International Journal of Pharma Medicine and Biological Sciences

ISSN 2278 – 5221 www.ijpmbs.com
Vol. 3, No. 2, April 2014

Research Paper

SPECIES RELATED DIFFERENCES IN *IN VITRO* METABOLISM OF PHENACETIN, A PROBE SUBSTRATE FOR CYP1A ENZYME, AND ENZYME KINETIC PARAMETERS OF PHENACETIN

Alice Varghese*, Jay Savai, Nancy Pandita and Ram Gaud[1]

*Corresponding Author:Alice Varghese ✉ alice.varghese@nmims.edu

The CYP1A P450 subfamily consists of two members, CYP1A1 and CYP1A2 in mouse, rat, dog, monkey and human. CYP1A shows a quite strong conservation among species with an identity to human higher that 80% in rat (83 and 80%, respectively for CYP1A1 and CYP1A2), mouse (83 and 80%, respectively for CYP1A1 and CYP1A2), dog (84% for CYP1A2) and monkey (95% for both CYP1A1 and CYP1A2). Drug-drug interactions may occur as a result of (a) induction of the expression of metabolizing enzymes or, (b) as a result of inhibition of enzyme activity or expression. Phenacetin is a recommended probe substrate for CYP1A for *in vitro* and *in vivo drug* interaction studies. Phenacetin has been found to be almost exclusively metabolized by CYP1A2 to its metabolite paracetamol in humans. The objective of the study was to investigate the metabolism of phenacetin to paracetamol and to determine the Km and Vmax values of phenacetin in mice, rat and pig liver microsomes. Phenacetin was metabolized to paracetamol in rat and mice liver microsomes. However, pig liver microsomes did not show metabolism of phenacetin to paracetamol, inspite of pigs reported to have CYP1A isozyme in livers. Enzyme kinetic parameters (Km and Vmax) for conversion of phenacetin in paracetamol in rat and mice liver microsomes were determined. Both rat and mice liver microsomes showed a linear formation of metabolite (paracetamol) in the concentration range of 5 – 160 µM and 40 – 100 µM of phenacetin, respectively. Km and Vmax values of phenacetin, for conversion to paracetamol, was found to be 54 µM and 0.0015 nmoles/min/mg protein, respectively in rat liver microsomes and 74 µM and 0.005 nmoles/min/mg protein, respectively in mice liver microsomes. The enzyme kinetic parameters will be useful to choose a concentration of phenacetin in vitro to carry out drug-drug interaction studies for CYP1A enzyme in rats and mice.

Keywords: CYP1A, Liver Microsomes, Rodents, Non-rodents, Phenacetin, Km, Vmax

INTRODUCTION

The most versatile enzyme system involved in the metabolism of xenobiotics is cytochrome P450. Inhibition of these enzymes often results in unexpected and sometimes severe adverse drug interactions, as the metabolic clearance of

[1] Shobhaben Pratapbhai Patel, School of Pharmacy and Technology Management, SVKM's NMIMS, Vile Parle (W), Mumbai 400056, India.

co-administered drugs can be altered dramatically (Ponnusankar *et al*., 2011). Inhibition of CYP enzymes can also be affected by natural products. CYP family consists of a number of isoforms which includes 3A, 2C, 2D, 2E and 1A. Out of all, CYP1A2 constitutes 13% of the total CYP content in the liver and plays an important role in the metabolic clearance of approximately 5% of currently marketed drugs. Human Cytochrome P450 1A2 (CYP1A2), a member of the Cytochrome P450 mixed function oxidase system, is one of the important enzymes involved in the metabolism of xenobiotics in the body. The actions of human cytochrome P450 1A2 may partly account for the carcinogenic effects of burned foods and cigarette smoke. Burning these substances alters amino acids and carbohydrates, producing heterocyclic amines. Many studies have reported inhibition data on CYP1A2 by many different herbs. For example, St John's wort, the components of *Ginkgo biloba* and flavonoids extracted from plants could decrease the activation of CYP1A2 (Liwei Hu *et al*., 2010; Guengerich, 1993).

