Screening Procedure for 21 Amphetamine-Related Compounds in Urine Using Solid-Phase Microextraction and Gas Chromatography–Mass Spectrometry

C. Battu, P. Marquet*, A. L. Fauconnet, E. Lacassie, and **G. Lachâtre**
Department of Pharmacology and Toxicology, University Hospital, 87042 Limoges Cedex, France

Abstract

A specific, sensitive, and rapid procedure for the screening of 21 amphetamine-related compounds in urine is developed using solid-phase microextraction (SPME) and gas chromatography–mass spectrometry. Very clean extracts are obtained in one step with SPME using silica fibers coated with a 100-µm polydimethylsiloxane stationary phase. Temperature, time, pH, and salt saturation are optimized to obtain consistent extraction. An excellent chromatographic separation of the underivatized analytes is obtained with a specially treated nonpolar capillary column (Supelco PTA-5, 30 m × 0.32-mm i.d., 0.5-µm film thickness) dedicated to amino compounds. Selected ion monitoring of three fragments per analyte and one for each of the three deuterated internal standards elicits a high selectivity and detection limits between 1 and 50 ng/mL (i.e., low enough to verify positive results obtained with immunochemical assays). The method is linear in a narrow range (from the detection limit up to 500 ng/mL) when all the amphetamines are assayed together but shows a good linearity up to 2000 ng/mL when the molecules are determined individually. Repeatability is not satisfactory for all compounds but could probably be improved by strictly controlling the extraction time (e.g., by automating the whole procedure using an autosampler). The use of SPME reduces the interference due to urinary low-volatility organic compounds and avoids the risks related to the use of organic solvents. To our knowledge, this technique is the first one allowing the sensitive determination of such a number of amphetamine analogs.

Introduction

Amphetamines and other phenylalkylamines are powerful stimulants of the central nervous system, abused both as a tool by occupational groups and as a mood enhancer by recreational users (1). They increase self-confidence and alertness and improve physical performance. Although widely prescribed some years ago as anorexic drugs for the treatment of obesity, their pronounced abuse potential has resulted in a drastic reduction of their medical use. Chronic abuse of amphetamines often leads to hallucinations and psychosis as well as to dysphoria and depression upon withdrawal (1). Therefore, their detection in biological fluids (above all in urine) is a major concern in toxicology, but also in occupational medicine, law enforcement administration, and other fields.

Most immunoassays for amphetamine-related drugs are designed to detect amphetamine and/or methamphetamine. However, most of these techniques also show significant cross-reactivity with some ring-substituted amphetamine derivatives, though not with all of them. On the other hand, because immunoassays are not perfectly specific, positive results must always be confirmed by a second, more specific method. Colorimetric techniques, ultraviolet (UV) and infrared spectrophotometric procedures, fluorescence detection, and thin-layer chromatography have been used for the determination of amphetamines but are now of historical interest only (2). Modern developments in chromatography largely superceded these techniques; numerous methods for the determination of phenylalkylamines in urine have been reported using high-performance liquid chromatography (HPLC) with UV (3), fluorescence (4,5), electrochemical (6), or photodiode array (7) detection or coupled with mass spectrometry (MS) (8). Gas chromatography (GC) with thermoionic (9), flame-ionization (10), or electron-capture (11) detectors or coupled with MS has also been used (12–17). Nevertheless, very few of these techniques were dedicated to a large number of these substances (5,10,18,19) and could be used as a screening procedure for amphetamine analogs.

The first aim of this study was to design a simple GC–MS procedure for the determination of a large number of amphetamine derivatives and related central nervous system (CNS) stimulants in urine samples, either for forensic purposes or as a confirmation technique for positive results obtained with immunochemical assays. The second aim was to take advantage of recent technological outcomes (such as new capillary columns dedicated to amino compounds or new solid-phase microextraction [SPME] devices) to make this procedure

* Author to whom correspondence should be addressed.

as simple and fast as possible. The use of SPME, which is simple, easy to automate, and does not require extraction solvents, has been reported for the detection of amphetamines (20,21), anorectic compounds (22), pesticides (29), and other drugs (23–28) in urine, but never for the simultaneous determination of such a number of amphetamine-related compounds.

