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Methadone metabolism by human placenta

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Abstract

Methadone pharmacotherapy is considered the standard for treatment of the pregnant heroin/opioid addict. One of the factors affecting the transfer kinetics of opioids across human placenta and their levels in the fetal circulation is their metabolism by the tissue. The aim of this investigation is to identify the enzyme(s) responsible for the metabolism of methadone, determine the kinetics of the reaction and the metabolites formed utilizing placental tissue obtained from term healthy pregnancies. Microsomal fractions of trophoblast tissue homogenates had the highest activity in catalyzing the metabolism of methadone. The product formed was identified by HPLC-UV as 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Inhibitors selective for cytochrome P450 (CYP) isozymes were used to identify the enzyme catalyzing the biotransformation of methadone. Aminoglutethimide and 4-hydroxyandrostenedione inhibited EDDP formation by 88 and 70%, respectively, suggesting that CYP19/aromatase is the enzyme catalyzing the reaction. This was confirmed by the effect of monoclonal antibodies raised against CYP19 that caused an 80% inhibition of the reaction. The apparent K_m and V_{max} values for the CYP19 catalyzed metabolism of methadone to EDDP were 424 ± 92 μM and 420 ± 89 pmol (mg protein)$^{-1}$ min^{-1}, respectively. Kinetic analysis of a cDNA-expressed CYP19 for the metabolism of methadone to EDDP was identical to that by placental microsomes. Taken together, these data indicate that CYP19/aromatase is the major enzyme responsible for the metabolism of methadone to EDDP in term human placentas obtained from healthy pregnancies.
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Keywords: Human placenta; Aromatase; Methadone; Metabolism; Pregnancy

1. Introduction

Methadone is the only approved pharmacotherapy for treatment of the pregnant heroin/opioid addict. Its use, since the late 1960s, has been the subject of numerous clinical trials, reports, and review articles that, for the most part, agree on the benefits of methadone maintenance programs in improving maternal and neonatal outcome. A review of this literature would be out of the scope of this manuscript.

Methadone is a synthetic, long-acting μ-agonist that undergoes first pass metabolism by its sequential *N*-demethylation to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) as shown (Fig. 1). Both EDDP and EMDP do not have analgesic activity [1]. Cytochrome P450 (CYP) 3A4 was identified as the major enzyme catalyzing the metabolism of methadone in human liver and intestine though the involvement of other CYP isoforms (namely CYP2D6, CYP1A2, CYP2C9, and CYP2C19) was not ruled out [2–5].

Several reports indicated that the metabolism of methadone in pregnant women is different from the non-pregnant. The differences were attributed to the changes in maternal physiology that accompany the onset of pregnancy to accommodate the growth and development of the feto-placental unit. During pregnancy, the placenta functions as an additional extra-hepatic site for biotransformation of drugs though the amount and activity of its metabolic enzymes is lower than that in the liver [6–9]. Therefore, the metabolic activity of the placenta could contribute to the changes in the pharmacokinetic-pharmacodynamic profile (bio-disposition) of drugs administered to the pregnant women. Indeed, methadone plasma

Abbreviations: EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP, 2-ethyl-5-methyl-3,3-diphenylpyrroline; CYP, cytochrome P450; LAAM, L-α-acetylmethadol; TCA, trichloroacetic acid; BUP, buprenorphine

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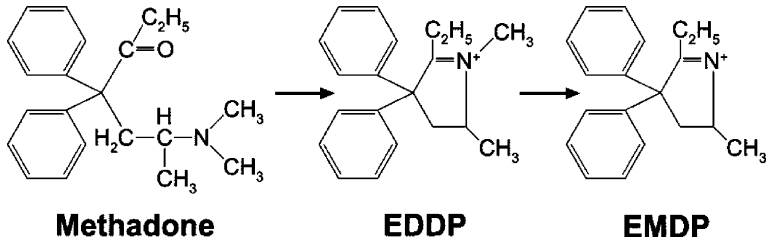

Fig. 1. The structure of the products formed by the sequential *N*-demethylation of methadone.

concentrations during the second and third trimesters were lower than after delivery and elimination of methadone in pregnant patients was significantly more rapid than in the non-pregnant [10,11]. These reports suggested that biotransformation of methadone during pregnancy may be accelerated as a result of enhanced maternal and fetal hepatic metabolism and (or) biotransformation of the drug by the placenta. As a result, the dose of methadone during pregnancy may have to be adjusted to enhance compliance and safety of the mother and newborn [10,11].

Pregnant women in methadone maintenance programs may require therapeutic interventions due to infections or other pregnancy-associated complications that dictate consideration of drug interactions. Changes in methadone clearance were observed in patients treated with cimetidine, erythromycin, ketoconazole, rifampin, and barbiturates [12,13]. Other sites for drug interactions can include key enzymes in placental biosynthesis of steroid hormones namely, CYP11A1 and CYP19, that are also involved in the metabolism of xenobiotics [14,15], buprenorphine, and L-α-acetylmethadol (LAAM) [16,17].

Taken together, these reports emphasize the need for information on the enzyme metabolizing methadone in human placenta and the products formed. This information will also allow a comparison of placental bio-disposition of methadone and buprenorphine as the latter is emerging as an alternative to treatment of the pregnant opioid-dependent woman. Therefore, the goal of this investigation is to identify and characterize the enzyme responsible for the metabolism of methadone in term human placenta.

