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Title Page

Identification of enzymes involved in the metabolism of 17α-hydroxyprogesterone Caproate (17-OHPC): An effective agent for prevention of pre-term birth.

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Running Title Page

In-vitro metabolism of 17α- hydroxyprogesterone caproate.

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Non Standard Abbreviations

17-OHPC**:** 17-α-hydroxyprogesterone caproate, FHH – Fresh human hepatocytes, HLM – Human liver microsomes. FMO – Flavin containing monooxygenase

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Abstract

Preterm delivery, that is delivery before 37 completed weeks of gestation, is the major determinant of neonatal morbidity and mortality. Until recently no effective therapies for prevention of preterm birth existed. In a recent multicentered trial, 17α-hydroxyprogesterone caproate (17-OHPC) reduced the rate of preterm birth by 33% in a group of high risk women. Limited pharmacologic data exist for this drug. The recommended dose is empiric, the metabolic pathways are not well defined especially in pregnant women, and the fetal exposure has not been quantified. In order to define the metabolic pathways of 17-OHPC we used human liver microsomes, fresh human hepatocytes and expressed enzymes. Human liver microsomes (HLM) in the presence of NADPH generated 3 metabolites; whereas 2 major metabolites were observed with fresh human hepatocytes (FHH). Metabolism of 17-OHPC was significantly inhibited by the CYP3A4 inhibitors ketoconazole and troleandomycin in HLM and FHH. Metabolism of 17-OHPC was significantly greater in FHH treated with the CYP3A inducers, rifampin and phenobarbital. Further, studies with expressed enzymes demonstrated that 17-OHPC is metabolized exclusively by CYP3A4 and CYP3A5. The caproic acid ester was intact in the major metabolites generated indicating that 17-OHPC is not converted to the primary progesterone metabolite, 17-α hydroxyprogesterone (HP). In summary, this study demonstrates that 17-OHPC is metabolized by CYP3A. Since, CYP3A is involved in the oxidative metabolism of numerous commonly used drugs; 17-OHPC may be involved in clinically relevant metabolic drug interactions with co-administered CYP3A inhibitors or inducers.

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Introduction

Preterm birth is the major determinant of neonatal morbidity and mortality ((Mattison, et al., 2001, Paneth, 1995). None of the numerous interventions to reduce the rate of preterm birth including labor inhibiting drugs or strategies such as home uterine monitoring have proven effective. Recent evidence from the Maternal–Fetal Medicine Units Network demonstrated that weekly injections of 250mg 17-OHPC reduced the rate of preterm birth in high risk women by 33% (Meis et al 2003). The dose used in this study was not based on the pharmacologic properties of 17-OHPC but rather on empiric doses used in clinical trials in the 1960s and 1970s. 17α-hydroxyprogesterone caproate (Fig.1A) is 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 (Fig.1C). In animal studies, 17-OHPC produces a longer lasting and more robust progestational effect on the endometrium than progesterone (Wu and Allen, 1959).

The mechanism of action of 17-OHPC is unknown but the progestational effect is likely related to its concentration. We have recently reported plasma concentrations in pregnant women with twins treated with 17-OHPC (Steve Caritis and Venkataramanan, 2007), but limited data exist on how this drug is metabolized and whether the drug or its metabolites reach the fetal circulation. The observation that only a third of cases benefit from treatment with 17-OHPC suggests that variability in 17-OHPC metabolism, or drug interactions may also contribute to the observed variability in clinical response after a fixed dosing regimen.

The purpose of the present study was to identify the various enzymes (cytochromes P450 and FMO enzymes) involved in 17-OHPC metabolism and to characterize the drug's metabolites.

Methods

Chemicals. 17α-hydroxyprogesterone caproate (Mol. Wt. 428.6) was a gift from Diosynth Inc, Chicago, The radioactive isotope of 17α-hydroxy [1, 2, 6, 7-^{3}H]-progesterone [1-^{14}C] caproate was custom synthesized by RTI International (Research Triangle Park, NC). Quinidine, sulfaphenazole, coumarin, ketoconazole, methimazole, α-naphthoflavone, testosterone, 6-β-hydroxytestosterone and NADPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Microsomes derived from baculovirus-infected insect cells were purchased from BD-Gentest (Woburn, MA, USA).

Human Liver Microsomes.

Human liver samples were obtained form Hepatocyte Transplantation Laboratory at the University of Pittsburgh. Donors of human liver tissue had no history of liver disease, but the liver was not used for transplantation.

Preparation of Human Liver Microsomes.

Liver pieces were dissected and kept in cold saline on ice. Liver microsomes were prepared by a standard differential centrifugation procedure with minor modifications (Court and Greenblatt, 1997, Nelson, et al., 2001). Briefly, liver pieces were homogenized with three volumes of a homogenization buffer (50 mM Tris-HCl buffer, 1.0% KCl and 1 mM EDTA, pH 7.4), using an electrical homogenizer (Polytron, Brinkman Instruments, N.Y., U.S.A.). The crude homogenate was centrifuged (Optima XL-100K ultracentrifuge, Beckman Instruments, Palo Alto, CA, USA) at 10,000g for 20 min at 4ºC. The supernatant was further centrifuged at 105,000g for 65 min at 4ºC to sediment the microsomes. The microsomes were reconstituted using a manual homogenizer (Wheaton, Millville, NJ, USA) in twice their weight of Tris HCl buffer (50 mM Tris-HCl buffer, pH 7.4) containing 20% glycerol. Aliquots (1.0 ml) were

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immediately kept in storage at -80ºC until used. The protein content was determined by Lowry's method (Lowry, et al., 1951) using bovine serum albumin (Fluka, USA, 98% pure) as a standard.

Microsomal incubations.

Optimal conditions for the evaluation of the metabolism of 17-OHPC were selected by varying the time of incubation (0 - 180 min) and the microsomal protein concentrations (0 – 2 mg/ml). Different concentrations of 17-OHPC (0 - 200 µg/ml in methanol, final concentration 1%) were incubated with human liver microsomes (0.5 mg/ml, optimum protein concentration) and $MgCl_2$ (10 mM) in 0.1mM phosphate buffer (pH 7.4). The final volume was allowed to equilibrate in a shaking water bath for 5 min at 37 ºC. 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 60 min of incubation (optimum incubation time), the reaction was stopped by immediately adding equal volume of cold methanol. The mixture was centrifuged at 700 RCF for 20 min and supernatant was injected into the HPLC.

Incubations of [^{3}H], [^{14}C] - 17-OHPC were also performed with human liver microsomes. Radiolabeled 17-OHPC was incubated with three individual human liver microsomal preparations for 10 mins using the abovementioned method and the samples analyzed directly by HPLC connected to a radioactivity detector.

Chemical Inhibition Studies.

Various CYP inhibitors were used in the study to identify CYPs which might be involved in the metabolism of 17-OHPC. Human liver microsomal preparations (n=3) were used for the studies with the incubations being performed in triplicate. Experiments were done at different 17-OHPC concentrations (0-200µg/ml expressed as 0-467 µM) spanning the concentrations seen clinically in order to identify the type of inhibition keeping the

inhibitor concentration constant. CYP isoform selective chemical inhibitors were used at the following concentration: CYP1A2 (α – naphthoflavone, 10 μM), CYP2A6 (coumarin, 20 μM), CYP2C9 (sulfaphenazole, 5 μM), CYP2D6 (quinidine, 5μM), CYP3A (ketoconazole, 1.0 μM), and FMO3 (methimazole, 200μM). Additionally, inhibition studies were also carried out by incubating 17-OHPC (25μM) with different concentrations of ketoconazole (0.01 to 10 μ) to determine the IC_{50} for the reaction. The formula used to determine the IC_{50} for microsomal incubations involving 17-OHPC and ketoconazole involves estimating % inhibition which was calculated as follows:

% Inhibition = [(17-OHPC $_{\textbf{without inhibitor}}$ - 17-OHPC $_{\textbf{with inhibitor}}$**) / (**17-OHPC $_{\textbf{without inhibitor}}$**)] * 100**

where, 17-OHPC $_{\text{without inhibitor}}$ is amount of 17-OHPC metabolized in the absence of ketoconazole relative to the total amount of 17-OHPC. 17-OHPC $_{\text{with inhibitor is}}$ amount of 17-OHPC metabolized in the presence of ketoconazole relative to the total amount of 17-OHPC. Subsequently, IC50 values or the inhibition concentration resulting in 50% inhibition of 17-OHPC metabolism, were determined from a plot of the % inhibition versus the logarithm of ketoconazole concentration.

In the case of mechanism based inhibitor like troleandomycin (TAO; 100μM), pre-incubation was done for 30 min at 37°C prior to adding the substrate (17-OHPC, 25μM). In inhibition experiments with ketoconazole (1, 10 μM) the substrate was co-incubated with inhibitor.

Expressed enzyme microsomal 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 pmole of each expressed enzyme tested was incubated for 60 mins with 17-OHPC and the samples analyzed using HPLC-UV.

Preparation of Human Hepatocytes:

Hepatocytes were prepared by a three-step collagenase perfusion technique (Strom, et al., 1996). Hepatocytes were plated on Falcon 6-well culture plates (1.5 x 10^6 cells per well), previously coated with rat tail collagen in HMM supplemented with 0.1 μM insulin, 0.1 μM 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.

Incubations with fresh human hepatocytes:

Briefly, hepatocytes were maintained in culture in the presence of the chemical under study or vehicle control (DMSO 0.1% or MeOH 0.1 %). On the day of the experiment, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and antifungal drugs. Drug stocks were prepared in methanol at 1000-fold incubation concentration (100 mM). Ten microliters of this 100 mM stock were added to a vial containing 10 ml of hepatocyte maintenance media (HMM). Reactions were started by incubating 6-well cell culture plates containing human hepatocytes (1.5 million cells/well) with the drug in HMM 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 (0.1 M, pH 7.4) and stored at -80°C for protein determination.

For acute inhibition experiments (n=4), human hepatocytes were co-incubated with 17-OHPC (25μM) in the presence and absence of inhibitors (troleandomycin and ketoconazole). The samples were incubated for 30 mins and collected as described above. The induction experiments were initiated 24 hrs after plating the cells. The hepatocytes (n=3) were incubated with the inducers (rifampin, phenobarbital and clotrimazole) for 4 days prior to adding HMM/ 17-OHPC (50μM) to estimate the effect of CYP3A induction on 17-OHPC metabolism.

