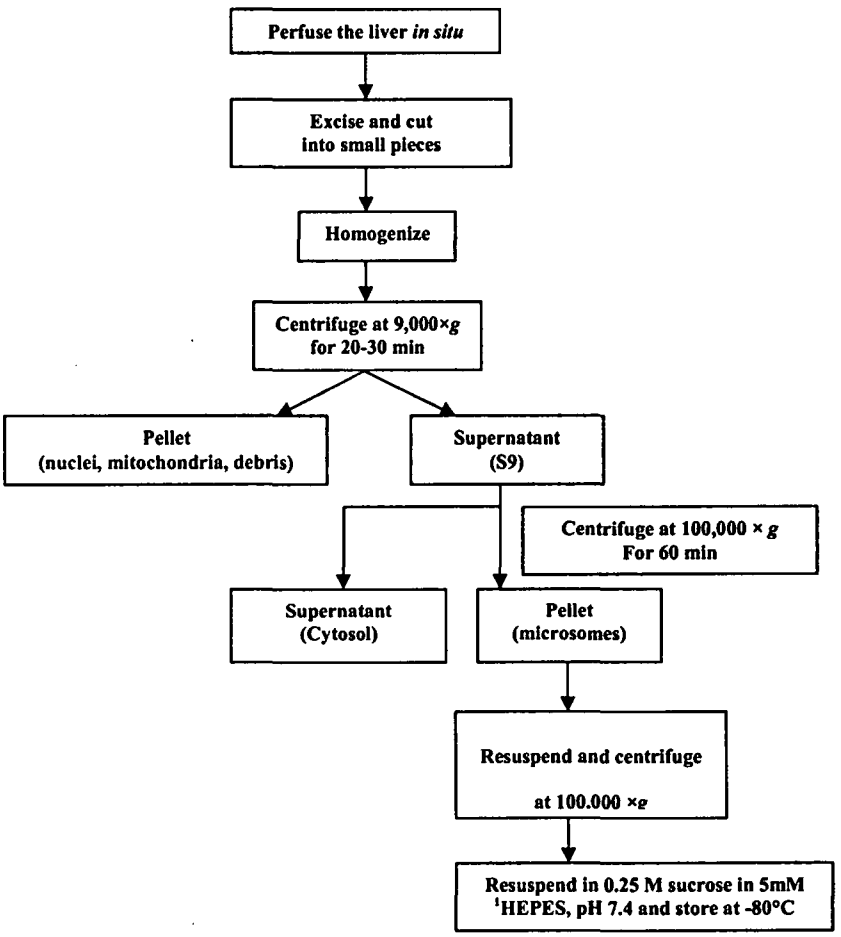
Chapter 2

Preparation of liver microsomes

2.1 Introduction

Liver is the principal organ involved in the metabolism of drugs and is rich in drug metabolizing enzymes. Liver homogenates on differential centrifugation at 9000 ×*g* and 1, 00,000 ×*g* form post mitochondrial (S9) and microsomal fractions, respectively. A generalized subcellular fractionation scheme is shown in Figure 1.

Figure 1. A generalized subcellular fractionation scheme [1].



[1]HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*)

Microsomes are subcellular fractions (mainly derived from endoplasmic reticulum) with abundant source of major drug-metabolizing enzymes, *e.g.* cytochrome P450s (CYPs), flavin mono-oxygenases, and glucuronosyltransferases [2].

The quality of microsomes is influenced by variables like composition of homogenization buffer, homogenization strokes and the force of centrifugation [3].

Average estimates of microsome yield per gram of wet liver tissue is 2-3 mg [4]. The estimation of protein content is usually performed by Lowry-folin or Bradford method. However, Bradford method is sensitive, easy and rapid compared to Lowry-folin.

CYPs are major contributors to drug metabolism. They are membrane proteins and are localized on the outer side of endoplasmic reticulum. Hence, the quality of microsomes can be assessed by determining the enzymatic activity of drug metabolizing families of CYPs (1, 2 and 3).

