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Short communication

Chiral separation and quantification of *R*/*S*-amphetamine, *R*/*S*-methamphetamine, *R*/*S*-MDA, *R*/*S*-MDMA, and *R*/*S*-MDEA in whole blood by GC-EI-MS

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Abstract

The enantioselective composition of the amphetamines is of interest, as the enantiomers show differences in their pharmacological effects and several methods for chiral separation of amphetamines have been described. Only a few methods have used whole blood as matrix and none of these separates both classic amphetamines (amphetamine and methamphetamine) and designer amphetamines (MDA, MDMA and MDEA). The aim of this study was, therefore, to develop a method for enantioselective analysis of AM, MA, MDA, MDMA, and MDEA in whole blood. The amphetamines were extracted from 0.5 g of whole blood by liquid–liquid extraction. After derivatization with *R*-MTPCl, the resulting diastereomers were separated by GC on a HP-5MS column and detected by SIM-MS. *R*-MTPCl was used as derivatization reagent because of the stability of this reagent and good separation of these analytes. Through the method, development time and temperature of the derivatization were optimized, and by admixture of 0.02% triethylamine it became possible to detect the amphetamines in adequately low concentrations as more analytes were derivatized. The method was validated and it was linear from 0.004 to 3 μg/g per enantiomer. The accuracy was within 91–115%, while the repeatability and reproducibility were ≤15% R.S.D. A method suitable for enantioselective separation and analysis of the amphetamines has been achieved, and the method was applied to analysis of whole blood samples originating from traffic and criminal cases and post mortem cases.

Keywords: Chiral separation; Amphetamines MDMA; Whole blood; GC/MS

1. Introduction

In recent years the use of amphetamines in Denmark has increased. The Danish police and the Department of Forensic Chemistry, University of Copenhagen wish to form a general view of the enantioselective composition of the amphetamines. The amphetamines are a group of structurally related compounds with limited legitimate use but vast potential for abuse, addiction and toxicity [1]. Abuse to combat fatigue or achieve euphoria exposes the individual to potentially serious toxicity risks [1]. The composition of drug is often not known to the user, and this may be of importance to the toxicity risks as the amphetamines are chiral compounds whose *S*-(+)-enantiomers exhibit greater pharmacologic potency than the corresponding *R*-(−)-enantiomers [1,2].

The most important chemical processes in the metabolism of the amphetamines are aromatic hydroxylation, N-dealkylation, O-demethylation and conjugation [3–5]. The major metabolites of MA are AM and 4-hydroxymethamphetamine, while the metabolites of MDMA and MDEA are MDA, 3,4-dihydroxymethamphetamine (HHMA), 4-hydroxy-3-methoxymethamphetamine (HMMA) and 4-hydroxy-3-methoxyamphetamine (HMA) [4,5]. The metabolism of the amphetamines is stereoselective; generally the *S*-enantiomer is more rapidly metabolized than the *R*-enantiomer. The enzyme CYP2D6 is enantioselective and catalyzes the β-hydroxylation of AM and the demethylations in the metabolism of MDMA. This results in

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R/*S* ratios >1 in biological fluids for AM and MDMA consumed as the racemate [6–8].

Several chiral derivatizing reagents have been used to derivatize the amphetamines, two of the most thoroughly described being *S*-(−)-*N*-trifluoracetylprolylchloride (*S*-TPCl) [1,9,10] and *R*-(−)-α-methoxy-α-trifluormethylphenylacetylchloride (*R*-MTPCl) [7,11,12].

A number of articles describe derivatisation with *S*-TPCl, including a work by Meyer et al. [1] presenting a method for derivatisation of AM in whole blood, and by Tetlow et al. [10] using urine as matrix for derivatisation and analysis of AM. An article by Hensley et al. [9] describes a method for derivatisation with *S*-TPCl of AM, MA, MDA, MDMA, and MDEA in urine. The use of *R*-MTPCl for derivatisation of amphetamines is presented by Fallon et al. [12], Pizzaro et al. [7] and Paul et al. [11] among others. The first two authors describe derivatisation of MDA and MDMA in respectively urine and plasma, while Paul et al. [11] has derivatised AM, MA, MDA, MDMA, and MDEA in urine.

This paper presents a sensitive and selective method for quantification of enantiomers of AM, MA, MDA, MDMA, and MDEA in whole blood. To our knowledge, it is the first time that a method for chiral separation and quantification of these five amphetamines in whole blood has been presented. In addition, the method is validated and applied to whole blood specimens from the Department of Forensic Chemistry.

