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Short Communication

IDENTIFICATION OF CYTOCHROME P450 ENZYMES INVOLVED IN THE METABOLISM OF 4′-METHYL-α-PYRROLIDINOPROPIOPHENONE, A NOVEL SCHEDULED DESIGNER DRUG, IN HUMAN LIVER MICROSOMES

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ABSTRACT:

4′-Methyl-α-pyrrolidinopropiophenone (MPPP) is a new drug of abuse. It is believed to have an abuse potential similar to that of amphetamines. Previous studies with Wistar rats had shown that MPPP was metabolized mainly by hydroxylation in position 4′ followed by dehydrogenation to the corresponding carboxylic acid. The aim of the study presented here was to identify the human hepatic cytochrome P450 (P450) enzymes involved in the biotransformation of MPPP to 4′-hydroxymethyl-pyrrolidinopropiophenone. Baculovirus-infected insect cell microsomes and human liver microsomes were used for this purpose. Only CYP2C19 and CYP2D6 catalyzed this hydroxylation. The apparent K_m and V_{max} values for the latter were 9.8 ± 2.5 μM and 13.6 ± 0.7 pmol/min/pmol P450, respectively. CYP2C19 was not saturable over the tested substrate range (2–1000 μM) and interestingly showed a biphasic kinetic profile with apparent $K_{m,1}$ and $V_{max,1}$ values of 47.2 ± 12.5 μM and 8.1 ± 1.4 pmol/min/pmol P450, respectively. Experiments with pooled human liver microsomes also revealed biphasic nonsaturable kinetics with apparent $K_{m,1}$ and $V_{max,1}$ values of 57.0 ± 20.9 μM and 199.7 ± 59.7 pmol/min/mg of protein for the high affinity enzyme, respectively. Incubation of 2 μM MPPP with 3 μM of the CYP2D6-specific inhibitor quinidine resulted in significant ($p < 0.01$) turnover inhibition (11.8 ± 1.6% of control). Based on kinetic data corrected for the relative activity factors, CYP2D6 is the enzyme mainly responsible for MPPP hydroxylation, confirmed by CYP2D6 inhibition studies.

4′-Methyl-α-pyrrolidinopropiophenone [MPPP[1], international nonproprietary name: 2-(pyrrolidine-1-yl)-1-(*p*-tolyl)propane-1-one] is a new designer drug that has appeared on the illicit drug market. MPPP has been scheduled in the German Act of Controlled Substances after large amounts of tablets were distributed for recreational use and seized by the police (Roesner et al., 1999). Routine drugs of abuse screenings do not allow the detection of MPPP in biosamples (Springer et al., 2002). Although little information about its pharmacological and toxicological properties is available, amphetamine-like effects can be predicted, since structurally similar anorectics like amfepramone and metamfepramone, drugs of abuse like cathinone and methcathinone, and antidepressants like bupropion are based on this mode of action (Bryant et al., 1983; Kalix and Glennon, 1986; Glennon et al., 1987; Martinez et al., 1998). Possible clinical effects of MPPP include tachycardia, hypertension, mydriasis, and tremor.

Previous in vivo studies in rats showed that MPPP was mainly metabolized by hydroxylation of the 4′-methyl group (Fig. 1) followed by dehydrogenation to the corresponding carboxylic acid (Springer et al., 2002). The aim of the study reported here was to identify the human hepatic cytochrome P450 (P450) enzymes involved in the hydroxylation and to determine the kinetic constants for this metabolic reaction.

[1] Abbreviations used are: MPPP, 4′-methyl-α-pyrrolidinopropiophenone; P450, cytochrome P450; MDPPP, 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone; HLM, human liver microsomes; RAF, relative activity factor; LC-MS, liquid chromatography-mass spectrometry; HO-MPPP, 4′-hydroxymethyl-α-pyrrolidinopropiophenone.

