

## INVOLVEMENT OF CYP3A4, CYP2C8, AND CYP2D6 IN THE METABOLISM OF (R)- AND (S)-METHADONE IN VITRO

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### ABSTRACT:

To clarify the oxidative metabolism of methadone (R)- and (S)-enantiomers, the depletion of parent (R)- and (S)-methadone and the formation of racemic 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine were studied using human liver microsomes and recombinant cytochrome P450 enzymes. Based on studies with isoform-selective chemical inhibitors and expressed enzymes, CYP3A4 was the predominant enzyme involved in the metabolism of (R)-methadone. However, it has different stereoselectivity toward (R)- and (S)-methadone. In recombinant CYP3A4, the metabolic clearance of (R)-methadone was about 4-fold higher than that of (S)-methadone. CYP2C8 is also involved in the metabolism of methadone, but its contribution to the metabolism of (R)-methadone was smaller than that of CYP3A4. But for the metabolism of (S)-methadone, the roles of CYP2C8 and CYP3A4 appeared equal. Although CYP2D6 is involved in the metabolism of (R)- and (S)-

methadone, its role was smaller compared with CYP3A4 and CYP2C8. Using clinically relevant concentrations of ketoconazole (1  $\mu$ M, selective CYP3A4 inhibitor), trimethoprim (100  $\mu$ M, selective CYP2C8 inhibitor), and paroxetine (5  $\mu$ M, potent CYP2D6 inhibitor), these inhibitors decreased the hepatic metabolism of (R)-[(S)-]methadone by 69% (47%), 22% (51%), and 41% (77%), respectively. However, inhibition of the metabolism of (R)- and (S)-methadone by paroxetine was due to inhibition not only of CYP2D6, but also CYP3A4 and, to a minor extent, CYP2C8. The present in vitro findings indicated that CYP3A4, CYP2C8, and CYP2D6 are all involved in the stereoselective metabolism of methadone (R)- and (S)-enantiomers. These data suggest that coadministration of inhibitors of CYP3A4 and CYP2C8 may produce clinically significant drug-drug interactions with methadone.

Methadone is a synthetic  $\mu$ -opioid receptor antagonist widely used for the treatment of opioid dependence and chronic pain (Farrell et al., 1994; Hagen and Wasylenko 1999). The clinically used *rac*-methadone is a mixture of (R)- and (S)-enantiomers in which the (R)-methadone is the major pharmacologically active form. The affinity of (R)-methadone for  $\mu$ - and  $\delta$ -opioid receptors and its analgesic activity are 10- and 50-fold higher, respectively, compared with those of (S)-methadone (Scott et al., 1948; Kristensen et al., 1995). Methadone shows considerable interindividual variability in its pharmacokinetics and pharmacodynamics (Boulton et al., 2001). In humans, methadone is extensively metabolized, and one major metabolic pathway is *N*-demethylation by P450<sup>1</sup> to EDDP (Sullivan and Due, 1973; Garrido and Troconiz, 1999). EDDP is pharmacologically inactive and subsequently undergoes spontaneous cyclization (Sullivan and Due, 1973). A previous in vitro study suggested that CYP3A4 is the predominant enzyme involved in the *N*-demethylation of both (R)- and (S)-methadone (Foster et al., 1999). Thus, the interindividual variations in expression of CYP3A4 have been thought to be the main

factor responsible for the interindividual variability of methadone disposition and, therefore, clinical effects (Garrido and Troconiz, 1999).

However, recent in vivo studies from healthy volunteers indicated that a potent CYP2D6 inhibitor, paroxetine (Crewe et al., 1992; Ball et al., 1997; Lam et al., 2002) significantly increased plasma concentrations of (R)- and (S)-methadone (Begre et al., 2002). In addition, in two poor metabolizers of CYP2D6, tested in 10 subjects, only (S)-methadone but not (R)-methadone was increased by paroxetine (Begre et al., 2002). This finding suggested that CYP2D6 is also involved in the metabolism of methadone and may preferentially be responsible for the metabolism of (R)-methadone. In agreement with this result, another selective serotonin reuptake inhibitor, fluoxetine, also a potent inhibitor of CYP2D6, stereoselectively increased the concentration of (R)- but not (S)-methadone (Eap et al., 1997). Although it is a nonselective inhibitor of CYP1A2, 2C, 2D6, and 3A4, fluvoxamine nonselectively increased concentrations of both (R)- and (S)-methadone (Eap et al., 1997). All these findings suggest that P450 isoforms other than CYP3A4, particularly CYP2D6, might be involved in the metabolism of methadone, and that they may have different stereoselectivity.

