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Investigation of stereoselective metabolism of amphetamine in rat liver microsomes by microdialysis and liquid chromatography with precolumn chiral derivatization

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Abstract

The utility of microdialysis as a quantitative sampling technique for in vitro drug metabolism studies was demonstrated by investigating the stereoselective metabolism of D-, L- and DL-amphetamine by the cytochrome P-450 enzymes. Microdialysates containing the isomers of amphetamine and its metabolite were derivatized with the fluorescent chiral derivatizing agent, (−)-fluorenylethyl chloroformate. The diastereoisomers were isocratically separated by liquid chromatography (LC) on a reversed-phase C_{18}, 3-μm (100×3.2 mm) column. The intra- and inter-assay relative standard deviation (R.S.D.) was below 10%. Michaelis–Menten parameters, K_m and V_{max} were obtained for the formation of both D- and L-hydroxyamphetamine from D-, L- and DL-amphetamine in the concentration range of 10–350 μ*M*.

Keywords: Microdialysis; Enantiomer separation; Amphetamine; Hydroxyamphetamine

1. Introduction

In recent years microdialysis has proven to be a very valuable tool for continuous, dynamic sampling of biological fluids or tissues to monitor real-time kinetic processes such as neurotransmitter release in brain. Although microdialysis has been used extensively as a research tool in the neurosciences, recently it has been applied for pharmacokinetic studies and in vivo monitoring of drug distribution or metabolism processes [1–6]. The inherent ability of microdialysis to extract the unbound analyte from a protein matrix can be immensely useful in sample clean-up for liquid chromatography (LC) analysis. Traditional sample clean-up methods involve protein removal by precipitation with an acid or an organic solvent followed by centrifugation. Most analytes require extraction from the supernatant into an organic phase after protein removal. The potential of microdialysis sampling has not been adequately exploited yet for sample clean-up for in vitro applications, such as pre-clinical drug metabolism studies. Only a few examples of in vitro applications have been reported [7,8]. The application described here demonstrates the utility of microdialysis for in vitro drug metabolism studies.

In the microdialysis sampling approach, the analyte is continually separated from the protein matrix by placing a semi-permeable membrane in the biological system and perfusing with a solution isotonic to the matrix. During perfusion the low-molecular-mass drugs or metabolites diffuse through the membrane into the perfusion phase and are carried away with the solution while proteins remain outside the membrane. The isolation of analyte from

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protein during dialysis quenches further metabolism by enzymes present in the matrix. This dual action of microdialysis is utilized in our study to investigate in vitro metabolism of amphetamine by liver microsomes. Although microdialysis obeys Fick's law of diffusion, it is a non-equilibrium convective process. Only a fraction of the drug or the analyte will diffuse into the perfusion medium. Therefore, it is essential to know the percentage recovery of the analyte in order to obtain quantitative data. The relative recovery of the analyte is defined as the ratio of the analyte concentration in the collected dialysate to the analyte concentration in the sample.

The US Food and Drug Administration (FDA) policy statement on the development of stereoisomeric drugs requires characterization of pharmacological activity of the individual enantiomers for the principal pharmacological effect [9]. Therefore, it is essential that individual enantiomers be examined separately in drug metabolism studies. In this study we have used microdialysis to extract amphetamine (Amp) and its metabolite, 4-hydroxyamphetamine (OH-Amp), from the microsomal matrix. Amphetamine, a chiral drug, is stereoselectively hydroxylated by the cytochrome P450 enzymes in rat liver microsomes. This *para*-hydroxylation is the major metabolic pathway in rat [10]. This process is pharmacologically significant because the metabolite, OH-Amp, contributes to the overall efficacy of Amp by affecting the central dopaminergic and serotonergic systems [11,12].

Chromatographic methods for primary amines, like amphetamine, require pre-column derivatization because of their poor optical absorbance properties. Chromatographic separation of enantiomers of these compounds can primarily be achieved by two methods. In the direct method the compound is achirally derivatized prior to separation on a chiral column. Although this approach offers good quantitative results, chiral columns frequently are less versatile and give lower plate counts. In the other approach, an achiral column separates the diastereomers formed by the pre-column derivatization of the analyte with a chiral derivatizing agent. We have utilized the latter approach in our experiments to separate Amp and OH-Amp enantiomers.