Correlation studies:

Testosterone 6β-hydroxylation was used as the marker for CYP3A activity (Chiba, et al., 1996). Formation rates of 17-OHPC metabolite (M2) were measured using microsomes (n=7) and fresh human hepatocytes (n=7) at a substrate concentration of 100 μM. These rates were correlated with 6-β-hydroxytestosterone formation activity to assess the involvement of CYP3A isoforms.

Analytical Procedure:

HPLC-UV: Analysis of the unmetabolized drug and the potential metabolites obtained from HLM based incubations was performed using a HPLC system equipped with UV detection. The HPLC system comprised of an autosampler (712 WISP, Waters) and solvent delivery system (Waters 501) attached to a UV detector (Waters 486). Chromatography was performed with a 4.6 x 250 mm, 100Å, 5μm Symmetry C18 (Waters, Milford, MA, USA) column. Isocratic elution was performed with a mobile phase of 90% (v/v) methanol in water at a flow rate of 0.8 ml/min, column temperature of 25°C and eluent monitored at 242 nm. The intra- and interday variation expressed as coefficient of variation did not exceed 10% in any of the assays. The concentration of 17-OHPC in samples was quantitated by comparing the peak areas in samples to a standard

curve of the pure drug. The metabolites were quantitated by expressing them in terms of 17-OHPC equivalents.

The concentration of 6β-hydroxytestosterone in the medium was measured by HPLC-UV method as previously described by Kostrubsky et al. (Kostrubsky, et al., 1999). The HPLC system comprised of a LiChrospher 100 RP-18 column (4.6 x 250 mm, 5 μm). 6β-hydroxytestosterone was eluted with a mobile phase of methanol/water (60:40, v/v) at a flow rate of 1.2 ml/min and the eluents were monitored at 242 nm. The concentration of the metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of the metabolite.

LC-MS/MS: Samples obtained from fresh human hepatocyte based incubations were analyzed by LC/MS (Thermo Electron, San Jose, CA, USA) system consisting of a Surveyor quaternary LC pump, a Surveyor autosampler, coupled to a triple quadrupole mass spectrometer (TSQ Quantum) and equipped with an atmospheric pressure ionization (API) electrospray (ESI) interface. Instrument control and data acquisition was performed with the Xcalibur software (Thermo Electron, 2.0). MS/MS conditions for the analytes were optimized by pump infusion of 17-OHPC stock solutions using the Quantum Tune Master® software (Thermo Electron). The HPLC column used was a Symmetry® C18 (150 × 2.1 mm, 3.5 μ) with an appropriate guard column (10 × 2.1 mm; Symmetry). The mobile phases used were: [A] – water (0.1% formic acid) and [B] – acetonitrile (0.1% formic acid). The total run time was 35 min at a flow rate of 0.2 ml/min. A gradient profile was used starting from a mobile phase containing 10% solution [B], increased linearly to 90% [B] over first 15 min, isocratic 90% [B] for 10 min, followed by returning to the initial condition of 10% [B] to achieve the base line.

Radio-HPLC: Analytical separations were achieved using conditions similar to abovementioned HPLC method. The metabolites were analysed using a Radiomatic

Model 525TR/FLO-ONE flow-through radioactivity detector (PerkinElmer Life Sciences, USA), and peak areas were integrated with Windows-based FLO-ONE version 3.61.

Data Analysis

Data are expressed as the mean ± SD. Student's t-test was used to assess the significance of results. IC_{50} was calculated using GraphPad Prism 4.0 (GraphPad Software Inc., USA).

Results

Metabolism of 17-OHPC by CYPs in human liver microsomal and fresh human hepatocyte preparations.

Incubation of 17-OHPC with human liver microsomes resulted in the generation of three ($M1\text{-}M3_{HLM}$) main metabolites wherein ($M2_{HLM}$) was the major metabolite. The formation of metabolites was observed to increase upto 60 minutes using a microsomal protein concentration of 0.5 mg/ml. Hence, the abovementioned conditions were used for all incubations unless specified otherwise. The formation of metabolites ($M1_{HLM}$ and $M3_{HLM}$) was low compared to $M2_{HLM}$, thus, accurate data could not be obtained for these minor metabolites and the paper focuses on the major metabolite ($M2_{HLM}$). Incubation of 17-OHPC with fresh human hepatocytes resulted in the generation of five metabolites ($M1\text{-}M5_{HH}$). The major metabolite generated was $M2_{HH}$ which was used to characterize 17-OHPC metabolism in fresh human hepatocytes. Fig. 2 depicts the chromatograms obtained using LC-UV and MS for analysis. Fig. 2 (A, B) shows the metabolism of 17-OHPC in microsomal incubates in the presence/ absence of NADPH. All the metabolites were formed in a NADPH-dependent manner only. Fig. 2 (C) shows the metabolism of 17-OHPC in fresh human hepatocytes incubated for 1 hour.

Incubation of radiolabeled 17-OHPC with HLM generated one major and two minor metabolite peaks (data not shown). The recovery of radioactivity from the incubations was 85-97%. The major metabolite constituted approximately 60-65% of the metabolized 17-OHPC and the two minor metabolites were estimated to be approximately 15%. The remaining 20% of the metabolite could not be accounted for due to the limitations of analytical method used. The two (***[^{14}C, ^{3}H]***) labels on the 17-OHPC molecule were observed to remain intact in the metabolites generated confirming that the caproate side chain or the ring structure was not cleaved during metabolism in human livers.

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Metabolic Profiles in Human Liver Microsomes and Hepatocytes

Metabolism of 17-OHPC was evaluated over time using human liver microsomes and hepatocytes. The concentrations of unmetabolized 17-OHPC and the major metabolite (expressed in terms of 17-OHPC equivalent) were determined over a time period of 0 – 180 mins incubation. Fig. 3 (A, B) shows that the concentration of 17-OHPC decreased in a time-dependent manner and the concentration of the metabolite increased proportionately. Approximately, 60% of the parent drug (17-OHPC) was metabolized within 60 minutes and $M2_{(HLM, HH)}$ accounted for almost 50% of metabolized 17-OHPC.

Identification of Human P450 Isoforms

Incubations with human liver microsomes: 17α-hydroxyprogesterone caproate was incubated with microsomes for 60 min in the presence of various representative inhibitors of P450 isoforms. A similar reaction was performed in the absence of the inhibitors, and the rate of major metabolite ($M2_{HLM}$) generated was compared between the two conditions to identify the isoforms responsible for 17-OHPC metabolism. As summarized in Fig. 4, α-naphthoflavone, sulfaphenazole, coumarin, quinidine and methimazole did not inhibit the metabolism of 17-OHPC by the corresponding CYPs. In contrast, the amount of $M2_{HLM}$ was significantly decreased in the presence of ketoconazole, indicating that the metabolism of 17-OHPC was markedly inhibited. The Vmax (0.29±0.02 nmole/min/mg) and Km (77.94±19.4μM) values for $M2_{HLM}$ were observed to decrease significantly in the presence of ketoconazole (Vmax=0.03±0.002, Km=15.38±3.9). An IC50 (0.17 μM) value for the inhibition of 17-OHPC metabolism by ketoconazole in human liver microsomes (n=3) was also calculated (Fig. 5).

Incubations with baculovirus expressed human CYP isoforms: Studies were also performed in baculovirus-infected insect cells expressing various CYPs. The metabolizing activity of each CYP3A isoform for 17 α-hydroxyprogesterone caproate

was compared to control microsomes, which were devoid of any CYP activity. The results indicate the involvement of CYP3A4/5 in the metabolism of 17-OHPC and the formation of the major metabolite ($M2_{HLM}$) was a result of CYP3A4/5 pathway (Table 1).

Incubations with Human Hepatocytes:

Inhibition Studies: Results obtained from inhibition experiments performed in HLM were confirmed in fresh adult human hepatocytes (Fig. 6) using chemical inhibitors for CYP3A (ketoconazole and troleandomycin). Troleandomycin and ketoconazole inhibited 17-OHPC metabolism ($M2_{HH}$ formation) by 75 and 89%, respectively, indicating involvement of CYP3A4/5.

Induction Studies: Induction studies were performed in fresh human hepatocytes to further confirm CYP3A to be the primary enzyme responsible for 17-OHPC metabolism. CYP3A inducers like Rifampin, Phenobarbital and Clotrimazole increased $M2_{HH}$ formation (Fig. 7). Rifampin showed the maximum (2.2 fold) induction in 17-OHPC metabolism. Phenobarbital showed 2.1 fold whereas clotrimazole showed the least, 1.2 fold induction in human hepatocytes.

Correlation studies: Rates of formation of 17-OHPC metabolite (M2) with liver microsomes and fresh adult human hepatocytes were measured at a substrate concentration of 100 μM. There was considerable interindividual variation in the values of metabolite generated (Fig. 8). Formation of $M2_{(HLM)}$ and $M2_{(HH)}$ correlated significantly with the testosterone 6-β-hydroxylation activity (CYP3A4) in both microsomes (r=0.89) and hepatocytes (r=0.91), respectively.

Discussion

To the best of our knowledge, this is the first report identifying the human hepatic enzymes, which play a major role in the metabolic pathway of 17-OHPC. The metabolism of 17-OHPC was evaluated using human liver microsomes, fresh human adult hepatocytes and recombinant systems expressing cytochrome P450s and FMOs. Incubation of 17-OHPC with heat-inactivated microsomes did not result in any loss of 17-OHPC or generation of any 17-OHPC metabolites. Significant metabolism of 17-OHPC however was seen in all the three systems that were tested. Generation of metabolites as well as loss of parent drug confirmed this observation. No metabolites were observed in the absence of NADPH thus confirming the metabolism to be CYP mediated. Incubation of 17-OHPC with human liver microsomes under the conditions for direct generation of conjugates did not alter 17-OHPC concentrations or yield any 17-OHPC metabolites, ruling out a direct role of UGT mediated pathway. However, studies evaluating the role of UGT as a sequential pathway in 17-OHPC metabolism are being carried out currently.

