Liquid Chromatographic Analysis of Amphetamine and Related Compounds in Urine Using Solid-Phase Extraction and 3,5-Dinitrobenzoyl Chloride for Derivatization

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Abstract

A chromatographic method for the analysis of amphetamine and related compounds in urine using 3,5-dinitrobenzoyl chloride (3,5-DNB) as a labeling reagent is presented. This assay is based on the employment of solid-phase extraction (SPE) cartridges for sample cleanup and derivatization. Experimental conditions are optimized for the simultaneous derivatization of ephedrine, norephedrine, pseudoephedrine, β-phenylethylamine, amphetamine, methamphetamine, and 3-phenylpropylamine. The derivatives formed are separated in a LiChrospher 100 RP_{18} (125 × 4-mm i.d., 5-µm film thickness) analytical column using a water–acetonitrile gradient elution and detected at 254 nm. Derivatization in C_{18} SPE disks is found to be the best option for analysis of urine samples; this method provides analyte conversions that are about 85–102% of those obtained by the analogous solution derivatization. Because the 3,5-DNB reagent is a strong π-acid, the described method can be used in combination with a Pirkle-type donor column for chiral analysis. The practicality of the described approach is illustrated by determining amphetamine enantiomers using a Supelcosil LC-(S)-naphtylurea (250 × 4.6-mm i.d., 5-µm film thickness) column and a mobile phase of *n*-hexane–acetonitrile–ethyl acetate. Under these conditions, good linearity and reproducibility are observed over the 0.5–10-µg/mL concentration range; the limit of detection is 50 ng/mL.

Introduction

The sensitive analysis of amphetamine and amphetamine-related compounds in biological fluids has become important because of the continual abuse of these drugs, and forensic and toxicologic laboratories are frequently requested to analyze their presence, especially in urine. High-performance liquid chromatography (HPLC) is the method of choice for the analysis of amphetamine-type compounds in biological samples. However, HPLC methods utilizing ultraviolet (UV) or fluorescence detection suffer from relatively poor sensitivity, and derivatization prior to the chromatographic step is often required. Moreover, possible differences in the pharmacological effects of drug enantiomers necessitate the development of chiral analytical methodologies. Therefore, the development of a derivatization procedure that improves both chromatographic detection and separation of enantiomers is an area of major interest in the analysis of amphetamines.

Chiral analysis of amphetamines has been accomplished in a nonchiral stationary phase by derivatizing with a chiral reagent. Successful results using the fluorogenic labels 9-fluorenylmethyl chloroformate-L-proline (1) and (+)-1(9-fluorenyl)acetyl chloroformate (2) have been described; derivatization using *o*-phthalaldialdehyde (OPA) and a chiral thiol such as *N*-acetyl-L-cysteine has also been reported (3). 2,3,4,6-Tetra-*o*-acetyl-β-D-glucopyranosyl isothiocyanate (4) and *N*-trifluoroacetyl-L-propyl chloride (5) have been used in combination with mass spectrometric and UV detectors, respectively. Other assays are based on the introduction in the analyte molecule of a specific site for interaction with a Pirkle-type chiral column. The introduced tag also converts the analyte to a more detectable species. Examples using the 3,5-dinitrobenzoyl chloride (3,5-DNB) (6) and 3-toluyl chloride (7) reagents have been reported.

On the other hand, enantiomeric analysis usually involves laborious treatment of samples when analyzing biological fluids because many endogenous compounds can lead to a pair of peaks, thus making the resolution of compounds of interest difficult. In addition, many chiral separations perform better in normal-phase conditions. Therefore, some kind of intermediate conditioning steps are required before injection into the chromatographic system when analyzing aqueous matrices (8).

We have recently demonstrated the possibility of integrating sample cleanup and derivatization in the same process by using C_{18} materials such as those commonly employed for analyte purification and preconcentration. The solid supports effect selective retention of the analytes and retention of the derivatives formed when a solution of derivatizing agent is flushed through them. In such a way, analytes can be purified, preconcentrated, and derivatized with minimum sample handling.

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This approach has been applied to the analysis of several amines, including amphetamine, with the derivatizing reagents 1,2-naphthoquinone-4-sulphonate, OPA, and 9-fluorenylmethyl chloroformate (9–11, R. Herráez-Hernández, P. Campíns-Falcó, A. Sevillano-Cabezo, and I. Trümpler. Derivatization of amines in solid-phase extraction supports with 9-fluorenylmethyl chloroformate for liquid chromatography. *Anal. Chim. Acta*, in press).

