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Determination of amphetamine and related compounds in urine using on-line derivatization in octadecyl silica columns with 9-fluorenylmethyl chloroformate and liquid chromatography

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Abstract

A method for the determination of amphetamine and related compounds in urine based on on-line derivatization with 9-fluorenylmethyl chloroformate (FMOC) and high-performance liquid chromatography is described. Derivatization is performed in a 20×2.1 mm I.D. column packed with a Hypersil ODS C_{18}, 30 μm stationary phase, which is also used for sample clean-up and enrichment of the analytes. Next, the derivatized analytes are transferred to a LiChrospher 100 RP-C_{18} (5 μm, 125×4 mm I.D.) analytical column for their separation and quantification, using reversed-phase conditions and fluorescence detection. The described assay was applied to the determination of norephedrine, ephedrine, pseudoephedrine, amphetamine, phenylpropylamine and methamphetamine at concentrations of 0.5–10.0 μg/ml. Analyte conversions were about 55–96% of those obtained by the off-line derivatization mode under similar conditions, resulting in limits of detection in the 5–25 ng/ml range.

Keywords: Amphetamine; Norephedrine; Ephedrine; Pseudoephedrine; Phenylpropylamine; Methamphetamine

1. 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 many analytical procedures have been developed for qualitative and quantitative purposes. In this respect, gas chromatographic (GC) methods have been traditionally recommended, due to the high sensitivity achieved (pg amounts of amphetamines). Moreover, GC coupled to mass spectrometric (MS) detection is the most powerful method for the identification of these compounds [1,2]. Major limitations are the requirement of prior derivatization and the cost involved. High-performance liquid chromatography (HPLC) is not so widely accepted because of the low UV absorbances of these compounds, and also because they have very little natural fluorescence. In an effort to improve analyte detectability, a number of methods involving pre-column or post-column derivatization have been developed. Successful examples using a variety of derivatization agents for UV, fluorescence or chemiluminescence detection have been reported [3–7]. However, most of these procedures involve extensive sample clean-up (many derivatization reagents are reactive towards matrix components in biological

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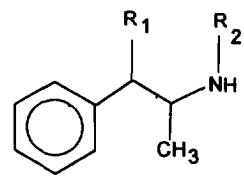
samples), solution derivatization of the purified extracts and re-extraction of the derivatized analytes to remove unreacted reagent, the final step of the analysis being the injection of the derivatives formed into the chromatographic system. Only a few procedures incorporate on-line derivatization of the samples. For example, Koning et al. [8] reported an automated method the derivatization of drugs with primary amino groups (including amphetamine), using naphthalenedialdehyde as the fluorogenic label. Maeder et al. [9] described an assay for the on-line derivatization of amphetamine-related compounds with 9-fluorenylmethyl chloroformate (FMOC) using UV detection. Both procedures were only applied to the analysis of aqueous standard solutions. Solid-phase reagents are an elegant alternative for the on-line derivatization of compounds in biological samples. These reagents have been used for direct injection of biofluids in HPLC using different tags [10–13]. However, the synthesis of the reagents is often laborious and periodic regeneration of the reagent may be required to obtain reproducible results.

Derivatization can also be performed in unmodified C_{18} cartridges, which effect retention of the analytes and retention of the derivatives formed when a solution of derivatizing agent is flushed through the cartridges. This has been illustrated by determining amphetamine and methamphetamine in urine using 1,2-naphthoquinone (NQS) for derivatization [14]. Although the analysis was facilitated (when compared with solution derivatization), off-line manipulation of the sample was still involved. We have recently illustrated the possibility of using a precolumn packed with a conventional ODS stationary phase for the on-line derivatization of amines in biological matrices [15]. The precolumn, which acted as a trapping column, was used to purify the sample and concentrate the analytes, and then, to retain the derivatized analytes formed, by injecting a solution of the derivatization reagent. Finally, the derivatives were transferred to the analytical column by means of a switching arrangement. The reliability of this approach was tested by using amphetamine, methamphetamine and β-phenylpropylamine as model compounds, and typical derivatization agents such as NQS, *o*-phthaldialdehyde (OPA) and FMOC. The main advantage of this methodology is that the derivatization reagent is used in solution form, and thus, conventional (unmodified) C_{18} packings can be used for both clean-up and derivatization; moreover, sample handling is eliminated. FMOC was found to be the reagent of choice for the derivatization of amines in urine because the set-up required was very simple, and also because satisfactory analyte conversion rates were obtained at ambient temperature in very short reaction times.

