

# Accelerated quantification of amphetamine enantiomers in human urine using chiral liquid chromatography and on-line column-switching coupled with tandem mass spectrometry

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Received: 12 August 2016 / Revised: 22 September 2016 / Accepted: 24 October 2016  
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**Abstract** Amphetamine (AM) is a powerful psychostimulant existing in two enantiomeric forms. Stereoselective analysis of AM in biosamples can assist clinicians and forensic experts in differentiating between abuse of illicitly synthesized racemic AM and ingestion of pharmaceutical AM formulations containing either S-AM or different proportions of the S- and R-enantiomers. Therefore, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for quantifying AM enantiomers in urine was newly developed. The method comprised dilution with water, followed by injection of the diluted sample onto an achiral C18 trapping column for purification and subsequent backflush elution to a chiral Lux 3  $\mu\text{m}$  AMP LC column by means of a switching valve. An isocratic mobile phase of 25 % acetonitrile in 0.1 M aqueous ammonia was used for enantiomeric separation. Injection, cleanup, and backflush of the next sample were performed before the previous sample had eluted from the analytical column, thus enabling simultaneous enantioseparation of up to three samples within the analytical column. This novel chromatographic concept allowed for increased sample throughput by accelerating both the sample preparation and the LC analysis. Analyte detection was accomplished by electrospray ionization in positive ion mode and selected reaction monitoring using a triple-stage quadrupole mass spectrometer. The method was successfully validated through assessment of its linearity, lower limit of quantification, accuracy and precision, selectivity, matrix effect, carry-over, dilution

integrity, and re-injection reproducibility. Linearity ranged from 0.05 to 25 mg/L for both enantiomers. Proof of the method included analysis of urine samples obtained from drug abusers and patients receiving an S-AM prodrug.

**Keywords** R-/S-amphetamine · Chiral separation · Urine · Column-switching chromatography · LC-MS/MS

## Introduction

Amphetamine (AM) is a potent stimulant of the central nervous system (CNS) and has been established as an effective treatment for attention deficit hyperactivity disorder (ADHD), as well as other CNS disorders such as narcolepsy. Its powerful stimulatory action does not only provide therapeutic efficacy, but it also responsible for AM's adverse effects and high potential for recreational abuse. As a result of the United Nations Convention on Psychotropic Substances of 1971, AM became a controlled substance in many countries and is available for treatment by prescription only [1–3]. Nevertheless, AM abuse is widespread, with global AM seizures fluctuating between about 20 and 33 t annually since 2009 [4].

As a molecule with a single chiral center, AM exists in two optically active forms, i.e., the S-(+)- (or d-) and R-(-)- (or l-) enantiomers, which differ in their pharmacological characteristics. While the R-enantiomer produces more cardiovascular and peripheral effects, the S-enantiomer has stronger stimulant properties. Illicit production of AM generally yields a 50:50 racemic mixture of the two enantiomers [5, 6]. Currently available pharmaceutical formulations of AM, on the other hand, primarily contain either S-AM (e.g., Dexedrine®, ProCentra®, Elvanse®) or different proportions of the S- and R-enantiomers (e.g., Adderall®, 3:1 mixture of S- and R-AM) [1, 2]. Thus, the determination of the enantiomeric composition of AM in

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biological samples may help to distinguish between the illicit consumption of street AM and the legitimate use of AM medication, which is of great relevance in clinical and forensic toxicology. With the approval of the S-AM prodrug Elvanse® (lisdexamfetamine [L-lysine-dextroamphetamine] dimesylate) at the beginning of 2014, AM-based medication for ADHD treatment also became available in Switzerland. Therefore, our laboratory finds itself confronted with a growing demand for an enantioselective method for the analysis of AM in forensically relevant biological matrices.

Various analytical methods have been reported for the enantioselective determination of AM in whole blood, plasma, urine, oral fluid, and hair. The most extensively used technique has been gas chromatography (GC) coupled with mass spectrometry (MS), comprising off-line derivatization with a chiral reagent and separation of the resulting diastereomeric derivatives on an achiral column, as recently reviewed [7]. Enantioseparation of AM after derivatization to diastereomers has also been achieved on conventional liquid chromatography (LC) stationary phases [8, 9]. A possible drawback of this approach is that such derivatizing reagents might be less than 100 % enantiomerically pure and that additional sample pretreatment steps are needed [10]. Using chiral stationary phases eliminates the need for tedious and time-consuming derivatization steps, enabling direct enantioseparation. Chiral LC methods using MS, UV, or fluorescence detection have been reported for the stereoselective analysis of AM in hair [11–13] and urine [14–16]. AM enantiomers have also been separated by capillary electrophoresis (CE) using chiral selectors as mobile phase additives and MS or UV detection [17–19]. However, compared to GC and LC, CE is much less commonly implemented in forensic laboratories.