In this study, we evaluated the potential of the derivatization technique in solid-phase extraction (SPE) cartridges with the 3,5-DNB reagent for the chromatographic analysis of amphetamine and other amine compounds. Conditions for the simultaneous derivatization and separation of ephedrine (EPE), norephedrine (NOREPE), pseudoephedrine (PSEPE), β-phenylethylamine (β-PEA), amphetamine (AMP), methamphetamine (MET), and 3-phenylpropylamine (PPA) were established under the conventional (solution) derivatization mode. The optimum procedure was subsequently adapted for their isolation and derivatization using SPE materials. On the basis of these studies, a method for separation and identification of amphetamines in urine is presented. Finally, the utility of the described procedure for the chiral analysis of amphetamine is described.

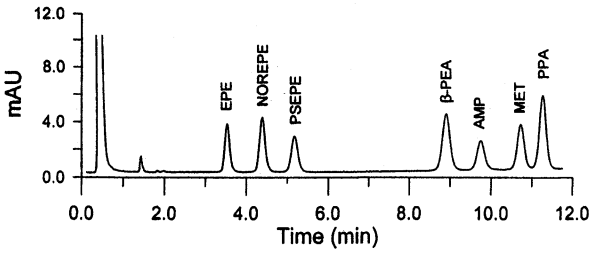

Figure 1. Chromatograms obtained for the amphetamines derivatized in solution: 0.50 mL of a standard solution containing 10.0 µg/mL of each compound plus 0.25 mL of 0.05M borate buffer (pH = 9.5) plus 0.25 mL of 20mM 3,5-DNB; reaction time, 5 min. Ephedrine (EPE), norephedrine (NOREPE), pseudoephedrine (PSEPE), β-phenylethylamine (β-PEA), amphetamine (AMP), methamphetamine (MET), and 3-phenylpropylamine (PPA).

Experimental

Apparatus

The chromatographic system that was used consisted of a quaternary pump equipped with an automatic sample injector (1050 series) (Hewlett-Packard, Palo Alto, CA). A diode-array detector (Hewlett-Packard, 1040 series) linked to a data system (Hewlett-Packard HPLC Chem Station) was used for data acquisition and storage. The chromatographic signal was monitored at 254 nm. All the assays were carried out at ambient temperature.

Reagents

All the reagents were of analytical grade. Acetonitrile, *n*-hexane, ethyl acetate, and methanol (Scharlau, Barcelona, Spain) were of HPLC grade. Ephedrine hydrochloride, pseudoephedrine hydrochloride, methamphetamine hydrochloride, β-phenylethylamine hydrochloride, and amphetamine sulphate were obtained from Sigma (St. Louis, MO). Norephedrine hydrochloride, PPA, 3,5-dinitrobenzoyl chloride, and triethylamine were obtained from Aldrich (Steinheim, Germany). Boric acid and sodium hydroxide (Panreac, Barcelona, Spain), pyridine (Fluka, Buchs, Switzerland), and sodium hydrogen carbonate (Probus, Badalona, Spain) were also used.

Different SPE columns were evaluated: C_{18}, C_8, C_2, cyclohexyl (CH), phenyl (PH), and cyanopropyl (CN) BondElut 100-mg/mL column cartridges (Scharlau). The 3M Empore C_{18} SPE disk cartridges (10 mm × 6 mL) were purchased from Varian (Harbour City, CA).