The aim of this work was to extend the derivatization technique in precolumns to a variety of amphetamine-related compounds of interest in forensic and toxicological fields (Fig. 1). Experimental conditions for the simultaneous retention and derivatization of interesting compounds have been optimized. On the basis of these experiments, a method for the separation and quantification of norephedrine, ephedrine, pseudoephedrine, amphetamine, phenylpropylamine and methamphetamine in urine is proposed.



	R_1	R_2
Norephedrine	-OH	-H
Ephedrine	-OH	$-CH_3$
Pseudoephedrine	-OH	$-CH_3$
Amphethamine	-H	-H
Methamphetamine	-H	$-CH_3$

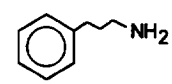
Phenylpropylamine 

Fig. 1. Chemical structure of amphetamine and related compounds under investigation.

2. Experimental

2.1. Apparatus

The chromatographic system used consisted of two quaternary pumps (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 Series) and a high-pressure six-port valve (Rheodyne Model 7000). A fluorescence (Hewlett-Packard, 1046 Series) detector linked to a data system (Hewlett-Packard HPLC Chem Station, Dos Series) was used for data acquisition and storage. The detector operated at an excitation wavelength of 264 nm and an emission wavelength of 313 nm.

2.2. Reagents

All the reagents were of analytical grade. Acetonitrile (Scharlau, Barcelona, Spain) was of HPLC grade. Ephedrine hydrochloride, pseudoephedrine hydrochloride, methamphetamine hydrochloride and amphetamine sulphate were obtained from Sigma (St. Louis, MO, USA). Norephedrine hydrochloride, phenylpropylamine and 9-fluorenylmethyl chloroformate were obtained from Aldrich (Steinheim, Germany). Sodium hydrogen carbonate (Probus, Badalona, Spain) and sodium hydroxide (Panreac, Barcelona, Spain) were also used.

2.3. Preparation of solutions

Stock solutions (1000 μg/ml) of each amine were prepared in water. Working solutions were prepared from the stock solutions by dilution with water. All solutions were stored in the dark at 2°C. FMOC solutions (20 m*M*) were prepared daily by dissolving the pure compound in acetonitrile. The hydrogen carbonate buffer (4%, w/v) was prepared by dissolving the appropriate amount of sodium hydrogen carbonate in water and then adjusting the pH to 10.0 with 10% NaOH (w/v).

2.4. Columns and mobile-phases

The pre-column (20×2.1 mm I.D.) was dry-packed with a Hypersil ODS-C_{18}, 30 μm, stationary phase (Hewlett-Packard, Darmstadt, Germany). A LiChrospher 100 PR-C_{18}, 5 μm, 125×4 mm I.D. column (Merck, Darmstadt, Germany) was used as the analytical column. Purified water was used for washing the precolumn during the clean-up step. A water–acetonitrile mixture in gradient elution mode was used as the mobile-phase for separation (at a flow-rate of 1.0 ml/min).

All solvents were filtered with a 0.45-μm Nylon membrane, (Teknokroma, Barcelona, Spain) and degassed with helium before use.

2.5. On-line derivatization

The set-up used for the on-line derivatization of the samples is shown in Fig. 2. At the beginning of each assay, 15 μl of sample were injected onto the trapping column, the switching valve being in position A. Matrix constituents were flushed-out by flushing the precolumn with water (delivered by pump 1). After sample injection, the autosampler withdrew 5 μl of 20 m*M* FMOC and 45 μl of water from separate vials. Mixing of the resulting solution was automatically performed by the autosampler. The diluted FMOC was then injected onto the precolumn. Next, 10 μl of sodium hydrogen carbonate buffer were injected. Cleaning of the samples was carried out during the dilution and injection of FMOC, and injection of the buffer. At 2.8 min, the switching valve was turned (to position B) and gradient elution in pump 2 was started. Different volumes of water for flushing the precolumn (V_f) were evaluated. Since the injection program was the same in all instances, V_f was modified by changing the flow in pump 1.

