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RESEARCH PAPER

Enantioselective quantitative analysis of amphetamine in human plasma by liquid chromatography/high-resolution mass spectrometry

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Abstract A method for the quantitative enantioselective analysis of amphetamine in human plasma by LC-HRMS is presented. High-resolution detection, alone and in combination with targeted MS/MS, was validated and compared to a highly sensitive GC-NICI-Method. Derivatization with (*S*)-*N*-(heptafluorobutyryl)-prolyl chloride was accomplished to yield derivatives suitable for enantioselective analysis of amphetamine on a nonchiral reversed phase column with MS-compatible mobile phase. Equal analytical performance was observed for the methods presented and the GC-NICI-MS method. A dynamic range of 4,000 was found for the established calibration curves. A fivefold deuterated analogue of both enantiomeres was used as an internal standard. Full validation data are given to demonstrate the usefulness of the assay, including specificity, linearity, accuracy and precision, autosampler stability, matrix effect, and prospective analytical batch size accuracy. The method has been successfully applied to pharmacokinetic profiling of the drug after oral application.

Keywords Bioanalytical methods · Chiral analysis · Drug monitoring · Drug screening · HPLC · Mass spectrometry

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Introduction

Amphetamine (ß-phenylisopropylamine) is a potent sympathomimetic that stimulates the central nervous system. It elevates synaptic levels of dopamine, norepinephrine, and serotonin [1], whereby dopamine is regarded as the main mediator of its stimulatory effects on behavior [2]. The main pharmaceutical application of amphetamine is in the treatment of attention deficit hyperactivity disorder (ADHD) [3], but also for patients with narcolepsy [4]. It is still widely used as a reference drug to evaluate the psychostimulatory potency of other agents. Due to its effect on the central nervous system, amphetamine is a widely consumed drug of abuse, thus evoking high interest in this drug and hence in its reliable analysis from forensic, toxicological, and pharmaceutical points of view, as high doses of amphetamine may lead to so-called amphetamine psychosis, a syndrome virtually indistinguishable from paranoid schizophrenia [5, 6]. (−)-Amphetamine is also a major metabolite of the monoamine oxidase B-inhibitor selegiline [7], famprofazone [8], and methamphetamine [9].

The enantiomeres of this chiral molecule have different pharmacokinetic and pharmacological properties. Thus, the (*S*)-enantiomer has fivefold higher psychostimulant activity than the (*R*)-form [10]. In forensic applications, enantiomeric composition may help to distinguish between the misuse of amphetamine and methamphetamine from the ingestion of medications like selegiline, L-methamphetamine, and famprofazone.

Enantiomeric analysis of amphetamine has been performed in several ways. Chiral derivatisation and analysis on nonchiral stationary phases was used for gas chromatography (GC) and GC-MS, using (*S*)-*N*-(trifluoroacetyl)-prolyl chloride [11–15], (*S*)-*N*-(heptafluorobutyryl)-prolyl chloride [16–19], (−)-menthyl chloroformate [20], and *N*-pentafluorobenzoyl-(*S*)-prolyl-1-imidazolide [21] derivatives. Liquid chromatography (LC) and LC-MS have also been used

with chiral columns to separate the enantiomeres of amphetamine on chiral stationary phases [10, 22, 23], or with reversed phase (RP) columns after chiral derivatisation using UV or fluorescence detection. A variety of agents have been described in the latter studies for diastereomer formation with amphetamine, among them (−)-menthyl chloroformate [24], 4-nitrophenylsulfonyl-L-proline [24], *o*-phtaldialdehyde/L-cysteine [24], (+)-1-phenylethyl isothiocyanate [25], and (−)-9-fluorenylethyl chloroformate [26–28]. While suitable for UV and fluorescence detectors, these latter methods cannot always easily be adopted for LC-MS detection due to compatibility problems with the mobile phase composition and hence the necessary chromatographic resolution. (*S*)-*N*-(heptafluorobutyryl)-prolyl chloride has so far not been used for chiral derivatisation of amphetamine enantiomeres and subsequent LC-MS analysis.

High-resolution mass spectrometry (HRMS) provides unsurpassed specificity due to drastically reduction of background interference. Being available only at dedicated laboratories, this technique has not gained widespread application so far. With the advent of Orbitrap mass spectrometers, this instrumentation has become affordable and downscaled to benchtop size, making application of HRMS more and more frequent.

For pharmacokinetic applications, robustness and short analysis time is a major concern, since they involve the processing of a large number of samples. Additionally, as children represent a considerable proportion of the target group for amphetamine medication, reduction of sample size is of critical importance. It was thus the aim of this study to elaborate and validate a LC-HRMS assay for the quantitative determination of amphetamine enantiomeres in human plasma that meets the requirements of sensitivity, specificity, speed and ruggedness for pharmacokinetic applications and drug monitoring, and to compare this method to detection with highly sensitive analysis by GC and negative ion chemical ionization (NICI) mass spectrometry.

