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Metabolism of 17α-hydroxyprogesterone caproate by hepatic and placental microsomes of human and baboons

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ABSTRACT

Recent data from our laboratory revealed the formation of an unknown metabolite of 17 hydroxyprogesterone caproate (17-HPC), used for treatment of preterm deliveries, during its perfusion across the dually perfused human placental lobule. Previously, we demonstrated that the drug is not hydrolyzed, neither *in vivo* nor *in vitro*, to progesterone and caproate. Therefore, the hypothesis for this investigation is that 17-HPC is actively metabolized by human and baboon (*Papio cynocephalus*) hepatic and placental microsomes. Baboon hepatic and placental microsomes were investigated to validate the nonhuman primate as an animal model for drug use during pregnancy. Data presented here indicate that human and baboon hepatic microsomes formed several mono-, di-, and tri-hydroxylated derivatives of 17-HPC. However, microsomes of human and baboon placentas metabolized 17-HPC to its mono-hydroxylated derivatives only in quantities that were a fraction of those formed by their respective livers, except for two metabolites (M16′ and M17′) that are unique for placenta and contributed to 25% and 75% of the total metabolites formed by human and baboon, respectively. The amounts of metabolites formed, relative to each other, by human and baboon microsomes were different suggesting that the affinity of 17-HPC to CYP enzymes and their activity could be species-dependent.

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1. Introduction

Preterm delivery (<37 weeks of gestation) is a leading cause of perinatal and neonatal morbidity and mortality. The benefits of 17α-hydroxyprogesterone caproate (17-HPC) in reducing the rate of recurrent preterm delivery in women with history of prior spontaneous preterm birth have been demonstrated in a recent clinical trial [1]. These findings renewed the interest of clinical and basic scientists in investigating the role of natural and synthetic progestins in preventing preterm delivery. However, reported data on the efficacy and clinical benefits of 17-HPC have been controversial [2,3].

During the last 3 years, the newly formed Obstetric-Fetal Pharmacology Research Units Network of NICHD sponsored investigations of the pharmacokinetics (PK) and pharmacodynamics (PD) of 17-HPC. At our site, University of Texas, the investigations focused on placental transfer and metabolism of 17-HPC as well as validating the nonhuman *Papio cynocephalus* (baboon) as an animal model for determining the PK and PD of drugs in the pregnant patient. The choice of the baboon is for the following reasons: its 95% DNA homology with humans [4]; anatomical and physiological similarities in fetal development [5]; placental shape, structure and maternal-fetal integration, organization and functions are almost

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identical with humans [6]. In addition, several CYP450 enzymes and their activities were identified and characterized in primates [7], and only a few interspecies biochemical differences were reported between them and humans [8–10].

Recent investigations in this laboratory on the *in vitro* hydrolysis of 17-HPC, radiolabeled in the progesterone [^{3}H] and caproate [^{14}C] revealed that the drug is neither hydrolyzed by human plasma, nor homogenates of human liver, term and preterm placentas [11]. Subsequent investigations on human placental transfer, metabolism and distribution of the radiolabeled 17-α-hydroxy-[^{3}H] progesterone [^{14}C] caproate utilizing dual perfused placental lobule revealed that 17-HPC was transferred to the fetal circuit, metabolized and retained by the placental tissue. The metabolite formed was more polar than the parent compound and was also transferred to the fetal circuit. Both the parent compound and its metabolite retained both [^{3}H] and [^{14}C] confirming its metabolism by a pathway that does not include its hydrolysis by human placental tissue [12]. To the best of our knowledge, data on 17-HPC metabolism via alternative pathway(s) are non-existent.

Human liver is the primary organ responsible for the metabolism of drugs and xenobiotics. However, during pregnancy, the placenta acts as a functional barrier, by virtue of its metabolic enzymes and efflux transporters, thus protecting the fetus from drugs and environmental toxins. Placental metabolic enzymes can contribute up to approximately 10% of the metabolism of a drug, which is in concert with the fetal circulation being approximately 4% of the maternal [13]. It should be noted that metabolite(s) formed by placental tissue should be more accessible to the fetal circulation than those formed by maternal liver.

Therefore, the hypothesis for this investigation is that 17-HPC is metabolized by human and baboon hepatic and placental microsomes.