2. Materials and methods

2.1. Chemicals and other supplies

All chemicals were purchased from Sigma Chemical Co. unless otherwise mentioned. Acetonitrile was purchased from Fisher Scientific Co. Methadone, its metabolites, and L-α-acetylmethadol (LAAM) were a gift from the National Institute on Drug Abuse drug supply unit. Monoclonal antibodies to CYP isoforms were a generous gift from Dr. Andrew Parkinson (Xenotech LLC). Rabbit antiserum to human placental aromatase was purchased from Hauptman–Woodward Institute. Properties of the antibodies and their effect on the activity of placental aromatase have been previously reported [18]. The cDNA-expressed CYP19 "supersomes" is commercially available from Gentest, and their characterization was described earlier [19].

2.2. Human placentas and derived preparations

Full-term human placentas from uncomplicated pregnancies were obtained from the labor and delivery ward at the University of Texas Medical Branch Hospital, Galveston, Texas, according to a protocol approved by the Institutional Review Board. Placentas of women who abused drugs during pregnancy were excluded from this investigation.

Approximately 40–50 g of villus tissue was excised at random from the placenta and rinsed with cold saline. The tissue was then minced and homogenized in two volumes of cold 0.1 M potassium phosphate buffer, pH 7.4 using a Tekmar SDT-1810, Tissumizer. The homogenate was subjected to differential centrifugation to prepare the following subcellular fractions: mitochondrial, 10,000 × *g* pellet; microsomal, 104,000 × *g* pellet; and the supernatant for the cytosolic. The mitochondrial and microsomal pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4). Protein content of the preparations was determined by a kit from Bio-Rad (Bio-Rad laboratories) using bovine serum albumin as a standard. Aliquots of the subcellular fractions were stored at −70 °C until analyses were performed within 2–3 weeks, and the enzymatic activity was not affected under these storage conditions.

2.3. Methadone metabolism

The activity of placental mitochondrial, microsomal, and cytosolic fractions in catalyzing the *N*-demethylation of methadone to EDDP was determined. Unless otherwise stated, the reaction components contained 100 mM potassium phosphate buffer, pH 7.4, 1 mg protein of the subcellular fraction and methadone at its final concentration of 1.25 mM (i.e., approximately 3× of its apparent K_m) in a total volume of 1 mL. The addition of the subcellular fraction to the components initiated the preincubation period of 5 min at 37 °C and the reaction was initiated by adding the NADPH-regenerating system made of the following: 0.4 mM NADP, 4 mM glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase, and 2 mM $MgCl_2$. This system contains glucose-6-phosphate

dehydrogenase that will convert, in presence of glucose-6-phosphate, the $NADP^+$ formed to NADPH required for the oxidase enzyme. The reaction components were then shaken at 37 °C for 20 min and reaction was terminated by the addition of 100 μL of 15% (w/v) trichloroacetic acid (TCA) containing 1 μg/mL LAAM as an internal standard. The reaction components were then centrifuged at 12,000 × *g* for 10 min and the amounts of EDDP formed was analyzed by HPLC as described below. The conversion of EDDP to EMDP was determined under identical experimental conditions. The control reaction was carried out utilizing TCA-denatured placental fraction. The effect of methadone, in the concentration range of 50–2000 μM, on reaction velocity was used to construct the saturation curve used to calculate the apparent K_m and V_{max} values. The remaining experimental conditions ensured that the rate of methadone *N*-demethylation was linear with protein concentration and incubation time.

2.4. Identification of the enzyme catalyzing the metabolism of methadone

Identification of the enzyme was achieved by utilizing (1) chemicals known as selective inhibitors of CYP isoforms and (2) monoclonal antibodies raised against purified human liver CYP isoforms.

2.4.1. Effect of chemical inhibitors on the biotransformation of methadone to EDDP

The effect of inhibitors selective for the different CYP isoforms on EDDP formation by microsomal fractions was determined by adding each to the reaction components. The final concentration of methadone in the reaction mixture was 500 μM, i.e., approximately equal to its apparent K_m value of 424 μM. The concentration used for each inhibitor was that equal to its reported IC_{50}, K_i, or apparent K_m values for a specific CYP isoform [20–22]. The following are the inhibitors used and the name of each is followed by its concentration and the name of the isozyme that it is selective for: α-naphthoflavone (0.1 μM), CYP1A; sulfaphenazole (10 μM), CYP 2C; quinidine (5 μM) CYP2D6; 4-methylpyrazole (25 μM), CYP2E1; nifedipine (200 μM), CYP3A4; ketoconazole (0.5 and 2.5 μM), CYP3A4; 4-hydroxyandrostenedione (1 and 10 μM), CYP19; and aminoglutethimide (1 and 10 μM) CYP19. Each inhibitor was simultaneously added with methadone to the reaction components and pre-incubated for 5 min at 37 °C. The NADPH-regenerating system was added to initiate the reaction, and the incubation was continued for another 20 min until terminated by the addition of 15% (w/v) TCA. All stock solutions of the inhibitors were made in methanol. The maximum final concentration of methanol in the reaction was 0.5%. The control reaction included all components except for the inhibitor and in presence of 0.5% methanol (v/v).

