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

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Abstract

A rapid method is described for the identification and determination of amphetamine and methamphetamine in human urine samples by liquid chromatography with UV–Vis detection. The samples were transferred onto a C_{18} solid-phase extraction column and chromatographed on a Hypersil ODS RP C_{18}, 5 μm (250 × 4 mm I.D.) with an acetonitrile–water elution gradient containing propylamine. Under these conditions, the amines are eluted with a short retention time. The procedure has been applied to the determination of amphetamine and methamphetamine in the range 0.3–4.0 μg/ml in spiked urine samples. The detection limits at 280 nm were 4 and 2 ng/ml for amphetamine and methamphetamine, respectively. The intra-day and inter-day precision and accuracy of the method were studied.

1. Introduction

Previously, sodium 1,2-naphthoquinone 4-sulfonate (NQS) has been used in the determination of amphetamines in alkaline solution on account of its capacity to react with amphetamines to form UV–Vis-absorbing compounds. In relation to spectrophotometric procedures, Gürkan [1] applied the reaction to the investigation of symphathomimetic amines, including amphetamines in pharmaceutical samples. In our previous work, we have proposed extraction–spectrophotometric procedures for the individual determination of amphetamine [2], methamphetamine [3] or both amines [4] in urine samples.

Only a few methods have been proposed in the literature for the determination of amphetamines in urine samples using NQS derivatization and HPLC separation, even though NQS provides the required sensitivity (4 ng/ml or 20 ng/ml) for the quantitative analysis of urine or plasma samples containing amphetamines [5–7]. Some characteristics of those methods which have been developed appear in Table 1 [6–10]. The structure of the products formed is:

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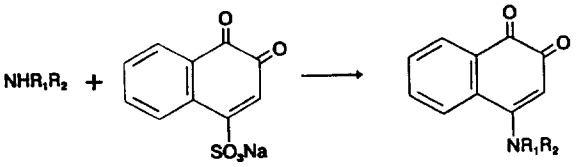
Table 1
Analytical properties of established procedures in which NQS is used as an amphetamine derivatizing agent in biological samples

Ref.	Source	Volume of sample (ml)	Sample clean-up	NQS derivatization				Injection volume (μl)	Separation			Detection	Detection limit
				Buffer	T (°C)	t (min)	Organic layer		Stationary phase	Eluent	Flow-rate (ml/min)		
6	Urine	50	L/L with *n*-hexane	Bicarbonate 2.7%	70	20	Chloroform (2 ml)	100	Lichrosorb Si 100 10 μm (25 cm × 2 mm I.D.)	Chloroform–ethyl acetate–ethanol–*n*-hexane (25:10:1:50)	1.2	UV–Vis 464 nm 280 nm	 5 ng 2 ng
7	Urine and plasma	20 10	S/L with XAD-2 resin	Bicarbonate 4%	70	20	Chloroform (2 ml)	20	Partisil (15 cm × 5 mm I.D.)	Chloroform–ethyl acetate–ethanol–*n*-hexane (25:35:1:50)	2.5	UV–Vis 248 nm	 2 ng
8	Biological fluids	0.05	–	Bicarbonate 0.7%	60	40	*n*-Hexane–ethyl ether (2:1, v/v) (1 ml)	5	μBondasphere C_{18} 5 μm 150 × 3.9 mm I.D. (40°C)	Acetonitrile–methanol–0.01 *M* H_2SO_4 (20:20:60)	0.8	Electro-chemical*	100 ng/ml (urine) 50 ng/ml (serum, plasma-salive)
9		0.5	L/L with hexane–ether (2:1, v/v)	1%	60	30	*n*-Hexane–ethyl acetate (1:1)	5					*10–20 ng/ml (serum, plasma-salive)
10	Urine samples	2.0	Comparative studied: L/L (*n*-hexane) and S/L	Bicarbonate 2%	70	20	Chloroform	25	LiChrospher Si-60, 5 μm, 125 mm × 4 mm I.D.	Chloroform–ethylacetate–ethanol–*n*-hexane (22:32:1:45)	2.0	UV–Vis 280 nm 450 nm	 2 ng 5 ng
This work	Urine samples	2.0	S/L C_{18}	Carbonate/bicarbonate 1% (pH 10)	Ambient	10	*n*-Hexane–ethyl acetate	50	Hypersil ODS RP-C_{18} 5μm 250 mm × 4 mm I.D.	Acetonitrile–water (gradient)	1.0	UV–Vis 280 nm	2 ng/ml (MET) 4ng/ml (AMF)

BNF = β-phenylethylamine, AMF = amphetamine, MET = methamphetamine. L = liquid, S = solid.