In addition to EDDP, six other metabolites of methadone have been identified. Urinary excretion of these metabolites has accounted for 43 to 83% of a given dose of methadone (Ånggard et al., 1975). Thus, a considerable amount of methadone is metabolized not via *N*-demethylation, but by other pathways. The objective of this study was to clarify the oxidative metabolism of (R)- and (S)-methadone by P450

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<sup>1</sup> Abbreviations used are: P450, cytochrome P-450; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; DDC, diethyldithiocarbamate; TAO, troleandomycin; HPLC, high-performance liquid chromatography.

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enzymes. To date, all the previous reaction phenotyping studies regarding methadone have been based on the formation of EDDP (Iribarne et al., 1996, 1998; Foster et al., 1999; Oda and Kharasch, 2001). In the present study, the reaction phenotyping of (*R*)- and (*S*)-methadone was conducted based on simultaneously monitoring the depletion of (*R*)- and (*S*)-methadone as well as the formation of EDDP in human liver microsomes and recombinant P450 isoforms using an *in vitro*  $t_{1/2}$  approach (Obach et al., 1997; Obach 1999).

### Materials and Methods

**Materials.** (*R*)- and (*S*)-Methadone, and *rac*-EDDP were provided by the National Institute on Drug Abuse Drug Supply program. *rac*-Methadone, fluvoxamine, tranlycypromine, trimethoprim, sulfaphenazole, diethyldithiocarbamate (DDC), ketoconazole, troleandomycin (TAO), and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Paroxetine was obtained from GlaxoSmithKline (Uxbridge, Middlesex, UK). *S*-Mephenytoin, pooled human liver microsomes (prepared from five male and five female human liver microsomal samples), and microsomes from baculovirus-infected cells engineered to express the cDNA encoding human CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were purchased from BD Gentest (Woburn, MA). Other chemicals and reagents were obtained from Fisher Scientific Co. (Pittsburgh, PA).

**Assays for the Oxidative Metabolism of Methadone.** The incubation mixture, in a final volume of 200  $\mu$ l, contained 0.5 mg/ml microsomal protein, 0.1 M sodium phosphate buffer (pH 7.4), 1.0 mM NADPH, 5 mM MgCl<sub>2</sub>, and 5  $\mu$ M (*R*)- or (*S*)-methadone (substrate stock solution dissolved in ethanol, and subsequently diluted using 0.1 M sodium phosphate buffer) in the presence or absence of one of the putative inhibitors. After incubation in a water bath (37°C) for 20 to 60 min, the reactions were terminated by adding 100  $\mu$ l of ice-cold acetonitrile containing 10  $\mu$ g/ml diphenhydramine as an internal standard, and the samples were cooled on ice for 15 min. The samples were then removed to glass tubes containing 0.2 ml of sodium carbonate buffer (1 M). The samples were then extracted with 3 ml of hexanes for 20 min. After centrifugation, the supernatant was evaporated to dryness. The residue was then reconstituted with 100  $\mu$ l of the mobile phase (75:25, v/v, acetonitrile/phosphoric acid buffer, pH = 4.5). Thereafter, 30  $\mu$ l of the sample was subjected to high-performance liquid chromatography (HPLC) analysis. The calculation of the *in vitro*  $t_{1/2}$  is based on an assumption of first-order kinetics. The depletion rates of (*R*)- and (*S*)-methadone, and the formation rate of EDDP were confirmed to be linear with respect to the microsomal protein concentration (up to 0.5 mg/ml) and various incubation times (0, 20, 40, and 60 min) used. A longer observation period, i.e., a longer than 75-min incubation time (15-min preincubation plus 60-min incubation) would be desirable for estimating  $t_{1/2}$  but would result in substantial loss of P450 enzyme activity, and the methadone metabolic kinetics would not follow first-order elimination, making any estimate of  $t_{1/2}$  erroneous. All human liver microsomal incubations were performed in duplicate. This experimental method (data generated from duplicate determinations) has been generally used in *in vitro* phenotyping studies (Kobayashi et al., 1999; Ma et al., 2000; Dierks et al., 2001; Wang et al., 2002). To increase confidence in the reproducibility of the experiments, a difference of less than 10% between the duplicate assays was used as the acceptance criterion.

Incubations with the recombinant P450 isoforms were performed using the same conditions as described for human liver microsomes, except that the mixture contained 10 pmol P450 (CYP1A2, 2C8, 2C9, 2C19, 2D6, or 3A4) and was incubated for 20 to 60 min. The depletion rates of (*R*)- and (*S*)-methadone and the formation rate of EDDP were linear over this incubation time. The incubations in the expressed enzymes were also performed in duplicate.

