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Fenproporex *N*-dealkylation to amphetamine—enantioselective in vitro studies in human liver microsomes as well as enantioselective in vivo studies in Wistar and Dark Agouti rats

Thomas Kraemer*, Thomas Pflugmann, Michael Bossmann, Nicole M. Kneller, Frank T. Peters, Liane D. Paul, Dietmar Springer, Roland F. Staack, Hans H. Maurer

Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Saarland, D-66421 Homburg (Saar), Germany

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Abstract

Fenproporex (FP) is known to be *N*-dealkylated to *R*(−)-amphetamine (AM) and *S*(+)-amphetamine. Involvement of the polymorphic cytochrome P450 (CYP) isoform CYP2D6 in metabolism of such amphetamine precursors is discussed controversially in literature. In this study, the human hepatic CYPs involved in FP dealkylation were identified using recombinant CYPs and human liver microsomes (HLM). These studies revealed that not only CYP2D6 but also CYP1A2, CYP2B6 and CYP3A4 catalyzed this metabolic reaction for both enantiomers with slight preference for the *S*(+)-enantiomer. Formation of amphetamine was not significantly changed by quinidine and was not different in poor metabolizer HLM compared to pooled HLM. As in vivo experiments, blood levels of *R*(−)-amphetamine and *S*(+)-amphetamine formed after administration of FP were determined in female Dark Agouti rats (fDA), a model of the human CYP2D6 poor metabolizer phenotype (PM), male Dark Agouti rats (mDA), an intermediate model, and in male Wistar rats (WI), a model of the human CYP2D6 extensive metabolizer phenotype. Analysis of the plasma samples showed that fDA exhibited significantly higher plasma levels of both amphetamine enantiomers compared to those of WI. Corresponding plasma levels in mDA were between those in fDA and WI. Furthermore, pretreatment of WI with the CYP2D inhibitor quinine resulted in significantly higher amphetamine plasma levels, which did not significantly differ from those in fDA. The in vivo studies suggested that CYP2D6 is not crucial to the *N*-dealkylation but to another metabolic step, most probably to the ring hydroxylation. Further studies are necessary for elucidating the role of CYP2D6 in FP hydroxylation.

Keywords: Amphetamine precursor drugs; Enantioselective; Metabolism; Cytochrome p450; Human liver microsomes; Dark Agouti rats

1. Introduction

Fenproporex (*R*,*S*-3-[(1-phenyl-2-propyl)-amino-]propionitril, FP) is a widely used anorectic. Previous in vivo studies in humans had shown two partially over lapping metabolic pathways for FP: ring alteration by single and double aromatic hydroxylation followed by methylation and side chain degradation by *N*-dealkylation to amphetamine (AM) [1]. The involvement of CYP2D6 in the formation of AM from such AM precursor drugs is still controversially discussed. Some authors tried to explain huge inter-individual differences in the amount of AM metabolically formed from such precursors by CYP2D6 polymorphism. This topic has been extensively reviewed [2,3].

The cytochrome P450 (CYP) family accounts for more than 90% of oxidative metabolic reactions of xenobiotics [4]. The involvement of particular cytochrome P450 enzymes in the biotransformation of a new drug has to be thoroughly investigated before it can be marketed. Such

Abbreviations: FP, Fenproporex (*R*,*S*-3-[(1-phenyl-2-propyl)-amino-]propionitril); AM, amphetamine; CYP, cytochrome P450; PM HLM, poor metabolizer genotype human liver microsomes; pHLM, pooled human liver microsomes; fDA, female Dark Agouti rats; PM, human CYP2D6 poor metabolizer phenotype; WI, male Wistar rats; EM, human CYP2D6 extensive metabolizer phenotype; mDA, male Dark Agouti rats; HFBP, *S*(−)-heptafluorobutyrylprolyl chloride; IS, internal standard; NICI, negative ion chemical ionization; GC–MS, gas chromatography–mass spectrometry; K_m, Michaelis–Menten constant; V_{max}, maximal turnover rate; BM, body mass; SPE, solid-phase extraction; AT, agilent technologies; WIQ, quinine pretreated Wistar rats; MA, methamphetamine

* Corresponding author. Tel.: +49 6841 1626430; fax: +49 6841 1626051.
E-mail address: thomas.kraemer@uniklinik-saarland.de (T. Kraemer).

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investigations allow to predict possible drug–drug interactions, inter-individual variations in pharmacokinetic profiles and increased appearance of side effects and serious poisonings [5]. However, such risk assessment is typically performed for newer substances intended for therapeutic use, but not for substances which have been on the market for decades such as FP. Therefore, the first aim of the work presented here was to identify the human hepatic CYPs catalyzing FP *N*-dealkylation to *R*(−)-AM and *S*(+)-AM and to determine the kinetic constants for these reactions. In addition, *R*(−)-AM and *S*(+)-AM formation from FP in single donor human liver microsomes (HLM) with CYP2D6 poor metabolizer genotype (PM HLM) should be compared with those in pooled human liver microsomes (pHLM) with and without addition of the CYP2D6 inhibitor quinidine.

The second aim was to study the actual in vivo influence of CYP2D6 on FP metabolism. For this purpose a rat model was chosen. Female Dark Agouti rats have been proposed as a model of the human CYP2D6 poor metabolizer phenotype (PM) allowing a preliminary screening for CYP2D6 substrates [6,7] and male Wistar rats as the corresponding model of the human CYP2D6 extensive metabolizer phenotype (EM) [7,8]. In addition, male Dark Agouti rats were used as an intermediate model between the PM and EM models [9]. These rat models have successfully been used by the author's working group also to investigate the role of CYP2D6 in human metabolism of the new designer drug TFMPP (1-(3-trifluoromethylphenyl)piperazine) [10]. After administration of FP, blood plasma levels of *R*(−)-AM and *S*(+)-AM in the above mentioned rats were compared in order to detect possible differences in pharmacokinetics in human PM and EM subjects. Such differences may become relevant in clinical and forensic toxicology or doping control for interpretation of analytical results, i.e. if a positive amphetamine result is claimed to be caused by such a precursor drug and not by illicit amphetamine.