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Materials and Methods

Materials. MPPP-HCl and MDPPP-HCl (3′,4′-methylenedioxy-α-pyrrolidinopropiophenone, international nonproprietary name: 1-(1,3-benzodioxol-5-yl)-2-(pyrrolidine-1-yl)propane-1-one) were provided by the Hessian State Criminal Office (Wiesbaden, Germany), before the compounds had entered the German Act of Controlled Substances. $NADP^+$ was obtained from Biomol (Hamburg, Germany), isocitrate, and isocitrate dehydrogenase from Sigma (Taufkirchen, Germany), all other chemicals and reagents from Merck (Darmstadt, Germany). The following microsomes were purchased from NatuTec (Frankfurt/Main, Germany): baculovirus-infected insect cell microsomes containing 1 nmol/ml CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 (Supersomes), wild-type baculovirus-infected insect cell microsomes (control Supersomes) and pooled human liver microsomes (HLM, 20 mg of microsomal protein/ml, 400 pmol of total P450/mg of protein). After delivery, the microsomes were thawed at 37°C, aliquoted, shock-frozen in liquid nitrogen, and stored at −80°C until use.

Microsomal Incubations. Incubation mixtures (final volume, 50 μl) consisted of 90 mM phosphate buffer (pH 7.4), 5 mM Mg^{2+}, 5 mM isocitrate, 1.2 mM $NADP^+$, 0.5 U/ml isocitrate dehydrogenase, 200 U/ml superoxide dismutase, and substrate at 37°C. The substrate was added after dilution of a 250 mM methanolic stock solution in buffer. The methanol concentration did not exceed 0.4% in any of the samples. Reactions were started by addition of the ice-cold microsomes and terminated with 5 μl of 60% (w/w) $HClO_4$. After addition of 1 μl of 0.1 mg/ml MDPPP as internal standard, the samples were centrifuged and the supernatants were transferred to autosampler vials.

Initial Screening Studies. To investigate the involvement of particular P450 enzymes in MPPP metabolism, 50 μM MPPP and 50 pmol/ml CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 were incubated for 30 min. For incubations with CYP2A6 or

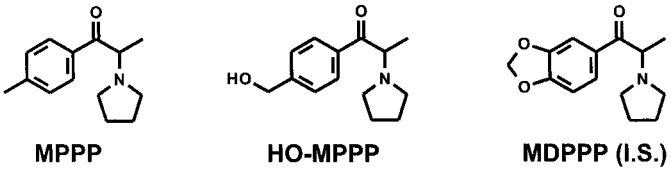

FIG. 1. *Structures of MPPP, its metabolite HO-MPPP, and the internal standard (I.S.) MDPPP.*

CYP2C9, phosphate buffer was replaced with 45 or 90 mM Tris buffer, respectively.

Kinetic Studies. Duration of and protein content for all incubations were in the linear range of metabolite formation (data not shown). Kinetic constants were derived from incubations with the following MPPP concentrations: 2, 5, 10, 15, 20, 30, 40, 75, 120, 200, 400, 700, and 1000 μM ($n = 2$, each). HLM and cDNA-expressed P450 protein contents were 0.5 mg/ml and 30 pmol P450/ml, respectively. Incubation times were 30 and 15 min for HLM and cDNA-expressed P450s, respectively. Less than 20% of substrate was metabolized in all incubations. Apparent K_m and V_{max} values for single enzymes were estimated by nonlinear regression according to the Michaelis-Menten equation.

$$V = \frac{V_{max} \times [S]}{K_m + [S]} \quad (1)$$

If visual inspection of the data points suggested that a two-site binding model was the more appropriate model, eq. 2 was applied for fitting the curve (Korzekwa et al., 1998).

$$V = \frac{V_{max,1} \times [S]}{K_{m,1} + [S]} + \frac{V_{max,2} \times [S]}{K_{m,2} + [S]} \quad (2)$$

Eadie-Hofstee plots were used to confirm biphasic kinetics (Clarke, 1998). The kinetic data were estimated using GraphPad Prism 3.02 software (San Diego, CA).

Calculation of Relative Activity Factors. To take into account differences in functional levels of redox partners between the two enzyme sources, the relative activity factor (RAF) approach was used (Crespi and Miller, 1999; Venkatakrishnan et al., 2000). The activities of CYP2C19 [specific substrate (*S*)-mephenytoin] and CYP2D6 (specific substrate bufuralol) in both, insect cell microsomes and HLM, were taken from the supplier's data sheets. The RAFs were calculated according to eq. 3 yielding 0.029 for CYP2C19 and 0.01 for CYP2D6.

$$RAF_{isoform} = \frac{\text{turnover rate for probe substrate in HLM[pmol/min/mg of protein]}}{\text{turnover rate for probe substrate in cDNA-expressed P450 [pmol/min/mg of protein]}} \quad (3)$$

