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Improved amphetamine and methamphetamine determination in urine by normal-phase high-performance liquid chromatography with sodium 1,2-naphthoquinone 4-sulphonate as derivatizing agent and solid-phase extraction for sample clean-up

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Abstract

Solid-phase extraction techniques were evaluated for the treatment of urine samples in the analysis of amphetamine and methamphetamine by normal-phase high-performance liquid chromatography with 1,2-naphthoquinone 4-sulphonate. Six different packing materials were tested, and the results obtained are compared with those obtained in a classical liquid–liquid extraction with *n*-hexane. Different clean-up eluents and the influence of pH of urine have been tested. The intra-day and inter-day precision, the accuracy of the method and the addition of β-phenylethylamine as internal standard were also studied.

1. Introduction

Sample clean-up and derivatization steps play a significant role in the amphetamine and methamphetamine determination by HPLC [1]. Sulphonate group displacement in an aromatic reagent can be the basis of a derivatization procedure for the determination of low concentrations of amines [2–6]. We have proposed extraction combined with spectrophotometric procedures for the individual determination of amphetamine [7] or methamphetamine [8] in urine samples with 1,2-naphthoquinone 4-sulphonate (NQS), selecting the best reaction conditions for each drug. The determination of amphetamine and methamphetamine in urine by the H-point standard additions method is also proposed [9]. Endo et al. [10] were the first in applying this reagent as derivatizing agent in the determination of both drugs in urine samples by normal-phase liquid chromatography.

Three pre-column derivatization reagents: *o*-phthalaldehyde, 4-chloro-7-nitrobenz-2,1,3-oxadiazole, NQS and two ion-pair reagents, i.e naphthalene-2-sulphonate and sodium dodecylsulphate have been investigated by Farrell and Jefferies [11]. These authors concluded that only with the method employing derivatization with

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NQS the required sensitivity (4 ng/ml or 20 ng/ml) for the quantitative analysis of urine or plasma samples containing amphetamines could be reached. Nakahara and co-workers [12,13] used NQS to analyze these drugs by reversed-phase liquid chromatography and electrochemical detection.

The most straight-forward method for sample preparation generally uses a liquid–liquid extraction before the derivatization step [14]. Solvents such as diethyl ether at strong basic pH and *n*-hexane have been used. These procedures are labour intensive operations and multistep extractions are necessary.

Farrell and Jefferies [11] have used solid-phase extraction for sample clean-up after derivatizing with NQS. The eluent used is chloroform–isopropanol (3:1, v/v), the volume required is 40 ml for urine or 20 ml for plasma, the recoveries for methamphetamine and amphetamine being 98 and 109%, respectively, for urine and 88 and 95%, respectively, for plasma. A column (Clean Screen-DAU, copolymeric bonded-phase silica column) extraction procedure has been described for the screening and confirmation of drugs in horse urine by thin-layer chromatography and gas chromatography–mass spectrometry, respectively [15]. Column extraction provided a broad coverage of drugs, separating the extracts into three fractions (acidic/neutral, steroids, basic). Patel et al. [16] isolated amphetamine and methamphetamine from urine using polymer-based C_{18} extraction cartridges. The extraction principle involves hydrophobic interaction using ion pairing with hexanesulfonic acid before sample application. Recently, Helmlin and Brenneisen [17] extracted psychotropic phenylalkylamine derivatives from urine samples on an Adsorbex SCX cation-exchange solid-phase extraction column.

This work shows the possibilities of the solid-phase extraction technique for sample clean-up for the determination of amphetamine and methamphetamine by HPLC using NQS as derivatizing agent. The packing materials employed are: C_{18}, C_8, C_2, cyclohexyl (CH), phenyl (PH) and cyano (CN). The influence of pH in the retention of the analytes has been tested. Different clean-up eluents are studied. The results obtained are compared with those found by a classical liquid–liquid extraction procedure with *n*-hexane.

