

Hepatic Metabolism of 17 α -hydroxyprogesterone Caproate in Mice, Rats, Dogs, Pigs, Rabbits and Baboons: A Comparison with Human Liver Microsomes and Human Hepatocytes

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Abstract

Hydroxyprogesterone caproate (17-OHPC) is used to prevent preterm labor in pregnant subjects. In the present study inter-species difference in the metabolism of 17-OHPC was evaluated using liver microsomes from mouse, rat, rabbit, pig, dog and baboon and compared with human liver microsomes and primary human hepatocyte cultures. In all the species evaluated, monohydroxylated 17-OHPC was observed to be the major metabolite. Significant inter-species difference in the rate of 17-OHPC metabolism was observed both qualitatively and quantitatively. Ketoconazole inhibited 17-OHPC metabolism in all the species evaluated confirming the dominant role of CYP3A (rats, rabbits, dog, pig, dog, baboon) and cyp3a (mice) in the metabolic pathway. It is concluded that monohydroxylated 17-OHPC is the major metabolite of 17-OHPC in various animal species. The baboon seems to provide the most suitable model for evaluating the pharmacokinetics of 17-OHPC in animals and screening for potential drug-drug interactions although interspecies variation exists in the disposition of 17-OHPC.

Keywords: Hydroxyprogesterone caproate; Interspecies; Microsomes; Hepatocytes; Enzyme kinetics

Introduction

Hydroxyprogesterone caproate (17-Hydroxyprog-4-ene-3-20-Dione, 17-OHPC), a synthetic hormone produced by the esterification with caproic acid at the 17 carbon position of the metabolite of the natural female sex hormone progesterone, is an agent used in the prevention of preterm delivery. It is a lipophilic molecule having a molecular weight of 428.7, a log p value of 4 with no apparent charges on the molecule.

Use of 17-OHPC has also been reported in the treatment of primary and secondary amenorrhea, metropathia hemorrhagica, infertility with inadequate corpus luteum function, habitual and threatened abortion [1-8].

There is robust progestational activity following administration of 17-OHPC which is much more prolonged and significantly higher as compared to 17 α -hydroxyprogesterone (17-OHP) [9]. However, the effectiveness of 17-OHPC for the prevention of preterm labor does not seem to be related to its progestational activity. In a recently reported study it was shown that 17-OHPC was not superior to progesterone in eliciting a hormonal response via the classic steroid receptor-mediated pathway [10]. The authors came to the conclusion based on studies showing binding to progesterone receptors, glucocorticoid receptors and expression of progesterone-responsive genes and observed no significant difference between 17-alpha hydroxyprogesterone caproate and progesterone. Thus, the mechanism of action of 17-OHPC in reducing preterm births is not known and studies are being carried out to evaluate other mechanisms, including active metabolites, different signaling pathways and anti-inflammatory effects.

We have previously shown that 17-OHPC is metabolized by CYP3A4 and CYP3A5 in humans leading to the generation of a monohydroxylated 17-OHPC (m/z=445.0) as the major metabolite. Further, it was also shown that the caproate side chain on the molecule remains intact in the metabolites formed [11,12]. Similar results have been previously reported by Wiener et al. in rat liver and human placental homogenates [13].

Limited documentation is available on the kinetics of 17-OHPC in humans or animals. The only study that had evaluated the pharmacokinetics of 17-OHPC was carried out in patients with endometrial carcinoma and used a nonspecific immunoassay to measure 17-OHPC [14]. This study reported a large variability in the serum concentration vs. time profile after a fixed intramuscular dose to different patients and the apparent half life of 17-OHPC appeared to be several days.

The pharmacokinetics of 17-OHPC in rats using radiolabeled drug was first reported by [13,15]. The study observed that 85% of the drug (or its metabolite) was eliminated within the first 8 days of injection of 17-OHPC (100 mg/kg) via the subcutaneous route. Almost 85% of the excreted drug (or its metabolite) was found in the feces and the remaining in the urine. Further, during the first 2 days of the study, most of the radioactivity (due to the drug or its metabolite) was observed in the liver and kidney. Detailed kinetics of 17-OHPC metabolism, the kinetics of 17-OHPC hydroxylation in animals has neither been evaluated nor compared among different animal species or between an animal species and humans.