Experimental

Chemicals and reagents

(*S*)-(+)-amphetamine (D-AMP) and (*R*)-(−)-amphetamine (L-AMP) were purchased as certified reference solutions from Cerilliant (USA). Heptafluorobutyric anhydride (HFBA) and (*S*)-proline were from Sigma (Vienna, Austria). (R)-α-methylbenzylamine of ChiraSelect grade was from Fluka (Vienna, Austria). All other substances, solvents, and reagents of analytical grade were from Merck (Darmstadt, Germany). Plasma samples were collected as part of a pharmacokinetic study and hence under approval of the corresponding authorities.

Preparation of (S)-*N*-(heptafluorobutyryl)-prolyl chloride (L-HPC)

(*S*)-*N*-(heptafluorobutyryl)-prolyl chloride (L-HPC) was prepared as described [29]. Then 1.2 g (10.4 mmol) of (*S*)-proline were dissolved in 30 mL ethyl acetate cooled to −20 °C. 2.5 mL (10 mmol) of cold HFBA were added and the mixture stirred for 1.5 h. After washing with 0.1 M HCl, the ethyl acetate was dried over anhydrous sodium sulfate and 2.8 mL (20 mmol) of triethyl amine were added. The solvent and excess triethyl amine were removed in vacuo. One hundred milliliters cold (−20 °C) solution of thionyl chloride in dichloromethane (2 M) were added, stirred on an ice bath for 15 min, and kept at room temperature for 2.5 h. Solvent and excess reagent were removed in vacuo and the residue dissolved in *n*-hexane. Optical purity was checked by derivatisation of (R)-α-methylbenzylamine of certified enantiomeric composition and was found to be 99.74 %.

Preparation of racemic d_5-amphetamine

Racemic d_5-amphetamine was prepared from (±)-amphetamine by acid-catalyzed exchange reaction using 5 % DCl in D_2O [30]. Briefly, racemic amphetamine sulphate (2 mg) was dissolved in 0.5 ml of 5 % DCl in D_2O, the glass vial capped tightly and held at 160 °C for 15 h. After cooling, the mixture was diluted with methanol and isotopic purity as well as enantiomer ratio checked by GC-NICI-MS.

Plasma sample preparation and derivatisation

Samples were thawed at room temperature and processed immediately after thawing. Fifty microliters of the methanolic solution of the internal standard, containing d_5-amphetamine free base (4 ng of each enantiomer), were pipetted into a 5-mL glass tube and 0.25 mL of plasma was added. After short, vigorous shaking, 0.2 mL of 0.3 M NaOH was added, followed by 2.5 mL of *n*-hexane. The tubes were shaken on a reciprocal shaker at low speed for 15 min and centrifuged at 3,000 rpm. The clear supernatant *n*-hexane phase was transferred to 5-mL glass tubes and 50 μL of the L-HPC reagent (1 mM in *n*-hexane) was added. The mixture was shaken and left at room temperature for 30 min. Thereafter, solvent was removed under a gentle stream of nitrogen at 40 °C. The dry residue was dissolved in 100 μL ethyl acetate for GC-NICI-MS or 150 μL methanol/water (60:40; *v*/*v*, containing 0.1 % formic acid) for LC-HRMS and transferred to autosampler vials. The vials were stored at −20 °C until analysis.

GC-NICI-MS analysis of amphetamine-L-HPC derivatives

GC-NICI-MS analysis of amphetamine-L-HPC derivatives was accomplished as previously described [19] with slight

adoptions to use only 250 μL of sample. An ISQ quadrupole mass spectrometer coupled to a TRACE GC Ultra (Thermo Scientific, Vienna) was used. The GC was fitted with a BPX5 fused silica capillary column (15 m×0.25 mm i.d., SGE). The injector was operated in the splitless mode at 280 °C. Helium was used as a carrier gas at a constant flow rate of 1.5 mL/min. Initial column temperature was 100 °C for 1 min, followed by an increase of 40 °C/min to 310 °C, and an isothermal hold of 2 min. The mass spectrometer transfer line was kept at 315 °C. NICI was performed with methane as a moderating gas. During single ion recording, *m/z* 368.1 and *m/z* 373.1 were recorded for target and internal standard, respectively, with a dwell time of 50 ms.

LC-HRMS analysis of amphetamine-L-HPC derivatives

A Q-Exactive high-resolution orbitrap mass spectrometer with a heated electrospray source coupled to an Accela 1250 HPLC pump (Thermo Scientific, Vienna) was used. The Hypersil Gold (Thermo Scientific, Vienna) reversed phase column (100×2.1 mm, 1.9 μm) was placed in a Maylab Mistraswitch thermostatic oven and kept at 35 °C. LC elution was accomplished at a flow rate of 300 μL/min starting with 40 % mobile phase A (0.012 % formic acid, 5 mM ammonium acetate) and 60 % mobile phase B (0.012 % formic acid in methanol, 5 mM ammonium acetate). A gradient to 100 % mobile phase B was run within 7 min, and mobile phase B continued isocratically for another 4 min. The column was then equilibrated at the starting conditions for 2 min before the next injections. Ten microliters of sample were used for analysis. Total analysis time per sample was 13 min. Identical conditions at a flow rate of 0.8 mL/min were employed for analysis on a chiral ß-cyclodextrin column (LiChroCART ChiraDex 250-4, 5 μ; Merck, Darmstadt).