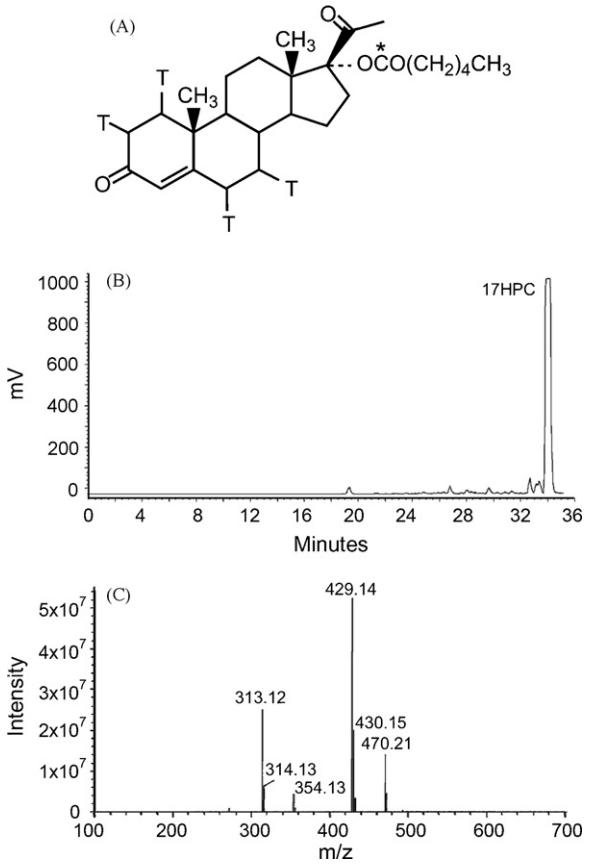

Fig. 1 – (A) The structure of 17α-hydroxyprogesterone caproate depicting the position of radioactive nucleotides [^{3}H and ^{14}C] in the 17-HPC utilized; T is for tritium and "*" for [^{14}C]. (B) Representative HPLC chromatograms for a standard of [^{3}H, ^{14}C]-17-HPC monitored in the ^{14}C channel and (C) mass spectrum of 17α-hydroxyprogesterone caproate.

2. Materials and methods

2.1. Chemicals and supplies

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned. Acetonitrile (Optima) and methylene chloride were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 17α-Hydroxy[1,2,6,7-^{3}H]-progesterone [1-^{14}C] caproate (Fig. 1A) was custom synthesized by RTI International (Research Triangle Park, NC, USA). The specific activity of 17-HPC was 26.3 mCi/mmol for [^{14}C] and 52.6 mCi/mmol for [^{3}H].

2.2. Human and baboon tissues

A pool of 15 donor human liver microsomes was purchased from CellzDirect (Austin, TX). Human placentas were obtained from term uncomplicated pregnancies of women delivering at the John Sealy Hospital, the teaching hospital of the University of Texas Medical Branch, Galveston, Tx, according to a protocol approved by the Institutional Review Board. Baboon placentas and livers were obtained by abdominal delivery and from animals sacrificed for herd reduction, respectively. Animal tissues were obtained according to an approved protocol by the Institutional Animal Care and Use Committee (IACUC) of the Southwest National Primate Research Center (San Antonio, TX, USA). Microsmal fractions were prepared from human placentas, baboon livers and placentas by differential centrifugation as previously described [14]. Pools of microsomes, utilized in this investigation, were prepared from 19 human and 12 baboon placentas and were used in all the experiments reported here.

2.2.1. Metabolism of 17-HPC by human and baboon microsomes

The activity of microsomes in metabolizing 17-HPC was determined in a reaction solution made of the following components at their respective final concentrations: 17-HPC, 60 μM; microsomal protein, 1 mg/ml of hepatic or 2 mg/ml of placental; 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 0.95 ml. The contents were preincubated for 5 min at 37 °C and the reaction initiated by the addition of 50 μl NADPH regenerating system (0.4 mM NADP, 4 mM glucose-6-phosphate, 1 U/ml glucose-6-phosphate dehydrogenase and 2 mM $MgCl_2$). The reaction was incubated at the same temperature for 60 min and terminated by the addition of

100 μl trichloroacetic acid (10%, w/v). Reaction samples at time 0 and 60 min incubation without substrate or NADPH-regenerating system served as controls. All reactions were performed at least three times in duplicates.

2.2.2. *Quantitative determination of 17-HPC metabolites formed by human and baboon microsomes*

The radiolabeled isotope ([^{3}H] 1.05 μCi and [^{14}C] 0.62 μCi) and unlabeled 17-HPC (60 μM) were combined, added to the reaction solution, and incubated under the same experimental conditions described above. The specific activity was then calculated. The radioactivity for the effluent of HPLC was monitored by an online detector in the [^{3}H] and [^{14}C] channels simultaneously. Fractions (0.5 ml) of the effluent were collected and the amount of each metabolite formed in each peak was determined and was calculated as percent of 17-HPC added to the reaction.

2.3. *Preparation of samples, extraction, and recovery*

17-HPC and its metabolites were twice extracted from the reaction solution with two volumes of methylene chloride and centrifuged at 3500 × *g* for 10 min. The organic layers obtained from the two extractions of each sample were combined, siphoned, and evaporated to dryness. The residue was reconstituted in 200 μl of 90% acetonitrile. Aliquot of the extract were injected into the HPLC column. The 17-HPC and its metabolites were resolved utilizing reverse phase HPLC and their structures tentatively identified by an online mass spectrometer and their amounts determined by their content of radioactivity.

The extent of 17-HPC recovery by the extraction method used was determined by adding a known amount 17-HPC to the reaction solution, extraction and analysis by liquid chromatography–mass spectrometry (LC/MS) as described above. The intensity of 17-HPC was determined and compared to that obtained after a direct injection of the same amount of 17-HPC into the LC/MS. The recovery of 17-HPC by extraction procedure used was 90%. This method was used because, to the best of our knowledge, the metabolites of 17-HPC detected in this investigation have not been reported earlier and hence there are no synthesized commercially available compounds.

Details for extraction and recovery of the metabolites are as follows: 17-HPC was incubated with hepatic microsomal proteins and the compounds extracted from the reaction solution. An aliquot of the extract was injected for LC/MS analysis and the intensity obtained for each compound was set at 100%. The same amount of the extract was added to the potassium phosphate buffer (pH 7.4) in the presence of NADPH regenerating system and microsomal proteins, extracted and analyzed by LC/MS. The mass intensity of each compound was compared to that set as 100%. Extraction recovery was found to be >85% for all the metabolites.

