Derivatization of tertiary amphetamines with 9-fluorenylmethyl chloroformate for liquid chromatography: determination of *N*-methylephedrine

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The fluorogenic reagent 9-fluorenylmethyl chloroformate (FMOC) was evaluated for the derivatization of tertiary amphetamines prior to liquid chromatographic analysis. Conditions for the derivatization were investigated, including the reaction time, the derivatization reagent concentration and the pH, using *N*-methylephedrine as a model compound. On the basis of these studies, a method for the quantification of *N*-methylephedrine is presented. The method involves derivatization with FMOC at ambient temperature and separation of the derivatives formed on a LiChrospher C_{18}, 5 μm, 125 × 4 mm id column using acetonitrile–water gradient elution. The proposed procedure shows good linearity, accuracy and reproducibility in the 1.0–25.0 μg mL^{-1} concentration range. The limit of detection was 0.1 μg mL^{-1} and the limit of quantification was 0.5 μg mL^{-1}. The utility of the assay was demonstrated by determining *N*-methylephedrine in water and urine samples.

Introduction

The rapid and sensitive determination of amphetamine and related compounds is an area of growing interest because these substances have become popular drugs of abuse. Different analytical techniques have been used for this purpose, including immunoassays, gas chromatography (GC), liquid chromatography (LC) and more recently capillary electrophoresis (CE).[1, 2] GC coupled with mass spectrometry (MS) seems to be the most reliable technique for the identification and determination of amphetamines in biological fluids, as it provides high levels of specificity and sensitivity. LC is also well suited for the determination of amphetamines in a variety of samples, but its application is limited by the poor sensitivity achieved with most LC detectors, particularly when analysing biological samples. For this reason, most LC methods incorporate a chemical reaction to improve sensitivity for UV, fluorescence or chemiluminescence detection. In this sense, numerous reagents have been proposed, including *o*-phthaldialdehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC), 3,5-dinitrobenzoyl chloride, dansyl chloride and 1,2-naphthoquinone-4-sulfonate.[2, 3] Although these reagents allow the determination of primary and secondary amphetamines at μg mL^{-1} levels, no satisfactory reagents are known for tertiary amphetamines, and only a few procedures for the derivatization of these compounds have been reported so far.[4]

Traditionally, the determination of tertiary amphetamines in biological samples by LC is based on the liquid–liquid extraction of the analytes from very large sample volumes and subsequent evaporation of the extraction solvent. These methods are time consuming and prone to errors, as there is a risk of losing the analytes during the evaporation step because of their volatility. We have recently reported a method for the on-line determination of primary, secondary and tertiary amphetamines based on the enrichment of the amphetamines in a trapping column prior to their chromatographic separation and UV detection at 210 nm.[5] The described procedure was very simple and rapid, as no sample manipulation was involved. However, although the method was suitable for many applications concerning the determination of amphetamines in biological fluids, the sensitivity for some of the amphetamines tested was

relatively low. In addition, some difficulties derived from the utilization at such low wavelengths were encountered, especially the allocation of the baseline for integration.

Among the numerous reagents that have been proposed, FMOC appears to be one of the most attractive for the determination of primary and secondary amphetamines, as the reactions proceed rapidly under mild conditions and the derivatives formed are highly fluorescent. Moreover, the stability of the FMOC derivatives is better than that obtained with other derivatizing agents typically used for amphetamines, such as OPA. Successful results have also been reported with other chloroformates such as (+)-1-(9-fluorenyl)ethyl chloroformate and *N*-9-fluorenylmethyl chloroformate–L-proline either in solution or as solid reagents.[6–11] These reagents have proved to be useful for the separation of the enantiomers of primary and secondary amphetamines, as the diastereomers formed can be separated in conventional (achiral) columns. Another chloroformate, 2-naphthyl chloroformate, was used by Gübitz and co-workers to derivatize some tertiary amines.[4] According to the workers, tertiary amines undergo dealkylation when heated with 2-naphthyl chloroformate, forming the corresponding carbamates with the resulting secondary amines. The method was successfully applied to the determination of some tertiary antihistamines. However, to our knowledge, this approach has not been used for the determination of tertiary amphetamines, most probably because the reagent is not commercially available, and also because the conditions required to obtain satisfactory conversion yields (heating for 1 h at 100 ºC) make this reaction unsuitable for routine analysis.