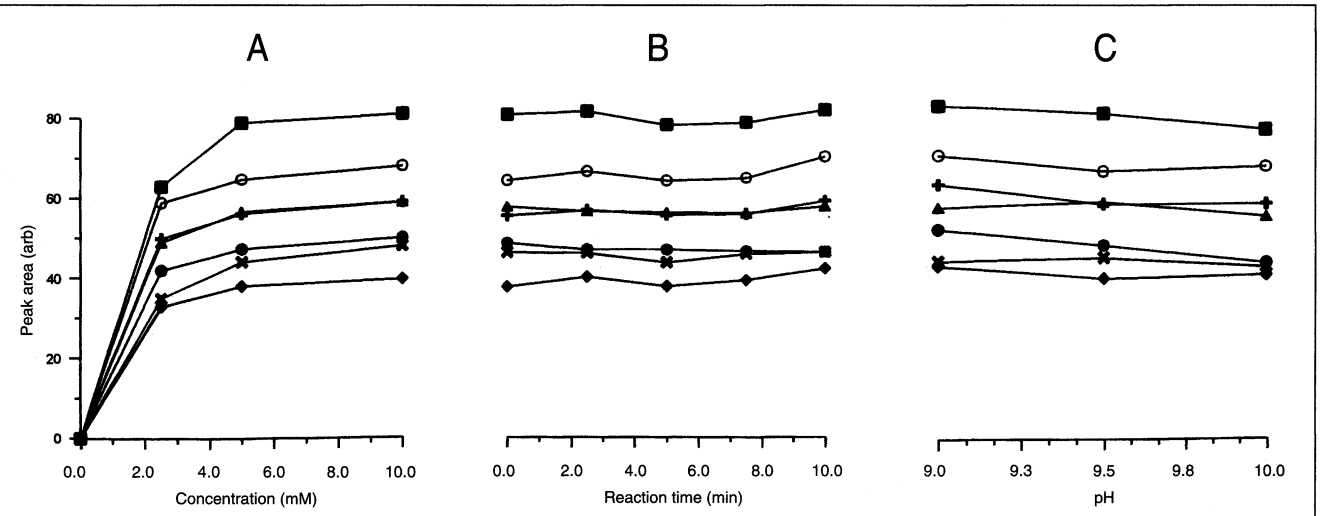

Figure 2. Analyte responses as a function of (A) concentration of 3,5-DNB (reaction time, 2 min); (B) reaction time (volume of 0.05M borate buffer [pH = 9.5], 0.25 mL; volume of 20mM 3,5-DNB, 0.25 mL); and (C) the pH of the borate buffer (volume of 0.05M borate buffer, 0.25 mL; volume of 20mM 3,5-DNB, 0.25 mL; reaction time, 5 min). In all instances, 0.50 mL of a standard mixture containing 10 µg/mL of each amphetamine was assayed. For other experimental details, see text. x = EPE, ▲ = NOREPE, ● = PSEPE, O = β-PEA, ♦ = AMP, + = MET, and ■ = PPA.

Preparation of solutions

Stock standard solutions (1000 µg/mL) of the analytes were prepared in water. Working solutions of the amines were prepared by dilution of the stock solutions with water. Stock standard solutions of 3,5-DNB (100mM) were prepared by dissolving the pure compound in acetonitrile. Working solutions of the reagent were prepared from the stock solutions by dilution with acetonitrile. The 0.05M sodium borate and sodium carbonate buffers were prepared by dissolving boric acid and sodium carbonate, respectively, in water. Next, the pH was adjusted to appropriate values with 10% NaOH (w/v). All solutions were stored in the dark at 2°C.

Columns and mobile phases

A LiChrospher 100 RP_{18} (125 × 4-mm i.d., 5-µm film thickness) (Merck, Darmstat, Germany) column was used for separation of the amphetamine derivatives. A water–acetonitrile mixture in gradient elution mode was used as the eluent at a flow rate of 1.5 mL/min. Different gradient elution profiles were investigated in order to optimize chromatographic resolution and analysis time. Although suitable resolution was observed under a variety of conditions, the best results were obtained by linearly increasing the acetonitrile content from 40% at 0–5 min to 50% at 10 min. After 10 min, the mobile phase composition was kept constant.

For the separation of the amphetamine–3,5-DNB enantiomers, a Supelcosil LC-(S)-naphtylurea (250 mm × 4.6-mm i.d., 5-µm film thickness) (Supelco, Bellefonte, PA) column was used. A mixture of *n*-hexane–ethyl acetate–acetonitrile (70:25:5, v/v) pumped at a flow rate of 1.0 mL/min was the eluent. All solvents were filtered with a 0.45-µm nylon membrane (Teknokroma, Barcelona, Spain) and degassed with helium before use.