Several indirect methods have been developed using various chiral derivatizing agents [13–15]. The disadvantage of using chiral reagents is the possibility of racemization during the derivatization. It is therefore very important to select a chiral agent that derivatizes the compound quantitatively under mild conditions and can be obtained in high optical purity. The chiral agent used here, (−)-1(9-fluorenyl)ethyl chloroformate (−) (FLEC) satisfies these requirements [16]. Hutchaleelaha et al. have used (−) FLEC for the isocratic separation of amphetamine and methamphetamine enantiomers [17]. This assay is highly sensitive due to the formation of fluorescent products upon derivatization with (−) FLEC.

2. Experimental

2.1. Materials

DL-Amphetamine sulfate, L-amphetamine, D-amphetamine sulfate, and NADPH were all purchased from Sigma (St. Louis, MO, USA). HPLC-grade tetrahydrofuran (THF) and (−) FLEC were obtained from Aldrich (Milwaukee, WI, USA). Hydroxyamphetamine was generously supplied by the Research Triangle Institute (Research Triangle Park, NC, USA) through the National Institute on Drug Abuse (NIDA). HPLC-grade ethyl acetate and pentane were purchased from Baxter Scientific (McGaw Park, IL, USA). All other reagents were of analytical grade or better and used as received. All the solvents were of HPLC grade. Sprague–Dawley rat liver microsomes were obtained from In Vitro Technologies (Baltimore, MD, USA) and kept at −80°C until use.

2.2. Instrumentation

HPLC was performed using a temperature-controlled BAS 200B integrated chromatographic system (Bioanalytical Systems (BAS), West Lafayette, IN, USA) with an internal electrochemical detector and an external BAS FL-45 fluorescence detector. A reversed-phase ODS column, 100×3.2 mm, 3 μm (BAS) was used for chromatographic separations. Samples were injected using a CMA 240 autoinjector with a 20-μl sample loop, combined with a CMA 210 microsampler. The chromatographic data acquisition and analysis were accomplished by BAS

ChromGraph software. Microdialysis was performed using 1-cm PAN DL probes, a CMA 100 syringe pump and a CMA 140 fraction collector, all from BAS.

2.3. Derivatization procedure

Dialysate samples were diluted 10-fold with 0.05 *M* phosphate buffer, pH 7.4, prior to derivatization. To a standard solution or a dialysate sample (50 μl) in a 1-ml glass vial, were added the following in order; 12.5 μl of 0.33 *M* phosphate buffer, pH 7.8, 62.5 μl acetonitrile (ACN), and 5 μl of 1 m*M* (−) FLEC in acetone. The mixture was incubated overnight at room temperature. After completion of the reaction, excess (−) FLEC was blocked by adding 7 μl of 0.1 *M* glycine solution and incubating for 30 min at room temperature. The derivatives were extracted by adding 200 μl pentane for Amp or 250 μl ethyl acetate for OH-Amp and vortexing for 2 min. After centrifugation for 10 min at 1500 *g*, 200 μl of the organic phase was removed and evaporated to dryness under nitrogen. The residue is reconstituted in 100 μl of 50% acetonitrile in water prior to assay.

2.4. Chromatographic procedure

The reconstituted samples were injected into the LC system using an autosampler with a 20-μl loop. The injection volume was 10 μl. Chromatographic separation of derivatized amphetamine was achieved isocratically on a C_{18} column at 40°C with a mobile phase consisting of 20 m*M* acetate buffer, pH 3.6, acetonitrile and THF. Mobile phase conditions were optimized to achieve short retention times with good resolution between enantiomers. The optimized composition of mobile phase for separation of amphetamine derivatives was 20 m*M* acetate buffer, pH 3.6–acetonitrile–THF (60:27.5:12.5, v/v), and for hydroxyamphetamine derivatives was 20 m*M* acetate buffer, pH 3.6–acetonitrile–THF (61.5:28:10.5, v/v). Flow-rate was 1 ml/min for both. Fluorometric detection was used for Amp with an excitation wavelength of 265 nm and an emission wavelength of 330 nm. Electrochemical detection was used for OH-Amp at 850 mV on a glassy carbon electrode with Ag/AgCl reference electrode. Stock solutions of D-Amp and DL-Amp (1 mg/ml) were prepared in water and L-Amp was prepared in methanol. Standards for in vitro recovery studies and calibrations were prepared daily from these stock solutions by diluting to desired concentrations in 0.05 *M* phosphate buffer (pH 7.4). All stock solutions were stored in a freezer.