In a previous paper, we proposed an improved method for determining amphetamine and methamphetamine in urine based on normal-phase HPLC, with sodium 1,2-naphthoquinone 4-sulfonate as a derivatizing agent and solid-phase extraction for sample clean-up [10]. Normal-phase conditions are reported to give satisfactory separation between these amines [6,7,10] but these modes of chromatography are not routinely used in clinical laboratories due to the difficulty in maintaining stable separations. In reversed-phase mode under isocratic conditions the resolution of these amines takes a very long time, and this procedure has been proposed with electrochemical detection [8,9]. The RP-HPLC separation of these reaction products with UV–Vis detection has not been proposed in the literature. Moreover, as can be seen in Table 1, all the procedures described require a very long time and high temperatures in order for the reaction between amines and NQS to take place. We have observed that this reaction is faster at lower temperatures if the pH is higher [4,5,11].

On the basis of these results, we have proposed in this work a simple and rapid procedure to determine amphetamine and methamphetamine in urine samples, under reversed-phase conditions using C_{18} solid-phase extraction columns for sample clean-up [10]. Gradient elution with acetonitrile–water in the presence of propylamine provides complete resolution between these drugs and endogenous compounds.

2. Experimental

2.1. Apparatus

A Hewlett-Packard 1014A 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 was coupled to a quaternary pump (Hewlett-Packard, 1050 Series) and automatic sample injector (Hewlett-Packard, 1050 Series). The column was a Hypersil ODS-C_{18}, 5 μm (250 × 4 mm I.D.) (Hewlett-Packard, Germany). The detector was set to collect a spectrum every 640 ms (over the range 220–600 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 of the urine samples with those previously obtained by injection of standards.

2.2. Reagents

All reagents were analytical grade. Methanol, acetonitrile, ethanol, *n*-hexane and ethyl acetate were HPLC grade from Scharlau (Barcelona, Spain). Water was distilled, deionized and filtered through 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain). Amine standard solutions were prepared by dissolving the pure compounds in water: amphetamine sulfate, methamphetamine hydrochloride from Sigma (St. Louis, MO, USA). The internal standard was 2.5 μg/ml phenylethylamine hydrochloride from Sigma. The bicarbonate solution was prepared by dissolving 1.0 g sodium hydrogen carbonate (Probus, Barcelona, Spain) in 100 ml distilled water. The pH was adjusted to 10 by adding a minimum amount of NaOH (5 *M*). 1,2-Naphthoquinone-4-sulfonic acid sodium salt from Sigma, stock solution (0.5% w/v), was prepared fresh for each experiment. Ammonium hydroxide (25%), hydrochloric acid, and sodium hydroxide, all from Probus, and propylamine from Fluka (Barcelona, Spain) were also used.

2.3. Standard solutions

The standard solution of each amine was prepared by dissolving 100 mg of the pure compound in 100 ml water. These stock solutions were then further diluted to yield appropriate working solutions for the preparation of the

calibration standards. All the solutions were stored in the dark at 2°C.

2.4. Derivatization

The procedure of amine derivatization with NQS was performed as follows: 1 ml carbonate/bicarbonate buffer at pH 10.0 (1%), 1 ml of NQS reagent and distilled water were added to different volumes of amine solution up to 3 ml. The sample mixture was maintained at ambient temperature (25–30°C) for 10 min. The mixture was then shaken with the same volume of organic solvent (*n*-hexane–ethyl acetate) for 2 min and was centrifuged for 5 min at 1500 *g*. The aqueous phase was discarded, the organic phase was evaporated, and the residue was reconstituted with 0.5 ml of acetonitrile–water (1:1). Finally, 50 μl of each sample was injected into the column via the auto sample injector.

This derivatization procedure was compared with the others proposed in the literature [6–7], one of which we tried out ourselves. Aliquots of 1 ml NQS (0.5%) and 1 ml bicarbonate solution (8%) were added to the amine solution, the sample mixture was heated at 70°C for 20 min and the reaction products treated as described above.