Thus, the objectives of the study presented here are 1) to investigate and compare the formation and kinetics of 17-OHPC hydroxy-metabolite in mouse, rat, rabbit, beagle dog, guinea pig and baboon liver microsomes, 2) to search for a suitable animal model for further study of 17-OHPC metabolism and pharmacokinetics and potential

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drug-drug interactions, and 3) to compare the kinetics and metabolism of 17-OHPC in animal species with human liver microsomes and primary human hepatocyte cultures. Further, the kinetics of 17-OHPC metabolism in humans will also be evaluated in detail using expressed human CYP3A isoforms.

Materials and Methods

Chemicals

17 α -hydroxyprogesterone caproate was a gift from Diosynth Inc (Chicago, IL, USA), and NADPH was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and HPLC grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animal and human microsomes

Microsomes derived from baculovirus-infected insect cells expressing human CYP isoforms (CYP3A4, 3A5 and 3A7) and pooled animal microsomes (female mice, female rat, male rabbit, female guinea pig and female beagle dog) were purchased from BD-Gentest (Woburn, MA, USA). Baboon liver microsomes were a gift from University of Texas Medical Branch, Galveston, Texas. Pooled baboon liver microsomes were made of 5 baboon liver microsomes.

Human Liver Microsomes: Human liver samples were obtained from the Hepatocyte Transplantation Laboratory at the University of Pittsburgh from liver from organs that could not be used for transplantation. Human liver microsomes were prepared by a standard differential centrifugation procedure [16,17] with minor modifications [11]. Pooled human liver microsomes were made from tissue from 6 human livers.

Preparation of isolated fresh human hepatocytes

Hepatocytes were prepared by a three-step collagenase perfusion technique [18]. Hepatocytes were plated on Falcon 6-well culture plates (1.5×10^6 cells), previously coated with rat tail collagen in Hepatocyte Maintenance Media (HMM, Lonza Walkersville, MD), supplemented with 0.1 μ M insulin, 0.1 μ M dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B and 10% bovine calf serum. After allowing the cells to attach for 4 to 6 hours, medium was replaced with serum free medium containing all of the supplements described above. Cells were maintained in culture at 37°C in an atmosphere containing 5% CO₂ and 95% air. After 24 hours in culture, unattached cells were removed by gentle agitation and the medium was changed. The medium was changed every 24 hours and the hepatocytes were maintained in culture for the experiment.

Microsomal incubations

Different concentrations of 17-OHPC (0-500 μ M in methanol) were incubated with human liver microsomes (0.5 mg/ml, optimum protein concentration) and MgCl₂ (10 mM) in 0.1 mM phosphate buffer (pH 7.4). The final volume was allowed to equilibrate in a shaking water bath for 5 min at 37°C. The reaction was initiated with the addition of NADPH (1 mM). In additional experiments, the incubations were also carried out in the absence of NADPH. After 20 min of incubation, the reaction was stopped by immediately adding equal volume of cold methanol. The mixture was centrifuged at 3000 rpm for 20 min and supernatant was injected into the HPLC.

Expressed enzyme incubations

The incubations (n=3) were carried similar to the method described for human liver microsomes. To evaluate the involvement of CYP isoforms in 17-OHPC metabolism, 20 pmol of expressed enzyme was incubated for 30 mins and samples injected into the HPLC.

Inhibition study

The effect of coincubation of CYP3A inhibitor, ketoconazole (KTZ) on 17-OHPC metabolism was evaluated in various animal species. The concentrations of KTZ were selected based on the Km values obtained from kinetic experiments utilizing liver microsomes from different animal species. 17-OHPC was incubated with or without KTZ at concentrations ranging from 0.01 μ M to 10 μ M, under the incubation conditions described earlier. Incubations without NADPH were used as the control values and the inhibition values were expressed as a percentage of the respective control values. The inhibitor potency was defined by IC₅₀ (50% inhibition of 17-OHPC metabolism compared with the control values).