2.5. Microdialysis

Microdialysis probes that can be used for in vitro metabolism studies are shown in Fig. 1. Loop probes with a 1-cm long polyacrylonitrile (PAN) membrane (30 000 molecular mass cutoff, 320 μm O.D.) were used in this application. Co-axial probes can be used for small sample volumes (<250 μl). The in vitro recoveries of the probe for Amp and OH-Amp were determined prior to actual metabolism studies. Each probe was soaked in distilled deionized water for at least 15 min prior to use. The probes were then flushed with distilled deionized water for 1 h followed by buffer for another 30 min. The probes were

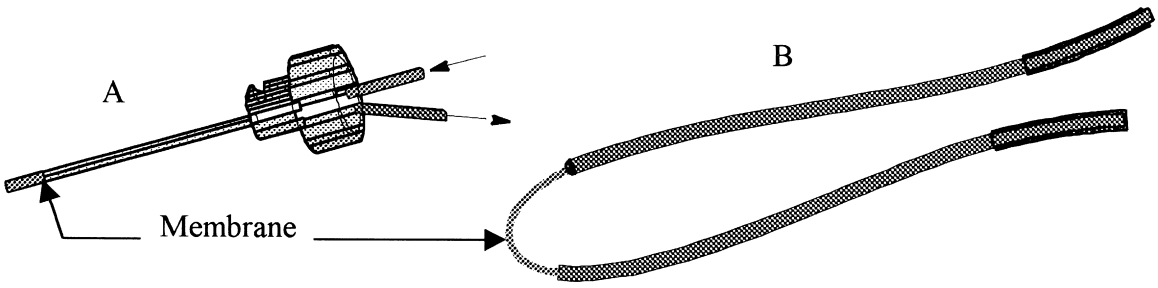


Fig. 1. Examples of microdialysis probes that can be used for microsomal incubations. (A) Co-axial probe, 2 or 4 mm membrane length for small volumes (~250 μl). (B) Loop probe, 1 cm membrane length for larger volumes (~1 ml).

immersed in standard solutions of Amp and OH-Amp and perfused with 0.05 *M* potassium phosphate buffer at pH 7.4 for 2 h at a flow-rate of 2 μl/min. Two 30-min samples were collected and derivatized. The relative recovery for each analyte was obtained from the equation: $\%R=100(C_{\text{dialysate}}/C_{\text{sample}})$ where $C_{\text{dialysate}}$ is the concentration of analyte in the dialysate and C_{sample} is the concentration of the analyte in the vial. The probes were thoroughly washed with water to remove any adsorbed analyte before they were used in metabolism studies.

2.6. Metabolism studies

Commercially obtained rat liver microsomes containing about 460 pmol of cytochrome P450s per mg of protein were used for incubations. The complete experimental setup for microsomal incubations is described elsewhere [18]. Briefly, the desired drug (concentration range 10–500 μ*M*) was incubated in a 1.5-ml polypropylene vial with 1 mg of microsomes in 0.05 *M* potassium phosphate buffer at pH 7.4 containing 10 m*M* magnesium chloride at 37°C with magnetic stirring. The microdialysis probe was placed in this solution and perfused with the same buffer as in the incubation mixture. After collecting two 5-min blank dialysates, the reaction was initiated by adding a 100-μl of 10 mg/ml NADPH. The final volume of the sample was 1 ml. Dialysate samples were collected every 10 min and derivatized as described previously.

The same incubations were carried out at different substrate concentrations ranging from 10 to 350 μ*M* to obtain Michaelis–Menten enzyme kinetics parameters (K_m and V_{max}) for the *para*-hydroxylation of Amp.