2.4.2. Inhibition of EDDP formation by monoclonal antibodies to CYP isozymes

Monoclonal antibodies against human liver CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1 and 3A4, and rabbit antiserum to human placental aromatase were used to identify the CYP isozyme responsible for the metabolism of methadone by placental microsomes. Each monoclonal antibody was added to the reaction components at its concentration causing 80% inhibition of the CYP isoform it was raised against. Microsomal protein, 0.2 mg, was pre-incubated with the antibody at room temperature for 15 min followed by the addition of methadone at its final concentration of 1.25 mM. The addition of the NADPH-regenerating system initiated the reaction that was incubated for 20 min at 37 °C then terminated by the addition of TCA. In the control reaction, mouse IgG replaced the monoclonal antibodies.

2.5. Metabolism of methadone by a cDNA-expressed CYP19 preparation

The kinetics for a cDNA-expressed CYP19 preparation for catalysis of the *N*-demethylation of methadone to EDDP was determined. The concentration of CYP19 was 40 pmol/mL of reaction volume. The effect of a range of methadone concentrations between 50 and 2000 μM on the velocity of EDDP formation was used to obtain the saturation curves necessary to calculate the apparent K_m and V_{max} for the reaction. The incubation conditions were identical to those mentioned above for determining the kinetics for *N*-demethylation of methadone by placental microsomal fractions. The rates of EDDP formation obtained were expressed as pmol of EDDP/pmol of CYP19.

2.6. The activity of placental CYP19/aromatase

The activity of CYP19/aromatase in catalyzing the conversion of testosterone to17β-estradiol was determined using placental mitochondrial and microsomal fractions. The following reaction components were pre-incubated for 5 min at 37 °C: 250 μg of protein (mitochondrial or microsomal) and 1.0 μM testosterone in a final volume of 1 mL of 0.1 M potassium phosphate buffer. The reaction was initiated by the addition of NADPH-regenerating system, incubated for 5 min and terminated by the addition of 100 μL of 10% (w/v) TCA. Estrone, 100 μL of a 10 μg/mL solution, was added to the reaction components as an internal standard, centrifuged at 12,000 × *g* for 10 min and the pellet discarded. The amount of 17β-estradiol in the supernatant was determined by HPLC as described below.

2.7. Western blot analysis of aromatase

Microsomal and mitochondrial fractions were prepared as described above. The amount of protein loaded for all

samples was 10 μg/well. Resolution of the samples was achieved using a 12% SDS–PAGE and electro-transferred to nitrocellulose membranes overnight at 4 °C using a constant potential of 25 V. The blots were probed, at room temperature, with a rabbit polyclonal antiserum against human aromatase (Hauptman–Woodward Medical research Institute), at a dilution of 1:2000 for 90 min. Goat anti rabbit IgG conjugated to horseradish peroxidase (Upstate) was used as secondary antibody at a dilution of 1:5000. Detection was achieved by an enhanced chemiluminescence system (Pierce), and the resulting signal was quantitated by spot densitometry using a digital imaging system (Alpha Innotech Corp.). Microsomes of cDNA-expressed aromatase (Gentest) were used as standards.

2.8. HPLC-UV

The HPLC systems used was made of a Waters 600E multi-solvent delivery system with a Waters 2487 dual wavelength absorbance detector and a Waters 717 autosampler and were controlled by a Waters Millennium chromatography manager (Waters). The stationary phase was a 250 mm × 4.6 mm Luna 5 μm C-18 column (Phenomenex).

2.8.1. Identification of EDDP

Separation and identification of EDDP was achieved by the method described by Iribarne et al. [4]. Briefly, the mobile phase was made of acetonitrile/water 35/65, v/v, containing 1% (v/v) triethylamine, and the pH was adjusted to 2.8 with orthophosphoric acid. Elution was at a flow rate of 1 mL/min and was monitored at a wavelength of 210 nm. Chromatographic peaks were identified by their retention times and compared to that of the standard compounds. The quantity of EDDP formed was determined using a standard curve made of six concentrations ranging between 0.25 and 2.0 μg/mL.

2.8.2. Determination of estradiol

The amount of 17β-estradiol formed was determined by the method described earlier [23]. The mobile phase was made of 0.1% triethylamine in acetonitrile/water (45:55, v/v), and the pH was adjusted to 3.5 by orthophosphoric acid. Isocratic elution was at a flow rate 1.2 mL/min and the eluent monitored at a wavelength of 280 nm. All calculations were based on the ratio of estradiol peak area to that of the internal standard (estrone).

2.9. Data analysis

All data are represented as mean ± S.D. Values for the apparent K_m and V_{max} were calculated from the saturation curves using Michaelis–Menten equation and nonlinear regression (SPSS 11.0 for windows software, SPSS Inc.).

Statistical analysis of the data on the effect of inhibitors on methadone metabolism was carried out using one-way ANOVA with Tukey's comparison and deemed significant if the P value was <0.05.

