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Rapid screening method for determination of Ecstasy and amphetamines in urine samples using gas chromatography–chemical ionisation mass spectrometry

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Abstract

The need for analytical screening tests more reliable and valid to detect amphetamine and related ''designer drugs'' in biological samples is becoming critical, due to the increasing diffusion of these drugs on the European illegal market. The most common screening procedures based on immunoassays suffer a number of limitations, including low sensitivity, lack of specificity and limited number of detectable substances. This paper describes a screening method based on gas-chromatography-mass–spectrometry (GC/MS) using positive chemical ionisation (PCI) detection. Methanol was used as reactant gas in the ionisation chamber. Molecular ions of different compounds were monitored, allowing a sensitivity of 5–10 ng/ml with high selectivity. The sensitivity of the method gives positive results in samples taken 48–72 h after intake of one dose of 50–100 mg. The method is simple and rapid. Sample preparation was limited to one liquid–liquid extraction, without any hydrolysis and derivatisation. Hydrolysis is critical to identify metabolites excreted as conjugates. Blank urine samples spiked with known amounts of amphetamine (AM), methylamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethylamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA) and methylenedioxyphenyl-*N*-methyl-2-butanamine (MBDB) were analysed. The method was successfully tested on real samples of urine from people, whose use of amphetamine was suspected, and results were compared with results obtained with immunoassays.

Keywords: Ecstasy; Amphetamines

1. Introduction

The diffusion of amphetamine and related ''designer drugs'' is dramatically increasing on the European illegal market. This trend is confirmed by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), indicating these drugs as the most widespread in Europe after cannabis [1].

Immunoassays like radioimmunoassays (RIA), enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA) are generally used in analytical procedures as screening techniques. Their results should be confirmed by specific techniques like gas-chromatography–mass-spectrometry (GC–

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MS). International guidelines [National Institute on Drug Abuse (NIDA), Substance Abuse and Mental Health Services Administration (SAMHSA), Guidelines for Federal Workplace drug testing] stated 1000 ng/ml of amphetamine and methylamphetamine as cut-off value for urine samples. This recommended value resulted in the development of immunochemical tests for detection of amphetamine and methylamphetamine in urine samples with very high cut-off suggested values (300–1000 ng/ml).

Immunochemical techniques suffer a number of limitations: (A) the capability of detection of many commercially available kits for methylenedioxyamphetamines is based on cross-reactivity. The number of detectable substances of this class is limited to methylenedioxyamphetamine (MDA), methylenedioxymethylamphetamine (MDMA) and in some cases methylenedioxyethylamphetamine (MDEA), but with decreased sensitivity (Table 1). (B) High cut-off values permit positive detection only for recent ingestion of high doses, resulting in the risk of false negative analyses [2,3]. (C) With cut-off values lower than the ones suggested the number of false positive results is high, due to common drugs like ephedrine, ranitidine and interfering substances in the biological matrix [4,5]. (D) Immunochemical assays are cost-effective only when many samples are analysed. Kraemer and Maurer published a complete review of procedures for analysis of amphetamine and related ''designer drugs'' based on clean-up of samples and GC–MS and HPLC–MS techniques [6]. More recent papers report methods for solid-phase micro-extraction (SPME) and for separation of enantiomers [7–10]. Methods based on chemical ionisation mass-spectrometry are reported as well [11–13]. Most of the procedures include hydrolysis and derivatisation steps and in some cases very expensive instruments are used.

This paper describes a simple and rapid GC–MS chemical ionisation screening method with good sensitivity and reasonable costs to detect amphetamine (AM), methylamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxymethylamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA) and methylenedioxyphenyl-*N*-methyl-2-butanamine (MBDB). Amphetamine and related ''designer drugs'' listed above are those most commonly encountered on the illegal market in Italy.