Experimental

Chemicals and reagents

NaOH, HCl, and K_2CO_3 were supplied by Prolabo (Paris, France). HPLC-grade methanol was from Carlo Erba (Milan, Italy). Pentadeuterated internal standards (ISs) methamphetamine-d_5, methylenedioxymethamphetamine-d_5 (MDMA-d_5), and methylenedioxyethylamphetamine-d_5 (MDEA-d_5) were purchased from Radian (Austin, TX). A mixed stock solution containing 10 mg/L of each IS was prepared in 0.2M HCl and kept at 4°C. It was stable for several months. Amphetamine sulfate, methamphetamine hydrochloride, 3,4-methylenedioxyamphetamine hydrochloride (MDA), methylenedioxymethamphetamine hydrochloride (MDMA), benzphetamine, methoxyphenamine, phentermine, 2,5-dimethoxy-4-bromoamphetamine (DOB), and 2,5-dimethoxy-4-methylamphetamine (DOM) were obtained from Sigma (St. Louis, MO). Methylephedrine, fencamfamine, and norephedrine were from Aldrich (Milwaukee, WI). Norfenfluramine hydrochloride and dexfenfluramine were from Servier (Paris, France). Pseudoephedrine was from Glaxo (Valbonne, France). Amfepramone hydrochloride was from Dexo (Nanterre, France). Mefenorex was from Pierre Fabre (Castres, France). Clobenzorex hydrochloride was from Roussel-Uclaff (Paris, France). Fenproporex was from R.P.R. Pharma Spécialités (Lisieux, France). Methylphenidate was from Ciba-Geigy (Paris, France), and methylenedioxyethylamphetamine (MDEA) was from Radian (Austin, TX). The structure of each analyte is developed in Figure 1. A 1-g/L stock solution was prepared for each compound by dissolution in methanol and stored at 4°C; they proved to be stable for several months. Working solutions were prepared by appropriate dilution in 0.2M HCl, then stored at 4°C and discarded after one week.

Sample preparation

Extraction was performed with extraction fibers coated with a 100-µm polydimethylsiloxane (PDMS) layer mounted on an SPME assembly (Supelco, Bellefonte, PA). Such fibers can either be plunged in liquid matrices or exposed to a gas. Because amphetamines are all semivolatile compounds, the second solution was chosen. Their volatilization in the headspace of an airtight vial was favored by pH adjustment, salt saturation, and heating of the sample. In a 10-mL vial, 900 µL of urine was added to 100 µL of the working solution of 21 amphetamines, 100 µL of the IS mixture, and 1 g potassium carbonate. The vial was rapidly sealed with a silicone septum and an aluminum cap. Then 100 µL of 1M NaOH were injected through the septum. The vial was heated at 80°C for 30 min in an aluminum block heater. During the last 10 min, the metallic needle of the SPME device, which contained the extraction fiber, was introduced in the vial through the septum, and the SPME fiber was exposed in the headspace. Finally, the needle was removed from the vial and inserted into the heated injection port of the GC–MS for the desorption step.

GC–MS

A Fisons (Paris, France) GC 800 equipped with a split–splitless injector was used in combination with a Fisons MD 800 mass selective detector operated in scan or selected ion monitoring (SIM) modes. Chromatographic separation was achieved on a PTA-5 fused-silica capillary column (30 m × 0.32-mm i.d., 0.5-µm film thickness [Supelco]). The GC operating conditions were as follows. The injector was used in the splitless mode at 200°C; the split opening time was 2 min. The oven temperature increased from 60 to 120°C at a rate of 30°C/min, then to 210°C at 5°C/min, and finally to 280°C at 30°C/min; this temperature was maintained for 5 min in order to elute the heaviest contaminants. The transfer line and detector temperature was 280°C. Helium was used as the carrier gas at a flow rate of 1.3 mL/min. Ionization was performed by electron impact at 70 eV.

Acquisition was realized in the SIM mode in order to enhance sensitivity. Three ions were selected for each compound (one for quantitation and two for confirmation), and one ion was selected for each IS. Because the total number of ions was important, acquisition was parted in four time periods during which different fragments were monitored: 14 ions for period 1, 12 for period 2, 19 for period 3, and 17 for period 4. A dwell time of 0.08 s, a span of 0.10 s, and an interchannel delay of 0.020 s were chosen. The whole procedure was controlled by a microcomputer equipped with MASSLAB software (Fisons Instruments), which was also devoted to data acquisition and processing.