2. Materials and methods

2.1. Chemicals and reagents

Following solutions of amphetamines were obtained from Cerilliant, Round Rock, TX, USA. Ethanol (99.9%) and triethylamine (Et_3N) were obtained from Merck (Darmstadt, Germany): methanolic solutions (1000 μg/ml free base) of racemic MA, MDA, MDMA, MDEA, and pure *R*-(−)-AM, *S*-(+)-AM, *R*-(−)-MA, *S*-(+)-MA; methanolic solution (1000 μg/ml) of racemic AM as the sulfate salt; methanolic solutions (100 μg/ml free base) of racemic AM-d_5, MA-d_5, MDA-d_5, MDMA-d_5, and MDEA-d_5. Ethyl acetate and 1-chlorobutane were obtained from Sigma-Aldrich (Steinheim, Germany). Acetonitrile was obtained from VWR International Ltd (Poole, Great Britain). MTPCl of +99% purity was obtained from Fluka Chemie (Bucsh, Switzerland). All solvents and reagents were of analytical grade or the highest purity available.

Acetonitrile and ethanol was dried over molecular sieves type 3A beads, respectively, type 4A beads for 24 h.

2.2. Biosamples

As a standard procedure in the laboratory whole blood from horses was used for development and validation of the procedure and was obtained from The Royal Veterinary and Agricultural High School of Denmark. The analytical results are similar.

Human whole blood samples were obtained from forensic cases taken in connection with traffic/violence offences and autopsies.

2.3. Sample preparation for GC–MS analysis

Aliquots (0.50 g) of whole blood were diluted with 500 μl of purified water. After addition of 25 μl of a solution of the racemic internal standards (IS) in purified water (3.2 μg/ml) and 500 μl of 1 M NaOH, the samples were whirlymixed for 10 s. Two milliliters of 1-chlorobutane which contained Et_3N (0.02%) was added to the tubes, and the solutions were mixed on a rotary shaker for 10 min and centrifuged (4000 rpm for 5 min). The organic phase were transferred to another set of glass tubes and used for derivatization.

A solution of *R*-(−)-MTPCl was prepared in dry acetonitrile (50 μl reagent/ml). For derivatization, approximately 25 μl was added to the organic phase. The glass tubes were capped and heated at 80 °C for 2 h. The solutions were cooled to room temperature, and 100 μl of dry ethanol was added to it. The glass tubes were recapped and heated at 70 °C for 15 min. The solutions were evaporated to dryness at room temperature under a nitrogen steam. The extracts were dissolved in 75 μl of ethyl acetate and 1 μl was injected into the GC–MS.

2.4. Enantioselective GC-EI-MS quantification

2.4.1. Apparatus

The samples were analyzed using an Agilent Technologies (AT) 6890N Series GC system combined with an AT 5973*Network* mass selective detector, an AT 7683 Series injector, and MSD ChemStation G1701DA, ver. D.01.02.16 15-June-2004.

2.4.2. GC conditions

GC conditions were as follows: splitless injection mode; HP5-MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness); injector port temperature, 250 °C; carrier gas, helium; flow rate, 0.8 ml/min; column temperature, 70–210 °C at 40 °C/min, 210–270 °C at 15 °C/min, 270–320 °C at 10 °C/min, and 320 °C in 10 min.

2.4.3. MS conditions

MS conditions were as follows: transfer line heater, 250 °C; electron ionization (EI, 70 eV); source temperature, 230 °C; solvent delay, 5 min; selected ion monitoring (SIM) mode; dwell-time, 50 ms. The SIM program was as follows: from 6.50 min, *m*/*z* 91, 189, and 260 for AM, and *m*/*z* 264 for AM-d_5; from 7.35 min, *m*/*z* 91, 189, and 274 for MA, and *m*/*z* 278 for MA-d_5; from 8.30 min, *m*/*z* 135, 162, and 189 for MDA, and *m*/*z* 167 for MDA-d_5; from 9.10 min, *m*/*z* 135, 189, and 274 for MDMA, *m*/*z* 278 for MDMA-d_5, *m*/*z* 135, 189, and 288 for MDEA, and *m*/*z* 293 for MDEA-d_5.

2.5. Assay validation

The GC–MS assay was validated for quantification of AM, MA, MDA, MDMA, and MDEA in whole blood with special

emphasis on the aspects of enantioselective analysis. The experimental design used for the validation experiments was based on the Washington Conference Report [13] and the one proposed by Peters et al. [2].