In this work, we present a straightforward and sensitive LC-MS/MS method which uses a new chiral stationary phase for separation and quantification of S- and R-AM in urine, a widely employed matrix for drug of abuse testing. We developed a novel chromatographic concept which allows for accelerated sample preparation and LC analysis without compromising the analytical quality, thus overcoming the drawbacks of time-consuming sample preparation procedures and long analytical run times from which previously reported methods have suffered. Main aspects of the presented concept are on-line sample purification using a trapping column and switching valve, and simultaneous analysis of several samples within the same analytical column. The validated method was applied to urine specimens obtained from forensic toxicology cases.

## Material and methods

### Chemicals and reagents

Standard solutions of (±)-AM, S-AM, R-AM (1 g/L), and (±)-AM-*d*<sub>5</sub> (100 mg/L, deuterium atoms on side chain) in

methanol were obtained from Cerilliant (Round Rock, TX, USA). The racemate was used for calibration and quality control, and the pure enantiomers for peak identification. Acetonitrile (99.9 %, HPLC gradient grade) was purchased from Acros Organics (Chemie Brunschwig, Basel, Switzerland) and ammonium hydroxide solution (25 %, for analysis EMSURE®) from Merck (Grogg Chemie, Stettlen, Switzerland). Water was purified in-house with a Milli-Q water system from Millipore (Zug, Switzerland). Blank urine was provided by healthy and abstinent volunteers.

### Calibration, quality control, and internal standard solutions

Serial dilution of the (±)-AM standard solution in acetonitrile yielded working solutions at 0.75, 1.5, 7.5, 15, 75, 150, and 375 mg/L per enantiomer for calibration and at 0.75, 2.25, 60, and 300 mg/L for quality control (QC). Separate dilution series were used for the preparation of calibration and QC working solutions. Adding 5 µL of working solution to 70 µL of blank urine created calibration samples at 0.05, 0.1, 0.5, 1, 5, 10, and 25 mg/L and QC samples at 0.05, 0.15, 4, and 20 mg/L per enantiomer. Calibration and QC samples were freshly prepared on each day of analysis. An internal standard working solution at 10 mg/L was obtained by 1:10 dilution of the (±)-AM-*d*<sub>5</sub> reference material in acetonitrile. All working solutions were stored at about –20 °C in polypropylene tubes.

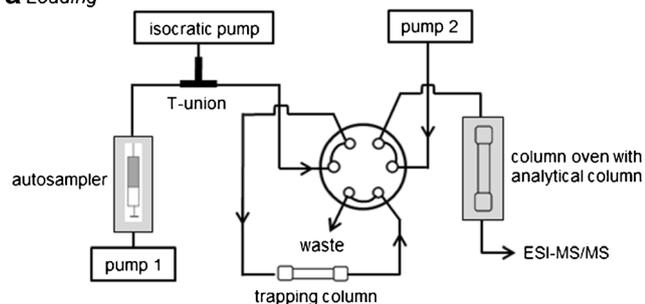
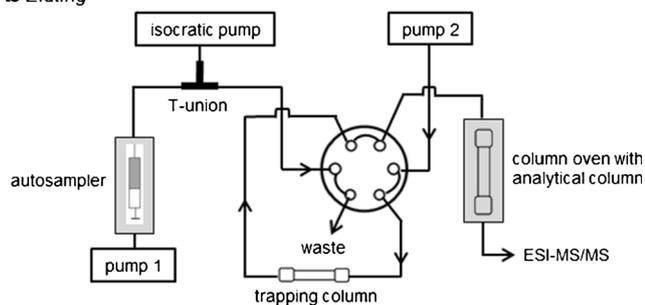
### Sample preparation

Fifteen microliters of internal standard working solution was added to 75 µL of urine sample (calibration, QC, or unknown sample) in an autosampler vial. After adding 1.41 mL of water, the mixture was thoroughly vortexed and 1.0 µL was injected into the column-switching LC-MS/MS system for quantitative analysis.

### Liquid chromatography

Column-switching liquid chromatography was performed using an UltiMate 3000 HPLC system (Dionex, Olten, Switzerland) equipped with two SRD-3600 solvent racks, two HPG-3200RS binary pumps, an ISO-3100SD isocratic pump, a WPS-3000TRS plate autosampler operated at 8 °C, and a TCC-3000RS column compartment set to 30 °C and fitted with a six-port switching valve. Mobile phase A was 0.1 M aqueous ammonia (pH 11) which was freshly prepared on each day of analysis, and mobile phase B was acetonitrile.