The orbitrap mass spectrometer was set to maximal resolution (140.000). The entrance quadrupole was set to a mass range from 428 to 435 at unit resolution. Detection of MH^+ for amphetamine-L-HPC and d_5-amphetamine-L-HPC was accomplished at *m/z* 429.1413 and *m/z* 434.1727, respectively. For MS/MS analysis, MH^+ ions of amphetamine and d_5-amphetamine were selected as precursors and fragmented by CID at a normalized collision energy of 35 %, followed by orbitrap detection at 70.000 resolution.

Analytical method validation

Calibration graphs were established in the range of 0.024 ng/mL plasma to 25 ng/mL plasma (L-AMP) and 0.098 ng/mL plasma to 100 ng/mL plasma (D-AMP) with 11 calibration points in duplicates. For this purpose, blank plasma was spiked with the appropriate amounts of L- and D-AMP and serial dilution of the highest calibrant with blank plasma. Standard solutions of L- and D-AMP were prepared in methanol and stored at −20 °C. Calibration curves were calculated by polynomial regression analysis (quadratic fit) weighting for $1/s^2$ (s=standard deviation of duplicates). For response function check, five individual calibration curves were measured and the coefficients of regression evaluated. Back-calculated values of all measured calibrants were correlated to their nominal values and correlation coefficients calculated. Different calibration ranges for L- and D-AMP were chosen due to the expected maximal levels of D-AMP after administration of dexamphetamine, thus demonstrating that the presented method can be adjusted to the needs of the analytical task.

Interday precision was determined at 0.024 (LOQ), 0.56, 4, and 20 ng/mL concentration levels (L-AMP) and 0.098 (LOQ), 2.24, 16, and 80 ng/mL concentration levels (D-AMP) by carrying 5 identical samples at each concentration level throughout the analytical sequence. Spiked samples were prepared from blank plasma. Intraday precision was determined at 0.024 (LOQ), 0.56, 4, and 20 ng/mL concentration levels (L-AMP) and 0.098 (LOQ), 2.24, 16, and 80 ng/mL concentration levels (D-AMP) by carrying 5 identical samples at each concentration level throughout the analytical sequence. Spiked samples were prepared from blank plasma. Accuracy of the methods was also tested at the abovementioned concentrations. Thus, the data from inter- and intraday precision measurements were used to calculate the deviation of the values measured from the actual spiked values. Specificity was tested by analyzing six different blank plasma samples. Freeze-thaw stability, long-term stability of stored samples, stock solution stability, and short-term (benchtop) stability have been evaluated previously [19].

Autosampler stability was determined by analyzing a set of spiked samples at different concentrations together with the corresponding calibration curve at two different days. The samples were thereby left at ambient temperature for 5 days until reanalysis.

Matrix effect was investigated using six lots of matrix including hemolyzed and hyperlipidemic sample matrix. For each analyte and the internal standard, the matrix factor (MF) was calculated in each lot of matrix by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with analyte at a concentration of 0.56 ng/mL (L-AMP) and 2.24 ng/mL (D-AMP) after extraction) to the peak area in the absence of matrix (pure solution of the analyte). The IS normalized MF was also calculated by dividing the MF of the analyte by the MF of the IS. The IS was added to the matrix or buffer together with the unlabelled target. Normalized MF were calculated for each sample separately.

For assessment of accuracy and precision at prospective analytical batch size, five replicates of spiked samples at 0.024 (LOQ), 0.56, 4, and 20 ng/mL concentration levels (L-AMP) and 0.098 (LOQ), 2.24, 16, and 80 ng/mL concentration levels (D-AMP) together with at least 90 blank plasma samples were extracted and chromatographed with a set of calibration standards in one single run. Quality control (QC) samples were

analyzed once before the blank plasma samples and again after analysis of 90 blank plasma samples. Accuracy was measured as bias (percent deviation of the calculated vs. the nominal values) and precision was expressed as coefficient of variation (%).

Results and discussion

LC-HRMS analysis of amphetamine-L-HPC derivatives

The method presented here provides a rapid and simple way for the enantioselective analysis of D- and L-AMP in plasma allowing processing of large sample batches. As children are a main target of this medication, small sample size is desirable. The high sensitivity of the orbitrap mass spectrometer allows the use of only 250 μL of plasma by keeping a LOQ of 0.024 ng/mL. Extraction and derivatisation are simple, and the alkaline *n*-hexane extract is very clean, even from a complex matrix like plasma. We have processed batches with up to 130 samples per day conveniently. Investment of time for this sample preparation is easily compensated by the benefit of clean extracts that result in lower insult of the chromatographic system.