2. Materials and methods

2.1. Materials

Fenproporex–hydrochloride (FP–HCl) was a kind gift of Professor Pfleger. Methanolic solution (1000 mg/L) of AM-d_{11} was obtained from Promochem. AM–HCl was obtained from Lipomed, quinine and quinidine were obtained from Promochem, $NADP^+$ was obtained from Biomol, isocitrate and isocitrate dehydrogenase from Sigma, all other chemicals and reagents from Merck. 0.1 M *S*-(−)-heptafluorobutyrylprolyl chloride (HFBP) in dichloromethane was synthesized according to ref. [11]. The following microsomes were from Gentest and delivered by NatuTec: baculovirus-infected insect cell microsomes (Supersomes) containing 1 nmol/mL human cDNA-expressed CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, or CYP3A4, wild-type baculovirus-infected insect cell microsomes (control Supersomes), pHLM (20 mg microsomal protein/mL, 400 pmol total CYP/mg protein) and PM HLM (20 mg microsomal protein/mL). After delivery, the microsomes were thawed at 37 °C, aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Microsomal incubations

Typical 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 $NADP^+$, 2 U/mL isocitrate dehydrogenase, 200 U/mL superoxide dismutase and substrate at 37 °C. The substrate was added after appropriate dilution of a 250 mM methanolic stock solution in buffer. The methanol concentration did not exceed 0.4%. Reactions were started by addition of the ice-cold microsomes and terminated with 10 μL of a 2 M aqueous trifluoroacetic acid solution. After addition of 5 μL of a 0.01 mg/mL solution of AM-d_{11} in methanol as internal standard (IS), 25 μL of carbonate buffer (5% w/v; pH 9) and 25 μL of derivatization reagent HFBP were added and the reaction vials were sealed and left on a rotary shaker at room temperature for 30 min. Thereafter, the HFBP derivatized analytes were extracted into 100 μL of ethyl acetate. An aliquot (3 μL) of the organic phase was analyzed by gas chromatography–negative ion chemical ionization–mass spectrometry (GC–NICI–MS).

2.3. Initial screening studies

In order to investigate the involvement of particular CYPs in FP dealkylation, incubations were performed with 200 μM FP and 50 pmol/mL CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 for 30 min. For incubations with CYP2A6 or CYP2C9, phosphate buffer was replaced with 45 mM and 90 mM Tris-buffer (pH 7.4), respectively, according to the Gentest manuals.

2.4. Enzyme 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 ($n = 2$ each) with the following FP concentration ranges (given for a single enantiomer), protein concentrations and incubation times 1–750 μM FP with 30 pmol CYP2B6, CYP2D6, CYP3A4, CYP1A2 for 10 min; and 1–750 μM FP with 0.5 mg HLM protein/mL for 15 min. Less than 20% of substrate were metabolized in all incubations. Apparent K_m and V_{max} values for single isoforms were estimated by nonlinear regression according to the

Michaelis–Menten equation

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

Eq. (2) for a two-site binding model was applied to the data of the pHLM experiments [12]

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

2.5. Chemical inhibition studies

The effect of 1 or 3 μM quinidine on AM formation was assessed in incubations containing 0.5 mg pHLM protein/mL and 50 μM FP for 10 min. Controls contained no quinidine, but the same amount of methanol to control for any solvent effects ($n = 6$, each). Significance of inhibition was tested by a one-tailed unpaired *t*-test.

2.6. Comparative studies between pHLM and PM HLM

Incubations were carried out at 50 μM FP for 10 min using either 0.5 mg pHLM or PM HLM protein/mL. Significance of differences in AM formation was tested by a one-tailed unpaired *t*-test.

2.7. Animals, treatments and collection of blood samples

The investigations were performed using blood samples of WI (Ch. River), of mDA or of fDA (both from Harlan–Winckelman) for toxicological diagnostic reasons according to the corresponding German law. The animals were housed in groups under controlled humidity and temperature (23 ± 1 °C) and a 12 h light–dark cycle (lights on 7:00 a.m. to 7:00 p.m.) with food and water ad libitum. A single dose of FP (3 mg/kg body mass, BM) in aqueous solution was administered to the rats by gastric intubation ($n = 5$ for each rat strain). Furthermore, WI were pretreated with quinine (80 mg/kg BM) [10,13] 3 h before administration of FP (3 mg/kg BM). The rats were housed in metabolism cages for 24 h after administration, having water ad libitum. Blood samples were taken from the tail vein 3, 6, 9, and 12 h after administration. All samples were directly analyzed as described below.

2.8. Sample preparation of rat blood plasma samples

The rat blood plasma samples were prepared according to a validated procedure [11] with modifications. Aliquots (0.2 mL) of rat blood plasma were diluted with 2 mL of purified water. After addition of 0.1 mL of a methanolic solution of the racemic IS AM-d_{11} (0.2 μg/mL) the samples were mixed (15 s) on a rotary shaker and loaded on HCX solid-phase extraction (SPE) cartridges previously conditioned with 1 mL of methanol and 1 mL of purified water. After extraction, the cartridges were washed with 1 mL of purified water, 1 mL of 0.01 M hydrochloric acid and 2 mL of methanol. Vacuum was applied until the cartridges were dry and the analytes were eluted with 1 mL of methanol/aqueous ammonia (98:2 v/v) into 1.5 mL reaction vials. The eluates were evaporated to dryness under a stream of nitrogen at 56 °C. After addition of 0.2 mL of aqueous carbonate buffer (5%, w/v; pH 9) and 6 μL of derivatization reagent HFBP, the reaction vials were sealed and left on a rotary shaker at room temperature for 30 min. Thereafter, 0.1 mL of cyclohexane was added, the reaction vials were sealed again and left on a rotary shaker for 1 min. The phases were separated by centrifugation (14,000 rpm, 1 min) and the cyclohexane phase (upper) was transferred to autosampler vials. Aliquots (3 μL) were injected into the GC–MS system.