The chromatographic setup is represented in Fig. 1. From a 100-µL autosampler loop, the prepared sample was loaded onto the trapping column (Gemini 5 µm C18 110 Å, 10 × 2 mm, Phenomenex, Torrance, CA, USA) with a 400 µL/min flow of 20 % B delivered by binary pump 1. To

**a Loading****b Eluting**

**Fig. 1** Configuration of the column-switching LC-MS/MS system. **a** Loading onto the trapping column and **b** elution to the analytical column. Arrows indicate flow direction. While chromatographic separation in isocratic mode is performed on one sample, the next sample is already loaded onto the trapping column

enhance the retention of the analytes on the trapping column, the injection solution was diluted via a T-union with a 200  $\mu\text{L}/\text{min}$  flow of mobile phase A delivered by the isocratic pump (Fig. 1a). After 1 min, the valve was switched and the retained analytes were transferred in backflush mode to the chiral analytical column (Lux 3  $\mu\text{m}$  AMP LC column, 150  $\times$  3.0 mm, Phenomenex, Torrance, CA, USA), fitted with a KrudKatcher Ultra in-line filter (Phenomenex, Torrance, CA, USA). Elution and detection of the analytes was performed under isocratic conditions with a 500  $\mu\text{L}/\text{min}$  flow of 25 % B delivered by binary pump 2. In the elution configuration (Fig. 1b), the flows of the isocratic pump and binary pump 1 were directed into the waste and were then reduced to 20  $\mu\text{L}/\text{min}$ . After 4 min of elution onto the analytical column, the valve was switched back to the loading position and the flow rates of the isocratic and binary pump 1 were increased to their initial values, without flow interruption on the analytical column. After re-equilibration for 1 min, the next sample was injected and the HPLC program was run again. The last run of a sequence was extended to 12 min in order to ensure elution of all samples from the analytical column. Details on the HPLC program for the column-switching chromatographic procedure are given in Table 1. Chromeleon software version 6.8 (Thermo Fisher Scientific, Waltham, MA, USA) was used to control the HPLC system.

**Table 1** HPLC program for the quantitative analysis of amphetamine enantiomers in urine

Time (min)	Binary pump 1 ( $\mu\text{L}/\text{min}$ ) <sup>a</sup>	Isocratic pump ( $\mu\text{L}/\text{min}$ ) <sup>b</sup>	Binary pump 2 ( $\mu\text{L}/\text{min}$ ) <sup>c</sup>	Position of switching valve
0.0	400	200	500	TC $\rightarrow$ waste
1.0	400	200	500	TC $\rightarrow$ AC
1.1	20	20	500	
4.9	20	20	500	TC $\rightarrow$ waste
5.0	400	200	500	
6.0 <sup>d</sup>	400	200	500	

TC Trapping column, AC analytical column

<sup>a</sup> Mobile phase was 0.1 M aqueous ammonia/acetonitrile, 80/20;  $v/v$

<sup>b</sup> Mobile phase was 0.1 M aqueous ammonia

<sup>c</sup> Mobile phase was 0.1 M aqueous ammonia/acetonitrile, 75/25;  $v/v$

<sup>d</sup> Twelve minutes for the last sample of an analysis sequence

To prevent the accumulation of possible impurities during the isocratic use, column washing was performed after injecting 15 urine samples by running a linear gradient from 20 to 95 % B and 25 to 95 % B, respectively (Table 2). The chromatographic system was held in the loading configuration (Fig. 1a) and a 1- $\mu\text{L}$  aliquot of mobile phase A was injected.

### Tandem mass spectrometry

A 5500 QTRAP® hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a Turbo V ion source (SCIEX, Brugg, Switzerland) was operated in selected reaction monitoring (SRM) mode for mass spectrometric detection. Electrospray ionization was performed in positive ion mode using the following settings: ion source voltage, 3500 V; source temperature, 600  $^{\circ}\text{C}$ ; curtain gas, 40; collision gas, medium; gas 1, 40; and gas 2, 60 (arbitrary units for the gas settings). Optimal SRM parameters were determined by direct infusion of a 100  $\mu\text{g}/\text{L}$  methanolic solution of ( $\pm$ )-AM and ( $\pm$ )-AM- $d_5$ , respectively, at a flow rate of 10  $\mu\text{L}/\text{min}$  using a syringe pump. Quantifier and qualifier mass transitions were selected on the basis of signal intensity. Monitored transitions were  $m/z$  136.1 $\rightarrow$ 91.0 (quantifier) and  $m/z$  136.1 $\rightarrow$ 119.0 (qualifier) for AM enantiomers, and  $m/z$  141.1 $\rightarrow$ 93.0 for AM- $d_5$  enantiomers. Collision energies were 26, 11, and 26 eV, respectively. Declustering potential, entrance potential, collision cell exit potential, and dwell time were 45 V, 10 V, 8 V, and 150 ms, respectively, for all transitions. Analyst software version 1.6.2 (SCIEX, Brugg, Switzerland) was used for data acquisition and processing. The HPLC system and the mass spectrometer were controlled separately. MS/MS data acquisition was started 2 min after sample injection and lasted for 6 min.

