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Sensitive enantiomer-specific high-performance liquid chromatographic analysis of methamphetamine and amphetamine from serum using precolumn fluorescent derivatization

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Abstract

In order to study the stereoselective disposition of methamphetamine (MAP), a widely abused drug, we have developed a sensitive HPLC assay to separate and quantitate the enantiomers of MAP and amphetamine (AP) in rat serum. Serum samples to which was added aniline sulfate (internal standard) were alkalized with 0.02 *M* carbonate buffer (pH 10.6) and extracted with ethyl acetate. Following back extraction with hydrochloric acid, neutralization, and reconstitution, the sample was derivatized with (−)-fluorenylethyl chloroformate overnight at room temperature. The derivatized products were separated following injection onto a reversed-phase C_{18} column. The mobile phase consisted of 0.02 *M* acetate buffer–acetonitrile–tetrahydrofuran (46:39:15, v/v). The fluorescent intensity of the effluent was monitored at excitation and emission wavelengths of 265 and 330 nm, respectively. The derivatized aniline, *R*-, *S*-AP, *R*- and *S*-MAP had retention times of 21.0, 22.6, 23.6, 27.7 and 29.0 min, respectively. Linear standard curves were obtained over the concentration range of 5–250 ng/ml. The inter-day and intra-day coefficients of variation for the assay of all four compounds at 12.5, 50.0 and 250 ng/ml were in the range of 2.1–18.6%. The method was applied to quantitate the concentrations of MAP and AP enantiomers in rat serum following a short term intravenous infusion of racemic MAP (15 mg/kg). There were no differences in serum concentrations of MAP enantiomers but the concentrations of *S*-AP were consistently greater than those of *R*-AP. These data suggest a stereoselective disposition for the formation and/or elimination of amphetamine.

1. Introduction

Virtually from the time of their development in the mid-1930s, the use of amphetamine (AP) and methamphetamine (MAP) have been plagued by problems of misuse and abuse within the populations of several countries. The effects of these drugs which are considered desirable by the user include: a sense of increasing energy, self-confidence and well-being; heightened awareness; loss of appetite; and euphoria [1]. Considerable research in recent years into the stereoisomeric differences between enantiomers of xenobiotics has indicated the differential pharmacodynamic properties and pharmacokinetic behavior of certain enantiomer pairs. The phar-

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macodynamic differences between the *R*- and *S*-isomer of AP and MAP have been known for some time with the *S*-isomer being approximately five times more active than the *R*-isomer in their effects on the central nervous system (CNS) [1]. However, there are limited and incomplete information concerning the disposition kinetics of MAP enantiomers. A major limitation to this understanding has been the use of nonspecific and insensitive assays and, for the most part, assays that are not selective for the enantiomers. The advantages to the examination of the disposition kinetics of the enantiomers following administration of a racemic mixture have been thoroughly reviewed [2–6]. An examination of the disposition kinetics of a compound which is measured as the racemate may be quite misleading in itself [2] and further may lead to a misinterpretation of the concentration–response relationship.

An investigation to completely characterize the pharmacokinetic profile of a compound usually requires the measurement of parent compound and its metabolite(s) in blood and urine. One of the difficulties in obtaining such measurements when performing pharmacokinetic studies in rodent has been the limitation of blood sample volume (~100 μl serum). Therefore, a sensitive analytical method that simultaneously quantitates the parent compound and its metabolite(s) in a single plasma sample is often required.

The use of chromatography to separate enantiomers can be accomplished with either direct or indirect approaches [7]. These methods have been applied in both GC and HPLC to separate the enantiomers of AP and MAP. The use of GC in the enantiomeric separation of MAP has been extensively reviewed [8]. Since we are interested in conducting disposition studies of MAP which may require the collection of eluent after chromatographic separation, GC was not our method of choice. As for HPLC, direct methods are usually performed by derivatization with achiral reagents prior to chromatography on a chiral column [9–12]. These assay methods were reported to provide excellent quantitative results when applied to both pharmaceutical [9,10] and biological samples [11,12]. Limitations of these methods include incomplete resolution between enantiomers [11], insufficient assay sensitivity [12] and the high cost associated with using a chiral column.