In this work, we evaluated the possibility of derivatizing tertiary amphetamines with FMOC prior to LC. For this purpose, the tertiary ampethamine *N*-methylephedrine (Fig. 1) was selected as a model compound. This substance, as many other ephedrines, is an ingredient in some pharmaceutical
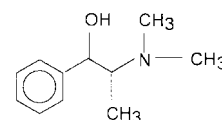
Fig. 1 Structure of *N*-methylephedrine.

preparations. Therefore, the determination of *N*-methylephedrine is often required in pharmaceuticals and also in biological fluids (*e.g.*, in doping control tests). The reliability of the described method was evaluated in terms of linearity, reproducibility, accuracy and sensitivity. As an example, the proposed procedure was applied to measure *N*-methylephedrine in water and urine. The possibility of using another chloroformate for the derivatization of the analyte, and the utility of the FMOC approach in the derivatization of other tertiary amphetamines, were also investigated.

Experimental

Apparatus

The chromatographic system consisted of a quaternary pump (1050 Series, Hewlett-Packard, Palo Alto, CA, USA), an automatic sample injector (Hewlett-Packard, 1050 Series) with a sample loop injector of 20 µL and a fluorescence detector (Hewlett-Packard, 1046 series) operated at 264 nm for excitation and 313 nm for emission. The detector was linked to a data system (Hewlett-Packard HPLC ChemStation) for data acquisition and storage. All the assays were carried out at ambient temperature.

Reagents

All the reagents were of analytical-reagent grade unless indicated otherwise. Acetonitrile and methanol of HPLC grade were purchased from Scharlau (Barcelona, Spain). *N*-Methylephedrine and *N*-methylpseudoephedrine were obtained from Sigma (St. Louis, MO, USA). 9-Fluorenylmethyl chloroformate, *N*-9-fluorenylmethyl chloroformate–L-proline and dicyclohexylcarbodiimide were obtained from Aldrich (Steinheim, Germany). Sodium hydroxide (Panreac, Barcelona, Spain) and sodium hydrogencarbonate (Probus, Badalona, Spain) were also used.

Preparation of solutions

Stock standard solutions of *N*-methylephedrine and *N*-methylpseudoephedrine (1000 µg mL^{-1}) were prepared in water. Working standard solutions of the amines were prepared by dilution of the stock standard solutions with water. Water was distilled, de-ionized and filtered through 0.45 µm nylon membranes (Teknokroma, Barcelona, Spain). All solutions were stored in the dark at 2 °C. FMOC solutions were prepared daily by dissolving the pure compound in acetonitrile. A 0.01 M hydrogencarbonate buffer was prepared by dissolving the appropriate amount of sodium hydrogencarbonate in water and adjusting the pH to the required value with 10% w/v sodium hydroxide solution.

Columns and mobile-phases

A LiChrospher 100 RP$_{18}$, 5 µm, 125 × 4 mm id column (Merck, Darmstadt, Germany) was used for separation of the derivatives formed. The mobile phase was acetonitrile–water. The chromatographic conditions used are summarized in Table 1. All solvents were filtered through 0.45 µm nylon membranes (Teknokroma) and de-gassed with helium before use.

Derivatization procedure

Derivatization with FMOC were carried out as follows (see Table 1): 0.125 mL of the samples and 0.125 mL of the

carbonate buffer were placed in 2 mL glass vials, then 0.250 mL of the reagent was added and the resulting mixture was left to react for a defined period of time. Finally, aliquots of 20 µL of the reaction solution were injected into the chromatographic system. Derivatizations were performed at ambient temperature and each sample was assayed in triplicate.