Method validation

Detection limits were determined by analyzing decreasing concentrations of the compounds in urine. The lowest concentration was established as that for which all the ions selected for a given compound could be differentiated from the background. Repeatability was assessed by extraction and determination of five aliquots of 100 ng/mL spiked urine in the same run. Calibration curves were constructed by spiking blank urine with the 21 amphetamines in order to obtain 0, 10, 20, 50, 100, 500, and 1000 ng/mL of each. Additionally, linearity was also studied for amphetamine, MDMA, DOM, and clobenzorex individually over a larger concentration range (up to 2000 ng/mL).

Results and Discussion

The *m/z* ratios selected in this study for the SIM mode used in the GC–MS procedure are given in Table I. Pseudomolecular ions, when present, were generally of low abundance in the given experimental conditions. The analytes studied contain

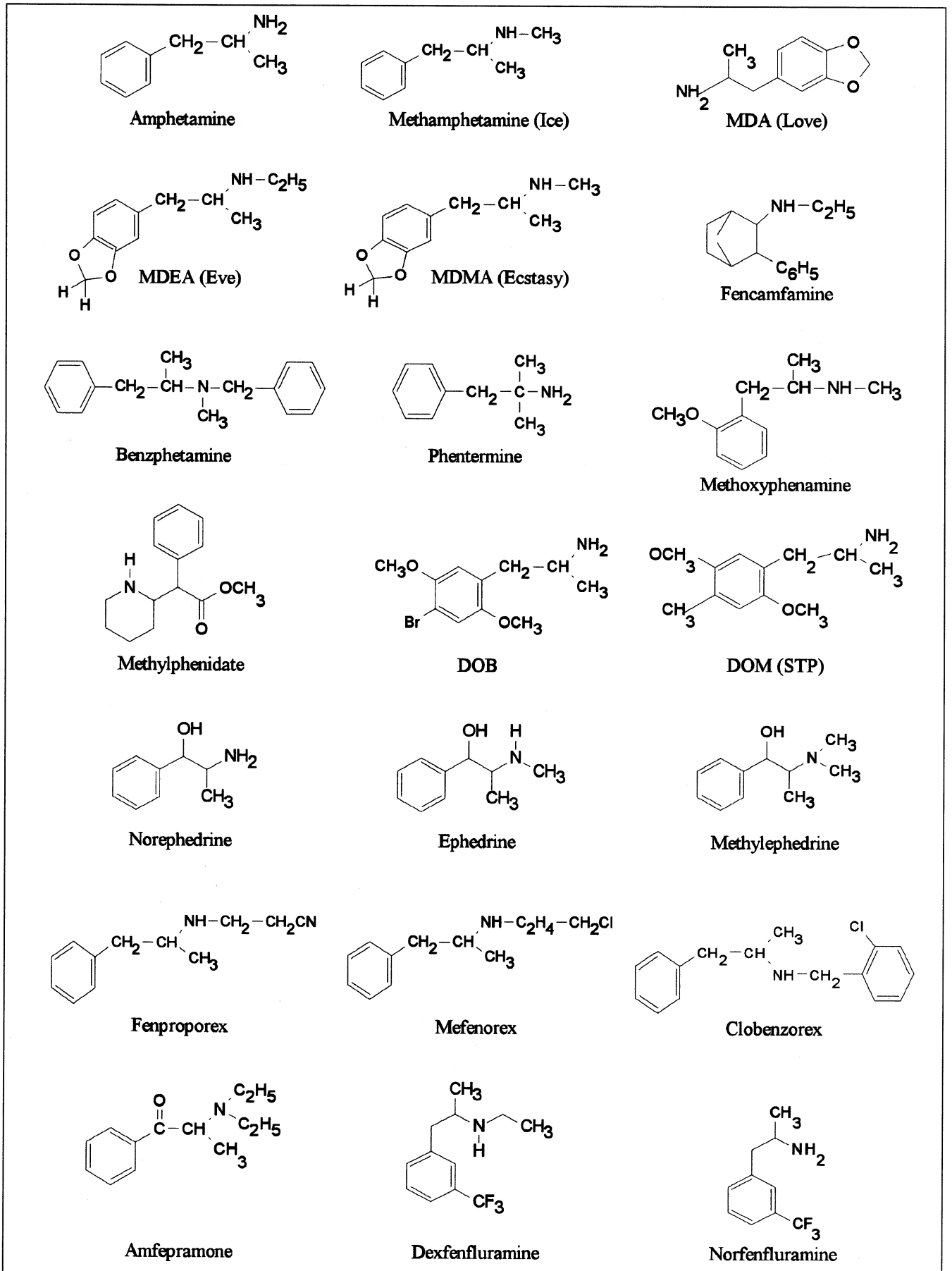

Figure 1. Chemical structures of the amphetamine-related drugs studied in this work.

