available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

The effect of methadone and buprenorphine on human placental aromatase

Olga L. Zharikova, Sujal V. Deshmukh, Tatiana N. Nanovskaya, Gary D.V. Hankins, Mahmoud S. Ahmed *

Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX, USA

ARTICLE INFO

Article history:
Received 17 November 2005
Accepted 27 December 2005

Keywords:
Human placenta
CYP19/aromatase
Methadone
Buprenorphine (BUP)
Estrogen formation
Pregnant opiate addict

Abbreviations:
BUP, buprenorphine
CYP, cytochrome P450
EDDP, 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine
EMDP, 2-ethyl-5-methyl-3,3-diphenylpyrroline
norBUP, norbuprenorphine
E_2, 17β-estradiol
16-OHT, 16α-hydroxytestosterone
E_3, estriol
TCA, trichloroacetic acid

ABSTRACT

Methadone and buprenorphine (BUP) are used for treatment of the pregnant opiate addict. CYP19/aromatase is the major placental enzyme responsible for the metabolism of methadone to 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and BUP to norbuprenorphine (norBUP). The aim of this investigation was to determine the effects of methadone and BUP on the activity of placental microsomal aromatase in the conversion of its endogenous substrates testosterone to 17β-estradiol (E_2) and 16α-hydroxytestosterone (16-OHT) to estriol (E_3). The conversion of testosterone and 16-OHT by human placental microsomes exhibited saturation kinetics, and the apparent K_m values were 0.2 ± 1 and 6 ± 3 μM, respectively. V_{max} values for E_2 and E_3 formation were 70 ± 16 and 28 ± 10 pmol/mg protein min, respectively. Also, data obtained revealed that methadone and BUP are competitive inhibitors of testosterone conversion to E_2 and 16-OHT to E_3. The K_i for methadone inhibition of E_2 and E_3 formation were 393 ± 144 and 53 ± 28 μM, respectively, and for BUP the K_i was 36 ± 9 and 6 ± 1 μM. The higher potency of the two opiates and their metabolites in inhibiting E_3 formation is in agreement with the lower affinity of 16-OHT than testosterone to aromatase. Moreover, the metabolites EDDP and norBUP were weaker inhibitors of aromatase than their parent compounds. The determined inhibition constants of methadone and BUP for E_3 formation by a cDNA-expressed CYP19 preparation were similar to those for placental microsomes. Therefore, data reported here suggest that methadone, BUP, and their metabolites are inhibitors of androgen aromatization in the placental biosynthesis of estrogens.

* *Corresponding author at:* Department of Obstetrics and Gynecology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0587, USA. Tel.: +1 409 772 8708; fax: +1 409 747 1669.
E-mail address: maahmed@utmb.edu (M.S. Ahmed).
0006-2952/$ – see front matter © 2006 Published by Elsevier Inc.
doi:10.1016/j.bcp.2005.12.035

1. Introduction

The human placenta assumes a crucial role in the maintenance of pregnancy and fetal organogenesis, growth, and development. The structure and localization of the placenta, as an interface between the maternal and fetal circulations, allow its regulation of nutrient uptake from the maternal circulation, exchange of gasses between the latter and fetal circulation, and elimination of fetal waste products. Moreover, the human placenta is responsible for the synthesis of specific polypeptide and steroid hormones that have endocrine and paracrine functions (e.g., chorionic gonadotropin, and estrogens). Our working hypothesis during the last 5 years has been, human placenta may act as a functional barrier protecting the fetus from the effects of drugs/opiates and xenobiotics. The placenta achieves this role, in part, by the activity of its metabolizing enzymes and efflux transporters. Therefore, our investigations focused on placental disposition of the two opiates used in treatment of the pregnant heroin/opiate addict — namely, methadone, and buprenorphine (BUP).

The transplacental transfer of methadone and BUP, as compared with the freely diffusible and non-metabolizable antipyrine, was investigated using the technique of dual perfusion of term placental lobule. The data obtained revealed that the rate of methadone transfer to the fetal circuit was higher ($29.4 \pm 4.6\%$) than that for BUP ($11.6 \pm 2.5\%$) [1,2]. The concentration ratio for BUP in the tissue/fetal and tissue/maternal circuits, when the drug was transfused in the maternal-to-fetal direction were 27.4 ± 0.4 and 13.1 ± 6.5, respectively. The concentration ratio for methadone in the tissue/fetal and tissue/maternal circuits under identical experimental conditions were 9.9 ± 1.2 and 6.5 ± 1.0, respectively. Therefore, it is apparent that a concentration gradient for each opiate is formed between placental tissue and both the maternal and fetal circuits and that it is higher for BUP than for methadone due to their retention by the tissue. If such a gradient exists in vivo, the concentration of either methadone or BUP in placental tissue could be significantly higher than its therapeutic levels in the maternal circulation. Accordingly, the effect of either methadone or BUP on the activity of placental metabolic enzymes should be greater than that assumed on the basis of its circulating concentration following administration of a therapeutic dose. This conclusion gained importance in light of the data obtained on the metabolism of methadone and BUP by placental tissue. These recent investigations in our laboratory revealed that the major placental enzyme responsible for the metabolism of methadone to 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and BUP to norbuprenorphine (norBUP) was microsomal CYP19/aromatase [3,4]. Moreover, earlier investigations identified CYP19 as the enzyme responsible for the metabolism of other endogenous placental compounds and xenobiotics [5–7].

On the other hand, the major enzyme responsible for the metabolism of methadone [8–10] and BUP by human hepatic microsomes [11–13] was identified as CYP3A4 although other CYP isozymes were not ruled out [10]. Two metabolites for methadone, namely EDDP and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP), were detected in human plasma and urine [14,15] as well as in mice bile [14]. However, to the best of our knowledge, the in vitro sequential demethylation of methadone to EDDP and EMDP was catalyzed only by human intestinal microsomal preparations [16].

It is well recognized that CYP19/aromatase is a key enzyme in the biosynthesis of estrogens by human placenta — specifically, the conversion of C_{19} androgens to C_{18} estrogens [17,18]. The role of estrogens in pregnancy is, and has been, the subject of numerous investigations. Reports indicated that estrogens regulate several placental functions crucial for the maintenance of pregnancy and fetal development such as trophoblast differentiation, uteroplacental blood flow, uterine growth and contractility, as well as progesterone biosynthesis [19–23]. During pregnancy, the placenta becomes the major source for 17β-estradiol (E_2), estriol (E_3), and estrone (E_1) in the maternal and fetal circulations. E_3 is produced exclusively by human placenta from fetal precursors and was considered a useful indicator of fetal well being [21,24,25]. In addition, lower levels of E_3 correlated with below normal fetal and placental weights [26,27], and it was suggested that estrogen levels should be monitored during pregnancy [21]. It is also important to note that the concentration of E_3 is lower in animals and humans under treatment with methadone [28–30].