2. Experimental

2.1. Apparatus

A Hewlett-Packard 1040A liquid chromatograph, equipped with a diode-array detector linked to a data system (Hewlett-Packard HPLC Chem Station, Palo Alto, CA, USA) was used for data acquisition and storage. The system consisted of a quaternary pump (Hewlett-Packard, 1050 Series) with a 25-μl sample loop injector. The column was a LiChrospher Si-60, particle size 5 μm, 125 × 4 mm I.D (Merck, Darmstadt, Germany). The detector was set to collect a spectrum every 640 ms (over the range 270–600 nm) and all the assays were carried out at ambient temperature. The identity of each compound was established by comparing the retention times and UV-Vis spectra in the urine samples with those previously obtained by injection of standards.

2.2. Reagents

All reagents were of analytical grade. Ethanol, ethyl acetate, chloroform and *n*-hexane were of HPLC grade from Scharlau (Barcelona, Spain). Amine standard solutions were prepared by dissolving the pure compounds in water. Amphetamine sulphate and methamphetamine hydrochloride were obtained from Sigma (St. Louis, MO, USA). The internal standard was β-phenylethylamine hydrochloride from Sigma. The bicarbonate solution was prepared by dissolving 8 g of sodium hydrogen carbonate from Probus (Barcelona, Spain) in 100 ml of distilled water. 1,2-Naphthoquinone-4-sulphonic acid sodium salt (Sigma), stock solution (0.5% w/v), was prepared freshly for each experiment and was stored in the dark. Ammonium hydroxide (25%), hydrochloric acid, sodium hydroxide and

sulphate anhydrous sodium were supplied by Probus.

2.3. Standard solutions

The standard solution of each amine was prepared by dissolving 100 mg of the pure compound in 100 ml of water. These stock solutions were then further diluted to yield the appropriate working solutions. All solutions were stored in the dark at 2°C.

2.4. Derivatization

Derivatization with NQS was performed as follows. Different volumes of the stock solution of the amines (amphetamine, methamphetamine and β-phenylethylamine) were added to 0.5 ml of bicarbonate solution (8%), 0.5 ml of NQS and distilled water up to 1.5 ml. The mixture was heated at 70°C for 20 min. After cooling, the mixture was shaken with the same volume of organic solvent (chloroform) for 2 min and was then centrifuged for 5 min at 1500 *g*. The aqueous phase was discarded, and sulphate anhydrous sodium was added to the organic solution to remove the water. The chloroform layer was filtered through 0.45-μm (13 mm diameter) Nylon filters from Teknokroma (Barcelona, Spain). Finally, 25 μl of each sample were injected onto the column using a Hamilton micro-syringe.

2.5. Mobile phase

The mobile phase was ethanol–chloroform–ethyl acetate–*n*-hexane (1:22:32:45, v/v). All the solutions were degassed with helium before use. The flow-rate was set at 2 ml min^{-1}. The chromatographic signal was monitored at 280 and 450 nm.

2.6. Urine samples

Urine samples (previously spiked or not with amphetamine and/or methamphetamine standard solutions and internal standard) were made between 7 to 11.4 pH values and centrifuged at 1500 g. The clear liquid was used for extraction procedure.

Liquid–liquid extraction

A 2-ml volume of urine sample in alkaline medium was subjected to liquid–liquid extraction with three 2-ml volumes *n*-hexane. A small amount (50 μl) of hydrochloride acid–ethanol (1:6, v/v) was added to the combined *n*-hexane extracts to convert the free amines into the hydrochlorides. Then the solvent was evaporated to dryness. The residue was derivatized as described above.

Solid-phase extractions

Six different Bond-Elut columns, 100 mg/ml, from Scharlau were evaluated for the extraction: C_{18}, C_8, C_2, cyclohexyl (CH) phenyl (PH) and cyanopropyl (CN). The solid-phase extraction columns were conditioned previously by drawing through 500 μl of methanol, followed by 500 μl of distilled water. Urine samples (2 ml) containing 100 μl of an aqueous solution of each amine (75 μg/ml) were transferred to the columns, and washed with 2.50 ml of distilled water to eliminate the biological matrix. Amines were eluted from the columns with 1 ml of methanol. Two ml of organic solvent were required when the sample was eluted with isopropanol–chloroform (1:3, v/v). The residue was derivatized as described above.

2.7. Recovery

Urine samples (2 ml) were spiked with amine standard solutions to give different concentrations in the 0.47–9.42 μg/ml range. These samples were subjected to the previously described extraction procedures. The percentage of drug recovered in a particular extraction was calculated by comparing the peak heights obtained for each drug in the spiked samples with the peak heights obtained for the standard samples after the derivatization procedure.