**Inhibition Studies.** The effects of 10 isoform-selective P450 inhibitors on the P450-mediated oxidative metabolism of (*R*)- and (*S*)-methadone were studied. Fluvoxamine (1  $\mu$ M) was used as a selective inhibitor of CYP1A2, tranlycypromine (1  $\mu$ M) for CYP2A6, trimethoprim (100  $\mu$ M) for CYP2C8, sulfaphenazole (4  $\mu$ M) for CYP2C9, *S*-mephenytoin (300  $\mu$ M) for CYP2C19, paroxetine (5  $\mu$ M) for CYP2D6, DDC (25  $\mu$ M) for CYP2E1, ketoconazole (1  $\mu$ M), and TAO (100  $\mu$ M) for CYP3A4 (Crewe et al., 1992; Newton et al., 1995; Eagling et al., 1998; Hickman et al., 1998; Rasmussen et al., 1998;

Taavitsainen et al., 2001; Wen et al., 2002). The stock solutions of the inhibitors were prepared in 0.1 M sodium phosphate buffer (pH 7.4) with, in some cases, minimal use of methanol or acetonitrile (final concentration less than 1%). DDC, TAO, paroxetine, and their matched controls (containing 1% methanol in the case of TAO) were preincubated with the incubation medium at 37°C for 15 min in the presence of 1.0 mM NADPH. After the preincubation, (*R*)- or (*S*)-methadone (final concentration 5  $\mu$ M) was added. In studies of time-dependent depletion of (*R*)- or (*S*)-methadone, ketoconazole (1  $\mu$ M), trimethoprim (100  $\mu$ M), paroxetine (5  $\mu$ M), and the noninhibitor controls were also preincubated with the incubation medium at 37°C for 15 min in the presence of 1.0 mM NADPH. Other inhibitors were incubated with (*R*)- or (*S*)-methadone without preincubation.

**Analytical Procedures.** The concentrations of (*R*)- and (*S*)-methadone and EDDP in incubations were measured by use of HPLC as described previously (Boulton and DeVane, 2000). The limit of quantification for (*R*)- and (*S*)-methadone and EDDP was 0.05  $\mu$ M, and the day-to-day coefficient of variation was below 10% at relevant concentrations ( $n = 5$ ).

**Data Analysis.** The *in vitro* elimination half-life ( $t_{1/2}$ ) was determined by the following equation (Obach et al., 1997; Obach 1999):

$$t_{1/2} = -0.693/k \quad (1)$$

where  $k$  is the slope of the linear regression from the natural logarithmic percentage of (*R*)- or (*S*)-methadone remaining versus incubation time (minutes). The *in vitro* intrinsic clearance ( $CL_{int}$ ) of (*R*)- and (*S*) methadone in human liver microsomal incubations was calculated using the following equation (Obach et al., 1997, Obach 1999):

$$CL_{int} = \frac{0.693}{t_{1/2}} \cdot \frac{\text{ml incubation}}{\text{mg microsomal protein}} \cdot \frac{45 \text{ mg microsomes}}{\text{g liver}} \cdot \frac{20 \text{ g liver}}{\text{kg b.wt.}} \quad (2)$$

The  $CL_{int}$  of (*R*)- or (*S*)-methadone in recombinant enzymes was calculated using the following equation:

$$CL_{int} = \frac{0.693}{t_{1/2}} \cdot \frac{\text{ml incubation}}{\text{pmol microsomes}} \cdot \frac{\text{P450 content}}{\text{mg microsomal protein}} \cdot \frac{45 \text{ mg microsomes}}{\text{g liver}} \cdot \frac{20 \text{ g liver}}{\text{kg b.wt.}} \quad (3)$$

where the P450 content for CYP2D6, CYP2C8, and CYP3A4 is 8, 22, and 142 pmol/mg microsomal protein, respectively (Shimada et al., 1994; Snauder and Lipscomb, 2000). Hepatic clearance was calculated by the well-stirred model (Houston, 1994):

$$CL_h = Q \cdot f_u \cdot CL_{int} / (Q + f_u \cdot CL_{int}) \quad (4)$$

where  $Q$  is the hepatic blood flow (20 ml/min/kg) (Lin and Lu, 1997) and  $f_u$  is unbound fraction of *R*-methadone (0.21) or *S*-methadone (0.14) in the blood (Boulton et al., 2001).

## Results

**Studies with Human Liver Microsomes.** Trimethoprim, paroxetine, ketoconazole, and TAO at the concentrations tested considerably inhibited the depletion of (*R*)- and (*S*)-methadone and the formation of EDDP from (*R*)- and (*S*)-methadone (Fig. 1). Fluvoxamine and sulfaphenazole also showed mild inhibitory effects toward the depletion of *S*-methadone; however, they did not consistently inhibit the formation of EDDP. Other chemical inhibitors tested showed negligible effects on the depletion of (*R*)- and (*S*)-methadone and the formation of EDDP (Fig. 1).

The *in vitro* depletion  $t_{1/2}$  of (*R*)- and (*S*)-methadone was considerably prolonged by 1  $\mu$ M ketoconazole, 100  $\mu$ M trimethoprim, and 5  $\mu$ M paroxetine (Table 1, Fig. 2). According to the well-stirred model, the extrapolated metabolic clearance of (*R*)- and (*S*)-methadone were markedly inhibited by these chemical inhibitors (Table 1).