**Table 2** HPLC program for column washing

Time (min)	Binary pump 1		Isocratic pump Flow ( $\mu\text{L}/\text{min}$ )	Binary pump 2		Position of switching valve
	% B	Flow ( $\mu\text{L}/\text{min}$ )		% B	Flow ( $\mu\text{L}/\text{min}$ )	
0.0	20	400	200	25	500	TC $\rightarrow$ waste
0.5	20	400	200	25	500	
1.5	95	400	20	95	500	
5.0	95	400	20	95	500	
5.5	20	400	200	25	500	
8.0	20	400	200	25	500	

TC trapping column

## Validation

Validation criteria included linearity, intra- and inter-assay accuracy and precision, lower limit of quantification (LLOQ), selectivity, matrix effect, carry-over, dilution integrity, and re-injection reproducibility. Method validation was conducted according to international guidelines [20, 21] and as previously reported [22].

Linearity was assessed by analyzing duplicate calibration curves on three different days ( $n = 6$ ). Calibration curves were generated by calculating the peak area ratio of analyte to internal standard for each analyte concentration ( $x$ ) and were fit by unweighted linear least-squares regression and linear least-squares regression applying a  $1/x$  and  $1/x^2$  weighting factor, respectively. The most appropriate calibration model was chosen by inspection of the percentage relative error (%RE) which compares the concentration calculated from the regression equation with the target value [23]. Linearity was acceptable if the correlation coefficient ( $r$ ) was at least 0.99 and calibrators quantified within  $\pm 15\%$  of the nominal concentration ( $\pm 20\%$  at LLOQ).

Accuracy and precision were evaluated at low, mid, and high QC level as well as at the LLOQ level. Intra-assay accuracy and precision were assessed with six replicates per level in a single run. For the determination of the inter-assay accuracy and precision, six replicates of each QC sample were prepared and analyzed on three different days ( $n = 18$ ). QC concentrations were calculated from daily calibration curves. Accuracy was defined as the percentage of the mean concentration of all replicates from the corresponding nominal concentration and was expected to be 85–115% (80–120% at LLOQ). Precision was expressed as percent coefficient of variation (% CV) of the calculated concentrations and should not exceed 15% (20% at LLOQ).

For evaluation of method selectivity, blank urine specimens from six different donors were analyzed to identify possible interferences from endogenous compounds. In addition, selectivity was assessed by spiking the different blank urine sources to the LLOQ level. Selectivity was demonstrated if any signal in the blank samples was  $\leq 20\%$  of the LLOQ and  $\leq 5\%$

internal standard response and if the LLOQ samples quantified within  $\pm 20\%$  of the nominal concentration.

Matrix effects were investigated through post-column infusion experiments. A methanolic solution containing ( $\pm$ )-AM and ( $\pm$ )-AM- $d_5$  at a concentration of 200  $\mu\text{g}/\text{L}$  each was infused continuously at a flow rate of 30  $\mu\text{L}/\text{min}$  via a T-union into the HPLC eluent during the LC-MS/MS analysis of blank urine samples from six different subjects. The signals of the monitored SRM transitions were examined for any suppression or enhancement over the entire chromatographic run.

Carry-over was assessed by injecting a blank specimen immediately after the highest calibration standard in three independent runs. Absence of carry-over was verified if any response in the blank specimens was  $\leq 20\%$  of the LLOQ and  $\leq 5\%$  of the internal standard response.

Dilution integrity was evaluated by diluting urine samples spiked with ( $\pm$ )-AM at a concentration of 75  $\text{mg}/\text{L}$  with blank urine to achieve 1:10 and 1:5 ( $v/v$ ) dilution ( $n = 6$  per dilution factor). Internal standard solution was added and samples were processed as described. Dilution integrity was considered acceptable if specimens quantified within  $\pm 15\%$  of expected diluted concentrations and with acceptable precision ( $\leq 15\%$  CV).

Re-injection reproducibility was examined with six replicates per QC level. These samples were analyzed immediately after sample preparation and were then stored at 8  $^{\circ}\text{C}$  in the autosampler. After 6 days, they were re-injected and quantified against the re-injected calibration curve. Reproducibility was proven if re-analyzed samples quantified within  $\pm 15\%$  of the first measurement.

## Application to authentic urine samples

The presented method was applied to 67 urine specimens which were obtained from forensic toxicology cases and were AM positive by our routinely used qualitative enzyme immunoassay (Immunanalysis, Pomona, CA, USA, 500  $\mu\text{g}/\text{L}$  cutoff for AM). In all the cases considered for this work, the positive immunoassay results for urine were followed by quantitative analysis of AM, methamphetamine (MA) and four

amphetamine-derived designer drugs in whole blood by a well-established GC-MS method in our laboratory. In 42 cases, both the urine and blood samples were positive for AM only. In two of these cases, the persons concerned had been treated with clinically relevant doses of Elvanse® (30–70 mg/day). In the other 40 cases with only AM present, AM abuse was assumed. In the remaining 25 cases, both the urine and blood samples were positive for AM and MA, indicating that the consumed drug was MA, with the detected AM being a metabolite of MA [24]. During the time between the immunological screening and the enantioselective analysis with the presented method (1–12 months), the urine samples were stored at  $-20\text{ }^{\circ}\text{C}$ .