2.8. Limit of detection

The limit of detection was established by using the standard procedure for a signal-to-noise ratio of 3 at a wavelengths of 280 and 450 nm. The values obtained were confirmed by analysis of urine spiked with the appropriate amount of amine to produce a concentration, after sample treatment, equivalent to the estimated limits of detection.

3. Result and discussion

The typical chromatograms obtained show peaks with retention times of 2.6, 3.7, and 4.9 min for methamphetamine, amphetamine and β-phenylethylamine, respectively.

3.1. Liquid–liquid extraction.

According to Ref. [10], we used *n*-hexane as the extraction solvent for amines from urine samples. The liquid–liquid procedure used is described in Ref. [7]. The volume of urine employed was 2 ml instead of the 50 ml required in the procedure described in Ref. [10]. In Fig. 1 are illustrated the chromatograms obtained from extracts of blank urine samples of a normal healthy volunteer and from extracts of urine samples previously spiked with amphetamine, methamphetamine and β-phenylethylamine. As can be seen, under the conditions employed the blank urine does not contain any peaks closely eluting to the peaks of the amines assayed; the results are similar for the two wavelength studied.

Different concentrations of methamphetamine, amphetamine and β-phenylethylamine were assayed, and the precision and recovery data for each drug are shown in Table 1. As can be seen, while the amphetamine and methamphetamine are recovered in percentages of ca. 90%, β-phenylethylamine is recovery in a low percentage.

Thus, the liquid–liquid extraction procedure provided good results for the recovery of amphetamine and methamphetamine, but not for β-phenylethylamine.

3.2. Solid-phase extraction.

Fig. 2A shows a chromatogram of a blank urine sample previously made pH 10 obtained

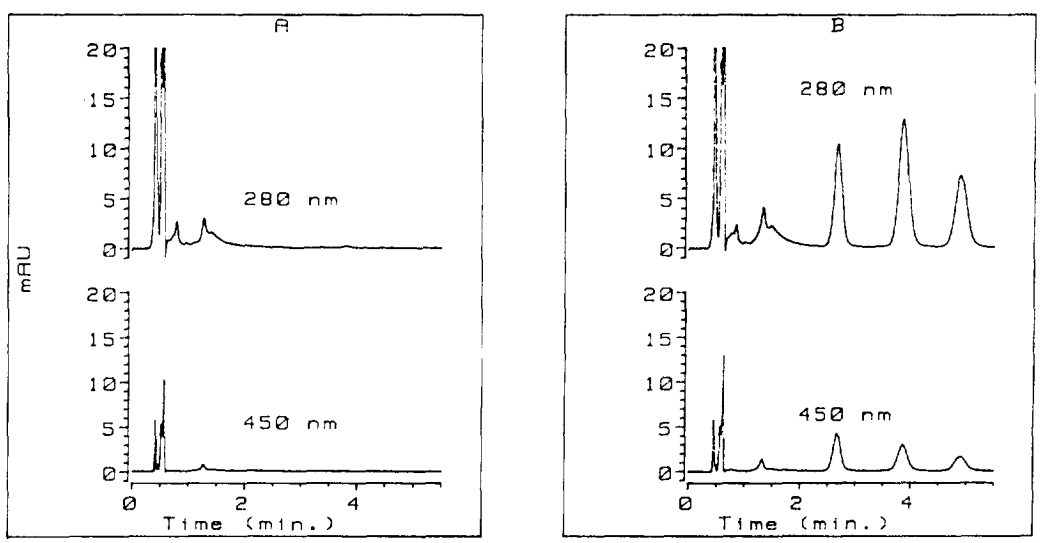

Fig. 1. Chromatograms at different wavelengths from (A) blank urine samples and (B) spiked urine samples after a liquid–liquid extraction under basic conditions (pH 10). Amine concentrations: injected 25 μl with 5.03 μg/ml of each amine.