Many indirect methods have been developed using various chiral derivatizing agents to facilitate the separation of the enantiomers of amphetamines. These derivatizating agents included 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [13,14], N-trifluoroacetyl-*l*-prolyl chloride (TPC) [15], (*S*)-(+)-benoxaprofen chloride [16], 1-[(4-nitrophenyl)sulfonyl]propyl chloride (NPSP-Cl) [17,18] and polymeric FMOC-L-proline [19]. These methods were not practical for our purposes due to the incomplete resolution of AP enantiomers [13–15], insufficient assay sensitivity [14,16] and the incapability to simultaneously quantitate AP and MAP enantiomers [17–19].

The purpose of this study was to develop a sensitive HPLC assay method that simultaneously quantitates the enantiomers of MAP and its metabolite, AP, in serum using precolumn derivatization. Application of this method to authentic serum samples collected after the administration of racemic MAP is described.

2. Experimental

2.1. Materials

Aniline sulfate, (−)-1-(9-fluorenyl)ethyl chloroformate [(−)-FLEC] and glycine were purchased from Aldrich (Milwaukee, WI, USA); racemic amphetamine sulfate from Sigma (St. Louis, MO, USA). *R*- and *S*-Amphetamine and racemic, *R*- and *S*-methamphetamine hydrochloride were generously supplied by the Research Triangle Institute (Research Triangle Park, NC, USA) through the National Institute on Drug Abuse. Baker Analyzed-grade *n*-pentane and ethyl acetate were obtained from J.T. Baker (Phillipsburg, NJ, USA). Tetrahydrofuran, acetone and acetonitrile were Omnisolve grade from E. Merk (Gibbstown, NJ, USA). Water was

freshly double distilled. All other chemicals and reagents used were of analytical grade.

2.2. Instrumentation

The chromatographic system consisted of a Beckman Model 504 autosampler (Berkely, CA, USA) with a 100-μl loop, a Water Associates (Milford, MA, USA) Model 501 pump, Model 470 fluorescence detector operating at an excitation wavelength of 265 nm and emission wavelength of 330 nm, a NEC PowerMate SX plus computer with an NEC Pinwriter P6200 printer and Maxima 820 Chromatographic Workstation software. The centrifugal evaporator was Savant Model AS 160 (Farmingdale, NY, USA).

FAB mass spectrometry was performed using a modified MAT 311A double focusing mass spectrometer (Finnigan MAT, San Jose, CA, USA). The analysis was done using a 15 kV Cs-ion gun for primary beam generation. The instrument was scanned from m/z 100 to m/z 2000 in 20 s, at a mass resolution of 1000 (10% valley). The samples were dissolved in glycerol and applied to the direct insertion probe without further preparation.

2.3. Standard solutions

Standard solutions of (*R*,*S*)-AP, (*R*,*S*)-MAP and aniline sulfate were prepared by dissolving in water to obtain a final concentration of 1 mg/ml (as the free base). Working solutions (5 μg/ml) were obtained by dilution from the standard solution. These solutions were stable for at least one year and were stored refrigerated at 8°C. All concentrations reported here are expressed as the free base.

2.4. Extraction procedure

Blank serum or serum spiked with racemic AP and racemic MAP or sample (0.1 ml) was placed into a 5-ml polypropylene tube to which was added 50 μl aniline sulfate solution (100 ng/ml water, internal standard). The mixture was alkalized with 200 μl 0.02 *M* carbonate buffer (pH 10.6) and extracted with 2 ml ethyl acetate. After the tube was capped, the extraction proceeded by shaking for 15 min followed by centrifugation at 1200 *g* for 5 min. The organic layer was transferred into a polypropylene tube and back-extracted with 200 μl 0.05 *M* hydrochloric acid. The tube was capped and shaken for 15 min followed by centrifugation at 1200 *g* for 5 min. The organic phase was aspirated at room temperature.

