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Stereoselective method development and validation for determination of concentrations of amphetamine-type stimulants and metabolites in human urine using a simultaneous extraction–chiral derivatization approach

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ABSTRACT

Amphetamine-type stimulants (ATS) are a group of chiral amine drugs which are commonly abused for their sympathomimetic and stimulant properties. ATS are extensively metabolised by hepatic cytochrome P450 enzymes. As metabolism of ATS has been shown to be highly stereospecific, stereoselective analytical methods are essential for the quantitative determination of ATS concentrations for both in vivo and in vitro studies of ATS metabolism. This paper describes a new stereoselective method for the simultaneous determination of amphetamine (AM), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), 4-hydroxy-3-methoxyamphetamine (HMA), 3,4-hydroxymethamphetamine (HHMA) and 3,4-hydroxyamphetamine (HHA) in human urine samples validated according to the United States Food and Drug Administration guidelines. In this method, analytes are simultaneously extracted and derivatized with *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (*R*-MTPCl) as the chiral derivatization reagent. Following this, the analytes were subjected to a second derivatization with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) which targets the hydroxyl groups present in HMMA, HMA, HHMA and HHA. The derivatized analytes were separated and quantified using gas chromatography–mass spectrometry (GC–MS). The method was evaluated according to the established guidelines for specificity, linearity, precision, accuracy, recovery and stability using a five-day protocol. Intra-day precision ranged from 0.89 to 11.23% RSD whereas inter-day precision was between 1.03 and 12.95% RSD. Accuracy values for the analytes ranged from −5.29% to 13.75%. Limits of quantitation were 10 μg/L for AM, MA, MDMA, HMA and HMMA and 2 μg/L for MDA, HMA and HHA. Recoveries and stability values were also within accepted values. The method was applied to authentic ATS-positive samples.

1. Introduction

Amphetamine (AM), methamphetamine (MA) and 3,4-methylenedioxymethamphetamine (MDMA) are drugs of abuse classified as amphetamine-type stimulants (ATS). ATS are consumed for their stimulant and hallucinogenic effects and have been shown to be highly neurotoxic and hepatotoxic [1]. Many ATS such as AM, MA and MDMA are racemic drugs which are stereoselectively metabolised in the liver by cytochrome P450 enzymes. Although chemically identical, enantiomers of ATS exhibit different pharmacokinetic and pharmacodynamic properties due to differences in binding affinities to their receptor sites [2]. Because of the significance of stereoselective metabolism of ATS, it is important that bioanalytical methods for ATS and related metabolites are able to differentiate between the individual enantiomers of ATS. The stereoselective metabolism of ATS has also been postulated as a potential reason for the documented inter-individual variability in toxicity and side-effects of MDMA and other related ATS [1]. Thus, the ability to quantitatively assay low concentrations of ATS stereoisomers is crucial to obtain an in-depth understanding of the pharmacokinetics of ATS metabolism and its related effects. In addition to this, the ability to identify ATS enantiomers is also important in forensic and toxicological applications as an aid to distinguish illicit consumption of ATS [3–6].

As enantiomers have identical physicochemical properties, conventional separation methods such as gas chromatography (GC)

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or liquid chromatography (LC) are unable to discriminate between enantiomers without the use of chiral chromatography or chiral derivatization prior to analysis [7]. Derivatization of enantiomers with a chiral derivatization reagent (CDR) is a commonly used approach for the analysis of stereoisomers. The reaction between an enantiomer and a CDR results in diastereomers which are amenable to separation and identification by normal chromatographic methods. An additional advantage of derivatization is that it improves sensitivity [8].

Several publications have reported the use of CDRs for stereospecific analysis of ATS in biological samples. Among CDRs commonly used are *S*-(−)-N-(heptafluorobutyryl)prolyl chloride (*S*-HFBPCl) [9–11], *S*-(−)-N-(trifluoroacetyl)prolyl chloride (*S*-TPC) [12–15] and *R*-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (*R*-MTPCl) [3,16–19]. Derivatization with *S*-HFBPCl results in diastereomers with excellent chromatographic properties and distinctive mass spectra, however the use of *S*-HFBPCl is disadvantageous as it is not commercially available and needs to be synthesized in the laboratory [20]. Even though *S*-TPC readily reacts with ATS, it has been reported to be of low enantiomeric purity [3] and is prone to racemisation during the derivatization process [21]. On the other hand, *R*-MTPCl readily reacts with primary and secondary amine enantiomers to form stable amide diastereomeric derivatives with excellent chromatographic properties [7]. It is also available in high enantiomeric purity which is essential for accurate quantitative chiral derivatization of analytes.

In this study, a chiral derivatization approach was used to separate and quantify ATS enantiomers using gas chromatography–mass spectrometry (GC–MS) with *R*-MTPCl as the CDR. However, in vivo, ATS such as MDMA are metabolised into catecholamine compounds containing polar hydroxyl groups which are difficult to analyse by GC–MS. Therefore, analysis of these metabolites require a second derivatization reaction targeted at the hydroxyl groups in order to analyse these metabolites with adequate sensitivity using GC–MS. The second derivatization was achieved using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA).

