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Amphetamine and methamphetamine determination in urine by reversed-phase high-performance liquid chromatography with simultaneous sample clean-up and derivatization with 1,2-naphthoquinone 4-sulphonate on solid-phase cartridges

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Abstract

A liquid–solid procedure is proposed for sample clean-up and derivatization of amphetamine and methamphetamine in urine samples. The reagent was 1,2-naphthoquinone 4-sulphonate, and a commercial C_{18} packing cartridge was used. The samples derivatized at room temperature were chromatographed on a 5-μm Hypersil ODS (250×4 mm I.D.) with an elution gradient of acetonitrile–water containing propylamine. Under these conditions, the amines were eluted with short retention times. The procedure was used to determine amphetamine, or methamphetamine with its metabolite amphetamine, in spiked urine samples. The detection limit (at a signal-to-noise ratio of 3) for amphetamine (0.1 μg/ml) was similar to that obtained with liquid–liquid derivatization and to those obtained with immobilized reagents on a polymeric solid support. The detection limit for methamphetamine (0.4 μg/ml) was higher than with the liquid–liquid procedure because of the lower reactivity on the cartridge. The precision and accuracy of the method were also studied.

Keywords: Amphetamine; Methamphetamine

1. Introduction

Amphetamine-type drugs are often abused and misused in sports. Forensic, hospital and toxicology laboratories are frequently requested to analyse for their presence, especially in urine samples. Sample clean-up and derivatization steps play a significant role in amphetamine and methamphetamine determination by HPLC [1]. The conventional methodology for derivatization involves a solution-based reaction: sodium 1,2-naphthoquinone 4-sulphonate (NQS), and *o*-phthaldehyde [2] and dansyl chloride [3] are used for amphetamine determination. Over the past decade, solid-phase reagents have become increasingly popular. Solid-phase reagents containing the 9-fluorenyl [4], 9-fluoreneacetyl [5], 3,5-dinitrophenyl [6] and 3,5-dinitrobenzoate [7] tags have been tested. Some characteristics of these procedures appear in Table 1.

The most straightforward method for sample clean-up involves liquid–liquid extraction before the derivatization step. We showed previously [1,8] that solid-phase extraction of urine samples offers advantages over liquid–liquid extraction for sample clean-up when NQS is used as the derivatizing agent. NQS provides the sensitivity needed (below 0.1 μg/ml) for quantitative analysis of urine samples containing amphetamines [1,8–10]. We have studied this de-

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Table 1
Analytical properties of established procedures in which NQS or immobilized reagents on polymeric supports are used as derivatizing agents for amphetamines

Ref.	Reagent	Analyte	Source	Sample clean-up	Derivatization				Injection volume (μl)	Detection	Detection limit
					Type	T (°C)	t (min)	Solvent			
[4]	9-F	AMP	Urine	–	Solid-phase	60	5	ACN–H_2O	10	UV/FL	0.1 ppm
[5]	9-FA	AMP	Plasma	–	Solid-phase	75	8	ACN–H_2O	20	FL	200 ng/ml
[6]	3,5-NP and NB (1:1)	AMP	Urine	–	Solid-phase	RT	2	ACN–H_2O	20	UV	1 ppm
[7]	3,5-NB	AMP	Urine	Solid-phase extrac.BEC	Solid-phase	RT	1/2	ACN–H_2O	200	UV	14 ng/ml
[9]	NQS	AMP MET	Urine	Solid-phase extraction XAD-2 resin	Liquid-phase	70	20	Chloroform–H_2O	20	UV	2 ng
[8]	NQS	AMP MET	Urine	Solid-phase extraction C_{18}	Liquid-phase	70	20	Chloroform–H_2O	25	UV	2 ng
[15]	NQS	AMP MET	Urine	Solid-phase extraction C_{18}	Liquid phase	RT	10	*n*-Hexane EtAc–H_2O	50	UV	4 ng/ml
This work	NQS	AMP MET	Urine	Solid-phase extraction C_{18}	Solid-phase extraction C_{18}	RT	10 15	Aqueous solution	20	UV	0.1 ppm 0.4 ppm