2.5. Derivatization

The aqueous layer was transferred into a 5-ml borosilicate glass tube and neutralized with 40 μl 0.25 *M* sodium hydroxide. The mixture was buffered with 50 μl 0.33 *M* phosphate buffer (pH 7.8). Acetonitrile (250 μl) and 25 μl (−)-FLEC (1 m*M* in acetone) were added and the tube was capped with a polyethylene closure. The reaction was allowed to proceed overnight at room temperature. After the reaction was complete, excess derivatizing agent was reacted with 30 μl glycine solution (100 m*M* in water). The derivatized products were extracted with 750 μl *n*-pentane by vortex-mixing for 2 min after the tube was capped. After centrifugation (5 min, 1200 *g*) the aqueous phase was removed and discarded and the organic layer was evaporated to dryness in a centrifugal evaporator. The residue was reconstituted with 50% acetonitrile (in water) before being injected onto the HPLC column.

2.6. Chromatographic procedures

Reconstituted sample (150 μl) was placed into an amber crimp top vial (0.7 ml) and capped. The autosampler was set to inject 100 μl onto the column. Reversed-phase HPLC separation was performed using an Adsorbosphere HS C_{18} column (3 μm, 150 × 4.6 mm I.D., Alltech Associates, Deerfield, IL, USA) at room temperature. The analytical column was preceded with a Direct-Connect column prefilter (Alltech Associates, Deerfield, IL, USA). The mobile phase was 0.02 *M* acetate buffer (pH 3.6)–ace-

tonitrile–tetrahydrofuran (46:39:15, v/v). The flow-rate of mobile phase was 1 ml/min.

2.7. Mass spectrometry

A 4-μl volume of racemic MAP or AP (equivalent to 4 μg of each enantiomer) was pipetted from a standard solution into a borosilicate glass test tube followed by 200 μl 0.05 *M* hydrochloric acid and 40 μl 0.25 *M* sodium hydroxide. To this mixture was added 50 μl 0.33 *M* phosphate buffer (pH 7.8), 250 μl acetonitrile and 15 μl 18 m*M* (−)-FLEC (in acetone). The reaction was allowed to proceed overnight. After the reaction was complete, the derivatized products were extracted into 2 ml *n*-pentane by vortex-mixing for 2 min. The mixture was centrifuged for 5 min at 1200 *g* and the organic layer was removed and evaporated to dryness. The residue was reconstituted in glycerol and subjected to mass spectrometry.

2.8. Standard calibration curves

Rat serum was spiked with aliquots of racemic AP and racemic MAP to provide final concentrations of 5, 12.5, 25, 50, 125 and 250 ng/ml per enantiomer. These serum samples were analyzed as described above. Standard calibration curves for each enantiomer were obtained by plotting peak-area ratio of the enantiomer derivative to that of internal standard derivative against concentration of enantiomer. The data were then analyzed by linear least-squares regression.

2.9. Validation of the method

The analytical method was evaluated to assess intra-day and inter-day variation. Serum spiked with racemic AP and racemic MAP (0.1 ml) at a concentration of each enantiomer of 12.5, 50 and 250 ng/ml were analyzed as described above. The concentration of each enantiomer was determined from standard curves prepared daily. Three samples of each concentration were assayed every day for three days.

2.10. Optimization of the derivatization conditions

pH of the reaction

Aliquots of a working solution which contained a mass of each enantiomer equivalent to the mass at concentrations of 12.5 and 250 ng/ml (in a 0.1-ml sample) were subjected to the derivatization reaction step using 0.33 *M* phosphate buffer at pH 7.0, 7.5, 7.8 and 8.2. A 25-μl volume of 1 m*M* (−)-FLEC and 250 μl acetonitrile were used and the reaction was allowed to proceed for 24 h followed by the extraction procedures described above. Peak areas of each enantiomer derivative at different pHs were compared.