Chromatographic resolution of the diastereomeres formed after derivatisation of L- and D-AMP with L-HPC on a conventional reversed phase column is the rate-limiting step in this type of enantioselective analysis, especially when the choice of mobile phase is restricted to electrospray-compatible compositions. We have therefore compared two modes of mass spectrometric detection: high resolution only (R=140.000) and targeted MS/MS analysis from the protonated precursor ions at R=70.000. The product ion mass spectra obtained for AMP-L-HPC and d_5-AMP-L-HPC are shown in Fig. 1. Besides loss of the *N*-carboxyamphetamine moiety, three specific fragment ions bearing the isotope label of the internal standard were formed: C_7H_7 (*m/z* 91.0548), C_9H_{11} (*m/z* 119.0861), and $C_9H_{14}N$ (*m/z* 136.1126), as well as their deuterated analogues at *m/z* 96.0862, *m/z* 124.1175, and *m/z* 141.1440. For quantitation, the summed intensities of these three ion traces were monitored. As seen in Fig. 2, baseline separation can be achieved under the conditions employed; the column temperature of 35 °C being crucial to this. This resolving power remained constant even after analysis of more than 150 samples on the same column without any washing or regeneration steps in between. This can be attributed to the careful conditioning during the LC method, which only moderately increases analysis time, but with the benefit of largely unattended operation. Even lower detection limits can be obtained, if desired, when larger plasma samples of 1 mL are extracted (results not shown). The HRMS method showed slight background interference near the L-AMP-L-HPC peak, which does not significantly affect quantitative performance, as shown below by the validation data. Virtually, no background is seen with the HRMS/MS method. The obtained chromatographic resolution is comparable to the results from analysis on a chiral stationary phase, where ideal peak shape was obtained for D-AMP and peak broadening for L-AMP. The elution order was L-AMP/D-AMP for GC-NICI-MS, D-AMP/L-AMP on the reversed phase column, and L-AMP/D-AMP on the ß-cyclodextrin column.

Analytical method validation

The calibration graphs established were linear within the tested range of 0.024 ng/mL plasma to 25 ng/mL plasma (L-AMP) and 0.098 ng/mL plasma to 100 ng/mL plasma (D-AMP). Proportionality of the response function was observed between 0.024 ng/mL plasma and 100 ng/mL plasma for both enantiomeres. For the needs of a pharmacokinetic application

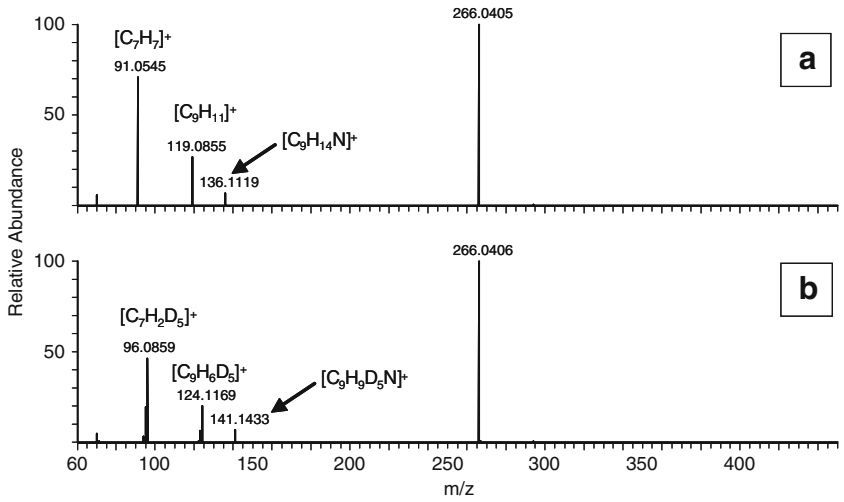

Fig. 1 HRMS/MS product ion mass spectra of the D- or L-amphetamine-L-HPC derivatives (**a**) and their pentadeuterated analogues (**b**). MS/MS spectra were obtained from the protonated MH^+ precursors and recorded at a resolution of 70.000

