was found to be less than 10 μM and so it was excluded from microsomal stability study. The protein concentration with Batch 1 microsome remained same (20.01±0.29) throughout the study period.

The microsomal stability data for atenolol, propranolol HCl, verapamil HCl, imipramine HCl, midazolam HCl andrographolide and 14-Deoxyandrographolide with batch 1 microsome is shown in Fig. 1. The results showed good day-to-day reproducibility and variation in percent stability was within ±5% of the average for each compound except for verapamil where 5.71% variation was observed.

Stability result with microsomes of different batches and vendors is shown in Table 2. The results indicated good batch to batch and vendor to vendor reproducibility. Atenolol showed maximum variability and that too was within 10% from batch to batch as well as vendor to vendor.

The effectiveness of reaction termination protocol for the present study was found to be satisfactory for both HPLC and fluorescence based read outs. HPLC based study indicated 100.12±1.13% (n = 3) effectiveness. The fluorometric results also demonstrated similar findings (Fig. 2).

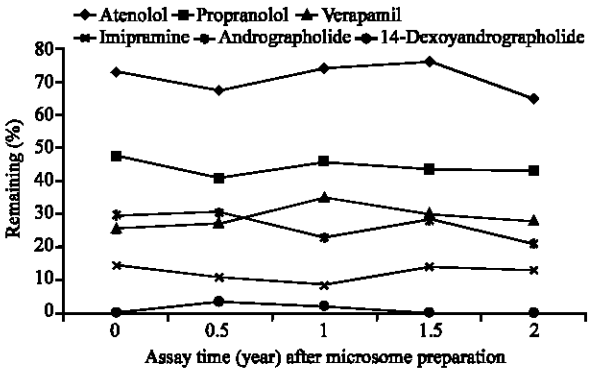

Fig.1: Reproducibility of microsomal stability over 2 years values are mean of 3 experiments

Table 2: Batch to batch and vendor to vendor variation of rat liver microsomes

	Parent compound remaining (%)					
	Vendor 1		Vendor 2		Vendor 3	
Compounds	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6
Atenolol	72.56±5.13	63.28±4.56	69.63±5.57	75.62±4.66	72.83±3.81	68.60±5.21
Propranolol	47.18±1.78	42.91±1.59	49.75±2.68	44.73±2.14	48.22±2.33	46.61±1.89
Verapamil	26.15±0.92	30.67±2.07	35.01±1.94	29.82±1.24	27.88±1.75	28.78±1.25
Imipramine	14.68±1.11	11.61±1.36	13.03±1.07	19.31±1.88	14.29±1.27	13.17±1.68
Andrographolide	28.74±1.84	31.81±2.05	32.62±1.55	26.11±0.97	32.52±0.95	30.01±1.08
14-Deoxyandrographolide	00.00±0.00	2.07±1.38	4.25±2.46	3.59±1.94	0.00±0.00	0.00±0.00

Values are Mean±SEM (n = 3)

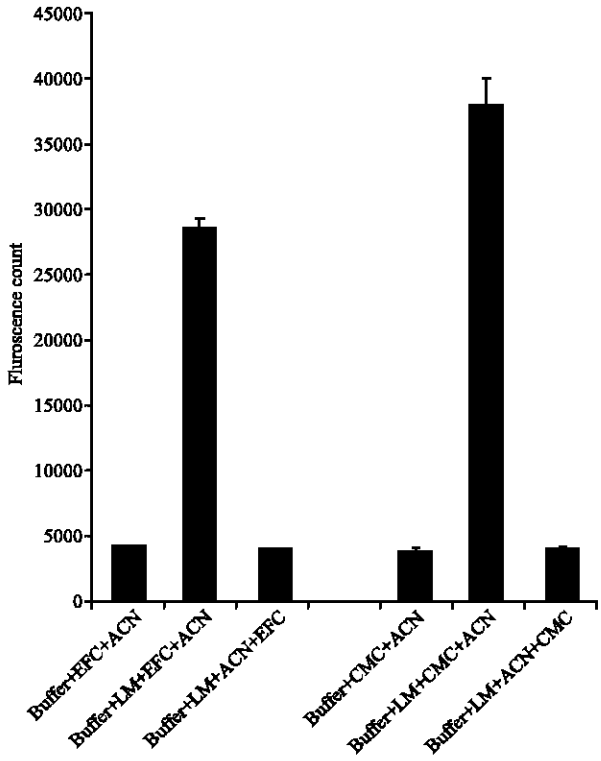

Fig. 2: Effectiveness of reaction termination in microsomal stability study. Values are Mean±SEM (n = 3); EFC: 7-Ethoxy-4-trifluoromethylcoumarine; CMC: 3-Cyano-7-methoxycoumarine; LM: Liver microsome; ACN: Acetonitrile