2.5. Mobile phase

A gradient of acetonitrile–water with an increasing acetonitrile content from 40% at zero time, 50% at 2.5 min and 70% at 3.5 min was used. After 3.5 min the acetonitrile content was kept constant. An aqueous solution was prepared by adding 2.5 ml of propylamine to 500 ml water. The solution was prepared daily, filtered through a 0.45 μm nylon membrane (Teknokroma) and degassed with helium before use. The flow-rate was 1 ml/min. The chromatographic signal was monitored at 280 and 450 nm.

2.6. Sample treatment

Concentrated ammonium (0.1 ml) was added to 2 ml of the urine sample, either spiked with amines or not, so that a urine sample of approximately pH 10 was obtained.

Extra-Sep C_{18} solid-phase extraction columns (Teknokroma) were conditioned with 1.0 ml methanol followed by 1.0 ml bicarbonate solution (pH 10). Urine samples [previously spiked with amphetamine and/or methamphetamine and 2.5 μg/ml β-phenylethylamine (I.S.)] were then drawn through the columns, and subsequently washed with 5 ml distilled water followed by 1 ml acetonitrile to eliminate the biological matrix. The analytes and the internal standard were eluted from the column with 2 ml methanol. A 100-μl volume of ethanol–concentrated hydrochloric acid (6:1) was added to the methanol sample and evaporated. The residues were derivatized as described above.

2.7. Recovery studies

Blank urine samples (2.0 ml) were spiked with amine standard solutions to give different concentrations in the range 0.3–4.0 μg/ml. These samples were subjected to the extraction and derivatization procedure previously described. The percentage of drugs recovered for a particular extraction was calculated by comparing the peak area obtained for each drug in the spiked samples with the peak area obtained for the standard samples after the derivatization procedure. Each concentration was assayed in sets of five.

2.8. Detection and quantification limits

The detection and quantification limits were estimated by analysis of solutions of decreasing concentration of each amine in the mixture sample. In this case, 3 ml of the extracting solvent (*n*-hexane–ethyl acetate, 1:1) were evaporated and reconstituted in 0.5 ml of acetonitrile–water (1:1). These concentrations were established as the concentrations required to generate a signal-to-noise ratio of 3 and 10, respectively. 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 detection or quantification limits.

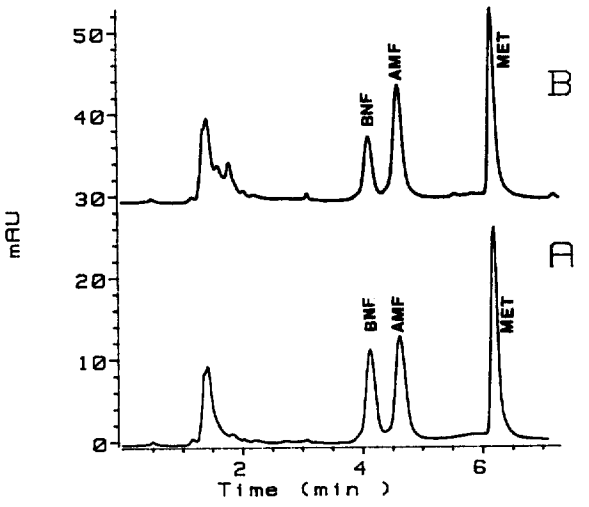


Fig. 1. Chromatograms at 280 nm of amine derivatives when the derivatization procedure is carried out (A) at 10 min with carbonate/bicarbonate buffer at pH 10.0 and ambient temperature, and (B) at 20 min with bicarbonate solution at pH 8.4 (8%) at 70°C. β-phenylethylamine (BNF) 1.5 μg/ml, amphetamine (AMF) 1.8 μg/ml and methamphetamine (MET) 2.1 μg/ml.