The fate of phenacetin and some of its metabolites have been examined in isolated rat hepatocytes. The major metabolites of phenacetin were paracetamol, free and conjugated, and phenetidine, and about 10% was lost. No N-hydroxyphenacetin was found, but experiments with N-hydroxyphenacetin as substrate showed that at low concentration (as might be formed from phenacetin) it disappeared very rapidly from cell suspensions. N-hydroxyphenacetin was metabolized to its conjugates, and to paracetamol, phenacetin and phenetidine, with a large proportion unaccounted for (McLean, 1978). Phenacetin is the USFDA recommended substrate for evaluation of CYP1A interaction potential of drugs in humans as an *in vitro* substrate (Guidance to industry, US-FDA, 2006). However, the enzyme kinetic parameters (Km and Vmax) of phenacetin in rodent and non-rodents have not been evaluated.

The objective of the present study was to estimate the metabolic pathway of phenacetin in rodents and non-rodents and to estimate the CYP1A enzyme kinetic parameters of phenacetin in mice, rat and pig liver microsomes.

MATERIALS AND METHODS

Chemicals and Equipments

All the solvents, chemicals and reagents used were of analytical grade and purchased locally. Phenacetin, paracetamol and caffeine were purchased from Sigma-Aldrich Ltd. Nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH) was purchased from SRL Labs Pvt. Ltd. HPLC grade methanol was purchased from Thermo Fischer Scientific India Pvt. Ltd. HPLC system consisted of a Perkin Elmer (Series 200), with an autosampler and PDA detector

Preparation of Solutions

Stock solutions of probe substrate, phenacetin was prepared in methanol at concentration 1, 2, 4, 8, 12, 16, 20 and 40 mM. Phosphate buffer (100 mM, pH 7.4) was used to make NADPH solution (10 mM).

Preparation of Rat Liver Microsomes

Rat Liver microsomes were isolated from male Swiss Wistar (150-200 g) strain rats based on the methods described by the calcium aggregation method (Savai *et al*., 2013). The experiment was performed as per the guidelines of Institutional Animal Care Committee constituted

as per the guidelines of the CPCSEA and the protocol (Protocol no. CPCSEA/IAEC/SPTM/P-59/2011) was duly approved by the Institutional Animal Ethics Committee. Briefly, the rats were euthanized by cervical dislocation and the livers (20 g) were quickly removed, perfused with 1.15% potassium chloride (KCl) solution and homogenized with four volumes (80 mL) of ice cold 10 mM Tris-HCl buffer containing 0.25 M Sucrose, pH 7.4, in a Potter glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 13,000×g for 10 min at 4ºC in a refrigerated centrifuge (Eppendorff) and the precipitate was discarded. To the supernatant, calcium chloride was added to yield a final concentration of 10 mM. The solution was stirred for 15-20 min and then centrifuged at 25,000 × g for 10 min at 4ºC. The firmly packed pellets of microsomes were resuspended by homogenization in 100 mM Tris-HCl buffer containing 20% w/v glycerol and 10 mM EDTA, pH 7.4. The microsomes were stored at –70ºC until use.

Preparation of Pig Liver Microsomes

The Pig Liver was brought from slaughter house. 40 g of excised Liver was thawed and weighed. To this excised liver, four volumes (160 mL) of Ice cold 10 mM Tris- HCl buffer containing 0.25 Sucrose, pH 7.4 was taken and homogenized in a homogenizer. Same procedure was followed as followed for isolation of rat liver microsomes. The microsomes were stored at –70ºC in a refrigerator.

Preparation of Mice Liver Microsomes

Livers were isolated from ten mice for isolation of microsomes. The experiment was performed as per the guidelines of Institutional Animal Care Committee constituted as per the guidelines of the CPCSEA and the protocol [Protocol no. CPCSEA/IAEC/SPTM/P-57/2012] was duly approved by the Institutional Animal Ethics Committee. 18 g of excised Liver was thawed and weighed. Same procedure was followed as followed for isolation of rat liver microsomes. The microsomes were stored at –70ºC in a refrigerator.

