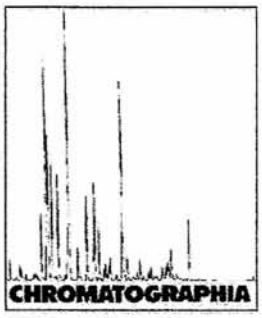
Separation of the Enantiomers of Primary and Secondary Amphetamines by Liquid Chromatography after Derivatization with (−)-1-(9-Fluorenyl)ethyl Chloroformate



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Key Words

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Designer drugs
Amphetamines

Summary

The chiral reagent (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) has been evaluated for the enantioselective analysis of amphetamines by liquid chromatography. For separation of the FLEC diastereomers conventional reversed-phase conditions were used. The conditions affording the best enantiomeric resolution and sensitivity were determined for amphetamine, methamphetamine, ephedrine, pseudoephedrine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), and 3,4-methylenedioxyethylamphetamine (MDE). All the amphetamines assayed could be separated with resolution factors ranging from 0.91 to 1.92. Although FLEC is typically used as a fluorogenic reagent, it was shown that UV detection is the best option for stereospecific analysis of the methylenedioxylated amphetamine derivatives (MDA, MDMA, and MDE), because different fluorimetric responses were obtained for the corresponding diastereomers. The utility of the proposed conditions for stereoselective analysis of amphetamines in different types of sample is discussed.

Introduction

The illicit consumption of amphetamine and related substances is increasing dramatically, especially among young people. The most frequently consumed compounds in this group are amphetamine itself, methamphetamine and ephedrine, and more recently the methylenedioxylated derivatives (also referred to as designer drugs) 3,4-methylenedioxymethamphetamine (MDMA, Adam, or Ecstasy), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxyethylamphetamine (MDE or Eve).

All amphetamines occur as a pair of enantiomers which can differ in their pharmacological activity and their rate of metabolism. Chiral discrimination between the isomers of these compounds is, therefore, important in pharmacokinetic and pharmacodynamic studies. Determination of the individual enantiomers is also very useful for distinguishing between therapeutic and illicit intake of some amphetamines [1]. Enantiomer composition can also aid identification of the synthetic pathways used for clandestine amphetamine preparation [2]. For these reasons, the enantioselective analysis of amphetamines is receiving increasing attention in clinical, toxicological, and pharmaceutical laboratories.

Although different assays based on gas chromatography (GC) [3, 4] and capillary electrophoresis (CE) [5, 6] have been reported, liquid chromatography (LC) has long been recognized as the method of choice for the stereospecific analysis of amphetamines [7]. In this respect derivatization with a homochiral reagent then separation of the diastereomers formed is the option preferred, particularly for analysis of amphetamines at trace levels, because derivatization also improves analyte detectability.

In recent years, several papers have been published on the use of different derivatizing reagents for the analysis of amphetamines by LC [7, 8]. According to these papers derivatization with a chloroformate seems to be one of the most attractive approaches for several reasons. First, chloroformates react with primary and secondary amphetamines very rapidly and under mild conditions [9, 10]. Second, the stability of the derivatives formed is better than that achieved with other reagents commonly used for amines, e.g. *o*-phthaldialdehyde (OPA) [11]. Finally, the derivatives obtained are highly fluorescent, enabling determination of traces of the analytes [9, 12, 13].

In chiral analysis several indirect methods based on the use of homochiral chlor-