2.4. *Analytical methods*

2.4.1. *HPLC analysis*

The HPLC system consisted of a Waters 600E multisolvent delivery system, a 2487 dual λ absorbance detector and a 717 autosampler controlled by Millennium chromatography manager (Waters, Milford, MA, USA). The samples were separated on a Waters Symmetry C18 column (5 μm, 150 mm × 4.6 mm). The mobile phases consisted of A: 30% acetonitrile containing 0.25% acetic acid and B: 90% acetonitrile. 17-HPC and its metabolites were eluted by a mobile phase gradient starting with 100% A for 5 min, followed by a linear increase to 81% B over a period of 25 min then maintained for an additional 10 min. The flow rate was 1.2 ml/min. For LC/MS analysis, the effluent from the column was transferred to the LC/MS interface with post-column splitting; approximately 840 μl of the effluent was directed to waste and 360 μl to the mass spectrometer. The amounts of radioactivity in 17-HPC and its metabolites were determined by a β-RAM flow-through on-line detector (model 4, IN/US Systems, Tampa, FL, USA) connected to the HPLC system and controlled by a ScintFlow SA software program (Tampa, FL, USA).

2.4.2. *LC/MS analysis*

The mass spectrometer (Waters EMD 1000 single-quadrupole; Milford, MA) was equipped with an electrospray ion interface (ESI) operated in positive mode and maintained at a temperature of 105 °C. The desolvation gas was liquid nitrogen with a flow rate of 55 l/h at a temperature of 300 °C. The mass resolution is 1 Da (1 amu). The capillary voltage was 2.5 kV, and the cone voltage was 27 V. Full mass scan from 100 to 750 Da was conducted at a rate of 1 scan/s. The molecular ion is *m/z* 429 for 17-HPC, selected-ion monitoring (SIM) at *m/z* 429 ($[M+H]^+$), *m/z* 445 ($[M+OH]^+$), *m/z* 461 ($[M+2OH]^+$) and *m/z* 477 ($[M+3OH]^+$) enabled mass spectral characterization of 17-HPC and its hydroxylated metabolites detected in the total ion current (TIC) chromatograms.

2.5. *Data analysis*

A pool of microsomal preparations from each organ was used to determine the metabolism of radiolabeled 17-HPC in three separate experiments. The mean of the data obtained for each organ was calculated and used for comparison.

3. Results

The structure of 17-HPC (Fig. 1A) reflects the position of the radio-nuclides [^{3}H] and [^{14}C]. The retention time for 17-HPC is 34 min (Fig. 1B). The mass spectrum of 17-HPC revealed its molecular ion $[M+H]^+$ at *m/z* 429 and its progesterone fragment at *m/z* 313 which is a result of a break of the ester bond between the acylcaproate and the hydroxyl- (C-17) of progesterone (Fig. 1C). The acetonitrile in the mobile phase (41 mass units) formed two adducts: one with the parent compound (17-HPC) and the second with the progesterone fragment as revealed by their mass ions at *m/z* 470 and 354, respectively.

3.1. *Metabolism of 17-HPC*

3.1.1. *The metabolites formed by human hepatic microsomes*

Incubation of 17-HPC in presence of human hepatic microsomes resulted in the formation of 21 metabolites as identified by their retention times (HPLC) and the simultaneous

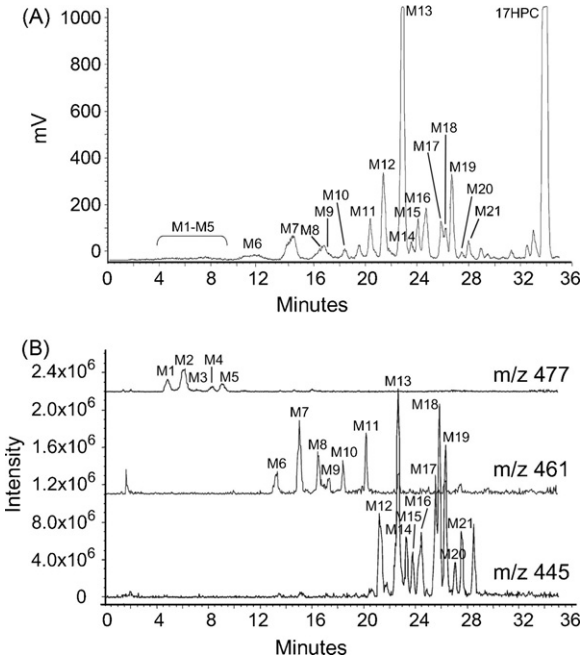

Fig. 2 – (A) Representative HPLC chromatograms for a reaction solution containing [^{3}H, ^{14}C]-17-HPC following its incubation in presence of human liver microsomes in presence of NADPH-regenerating system as monitored in the ^{14}C channel. (B) Extracted ion chromatograms of the metabolites formed (*m/z* 445, 461, and 477) by human liver microsomes and as revealed by HPLC/MS.

monitoring of their radioactivity in the [^{3}H] and [^{14}C] channels. Moreover, the formation of the metabolites in the reaction solution required the presence of an NADPH-regenerating system indicating that the reaction is catalyzed by one or more of the cytochrome P450 isozyme(s). A chromatogram of the components of the reaction solution obtained by monitoring the [^{14}C] channel is shown (Fig. 2A). Each of the 21 metabolites formed retained both [^{3}H], not shown, and [^{14}C] radio-nuclides in the same ratio as 17-HPC (approximately 2:1) indicating that the ester bond was not hydrolyzed.