At the end of each assay, the valve was turned to the original position to regenerate and re-equilibrate both the precolumn and the analytical column. Rotation of the valve was performed manually.

For the off-line derivatizations, 0.15 ml of sample was added to 0.45 ml of water and 0.05 ml of 20 m*M* FMOC. Finally, 0.10 ml of hydrogen carbonate buffer was added to the mixture and the resulting solution was injected into the analytical column.

In all instances, derivatizations were carried out at ambient temperature and each sample was assayed in triplicate.

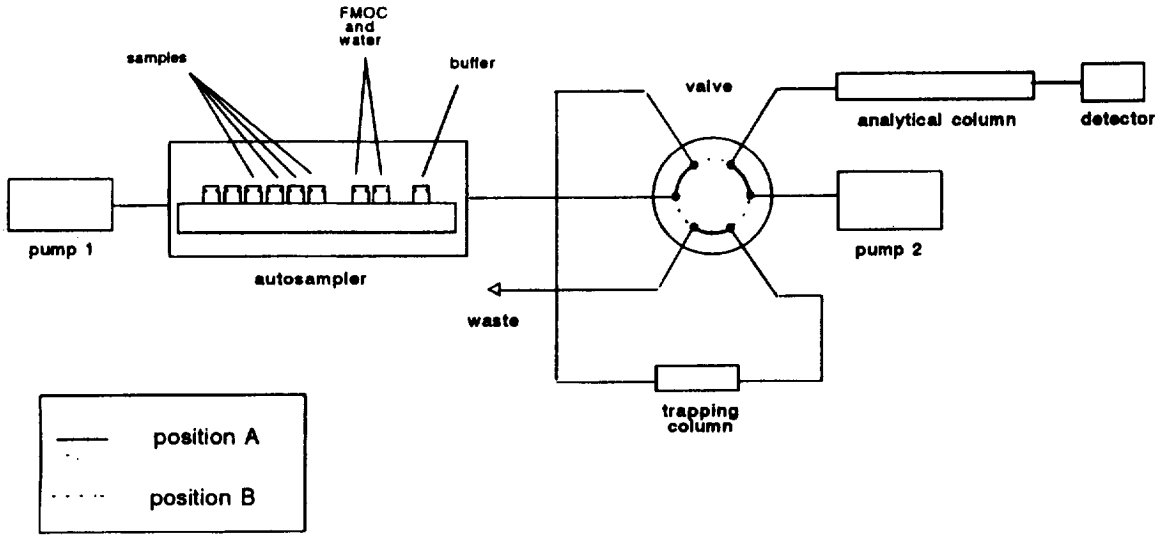


Fig. 2. Schematic representation of the system used for the on-line clean-up plus derivatization of amphetamine and related compounds in urine.

2.6. Recovery studies

Retention of the amphetamines in the trapping column was evaluated by comparing peak areas obtained for a particular assay (at a concentration of analyte of 5.0 μg/ml) with those obtained when flow in pump 1 was stopped immediately after sample injection (V_f=0 ml); these areas were assumed to represent 100% retention of the analytes.

The percentage of drug recovered after clean-up and derivatization was evaluated by comparing the peak areas obtained for a particular assay in the described system (at a concentration of analyte of 5.0 μg/ml) with those obtained for direct injections of standards containing an equivalent amount of drug, after off-line derivatization.

In all cases, each sample was assayed in triplicate.

2.7. Urine samples

Untreated urine samples were spiked with the analytes, reproducing concentrations in the 0.5–10.0 μg/ml range. Volumes of 1 ml of these samples were placed into glass injection vials, and 15-μl samples were injected onto the chromatographic system. Each sample was assayed in triplicate.