Abbreviations: 9-F=9-fluorenyltagged; 9-FA=9-fluoreneacetytagged; 3,5-NP=3,5-dinitrophenyltagged; NB=p-nitrobenzoyltagged; 3,5-NB=3,5-dinitrobenzoyltagged; NQS=1,2-naphthoquinone 4-sulphonate; AMP=amphetamine; MET=methamphetamine; BEC=Bond Elut Certify; RT=room temperature; ACN=acetonitrile; EtAc=ethyl acetate; UV=ultraviolet; FL=fluorescence.

rivatization reaction previously [11–14] and have observed that it depends on time, temperature and pH; we therefore proposed a derivatization procedure requiring only 10 min and room temperature [15] (see Table 1). Reversed-phase instead of normal-phase chromatography with UV–Vis detection is another improvement described in that report.

Recently, we studied the derivatization of amphetamine or methamphetamine on the solid-phase cartridges with NQS reagent and applied this method to the analysis of pharmaceutical samples using a UV–Vis spectrophotometer [16]. The optimum conditions for these determinations were studied and found to be as follows: carbonate–bicarbonate buffer (pH 10.0), 10 min reaction time at 25°C and 0.5% (w/v) NQS concentration.

In the present study we have optimized the derivatization procedure on C_{18} cartridges for urine samples analyzed using a reversed-phase HPLC method with UV–Vis detection. Different conditions for the derivatization reaction were tested: derivative-reaction times, NQS reagent concentrations and volumes of the eluting solvent (acetonitrile–water, 1:1). A liquid–solid procedure is proposed here instead of the two-stage discontinuous procedure involving solid-phase extraction for sample cleanup and liquid–liquid derivatization.

2. Experimental

2.1. Apparatus

A Hewlett-Packard 1014A liquid chromatograph equipped with a diode-array detector was used to collect data. It was coupled to a quaternary pump (Hewlett-Packard, 1050 Series) with a 20-μl sample loop. All data were stored with a linked data system (Hewlett-Packard HPLC Chem. Station, Palo Alto, CA, USA). The column was a 5-μm Hypersil ODS (250×4 mm I.D.) (Merck, Darmstadt, Germany). Signal detection was set between 250 and 500 nm every 640 ms, and all the assays were performed at room temperature. The identity of each compound was established by comparing the retention times and UV–Vis spectra of the peaks in the urine samples with those previously obtained by injection of standards.

2.2. Reagents

All reagents were of analytical grade. Methanol and acetonitrile were of HPLC grade from Scharlau (Barcelona, Spain). Water was distilled, deionized and filtered through 0.45-μm (13 mm diameter) nylon membranes from Teknokroma (Barcelona, Spain). The 1% and 8% bicarbonate buffer solutions were prepared by dissolving 1.0 g or 8.0 g, respectively, of sodium hydrogencarbonate from Probus (Barcelona, Spain) in 100 ml of distilled water. The pH was adjusted to 10.0 by adding a minimum of 10 *M* NaOH (Probus). Amphetamine sulphate and methamphetamine hydrochloride were from Sigma (St. Louis, MO, USA). The internal standard, β-phenylethylamine hydrochloride, was also from Sigma. Stock solutions (0.5%, 1% or 2%) of the derivatization reagent 1,2-naphthoquinone 4-sulphonic acid sodium salt (NQS) from Sigma, were prepared fresh for each derivatization reaction and stored in the dark. Propylamine from Fluka (Barcelona, Spain) and hydrochloric acid from Probus were also used.

The columns were Bond-Elut C_{18} 200 mg from Varian (Barcelona, Spain).

2.3. Standard solutions

The standard solution of methamphetamine hydrochloride was 300 μg/ml and the amphetamine sulphate standard solution was 100 μg/ml. The solution of the internal standard was prepared by dissolving 5 mg of β-phenylethylamine hydrochloride in 100 ml of distilled water. These stock solutions were then further diluted to yield appropriate working solutions for the preparation of the calibration standards and the calibration urine samples. All solutions were stored in the dark at 2°C.