3. Results

3.1. Methadone metabolite(s)

Separation of methadone and its metabolite EDDP was achieved by HPLC according to the method described. A chromatogram showing the retention times of the opioid and its metabolite is presented in Fig. 2A. Aliquots of the reaction supernatant containing methadone and the

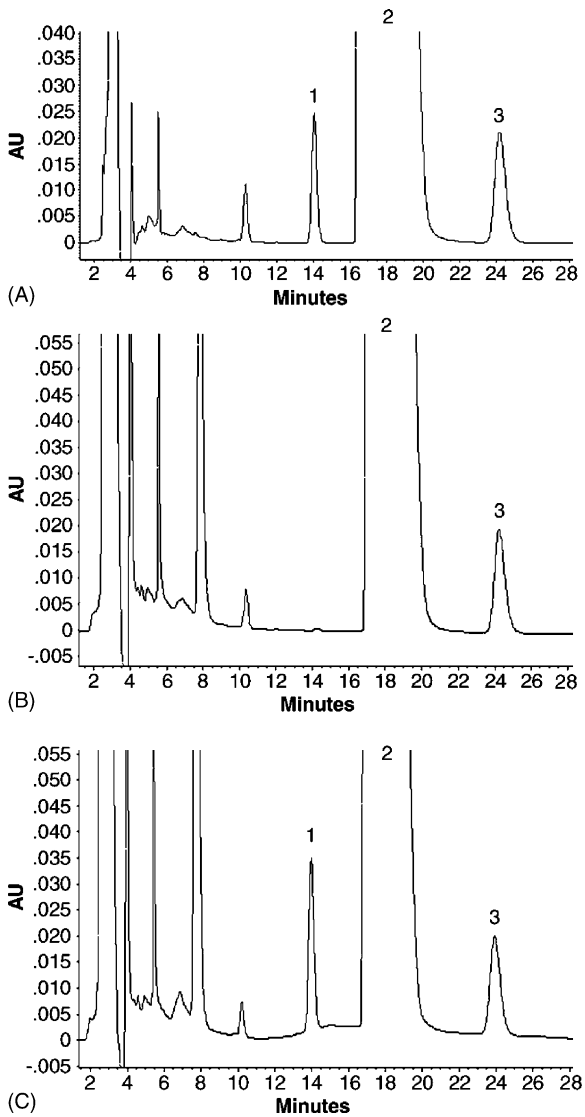

Fig. 2. (A) An HPLC chromatogram for the retention times of standards: (1) EDDP (14 min); (2) methadone (18 min); (3) LAAM (24.5 min). The latter compound is used as an internal standard. A chromatogram of the reaction components: (B) in absence and (C) in presence of the NADPH-regenerating system. EDDP is the only metabolite formed in presence of the co-enzyme (C).

microsomal fraction in absence (Fig. 2B) and presence (Fig. 2C) of the NADPH-regenerating system are shown. The chromatogram revealed the formation of the metabolite EDDP (14 min) in the presence of NADPH suggesting that a CYP isozyme might be catalyzing the reaction. The metabolite EMDP was not detected under this experimental conditions. Also, EMDP was not detected when EDDP was used as a substrate instead of methadone.

The catalytic activity of the mitochondrial and cytosolic fractions was compared to the microsomal. Eight placentas were used for this comparison and equal protein concentration of each fraction was utilized. Data obtained revealed that the highest rates for *N*-demethylation of methadone was in the microsomal fraction (432 ± 176 pmol (mg protein)$^{-1}$ min^{-1}) followed by the mitochondrial (252 ± 128 pmol (mg protein)$^{-1}$ min^{-1}) and the cytosolic (58 ± 20 pmol (mg protein)$^{-1}$ min^{-1}).

3.2. Methadone metabolism by microsomal preparations

Preliminary data on the reaction conditions revealed that the amount of EDDP formed was linear with protein concentration and time (up to 3 mg/mL and 20 min, respectively). The rate of EDDP formation was dependent on methadone concentration and exhibited typical Michaelis–Menten saturation kinetics (Fig. 3). Analysis of the hyperbolic saturation curves obtained from seven microsomal preparations revealed a mean apparent K_m and V_{max} values of 424 ± 92 μM and 420 ± 89 pmol (mg protein)$^{-1}$ min^{-1}, respectively.

Eadie–Hofstee plot of the data (Fig. 3, insert) indicated that *N*-demethylation of methadone exhibited monophasic

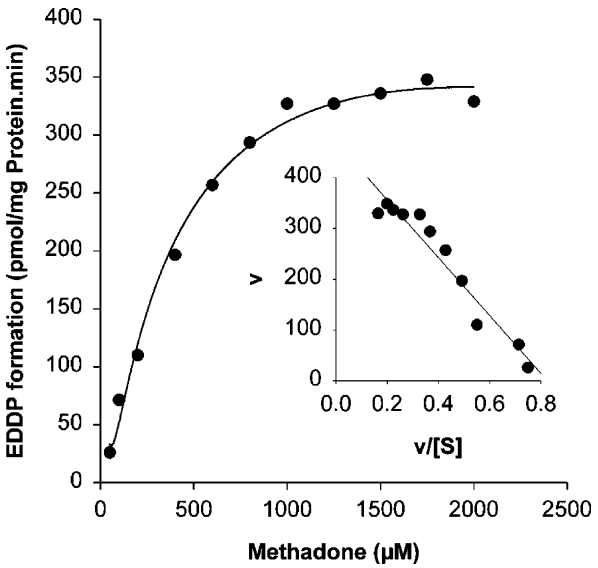

Fig. 3. A plot of the relation between increasing methadone concentrations and the rate of methadone metabolism by placental microsomes indicates saturation kinetics. The apparent K_m and V_{max} values calculated from the saturation curve were 424 ± 92 μM and 420 ± 89 pmol (mg protein)$^{-1}$ min^{-1}. (Inset) Monophasic kinetics was confirmed using Eadie–Hofstee plots of reaction velocity (v) against $v/[S]$.