Reliability and reproducibility of the AM enantiomer concentrations in authentic urine samples were verified by re-analyzing a random selection of 23 samples after storage at about  $-20\text{ }^{\circ}\text{C}$  for 2 to 3 weeks. The difference between the initial concentration and the concentration measured during re-analysis should not exceed 20 % of their mean for at least 67 % of the repeats, as it is generally required for valid sample re-analysis [21].

## Results and discussion

### Method development and analytical performance

We have developed an analytical method for the enantioselective determination of R- and S-AM in human urine. Our goals of method development were minimal sample preparation and a short analysis time. To meet these criteria, we used a column-switching HPLC system consisting of an achiral reversed-phase trapping column and a chiral analytical column combined with tandem mass spectrometry. In comparison to existing GC-MS methods [6, 25–29], chiral LC-MS/MS enables direct separation and analysis of AM enantiomers, rendering time-consuming sample derivatization steps unnecessary.

Sample purification was achieved on-line by using a trapping column, limiting off-line sample pretreatment to a simple dilution step with water. With the mobile phases described, the analytes were retained and concentrated on the trapping column, while salts and other urine constituents were washed away to waste. Compared to the off-line solid-phase extraction and liquid-liquid extraction procedures commonly used for preparing urine samples for stereoselective AM analysis [6, 16–19, 25–29], on-line sample cleanup has the advantages of being time- and cost-efficient and less prone to sample contamination, human error, and reproducibility problems. In addition, the use of a trapping column allows for large injection volumes without affecting the chromatographic performance of the analytical column, thus making the presented method easily transferable between different mass spectrometers by

simply adjusting the injection volume according to the instrument's sensitivity.

The AM enantiomers were retained as racemic mixture on the achiral C18 trapping column. Switching of the valve backflushed the enriched and cleaned analytes and transferred them onto the analytical column for enantioselective separation and detection. Chiral separation was achieved on a Lux 3  $\mu\text{m}$  AMP LC column containing a novel stationary phase which was specifically designed for the chiral analysis of AM and substituted AMs. Various mobile phase conditions were investigated to optimize analyte trapping and enantioseparation. Enantioselectivity was found to be pH dependent and was optimal under strongly alkaline conditions. Therefore, 0.1 M aqueous ammonia (pH 11) was used for pH adjustment of the mobile phase. The employed Gemini C18 trapping column is characterized by enhanced stability under extreme pH conditions and offers good retention of basic compounds at an alkaline pH value. Binary mixtures of aqueous ammonia and acetonitrile or methanol as well as ternary mixtures of aqueous ammonia, acetonitrile, and methanol were tested in the isocratic and gradient elution mode. Enantioseparation was better in the isocratic elution mode than with gradient elution, and methanol as organic modifier produced somewhat higher enantioselectivity than acetonitrile and acetonitrile/methanol mixtures. However, acetonitrile gave sharper peaks, shorter retention times, and lower backpressure while still providing sufficiently high enantioselectivity. Thus, isocratic conditions with acetonitrile as organic solvent were chosen.

Column washing by running a gradient was only carried out after every 15 injections, which saved column re-equilibration time and proved to be sufficient to avoid accumulation of impurities. Performing isocratic elution and intermittent column washing allowed for simultaneous analysis of several samples on the analytical column. Injection, cleanup, and backflush of a sample were carried out before the previous sample had completely eluted from the analytical column, resulting in up to three samples migrating through the analytical column at once. Careful optimization of the injection and valve switching times (Table 1) ensured that no intermixing of the sample bands took place. Using this approach, an average sample analysis time of 6.4 min could be achieved. Compared to a conventional sequential analysis with the same HPLC system, this multiple-injection-approach significantly increases sample throughput, resulting in a shorter analysis time than the existing methods for stereoselective determination of AM in urine [6, 14–19, 25–29]. Other approaches such as parallel analysis with several columns would require additional HPLC columns, additional pumps, and a second valve.

The presented method achieved sufficient separation of the two AM enantiomers with average retention times of 1.92 and 2.90 min, respectively. Retention times were highly reproducible and stable over a large number of injections. By analyzing

enantiomerically pure standards, the earlier eluting peak was identified as R-AM (Fig. 2).

## Validation

Evaluation of relative percentage errors showed that linear least-squares regression with a  $1/x$  weighting factor was the most appropriate calibration model. The LLOQ, defined as the lowest concentration that can be quantified with acceptable accuracy (80–120 %) and precision ( $\leq 20$  % CV), was determined to be 0.05 mg/L for both AM enantiomers. An extracted ion chromatogram of a urine sample spiked at the LLOQ level is presented in Fig. 2b. The method was linear over the tested calibration range (0.05–25 mg/L for both AM enantiomers) with correlation coefficients  $r \geq 0.994$  when employing  $1/x$  weighting. Back-calculated concentrations of the calibration samples were within  $\pm 13.2$  % of the target concentrations ( $\pm 17.2$  % at LLOQ).