3. Results and discussion

3.1. Chromatography

Different extraction organic phases and mobile phase conditions were required for Amp and OH-Amp for good chromatographic separation. Specifically, pentane was used for the extraction of Amp derivatives, whereas ethyl acetate was used for more polar OH-Amp derivatives. Slightly more polar mobile phase conditions were needed for the separation of OH-Amp enantiomers. Resolution of enantiomers was greatly affected by the composition of the mobile phase, especially by the THF content. Since different organic phases were used for the extraction of Amp and OH-Amp derivatives, in vitro recoveries of the dialysis probes were evaluated for each compound separately.

Since the fraction of substrate that gets hydroxylated by cytochrome P450 is low, monitoring the formation of OH-Amp was necessary. Only 0.002% of the racemic amphetamine was converted to OH-Amp in rat liver microsomes [19]. Dingell and Bass have failed to detect any metabolism of Amp in liver microsomes when disappearance of Amp was monitored [20]. Therefore, conditions were selected to optimize the detection of OH-Amp peaks. By using electrochemical detection, only OH-Amp in dialysates was detected, minimizing the possible appearance of reaction side-products and possible Amp peaks. The detection limit by electrochemical detection was found to be 0.2 pmol of OH-Amp. The assay was linear in the studied range, 5–200 ng/ml of OH-Amp. Table 1 lists the inter- and intra-assay variation at two concentrations of OH-Amp.

The elution order of Amp diastereomers was

Table 1
Intra- and inter-assay variation of the hydroxyamphetamine analysis

Isomer	Concentration (ng/ml)	Intra-assay R.S.D. (%) ($n=5$)	Inter-assay R.S.D. (%) ($n=5$)
L-Hydroxyamphetamine	25	7.09	7.43
D-Hydroxyamphetamine		9.75	4.68
L-Hydroxyamphetamine	150	9.21	3.82
D-Hydroxyamphetamine		5.72	3.14

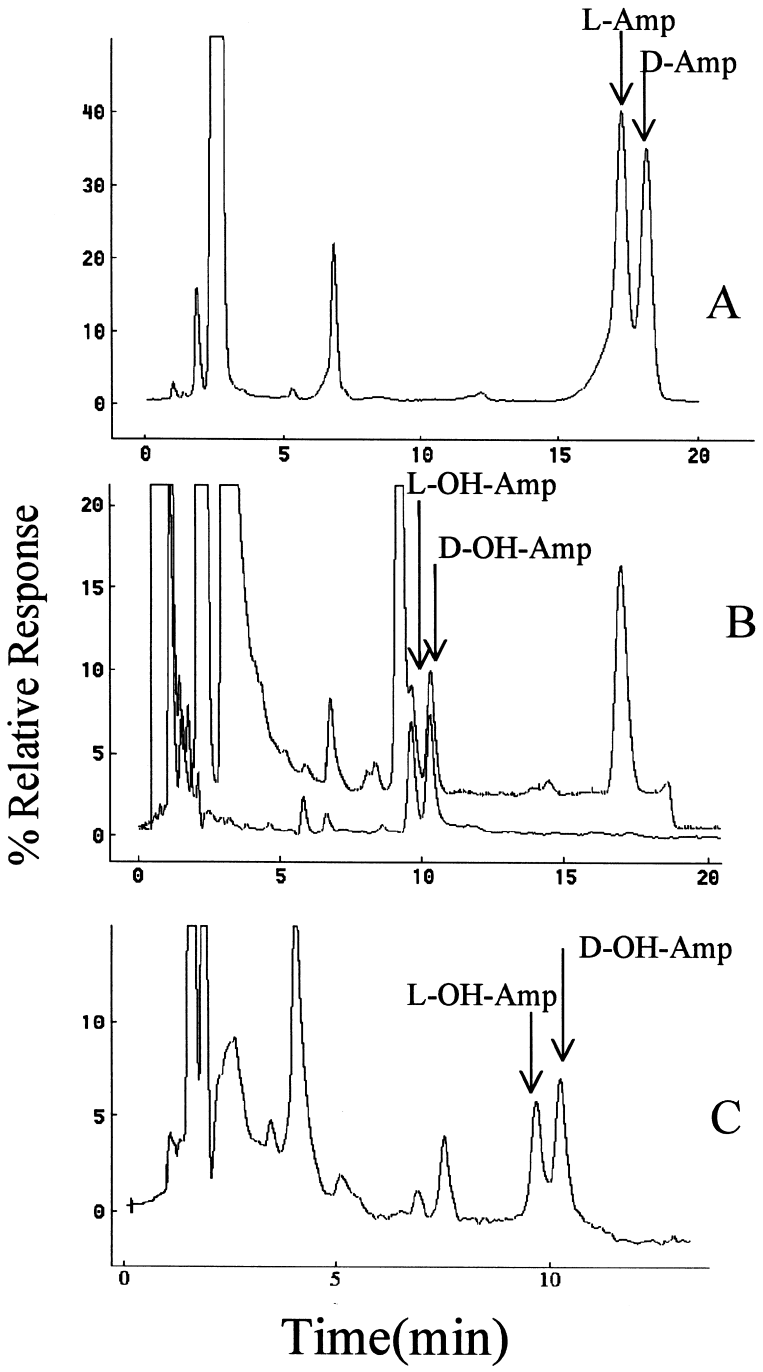
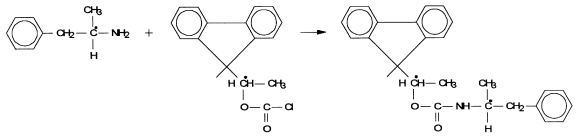