Prior to derivatization and GC–MS analysis, a sample clean-up step is generally required. Several papers have reported the use of solid-phase extraction (SPE) for the isolation of analytes from the sample matrix, followed by derivatization of the dried extract [5,18,22]. However, the use of SPE requires several steps to be performed. The novelty of this method is that we have implemented a new approach using a simultaneous liquid–liquid extraction and derivatization methodology to simplify sample preparation and decrease the analytical time. This approach represents an improvement over other published studies [16,17,19,21,22] which also use *R*-MTPCl as a derivatization reagent in a separate step after isolation of the analytes from the matrix. We have previously demonstrated that such an approach was able to simultaneously extract and derivatize MDMA, AM, MA and 4-hydroxy-3-methoxymethamphetamine (HMMA) from human urine samples and subsequently a fractional factorial design was used to optimize the recovery and the sensitivity of our method [23]. Based on the findings from our previous work, we now describe a new, stereoselective assay for AM, MA, MDMA, 3,4-methylenedioxyamphetamine (MDA), HMMA, 4-hydroxy-3-methoxyamphetamine (HMA), 3,4-hydroxymethamphetamine (HHMA) and 3,4-hydroxyamphetamine (HHA) in human urine samples which has been validated according to the United States Food and Drug Administration (FDA) guidelines [24]. Of these analytes, AM, MA, MDMA and MDA are commonly abused ATS, whereas HMMA, HMA, HHMA and HHA are metabolites resulting from the metabolism of MDMA. The simultaneous determination method is fast, economical and is applicable to the routine analysis of human samples.

2. Materials and methods

2.1. Reagents and materials

Methanolic drug standards of racemic AM, MA, MDA and MDMA (1000 μg/mL of free base) and AM-d5, MA-d5 and MDMA-d5 (100 μg/ml of free base) were purchased from Cerilliant (Round Rock, TX, USA). HMMA, HMA, HHA and HHMA were synthesized in our lab according to the method published by Forsling et al. [25]. Dihydroxybenzylamine (DHBA) and hexane were obtained from Fisher Scientific (Hampton, NH, USA). Ethyl acetate was purchased from Merck (Darmstadt, Germany). β-Glucuronidase from *Helix pomatia* with glucuronidase and sulfatase activity, triethylamine (TEA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) and sodium metabisulfite (SMBS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The chiral derivatization reagent *R*-MTPCl (ChiraSelect grade, 99% purity, 99.5:0.5 enantiomeric ratio) was obtained from Sigma–Aldrich, whereas MSTFA was obtained from Chem Fabrik Karl Bucher GMBH (Waldstetten, Germany). All reagents and solvents used were of analytical grade.

2.2. Sample preparation

Urine samples (2 mL) in PTFE-lined screw cap culture tubes were spiked with 25 ng each of AM-d5, MA-d5, MDMA-d5 and 10 ng each of MDA-d5 and DHBA as internal standards. A mixture of 200 μL of 250 mM SMBS and 200 μL of 250 mM EDTA was added to preserve the dihydroxylated compounds. The samples were then adjusted to pH 9 with 1 mL 1 M sodium carbonate buffer. TEA (5 μL) was added and the sample was vortex-mixed thoroughly. A solution of 3 mL hexane–ethyl acetate (2:1) containing 100 μL of 1% *R*-MTPCl was then added and the sample was vortexed for 1 min. Following this, the samples were mixed on a rotary mixer for 20 min at 40 rpm. Subsequently the samples were centrifuged at 3000 rpm for 5 min and the upper organic layer was transferred using a glass Pasteur pipette to another culture tube. The samples were dried on a heating block at 40 °C under a gentle flow of oxygen-free nitrogen gas to dryness. The residue was reconstituted in 50 μL of MSTFA and further incubated on a heating block at 80 °C for 20 min. After cooling to room temperature, the samples were transferred to autosampler vials before injecting into the GC–MS.

2.3. GC–MS analysis

All samples were analysed using an Agilent (Santa Clara, CA, USA) 6890 series gas chromatograph equipped with a model 5973 mass selective detector. Injections were performed using an Agilent auto sampler (model 7893) and injector. Separations were achieved using a HP-5ms column (20 m, 250 μm i.d., 0.25 μm film thickness). The GC parameters were helium as carrier gas with a column flow of 1.0 mL/min. The oven temperature programme was as follows: held at 100 °C for 1 min, to 238 °C at 15 °C/min and held for 3.5 min, to 310 °C at 40 °C/min and held for 1 min for a total run time of 16.5 min. Samples were introduced using a 2 μL split injection with injector port temperature at 250 °C, a split ratio of 20:1 and a split flow of 20 mL/min. The MS parameters were electron impact ionization with an ion source temperature of 230 °C, transfer line temperature at 280 °C and a solvent delay time of 5 min. Analysis was performed in selected ion monitoring (SIM) mode. The mass/charge (*m*/*z*) values monitored in SIM mode are listed in Table 1.