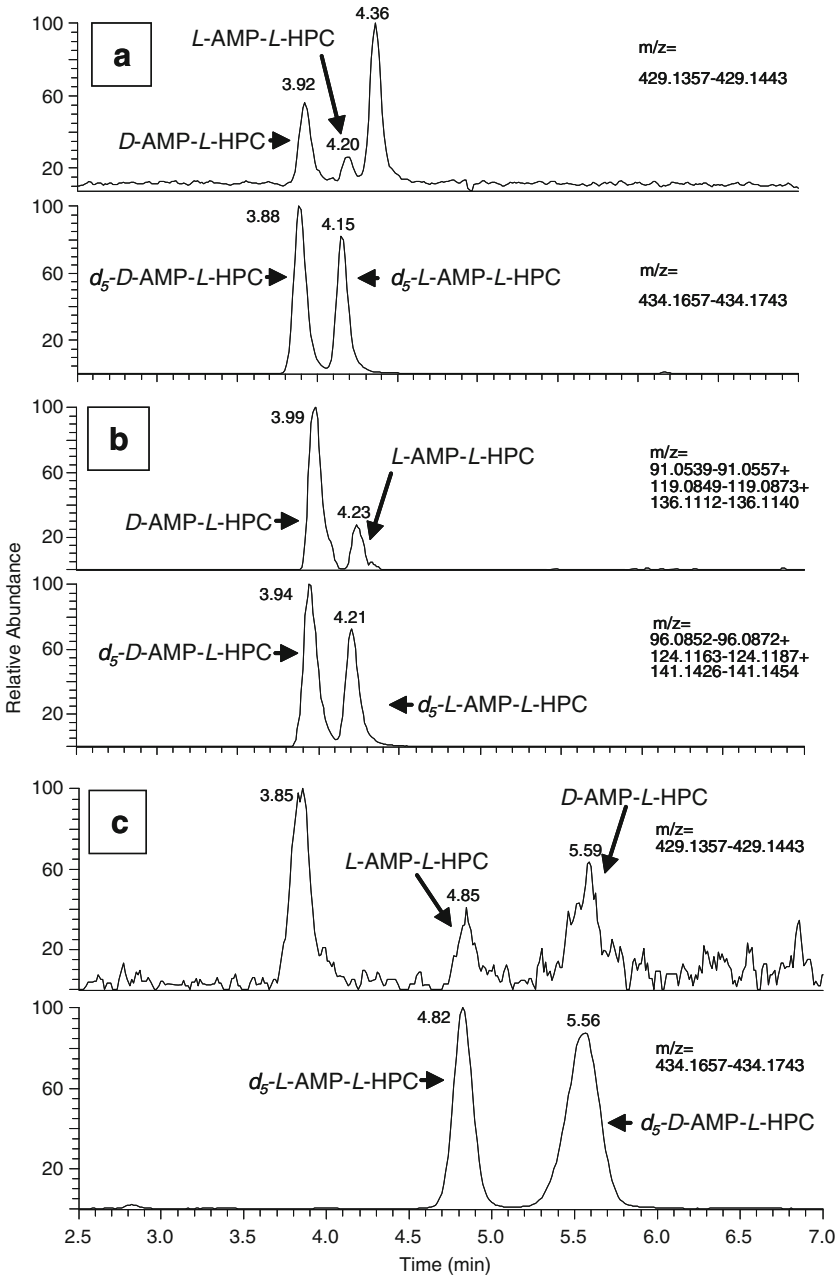

Fig. 2 Chromatograms obtained after analysis of plasma containing 0.024 ng/mL of L-amphetamine and 0.098 ng/mL of D-amphetamine. Samples were converted to the L-HPC derivatives and analyzed **a** by LC-HRMS or **b** by LC-HRMS/MS on a reversed phase column, and **c** by LC-HRMS on a ß-cyclodextrin column

(administration of dexamphetamine), however, calibration ranges were set as indicated above and the method validated within that range. Mean regression coefficients (r^2, n=5) were 0.99972 (D-AMP) and 0.99927 (L-AMP) with the HRMS method, and 0.99965 (D-AMP) and 0.99987 (L-AMP) with the HRMS/MS method. The mean regression equations from five calibration curves were as follows: $Y=-8.2967^{-4}$ $(\pm 0.00411)+0.02841$ $(\pm 3.80616^{-4}) \times X - 1.25385^{-5}$ $(\pm 3.94046^{-6}) \times X^2$ (D-AMP, HRMS); $Y=-0.00224$ $(\pm 0.0019)+0.03314$ $(\pm 7.04625^{-4}) \times X - 7.64652^{-5}$ $(\pm 2.91795^{-6}) \times X^2$ (L-AMP, HRMS); $Y=0.01055$ $(\pm 0.00444)+0.07831$ $(\pm 0.00146) \times X - 1.51064^{-4}$ $(\pm 5.8973^{-5}) \times X^2$ (D-AMP, HRMS/MS); $Y=0.00521$ $(\pm 0.00235)+0.11049$ $(\pm 8.69072^{-4}) \times X - 7.40753^{-5}$ $(\pm 3.59895^{-5}) \times X^2$ (L-AMP, HRMS). Correlation (r^2) of back-calculated values with their respective nominal values (means of five calibration curves) were 0.99955 (D-AMP, HRMS), 0.99977 (L-AMP, HRMS), 0.99988 (D-AMP, HRMS/MS), and 0.99959 (L-AMP, HRMS/MS). The data demonstrate the extraordinary analytical power of the orbitrap HRMS system, as quantification can be achieved over a dynamic calibration range of 4,000.

The coefficients of inter- and intraday variation (precision) and accuracy of the spiked samples are presented in Tables 1 and 2. It can be seen from these data that the method provides a highly precise and accurate assay for L-and D-AMP in

Table 1 Intraday precision and accurracy

Intraday precision and accuracy for L-AMP determination								
	Nominal concentration (ng/mL plasma)							
	0.024		0.560		4.000		20.000	
	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS
Mean	0.024	0.024	0.553	0.567	4.066	3.973	20.841	20.150
s.d.	0.0014	0.00029	0.032	0.027	0.13	0.12	0.84	0.48
CV (%)	5.95	1.24	5.79	4.78	3.29	2.97	4.05	2.39
Accuracy (%)	−1.73	−2.07	−1.23	1.29	1.64	−0.68	4.20	0.75
Intraday precision and accuracy D-AMP determination								
	Nominal concentration (ng/mL plasma)							
	0.098		2.240		16.000		80.000	
	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS
Mean	0.097	0.098	2.222	2.294	15.662	15.740	75.012	79.078
s.d.	0.001	0.001	0.083	0.089	0.51	0.33	1.72	2.66
CV (%)	1.05	1.07	3.75	3.90	3.23	2.07	2.30	3.37
Accuracy (%)	−0.79	−0.31	−0.80	2.42	−2.11	−1.63	−6.24	−1.15