2.9. Enantioselective GC–NICI–MS quantification

2.9.1. Apparatus

The samples were analyzed using an Agilent Technologies (AT) 6890 Series GC system combined with an AT 5973 network mass selective detector, an AT 7683 series injector and an AT enhanced Chem Station G1701CA version C.00.00 21 December 1999.

2.9.2. GC conditions

Splitless injection mode; column, 5% phenyl methyl siloxane (HP-5MS, 30 m × 0.25 mm i.d., 250 nm film thickness); injection port temperature, 280 °C; carrier gas, helium; flowrate, 1 mL/min; column temperature, 100 °C raised to 180 °C at 30 °C/min, to 230 °C at 5 °C/min, to 310 °C at 30 °C/min.

2.9.3. MS conditions

Transfer line heater, 280 °C; NICI, methane (2 mL/min); source temperature, 150 °C; solvent delay, 9 min; selected ion monitoring (SIM) mode; 9–11 min *m*/z 399 (target ion), 379, 439 for AM-d_{11}; 388 (target ion), 368, 428 for AM. The enantiomers of AM were quantified by comparison of their peak area ratios (enantiomer of analyte versus corresponding enantiomer of the IS) to calibration curves in which the peak area ratios of spiked calibration standards had been plotted versus their concentrations using a linear regression model.

2.10. Statistical analysis

All statistics were calculated using GraphPad Prism 3.02 software designed for nonlinear regression analysis. The Michaelis–Menten parameters K_m and V_{max} were calculated by fitting kinetic data to a one- or two-site binding model. Significance of inhibition and differences in metabolite formation were determined using a one-tailed unpaired *t*-test.

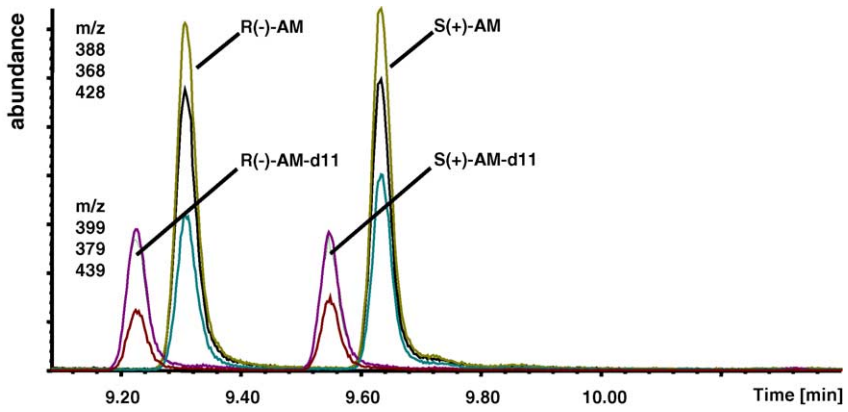

Fig. 1. Typical merged mass fragmentograms of a WI plasma sample after SPE and derivatization with HFBP.

3. Results

3.1. GC–MS procedures

The mass fragmentograms in Fig. 1 show that the applied GC–MS conditions provided baseline separation of the derivatized AM enantiomers. The figure shows typical merged mass fragmentograms of a WI blood plasma sample after SPE and derivatization with HFBP. The chosen target ions were selective for the analytes under these conditions as proven with blank samples (control blood samples or microsomes without substrate and IS).

3.2. Initial screening studies

Fig. 2 illustrates that among the nine CYPs tested only CYP1A2, CYP2B6, CYP2D6 and CYP3A4 catalyzed the *N*-dealkylation of FP to the *R*(−)-enantiomer of AM (black bars) and to the *S*(+)-enantiomer of AM (grey bars). AM was not detectable in incubations with the remaining recombinant CYPs or with insect cell control microsomes.

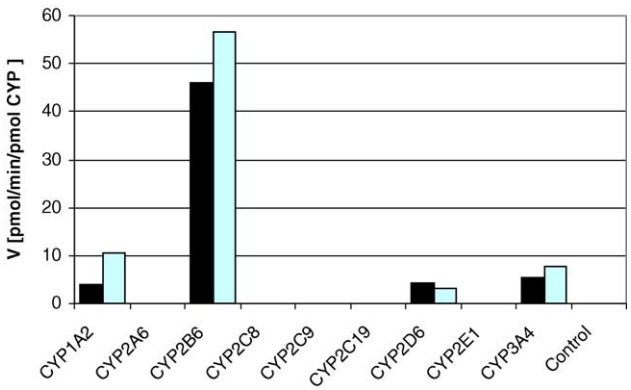

Fig. 2. Formation rates (*V* in [pmol/min/pmol CYP]) of AM from FP (200 μM FP) with 50 pmol/mL of the given individual CYPs and with insect cell control microsomes ($n = 2$). The black bars correspond to the *R*(−)-enantiomer and the grey bars to the *S*(+)-enantiomers.

3.3. Kinetic studies

All incubations were carried out at initial rate conditions, a prerequisite for Michaelis–Menten kinetics. All of the kinetics of the investigated reaction with single recombinant CYPs showed a typical hyperbolic profile, as shown in Fig. 3. The kinetic parameters (apparent K_m, V_{max}) for these reactions are also given in Fig. 3, as well as the corresponding values for intrinsic clearances (Cl_{int}). For incubations with recombinant CYPs, the parameters were estimated using Michaelis–Menten Eqs. (1) and (2) was used for incubations with pHLM, as more than one isoform was capable to catalyze the reaction. The apparent $K_{m,\,1}$ and $V_{max,\,1}$ values for the *S*(+)-enantiomer in pHLM were 50 μM and 54.2 pmol/min/mg protein. The apparent $K_{m,\,1}$ and $V_{max,\,1}$ values for the *R*(−)-enantiomer were 75 μM and 141.6 pmol/min/mg protein.

