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Aqueous alkylchloroformate derivatisation and solid-phase microextraction: determination of amphetamines in urine by capillary gas chromatography

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Abstract

The alkylchloroformate derivatisation and solid-phase microextraction of amphetamine and methamphetamine directly in urine samples prior to capillary gas chromatographic analysis is described. The alkylchloroformate reagent was added to the urine sample, which was adjusted to pH 10.8, and an internal standard was added. The resulting products were water-stable carbamates that were extracted without organic solvent. The polydimethylsiloxane coated fibre was inserted into the modified sample and agitated for 14 min. The fibre with the extracted derivatisation products was injected into the capillary gas chromatograph. The extracted carbamates were evaporated at 300°C in the split–splitless injection port of the gas chromatograph, separated on a methylsilicone capillary column and detected by either a nitrogen–phosphorus detector or by mass spectrometry. The method was shown to be reproducible with a detection limit of 50 ng/ml of amphetamine and methamphetamine in urine. © 1997 Elsevier Science B.V.

Keywords: Alkylchloroformate; Amphetamines

1. Introduction

In solid-phase microextraction (SPME), the solid phase is a non-volatile sorbent coated on a silica fibre. The fibre is mounted in a syringe-like device for protection [1–3]. SPME integrates sampling, extraction, concentration and sample introduction into a single step. SPME is based on the partitioning of organic compounds between an aqueous sample phase and the organic polymer phase. Several polymeric phases are commercially available for SPME; polydimethylsiloxane (PDMS), polyacrylate, Carbowax–divinylbenzene and polydimethylsiloxane–divinylbenzene. The amount of analyte extracted depends on the partition coefficients of the analytes. SPME was designed for the quantitative analysis of trace constituents in water samples and has been successfully used in the determination of volatile drugs in biological samples, e.g. urine and plasma [4–15]. However, the sensitivity and reproducibility obtained in the determination of more polar/less volatile drugs by SPME has been poor. Improvement of efficiency and sensitivity in SPME of polar analytes can be performed by derivatisation, which converts polar analytes into their less polar analogues and thereby increases their partitioning into the SPME coating [16].

Due to the widespread abuse of amphetamine and

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methamphetamine, drug testing for amphetamines in urine is routinely done in forensic toxicology. Determination of amphetamines in urine has been performed by capillary gas chromatography (GC), including head-space SPME, GC–mass spectrometry (GC–MS) [15,17–21] and by high-performance liquid chromatography (HPLC) [22–25]. Lately, SPME has been combined with HPLC analysis [26,27]. With the commercially available instrumentation, SPME–HPLC is an off-line technique that requires manual handling and is less suited for routine analysis than automated SPME–GC when a high sample throughput is required.

Head-space GC analysis is less advantageous as it requires specialised equipment that is not readily available in every laboratory. In order to improve the chromatography of amphetamines, most GC and GC–MS methods include a derivatisation step. The presence of an organic solvent is required in the derivatisation process performed with reagents such as heptafluorobutyric anhydride, trichloroacetic anhydride and trifluoroacetic anhydride. The derivatisation requires several steps including heating, evaporation of urine extracts and excess reagent.

The derivatisation of amines by alkylchloroformates in an aqueous environment was extensively studied in the early 1980s [28–30] (Fig. 1). The reaction of amines with alkylchloroformates has been reported to be rapid and the resulting carbamates are water-stable, thus allowing the direct derivatisation of amphetamines in the biological sample [19–21]. Amphetamine and methamphetamine were chosen as model compounds for SPME and direct alkylchloroformate derivatisation.

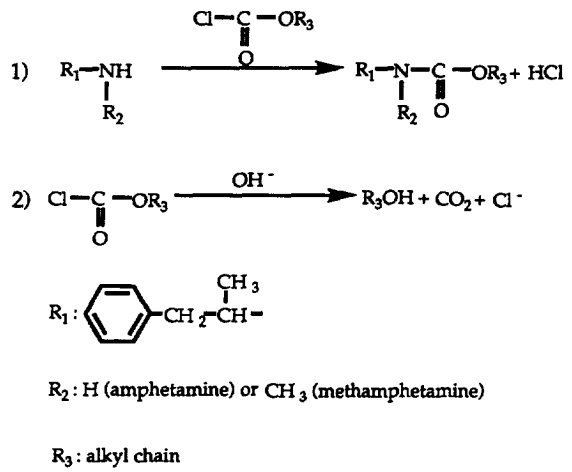

Fig. 1. (1) Reaction of an amine with alkylchloroformate and (2) hydrolysis of alkylchloroformate reagent.