3. Results and discussion

3.1. Chromatographic separation of amine derivatives

An acetonitrile–water mixture in gradient elution mode was selected, in the analytical column, for the separation of the derivatives formed [15]. Different gradient profiles and flow-rates were tested to achieve peak shapes and resolution that were comparable to those obtained for direct injection of the off-line derivatized samples. Under all conditions tested, amphetamine and phenylpropylamine were partially overlapped. In order to improve the resolution, we tested a longer (250×4 mm I.D. Hypersil, 5 μm, ODS C_{18}) column, but the analysis time was unacceptably high. Best separation in a reasonable analysis time was achieved with the elution program summarized in Table 1.

In Fig. 3 the chromatograms obtained under the selected conditions for blank (water) and standard mixtures of amphetamines are shown. This figure also shows the chromatogram obtained for the tested mixture after off-line derivatization. In all instances, different side-products were observed. The peak at 6.1 min most probably corresponds to the excess of reagent and/or the hydrolyzed FMOC [9,17,18].

Table 1
Time schedule and conditions used in the determination of amphetamine and related compounds in urine

Valve position	Cumulative time (min)	Trapping column	Analytical column
A	-	Conditioning: 1 ml of water	Conditioning: 40:60 (v/v) acetonitrile–water (at a flow-rate of 1.0 ml/min)
	0	Sample injection: 15 μl	
	0–2.8	Sample clean-up[a]: 1 ml of water (at a flow-rate of 0.35 ml/min) Dilution of the FMOC solution: 5 μl of 20 m*M* FMOC + 45 μl of water Injection of the FMOC solution Injection of the buffer: 10 μl	
B	2.8	Transfer of the derivatives	
	2.8–22.8	Analytical separation — 40:60 (v/v) acetonitrile–water at 2.8 min — 70:30 (v/v) acetonitrile–water at 17.8 min — 100% acetonitrile at 22.8 min	
A	25	End	

[a] Clean-up is performed during dilution and injection of the FMOC and injection of the buffer steps.

Although this peak is very intense due to the relatively high amount of FMOC used, it eluted always at retention times lower than those observed for the analytes. Therefore, under the present conditions, elimination of excess reagent was not necessary. Another peak was observed in all chromatograms at 11.3 min, although the intensity of this peak in the off-line approach was much lower. This peak could also be due to a degraded or to a condensated form of FMOC [9]. An additional peak was observed at 14.2 min, when the reaction was carried out in the trapping column. Since this latter peak was observed even after processing water, it is most probably due to the presence of an impurity in the precolumn packing or to the degradation of the packing produced by the buffer solution [15]. Nevertheless, under the proposed gradient elution, no interferences with interesting peaks were observed.

3.2. Derivatization conditions

3.2.1. Reaction time

The reaction between FMOC and both primary and secondary amino groups is very fast, and the complete reaction is observed within a few min, at basic pH [9,16–18]. We observed that for a molar ratio of FMOC to amine that was higher than 100, increasing the reaction time from 0 to 5 min causes a slight improvement in the responses of the analytes. This means that losses in sensitivity when the derivatives are transferred to the analytical column immediately after injection of the derivatizing solution can be considered negligible. In our case, a delay of 0.1–0.3 min, (depending on the flow in pump 1), was found to be necessary for the reagent to reach the trapping column.

3.2.2. Derivatization reagent

Indeed, the responses can also be increased by increasing the amount of reagent injected. However, there are two major problems when increasing the excess of FMOC. First, peak responses for undesirable peaks increase as the amount of FMOC is increased; moreover, significant band-broadening was observed for a large excess of FMOC. As a consequence, severe overlapping between the side-product eluted at 11.3 min (see Fig. 3) and norephedrine and ephedrine occurred. Secondly, FMOC exhibits a low solubility in water. Although acetonitrile has been reported to be an excellent medium for FMOC derivatizations [10], responses much lower than expected were observed for norephedrine and ephedrine (and to a lesser degree for pseudoephedrine) when solutions of FMOC were prepared in acetonitrile. This can be explained by partial elution of interesting compounds produced when the acetonitrile is flushed through the trapping column, even for FMOC volumes as small as 15 μl. This is in agreement with the fact that the effect is more important for norephedrine, ephedrine and pseudo-

