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Simultaneous determination of amphetamine and its analogs in human whole blood by gas chromatography–mass spectrometry

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Abstract

A sensitive and specific gas chromatography–mass spectrometry (GC–MS) method for the determination of amphetamine (AM), methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA) in whole blood was designed, using the respective pentadeuterated analogs of the analytes as internal standards (I.S.). After alkalinisation of blood samples, the amphetamines were extracted using diethyl ether, derivatized with heptafluorobutyric anhydride, then purified by successive washings with deionized water and 4% NH_4OH. Extraction recoveries were 85.2% for AM, 90.9% for MA, 76.5% for MDA, 84.1% for MDMA and 63.6% for MDEA. Chromatographic separation was performed on a non-polar 30 m×0.32 mm HP 5 MS capillary column using a temperature program. Detection was carried out in the electron-impact, selected ion-monitoring mode, using three mass-to-charge ratios for each analyte and one for each I.S. Limits of detection ranged from 0.5 to 8 ng/ml and limits of quantification were 10 ng/ml for AM, MDMA and MDEA; 20 ng/ml for MA; and 50 ng/ml for MDA. The method was linear from this limit up to 1000 ng/ml for all analytes, with good intra-assay precision and good intermediate precision and accuracy over these ranges. There was no interferences from other sympathomimetic drugs such as ephedrine, norephedrine or methoxyphenamine. This method is thus suitable for clinical and forensic toxicology, as well as for doping control.

Keywords: Amphetamines; Methamphetamine; Methylenedioxyamphetamine ; Methylenedioxymethamphetamine; Methylenedioxyethylamphetamine

1. Introduction

Amphetamines are CNS-stimulant drugs frequently abused by sportsmen, drug addicts or for recreation by occasional users. Apart from amphetamine (AM) and methamphetamine (MA), the main illicit street-drugs, so called entactogens, are methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA) and methylenedioxyethylamphetamine (MDEA). When overdosed, they can be responsible for hallucinations, paranoid delirium, seizures, coma or even death. Chronically administered, they induce physical and psychic dependence, as well as tolerance [1].

Therefore, it is important for laboratories involved in clinical, forensic, sport or occupational toxicology to design specific and quantitative analytical techniques for these compounds, in order either to verify

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positive results obtained by commonly used immunochemical techniques in urine, or to directly detect and quantitate entactogens in biological media other than urine, the most frequent of which is blood.

Gas chromatography has been most frequently proposed for that purpose, using many different derivatizing reagents: trifluoroacetic anhydride (TFAA) [2–4], *N*-methyl-*N-tert.*-butyldimethylsilyl trifluoroacetamide (MTBSTFA) [5], pentafluoropropionic anhydride (PFPA) that allows spectral differentiation of the stereoisomers MBDB, MDEA and dimethyl-MDA [6,7], heptafluorobutyric acetyl (HFB) [8] or anhydride (HFBA) [9,10], etc. These polyfluorated reagents generally provide good limits of detection for amphetamines, because they give high-mass derivatives and thus better signal-to-noise ratios. In this respect, HFBA (or HFB) yields the heaviest derivatives and has been long reported to give excellent detection limits for amphetamine in biological fluids [11].

Up to now, few quantitative techniques dealing with entactogens have been proposed: one was devoted to AM, MA, MDA and MDMA determination in hair, using PFPA-PFPOH derivatization and gas chromatography–mass spectrometry (GC–MS), and achieving limits of detection (LOD) of 0.05–0.1 ng/mg [6]; another one, using liquid–liquid extraction and derivatization with TFAA yielded poor LOD values for MDA, MDMA and MDEA in whole blood (200–500 ng/ml) [2].

The aim of the present study was to design a sensitive and validated GC–MS method for the determination of AM, MA, MDA, MDMA and MDEA in whole blood, applicable to clinical, occupational or forensic purposes.