2. Experimental

2.1. Chemicals and reagents

D,L amphetamine HCl and D,L methylamphetamine HCl (SALARS, Como, Italy); D,L methylenedioxyamphetamine HCl (MDA), D,L methylenedioxymethyl-amphetamine HCl (MDMA), D,L methylenedioxyethylamphetamine HCl (MDEA) and methylenedioxyphenyl-*N*-methyl-2-butanamine HCl (MBDB) (LIPOMED, Arleshein, Switzerland); the internal standards methoxyphenamine HCl and *N*-methyl-bis-trifluoroacetamide (MBTFA) (Sigma–Aldrich, Milan, Italy). Sodium hydroxide, sodium chloride, ethyl acetate and methanol (Carlo Erba, Milan, Italy). *tert*-Butylmethyl ether (Merck, Darmstadt, Germany).

Table 1
Cross-reactivity of some immunoassays

Methods	FPIA A/M II	Emit II	Roche online	Emit dau monocl	Emit Dau policlon	Cedia dau AMF	Cedia dau ecstasy
Cut-off[a]	300	1000	1000	1000	2000	1000	300
Cross-reactivity (%)[b]							
d-A	100	100	100	250	250	100	0.05
d-MA	100	100	95	100	100	100	0.06
MDA	70	33	33	100	2	2	57
MDMA	63	6	0–2	33	10	69	100
MDEA	46	13	–	30	–	–	129
MBDB	–	–	–	–	–	–	24

[a] Values suggested by producers in ng/ml.
[b] Cross-reactivity (%) referred to the reported cut-off value.

2.2. Preparation of standard solutions

Standard solutions of the six compounds of interest and of the internal standard (I.S.) were prepared in methanol (1 mg/ml) and stored at −20 °C. Mixtures of the six compounds were prepared at 0.5, 1 and 5 μg/ml. The I.S. solution of methoxyphenamine was prepared at 2 μg/ml.

2.3. Urine samples

Human blank urine was taken from 20 volunteers (14 males and six females) between 18 and 50 years

Table 2
Ion trap analytical parameters

Emission current (μA)	10
Count threshold	1
Multiplier offset (V)	0
Scan time (s/scan)	0.5
Chemical ionisation	
CI storage level (*m*/*z*)	15.0
Ejection amplitude (V)	10
Max. ionization time (s)	2000
Max. reaction time (ms)	4
Target tic (counts)	5000
Background mass (*m*/*z*)	55