The aim of this study was to demonstrate the potential of SPME and alkylchloroformate derivatisation directly in the biological sample without any prior sample preparation. In addition, the three different alkylchloroformates: methyl-, propyl- and butyl- were studied with respect to e.g. reactivity, stability and reproducibility.

2. Experimental

2.1. Chemicals

Amphetamine sulphate and methamphetamine hydrochloride were obtained from Norsk Medisinaldepot (Oslo, Norway). Methoxyphenamine was supplied by Sigma (St. Louis, MO, USA). Propylchloroformate and butylchloroformate were purchased from Aldrich (Milwaukee, MI, USA) and methylchloroformate was from Fluka (Buchs, Switzerland). Potassium hydrogen carbonate, potassium carbonate, sodium chloride, hexane and chloroform were supplied by Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standards

Stock standard solutions (1 mg/ml) of amphetamine, methamphetamine and methoxyphenamine (internal standard, I.S.) were prepared in methanol. Urine samples that had been spiked with amphetamine and methamphetamine (0.1–10 μg/ml) were prepared from stock solutions. The spiked urine samples were prepared freshly prior to analysis.

2.3. Sample pretreatment and derivatisation

Methoxyphenamine (I.S.; 5 μg/ml) and 300 μl of a solution consisting of 2.5 *M* K_2CO_3–$KHCO_3$ buffer and 0.5 g of NaCl (final concentration 5.5 *M*, pH 10.8) were added to a urine sample (1200 μl). The mixture was agitated before 2, 4 or 16 μl of

either butyl-, propyl- or methylchloroformate, respectively, were added and the mixture was vortex-mixed for 1 min.

2.4. Automated SPME and capillary GC analysis

Automated SPME was performed using a Varian 8200 CX GC autosampler (Varian, Walnut Creek, CA, USA) equipped with a PDMS-coated fibre (film thickness, 100 μm; Supelco, Bellefonte, PA, USA). Prior to use, the PDMS-coated fibres were conditioned at 250°C for 30 min under nitrogen and the fibres were checked for impurities prior to GC analysis. The derivatised amphetamines were extracted by immersion of the PDMS fibre in the urine sample. Extraction was performed for 14 min, during which time, the fibre was agitated to enhance partitioning. The capillary gas chromatograph was equipped with an SPB-1 polydimethylsiloxane column (30 m×0.25 mm I.D., 0.25 μm film thickness; Supelco) and a nitrogen–phosphorus detector (NPD). The temperatures of the injector and detector were 300 and 250°C, respectively. Helium was used as the carrier gas at a flow-rate of 1 ml/min (180°C). The detector gases were hydrogen (4.2 ml/min) and air (171 ml/min). Helium was used as the make-up gas at a flow-rate of 6.8 ml/min.

The extracted analytes were thermally desorbed from the PDMS-coating into the heated (300°C) splitless injector and into the capillary GC system for chromatographic separation and detection. After desorption (1 min), the SPME fibre was removed from the injection port and the split vent was opened. Chromatographic separation was achieved by temperature programming. The chromatograms were recorded using a Varian Star Chromatography Workstation, version 4.5.

A new sample could be injected every 15 min and a total of 96 samples could be analysed in 24 h.

2.5. Liquid–liquid extraction and determination of the amount extracted

A 200-μl volume of hexane–chloroform (3:1, v/v), after alkalisation with 50 μl of 0.25 *M* K_2CO_3–$KHCO_3$ buffer (adjusted to pH 12), was added to a urine sample (200 μl) that had been spiked with amphetamine, methamphetamine and methoxyphenamine (I.S.; 20 μg/ml). For the derivatisation step, either 1 μl of butylchloroformate, 1 μl of propylchloroformate or 3 μl of methylchloroformate were added to the mixture. The sample was vortex-mixed for 1 min. The aqueous bottom phase was removed and the organic phase was centrifuged twice at 3000 rpm for 5 min and the remaining aqueous phase was removed. The organic phase was concentrated to 50 μl under a stream of nitrogen, and 1 μl was injected into either a GC–NPD or a GC–MS system. The amount of amphetamine, methamphetamine and methoxyphenamine (I.S.) extracted was determined by comparison of the peak heights obtained by liquid–liquid extraction and the peak heights obtained by SPME. For separation of the derivatised amphetamines, the temperature was held at 180°C for 1 min and then increased by 20°C/min to 300°C, and held constant for 1 min.