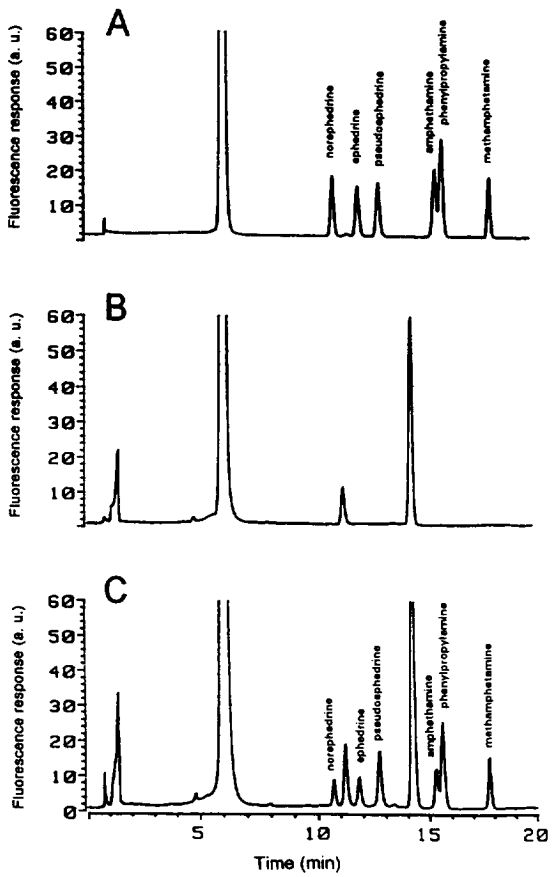


Fig. 3. Chromatograms of an aqueous mixture of the amphetamines derivatized off-line (A); a blank (water) sample derivatized on-line (B) and an aqueous mixture of the amphetamines derivatized on-line (C). (Note that the retention times given are from the rotation of the switching valve). Concentration of analytes, 5 μg/ml. For experimental details, see Section 2. a.u.= arbitrary units.

ephedrine, which are the most polar analytes, due to the presence of the –OH group in their chemical structure (Fig. 1).

In order to reduce losses of the analytes, we tested different acetonitrile–water mixtures for preparation of the derivatizing reagent. Suitable responses for all analytes were observed for 2 m*M* FMOC [dissolved in acetonitrile–water (10:90, v/v)], when the reagent was freshly prepared. However, significant reductions in responses were observed for successive injections. For example, reductions of about 22% in peak areas obtained for pseudoephedrine were observed 8 h after reagent preparation. Consequently, for the final procedure, a 20 m*M* FMOC solution was prepared in acetonitrile and diluted on-line with water before each injection. This step was automatically performed by the autosampler. The best sensitivity for most analytes was achieved by diluting 5 μl of 20 m*M* FMOC with 45 μl of water.

Under such conditions, the molar ratio of FMOC to analyte was in the 2200–100 range for concentrations of analyte in the 0.5–10 μg/ml range. We observed linear responses for aqueous solutions of the analytes in the tested concentration range. The responses obtained for each analyte were also independent (within experimental variations) of the presence of other amines, which means that different compounds can be simultaneously processed.

3.2.3. Buffer solution

Initially, we tested carbonate buffers at different pHs between 9.5 and 10.3, but no significant differences in the chromatograms were observed. Peaks corresponding to side-products were also observed when a borate buffer was used instead of a carbonate buffer. In previous studies [15], the derivatization reagent and the buffer were mixed before injection in the trapping column. We have seen that for a given volume of FMOC, the peaks corresponding to side-products were less intense when the FMOC and the buffer solutions were injected in separated steps, which suggests that FMOC is rapidly degraded in the presence of the buffer.