3. Results and discussion

Different gradient elution conditions were tested in order to obtain a suitable sensitivity for detecting or determining these amines using a Hypersil ODS-C_{18} column. The best results were obtained with a mobile phase consisting of acetonitrile–water (containing propylamine), and gradient elution mode to obtain an appropriate resolution in a short time. Different propylamine concentrations were assayed, and the best results were obtained between 0.048 and 0.065 *M*. Higher concentrations did not improve the resolution. After optimizing the chromatographic conditions, we then studied the different procedures for sample derivatization. Fig. 1 shows the chromatograms obtained for β-phenylethylamine, amphetamine and methamphetamine at (A) 10 min at ambient temperature with carbonate/bicarbonate buffer at pH 10.0, and (B) 70°C, 20 min with 8% bicarbonate solution at pH 8.4. As can be seen, the sensitivities obtained for amphetamine and methamphetamine are similar in the two procedures. We observed from their absorption spectra that the amine-NQS reaction products obtained by working with the two different procedures were the same. On the basis of these results, we selected pH 10 for optimum derivatization. The typical chromatograms obtained show peaks with retention times of 4.1, 4.7 and 6.3 min for β-phenylethylamine, amphetamine and methamphetamine, respectively.

The employment of solid-phase extraction columns for sample treatment provides clean extracts of the drug for analysis and the recoveries of these amines are greater than those obtained for the same samples using a conventional liquid–liquid extraction procedure; see, for example, the results for β-phenylethylamine in

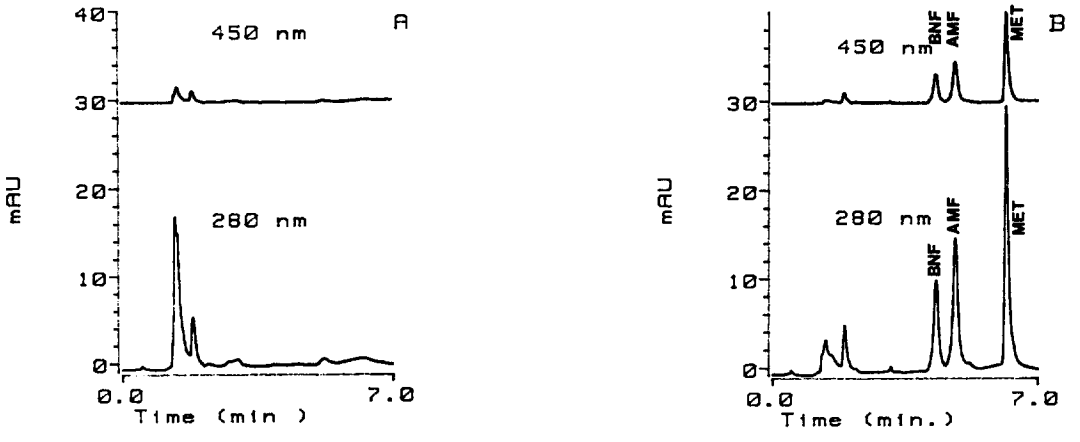


Fig. 2. Chromatograms at 280 and 450 nm of (A) a blank urine sample and (B) a urine sample spiked with 3.05 μg/ml amphetamine (AMF), 3.35 μg/ml methaphetamine (MET) and 2.5 μg/ml β-phenylethylamine (BNF).

Table 2
Recovery percentages for different amine concentrations in urine samples using C_{18} solid-phase extraction columns and methanol as an elution solvent

Concentration added (μg/ml)			Recovery (mean ± S.D.) (%)					
BNF	AMF	MET	280 nm			450 nm		
			BNF	AMF	MET	BNF	AMF	MET
2.5	0.31	0.33	100.8 ± 6.2	72.2 ± 6.0	104.0 ± 7.0	102.2 ± 5.0	83.0 ± 8.0	96.8 ± 9.0
	0.62	0.68	104.8 ± 2.4	72.0 ± 7.2	76.6 ± 4.6	98.8 ± 7.0	83.2 ± 7.2	87.3 ± 3.2
	1.24	1.36	101.9 ± 2.3	86.2 ± 7.6	86.2 ± 7.1	95.0 ± 6.2	96.2 ± 8.0	87.3 ± 7.7
	1.84	2.00	96.2 ± 3.2	93.2 ± 7.0	84.8 ± 7.0	101.5 ± 0.2	99.2 ± 8.0	88.1 ± 7.7
	2.47	2.68	96.3 ± 3.5	86.7 ± 4.3	86.1 ± 5.1	100.3 ± 8.2	94.2 ± 9.3	87.5 ± 7.6
	3.06	3.35	100.6 ± 8.5	100.3 ± 3.5	89.4 ± 5.0	102.7 ± 4.0	104.2 ± 9.0	93.5 ± 4.3
	3.67	4.02	90.0 ± 6.4	100.1 ± 4.0	90.0 ± 4.0	100.2 ± 3.0	96.3 ± 3.0	92.3 ± 4.2
		Mean	99 ± 5	87 ± 11	88 ± 8	100 ± 3	94 ± 8	90 ± 4

Number of samples tested (n) is 5. BNF = β-phenylethylamine, AMF = amphetamine, MET = methamphetamine.