At present time, advancement in combinatorial chemistry, predictive modeling and parallel synthesis has significantly increased the number of potential active compounds entering drug discovery process. However a large portion fails in the later stage of development due to unfavorable pharmacokinetic properties, specially unpredictive effects of human metabolism and toxicity (Xu *et al*., 2002; Di *et al*., 2003; Trubetskoy *et al*., 2005). Thus the current strategy incorporates early measurement of metabolism not only to increase success rate but also to guide medicinal chemists to modify the compounds to get metabolically stable compounds. However, there are differences in stability data among different laboratories due to changes in reaction protocol (Xu *et al*., 2002; Di *et al*., 2003; Jenkins *et al*., 2004). It is also reported that with same assay protocol, wide variability, as much as 100% was observed when rat liver microsomes of different vendors were used (Di *et al*., 2003). Microsome of same vendor also showed wide differences in stability data and Batch to batch variation, for some compounds were more than 40% (Di *et al*., 2003). Such variations make it difficult to use the data to rank the compounds. It is possible that such changes may be due to changes in animal strain, age, sex, variation in microsome preparation protocol and stability of microsome at storage condition. So, the present study was undertaken to check differences in stability with microsomes of different batches as well as different vendors where type of animal, microsome preparation protocol and storage condition remained same. This is note-worthy that the suppliers of animals for vendor 1, 2 and 3 (as described above) were different. As, in such studies, laboratory to laboratory and person to person variations are important total 8 researchers were involved in microsome preparation and/or performing the stability experiments in 3 different laboratories.

It is known that rat liver microsomes can be stored frozen at -80°C for more than 1 year (Lin and Rodrigues, 2001). In the present study microsomes were found to be stable at -80°C for at least upto 2 years. However, with the present microsomal stability protocol, it is required to determine solubility of the test compounds and those having solubility 10 μM or above can be considered for metabolism study. For example, Tamoxifen was excluded from the study due to this issue. However such compounds can be tested at very low concentration e.g., 1 μM where HPLC alone may not be the right instrument due to sensitivity issue at very low concentration and costly instruments like LC-MS/MS needs be used (Xu *et al*., 2002; Di *et al*., 2003).

The present study with atenolol, propranolol HCl, verapamil HCl, imipramine HCl, midazolam HCl andrographolide and 14-Deoxy andrographolide showed good day-to-day reproducibility (Fig. 1) for the entire study period of 2 years and variation in stability data was within ±5% of the average for all compounds except for verapamil where 5.71% variation was observed. Results with different batches of microsome (Table 2) also indicated good batch to batch and vendor to vendor reproducibility where atenolol showed maximum variability and that too was within 10% from batch to batch as well as vendor to vendor. So the data are indicative of significant less variability than reported (upto 100% for vendor to vendor and 40% for batch to batch) (Di *et al*., 2003). This indicates that variability can be minimized using same type (strain, sex etc) of animals as well as same microsome preparation and assay protocol. However, best type or strain of animal to be used to get reliable data for predicting metabolism in human is not yet established as rate and extent of metabolism differs based on animal species (Di *et al*., 2003; Li, 2004; Ito and Houston, 2004; Giuliano *et al*., 2005). However in all such *in vitro* studies data are used to rank the compounds based on their metabolic stability using microsomes of same animal species.

For microsomal experiments, in general, reaction is terminated by adding 2-3 fold of ice cold acetonitrile

compared with reaction volume. In such cases detection involves use of costly instrument LC-MS/MS. Due to less sensitivity of HPLC compared to LC-MS/MS, reaction was terminated with lesser volume of acetonitrile to avoid sample dilution. So, the effectiveness of reaction termination protocol was tested by HPLC where it is expected that a high turn over compound, imipramine will continue to get metabolized if reaction is not quenched efficiently. The results indicated no such metabolism which was further confirmed by fluorimetric study. EFC and CMC are fluorescent substrates that are efficiently metabolized by liver microsomal enzymes to yield products with altered fluorescent properties, usually increased fluorescent intensity (Trubetskoy *et al*., 2005). Cytochrome P_{450} converts 7-Ethoxy-4-trifluoromethylcoumarine (EFC) and 3-Cyano-7-methoxycoumarine (CMC) to 7-Hydroxy-4-trifluoromethylcoumarine (HFC) and 3-Cyano-7-hydroxycoumarine (CHC) respectively which are measured fluorometrically. The results (Fig. 2) also demonstrated that reaction termination protocol was acceptable.

The present study indicated that microsomes can be stored at -80°C for at least upto 2 years and also the conventional HPLC can provide useful microsomal stability data.

As microsomes are utilized for ranking the compounds according to their metabolic susceptibility, it is advisable to prepare a large stock of microsome, preferably in-house, from same type of animal. It is also a good practice to check stability of some standard compounds (quality control) whenever new batch to be used and adjust assay conditions (e.g., microsome concentration, incubation period) to normalize the assay results to avoid batch to batch and vendor to vendor variation so that appropriate stability ranking can be done.

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