Analysis of urine samples

Untreated urine samples were spiked with the amphetamines to give concentrations in the range 1.0–25.0 µg mL^{-1}. According to previous studies, C$_{18}$ solid-phase extraction cartridges (Bond Elut, 100 mg mL^{-1}; Varian, Harbor City, CA, USA) were employed for sample clean-up.[12, 13] Sample conditioning was carried out as follows: the cartridges were conditioned by drawing through them 1 mL of methanol followed by 1 mL of water; next, 0.25 mL of the samples were drawn through the cartridges and endogenous compounds were flushed out with 2 mL of water. The cartridges were then dried with air. The analyte was subsequently eluted from the cartridges with 0.25 mL of acetonitrile. The extracts were subjected to the derivatization procedure described previously (see Table 1), but the volumes of buffer and reagent used were 0.25 and 0.5 mL, respectively. Finally, 20 µL aliquots of the extracts were injected into the chromatograph. Each sample was assayed in triplicate.

Results and discussion

Optimization of the derivatization procedure

Previous experiments under a variety of conditions showed that derivatization of *N*-methylephedrine with FMOC led to two main products of reaction. Although in the present work the mechanism of the reaction was not investigated, the results obtained are in agreement with the reaction scheme proposed by Gübitz and co-workers,[4] which is shown in Fig. 2. In their scheme, the reaction between a tertiary amine and a chloroformate may lead to different carbamates depending on the group split off. For *N*-methylephedrine, dealkylation can affect only two groups (see Fig. 1); consequently, two products of reaction can be expected in the derivatization of this compound.

Table 1 Conditions used for derivatization of *N*-methylephedrine

Step	Conditions
Derivatization procedure	0.125 mL of sample + 0.125 mL of buffer (10 mM carbonate at pH 9.5) + 0.250 mL of FMOC (15 mM) Time of reaction: 15 min Temperature: ambient
Chromatographic separation	Gradient elution: 100% water at 0–5 min 100% acetonitrile at 15–20 min Flow rate: 1.0 mL min^{-1}
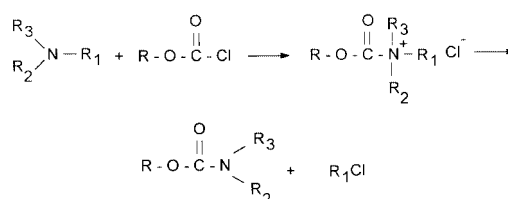
Fig. 2 Scheme of the reaction of chloroformates with tertiary amphetamines.

Fig. 3 shows a typical chromatogram obtained for *N*-methylephedrine after reaction with FMOC. As can be seen, the response obtained for the first eluting derivative is higher than that observed for the last eluting derivative. The same behaviour was also observed under a wide variety of derivatization conditions. For this reason, the elution conditions were selected in order to provide satisfactory resolution of the first eluting derivative in the minimum time of analysis. The best results were obtained by increasing the acetonitrile content from 0% at 0–5 min to 100% at 15 min, at a mobile phase flow rate of 1.0 mL min^{-1}; from 15 to 20 min, the mobile phase was acetonitrile. Under such conditions partial co-elution between the last eluting derivative (retention time 17.2 min) and peaks corresponding to unreacted FMOC was observed. However, the peak selected for quantification of *N*-methylephedrine (retention time 11.2 min) was completely resolved from possible interfering peaks (see Fig. 4).

The effect of the experimental conditions affecting the derivatization process was investigated using *N*-methylephedrine at a concentration of 25.0 μg mL^{-1}, the highest concentration assayed. First, the effect of the reaction time was examined by allowing the derivatization to proceed for times ranging from 1 to 50 min; in this study the concentration of reagent was 20 mM and the pH of the buffer was 10.5. The results are depicted in Fig. 5(a). As can be observed, the analytical signals increased as the time of reaction increased within the tested interval. In contrast, in previous studies we found that reaction between FMOC and primary and secondary amphetamines under comparable conditions reached a maximum in only 2–10 min.[10] This suggests that the reaction with

the tertiary amine *N*-methylephedrine proceeds more slowly than reaction with primary and secondary amphetamines, which may be caused by steric hindrance.[6] The calculated rate constant was about six times lower than that observed for amphetamine. As a compromise between sensitivity and time of analysis, a time of reaction of 15 min was selected for further experiments.

The influence of the concentration of FMOC in the reaction yields was examined in the range 5–20 mM. The other experimental conditions were as indicated above. As shown in Fig. 5(b), the analytical responses increased with increasing FMOC concentration up to 15 mM. A further increment did not significantly improve the analytical signal. Consequently, 15 mM was the concentration selected for subsequent experiments.