2.4. Assay validation

The developed method was validated according to the guidelines specified by the FDA [24]. Based on these guidelines, the

Table 1
Mass-to-charge values monitored in SIM mode for GC–MS analysis.[a]

Analytes	Mass-to-charge values (*m*/*z*)
AM	260,119, 189
AM-d5	264, 124, 189
MA	274, 91, 189
MA-d5	278, 92, 189
MDA	162, 189, 135
MDA-d5	167, 189, 135
MDMA	162, 189, 274
MDMA-d5	164, 189, 278
DHBA	267, 179, 189
HMMA	236, 189, 274
HMA	236, 189, 260
HHMA	294, 189, 274
HHA	294, 189, 260

[a] Underlined *m*/*z* values represent the ions used for quantification.

following parameters were determined for all analytes: method selectivity, linearity, accuracy, precision, recovery, limits of detection and quantitation as well as sample stability.

2.4.1. Preparation of solutions

An aqueous working solution containing 10 μg/mL of AM, MA, MDMA, HMMA and HMMA and 2 μg/mL of MDA, HMA and HHA respectively was prepared in distilled water containing 3% SMBS and 3% EDTA for the preservation of dihydroxylated compounds. An aqueous internal standard working solution containing 1 μg/mL of AM-d5, MA-d5, MDA-d5, MDMA-d5 and DHBA respectively was also prepared. All working solutions were protected from light and stored at 4 °C.

2.4.2. Preparation of QC samples

The QC concentrations used for validation were selected according to the recommendations by the FDA guidelines which suggest a minimum of three concentrations: one within three times the LLOQ concentration, one near the center and one near the upper boundary of the calibration curve [24]. The quality control samples were prepared by spiking the appropriate volume of working solution in drug-free blank urine. Two millilitres were then aliquoted into screw-cap culture tubes and stored at −20 °C in the dark until required. The concentration levels prepared for the QC samples were (1) lower limit of quantification QC (LLOQ) containing 10 μg/L AM, MA, MDMA, HMMA, HHMA and 2 μg/L MDA, HHA, HMA, (2) medium QC (MED) containing 200 μg/L AM, MA, MDMA, HMMA, HHMA and 40 μg/L MDA, HHA, HMA and (3) high QC (HIGH) containing 500 μg/L AM, MA, MDMA, HMMA, HHMA and 100 μg/L MDA, HHA, HMA respectively.

2.4.3. Preparation of R-MTPCl

Aliquots of 10 μL *R*-MTPCl were transferred to 4.0 mL amber glass vials, sealed with PTFE-lined screw caps and stored at −20 °C. When required, a vial was thawed to room temperature and diluted with 1 mL of hexane to give a 1% solution of *R*-MTPCl in hexane.

2.4.4. Method selectivity and specificity

For determining method selectivity and specificity, blank urine samples that have previously been confirmed to be free from the drugs were assayed in this method using an in-house validated toxicological screen.

For determination of selectivity, blank urine samples from six different donors were analysed to identify any possible interferences from endogenous compounds.

As for method specificity, first a blank urine sample containing only internal standards was also analysed to rule out the possibility of cross-contribution from the internal standard ions to the analyte ions [26]. Finally, blank urine samples spiked with amphetamine-type stimulants (ephedrine, pseudoephedrine, phentermine and pholedrine) and other commonly abused drugs (cannabis and cocaine) at 1000 μg/L each were analysed to identify any possible interferences with the peaks of interest.

2.4.5. Linearity

The linearity was tested between 10 and 500 μg/L for single enantiomers of AM, MA, MDMA, HMMA and HHMA using an 8-point calibration curve (10, 25, 50, 100, 200, 300, 400 and 500 μg/L). For MDA, HHA and HMA, the linear range tested was 2–100 μg/L using an 8-point calibration curve (2, 5, 10, 20, 40, 60, 80, 100 μg/L). A total of three replicates were performed for each concentration level over three days. Mean peak area ratios of analyte to its respective internal standard were determined and the regression model was fitted using a weighted (1/concentration) least-squares regression analysis. The mean back-calculated concentrations of each level were required to be within the range of ±15% of the nominal concentration for all concentration levels with the exception of the lowest calibration level which was required to be within ±20% of the nominal concentration. A set of calibration samples were also prepared and analysed with each batch of analytical and validation samples to obtain a daily calibration curve.

2.4.6. Accuracy, precision and recovery

Accuracy and precision were determined by analysing replicate QC samples ($n = 5$ for each QC level) over five days. Peak-area ratios were calculated and the concentration of each analyte was determined from the daily calibration curves that were prepared for each day of analysis. Accuracy was determined from the percent deviation of the mean determined concentration from the true concentration value.

Precision was determined and expressed as percentage relative standard deviation, %RSD (where %RSD = standard deviation/mean × 100%). Both inter- and intra-day precisions were determined. The accuracy of all QC samples was required to be within the range of ±20% for the LLOQ QC and ±15% for all other QC concentrations while both inter- and intra-day precisions should not be more than 20% for the LLOQ QC level and not more than 15% for all other QC concentrations [24].

Recovery of the samples was calculated at three QC concentrations ($n = 3$) by comparing mean absolute peak areas of extracted samples with the mean absolute peak areas obtained from direct injection of the non-extracted, derivatized samples of the same concentration representing 100% recovery.