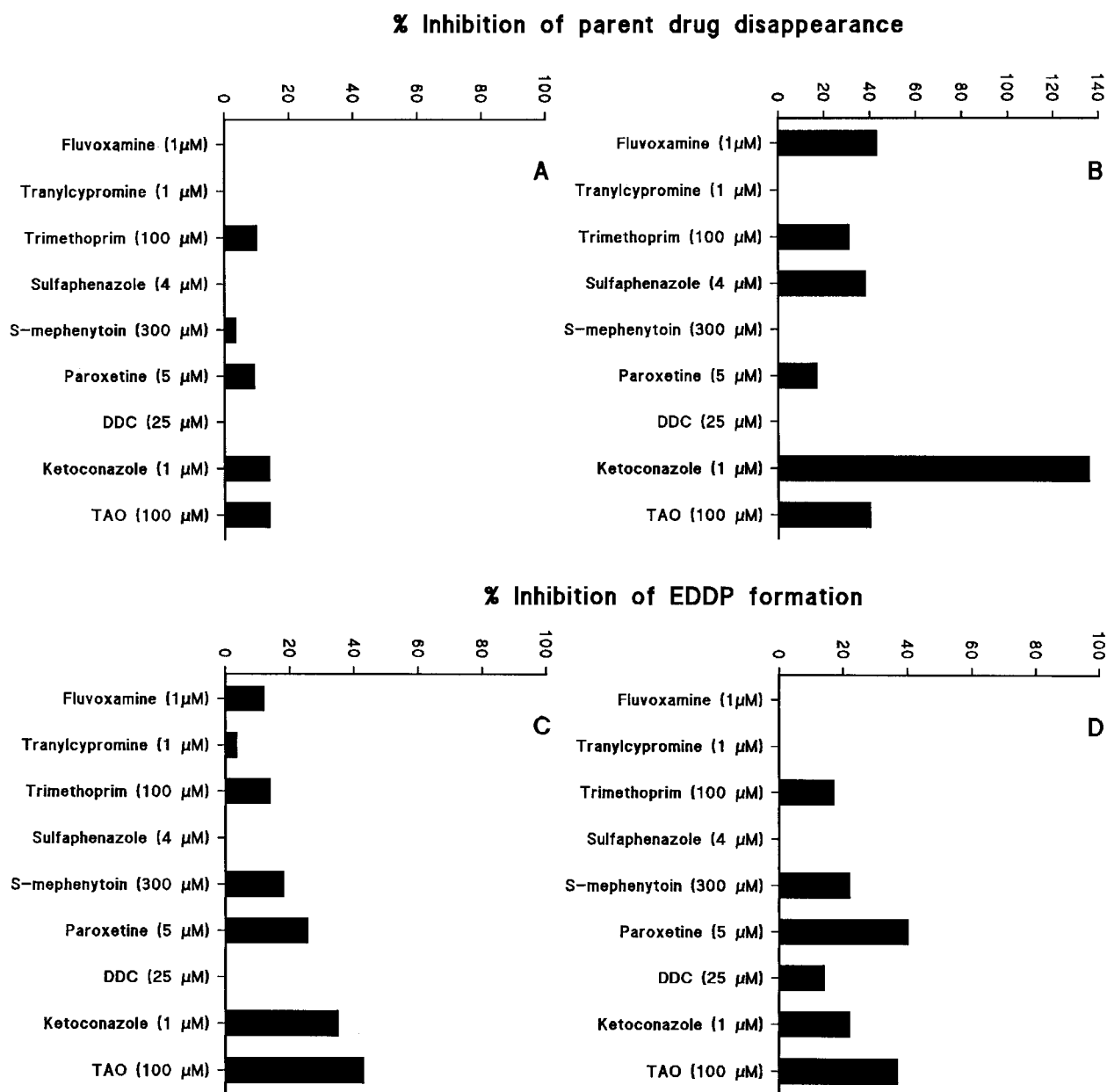


FIG. 1. Percentage inhibition of the depletion of (*R*)- (A) and (*S*)-methadone (B), and the formation of EDDP from (*R*)- (C) and (*S*)-methadone (D) by selective inhibitors of eight P450 isoforms in human liver microsomes (data represent the mean of duplicate incubations).

The percentage inhibition of parent drug disappearance (PD) was calculated according to the following equation:  $PD = (C_i - C_0)/C_0 \times 100\%$ , where  $C_i$  and  $C_0$  were the concentrations of *R*- or *S*-methadone in incubations after 60 min incubation of *R*- or *S*-methadone in the presence or absence of a chemical inhibitor, respectively. The percentage inhibition of EDDP formation (PF) was calculated according to the following equation:  $PF = (E_0 - E_i)/E_0 \times 100\%$ , where  $E_i$  and  $E_0$  were the concentrations of EDDP in incubations after 60-min incubation of *R*- or *S*-methadone in the presence or absence, respectively, of a chemical inhibitor.

