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A rapid and sensitive liquid chromatography-tandem mass spectrometry method for the determination of amphetamine and related designer drugs in urine

Une méthode par chromatographie liquide couplée à la spectrométrie de masse en tandem rapide et sensible pour le dosage de l'amphétamine et de drogues de synthèse dans les urines

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SUMMARY

A method for the direct analysis of six amphetamine compounds in urine was developed using liquid chromatography tandem mass spectrometry (LC-MS/MS). We added 90 µl of a solution of internal standards (1 µg/mL of d_5-AMP, d_5-MET, d_5-MDA, d_5-MDMA, d_5-MDEA and d_5-MBDB) to 10 µl of urine followed by vortex-mixing and centrifugation. The sample solutions were analyzed by LC-MS/MS in the MRM mode after separation on a reversed-phase C18 column using gradient elution. Separation and detection of all compounds was accomplished within eight minutes. Linearity was established for all compounds, from 78 to 100000 ng/mL. Correlation coefficients for all analytes exceeded 0.998.The lower limit of quantification was 10 ng/mL for all compounds, except for AMP and MDA (78 ng/mL). Within-day imprecision (CV%) and between-day CVs (78, 625 and

RÉSUMÉ

Une méthode pour l'analyse directe de six amphétaminiques dans les urines a été développée en utilisant la chromatographie liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS). Nous avons ajouté 90 µl d'un mélange d'étalons internes (1 µg/mL de d_5-AMP, de d_5-MET, de d_5-MDA, de d_5-MDMA, de d_5-MDEA et de d_5-MBDB) à 10 µl d'urine, mélangé par vortex et centrifugé. Les échantillons ont été analysés par LC-MS/MS en mode MRM après séparation sur une colonne C18 à phase inverse en utilisant un gradient d'élution. La séparation et la détection de tous les composés ont été accomplies en huit minutes. La linéarité a été établie pour tous les composés, de 78 à 100000 ng/mL. Les coefficients de corrélation étaient supérieurs à 0.998. Les limites de quantification étaient inférieures à 10 ng/mL, sauf pour l'amphétamine et la MDA (78 ng/mL). La répéta-

10000 ng/mL) ranged from 2.62 to 16.26% and from 0.86 to 11.98%, respectively. Accuracy (bias%) lay between 0.16 and 7.17 %. The peak areas of the amphetamines added to urine fell in the range 85-115% compared to standard solutions in methanol/water, except for AMP and MDA. Carry-over was negligible and stability after storage at room temperature for up to 24h was acceptable. In conclusion, the presented method allows the accurate, precise and rapid determination of six amphetamine compounds in urine over a wide analytical range.

KEY-WORDS
Amphetamines, MDMA, liquid chromatography, tandem mass spectrometry, urine.

bilité (CV%) et la reproductibilité variaient respectivement entre 2.62 et 16.26% et entre 0.86 et 11.98%. En ce qui concerne l'exactitude, le pourcentage de biais à 78 et 10000 ng/mL variait entre 0.16 et 7.17 %. La surface des pics des amphétamines ajoutées à de l'urine variait entre 85 et 100% de celle des amphétamines dissous dans un mélange d'eau et de méthanol, excepté pour l'amphétamine et le MDA. Le carry-over était négligeable et la stabilité (156 et 5000 ng/mL) après stockage à la température ambiante pendant 24h était acceptable. En conclusion, la méthode présentée permet la détermination exacte, précise et rapide de six amphétaminiques dans les urines sur une plage de concentration large.

MOTS-CLÉS
Amphétamines, MDMA, chromatographie en phase liquide, spectrométrie de masse en tandem, urine.

Introduction

In the last few decades, amphetamine designer drugs have gained popularity as recreational drugs and they are used mainly for their stimulating effects, especially in gatherings known as raves and in the dancing scene (1,2). Monitoring of amphetamines and designer drugs in human urine is successfully used for clinical and forensic applications.

For most clinical and forensic applications, initial screening is performed by an immunoassay, and presumptive positive samples are confirmed by a more specific method. To date, the confirmation of amphetamines in urine samples is mainly performed by gas chromatography-mass spectrometry (GC-MS)(3). Despite the many advantages of GC-MS, such as the high sensitivity and specificity and its widespread availability, it does have limitations. One of them, linked to amphetamines, is that the compounds with the amphetamine core structure have base peaks at low masses, resulting in interference from biological background. This can be overcome by the use of extraction from the biological fluid, followed by derivatisation, a step also needed for improving the GC-properties of the compounds. A major drawback of derivatisation, specifically in a routine laboratory with a large number of samples to be analysed in a short time, is that the procedure becomes laborious and time-consuming.