2.4.7. Limits of quantitation (LOQ)

In this study, the LOQ for all analytes were defined as the lowest concentration of the calibration curve that routinely demonstrates (1) an identifiable, discrete and reproducible peak with accuracy of 80–120% and %RSD of not more than 20% and (2) a signal-to-noise ratio (S/N) of not less than five.

2.4.8. Sample stability

Sample stability was evaluated using QC samples at two concentrations: LLOQ (10 μg/L AM, MA, MDMA, HMMA, HHMA and 2 μg/L MDA, HHA, HMA) and HIGH (500 μg/L AM, MA, MDMA, HMMA, HHMA and 100 μg/L MDA, HHA, HMA). Freeze–thaw stability was evaluated by freezing QC samples ($n = 3$ for each concentration) for 24 h at −20 °C followed by complete thawing at room temperature. This freeze–thaw cycle was repeated for another two times after which the samples were processed and analysed as described. Short-term stability was determined by analysing QC samples ($n = 3$ for each concentration) that had been left on the bench-top at ambient temperature for 8 h prior to sample preparation and analysis. Long-term stability was assessed by analysis of QC samples ($n = 3$ for

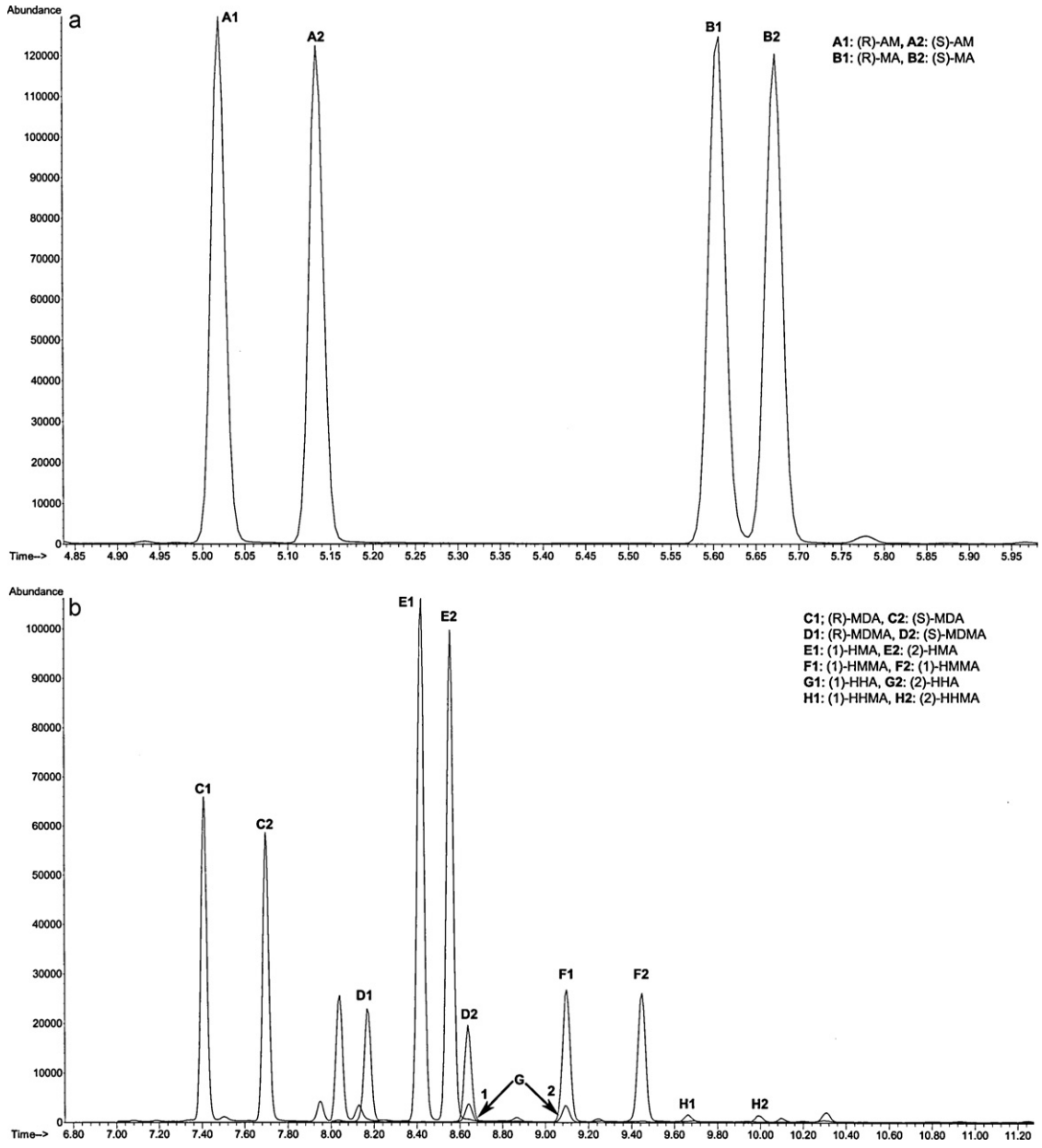

Fig. 1. (a) Extracted ion chromatogram for AM and MA at LOQ. (b) Extracted ion chromatogram for MDA, MDMA, HMMA, HMA, HHMA and HHA at LOQ.

each concentration) after three months and six months of storage at −20 °C each time.