**Studies with Recombinant P450 Isoforms.** Time-dependent formation of EDDP and depletion of (*R*)- and (*S*)-methadone were observed in recombinant CYP2C8, CYP2D6, and CYP3A4 (Table 2, Fig. 3). The CYP2D6 showed the highest catalytic capacity in the formation of EDDP from both (*R*)- and (*S*)-methadone among the three recombinant P450 isoforms tested. However, considering the hepatic content of each P450 isoform, the  $CL_{int}$  value for the (*R*)- and (*S*)-methadone was ranked as: CYP3A4 > CYP2C8 > CYP2D6 (Table 2). Formation of EDDP and depletion of (*R*)- and (*S*)-methadone were not observed in recombinant CYP1A2, CYP2C9, and CYP2C19 by incubating (*R*)- or (*S*)-methadone with the incubation medium for up to 1 h.

Preincubation of 5 μM paroxetine with NADPH for 15 min strongly inhibited the CYP2D6-mediated formation of EDDP from (*R*)- and (*S*)-methadone (Fig. 4). In addition, it also moderately inhibited CYP3A4 and mildly inhibited CYP2C8-mediated formation of EDDP (Fig. 4).

### Discussion

The present in vitro study indicated that CYP3A4, CYP2C8, and CYP2D6 are all involved in the metabolism of (*R*)- and (*S*)-methadone in human liver microsomes, and CYP3A4 had a different stereoselectivity toward the (*R*)- and (*S*)-enantiomeric forms. The  $CL_{int}$  of CYP3A4 for (*R*)-methadone was about 4-fold higher than that for

TABLE 1

Inhibition of metabolism of *R*- and *S*-methadone by ketoconazole, trimethoprim, and paroxetine after incubation of clinically relevant concentrations of the inhibitors with 5  $\mu\text{M}$  (*R*-) or (*S*-)methadone in human liver microsomes

	<i>R</i> -Methadone				<i>S</i> -Methadone			
	$t_{1/2}^a$	$CL_{int}^b$	$CL_h^c$	% Inhibition	$t_{1/2}^a$	$CL_{int}^b$	$CL_h^c$	% Inhibition
	min	ml/min/kg			min	ml/min/kg		
Control	68	18.4	3.2		45	27.7	3.2	
Ketoconazole (1 $\mu\text{M}$ ) <sup>d</sup>	247	5.0	1.00	69	105	11.9	1.54	47
Trimethoprim (100 $\mu\text{M}$ ) <sup>d</sup>	91	13.7	2.52	22	102	12.2	1.58	51
Paroxetine (5 $\mu\text{M}$ ) <sup>d</sup>	124	10.06	1.91	41	231	5.4	0.73	77

<sup>a</sup> In vitro elimination half-life determined by eq. 1 (see *Data Analysis* under *Materials and Methods* for details).

<sup>b</sup> In vitro intrinsic clearance calculated by eq. 2.

<sup>c</sup> Hepatic clearance calculated by the well-stirred model (eq. 4).

<sup>d</sup> The inhibitor concentrations 1  $\mu\text{M}$ , 100  $\mu\text{M}$ , and 5  $\mu\text{M}$  for ketoconazole, trimethoprim, and paroxetine, respectively, used in the incubation were calculated using the plasma concentrations of ketoconazole (1  $\mu\text{M}$ ), trimethoprim (20  $\mu\text{M}$ ), and paroxetine (0.18  $\mu\text{M}$ ) multiplied by their respective liver/plasma partition ratios of 1, 6.5, and 26.2 for ketoconazole, trimethoprim, and paroxetine, respectively (von Moltke et al., 1995, 1998; Lam et al., 2002; Wen et al., 2002).