Incubations with fresh human hepatocytes

Briefly, hepatocytes were maintained in culture in the presence of the chemical under study or vehicle control (MeOH 0.1%). On the day of the experiment, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and antifungal drugs (HMM blank). Solutions with different concentrations of 17-OHPC were made by diluting stocks (1000 X in methanol) with HMM blank. Reactions were started by incubating human hepatocyte cultures (1.5 million cells/ml) with the 17-OHPC solutions for 60 min. At the end of that time, 1 mL of medium was sampled and stored at -80°C analysis. The remaining media was aspirated, and the cells were harvested in phosphate buffer (pH 7.4) and stored at -80°C for protein determination.

Analytical procedure (LC-MS)

The HPLC system used for the analysis of 17-OHPC and its metabolites was a Waters 2695 model (Waters Corporation, MA, USA). Separation was performed on Waters C18 Symmetry (3.5 μ m, 2.1 \times 150 mm) analytical column at 40°C with a Waters C18 Symmetry (2.1 \times 10 mm) guard column. The mobile phases used were: [A] - 5% methanol in water containing 0.1% formic acid and [B] - methanol containing 0.1% formic acid. The total run time was 45 min at a flow rate of 0.2 ml/min. A gradient profile was used starting from a mobile phase containing 50% solution [B], increased linearly to 95% [B], and followed by returning to the initial condition of 50% [B] to achieve the base line. Medroxyprogesterone acetate was used as the internal standard [19].

Analysis was performed on a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with positive electrospray ionisation mode. For all analytes and internal standard, MS settings used were as follows: capillary voltage 3.5 kV; source temperature 100°C; desolvation temperature 300°C; cone gas flow (L/hr) 50; desolvation gas flow (L/hr) 550; argon pressure 20 \pm 10 psig; nitrogen pressure 100 \pm 20 psig. The LC-MS system was controlled and data collected with Masslyn^x version 4.1.

Data analysis

Data are expressed as the mean \pm SD. Apparent kinetic parameters for 17-OHPC metabolism were estimated by the non-linear least square regression analysis using GraphPad Prism 4.0 (GraphPad Software Inc.). Half life estimates were obtained by WinNonlin 4.1 (Pharsight Corp.) using loss of 17-OHPC as the measure.

Results

Metabolism of 17 α -hydroxyprogesterone caproate (17-OHPC)

Metabolic profiles generated by incubation of 17-OHPC with microsomes derived from various species (Mouse, Rat, Rabbit, Pig,

Dog and Baboon) were compared with human liver microsomes and primary human hepatocytes. The profile of major metabolites (Figure 1) analyzed by LCMS revealed a similarity amongst the animal species and humans. Extensive metabolism of 17-OHPC was observed in all the species tested. The metabolism was observed to be NADPH dependent, with no metabolite being observed in its absence (Figure