2. Experimental

2.1. Chemicals and reagents

Amphetamine sulfate, methamphetamine, MDA and MDMA were purchased from Sigma (St. Louis, MO, USA), MDEA and 100 mg/l methanolic solutions of *d*5-amphetamine, *d*5-methamphetamine, *d*5-MDA, *d*5-MDEA and *d*5-MDMA from Radian Corporation (Austin, TX, USA). Stock solutions of drugs were prepared at 1 g/l in methanol and stored, together with internal standard solutions, at +4°C. A 1-mg/l mixed solution of the five analytes and a 5-mg/l mixed solution of their five deuterated analogs were prepared in 0.2 *M* hydrochloric acid and kept at +4°C for a maximum of 4 weeks.

Diethyl ether, methanol, isopropanol, ethyl acetate, HCl and NaOH, all of HPLC grade, were purchased from Prolabo (Paris, France); NH_4OH from Sigma; *n*-hexane for HPLC from Carlo-Erba (Milan, Italy); heptafluorobutyric anhydride (HFBA) from Pierce (Supelco, Paris, France).

2.2. Extraction

The analysis can be performed from whole blood collected with or without anticoagulants, as well as from hemolyzed whole blood (post mortem or defrost blood). As the amphetamines studied are not directly metabolized by sulfo- or glucuroconjugation in the liver, no enzymatic hydrolysis was required prior to extraction.

To a 1-ml blood sample were added 20 μl of internal standard (5 mg/l), 0.5 ml of 1 *M* NaOH and 5 ml of diethyl ether in a 15-ml round-bottomed glass tube. Extraction was performed by reciprocating agitation during 15 min, followed by 5 min centrifugation at 3000 rpm (900 *g*). The organic layer was transferred to a 10-ml conical glass tube containing 100 μl of an isopropanol–HCl mixture (99:1; v/v), and evaporated at 30°C under a gentle stream of nitrogen. To the dry extract were added 100 μl HFBA and 50 μl ethyl acetate, then derivatization was carried out at 70°C for 20 min. After cooling, the derivatized extract was evaporated at 30°C under a nitrogen stream, redissolved in 400 μl hexane and 200 μl purified water. After 30 s vortexing and 5 min centrifugation, the aqueous phase was discarded and 200 μl of 4% NH_4OH added. After vortexing and centrifugation the organic layer was transferred to another 10-ml conical glass tube, evaporated to dryness and the dry extract reconstituted in 50 μl ethyl acetate, of which 1 μl was injected into the chromatographic system.

2.3. Gas chromatography–mass spectrometry

Separation was performed using a Hewlett-Packard model 5890 series II gas chromatograph,

equipped with a split/splitless injector and an HP 5 MS 30 m×0.32 mm I.D., 0.25 μm film-thickness capillary column. The carrier gas was helium, delivered at a regulated head-pressure of 50 kPa. The split/splitless injector was heated at 280°C and used in the splitless mode; the split opening time was set at 30 s. The oven temperature was initially set at 60°C for 1 min, increased to 140°C at 15°C/min, then to 212°C at 30°C/min, maintained at this temperature for 3 min and at last rapidly brought to 285°C in order to clean the column. Interface temperature was set at 280°C, inducing a source temperature of approximately 175°C. Detection was performed with a Hewlett-Packard MSD 5972 mass detector, operated in the electron-impact (70 eV), selected-ion monitoring (SIM) mode. The electron multiplier was set at 2200 V. One quantitation and two or three confirmation ions (as some are common to two or more analytes) were selected for each analyte, whereas only one ion was recorded for each internal standard (Table 1). These mass-to-charge ratios were carefully selected to avoid all those belonging to both analyte and internal standard spectra, because each analyte co-elutes with its deuterated analog. Analytes were subsequently identified by their relative retention time and by the ratios of their respective confirmation ions to their quantitation ion.

2.4. Validation procedure

For the analytical validation, the guiding principles established during a conference on "method validation for the quantitation of drugs in biological media" were followed [12].

Recoveries were determined in triplicate, using drug-free whole blood spiked at 100 ng/ml of each analyte. Intra-assay precision was determined at 20 and 500 ng/ml by extraction and analysis, on the same day, of five aliquots of spiked whole blood for each concentration. For the intermediate ('inter-assay') precision [13] and accuracy assessment, drug-free blood samples, spiked at 0, 10, 20, 50, 100, 200, 500 and 1000 ng/ml, were prepared in advance in 10-ml volumetric flasks, 1-ml aliquots thereof were stored at −20°C until analysis; then a set of calibrating samples were analyzed each day for 5 days. The limit of quantification (LOQ) of a given analyte was defined as the lowest concentration yielding a coefficient of variation (C.V.) lower than 20% (precision) and a deviation of less than 15% from the nominal value (inaccuracy). The limit of detection (LOD) was evaluated as the lowest concentration giving a chromatographic signal-to-noise ratio higher than 5.