The influence of the volume of carbonate buffer on the responses was studied for volumes in the 5–25 μl range. We observed that, in order to minimize peaks of side-products, the volume of buffer should be as low as possible. As an example, Fig. 4 shows the chromatograms obtained for norephedrine when using volumes of buffer of 10 μl (A) and 25 μl (B). As can be seen from this figure, there is no significant difference in the responses obtained for norephedrine, but peaks at 11.3 and 14.2 min increase as the volume of buffer is increased. A 10-μl volume of buffer yielded satisfactory derivatization rates, with minimum peak intensity for side-products (Fig. 4C).

3.3. Recovery

Peak areas of the analytes, when using different

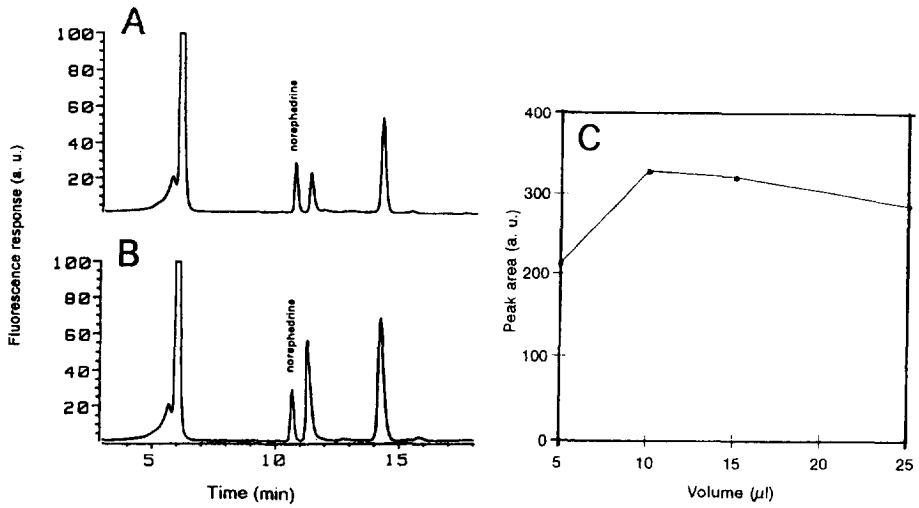


Fig. 4. Chromatograms obtained for norephedrine when using volumes of carbonate buffer, pH 10, of 10 μl (A) and 25 μl (B). The figure also shows the effect of the volume of buffer on peak areas obtained for norephedrine (C). (The retention times given are from the rotation of the switching valve). Concentration of norephedrine = 10 μg/ml. For experimental details, see Section 2. a.u.=arbitrary units.

volumes of water for flushing (V_f) the precolumn, are shown in Fig. 5. As can be seen from this figure, retention is virtually independent for most analytes for V_f in the 0–2.0 ml range, except for norephedrine and ephedrine which are less retained. In order to obtain suitable selectivity and sensitivity, the precolumn was flushed with 1 ml of water (flow-rate in pump 1 was set at 0.35 ml/min). In this way, retention for most analytes is almost complete, with the exception of norephedrine, as can be seen in Table 2. This table also shows a comparison between the peak areas obtained under the selected conditions and those obtained when samples were derivatized off-line (for equivalent reagent and analyte concentrations). It can be seen from this table that derivatizations in the precolumn are less efficient than the homologous solution derivatizations. The percentages of drugs transformed in the precolumn

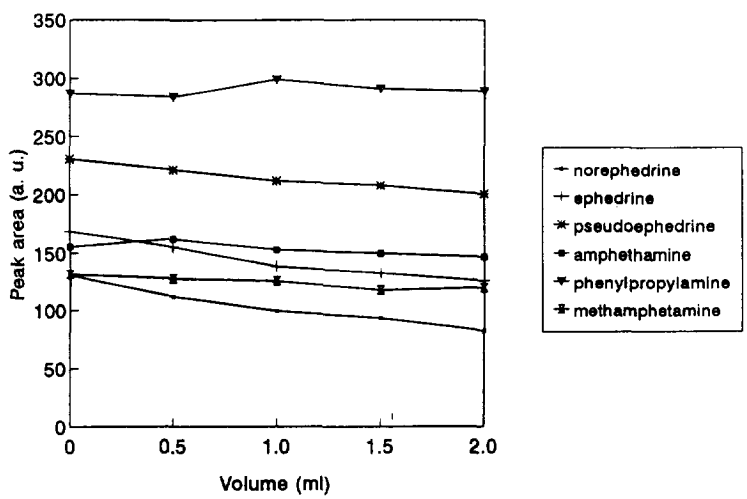


Fig. 5. Effect of the volume of flushing eluent (used for cleaning of the samples) on peak areas. Concentration of analyte = 5 μg/ml (in water). For experimental details, see Section 2. a.u.=arbitrary units.