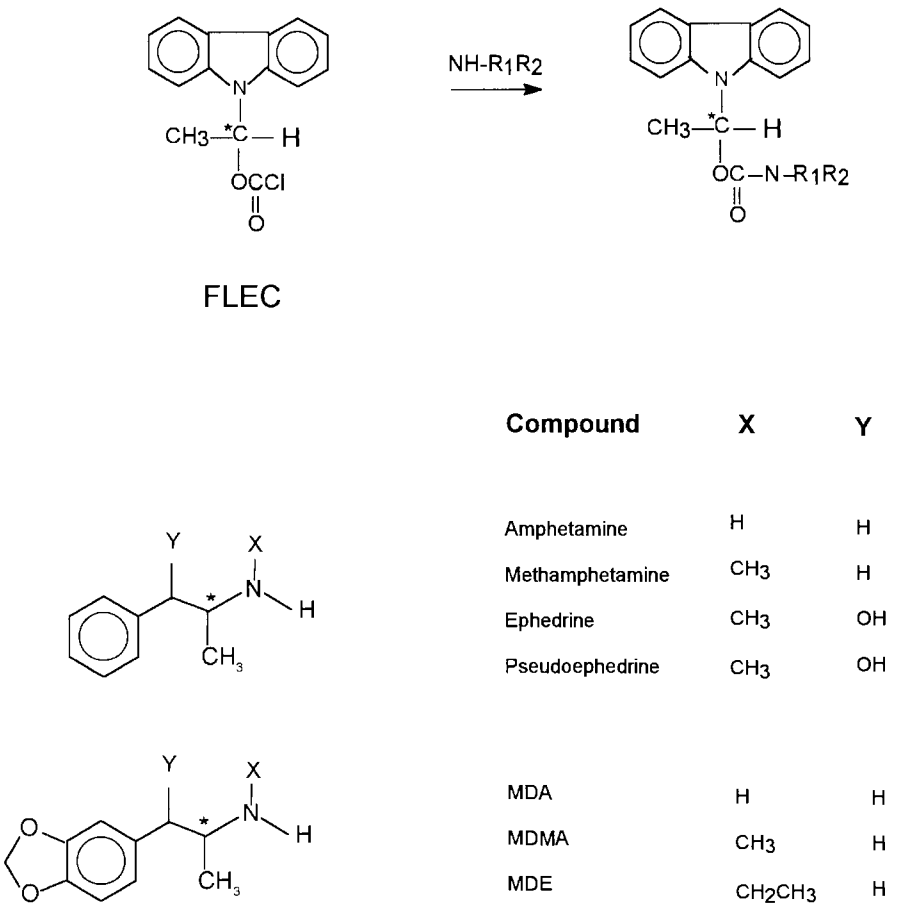

Figure 1. Reaction of amphetamines with FLEC and the chemical structures of the amphetamines investigated (the asterisks denote the chiral centres).

oformates have been reported, specially for analysis of the enantiomers of amino acids [14]. One of the reagents in this group, 9-fluorenylmethyl chloroformate-L-proline (FMOC-L-Pro), has been used to resolve the enantiomers of amphetamine, ephedrine, and pseudoephedrine [15], and as a solid-phase reagent for the derivatization and separation of amphetamine enantiomers [16]. The FMOC-L-Pro diastereomers could always be readily separated, but previous activation of the reagent was necessary. Kinetic resolution was, moreover, observed for pseudoephedrine enantiomers [15].

Another chloroformate extensively used in chiral separations is 1-(9-fluorenyl)ethyl chloroformate (FLEC). The main advantage of FLEC over FMOC-L-Pro is that no previous treatment is needed. Derivatization with FLEC has been successfully used to investigate the stereoselective metabolism of amphetamine [17]. The simultaneous determination of amphetamine and methamphetamine after formation of their FLEC diastereomers has been reported [18]. This reagent has not, however, been applied to the analysis of the enantiomers of many other amphetamines of interest (e.g. the designer drugs).

In this study we have evaluated the possibility of using FLEC as a general reagent for differentiation between the enantiomers of amphetamines by LC. For separation of the FLEC diastereomers conventional reversed-phase conditions were used. The effect of experimental conditions on resolution was investigated for a wide variety of primary and secondary amphetamines – amphetamine, methamphetamine, ephedrine, pseudoephedrine, MDA, MDMA, and MDE (Figure 1).

Experimental

Apparatus

Chromatography was performed with a quaternary pump (Hewlett-Packard (HP) 1050 Series; Agilent, Palo Alto, CA, USA), an automatic sample injector (HP1050 Series) with a 100-µL sample loop, and a fluorescence detector (HP1046 Series) or a UV diode-array detector (HP 1040 Series). The detectors were linked to a data system (Agilent HPLC Chem Station) for data acquisition and storage. Unless otherwise stated the fluorescence detector was operated at 212 nm for excitation and at 313 nm for emission whereas the diode-array detector was operated at 265 nm.

Reagents

All reagents were of analytical-reagent grade. Racemic amphetamine sulphate, methamphetamine hydrochloride, 3,4-methylenedioxyamphetamine, 3,4-methylenedioxymethamphetamine, and 3,4-methylenedioxyethylamphetamine, and (+)-amphetamine sulphate, (+)-methamphetamine hydrochloride, (–)-ephedrine hydrochloride, (+)-ephedrine, (–)-pseudoephedrine, and (+)-pseudoephedrine hydrochloride were obtained from Sigma (St. Louis, MO, USA). (–)-1-(9-Fluorenyl)-ethyl chloroformate, 0.5% (*w/v*) solution in acetone (equivalent to a concentration of 18 mM), was purchased from Acros (Geel, Belgium). Methanol (Scharlau, Barcelona, Spain) and acetonitrile (T.J. Baker, Deventer, The Netherlands) were of HPLC grade. Sodium hydrogen carbonate, sodium acetate, acetic acid, phosphoric acid (Probus, Badalona, Spain), sodium hydroxide (Panreac, Barcelona, Spain), and sodium dihydrogen phosphate monohydrate (Merck, Darmstadt, Germany) were also used.

Preparation of Solutions

Stock standard solutions of racemic amphetamine, methamphetamine, MDA, MDMA, and MDE (100 µg mL^{-1} of each enantiomer), and (+)-amphetamine, (+)-methamphetamine, (+)-ephedrine, (–)-ephedrine, (+)-pseudoephedrine, and (–)-pseudoephedrine (100 µg mL^{-1}) were prepared in water. Racemic ephedrine and pseudoephedrine were prepared by mixing equal volumes of the stock solutions of the individual enantiomers. Working solutions of the analytes were prepared by dilution of the stock solutions with water. Water was distilled, deionized, and filtered through 0.45-µm nylon membranes (Teknokroma, Barcelona, Spain). All solutions were stored in the dark at 2 °C.