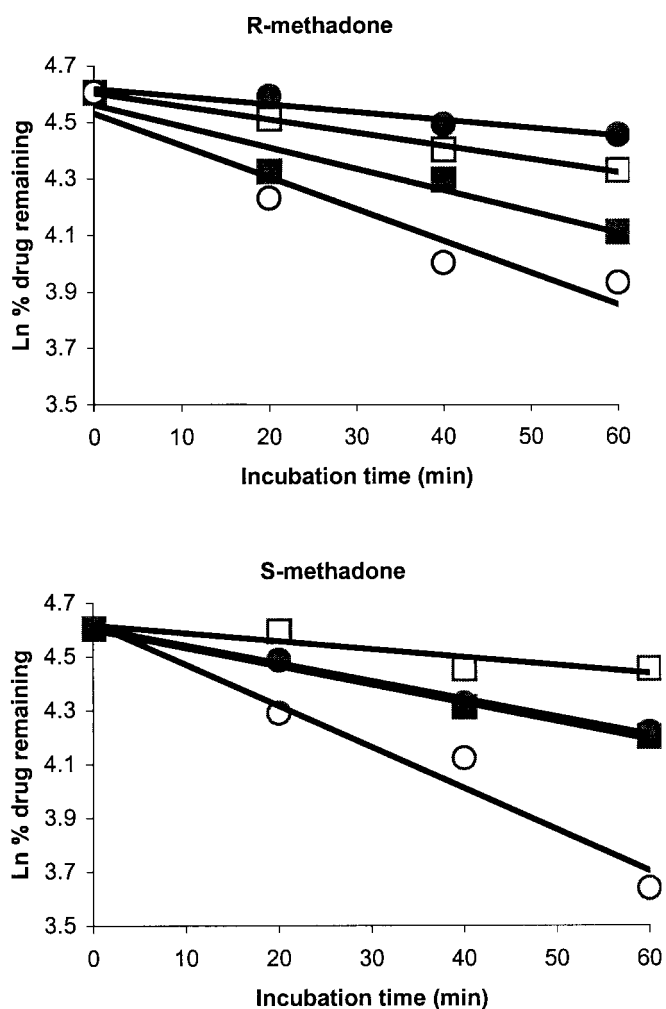


FIG. 2. Inhibitory effects of 1  $\mu\text{M}$  ketoconazole (solid circles), 100  $\mu\text{M}$  trimethoprim (solid squares), and 5  $\mu\text{M}$  paroxetine (open squares) on the depletion of (*R*-) (upper panel) and (*S*-)methadone (lower panel).

Open circles, control incubations containing 1% methanol matched with that of inhibitors. The inhibitors and the noninhibitor controls were also preincubated with the incubation medium at 37°C for 15 min in the presence of 1.0 mM NADPH.

(*S*-)methadone, whereas the CYP2C8 and CYP2D6 showed only a marginally different catalytic capacity toward the (*R*-) and (*S*-)enantiomers (Table 2). Consistent with these results, the CYP3A4, CYP2C8, and CYP2D6 inhibitors ketoconazole, TAO, trimethoprim, and paroxetine, at their respective concentrations tested, considerably

inhibited the depletion of (*R*-) and (*S*-)methadone and the formation of EDDP in human liver microsomes.

The 1  $\mu\text{M}$  ketoconazole concentration used in the inhibition studies is clinically relevant (von Moltke et al., 1998) and is selective toward CYP3A4 (Newton et al., 1995). The recombinant enzyme and inhibition results consistently indicated that CYP3A4 is stereoselectively involved in the metabolism of methadone. It is a predominant enzyme involved in the metabolism of (*R*-)methadone; its contribution to the metabolism of (*S*-)methadone is somewhat smaller compared with that of (*R*-)methadone and is similar to that of CYP2C8 (Tables 1 and 2).

The involvement of CYP3A4 in the metabolism of methadone has been confirmed in previous studies (Iribarne et al., 1996, 1998; Foster et al., 1999; Boulton et al., 2001; Oda and Kharasch, 2001). The possible involvement of CYP2C8 and CYP2D6 in the metabolism of methadone has not been thoroughly studied. In addition, Foster et al. (1999) reported that the involvement of CYP3A4 in the *N*-demethylation of methadone is nonstereoselective. A reaction phenotyping based on monitoring EDDP formation rather than the parent compound depletion was employed in these studies (Iribarne et al., 1996, 1998; Foster et al., 1999; Oda and Kharasch, 2001). Considering that methadone is not exclusively metabolized via the *N*-demethylation pathway (about 43–83% of doses of methadone are not metabolized via the *N*-demethylation pathways; Anggard et al., 1975), the reaction phenotyping based on the formation of EDDP may not correctly reflect the real enzyme mapping of methadone.

In the present study, CYP2C8 has been identified to be important in the metabolism of both (*R*-) and (*S*-)methadone, with the role being more important in the metabolism of (*R*-)methadone. The involvement of CYP2C8 in the *N*-demethylation of *rac*-methadone has been observed by Iribarne et al. (1996). CYP2C8 is expressed at relatively high levels (6–7% of total P450 content) in human liver (Romkes et al., 1991), and its importance in drug metabolism has recently been recognized (Ong et al., 2000). It is primarily responsible for the metabolism of, for example, paclitaxel (Taxol), cerivastatin, rosiglitazone, and troglitazone, and also involved in the metabolism of zopiclone, carbamazepine, verapamil, and amiodarone (Ong et al., 2000). Trimethoprim has recently been identified to be an effectively selective inhibitor of CYP2C8 at a concentration of 100  $\mu\text{M}$  (Wen et al., 2002). The 100  $\mu\text{M}$  concentration of trimethoprim tested in the inhibition study is also clinically relevant (estimated by multiplying the total plasma concentration of 15  $\mu\text{M}$  trimethoprim with a liver/plasma partition ratio of 6.5) (Craig and Kunin, 1973; Moore et al., 1996). The current results therefore suggest a possible drug-drug interaction between trimethoprim and methadone in vivo. The metabolic clearance of (*R*-) and (*S*-)methadone would be predicted to