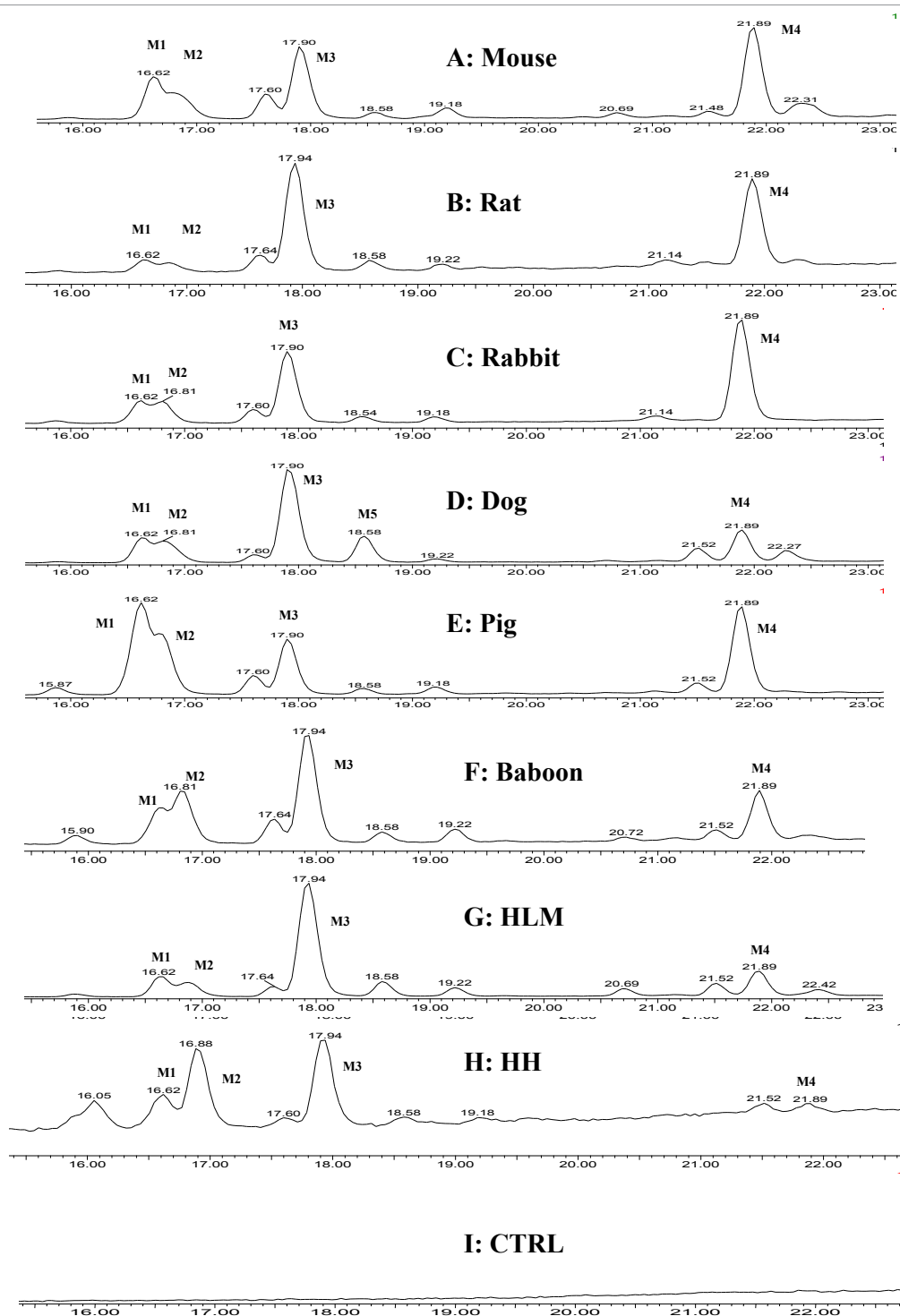


Figure 1: LCMS chromatograms depicting 17-OHPC metabolite (M1-M4) profile generated from incubations in various species, namely, Mouse (A, liver microsomes), Rat (B, liver microsomes), Rabbit (C, liver microsomes), Dog (D, liver microsomes), Pig (E, liver microsomes), Baboon (F, liver microsomes), Human (G, liver microsomes and H, primary hepatocytes) and CTRL (I).

1I) thus, indicating the involvement of cytochrome P450s in the metabolism of 17-OHPC. Four peaks were observed to be common in all species including humans, and constituted the major metabolites (M1-M4) having $m/z=445$. The fourth metabolite (M4) was not the major metabolite in human hepatocytes. A fifth metabolite (M5) was one of the major metabolites only in dog. All five metabolites (M1-M5) were monohydroxylated derivatives of 17-OHPC, with the hydroxyl moiety attached at different positions on the molecule, thus explaining the different retention times, but same m/z .

The metabolite profile generated by baboon liver (Figure 1F) was very similar to the human microsomes (Figure 1G) and hepatocytes (Figure 1H) with M3 being the major metabolite having a retention time of 17.94 mins. Profile generated by rat liver (Figure 1B) microsomes was also, qualitatively, similar to human with M3 being the major metabolite. Metabolite profile in dog liver microsomes (Figure 1D) was similar to humans except the formation of M5 which was observed to be generated in significant amounts. M1 was the major metabolite generated in pig (Figure 1E); M4 was the major metabolite in rabbit (Figure 1C) and Mouse (Figure 1A) liver microsomes. No metabolite formation was observed in the control sample (Figure 1I) when NADPH was not added.

A similar profile depicting the generation of three major metabolites having $m/z=445.0$ was observed in incubations involving expressed CYP3A4/3A5 system. Further, M3 was the major metabolite generated by both isoforms evaluated (Figure 2). Although M4 was observed in

both isoforms, however, the amounts generated were insufficient to characterize the kinetics.