Therefore, it appears that human placenta could be a target for drug interactions in pregnant women under treatment with methadone or BUP. The aim of this investigation was to determine the effects of these two opiates on the activity of term placental CYP19/aromatase in the conversion of its endogenous substrates testosterone to E_2 and 16α-hydroxytestosterone (16-OHT) to E_3.

2. Material and methods

2.1. Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo) unless otherwise indicated. BUP, norBUP, methadone and EDDP were a gift from the drug supply unit of the National Institute on Drug Abuse. Acetonitrile was purchased from EM Science (Gibbstown, NJ). The cDNA-expressed CYP19 supersomes, commercially available from Gentest were utilized. Properties of the supersomes were reported previously [31].

2.2. Clinical material

All placentas were obtained immediately after delivery, from term healthy pregnancies, according to a protocol approved by the Institutional Review Board of the University of Texas Medical Branch at Galveston. Placentas of drug abusing women were excluded.

Villous tissue was excised, rinsed with ice-cold saline, and homogenized in 0.1 M potassium phosphate buffer pH 7.4 (Ultra Turrax, Staufen, Germany). The homogenate was used to prepare crude subcellular fractions (mitochondrial and microsomal) by differential centrifugation. The microsomal fraction was prepared by resuspending the $10{,}000 \times g$ pellet in 0.25 M sucrose buffer (pH 8), centrifuging at $104{,}000 \times g$, and

the pellet obtained was resuspended in 0.1 M potassium phosphate buffer (pH 7.4). Protein content of the fraction was determined using a commercially available reagent (Bio-Rad Laboratories kit, Hercules, CA) and BSA as a standard. Aliquots of the subcellular fraction were stored at −80 °C until used. A pool of 15 microsomal fractions was prepared and used in all experiments.

2.3. Activity of aromatase (CYP19)

2.3.1. 17β-Estradiol formation

The activity of placental microsomal fractions (0.25 mg protein) in catalyzing the conversion of testosterone to 17β-estradiol was determined in a total reaction volume of 1 mL potassium phosphate buffer (pH 7.4). Increasing concentrations of testosterone were added to the reaction solution, (highest concentration, 2.0 μM), and preincubated for 5 min at 37 °C. The reaction was initiated by the addition of NADPH regenerating system (NADP 0.4 mM, glucose-6-phosphate (G-6-P) 4 mM, G-6-P dehydrogenase 1 U/mL, and 2 mM $MgCl_2$) and incubated for 5 min at the same temperature. The reaction was terminated by the addition of 100 μL of a 10% (w/v) trichloroacetic acid and placed on ice. Estrone, 100 μL of 10 μg/mL solution, was added to each tube as an internal standard. The precipitated protein was separated by centrifugation at 12,000 × *g* for 10 min and the resulting supernatant extracted with 1.5 mL butyl chloride. The organic layer was separated, evaporated, and the residue resuspended in 200 μL of the HPLC mobile phase used to determine the amounts of E_2 formed as described below. Data reported on the kinetics of E_2 formation are the mean of three experiments.

2.3.2. Estriol formation

The activity of the pool of placental microsomal fractions in catalyzing the conversion of 16-OHT to E_3 was determined under identical reaction conditions to those described for E_2 formation except for the following: the highest concentration of 16-OHT was 50 μM, the incubation period was 15 min, and chlorimipramine (25 μL of 10%, w/v) was used as the internal standard, and the supernatant obtained after centrifugation was analyzed to determine the amounts of E_3. The mean of the results obtained from three experiments on the kinetics of E_3 formation is reported.

The apparent K_m and V_{max} values were calculated from the saturation curves of testosterone and 16-OHT using Michaelis-Menten equation and nonlinear regression.

2.4. Effect of the opiates on aromatase activity

2.4.1. Effect of the opiates on estrogen formation by placental microsomal fraction

The effect of BUP, methadone, and their metabolites on the aromatization of testosterone to E_2 and 16-OHT to E_3 by placental microsomal fractions was investigated. The IC_{50} value for the opiates were calculated from data obtained from three experiments for each as described below. The concentrations of steroid substrates were equivalent to their apparent K_m values determined in our laboratory (0.2 μM for testosterone and 6.0 μM for 16-OHT), and each opiate was added at a range of concentrations. For E_2 formation, the concentrations of opiates used were: methadone, 100–2000 μM; EDDP, 100–1000 μM; BUP, 10–200 μM; norBUP, 10–400 μM. For E_3 formation, the concentrations were: methadone and EDDP, 100–1000 μM; BUP, 1–100 μM; norBUP, 10–200 μM. Each IC_{50} value was calculated from plots of the percent of the product formed (i.e., in the absence of inhibitor) versus either the concentration of the inhibitor or the log of its concentration.

2.4.2. Kinetics of aromatase inhibition by opiates in placental microsomal fractions

The type of inhibition caused by each opiate, competitive or non-competitive, was determined in the presence and absence of each opiate, and the following ranges of substrate concentrations: testosterone, 100–800 nM; 16-OHT, 3–12 μM. For each reaction, zero time served as blank, and in the control, the opiate was replaced by an equal volume of the solvent. The data obtained were plotted as the reciprocal of the concentration of product formed versus the reciprocal of substrate concentration in the absence and presence of at least three concentrations of each inhibitor.

The constant of inhibition (K_i) of each opiate was determined by Dixon plots of data obtained on the effect of a range of opiate concentrations in the presence of two or three substrate concentrations with one of them equal to its apparent K_m value. The concentration range of each opiate was as follows: for E_2 formation — methadone, 500–1000 μM; BUP, 10–200 μM; norBUP, 50–400 μM and for E_3 formation — methadone, 100–500 μM; BUP, 5–25 μM; EDDP, 200–750 μM; norBUP, 25–100 μM. K_i values were estimated from plotting the reciprocal of the velocity of estrogens (E_2, E_3) formation versus inhibitor concentrations as an intercept of lines, representing two or three substrate concentrations. The values reported for each of the K_i of each opiate is the mean of the data obtained from three experiments.

The K_i for EDDP inhibition of E_2 formation was calculated from its IC_{50} values using the equation $IC_{50} = K_i \times (1 + [S]/K_m)$ [32]. The IC_{50} for EDDP was determined experimentally as described in Section 2.4.1.

2.4.3. Kinetics for the inhibition of cDNA-expressed aromatase activity by methadone and buprenorphine

The effect of methadone and BUP on E_3 formation by a cDNA-expressed CYP19 preparation "supersomes" was investigated and the K_i values for the opiates determined. The concentration of CYP19 was 10 pmol/250 μL of reaction volume. The reaction conditions and the concentration of methadone and BUP were identical to those described above for placental microsomes. The substrate concentration was equal to its K_m and $2 \times K_m$ (6 and 12 μM). The rates of E_3 formation are expressed as pmol of E_3/pmol of CYP19. The K_i values were determined by Dixon plots of the data obtained from three experiments.