Headspace solid phase micro-extraction (SPME) is one potential solution to minimize the time spent by technical staff preparing samples for GC-MS analysis (4). The disadvantages, on the other hand, are the need for special equipment, the carry-over effect and the need for conditioning of the fibre before use. These limitations of GC-MS led to investigate alternative approaches for analysing amphetamines in biological fluids.

In the last few years, liquid chromatography coupled to mass spectrometry (LC-MS) has developed rapidly in forensic and clinical applications (5,6). Several LC-MS interface types are described. Today, however, two relatively robust LC-MS interface types are most frequently used, the atmospheric-pressure ionisation techniques, electrospray (ESI) and atmospheric-pressure chemical ionisation (APCI). LC-MS offers a higher sensitivity and specificity and reduces sample preparation required with GC-MS because relatively non-volatile compounds can be analysed and no derivatisation is necessary.

A further development is the combination of two mass spectrometers with an interposed collision cell. This characterizes LC-tandem mass spectrometry (LC-MS/MS), which generally provides superior limit of quantification (LOQ), sensitivity and improved selectivity. An extra advantage of MS-MS, in respect of MS, is the ability to shorten the chromatographic run-time dramatically.

This paper describes the validation of a liquid chromatography-APCI-tandem mass spectrometry method (LC-APCI-MS/MS) for simultaneous analysis of six amphetamine compounds in urine. This method is based on the method of Nordgren et al.(7).

Materials and Methods

Chemicals and reagents

Standard solutions of amphetamine (1 mg/mL), methamphetamine (1 mg/mL), 3,4-methylenedioxyamphetamine (MDA) (1 mg/mL), 3,4- methylenedioxymethamphetamine (MDMA) (1 mg/mL), 3,4-methylenedioxyethylamphetamine (MDEA) (1 mg/mL), N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) (1mg/mL), and d_5-deuterated analogues (100

µg/mL) used as internal standards (IS) in methanol were obtained from Cerilliant (Austin, Texas). Methanol (absolute) and water for LC-MS were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate (p.a) was supplied by Sigma-Aldrich (Bornem, Belgium).

Instrumentation and MS/MS conditions

An Agilent 1100 series HPLC system (Agilent Technologies) consisting of a pump, column oven, autosampler and degasser were used for solvent delivery and sample introduction. The injected volume was 20 µl. Analytes were separated at 40°C on a 2.1 x 30 mm Zorbax SB-C18 Rapid Solution column (Agilent Technologies). The column was eluted at a flow rate of 0.3 mL/min and developed with gradient elution as follows: 0-0.2 min, 95%A/5%B; 1.2-4.5 min, 5%A/95%B and 4.8-8 min 95%A/5%B (A: H_2O + 2mM ammoniumacetate, B: MeOH + 2 mM ammoniumacetate).

The LC-MS/MS system consisted of an API 2000™ triple-quadrupole mass spectrometer equipped with an APCI interface (Applied Biosystems/MDS Sciex, Langen, Germany) used in the positive-ion mode. The six amphetamine compounds were detected in the multiple-reaction monitoring mode. Two MRM transitions for each substance were monitored to provide sufficient identification of the amphetamine compounds. The chosen MRM transitions for each amphetamine compound and d_5-deuterated analogue are summarised in table 1. The entrance potential varied from 5.5 V to 9 V, the collision cell entrance potential varied from 14 to 20 V and the cell exit potential was set at 2 V or 4 V, according to the analyte.

Analyst Software (Ver. 1.3.1; Applied Biosystems/MDS Sciex) was used for HPLC system control, data acquisition, and data processing.

Table I : *Retention time, parent ion and the chosen daughter ions for each amphetamine compound and* d_5*-deuterated analogue*

Amphétamines	Retention time (min)	M+1	MRM 1	MRM 2
AMP	4.20	136.079	91.05	65.05
MET	4.36	150.078	91.05	119.15
MDA	4.29	180.119	135.15	133.05
MDMA	4.38	194.085	163.05	105.05
MDEA	4.50	208.068	163.05	105.15
MBDB	4.61	208.068	135.05	177.15
d_5-AMP	4.17	141.112	93.35	
d_5-MET	4.35	155.09	92.35	
d_5-MDA	4.26	185.114	110.15	
d_5-MDMA	4.35	199.136	165.15	
d_5-MDEA	4.48	213.075	163.05	
d_5-MBDB	4.60	199.136	165.15	

Calibration standards and internal standard mix-solution

Calibration standards were prepared in drug-free urine from methanolic stock solutions, containing all amphetamine compounds at a concentration of 1 mg/mL. The concentrations of the calibration standards were 78.125, 156.25, 312.5, 625, 1250, 5000, 10000, 20000, 30000, 40000, 50000, 60000, 70000, 80000, 90000 and 100000 ng/mL.