Multiple approaches were used to identify the cytochrome P450 enzymes involved in the metabolism of 17α-hydroxyprogesterone caproate. Metabolism of medroxyprogesterone acetate (MPA), a potent progestogenic compound, has been reported in literature (Kobayashi, et al., 2000) to be catalyzed mainly by CYP3A4. Since, MPA is structurally similar to 17-OHPC it was expected that the metabolic pathways for both compounds would likely coincide. The results obtained confirmed this expectation. In human liver microsomes, 17α-hydroxyprogesterone caproate was metabolised to one major and two minor metabolites. CYP inhibition experiments indicated CYP3A4/5 to form the main metabolic pathway. Ketoconazole (CYP3A inhibitor) at 1.0 μM inhibited 90% of 17-OHPC biotransformation in human liver microsomes. These findings and the lack of

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effect of α – naphthoflavone, quinidine, coumarin, sulphaphenazole (inhibitors for CYP1A2, CYP2D6, CYP2A6, and CYP2C9) suggest a major role for CYP3As in 17-OHPC metabolism.

In fresh human adult hepatocytes, five ($M1\text{-}5_{HH}$) metabolites were observed on incubation of 17-OHPC with $M2_{HH}$ being the major metabolite. Ketoconazole and Troleandomycin (a known CYP3A inhibitor) significantly inhibited 17-OHPC metabolism. Inducers of CYP3A namely; rifampicin, phenobarbital and clotrimazole significantly increased 17-OHPC metabolism in comparison to control. Thus, the results in human liver microsomes were successfully reproduced in fresh human hepatocytes. This showed CYP3A to play the major role in 17-OHPC metabolism in hepatocytes as well.

To further confirm our results we conducted experiments to check the ability of expressed enzyme systems to catalyze the biotransformation of 17-OHPC. CYP3A isoforms were the only enzyme systems that metabolized 17-OHPC significantly. The enzyme activity for 17-OHPC metabolism was observed to be higher for CYP3A5 isoform than CYP3A4. The reason for the differential activity is not known at this time.

The CYP3A subfamily is known to be expressed most abundantly (i.e., from 10-60% of total CYPs) in human liver and plays a pivotal role in the oxidative metabolism of many clinically important drugs. Among the CYP3A isoforms tested (i.e., CYP3A4 and 3A5), CYP3A4 is the major isoform in adult humans. CYP3A5 is polymorphically expressed in approximately 10 to 20% of the adult liver (Wrighton, et al., 1990). Overall, we can predict that CYP3A4 would be the major CYP isoform responsible for the hepatic metabolism of 17-OHPC in the majority of adult patients given 17-OHPC. Given that CYP3A5 has higher activity for 17-OHPC, genetic polymorphism in CYP3A5 may have a significant role in 17-OHPC metabolism and pharmacokinetics, but this remains to be

evaluated. It has been proposed that the prolonged and more potent action of 17-OHPC over progesterone involves the cleavage of 17-OHPC molecule to 17-OHP (Fig.1B) and release of free caproic acid. It was also suggested that caproic acid could affect genomic pathways and hence have an effect of progesterone signaling pathways (Attardi, et al., 2007). However, results of radio-HPLC based method confirmed that the structure of 17-OHPC remained intact during metabolism by human enzymes. Our observation does not support the hypothesis that 17-OHPC is a prodrug that gets metabolized to progesterone or hydroxy progesterone and thus prevents pre-term labor. Further, LC-MS based analysis of the major metabolite ($M2_{HLM}$, m/z=445) generated from incubations in human liver microsomes indicated towards a possible mono-hydroxylation or an oxidation mechanism although di- and tri-hydroxy metabolites (data not shown) were also observed in human liver microsomes. Identity of the major metabolite ($M2_{HH}$) generated from human hepatocytes needs to be elucidated although preliminary data based on retention time and LCMS data analysis indicates $M2_{HH}$ (m/z=445) to be similar to $M2_{HLM}$. Similar results have recently been reported by Yan et al. (Yan, et al., 2008). The study, performed in human liver microsomes, reported the lack of 17-OHPC cleavage to release caproate ester based on radio-HPLC experiment. Further, generation of mono-, di- and tri-hydroxylated metabolites were also observed. Our study reports the production of three main metabolites from human liver microsomal incubation whereas the Yan study reported the formation of 21 metabolites. This can be attributed to our study being based on a simpler, isocratic LC method with UV detection as compared to a gradient LC method with MS (single quadrupole) detection used by the previous study. However, $M2_{HLM}$ (m/z =445.0) is the major metabolite (>60%) reported by both studies and this was the primary focus of this publication.

Our study demonstrates that the metabolism of 17-OHPC is predominantly mediated by CYP3A isoforms, mainly CYP3A4. Given that the activity of CYP3A enzyme is known to vary between subjects, one would expect large variation in the pharmacokinetics of 17-OHPC in pregnant subjects. Indeed in women pregnant with twins we noted a large variation in concentrations despite a fixed dosing regimen (Steve Caritis and Venkataramanan, 2007). CYP3A4 plays a major role in the metabolism of various drugs due to its abundance in the liver and its broad substrate specificity. Numerous clinically important drugs are known as substrates of CYP3A4 (Rendic and Di Carlo, 1997). Thus, further in-vitro and clinical studies are required to assess 17-OHPC associated clinically relevant metabolic drug interaction with any co-administered CYP3A4 substrates/ inhibitors.

Pregnancy is a dynamic state of the human body which is characterized by significant variations in the physiology and metabolism. Changes in the metabolizing activity of CYPs especially CYP3A4 has been reported in literature (Tracy, et al., 2005). Activity of CYP3A4 has been shown to increase significantly in all trimesters in humans. Further, the expression level of CYP3A isoforms varies from individual to individual. Thus, we expect significant inter-individual fluctuations in 17-OHPC plasma concentrations in pregnant patients over time. In clinical studies, 17α-Hydroxyprogesterone Caproate is administered as a fixed dose regimen of 250mg weekly. The therapy was observed to be effective in 33% of the patients in the study of Meis et al. It is possible that the low success rate of 17-OHPC may be attributable to the significant variation in CYP3A mediated metabolism of 17-OHPC in these patients. On the basis of the abovementioned facts, it may be necessary to investigate various dosing regimen and individualize the therapy with 17-OHPC. Monitoring 17-OHPC plasma levels and adjustment of dose accordingly may be needed to improve therapeutic outcomes.

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References

Attardi BJ, Zeleznik A, Simhan H, et al. Comparison of progesterone and glucocorticoid receptor binding and stimulation of gene expression by progesterone, 17-alpha hydroxyprogesterone caproate, and related progestins. Am J Obstet Gynecol. 2007;197:599 e591-597.

Chiba M, Hensleigh M, Nishime JA, et al. Role of cytochrome P450 3A4 in human metabolism of MK-639, a potent human immunodeficiency virus protease inhibitor. Drug Metab Dispos. 1996;24:307-314.

Court MH, Greenblatt DJ. Molecular basis for deficient acetaminophen glucuronidation in cats. An interspecies comparison of enzyme kinetics in liver microsomes. Biochem Pharmacol. 1997;53:1041-1047.

Kobayashi K, Mimura N, Fujii H, et al. Role of human cytochrome P450 3A4 in metabolism of medroxyprogesterone acetate. Clin Cancer Res. 2000;6:3297-3303.

Kostrubsky VE, Ramachandran V, Venkataramanan R, et al. The use of human hepatocyte cultures to study the induction of cytochrome P-450. Drug Metab Dispos. 1999;27:887-894.

Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265-275.

Mattison DR, Damus K, Fiore E, et al. Preterm delivery: a public health perspective. Paediatr Perinat Epidemiol. 2001;15 Suppl 2:7-16.

Nelson AC, Huang W, Moody DE. Variables in human liver microsome preparation: impact on the kinetics of l-alpha-acetylmethadol (LAAM) n-demethylation and dextromethorphan O-demethylation. Drug Metab Dispos. 2001;29:319-325.

Paneth NS. The problem of low birth weight. Future Child. 1995;5:19-34.

Rendic S, Di Carlo FJ. Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. Drug Metab Rev. 1997;29:413-580.

Steve Caritis, Venkataramanan R. Pharmacokinetics of 17-alpha hydroxyprogesterone caproate in women with twin gestation. Reproductive Sciences Supplement. 2007;14:

Strom SC, Pisarov LA, Dorko K, et al. Use of human hepatocytes to study P450 gene induction. Methods Enzymol. 1996;272:388-401.

Tracy TS, Venkataramanan R, Glover DD, et al. Temporal changes in drug metabolism (CYP1A2, CYP2D6 and CYP3A Activity) during pregnancy. Am J Obstet Gynecol. 2005;192:633-639.

Wrighton SA, Brian WR, Sari MA, et al. Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). Mol Pharmacol. 1990;38:207-213.

Wu DH, Allen WM. Maintenance of pregnancy in castrated rabbits by 17-alpha-hydroxy-progesterone caproate and by progesterone. Fertil Steril. 1959;10:439-460.

Yan R, Nanovskaya TN, Zharikova OL, et al. Metabolism of 17alpha-hydroxyprogesterone caproate by hepatic and placental microsomes of human and baboons. Biochem Pharmacol. 2008; In Press.

Footnotes:

Grant Support: This work is supported in part by NICHD grant HD-047905-2.

Legends for Figures

Fig. 1

Chemical Structures of:

A)

Product Name: 17α-Hydroxyprogesterone caproate (17 α- hydroxy-4-pregnene-3, 20-dione hexanoate)

B)

Product Name: 17α-Hydroxyprogesterone (17 α- hydroxy-4-pregnene-3, 20-dione)

C)

Product Name: Progesterone (4-pregnene-3, 20-dione)

Fig. 2

Chromatograms illustrating the metabolism of 17-OHPC by human liver microsomal preparations.

A) Chromatogram (HPLC-UV) showing retention times and absorption peaks for metabolites 1, 2, 3 ($M1_{HLM}$, $M2_{HLM}$, $M3_{HLM}$) and 17-OHPC having retention times of 4.84, 5.10, 5.94 and 8.55 respectively, after microsomal incubation in the presence of NADPH.

B) Chromatogram (HPLC-UV) depicting incubation of 17-OHPC with HLM in the absence of NADPH.

C) Chromatogram (LC-MS) showing absorption peaks for metabolites ($M1_{HH}$, $M2_{HH}$, $M3\text{-}5_{HH}$) and 17-OHPC after incubation with Fresh Human Hepatocytes (FHH) for 60 minutes. The retention times for M1, M2 and 17-OHPC were observed to be 6.64, 7.99 and 21.71 respectively.