2.5. Analysis of 17β-estradiol and estriol formation

The amounts of E_2 and E_3 formed were determined by HPLC/UV according to the method of Taniguchi et al. [33] with slight modifications to resolve E_2 from E_3 using a 250 × 4.6 mm Luna 5 μM C_{18} chromatography column (Phenomenex, Torrance,

Calif). The mobile phase used for analysis of E_2 was acetonitrile:water (45:55, v/v) containing 0.1% (v/v) triethylamine at a pH of 3.0 adjusted with orthophosphoric acid. Isocratic elution was performed at a flow rate of 1.2 mL/min and the eluent monitored at a wavelength of 200 nm. The mobile phase used for E_3 was made of acetonitrile:water (35:65, v/v) containing 0.2% (v/v) triethylamine at a pH of 3.5. The flow rate was maintained at 0.5 mL/min for the first 15 min of the run time and then changed to 1 mL/min for the remaining period, and the compound was detected at a wavelength of 280 nm.

2.6. Statistical analysis

Statistical analysis of data on the effect of the opiates on aromatase activity was carried out using ANOVA with multiple comparison analysis compared with zero inhibitor concentration.

3. Results

3.1. The conversion of testosterone to 17β-estradiol and 16α-hydroxytestosterone to estriol by placental microsomal fractions

Testosterone and 16-OHT are metabolic intermediates in the biosynthesis of estrogens in human placenta and are the naturally occurring substrates for the enzyme CYP19/aromatase. The rate of formation of the two products E_2 and E_3 was dependent on the concentration of their respective substrates and exhibited saturation kinetics (Fig. 1A and B). Analysis of the data obtained revealed apparent K_m values for testosterone and 16-OHT of 0.2 and 6 μM, respectively. These data suggest that the enzyme responsible for each of the two reactions is likely to be a CYP isozyme with higher affinity to testosterone (approximately 30 times higher) than to 16-OHT. Also, the maximum velocity for E_2 formation was approximately three times that for E_3 formation (70 ± 16 versus 28 ± 10 pmol mg^{-1} protein min^{-1}, respectively).

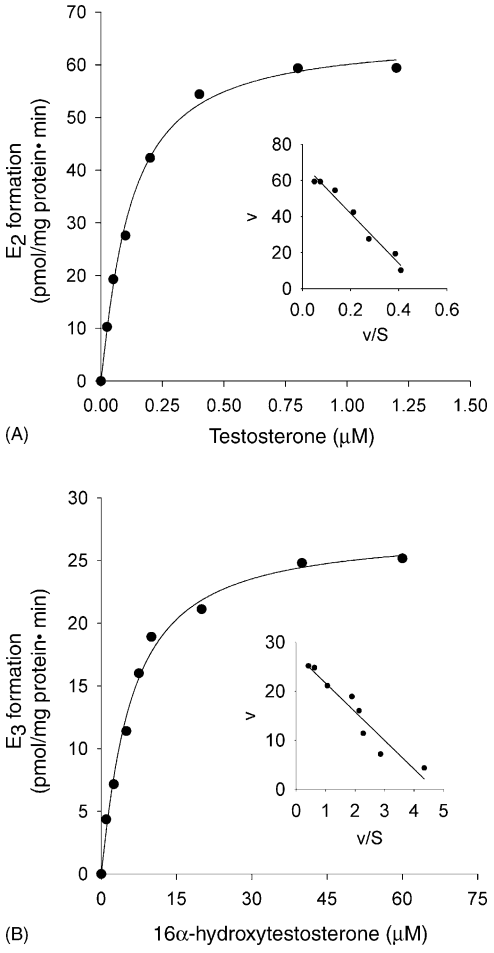

Fig. 1 – Plots of the relation between increasing concentrations of the androgens (A) testosterone or (B) 16α-hydroxytestosterone and the rate of estrogen formation (17β-estradiol [E_2] or estriol [E_3], respectively) by a pool of placental microsomal fractions indicate saturation kinetics. The insets, Eadie-Hofstee plots of reaction velocity (v) against $v/[S]$ confirm monophasic kinetics for the formation of E_2 and E_3. Each point represents the mean of three experiments. Analysis of the data obtained revealed the apparent K_m values for testosterone and 16α-hydroxytestosterone of 0.2 and 6 μM, respectively, and V_{max} values for E_2 and E_3 formation of 70 ± 16 and 28 ± 10 pmol mg^{-1} protein min^{-1}, respectively.

3.2. Effects of methadone, buprenorphine, and their metabolites on the activity of CYP19/aromatase

3.2.1. Methadone and EDDP

Both methadone and EDDP inhibited the formation of E_3 but only methadone had an effect on E_2 formation (Fig. 2A and B). Methadone, at a concentration of 500 μM, inhibited the formation of E_2 and E_3 by 40 and 90%, respectively. On the other hand, the concentration of 500 μM EDDP did not affect E_2 formation but inhibited that of E_3 by approximately 60%. The IC_{50} values calculated for the effect of methadone and its metabolite EDDP (Fig. 2A and B) are cited in Table 1.

3.2.2. Buprenorphine and norBUP

Both BUP and norBUP inhibit the conversion of testosterone to E_2 and 16-OHT to E_3 and were more potent inhibitors of E_3 than E_2 formation (Fig. 3A and B). BUP at a concentration of 100 μM inhibited E_3 formation by 90 and E_2 by 60%. On the other hand, an equimolar concentration of norBUP (100 μM) inhibited E_3 and E_2 formation by approximately 50 and 30%, respectively. It is also apparent that BUP is more potent than its metabolite norBUP in inhibiting E_2 and E_3 formation. The IC_{50} values calculated for BUP inhibition of E_2 and E_3 formation were 80 and 7 μM, respectively, while the corresponding values for norBUP were 176 and 103 μM (Table 1).

3.3. Kinetics of aromatase inhibition by opiates

3.3.1. Methadone and buprenorphine

Lineweaver-Burk plots of the data obtained revealed that methadone and BUP are competitive inhibitors for the binding of both testosterone and 16-OHT to aromatase (Fig. 4A–D).