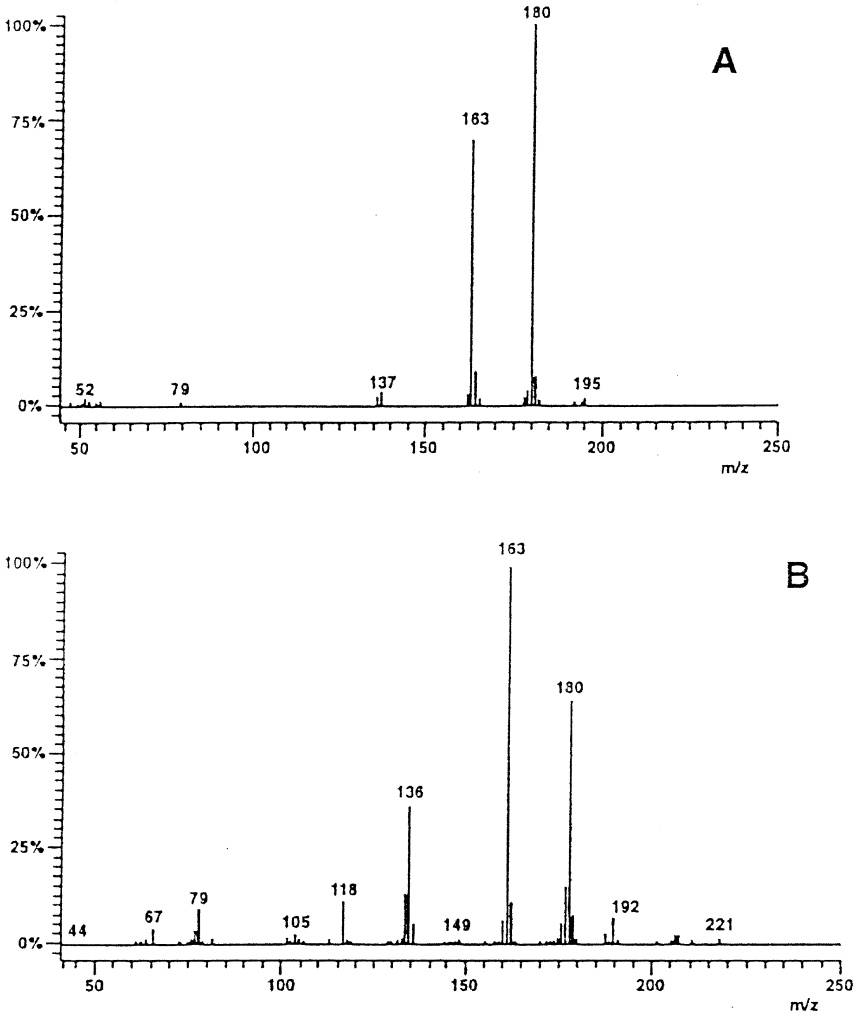

Fig. 1. Chemical ionisation mass spectra of MDA. Reactant gas (A) methanol, (B) acetonitrile.

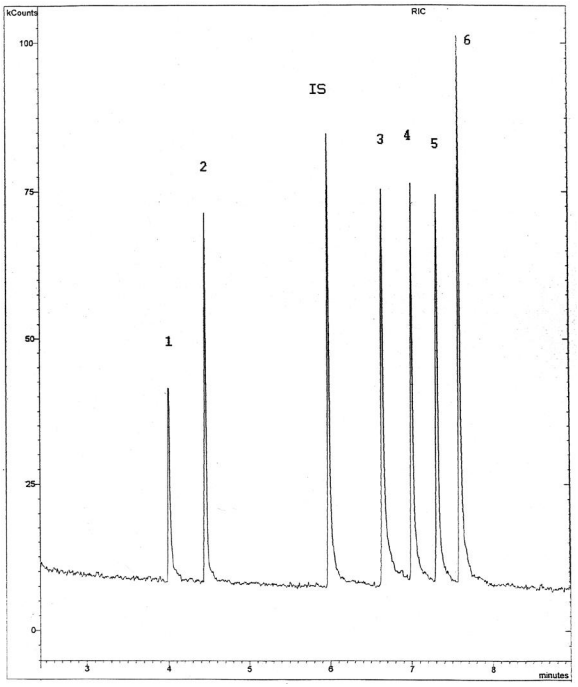

Fig. 2. Chromatogram (GC–MS chemical ionisation mass-spectrometry, reactant gas methanol) of a standard mixture in methanol (20 ng/μl): (1) AM, (2) MA, (3) MDA, (4) MDMA, (5) MDEA, (6) MDBD, I.S. methoxyphenamine. See text for analytical conditions.

old. All the volunteers declared they had not used amphetamine and ''designer drugs''. Twelve samples to be screened were collected from the Rehabilitation Centre in drug of abuse detoxification programs of the Ospedale degli Infermi, Rimini (Italy). The pH of samples ranged from 5.5 to 6.5. Samples were stored at −20 °C before analyses. All samples were analysed to check for interference due to the biological matrix. Blank samples of urine were spiked with a mixture of the six compounds of interest at concentrations of 2, 5, 10, 20 and 50 ng/ml adding standard methanolic solutions.

2.4. Sample preparation from urine

A 1-ml volume of urine was basified with sodium hydroxide 0.5 *M*, after adding 20 ng of I.S. and 200 mg of sodium chloride. The samples were then extracted with 3 ml of *tert.*-butyl methyl ether shaking with a vortex for 2 min. The organic layer was evaporated to dryness under nitrogen flux at *T* lower than 40 °C. The residue was dissolved with 30 μl of ethyl acetate and 1 μl of the solution was analysed by GC–MS.