Fig. 2. Representative chromatograms illustrating the separation of the derivatives under the chromatographic conditions described in the text. (A) Amphetamine standard (5 ng/ml), fluorescence detection (10 units full scale). (B) Hydroxyamphetamine standard (5 ng/ml); the upper trace in (B) is fluorescence detection (10 units full scale) and the lower trace is electrochemical detection (2 nA full scale). (C) Dialysate from an incubation mixture containing 100 μ*M* DL-Amp, electrochemical detection 2 nA range.

determined by separately derivatizing pure D- and L-enantiomers of Amp. The same elution order was presumed to be the order for OH-Amp enantiomers. With an optimized mobile phase composition of 20 m*M* acetate buffer, pH 3.6–ACN–THF (60:27.5:12.5, v/v), L- and D-Amp eluted at 17.1 and 18.0 min, respectively. The optimized mobile phase composition for L- and D-OH-Amp was acetate–ACN–THF (62.5:27.5:10, v/v), and the retention times were 9.5 and 10.2 min, respectively. Fig. 2A,C illustrates representative chromatograms of standards of derivatized Amp and OH-Amp and a chromatogram of a dialysate sample from a microsomal incubation. Fig. 2B compares the chromatograms obtained for OH-Amp by two detection methods. Electrochemical detection was less sensitive to impurities because of its selectivity. Complete resolution of D- and L-isomers was obtained by electrochemical detection, whereas in fluorescence detection an impurity peak overlaps with the L-OH-Amp peak.

3.2. Derivatization procedure

The reaction of the (−) FLEC reagent with amphetamine occurs at room temperature under basic conditions as shown.



Addition of glycine and subsequent extraction of the Amp derivatives into pentane removes the excess (−) FLEC reagent as the glycine derivative in aqueous phase. A slightly more polar organic phase is required to extract the more hydrophilic OH-Amp derivatives. In order to optimize the derivatization time, 50-μl aliquots of 10 ng/ml DL-Amp were derivatized as described before for different time intervals (1, 3, 5, 6, 7, 16, 24 h). The samples were then extracted and assayed. Peak areas for both L- and D-isomers were compared as in Fig. 3. The