The mean calculated concentrations of the three replicates were determined and compared with the nominal concentration for each QC. The samples were considered stable if the accuracy was within 80–120% and precision <15%.

2.4.9. Application of the method

The developed method was applied to the analysis of human urine samples which were previously analysed using a non-stereoselective analytical method and confirmed to be positive for the presence of ATS. Eleven separate human urine samples that were received by our Forensic laboratory over a period of five years and stored at −20 °C were analysed. As the ring-hydroxylated metabolites (HMMA, HMA, HHA, and HHMA) are excreted in urine as glucoronide conjugates, the urine samples were subjected to enzymatic hydrolysis. In brief, the urine samples were adjusted to pH 5.0 with 1 M sodium acetate buffer and incubated with 30 μL of a solution containing 250,000 Fishman units/mL β-glucuronidase and 2500 Fishman units/mL sulfatase from *H. pomatia* at 55 °C for 4 h. The samples were then allowed to cooled to room temperature before being analysed using the developed validated method.

2.4.10. Analysis of samples with concentrations over the calibration limit

The positive human urine samples used in this study were found to have analyte concentrations that exceeded the highest concentration of the calibration curve. Therefore, to analysis of these samples, a sample dilution approach was used. Analyte concentrations were first estimated by extrapolation of the calibration curve. Next, samples were diluted with a sufficient volume of blank urine in order to ensure that the concentration of the analyte falls within range of the calibration curve. The samples were then reassayed together with QC samples ($n = 3$) that were spiked with identical concentrations of analytes and were also subjected to the same dilution and sample treatment. The QC samples were used to ensure

Table 2
Linearity data for analytes.

Replicates	Intercept	Slope	Correlation coefficient	Intercept	Slope	Correlation coefficient
AM	(*R*)-AM			(*S*)-AM		
Day 1	0.128	157	0.999	0.086	153	0.999
Day 2	0.250	147	0.999	0.240	148	0.999
Day 3	0.148	156	0.999	0.108	152	0.999
MA	(*R*)-MA			(*S*)-MA		
Day 1	0.330	169	0.999	0.212	161	0.999
Day 2	0.282	164	0.994	0.311	154	0.994
Day 3	0.421	156	0.999	0.555	148	0.999
MDA	(*R*)-MDA			(*S*)-MDA		
Day 1	0.069	1021	0.997	0.158	954	0.998
Day 2	0.796	947	0.995	0.875	993	0.995
Day 3	0.812	880	0.998	1.03	925	0.997
MDMA	(*R*)-MDMA			(*S*)-MDMA		
Day 1	0.161	487	0.999	−0.200	441	0.993
Day 2	2.520	710	0.997	−0.105	797	0.999
Day 3	−0.660	492	0.994	1.07	478	0.999
HMA[a]	(1)-HMA			(2)-HMA		
Day 1	0.787	789	0.995	0.859	809	0.997
Day 2	0.957	670	0.997	0.904	628	0.995
Day 3	0.318	826	0.995	0.423	776	0.997
HMMA[a]	(1)-HMMA			(2)-HMMA		
Day 1	0.428	477	0.999	−0.584	597	0.999
Day 2	1.26	332	0.997	0.499	335	0.998
Day 3	0.621	379	0.998	−0.656	507	0.998
HHA[a]	(1)-HHA			(2)-HHA		
Day 1	−0.0046	4.46	0.996	0.00183	4.01	0.997
Day 2	−0.0172	4.74	0.993	−0.0101	4.18	0.995
Day 3	−0.0205	4.58	0.995	−0.0001	4.07	0.995
HHMA[a]	(1)-HHMA			(2)-HHMA		
Day 1	0.00159	3.04	0.998	0.0021	2.86	0.997
Day 2	0.00098	7.13	0.992	−0.0065	6.81	0.992
Day 3	0.00232	2.48	0.997	0.0173	2.51	0.995

[a] Stereoisomers are labeled according to order of elution rather than stereoisomer configuration due to lack of authentic single isomer standards.

accuracy and precision was within the limits required by the FDA guidelines.

3. Results and discussion

A simultaneous extraction–derivatization approach allowed a simple, rapid and sensitive stereoselective analysis of ATS in human urine samples. The sensitivity and detection limits of this method were comparable to previously published studies [5,18,22,27]. However, these methods use SPE as a sample preparation method whereas our novel simultaneous extraction–derivatization approach offers a simplified experimental procedure, resulting in the elimination of potential experimental errors as well as significantly shortening sample preparation times.

Hydrolysis of the samples prior to derivatization and analysis was necessary as ATS metabolites are eliminated in the form of glucuronic or sulfuric acid conjugates. Both enzymatic and acid hydrolysis were investigated. Mueller et al. [28] have found that acid hydrolysis maximises cleavage of both glucuronic and sulfuric acid conjugates for MDMA metabolites in human samples. However in our hands, acid hydrolysis was found to cause a marked decrease in recovery of AM and MA and the presence of acid also interfered with the derivatization of analytes. Thus, for cleavage of conjugates, β-glucuronidase from *H. pomatia* with glucuronidase and sulfatase activity was employed instead.