2.5. Apparatus and analytical conditions

The immunoassay apparatus was an Olympus AU 400 clinical chemistry analyser. The immunoassay was the Olympus DAU Amphetamines (Olympus Diagnostica GmbH, Hamburg, Germany).

The GC was a Varian 3400 CX connected to a Varian Saturn 2000 ion trap mass spectrometer (Varian, Palo Alto, CA, USA). The GC conditions were as follows: split injection mode (15:1); column Restek capillary Rtx-5MS (20 m×0.25 mm I.D.), 95% dimethyl–5% diphenyl polysiloxane, film thickness 0.25 μm; injection port temperature, 250 °C; carrier gas, helium; flow rate, 0.9 ml/min; column temperature, programmed from 70 to 280 °C, 20 °C/min, initial time 1 min. The MS conditions were as follows: transfer line, 270 °C; ion source temperature 150 °C; manifold temperature, 80 °C. Methane, methanol and acetonitrile were used as reactant gas into the MS source. Protonated quasi-molecular ion (M+1) of the six compounds of interest and of the I.S. were monitored (*m/z* 136, 150, 180, 194, 208) using five scan segments with windows of three amu width. Other analytical conditions as in Table 2.

3. Results and discussion

3.1. GC–MS–chemical ionisation

Electron impact mass spectra of compounds with amphetamine-like structure have base peaks at low mass (*m/z* 44, 58, 72) of poor selectivity. A screening method based on the acquisition of these ions in selected ion monitoring (SIM) or selected ion storage (SIS) gives ion chromatograms with a strong interference from biological background, lacking sensitivity and selectivity. This problem could be overcome by derivatizing, resulting in better spectra, but a derivatization step increases the time of analysis.

Positive chemical ionisation is a soft ionisation technique and gives simple spectra, dominated by the