2.2 Experimental

2.2.1 Materials

Chemicals

Tris(hydroxymethyl)aminomethane (Tris base), KCl, $MgCl_2 \cdot 6H_2O$ and bovine serum albumin (BSA) were purchased from Sisco Research Laboratory (Mumbai, Maharashtra, India). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from USB Corporation (Cleveland, Oh., USA.) Phenylmethanesulphonylfluoride (PMSF) and male human liver microsomes were procured from Sigma (St. Louis, Mo., USA).Glycerol was purchased from Ranbaxy (New Delhi, India). Bradford reagent and diethyl ether were procured from Loba Chemie (Mumbai) and TKM Pharma (Hyderabad, AP, India), respectively.

Instruments

Potter Elvehjem homogenizer, bench-top ultracentrifuge (Beckman Coulter, Brea, CA, USA), Perfusion pump, Shimadzu UFLC pump (LC-20AD) with degasser (DGU-20A3) and autosampler (SIL-HTc) coupled to API 4000 Q trap (Applied Biosystems, Toronto, Canada) mass spectrometry was used for liquid chromatography tandem mass spectrometric analysis (LC-MS/MS).

2.2.2 Animals

Institutionally bred male Sprague Dawley rats (225±25 g) housed in an environmentally controlled room (12 hours light and 12 hours dark with ambient temperature and humidity) were procured and kept for overnight fasting and water was given *ad libitum*. Animals were handled in compliance with the Institutional Animals Ethics Committee of CDRI.

2.2.3 Preparation of Buffer (Homogenization and Re-suspension)

a) Homogenization

Tris base	0.1M
KCl	0.125 M
EDTA-2Na	1 mM

Dissolved in MilliQ water, adjusted pH to 7.4 with concentrated HCl and filtered.

b) Re-suspension buffer

Tris base	0.1M
KCl	0.125 M
EDTA-2Na	1 mM
PMSF	1 mM
Glycerol	20 % (v/v)

Dissolved in MilliQ water, adjusted pH to 7.4 with concentrated HCl and filtered.

2.2.4 Sub-cellular fractionation

Abdominal viscera of ether anesthetized rats were exposed and livers were perfused *in situ* through cannulation of hepatic portal vein. S9 and microsomal fractions were prepared by the reported method [3] with some modifications. Briefly, perfusion and homogenization (1:4 w/v) were performed with ice cold 0.1 M Tris buffer pH 7.4, 0.125 M KCl, and 1 mM EDTA-2Na. The homogenate was centrifuged using bench-top ultracentrifuge (Beckman Coulter, Brea and California, USA) at 9000×g and 1, 00,000×g for 20 min. and 60 min., respectively. Microsomal pellet was re-suspended in resuspension buffer (0.1 M Tris pH 7.4, 0.125 M KCl, 1 mM EDTA-2Na, 1 mM PMSF and 20% (v/v) glycerol).

2.2.5 Protein estimation

Protein concentration was determined using Bradford reagent. Briefly, fresh BSA standard solution of concentration 1mg/mL was prepared. A working stock solution of 200 µg/mL was prepared from 1 mg/mL. Subsequent dilutions of protein standards ranging from 20 µg/mL to 80 µg/mL were prepared from working stock solution.

Simultaneously, unknown protein samples of different dilutions were also prepared. To 0.1 mL of the sample 1 mL of Bradford reagent was added and gently vortex-mixed and incubated for 5 minutes. Absorbance was recorded at 595 nm. Table 1 shows the assay setup.