Both AM enantiomers demonstrated acceptable accuracies and precisions at all QC concentrations (Table 3). Accuracy ranged from 90.1 to 103.0 % and precision was less than 14.1 % CV for the two AM enantiomers at each QC level.

The method proved to be selective. No significant endogenous interferences could be detected at the expected retention times of the AM and AM- $d_5$  enantiomers in any of the tested blank urine samples, and quantification at the LLOQ level was within 12.2 % for both enantiomers in all six urine sources. An extracted ion chromatogram for a blank urine specimen is shown in Fig. 2a.

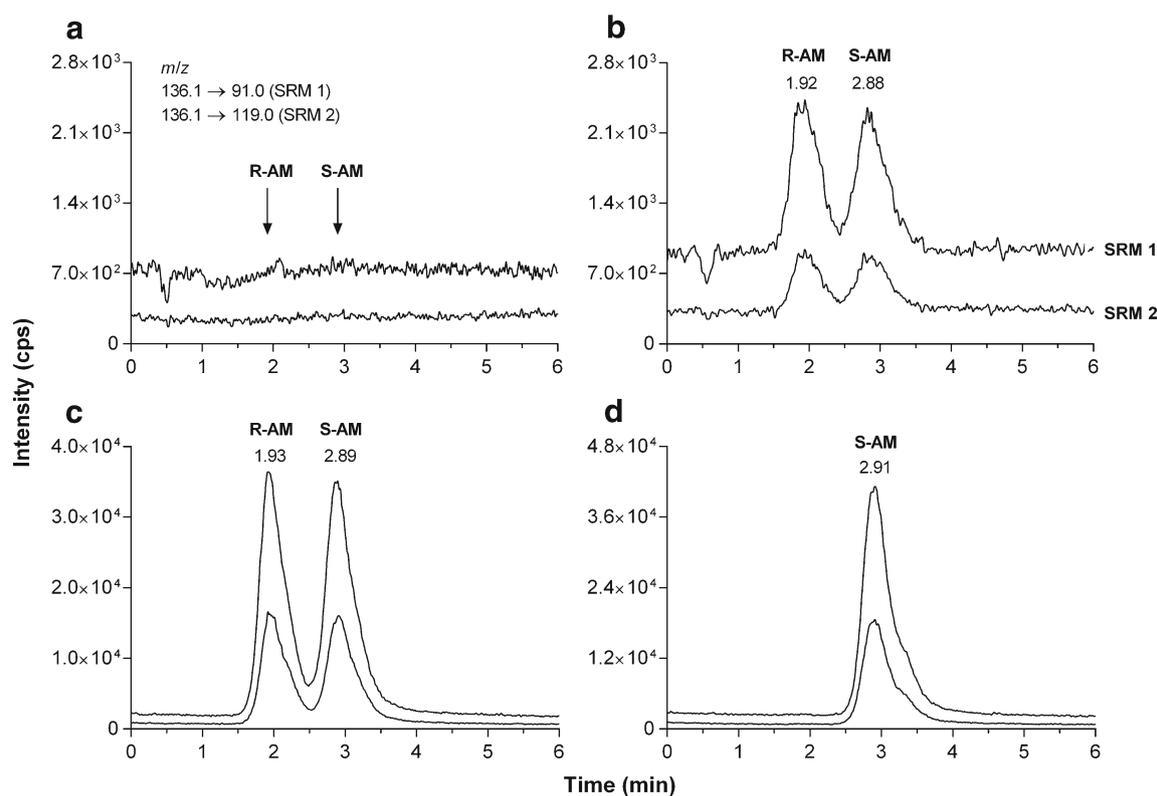
No significant changes of the ion intensities were observed in the infusion profiles of AM and AM- $d_5$  after injection of blank urine samples, indicating that matrix effects were negligible.

Any signal detected in blank specimens injected after the highest calibration standard was insignificant, demonstrating absence of carry-over.

Samples spiked at concentrations exceeding the highest calibration standard could be accurately quantified when diluted either 5- or 10-fold in blank urine. Accuracy was better than 88.3 % and precision was less than 4.1 % CV for both AM enantiomers.

Re-injection reproducibility was acceptable. Processed samples were within  $\pm 14.7$  % of initially measured values when re-analyzed after storage at 8 °C for 6 days.

The stability of AM in urine under different storage conditions has been demonstrated previously [25, 30, 31] and was therefore not assessed with the presented method.