For recovery testing, standards with concentrations of 78.125, 625 and 10000 ng/mL were made in H_2O/MeOH (50/50) solution from the same stock solutions (1mg/mL).

The internal standard-mix solution (1 µg/mL of each amphetamine) was prepared by dilution of 10µl from each d_5-deuterated analogue in 10 mL distilled water.

All standards were stored at 4°C and were allowed to come to room temperature, vortex-mixed and centrifuged prior to analysis.

Sample preparation

Sample preparation was minimal and consisted of adding 90 µl of the internal standard-mix solution to 10 µl of sample (calibration standards and standards made in H_2O/MeOH (50/50) solution). After vortex-mixing and centrifugation (2 min at 13000g), 85 µl of the sample solution was pipetted into crimp-cap autosampler vials and placed in the autosampler.

Validation experiment

Method validation, including studies of imprecision (within-day and between-day), accuracy, linearity, stability, carry-over, recovery and the determination of the limit of detection (LOD) and quantification (LOQ) of the LC-MS/MS method was performed according to the FDA recommendations (8).

Results and discussion

Figure I shows a typical LC-MS/MS chromatogram of a patient urine sample containing 11400 ng/mL AMP, 5633 ng/mL MDMA and 281 ng/mL MDA.

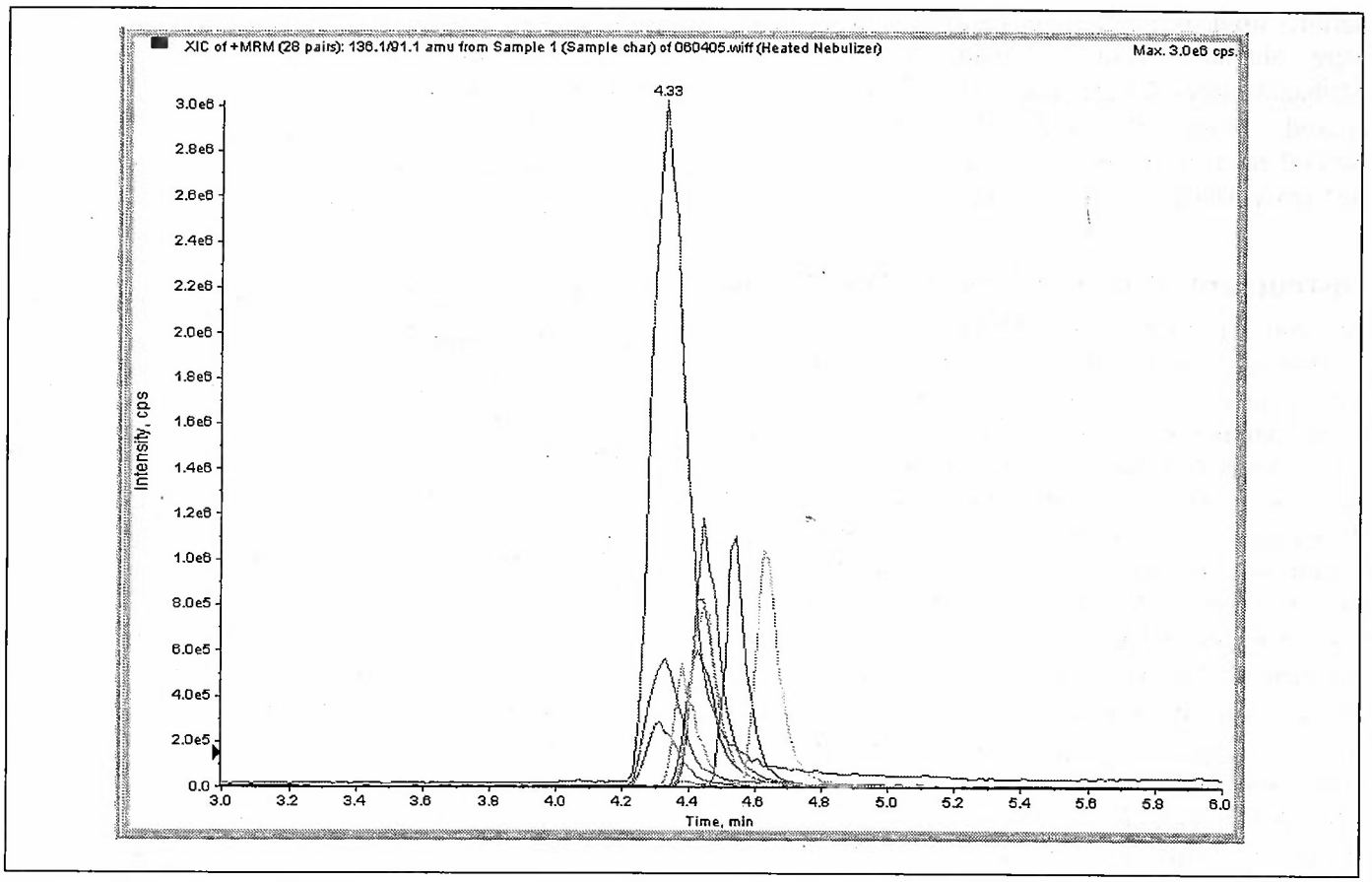

***Figure 1** : LC-MS/MS chromatogram of a patient urine sample containing AMP (11400 ng/mL), MDMA (5633 ng/mL) and MDA (281 ng/mL). The retention times (RT) of the internal standards are 4.33 min (d_5-AMP), 4.43 min (d_5-MET), 4.38 min (d_5-MDA), 4.43 min (d_5-MDMA), 4.54 min (d_5-MDEA), and 4.63 min (d_5-MBDB).*

Calibration curves

To construct calibration curves, a set of eight urine samples spiked with the amphetamine compounds at concentrations ranging from 78 ng/mL to 10000 ng/mL were used. The calibrators were measured for five consecutive days. All calibration curves showed linearity for all amphetamine compounds and correlation coefficients exceeded 0,998.

LOD and LOQ