Kinetics of 17-OHPC metabolism

The formation of monohydroxylated metabolites (M1-M4) followed Michaelis Menten kinetics in all the species studied. Further, a monophasic behavior was observed indicating that only one enzyme was primarily involved in the metabolism of 17-OHPC. The results were confirmed by analysis using GraphPad Prism (one enzyme vs. two enzyme and Michaelis Menten vs Hill's equation) and affinity constant (K_m) and the maximum enzyme velocity (V_{max}) were calculated. The kinetic parameters are reported in table 1. The rank order of metabolites generated in various species was: Mouse (M4>M3>M1>M2), Rabbit (M4>M3>M2>M1), Pig (M1>M2>M4>M3), Dog and Baboon (M3>M2>M1>M4) and Human and Rat (M3>M4>M1>M2). The rank order observed in human hepatocytes depicted M3 as the major metabolite followed by M2 and M1.

The rank order of observed V_{max} for M3 in microsomes (the major metabolite in humans) from different species was Human>Dog>Baboon>Pig>Mouse>Rabbit>Rat.

Monophasic, michaelis menten kinetics was observed for the monohydroxylated metabolites generated by expressed CYP3A4 and CYP3A5 isoforms (Figure 3). The calculated kinetic parameters are reported in table 2. The rank order observed in both isoforms was similar with M3>M1>M2. Although similar V_m values were observed for CYP3A4 and CYP3A5, however, CYP3A5 had significant lower K_m

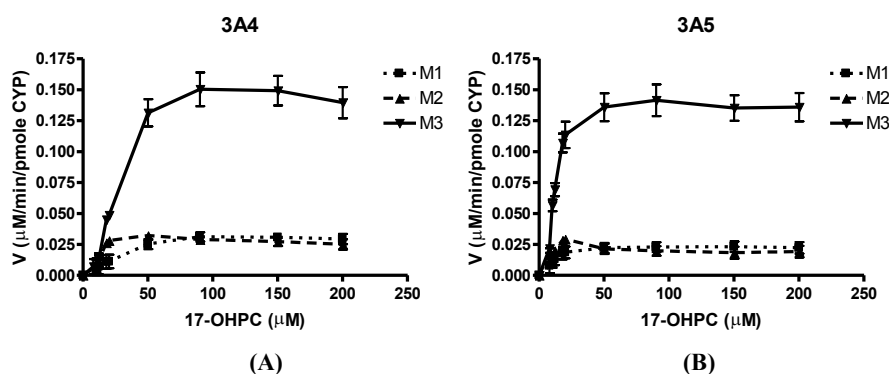


Figure 2: Kinetic profiles of 17-OHPC metabolites, M1 (■), M2 (▲), M3 (▼) observed in expressed CYP3A4 and CYP3A5. All the experiments were performed in triplicate. The metabolites levels are expressed in terms of 17OHPC equivalents.

	M1		M2		M3		M4	
	V_m	K_m	V_m	K_m	V_m	K_m	V_m	K_m
MOUSE	0.34 ± 0.05	86.25 ± 34.0	0.28 ± 0.03	48.12 ± 18.1	0.52 ± 0.07	64.1 ± 27.7	0.59 ± 0.07	239.2 ± 55.1
Rat	0.04 ± 0.01	59.02 ± 16.5	0.03 ± 0.01	17.3 ± 8.6	0.33 ± 0.02	74.2 ± 8.1	0.19 ± 0.02	53.12 ± 14.2
Rabbit	0.14 ± 0.01	50.22 ± 10.2	0.16 ± 0.01	21.4 ± 3.3	0.50 ± 0.02	71.0 ± 5.3	0.59 ± 0.07	122.8 ± 39.1
Dog	0.31 ± 0.06	115.85 ± 38.8	0.39 ± 0.06	76.2 ± 32.0	1.20 ± 0.14	75.1 ± 32.0	0.12 ± 0.03	128.5 ± 68.5
Pig	1.02 ± 0.32	73.0 ± 21.1	0.56 ± 0.12	53.6 ± 19.7	0.57 ± 0.1	90.21 ± 22.6	0.65 ± 0.08	120.1 ± 35.5
Baboon	0.16 ± 0.03	59.2 ± 8.8	0.26 ± 0.04	37.78 ± 6.5	0.68 ± 0.1	80.5 ± 15.2	0.15 ± 0.01	162 ± 22.1
Human	0.36 ± 0.1	96.1 ± 10.1	0.26 ± 0.02	41.2 ± 7.1	1.51 ± 0.4	78.4 ± 14.1	0.62 ± 0.05	674 ± 73.15
Hepatocytes	0.39 ± 0.13	35.58 ± 40.1	0.97 ± 0.26	56.1 ± 38.7	1.7 ± 0.52	67.6 ± 49.4	ND	ND