2.6. Capillary GC–MS analysis

The mass spectrometer was a Fisons MD 800 (Micromass, Manchester, UK) connected to a Fisons GC 8065 equipped with a HP-1 methylsilicone capillary column (12 m×0.2 mm I.D., 0.33 μm film thickness). The MS instrument was operated in the electron impact mode at 70 eV with an ion source temperature of 275°C and was scanned in the mass range from 40 to 300.

2.7. Validation of the method

The calibration graphs for the determination of amphetamine and methamphetamine were based on peak-height measurements versus peak-height of the I.S. The limit of detection was determined at a signal-to-noise ratio of three ($S/N=3$).

3. Results and discussion

3.1. Derivatisation

The majority of drugs are compounds with polar entities and limited volatility. In order to improve the chromatography of these compounds, a derivatisation step has to be included prior to capillary GC analysis. The success of SPME in the bioanalysis of

drugs by capillary GC is dependent upon the possibility of derivatising the analytes prior to analysis. Derivatisation can be performed in the sample matrix, in the fibre coating or in the GC injection port [16]. During this study, the derivatisation of amphetamine, methamphetamine and methoxyphenamine (I.S.) with methyl-, propyl- and butylchloroformate in urine was studied. During method development, the amount of derivatisation reagent and the derivatisation time were optimised. The addition of 2, 4 or 16 μl of butyl-, propyl- and methylchloroformate, respectively, was found to be sufficient for the complete derivatisation of the amphetamines in urine within 1 min. No trace of underivatised amphetamine, methamphetamine and/or methoxyphenamine could be detected by either GC–NPD or GC–MS. Any further increase in the amount of alkylchloroformate reagent resulted in a reduced life-time of the SPME coating. The optimum pH for the derivatisation has been reported to be 10.8 [8], a pH at which amphetamine, methamphetamine and methoxyphenamine (I.S.) are predominately non-ionised (pK_a values of 9.9, 10.1 and 10.1, respectively). Altering the pH in the range 9.5–14 had no significant effect upon the derivatisation process. At pH>11, the life-time of the SPME coating was significantly reduced. Excess derivatisation reagent was not significantly extracted by the SPME coating and was subsequently hydrolysed to the corresponding alcohol by a secondary reaction (Fig. 1). The derivatives remained stable for more than 24 h at room temperature.

3.2. SPME

At the beginning of the study, the SPME of underivatised amphetamines was studied by immersion of the SPME fibre in urine samples. Several different SPME coatings including PDMS, polyacrylate, PDMS–divinylbenzene and carbowax–divinylbenzene were evaluated. The amount of amphetamine and methamphetamine extracted was found to be insufficient for forensic toxicology purposes. Head-space SPME analysis of the amphetamines in urine has previously been reported [18]. Head-space SPME was not considered to be a practical alternative in this study as the procedure required special instrumentation and could not be automated with the autosampler used for SPME in our laboratory.

The alkylchloroformate derivatisation of amphetamine and methamphetamine converted the amphetamines into less polar compounds. During method development, the PDMS coating was found to be more efficient and robust in the extraction of derivatised amphetamines than the polyacrylate, PDMS–divinylbenzene and carbowax–divinylbenzene coatings. The addition of sodium chloride to the urine sample was found to further enhance the partitioning of the analytes onto the PDMS coating. Maximum partitioning was achieved when a 1.2-ml urine sample was added of 300 μl of a carbonate buffer (5.5 *M*). The SPME sorption curves of amphetamine and methamphetamine in urine after derivatisation with propyl- and butylchloroformate are shown in Fig. 2. The samples were automatically agitated during the extraction. A sorption time of 14 min was chosen as the partitioning had reached an equilibrium and the limit of detection was more than sufficient for use in forensic toxicology. The SPME fibres had to be replaced every 100 samples, due to a decrease in reproducibility and enrichment. The life-time of the fibre was enhanced by immersion of the fibre in pure water when not in use and by placing pure water samples in between the urine samples to be analysed.