Columns and Mobile Phases

All chromatographic assays were performed on a 125 mm × 4 mm i.d., 5 μm particle, LiChrospher 100 RP_{18} column (Merck). Mixtures of methanol with 0.05 M acetate buffer (pH 4.5–7.0), of acetonitrile with 0.05 M acetate buffer (pH 4.5), and of methanol with 0.05 M phosphate buffer (pH 3.0) were tested as mobile phases. The acetate buffer was prepared by dissolving sodium acetate in water then adjusting the pH by addition of concentrated acetic acid. The phosphate buffer was prepared by dissolving sodium dihydrogen phosphate monohydrate in water then adjusting the pH by addition of the minimum volume of concentrated phosphoric acid.

Before use all solvents were filtered through 0.45-μm nylon membranes (Teknokroma, Barcelona, Spain) and degassed with helium.

Derivatization Procedure

Standard solutions of the analytes (125 μL) were placed in 2-mL glass vials, and mixed with 125 μL carbonate buffer. FLEC solution (250 μL) was then added and the resulting mixture was left to react for a defined period of time. Finally, the reaction mixture (50 μL) was injected into the chromatograph.

The solutions of reagent were prepared daily by dilution of the original solution of FLEC with methanol. The final concentration of FLEC in such solutions ranged from 0.5 mM to 10 mM. The carbonate buffer, 1% (*m/v*), was prepared by dissolving sodium hydrogen carbonate in water then adjusting the pH to the required value by addition of 10% (*m/v*) NaOH.

All derivatization was performed at ambient temperature and each sample was assayed in triplicate.

Analysis of Urine Samples

Untreated urine samples were spiked with stock solutions of the MDA to give concentrations of 5.0 and 10.0 μg mL^{-1} (each isomer). Solid-phase extraction (SPE) on BondElut C_{18} cartridges (200 mg mL^{-1}, Varian, Harbor City, CA, USA) was used for sample clean-up. Conditions for SPE were selected accordance with previous work [10, 13]. The cartridges were conditioned by passage, under vacuum, of 1 mL methanol then 1 mL water; the samples (1 mL) were then sucked through the cartridges, endogenous compounds were flushed out with 1 mL water, and the cartridges were then dried with air. The analytes were then desorbed with acetonitrile-0.01 M phosphate buffer, pH 3 (1:1, *v/v*; 1 mL) and collected in 2-mL glass vials. Finally, the extracts (125 μL) were placed in 2-mL glass vials and subjected to the derivatization procedure described above.

The phosphate buffer used for desorption of the analytes from the cartridges was prepared as indicated above.

Results and Discussion

Optimization of the Elution Conditions

Initially the possibility of resolving the diastereomers obtained by derivatization of the analytes with FLEC was investigated. Standard solutions containing racemic mixtures of the analytes at a concentration 25.0 μg mL^{-1} (each enantiomer) were derivatized with 4.5 mM FLEC for 10 min; the pH of the carbonate buffer was 10.0. The reaction mixtures were then chromatographed on the LiChrospher C_{18} column, using isocratic elution with mixtures of methanol with 0.05 M acetate buffer (pH 4.5), of acetonitrile with 0.05 M acetate buffer (pH 4.5), or of methanol with 0.05 M phosphate buffer (pH 3.0). For each mixture different mobile phase compositions and flow rates (ranging from 0.5 to 1.5 mL min^{-1}) were evaluated. The chromatograms were recorded by means of fluorescence detection.

As expected, comparison of the chromatograms obtained for a blank (water) and for standard solutions of the analytes revealed that all the amphetamines tested reacted with the FLEC reagent, producing intense chromatographic peaks. Although for the same amount (%) of buffer in the mobile phase the retention times were similar, the best peak profiles were obtained by use of methanol-acetate buffer as mobile phase. Neither retention nor peak profiles were significantly affected by the pH of the acetate buffer within the range 4.5–7.0. Consequently a mixture of methanol and 0.05 M acetate buffer, pH 4.5, was used as mobile phase in further experiments.

The chromatograms obtained for blanks and samples also contained different peaks corresponding to excess reagent and its degradation or condensation by-products [9]. Most of these peaks, however, eluted at retention times much shorter than those of the analytes and, therefore, did not interfere. The derivatives obtained from the analytes were, in fact, strongly retained by the column, and even for the highest mobile phase flow rate tested 60% methanol in the mobile phase was necessary for elution of the more retained analytes in reasonable times (< 60 min).

For all the amphetamines tested it was possible to find chromatographic conditions enabling at least partial resolution of the corresponding FLEC diastereomers. The derivatives formed by ephedrine and pseudoephedrine were less retained than those from the other analytes; this was because of the higher polarity of these compounds, owing to the presence of the -OH substituent in their molecules (Figure 1). As is apparent from Figure 2, the diastereomers obtained from both compounds were completely resolved by use of methanol-acetate buffer, 40:60 (*v/v*), as mobile phase at a flow rate of 1 mL min^{-1}. The same elution conditions could also be used to separate the diastereomers formed by the other amphetamines, although the times required to elute these compounds were unacceptably long (especially for the diastereomers of MDMA and MDE) and poor peak shapes were usually obtained.