Table 2 Intraday precision and accurracy

Interday precision and accuracy for L-AMP determination								
	Nominal concentration (ng/mL plasma)							
	0.024		0.560		4.000		20.000	
	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS
Mean	0.024	0.024	0.563	0.564	4.070	3.992	20.406	20.528
s.d.	0.00053	0.00036	0.017	0.0091	0.040	0.067	0.43	0.34
CV (%)	2.23	1.50	2.99	1.61	0.98	1.69	2.13	1.65
Accuracy (%)	−1.28	−0.85	0.55	0.70	1.75	−0.19	2.03	2.64
Interday precision and accuracy D-AMP determination								
	Nominal concentration (ng/mL plasma)							
	0.098		2.240		16.000		80.000	
	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS	HRMS	MS/MS
Mean	0.097	0.098	2.213	2.272	15.846	15.907	78.987	80.612
s.d.	0.0010	0.00020	0.015	0.041	0.12	0.17	2.63	4.05
CV (%)	1.04	0.17	0.66	1.81	0.74	1.06	3.33	5.03
Accuracy (%)	−1.13	−0.50	−1.19	1.40	−0.96	−0.58	−1.27	0.77

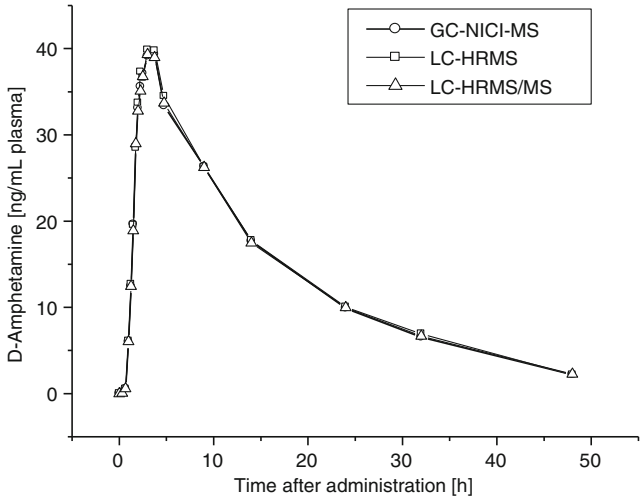

Fig. 3 Pharmacokinetic profile of D-amphetamine from a human volunteer receiving 20 mg of dexamphetamine orally. Samples were collected at the indicated time points and processed as described under methods. Analysis of samples was performed by GC-NICI-MS, LC-HRMS, and LC-HR-MS/MS

human plasma, with both, the HRMS and HRMS/MS method being equally suitable. This can be attributed at least in part to the use of stable isotope-labeled internal standards. Mass spectrometry in combination with stable isotope dilution is a very powerful tool in external quality assessment schemes, and assays based on this technique can be regarded as reference procedures to validate other analytical methods.

Six different blank matrices were checked for interferences. In none of the samples, there was background contribution above 25 % LOQ at the retention times of targets and internal standards, respectively.

For autosampler stability, the mean concentrations of samples chromatographed immediately after sample preparation and 5 days later were measured and the deviation from immediately analyzed samples were 0.74 % (2.24 ng/mL), 0.54 % (16 ng/mL), and −1.50 (80 ng/mL) (D-AMP, HRMS); 4.96 % (0.56 ng/mL), −2.22 % (4 ng/mL), and −3.43 (20 ng/mL) (L-AMP, HRMS); 0.21 % (2.24 ng/mL), 1.74 % (16 ng/mL), and 4.36 (80 ng/mL) (D-AMP, HRMS/MS); and 1.27 % (0.56 ng/mL), −1.61 % (4 ng/mL), and 3.17 (20 ng/mL) (L-AMP, HRMS/MS). Thus, D- and L-amphetamine-L-HPC derivatives are stable to repeated analysis conditions with both of the described methods.

Evaluation of the matrix effect resulted in mean internal standard-normalized matrix factors for normal, lipidemic, and hemolytic matrix of 1,00±0.007 (L-AMP, HRMS), 1,00±0.022 (D-AMP, HRMS), 0.99±0.013 (L-AMP, HRMS/MS), and 1,01±0.017 (D-AMP, HRMS/MS). This demonstrates that assay performance is not influenced by plasma matrix composition and no significant matrix effect can be observed. The matrix effect measured herein is comparable to that of the GC-NICI-MS method which was determined as 1,05±0.061 (L-AMP) and 1,05±0.033 (D-AMP). Again, this must be attributed to the sample preparation and clean-up procedure, making this assay robust against variations of matrix composition.