3.4. Chemical inhibition studies

In order to investigate the possible importance of CYP2D6 in FP *N*-dealkylation to AM, the CYP2D6 specific inhibitor quinidine (1 or 3 μM) was added to incubation mixtures and the metabolite formation rates of *R*(−)-AM and *S*(+)-AM compared to those in incubations without inhibitor. Fig. 4 shows that in presence of 1 μM inhibitor and 50 μM FP the metabolite formation of both enantiomers was not significantly inhibited. In presence of 3 μM inhibitor and 50 μM FP the metabolite formation of the *S*(+)-enantiomer was significantly (*P* value 0.0145) inhibited.

3.5. Comparative studies between pHLM and PM HLM

In order to further investigate the importance of CYP2D6 in FP *N*-dealkylation to AM and to demonstrate differences in PM and EM subjects, the metabolite formation rate of *R*(−)-AM and *S*(+)-AM in pHLM was compared to that in PM HLM. However, there were no

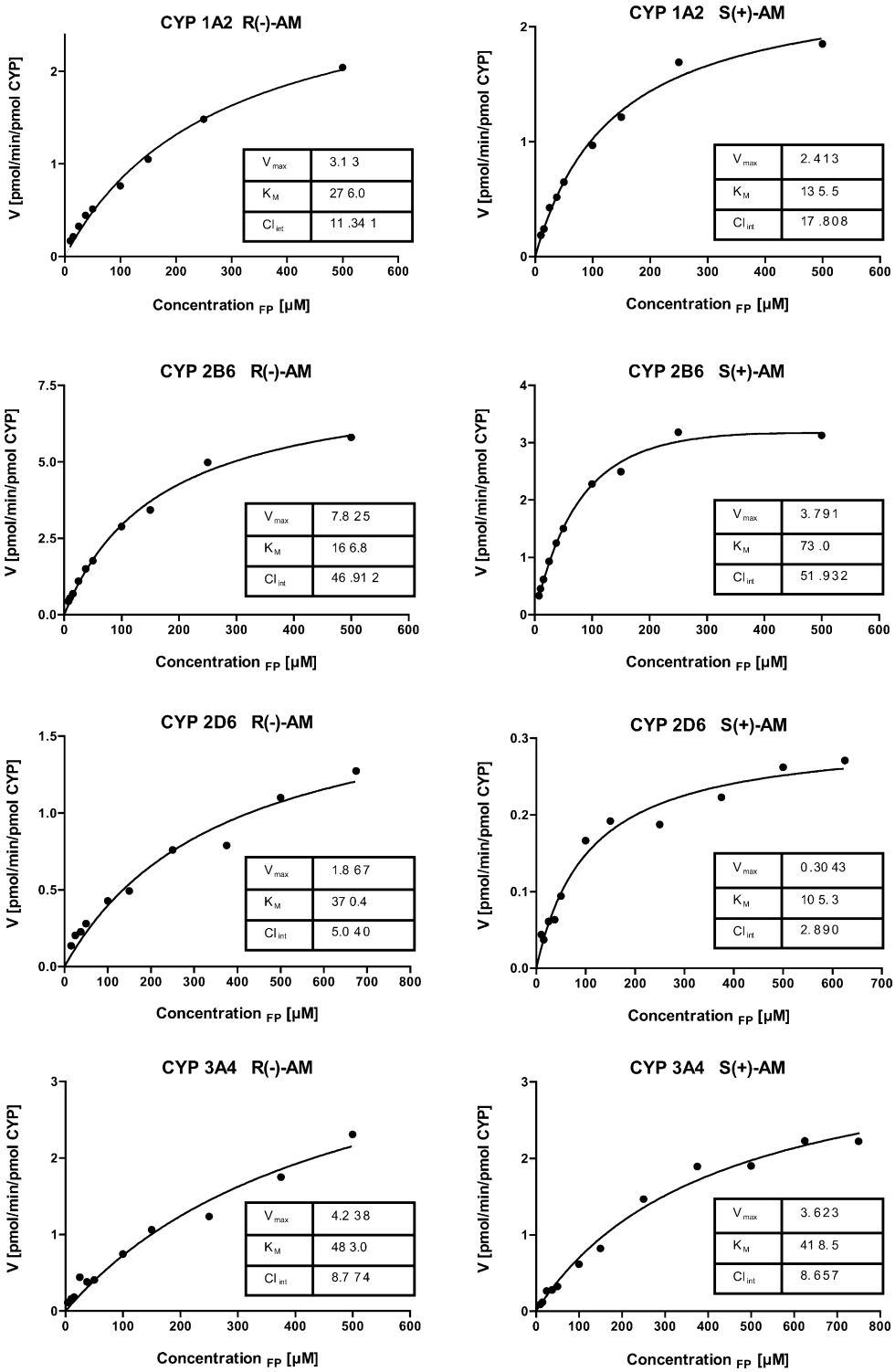

Fig. 3. Michaelis–Menten plots for FP *N*-dealkylation catalyzed by CYP1A2, CYP2B6, CYP2D6 and CYP3A4. Results for formation of *R*(−)-AM are given on the left side each, for *S*-(+)-AM on the right side each. Values represent the mean of duplicate incubations. Curves were calculated by nonlinear regression according to Eq. (1) (one-site binding model). V_{max} values given in pmol/min/mg protein; K_m values in μM and intrinsic clearances in nL/min/mg protein.

significant differences in the metabolite formation rates in PM HLM and pHLM as can be seen in Fig. 4. Metabolite formation of the *S*(+)-enantiomer was significantly higher in PM HLM than in the pHLM with 3 μM quinidine (*P* value 0.0151).