Both primary and secondary amines were found to readily react with *R*-MTPCl. However, under the conditions used, the hydroxylated ring of the metabolites HMMA, HMA, HHMA and HHA was not derivatized and therefore interfered with the GC–MS analysis. Because of this, a second derivatization step to eliminate the polar hydroxyl groups was necessary. MSTFA was the derivatization agent of choice as it readily silylates phenolic hydroxyl groups and the excess reagent does not interfere with chromatographic analysis.

Using this novel method, the analytes were detected with adequate resolution and sensitivity by GC–MS (Fig. 1). Using single isomer authentic standards for comparison, the order of elution of enantiomers of AM, MA, MDA and MDMA were found to be the *R*-stereoisomer followed by the *S*-stereoisomer. For HMA, HMMA, HHA and HHMA however, authentic single isomer standards were not commercially available and as a result, the elution order could not be determined. Thus, for analysis, the peaks on the chromatogram are denoted by numbers representing the order of elution.

Table 3
Inter- and intra-day precision data for QC samples.

Analyte	Nominal concentration (μg/mL)	Intra-day precision, %RSD, $n = 5$ (%)		Inter-day precision, %RSD, $n = 5$ (%)	
AM		(*R*)-AM	(*S*)-AM	(*R*)-AM	(*S*)-AM
	0.010	4.61	4.52	6.83	8.25
	0.200	2.06	2.02	3.80	2.71
	0.500	0.96	0.89	3.11	1.03
MA		(*R*)-MA	(*S*)-MA	(*R*)-MA	(*S*)-MA
	0.010	6.93	5.31	9.99	10.75
	0.200	5.02	5.01	6.55	6.50
	0.500	1.39	2.47	4.76	7.61
MDA		(*R*)-MDA	(*S*)-MDA	(*R*)-MDA	(*S*)-MDA
	0.002	9.09	9.11	12.29	10.94
	0.080	4.80	5.51	6.56	7.27
	0.500	4.46	2.03	4.57	4.33
MDMA		(*R*)-MDMA	(*S*)-MDMA	(*R*)-MDMA	(*S*)-MDMA
	0.010	6.70	3.05	4.37	5.13
	0.200	4.36	3.91	6.51	6.66
	0.500	1.55	3.10	3.26	4.37
HMA[a]		(1)-HMA	(2)-HMA	(1)-HMA	(2)-HMA
	0.002	5.61	4.64	8.66	11.86
	0.080	6.25	5.26	6.19	9.94
	0.100	4.74	3.94	8.62	4.37
HMMA[a]		(1)-HMMA	(2)-HMMA	(1)-HMMA	(2)-HMMA
	0.010	4.25	4.59	7.87	8.43
	0.200	2.12	2.34	4.94	5.86
	0.500	1.22	1.44	2.43	2.58
HHA[a]		(1)-HHA	(2)-HHA	(1)-HHA	(2)-HHA
	0.002	9.34	10.33	11.23	12.09
	0.080	5.23	3.48	6.34	4.67
	0.100	2.45	1.10	3.24	2.45
HHMA[a]		(1)-HHMA	(2)-HHMA	(1)-HHMA	(2)-HHMA
	0.010	10.23	11.23	11.22	12.95
	0.200	6.87	7.34	7.98	8.43
	0.500	3.53	4.98	5.32	5.76

[a] Stereoisomers are labeled according to order of elution rather than stereoisomer configuration due to lack of authentic single isomer standards.

3.1. Method selectivity

Blank urine samples from six different donors were analysed and checked for possible ion cross-contribution. None of the blank urine samples showed any of the analyte ions at their respective retention times. Blank urine samples enriched with ephedrine, pseudoephedrine, phentermine, pholedrine, cannabis and cocaine at 1000 μg/L also did not show any interfering ion contributions. Thus, it was concluded that the method is selective for the analytes tested and is not affected by any cross-contribution from the sample matrix or other drugs possibly present in the sample matrix.

3.2. Linearity

The described method was linear within the concentration range tested for all the analytes. Using a weighted (1/concentration) least-squares regression, mean ($n = 3$) correlation coefficients, R^2 for all analytes were ≥0.992. The correlation coefficients and linear equations for each of the analytes are shown in Table 2.

3.3. Precision, accuracy and recovery

All analytes demonstrated acceptable precisions, accuracies and recoveries at all concentrations. Both MED and HIGH QC samples had intra- and inter-day precisions below <10%, whereas the LLOQ QC samples were slightly higher but well within the accepted limit of not more than 15% (Table 3).

The analytes also exhibited acceptable accuracy levels (Table 4), with mean calculated concentrations for the QC samples rarely deviating from the nominal concentration by more than ±10%. Again, the percentage deviation of the LLOQ QC samples was slightly higher compared to the MED and HIGH QC samples but below 15% as per the recommendations of the FDA [24].