3.3. Capillary GC analysis

Due to the high volatility of amphetamine and methamphetamine, interference from volatile matrix components can be encountered, as mentioned above. The formation of less volatile compounds by derivatisation of the amphetamines may increase the retention sufficiently to avoid interferences. Alkylchloroformate-derivatised amphetamine and methamphetamine (Fig. 1) are less volatile than underivatised amphetamine and methamphetamine and an increase in their retention was observed, compared to the retention of the unchanged amphetamines. A satisfactory separation was achieved after derivatisation of the amphetamines using propyl- and butylchloroformate on the methylsilicone capillary column within 8 min. Chromatograms of amphetamine and methamphetamine in urine after derivatisation using propyl- and butylchloroformate,

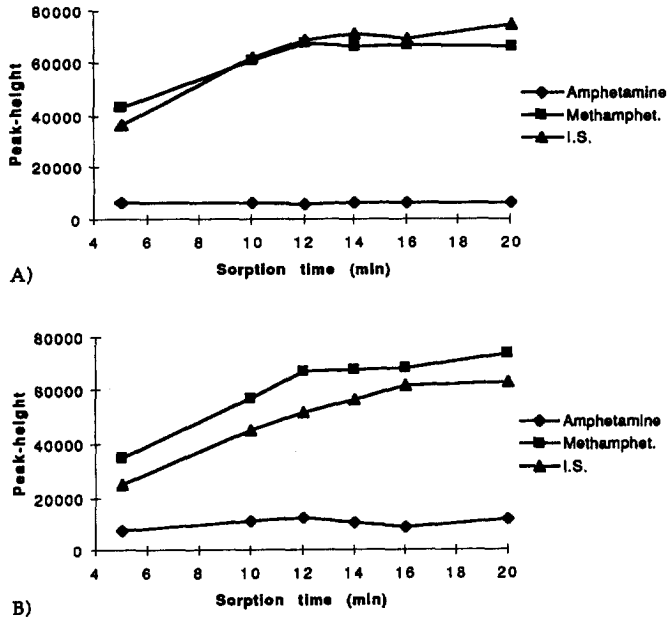

Fig. 2. SPME sorption curves for amphetamine, methamphetamine and methoxyphenamine (I.S.) after derivatisation with (A) propylchloroformate and (B) butylchloroformate.