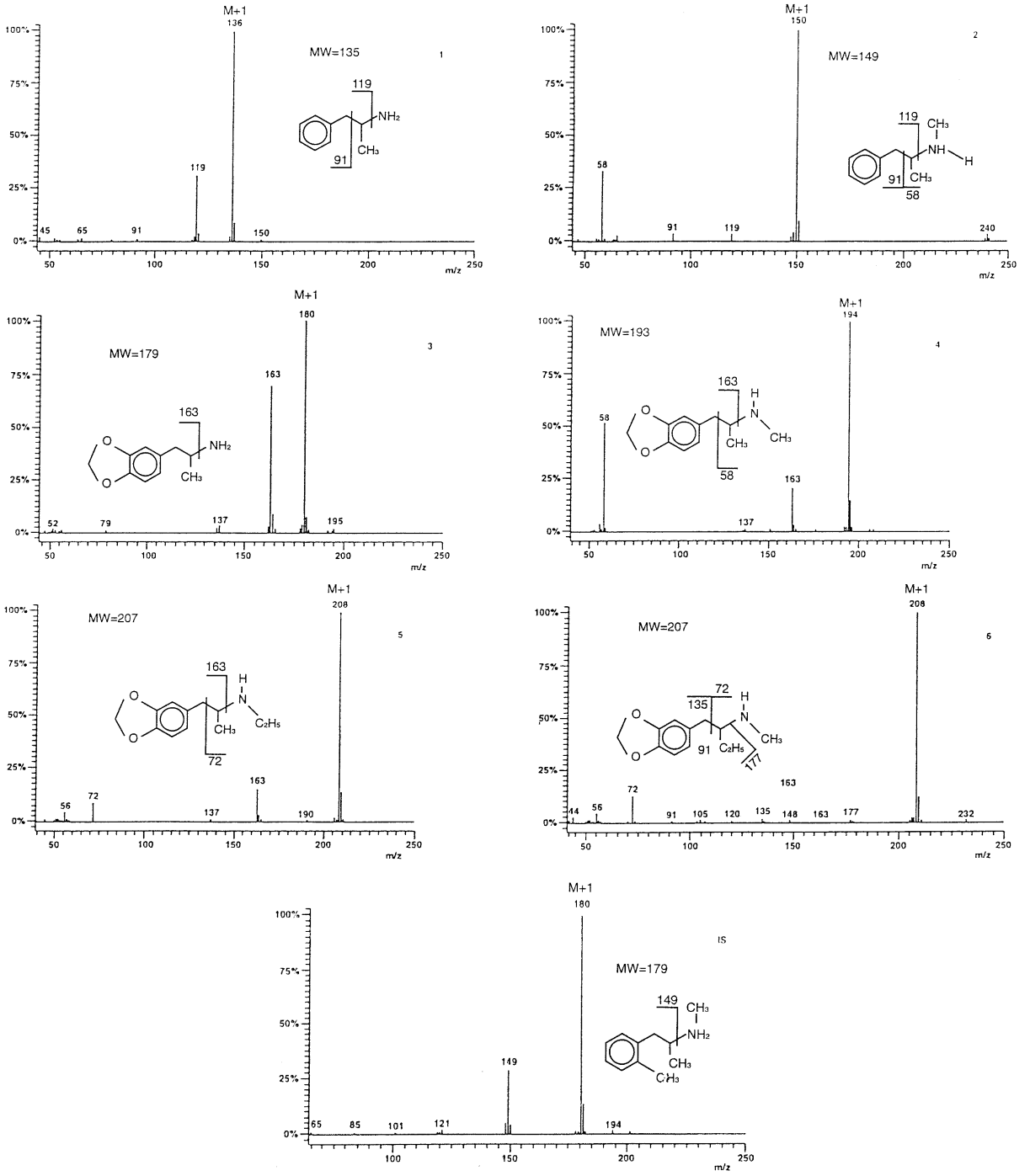

Fig. 3. Chemical ionisation mass spectra of: (1) AM, (2) MA, (3) MDA, (4) MDMA, (5) MDEA, (6) MDBD and I.S. methoxyphenamine. Reactant gas methanol.

Table 3
Retention times of compounds of interest relative to methoxyphenamine

Sample	GC–MS
AM	0.67
MA	0.75
I.S.	1
MDA	1.11
MDMA	1.17
MDEA	1.22
MBDB	1.27

protonated quasi-molecular ion (M+1). Monitoring these quasi-molecular ion allows more selective and sensitive detection, due to the lower interfering effect of the biological background.

Methane, methanol and acetonitrile were tested as reagent gas in the ion trap source. Methanol was found to be superior because spectra exhibit less fragmentation. Probably the $MeOH_2^+$ reactant ion from methanol (Fig. 1) has a proton affinity closer to the target molecules than the reactant ions from methane or acetonitrile, giving a less exothermic protonation reaction. All the spectra are very simple, with the quasi-molecular ion $[M+H]^+$ as base peak. The other fragments originate from the cleavage in α to the aminic group, leaving the charge on the aromatic function (*m*/*z* 119, 149, 163) or by β cleavage next to the aminic group, leaving the charge on the fragment containing the heteroatom (*m*/*z* 44, 58, 72; Figs. 2 and 3).

3.2. Selectivity and limit of detection (LOD)

The selectivity of the method was demonstrated analysing 20 blank samples of urine. The analyses showed no significant interference at the retention time of the compounds of interest (see relative retention times in Table 3), as no peaks were found. LOD was 10 ng/ml of urine for amphetamine and MDA and 5 ng/ml for the other target molecules. These values are suitable for a rapid and simple screening procedure. The *S*/*N* ratio was calculated on 20 blank samples. LOD was calculated with the mean value plus three standard deviation (SD). Three samples of urine spiked with the six compounds of interest (20, 15, 10, 5 and 2 ng/ml) were analysed following the procedure described above under routine conditions. The different values of LOD are caused by the different chromatographic behaviour of the compounds. The liners used were deactivated and the injector had to be cleaned after 100 samples to avoid a decrease in sensitivity, especially for amphetamine and MDA. Based on the published pharmacokinetic data [14–19], the sensitivity would allow positive results with urine samples collected up to 48–72 h after intake of one dose of 50–100 mg.