The limit of detection (LOD), defined as a signal-to-noise ratio of 3, and the limit of quantification (LOQ), defined as a signal-to-noise ratio of 10, were calculated by a script in the Analyst Software. The LOD and LOQ were 4.9 ng/mL and 9.8 ng/mL, respectively for each analyte except for amphetamine and MDA (table II). The LOQ's are lower than the current recommended urine confirmation cut-off levels (9). However if for certain applications higher sensitivity is necessary some modifications can be tried out: increasing the injected volume, increasing the sample/internal standard ratio, lowering the number of MRM-transitions or another procedure (e.g. with extraction) can be applied.

***Table II** : Limit of detection and limit of quantification for each amphetamine compound.*

Amphétamines	LOD (ng/mL)	LOQ (ng/mL)
AMP	39.1	78.1
MET	4.9	9.8
MDA	39.1	78.1
MDMA	4.9	9.8
MDEA	4.9	9.8
MBDB	4.9	9.8

Imprecision

Imprecision (CV%) was evaluated by analysing three calibrator samples with a low (78 ng/mL), medium (625 ng/mL) and high (10000 ng/mL) concentration of each amphetamine on the same day in five replicates (within-day imprecision) and over five consecutive days (between-day reproducibility). The within-day CVs ranged from 2.62 to 16.26%, the between-day CVs from 0.86 to 11.98% (table III). So, data for imprecision were within required limits of 20% at the

Tableau III : *Imprecision (CV%), bias (%) and recovery (%) determined for three calibration standards with low (78 ng/mL), medium (625 ng/mL) and high (10000 ng/mL) concentration of each amphetamine compound, respectively (n=5).*

	Imprecision (CV%)						Accuracy (bias%)			Recovery (%)		
	Within-day CV			Between-day CV								
	78 ng/mL	625 ng/mL	10000 ng/mL	78 ng/mL	625 ng/mL	10000 ng/mL	78 ng/mL	625 ng/mL	10000 ng/mL	78 ng/mL	625 ng/mL	10000 ng/mL
AMP	16.26	4.76	3.86	7.07	4.39	0.86	2.5	1.0	1.6	61	78	70
MET	3.39	2.94	3.35	3.83	4.14	1.29	2.4	0.5	2.4	110	109	99
MDA	3.42	4.60	3.41	10.42	3.05	1.89	7.7	1.3	0.2	94	93	75
MDMA	6.80	2.77	4.42	11.98	3.57	1.61	4.1	0.2	1.4	108	108	95
MDEA	8.17	3.53	5.36	10.98	3.91	1.29	3.0	0.2	0.8	101	102	92
MBDB	10.76	5.68	2.62	2.40	2.39	2.20	1.1	3.4	0.4	102	105	98

lowest concentration and below 15% at higher concentrations.