Table I. Principal Analytical Parameters of the 21 Amphetamine-Related Compounds Studied

Compound	Retention time (min)	Selected ions* (*m/z*)	Detection limit (ng/mL)	Repeatability at 100 ng/mL (five replicates) (Coefficient of variation [%])
Amphetamine	3.608	**65**, 91, 120	50	21.88
Norfenfluramine	3.701	109, 159, **184**	10	21.16
Phentermine	3.912	58, 91, **134**	1	25.81
Methamphetamine	4.075	58, 91, **89**	10	4.74
Dexfenfluramine	4.613	72, 159, **216**	10	12.89
Norephedrine	6.416	**71**, 79, 105	50	18.21
Pseudoephedrine	6.636	**58**, 71, 77	10	6.59
Methoxyphenamine	6.636	58, 65, **91**	10	23.22
Methylephedrine	7.077	56, **72**, 105	50	6.35
MDA	7.945	51, 77, **136**	10	27.98
Amfepramone	8.580	72, 77, **100**	10	9.61
MDMA	9.119	**58**, 77, 135, 136	10	4.54
MDEA	10.008	**72**, 77, 135, 136	10	13.03
Mefenorex	10.135	91, **120**, 122	10	7.70
Fenproporex	10.516	56, 65, **97**	10	13.54
DOM	10.896	69, 151, **166**	10	11.13
Fencamfamine	11.992	91, **98**, 115, 215	10	10.50
Methylphenidate	12.323	56, **84**, 115	10	9.36
DOB	14.187	85, **230**, 232	50	24.66
Benzphetamine	16.142	91, **148**	10	1.33
Clobenzorex	17.615	91, **125**,168	10	1.53

* Quantitation ions of each compound are shown in bold.

amino and hydroxyl groups (Figure 1), which usually require a derivatization when the separation is to be performed on columns coated with a "classical" methyl–phenyl silicone phase for nonpolar compounds; the PTA-5 column, based on a 5% phenyl–95% methyl silicone bonded phase specially deactivated to avoid adsorption of polar (in particular, amino) compounds, was chosen in order to overcome such a derivatization step in the sample workup. The total ion chromatogram of a urine sample spiked with 100 µg/L of each of the 21 CNS stimulants is presented in Figure 2. Clean chromatograms were obtained showing thin, symmetric chromatographic peaks, and most drugs were resolved, except norephedrine and pseudoephedrine on one hand and fencamfamine and methylphenidate on the other hand. However, their peaks were clearly resolved in the chromatograms reconstructed from their respective selected ions (Figure 3), owing to distinct *m/z* ratios. Thus, the incomplete chromatographic separation did not impair the correct detection and identification of the 21 amphetamines. Despite the presence of numerous biogenic organic compounds at high concentrations in urine, comparison of the total ion chromatogram of a blank urine