2.4. Sample treatment and derivatization

The solid-phase extraction columns (C_{18} Bond-Elut) were previously conditioned by adding 1 ml of methanol, followed by 1 ml of bicarbonate buffer (1%) at pH 10. Afterwards, 2 ml of the sample solutions (water or urine samples previously spiked with analytes) containing different amine concentrations were transferred to the column and washed

with 3 ml of distilled water. The following derivatization procedure was carried out: 0.5 ml of NQS reagent (0.5%, 1% or 2.0%) was run with 0.5 ml bicarbonate solution (1%) at pH 10.0 through the column that contains the analytes. After 10 min at room temperature for amphetamine and 15 min when methamphetamine was also present, the columns were washed with 3 ml of distilled water. The reaction products (amine-NQS) were eluted from the columns with 1 ml of acetonitrile–water (1:1). Finally, 20 μl of each sample were injected onto the column using a Hamilton micro-syringe.

2.5. Mobile phase

Two gradients of acetonitrile–water with an increasing acetonitrile content were used. Gradient A: 40% at zero time, 50% at 2.5 min, 70% at 3 min. After 3 min the percentage of acetonitrile was kept constant. A second mobile phase, gradient B, was used to improve the peak shape of methamphetamine: 40% at zero time, 50% at 2.5 min, 70% between 3 and 4.5 min and 85% at 5.5 min. After 5.5 min, the percentage of acetonitrile was kept constant. The chromatograms were recorded for up to 7 min. The water solution was prepared by adding 2.5 ml propylamine in 500 ml of water.

The solutions of water and acetonitrile were prepared daily, filtered through a 0.45-μm Nylon membrane and degassed with helium before use. The flow-rate was 1.0 ml/min.

2.6. Urine sample treatment

A 0.4-ml volume of bicarbonate buffer (8%) at pH 10 was added to 2 ml of the urine sample, spiked with amines or unspiked, and the mixture was centrifuged at 1500 *g* for 2 min. Finally, 2 ml of urine sample were taken from the clear liquid obtained and run through the column as described above.

3. Results and discussion

In previous work [16] we studied spectrophotometrically the solid-phase extraction-reaction of the amphetamine or methamphetamine standards with NQS as derivatization reagent and C_{18} support. The results obtained by this procedure are similar to those obtained with the liquid–liquid procedure [10–13]. The reaction products are the same, as indicated by their spectra, and the sensitivity for the analytes tested is similar.

In this work we describe the solid-phase procedure for urine samples. Previously, the reaction was tested when the amines were processed together. Different NQS concentrations were assayed (0.5, 1 and 2%, w/v). For the amphetamine and β-phenylethylamine standards, the area values obtained were similar regardless of the amount of methamphetamine present for the three reagent concentrations. It can be seen that the secondary amine methamphetamine is the one that depends the most on the reagent concentration. The areas for methamphetamine increased by almost 40% by increasing the reagent concentration up to 2% when the reaction time was 10 min. Higher reagent concentrations were not tested because the reactive products of the urine matrix with NQS have a negative influence on the column lifetime.

Fig. 1 shows the influence of the reaction time on the analytical signal when the amines were derivatized together: the areas of standards of primary amines (β-phenylethylamine and amphetamine) remain almost constant. The analytical signal of methamphetamine (secondary amine) varies much more with the reaction time than that of the others. The reaction rate is higher for methamphetamine with the liquid–liquid procedure [8,15]. Similar

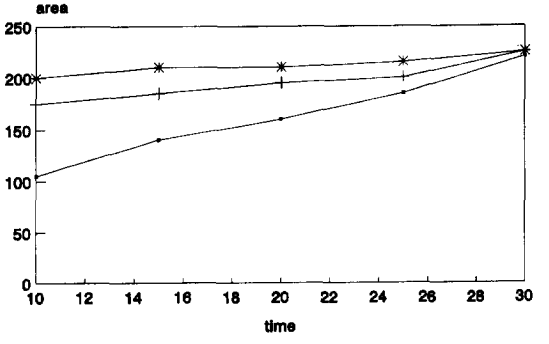

Fig. 1. Influence of the derivatization time (min) on the analytical signal. Conditions: 2% NQS, (*) β-phenylethylamine 3.2 μg/ml, (+) amphetamine 3.0 μg/ml and (·) methamphetamine 9.6 μg/ml.

behaviour has been described for secondary amines in studies using polymeric reagents when the detection-sensitive tags are immobilized on solid supports [4]. The reaction rate is higher for methamphetamine when it is alone [16], but this never happens in urine samples.