Another parameter affecting the derivatizations with FMOC is the pH. For this reason, the effect of the pH of the buffer was examined within the range 7.5–12.0 (for a reaction time of 15 min and using 15 mM FMOC). As shown in Fig. 5(c), the best results were obtained at pH 10.5. Higher pHs resulted in a decrease in the reaction yield, most probably due to the hydrolysis of the reagent.[11] Since the responses obtained at pH 9.5 were slightly lower than those obtained at pH 10.5, and in order to prevent hydrolysis, a pH of 9.5 was selected as the best option for the derivatization of *N*-methylephedrine.

It should be noted that the reaction yields could also be improved by increasing the temperature of the reaction. However, different studies demonstrated that FMOC and related derivatives exhibit limited stability at high temperature.[6] For this reason, in the present study ambient temperature was preferred for derivatization.

On the basis of the above results, the conditions finally selected for the determination of *N*-methylephedrine are those summarized in Table 1. The proposed conditions were applied
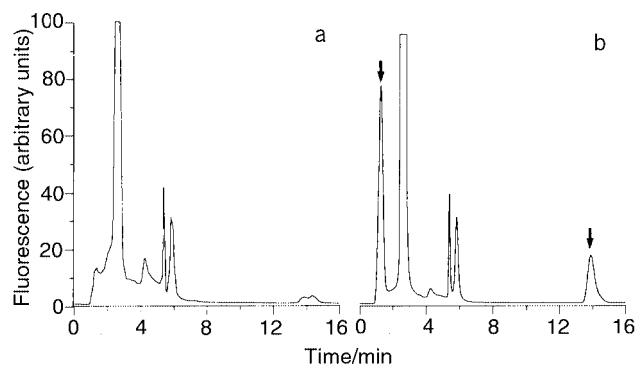
Fig. 3 Chromatograms obtained for (a) a blank (water) and (b) a solution of *N*-methylephedrine (25.0 μg mL^{-1}) derivatized with FMOC; the arrows indicate the peaks corresponding to the analyte. Concentration of FMOC, 20 mM; time of reaction, 25 min; pH of the buffer, 10.5; elution conditions, acetonitrile–water (1 + 1 v/v) at a flow rate of 1 mL min^{-1}. For other experimental details, see text.
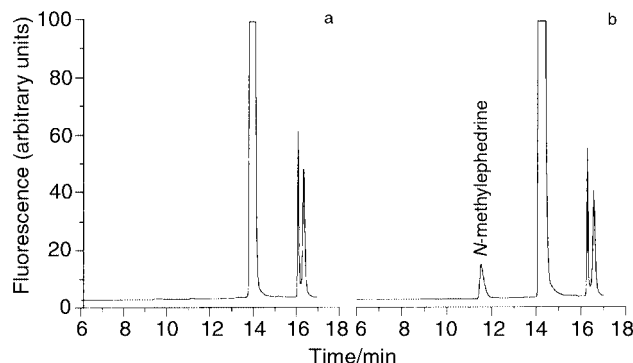
Fig. 4 Chromatograms obtained for (a) a blank (water) and (b) a solution of *N*-methylephedrine (5.0 μg mL^{-1}) under conditions summarized in Table 1.
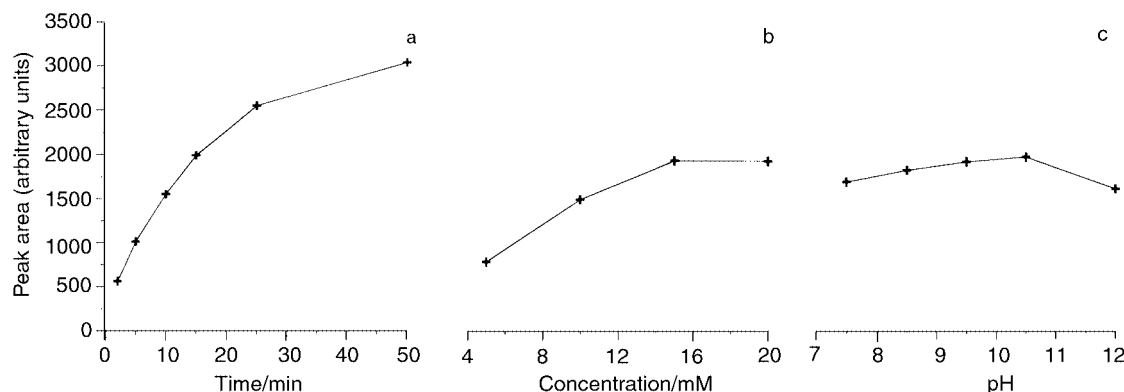
Fig. 5 Effect of experimental conditions in the derivatization of *N*-methylephedrine (25.0 μg mL^{-1}). (a) Effect of the time of reaction; concentration of FMOC, 20 mM; pH of the buffer, 10.5. (b) Effect of the concentration of FMOC; time of reaction, 15 min; pH of the buffer, 10.5. (c) Effect of the pH of the buffer; time of reaction, 15 min; concentration of FMOC, 15 mM. For other experimental details, see text.