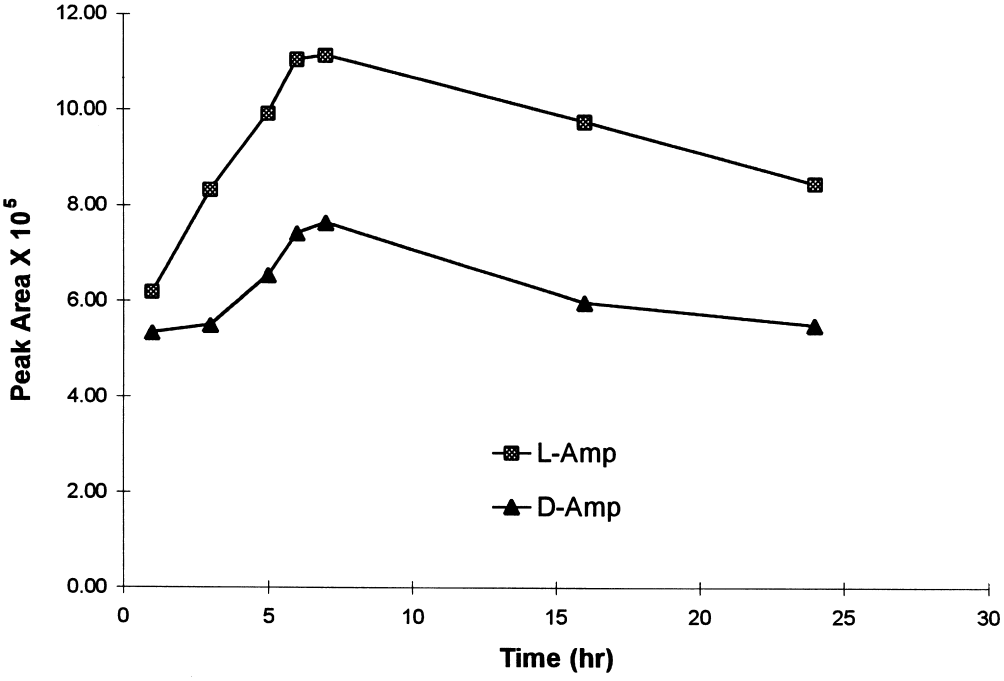


Fig. 3. Optimization of the derivatization time with FLEC. Samples of 10 ng/ml DL-Amp (50 μl) were derivatized at room temperature with 1 m*M* (−) FLEC as described in the text for different time intervals. Peak areas for both D- and L-isomers were compared as a function of time.

results indicate a slight difference in reaction rates for the L- and D-isomers. The optimum derivatization time is about 7 h. The peak area decreases slightly after this time possibly due to the instability of the derivatives at room temperature. For convenience, the samples were derivatized overnight (~16 h) in this study. At this reaction time, the decrease in peak area from the optimum time was 20% for D-isomer and 27% for L-isomer.

3.3. Metabolism of amphetamine

Traditional sampling techniques for studying microsomal incubations or pharmacokinetics use discontinuous sampling at specified time intervals. Microsomal studies are normally carried out by incubating the parent drug at 37°C with the liver microsomes containing cytochrome P450 enzymes. The reaction is usually started by adding the enzyme cofactor, NADPH or an NADPH-generating system. After a fixed time interval the reaction is terminated by adding a quenching agent or an inhibitor to stop the enzymatic process. The samples drawn from the microsomal mixture need to be cleaned up to remove protein prior to the analytical system. Since samples collected by microdialysis are protein-free, further sample clean-up is not required before analysis.

The relative in vitro recoveries (mean±S.D. for $n=5$) of the dialysis probe for D- and L-Amp (1 μg/ml) are 55.6±2.3 and 52.1±1.7% ($n=5$), respectively. The recoveries for OH-Amp (1 μg/ml) are significantly higher; 77.5±2.2% for L-isomer and 81.6±2.4% for D-isomer. Metabolic profiles obtained for D- and L-OH-Amp, shown in Fig. 4 indicate that the hydroxylation is linear up to about 60 min. Initially both metabolites are formed in approximately the same amounts and, as the reaction proceeds further, the amount of L-OH-Amp becomes significantly higher. It has been observed that in rats, the