3.3. Recovery and robustness

Recovery of the procedure was evaluated comparing chromatographic results (peak areas) of a mixture of A, MA, MDA, MDMA, MDEA and MBDB in a standard solution and after extraction of a urine sample (1 ml) spiked having the same concentration (15 ng/ml) (Fig. 4). I.S. was added prior to evaporating the sample. The extraction tests were repeated three times during the same day. Recovery was between 62 and 66% for A and MDA and between 73 and 85% for the other four compounds, comparable to published data [12,20]. The evaporation step is critical and attention must be paid to avoid analyte losses.

Robustness was tested for 3 months analysing every week two urine samples spiked at 10 and 20 ng/ml, respectively. Analyses were carried out in triplicate and performed by three different operators. The mass spectrometer allowed easy and rapid CI detection, giving the opportunity to perform CI analysis after EI analysis in a few minutes without time-consuming recalibration.

The split injection avoids rapid decrease in column efficiency, resulting in more samples analysed before column changing. Urine samples from 12 people collected from the Rehabilitation Centre in drug of abuse detoxification programs of the Ospedale degli Infermi, Rimini (Italy) were analysed using the immunoassay. The numbers in the second column in Table 4 cannot be used as quantitative results [21] but need a Bayesian statistical interpretation. Only the results higher than the cut-off have a reasonable positive predictive value and are confirmed to avoid the reporting of false positive results [22,23]. The Olympus DAU Amphetamine kit has a suggested cut-off value of 1000 ng/ml. For this reason the only