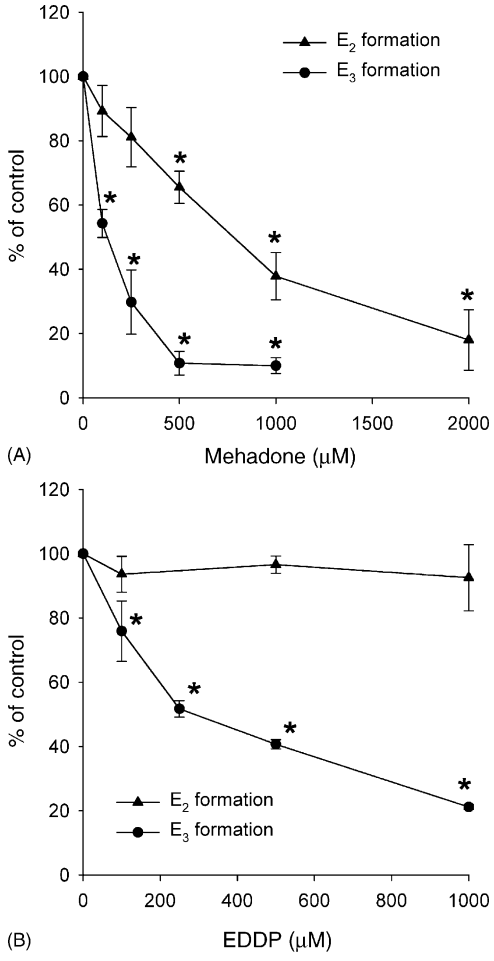

Fig. 2 – The effects of increasing concentrations of (A) methadone and (B) EDDP on 17β-estradiol (E_2) and estriol (E_3) formation. The opiates were pre-incubated with the steroid substrates at 37 °C for 5 min. The reaction was initiated by the addition of an NADPH regenerating system and the incubation continued for another 5 min in case of E_2 formation and 60 min for E_3. The concentrations of the substrates were 0.2 μM for testosterone and 6.0 μM for 16α-hyroxytestosterone. The rates for metabolite (E_2 or E_3) formation are expressed as percent of control (absence of an opiate). Each data point represents the mean ± S.D. of three experiments. *Statistical significance of $P < 0.05$.

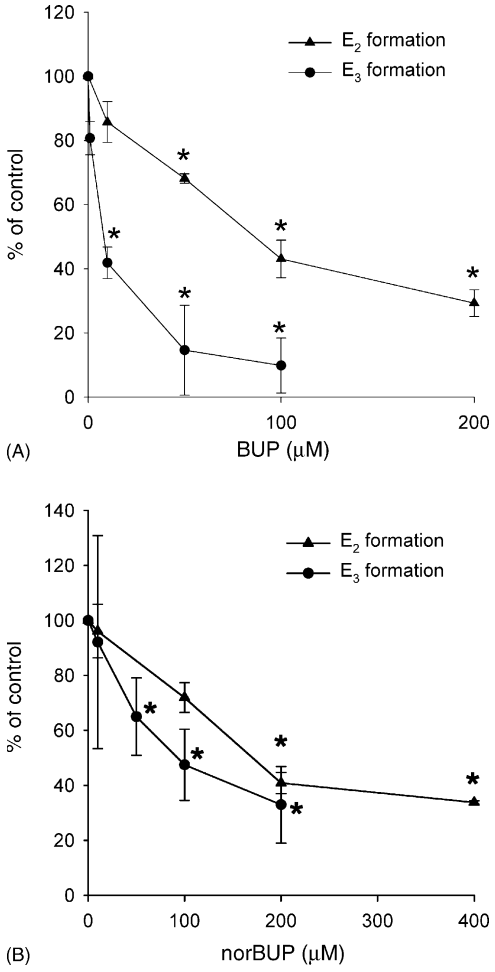

Fig. 3 – The effects of increasing concentrations of (A) BUP and (B) its metabolite norBUP on 17β-estradiol (E_2) and estriol (E_3) formation. The experimental conditions are identical to those described in Fig. 2. The rates of product formation (E_2 or E_3) are expressed as percent of control. Each data point represents the mean ± S.D. of three experiments. *Statistical significance of $P < 0.05$.

The K_i values for methadone and BUP inhibition of E_2 and E_3 formation were calculated by Dixon plots (Table 1, Fig. 5A and B). It is apparent from the K_i values for methadone and BUP that they are lower for the inhibition of the conversion of 16-OHT to E_3 than for testosterone to E_2. In all cases, it appears that BUP has approximately 10 times greater affinity to the enzyme than methadone.

A commercially available preparation of cDNA-expressed CYP19 was used to determine the K_i values for methadone and BUP inhibition of E_3 formation under experimental conditions similar to those described above for the pool of placental microsomal fractions. Dixon plots of the data obtained revealed K_i values (Fig. 6). These K_i values are in agreement with those obtained for the pool of placental microsomal fractions (Table 1) and confirm that the enzyme catalyzing the reactions in both cases is most likely the same.

3.3.2. *EDDP and norBUP*

The type of inhibition caused by EDDP and norBUP on the formation of E_2 and E_3 was determined using identical experimental conditions to those described for their parent compounds. EDDP, at a concentration of 1 mM, had no effect on the conversion of testosterone to E_2 but it was a competitive inhibitor of 16-OHT. However, norBUP was a competitive inhibitor of testosterone and 16-OHT conversion to E_2 and E_3, respectively.

The K_i for EDDP inhibition of the conversion of 16-OHT to E_3 and norBUP inhibition of testosterone conversion to E_2 and 16-OHT to E_3 was determined by Dixon plots of data obtained utilizing experimental conditions identical to those for their respective parent compounds. The K_i values obtained are cited in Table 2. It is apparent that the affinity of the opiate

Table 1 – The inhibition constants for the opiates and their metabolites

Opiate	Inhibition of testosterone conversion to 17β-estradiol		Inhibition of 16α-hydroxytestosterone conversion to estriol		
	Pool of placental microsomes[a]		Pool of placental microsomes[a]		cDNA-expressed CYP19
	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	K_i (μM)
Buprenorphine	80 ± 14	36 ± 9	7 ± 2	6 ± 1	9 ± 3
Norbuprenorphine	176 ± 22	131 ± 39	103 ± 40	56 ± 13	ND
Methadone	613 ± 44	393 ± 144	144 ± 58	53 ± 28	58 ± 26
EDDP	>2000	[b]	295 ± 27	161 ± 36	ND

Data represented are mean ± S.D. of three experiments. ND indicates "not determined"; EDDP, 2-ethylidine-1,5-dimethyl-3,3-diphenylpyrrolidine.

[a] Pool of microsomal fractions from 15 term placentas.

[b] K_i was calculated from the determined IC_{50} as 18,445 ± 15,859 μM.

metabolites to aromatase is less than that of their parent compounds. However, the metabolites are similar to the parent compounds in having higher affinity for aromatase conversion of 16-OHT to E_3 than that for testosterone to E_2. Also, norBUP has higher affinity to CYP19 than EDDP.