Determination of Protein Content of Rat, Mice and Pig Liver Microsomes

Protein concentrations of all species of microsomes were determined by Folin Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard. The optical density was recorded on a Perkin Elmer UV/vis spectrophotometer at 625 nm.

Incubation of Phenacetin in Rat, Mice and Pig Liver Microsomes

Phenacetin was incubated at a final concentration of 60 µM in rat, mice and pig liver microsomes to evaluate the metabolism of phenacetin in these species. Briefly, a standard 100 µL incubation mixture contained protein (1 mg/mL), NADPH (1 mM) in 0.1 M phosphate buffer (pH 7.4) and phenacetin (60 µM). All reactions were initiated with NADPH and incubated at 37ºC for 60 min. The reaction was stopped by adding 100 µL of methanol containing Caffeine (100 µg/mL). Caffeine was used as an internal standard. The samples were centrifuged at 4000 rpm for 10 min at 4ºC. The supernatant were analyzed by HPLC. Samples were run on a C18 column and mobile phase used was (A) Water and (B) methanol and was pumped at a flow rate of 1 mL/min. The gradient program used was time: %B – 0 /10; 5/95; 15/95; 18/10; 20/10. Detection of paracetamol and phenacetin was accomplished by UV absorbance at a wavelength of 240 nm. Retention times of paracetamol, caffeine (IS) and phenacetin was

6.9, 7.7 and 9.1 min, respectively.

Km and Vmax Determination of Phenacetin in Rats and Mice

Enzyme kinetics defined by two constants, Km and Vmax, play an important role to understand enzyme activity on the macroscale and to understand the effects of different types of enzyme inhibitors.

The maximal velocity, or V_{max}, is the rate of the reaction under these conditions. V_{max} reflects how fast the enzyme can catalyze the reaction.

An enzyme's K_m describes the substrate concentration at which half the enzyme's active sites are occupied by substrate. A high K_m means a lot of substrate must be present to saturate the enzyme, meaning the enzyme has low affinity for the substrate. On the other hand, a low K_m means only a small amount of substrate is needed to saturate the enzyme, indicating a high affinity for substrate (Price, 1985).

Phenacetin was used as the probe substrate to evaluate the activity of CYP1A in rats and mice (Guidance to industry, US-FDA, 2006). Km value for phenacetin in RLM was determined in-house by Lineweaver-Burk plot (Lineweaver *et al*., 1934). Km value for CYP1A probe substrate (Phenacetin) was determined by incubating different concentrations of substrate (5-200 µM) in rat and mice liver microsomes. Briefly, a standard 100 µL incubation mixture contained protein (1 mg/mL), NADPH (1 mM) in 0.1 M phosphate buffer (pH 7.4) and phenacetin (5-200 µM). All reactions were initiated with NADPH and incubated at 37°C for 60 min. The reaction was stopped by adding 100 µL of methanol containing Caffeine (100 µg/mL). Caffeine was used as an internal standard. The samples were centrifuged at 4000 rpm for 10 min at 4°C. The supernatant were analyzed by HPLC using the method.

Km value for phenacetin in rats was calculated using the Lineweaver-Burk plot 1/[V] Vs 1/[S]; where [V] is the velocity of reaction in nmoles/min/mg and [S] is the substrate concentration (µM) was obtained. The Lineweaver-Burk double reciprocal plot rearranges the Michaelis-Menten equation as:

$$1 / v = 1 / Vmax + Km / Vmax \times 1 / [S]$$

The *y*-intercept of such a graph is equivalent to the inverse of V_{max} and the *x*-intercept of the graph represents $-1/K_m$.