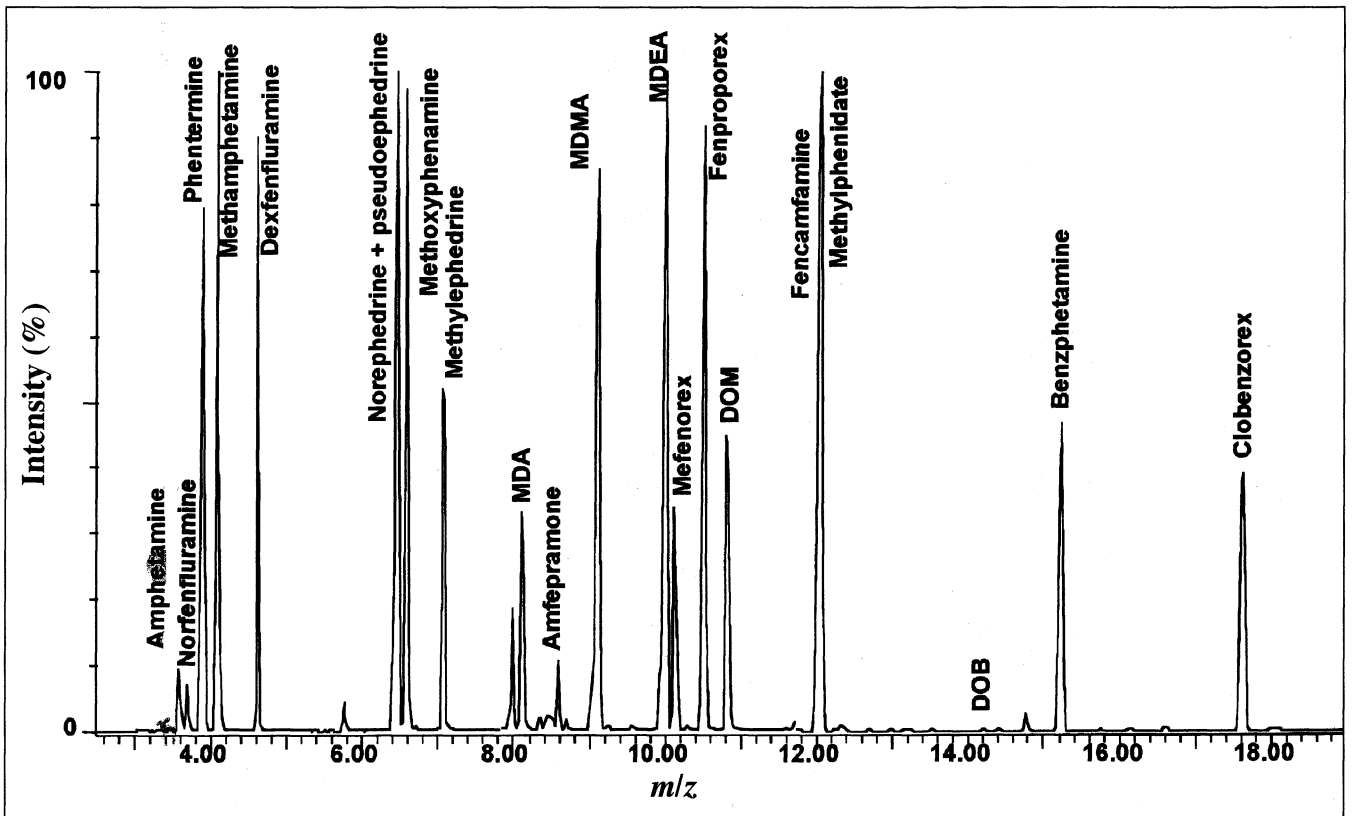

Figure 2. Total ion chromatogram of a urine sample extract spiked with 21 CNS stimulants at 1000 µg/L.

sample with that of the same urine supplemented with the 21 analytes showed only a few peaks from endogenous compounds, none of which interfered with the analytes.

SPME is based on the adsorption of organic compounds on a stationary phase coating a fine rod of fused silica. Then the desorption of the analytes from the polymeric layer is directly realized into the carrier gas stream of a heated GC injector or in the mobile phase of an HPLC device. The fiber can be used repeatedly for many extraction cycles (30). Generally, nonpolar compounds are more likely to be adsorbed on nonpolar coatings. Selectivity is further gained by the proper choice of polymeric phase. Four different types of fibers are commercially available: 7-, 30-, and 100-µm PDMS-coated fibers and 65-µm PDMS–divinylbenzene-coated fibers. The PDMS fibers show excellent selectivity for volatile compounds (31) such as amphetamines (30). Generally, the thicker the SPME phase is, the higher the capacity for the extracted organics. On the other hand, the reduction of the film thickness from 100 to 7 µm allows the bound phase to be more stable at higher temperatures and elicits the desorption of compounds with higher boiling points because the diffusion out of the 7-µm coating is much easier than desorption out of the 100-µm coating (30). Because 100-µm PDMS fibers were found to be more efficient for the semivolatile analytes studied herein, the carry-over of amphetamines from an injection to the following one was checked by extracting a urine sample spiked with 500 µg/L of the 21 amphetamines, exposing the fiber in the heated injector for 20 min, then exposing it for another 20 min without cleaning the fiber of the SPME device. No amphetamines were found in the second run, confirming that carry-over was completely prevented by heating the fiber at 200°C for 20 min in the injection port of the GC, which was done for each injection thereafter. Because volatilization of compounds from a liquid matrix depends strongly on some basic parameters such as pH or salt concentration, repeatable extraction recoveries can only be obtained when these parameters are strictly standardized. They were tested and optimized for the present application; the most appropriate pH was obtained with 100 µL of 1M NaOH (0, 50, 100, and 200 µL of 1M NaOH and 100 µL of 5M NaOH were tested). The salting-out effect was most effective using a complete saturation with K_2CO_3 (1 g in 1 mL). Then the heating temperature of the headspace vial was set at 60, 70, and 80°C for 20 min; the best results were obtained for 80°C, as was also reported in a previous study (32). The suitable time for exposing the fiber in the headspace above the sample was found to be 10 min after testing three different times (5, 10, and 20 min).