Table 1. Sample preparation for the determination of protein concentration

S No.	*BSA concentration (µg/mL)*	*Sample Volume µL*	*Bradford Reagent µL*
1	20	0.1	1
2	30	0.1	1
3	40	0.1	1
4	50	0.1	1
5	60	0.1	1
6	70	0.1	1
7	80	0.1	1
8	Unknown Protein	0.1	1

Calculation

Standard curve was obtained using GraphPad Prism 4.0 software. Data was fit to linear regression equation.

$$Y = mx + c \qquad \textbf{Equation 1}$$

2.2.6 Determination of Phenacetin-o-deethylase activity

Phenacetin-O-deethylase activity (paracetamol formation) was determined using liquid chromatography coupled to mass spectrometry (LCMS/MS). Coumarin was used as an internal standard. Varying concentrations of phenacetin (10 µM to 80 µM) were incubated with freshly prepared in-house male Sprague Dawley rat liver microsomes. The experiment was performed in triplicate. Paracetamol (phenacetin-O-deethylation) formation was quantified by using validated developed bio-analytical method. Assay conditions are summarized in Table 2.

Table 2. Assay conditions for CYP1A2 activity using Phenacetin-o-deethylation reaction

Analytical conditions	
Analyte	Paracetamol
Internal Standard (concentration)	Coumarin
Column	Phenomenex Luna C18 (50 ×2.00 mm, 3 µm)
Flow rate	0.4 (mL/min)
Injection Volume	20 µL
Mobile Phase	ACN , 0.1% formic acid
Gradient, %B (min)	98(0.01)-98(0.5)-2(5.0)-98(10)
Mass spectrometry conditions	
Polarity	Positive
IS[a]	5500V
Temperature	350°C
DP[b]	65V
CE[c]	30V
MRM transitions **Paracetamol** **Coumarin**	 m/z 152-m/z 110 m/z 147-m/z 103
Incubation conditions	
Microsomes	0.4 mg/mL
Incubation time	30 min
Phenacetin concentration	10-80 µM
Standard curve	0.5-10 µM
Sample Preparation	1:1 Protein precipitation with ice cold ACN

[a]IS, Ion Spray Voltage; [b]DP, Declustering potential; [c]CE, Collision energy

Calculation

(a) Phenacetin-o-deethylase activity of CYP1A2 was calculated by fitting the processed data to Michaelis-Menten equation of GraphPad prism 4 software.

$$v_0 = \frac{V_{max} \times [S]}{K_m + [S]} \qquad \textbf{Equation 2}$$

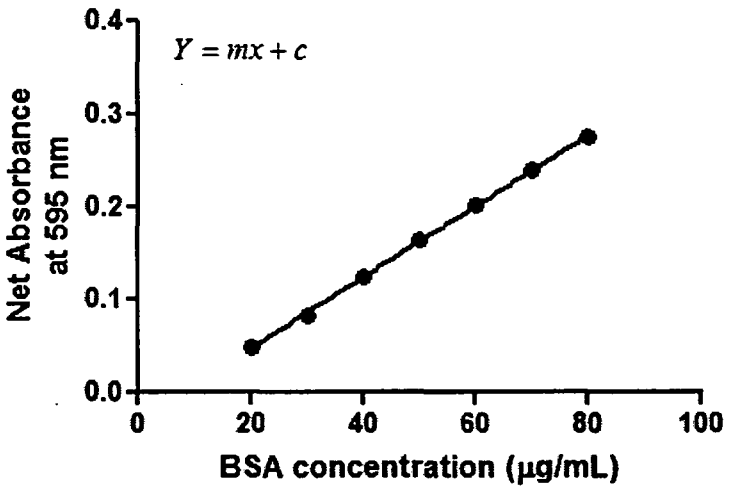
Where, V_{max} is the maximum enzyme velocity, K_m is the Michaelis-Menten constant and is defined as the substrate concentration needed to achieve a half maximum enzyme velocity and $[S]$ is the substrate concentration.

2.3 Results & discussions

Microsomes were prepared by differential centrifugation of the perfused rat liver. Perfusion was performed to remove serum proteins and hemoglobin. It was ascertained that the number of homogenization strokes were optimal for microsome preparation but prevented nuclear and mitochondrial fragmentation [4].