(−)-FLEC concentration

Derivatization of the two concentrations was carried out using 0.33 *M* phosphate buffer pH 7.8 and 250 μl acetonitrile with different concentrations of (−)-FLEC (25 μl 0.4 or 1.0 or 2.0 m*M*). The reaction proceeded for 24 h and the subsequent steps were performed as described above. Peak areas of each enantiomer derivative at different concentrations of derivatizing agent were compared.

Reaction time

Two concentrations of enantiomers as described above were derivatized using 0.33 *M* phosphate buffer pH 7.8, 25 μl 1 m*M* (−)-FLEC and 250 μl of acetonitrile but the reaction was allowed to proceed for different times (1, 2, 6, 24 and 48 h). The mixtures were then treated as described above. Peak areas of each enantiomer derivative at different reaction times were compared.

2.11. Application to animal studies

A male Fisher rat weighing 216 g (Harlan Sprague–Dawley, Indianapolis, IN, USA) was given a short term intravenous infusion (over 90 s) of racemic methamphetamine hydrochloride (15 mg base/kg, 5 mg base/ml saline) via a

jugular vein catheter which was surgically implanted and exteriorized 2 days prior to the study. Blood samples were obtained prior to and at selected times following dosing. Approximately 0.3 ml blood was obtained at each time, after 0.1 ml fluid had been withdrawn (which was subsequently reinjected and followed by 0.3 ml saline) in order to avoid an artifact caused by sample contaminated by saline or blood trapped in the cannula. Serum was obtained from blood and stored at −20°C before being assayed. The quantitative analysis of these samples was performed using 0.1 ml of serum followed by the extraction and derivatization procedures described above. Standard calibration curves were constructed by the method described above. The concentrations of the unknown samples were obtained from the relationship between peak area ratio (for each enantiomer) vs. enantiomer concentration. A standard curve was prepared daily.

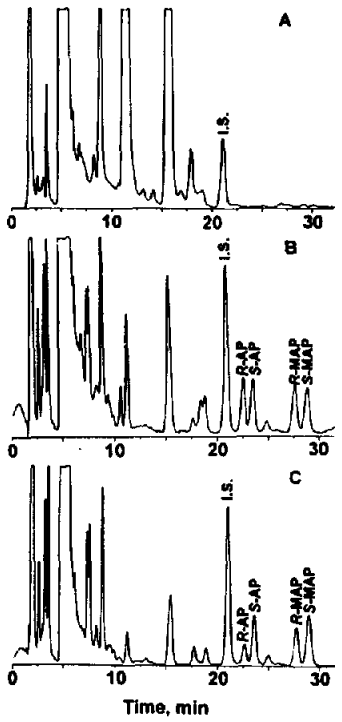

Fig. 1. Representative high-performance liquid chromatograms illustrating the separation of the derivatives of the enantiomers of methamphetamine (MAP) and amphetamine (AP) after treatment of serum samples and under the chromatographic conditions outlined in the text. (A) Blank serum spiked with aniline sulfate (I.S.); (B) blank serum spiked with each enantiomer at a concentration of 25 ng/ml; (C) authentic serum sample obtained from a rat 4 h following a short term i.v. infusion of racemic MAP (15 mg/kg). The concentrations of *R*-AP, *S*-AP, *R*-MAP asnd *S*-MAP are 11.6, 32.5, 30.1 and 32.8 ng/ml, respectively.

3. Results

3.1. Chromatography

Fig. 1 illustrates the chromatograms obtained for blank serum spiked with internal standard, serum spiked with 50 ng/ml of racemic MAP and racemic AP and serum from a rat taken 4 h after a short term i.v. infusion of 15 mg/kg racemic MAP. The retention times of the derivatives of aniline, *R*-AP, *S*-AP, *R*-MAP and *S*-MAP were 21.0, 22.6, 23.6, 27.7 and 29.0, respectively. The capacity factor (k') for the corresponding compounds were 9.72, 10.53, 11.01, 13.13 and 13.75, respectively. Both diastereomers of AP and MAP have a selectivity factor (α) of 1.05. The resolution (R_S) of diastereomers of AP and MAP was 0.87 and 1.03, respectively. Blank serum showed no interfering peaks for the compounds of interest. Peaks eluting early in the chromatogram correspond to reaction side-products. The elution order of each individual diastereomer was established by derivatizing each pure enantiomer of AP and MAP. In each case, the *R*-enantiomer eluted before the *S*-enantiomer.