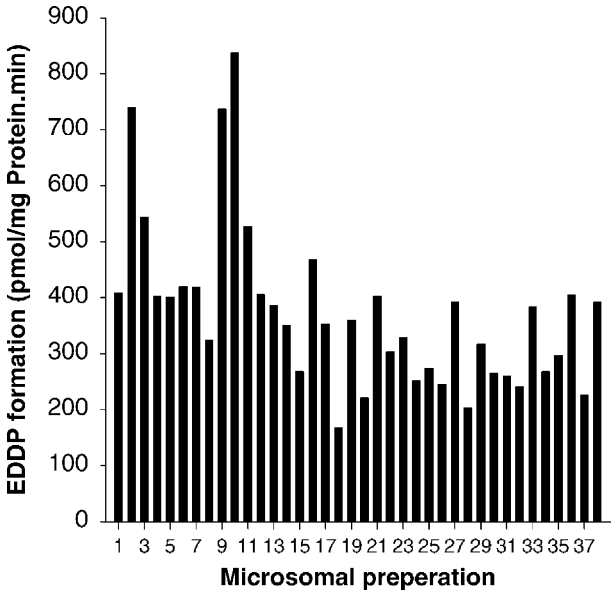

Fig. 4. The variations observed in the reaction velocity for *N*-demethylation of methadone by microsomal preparations from 38 term placentas.

enzyme kinetics in the range of methadone concentrations tested.

The *N*-demethylation activity of microsomal preparations from 38 placentas ranged between 184 and 839 pmol (mg protein)$^{-1}$ min^{-1} (Fig. 4). However, the distribution of the enzyme activity, in pmol (mg protein)$^{-1}$ min^{-1}, was as follows: <250, 10% of the preparations; 250–500, 82%; and >500, 8%. This range of activity could be attributed to the variations between placentas obtained from different women (interplacental variations).

3.3. Inhibition of methadone N-demethylation

The effect of compounds that are selective inhibitors of CYP isoforms on EDDP formation was investigated in five placental preparations. In each reaction, the final concentrations of methadone was 500 μM and each inhibitor was 10× its reported IC_{50} value for its specific CYP isozyme. The maximum inhibition of EDDP formation was observed in presence of 10 μM aminoglutethimide (88% of control) and 4-hydroxyadrostenedione (70% of control) (Fig. 5). Both compounds are selective for CYP19 and the inhibition was statistically significant ($P < 0.001$). On the other hand, the following compounds, at their concentrations shown in Fig. 5, did not affect EDDP formation: quinidine (CYP2D6), sulfaphenazole (CYP2C9), α-naphthoflavone (CYP1A), 4-methylpyrazole (CYP2E1), ketoconazole, and nifedipine (CYP3A). These data suggest that CYP19 is the microsomal enzyme catalyzing the biotransformation of methadone to EDDP.

Antibodies raised against human CYP isoforms were utilized to confirm the identity of the microsomal enzyme catalyzing the biotransformation of methadone to EDDP. A pool of microsomal fractions prepared from 12 placentas

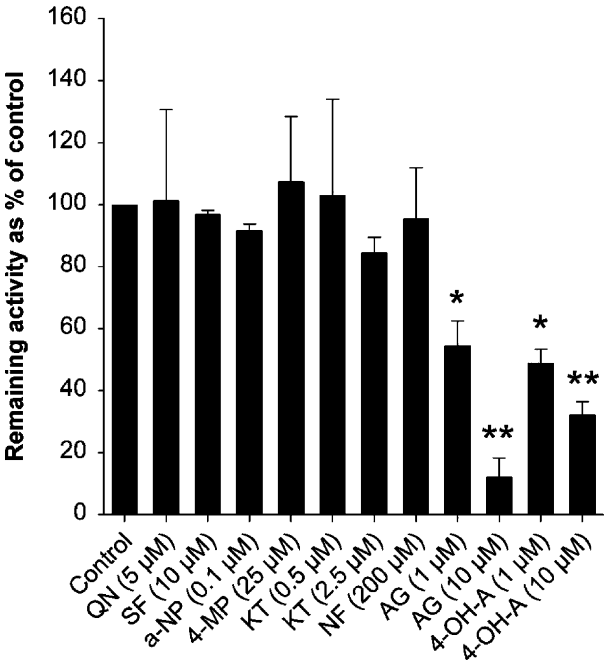

Fig. 5. The effect of chemical inhibitors on methadone *N*-demethylation by placental microsomes. The inhibitors are: quinidine, QN; sulfaphenazole, SF; α-naphthoflavone, α-NP; 4-methylpyrazole, 4-MP; ketoconazole, KT; nifedipine, NF; aminoglutethimide, AG; and 4-hydroxyandrostenedione, 4-OH. The final concentration of methadone in the reaction was 500 μM, i.e., equal to its apparent K_m. The rates of metabolite formation are expressed as percent of control (absence of inhibitors) and represent the mean ± S.D. of three to six experiments. Only AG and 4-OH-A caused a significant inhibition of methadone *N*-demethylation.