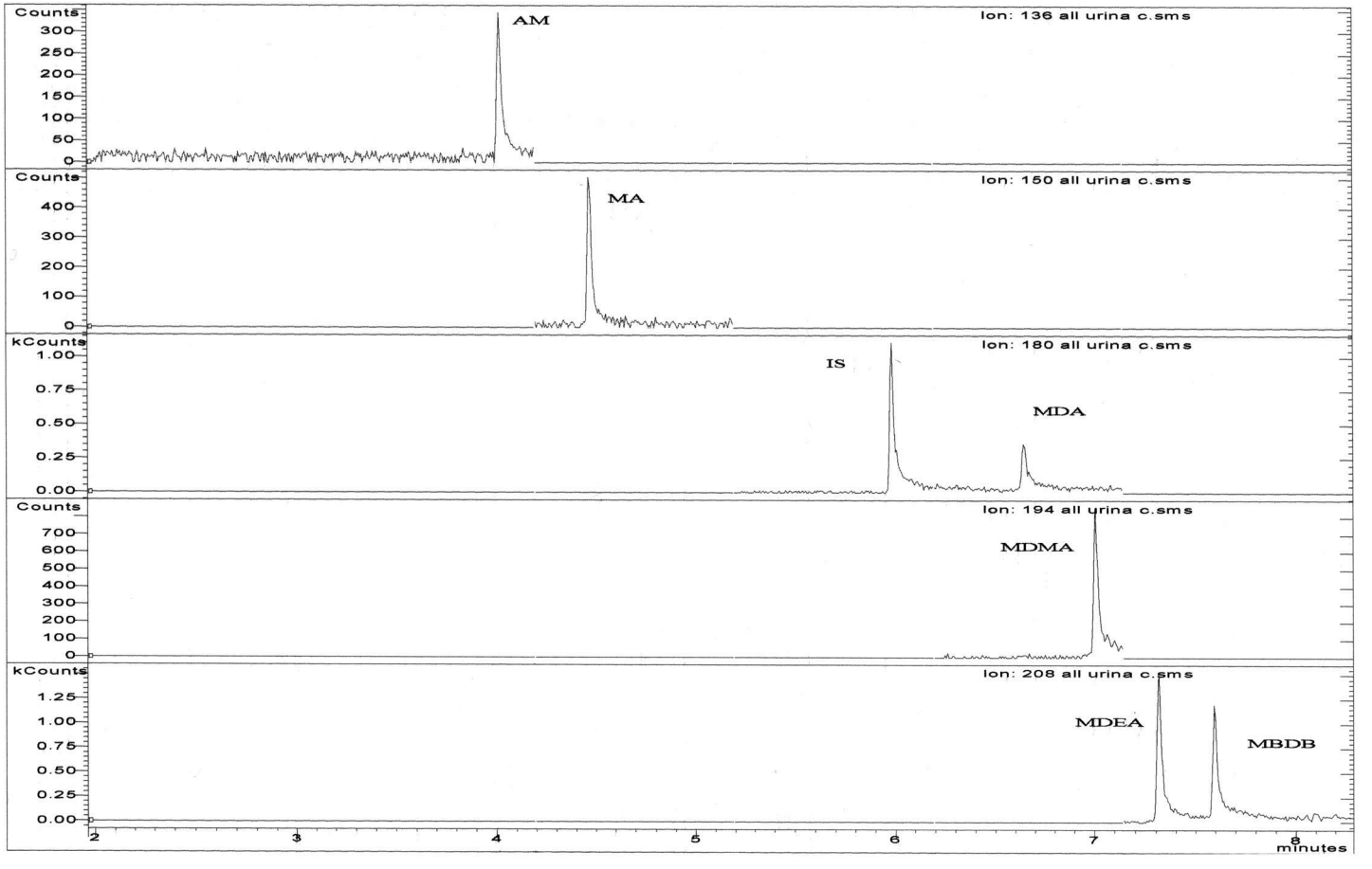

Fig. 4. Urine (1 ml) spiked with a mixture of the six compounds of interest (15 ng/ml) and I.S. methoxyphenamine (20 ng/ml). Ion chromatograms of protonated quasi-molecular ion (M+1).