V_{max} values of the MPPP hydroxylation obtained from incubations with cDNA-expressed P450s were then multiplied with the corresponding RAF leading to a value, which is defined as "contribution".

$$Contribution_{isoform} = V_{max} \text{ for MPPP hydroxylation (with cDNA-expressed P450)} \times RAF_{isoform} \quad (4)$$

The percentage of intrinsic clearance by a particular enzyme was derived from calculations according to eq. 5, where intrinsic clearance equals contribution/apparent K_m.

$$\text{Percentage of clearance by isoform} = clearance_{isoform} \div \sum clearances_{isoform} \times 100\% \quad (5)$$

Chemical Inhibition Studies. The effect of 3 μM quinidine on HO-MPPP formation was assessed in incubations containing 0.5 mg of HLM protein/ml and 2 μM MPPP. Controls contained no quinidine, but the same amount of methanol ($\ll$0.1%) to cancel out any solvent effects ($n = 6$ each). A one-tailed unpaired *t* test with Welch's correction was used to test for significance of inhibition using GraphPad Prism 3.02 software.

Liquid Chromatography-Mass Spectrometry (LC-MS) Conditions and Quantification of Metabolite. MPPP, HO-MPPP, and MDPPP were separated and quantified using an Agilent Technologies (Waldbronn, Germany) AT 1100 series atmospheric pressure chemical ionisation electrospray LC-mass spectrometric detection, SL version, and a LC-mass spectrometric detection ChemStation using the A.08.03 software.

LC conditions. Gradient elution was achieved on a Merck LiChroCART column (125 × 2 mm i.d.) with Superspher60 RP Select B as stationary phase and a LiChroCART10–2 Superspher60 RP Select B guard column. The mobile phase consisted of ammonium formate (5 mM, adjusted to pH 3 with formic acid) (eluent A) and acetonitrile (eluent B) according to Maurer et al. (2002). The gradient and the flow rate were as follows: 0 to 3 min 15% B (flow, 0.4 ml/min), 3 to 5 min 40% B (flow, 0.4 ml/min), 5 to 8 min 90% B (flow, 0.6 ml/min), 8 to 8.75 min 90% B (flow, 0.9 ml/min), 8.75 to 10 min 15% B (flow, 0.4 ml/min). The injection volume was 2 μl.

Electrospray conditions. The following atmospheric pressure chemical ionization electrospray inlet conditions were applied: drying gas (7000 ml/min, 300°C) and nebulizer pressure (25 psi) (both nitrogen); capillary voltage (4000 V); drying gas temperature (set at 300°C), vaporizer temperature (set at 400°C); corona current (5.0 μA); positive selected-ion monitoring mode; fragmentor voltage (100 V).

MS conditions. For quantification, the following target ions (*m/z*) were used in the selected-ion monitoring mode: time window 1.55 to 4 min, 234 for HO-MPPP; time window 4 to 6 min, 248 for the internal standard MDPPP; time window 6 to 8 min, 218 for MPPP.

Metabolite quantification. The concentration of HO-MPPP in the incubation mixture was estimated from the known concentration of the internal standard MDPPP, because reference substance of HO-MPPP for a more accurate quantification was not available.

Results and Discussion

LC-MS Procedure. HO-MPPP was not available for its validated determination. However, the peak areas of HO-MPPP and MDPPP were about equal, if their concentrations were the same. This could be concluded from the following observations: many LC-MS runs showed that the peak areas of the internal standard MDPPP and MPPP were about the same, if injected in equimolar amounts. Furthermore, the peak areas of MPPP and HO-MPPP were essentially equal in incubations, in which half of the substrate (half of the initial peak area) had been hydroxylated to HO-MPPP. The levels of HO-MPPP were estimated in MDPPP equivalents and as a first approximation, MDPPP equivalents and HO-MPPP levels may be about the same. All formation rates should be understood as such. In addition, the apparent K_m values and conclusions concerning the percentage of clearance by a specific P450 enzyme are not affected by this quantification.

Initial Screening Studies. Among the nine P450 enzymes tested for possible HO-MPPP formation from MPPP, only CYP2C19 and CYP2D6 were markedly capable of catalyzing this reaction. CYP1A2, CYP2B6, and CYP2C9 exhibited only very marginal turnover rates, and their kinetic profile was not investigated in detail for this reason.