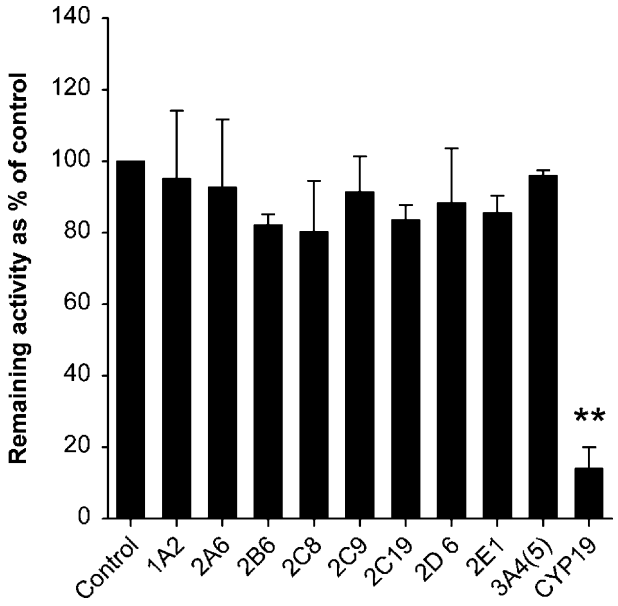

Fig. 6. The effect of antibodies raised against human CYP450 isozymes on the *N*-demethylation of methadone by placental microsomes. The rate of metabolite formation was expressed as percentage of control (absence of antibodies). The values are given as mean ± S.D. of three to six experiments. Antibodies raised against CYP19 caused >80% inhibition of EDDP formation.

was utilized in these experiments and each antibody was tested in triplicates. Antibodies to CYP19 were the most potent and caused an 80% inhibition of EDDP formation. Antibodies against 2B6, 2C8 and 2C19 caused <20% inhibition, while the effect of the remaining antibodies did not reveal any effect on EDDP formation (Fig. 6).

Therefore, compounds that are selective inhibitors of CYP19 and antibodies raised against the same isozyme caused the most pronounced inhibition of methadone metabolism to EDDP. Taken together, these data strongly suggest that CYP19 is the enzyme catalyzing the metabolism of methadone.

3.4. Kinetic constants for N-demethylation of methadone by cDNA-expressed CYP19

The saturation curves for the *N*-demethylation of methadone to EDDP by the cDNA-expressed CYP19 were used to calculate the apparent K_m and V_{max} and reveled the following values, 334 ± 91 μM and 6.3 ± 2.3 pmol (pmol CYP19)$^{-1}$ min^{-1}, respectively. This apparent K_m value is similar to that determined for the placental microsomal fractions catalyzing the same reaction.

Taken together, three different approaches to the identification of the enzyme catalyzing the metabolism of methadone indicated that it is CYP19/aromatase.

3.5. Aromatization of testosterone to estradiol by placental subcellular fractions

The activity of CYP19/aromatase in catalyzing the aromatization of testosterone (its natural substrate) to estradiol in microsomal and mitochondrial fractions prepared from nine placentas was determined. The mean ratio of mitochondrial to microsomal CYP19 activity was 0.52 ± 0.2. The *N*-demethylation of methadone by the same placental preparations was determined, and the mean activity ratio for the same fractions was 0.43 ± 0.16.

CYP19 protein expression in the mitochondrial and microsomal fractions from nine placentas was compared to that of the cDNA-expressed isozyme using Western blots (data for four placentas are shown in Fig. 7). The

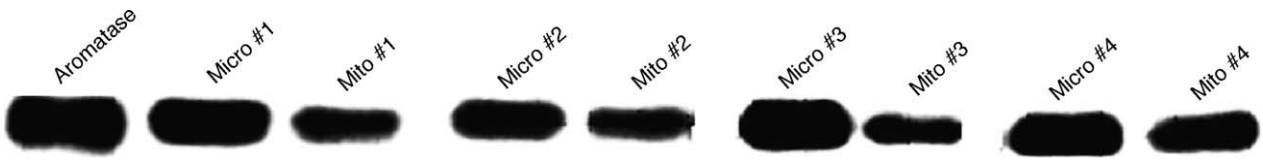

Fig. 7. The expression of microsomal and mitochondrial aromatase as revealed by Western blots. In general, the intensity of the bands corresponding to aromatase in the mitochondrial preparations were approximately half that of the microsomal.