Protein content was estimated using Bradford reagent with BSA as the standard and was adjusted to 10 mg/mL prior to storage at -80°C. The data shown in Figure 2 was fit to linear regression equation (1) and the coefficients of correlation (r) were better than ≥ 0.99.

Figure 2. Standard curve of Net Absorbance at 595 nm versus BSA concentration



Cytochrome P450 1A2 is an important drug metabolising enzyme of liver. It is responsible in the O-deethylation of phenacetin. Phenacetin-o-deethylase activity is extensively used for rapid characterization and quantification of cytochrome P450 1A2 in *in vitro* drug metabolic studies [5]. Hence, an assay condition was optimized to determine CYP1A2 activity in liver microsomes using reported LCMS/MS method with some modifications [6].

The product ion spectra and typical MRM chromatograms of paracetamol and IS are shown in figure (Figure 3). It was observed that electro-spray ionization (ESI) of phenacetin caused in-source fragmentation. Moreover, it has been reported that electro-spray ionization (ESI) of phenacetin causes in-source fragmentation [7]. Hence, optimization of liquid chromatography conditions was important (chromatographic separation between phenacetin and paracetamol). The retention time (R_t) of paracetamol and coumarin was 1.4 and 4.6 minutes, respectively (Figure 4).

Figure 3. Product ion spectra of (a) paracetamol, (b) IS and (c) phenacetin

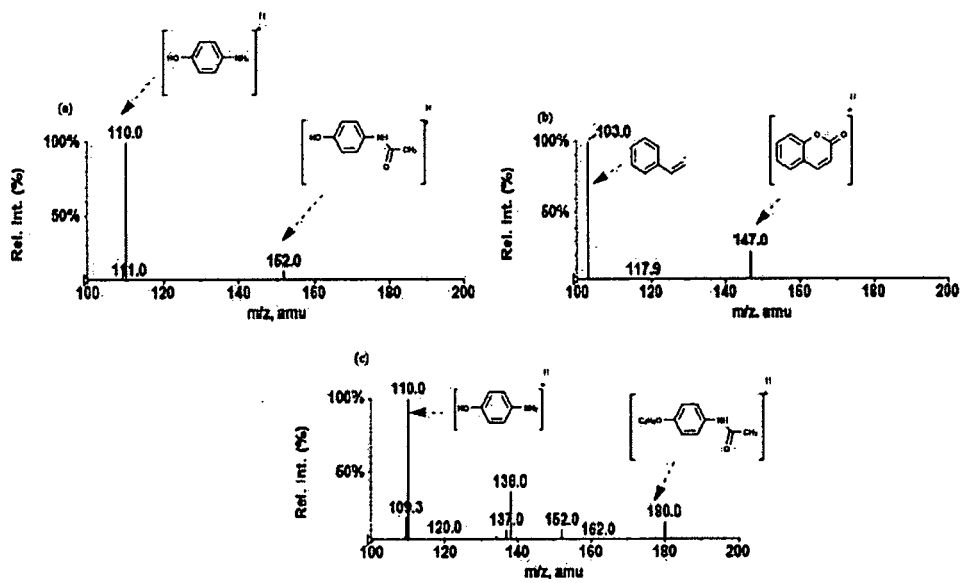
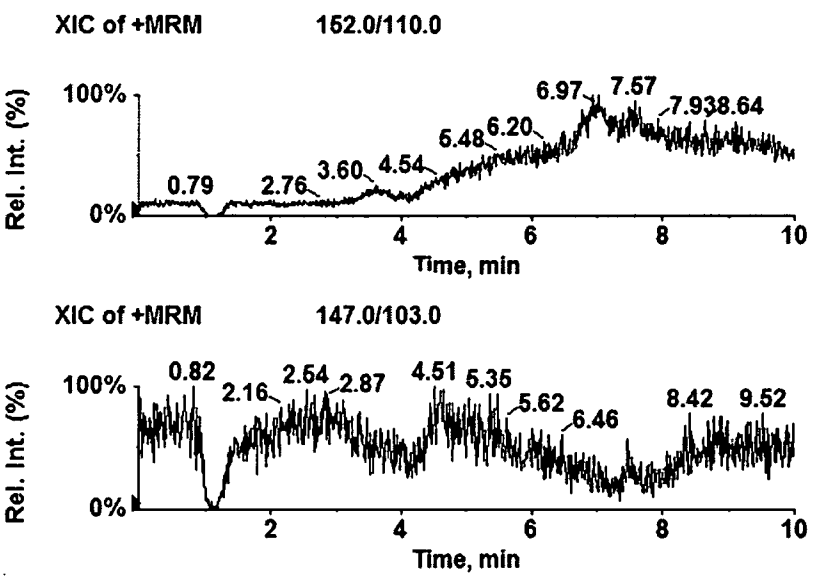
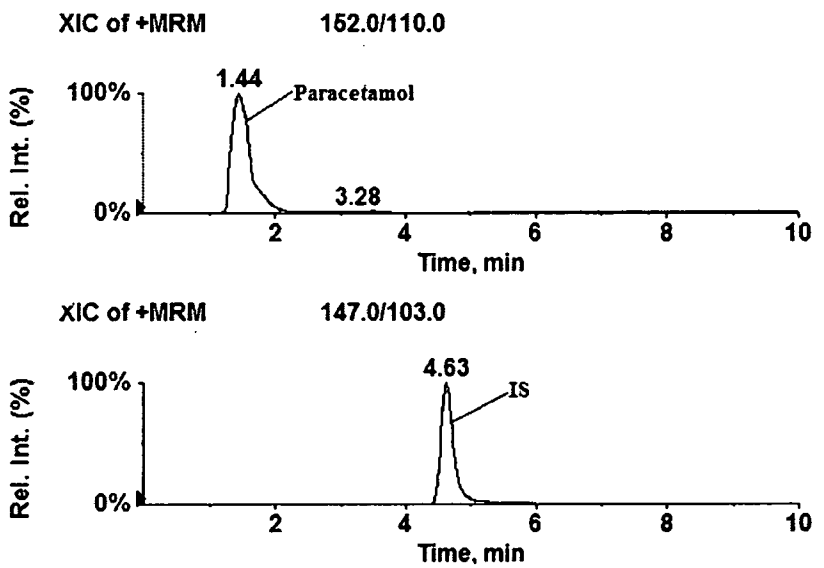