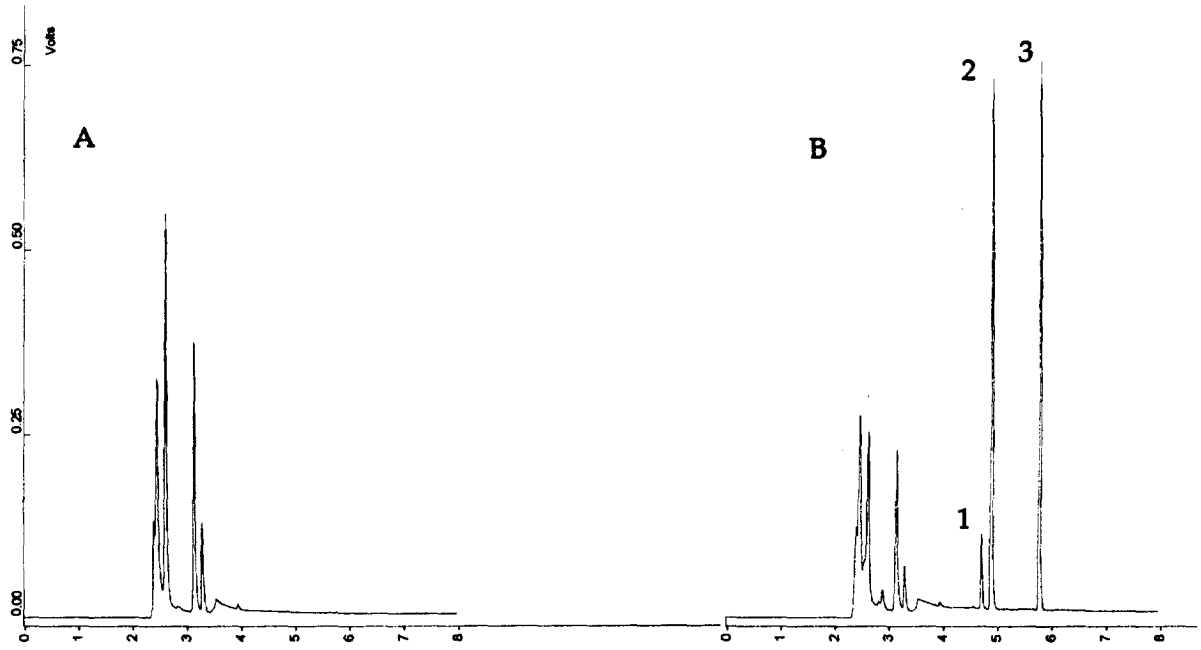

Fig. 3. Chromatograms of (A) a drug-free urine sample and (B) a urine sample that was spiked with amphetamine and methamphetamine (5 μg/ml) and methoxyphenamine (5 μg/ml, I.S.) after derivatisation with propylchloroformate. Chromatograms of (C) a drug-free urine sample and (D) a urine sample that was spiked with amphetamine and methamphetamine (5 μg/ml) and methoxyphenamine (5 μg/ml, I.S.) after derivatisation with butylchloroformate. Peaks: 1=amphetamine, 2=methamphetamine and 3=methoxyphenamine (I.S.).

respectively, are shown in Fig. 3. A satisfactory separation was not achieved for the methylchloroformate-derivatised amphetamines as they eluted early in the chromatogram and partially overlapped with endogenous compounds.

3.4. GC–MS analysis

Mass spectra of the butylchloroformate derivatives of amphetamine, methamphetamine and methoxyphenamine, respectively, are shown in Fig. 4, over the mass range 40–300. Each of the mass spectra were unique, allowing a positive identification. The mass spectra of the propylchloroformate derivatives of amphetamine and methamphetamine concurred with mass spectra previously published [19].

3.5. Determination of the amount extracted

The absolute amount of amphetamine and methamphetamine extracted from 1.2 ml of urine after derivatisation using propyl- and butylchloroformate was determined by comparing the results with those obtained by syringe injection of an aliquot of the organic phase after liquid–liquid extraction (Table 1). Increasing the alkyl chain length of the derivatisation reagent, from propyl to butyl, enhanced the amount of amphetamine extracted, due to an increase in hydrophobicity. A similar enhance-