to the derivatization of aqueous solutions containing *N*-methylephedrine in the range 1.0–25.0 µg mL^{-1}. For these concentrations the molar concentration ratio of FMOC to analyte was approximately within the range 300–7200. The values are clearly higher than those required to derivatize primary and secondary amphetamines under analogous conditions.[10,11] For such amphetamines, nearly quantitative conversions can be achieved with molar ratios of about 100–2000. As has been suggested earlier, this behaviour can be explained by the steric difficulties in the reaction with the tertiary amino group.

Determination of *N*-methylephedrine in water

Linearity, reproducibility and accuracy studies. The reliability of the described method was tested by analysing aqueous solutions containing *N*-methylephedrine in the range 1.0–25.0 µg mL^{-1}. The linearity was evaluated by plotting the peak area against the concentration of *N*-methylephedrine. The results are summarized in Table 2. As can be seen, derivatization under the proposed conditions resulted in a linear dependence of *N*-methylephedrine responses on concentration.

The reproducibility was tested at different concentrations through the consecutive injection of aliquots of the samples. The results obtained are also given in Table 2 and demonstrate that the proposed procedure provides satisfactory reproducibility, thus making the addition of an internal standard unnecessary. The intra- and inter-day RSDs ranged from 3 to 9% and from 9 to 13%, respectively; these values are comparable to those reported for other LC methods proposed for *N*-methylephedrine.[14,15]

The accuracy of the method was established by calculating the relative errors obtained in the quantification of aqueous solutions containing different concentrations of the analytes within the tested concentration range. The results are given in Table 3. The method provides concentrations close to the real values, with relative errors ranging from −5 to +2%.

Limit of detection and limit of quantification. The limit of detection (LOD), established for a signal-to-noise ratio of 3, was 0.10 µg mL^{-1}. Although with the present procedure quantitative conversion of the analyte is not achieved (as derivatization gives different products), the sensitivity can be considered satisfactory, and the LOD obtained is comparable to (and sometimes lower than) those reported for other LC methods.[5,14,15] However, the minimum detectable amount of *N*-methylephedrine by the present procedure is clearly lower than that reported for the other methods, which indicates that the derivatization with FMOC under the proposed conditions significantly improved the sensitivity. Based on the literature, we can conclude that the present method is sensitive enough for most applications concerning the detection and quantification of *N*-methylephedrine at therapeutic levels and also in doping control tests.[14,15] On the other hand, the FMOC approach offers better sensitivity in the determination of primary and secondary amphetamines, which is in agreement with the results reported above.[11] The limit of quantification (LOQ), established for a signal-to-noise ratio of 10, was 0.5 µg mL^{-1}.

Selectivity. The proposed method was also applied to the derivatization of other amphetamines in order to establish possible interferences. The compounds examined were 3-phenylpropylamine (retention time t_r = 14.08 min), norephedrine (t_r = 15.29 min), ephedrine (t_r = 15.69 min), pseudoephedrine (t_r = 15.84 min), ß-phenylethylamine (t_r = 16.11 min), amphetamine (t_r = 16.44 min) and methamphetamine (t_r = 17.06 min). Therefore, no interferences with *N*-methylephedrine were observed.