Figure 4. Typical MRM chromatograms of paracetamol and IS in (a) rat blank liver microsome and (b) in rat liver microsome spiked with paracetamol and IS (c) after 0 minutes of incubation of Phenacetin and (d) after 30 minutes of incubation of Phenacetin.

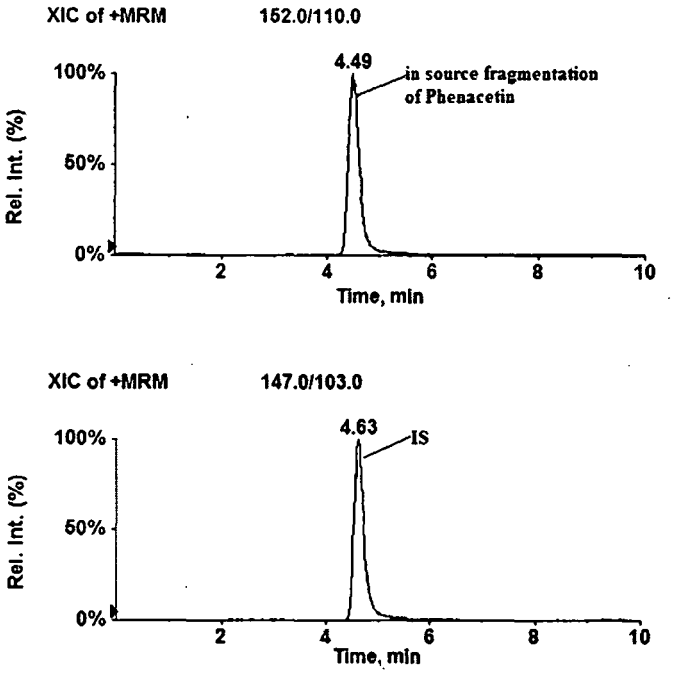
(a)