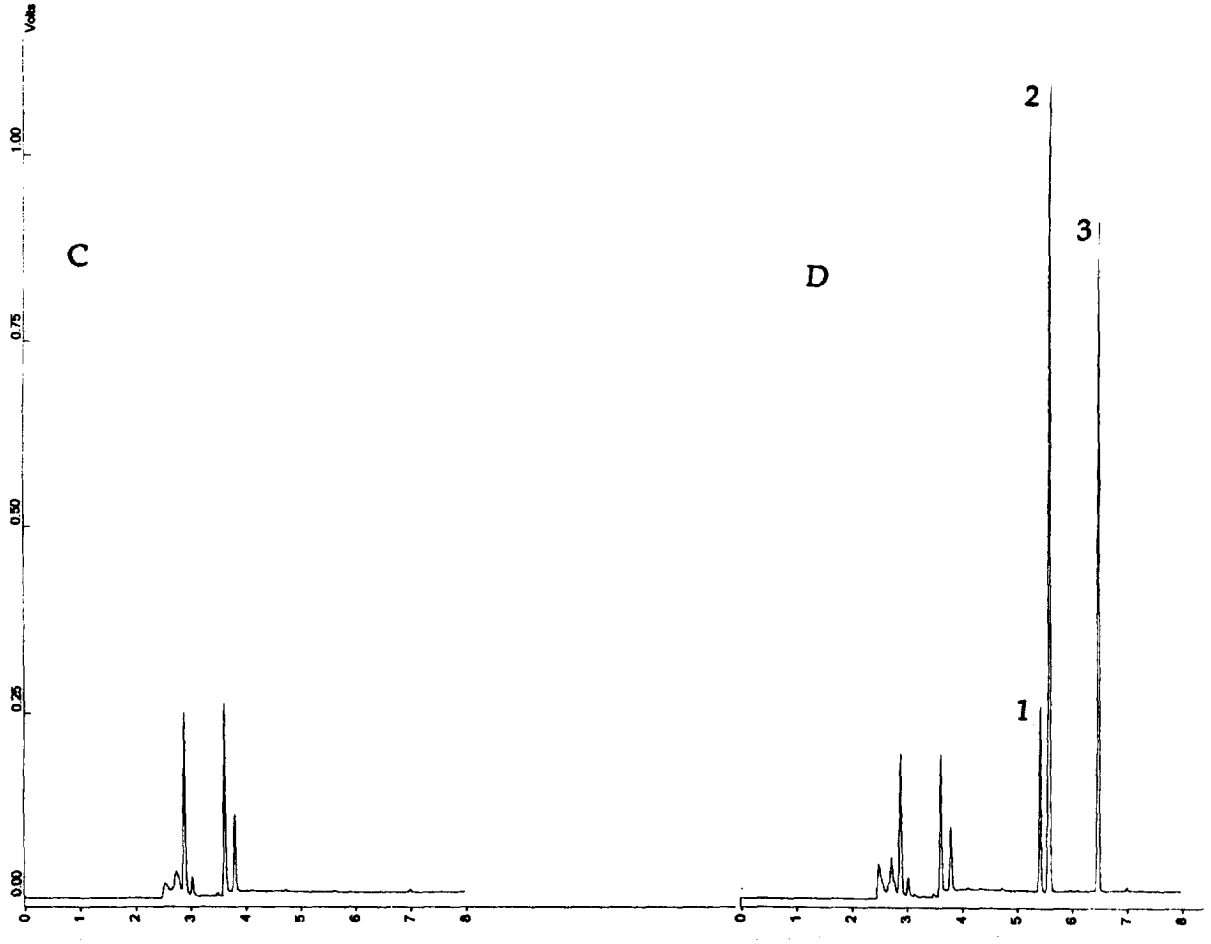

Fig. 3. (*continued*)