The detection limit was 10 ng/mL for most of the CNS stim-

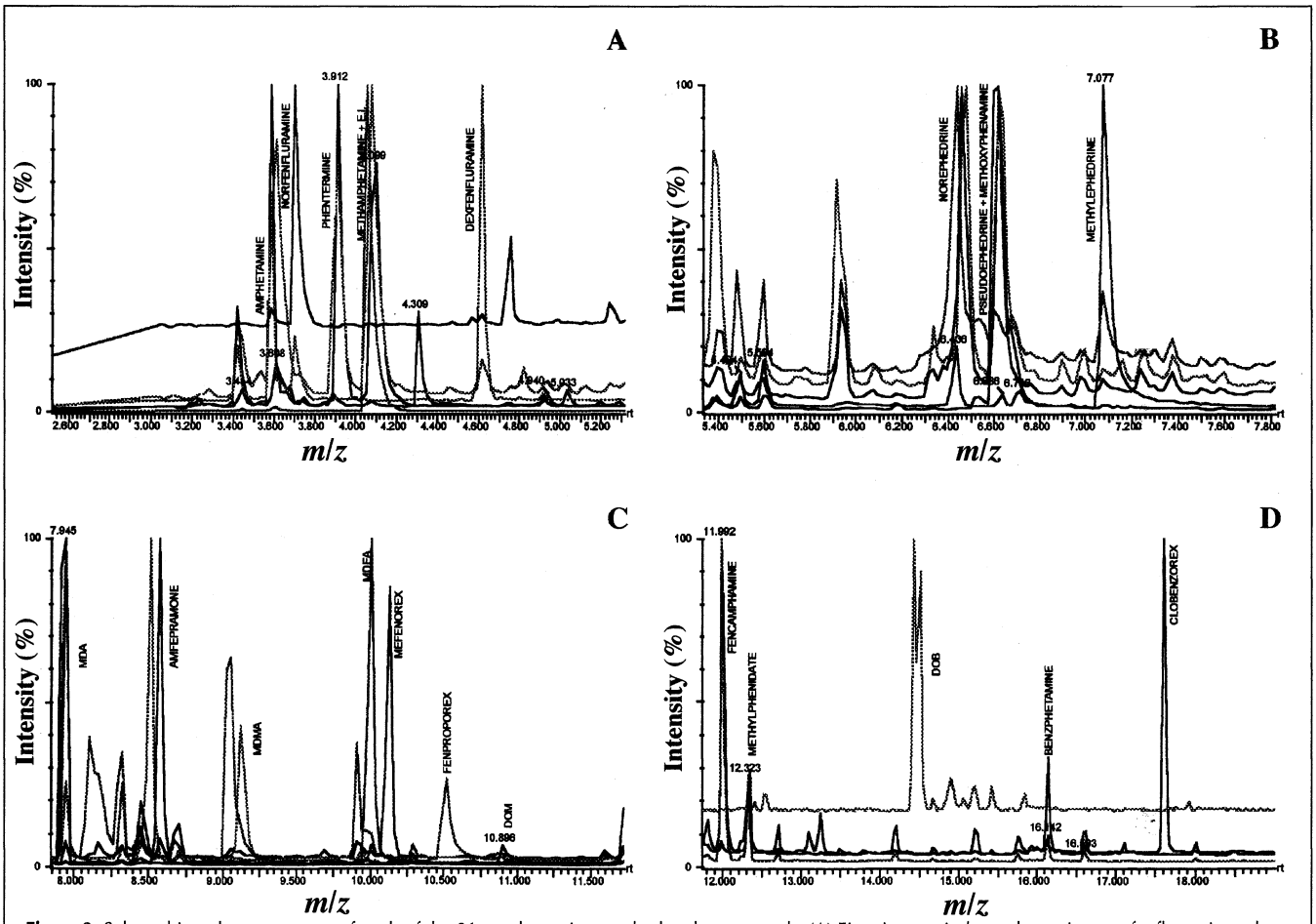

Figure 3. Selected ion chromatograms of each of the 21 amphetamines and related compounds. (A) First time period: amphetamine, norfenfluramine, phentermine, methamphetamine, methamphetamine-d_5, and dexfenfluramine. (B) Second time period: norephedrine, pseudoephedrine, methoxyphenamine, and methylephedrine. (C) Third time period: MDA, amfepramone, MDMA, MDEA, mefenorex, fenproporex, and DOM. (D) Fourth time period: fencamfamine, methylphenidate, DOB, benzphetamine, and clobenzorex.