Different methamphetamine concentrations with different β-phenylethylamine and amphetamine concentrations were examined in this study: their analytical signals varied linearly with the concentration under identical reaction conditions.

Fig. 2 shows chromatograms of (A) blank urine and (B) spiked urine samples when different concentrations of NQS and reaction times were used. As can be seen in Fig. 2A, the endogenous compounds were eluted within 3 min, regardless of the concentration used. Under our working conditions, the internal standard β-phenylethylamine, was eluted in 3.6 min, and amphetamine and methamphetamine were eluted in 4.1 and 5.6 min, respectively.

The percentage of reaction products recovered for a particular extraction–derivatization was calculated by comparing the peak-area obtained for each drug in the spiked samples with that of the standard samples. Table 2 shows the results obtained for different concentrations of the amines tested. Good recoveries were obtained for the primary amines when the concentration of NQS was 1.0 or 2.0% and the reaction time was 10 or 15 min, respectively. We chose a reaction time of 15 min as a compromise between the sensitivity of the methamphetamine determination and the speed of analysis. Lower concentrations of NQS (0.5%, as in the liquid–liquid procedure) gave lower efficiency. The recovery for methamphetamine, from the urine was no more than 60% even under the best conditions, i.e. 2% of NQS, 15 min and gradient B (see Section 2 and Fig. 2B).

The calibration graphs at 280 nm for the amphetamine were linear between 0.9 and 9.5 μg/ml for the two sets of conditions tested. The slopes of these calibration graphs were 55.97 (1% NQS, 10 min, gradient a), r=0.999, and 68.38 (2% NQS, 15 min, gradient b), r=0.998, for standards and 48.48 (r=0.991) and 68.71 (r=0.991) for urine samples. They were similar to those obtained by the liquid–liquid procedure [15] and by the liquid–solid procedure with amphetamine alone [16]. The intercepts were −9.3, −16.1 and −11.3 and −20, respectively. For

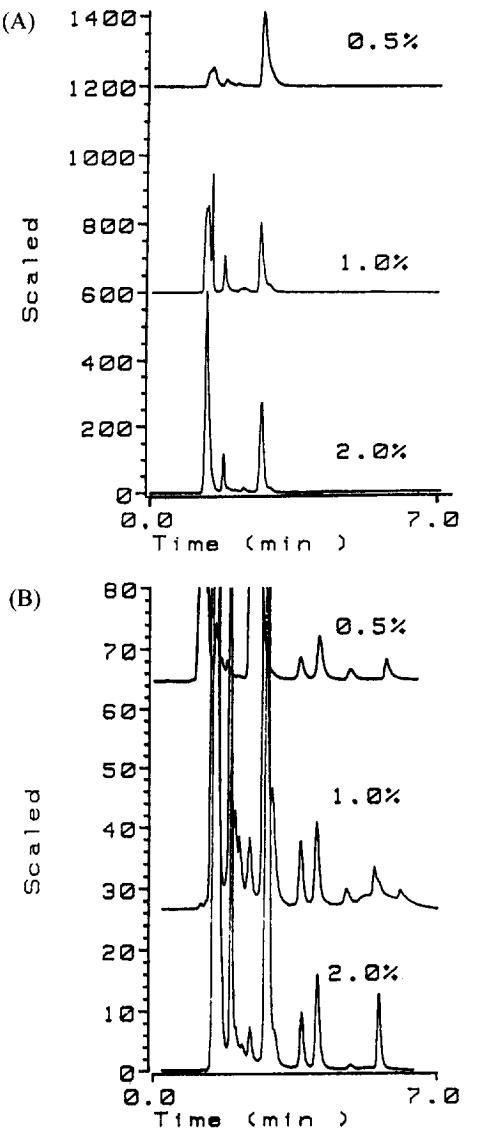

Fig. 2. Chromatograms at 280 nm of (A) blank urine and (B) urine samples spiked with β-phenylethylamine (3.2 μg/ml), amphetamine (4.1 μg/ml) and methamphetamine (12.3 μg/ml) using different NQS reagent concentrations.