Application to urine samples

The applicability of the method was evaluated by determining *N*-methylephedrine in urine. For sample clean-up, C$_{18}$ solid-phase extraction cartridges were used. Preliminary assays demonstrated that the proportion of drug recovered under the described conditions was 103 ± 7% (*n* = 3), which is in concordance with previous results.[5,12,13] The volume of sample and the volume of methanol used to desorb the purified analyte from the cartridges were the same, so the concentration of the analyte in the samples and in the collected extracts was approximatedly the same. For this reason, and since 0.125 mL of methanol provided unsatisfactory reproducibility, the volume of sample was 0.250 mL.

As an example, in Fig. 6 are shown the chromatograms obtained for blank urine and urine spiked with *N*-methylephedrine at a concentration of 5.0 µg mL^{-1}. As can be observed, the sample treatment provided satisfactory selectivity. It is interesting that methods based on the measurement of underivatized *N*-methylephedrine in biological samples involve UV detection at low wavelengths (< 215 nm). In such methods, and in order to achieve suitable selectivity, very efficient sample clean-up is required, *e.g.*, by multiple liquid–liquid extraction.[15] In the present method, a very simple solid-phase extraction procedure provides excellent selectivity. In addition, problems derived from baseline distortions are avoided.[5] On the other hand, the LOD found for this kind of sample was the same found for aqueous solutions.

The practicability of the proposed method for the determination of *N*-methylephedrine in urine was evaluated by analysing

Table 3 Accuracy for the determination of *N*-methylephedrine in water and urine (*n* = 3)

| Sample	Concentration added/ µg mL^{-1}	Concentration determined/ µg mL^{-1}	Error (%)
Water	2.5	2.27 ± 0.13	−5
	5.0	4.8 ± 0.4	−4
	10.0	10.2 ± 0.5	2
Urine	50	5.3 ± 0.3	6
	15.0	16 ± 1	7

Table 2 Linearity and reproducibility for the determination of N-methylephedrine

Linearity (*n* = 15)		Reproducibility: RSD (%)					
		Intra-day precision[b]			Inter-day precision[c]		
y = *ax* + *b*	$t_{calculated}$[a]	2.5 µg mL^{-1}	10.0 µg mL^{-1}	15.0 µg mL^{-1}	2.5 µg mL^{-1}	10.0 µg mL^{-1}	15.0 µg mL^{-1}
a ± S_a = 188 ± 8	232	9	3	3	13	9	10
b ± S_b = −120 ± 60							

[a] $t_{calculated}$ = a/S_a; $t_{tabulated}$ = 3.01 for a confidence level of 99 % and 13 degrees of freedom. [b] *n* = 3. [c] *n* = 9.
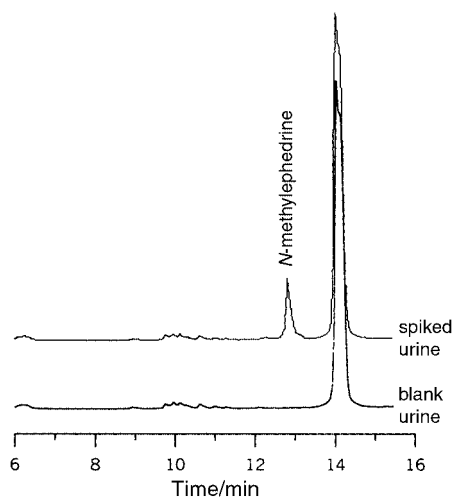
Fig. 6 Chromatograms obtained for blank urine and urine spiked with *N*-methylephedrine (5.0 μg mL^{-1}) under the proposed conditions.
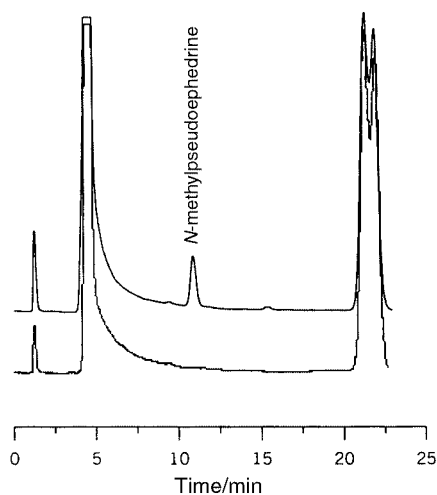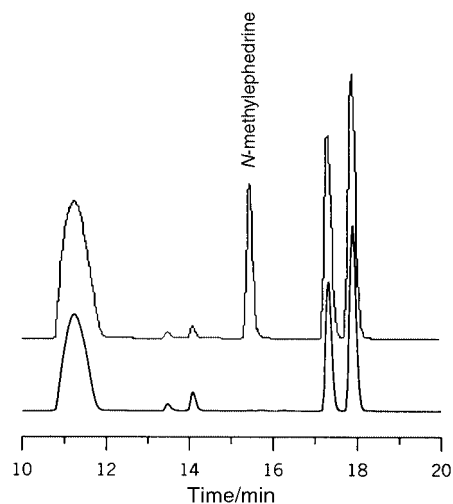