3.6. Analysis of rat blood plasma samples

The concentrations of *R*(−)-AM and *S*(+)-AM were determined in rat blood plasma samples. The complete data on blood plasma concentrations of *R*(−)-AM and *S*(+)-

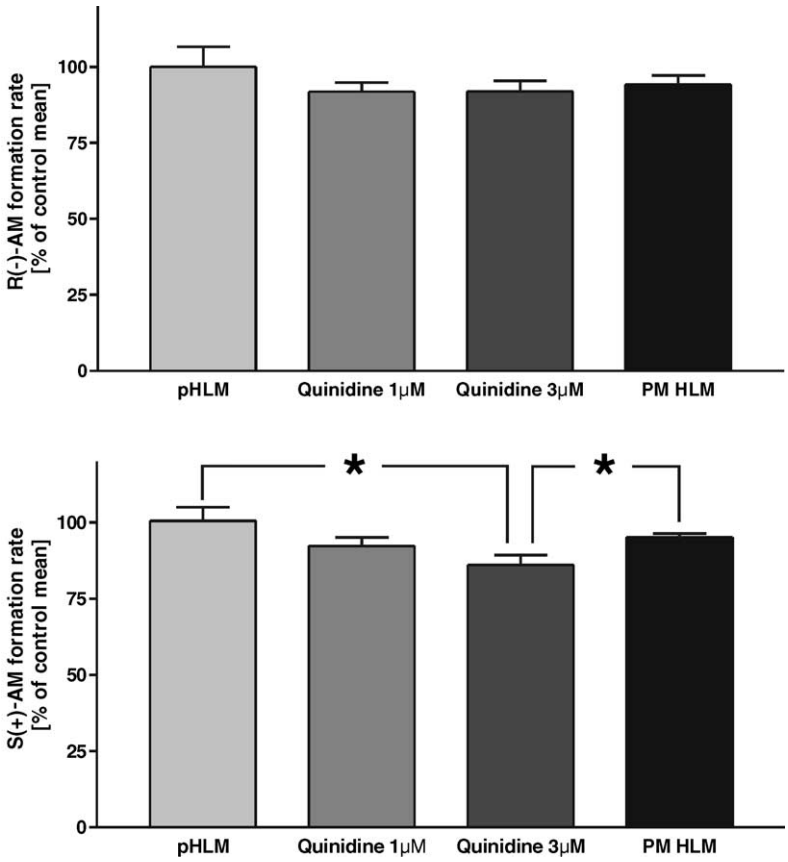

Fig. 4. AM formation (*R*(−)-enantiomer upper part, *S*(+)-enantiomer lower part) from FP in PM HLM (right bar) and effect of 1 or 3 μM quinidine on metabolite formation in pHLM (middle bars) in incubation mixtures containing 50 μM FP. Controls with pHLM without inhibitor (left bar) were set to 100%. Each bar represents the mean of six incubations ± standard error of the mean. (* represents the *P* value 0.01–0.05).

AM in all groups (fDA, mDA, WIQ and WI) at all points in time is given in Fig. 5 together with the results of the *t*-tests, showing the level of significance for the differences in AM formation between the groups. The blood plasma levels of both AM enantiomers in fDA and in mDA were significantly higher than those of WI at all tested points in time. The AM blood plasma levels in mDA always lay in between fDA and WI. Pretreatment of WI with quinine (WIQ) led to significantly higher blood plasma levels at all time points compared to untreated WI.

Fig. 6 shows the changes over time in *R* to *S* ratios of the concentrations of the AM enantiomers determined in plasma samples of WIQ, fDA and WI. The corresponding *R*/*S* ratios for both, fDA and WIQ, are significantly higher than those for WI. The *R*/*S* ratios of fDA and WIQ are not significantly different from each other with the exception of the ratios at 9 h.

4. Discussion

The human hepatic CYPs involved in FP *N*-dealkylation were identified using microsomal preparations from different sources: recombinant CYP enzymes, pHLM and PM HLM. This study design has already been described for other drugs like e.g. new designer drugs [10,14–17]. The initial screening studies with the nine most abundant human hepatic CYPs were performed to identify their possible role in FP *N*-dealkylation. According to the supplier's advice, the incubation conditions chosen were adequate to make a statement on a general involvement of a particular CYP enzyme. The data revealed that CYP1A2, CYP2B6, CYP2D6 and CYP3A4 were capable of catalyzing the monitored reaction for both enantiomers of FP each. None of the isoforms, which catalyzed the reaction, acted enantiospecifically, i.e. that it catalyzed the *N*-dealkylation of only one enantiomer. The kinetic profiles of the *N*-dealkylation catalyzed by these particular CYPs and pHLM were further investigated. Kinetic assays with these single CYPs and pHLM were performed under initial rate conditions, a prerequisite for Michaelis–Menten kinetics [12].

As expected, classical hyperbolic Michaelis–Menten plots (Fig. 3) were found using recombinant CYPs. The apparent K_m and V_{max} values of the investigated CYPs were calculated by nonlinear regression fit according to Eq. (1). Visual inspection of the plots (e.g. for the *R*-(−)-enantiomers by CYP2D6 and CYP3A4) suggested biphasic enzyme character. However, the corresponding Eadie–Hofstee plots did not sufficiently support biphasic