RESULTS

Protein Content of Rat, Mice and Pig Liver Microsomes

Protein concentrations were determined by Folin Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard. Protein content of rat, mice and pig liver microsomes were found to be 15.5 ± 0.2 mg/mL, 9.67 ± 0.22 mg/mL and 7.7 ± 0.18 mg/mL, respectively

Metabolism of Phenacetin in Rat, Mice and Pig Liver Microsomes

Phenacetin is reported to be metabolized to paracetamol by CYP1A2 enzyme in humans. In rat liver microsomes, phenacetin showed metabolism to paracetamol and also showed presence of another metabolite (retention time = 6.2 min). A representative chromatogram of phenacetin incubated in rat liver microsomes is shown in Figure 1. Mice liver microsomes also showed a similar metabolism profile of phenacetin shown in Figure 2. Phenacetin in pig liver microsomes however did not show any metabolism to paracetamol, but shows presence of unknown metabolite in Figure 3.

Figure 1: Representative Chromatogram of Formation of Paracetamol and Unknown Metabolite from Phenacetin after Incubation for 60 mins. in Rat Liver Microsomes

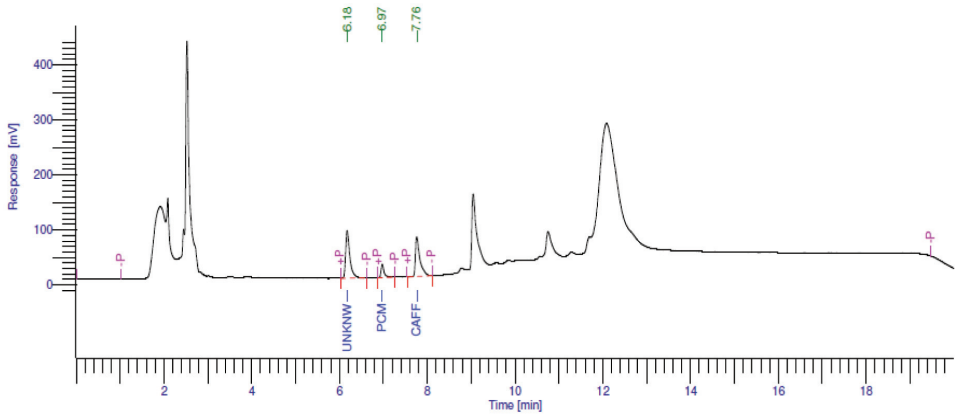


Figure 2: Representative Chromatogram of Formation of Paracetamol and Unknown Metabolite From Phenacetin After Incubation for 60 mins. in Mice Liver Microsomes

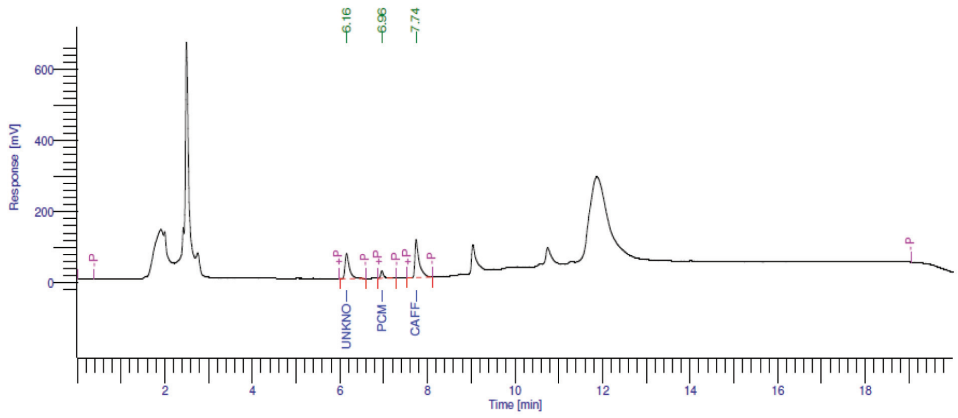
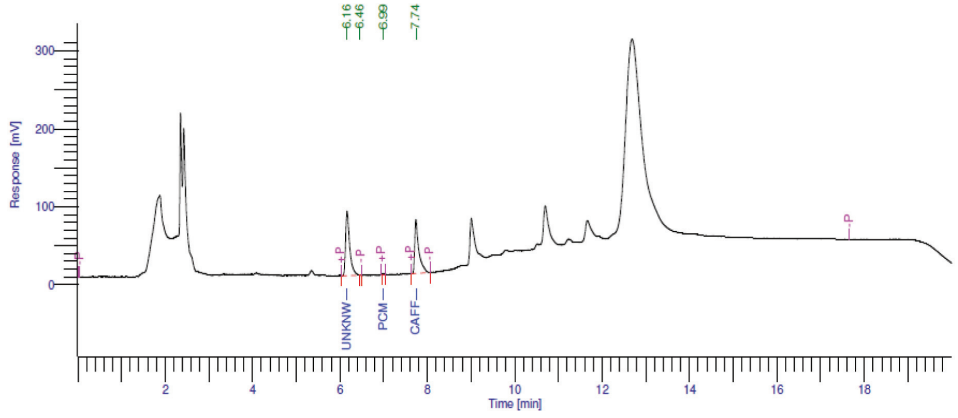