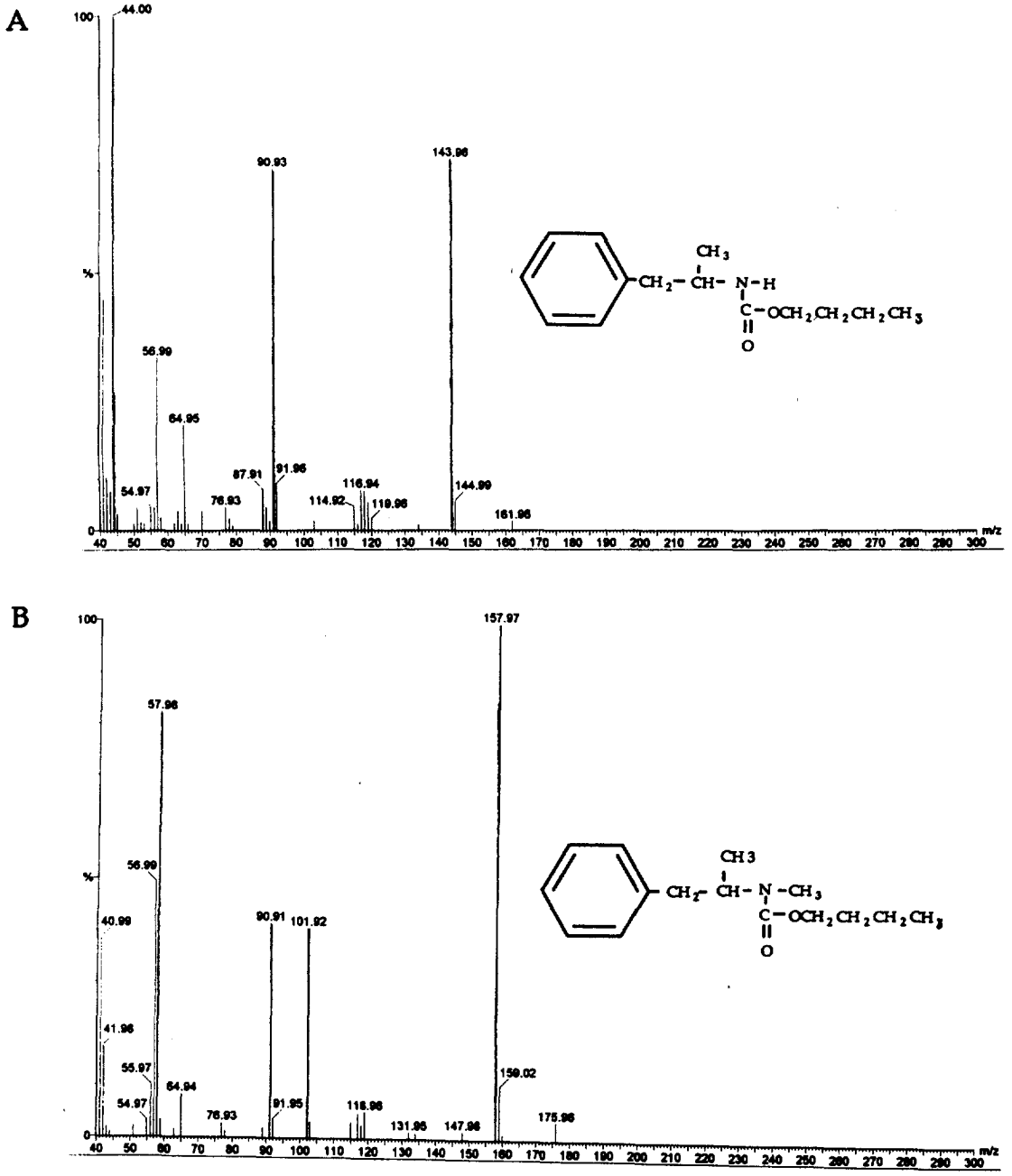

Fig. 4. Mass spectra of the butylchloroformate derivatives of (A) amphetamine, (B) methamphetamine and (C) methoxyphenamine (I.S.).

ment was not found for methamphetamine, as the amount of the propyl derivative extracted exceeded the amount of the butyl derivative that was extracted. After propylchloroformate derivatisation and SPME, 2.0 and 6.8% of the initial amount of amphetamine and methamphetamine were extracted by the PDMS coating. After butylchloroformate derivatisation and SPME, 2.3 and 5.8% of the initial amounts of

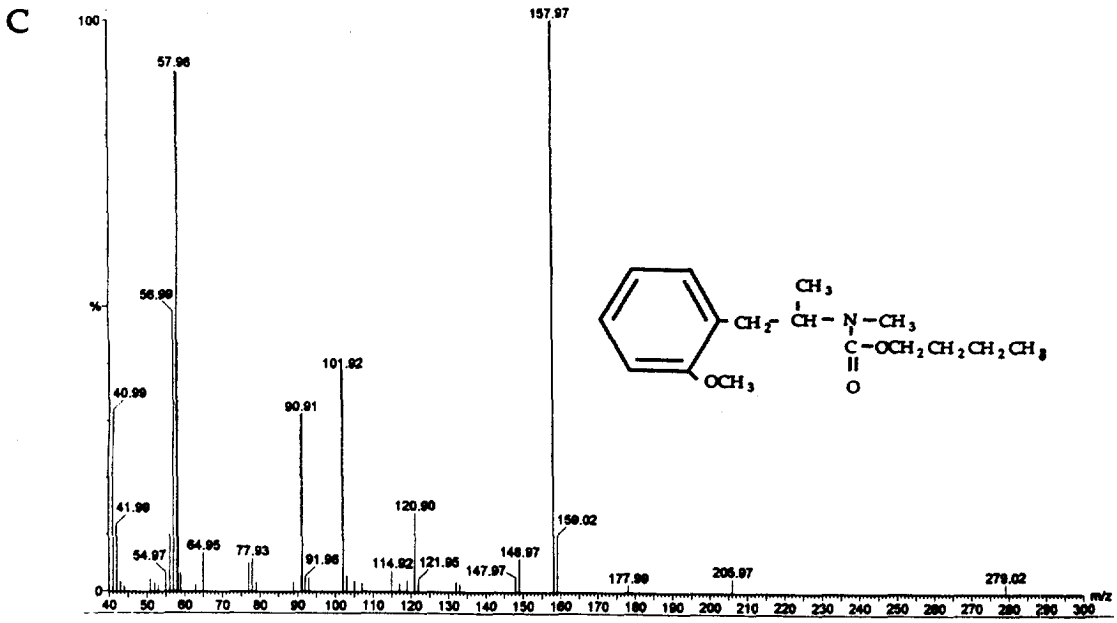

Fig. 4. (*continued*)

amphetamine and methamphetamine were extracted by the PDMS coating.