Accuracy

The accuracy of this method for each amphetamine compound was obtained by analyzing the same three calibration standards as mentioned in the paragraph 'imprecision' over five consecutive days. As indicated in table III, the calculated concentration of each compound agreed well with the expected values.

Recovery

The recoveries were obtained by comparing the peak areas of spiked urine with those of the same concentrations of the analytes in H_2O/MeOH (50/50) solution. Three concentrations were tested (78 ng/mL; 625 ng/mL; 10000 ng/mL) in five-fold. The results are presented in table III. We observed good agreement (< 15% deviation) for most analytes, except for amphetamine (all concentrations) and MDA (only the highest concentration).

Carry-over

Carry-over was evaluated by injecting a blank urine specimen containing the internal standards immediately after a sample that contained 10 000 ng/mL of each amphetamine compound. Carry-over was less than 0.32% and the results are shown in table IV. Although the carry-over is low, the confirmation cut-off of 200 ng/mL could be reached after a sample containing 60000 ng/mL of an amphetamine, which occurs occasionally.

Table IV : *Carry-over (%) in a blank urine sample analyzed after a calibration standard with a concentration of 10000 ng/mL.*

Amphétamines	Carry-over (%)
AMP	0
MET	0.22
MDA	0.06
MDMA	0.22
MDEA	0.32
MBDB	0.26

Linearity above 10000 ng/mL

Standards with concentrations between 20000 ng/mL and 100000 ng/mL, made in drug-free urine samples, were used to determine linearity above 10000 ng/mL. The linearity was evaluated by dividing the observed value of each standard by the expected value of each standard to determine the percentage of the expected result for each concentration. The percentages of the expected results for the amphetamines were between 91% and 107% (table V).

Stability

For stability studies, two calibration standards (calibration standard 2 with a concentration of 156 ng/mL and calibrator 7 with a concentration of 5000 ng/mL) were each split into 10 aliquots, with five aliquots assayed immediately and the other five stored for up to 24 h at room temperature. The means of the five determinations for each calibrator, before and after storage were then compared. The data are given in table VI.

Tableau V : *Linearity obtained by dividing the observed value of each standard by the expected value of each standard and multiplied by 100. The deviations (%) were below 10%.*

Amphetamine	Linearity as deviation (%) of the observed value to the expected value								
	20000 ng/mL	30000 ng/mL	40000 ng/mL	50000 ng/mL	60000 ng/mL	70000 ng/mL	80000 ng/mL	90000 ng/mL	100000 ng/mL
AMP	107	104	103	103	101	98,7	101	97,2	92,3
MET	107	106	104	106	104	100	100	97.4	95.4
MDA	91.8	95	96	103	102	104	105	104	97.4
MDMA	104	106	103	106	99.7	102	102	100	94
MDEA	109	101	106	107	99.1	101	99.7	100	94.8
MBDB	107	105	99.2	104	104	101	102	102	92.7

Table VI : *Stability of two calibration standards with a concentration of 156 ng/mL and 5000 ng/mL, respectively after a 24 h storage at room temperature. The means of five determinations for each compound, before and after storage were subtracted and divided by the mean of the corresponding results obtained before storage.*

Amphétamine	Stability (%)	
	156 ng/mL	5000 ng/mL
AMP	6.8	-1.78
MET	1.43	-0.26
MDA	-3.31	-14.66
MDMA	-3.45	0.07
MDEA	-11.99	-5.04
MBDB	-15.87	-13.57

Conclusion

We have developed and validated a LC-MS/MS method for the simultaneous determination of six amphetamine compounds in urine samples. The sample pre-treatment is fast and simple, requiring no derivatisation. The LOQs are much lower than recommended urine confirmation cut-off levels, i.e. this method is sensitive enough for routine confirmation. Accuracy and imprecision fulfil the criteria of < 20% at a concentration equal to the LOQ and < 15% at higher concentrations. Good recoveries and linearity over a wide analytical range were obtained. Carry-over is minimal. Separation and detection of all compounds was accomplished within eight minutes. The main advantages of the present method lie in its simple sample preparation, reliable results and short analysis time.

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