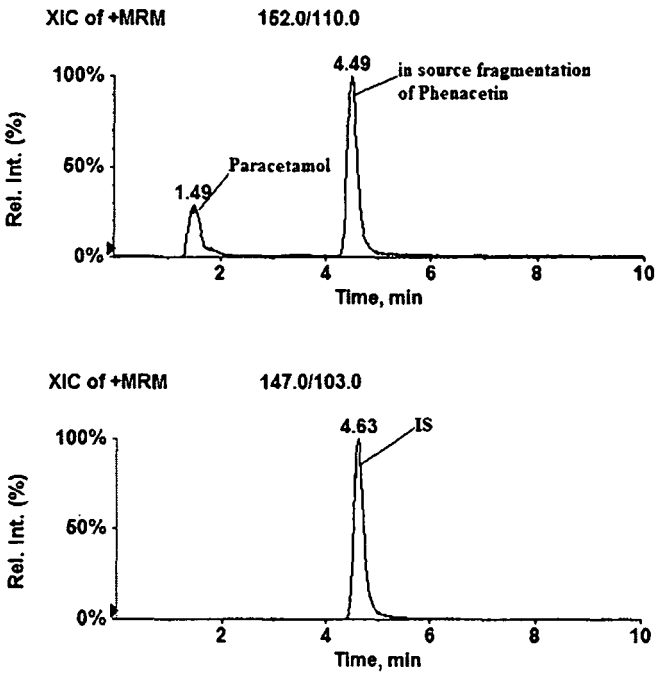
(b)



(c)


XIC of +MRM
152.0/110.0
4.49
in source fragmentation
of Phenacetin
100%
50%
0%
Rel. Int. (%)
2
4
6
8
10
Time, min
XIC of +MRM
147.0/103.0
4.63
IS
100%
50%
0%
Rel. Int. (%)
2
4
6
8
10
Time, min

(d)


XIC of +MRM
152.0/110.0
4.49
in source fragmentation
of Phenacetin
Paracetamol
1.49
100%
50%
0%
Rel. Int. (%)
2
4
6
8
10
Time, min
XIC of +MRM
147.0/103.0
4.63
IS
100%
50%
0%
Rel. Int. (%)
2
4
6
8
10
Time, min

It was observed that 0.4 mg/mL of protein and an incubation time of 30 minutes were optimal for assay.

The calibration curve was constructed using six calibration standards (0.5–10 μM) and was found to be reproducible. Calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte / peak area IS) versus concentration and using linear regression equation (equation 1) with $1/X^2$ as the weighing factor. The coefficient of correlation was found to be ≥ 0.99 for the range 0.5-10 μM (Figure 5).

Accuracy and precision data for intra-day and inter-day are presented in Table 3. The assay values on both the occasions (intra-day and inter-day) were found to be within the accepted variable limits.

Figure 5. Calibration standard curve of paracetamol.