Ref. [10]. The extraction was carried out with C_{18} columns, using methanol as the elution solvent and with the urine sample at pH 10.

Fig. 2 shows chromatograms of (A) blank and (B) spiked urine samples. Under the working conditions, the endogenous compounds are primarily eluted within 3 min. By comparing Figs. 2A and 2B it can be seen that, with the proposed sample treatment and the elution conditions, the chromatograms are free from endogenous compounds which may interfere with the identification or determination of these amines.

The extraction recoveries for the different amines and concentrations tested with a C_{18} packing are given in Table 2. The efficiency and precision obtained in the sample clean-up step are adequate, with mean recoveries for amphetamine and methamphetamine of approximately 90% for both amines, in the concentration range 0.3–4.0 μg/ml. For the internal standard the

Table 3
Amine concentrations observed in urine samples

Concentration added (μg/ml)			Observed concentration in urine samples (mean ± S.D.) (μg/ml)							
BNF	AMF	MET	280 nm				450 nm			
			AMF		MET		AMF		MET	
			A	B	A	B	A	B	A	B
2.5	0.31	0.33	0.32 ± 0.17	0.27 ± 0.05	0.33 ± 0.05	0.32 ± 0.05	0.31 ± 0.06	0.31 ± 0.07	0.35 ± 0.04	0.35 ± 0.04
	0.62	0.68	0.62 ± 0.11	0.62 ± 0.09	0.66 ± 0.06	0.64 ± 0.06	0.59 ± 0.06	0.56 ± 0.06	0.66 ± 0.04	0.69 ± 0.09
	1.24	1.36	1.20 ± 0.04	1.20 ± 0.06	1.36 ± 0.05	1.33 ± 0.05	1.23 ± 0.07	1.16 ± 0.07	1.37 ± 0.10	1.37 ± 0.10
	1.84	2.00	1.87 ± 0.13	1.70 ± 0.13	1.96 ± 0.16	1.91 ± 0.14	1.83 ± 0.24	1.77 ± 0.12	1.98 ± 0.15	1.98 ± 0.15
	2.47	2.68	2.25 ± 0.11	2.24 ± 0.11	2.64 ± 0.15	2.60 ± 0.15	2.34 ± 0.12	2.30 ± 0.18	2.68 ± 0.13	2.69 ± 0.13
	3.06	3.35	3.01 ± 0.04	3.00 ± 0.02	3.32 ± 0.14	3.36 ± 0.11	3.07 ± 0.15	2.83 ± 0.04	3.40 ± 0.03	3.38 ± 0.04
	3.67	4.02	3.21 ± 0.16	3.2 ± 0.16	3.90 ± 0.24	3.85 ± 0.31	3.67 ± 0.23	3.52 ± 0.30	4.00 ± 0.2	3.97 ± 0.17

Values obtained via (A) calibrations with urine samples and (B) calibrations with standard solutions treated in the same way as the urine samples; $n = 5$. BNF = β-phenylethylamine, AMF = amphetamine, MET = methamphetamine.

mean recovery obtained was 99% ($n = 35$), similar to the value obtained for the analytes. The calibration graphs (area vs. concentration) for both standards and urine samples were linear over the working range, the intercept being essentially zero. The slopes of these calibration graphs were 78.467 ($r = 0.9992$) and 105.399 ($r = 0.9991$) for amphetamine and methamphetamine respectively for standards and 68.238 (0.999) and 90.599 (0.999) for urine samples. The slopes for both calibration graphs are statistically equivalent if the mean percentage of recovery is considered. Therefore, the matrix of the sample does not modify the slope of the calibration graph obtained with standards, and when the standards are processed in the same way as the urine samples the slope obtained is the same.

Control urine samples from different volunteers were spiked with amphetamine and methamphetamine and tested to determine these amine levels (Table 3). The concentrations were calculated from the calibration graph corresponding to a random urine sample spiked with analytes, and the calibration graph obtained with standards treated as the urine sample in the presence of I.S. (2.5 μg/ml). The precision between samples is generally good, and therefore it seems that determination is independent of the matrix of the urine samples for all cases tested.