D) Chromatogram (LC-MS) showing absorption peak for 17-OHPC after incubation with Fresh Human Hepatocytes (FHH) for 1 minute. No metabolite formation was detected. The retention time for 17-OHPC was 21.97.

Fig. 3

Metabolism of 17-OHPC by human hepatocytes and human liver microsomes (n=3).

A) Incubation of 17-OHPC (150µM) with Fresh Adult Human Hepatocytes generated five metabolites (M1-5_{HH}). The major metabolite ($M2_{HH}$, ■) showed a time dependent increase throughout the incubation which corresponded with a decrease in the amount of 17-OHPC (▲).

B) Incubation of 17-OHPC (50µM) with Human Liver Microsomes generated metabolites 1, 2 and 3. Metabolite 2 ($M2_{HLM}$, ■) was the major metabolite which depicted time dependent increase in concentration corresponding to a decrease in 17-OHPC concentration (▲).

The amount of metabolite has been expressed in terms of 17-OHPC equivalents.

Fig. 4

Ketoconazole inhibits 17-OHPC metabolism.

Of the various inhibitors evaluated, namely, Coumarin (CYP2A6, ■), α-Naphthoflavone (CYP1A2, ▲), Sulfaphenazole (CYP2C9, ▼), Quinidine (CYP2D6, □), Ketoconazole (CYP3A, ♦), and Methimazole (FMO3, ●), only Ketoconazole (1µM) demonstrated significant inhibition of 17-OHPC (0-200 µg/ml expressed as 0-467µM) metabolism in human liver microsomes (n=3) when compared to control (No NADPH, Δ). This indicated the role of CYP3A isoforms in metabolizing 17-OHPC and the potential involvement of CYP3A4. Approx. 80% inhibition of 17-OHPC was observed at 1 µM Ketoconazole (* $p < 0.05$). V (µmoles/min/mg microsomal protein) denotes the major metabolite formation ($M2_{HLM}$) expressed in terms of 17-OHPC equivalents.

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Fig. 5

Estimation of IC_{50}

IC_{50} value for the ketoconazole mediated inhibition of 17-OHPC (25μM) metabolism was calculated to be 0.17 μM in human liver microsomes (n=3). Inhibition of 17-OHPC metabolism was evaluated by estimating the formation of $M2_{HLM}$.

Fig. 6

CYP3A4/5 metabolize 17-OHPC in Fresh Adult Human Hepatocytes

Ketoconazole (**K**, 1 and 10μM) and Troleandomycin (**TAO**, 100μM) demonstrated significant ($p<0.05$) inhibition of 17-OHPC (25μM) metabolism in Fresh Human Hepatocytes (n=4). This indicated the role of CYP3A isoforms in metabolizing 17-OHPC and the potential involvement of CYP3A4. Inhibition of 17-OHPC metabolism was evaluated by estimating the formation of $M2_{HH}$.

Fig. 7

CYP3A inducers increase 17-OHPC metabolism in Fresh Adult Human Hepatocytes

Incubation of fresh human hepatocytes (n=3) with specific CYP3A4 inducers, namely, Rifampin ($p<0.05$), Phenobarbital ($p<0.05$) and Clotrimazole ($p=0.054$) increased the metabolism of 17-OHPC (50μM) as compared to control (DMSO). The estimation of induction was based on the generation of the major metabolite ($M2_{HH}$) in hepatocytes.

Fig. 8

Correlation analysis of 17-OHPC metabolite M2 with CYP3A dependent 6β-hydroxytestosterone formation.

(A) Correlation analysis in fresh human hepatocytes (FHH); n=7

(B) Correlation analysis in human liver microsomes (HLM); n=7

CYP3A activity was estimated by incubating 17-OHPC (100μM) and Testosterone (100 μM) in individual human liver microsomal preparations and fresh human hepatocyte cultures. The experiment was carried out in triplicate in both cases.

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Table 1: Identification of cytochrome P450s involved in the metabolism of 17-OHPC.

Expressed CYPs	Metabolite ($M2_{HLM}$) Generated (pmole/pmole CYP/min)	17-OHPC Metabolized (pmole/pmole CYP/min)
CTRL	ND	ND
1A1	ND	ND
1A2	ND	ND
1B1	ND	ND
2A6	ND	ND
2B6	ND	ND
2C8	ND	ND
2C9*1	ND	ND
2C9*2	ND	ND
2C9*3	ND	ND
2C18	ND	ND
2D6*1	ND	ND
2D6*10	ND	ND
2E1	ND	ND
19	ND	ND
FMO1	ND	ND
FMO3	ND	ND
FMO5	ND	ND
CYP3A4	27.4±1.2	64.5±0.1
CYP3A5	57.1±3.7	68.4±0.4

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Mean ± SD of triplicate in-vitro preparations.

Amount of metabolite is expressed in terms of HPC equivalent. Significant levels of metabolite were detected on incubation of 17-OHPC (100 μM) with expressed CYP3A4/5 whereas no metabolite could be detected for other CYPs.