2.5.1. Preparation of solutions

Aqueous analytical standard solutions containing both racemic AM, MA, MDA, MDMA, and MDEA (0.002, 0.004, 0.5, 1, 2, and 3 μg/g of each enantiomere) were prepared from methanolic stock solutions (1000 μg/ml). Aqueous analytical standard solution of the IS (3.2 μg/ml) was prepared from methanolic stock solutions (100 μg/ml) of racemic AM-d_5, MA-d_5, MDA-d_5, MDMA-d_5, and MDEA-d_5. All solutions were stored at 5 °C.

2.5.2. Preparation of QC samples

For preparation of QC samples, 0.50 g aliquots, whole blood was spiked at three levels: QC low (0.008 μg/g), QC medium (1 μg/g), and QC high (2 μg/g). The QC samples were stored at −18 °C in separate tubes containing 0.50 g.

In all analysis sequences QC samples were analyzed and quantified to approve the sequence.

2.5.3. Selectivity

Whole blood from three post mortem cases and two criminal offence cases, all negative cases concerning amphetamines, were analyzed to control that no peaks interfere with the detection of analytes or the IS. Blank whole blood samples enriched with other sympathomimetics amines (ephedrine and norpseudoephedrine) were controlled for interferences. Two whole blood samples, which were partially decomposed, were controlled for β-phenylethylamine that might interfere with the detection of analytes and the IS. The samples were analyzed in SIM mode.

Four zero samples (blank sample plus IS) were analyzed to quantitate the amount of analyte ions in the respective peaks of the IS.

2.5.4. Linearity

Aliquots of blank whole blood (0.50 g) were enriched with 500 μl of the corresponding analytical standard solutions to obtain enriched calibration samples at concentrations of 0.002, 0.004, 0.5, 1, 2, and 3 μg/g of each enantiomere of AM, MA, MDA, MDMA, and MDEA. Replicates ($n = 2$) at each concentration were analysed.

Daily calibration curves using the concentrations 0.004, 1.5, and 3 μg/g (two injections per concentrations) were prepared with each batch of validation and authentic samples. The samples to the daily calibration curves were prepared in the same way as the QC samples and stored at −18 °C.

2.5.5. Analytical repeatability, reproducibility, accuracy and recovery

QC samples (low, medium and high) were analyzed as described above in duplicate on every 2–6 days. The concentrations of the five amphetamines in the QC samples were calculated on the basis of the daily prepared calibration curves. The enantiomers were quantified by comparison of their peak-area ratios (enantiomer of analyte versus corresponding enantiomere of the IS) with calibration curves in which the peak area ratios of the enriched calibration samples were plotted versus their concentrations.

Analytical repeatability was calculated for each enantiomere as %R.S.D. for each duplicate with-in the same day. Reproducibility (within-lab) was calculated by one-way ANOVA using day as the grouping variable and as %R.S.D. Analytical accuracy was calculated as the percentage of the mean calculated concentration from the spiked concentration. The extraction efficiency was determined for MDMA, as this analyte is representative for the five amphetamines.

2.5.6. Verification of LOQ

Samples ($n = 5$) with a concentration of 0.004 μg/g were analyzed to determine whether the criteria established for LOQ (analytical accuracy within 100 ± 20% of the nominal value and R.S.D.% < 20%) were meet at this concentration. Aliquots of 0.50 g whole blood were added 500 μl of a standard solution (0.004 μg/ml of each enantiomere of the five amphetamines) and left for 30 min. The samples were analyzed.

2.5.7. Stability

For estimation of the stability of the derivatized constitute under the conditions of GC–MS analysis, all QC samples were extracted and derivatized. Caffeine was used as a correction factor, and the extracts were dissolved in 75 μl 0.75 mg/ml caffeine in ethyl acetate. The extracts was transferred to autosampler vials and injected under the conditions of a regular run. The sequence was repeated after 24 h.

3. Results and discussion

The developed method succeeded in separating the enantiomers of the five amphetamine derivatives. The following elution order was determined of the derivatives: AM, MA, MDA, MDMA, and MDEA. By analyzing enantiomerically enriched *R*-AM, *S*-AM, *R*-MA, and *S*-MA standards, it was observed that the *R*-enantiomers eluted before the *S*-enantiomer. Because of the lack of appropriate enantiomeric standards, MDA, MDMA, and MDEA enantiomers could not be assigned to the corresponding chromatographic peaks. Paul et al. [11] showed that the *R*-enantiomers of these analytes also eluted before the corresponding *S*-enantiomers, whereby this was applied to all five amphetamines. Representative extracted ion chromatograms are presented in Fig. 1.