Solution derivatizations

For the solution derivatization method, 0.50 mL of the samples (containing 10.0 µg/mL of the analytes) was placed into 2-mL injection glass vials. Next, aliquots of 0.10, 0.25, or 0.35 mL of the buffer solutions were added to the samples. The mixture was further diluted to 1 mL with the 3,5-DNB derivatization reagent (5–40mM). In some instances, 10^{-3}mM pyridine or 10^{-3}mM triethylamine were added to the samples prior to derivatization instead of a buffer solution. The mixtures were then allowed to react for a defined period of time (0, 2.5, 5.0, 7.5, and 10 min). Finally, 25 µL of the mixtures were injected into the analytical column. Each sample was assayed in triplicate.

Table I. Conditions Used for Derivatization of Amphetamines into SPE Cartridges

Process	Conditions
Conditioning the cartridge	1 mL methanol
	+
	1 mL water
Sample loading	0.50 mL
Cleaning*	1 mL of borate buffer (pH = 9.5)
Drying the cartridge	vacuum (2 min)
Derivatization plus desorption	0.25 mL of 20mM 3,5-DNB
	+
	0.25 mL acetonitrile

* When processing urine samples.

Isolation and derivatization using SPE cartridges

The SPE column and disk cartridges were conditioned previously by drawing with 1.0 mL of methanol followed by 1.0 mL of water. Samples (0.50 mL) were placed into the cartridges. The cartridges were then flushed with 0.25–2.0 mL of 0.05M buffer solutions. Next, the cartridges were dried under a vacuum for 2 min (at 0.4 bar). Single 0.25-mL aliquots of derivatizing reagent and acetonitrile were drawn in succession through the cartridges (by flushing with air using a 10-mL syringe) and collected into 2-mL glass vials. Finally, 25 µL of the collected extracts was injected into the chromatographic system.

Derivatization rates into the cartridges were evaluated by comparing peak areas obtained for a particular assay (at an analyte concentration of 5.0 µg/mL) with those obtained for samples that contained an equivalent amount of the drug and were derivatized in the solution mode. Each sample was assayed in triplicate.

Urine samples

Untreated urine samples were spiked with the analytes at a concentration of 5 µg/mL. Volumes of 0.50 mL of these samples were placed into conditioned column or disk cartridges, and the matrix components were eliminated with 1.0 mL of borate buffer (pH = 9.5). The retained analytes were then processed as described above, and 25 µL was injected onto the chromatographic system. Each sample was assayed in triplicate.

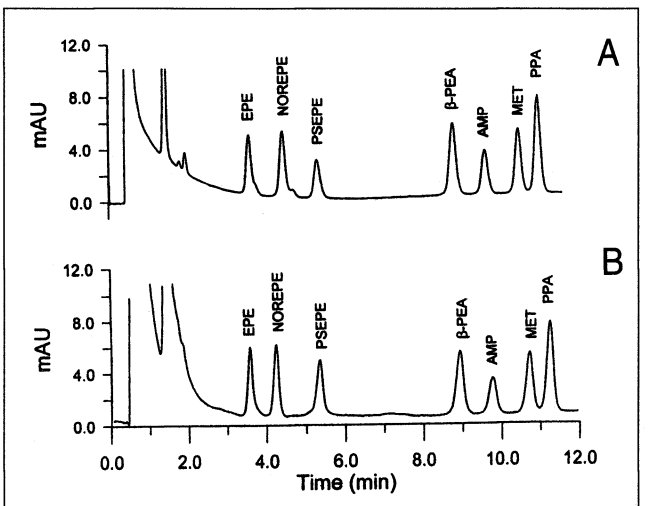

Figure 3. Chromatograms obtained for a standard solution of the amphetamines (containing 5.0 µg/mL of each amine) derivatized in C_{18} SPE (A) column and (B) disk cartridges. Conditions used for derivatization were those summarized in Table I. Ephedrine (EPE), norephedrine (NOREPE), pseudoephedrine (PSEPE), β-phenylethylamine (β-PEA), amphetamine (AMP), methamphetamine (MET), and 3-phenylpropylamine (PPA).

Analysis of amphetamine enantiomers in urine

Untreated urine samples were spiked with amphetamines; concentrations for each enantiomer were reproduced in the 0.5–10.0-µg/mL range. These samples were processed in C_{18} SPE disk cartridges as described above. Each sample was assayed in triplicate.

Results and Discussion

Optimization of the derivatization conditions

We studied the effect of the experimental parameters on the derivatization rates when derivatizing in the solution form.