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Fig. 1A

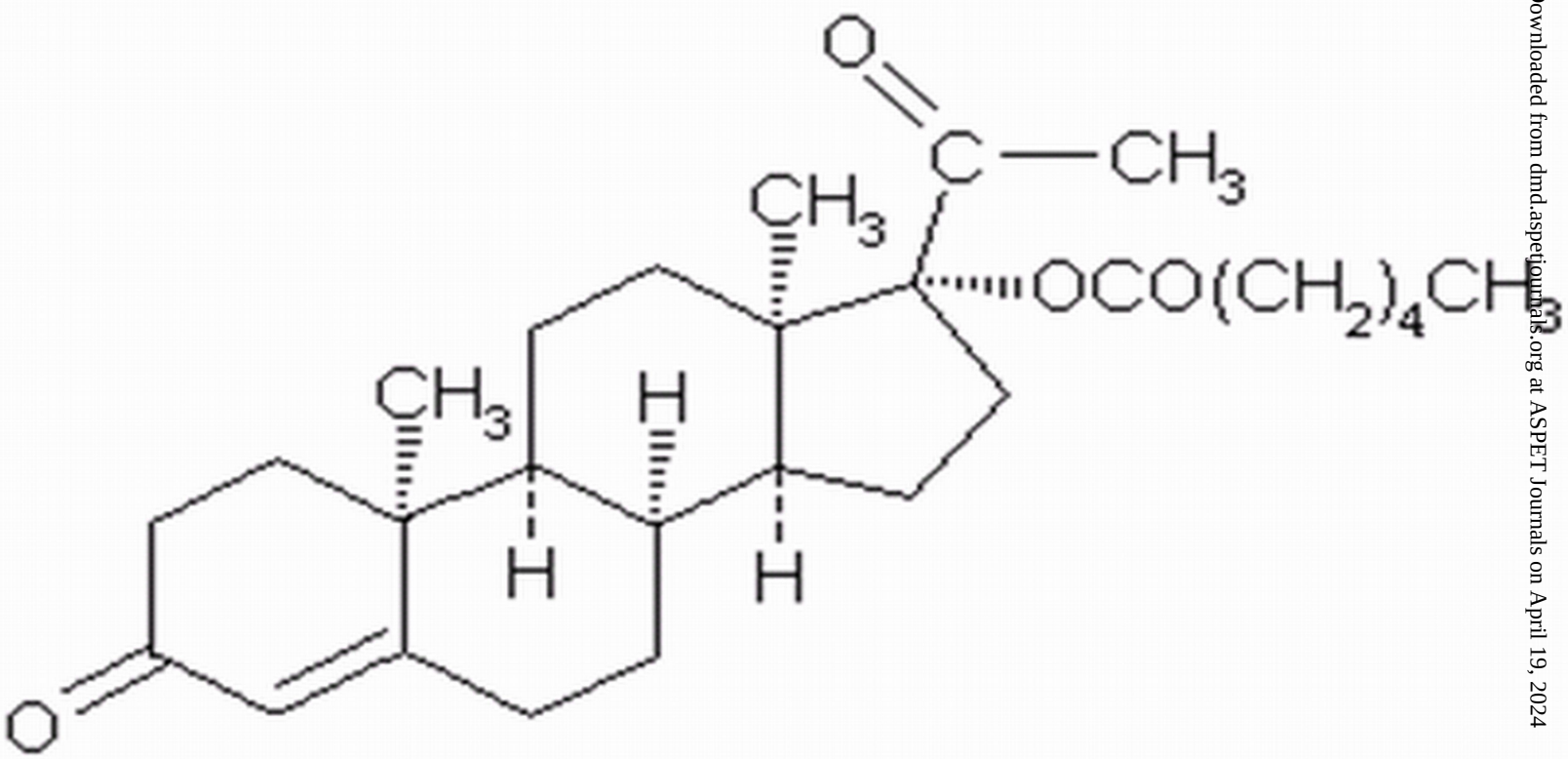

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Fig. 1B

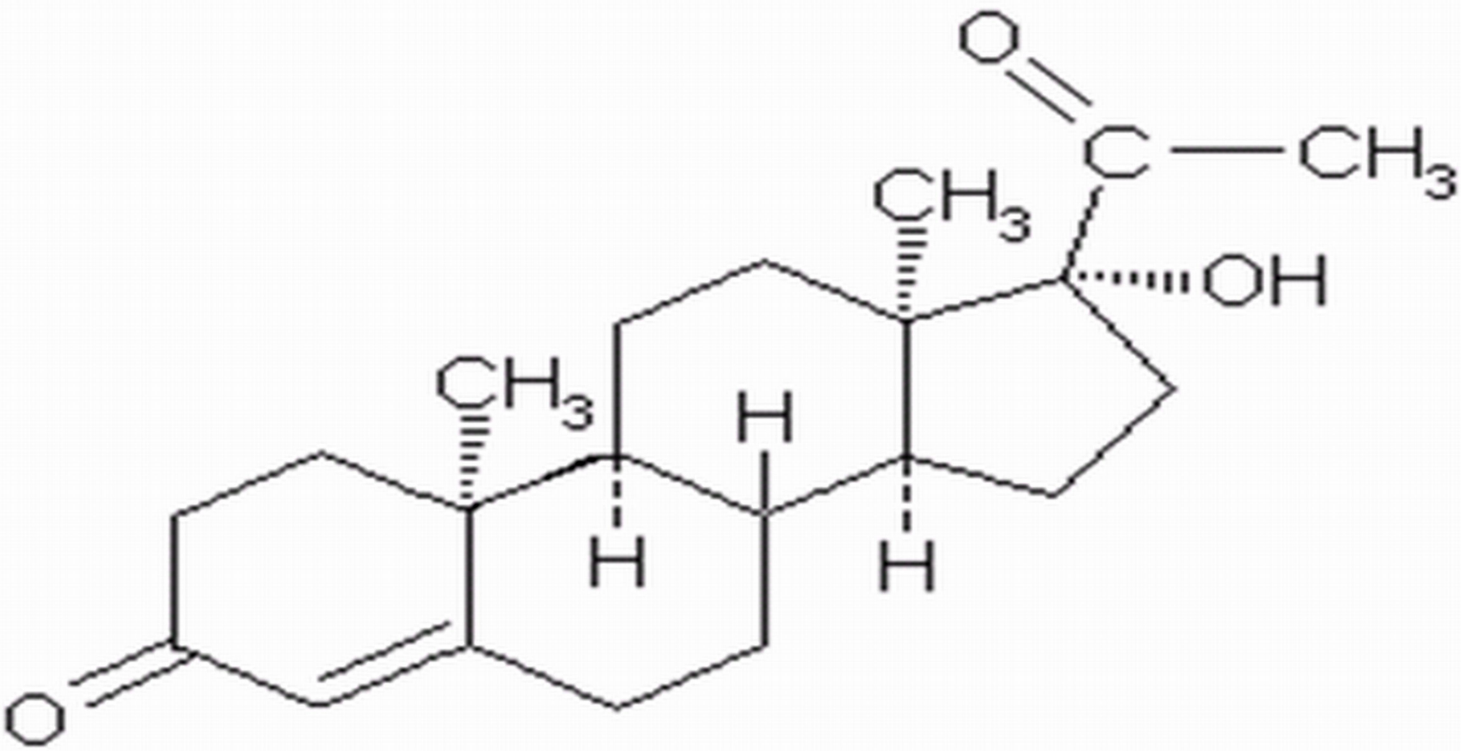

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Fig. 1C

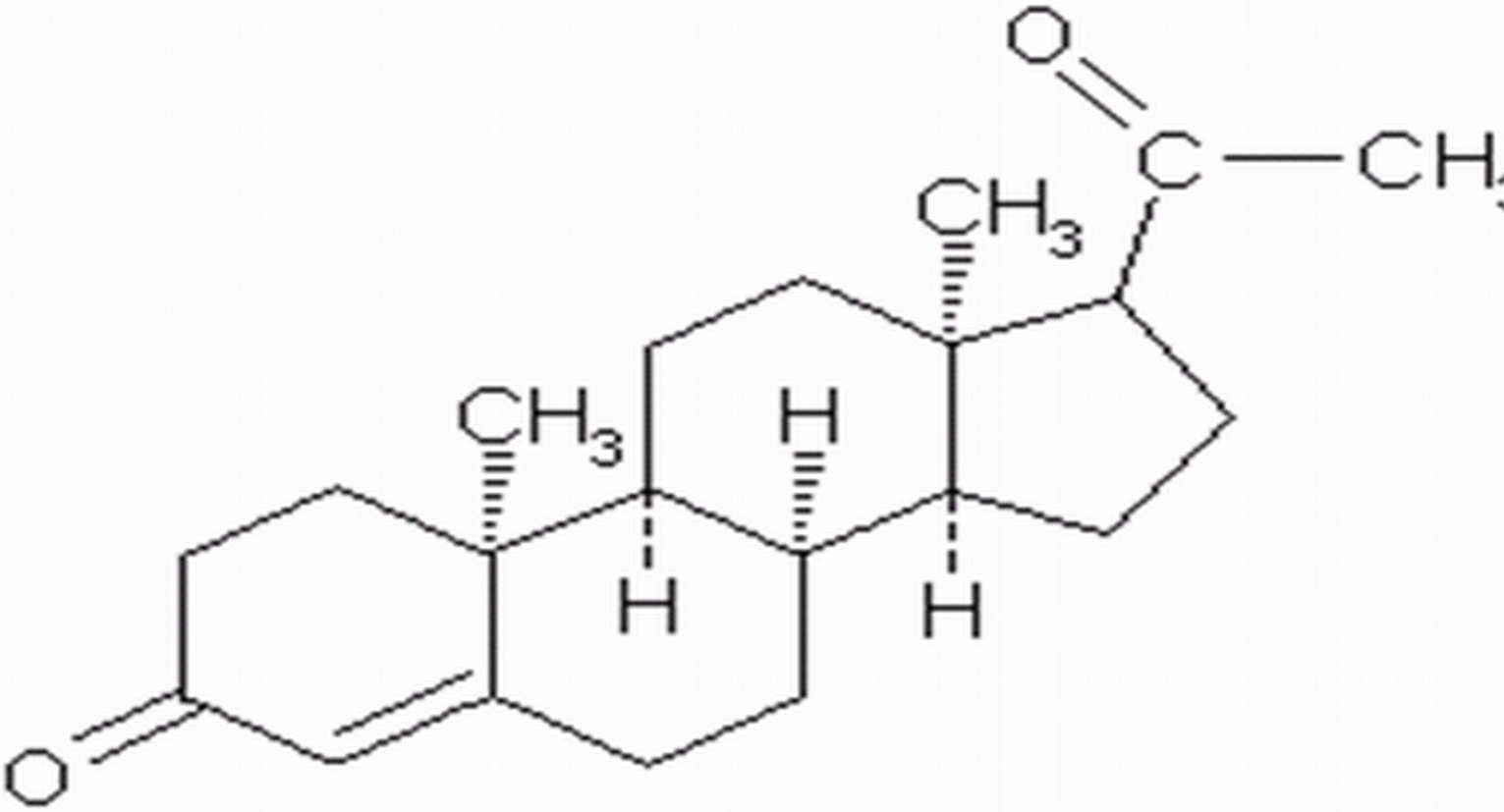

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Fig. 2A