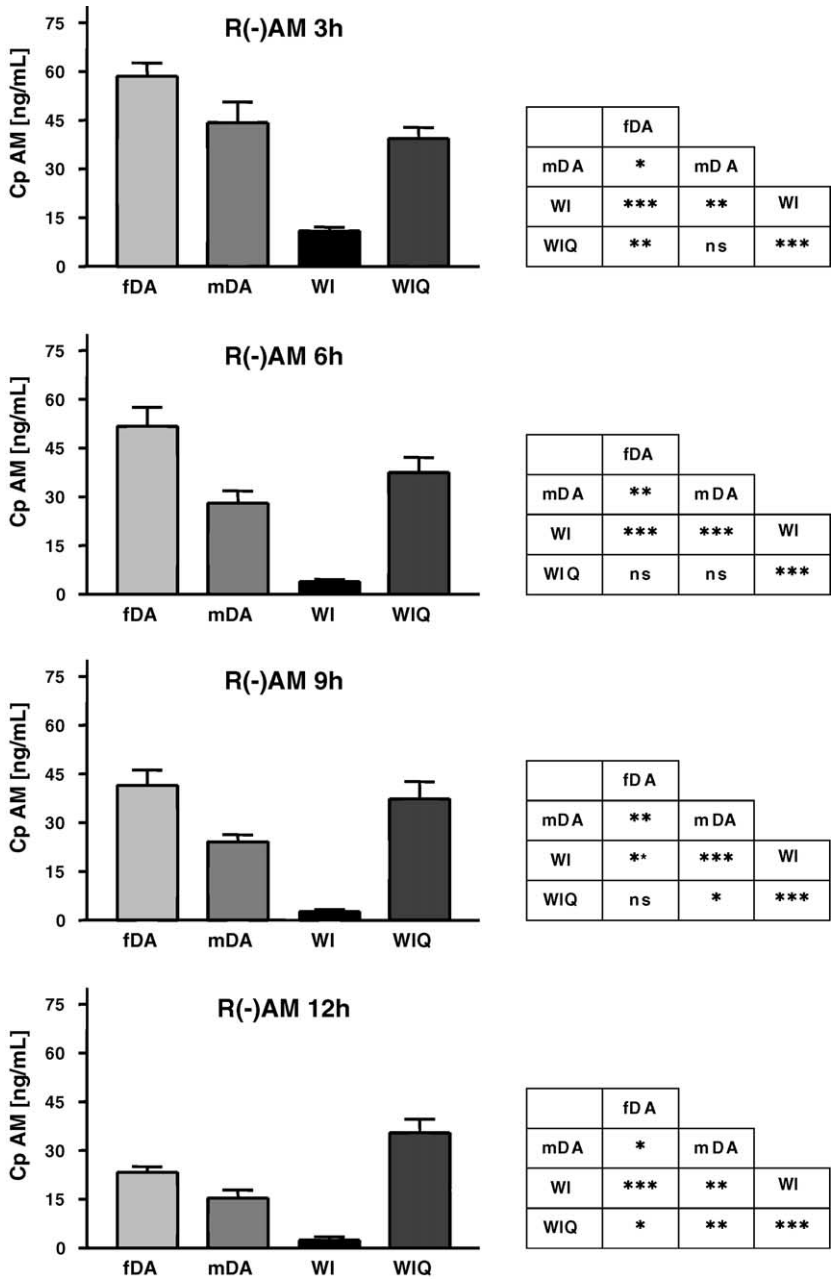

Fig. 5. *R*(−)-AM and *S*(+)-AM concentrations determined in blood plasma (Cp) of fDA, mDA, WI and WIQ after solid-phase extraction and HFBP derivatization taken 3, 6, 9, and 12 h after administration. Each bar represents the mean of at least four samples ± standard error of the mean. The tables on the right part of the figure give the level of significance for the differences between the groups. (* represents the *P* value 0.01–0.05; ** represents the *P* value 0.001–0.01; *** represents the *P* value < 0.001; ns: not significant; *P* value > 0.05).

kinetics and the fit into a two-site binding model did not provide reasonable results. As given in Fig. 3, the differences in the K_m and V_{max} values for the *S*(+)- and *R*(−)-enantiomers were not too big. The K_m values were slightly higher for the *R*(−)-enantiomer (about 1.2–3.5-fold) and the V_{max} values for the *R*(−)-enantiomers were also higher than for the *S*(+)-enantiomers (up to six-fold). If at all, the *N*-dealkylation of FP to AM is slightly enantioselective, preferring the *S*(+)-enantiomer. This conclusion is further supported by the results of the in vivo experiments with different rat strains (see discussion on the *R*/*S* ratios in rat blood plasma samples). Using the data on intrinsic clearance for the particular isoforms the percentage of contribution of each isoform to the entire microsomal clearance in vitro was calculated. The highest contribution was found for CYP2B6 (65% for *R*(−) and 72% for the *S*(+)-enantiomer) and the lowest values were found for CYP2D6 (7 and 4% for *R*(−) and *S*(+)-enantiomers, respectively). Nevertheless, it should be taken into account that this data does not reflect in vivo conditions. Differences in intrinsic activity (per unit of CYP) between cDNA-expressed CYP isoforms and human liver enzymes