V_m : $\mu\text{M}/\text{min}/\text{mg}$ protein
 K_m : μM
 ND – Not Detectable

Table 1: Kinetic parameters for 17-OHPC hydroxylated metabolites (M1-M4) observed in Rabbit, Rat, Dog, Mouse, Pig, Baboon, Human Liver Microsomes and Human Hepatocytes. All the experiments were performed in triplicate and the values are reported as mean \pm SD. The kinetic parameters were calculated by expressing metabolite levels in terms of 17-OHPC equivalents.

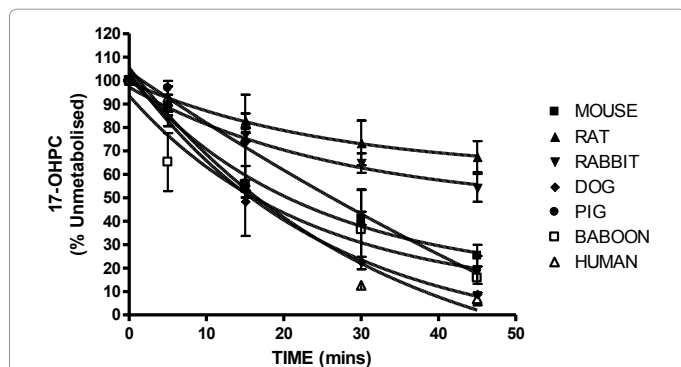


Figure 3: Kinetic profile of loss of 17-OHPC via CYP mediated metabolic pathway in liver microsomes obtained from various animal species, including, Mouse (■), Rat (▲), Rabbit (▼), Dog (◆), Pig (●), Baboon (□) and Human (△). All the experiments were performed in duplicate and the values are reported as mean \pm SD.

	M1		M2		M3	
	V_m	K_m	V_m	K_m	V_m	K_m
CYP 3A4	0.04 \pm 0.006	40.1 \pm 16.1	0.032 \pm 0.003	10.5 \pm 4.8	0.20 \pm 0.02	57.5 \pm 14.7
CYP 3A5	0.025 \pm 0.003	10.1 \pm 5.1	0.021 \pm 0.002	1.5 \pm 1.8	0.16 \pm 0.01	14.5 \pm 2.7

V_m : μ M/min/ pmol CYP
 K_m : μ M

Table 2: Kinetic parameters for 17-OHPC hydroxylated metabolites (M1-M3) in expressed CYP3A4 and CYP3A5. All the experiments were performed in triplicate and the values are reported as mean \pm SD.

values than CYP3A4. This indicated that 17-OHPC had much higher affinity for CYP3A5 than for CYP3A4.

The half life for 17-OHPC metabolism was calculated based on loss of 17-OHPC as the measure. Human liver was observed to have the fastest rate of 17-OHPC elimination and rat liver showed the slowest rate. The rank order of half lives for loss of 17-OHPC in the microsomal preparations was – Rat>Rabbit>Mouse>Pig>Baboon>Dog>Human (Figure 3 and Table 3).

The metabolite profile (Figure 4) generated in the 17-OHPC clearance study showed a decline in metabolites with time. The decline was highly significant in the case of human liver microsomes. A similar profile was seen in the case of baboon liver and to some extent in pig, dog and rabbit liver although the decrease in the amount of metabolite was not as significant as observed in the case of human liver. An increase in the amount of metabolites generated with time was observed in the case of rat and mouse although the increase was not linear with time, possibly due to the lack of substrate i.e. unmetabolized 17-OHPC. These observations may indicate predominance of sequential metabolism in certain species.