**Fig. 2** SRM ion chromatograms of **a** blank urine (arrows indicate expected retention times); **b** analytes at the lowest calibration level (0.05 mg/L per enantiomer); **c** an authentic urine specimen from an AM

abuser (R-AM, 1.54 mg/L; S-AM, 1.40 mg/L); and **d** an authentic urine specimen from a patient treated with S-AM (R-AM, 0 mg/L; S-AM, 1.51 mg/L)

**Table 3** Accuracy and precision data. Analyte concentrations of LLOQ and low, mid, and high QC samples were 0.05, 0.15, 4, and 20 mg/L, respectively

QC level	Intra-assay ( <i>n</i> = 6)		Inter-assay ( <i>n</i> = 18)	
	Accuracy (% recovery)	Precision (% CV)	Accuracy (% recovery)	Precision (% CV)
R-Amphetamine				
LLOQ	98.0	14.1	92.8	11.2
Low	92.2	5.5	90.1	4.6
Mid	99.2	6.1	94.8	6.6
High	99.8	4.6	96.3	5.3
S-Amphetamine				
LLOQ	103.0	6.8	96.4	8.7
Low	94.0	5.9	91.3	4.7
Mid	93.5	10.8	94.4	7.5
High	96.9	5.3	95.1	5.9

### Proof of applicability

The method was applied to the analysis of 67 forensic urine samples. In each of the 40 samples obtained from suspected AM abusers, both AM enantiomers were present (Table 4,

samples 1–40). Median (range) concentrations were 3.99 mg/L (0.50–13.4 mg/L) for R-AM and 2.68 mg/L (0.46–9.77 mg/L) for S-AM. R/S-AM ratios were calculated as the concentration of the R-enantiomer divided by the concentration of the S-enantiomer. The median R/S ratio was 1.25 (0.97–1.94), indicating a

**Table 4** Concentrations of AM enantiomers and R/S-enantiomer ratios determined in 67 urine samples obtained from suspected AM abusers (samples 1–40), subjects treated with an S-AM prodrug (samples 41–42), and suspected MA abusers (samples 43–67)

Sample number	R-AM (mg/L)	S-AM (mg/L)	R/S ratio	Sample number	R-AM (mg/L)	S-AM (mg/L)	R/S ratio	Sample number	R-AM (mg/L)	S-AM (mg/L)	R/S ratio
1	1.19	0.80	1.49	24	8.22	7.78	1.06	47	0.16	1.11	0.14
2	7.44	7.02	1.06	25	8.79	7.08	1.24	48	0.06	2.23	0.03
3	1.54	1.40	1.10	26	1.16	1.02	1.14	49	<LLOQ	3.00	–
4	8.76	6.61	1.33	27	6.89	6.80	1.01	50	<LLOQ	22.90	–
5	7.41	6.61	1.12	28	1.15	0.90	1.28	51	0.40	13.30	0.03
6	3.25	2.52	1.29	29	9.20	7.22	1.27	52	3.26	14.70	0.22
7	2.75	2.55	1.08	30	2.13	1.15	1.85	53	0.23	0.50	0.47
8	6.43	5.12	1.26	31	7.46	6.04	1.24	54	n.d.	2.94	–
9	1.49	0.77	1.94	32	2.33	1.85	1.26	55	0.10	1.53	0.07
10	0.82	0.72	1.14	33	12.60	9.64	1.31	56	n.d.	6.69	–
11	10.80	8.17	1.32	34	4.73	4.39	1.08	57	5.69	22.60	0.25
12	5.54	4.46	1.24	35	5.50	3.68	1.49	58	<LLOQ	0.44	–
13	1.37	0.82	1.68	36	12.20	9.77	1.25	59	n.d.	0.76	–
14	0.50	0.46	1.09	37	4.03	2.52	1.60	60	0.50	18.70	0.03
15	8.28	6.44	1.29	38	2.41	2.11	1.14	61	0.43	20.00	0.02
16	3.94	2.80	1.41	39	1.01	0.97	1.04	62	0.80	15.90	0.05
17	13.40	8.43	1.59	40	0.63	0.65	0.97	63	0.60	1.45	0.41
18	1.62	1.17	1.38	41	n.d.	1.51	–	64	<LLOQ	2.99	–
19	2.32	2.36	0.98	42	<LLOQ	1.35	–	65	0.12	16.70	0.01
20	6.50	5.58	1.16	43	n.d.	6.40	–	66	<LLOQ	1.02	–
21	5.07	3.75	1.35	44	0.34	1.75	0.19	67	n.d.	7.94	–
22	3.09	2.03	1.52	45	<LLOQ	4.14	–				
23	0.60	0.55	1.09	46	1.32	6.49	0.20				

n.d. not detected, <LLOQ below limit of quantification

predominance of the R-enantiomer. The expected urinary R/S ratio for abusers of illicit racemic AM would be 1. The observed predominance of the R-enantiomer may be explained by the stereoselective metabolism of AM, with the S-enantiomer being metabolized faster than the R-enantiomer, which results in higher concentrations of R-AM [32, 33]. In accordance with our observation, Tetlow et al. [6] reported an average urinary R/S ratio of  $1.19 \pm 0.19$  for AM abusers and Nyström et al. [34] found R/S ratios  $>1$  for AM in blood and hair samples from a patient receiving AM racemate.