Fig. 7 Chromatograms obtained for (a) a blank (water) and (b) a solution of *N*-methylpseudoephedrine (25.0 μg mL^{-1}) under conditions summarized in Table 1.

urine spiked at concentrations of 5.0 and 15.0 μg mL^{-1}. The concentration of the analyte was calculated from the calibration curves obtained for the aqueous solutions, and taking into account the percentage of analyte recovered after sample clean-up. The levels of *N*-methylephedrine found are given in Table 3. The values found are close to the real values, and the relative errors are of about the same magnitude as those found in the analysis of aqueous solutions. Therefore, the present method can also be applied to the determination of *N*-methylephedrine in urine. Additional experiments with plasma samples demonstrated that adequate selectivity is also achieved when applying the proposed conditions to plasma samples (data not shown).

Derivatization of other tertiary amphetamines

The possibility of applying the proposed method to other tertiary amphetamines was also investigated. For this purpose, the tertiary ephedrine *N*-methylpseudoephedrine was used and Fig. 7 shows the chromatograms obtained. This figure shows a peak corresponding to a derivative of the analyte at a retention time of 11.1 min. Therefore, the FMOC approach can be extended to the derivatization of other tertiary amphetamines.

Fig. 8 Chromatograms obtained for (a) a blank (water) and (b) a solution of *N*-methylephedrine (25.0 μg mL^{-1}) derivatized with FMOC–L-proline. For other experimental details, see text.

Derivatization of *N*-methylephedrine with other chloroformates

Finally, we evaluated the possibility of using another chloroformate to derivatize *N*-methylephedrine. The reagent used was FMOC-L-proline in combination with the coupling agent dicyclohexylcarbodiimide. Derivatization was performed according to a previously described procedure.[16] The chromatographic conditions were the same as used for the FMOC method. As can be deduced from Fig. 8, the analyte also reacted with FMOC-L-proline, giving a peak at 15.3 min. No other peaks were detected at retention times lower than 20 min. Therefore, the derivatization of the analyte with a chiral chloroformate could be used for chiral analysis, through the diastereomers obtained by substitution of one of the methyl groups (see Fig. 1).

Conclusions

This work demonstrates that FMOC and analogous compounds can be used for the determination of tertiary amphetamines under mild conditions. Although the derivatization is not quantitative and proceeds more slowly than reactions with primary and secondary amphetamines under comparable conditions, the FMOC method is more sensitive than other LC assays based on the determination of underivatized *N*-methylephedrine. The proposed procedure provides adequate linearity, reproducibility and accuracy in the quantification of *N*-methylephedrine in aqueous solutions. Moreover, it can be easily adapted to the determination of *N*-methylephedrine in typical biofluids, such as urine, with improved selectivity. The analysis time is about 35 min (40 min for biofluids). Although this value is relatively high, the proposed method is more rapid and simpler than methods involving liquid–liquid extraction and solvent evaporation steps.

The FMOC method can also be extended to the derivatization of other tertiary amphetamines. In the present instance, satisfactory results were obtained for *N*-methylpseudoephedrine.

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