Inhibition study

We have previously shown in human liver microsomes and primary human hepatocytes that CYP3A isoforms are the major enzymes involved in the metabolism of 17-OHPC. To evaluate whether the CYP3A or cyp3a isoform subfamily is the major pathway in other animal species we performed *in vitro* microsomal incubations using a typical inhibitor, ketoconazole (KTZ). The results of incubating KTZ with microsomes are shown in figure 5. The IC_{50} (\pm SD) values estimated for inhibition of 17-OHPC metabolism in various species were – Human (0.20 \pm 0.03), Pig (0.27 \pm 0.03), Baboon (0.45 \pm 0.04),

Mouse (0.23 \pm 0.06), Rat (1.34 \pm 0.07), Rabbit (0.067 \pm 0.02) and Dog (8.5 \pm 1.2). A concentration dependent inhibition of 17-OHPC metabolism was observed in all the species investigated. The maximum amount of inhibition observed in various microsomal incubations was – Human (91%), Pig (93%), Baboon (92%), Mouse (93%), Rat (91%), Rabbit (98%) and Dog (83%). These observations indicate that CYP3A/cyp3a are the major enzymes responsible for the metabolism of 17-OHPC in mouse, rat, rabbit, dog, pig, baboon and human.

Discussion

Previously reported studies involving elucidation of 17-OHPC metabolism have shown the drug to be extensively metabolized by human liver and CYP3A to be the major enzyme responsible for metabolism of 17-OHPC [11,12]. Monohydroxylated-17OHPC, mediated by CYP3A, has been reported to be the major metabolic product of metabolism. The present study was performed to evaluate and compare the metabolism of 17-OHPC in various animal species, namely, Mouse, Rat, Rabbit, Pig, Dog and Baboon with Human. The

	MLM	RatLM	RbLM	DLM	PLM	BLM	HLM
T_{half} min	55 \pm 11	289 \pm 82	128 \pm 16	31 \pm 3	44 \pm 13	41 \pm 2	29 \pm 8

Table 3: Half life estimates of 17-OHPC by microsomes derived from livers of various animal species. All the experiments were performed in duplicate and the values are reported as mean \pm SD.

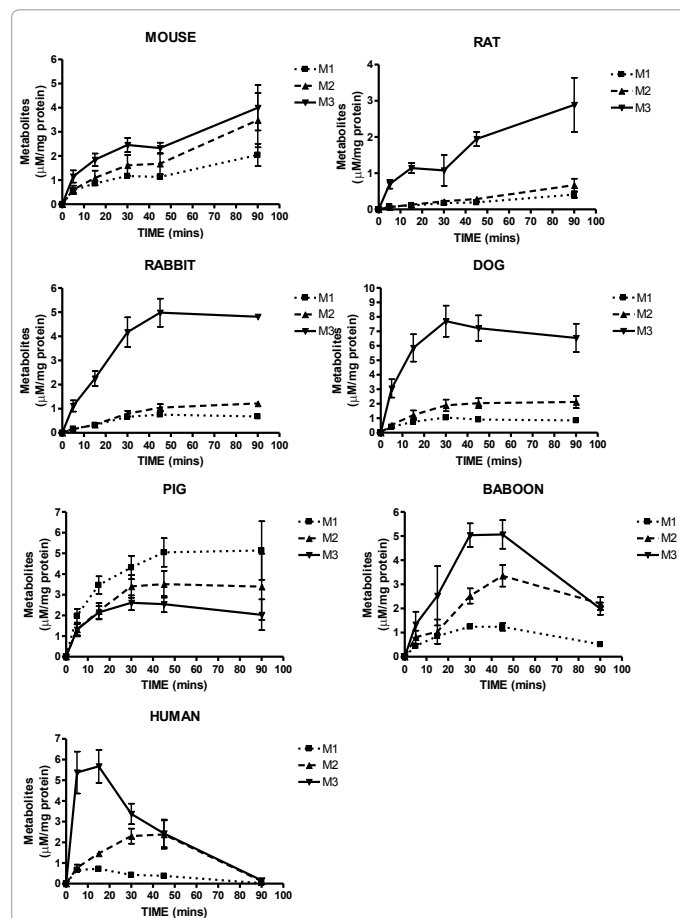
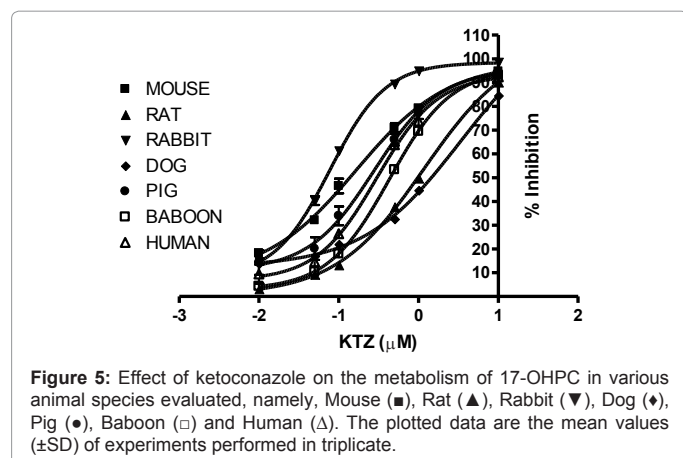