Concentration of 3,5-DNB

The effect of 3,5-DNB concentration on the reaction rate was evaluated in the 1.0–10.0mM range. Under such conditions, unreacted 3,5-DNB did not interfere with peaks corresponding to the amine derivatives (Figure 1). However, concentrations of derivatization reagent higher than 10mM produced an intense peak that partially overlapped those of the EPE, NOREPE, and PSEPE derivatives, so they were not investigated. The reaction time was 5 min, and 0.25 mL of 0.05M borate buffer (pH = 9.5) was added to the samples. For samples containing 10.0 µg/mL (5 µg/mL in the final solution), the minimum concentration of 3,5-DNB necessary to achieve constant analyte conversions was 5mM (Figure 2A).

Reaction time

The effect of the reaction time on analyte responses was evaluated by mixing 0.50 mL of aqueous samples with 0.25 mL of 0.05M borate buffer (pH = 9.5) and 0.25 mL of 20mM 3,5-DNB (5mM in the final solutions). The resulting mixtures were allowed to react for a defined period of time in the 0–10.0-min interval and then chromatographed. The results are shown in Figure 2B. As can be seen from the figure, 3,5-DNB reacted rapidly with all the amines under investigation. In all instances, stable responses were observed in the 0–10-min time interval. Therefore, under these conditions, the reaction time was not a critical parameter in the derivatization of aqueous solutions of the compounds.

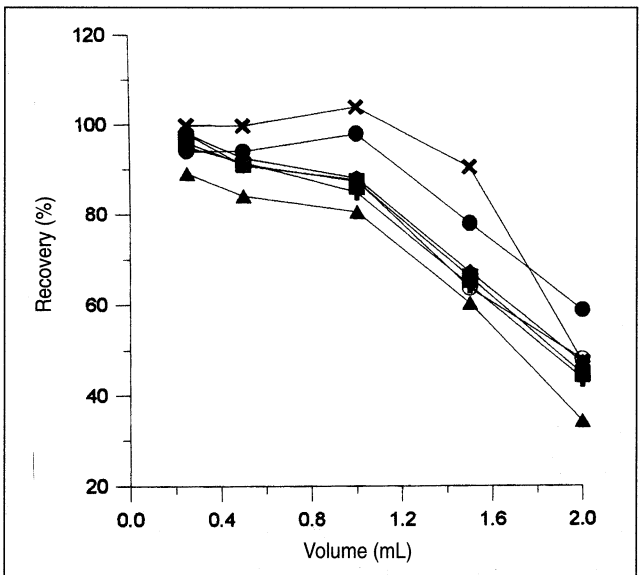

Figure 4. Effect of the buffer volume used for cleaning the samples (standard solution of amines containing 5.0 µg/mL of each compound) on analyte recoveries. Conditions used for derivatization were those summarized in Table I. For other experimental details, see text. x = EPE, ▲ = NOREPE, ● = PSEPE, O = β-PEA, ♦ = AMP, ✚ = MET, and ■ = PPA.

Buffer solution

In order to keep the analytes as unprotonated amines, reactions involving the 3,5-DNB reagent required a basic medium. In most procedures, the pH of the reaction mixture is adjusted to a basic value by adding an appropriate buffer to the samples; derivatization yields can also be improved by adding a tertiary amine to the reaction mixture (6,8). We initially compared both possibilities. In this study, 0.05M borate and carbonate buffers (pH = 9.5) and 10^{-3}mM pyridine and triethylamine were investigated. The best reaction yields were obtained with a borate buffer; however, no significant differences in the 9.0–10.0 pH range were observed (Figure 2C). The addition of 0.25 mL of borate buffer (pH = 9.5) and 0.25 mL of 20mM 3,5-DNB to the samples (0.5 mL) was selected as the best option for derivatization of the analytes in solution.

It should be noted that because the derivatizing reagent was prepared in acetonitrile, the final percentage of this solvent in the reaction vials was 25%. We observed that the presence of acetonitrile in the derivatization medium was necessary to obtain suitable conversions of the analytes. For example, responses for amphetamine were about 5% of those observed under the optimized conditions, in which the reaction was performed in a solution that contained 1% acetonitrile. If the final percentage of acetonitrile was higher than 20%, analyte responses were not dependent on the acetonitrile content (data not shown).