4. Discussion

Methadone and buprenorphine are used in maintenance/treatment programs for pharmacotherapy of the pregnant opiate addict. The patient, in most cases, joins the treatment

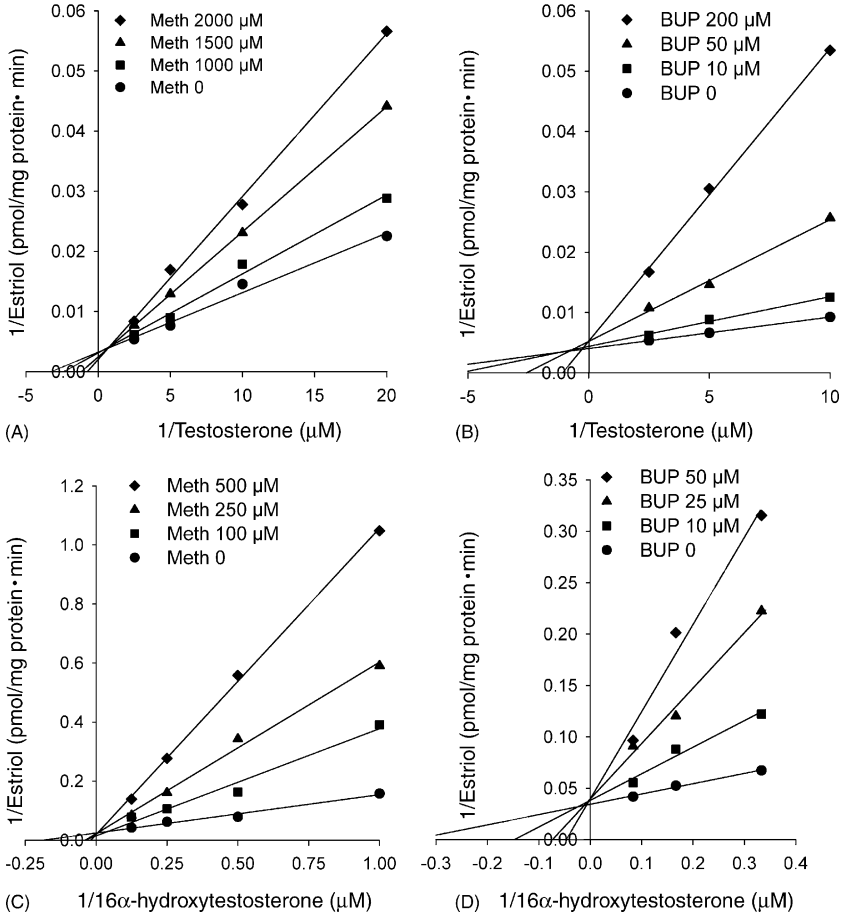

Fig. 4 – Lineweaver-Burk plots of the data to determine the type of inhibition for (A and B) 17β-estradiol (E_2) and (C and D) estriol (E_3) formation. The reciprocal of the rate of estrogen formation is plotted versus the reciprocal of substrate concentration in the presence and absence of the inhibitor (methadone [Meth] and buprenorphine [BUP]). For E_2 formation, testosterone was used at four concentrations: 0.1, 0.2, 0.4, and 0.8 μM (1/2, 1, 2, and 4 × K_m). For E_3 formation, 16α-hydroxytestosterone was used at three concentrations: 3, 6, and 12 μM (1/2, 1, and 2 × K_m). In both reactions the opiate did not have an effect on the V_{max} value but increased the apparent K_m of the reaction indicating competitive inhibition.

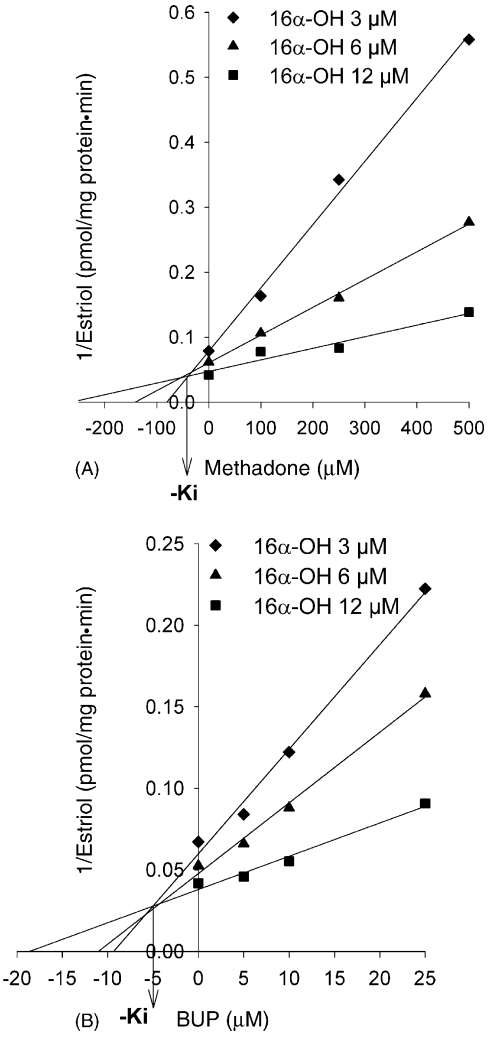

Fig. 5 – Dixon plots of the data obtained to determine K_i for (A) methadone and (B) buprenorphine inhibition of 16α-hydroxytestosterone (16-OHT) conversion to estriol. The reciprocal of the velocity for estriol formation is plotted against the concentration of the opiate in the presence of three different substrate concentrations (1/2, 1, and 2 × K_m). Each substrate concentration was incubated in the presence of placental microsomes, NADPH regenerating system, and increasing concentrations of either methadone (100, 250, 500 μM) or BUP (5, 10, 25 μM) for a period of 60 min.

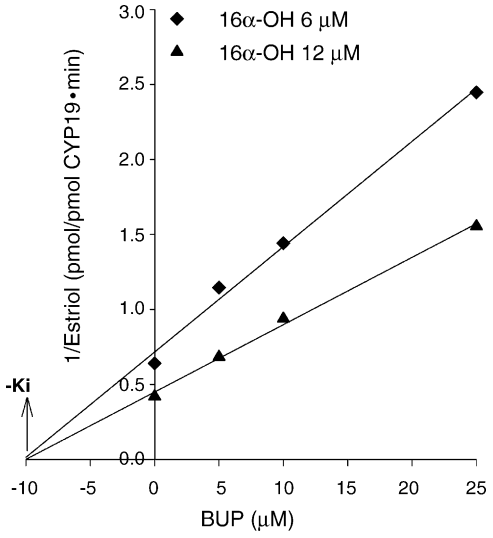

Fig. 6 – Dixon plot of the data obtained to determine buprenorphine (BUP) constant of inhibition (K_i) for the conversion of 16α-hydroxytestosterone (16-OHT) to estriol by a commercially available cDNA-expressed CYP19. Two substrate concentrations were used: equal to and 2× apparent K_m value. All other experimental conditions were identical to those described in Fig. 5.

program in the beginning of the first trimester and continues until delivery. The administered dose of methadone varies between 40–150 and 4–24 mg/day for BUP [34,35] according to the patient condition. However, adjustment of the administered opiate dose with the progress of gestation might be necessary for certain individuals. Evidence on improving maternal and neonatal outcome of patients under treatment has been extensively reported and a review of these data would be beyond the scope of this report. Nevertheless, a controversy exists on whether the dose of the administered opiate correlates with the incidence and/or intensity of neonatal abstinence syndrome [36].