Considerable reduction of analysis time without significant loss of resolution was achieved for amphetamine, methamphetamine, and MDA by increasing the amount of methanol to 70% (for the same mobile phase flow rate). The chromatograms obtained for these compounds are also depicted in Figure 2, in which it is apparent that the derivatives of amphetamine and methamphetamine were fairly well separated, but not to baseline, whereas the diastereomers obtained from MDA were completely resolved. For the most retained FLEC-MDMA and FLEC-MDE derivatives the best enantiomer resolution in the minimum analysis time was obtained by use of a mobile phase containing 75% methanol at the same flow rate (Figure 2).

On the basis of these results it can be concluded that the best chromatographic conditions for each analyte are those summarized in Table I. It is interesting to note that different peak responses were obtained from the diastereomers obtained from racemic MDA, MDMA, and MDE (Figure 2). Analysis of solutions contain-

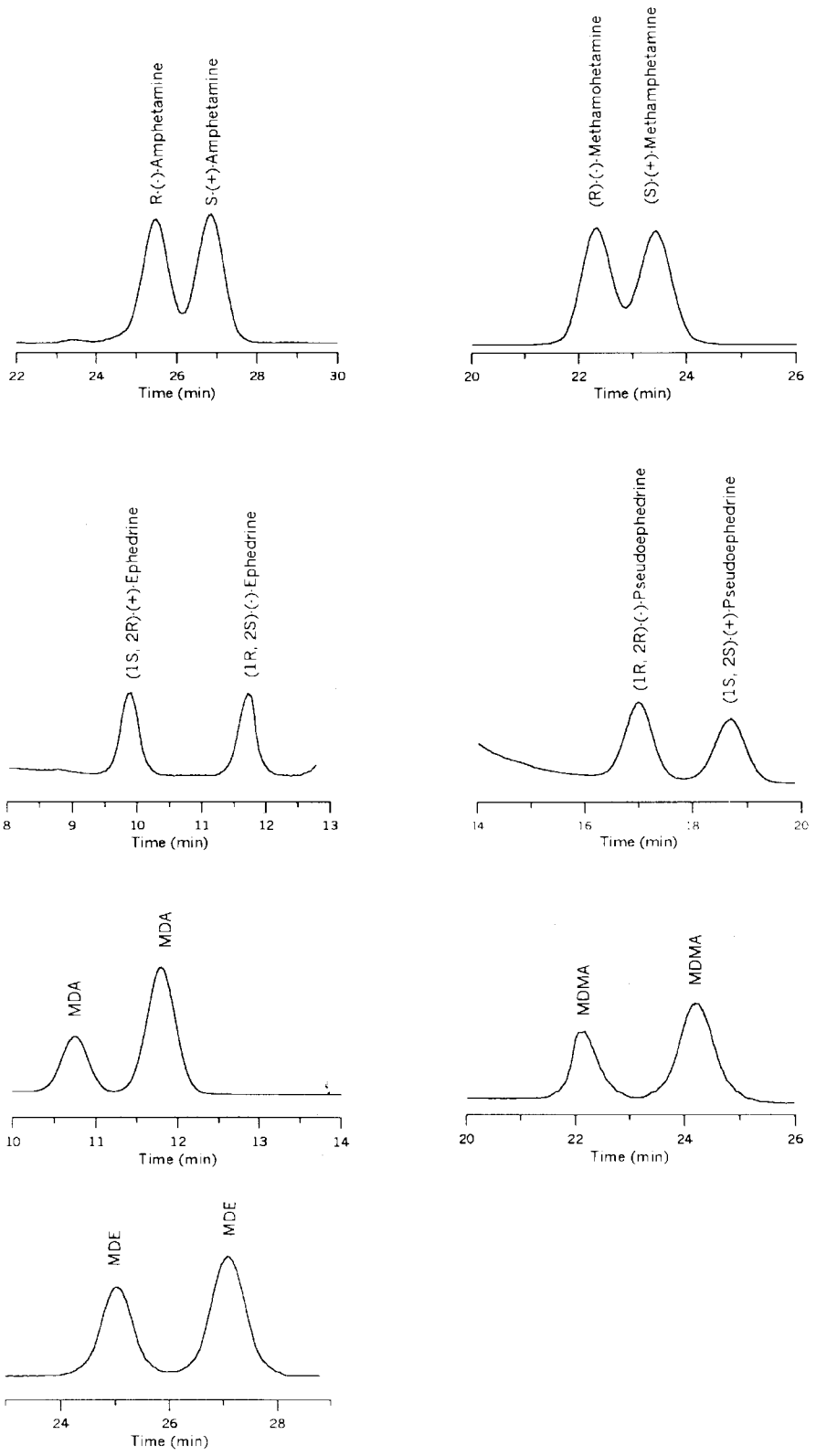

Figure 2. Chromatograms obtained from samples of racemic amphetamines under the elution conditions listed in Table I. Detection: fluorimetric, $\lambda_{excitation}$ = 212 nm, $\lambda_{emission}$ = 313 nm. Concentration of analytes, 25 μg mL^{-1}, each enantiomer.

ing individual enantiomers of ephedrine, pseudoephedrine, amphetamine, and methamphetamine revealed that the derivatives obtained from the *S* enantiomers interacted more strongly with the stationary phase than those obtained from the *R* isomers.