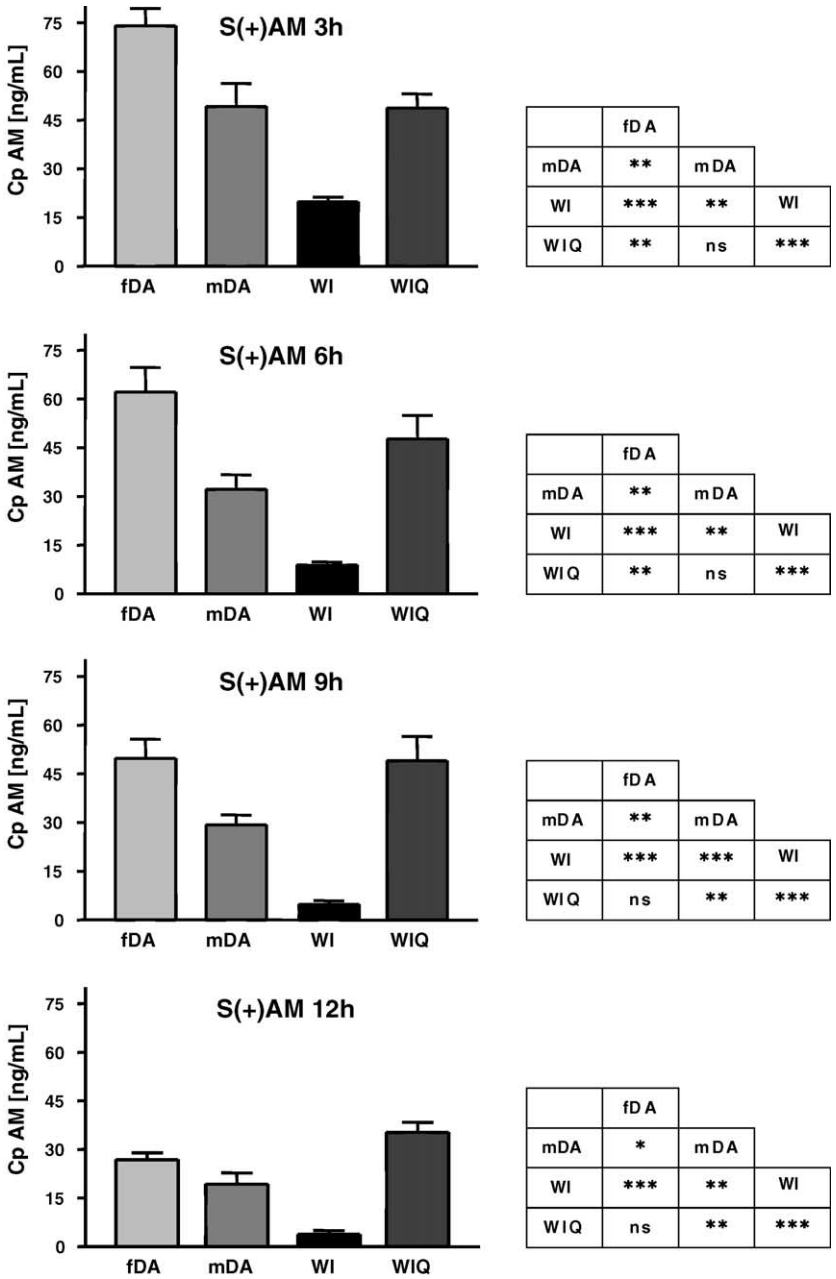

Fig. 5. (*Continued*).

complicate the prediction of in vivo drug clearance by in vitro data.

As more than one enzyme was involved in FP *N*-dealkylation, Eq. (2) was used to fit into the data points of pHLM experiments. In order to investigate the role of CYP2D6 in FP *N*-dealkylation, inhibition studies with the CYP2D6 specific chemical inhibitor quinidine [8] were performed. The concentrations of the inhibitor (1 or 3 μM) were based on average literature data [12,18,19]. The substrate concentration examined was 50 μM, which corresponded to the calculated slightly lower of the two $K_{m,\,1}$ values of the enantiomers in pHLM. The results showed that at this substrate concentration the overall turnover was not significantly inhibited by 1 μM quinidine. The slight inhibition for the *S*(+)-enantiomer at 3 μM quinidine may be explained by the missing specificity of the inhibitor at this relatively high concentration. A further support for this hypothesis is that this formation rate is even significantly lower than that of PM HLM. If only CYP2D6 was inhibited, the formation rate should be that of PM HLM. The all in all missing inhibition can be explained by the fact that three other isoforms were also able to catalyze this reaction showing similar affinities towards FP and even higher capacities. Consequently, the metabolite formation rate of PM HLM was also not significantly different from that of pHLM (Fig. 4).

In conclusion, the in vitro results suggested that CYP2D6 obviously was not the major isoform responsible

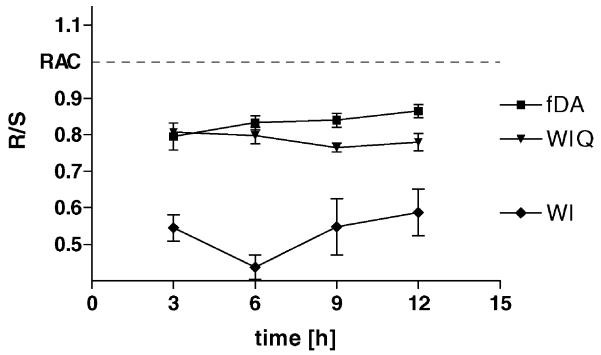

Fig. 6. Changes over time in *R* to *S* ratios of the concentrations of the AM enantiomers determined in plasma samples of WIQ, fDA and WI. Each point represents the mean of at least four samples ± standard error of the mean. (RAC, *R* to *S* ratio for racemic mixture, 1.0).

for the formation of AM from FP. Unfortunately, in vivo experiments with humans showing different CYP2D6 phenotypes were not possible for ethical reasons. Therefore, experiments were performed with different rat strains, which are accepted models of the human PM and EM [6–10]. The results concerning the blood plasma levels of the AM enantiomers after administration of FP to the different rat groups (fDA, mDA, WI) were surprising. At all points in time, both enantiomers of AM showed significantly higher concentrations in the PM model (fDA) than in the EM model (WI). The blood plasma levels in mDA lay between these two groups. The presence of CYP2D obviously did not lead to higher but to markedly lower AM levels in the corresponding rats. For further confirmation, that these differences could be attributed to the differences in CYP2D activity, WI rats were pretreated using the CYP2D specific inhibitor quinine before administration of FP [8,20]. Actually, pretreatment with quinine and thereby inhibition of CYP2D activity resulted in significantly higher AM blood plasma levels for both enantiomers at all points in time. The complete data on blood plasma concentrations of *R*(−)- and *S*(+)-AM in all groups (fDA, mDA, WIQ and WI) at all points in time can be observed in Fig. 5 together with the results of the *t*-tests, showing the level of significance for the differences in AM formation between the groups.