The working hypothesis for investigations in our laboratory during the last 7 years has been, and is, one of the variables that could affect the incidence and intensity of neonatal abstinence syndrome is the concentration of methadone or BUP in the fetal circulation and the latter will depend on placental disposition of the opiate. The human placenta acts as an interface between the maternal and fetal circulations and was considered to be a "barrier". However, this barrier does not affect drugs with a molecular weight <1000. These drugs, including opiates, are transferred from one circulation to the other by passive diffusion and according to their physicochemical properties. However, drugs can also be transferred from the maternal circulation by uptake transporters and from the tissue back to the maternal circulation by efflux transporters localized in the trophoblast layer of the tissue. In addition, the human placenta is also capable of metabolizing drugs and xenobiotics.

Aromatase is the major enzyme metabolizing methadone and BUP to EDDP and norBUP, respectively [3,4] and is also the key enzyme in the biosynthesis of estrogens by the human placenta; it is the main source of these hormones during pregnancy [25,37]. Thus, it became apparent that the human placenta could be a site for drug interactions between the administered methadone or BUP and the conversion of androgens to estrogens by aromatase. The goal of this investigation was to determine the effects of methadone, BUP, and their metabolites EDDP and norBUP on the in vitro conversion of testosterone to E_2 and 16-OHT to E_3 by human placental microsomal fractions.

The conversion of testosterone to E_2, by a pool of 15 placental microsomal fractions revealed typical substrate saturation kinetics with an apparent K_m of 0.2 μM (Fig. 1A) and required the presence of saturating concentration of

NADPH, suggesting that the reaction is catalyzed by a CYP450 enzyme. This data is in agreement with that reported earlier on the kinetics of the reaction [37,38]. Similarly, the conversion of 16-OHT to E_3 by the same pool of placental microsomes also required the presence NADPH. Analysis of the data obtained revealed substrate (16-OHT) saturation kinetics and an apparent K_m of 6 μM. Moreover, the apparent K_m values determined in our laboratory are in agreement with earlier reports indicating that the affinity of testosterone and androstenedione to aromatase is higher than their hydroxylated derivatives [38–40].

An earlier report [4] from our laboratory provided data on the metabolism of methadone (apparent K_m value, 424 ± 92 μM) to EDDP by aromatase. Data cited in this report indicated that methadone is a more potent inhibitor for the conversion of 16-OHT to E_3 than testosterone to E_2 as revealed by the respective K_i values of 53 and 393 μM (Fig. 2A; Table 1).

BUP is also metabolized to norBUP by term human placental CYP19 and the apparent K_m value reported was 12 ± 4 μM [3]. The data cited in Table 2 indicate that BUP was also a more potent inhibitor of 16-OHT conversion to E_3 than testosterone to E_2 as revealed by the respective K_i values of 6 ± 1 and 36 ± 9 μM. Moreover, analysis of the type of inhibition caused by methadone and BUP revealed that it was competitive (Fig. 4A–D). It is apparent that both methadone and BUP are more potent inhibitors of the reactions where the natural substrate had a lower affinity to aromatase — namely, 16-OHT (Table 1).

The effects of methadone and BUP on the activity of a commercially available preparation of cDNA-expressed CYP19 in conversion of 16-OHT to E_3 was compared to the effects of the opiates on the pool of placental microsomal fractions used in this investigation. Analysis of the data obtained revealed that methadone and BUP inhibited the reaction catalyzed by the cDNA-expressed CYP19 with K_i values of 58 ± 26 and 9 ± 3 μM, respectively. These inhibition constants are in agreement with those obtained for the same reaction catalyzed by the pool of placental microsomal fractions (Table 1), confirming that the enzyme affected in the preparation is aromatase.

Our laboratory reported on the metabolism of methadone to EDDP by aromatase and that EMDP was not detected under the experimental conditions used [4]. Similarly, hepatic microsomes metabolized methadone to EDDP only [8,9]. However, it should be noted that the sequential demethylation of methadone by intestinal microsomes to EDDP and EMDP was also reported [16]. At this time, it is unclear whether the sequential demethylation of methadone and the detection of EDDP and EMDP is tissue specific or is due to the experimental conditions used by the different investigators. Therefore, the effect of EDDP only was investigated and the data revealed that it had no effect on the conversion of testosterone to E_2 but inhibited the conversion of 16-OHT to E_2 with a K_i of 161 ± 36 μM.

An earlier investigation of BUP metabolism by placental CYP19 revealed its dealkylation to norBUP as detected by HPLC/MS [3]. Contrary to EDDP, norBUP inhibited the formation of both E_2 and E_3. NorBUP was also a more potent inhibitor of 16-OHT conversion to E_3 than testosterone to E_2 as revealed by the determined K_i values of 56 ± 13 and 131 ± 39 μM, respectively. Moreover, analysis of our data indicated that both EDDP and norBUP were competitive inhibitors of the natural substrates of CYP19. Therefore, it is apparent that EDDP and norBUP are poor substrates of aromatase and consequently are weaker inhibitors of estrogen formation than their parent compounds. In addition, the potency of EDDP and norBUP is similar to their parent compounds in inhibiting E_3 more than E_2 formation as discussed above and could also be explained by the lower affinity of 16-OHT than testosterone to CYP19.

The characteristics of the active site of aromatase and the binding properties of its substrates and inhibitors as well as the mechanism of aromatization has been the subject of numerous investigations that provided insight into the role of the enzyme in the biosynthesis of estrogens by the placenta and other tissues. Initial reports suggested the presence of aromatase isozymes in steroidegenic tissues [39] and were followed by others, suggesting one enzyme with two interactive binding sites or one binding site for all androgens [38,41–43]. Moreover, a full-length cDNA insert complementary to mRNA encoding human CYP450 aromatase was reported. The expressed protein was similar in size to the human placental enzyme and catalyzed aromatization of C_{19} steroids [44]. A discussion of the data in the above-mentioned reports would be out of the scope of the aim of this work, which is to investigate the in vitro effects of methadone and BUP on estrogen formation by placental microsomes rather than opiate–androgen interactions at the binding site of the enzyme. Therefore, on the basis of the data cited in this report, it can be concluded that methadone, BUP, and their respective metabolites EDDP and norBUP are competitive inhibitors of androgens aromatization in the human placenta.