In the two urine specimens from subjects prescribed Elvanse® at a dosage of 30–70 mg/day (Table 4, samples 41 and 42), S-AM concentrations were determined to be 1.51 and 1.35 mg/L, respectively. R-AM was undetectable in the first sample, whereas in the second sample R-AM was detected at a concentration of  $<LLOQ$ . Since Elvanse® (lisdexamfetamine [L-lysine-dextroamphetamine] dimesylate) is a prodrug of S-AM [35], the R-enantiomer was not expected to be present in these urine samples. However, the measured R-AM concentration was very low, suggesting that it might be due to enantiomeric impurities arising from the drug manufacture itself. Lisdexamfetamine is described as a single-enantiomer drug, but without providing purity specifications [36]. To our knowledge, enantioselective quantification of AM in biological fluids after lisdexamfetamine administration has not yet been performed and thus no data are available to confirm our assumption. As a comparison, Tetlow et al. [6] and George et al. [5] reported average urinary R/S-AM ratios of 0.13 and 0.08, respectively, for subjects being treated with a pharmaceutical preparation of S-AM which contained approximately 5 % R-AM impurity. Based on their data, they proposed that an R/S ratio  $<0.3$  and  $<0.2$ , respectively, would suggest patient compliance. We observed an R/S ratio significantly lower than these cutoffs and thus both subjects were assumed to be compliant with their lisdexamfetamine treatment and abstinent from illicit racemic AM. Thevis et al. [37] analyzed urine samples provided by a patient receiving 30 mg of Elvanse® once-daily (corresponding to 17.3 mg of lisdexamfetamine free base) by means of an achiral LC-MS/MS method and reported ( $\pm$ )-AM concentrations of 0.04, 0.11, 0.45, and 1.15 mg/L at 3, 6, 9, and 11 h post-administration. Therefore, we conclude that the urinary concentrations of the herein measured AM enantiomers are plausible.

In 80 % of the urine samples obtained from individuals suspected of MA abuse, both AM enantiomers were detected, although a strong predominance of the S-form was observed (Table 4, samples 43–67). In these cases, the median (range) concentrations were 0.20 mg/L ( $<LLOQ$ –5.69 mg/L) for R-AM and 3.57 mg/L (0.44–22.9 mg/L) for S-AM. R/S ratios in samples exhibiting concentrations above the LLOQ ranged from 0.01 to 0.47 (median 0.10). Five samples collected from suspected MA abusers contained only S-AM, with concentrations ranging from 0.76 to 7.94 mg/L. The total concentrations

of the AM enantiomers were in the range of those measured by Kumihashi et al. [38] in urine samples from MA abusers. As reported for AM, the S-enantiomer of MA was found to be metabolized at a greater rate than the R-enantiomer [24]. Thus, more S-MA is converted to S-AM than R-MA to R-AM. Keeping this in mind, the measured urine samples from suspected MA abusers can be classified into three groups according to the observed stereoisomeric profiles of AM and the assumed composition of the consumed MA: (I) only S-AM present, this indicates misuse of pure S-MA; (II) R/S ratios between 0.01 and 0.07 or R-AM concentrations  $<LLOQ$ , this might be due to intake of S-MA containing small amounts of R-MA; (III) R/S ratios between 0.14 and 0.47, consumption of racemic MA. This classification is supported by the findings reported by Li et al. [24], Nagai et al. [39], and Wang et al. [40].

From the results described here, it can be seen that the measured urinary R/S-AM ratios proved useful for discriminating between subjects using illicit racemic AM and those undergoing S-AM treatment. However, when reviewing laboratory results, it must be taken into consideration that the presence of AM in urine can be the result of MA metabolism. We observed that MA abuse can yield very similar urinary R/S-AM ratios as intake of S-AM medication. Therefore, in critical cases, MA should be identified as well. Careful consideration must also be given to the existence of AM enantiomers eliminated in urine as the metabolites of therapeutic drugs such as selegiline and famprofazone [41].

Incurred sample re-analysis was performed with a random selection of 23 authentic urine samples. The percentage difference between the original and analyzed R-AM concentrations was within 20 % for 82.6 % of the repeated samples. For S-AM, 91.3 % of the repeats were within 20 %. Thus, both enantiomers met the predetermined criteria, further demonstrating the reliability and reproducibility of the presented method.

## Conclusion

In this paper, a novel LC-MS/MS method for the quantification of AM enantiomers in human urine is described, which offers significant advantages in overall speed and simplicity over existing methods. The presented method proved to be sensitive, selective, accurate, and precise and was successfully used on authentic urine samples. The enantioselective determination of AM in urine can help clinicians and forensic experts to differentiate between illegal and legitimate use of AM.

The chromatographic concept presented in this work allows for minimal sample pretreatment and increased sample throughput in chiral HPLC under isocratic conditions and thus presents an attractive cost-effective approach for stereoselective pharmacokinetic studies which are an important issue in therapeutic drug development and usually involve a large number of samples.

### Compliance with ethical standards

**Informed consent** Informed consent was obtained from all individuals providing urine samples.

**Conflict of interest** The authors declare that they have no conflict of interest.

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