The selectivities and resolution factors obtained under the optimized elution conditions (given in Table I) are listed in Table II. The worst results were obtained for amphetamine (R = 0.97) and methamphetamine (R = 0.91); the separation efficiencies obtained were, even so, comparable with those reported previously after use of the same derivatization reagent [17, 18]. The main advantage of the conditions proposed in this work is that elimination of unreacted FLEC and sub-products was not necessary; this simplifies the overall analytical procedure.

The only difference between the methamphetamine molecule and the ephedrines is the presence of the hydroxy substituent on the carbon atom adjacent to the chiral centre (Figure 1). Unlike the FLEC-methamphetamine derivatives, however, the diastereomers formed by the two ephedrines tested could be completely resolved. This might be explained by conformational differences between the diastereomers as a result of the formation of intramolecular hydrogen bonds between the hydroxy substituent and the FLEC group [14]. The diastereomers formed by the methylenedioxylated amphetamines could be also be resolved to baseline. This means that, compared with amphetamine or methamphetamine, the presence of the additional ring in the methylenedioxylated amphetamines has a positive effect on resolution; this can be explained by the substantial conformational rigidity of the molecules of the diastereomers obtained (Figure1).

Derivatization Conditions

The effect of the derivatization conditions was evaluated by processing racemic samples of the analytes at a concentration of 50.0 μg mL^{-1} (25.0 μg mL^{-1} of each enantiomer). According to the literature the main conditions affecting derivatization with chloroformates are the concentration of reagent, the reaction time, and the pH of the reaction medium [9, 10, 12]. Although absolute yields were not established (because external standards of the FLEC-analyte derivatives were not available), the effect of those conditions on analyte conversion was investigated by varying each condition while keeping the others constant.

The effect of the reaction time between 2 and 30 min was investigated for all the amphetamines. The concentration of reagent was 4.5 mM and the pH of the carbonate buffer was 10.0. The results obtained indicated that all the analytes re-

Table I. Optimized conditions for separation and detection of the FLEC diastereomers. Fluorescence (FL) detection: $\lambda_{excitation}$ = 212 nm, $\lambda_{emission}$ = 313 nm; UV detection: λ = 265 nm.

Analyte	Mobile phase	Flow rate	Detection
Ephedrine	40:60 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	FL
Pseudoephedrine	40:60 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	FL
Amphetamine	70:30 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	FL
Methamphetamine	70:30 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	FL
MDA	70:30 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	UV
MDMA	75:25 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	UV
MDE	75:25 (*v/v*) methanol-0.05 M acetate buffer (pH 4.5)	1 mL min^{-1}	UV

acted rapidly, and constant analyte responses were obtained within the 10 to 30 min time interval.

To investigate the effect of the concentration of FLEC, reagent concentrations of 0.5, 4.5, 8.0, and 10.0 mM were evaluated. Higher concentrations could not be used, because of the limited solubility of the reagent and/or products in the reaction medium. The reaction time was 10 min and the pH of the carbonate buffer was 10.0. Increasing the concentration of FLEC from 0.5 to 4.5 mM increased the responses to the analytes for all the amphetamines assayed. Increasing the concentration further, however, resulted in little improvement (< 5%).

Finally, the effect of pH on analyte conversion was studied within the range 8.5–10.5. The reaction time was 10 min and the concentration of FLEC in the derivatization solution was 4.5 mM. The best results were obtained at pH 9.5–10.0. At pH 10.5, peak signals decreased slightly, most probably because of hydrolysis of the reaction products [9, 12], whereas at pH < 9.5 poor responses were obtained for most analytes.

In accordance with the results presented above the procedure selected for derivatization of the analytes was mixing of 125 µL sample, 125 µL carbonate buffer (pH 10.0), and 250 µL 4.5 mM FLEC and reaction for 10 min. The resulting mixture was chromatographed. It should be noted that the reaction solvent was 50:37.5:12.5 (*v/v*) water-methanol-acetone. The use of this amount of methanol prevented the formation of two phases in the reaction mixture and precipitation of the reagent and/or products. Solvents other than methanol were not investigated.

Detection of the Methylenedioxylated Amphetamines

Under the optimized derivatization conditions the different peak signals obtained from the diastereomers obtained from identical amounts of the two enantiomers could be successfully used for quantitative purposes. Derivatization furnishing equivalent peak responses are, however, highly desirable, particularly when external standards of the corresponding derivatives are not readily available. Consequently, further studies were performed with the methylenedioxylated amphetamines to find conditions giving equivalent peak signals.

It has already been demonstrated that derivatization of racemic pseudoephedrine with the chiral chloroformate FMOC-L-Pro led to different peak responses [15]. Differences between the areas (or heights) of the peaks of the two FMOC-L-Pro-pseudoephedrine derivatives were reduced by increasing the concentration of reagent in the reaction medium or by increasing the reaction time. The differences were, therefore, a consequence of the different rates of reaction of the pseudoephedrine enantiomers with the reagent. As stated above, however, increasing the concentration of FLEC and the reaction time had a negligible effect on the responses obtained for the analytes. Thus the ratio *peak area of the first-eluting isomer/peak area of the last-eluting isomer* for the diastereomers of MDA, MDMA and MDE was not substantially modified by varying the derivatization conditions, as is apparent from Table III.