are higher than 65%, except for norephedrine (which is about 50%). However, this low percentage is also due to the low retention of the analyte on the trapping column.

No significant differences in reaction rates for primary and secondary amines are observed, which is most probably due to the fact that the reaction between FMOC and the analytes is very rapid, and also due to the large excess of reagent used. It should be noted that values in Table 2 compare the reaction rates obtained under the described conditions with those obtained when the reaction was performed off-line on equivalent quantities of FMOC and amines (which would also not react 100%).

The final recommended procedure for derivatization of amphetamine-related compounds is summarized in Table 1. As an example, typical chromatograms obtained for blank urine and urine spiked with a mixture of amphetamines are shown in Fig. 6. As can be seen from this figure, the selectivity is fully satisfactory, since endogenous urinary compounds are eluted at retention times lower than those of the analytes.

3.4. Analysis of urine samples

On the basis of the above studies, the determination of interesting compounds in urine, at their therapeutical levels, was performed. In Table 3, the relevant analytical data obtained with the procedure described in Section 2 are shown. As can be seen in this table, interesting compounds can be determined

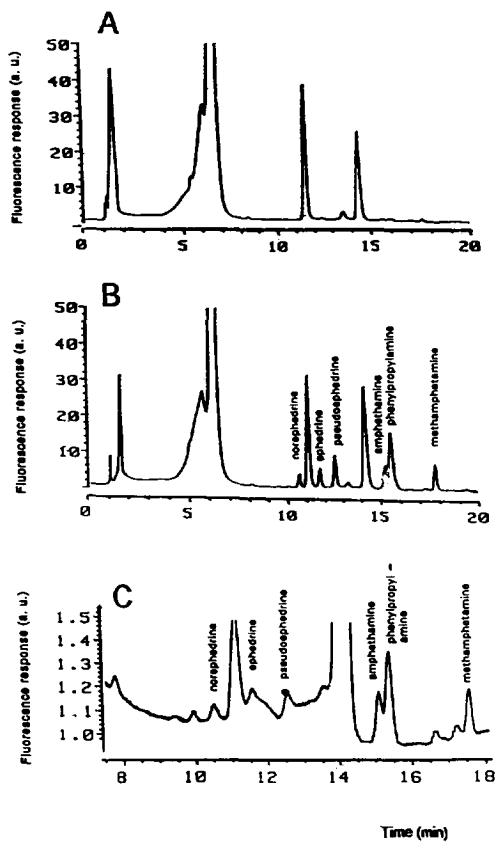


Fig. 6. Chromatograms obtained for blank urine (A) and urine spiked with a mixture of the tested compounds at concentrations of 2.5 μg/ml (B) and 0.05 μg/ml (C). (The retention times given are from the rotation of the switching valve). For experimental details, see Section 2. a.u.=arbitrary units.

Table 2
Comparison between the analytical responses obtained for amphetamines in water when flushing the precolumn with 1.0 and 0 ml of water ($n=3$)

Compound	$\frac{\text{Peak areas at } V_f = 1.0 \text{ ml}}{\text{Peak areas at } V_f = 0 \text{ ml}} \times 100$	$\frac{\text{Peak areas off-line derivatization}}{\text{Peak areas on-line derivatization}} \times 100$
Norephedrine	76±2	51±6
Ephedrine	88±6	88±7
Pseudoephedrine	92±2	87±8
Amphetamine	98±6	77±7
Phenylpropylamine	104±4	66±4
Methamphetamine	97±4	79±6

The table also shows the responses obtained under the off-line and the on-line (V_f=1 ml/min) approaches. Analyte concentration = 5 μg/ml. For experimental details, see Section 2.