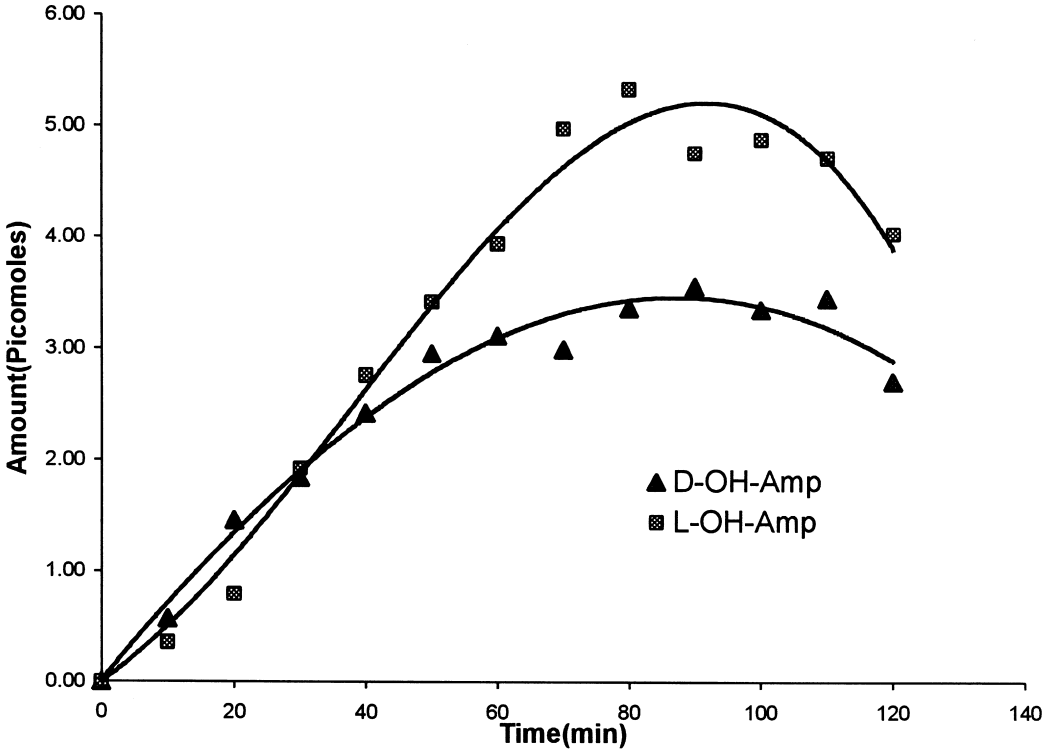


Fig. 4. Metabolic profiles of Amp metabolites; DL-Amp (200 μ*M*) was incubated with rat liver microsomes at 37°C. Total volume of the sample was 1 ml.

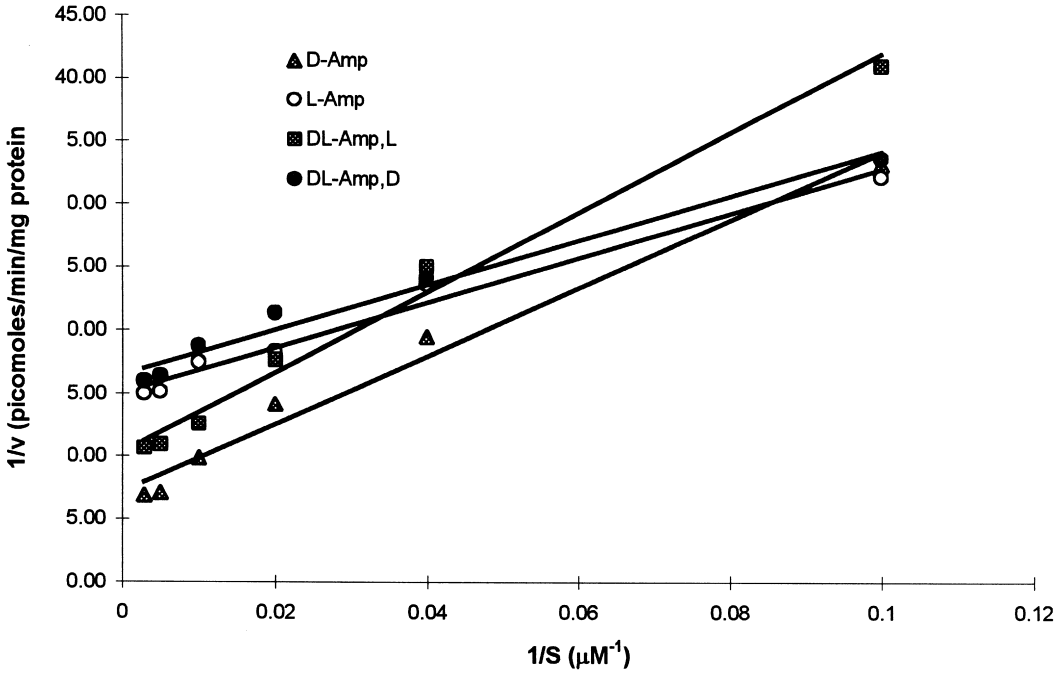


Fig. 5. Lineweaver–Burk enzyme kinetics plots for *para*-hydroxylation of DL-Amp, D-Amp and L-Amp for the concentration range 10–350 μ*M*.