Further analysis of the same reaction solution by LC/MS revealed that the 21 metabolites could be divided into three groups according to the number of hydroxyl groups introduced into 17-HPC as revealed by their corresponding molecular ions $[M + H]^+$ (Fig. 2B). These three groups are as follows: Group I, trihydroxy- (*m/z* 477); Group II, dihydroxy- (*m/z* 461) and group III, monohydroxy- (*m/z* 445). Furthermore, the fragmentation pattern of the hydroxylated compounds lead us to subdivide the group into subgroups according to the position of the hydroxyl group being in the progesterone (a) or caproate (b) moiety of 17-HPC (Fig. 3A–C).

Group I (trihydroxy-17-HPC) includes five metabolites (M1–M5) with the lowest (by comparison to the other two groups) retention times between 4 and 10 min (Fig. 2A and B). The mass spectrum of each of these compounds revealed the presence of an ion (*m/z* 477), which corresponds to the introduction of three hydroxyl groups (48 mass units) to the parent compound (*m/z* 429) (Fig. 3A, Ia,b). The fragmentation pattern of the trihydroxylated 17-HPC revealed the presence of two ions corresponding to the dihydroxy-progesterone (*m/z* 345) and monohydroxy-caproate (*m/z* 133). Three metabolites (M1–M3; Fig. 3A, Ia,b) exhibited the fragment ion at *m/z* 329, which corresponds to hydroxyprogesterone. The fragmentation of the other two metabolites (M4 and M5; Fig. 3A, Ia,b) revealed the presence of one ion at *m/z* 327, which corresponds to the loss of one molecule of water from the dihydroxy-progesterone (*m/z* 345).

Group II (dihydroxy-17-HPC) includes six metabolites, namely, M6–M11 with retention times ranging between 10 and 21 min (Fig. 2A and B). Evidence for the introduction of two hydroxyl groups is provided by their mass spectra revealing a molecular ion at *m/z* 461. Moreover, the mass spectra for five of these metabolites (M6–M10) revealed the presence of mass ions at *m/z* 329 (monohydroxy-progesterone) and *m/z* 370 (the acetonitrile adduct of mono-hydroxy-progesterone) (Fig. 3B, IIa,b). The presence of a fragment ion at *m/z* 133 indicates that a hydroxyl group was introduced into the acylcaproate of 17-HPC. The sixth metabolite of the dihydroxy-progesterone compounds is M11. The mass spectrum of M11 (Fig. 3B, IIa) revealed the presence of its molecular ion at *m/z* 461 and its acetonitrile adduct (*m/z* 502). Furthermore, the presence of an ion at *m/z* 345 indicates that both hydroxyl groups were introduced into the progesterone nucleus of 17-HPC while the presence of a molecular ion at *m/z* 327 (one molecule of water less than dihydroxy-progesterone) could be explained by the loss of one of the two hydroxyl groups in the progesterone moiety and an adjacent hydrogen.

Group III (monohydroxy-17-HPC) includes 10 metabolites, namely, M12–M21, and had the longest retention times, which ranged between 21 and 30 min (Fig. 2A and B). Their mass spectra revealed the presence of an ion at *m/z* 445 (Fig. 3C, IIIa and IIIb) indicative of the introduction of one hydroxyl group into 17-HPC. The fragmentation pattern of four of these 10 metabolites, namely, M12–M15, revealed an ion at *m/z* 313, progesterone, and its adduct with acetonitrile at *m/z* 354, which were also present in the mass spectrum of 17-HPC (Figs. 1C and 3C, IIIb). Therefore, the hydroxyl group introduced into 17-HPC was most likely in its caproate moiety. On the other hand, the fragmentation pattern of the six remaining metabolites (M16–M21) revealed the presence of a molecular ion at *m/z* 329 indicating that the hydroxyl group was introduced into the progesterone moiety of 17-HPC (Fig. 3C, IIIa).

3.1.2. *The metabolites formed by baboon hepatic microsomes*

The metabolism of 17-HPC by baboon hepatic microsomes revealed the formation of 21 compounds that are identical to those formed by human hepatic microsomes as determined by their retention times and mass spectra. These data suggests that the metabolism of 17-HPC by the hepatic microsomes of the nonhuman primate is identical to that of humans.

3.1.3. *The amounts of 17-HPC metabolites formed by human and baboon livers*

The incubation of 17-HPC in presence of human liver microsomes resulted in the metabolism of approximately 55% of the drug as determined by radionuclide content (Table 1). The amounts of the metabolites in Groups I, II,