3.6. Validation of the method

A five-point calibration curve was set up in the range 0.1–10 μg/ml of amphetamine and methamphetamine in urine. The linear equations for the calibration curves for the derivatisation of amphetamine were $y=0.0267x+0.0005$ and $y=0.0779x-0.0041$ with propyl- and butylchloroformate, respectively. The calibration equations for methamphetamine were found to be $y=0.2028x+0.0005$ and $y=0.2299x+0.0335$ with propyl- and butylchloroformate, respectively (y=peak height ratio, x=concentration of amphetamines in urine). The correlation coefficients were $r=0.9996$ or better. The results of intra- and inter-assay validation are shown in Tables 2 and 3. The relative standard deviations were between 2.14 and 20.30% ($n=6$). The method was found to be highly reproducible for the determination of methamphetamine. The lower reproducibility in the determination of derivatised amphetamine was due to variations in the amount extracted onto the SPME fibre. The limit of detection at $S/N=3$ was 50 ng/ml for amphetamine and methamphetamine in urine. The sensitivity was found to be equivalent to that observed with existing methods based on HPLC and GC–MS [19,31] and was sufficient for the analysis of real-life samples.

Table 1
Absolute amount of amphetamine, methamphetamine and methoxyphenamine adsorbed, expressed as ng on a SPME fibre

	Propyl derivatives (ng)	Butyl derivatives (ng)
Amphetamine	120	140
Methamphetmaine	410	350
Methoxyphenamine (I.S.)	320	570

4. Conclusions

The potential of solvent-free derivatisation and extraction of amines in aqueous sample matrices combined with SPME has been demonstrated. A method based on alkylchloroformate derivatisation

Table 2
Intra- and inter-assay variations after derivatisation with propylchloroformate and SPME, expressed as the mean of parallel samples±standard deviation (S.D.) and relative standard deviation (R.S.D.)

Drug	Concentration added (μg/ml)	Measured concentration (mean±S.D.) (μg/ml)	R.S.D. (%)
Intra-assay (n=6)			
Amphetamine	1.00	0.58±0.002	13.15
	4.00	2.67±0.01	12.81
	7.00	5.19±0.01	6.24
Methamphetamine	1.00	0.97±0.02	10.88
	4.00	3.63±0.03	4.17
	7.00	6.70±0.03	2.14
Inter-assay (n=6)			
Amphetamine	1.00	0.60±0.12	20.30
	4.00	2.50±0.32	12.76
	7.00	4.68±0.91	19.47
Methamphetamine	1.00	0.95±0.03	3.33
	4.00	3.79±0.10	12.87
	7.00	6.74±0.27	4.05

Table 3
Intra- and inter-assay variations after derivatisation with butylchloroformate and SPME, expressed as the mean of parallel samples±standard deviation (S.D.) and relative standard deviation (R.S.D.)

Drug	Concentration added (μg/ml)	Measured concentration (mean±S.D.) (μg/ml)	R.S.D. (%)
Intra-assay (n=6)			
Amphetamine	1.00	0.91±0.004	5.73
	4.00	3.14±0.03	12.48
	7.00	6.84±0.06	10.94
Methamphetamine	1.00	0.85±0.01	4.44
	4.00	4.02±0.05	5.50
	7.00	6.59±0.16	10.32
Inter-assay (n=6)			
Amphetamine	1.00	0.89±0.16	14.39
	4.00	2.93±0.55	18.85
	7.00	4.62±0.55	11.97
Methamphetamine	1.00	0.88±0.05	6.14
	4.00	3.92±0.25	6.39
	7.00	7.26±0.59	8.15

and SPME was developed for the determination of amphetamine and methamphetamine in urine. SPME and derivatisation with propyl- and butylchloroformate was found to be reproducible and offered sufficient enrichment for bioanalysis.

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