Derivatization in SPE cartridges

The optimum procedure found for the solution derivatization mode was adapted for the isolation and derivatization of these compounds using SPE materials. In this study, C_{18} SPE

Table II. Recoveries of Amphetamines from Water and Urine after Cleanup and Derivatization into C_{18} SPE Disk Cartridges (Three Replicates)

Compound	Recovery (%) Water*	Recovery (%) Urine†	Detection limit in urine (ng/mL)
Ephedrine	103 ± 4	102 ± 9	25
Norephedrine	80.1 ± 0.7	95 ± 6	25
Pseudoephedrine	98 ± 2	100 ± 4	25
β-phenylethylamine	88 ± 4	86 ± 6	10
Amphetamine	83 ± 3	89 ± 6	25
Methamphetamine	88 ± 4	86 ± 3	25
3-Phenylpropylamine	87 ± 4	85 ± 6	10

* Determined from one sample.
† Determined from two samples.

columns were used. The columns were conditioned with methanol and water, and amphetamines (sample volume, 0.50 mL) were then trapped in the cartridges.

The usual treatment when derivatizing in solid supports is to trap the analytes in the SPE material. The endogenous compounds are then sent to waste by flushing the solid support with an appropriate solvent. Next, a solution of the derivatizing reagent is flushed through the packing. Analyte and reagent are allowed to react for a given period of time. Finally, the unreacted reagent is flushed out, and the derivatives are desorbed from the packing and chromatographed (11). In the present instance, because the derivatization reaction was very rapid and elimination of the unreacted reagent was found to be unnecessary, flushing an appropriate volume of the 3,5-DNB prepared in acetonitrile simultaneously brought about formation and desorption of the derivatives from the C_{18} column cartridges. Satisfactory responses were observed when 0.25–1.0 mL of 5mM 3,5-DNB reagent was flushed through the cartridges.

In contrast to the solution derivatization mode, the presence of a buffer in the collected extracts did not improve the analytical signals, which indicated that the analytes were in the cartridges in the appropriate (unprotonated) form. On the other hand, variations of the percent of analytes converted as a function of the concentration of 3,5-DNB and as a function of the reaction time were similar to those observed for the solution derivatization.

Although most of the derivatives were completely eluted from the C_{18} column cartridges, variable fractions of the 3,5-DNB remained in the cartridges after the reagent was flushed. In other words, the final amount of 3,5-DNB in the collected extracts varied between assays. Better reproducibility was obtained when the column cartridges were flushed with the derivatizing solution and then with acetonitrile. In such a way, the final concentration of 3,5-DNB in the extracts was found to be fairly constant and independent on the cartridge design (dead volume) and on the amount of SPE material (loading capacity). In order to achieve maximum analyte detectability, volumes of 3,5-DNB and acetonitrile should be adjusted to a minimum. In the present instance, satisfactory reproducibility was obtained by flushing 0.25 mL of each derivatization reagent followed by 0.25 mL of acetonitrile.

The described procedure was also satisfactory for the derivatization of analytes in column cartridges packed with the other packing materials under investigation, as well as for derivatization in C_{18} SPE disks. The final recommended procedure for derivatization of the amphetamines in SPE supports is shown in Table I. Under such conditions, the percentages of drugs recovered were comparable to those obtained by the solution derivatization mode. In most instances, the highest values corresponded to norephedrine, whereas the lowest recoveries were observed when using C_{18} SPE disks, with values in the 84–99% interval.

As an example, Figure 3 shows the chromatograms obtained for standard mixtures of amphetamines processed in the C_{18} column and C_{18} disk cartridges. By comparing Figures 1 and 3, it can be deduced that the main difference between the solution and the solid support mediated reactions is the presence of different byproducts when using the latter approach. This is most probably due to the presence of impurities in the packing material that react with the derivatization reagent, especially at basic pH. This is the most serious limitation of the derivatization in solid supports approach, although, in some instances, acidification of the collected extracts eliminates unwanted products (R. Herráez-Hernández, P. Campíns-Falcó, A. Sevillano-Cabezo, and I. Trümpler. Derivatization of amines in solid-phase extraction supports with 9-fluorenylmethyl chloroformate for liquid chromatography. *Anal. Chim. Acta*, in press). In the present study, most byproducts eluted between