The fragmentation patterns for the *R*- and *S*-enantiomers were similar. The ion fragments that showed the highest intensity were selected as part of the drug identification. Structures and fragmentation patterns of the amphetamines derivatized with *R*-MTPCl are shown in Fig. 2. The selected ion fragments and retention times for each enantiomer are presented in Table 1. The ion marked with (Q) is used for quantification.

The analytes are identified from the relative ion intensities for the three selected ion fragments (I1 (Q), I2, I3). The ratios

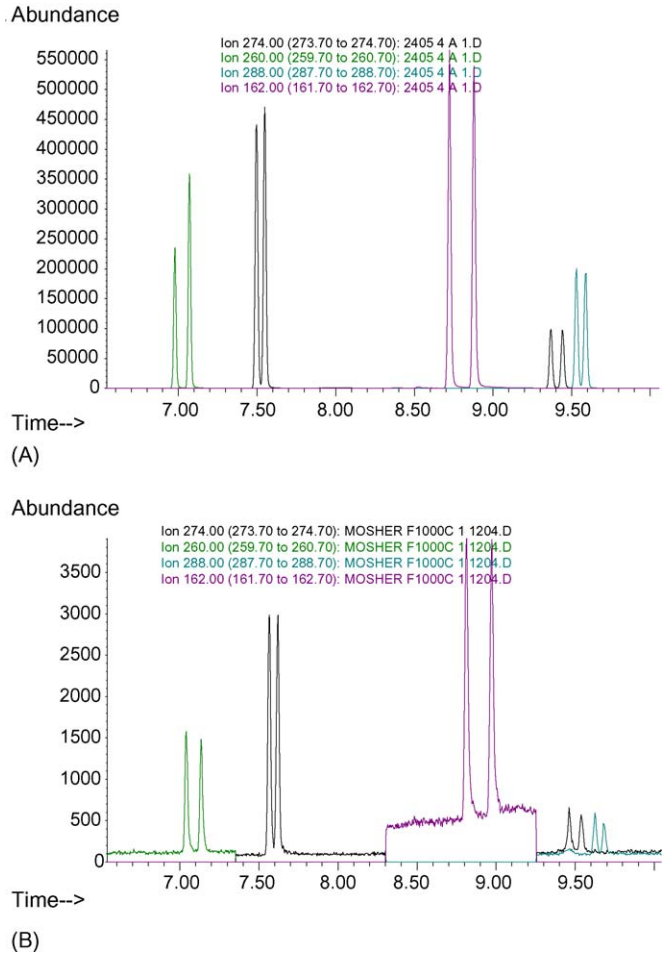

Fig. 1. Extracted ion chromatogram of *R*-/*S*-AM, *R*-/*S*-MA, *R*-/*S*-MDA, *R*-/*S*-MDMA, and *R*-/*S*-MDEA as *R*-MTPCl derivatives in whole blood concentrations at (A) 2 μg/g and (B) 0.002 μg/g, respectively.

should be within the following: I2/I1 ± (0.1 × I2/I1 + 10%) and I3/I1 ± (0.1 × I3/I1 + 10%) [14].

3.1. Sample preparation

In the reaction of primary and secondary amines with *R*-MTPCl an amide is formed together with HCl. The acid is neutralized by an amine acting as a Brønsted base forming the corresponding hydroclorides. The protonated amines are unable to react with *R*-MTPCl and addition of another Brønsted base is necessary to allow complete derivatization of the amphetamines. As described by Pizarro et al. [7] triethylamine (0.02%) was added to the extraction solvent, and a noticeable increase in signal intensity was observed.

The influence of the temperature and time during derivatization was investigated, and 2 h at 80 °C was found to be the best derivatization conditions. After derivatization the excess derivatization reagent was decomposed by anhydrous ethanol.

Table 1
Diagnostic ions for the enantiomers of the amphetamines and internal standards

T_R (min) *R*-/*S*-	Analyte	Ions
6.92/7.01	AM	91, 189, 260 (Q)
	d_5-AM	189, 264 (Q)
7.43/7.49	MA	91, 189, 274 (Q)
	d_5-MA	189, 278 (Q)
8.65/8.80	MDA	135, 189, 260 (Q)
	d_5-MDA	189, 264 (Q)
9.28/9.36	MDMA	135, 189, 274 (Q)
	d_5-MDMA	189, 278 (Q)
9.45/9.50	MDEA	135, 189, 288 (Q)
	d_5-MDEA	189, 293 (Q)

T_R is the retention time and Q is the ion used for quantification.