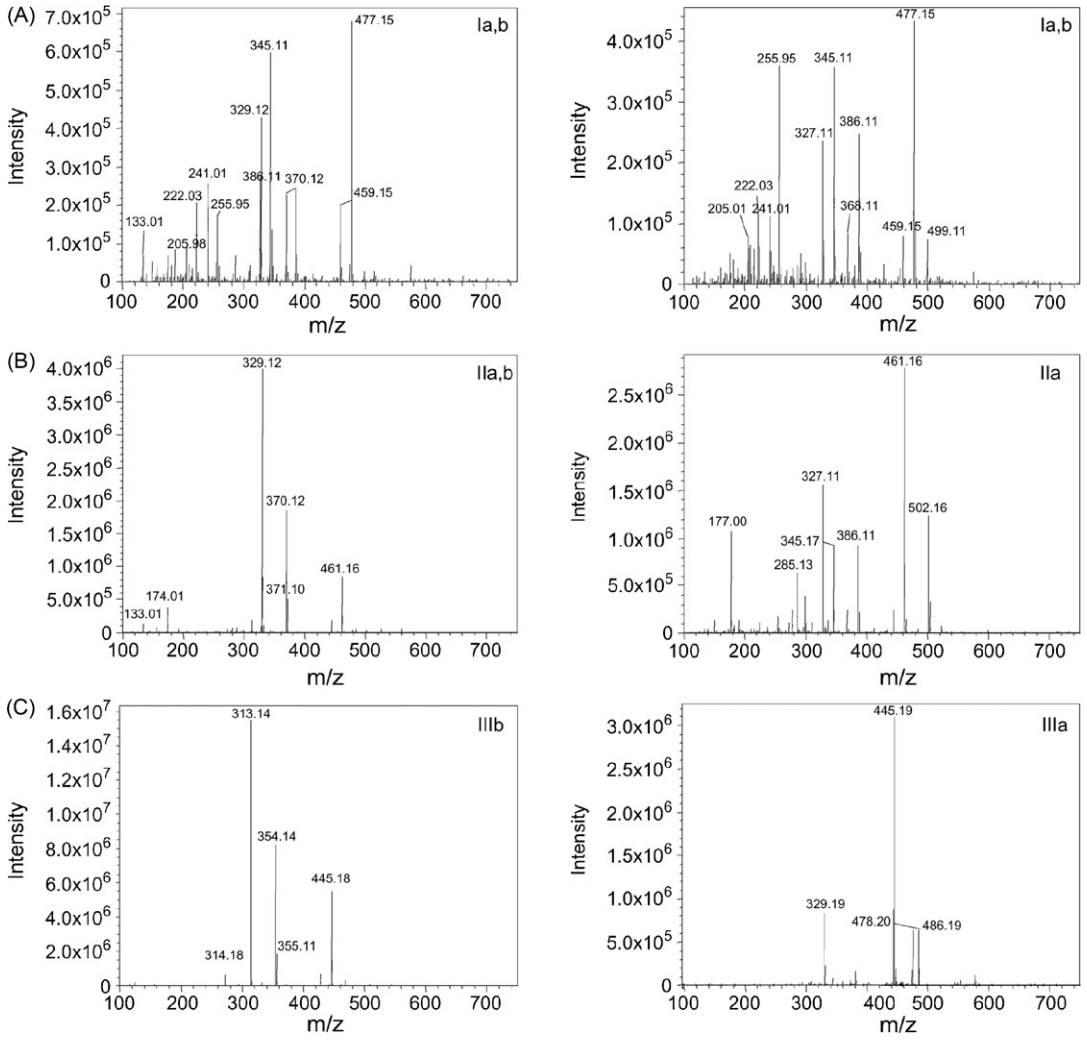

Fig. 3 – Mass spectra of 17-HPC metabolites formed by human hepatic microsomes. The metabolites formed are divided into three main groups (I, II, and II) according to the number of OH groups introduced and sub-groups (a and b) according to the position of OH group being in the progesterone or caproate moieties, respectively: (A) I, tri-hydroxylation (Ia,b: M1–M3; Ia,b: M4–M5); (B) II, di-hydroxylation (IIa,b: M6–M10; IIa: M11); (C) III, mono-hydroxylation (IIIb: M12–M15; IIIa: M16–M21).

and III accounted for <3, 21, and 76%, respectively, of the total metabolites formed.

For Group I (M1–M5), the two major metabolites in this group (M1 and M4) accounted for 65% of the total amounts of tri-hydroxylated metabolites.

For Group II (M6–M11), the amount of M7 represented 32% of the total metabolites formed in this group followed by M8 and M11, which contributed 19% and 23%, respectively (Table 1).

For Group III (M12–M21), M13 contributed 50% of the mono-hydroxylated 17-HPC; thus, it is the major metabolite formed in this group, while M20 contributed the least (1%) (Table 1).

Therefore, the top five metabolites in the amounts formed by human liver microsomes contributed to approximately 70% of the total and were as follows (in descending order): M13, monohydroxy- (38%); M12, monohydroxy- (11%); M19, mono-hydroxy- (8%); M7, dihydroxy- (7%); and M16, monohydroxy- (6%). Each of the remaining 16 metabolites contributed <5% of the total (Fig. 4A).

In the presence of baboon liver microsomes, under identical experimental conditions to those used for human, only 33% of 17-HPC was metabolized and resulted in the formation of the same compounds observed for human liver microsomes. Moreover, the distribution of each group of metabolites formed was also similar to that of humans.

For Group I, the trihydroxy-17-HPC metabolites contributed approximately 2% of the total formed while the di-hydroxy- (Group II) and monohydroxy- (Group III) contributed 19% and 79%, respectively (Fig. 4A).

Therefore, the mono-hydroxylated derivatives of 17-HPC contributed to the majority of the metabolites formed by baboon liver, which were also identical to those formed by human hepatic microsomes.

For Group II, M11 contributed approximately 27% of the di-hydroxylated metabolites (Table 1).