Table 3
Analytical data for the determination of amphetamine and related compounds in urine

Compound	Recovery[a] (n=3) (%)	Linearity			Intra-day precision[a] (n=6) (%)	Inter-day precision[a] (n=15) (%)	Limit of detection (ng/ml)
		$y=a+bx$	S_{xy}	n			
Norephedrine	55 ±5	$a=-1.6\pm1.3$ $b=16.6\pm0.2$	3.1	14	3	9	25
Ephedrine	91 ±3	$a=5.9\pm2.8$ $b=12.1\pm0.6$	6.3	15	6	7	25
Pseudoephedrine	95.7±0.8	$a=2.5\pm2.2$ $b=14.5\pm0.4$	4.9	15	7	7	10
Amphetamine	75 ±3	$a=6.0\pm3.7$ $b=18.8\pm0.7$	7.9	15	3	9	10
3-Phenylpropylamine	68 ±5	$a=4.5\pm5.6$ $b=34.5\pm0.39$	12.8	15	2	10	5
Methamphetamine	83 ±5	$a=10.1\pm1.0$ $b=12.5\pm0.4$	4.8	15	3	8	10

For experimental details, see Section 2.
[a]Determined at half of the highest concentration in the tested range.

with satisfactory linearity in the 0.5–10.0 μg/ml range, thus covering the therapeutical interval [19]. Most of the previous assays proposed for the off-line derivatization of amphetamines in biological fluids reported relative standard deviations in the 4–10% range [4,7,13]. Reproducibilities reported for on-line derivatization of amphetamines in water, using FMOC in solution, are in the 5–14% interval [9], whereas the on-line derivatization of amphetamine in urine using FMOC in a solid-phase form provided a relative standard deviation of about 10% [10]. Therefore, the reproducibility obtained under the present assay conditions is comparable to that of most HPLC assays (see Table 3).

In contrast, methods using off-line solution derivatizations reported limits of detection in the low ng/ml range [19]. However, these methods usually involved liquid–liquid extraction, effecting re-concentration of the derivatives. On-line procedures seem to be less sensitive, and the limits of detection are in the 0.02–1.0 μg/ml range [8,11]. With the described assay, the limits of detection (for a signal-to-noise ratio of three) are in the 5–25 ng/ml range and thus can be considered acceptable. Better sensitivity can be achieved for some amphetamines by injecting larger volumes of samples, especially for amphetamines which exhibit a good retention on the precolumn [15]. However, injection of larger sample volumes may require longer flushing stages to obtain satisfactory selectivity, which would result in losses of the more polar analytes. Larger injection volumes may also reduce the operative life of the precolumn.

In the present assay, untreated urine was injected directly into the system, but effective regeneration of the trapping column was achieved in a few min by flushing with 100% acetonitrile. This means that several samples could be used without replacing the precolumn. However, in order to ensure a satisfactory analytical column lifetime, we replaced the trapping column every 40–50 injections.

4. Conclusions

This study illustrates the potential of conventional ODS precolumns for the on-line sample clean-up and derivatization of amphetamine and related compounds in the analysis of biological samples prior to LC analysis. With the described approach, analyte conversions are about 55–96% of those obtained by the off-line derivatization mode under similar conditions, and the sensitivity can be considered satisfactory for most applications. The total analysis takes about 28 min, the limiting step being the time required for chromatographic analysis.

The main advantages of the described procedure over previously reported HPLC methods are that off-line steps for sample conditioning, re-concen-

tration or elimination of the unreacted reagent excess are not involved, and the whole procedure can be fully automated. In addition, the assay is very simple, since only conventional ODS columns are required, and FMOC is used as a solution (thus avoiding laborious synthesis of FMOC-tagged solid-phase reagents). The major limitation of the described procedure is that it can only be applied to the determination of primary and secondary amines. Thus, metabolites with a tertiary amine structure cannot be determined by the proposed conditions.

Acknowledgments

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