Table 1
Recovery percentages of the amphetamines after liquid–liquid extraction under basic conditions (pH 10)

Concentration$_{added}$ (μg/ml)			Recovery (%)					
			280 nm			450 nm		
β-Phenylethylamine	Methamphetamine	Amphetamine	β-Phenylethylamine	Methamphetamine	Amphetamine	β-Phenylethylamine	Methamphetamine	Amphetamine
–	2.66	–	–	98.4	–	–	–	–
–	5.33	–	–	93.0	–	–	–	–
–	8.00	–	–	78.0	–	–	76.0	–
–	10.66	–	–	82.8	–	–	82.8	–
–	13.30	–	–	81.1	–	–	87.5	–
3.5	8.00	–	44.7	87.8	–	44.6	91.5	–
3.5	10.66	–	41.3	84.9	–	40.3	88.9	–
3.5	–	–	48.2, 38.6, 45.3, 36.5	–	–	43.5, 37.4, 45.1	–	–
5.03	5.03	5.03	53.0	81.7	89.3	50.7	81.5	85.3
			58.6	87.4	98.7	58.4	87.7	95.3
			44 ± 8	86 ± 6		44 ± 8	86 ± 5	

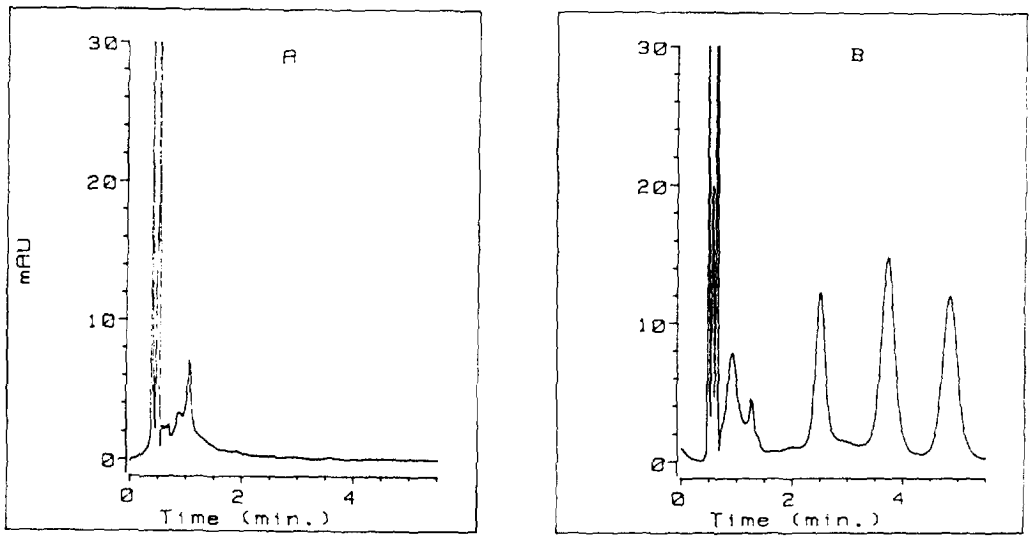

Fig. 2. Chromatograms at 280 nm from (A) blank urine samples and (B) from spiked urine samples extracted in different columns packing under basic conditions (pH 10). Elution solvent methanol.

with the C_{18} solid-phase extraction column. The chromatogram of a mixture of amphetamine, methamphetamine and β-phenylethylamine obtained with such a packing is shown in Fig. 2B. For the other packings the chromatograms obtained are similar. The percent recoveries obtained for the analytes are shown in Table 2 for the different packing tested. The precision of the method is similar to that shown by the liquid–liquid extraction. However, the recoveries are higher for β-phenylethylamine, and similar for methamphetamine and amphetamine. The chromatograms show similar background peaks corresponding to urinary endogenous compounds as the chromatograms obtained by the liquid–liquid extraction procedure. As can be seen in Table 2, amphetamine is completely recovered with CN, PH, C_8, CH, C_2 and C_{18} packings. Methamphetamine is well recovered with all packings tested, while for β-phenylethylamine the CN packing gives the lowest recovery. We selected packing C_{18} for subsequent studies. The percent recoveries were independent of the wavelength used. Due to the higher sensitivity obtained a 280 nm wavelength was chosen. Lower wavelengths were not used because of the interference of the blank urine and the high background corresponding to the solvent used.