methamphetamine in the 3–30 μg/ml range, the slopes were: 10.1 (1% NQS, 10 min, gradient A), r=0.997, and 24.24 (2% NQS, 15 min, gradient B), r=0.999, for standards and 5.6 (r=0.9) and 14.19 (r=0.993) for urine samples. The intercepts were −13.9, −32 and −7.9, −40.0, respectively. The slopes for both urine and standard calibration graphs

Table 2
Recovery percentages for different amine concentrations in urine samples

NQS concentration (%)	Recovery (%)				
	AMP		MET		BFN
	Without IS	With IS	Without IS	With IS	
0.5	39±5 n=20	–	12±2 n=10	–	30±4 n=20
1.0	87±7 n=30	86±5 n=30	55±5 n=18	57±4 n=18	105±10 n=19
2.0	100±8 n=20	102±7 n=20	59±5 n=16	60±4 n=16	96±8 n=16

Abbreviations: AMP=amphetamine; MET=methamphetamine; BNF=β-phenylethylamine; IS=internal standard.

are statistically equivalent if the mean percentage of recovery is considered. The high intercepts of some methamphetamine calibration plots can be explained by the elution profile.

Control urine samples from different volunteers were spiked with amphetamine and methamphetamine and tested to determine these amines using two sets of conditions (Table 3 and Table 4). The concentrations were calculated from the calibration graphs with or without internal standard (β-phenylethylamine) corresponding to a random urine sample spiked with analytes. Each replicate was made with a different urine sample and on different days. The precision and accuracy for the samples are generally good, and therefore it seems that the determination does not depend on the urine matrix in any of the cases tested. Since the matrix of the samples does not disturb the determinations, an estimation of the inter-day precision can be obtained from these data.

More accurate and precise results were obtained when the internal standard was directly added to the samples. Both sets of conditions are good for amphetamine, while the best conditions for metham-

Table 3
Determination of amphetamine and methamphetamine

Concentration added (μg/ml)		Concentration found (μg/ml) (% relative error)			
		Without IS		With IS	
AMP	MET	AMP	MET	AMP	MET
0.9	6.0	0.9 (0.0)	6.1 (1.7)	1.0 (11.1)	6.2 (3.3)
1.9	9.0	2.2 (15.8)	9.9 (10.0)	1.9 (0.0)	8.9 (−1.1)
		1.8 (−5.3)	9.2 (2.2)	1.9 (0.0)	9.4 (4.4)
2.8	12.0	3.3 (17.9)	10.8 (−10.0)	2.9 (3.6)	10.8 (−10.0)
		2.7 (−3.6)	12.7 (5.8)	2.7 (−3.6)	12.5 (4.2)
3.8	14.9	4.2 (10.5)	–	3.8 (2.6)	–
		3.7 (−2.6)	15.4 (3.4)	3.6 (−5.3)	15.8 (6.0)
4.7	17.9	4.4 (−6.4)	18.1 (1.1)	4.5 (−4.3)	16.3 (−8.9)
		4.4 (−6.4)	20.9 (16.8)	4.6 (−2.1)	19.2 (7.3)
7.6	20.9	7.4 (−2.6)	–	7.4 (−2.6)	–
		7.9 (4.0)	22.0 (5.2)	7.7 (1.3)	21.6 (3.3)
8.5	26.9	10.3 (21.2)	–	8.7 (2.3)	–
		8.4 (−1.2)	30.0 (11.5)	8.5 (0.0)	25.0 (−7.1)
9.4	29.9	9.8 (4.2)	–	9.5 (1.1)	–
		9.3 (−1.1)	33.6 (12.4)	9.6 (2.1)	33.6 (12.4)

Conditions: 1% NQS, reaction time 10 min, gradient A and 280 nm. Abbreviations: AMP=amphetamine; MET=methamphetamine; IS=internal standard.