For Group III, M12 and M13 contributed to 68% of the total mono-hydroxylated metabolites. M19 represented 10% and each of the remaining seven monohydroxy-17-HPC

Table 1 – The amounts of 17-HPC metabolites formed by human and baboon hepatic and placental microsomes

Metabolites group	Metabolite	Amounts of metabolite formed							
		Human liver		Baboon liver		Human placenta		Baboon placenta	
		Pico mole	% of each group	Pico mole	% of each group	Pico mole	% of each group	Pico mole	% of each group
I (trihydroxy-17-HPC)	M1	67.5	29.1	34.6	31.9	–	–	–	–
	M2–3	51.5	22.2	25.4	23.5	–	–	–	–
	M4	84.4	36.4	37.4	34.5	–	–	–	–
	M5	28.7	12.4	10.7	9.9	–	–	–	–
II (dihydroxy-17-HPC)	M6	219.8	12.8	143.4	15.9	–	–	–	–
	M7	544.8	31.8	211.7	23.5	–	–	–	–
	M8	326.8	19.1	175.1	19.4	–	–	–	–
	M9	131.6	7.7	63.3	7.0	–	–	–	–
	M10	102.5	6.0	60.8	6.8	–	–	–	–
	M11	389.4	22.7	246.1	27.3	–	–	–	–
III (monohydroxy-17-HPC)	M12	886.0	14.2	1160.5	29.7	33.0	7.9	8.2	7.8
	M13	3138.4	50.3	1499.6	38.4	156.8	37.3	13.0	12.4
	M14	102.4	1.6	73.4	1.9	127.3	30.3	6.1	5.8
	M15	272.3	4.4	106.6	2.7	–	–	–	–
	M16	463.9	7.4	227.3	5.8	–	–	–	–
	M16′–17′	–	–	–	–	103.0	24.5	77.7	74.0
	M17	269.9	4.3	116.2	3.0	–	–	–	–
	M18	259.6	4.2	131.1	3.4	–	–	–	–
	M19	686.7	11.0	403.9	10.3	–	–	–	–
	M20	72.9	1.2	72.8	1.9	–	–	–	–
	M21	82.0	1.3	118.0	3.0	–	–	–	–
Total	8181	–	4918	–	420	–	105	–	

metabolites (M14–M18, M20, and M21) accounted for <6% (Table 1).

The top two metabolites, in amount, formed by baboon liver were M12 and M13, which contributed to 30% and 38%, respectively, of the total formed (Fig. 4A).

3.1.4. *The metabolites formed by human placental microsomes*

Human placental microsomes metabolized 17-HPC to five compounds that retained both radioactive nuclides and required the presence of the NADPH-regenerating system (Fig. 5A). The HPLC chromatogram of the reaction solution revealed, on basis of retention times and mass spectra, the presence of M12, M13, and M15 (Figs. 2A, 5A and 3C, IIIb). Therefore, each of the three metabolites is a mono-hydroxy-17-HPC with the hydroxyl group having been introduced into the acylcaproate (Group IIIb).

The fourth and fifth metabolites had retention times of 25.3 and 25.9 min, respectively, and are designated M16′ and M17′. The designation with (′) is due to the fact that the particular

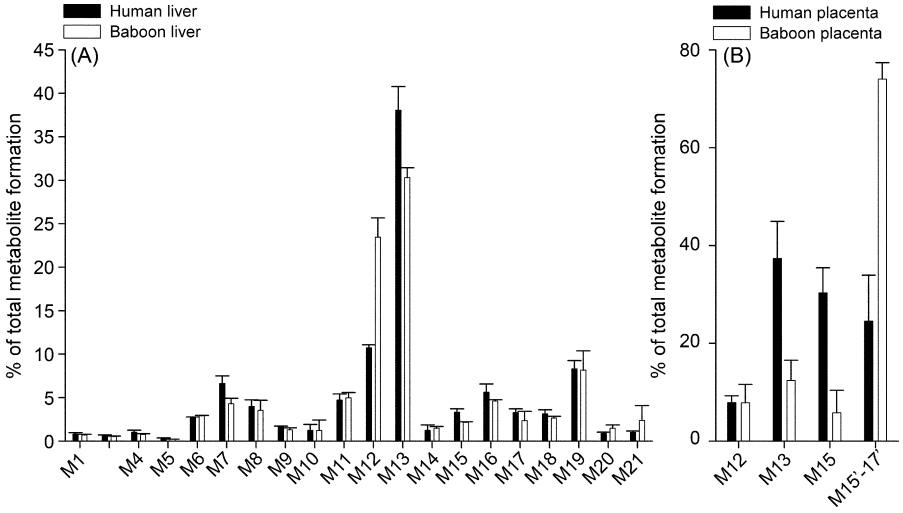

Fig. 4 – Histograms illustrating the amount of each metabolite of 17-HPC as percent of the total formed by each of the four microsomal preparations: (A) human and baboon liver and (B) human and baboon placenta. Each histogram represents the mean for data obtained from three experiments.