3.2. Mass spectrometry

In an attempt to confirm the structure of the derivatization products formed from the reaction between MAP and AP enantiomers and (−)-FLEC, mass spectrometric analysis was performed using fast-atom bombardment (FAB) ionization. Fig. 2 illustrates the mass spectra of the MAP and AP derivatives. For the MAP derivative, the m/z at 386 represents the MH^+ for the protonated MAP-FLEC complex. The

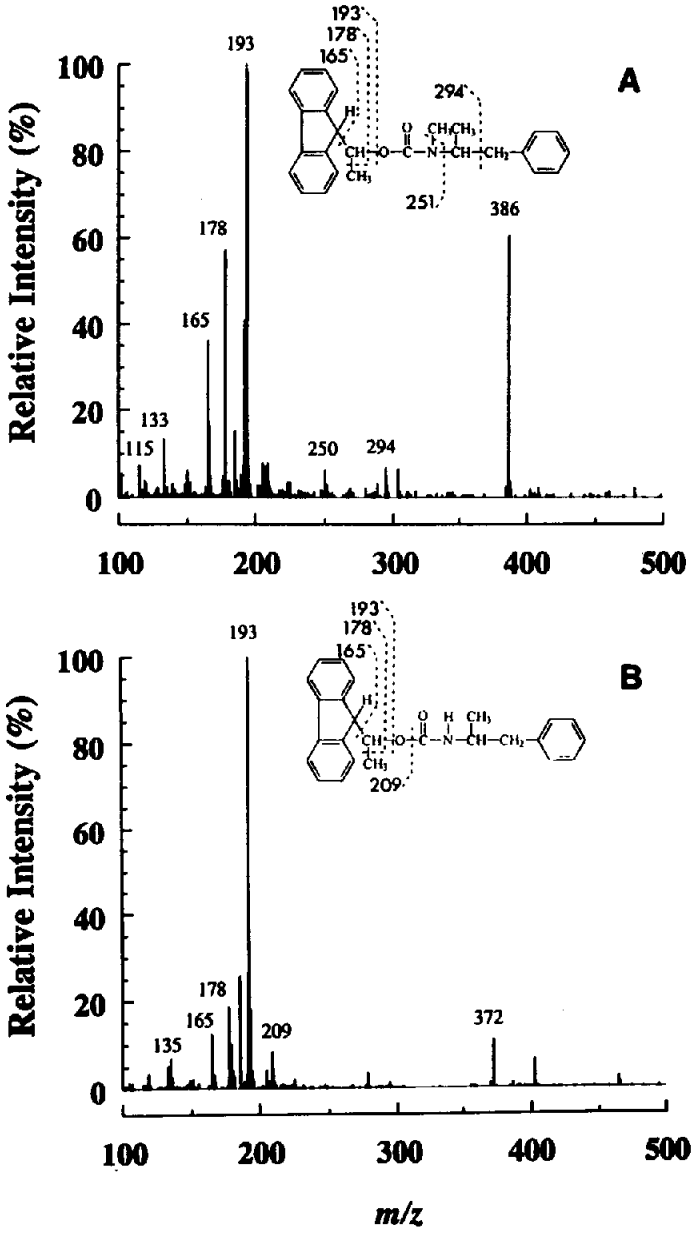

Fig. 2. Reconstructed FAB ionization mass spectra of (−)-FLEC derivatives of (A) MAP and (B) AP.