Table 4
Determination of amphetamine and methamphetamine

Concentration added (μg/ml)		Concentration found (mean±S.D.) (μg/ml) (% relative error)			
		Without IS		With IS	
AMP	MET	AMP	MET	AMP	MET
1.9	6.1	$1.9\pm0.1_5$; (0.0)	6.5±0.4; (6.6)	$1.9\pm0.1_5$; (0.0)	$6.3\pm0.3_5$; (3.3)
3.7	12.3	3.5±0.2; (5.7)	11.7±0.7; (−4.-9)	$3.7\pm0.1_5$; (0.0)	11.7±0.6; (−4.-9)
5.6	18.4	$5.2\pm0.2_5$; (7.1)	17.7±0.9; (−3.-8)	5.6±0.2; (0.0)	17.7±0.9; (−3.-8)
7.4	24.6	7.4±0.4; (0.0)	25.1±0.8; (2.0)	7.7±0.3; (4.1)	24.7±0.5; (0.0)
2.1	–	2.4; (14.3)	–	2.1; (0.0)	–
		2.5; (19.0)		2.1; (0.0)	
4.1	–	4.1; (0.0)	–	4.0; (−2.4)	–
		4.3; (4.9)		4.0; (−2.4)	
6.2	–	6.0; (−3.2)	–	5.8; (−6.5)	–
		6.3; (1.6)		5.9; (−1.6)	
8.2	–	8.5; (3.7)	–	8.2; (0.0)	–
		8.8; (7.3)		8.0; (−2.4)	

Conditions: 2% NQS, reaction time 15 min, gradient B and 280 nm. Number of replicates $n=4$, except when specified values. Abbreviations: AMP=amphetamine; MET=methamphetamine; IS=internal standard.

phetamine were 2% NQS, 15 min and gradient B, as can be seen in Table 3 and Table 4. The detection limit for amphetamine was similar to that obtained with the liquid–liquid procedure [8,10], and to those obtained with immobilized reagents on a polymeric solid support (Table 1). A larger injection volume or evaporation of the solvent after elution of the reagent products would have allowed lower concentrations to be detected, as in Ref. [15].

The detection limit for methamphetamine is higher due to the lower reactivity on the cartridge under the experimental conditions used. This could be explained by the fact that it is a secondary amine. However, in medically relevant samples the methamphetamine concentration is always much higher than the concentration of its metabolite amphetamine. The methamphetamine to amphetamine ratio is generally between 4 and 10 in urine samples [17–19].

Samples with different ratios and with concentrations similar to those found in some urine samples were assayed, and the method gave good results (Table 3 and Table 4).

Bond-Elut C_{18} columns could be used for up to 3 months using standard samples or pharmaceutical samples [16]. They were washed with 5 ml of methanol that contains 1 *M* hydrochloric acid. With this treatment the columns could be used with urine samples up to four times without losing sensitivity.

4. Conclusions

Solid-phase derivatization can be done more quickly than the derivatization outside the column after having separated the analytes in the cartridge from the endogenous compounds. The derivatization outside column can be done in 1 h, whereas the derivatization on the solid-phase is ready in a quarter of an hour. Furthermore, with a vacuum station 10 or more samples can be processed together. The time of the analysis is therefore markedly reduced. Using the derivatization on the column, it is not necessary to evaporate the eluate.

We showed that amphetamine determination is very easy and reproducible using our procedure; the response signal of this amine is similar to the one given when it is treated by the liquid–liquid procedure. The efficiency of this solid-phase method is good for this analyte. The detection limit is similar to that obtained by the procedures with polymeric reagents specially prepared for the solid-phase derivatization (Table 1). In our procedure a commercial C_{18} cartridge was used and the sample clean-up and derivatization steps were done on the same support.

In order to obtain good results methamphetamine determination requires a much larger excess of NQS than needed with primary amine amphetamine. This is evident from the strong dependence of the

methamphetamine analytical signal on the NQS reagent concentration and reaction time.

We also showed that methamphetamine can be determined in urine samples, with a detection limit of 0.4 μg/ml. However, we are currently trying to optimize the sensitivity of the procedure for methamphetamine.

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