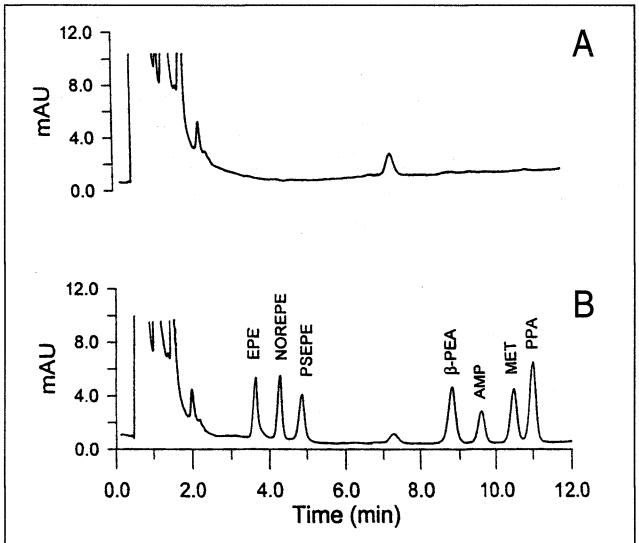

Figure 5. Chromatograms obtained for (A) blank urine and (B) urine spiked with 5.0 µg/mL of each amphetamine. Conditions used for isolation and derivatization were those summarized in Table I. Ephedrine (EPE), norephedrine (NOREPE), pseudoephedrine (PSEPE), β-phenylethylamine (β-PEA), amphetamine (AMP), methamphetamine (MET), and 3-phenylpropylamine (PPA).

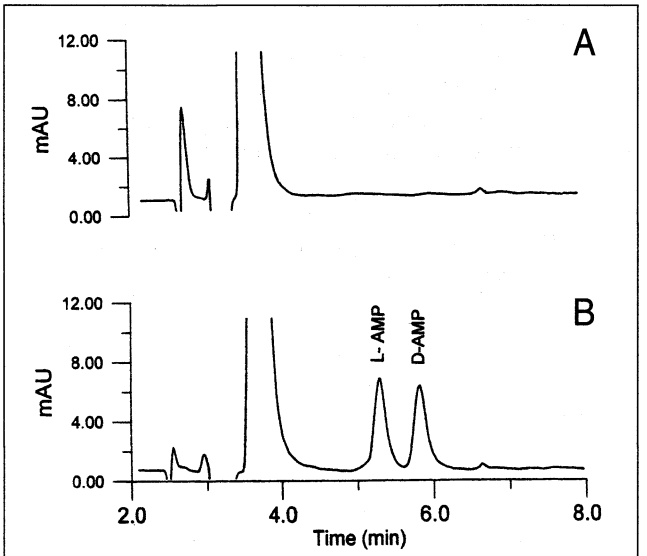

Figure 6. Chromatograms obtained for (A) blank urine and (B) urine spiked with 10.0 µg/mL racemic amphetamine (5 µg/mL of each enantiomer). Conditions used for isolation and derivatization were those summarized in Table I.

0.5 and 2.0 min, and therefore, they did not interfere with the interesting peaks. However, when using extraction columns, a peak eluting at a retention time close to that of the norephedrine derivative was observed, which explains the high recoveries obtained for this compound (100–117%). No similar peak was produced by the C_{18} extraction disks.

Derivatization of amphetamines in urine

Derivatization of amphetamines in urine was performed as described in Table I, but urinary endogenous compounds were eliminated from the column or from the disk cartridges before the derivatization step. In order to keep the amphetamines in the unprotonated form, a 0.05M borate buffer (pH = 9.5) was used in the cleanup stage.

C_{18} extraction disks provided the best selectivity when analyzing urine samples. In addition, no interferences with byproducts produced by the cartridge itself were observed (as stated above). Therefore, in further experiments, C_{18} disks were used for the isolation and derivatiation of amphetamines.

Retention of the analytes was evaluated by washing the disk cartridges with different volumes of buffer after sample loading. Recoveries were calculated by comparing peak areas with those obtained when the analytes were derivatized after sample loading (washing volume, 0 mL). The results obtained are shown in Figure 4. As can be seen from this figure, most amphetamines were satisfactorily retained when flushing the disks with 0.25–1.0 mL of buffer. Therefore, low responses obtained for amphetamine derivatives, with respect to those observed when derivatizing in SPE columns, cannot be explained by low retention of these compounds into the C_{18} SPE disks. This indicated that the reaction performed slightly worse in the C_{18} SPE disks.