MH^+ of the AP-FLEC complex occurs at m/z 372. Both MAP and AP derivatives have ions at m/z 193 as their base peak. This ion resulted from cleavage of the alkyl oxygen bond. The results also confirmed that the reaction of (−)-FLEC and MAP and AP yielded derivatized products that have a molecular ratio of 1:1.

3.3. Optimization of derivatization conditions

Effect of pH on the maximum reaction yield was measured by determining peak areas of the derivatized compounds. Peak areas of the derivatized MAP enantiomers increased as pH increased from 7.0 to 7.5 and then remained

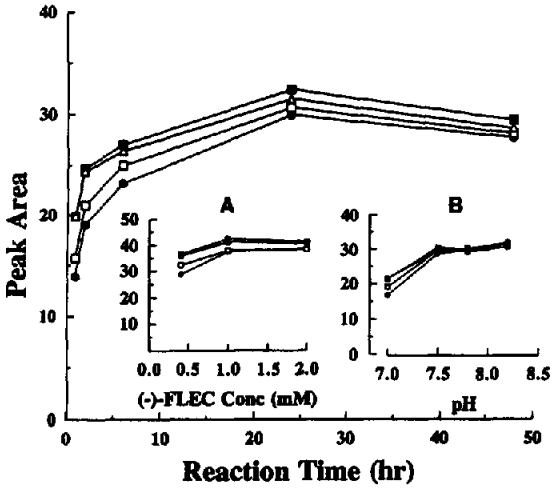

Fig. 3. Results of experiments to optimize the derivatization procedure. Peak area of derivatized MAP and AP enantiomers as a function of reaction time with (−)-FLEC (1 m*M*) at room temperature and at pH 7.8. Concentration of each enantiomer was 250 ng/ml. Each value represents the mean of 3 determinations. Inset graph A is a plot of peak area as a function of (−)-FLEC concentration (enantiomer concentration, 250 ng/ml; pH 7.8; room temperature; reaction time 24 h). Inset graph B is a plot of peak area as a function of aqueous pH (enantiomer concentration, 250 ng/ml; (−)-FLEC concentration, 1 m*M*; room temperature; reaction time 24 h). Key: (●) *R*-AP, (□) *S*-AP, (△) *R*-MAP, (■) *S*-MAP.

constant up to pH 8.2. Peak areas of derivatized AP enantiomers increased as pH increased from 7.0 to 7.8 (Fig. 3). However, the peak areas of derivatized aniline remained relatively constant over this pH range (data not shown).

The dependence of the maximum reaction yield upon (−)-FLEC concentration is shown in Fig. 3. Peak areas of the derivatives of MAP enantiomers are independent of (−)-FLEC concentration over the range examined. The peak areas of derivatized AP enantiomers increase as (−)-FLEC concentration increases from 0.4 to 1 m*M* with no change thereafter up to 2 m*M* (−)-FLEC. Once again, peak areas of derivatized aniline were relatively constant at these three concentrations (data not shown).

As reaction times increase, peak areas of all compounds increased and became relatively constant after *ca.* 6 h (Fig. 3). This reaction time is much longer than those reported in the literature. For practical purposes we allowed the reaction to proceed overnight (15–20 h).

The results noted were identical for both high and low concentrations of MAP and AP enantiomers.

3.4. Standard calibration curves

Standard calibration curves of *R*-, *S*-AP, *R*- and *S*-MAP were constructed. These plots were linear over the range of 5–250 ng/ml. The parameters of the linear regression equations describing the relationship between peak-area ratio and concentration are shown in Table 1. The curves for the enantiomers of each compound were virtually superimposable. The curves reproduced well from day-to-day.