Kinetic Studies. CYP2D6 showed a typical hyperbolic metabolite formation profile (Fig. 2A). Apparent K_m for CYP2D6 was determined to be 9.8 ± 2.5 μM, and V_{max} was estimated to be 13.6 ± 0.7 pmol/min/pmol P450. The data points for 1000 μM substrate concentrations lay somewhat below the 400 and 700 μM ones, so that substrate inhibition could be discussed, but the corresponding Eadie-Hofstee plot (not shown) did not back this assumption. With roughly 80% of the net clearance, CYP2D6 should be the predominant enzyme responsible for MPPP hydroxylation. Visual inspection of the CYP2C19 Michaelis-Menten plot (Fig. 2B) gave evidence that the enzyme was not saturated even at the highest substrate concentration. A linear increase in the turnover rate from 200 to 1000 μM was

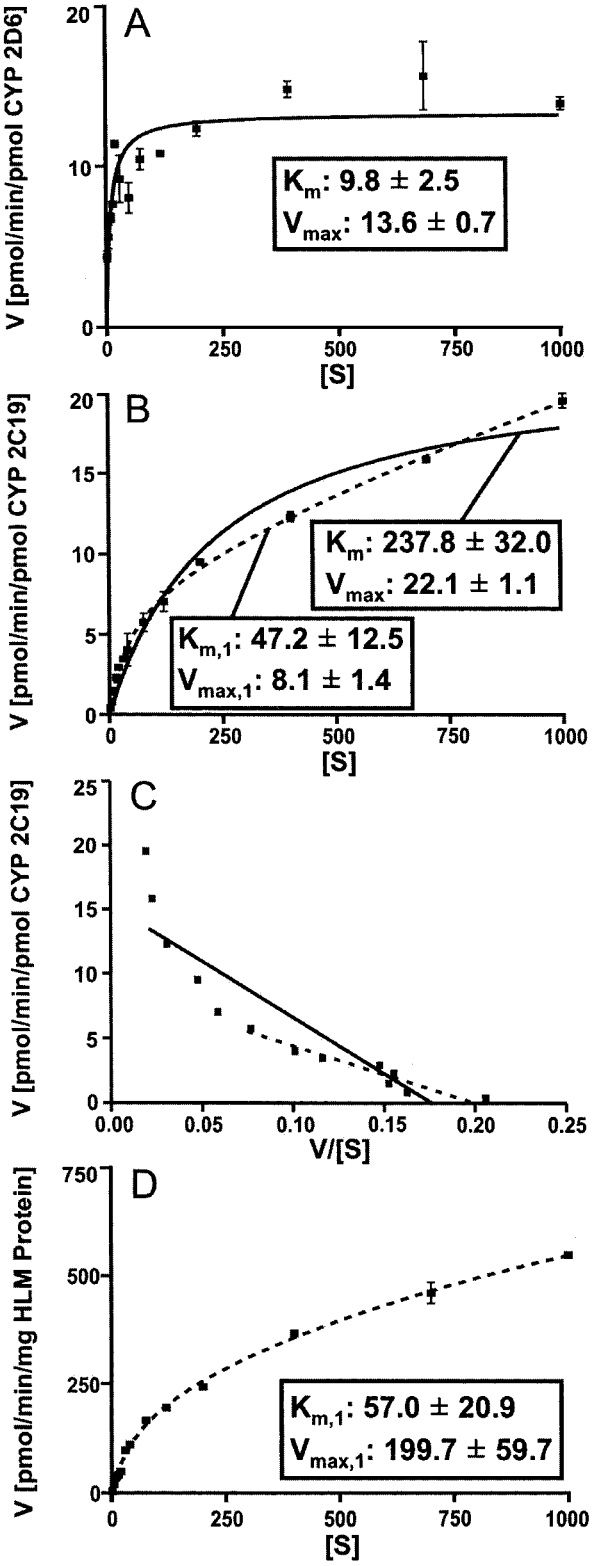

FIG. 2. *Michaelis-Menten plots (A, B, and D) and Eadie-Hofstee plot (C) for CYP2D6 (A), CYP2C19 (B and C), and HLM (D).*