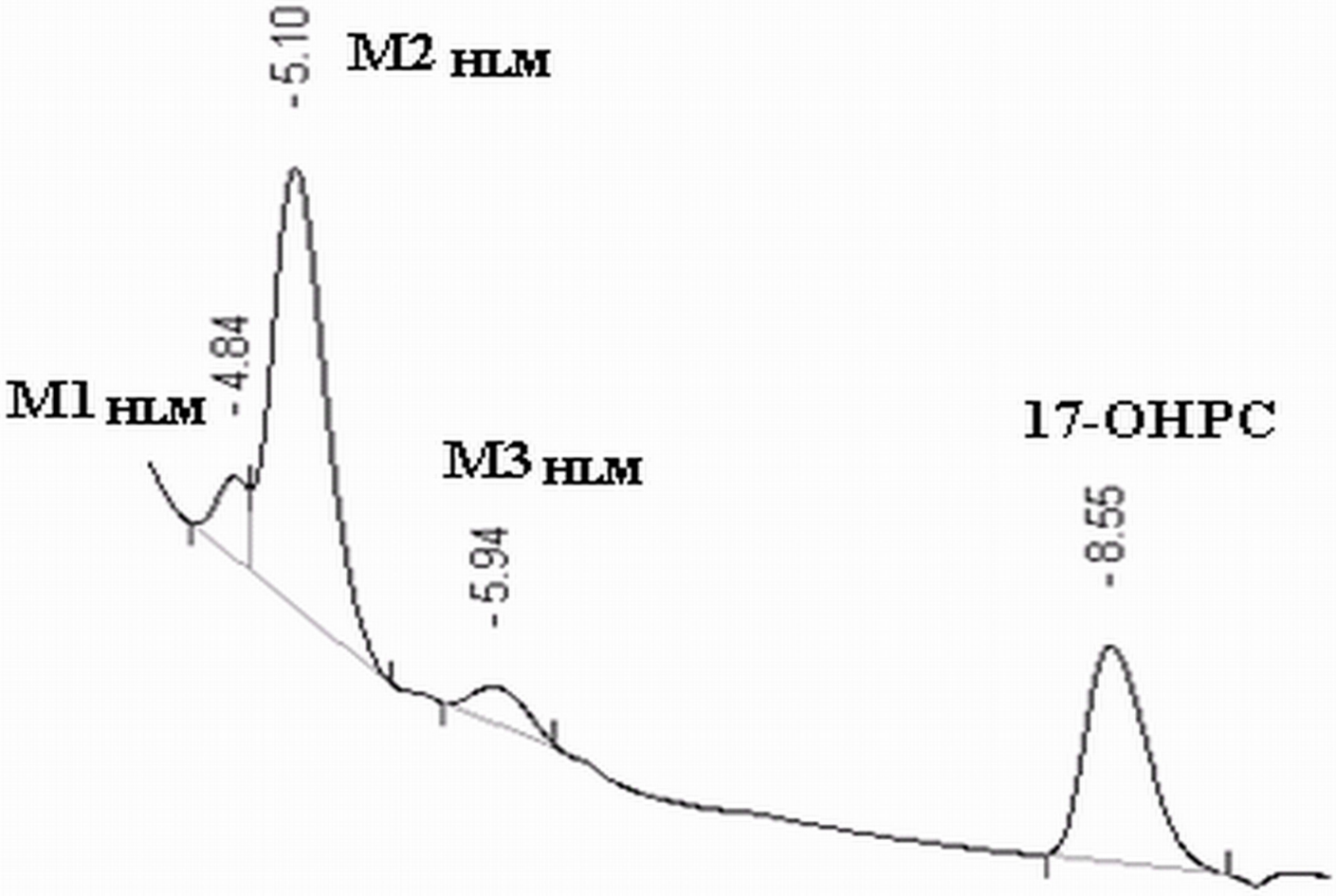

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Fig. 2B

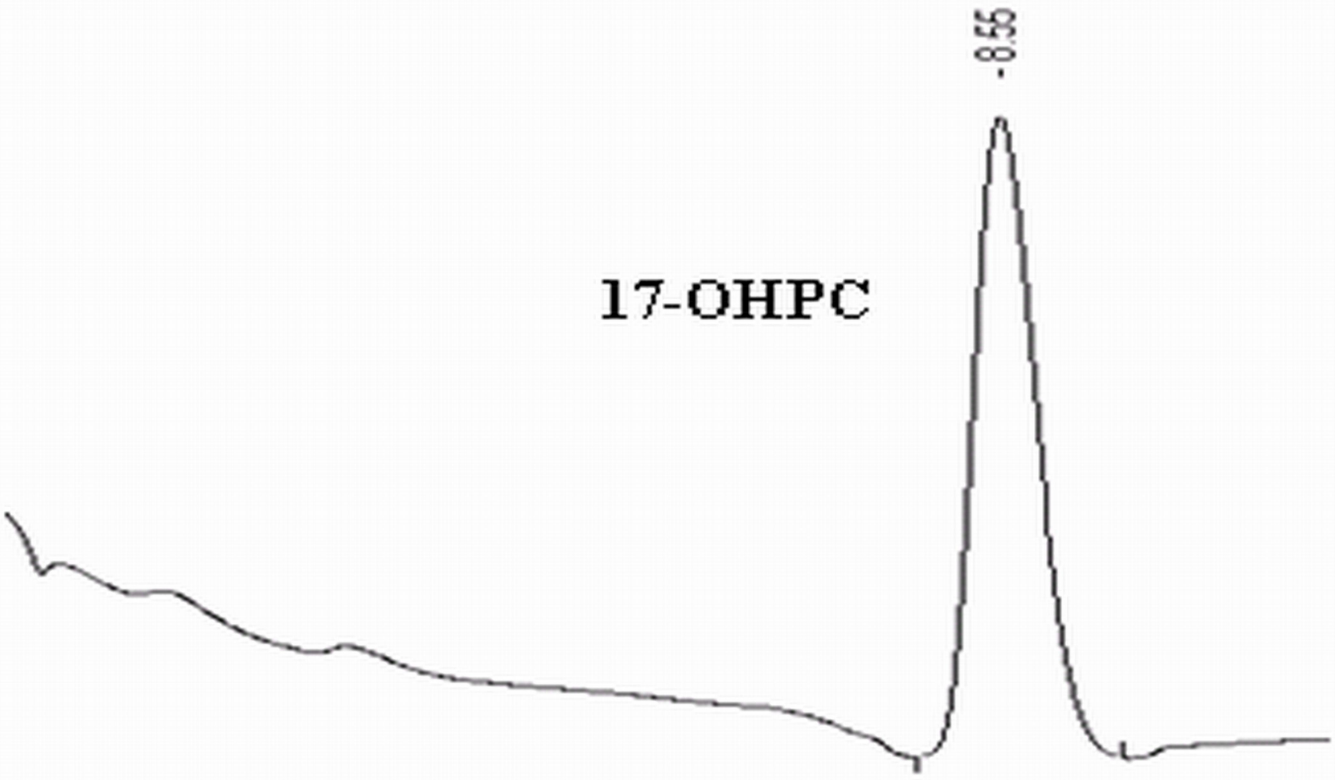

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Fig. 2C

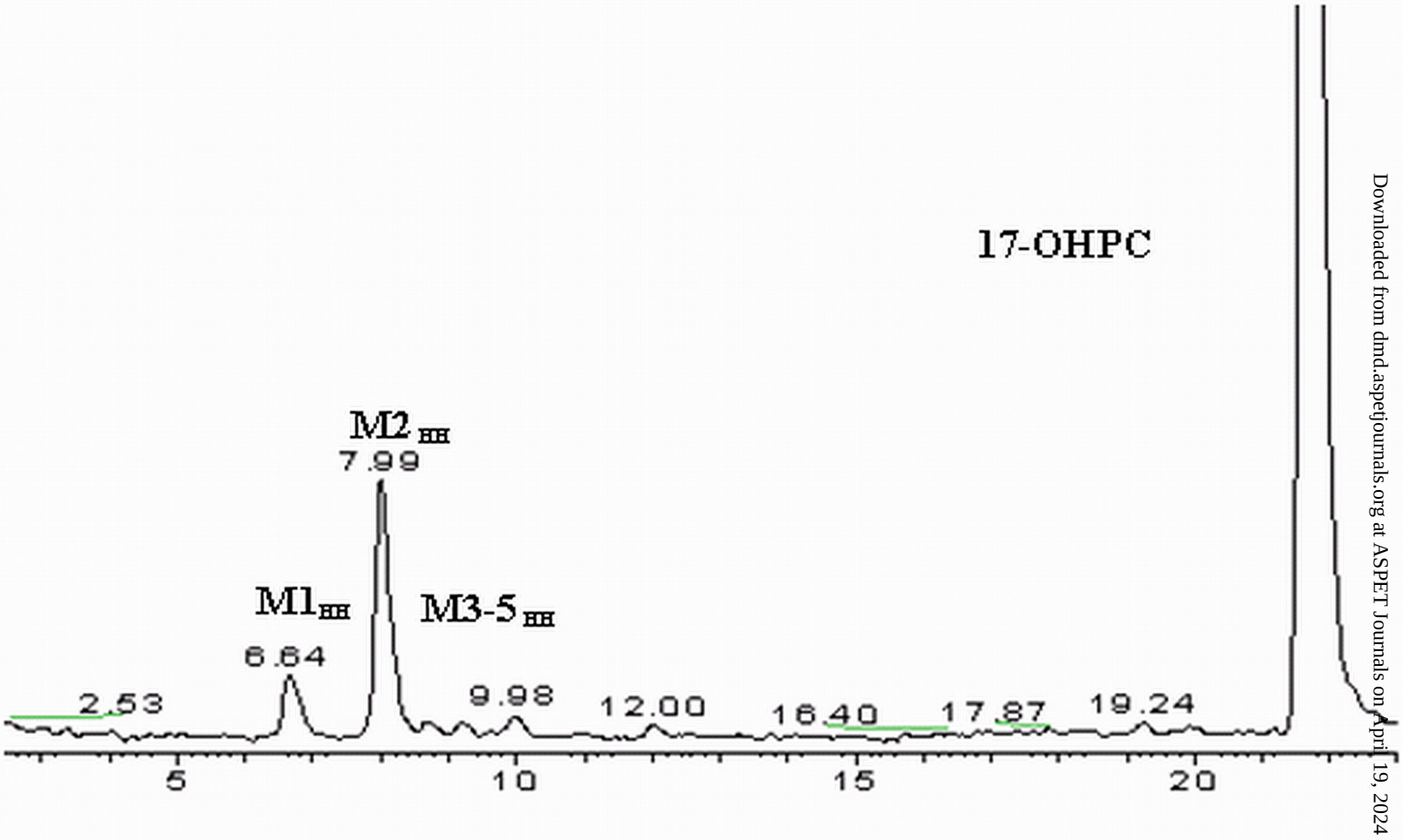

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Fig. 2D

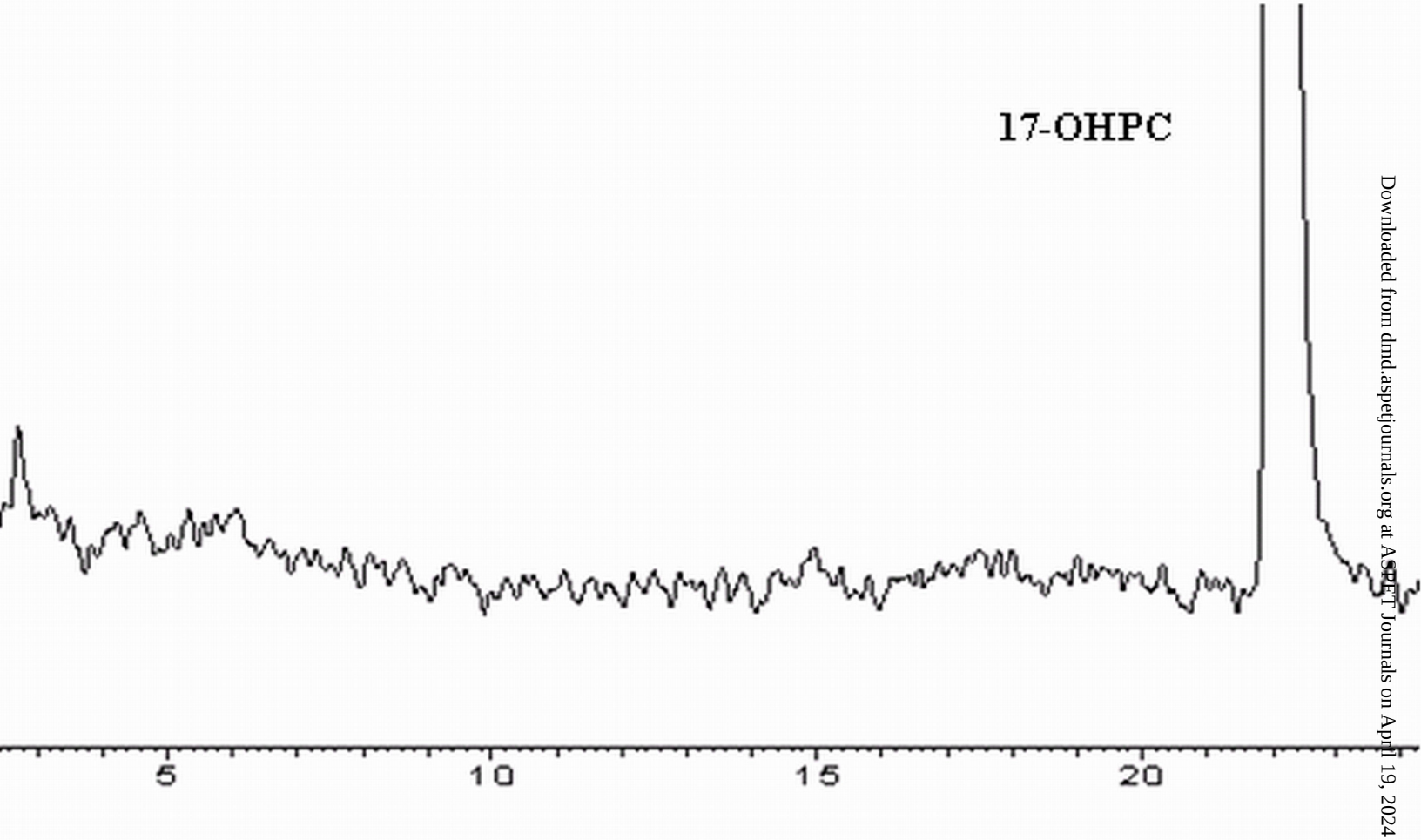