The results may be explained by the presence of another metabolizing step which is catalyzed by CYP2D isoforms and which leads to other metabolites than AM. Previous in vivo studies in humans or rats had shown two partially overlapping metabolic pathways for FP: besides the side chain degradation by *N*-dealkylation to amphetamine, ring alteration by aromatic hydroxylation [1,21]. If the latter reaction was (mainly) catalyzed by CYP2D6, the higher AM levels in PM subjects could be explained by the lack of hydroxylation of AM as well as of the parent compound FP thus leading to higher levels of unchanged AM and unchanged FP, which for its part could be dealkylated to AM. At least for the hydroxylation of AM and its *N*-methyl analogue methamphetamine (MA), involvement of CYP2D isoforms had been shown in the literature [2,13,20,22–24]. For selegiline (*R*-(−)-*N*-methyl-(1-phenyl-2-propyl)-2-propinylamin), a further AM/MA precursor, which is *N*-dealkylated to MA and AM, involvement of CYP2D isoforms in the hydroxylation step is at least discussed [25]. These authors did not find significant differences in the pharmacokinetic parameters of selegiline, desmethylselegiline, and bisdealkylselegiline (AM) between poor metabolizers and extensive metabolizers. However, the area under the serum concentration–time curve (AUC) values of the *N*-dealkyl metabolite *R*(−)-MA were, on average, 46% higher in poor metabolizers than in extensive metabolizers. They suggested an involvement of CYP2D6 in a later phase of selegiline elimination, especially in the ring hydroxylation. In this study, one ultrarapid metabolizer was also tested. He did not differ from the other volunteers with regard to pharmacodynamics and pharmacokinetics with the exception of a low area under the serum concentration–time curve of *R*(−)MA.

Hydroxy metabolites of FP or AM could not be determined in the blood plasma samples of the different rat models, because of the very low concentrations. Under in vivo conditions, the hydroxylated metabolites are conjugated immediately after functionalization and excreted. Indeed, it could be shown in a preliminary study on urine samples of the different rat strains, which had been collected during our studies, that hydroxylated metabolites were almost completely missing in the urine samples of fDA and WIQ, whereas hydroxylated metabolites predominated in urine samples of WI.

The differences in the enantiomeric ratios of *R*(−)-AM versus *S*(+)-AM between WI on the one hand and fDA and WIQ on the other hand (Fig. 6) may also be explained by the hydroxylation reaction. As already deduced from the kinetic data found for the different isoforms involved in *N*-dealkylation of FP, differences in the enantiomeric composition of AM should be low. The fact that the ratio does not exactly correspond to the racemic ratio of 1.0 may be explained by the slightly higher affinities of *S*(+)-FP to the isoforms involved in *N*-dealkylation or, of course, by competing phase one metabolic reactions or other enantioselective processes (conjugation, distribution). The significantly lower *R*/*S* ratios in the WI group (mean of 0.53 versus 0.83 in fDA) cannot be explained by the fact that the differences in K_m values for the *R* and *S* enantiomers are higher for CYP2D6 (3.5-fold) than for the other isoforms (1–2.2-fold). If this was true, CYP2D6 should have a marked influence on overall AM formation. As already discussed, CYP2D6 does not play an important role in AM formation. In addition, the specific ‘‘switching-off’’ of CYP2D6 in the Wistar rats by quinine leads to the same *R*/*S* ratios as in the fDA group. This proves, that only one isoform (namely CYP2D6) should be responsible for the differences in the *R*/*S* ratios. Therefore, the significantly lower *R*/*S* ratios in the WI group may be explained by another CYP2D catalyzed and enantioselective reaction.

This could be the ring hydroxylation. However, further studies on the enzyme kinetics of FP hydroxylation are necessary to finally prove the hypothesis for FP.

Concerning the interpretation of positive AM results in blood e.g. in clinical and forensic toxicology or especially in driving under the influence of drugs (DUID) cases the *R*/*S* ratios of AM concentrations in blood may be useful for differentiation of legal intake of AM precursors from illicit use of AM. The *R*/*S* ratios of AM concentrations in blood after intake of racemic AM usually are greater than 1.0 [11,26] whereas the corresponding ratios of the AM enantiomers metabolically formed from precursors should be markedly lower than 1.0 for EM subjects and slightly lower than 1.0 for PM subjects (at least shortly after ingestion). However, this hypothesis is yet to be investigated.

The fact that the in vitro results concerning AM formation in pHLM, PM HLM, and quinidine treated pHLM do not reflect the in vivo results found in the corresponding rat models, may be explained by the much lower extent of hydroxylation under in vitro conditions. It should be kept in mind that under in vivo conditions the formed hydroxy metabolites are conjugated immediately after functionalization and thus are removed from the reaction equilibrium. Conjugation does not take place under the applied in vitro conditions. Indeed, preliminary studies on the hydroxylation in the applied in vitro systems showed very low concentrations of hydroxylated FP compared to those of AM. Further studies including development of highly sensitive analytical methods for determination of hydroxylated FP are necessary to finally explain those findings.

In summary, the studies showed that FP *N*-dealkylation is catalyzed by four different CYP isoforms: CYP1A2, CYP2B6, CYP2D6 and CYP3A4. This metabolic reaction seems to be slightly enantioselective. Regardless which isoform plays the major role in FP *N*-dealkylation, it should be kept in mind that inter-individual differences may be caused by each of these isoforms. CYP1A2 is known to be inducible e.g. by cigarette smoking [27], CYP2D6 is expressed polymorphically [28], CYP3A expression varies as much as 40-fold in liver [29] and CYP2B6 has recently been described as being polymorphically expressed [30–33]. The animal studies presented here indicated that hydroxylation reactions are probably responsible for the differences in PM and EM subjects concerning AM levels. CYP2D6 poor metabolizers, who account for about 7% of the Caucasian population [34,35], might exhibit higher AM blood plasma concentrations after intake of FP. This might be an issue in clinical and forensic toxicology or doping control. Furthermore, simultaneous intake of potent CYP2D6 inhibitory drugs, such as fluoxetine or paroxetine [36], might also lead to elevated AM blood plasma concentrations by preventing hydroxylation. Whether this genetic polymorphism and/or drug interactions actually are of relevance for FP pharmacokinetics, cannot be assessed at the moment due to lack of sufficient authentic human data.

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