Figure 3: Representative Chromatogram of Formation of Only Unknown Metabolite from Phenacetin After Incubation for 60 Mins. in Pig Liver Microsomes



Enzyme Kinetic Parameters of Phenacetin Metabolism to Paracetamol in Rat and Mice Liver microsomes

Rat Liver Microsomes

The average velocities of the enzymatic reactions of phenacetin in nmoles/min/mg protein in rat liver microsomes are summarized in Table 1.

The Lineweaver-Burk (double reciprocal) plot of phenacetin in rat liver microsomes was obtained by plotting 1/[S] vs. 1/[V] and is represented in Figure 4.

The *y*-intercept of such the graph is equivalent to the inverse of V_{max} and the *x*-intercept of the graph represents $-1/K_m$. By substituting the values obtained from the equation of the plot in Figure 4, the Km value of phenacetin was found to be 54 µM and V_{max} of the reaction was found to be 0.0015 nmoles/min/mg protein in rat liver microsomes.

Mice Liver Microsomes

The average velocities of the enzymatic reactions of phenacetin in nmoles/min/mg protein in mice liver microsomes are summarized in Table 2.

The Lineweaver-Burk (double reciprocal) plot of phenacetin in mice liver microsomes was obtained by plotting 1/[S] vs. 1/[V] and is represented in Figure 5.

The *y*-intercept of such the graph is equivalent to the inverse of V_{max} and the *x*-intercept of the graph represents $-1/K_m$. By substituting the values obtained from the equation of the plot in Figure 5, the Km value of phenacetin was found to be 74 µM and V_{max} of the reaction was found to

Table 1: Average Reaction Velocities of Conversion of Phenacetin to Paracetamol in Rat Liver Microsomes

Phenacetin [S] (µM)	Area Ratio (Area of Phenacetin/Area of Caffeine)	Conc (nmoles/mL)	[V] (nmoles/min/mg)
5	0.007213803	0.007	0.014427607
10	0.016250286	0.016	0.032500573
20	0.022609834	0.023	0.045219668
40	0.033694823	0.034	0.067389646
80	0.042482733	0.042	0.084965466
160	0.082747293	0.083	0.165494586
200	0.069588114	0.070	0.139176229

Table 2: Average Reaction Velocities of Conversion of Phenacetin to Paracetamol in Mice Liver Microsomes

Phenacetin (µM)	Area Ratio	Conc (nmoles/mL)	V (nmoles/min/mg)
40	0.112270508	0.112	0.001871175
60	0.136635618	0.137	0.00227726
80	0.173151903	0.173	0.002885865
100	0.173826901	0.174	0.002897115

Figure 4: Lineweaver-Burk (Double Reciprocal) Plot of 1/Velocity(nmoles/min/mg protein) Vs 1/Phenacetin [S] for Rat Liver Microsomes