3.5. Validation of the method

The intra-day and inter-day variation of the assay are reported in Table 2. The intra-day coefficients of variation for the enantiomers of AP were in the range of 4.6–11.7% and those

Table 1
Calibration parameters of standard curves for the analysis of enantiomers of amphetamine and methamphetamine in rat serum

Compound	Slope (±S.D.) (ml/ng)	Intercept (±S.D.) (ng/ml)	r^2
R-Amphetamine	0.017 (±0.005)	0.027 (±0.020)	0.997
S-Amphetamine	0.019 (±0.006)	−0.021 (±0.038)	0.998
R-Methamphetamine	0.022 (±0.006)	0.013 (±0.031)	0.998
S-Methamphetamine	0.022 (±0.006)	−0.014 (±0.031)	0.999

Mean values are reported for 3 standard curves and each curve is based upon 6 concentrations.

Table 2
Intra-day and inter-day reproducibility of the analysis of the enantiomers of amphetamine and methamphetamine in rat serum

Compound	Conc. added (ng/ml)	Intra-day reproducibility		Inter-day reproducibility	
		Conc. found (%C.V.)[a] (ng/ml)	%Bias[b]	Conc. found (%C.V.)[c] (ng/ml)	%Bias
R-Amphetamine	250	259.3 (5.56)	3.72	240.8 (12.5)	−3.67
	50.0	50.75 (11.7)	1.50	46.28 (13.8)	−7.44
	12.5	11.84 (11.3)	−5.28	13.39 (18.6)	7.12
S-Amphetamine	250	265.3 (4.56)	6.15	244.1 (11.6)	−2.38
	50.0	51.92 (6.61)	3.84	47.98 (10.0)	−4.04
	12.5	12.97 (7.95)	3.76	13.86 (13.0)	10.9
R-Methamphetamine	250	263.1 (3.99)	5.24	247.6 (9.49)	−0.96
	50.0	50.62 (9.51)	1.24	48.12 (8.70)	−3.76
	12.5	11.84 (5.81)	−5.28	13.75 (18.3)	10.0
S-Methamphetamine	250	263.3 (3.54)	5.32	246.3 (9.27)	−1.48
	50.0	49.58 (4.70)	−0.84	47.72 (6.18)	−4.56
	12.5	12.16 (2.09)	−2.72	13.41 (11.8)	7.28

[a] Mean of 3 samples.
[b] %Bias = [(found − added) · 100]/added
[c] Mean of 9 samples.

for MAP were 2.1–9.5% at concentrations of 12.5, 50.0 and 250 ng/ml. The inter-day coefficients of variation for the enantiomers of AP were 6.2–18.3% over the same concentration range.

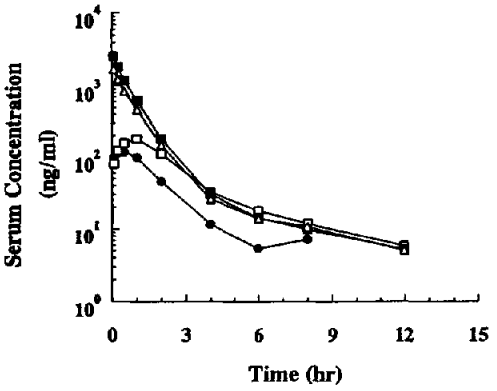

Fig. 4. Serum concentration–time profile of enantiomers of MAP and AP after a short term i.v. infusion of racemic MAP (15 mg/kg) into one rat. Key: (●) *R*-AP, (□) *S*-AP, (△) *R*-MAP, (■) *S*-MAP.

3.6. Application in animal studies

The assay was applied to the analysis of authentic samples obtained after a short term i.v. infusion of MAP into one male Fisher rat. Fig. 4 illustrates the serum concentration–time profile of the enantiomers of MAP and AP. There are no obvious differences in the serum concentrations of the MAP enantiomers. However, serum concentrations of *S*-AP were higher than those of the *R*-enantiomer. These data suggest that there are stereoisomeric differences in the metabolic formation of AP from MAP and/or differences in the subsequent elimination of AP.