Accuracy at prospective analytical batch size has been estimated for a 90-sample batch. Deviations of samples analyzed after sample batch from early analyzed samples were 1.87 % (2.24 ng/mL), 1.00 % (16 ng/mL), and −1.45 (80 ng/mL) (D-AMP, HRMS); −1.49 % (0.56 ng/mL), −1.78 % (4 ng/mL), and −3.15 (20 ng/mL) (L-AMP, HRMS); −0.48 % (2.24 ng/mL), 0.09 % (16 ng/mL), and 2.50 % (80 ng/mL) (D-AMP, HRMS/MS); and −1.13 % (0.56 ng/mL), 1.23 % (4 ng/mL), and 0.92 (20 ng/mL) (L-AMP, HRMS/MS). The method is thus suitable for analyzing batch sizes up to 130 samples, including calibrants and quality control samples.

We have applied the method described herein to the analysis of D- and L-amphetamine during pharmacokinetic profiling of the drug. Figure 3 shows a typical time course from a human volunteer receiving 20 mg of dexamphetamine (D-AMP) orally as a fast-release formulation and subsequent analysis by GC-NICI-MS, LC-HRMS and LC-HRMS/MS. These data are part of a pharmacokinetic bioequivalence study which has been approved by the responsible authorities. As can be seen, the time course is virtually identical for all three methods. Values obtained by the LC-MS methods and the GC-NICI method showed a linear regression coefficient r^2= 0.99965 and slope B=0.9950 (HRMS/MS), as well as r^2= 0.99902 and slope B=1.01486 (HRMS). It is a beneficial feature of the L-HPC derivative that processed samples can be analyzed by GC and LC as well, thus allowing direct comparison of method performance from the same set of samples. It should be noted that the method described was also applied to the analysis of D- and L-amphetamine after oral intake of racemic amphetamine with unattenuated assay performance. The results of this pharmacokinetic study will be published separately.

Conclusions

The analysis of (*S*)-amphetamine and (*R*)-amphetamine from human plasma is of major interest in pharmaceutical research and toxicological monitoring of the drug. Enantiomeric separation by covalent chiral derivatisation allows the use of nonchiral chromatographic systems, an approach widely used in enantioselective GC-analysis. The use of L-HPC as a chiral label for amphetamine in LC-MS analysis has not been described so far. High-resolution mass spectrometry adds an additional dimension to specificity and allows detection of amphetamine by the described methods with accuracy, precision, and sensitivity comparable to the highly sensitive GC-NICI-method. The use of a stable isotope labeled internal standards adds an additional dimension of specificity and selectivity to the mass spectrometric detection, thereby also

compensating ideally for losses during sample work-up procedure and derivatisation sequence. We have successfully applied this method to the bulk analysis of plasma samples for a preliminary pharmacokinetic study, demonstrating its ability for routine measurements