In a recent report, placental transfer and retention of methadone was investigated utilizing the ex vivo model system of dual perfusion of the placental lobule. Methadone was transfused at a concentration range of 100–400 ng/mL corresponding to 0.3–1.3 μM [2], which is equal to that reported for its level in the maternal circulation following the administration of a range of therapeutic doses [34]. The data obtained revealed that methadone was retained and accumulated by the transfused placental tissue. The amount of methadone retained by the tissue formed a concentration gradient that is eight times that in the maternal circuit of the ex vivo model system used [2]. In each experiment, the weight of the transfused tissue/lobule ranges between 13 and 16 g and the volume of the maternal circuit is 250 mL. In vivo at term, the maternal blood volume is approximately 6–7 L and the weight of the placenta is 400–500 g. It is apparent that the ratio of maternal circulation volume to placental tissue weight in vivo is similar to that in the ex vivo model system. Therefore, the concentration of methadone in placental tissue in vivo could be in the range of 2–10 μM. The apparent K_i values for methadone determined in this investigation for inhibition of E_3 and E_2 formation by aromatase are 50 and 400 μM, respectively. Accordingly, it is reasonable to assume that the concentration of methadone in placental tissue in vivo could affect the activity of aromatase in the conversion of 16-OHT to E_3 more than testosterone to E_2. Similarly, the above calculations and assumptions could be applied for BUP that was transfused, utilizing the same model system, at a range of

concentrations between 0.5 and 30 ng/mL corresponding to 0.001–0.06 μM [1]. This range of concentrations was reported in the maternal circulation of women under treatment with BUP [35]. However, there is an important and significant difference between methadone and BUP — the amount of BUP retained by the transfused placental lobule could form a concentration gradient that is 20 times that in the maternal circuit [1] (i.e., approximately twice that formed by methadone). If true in vivo, the concentration of BUP in placental tissue could reach 1.2 μM. In addition, BUP was a more potent inhibitor of aromatase than methadone with an apparent K_i values for E_2 and E_3 formation of 36 and 6 μM, respectively (Table 1). Therefore, the data cited here suggest that BUP administered to women during pregnancy could also affect placental biosynthesis of estrogens.

In conclusion, our previous data on the transplacental transfer of methadone and BUP, as well as on their retention and accumulation by the tissue, were obtained utilizing an ex vivo model system. More over, the concentrations used for each opiate to calculate its K_i values were much higher than their levels in the maternal circulation of pregnant women under treatment. However, in view of the K_i values determined, as well as the placental tissue accumulation of BUP and methadone, it is reasonable to assume that each opiate might affect estrogen biosynthesis in vivo. It is also reasonable to assume that the metabolites, EDDP and norBUP, whether formed by placental CYP19 or maternal hepatic CYP3A4, could also affect steroidegenesis in various tissues. These may merely be reasonable assumptions, but they are validated by earlier reports on lower levels of estriol in pregnant women under treatment with methadone [30] and in light of similar data obtained using animal models (mice and rats) treated acutely and chronically with methadone [28,29]. Unfortunately, most likely due to the rather recent use of BUP for treatment of the pregnant women, there are no reports, to the best of our knowledge, on estrogen levels of this patient population. Therefore, clinical investigations of women under treatment with BUP during pregnancy should provide information on their levels of estrogens.

Acknowledgements

The authors appreciate the support of the National Institute on Drug Abuse drug supply program for providing methadone, EDDP, BUP, and norBUP. The authors appreciate the assistance of the medical staff, the Chairman's Research Group, and the Publication, Grant, and Media Support area of the Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, Texas. This work was supported by a grant from the National Institute on Drug Abuse to Mahmoud S. Ahmed (DA-13431).

REFERENCES

[1] Nanovskaya TN, Deshmukh S, Brooks M, Ahmed MS. Transplacental transfer and metabolism of buprenorphine. J Pharmacol Exp Ther 2002;300(1):26–33.

[2] Nekhaeva IA, Nanovskaya TN, Deshmukh S, Zharikova OL, Hankins GDV, Ahmed MS. Bidirectional transfer of methadone across human placenta. Biochem Pharmacol 2005;69:187–97.

[3] Deshmukh SV, Nanovskaya TN, Ahmed MS. Aromatase is the major enzyme metabolizing buprenorphine in human placenta. J Pharmacol Exp Ther 2003;306:1099–105.

[4] Nanovskaya TN, Deshmukh SV, Nekhaeva IA, Zharikova OL, Hankins GDV, Ahmed MS. Methadone metabolism by human placenta. Biochem Pharmacol 2004;68:583–91.

[5] Osawa Y, Higashiyama T, Shimizu Y, Yarborough C. Multiple functions of aromatase and the active site structure; aromatase is the placental estrogen 2-hydroxylase. J Steroid Biochem Mol Biol 1993;44(4–6):469–80.

[6] Toma Y, Higashiyama T, Yarborough C, Osawa Y. Diverse function of aromatase: O-deethylation of 7-ethoxycoumarin. Endocrinology 1996;137(9):3791–6.

[7] Osawa Y, Higashiyama T, Toma Y, Yarborough C. Diverse function of aromatase and the N-terminal sequence deleted form. J Steroid Biochem Mol Biol 1997;61(3–6):117–26.

[8] Iribarne C, Berthou F, Baird S, Dreano Y, Picart D, Bail JP, et al. Involvement of cytochrome P450 3A4 enzyme in the N-demethylation of methadone in human liver microsomes. Chem Res Toxicol 1996;9:365–73.

[9] Moody DE, Alburges ME, Parker RJ, Collins JM, Strong JM. The involvement of cytochrome P450 3A4 in the demethylation of L-α-acetylmethadol (LAAM) nor LAAM, and methadone. Drug Metab Dispos 1997;25:1347–53.

[10] Foster DJ, Somogyi AA, Bochner F. Methadone N-demethylation in human liver microsomes: lack of stereoselectivity and involvement of CYP3A4. Br J Clin Pharmacol 1999;47:403–12.

[11] Cone EJ, Gorodetzky CW, Yousefnejad D, Buchwald WF, Johnson RE. The metabolism and excretion of buprenorphine in humans. Drug Metab Dispos 1984;12:577–81.

[12] Iribarne C, Picart D, Dreano Y, Bail JP, Berthou F. Involvement of cytochrome P450 3A4 in N-dealkylation of buprenorphine in human liver microsomes. Life Sci 1997;60:1953–64.