TABLE 2

Rates of metabolic formation of EDDP from (R)- and (S)-methadone enantiomers, and the in vitro  $t_{1/2}$  and  $CL_{int}$  of (R)- and (S)-methadone in the recombinant CYP2C8, CYP2D6, and CYP3A4

	R-Methadone			S-Methadone		
	Formation of EDDP	In Vitro $t_{1/2}$ <sup>a</sup>	$CL_{int}$ <sup>b</sup>	Formation of EDDP	In Vitro $t_{1/2}$ <sup>a</sup>	$CL_{int}$ <sup>b</sup>
	pmol/min/pmol P450	min	ml/min/kg	pmol/min/pmol P450	min	ml/min/kg
CYP2C8	0.46	61	4.6	0.26	78	3.6
CYP2D6	0.58	165	0.6	0.96	103	1.0
CYP3A4	0.26	89	20	0.035	365	4.8

<sup>a</sup> In vitro  $t_{1/2}$  determined by eq. 1 (see Data Analysis under Materials and Methods for details).

<sup>b</sup> In vitro  $CL_{int}$  calculated by eq. 3.

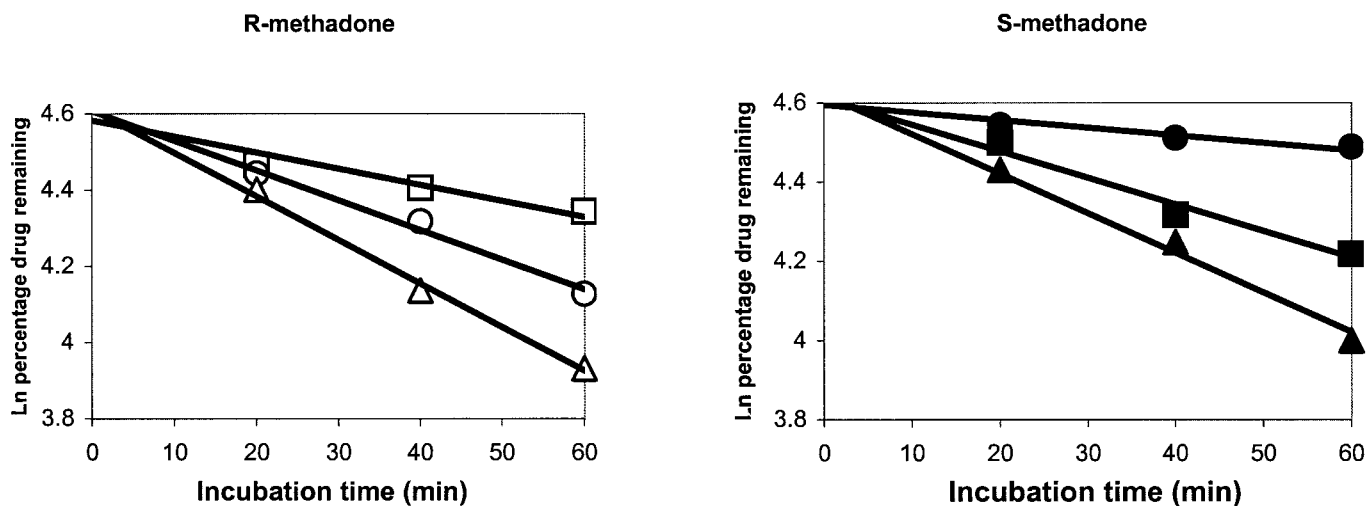


FIG. 3. Time-dependent metabolic depletion of (R)- and (S)-methadone ( $5 \mu\text{M}$ ) in recombinant CYP3A4 (open or solid circles), CYP2C8 (open or solid triangles), and CYP2D6 (open or solid squares).

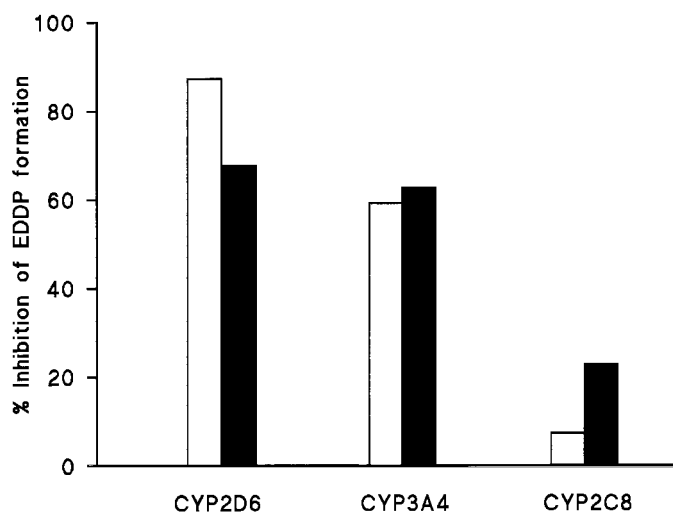


FIG. 4. Inhibitory effects of  $5 \mu\text{M}$  paroxetine on the CYP2D6-, CYP3A4-, and CYP2C8-mediated formation of EDDP from (R)- (open bars) and (S)-methadone (solid bars).