3. Results and discussion

In the chromatographic conditions described, AM, MA, MDA, MDEA and MDMA were perfectly resolved (Table 1). No interferences could be noted, in particular from other sympathomimetic drugs such as ephedrine (t_R=9.32 min), pseudoephedrine (t_R= 9.64 min) or methoxyphenamine (t_R=10.11), the HFBA derivatives of which share common mass

Table 1
Retention time, quantitation and confirmation m/z ratios of the five analytes and their respective internal standards

Compounds	Retention time (min)	Quantitation ion (amu)	Confirmation ions (amu)
*d*5-AM (E.I.)	8.38	244	–
AM	8.40	240	118, 91
*d*5-MA (E.I.)	9.10	258	–
MA	9.12	254	210, 169, 91
*d*5-MDA (E.I.)	10.53	380	–
MDA	10.55	375	240, 162, 135
*d*5-MDMA (E.I.)	10.46	258	–
MDMA	10.48	254	389, 210, 162
*d*5-MDEA (E.I.)	11.84	273	–
MDEA	11.86	268	403, 240

AM, amphetamine; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine; MDEA, methylenedioxyethylamphetamine.

fragments with methamphetamine [14] or MDMA (*m/z* 254, 210, 169 and 91). The chromatograms of reagent blank, of blank blood and of drug-free whole blood spiked with the five amphetamines at 100 ng/ml, are presented in Fig. 1. The five pentadeuterated internal standards showed no isotopic contributions to the *m/z* ratios selected for their respective analyte, contrary to other deuterated analogs, such as *d*3- or phenyl-*d*5-amphetamine, HFB-, PFP- or TFA-derivatives of *d*6- or *d*10-methamphetamine, as recently reported [15].

The liquid–liquid extraction used could not avoid evaporation steps, which are critical for the recovery of such volatile molecules as amphetamines. Nevertheless, loss of analytes was minimized by acidification of the medium to convert base forms to hydrochlorides, by addition of isopropanol in order to slow down the solvent evaporation and by rapidly withdrawing the dry extracts from the nitrogen stream. The recoveries thus obtained were satisfactory, ranging between 63.6±8.5% for MDEA and 90.9±6.0% for MA (Table 2).

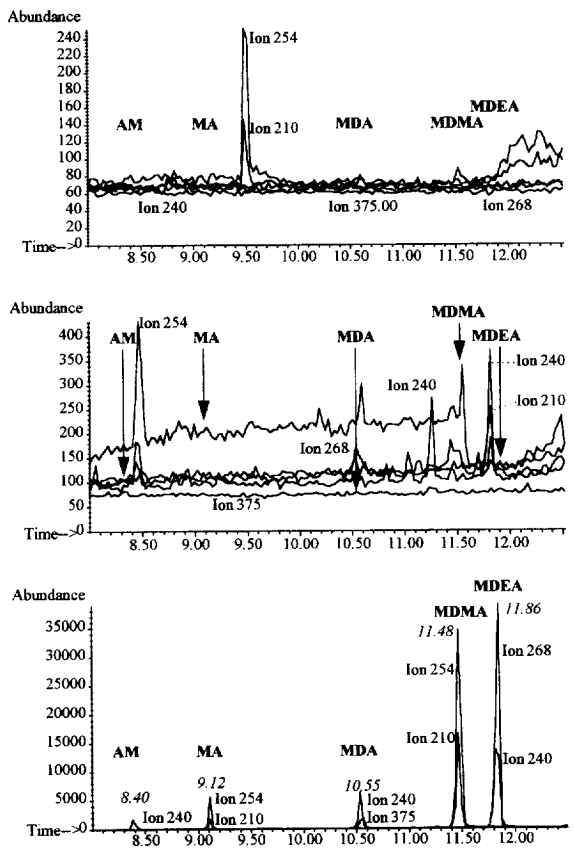

Fig. 1. Chromatograms of reagent blank (top), of blank blood (middle) and of drug-free whole blood spiked with 100 ng/ml AM, MA, MDA, MDMA and MDEA (bottom). Note the difference between intensity scales.