The results in Table III suggest that the diastereomers formed by the methylenedioxylated amphetamines have different detection properties rather than different reaction rates. It is, on the other hand, apparent from Table III that the ratios obtained for the three amphetamines were quite similar, which indicates that the different peak responses do not depend significantly on the substituent on the nitrogen atom (Figure 1). In other words, differences between the fluorimetric responses of the diastereomers seem to be related to the methylenedioxylated moiety.

Table II. Separation data obtained for the FLEC diastereomers under optimized conditions.

Compound	α*	R_S**
Ephedrine	1.23	1.92
Pseudoephedrine	1.11	1.27
Amphetamine	1.06	0.97
Methamphetamine	1.04	0.91
MDA	1.12	1.24
MDMA	1.18	1.31
MDE	1.09	1.21

* separation factor; ** resolution.

The formation of diastereomers with different detection properties has previously been reported by Krull et al. [19], who tested FMOC-L-Pro for separation of the enantiomers of a wide variety of amines and aminoalcohols. When fluorimetric detection was used different peak signals were observed only for the diastereomers obtained from naphthylethylamine. The authors explained this behaviour as a consequence of a quenching phenomenon, owing to intramolecular rearrangement in one of the diastereomers. This could also be the reason for the results obtained in this study, taking into account the similar structures of naphthylethylamine and the methylenedioaxylated amphetamines (with an additional ring in their molecules). The above explanation is, moreover, in agreement with the fact that similar peak responses were observed for racemic samples of MDA, MDMA, and MDE when the chromatograms were monitored with the UV detector (Table III). As an example, the chromatograms obtained from the FLEC-MDA derivatives by use of both fluorimetric and UV detection are depicted in Figure 3.

On the basis of these results, it can be concluded that UV detection is the best option for the enantiomeric analysis of the methylenedioxylated amphetamines. In this study the chromatograms were monitored at different wavelengths between 220 and 300 nm; the best analyte-to-baseline responses were obtained at 265 nm (Table I).

Table III. Relative peak areas obtained for racemic MDA, MDMA, and MDE with fluorimetric detection (FL) or with UV detection ($n = 3$).

Compound	FL detection			UV detection
	Concentration of FLEC (mM)	Reaction time (min)	A_1/A_2*	A_1/A_2*
MDA	4.5	10	0.524 ± 0.007	1.005 ± 0.003
	4.5	30	0.53 ± 0.03	–
	7.25	10	0.51 ± 0.03	–
	10.0	10	0.49 ± 0.02	–
MDMA	4.5	10	0.53 ± 0.02	0.998 ± 0.007
MDE	4.5	10	0.55 ± 0.03	1.01 ± 0.01

* (peak area of first-eluting isomer)/(peak area of last-eluting isomer).

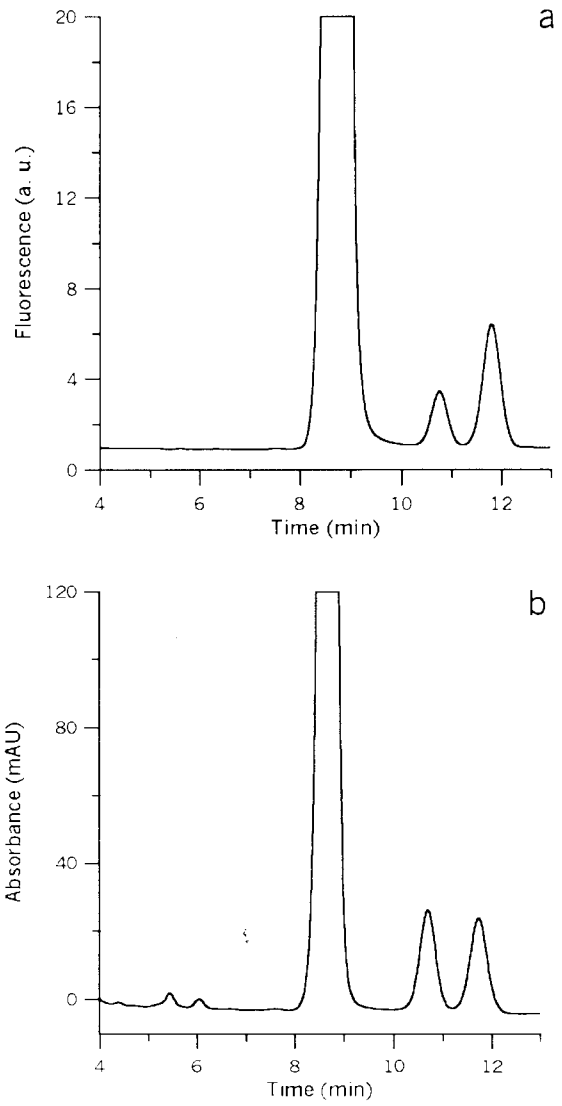

Figure 3. Chromatograms obtained from racemic MDA (25 μg mL^{-1}, each enantiomer) using (**a**) fluorimetric ($\lambda_{excitation}$ = 212 nm, $\lambda_{emission}$ = 313 nm), and (**b**) UV detection (265 nm). The peak at 8.4 min is that of unreacted FLEC.