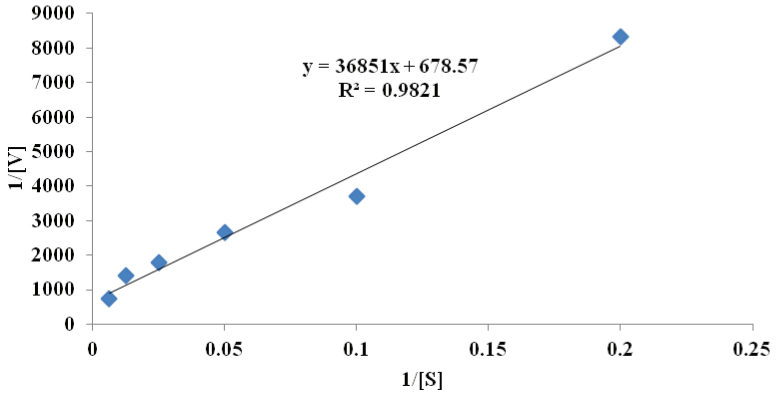
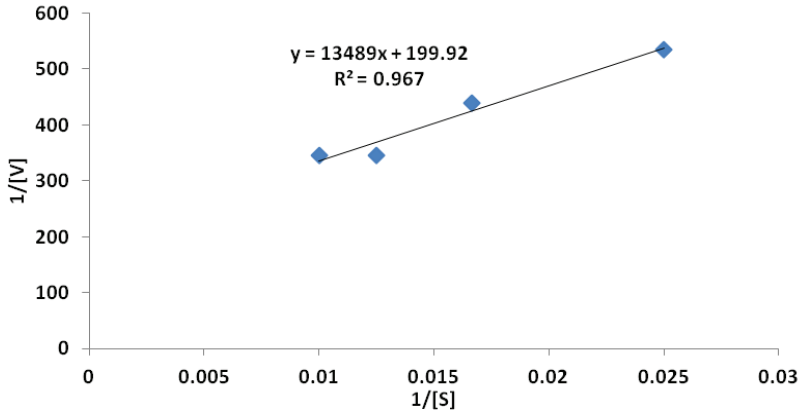


Figure 5: Lineweaver-Burk (Double Reciprocal) Plot of 1/velocity(nmoles/min/mg protein) Vs 1/Phenacetin [S] for Mice Liver Microsomes



be 0.005 nmoles/min/mg protein in mice liver microsomes.

DISCUSSION

The CYP1A enzymes are involved in the metabolism of several carcinogens such as aromatic and heterocyclic amines, estrogens, mycotoxins and xanthines. These enzymes are also involved in the metabolism of several antidepressant and analgesic drugs. Cytochrome P450 (CYP)1 family members, CYP1A1, CYP1A2 and CYP1B1 which are under the transcriptional regulation of the AhR receptor, are known for their induction by and catalysis of the ubiquitous polyaromatic hydrocarbons (PAHs) present in cigarette smoke, industrial dyes and agricultural pesticides. Expressed in different amounts in the liver (CYP1A2) and extra-hepatic 11 organs (CYP1A1 and CYP1B1) these enzymes in particular CYP1A1 and CYP1B1 catalyze critical conversions to form the ultimate carcinogen (Badala *et al*., 2011). Phenacetin is a recommended probe substrate for CYP1A for *in vitro* and *in vivo* interaction studies. Phenacetin has been found to be almost exclusively metabolized by CYP1A2 to its metabolite

paracetamol in humans (Figure 6) (Nelson *et al*., 2003). An understanding of CYP1A regulation is important for determining and assessing chemical carcinogenesis.

CYP1A P450 subfamily consists of two members, CYP1A1 and CYP1A2 in mouse, rat, dog, monkey and human. CYP1A shows a quite strong conservation among species with an identity to human higher that 80% in rat (83 and 80%, respectively for CYP1A1 and CYP1A2), mouse (83 and 80%, respectively for CYP1A1 and CYP1A2), dog (84% for CYP1A2) and monkey (95% for both CYP1A1 and CYP1A2) (Mugford, 1998; Marcella 2006; Mandlekar 2007).