L-isomer of OH-Amp is excreted in urine to a greater extent than the D-isomer [21]. Gunne and Galland [22] have suggested that D-OH-Amp becomes further hydroxylated to *p*-hydroxynorephedrine by dopamine-β-hydroxylase, an enzyme present in sympathetic nerves. Since the presence of this enzyme in microsomes is not known, it is unlikely that the further hydroxylation accounts for the lower amount of D-OH-Amp produced during the microsomal reaction. Therefore, the difference in the extent of excretion of D-and L-isomers of OH-Amp can be partially due to the difference in rates of para hydroxylation by cytochrome P450 enzymes.

Hydroxylation of Amp followed Michaelis–Menten kinetics in the concentration range (10–350 μ*M*) studied here. Substrate inhibition occurs at higher concentrations of Amp [23]. Substrate concentration–velocity data were obtained from the first 15 min of the metabolic profiles of OH-Amp at different Amp concentrations. Enzyme kinetics parameters obtained by fitting these substrate concentration and velocity data to Lineweaver–Burk plots in Fig. 5 show that hydroxylation of L-isomer from both L-Amp and the DL-Amp has a lower rate compared to the D-isomer (Table 2). These data indicate the presence of a specific stereoselective pathway for the metabolism of Amp in rats. Although the values reported for K_m and V_{max} in the literature are not in agreement, they have consistently shown that the K_m for L-isomer is lower than for the D-isomer [23–25].

Table 2
Michaelis–Menten kinetics parameters for amphetamine metabolites

Metabolite	K_m (μ*M*)	V_{max} (pmol min^{-1} per mg protein)
D-OH-Amp	36.6±1.8	138.8±4.2
L-OH-Amp	12.2±0.6	66.9±1.0
D-OH-Amp, from DL-Amp	31.7±1.5	105.5±6.0
L-OH-Amp, from DL-Amp	10.7±0.5	59.4±4.9

All values are mean±S.E.M. for $n=4$.

4. Conclusions

The utility of microdialysis sampling for quantitative evaluation of drug metabolism kinetics has been demonstrated here by investigating the stereoselective metabolism of amphetamine. In addition to obtaining protein-free samples, microdialysis provides a way to reduce the number of test samples or subjects used in a single experiment. When studying the time course of a metabolism process, conventional sampling techniques require a fresh incubation sample for each time point. With microdialysis the whole time profile can be obtained from a single incubation sample due to the continuous extraction of analytes without any fluid removal or sample quenching. This is a very valuable feature when metabolism studies need to be done with costly human liver microsomes or expressed enzymes.

This study has shown that the L-Amp is metabolized to a larger extent than the D-Amp. This observation is in accordance with the claim that L-Amp has less pharmacological activity than D-Amp [26,27]. The Michaelis–Menten parameters are useful in determining the intrinsic in vitro clearance, CL_{int} which is defined as the ratio of V_{max} to K_m at substrate concentrations lower than K_m.

In an earlier study we have shown that for analytes that do not require complicated derivatization schemes for detection as in this application, microdialysis sampling can be automated with an on-line valve [18]. Injecting sample on-line eliminates the need to store samples and minimize sample degradation. The only requirement for this type of on-line assay is shorter chromatographic run times than the microdialysis sampling time. The temporal resolution of the assay, which depends on the sampling time, can be improved by coupling LC with tandem mass spectrometry (LC–MS–MS). Since complete baseline LC separation of the analytes in the sample is not necessary for MS–MS analysis, the chromatographic separation time could be dramatically shortened. This type of an automated system is potentially very useful for high throughput screening of combinatorial drug candidates.

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