Data points in A, B, and D represent means (solid squares) and ranges (error bars) of two individual measurements. Solid curves in A and B were calculated by nonlinear regression according to eq. 1 (one-site binding model), dotted curves in B and D according to eq. 2 (two-site binding model). Solid regression line in C covers the whole substrate concentration range, dotted regression line covers MPPP concentrations from 2–120 μM. The inserts show the calculated kinetic constants. Units are μM for K_m, pmol/min/pmol P450 (A and B), or pmol/min/mg of protein (D) for V_{max}.

observed, and the concave course of the corresponding Eadie-Hofstee plot (Fig. 2C) clearly demonstrated atypical, biphasic, kinetics (Korzekwa et al., 1998). Therefore, CYP2C19 kinetic parameters were estimated by fitting the data into eq. 2 for a two-site binding model (Fig. 2B, dotted line), which gave significantly better results (F-test, $p < 0.0001$) than the one-site binding equation (cf. solid line in Fig. 2B). The latter returned 237.8 ± 32.0 μM for apparent K_m and 22.1 ± 1.1 pmol/min/pmol P450 for V_{max}, whereas the former resulted in an apparent $K_{m,1}$ of 47.2 ± 12.5 μM and a $V_{max,1}$ of 8.1 ± 1.4 pmol/min/pmol P450. With the linear increase of the turnover rates at higher substrate concentrations, apparent $K_{m,2}$ and $V_{max,2}$ are difficult to estimate. Such profiles, despite using single enzyme sources, have already been observed for CYP1A1 with aminopyrine (Inouye et al., 2000), CYP1A2 with 1-methoxy-4-nitrobenzene (Miller and Guengerich, 2001), CYP2C9 with naproxene (Korzekwa et al., 1998; Hutzler and Tracy, 2002), and CYP3A4 with levo-α-acetylmethadol or naphthalene (Korzekwa et al., 1998; Oda and Kharasch, 2001), but not for CYP2C19. If these kinds of biphasic plots are obtained, Korzekwa et al. (1998) proposed that two substrate molecules at one time have access to the reactive oxygen. This was more likely, if the binding pocket is large and the substrate molecules are small, as it is the case with MPPP.

As more than one enzyme was involved in MPPP hydroxylation, eq. 2 was used to fit into the data points of the HLM experiments (Fig. 2D). Apparent $K_{m,1}$ and $V_{max,1}$ values were 57.0 ± 20.9 μM and 199.7 ± 59.7 pmol/min/mg of protein, respectively. Apparent $K_{m,2}$ and $V_{max,2}$ could not be estimated with nonlinear regression. Apparent K_m values for CYP2D6 and the high affinity enzyme of HLM differed somewhat from each other. This may be due to the fact that unspecific protein binding was greater in HLM (0.5 mg/ml) compared with CYP2D6 microsomes (0.2 mg/ml).

Chemical Inhibition Studies. To underline the importance of CYP2D6 in MPPP metabolism, the CYP2D6-specific inhibitor quinidine (3 μM) was added to incubation mixtures, and the rate of metabolite formation was compared with incubations without the inhibitor. The concentration of the inhibitor was based on average literature data (Clarke, 1998). The inhibition experiments were performed with 2 μM MPPP, because plasma levels are expected to be in this range. HO-MPPP formation was inhibited by approximately 88% ($p < 0.001$). This remarkable inhibition was consistent with the observation that CYP2D6 accounted for about 80% of the net intrinsic clearance of MPPP. With pooled human liver microsomes representing an average, these figures are not absolute and can vary substantially among individuals. Usually, the accuracy of predictions of P450 enzyme contribution to a particular reaction can be easily assessed by comparison with chemical inhibition data in HLM. At a given substrate concentration, a P450 enzyme-specific inhibitor should reduce the formation rate of the metabolite in HLM by approximately the same fraction that the particular P450 is estimated to account for (Stormer et al., 2000).

RAF-corrected kinetic studies as well as inhibition experiments at lower MPPP concentrations demonstrated that CYP2D6 contributed markedly to the clearance of the designer drug. CYP2D6 poor metabolizers, which account for about 7% of the Caucasian population (Bertilsson, 1995; Smith et al., 1998), might therefore exhibit lower clearances than extensive metabolizers. This should not be true for CYP2C19 poor metabolizers (frequency of occurrence 3% in Caucasians) (Bertilsson, 1995; Smith et al., 1998) for obvious reasons. Simultaneous intake of strong CYP2D6 inhibitory drugs might also lead to a decreased clearance of MPPP and elevated plasma concentrations. It cannot be concluded that these genetic polymorphisms and

drug interactions are of clinical relevance and will require further studies.