In order to achieve the best sensitivity, a volume of 1.0 mL was used in further experiments. Table II summarizes the overall recoveries obtained for amphetamines under the cleanup and derivatization conditions (Table I). As observed from Table II, responses obtained for urine samples were similar to those found for aqueous standard solutions of amphetamines, which indicates that neither the retention nor the derivatization of the analytes was affected by the matrix. Table II also shows the limits of detection (for a signal-to-noise ratio of 3) found in urine samples. The sensitivity was comparable to that achieved by other UV assays (6,12). Figure 5 shows the chromatograms obtained for blank urine and urine spiked with a mixture of the tested amphetamines. As can be deduced from this figure, the assay offers satisfactory selectivity.

Analysis of amphetamine enantiomers in urine

Despite their popularity in other areas, Pirkle-type stationary phases have limited application in drug analysis because they perform better under normal-phase conditions, whereas HPLC drug analysis in biological fluids usually involves reversed-phase conditions (6,13). One advantage of the described procedure is that it combines sample cleanup in reversed-phase conditions and derivatization in a 100% acetonitrile solvent. Therefore, derivatization under the described procedure is also well-suited for chiral separations. The utility of the described approach in chiral analysis was evaluated by analyzing amphetamine enantiomers in urine at therapeutic concentrations.

Amphetamine–3,5-DNB enantiomers were satisfactorily resolved in a typical Pirkle-type column with a hexane–ethyl acetate–acetonitrile mixture (70:25:5, v/v), pumped at a flow rate of 1.0 mL/min. As observed in the achiral separation, peaks corresponding to the unreacted 3,5-DNB and peaks produced by the C_{18} disks were eluted with low retention times. Moreover, the selectivity when analyzing urine samples was also satisfactory, as can be seen in Figure 6.

Table III summarizes some relevant analytical data obtained with the present procedure. Linearity and reproducibility (obtained by one analyst) were suitable over the 0.5–10.0-µg/mL range. The method is less sensitive than the achiral approach, which can be explained by the baseline distortions observed at 254 nm when using the chiral column and normal-phase elution conditions for separation (see Figure 6). However, analyte detectability is comparable to that achieved by most HLPC assays using UV detection proposed for the analysis of amphetamine enantiomers (4,6). Therefore, the described approach can be considered satisfactory for most applications. Pirkle-type columns have been successfully applied to the separation of enantiomeric forms of several primary and secondary amines after derivatization (7). This suggests that application of the described procedure to the chiral analysis of other amphetamine-type compounds could be possible. This approach is currently under investigation in our laboratory.

Table III. Analytical Data for the Derivatization of Amphetamine Enantiomers in Urine Using C_{18} SPE Disks

Enantiomer	Linearity* $A = b + aC$ (10 replicates)	Intraday precision† (%) (six replicates)	Interday precision† (%) (12 replicates)	Detection limit (ng/mL)
L-Amphetamine	22.62 + 6.245 C (r = 0.998)	3	7	50
D-Amphetamine	17.66 + 7.031 C (r = 0.9990)	3	6	50

* A represents the peak area (arbitrary units), a and b are the slope and intercept of the calibration graph, respectively, and c is the concentration of amphetamine (µg/mL). r is the regression coefficient.
† Determined at half of the highest concentration in the tested range.

Conclusion

The combination of 3,5-DNB and C_{18} SPE disks is a viable alternative to conventional (solution) derivatizations in the analysis of amphetamines in urine. Although conversion yields were comparable to those obtained by the analogous solution derivatizations, purification and derivatization of the analytes were greatly simplified. In addition, the derivatives were formed in

solutions that are amenable to Pirkle-type enantioselective separations. Therefore, intermediate conditioning steps before injection into the chromatographic system were not required. The main limitation was the volume required to desorb the derivatized analytes from the cartridges with satisfactory reproducibility. C_{18} SPE disks provided the best selectivity for the analysis of urine samples. In this way, the analysis of amphetamines can be performed with satisfactory sensitivity and reproducibility and a minimum of sample handling.

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