Farrell and Jefferies [11], studied the derivatization of amines with different reagents (such as NQS). In their study the urine samples were made alkaline to pH 11.4, and the solutes were eluted with chloroform–isopropanol (3:1, v/v) in the sample clean-up step. According to this, we studied the influence of the urine pH in the extraction procedure. The pH range studied was 7–11.4. Fig. 3A shows chromatograms of blank urine samples obtained at different pH values using solid-phase extraction C_{18} columns and methanol as elution solvent. As can be seen, the chromatogram of the blank urine shows a peak very close (t_R = 3.2 min) to that of amphetamine (t_R = 3.7 min) for the highest pH assayed (pH 11.4). However, the area of this peak was lower when the sample was eluted with chloroform–isopropanol (3:1, v/v). Fig. 3B shows the chromatograms obtained for a urine sample spiked with amphetamine, methamphetamine and β-phenylethylamine at different pH values. Methamphetamine recovery was influenced by the urine pH, and the best recovery was obtained by working at a pH close to 10. In-

Table 2
Recovery percentages of amphetamines in different solid-phase extraction columns tested under basic conditions (pH 10)

Column	Recovery (%)					
	280 nm			450 nm		
	β-Phenylethylamine	Methamphetamine	Amphetamine	β-Phenylethylamine	Methamphetamine	Amphetamine
PH	92 ± 1 (n = 3)	83 ± 5 (n = 3)	105 ± 9 (n = 4)	88 ± 4 (n = 3)	78 ± 4 (n = 4)	102 ± 8 (n = 4)
C_8	85 ± 7 (n = 4)	75 ± 7 (n = 4)	95 ± 2 (n = 3)	88 ± 3 (n = 3)	84 ± 6 (n = 3)	95 ± 6 (n = 3)
C_2	88 ± 5 (n = 4)	85 ± 2 (n = 3)	100 ± 5 (n = 3)	85 ± 5 (n = 3)	86 ± 4 (n = 3)	97 ± 5 (n = 4)
CN	45 ± 4 (n = 2)	83 ± 1 (n = 2)	91 ± 3 (n = 2)	46 ± 1 (n = 3)	85 ± 3 (n = 3)	93.5 (n = 1)
CH	87 ± 6 (n = 4)	81 ± 1 (n = 3)	99 ± 7 (n = 4)	82 ± 4 (n = 3)	91 ± 8 (n = 2)	96 ± 7 (n = 3)
C_{18}	94 ± 7 (n = 8)	80 ± 3 (n = 4)	104 ± 6 (n = 8)	91 ± 7 (n = 6)	84 ± 5 (n = 5)	102 ± 6 (n = 5)

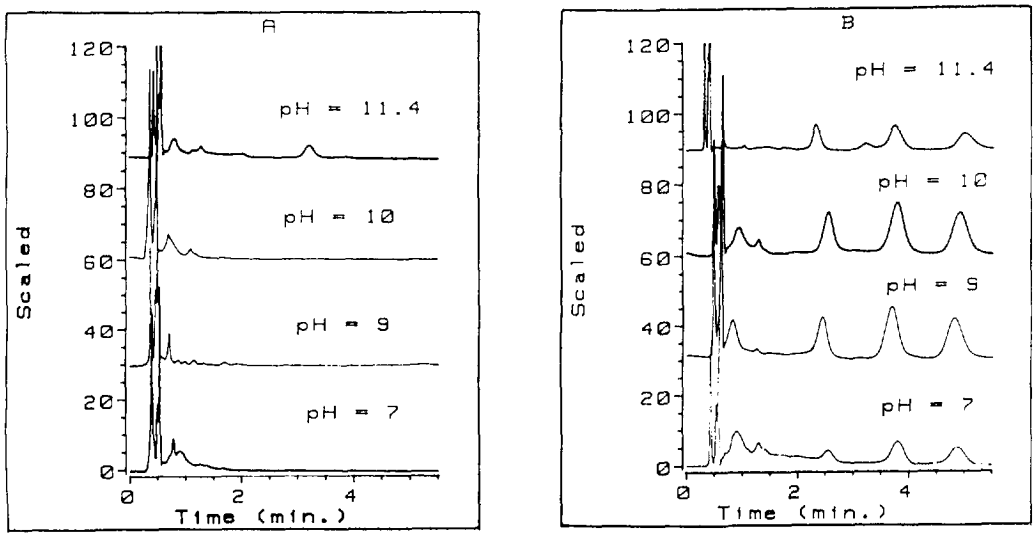

Fig. 3. Chromatograms at 280 nm from (A) blank urine samples and (B) urine samples spiked with a mixture of amines at different pH values and extracted in C_{18} columns. Elution solvent methanol. Amine concentrations: injected 25 μl with 5.03 μg/ml of each amine.