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References

Bertilsson L (1995) Geographical/interracial differences in polymorphic drug oxidation. Current state of knowledge of cytochromes P450 (CYP) 2D6 and 2C19. *Clin Pharmacokinet* **29:**192–209.

Bryant SG, Guernsey BG, and Ingrim NB (1983) Review of bupropion. *Clin Pharm* **2:**525–537.

Clarke SE (1998) In vitro assessment of human cytochrome P450. *Xenobiotica* **28:**1167–1202.

Crespi CL and Miller VP (1999) The use of heterologously expressed drug metabolizing enzymes–state of the art and prospects for the future. *Pharmacol Ther* **84:**121–131.

Glennon RA, Yousif M, Naiman N, and Kalix P (1987) Methcathinone: a new and potent amphetamine-like agent. *Pharmacol Biochem Behav* **26:**547–551.

Hutzler JM and Tracy TS (2002) Atypical kinetic profiles in drug metabolism reactions. *Drug Metab Dispos* **30:**355–362.

Inouye K, Mizokawa T, Saito A, Tonomura B, and Ohkawa H (2000) Biphasic kinetic behavior of rat cytochrome P-4501A1-dependent monooxygenation in recombinant yeast microsomes. *Biochim Biophys Acta* **1481:**265–272.

Kalix P and Glennon RA (1986) Further evidence for an amphetamine-like mechanism of action of the alkaloid cathinone. *Biochem Pharmacol* **35:**3015–3019.

Korzekwa KR, Krishnamachary N, Shou M, Ogai A, Parise RA, Rettie AE, Gonzalez FJ, and Tracy TS (1998) Evaluation of atypical cytochrome P450 kinetics with two-substrate models: evidence that multiple substrates can simultaneously bind to cytochrome P450 active sites. *Biochemistry* **37:**4137–4147.

Martinez M, Mercado O, Santamaria A, Galvan S, Vazquez M, Bucio V, Hall C, Hernandez R, Hurtazo A, Pego E, Rodriguez F, Salvatierra R, Sosa A, and Rios C (1998) The action of amfepramone on neurochemical and behavioral markers in rats. *Proc West Pharmacol Soc* **41:**125–127.

Maurer HH, Kratzsch C, Weber AA, Peters FT, and Kraemer T (2002) Validated assay for quantification of oxcarbazepine and its active dihydro metabolite 10hydroxy carbazepine in plasma by atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. *J Mass Spectrom* **37:**687–692.

Miller GP and Guengerich FP (2001) Binding and oxidation of alkyl 4-nitrophenyl ethers by rabbit cytochrome P450 1A2: evidence for two binding sites. *Biochemistry* **40:**7262–7272.

Oda Y and Kharasch ED (2001) Metabolism of levo-alpha-Acetylmethadol (LAAM) by human liver cytochrome P450: involvement of CYP3A4 characterized by atypical kinetics with two binding sites. *J Pharmacol Exp Ther* **297:**410–422.

Roesner P, Junge T, Fritschi G, Klein B, Thielert K, and Kozlowski M (1999) Neue synthetische Drogen: Piperazin-, Procyclidin- und alpha-Aminopropiophenonderivate. *Toxichem Krimtech* **66:**81–90.

Smith DA, Abel SM, Hyland R, and Jones BC (1998) Human cytochrome P450s: selectivity and measurement in vivo. *Xenobiotica* **28:**1095–1128.

Springer D, Peters FT, Fritschi G, and Maurer HH (2002) Studies on the metabolism and toxicological detection of the new designer drug 4′-methyl-alpha-pyrrolidinopropiophenone (MPPP) in urine using gas chromatography-mass spectrometry. *J Chromatogr B* **773:**25–33.

Stormer E, von Moltke LL, and Greenblatt DJ (2000) Scaling drug biotransformation data from cDNA-expressed cytochrome P-450 to human liver: a comparison of relative activity factors and human liver abundance in studies of mirtazapine metabolism. *J Pharmacol Exp Ther* **295:**793–801.

Venkatakrishnan K, von Moltke LL, Court MH, Harmatz JS, Crespi CL, and Greenblatt DJ (2000) Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches. *Drug Metab Dispos* **28:**1493–1504.