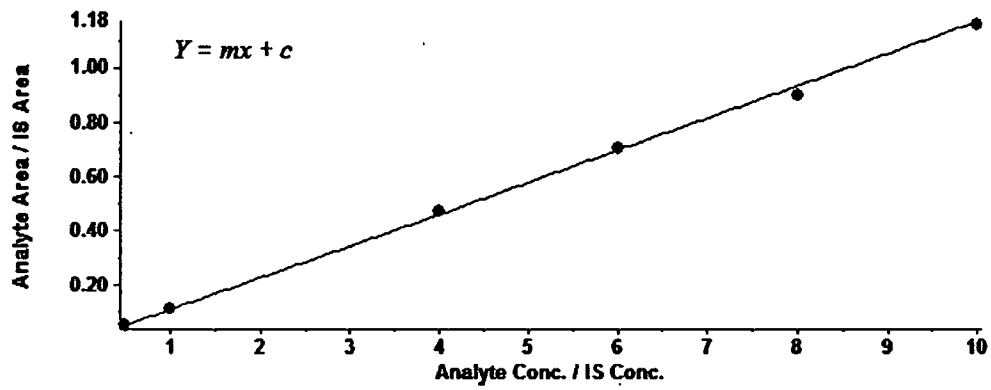


Table 3. Intra-day and inter-day accuracy and precision of Paracetamol in rat liver microsomes (n = 3 days with six replicates per day)

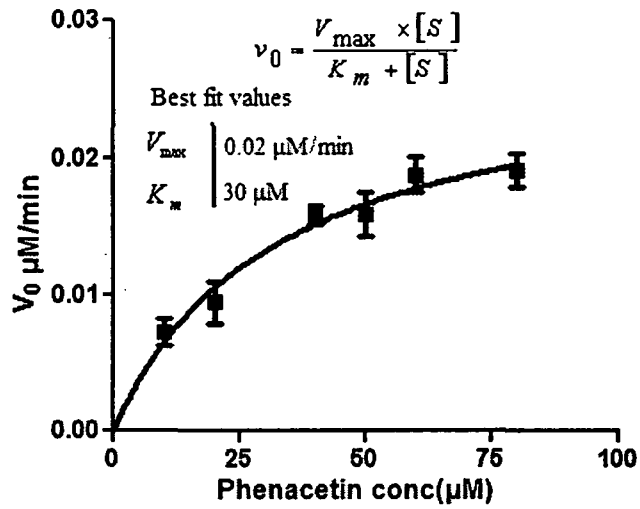
QC[c] (μM)	Accuracy (% bias)[a]		Precision (% RSD)[b]	
	Intra-day	Inter-day	Intra-day	Inter-day
LQC (1.0)	-4.05	-4.06	5.21	3.63
MQC (5)	-7.16	-4.59	3.49	2.22
HQC (9)	-6.46	-7.03	2.48	3.97

[a] % bias is [(Final concentration-Nominal concentration) / Nominal concentration]*100
[b] % RSD is (Standard deviation / Mean)
[c] QC is quality control;LQC is Low quality control; MQC is Medium quality control; HQC is High quality control

Phenacetin-o-deethylation exhibits biphasic kinetics. However, at concentrations below 100 μM it exhibits Michaelis-Menten kinetics specific to CYP1A2 activity [8]. It has also been reported that phenacetin-o-deethylation activity of CYP1A2 is similar in human and rat liver microsomes [9].

Data was fit to Michaelis-Menten equation with weighting *1/Y* (equation 2). The plot illustrated in Figure 6. shows results, which is coherent with a kinetics following a Michaelis-Menten enzymatic reaction. The non-linear regression results $r \geq 0.97$ show that the observed V_{max} and K_m for Phenacetin was 0.02 μM/min and 30 μM, respectively. The calculated value of K_m was in the reported range 10 μM to 50 μM [8]. Intra-assay precision of reaction velocity was 0.11%.

Figure 6. Substrate saturation curve for CYP1A2 mediated phenacetin –o-deethylase activity in in-house prepared rat liver microsomes. Each point in the curve represents the mean ± S.E. of the triplicate determinations.



2.4 Conclusion

Microsomes are an ideal preparation to study the relationships between enzyme structure, protein-protein and lipid-protein interactions, and the functional properties of membrane bound enzymes.

Microsomes were prepared by the reported method with some modifications. Protein concentration and quality of microsomes was assessed by Bradford reagent and Phenacetin-o-deethylase activity of CYP1A2, respectively. The in-house prepared rat liver microsomes were found to be optimal for prospective experiments.

Reference

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