ulants, except for amphetamine, norephedrine, methylephedrine, and DOB, for which it was 50 ng/mL, and for phentermine, for which it was as low as 1 ng/mL (Table I). Repeatability was not very good, but this may be due to time variations during the extraction; the heating time at 80°C and the adsorption time must be perfectly controlled. The full automation of the extraction process in combination with the control of the GC system is possible using an autosampler, which would probably improve these results. The calibration curves were linear over a restrictive range (from 10 or 50 ng/mL up to 500 ng/mL, depending on the analytes). For concentrations exceeding 500 ng/mL, the response deviated from linearity, probably because of an overloading of the polymeric phase by high concentrations of 21 analytes and three ISs. In these restrictive ranges, the correlation coefficients were satisfactory, ranging between 0.902 and 1.000. Methamphetamine, MDMA, and MDEA gave the best results, probably because their respective deuterated analog was used as an IS. This clearly demonstrates that the use of an IS with close structural similarity and chemical properties to the analyte of interest can provide sound analytical results. When amphetamine, MDMA, DOM, and clobenzorex were analyzed individually, the saturation of the polymeric phase was avoided, and the calibration curves were linear up to 2000 ng/mL; correlation coefficients were between 0.939 and 0.965.

SPME, achieved without organic solvents, proved effective for the extraction of amphetamine-related compounds. Furthermore, only a small sample volume was necessary, and the fibers could be used repeatedly. SPME eliminated the need for evaporation steps, which generally decrease the recovery of these semivolatile compounds, and proved to be relatively insensitive to matrix effects. A large range of linearity could be obtained for single analytes (which is generally the case in clinical samples), and repeatability would probably be better if standard parameters were carefully controlled. In addition, the use of a specially deactivated capillary column for amino compounds prevented the usually long, tedious derivatization step.

A previously published SPME–GC–MS method for the determination of amphetamine and methamphetamine in urine using the same type of fiber yielded an excellent linearity (up to 100 µg/mL) and a detection limit of 100 ng/mL for each (versus 2000 ng/mL with a simple headspace gas sampling, realized in the same conditions) (20). A preliminary report with little quantitative data showed the applicability of SPME to MDMA and MDEA at levels higher than 300 ng/mL (21). Another published technique using 5 mL of urine, direct injection of headspace gas, and positive chemical ionization MS yielded detection limits of 10 ng/mL for the same two amphetamines (32). The analytical technique presented herein gave detection limits almost equivalent to the latter but for 21 molecules simultaneously, using only 1 mL of urine. It also compares favorably with the previously published procedures for the simultaneous determination of numerous amphetamine analogs, which yielded detection limits from 20 to 100 ng/mL for four amphetamines (18), 4 ng/mL for amphetamine, and 7 ng/mL for methamphetamine; those of nine other analogs were not determined (10). Detection limits in the 200–500 ng/mL range were achieved for seven sympathomimetic amines (19); detection limits between 5 and 25 ng/mL were achieved for ephedrine, pseudoephedrine, norephedrine, amphetamine, methamphetamine, and 3-phenylpropanolamine (5); and detection limits of 5–50 ng/mL were achieved for the qualitative GC–MS identification of MDA, MDMA, MDEA, MBDB, and related metabolites (33).

Conclusion

To our knowledge, the analytical procedure presented here is the first one allowing the simultaneous determination of such a number of amphetamine analogs. It is simple, fast, and sensitive, even with a low-volume sample. Moreover, it is easy to automate by use of an autosampler, which would allow more precision. In our experience, it proved suitable as a confirmation analysis for positive results obtained with immunoassays, but it is also convenient as a first-line detection technique for amphetamines and related compounds in urine (e.g., drug-testing in sports, law enforcement, or forensic toxicology) when rapid, specific results are required. Lastly, it avoids the risks linked to the use of organic solvents.

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