[13] Kobayashi K, Yamamoto T, Chiba K, Tani M, Shimada N, Ishizaki T, et al. Human buprenorphine N-dealkylation is catalyzed by cytochrome P450 3A4. Drug Metab Dispos 1998;26:812–8.

[14] Pohland A, Boaz HE, Sullivan HR. Synthesis and identification of metabolites resulting from the biotransformation of DL-methadone in man and in the rat. J Med Chem 1971;14:194–7.

[15] Alburges ME, Huang W, Foltz RL, Moody DE. Determination of methadone and its N-demethylation metabolites in biological specimens by gas chromatography/positive ion chemical ionisation–mass spectrometry. J Anal Toxicol 1996;20:362–8.

[16] Oda Y, Kharasch E. Metabolism of methadone and *levo*-α-acetylmethadol (LAAM) by human intestinal Cytochrome P450 (CYP3A4): potential contribution of intestinal metabolism to presystemic clearance and bioactivation. J Pharmacol Exp Ther 2001;298:1021–31.

[17] Ryan KJ. Conversion of androstendione to estrone by placental microsomes. Biochim Biophys Acta 1958;27(3):658–9.

[18] Thompson EA, Siiteri PK. The involvement of human placental microsomal cytochrome P-450 in aromatization. J Biol Chem 1974;249(17):5373–8.

[19] Pepe GJ, Albrecht ED. Actions of placental and fetal adrenal steroid hormones in primate pregnancy. Endocr Rev 1995;16(5):608–48.

[20] Buster JE, Carson SA. Endocrinology and diagnosis of pregnancy. In: Gabbe SG, Niebyl JR, Simpson JL, editors. Obstetrics normal and problem pregnancies, vol. 2. New York: Churchill-Livingstone; 1996. p. 31–64.
[21] Goodwin T, Murphy MD. A role of estriol in human labor, term and preterm [hormonal pathways of preterm birth]. Am J Obstet Gynecol 1999;180:208S–13S.
[22] Speroff L, Glass RH, Kase NG. Clinical gynecologic endocrinology and infertility, 6th ed., Baltimore: Lippincott Williams & Wilkins; 1999.
[23] Rama S, Petrusz P, Rao AJ. Hormonal regulation of human trophoblast differentiation: a possible role for 17β-estradiol and GnRH. Mol Cell Endocrinol 2004;218:79–94.
[24] Siiteri PK, MacDonald PC. Placental estrogen biosynthesis during human pregnancy. J Clin Endocrinol 1966;26:751–61.
[25] Norman AW, Litwack G. Hormones, 2nd ed., San Diego: Academic Press; 1977.
[26] Hardy MJ, Humeida AK, Bahijri SM, Basalamah AH. Late third trimester unconjugated serum oestriol levels in normal and hypertensive pregnancy: relation to birth weight. Br J Obstet Gynaecol 1981;88(10):976–82.
[27] Kaijser M, Granath F, Jacobsen G, Cnattingius S, Ekbom A. Maternal pregnancy estriol levels in relation to anamnestic and fetal anthropometric data. Epidemiology 2000;11(3):315–9.
[28] Bui QQ, Tran MB, West WL. Acute and subchronic effects of methadone on the blood hormonal levels of pregnant and non-pregnant Charles River CD-1 mice. Pediatr Pharmacol (New York) 1983;3(2):69–78.
[29] Bui QQ, Tran MB, West WL. Evidence for hormonal imbalance after methadone treatment in pregnant and pseudopregnant rats. Proc Soc Exp Biol Med 1983;173(3):398–407.
[30] Facchinetti F, Comitini G, Petraglia F, Volpe A, Genazzani AR. Reduced estriol and dehydroepianrosterone sulphate plasma levels in methadone-addicted pregnant women. Eur J Obstet Gynecol Reprod Biol 1986;23(1–2):67–73.
[31] McNamara J, Stocker P, Miller VP, Patten CJ, Stresser DM, Crespi C. CYP19 (aromatase): characterization of the recombinant enzyme and its role in the biotransformation of xenobiotics; 1999. Available at: http://www.Gentest.com/pdf/post-016.pdf.
[32] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of enzymatic reaction. Biochem Pharmacol 1973;22:3099–108.
[33] Taniguchi H, Feldman HR, Kaufmann M, Pyerin W. Fast liquid chromatographic assay of androgen aromatase activity. Anal Biochem 1989;181:167–71.
[34] Drozdick III J, Berghella V, Hill M, Kaltenbach K. Methadone through levels in pregnancy. Am J Obstet Gynecol 2002;187:1184–8.
[35] Chawarski MC, Schottenfeld RS, O'Connor PG, Pakes J. Plasma concentration of buprenorphine 24 and 72 h after dosing. Drug Alcohol Depend 1999;55:157–63.
[36] Johnson RE, Jones HE, Fischer G. Use of buprenorphine in pregnancy: patient management and effects on the neonate. Drug Alcohol Depend 2003;70:S87–101.
[37] Reed KC, Ohno S. Kinetic properties of human placental aromatase. Application of an assay measuring ^3H_2O release from 1β,2β-^{3}H-androgens. J Biol Chem 1976;251(6):1625–31.
[38] Kellis JT, Vickery LE. Purification and characterization of human placental aromatase cytochrome P-450. J Biol Chemistry 1987;262(9):4413–20.
[39] Canick JA, Ryan KJ. Cytochrome P-450 and the aromatization of 16α-hydroxytestosteron and androstendion by human placental microsomes. J Mol Cell Endocrinol 1976;6:105–15.
[40] Numazawa M, Konno T, Furihata R, Ishikawa S. Determination of aromatization of 19-oxygenated 16α-hydroxyandrostendione with human placental microsomes by high-performance liquid chromatography coupled with coulometric detection. J Steroid Biochem 1990;36(4):369–75.
[41] Purohit A, Oakey RE. Evidence for separate sites for aromatization of androstendione and 16α-hydroxyandrostendione in human placental microsomes. J Steroid Biochem 1989;33(3):439–48.
[42] Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 1994;15(3):342–55.
[43] Numazawa M, Tachibana M, Mutsumi A, Yoshimura A, Osawa Y. Aromatization of 16α-hydroxyandrostenedione by human placental microsomes: effect of preincubation with suicide substrates of androstendione aromatization. J Steroid Biochem Mol Biol 2002;81:165–72.
[44] Corbin CJ, Graham-Lorence S, McPhaul M, Mason JI, Mendelson CR, Simpson ER. Isolation of full-length cDNA insert encoding human aromatase system cytochrome P-450 and its expression in nonsteroidogenic cells. Proc Natl Acad Sci USA 1988;85(23):8948–52.