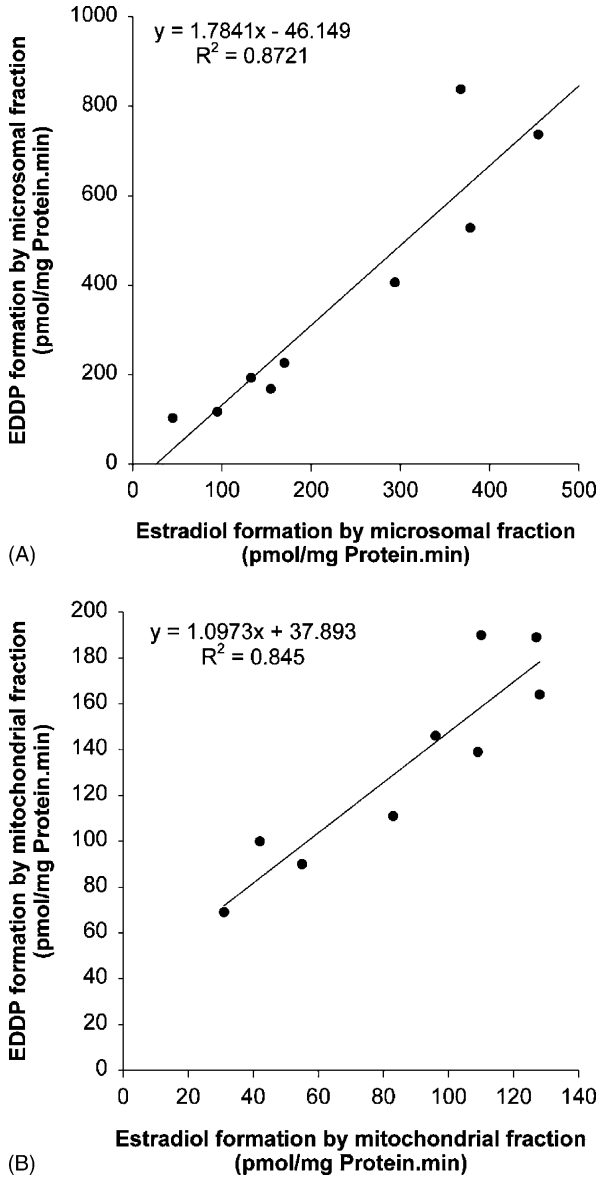

Fig. 8. Correlation between *N*-demethylation of methadone and aromatization of testosterone in (A) the microsomal and (B) the mitochondrial preparations.

data revealed that aromatase expression in the mitochondrial fraction was half that of the microsomal.

An examination of the correlation between CYP19 activities in its demethylation of methadone to EDDP and its conversion of testosterone to estradiol, in nine placental microsomal and mitochondrial preparations revealed R^2 values of 0.87 and 0.85, respectively (Fig. 8A and B).

Taken together, these data indicate that CYP19/aromatase is involved in the *N*-demethylation of methadone in placental microsomal and mitochondrial fractions.

4. Discussion

Methadone maintenance programs are considered the standard for pharmacotherapy of the heroin/opioid addict, including the pregnant. However, information on its biodisposition by human placenta is scarce to nonexistent. This report provides information on the identification of the major enzyme responsible for the metabolism of methadone in term placentas obtained from healthy pregnancies without obstetrical complications.

Microsomal preparations from human liver catalyzed the *N*-demethylation of methadone to EDDP but EMDP was not detected by the analytical methods used [2–4]. The concentration of EMDP, when present in human urine, was reported as <5% of that for EDDP, leading to difficulties in its determination because of the detection limits for the methods used [24–26]. The major enzyme responsible for the metabolism of methadone by human liver microsomal fractions was identified as CYP3A4 [2,4]. In human intestine, the major enzyme responsible for the metabolism of methadone to EDDP is also CYP3A4 but small amounts of EMDP were detected and attributed to the increase in the detection limits of the method used namely, liquid chromatography-mass spectrometry instead of HPLC-UV [27]. CYP3A4 was also responsible for the metabolism of the opioids buprenorphine and LAAM in the human liver and intestine [2,5,27–29]. However, in term human placenta, CYP19 was identified as the major enzyme responsible for the *N*-dealkylation of buprenorphine to norbuprenorphine and the *N*-demethylation of LAAM to norLAAM [16,17].

In our initial experiments to identify the major enzyme responsible for the metabolism of methadone in human placenta, it was assumed that it could be a microsomal CYP450 isozyme because it required the presence of NADPH-regenerating system. Moreover, placental CYP19 metabolized LAAM which is a congener of methadone [17]. The activity of microsomal preparations in metabolizing methadone was determined in 38 term placentas and revealed a range of four- to six-fold (Fig. 4). The only metabolite detected under our experimental conditions and utilizing the HPLC-UV method described was EDDP (Fig. 2). EMDP was not detected in any of the placental preparations, i.e., no sequential demethylation of methadone was observed. Similar lack of detectable sequential demethylation was reported for LAAM to norLAAM only (no dinorLAAM) by placental microsomal CYP19 [17]. It appears that placental metabolism of methadone and its congener LAAM by CYP19 is different from that reported for their hepatic metabolism by CYP3A4. Another similarity between the metabolism of methadone and that of its congener LAAM as well as the opioid buprenorphine is the four- to five-fold range of enzymatic activities between different placentas [16,17].