4. Discussion

Before developing the current assay, we evaluated two published separation methods. Initially, we attempted to adopt a technique using derivatization with GITC prior to a normal-phase separation [13]. The detection limit for this assay

was inadequate for our purposes. Further, the derivatization process from the extracted biological samples was found to be poorly reproducible. We then examined a separation method developed by Wainer *et al.* [10] who utilized (*R*)-N-(3,5-dinitrobenzoyl)phenyl glycine as the chiral stationary phase. Although their method achieved excellent resolution for both MAP and AP enantiomers, it did not meet our sensitivity needs and it required the synthesis of a derivatizing reagent. As both assays employed UV detection, our next approach was to examine methods which utilized chiral fluorescent derivatizing agents to improve the sensitivity of the assay.

It has been shown that (+)-1-(9-fluorenyl)-ethyl chloroformate [(+)-FLEC], and its enantiomer, (−)-FLEC, were good derivatizing agents for amino acids and amines [20–23] and are commercially available with 99.5% purity. The derivatization of amino acids and amine compounds with (+)- or (−)-FLEC could be achieved under mild conditions [20–23]. This is crucial since racemization of chiral reagents at elevated temperature may occur [20]. The pH of the reaction was in the range of 7.85–8.5. An excess amount of FLEC was present and the reaction times were less than 30 min. We have used these data as a guideline to optimize the derivatization conditions. We have found that at substrate concentrations of 12.5 and 250 ng/ml, the derivatization reaction reached maximum yield when performed under room temperature overnight (>6 h) at pH 7.8 with a concentration of (−)-FLEC of 1 m*M*. We conclude that all concentrations within that range, such as used in the standard curve, will behave in a manner similar to the two test concentrations.

One major concern in using a chiral derivatizing reagent is the completeness of the reaction because enantiomers can react with the chiral reagent at different rates. We have shown that the reaction of enantiomers of AP and MAP with (−)-FLEC is identical (see Fig. 3) and achieved maximum yield under these conditions. Aniline sulfate was chosen as the internal standard because it contains an amino functional group that can react with (−)-FLEC under the same reaction conditions as MAP and AP. The peak areas of the aniline derivative were independent of different derivatization conditions suggesting that the reaction of aniline with (−)-FLEC was quantitatively the same. These chemical properties facilitate the reproducibility of this assay method.

(−)-FLEC's mass, in molar terms, was approximately 25 fold greater than that of MAP, AP and I.S. combined. Like all chloroformates, (−)-FLEC is reactive toward a variety of nucleophilic species such as moisture from the air and residual silanol groups on bonded silica columns. We have found that direct injection of nonreacted (−)-FLEC significantly reduced column longevity. To avoid this problem, nonreacted (−)-FLEC must be eliminated prior to injection. The methods that were used to remove (−)-FLEC included: passage of the reaction mixture through a C_{18} solid-phase extraction cartridge [21], extraction of nonreacted FLEC with *n*-pentane [20] and reaction of FLEC with an amino acid followed by *n*-pentane extraction [22]. The method that we ultimately applied required the addition of L-glycine to react with the excess amount of (−)-FLEC. The derivatized products which were in the uncharged form were subsequently extracted into *n*-pentane. This final extraction procedure also resulted in fewer late-eluting and interfering peaks.

Due to the high reactivity of (−)-FLEC, the cleanliness of all reagents was essential. Freshly double distilled water should be used. All reagents should be freshly prepared or used within a week.

Since (−)-FLEC can react with other components in serum samples which may result in extra peaks in the chromatogram, a sample clean-up procedure is needed. This was achieved by using liquid–liquid extraction. The addition of hydrochloric acid in the back-extraction step results in formation of the hydrochloride salt which prevents loss of MAP and AP by evaporation.

In summary, a precolumn derivatization HPLC method was developed to simultaneously separate and quantitate the serum concentrations of MAP and AP enantiomers. The fluorescent compound, (−)-FLEC, was used as a derivatiz-

ing agent. The derivatized products were stable at room temperature for several days. The method was reproducible and sensitive enough for the pharmacokinetic studies of MAP and AP. Application to a disposition kinetic study in the rat indicated no differences in the serum concentration of MAP enantiomers but those of *S*-AP were greater than the *R*-AP.

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