dependent of the eluent solvent used, we selected pH 10 because the recovery of the analytes studied was higher (especially the methamphetamine recovery) and the chromatograms of blank urine only show minor background peaks.

We performed a comparative study of the elution solvent, using chloroform–isopropanol (3:1, v/v) and methanol under the same conditions. The volume required for the elution of the analytes with chloroform–isopropanol (3:1, v/v) was four ml (using C_{18} Bond-Elut columns 200 mg/ml), two ml being not enough. However, recovery of the analytes was approximately 100% when 2 ml of methanol was used as elution solvent.

In Table 3 are shown the percentage recoveries obtained for different concentrations of the amines (β-phenylethylamine, amphetamine and methamphetamine) by using as eluent chloroform–isopropanol (3:1, v/v) and methanol as elution solvent. As can be seen, at 2.5 μg/ml there are no significant differences between the results obtained for both solvents. In these cases, the recovery obtained for low methamphetamine concentrations was lower than that obtained for the other concentrations tested. This effect was also observed for the highest concentrations studied (12.58 μg/ml) and it was independent of the solvent used for the elution. The recovery obtained for β-phenylethylamine and amphetamine was similar for all the concentrations studied.

The slopes of the calibration graphs obtained for amphetamine and methamphetamine in urine samples are similar to those obtained for these analytes in standard samples taking into account the recovery, so their is no matrix effect and the analyte concentration could be calculated from the calibration graph with standards. The relative errors found are acceptable in all instances.

The intra-day and inter-day precision and percent error of the method were determined by replicate measurement of urine spiked with amphetamine and methamphetamine on the initial day of preparation and on subsequent days. The data are listed in Table 4. We used the calibration graphs with and without internal standard (β-phenylethylamine) to test these analytical parameters. The eluent used in the sample clean-up step was methanol. As can be seen, suitable results are obtained in both cases. However, the accuracy of the method is improved

Table 3
Recovery percentages for different amine concentration in urine samples by using solid-phase extraction C_{18} and different eluent solvents

Concentration$_{added}$ (μg/ml)			Recovery (%)											
BFN	MET	AMP	280 nm						450 nm					
			β-Phenylethyl-amine		Metham-phetamine		Amphetamine		β-Phenylethyl-amine		Metham-phetamine		Amphetamine	
			A	B	A	B	A	B	A	B	A	B	A	B
5.03	0.63	0.63	91.1	99.0	58.6	69.2	128.0	108.0	89.4	97.8	50.9	56.0	92.2	103.5
			–	91.5	–	52.2	–	104.9	–	88.4	–	31.5	–	80.0
	2.52	2.52	98.4	95.7	81.1	75.8	99.8	101.1	96.7	90.4	80.9	71.9	91.9	88.5
			93.4	88.0	79.2	73.9	95.1	93.6	93.6	85.2	77.6	61.9	87.5	87.3
	5.03	5.03	90.4	73.5	80.9	84.0	105.1	78.8	89.1	68.6	81.9	79.8	100.7	92.2
			89.0	90.8	78.1	84.4	95.6	107.2	76.3	61.2	66.0	76.6	89.2	80.6
	8.81	8.81	92.5	90.8	84.3	86.6	106.7	115.6	92.0	84.9	80.4	88.2	106.9	109.1
			85.3	85.9	80.4	84.8	105.2	108.2	85.6	73.4	79.3	59.2	105.3	83.1
	12.58	12.58	93.5	101.0	61.4	76.6	104.3	105.5	90.3	93.2	55.8	80.5	105.6	103.6
			84.3	94.7	53.1	–	100.0	98.1	85.6	91.1	53.8	–	101.4	99.6

(A) 2 ml of methanol, (B) 4 ml of chloroform–isopropanol (3:1, v/v). BFN = β-phenylethylamine, AMP = amphetamine, MET = methamphetamine.