The limits of detection obtained with the present method are as follows: AM, 1 ng/ml; MA, 2 ng/ml; MDA, 8 ng/ml; MDMA, 1 ng/ml; MDEA, 0.5 ng/ml. As a comparison, the best previously reported LOD values for AM were 2 ng/ml in plasma with GC–NPD [16], 4 ng/ml in plasma with GC–FID [17], 5 ng/ml in blood with GC–ECD [11] and 5 ng/ml in urine with GC–MS [6,15,18]; for MA, the best reported LODs were 2 ng/ml in plasma with GC–NPD [16] and 3 ng/ml in urine with GC–MS [6]; for MDA, MDMA and MDEA in whole blood, only one published technique could be found, reporting LOD values between 200 and 500 ng/ml [11].

The intra-assay and inter-assay precision study showed that the present method was accurate, repeatable and reproducible over the range of concentrations usually found in clinical or forensic blood samples. LOQ, derived from inter-assay experiments as the lowest concentration giving a intermediate precision C.V. lower than 20% and an inaccuracy lower than 15%, was 10 ng/ml for AM, MDMA and MDEA, 20 ng/ml for MA and 50 ng/ml for MDA (Table 2). These results might have been better for low concentrations if weighted linear regression was available in the software used. Linearity, precision and accuracy were excellent for each analyte, from its LOQ up to 1000 ng/ml.

4. Conclusion

This paper presents a validated method for the specific, sensitive and precise whole blood determination of amphetamine and four of its analogs mainly found on the illicit market. This technique also applies to urine samples, with even better recoveries and limits of detection (data not shown). It is therefore suitable for clinical toxicology, for the confirmation of doping in sports, of driving under the influence of drugs or for other forensic applications.

Table 2
Validation results of the GC–MS quantitative determination of amphetamines in whole blood

Compounds		Intra-assay precision ($n=5$), C.V. (%)	Inter-assay study ($n=5$)		
Concentration added (ng/ml)	Extraction recovery (%)		Mean concentration found (ng/ml)	Precision C.V. (%)	Inaccuracy (%)
AM					
10	—	—	10.9	15.2	9.0
20	—	11.9	19.7	17.0	1.4
50	—	—	50.8	7.2	1.7
100	85.2	—	99.4	9.2	0.6
200	—	—	206.1	10.9	3.1
500	—	11.0	492.6	8.3	1.5
1000	—		997.9	2.0	0.2
			$r=0.99675$		
MA					
10	—	—	11.7	22.5	17.3
20	—	12.7	22.4	17.9	12.1
50	—	—	50.9	6.8	1.8
100	90.9	—	98.7	5.9	1.3
200	—	—	190.5	3.4	4.7
500	—	4.8	461.0	11.5	7.8
1000	—	—	981.1	2.9	1.9
			$r=0.99025$		
MDA					
10	—	—	11.85	42.2	18.5
20	—	9.9	21.4	27.2	7
50	—	—	45.3	7.9	9.4
100	76.5	—	102.7	4.9	2.7
200	—	—	176.1	12.1	11.9
500	—	8.7	552.3	9.5	10.5
1000	—	—	1001.5	2.6	0.2
			$r=0.98500$		
MDMA					
10	—	—	9.3	10.8	7.3
20	—	5.1	20.5	9.1	2.5
50	—	—	47.9	8.6	4.2
100	84.1	—	102.1	5.6	2.1
200	—	—	186.1	5.8	7.0
500	—	9.4	526.7	10.1	5.3
1000	—	—	1001.8	1.0	0.2
			$r=0.99925$		
MDEA					
10	—	—	8.88	13.0	11.2
20	-	5.0	20.9	9.3	4.5
50	—	—	46.9	3.6	2.3
100	63.6	—	102.1	3.4	2.1
200	—	—	188.6	5.7	5.7
500	—	2.1	505.5	2.2	1.1
1000	—	—	1002.3	1.9	0.2
			$r=0.99875$		

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