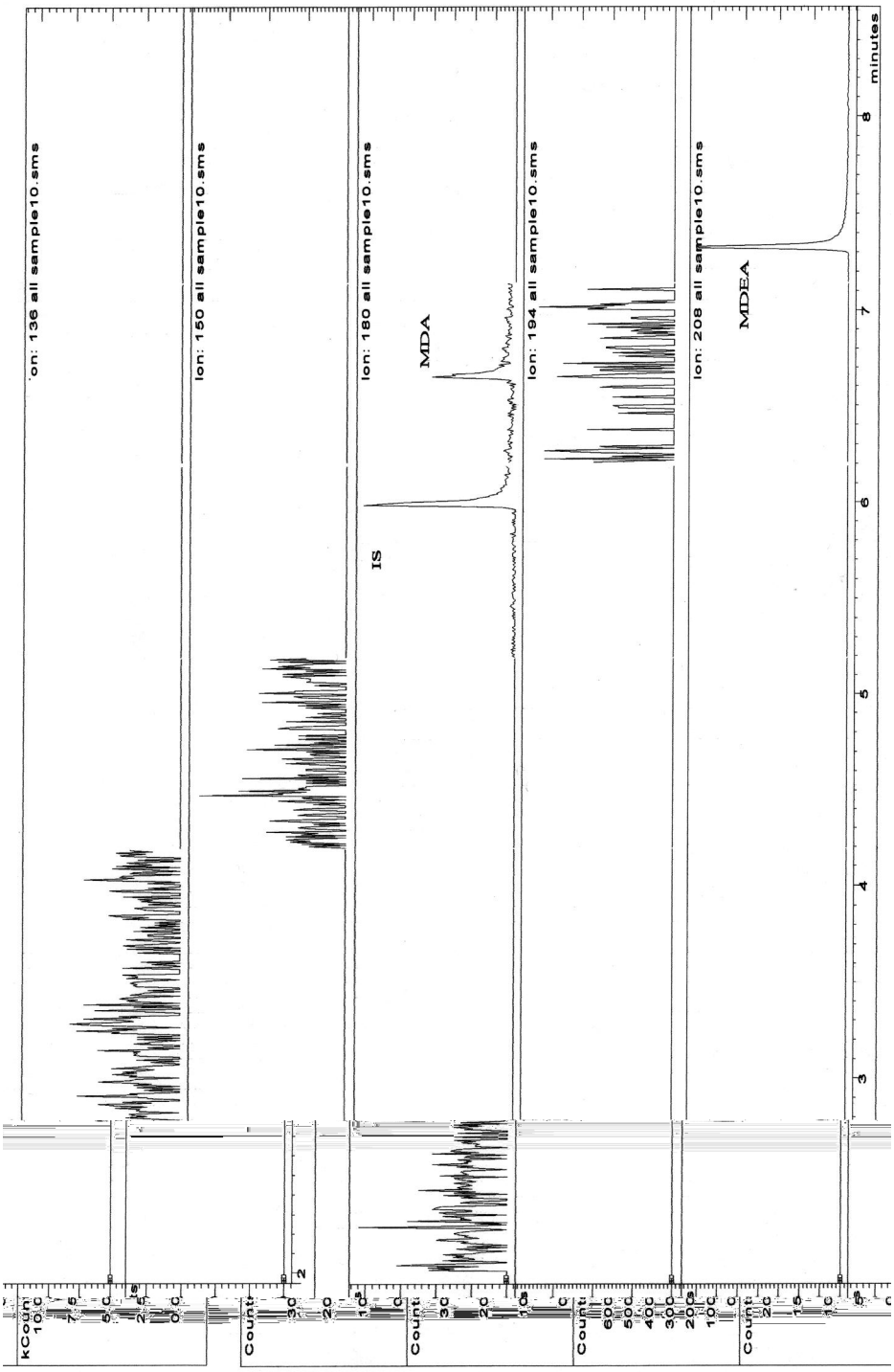

Fig. 5. Analysis of a real sample of urine containing MDEA and MDA.

Table 4
Results of screening analyses by immunoassay and GC–MS (CI)

Sample	Olympus DAU amphetamines (ng/ml)	GC–MS
1	98	Negative
2	117	Negative
3	79	Negative
4	56	Negative
5	268	Negative
6	6879	Negative
7	1484	MDEA, MDA
8	236	MDMA, MDA
9	925	AM
10	1873	AM
11	737	MDEA, MDA
12	294	MDEA, MDA

positive samples for the screening test are no. 6, 7 and 10. All the other samples should be considered as negative (Table 4). The use of the GC–MS procedures (Fig. 5) described permitted to obtain six positive results (no. 7, 8, 9, 10, 11 and 12) instead of three obtained by immunoassays and to determine that sample no. 6 is negative (Table 4). In order to confirm the positive results, extracts from samples were taken to dryness under a nitrogen stream at 40 °C, samples were derivatised with 50 μl of MBTFA at 70 °C during 20 min, then 2 μl were analysed by GC–MS and EI spectra were recorded [24]. MBTFA is a potent acylating agent, producing TFA derivatives with a significant EI spectra. The GC–MS analysis permits the identification of the compounds of interest, despite the absence of molecular ion in the spectra. All six samples having positive results after the GC–MS screening (no. 7, 8, 9, 10, 11 and 12) were confirmed.

Sample no. 6 was analysed following the same confirmation method [24]. The analysis obviously confirmed the negative results and examination of mass spectra did not permit to identify the peaks but ruled out all the drugs of abuse forensically monitored in Italy.

4. Conclusions

The present paper describes a qualitative screening method for amphetamine and related ‘‘designer drugs’’. The procedure is alternative to the immunoassays available on the market due to its better sensitivity and selectivity. With a limited number of samples the method is particularly cost-effective. The extraction is very simple and fast and no derivatisation is necessary, resulting in analysis times of less than 30 min. The method is a valid alternative to the traditional immunoassays in forensic toxicology for the compounds studied.

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