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Fig. 3A

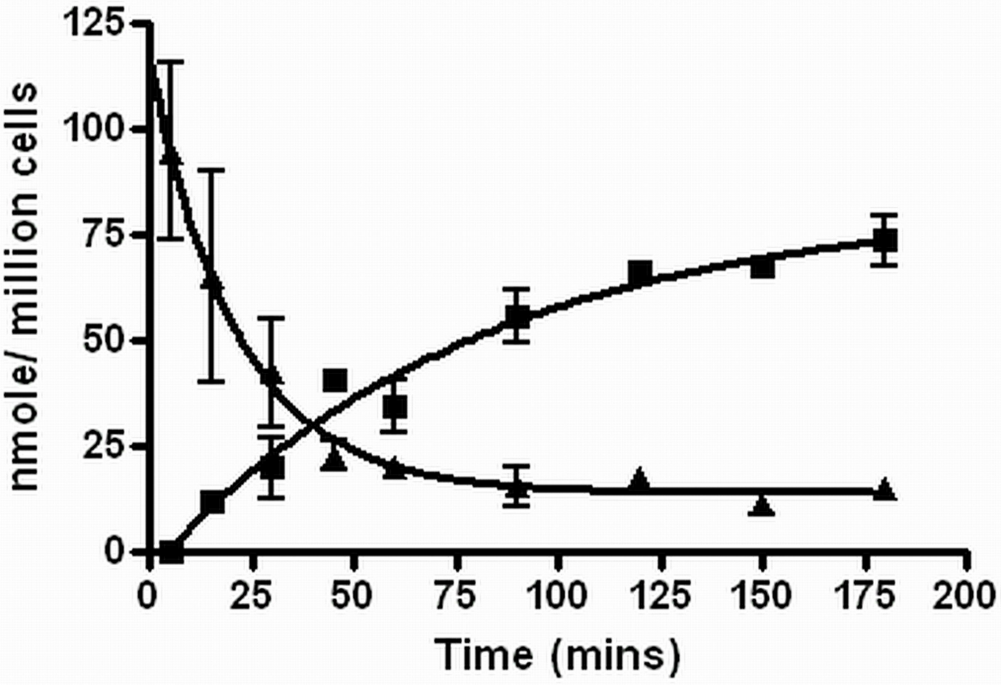

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Fig. 3B

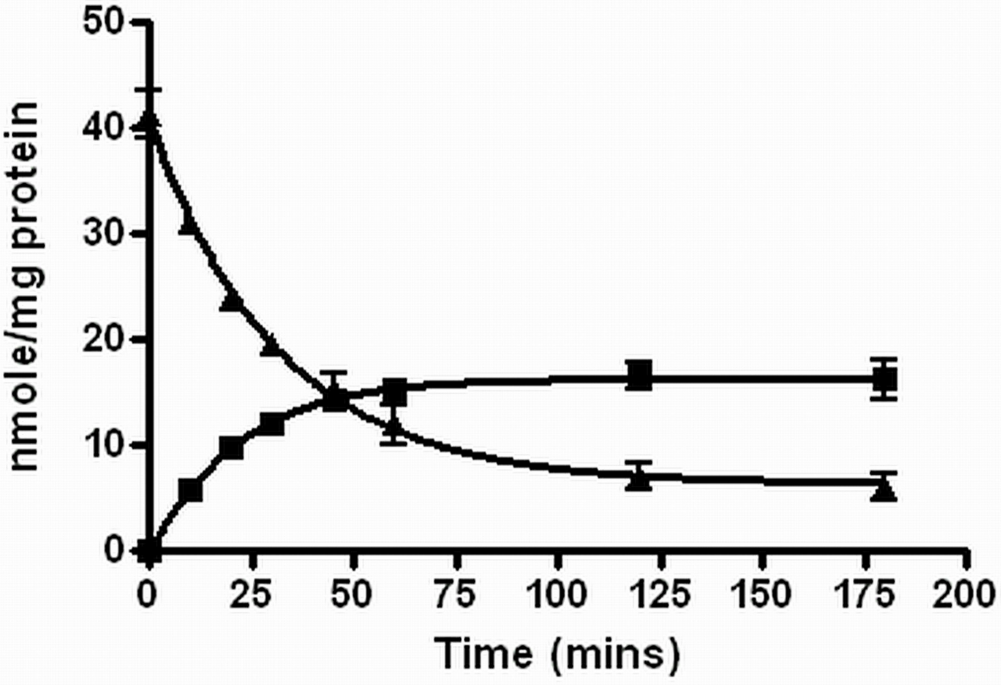

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Fig. 4

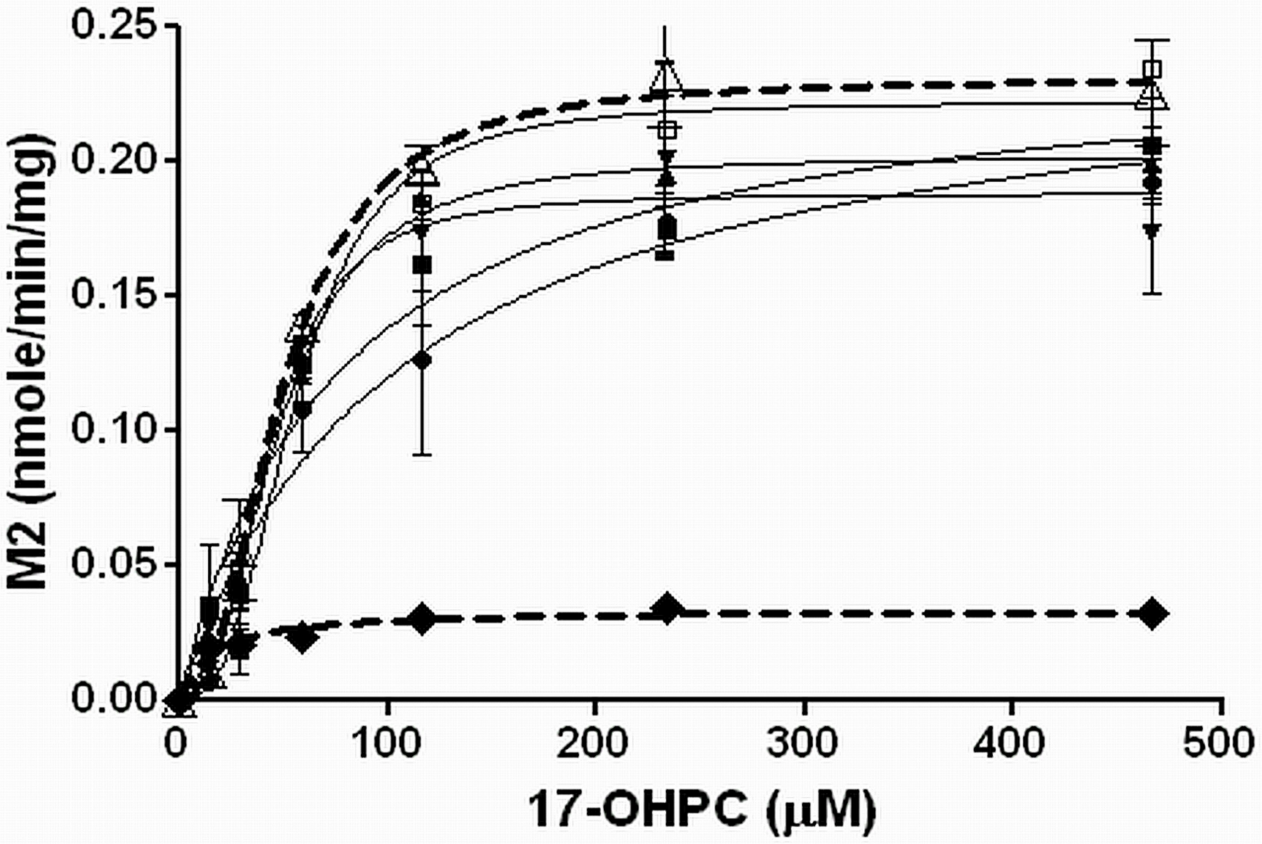

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Fig. 5

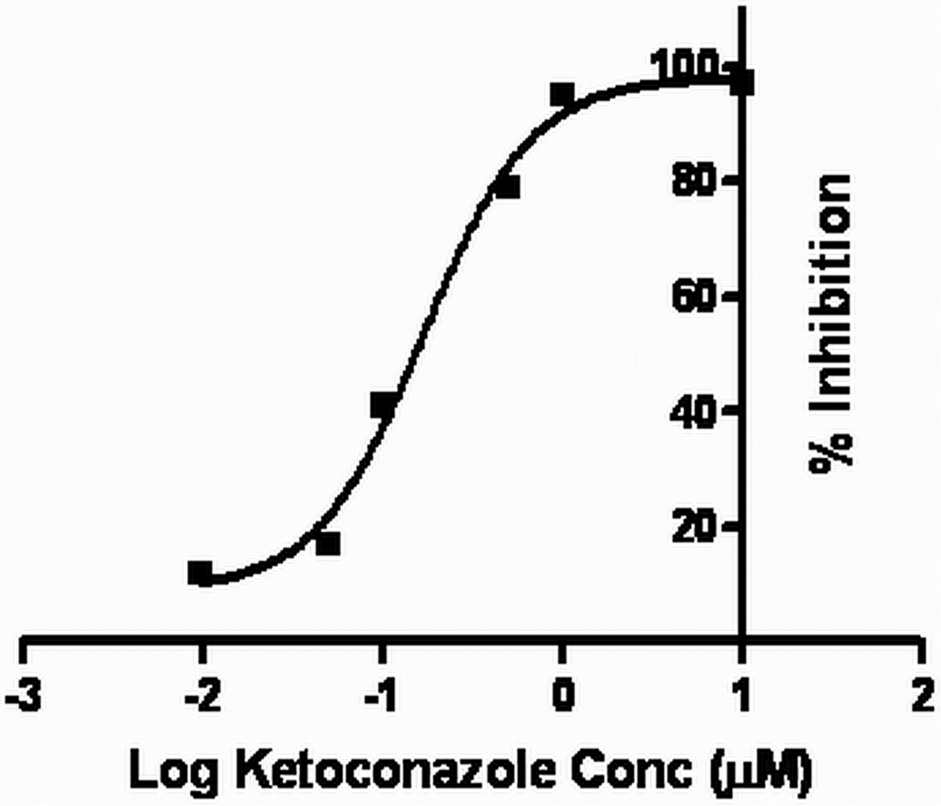