Recovery was >80% for AM, MA, MDA, MDMA, HMA and HMMA (Table 4). It was observed that the primary amines AM, MDA and HMA achieved slightly higher recovery values when compared to the secondary amines MA, MDMA and HMMA. This is likely due to the fact that *R*-MTPCl reacts more favourably with primary rather than secondary amines. As a simultaneous extraction–derivatization approach was used, the rate of extraction would also be correlated to the rate of derivatization reaction. Thus, recovery values for secondary amines are observed to be lower compared to primary amines due to the lower rate of derivatization. The dihydroxylated metabolites HHA and HHMA showed lower recovery values (50–70%). This may be due to the highly polar and hydrophilic nature of these amines which result in lower recovery from the matrix [29]. Although the recovery for HHA and HHMA

Table 4
Accuracy and recovery data for QC samples.

Analyte	Nominal concentration (μg/mL)	Accuracy, $n = 5$ (%)		Recovery, $n = 5$ (%)	
AM		(*R*)-AM	(*S*)-AM	(*R*)-AM	(*S*)-AM
	0.010	3.83	2.93	98	98
	0.200	−0.63	−0.51	92	95
	0.500	0.96	0.79	92	92
MA		(*R*)-MA	(*S*)-MA	(*R*)-MA	(*S*)-MA
	0.010	6.09	6.09	86	87
	0.200	5.02	5.01	88	88
	0.500	1.39	2.47	87	84
MDA		(*R*)-MDA	(*S*)-MDA	(*R*)-MDA	(*S*)-MDA
	0.002	11.10	13.75	99	99
	0.080	−1.63	−2.42	98	99
	0.100	6.48	6.33	98	98
MDMA		(*R*)-MDMA	(*S*)-MDMA	(*R*)-MDMA	(*S*)-MDMA
	0.010	11.14	11.12	82	81
	0.200	−3.72	−5.29	83	84
	0.500	−0.83	−3.45	81	85
HMA[a]		(1)-HMA	(2)-HMA	(1)-HMA	(2)-HMA
	0.002	7.50	9.10	91	90
	0.080	1.49	−1.50	92	90
	0.100	4.76	3.94	90	90
HMMA[a]		(1)-HMMA	(2)-HMMA	(1)-HMMA	(2)-HMMA
	0.010	6.42	7.34	80	80
	0.200	−2.98	−3.01	80	81
	0.500	1.24	1.98	81	81
HHA[a]		(1)-HHA	(2)-HHA	(1)-HHA	(2)-HHA
	0.002	11.98	10.87	68	66
	0.080	4.98	6.34	69	66
	0.100	2.87	4.97	68	67
HHMA[a]		(1)-HHMA	(2)-HHMA	(1)-HHMA	(2)-HHMA
	0.010	12.97	13.01	50	50
	0.200	5.34	4.43	49	48
	0.500	2.34	3.22	51	51

[a] Stereoisomers are labeled according to order of elution rather than stereoisomer configuration due to lack of authentic single isomer standards.

was quite low compared to the other analytes, the acceptable precision and accuracy data indicate that low recovery does not have a significant negative effect on the quantitation of these analytes.

3.4. Limits of quantitation

For all analytes, the determined LOQ was the lowest concentration of the respective calibration curve. From Tables 2 and 3, it can be seen that the LOQ has accuracy and precision well within the accepted values and is reproducible. All analytes had an S/N value of more than five, with the lowest S/N value detected being 35 for HHMA.

3.5. Sample stability

Sample stability studies were performed to identify any possible deterioration or sample loss due to storage and temperature variations. QC samples at two concentrations ($n = 3$ per concentration) were analysed using the developed method. The results from the stability experiments indicate that the samples are stable under normal experimental conditions (Table 5). In addition, the samples were shown stable up to six months while being stored at −20 °C.

3.6. Application of the method

Eleven ATS-positive urine samples were analysed using the described method and the results are shown in Table 6. The assayed urine samples were not creatinine normalized, however the specific gravities for each sample were within the normal range of 1.010–1.025 g/mL. AM and MA were present in all of the samples. Initially, the concentrations of AM and MA were found to be several orders of magnitude higher than the upper limit of the calibration curve. To overcome this problem, samples were diluted and reanalysed as described in Section 2.4.10. By analysing the QC samples using this approach, it was found that the method accuracy and precision were not affected by dilution of the original sample.

MDA, MDMA, HMA, HMMA, HHA and HHMA were not detected in any of the samples therefore quantification results for these analytes could not be shown. For AM, all samples showed higher concentrations of (S)-AM when compared to (*R*)-AM. (*R*)-AM was detected in six out of 11 samples, but out of these, five were insignificant as the concentrations were below the quantitation limit of the method.

In all the samples obtained from drug abusers, MA was found at higher concentrations than AM, indicating that MA was the drug being abused by the sample donor, whereas the AM present in the samples is a result of the metabolism of MA. All the samples con-

Table 5
Freeze–thaw, short-term, three months and six months stability data.