Reproducibility and Sensitivity Studies

Reproducibility was evaluated by measuring the peak areas for each isomer at a concentration of 10.0 μg mL^{-1}. As is apparent from Table IV, under the derivatization, separation and detection conditions proposed satisfactory reproducibility was obtained for all the analytes tested – relative standard deviations were in the range 4–8%.

The limits of detection (for a signal-to-noise ratio of 3) were established for each analyte under the optimized conditions listed in Table I. For amphetamine and methamphetamine the limits of detection (*LOD*) were also established by use of UV detection at 265 nm. As observed from Table IV, the *LOD* obtained for those amphetamines detected by fluorescence were 50–100 ng mL^{-1}. For the methylenedioxylated amphetamine derivatives the UV *LOD* were only 2 to 4 times greater whereas *LOD* for amphetamine and methamphetamine when using UV detection were approximately the same as those obtained by use of fluorescence detection. It can be concluded that although FLEC is typically used as a fluorogenic reagent use of UV detection does not result in significant loss of sensitivity.

The *LOD* in Table IV are approximately one order of magnitude higher than those achieved by derivatization with achiral chloroformates and use of fluorescence detection [12, 13]; they are comparable with those reported by other LC methods involving pre-column chiral derivatization (provided that no analyte preconcentration is effected). For example, *LOD* obtained for amphetamine by use of this method are the same as values obtained by derivatization with the fluorogenic reagent OPA and the chiral thiol *N*-acetyl-L-cysteine (NAC) [20]. No signal discrimination between the OPA-NAC diastereomers was observed, however (and, therefore, fluorescence detection could be used), and so *LOD* obtained for MDA isomers were lower than the values obtained by use of this method. It should be noted that the reagent OPA reacts with primary amines only. The main advantage of this method over the OPA-NAC approach is, therefore, that it can be used for resolution of the enantiomers of primary and secondary amphetamines.

The sensitivity is clearly better than that of LC methods developed for enantioselective analysis of underivatized amphetamines (e.g. methods based on use of β-cyclodextrins as chiral selectors) [21].

Table IV. Reproducibility and limits of detection obtained under the proposed conditions.

Compound	Within-day precision* (%, $n = 5$)	Limit of detection (ng mL^{-1})	
		FL detection	UV detection
(1*S*,2*R*)-Ephedrine	5	50	–
(1*R*,2*S*)-Ephedrine	6	50	–
(1*R*,2*R*)-Pseudoephedrine	6	50	–
(1*S*,2*S*)-Pseudoephedrine	8	50	–
(*R*)-Amphetamine	5	50	100
(*S*)-Amphetamine	5	50	100
(*R*)-Methamphetamine	6	100	100
(*S*)-Methamphetamine	7	100	100
MDA – first eluting isomer	5	–	100
MDA – last eluting isomer	6	–	100
MDMA – first eluting isomer	5	–	150
MDMA – last eluting isomer	4	–	150
MDE – first eluting isomer	7	–	200
MDE – last eluting isomer	6	–	200

* Determined at a concentration of 10.0 μg mL^{-1}.

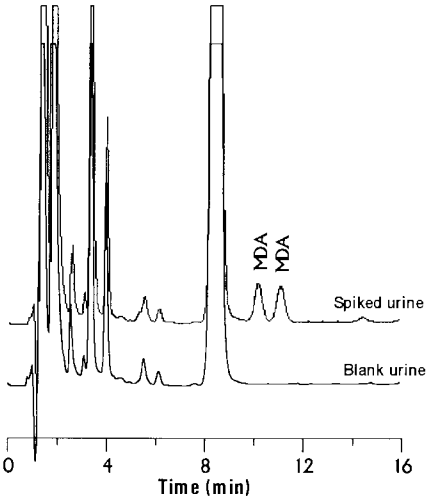

Figure 4. Chromatograms obtained, by use of the proposed procedure, from extraction of blank urine and from urine spiked with MDA (5 μg mL^{-1} of each enantiomer).

Table V. Results obtained from the determination of MDA in water and urine samples.

Compound	Linearity ($n = 8$)	Accuracy (n = 3)		
		Concentration added (μg mL^{-1})	Concentration determined (μg mL^{-1})	
			Water	Urine
MDA – first eluting isomer	$y = -(9 \pm 10) + (21.2 \pm 0.7)x$; $R^2 = 0.997$	5.0	4.9 ± 0.3	5.0 ± 0.4
		10.0	9.9 ± 0.6	10.5 ± 0.9
MDA – last eluting isomer	$y = -(7 \pm 8) + (19.9 \pm 0.6)x$; $R^2 = 0.997$	5.0	4.8 ± 0.3	4.9 ± 0.2
		10.0	10.0 ± 0.5	10 ± 1

Utility

The reliability of these conditions for determination of the amphetamines was evaluated by using MDA as an example. Linearity was evaluated by analysis of standard solutions of racemic MDA in water, in which the concentrations of each isomer ranged from 1.0 to 25.0 μg mL^{-1}. The calibration graphs were obtained by plotting peak area against concentration of MDA. As is apparent from Table V, linearity was good within the interval tested. Calibration plots were used to determine the concentration of standard solutions of racemic MDA at two different concentrations within the working interval. The results obtained indicated that the accuracy of the method was satisfactory (Table V).