3.2. Validation results

3.2.1. Selectivity

Five blank whole blood samples were prepared and analyzed as described above. No peaks were detected in the SIM mode of the method; therefore, no endogenous compounds were found to interfere with the analysis.

In two partially decomposed whole blood samples a peak corresponding to β-phenylethylamine was detected with a retention time similar to those of the AM enantiomers. As β-phenylethylamine does not show the mass fragment *m*/*z* 260 no interference with the quantification of AM was observed.

No interference was detected in any of the enriched samples containing other sympathomimetic amines (ephedrine and norpseudoephedrine). No signals of amphetamines were detected in the zero samples containing only internal standard and limit of blank (LOB) was set to zero.

3.2.2. Linearity

The method was linear from 0.004 to 3 μg/g for *R*-/*S*-AM, *R*-/*S*-MA, *R*-/*S*-MDMA, and *R*-/*S*-MDEA. This range covers concentrations that are to be expected in blood from abusers and toxic levels. The slopes of the calibration curves were between 9.0 and 15 with correlation coefficients >0.995 for all analytes. All curves were fitted to origin (0,0).

3.2.3. Analytical repeatability, reproducibility, accuracy and recovery

QC samples were analyzed in duplicate on every second day in a period of 6 days. The results for analytical repeatability were within the acceptance limit of R.S.D. < 15% (R.S.D. < 20% at LOQ). The obtained data ranged from 0.13 to 13%. The results of the ANOVA and the calculated R.S.D. for reproducibility (within lab) confirmed the reliability of the developed method. The results for reproducibility were within the criteria with data ranging from 1.7 to 13%. Three results from the ANOVA concerning MDA did not meet with the criteria for the *F*-test, but compared with the calculated %R.S.D. the reproducibility of the method was satisfying. Analytical accuracies were found to be within 90.5–114.7%, which is within the criteria. The accuracy results for *R*-MDA at the low concentration level were 122%.

The extraction efficiency for MDMA was found to be higher than 95%, which is very acceptable and assumed to represent the level for all analytes.