Table 4
Found concentration of amines in urine samples by using β-phenylethylamine as internal standard and without internal standard

280 nm				450 nm			
Amphetamine (5.03 μg/ml)		Methamphetamine (5.03 μg/ml)		Amphetamine (5.03 μg/ml)		Methamphetamine (5.03 μg/ml)	
Con. (μg/ml)	Er. (%)	Con. (μg/ml)	Er. (%)	Con. (μg/ml)	Er. (%)	Con. (μg/ml)	Er. (%)
Concentration found in urine samples with I.S. (β-phenylethylamine)							
4.99	−0.79	4.99	−0.79	5.00	−0.59	4.84	−3.77
5.00	−0.59	4.90	−2.58	5.16	+2.58	4.82	−4.17
5.23	+3.98	5.10	+1.39	5.00	−0.59	5.10	+1.39
5.38	+6.96	5.40	+7.36	5.30	+5.36	4.98	−0.99
4.99	−0.79	4.80	−4.57	5.03	0.00	4.70	−6.56
4.90	−2.58	5.34	+6.16	5.45	+8.35	5.26	+4.57
5.03	0.00	4.95	−1.59	4.98	−0.99	4.15	−17.49
4.98	−0.99	4.90	−2.58	4.96	−1.39	4.35	−13.51
5.07 ± 0.13[a] (s.d.)		5.00 ± 0.10[a]		5.05 ± 0.09[a]		4.92 ± 0.15[a]	
5.06 ± 0.15		5.05 ± 0.21		5.11 ± 0.18		4.78 ± 0.37	
Found concentration in urine samples without I.S.							
5.03	0.00	5.40	+7.36	5.08	+0.99	5.00	−0.59
5.40	+7.36	5.32	+5.76	5.70	+13.32	5.04	−0.19
5.60	+11.33	5.50	+9.34	5.65	+12.32	5.40	+7.35
4.98	−0.99	4.60	−8.54	5.07	+0.79	4.88	−2.98
5.41	+7.55	4.50	−10.54	5.17	+2.78	4.92	−2.19
5.60	+11.33	4.80	−4.57	5.17	+2.78	4.17	−17.09
5.14	+2.19	4.37	−13.12	4.85	−3.58	4.40	−12.52
5.00	−0.59	4.63	−7.95	4.85	−3.58	4.50	−10.53
5.34 ± 0.29[a]		5.41 ± 0.09[a]		5.47 ± 0.34[a]		5.15 ± 0.22[a]	
5.27 ± 0.26		4.89 ± 0.45		5.19 ± 0.30		4.79 ± 0.40	

The concentration of amphetamine and methamphetamine in the sample is 5.03 μg/ml.[a] Correspond to the accuracy and intra-day precision.

when the internal standard is directly added to the samples, the precision being good. The detection limits obtained were the same as those reported by Endo et al. [10] and Farrell and Jefferies [11], although the volume of urine required by our procedure is lower.

4. Conclusions

The application of solid-phase extraction columns for sample treatment gives good results in the determination of amphetamine and methamphetamine in urine samples by normal-phase liquid chromatography with NQS as derivatizing agent. Methamphetamine and amphetamine are well recovered with all packings tested, while for β-phenylethylamine the CN packing is giving the worst results. For this amine solid-phase extraction gives recoveries two-fold those obtained with a liquid–liquid extraction procedure with *n*-hexane. This is important for its use as internal standard. A pH of 10 was selected for the sample clean-up step because the recovery of the analytes was high and the chromatograms of blank urine show only minor background peaks. Methanol instead of chloroform–isopropanol (3:1, v/v) is proposed as elution solvent. The accuracy and precision of the procedure are good. Solid-phase extraction techniques are

rapid (the time needed for a liquid–liquid extraction is approximately five times longer than that required by a solid-phase extraction), simple and give good recoveries for the three amines tested. Furthermore a single extraction step is effective for all analytes. Therefore, these techniques are advantageous over liquid–liquid extraction in the analysis of amphetamine and methamphetamine and their mixtures or in screening procedures.

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