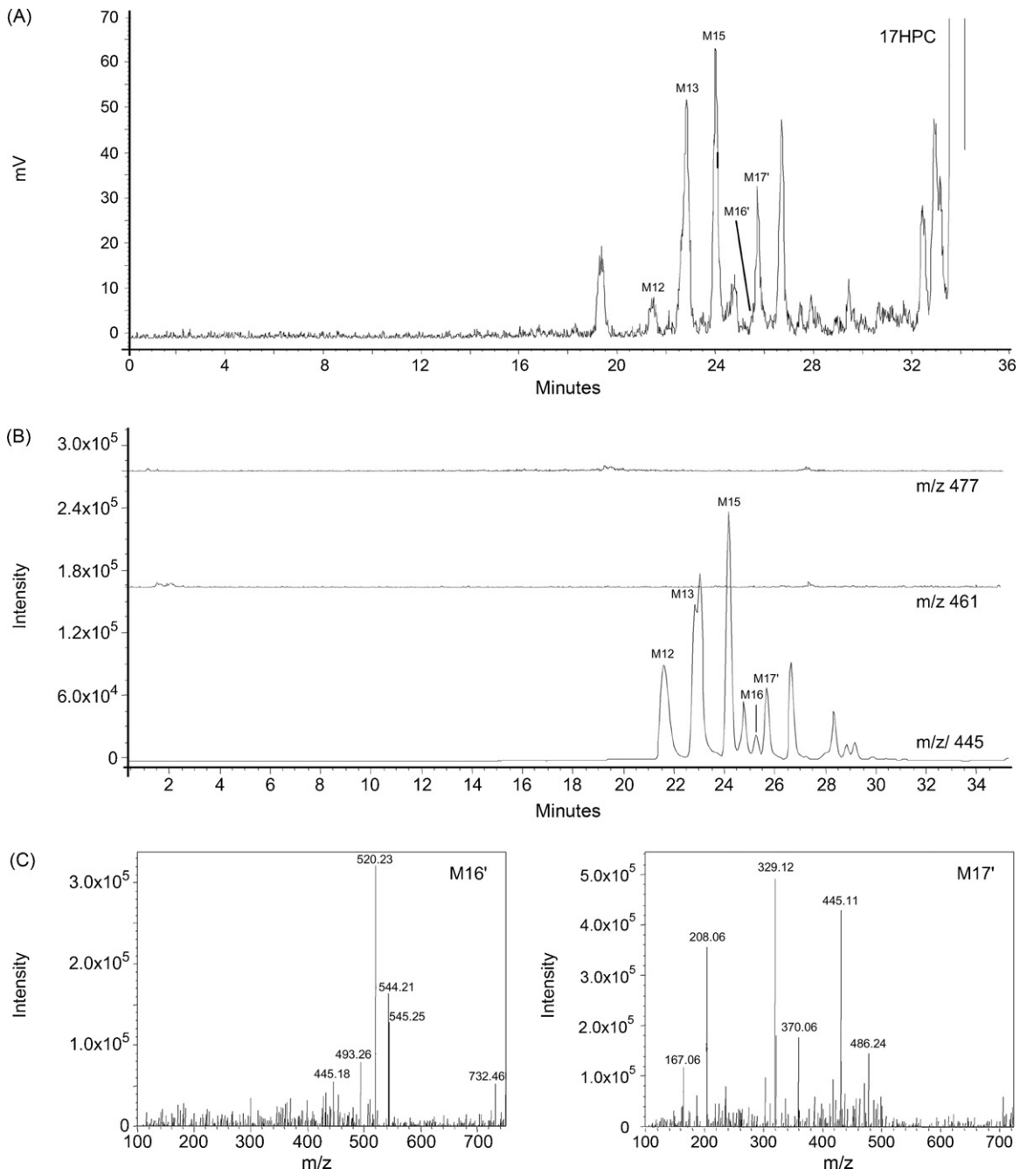

Fig. 5 – (A) HPLC chromatograms for the reaction solution of [^{3}H, ^{14}C]-17-HPC following its incubation with human placental microsomes and monitored in ^{14}C channel. (B) Extracted ion chromatograms of the metabolites formed by human placental microsomes as revealed by HPLC/MS (*m/z* 445, 461, and 477). (C) Mass spectra of 17-HPC metabolites formed by human placental microsomes.

compound was not identified in the reactions catalyzed by baboon and human liver microsomes (Figs. 2A and B and 5A and B). The mass spectrum of M16′ revealed the ion *m/z* 445 but with relatively low intensity and a base peak of the ion *m/z* 520. Therefore, M16′ could be a mono-hydroxylated 17-HPC but could not be ascertained by the data obtained in this investigation. On the other hand, the mass spectrum of M17′ revealed the molecular ion at *m/z* 445 and the fragment ion at *m/z* 329 that are characteristic of hydroxyl-progesterone (Fig. 5C).

3.1.5. *Metabolites formed by baboon placental microsomes*

The metabolism of 17-HPC by baboon placental microsomes revealed the formation of five mono-hydroxylated metabolites that are identical to those formed by human placental microsomes. Therefore, it appears that the

metabolism of 17-HPC by human and baboon placentas is identical.

3.1.6. *Amounts of 17-HPC metabolites formed by human and baboon placentas*

Approximately 3% of the amount of 17-HPC was metabolized by human placental microsomal proteins (Table 1). The amount of M13 and M15 formed accounted for 68% of the total (Table 1 and Fig. 4B). The metabolites designated M16′ and M17′ were not well separated as is apparent from their radiochromatograms and accordingly their amount was calculated on basis of radioactivity under one chromatographic peak representing both of them. The amount of each metabolite formed as a percentage of the total was as follows: M13, 37%; M15, 30%; M16′, and M17′, 25%; and M12, 8%.

Baboon placental microsomes metabolized less than 1% of 17-HPC under identical experimental conditions to those for human placental microsomes (Table 1). The metabolites M16′ and M17′ contributed to approximately 74% of the total. The percent amounts of the three remaining metabolites were as follows: M12, 8%; M13, 12%; and M15, 6% (Table 1 and Fig. 4B).

4. Discussion

The aim of this investigation was to determine whether 17-HPC is metabolized by human and baboon hepatic and placental microsomes. A recent investigation in our laboratory revealed that 17-HPC was not hydrolyzed *in vitro* by human plasma and homogenates of human livers and placental trophoblast tissue [11]. Moreover, the drug was not hydrolyzed during its transfer across the dually perfused placental lobule but an unknown metabolite of 17-HPC was formed and partially identified [12]. These data suggested the presence of a metabolic pathway in placental trophoblast tissue that biotransforms 17-HPC without hydrolysis of the ester bond between progesterone and caproate. Therefore, we investigated the metabolism of 17-HPC by human hepatic and placental microsomes. Moreover, the need for *in vivo* investigations of the effects, PK and PD of the drug during pregnancy required an adequate animal model for obvious ethical and safety considerations and the baboon was chosen for the reasons cited earlier (Section 1) in detail. Accordingly the metabolism of 17-HPC by baboon hepatic and placental microsomes was also investigated to validate the primate as an animal model.