In order to evaluate the precision and accuracy of the method, different samples from different subjects were spiked with amphetamine and methamphetamine in two concentrations. Each urine sample was assayed on the initial day of preparation and on various other days. The intra-day and inter-day precision of the method were determined. The results are shown in Table 4. The relative error corresponds to the samples tested on the days other than the initial day. We used the calibration graphs with and without I.S.

Table 4
Amine concentrations observed (at 280 nm) in urine samples both with an I.S. present and without an I.S. via calibration graphs for urine samples (β-phenylethylamine)

AMT 1.24 μg/ml		AMT 3.05 μg/ml		MET 1.36 μg/ml		MET 3.35 μg/ml	
Conc. (μg/ml)	Error (%)	Conc. (μg/ml)	Error (%)	Conc. (μg/ml)	Error (%)	Conc. (μg/ml)	Error (%)
With I.S. (β-phenylethylamine)							
1.19	−4.03	2.92	−4.26	1.32	−2.22	3.41	−1.79
1.15	−7.26	2.94	−3.61	1.38	+2.22	3.33	−0.50
1.18	−4.84	2.76	−9.51	1.35	−0.74	3.38	+0.90
1.20	−3.23	2.52	−17.37	1.32	−2.22	3.22	−3.88
1.17	−5.65	3.01	+1.31	1.34	−1.47	3.32	−0.909
1.12	−9.67	–	–	1.41	+3.67	–	–
1.22	−1.66	–	–	1.16	−14.71	–	–
1.17 ± 0.02*		2.87 ± 0.09*		1.35 ± 0.03*		3.37 ± 0.04*	
1.18 ± 0.03		2.83 ± 0.20		1.33 ± 0.08		3.33 ± 0.07	
Without I.S.							
1.10	−11.29	2.55	−16.39	1.37	+0.74	3.30	−1.49
1.10	−11.29	2.77	−9.18	1.40	+2.94	3.33	−0.60
1.20	−3.23	2.81	−7.86	1.35	−0.74	3.38	+0.90
1.22	−1.61	2.44	−20.00	1.43	+5.15	3.28	−2.09
1.10	−11.3	3.12	+2.30	1.19	−12.50	3.52	+5.07
1.20	−3.23	3.33	+9.18	1.28	−5.88	3.61	+7.76
1.03	−16.94	–	–	1.34	−1.47	–	–
1.13 ± 0.06*		2.71 ± 0.14*		1.37 ± 0.03*		3.34 ± 0.04*	
1.14 ± 0.07		2.84 ± 0.34		1.34 ± 0.08		3.40 ± 0.13	

AMF = amphetamine, MET = methamphetamine. Asterisk corresponds to the accuracy and intra-day precision.

(β-phenylethylamine) to test these analytical parameters. As can be seen, suitable results are obtained in both cases. However, the accuracy of the method is improved when the internal standard is added to the samples immediately.

The limit of detection (for a signal-to-noise ratio of 3) corresponds to an injected amount of 0.8 and 0.4 ng, which is equivalent to an amphetamine and methamphetamine concentration in urine of 4 ng/ml and 2 ng/ml respectively. The quantification limits are 10 ng/ml for both drugs.

Fisher and Bourque [12] have proposed a procedure based on precolumn derivatization at room temperature with a polymeric reagent and reversed-phase HPLC to analyse amphetamine in urine. The proposed NQS-based method provides shorter analysis time and smaller detection limits than those reported by Fisher and Bourque.

4. Conclusion

A reversed-phase HPLC method for the identification and determination of amphetamine and methamphetamine in urine samples has been developed. The chromatographic conditions were optimized to obtain good sensitivity and separation of the analytes in a short time. The assay is rapid, requiring only 10 min for the derivatization procedure at ambient temperature, as opposed to 20 min at 70°C proposed in the literature. Moreover, the use of solid-phase extraction columns saves time in the sample preparation and provides high analytes recoveries. Reversed-phase HPLC instead of normal-phase chromatography, with UV–Vis detection, represents a further improvement in the quantitative analysis of these drugs. Reversed-phase HPLC provides similar detection limits to those obtained with normal-phase HPLC, as can be seen in Table 1.

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