References

1. Madras BK, Miller GM, Fischman AJ (2005) The dopamine transporter and attention-deficit/hyperactivity disorder. Biol Psychiatry 57(11):1397–1409
2. Boutrel B, Koob GF (2004) What keeps us awake: the neuropharmacology of stimulants and wakefulness-promoting medications. Sleep 27(6):1181–1194
3. Elia J, Borcherding BG, Potter WZ, Mefford IN, Rapoport JL, Keysor CS (1990) Stimulant drug treatment of hyperactivity: biochemical correlates. Clin Pharmacol Ther 48(1):57–66
4. Mitler MM, Aldrich MS, Koob GF, Zarcone VP (1994) Narcolepsy and its treatment with stimulants. ASDA standards of practice. Sleep 17(4):352–371
5. Angrist BM, Gershon S (1970) The phenomenology of experimentally induced amphetamine psychosis–preliminary observations. Biol Psychiatry 2(2):95–107
6. Griffith JD, Cavanaugh J, Held J, Oates JA (1972) Dextroamphetamine. Evaluation of psychomimetic properties in man. Arch Gen Psychiatry 26(2):97–100
7. Magyar K (2011) The pharmacology of selegiline. Int Rev Neurobiol 100:65–84
8. Greenhill B, Valtier S, Cody JT (2003) Metabolic profile of amphetamine and methamphetamine following administration of the drug famprofazone. J Anal Toxicol 27(7):479–484
9. Schepers RJ, Oyler JM, Joseph RE Jr, Cone EJ, Moolchan ET, Huestis MA (2003) Methamphetamine and amphetamine pharmacokinetics in oral fluid and plasma after controlled oral methamphetamine administration to human volunteers. Clin Chem 49(1):121–132
10. Herraez-Hernandez R, Campins-Falco P, Verdu-Andres J (2002) Strategies for the enantiomeric determination of amphetamine and related compounds by liquid chromatography. J Biochem Biophys Methods 54(1–3):147–167
11. Ellerbe P, Long T, Welch MJ (1993) The determination of amphetamine and methamphetamine in a lyophilized human urine reference material. J Anal Toxicol 17(3):165–170
12. Hensley D, Cody JT (1999) Simultaneous determination of amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), and methylenedioxyethylamphetamine (MDEA) enantiomers by GC-MS. J Anal Toxicol 23(6):518–523
13. Tao QF, Zeng S (2002) Analysis of enantiomers of chiral phenethylamine drugs by capillary gas chromatography/mass spectrometry/flame-ionization detection and pre-column chiral derivatization. J Biochem Biophys Methods 54(1–3):103–113
14. Tetlow VA, Merrill J (1996) Rapid determination of amphetamine stereoisomer ratios in urine by gas chromatography-mass spectroscopy. Ann Clin Biochem 33(Pt 1):50–54
15. Maurer HH, Kraemer T (1992) Toxicological detection of selegiline and its metabolites in urine using fluorescence polarization immunoassay (FPIA) and gas chromatography-mass spectrometry (GC-MS) and differentiation by enantioselective GC-MS of the intake of selegiline from abuse of methamphetamine or amphetamine. Arch Toxicol 66(9):675–678
16. Srinivas NR, Cooper JK, Hubbard JW, Midha KK (1989) Isothermal capillary gas chromatography with electron-capture detection of heptafluorobutyryl-L-prolyl derivatives of chiral amphetamines. J Chromatogr 491(1):262–264
17. Srinivas NR, Hubbard JW, Hawes EM, McKay G, Midha KK (1989) Enantioselective gas chromatographic assays with electron-capture detection for methoxyphenamine and its three primary metabolites in human urine. J Chromatogr 487(1):61–72
18. Lim HK, Su Z, Foltz RL (1993) Stereoselective disposition: enantioselective quantitation of 3,4-(methylenedioxy) methamphetamine and three of its metabolites by gas chromatography/electron capture negative ion chemical ionization mass spectrometry. Biol Mass Spectrom 22(7):403–411
19. Leis HJ, Rechberger GN, Fauler G, Windischhofer W (2003) Enantioselective trace analysis of amphetamine in human plasma by gas chromatography/negative ion chemical ionization mass spectrometry. Rapid Commun Mass Spectrom 17(6):569–575
20. Hughes RO, Bronner WE, Smith ML (1991) Detection of amphetamine and methamphetamine in urine by gas chromatography/mass spectrometry following derivatization with (-)-menthyl chloroformate. J Anal Toxicol 15(5):256–259
21. Matin SB, Wan SH, Knight JB (1977) Quantitative determination of enantiomeric compounds. I–simultaneous measurement of the optical isomers of amphetamine in human plasma and saliva using chemical ionization mass spectrometry. Biomed Mass Spectrom 4(2):118–121
22. Mortier KA, Maudens KE, Lambert WE, Clauwaert KM, Van Bocxlaer JF, Deforce DL, Van Peteghem CH, De Leenheer AP (2002) Simultaneous, quantitative determination of opiates, amphetamines, cocaine and benzoylecgonine in oral fluid by liquid chromatography quadrupole-time-of-flight mass spectrometry. J Chromatogr B 779(2):321–330
23. Shpigun OA, Shapovalova EN, Ananieva IA, Pirogov AV (2002) Separation and enantioseparation of derivatized amino acids and biogenic amines by high-performance liquid chromatography with reversed and chiral stationary phases. J Chromatogr A 979(1–2):191–199
24. Miller KJ, Gal J, Ames MM (1984) High-performance liquid chromatographic resolution of enantiomers of 1-phenyl-2-aminopropanes (amphetamines) with four chiral reagents. J Chromatogr 307(2):335–342
25. Kleidernigg OP, Lindner W (1997) Synthesis of new stable aliphatic isothiocyanate-based chiral derivatizing agent and application to indirect separation of chiral amino and thiol compounds. Chromatographia 44(9–10):465–472
26. Hutchaleelaha A, Sukbuntherng J, Chow HH, Mayersohn M (1994) Disposition kinetics of D- and L-amphetamine following intravenous administration of racemic amphetamine to rats. Drug Metab Dispos 22(3):406–411
27. Hutchaleelaha A, Walters A, Chow HH, Mayersohn M (1994) Sensitive enantiomer-specific high-performance liquid chromatographic analysis of methamphetamine and amphetamine from serum using precolumn fluorescent derivatization. J Chromatogr B Biomed Appl 658(1):103–112
28. Sukbuntherng J, Hutchaleelaha A, Chow HH, Mayersohn M (1995) Separation and quantitation of the enantiomers of methamphetamine and its metabolites in urine by HPLC: precolumn derivatization and fluorescence detection. J Anal Toxicol 19(3):139–147
29. Goodnough DB, Lutz MP, Wood PL (1995) Rapid and sensitive procedure for the separation and quantitation of D- and L-serine in rat brain using gas chromatography-mass spectrometry. J Chromatogr B Biomed Appl 667(2):223–232
30. Leis HJ, Windischhofer W, Wintersteiger R (1994) Quantitative measurement of amphetamine in human plasma by gas chromatography/negative ion chemical ionization mass spectrometry using (2H5)amphetamine as internal standard. Biol Mass Spectrom 23(10):637–641