The objective of our study was to investigate the difference in metabolism of phenacetin in rat, mice and pig liver microsomes. The study also involved determination of enzyme kinetic parameters of conversion of phenacetin to paracetamol in these species, since they have not been reported.

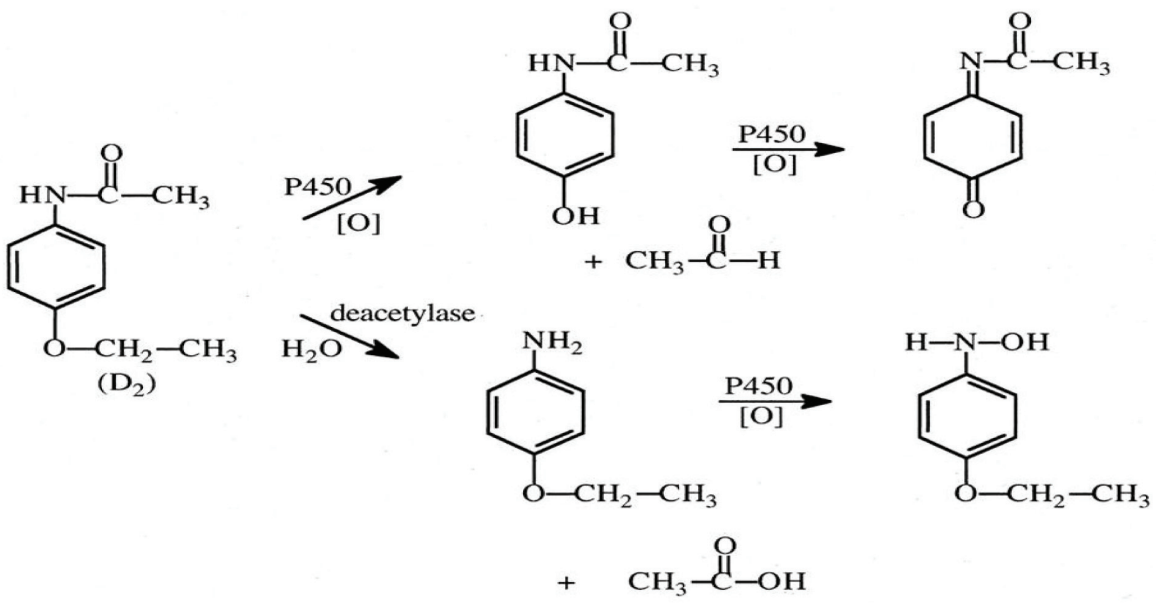
Phenacetin showed metabolism to paracetamol in rat and mice liver microsomes, however phenacetin did showed any conversion to paracetamol in pig liver microsomes.

It is reported that CYP1A activity is also present in porcine liver micromes by ethoxy- and methoxy- resorufin O-dealkylation (EROD and MROD) (Nebbia *et al*., 2003).

Though porcine liver has been reported presence of CYP1A enzyme, there was no metabolism of phenacetin to paracetamol, highlighting the difference is substrate specificity in pigs.

All three species showed presence of an unknown metabolite at retention time of 6.1 min, which needs to be characterized. Pig liver microsomes showed presence of only unknown metabolite. This unknown metabolite is absent when phenacetin is incubated in human liver microsomes.

Figure 6: Partial Scheme of Metabolism of Phenacetin



Enzyme kinetic parameters (Km and Vmax) for CYP1A enzyme for conversion of phenacetin to paracetamol were calculated in rat and mice liver microsomes only, since pig liver microsomes did not show conversion of phenacetin to paracetamol. Km and Vmax values of phenacetin, for conversion to paracetamol, was found to be 54 µM and 0.0015 nmoles/min/mg protein, respectively in rat liver microsomes and 74 µM and 0.005 nmoles/min/mg protein, respectively in mice liver microsomes. Formation of paracetamol from phenacetin in rat and mice liver microsomes was linear in the range of 5 – 160 µM and 40 – 100 µM of phenacetin, respectively.