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Fig. 6

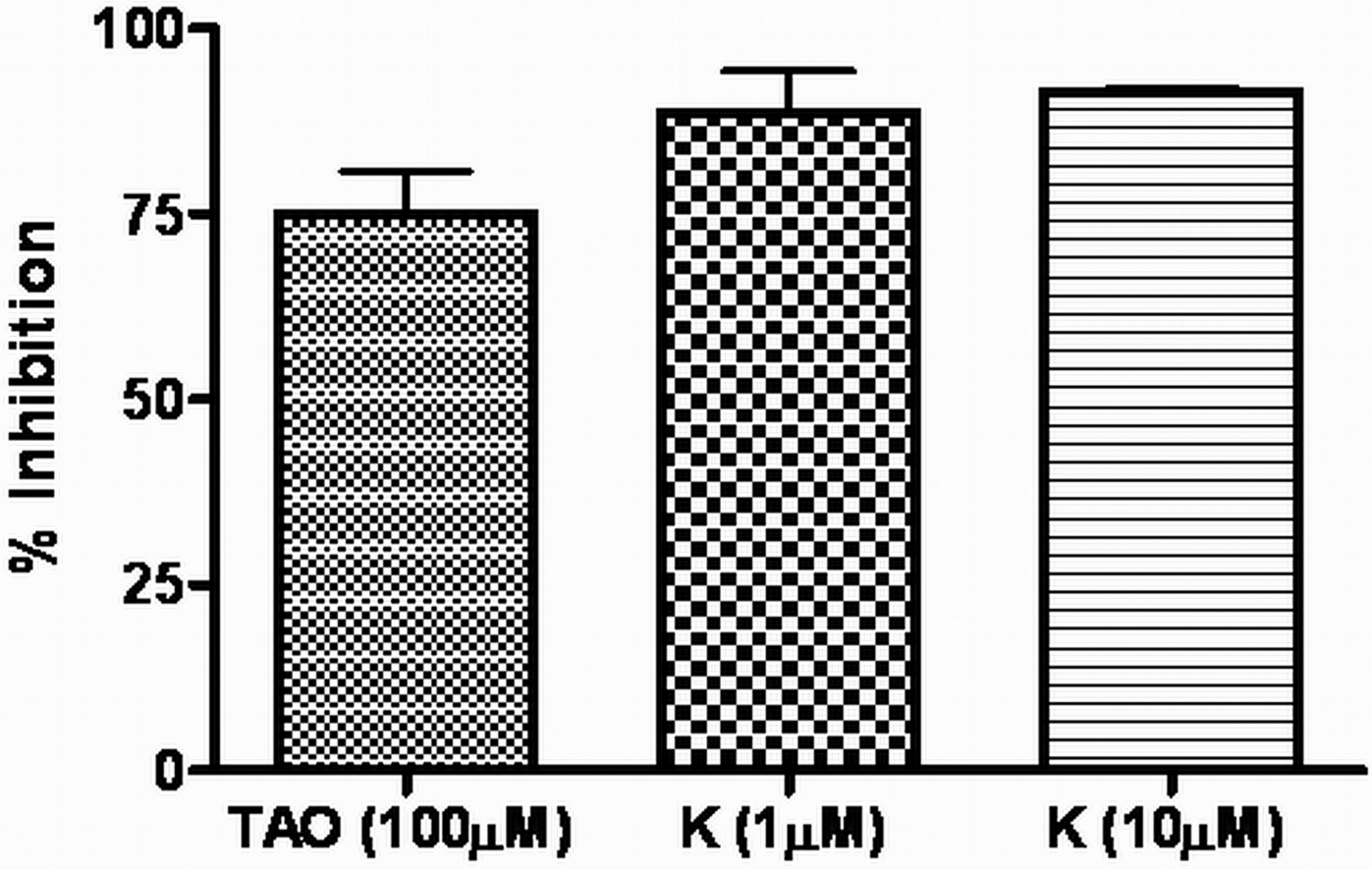

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Fig. 7

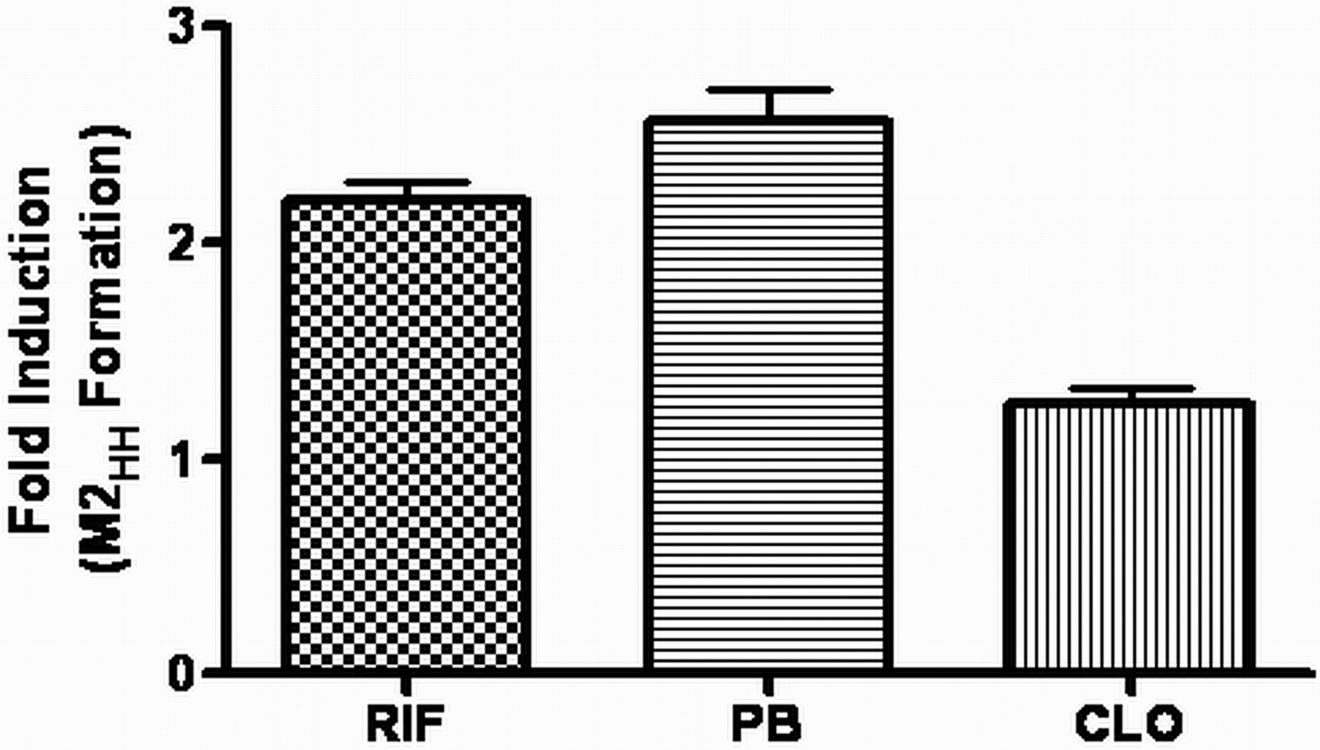

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Fig. 8A

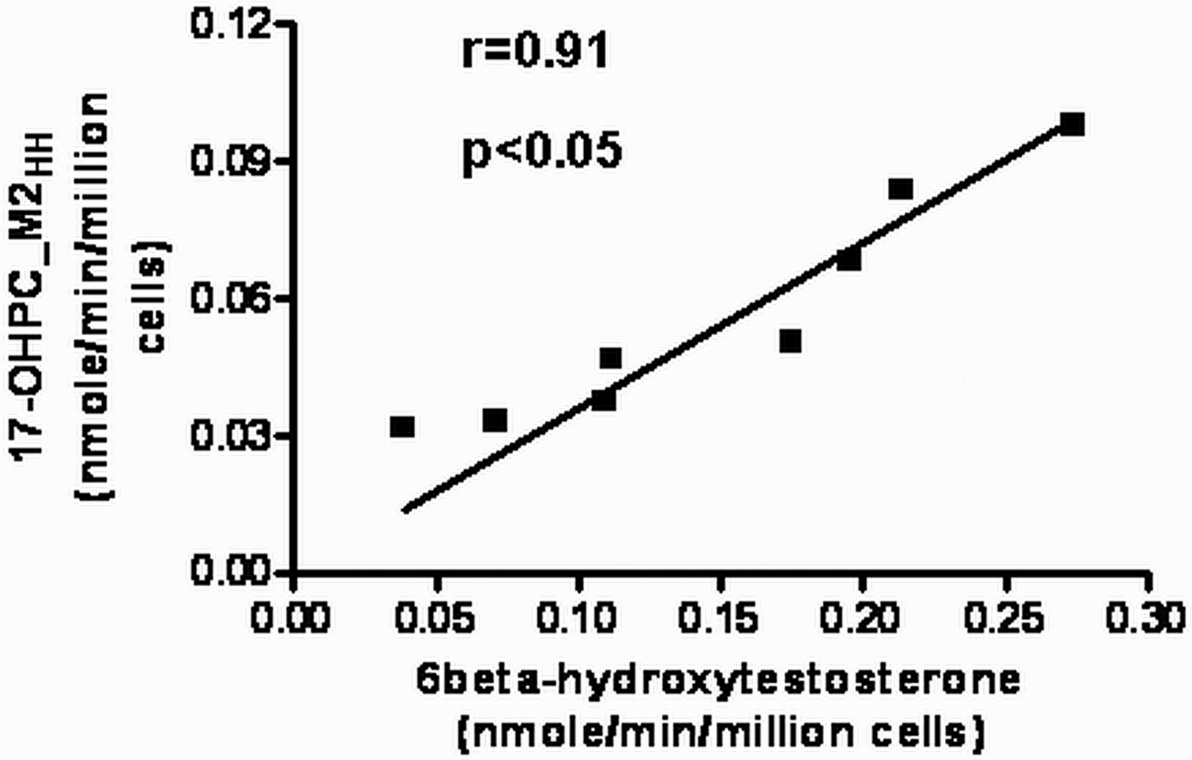

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Fig. 8B