Figure 4: Kinetic profiles of 17-OHPC metabolites, M1 (■), M2 (▲) and M3 (▼) observed in various animal species. All the experiments were performed in triplicate. The metabolites levels are expressed in terms of 17OHPC equivalents.



objective was to find a suitable animal model for conducting *in vivo* pharmacokinetic and drug-drug interaction studies.

The profile generated in all the species tested indicated the formation of mono-hydroxylated 17-OHPC compounds as major metabolites. This confirmed CYP3A/cyp3a to be the major metabolic route for 17-OHPC. The qualitative profile of monohydroxylated metabolites formed by baboon and rat liver was very similar to humans with M3 being the major metabolite. However, based on the rank order of the metabolites generated (M1-M4), rat microsomes depicted a profile which was closer to humans (M3>M4>M1>M2) than baboons (M3>M2>M1>M4).

Based on the kinetic parameters calculated from the microsomal incubations for the major monohydroxylated metabolite generated in humans (M3), it was observed that the V_m (and K_m) for dog followed by baboon was the closest to humans with rat showing the lowest values for the parameters.

Estimation of half life of 17-OHPC from loss of drug experiments indicated that human liver had the fastest rate followed by dog and baboon. Further, the metabolite profile generated during clearance study indicated a significant decline in the metabolites with time produced from human and baboon and to some extent in the dog and pig liver microsomes. The decline in the metabolites can be attributed to possible sequential metabolism of monohydroxylated metabolites to generate numerous di- and tri-hydroxylated metabolites. A significant increase in the amount and number of di- and tri-hydroxylated metabolites was observed with time in human microsomes although the quantities observed were too small to be quantified.

We have previously reported ketoconazole to be an inhibitor of CYP3A in human liver microsomes and hepatocytes [11]. The IC_{50} value in human microsomes observed in this study was similar to the previous study. Significant, dose dependent inhibition of 17-OHPC metabolism was observed with the CYP3A inhibitor, ketoconazole in all the species that were evaluated. The IC_{50} values observed were <1 μ M in case of Human, Pig, Baboon, Mouse and Rabbit. This confirmed the major role played by CYP3A/cyp3a in 17-OHPC metabolism since KTZ is reported to be selective for this isoform at concentrations below 1 μ M [20]. An IC_{50} value higher than 1 μ M was observed in the case of Rat and Dog. This suggests the possibility of other CYP isoforms being involved in 17-OHPC metabolism due to the differential selectivity of ketoconazole in different species at higher concentrations.

In conclusion, the study shows that CYP3A/cyp3a is the major

pathway by which the monohydroxylated metabolites of 17-OHPC are formed by various animal species although, the position of hydroxyl substitution in the major metabolite was observed to be different for baboon, rats, dogs, humans (M3); Pig (M1) and Rabbit, Mouse (M4). Further, the baboon and rat seem to provide suitable models for evaluating the pharmacokinetics of 17-OHPC in animals and screening for potential drug-drug interactions.

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