The possibility of using the FLEC approach for the enantioselective analysis of amphetamines in biological fluids was evaluated by processing urine spiked with the analytes at concentrations typically encountered in biological samples [5]; SPE on packed C_{18} cartridges was used for sample clean-up [10, 13]. The selectivity was found to be satisfactory even when using UV detection. As an illustrative example, Figure 4 shows the UV chromatograms obtained for blank urine and urine spiked with 5.0 μg mL^{-1} MDA (each enantiomer). Recoveries of MDA from the samples were 98 ± 2% and 95 ± 4% for the first- and last-eluting isomers, respectively. Although the FLEC reagent can react with other urinary compounds containing amino groups, these results suggest that most of these are eliminated by the proposed clean-up procedure. In this way the FLEC-to-analyte concentration ratio is kept high enough to ensure adequate conversion of the analyte. Under the conditions proposed the method can be applied to urine samples containing concentrations of unchanged MDA of, at least, 20 μg mL^{-1} (the highest concentration assayed). For this concentration the FLEC/MDA concentration ratio was approximately 80.

Finally, the accuracy of the determination of MDA in urine was studied. Samples were subjected to the proposed extraction and derivatization procedures and then chromatographed. The concentrations of each isomer in the samples were calculated from the calibration plots obtained for the aqueous solutions, taking into account the percentages of analytes recovered. The results obtained (Table V) were comparable with those obtained for aqueous samples, which indicates that the method can be adapted to the enantioselective analysis of MDA in urine.

Although not tested the separation efficiencies and sensitivities achieved for the other amphetamines suggest that the FLEC method might be also suitable for chiral recognition and quantification of these compounds in a wide variety of samples such as pharmaceuticals [15], biological samples [5, 10], or illicit formulations [22]. Even for amphetamine and methamphetamine the resolution can be regarded as sufficient for most applications, although more selective alternatives, e.g. β-cyclodextrin-mediated CE, might be necessary for quantification of amphetamine and methamphetamine in samples containing very different concentrations of the two enantiomers (for example, in studies performed to check the enantiomeric purity).

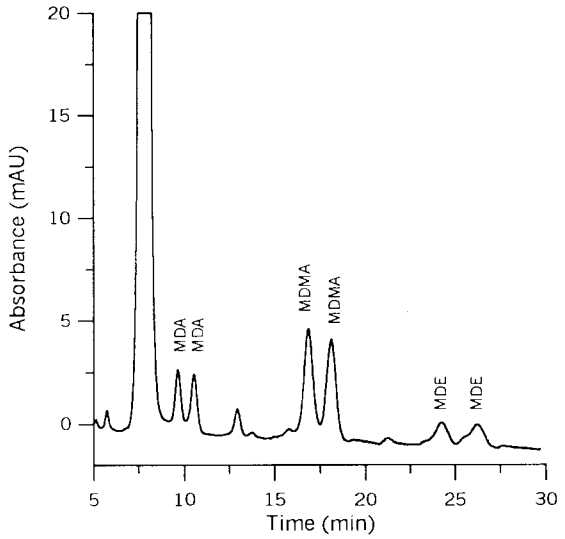

Figure 5. Chromatograms obtained from a mixture of racemic MDA, MDMA, and MDE. Mobile phase, methanol-0.1 M acetate buffer (pH 4.5), at a flow rate of 1 mL min^{-1}; gradient elution program: 0–15 min, 70% methanol; at 30 min, 80% methanol. Detection: UV at 265 nm. Concentration of the analytes, 10 μg mL^{-1} of each enantiomer.

It is interesting to note that the proposed conditions could be adapted for simultaneous analysis of two or more amphetamines, which might be of interest in some applications. Examples are the determination, in urine, of methamphetamine and its metabolite amphetamine, MDMA and its metabolite MDA, and MDE and its metabolite MDA [7], or the simultaneous determination of methamphetamine, ephedrine, and pseudoephedrine to establish the synthetic pathway used for clandestine formulation of methamphetamine [2].

Conclusions

This work shows that precolumn derivatization with FLEC is a valid alternative for enantioselective analysis of a wide variety of primary and secondary amphetamines by LC. In this study, the FLEC diastereomers obtained for all the compounds tested were satisfactorily resolved under typical reversed-phase conditions and problems encountered with other chiral chloroformates, e.g. FMOC-L-Pro (for example, kinetic resolution) were avoided. Although FLEC has been proposed as a sensitive fluorimetric reagent, it was shown that UV detection is the most reliable option for analysis of methylenedioxylated amphetamines, particularly for quantitative studies.

Owing the large differences between analyte polarity, the chromatographic conditions should be selected for each amphetamine, to achieve maximum separation efficiency in the minimum analysis time. Conditions can, nevertheless, be optimized to achieve the simultaneous separation of two or more amphetamines in a single chromatographic run. As an example, Figure 5 shows the chromatogram obtained for a mixture of the designer drugs included in this study. It should be remarked that as far as we are aware this is the first method described for stereospecific analysis of amphetamine-derived designer drugs involving derivatization with a detector-sensitive reagent. The FLEC approach can also be adapted to the analysis of amphetamines in biological fluids, e.g. urine, by incorporation of SPE on relatively non-selective C_{18} cartridges.

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