To achieve these goals, we developed an LC/MS method to separate and identify the metabolites of 17-HPC formed by hepatic and placental microsomal preparations from humans and baboons. Under the conditions of the HPLC method developed, 17-HPC and its metabolites were well separated and eluted within 35 min of their introduction into the column. The use of the dual-labeled 17-HPC with [^{3}H] in the progesterone and [^{14}C] in the caproate allowed the monitoring of the ester bond and enhanced the detection limits. It also allowed us to confirm that all the metabolites formed in presence of any of the four microsomal preparations retained both nuclides in the same ratio (2:1) as in the parent compound. This finding further confirms our previous report that 17-HPC is not hydrolyzed *in vitro*. Moreover, all the metabolites formed in the reaction solutions required an NADPH regeneration system indicating that they were catalyzed by one or more CYP isozymes. In addition, the radioactivity allowed the quantitative determination of the metabolites formed, relative to each other and to the substrate 17-HPC, due to the lack of synthesized compounds with identical molecular weight/structure. The molecular weights and fragmentation patterns of the metabolites were determined by LC/MS. Therefore, our data indicate that all the metabolites are hydroxylated derivatives of 17-HPC.

The metabolism of 17-HPC by human hepatic microsomes was extensive, and consequently, the metabolites were divided into three main groups according to the number of hydroxyl groups introduced, namely, mono-, di-, and trihydroxylated. The mass spectra of the fragmentation pattern for the monohydroxy-17-HPC revealed that a hydroxyl group can be introduced either in the progesterone or the caproate. For the dihydroxy-17-HPC, both hydroxyl groups could be introduced into the progesterone or one in the progesterone and the second into the caproate. For the trihydroxy-HPC, two hydroxyl groups were introduced in progesterone and one in the caproate. Therefore, it is apparent that the exact position of the hydroxyl groups introduced into 17-HPC cannot be identified at this time.

The *in vitro* hydroxylation of naturally occurring steroids, including progesterone, has been reported by other investigators as follows: progesterone [15–17], testosterone [15,16,18], androstedione [16], estradiol [19] and esterone [20]. The hydroxylation of synthetic steroid derivatives has also been reported: medroxyprogesterone acetate [21]; Org 4060 and Org 30659 [22]. The hydroxylation of steroids by human hepatic microsomes is possible in the C-2, -4, -6, -15, -16, and -21 positions of the progesterone moiety. Therefore, it appears that *in vitro* hydroxylation of the progesterone moiety of 17-HPC, by hepatic and placental microsomes, could occur in one or more of the positions mentioned above. Moreover, it appears that the introduction of more than one hydroxyl group into the progesterone molecule is also possible. However, to the best of our knowledge, no reports have been made on the introduction of two hydroxyl groups into progesterone or the progesterone moiety of other steroids. Therefore, it could be speculated that the esterification of progesterone with caproate could have resulted in making 17-HPC a ''better'' substrate for CYP isozyme(s) that facilitated its further hydroxylation with a second and possibly a third hydroxyl group. This speculation is based on our data indicating that the majority of 17-HPC metabolites (M1–M5, M6–10, M12–M15) are monohydroxylated in the caproate. Moreover, the mono-hydroxy metabolites (M12–M15 by liver; M12, M13, and M15 by placenta) with the hydroxyl group in the caproate contributed between 53% and 75% of the total metabolites formed with the exception of baboon placental microsomes, which accounted for 26%. Therefore, it appears that one or more CYP isozymes may have a preference to the hydroxylation of caproate over progesterone. One explanation could be offered namely, the presence of caproate in the C-17 position, which hinders the hydroxylation of the progesterone molecule. Indeed, the introduction of an acetoxy group in the C-17 of progesterone diminished the metabolism of progesterone by rabbit liver microsomes [23]. However, the length of

the fatty acid side chain is of importance since the hydroxylation of medroxy-progesterone acetate in human plasma was mainly in the progesterone molecule only (none on the acetate) [24].

The metabolites of 17-HPC formed by baboon liver microsomes revealed mass spectral fragmentation pattern and their retention times on HPLC column identical to those formed by human liver microsomes. Therefore, it is reasonable to conclude that the metabolites of 17-HPC formed by human and baboon hepatic microsomes are the same except for chirality that cannot be determined under the experimental conditions utilized in this investigation.

The extent of 17-HPC metabolism by human placentas was much lower than that by the liver. The fragmentation patterns of the five metabolites detected revealed the presence of monohydroxy-17-HPC only. Two of the metabolites (M16′ and M17′) were formed by placental but not hepatic microsomes, suggesting that the CYP isozymes present in the placenta may be different.

In conclusion, data presented in this report indicate that 17-HPC is metabolized to numerous hydroxylated derivatives of progesterone and caproate without hydrolysis of the parent compound. However, the amounts of metabolites formed by both human and baboon placental microsomes are a fraction of those formed by their respective livers with the exception of M16′ and M17′, which were only formed by placentas. Moreover, the metabolites formed by human and baboon microsomes were identical but their quantities, relative to each other, were not. These findings, if true *in vivo*, indicate that differences in the CYP isozymes of the baboon and human may exist and is currently under investigation in our laboratory.

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