			AM (*R*)-	AM (*S*)-	MA (*R*)-	MA (*S*)-	MDA (*R*)-	MDA (*S*)-	MDMA (*R*)-	MDMA (*S*)-	HMA[a] (1)-	HMA[a] (2)-	HMMA[a] (1)-	HMMA[a] (2)-	HHA[a] (1)-	HHA[a] (2)-	HHMA[a] (1)-	HHMA[a] (2)-
Freeze–thaw stability	LLOQ	Precision, %RSD	3.34	3.02	5.45	5.41	8.02	9.10	4.45	4.98	5.13	5.34	5.95	5.64	9.90	9.95	11.83	11.45
		Accuracy, %	2.10	1.95	4.34	4.10	9.87	9.87	10.01	10.10	8.34	8.45	7.32	7.81	11.76	12.01	10.88	10.98
	HIGH	Precision, %RSD	0.65	0.85	0.89	0.85	4.11	5.01	2.23	2.43	4.33	3.43	1.98	1.45	5.01	5.34	6.81	6.09
		Accuracy, %	−0.51	−0.66	2.01	2.11	3.98	3.45	2.01	2.22	1.96	1.68	2.81	2.94	4.67	2.76	2.55	2.45
Short-term stability	LLOQ	Precision, %RSD	2.22	3.13	4.54	4.89	7.86	5.87	4.12	4.39	6.78	6.98	7.98	7.64	10.09	10.34	12.10	13.87
		Accuracy, %	1.87	2.98	4.23	4.44	8.87	8.45	3.12	4.23	8.78	7.56	8.87	8.85	12.45	12.78	13.08	12.97
	HIGH	Precision, %RSD	0.95	1.34	2.87	2.86	3.12	3.44	2.54	2.76	4.11	3.92	2.34	2.38	5.84	5.86	5.12	5.45
		Accuracy, %	0.89	0.82	3.65	3.45	4.55	4.67	3.31	3.64	2.98	2.78	4.87	4.78	5.87	5.43	3.87	4.23
Long term stability (three months)	LLOQ	Precision, %RSD	2.19	2.67	4.12	4.87	6.87	7.08	5.10	5.09	7.33	7.45	7.89	7.55	11.41	11.72	12.98	12.54
	HIGH	Accuracy, %	0.87	0.91	3.98	2.76	9.34	9.22	2.87	2.98	10.98	11.87	5.23	5.22	12.98	12.78	13.12	13.98
		Precision, %RSD	1.76	1.68	4.11	4.01	4.12	4.87	1.87	1.82	3.98	4.09	1.76	1.54	4.08	3.89	4.87	4.75
		Accuracy,%	1.45	1.56	2.67	2.69	6.37	6.69	2.09	1.56	1.98	1.94	3.24	3.33	6.00	6.15	2.11	2.27
Long term stability (six months)	LLOQ	Precision, %RSD	1.97	1.89	4.34	3.49	10.22	10.67	4.12	4.47	7.01	6.69	8.45	8.15	10.45	10.88	12.56	12.51
		Accuracy, %	1.23	1.53	5.55	5.58	11.74	11.76	4.41	4.53	9.75	7.31	8.77	7.45	13.20	12.99	13.45	13.12
	HIGH	Precision, %RSD	1.03	0.98	2.01	2.21	3.97	3.67	1.34	1.53	4.41	4.21	1.56	1.62	3.31	3.10	4.10	4.12
		Accuracy, %	1.91	1.67	2.45	2.44	6.21	6.09	2.24	2.78	3.11	3.98	3.76	3.58	5.56	5.12	5.69	5.76

[a] Stereoisomers are labeled according to order of elution rather than stereoisomer configuration due to lack of authentic single isomer standards.

Table 6
ATS concentrations detected in ATS-positive urine samples.

Sample	Calculated concentrations (μg/mL)			
	(*R*)-AM	(*S*)-AM	(*R*)-MA	(*S*)-MA
A	<LOQ[a]	0.96	0.03	5.13
B	<LOQ[a]	3.19	0.05	12.64
C	<LOQ[a]	3.12	ND[b]	8.22
D	ND[b]	1.82	ND[b]	2.15
E	ND[b]	0.51	0.02	0.87
F	ND[b]	0.82	ND[b]	3.06
G	<LOQ[a]	0.32	0.06	8.59
H	0.18	0.92	3.59	3.71
I	<LOQ[a]	3.29	0.03	13.67
J	ND[b]	0.08	0.18	5.13
K	ND[b]	3.13	<LOQ[a]	15.41

[a] <LOQ – below limit of quantitation.
[b] ND – not detected.

tained (*S*)-MA whereas only seven samples contained (*R*)-MA. In all of the samples except for sample H, the concentration of (*S*)-MA is very much higher than (*R*)-MA. This is to be expected, as most illegal consumption of MA is by smoking of the crystalline form of (*S*)-MA. Only sample H demonstrated a 1:1 ratio of (*R*)- to (*S*)-methamphetamine, indicating that the ingested drug was in racemic form. However, this is not consistent with abuse of crystalline MA and indicates either the ingestion of non-crystalline MA or possibly ingestion of one of several therapeutic drugs such as famprofazone [30] that produce MA as a metabolite.

4. Conclusion

This novel and validated method is sensitive and has demonstrated to be within the guidelines specified by the FDA for bioanalytical methods. The assay is reproducible and accurate for simultaneous determination of enantiomeric concentrations of AM, MA, MDA, MDMA, HMMA, HMA, HHA and HHMA. The method has good applicability in toxicological, forensic and pharmacological studies.

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