The information provided by our study is useful to decide concentration of phenacetin to be used as a probe substrate for CYP1A drug-drug, drug-food and drug-herb interactions in rats & mice.

ACKNOWLEDGMENT

The authors would like to thank Department of Bio-technology, New Delhi, India for providing financial support through project grant [DBT Project no: BT/PR14460/PBD/17/703/2010] for the present research work to Shobhaben Pratapbhai Patel, School of Pharmacy and Technology Management, SVKM's NMIMS. The authors would like to thank Dr. R. S. Gaud; Dean of SPP-SPTM, NMIMS, Mumbai for providing support and necessary facilities.

REFERENCES

1. Badala S *et al*. (2011), "Cytochrome P450 1 enzyme inhibition and anticancer potential of chromene amides from *Amyris plumier*", *Fitoterapia*, Vol. 82, pp. 230-236.
2. Guengerich P F (1993), "Cytochrome P450 Enzymes", *American Scientist*, Vol. 81, pp. 440-447.
3. Guidance for Industry Drug Interactions Studies (2006), US-FDA Clinical Pharmacology.
4. Lineweaver H and Burk D (1934), "The Determination of Enzyme Dissociation Constants", *Journal of the American Chemical Society*, Vol. 56, pp. 658-666.
5. Liwei Hu, Wen Xu, Xi Zhang, Juan Su, Xinru Liu, Haiyun Li and Weidong Zhang (2010), "*In vitro* and *in vivo* evaluations of Cytochrome P450 1A2 interactions with nuciferine", *J Pharm Pharmacol*, Vol. 62, pp. 658-662.
6. Lowry O H, Rosenbrough N J, Farr A L and Randall R J (1951), "Protein measurement with the Folin Phenol Reagent", *J. Biol. Chem.*, Vol. 193, pp. 265-275.
7. Mandlekar V, Rose V, Cornelius U, Sleczka B, Caporuscio C, Wang J and Marathe P (2007), "Development of an in vivo rat screen model to predict pharmacokinetic interactions of CYP3A4 substrates", *Xenobiotica*, Vol. 37, pp. 923-942.
8. Marcella M, Geny G and Kanter R (2006), "Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction", *Expert Opin. Drug Metab. Toxicol*, Vol. 2, pp. 875-894.
9. McLean S (1978), "Metabolism of phenacetin and N-hydroxyphenacetin in isolated rat hepatocytes", *Naunyn Schmiedebergs Arch Pharmacol*., Vol. 305, pp. 173-180.
10. Mugford CA, Kedderis GL (1998), "Sex-dependent metabolism of xenobiotics", *Drug Metab Rev*, Vol. 30, pp. 441-498

11. Nebbia C, Dacasto M, Rossetto Giaccherino A, Guiliano Albo A and Carletti M. (2003), "Comparative expression of liver cytochrome P-450 dependent monooxygenases in the horse and in other agricultural and laboratory species", *The Vet. J.*, Vol. 165, pp. 53-64.

12. Nelson S and Trager W (2003), "The use of deuterium isotope effects to probe the active site properties, mechanism of cytochrome p450-catalyzed reactions, and mechanisms of metabolically dependent toxicity", *Drug Metab Dispo*., Vol. 31, pp. 1481-1497.

13. Ponnusankar S, Pandit S, Babu R, Bandyopadhyay A and Mukherjee P K (2011), "Cytochrome P450 inhibitory potential of Triphala—A Rasayana from Ayurveda", *J Ethnopharmacol*., Vol. 133, pp. 120-125.

14. Price NC (1985), "The determination of Km from Lineweaver-Burk plots", *Biochem. Edu.*, Vol. 13, p. 81.

15. Savai J, Varghese A and Pandita N (2013), "Lack of the cytochrome P450 3A interaction of methanolic extract of Withania somnifera, Withaferin A, Withanolide A and Withanoside IV", *J Pharm Negative Results*, Vol. 4, pp. 26-32.