The effect of increasing concentrations of methadone on the rate of EDDP formation exhibited hyperbolic Michaelis–Menten saturation kinetics. Analysis of the data revealed an apparent K_m of 424 ± 92 μM. An Eadie–Hofstee plot of the data indicated monophasic kinetics and suggested that either a single enzyme is catalyzing the biotransformation of methadone to EDDP (Fig. 3), or

multiple enzymes with similar affinities to the substrate. Similar monophasic kinetics for the metabolism of methadone by hepatic microsomes was reported [3]. However, in human intestine, the metabolism of methadone by microsomal CYP3A4 exhibited biphasic kinetics [27].

The intrinsic clearance of methadone by human placental microsomes was $8.5 \times 10^{-4} \pm 2.6$ mL (mg protein)$^{-1}$ min^{-1} and is one order of magnitude less than that reported for intestinal microsomes ($11.3 \times 10^{-3} \pm 1.1$ mL (mg protein)$^{-1}$ min^{-1}) [27]. The latter clearance was comparable to that for hepatic microsomes and suggests that placental metabolism of methadone is significantly less than that by the liver and or intestine.

In this report, three experimental approaches were utilized to identify the major placental enzyme responsible for *N*-demethylation of methadone to EDDP namely; the effect of chemical inhibitors selective for specific CYP isoforms; the effect of monoclonal antibodies against human CYP isoforms; and comparing the kinetics of the reaction in placentas to that catalyzed by a cDNA-expressed preparation of CYP19. Aminoglutethimide and 4-hydroxyandrostenedione, at their concentrations that are most selective for CYP19, were the most potent inhibitors of EDDP formation (88 and 70% inhibition, respectively). These data suggests that CYP19/aromatase is the major placental enzyme responsible for *N*-demethylation of methadone (Fig. 5).

The effect of monoclonal antibodies on the metabolism of methadone by placental microsome revealed that those raised against human placental CYP19 were the most potent and caused approximately 80% inhibition of EDDP formation (Fig. 6). Antibodies against CYP2B6, 2C8, and 2C19 caused <20% inhibition while those against 1A2, 2A6, 2C9, and 2E1, and 3A4/5 had no effect. The lack of a total (100%) inhibition of CYP19 demethylation of methadone to EDDP by either the chemical inhibitors or the monoclonal antibodies suggests that CYP19 might not be the only enzyme responsible for the metabolism of methadone.

The apparent K_m values for methadone metabolism by placental microsomal preparations and by the cDNA-expressed CYP19 were similar. Taken together, the inhibition experiments and the similarity of the kinetics between of the cDNA-expressed CYP19 and that of the placenta indicate that aromatase is the major enzyme involved in the *N*-demethylation of methadone in human placenta.

The activity of placental mitochondrial fraction in the *N*-demethylation of methadone was approximately 50% of the microsomal. A similar distribution for the activity of CYP19 between placental microsomal and mitochondrial fractions was reported earlier for the conversion of androgens to estrogens [30]. The activity of placental mitochondrial and microsomal CYP19 in the aromatization of its natural substrate testosterone to estradiol was determined. The ratios for the enzymatic activities of the mitochondrial to microsomal metabolism for both substrates were approximately 0.5. Moreover, the expression of aromatase in the mitochondrial fraction as determined by Western blots was 50% of that in the microsomal fraction (Fig. 7). These data indicate that aromatase activity is present in the placental mitochondrial fractions and is approximately half of that in the microsomal. In addition, the correlation between the *N*-demethylation of methadone in the microsomal and mitochondrial fractions with the aromatization of testosterone to estradiol had an R^2 value of 0.87 and 0.84, respectively (Fig. 8A and B). This correlation is consistent with CYP19 being the enzyme catalyzing the aromatization and the *N*-demethylation reactions.

The data, from our laboratory and as reported by others, do not provide evidence for the presence of CYP19 in placental mitochondria and our experimental conditions cannot rule out contamination of both the microsomal and mitochondrial fractions with each other. However, the focus of our investigation was to identify the major placental enzyme responsible for the metabolism of methadone rather than its subcellular localization. Our ultimate goal is a better understanding of the placental role in the bio-disposition of the opioids used for treatment of the pregnant woman.

Data cited in this report on the metabolism of methadone by CYP19 indicate its role as xenobiotic-metabolizing enzyme in human placenta. Recent data indicated that a concentration of 0.24 mg/L methadone in maternal circulation, or more, is necessary to prevent fetal and maternal withdrawal and that it can be achieved by a dose of the drug ranging between 50 and 150 mg with occasional need for higher doses during the third trimester [31]. Our data revealed that the activity of term placental preparations could vary by four- to six-folds in the rates of methadone metabolism. This variation could be one of the contributing factors for the increased metabolism of methadone during pregnancy, especially during the third trimester, and emphasizes appropriate methadone dosing to prevent maternal and fetal/neonatal withdrawal. Moreover, it is unclear whether the metabolism of methadone or BUP by placental CYP19 could affect its role in placental biosynthesis of estrogens.

In summary, CYP19/aromatase is a key enzyme in human placental steroidogenesis specifically the aromatization of androstenedione and testosterone to estrogens [32]. The enzyme was also implicated in the biotransformation of xenobiotics [15,33]. Our laboratory, reported on the metabolism of buprenorphine [16] and LAAM [17] by placental CYP19 and the same enzyme also metabolizes methadone. Taken together, it can be concluded that CYP19/aromatase is the major enzyme responsible for the metabolism of the three opioids in term human placenta.

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