Paroxetine was preincubated with NADPH for 15 min before adding  $5 \mu\text{M}$  (R)- or (S)-methadone (see Materials and Methods for details).

decrease by about 22% and 51%, respectively, from coadministration of therapeutic doses of trimethoprim. This would result in a substantial increase in methadone plasma concentration.

The role of CYP2D6 in the metabolism of methadone is definitely established by these data but with a somewhat smaller role compared

with those of CYP3A4 and CYP2C8 according to the recombinant enzyme results (Table 2). The  $5 \mu\text{M}$  paroxetine used in human liver microsomal incubations was thought to approximate the hepatic concentration of paroxetine by multiplying the average plasma concentration of paroxetine ( $0.18 \mu\text{M}$ ; Lam et al., 2002) by a liver/plasma partition ratio of 26.2 (von Moltke et al., 1995). However, the  $5 \mu\text{M}$  paroxetine used not only strongly inhibited the CYP2D6-mediated EDDP formation, but also inhibited CYP3A4 and, mildly, CYP2C8 from (R)- and (S)-methadone (Fig. 4). Thus, the inhibition of metabolic clearance of (R)- and (S)-methadone by paroxetine observed in the present study and a recent in vivo study (Begre et al., 2002), is due to inhibition not only of CYP2D6, but also CYP3A4, and to a minor extent, CYP2C8. Although quinidine was originally tested in the current study as a selective inhibitor of CYP2D6 (Newton et al., 1995), it was found to greatly interfere with the analysis of EDDP by HPLC.

The nonspecific binding of (R)- and (S)-methadone to the  $0.5 \text{ mg/ml}$  microsomal protein was found to be significant. About 32 and 48% of (R)- and (S)-methadone were found to bind to the microsomal protein (ultrafiltration method; Wang et al., 2002). Although the binding of ketoconazole, trimethoprim, and paroxetine to the incubation matrices were not measured in the present study, they may also bind to some extent to the incubation matrices. Therefore, a limitation of the current inhibition results is that some underestimation of the degree of inhibition is possible. Another limitation is the lack of multiple determinations from several independent experiments. However, all the results met the acceptance criterion of less than a 10% difference in duplicate assays. Although this result increases confi-

dence in the reproducibility of the data, independent confirmation from other laboratories is always desirable.

Finally, these results are consistent with our previous findings in humans (Boulton et al., 2001). The  $CL_{int}$  of *R*-methadone in human liver microsomes in the absence of inhibitors (control) was 18.4 (ml/min/kg) compared with 27.7 (ml/min/kg) for (*S*)-methadone (Table 1). In eight women given a single oral dose of *rac*-methadone, the oral clearance of *R*-methadone was similarly slower than that of *S*-methadone ( $4.01 \pm 2.49$  l/h versus  $20.7 \pm 16.9$  l/h), although considerable variability was present. The relatively slower intrinsic clearance of *R*-methadone was reflected by a greater area under the plasma concentration versus time curve for six of eight women. In addition, the activity of CYP3A4 as measured by an in vivo ratio of urinary 6- $\beta$ -hydroxycortisol to cortisol was a better predictor of the pharmacokinetics of (*R*)- than of (*S*)-methadone. This would be expected from the present in vitro data demonstrating a lesser involvement of CYP3A4 in the metabolism of (*S*)-methadone (Tables 1 and 2). The present results help clarify our human data by documenting the involvement of additional P450 isoforms in the stereoselective metabolism of methadone.

In conclusion, the present study showed evidence that CYP3A4, CYP2C8, and CYP2D6 are all involved in the metabolism of methadone. However, their contribution to the (*R*)- and (*S*)-enantiomers is different. The effective CYP3A4 and CYP2C8 inhibitors may inhibit the metabolism of (*R*)- and (*S*)-methadone in vivo. The clinically observed interaction between methadone and paroxetine is due to inhibition of CYP2D6- and CYP3A4-mediated methadone metabolism by paroxetine. Other serotonin reuptake inhibitors such as fluoxetine and fluvoxamine may also inhibit the metabolism of methadone by inhibiting the catalytic activities of CYP3A4 and CYP2D6.

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