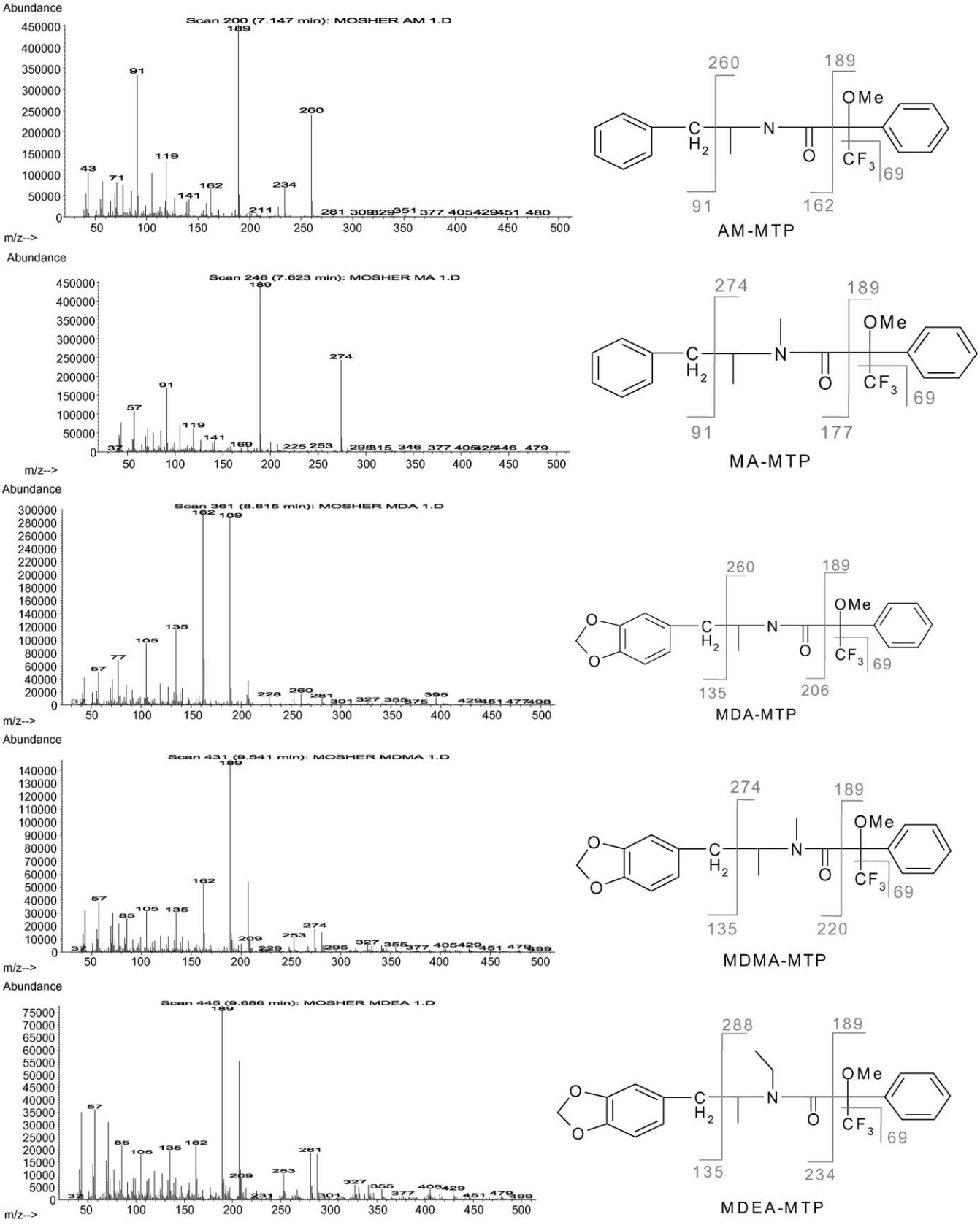

Fig. 2. Structure and mass fragmentation of the amphetamines derivatized with *R*-MTPCl.

Table 2
Results from a forensic case obtained with the developed method and the currently used non-chiral method

	Enatiomeric concentration	Total concentration (μg/g)	Results obtained with a non-chiral method (μg/g)
R-AM	0.084 μg/g	0.149	0.27
S-AM	0.065 μg/g		
R-MA	0.039 μg/g	0.052	0.06
S-MA	0.013 μg/g		
R-MDA	0.009 μg/g	0.009	0.01
S-MDA	<LOQ		
R-MDMA	0.048 μg/g	0.048	0.05
S-MDMA	–		

3.2.4. Verification of LOQ

The LOQ of the analyte is the lowest concentration that can be detected with a signal-to-noise ratio >10. Analytical accuracy should be within 100 ± 20% and the repeatability R.S.D. < 20%. For *R*-/*S*-AM, *R*-/*S*-MA, *R*-/*S*-MDMA, and *R*-/*S*-MDEA the LOQ was verified to 0.004 μg/g. The results for analytical accuracy for *R*-MDA ranged from 105.3 to 138.5% with two results beyond the acceptance limit. Since the results for repeatability are within the criteria it was estimated that the method was usable. Futhermore, MDA is usually only seen as the metabolite of MDMA and MDEA, and is therefore less frequently the main analyte.

3.2.5. Stability

The derivatized amphetamines showed to be stabile for at least 24 h at room temperature.

3.3. Application of method to forensic cases

The method was applied to whole blood from four forensic cases. One case originated from a 22 years old man who was suspected to be driving under the influence of drugs. The results of the analysis obtained with the developed method is shown in Table 2 as well as the racemic results obtained by the currently used non-chiral method [15,16]. The total concentration obtained with the developed method is in accordance with the results obtained by the non-chiral method, bias being below 30%. The difference in concentrations may be due to instability of the samples, as there were more than 6 months between the determinations. This is more pronounced for AM as it is the most volatile of the amphetamines. Interlaboratory variations also contribute.

It appeared that the amphetamines had been ingested as racemates in all four cases.

It was possible to see the effects of the stereoselective metabolism of the amphetamines in the authentic samples as the *R/S* ratio for most enantiomers are >1. This indicates that the *R*-enantiomer is metabolised faster than the *S*-enantiomer.

An analysis of 10 different seizures (tablets or powders obtained from the police) containing amphetamines showed that one of the seizures contained pure *S*-MA, but more samples in a defined period need to be analyzed to evaluate the prevalence of this.

4. Conclusion

The method described allowed chiral separation and quantitation of *R*/*S*-AM, *R*/*S*-MA, *R*/*S*-MDA, *R*/*S*-MDMA, and *R*/*S*-MDEA in whole blood by GC-EI-MS. Applicability was demonstrated by application to forensic cases.

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