Chiral determination of amphetamine and related compounds using chloroformates for derivatization and high-performance liquid chromatography

The Analyst

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Received 2nd April 1998, Accepted 21st July 1998

The enantiomeric determination of amphetamine and various amphetamine-type compounds by liquid chromatography after chiral derivatization with 9-fluorenylmethyl chloroformate-L-proline (FMOC-L-Pro) is reported. The results obtained were compared with those achieved after achiral derivatization with 9-fluorenylmethyl chloroformate and subsequent separation of the derivatives on a β-cyclodextrin chiral stationary phase. Conditions for the derivatization of amphetamines with FMOC-L-Pro were investigated, including the effect of the derivatization reagent concentration, pH and reaction time, using amphetamine, ephedrine and pseudoephedrine as model compounds. On the basis of these studies, possible conditions for the determination of each amphetamine are indicated. To demonstrate the utility of the proposed procedures, data on linearity, repeatability and sensitivity are given. Results of the determination of ephedrine enantiomers in different pharmaceutical samples are also presented.

Because of the increasing popularity of amphetamines as drugs which act as central nervous stimulants, the determination of these compounds is receiving increasing attention in the clinical, toxicological and pharmaceutical fields. In this respect, and as the enantiomers of amphetamines have different pharmacological and toxicological properties, the development of chiral methodologies is an area of major interest.

High-performance liquid chromatography (HPLC) is the method of choice for the enantiomeric determination of amphetamine and amphetamine-type compounds and a number of chromatographic procedures have been proposed using both direct and indirect methods.[1] For example, successful enantioresolution has been achieved for different amphetamines by using chiral stationary phases such as cyclodextrins (CDs),[2–5] α_1-acid glycoprotein[6] or functionalized cellulose phases.[1] Generally, no derivatization of the analytes is necessary to achieve suitable resolution, but in some instances achiral derivatization (simple acylation of the amino group or formation of ion pairs) greatly improves the separation.[1] Other assays are based on the introduction into the analyte molecule of a specific site for interaction with a Pirkle-type chiral column. Suitable resolution has been obtained with the reagents 3,5-dinitrobenzoyl chloride[7,8] and 3-toluoyl chloride,[9] which also facilitate analyte detectability.

In spite of recent advances in the development of chiral stationary phases, indirect methods are still preferred for the determination of amphetamines, because derivatization is a common and often obligatory step in most chromatographic methods, owing to the low UV absorbance of these compounds and also to their very low natural fluorescence. Therefore, derivatizations are designed not only to achieve highly sensitive fluorimetric or photometric detection, but also to avoid the employment of chiral columns in the chromatographic step. This possibility has been widely illustrated for a variety of amines and amino acids by using *o*-phthaldialdehyde (OPA) in combination with a chiral thiol.[1,10] We have recently reported an HPLC method for the automated determination of amphetamine enantiomers in urine, based on the employment of OPA and the homochiral thiol *N*-acetyl-L-cysteine.[11] Unfortunately, the OPA method can only be applied directly to the derivatization of primary amines.

Among the numerous derivatizing agents proposed in the literature, 9-fluorenylmethyl chloroformate (FMOC) appears to be a very attractive reagent for primary and secondary amino acids and amines prior to HPLC, because the reactions proceed rapidly under very mild conditions, and the derivatives formed are stable and highly fluorescent.[12,13] Derivatizations with FMOC have also been used in combination with CD chiral stationary phases for enantiomeric separations. This possibility has been applied to the enantioresolution of different amino acids and peptides,[14,15] but no similar attempt has been reported for FMOC-amphetamines. Enantioseparation of underivatized amphetamines on β-CD columns has also been described.[2] However, the sensitivity achieved would be unsatisfactory for most real applications.

As an alternative to the employment of chiral stationary phases, chiral chloroformates can be used to form the diastereomers of compounds of clinical interest. Successful results have been obtained for various drugs such as β-adrenoceptor blocking agents and methamphetamine by using (+)-1-(fluorenyl)ethyl chloroformate or (−)-menthyl chloroformate as reagents.[16–20] A solid-phase reagent containing the 9-fluorenylmethyl chloroformate-L-proline (FMOC-L-Pro) tag has also been used for the resolution of amphetamine enantiomers on a C_{18} analytical column.[21,22] However, to our knowledge, FMOC-L-Pro has not yet been used for the solution derivatization of amphetamines.

In this work, we have evaluated the possibility of resolving the enantiomers of amphetamines by using two different approaches: derivatization with FMOC and separation on a chiral stationary phase; and derivatization with FMOC-L-Pro and subsequent separation on a conventional C_{18} column. A β-CD column was selected for the separation of the FMOC derivatives, because CDs are the chiral selectors most commonly used (as column packings or as additives to the mobile phase) for the enantioresolution of amphetamines in both chromatography and capillary electrophoresis.[2–5] The primary amine amphetamine and the secondary amines ephedrine and pseudoephedrine were selected as model compounds (Fig. 1). Conditions for the solution derivatization of these compounds with FMOC-L-Pro were also optimized. On the basis of these studies, the optimum derivatization and separation conditions

for each amphetamine are indicated. As an example, the proposed conditions were applied to measure ephedrine enantiomers in different pharmaceutical formulations.

Experimental

Apparatus

The chromatographic system used consisted of a quaternary pump (Hewlett-Packard, 1050 Series, Palo Alto, CA, USA), and an automatic sample injector (Hewlett-Packard, 1050 Series) equipped with a sample loop injector of 100 μL. For detection, a fluorescence detector (Hewlett-Packard, 1046 Series) and a diode-array UV detector (Hewlett-Packard, 1040 Series) linked to a data system (Hewlett-Packard HPLC Chem. Station) were coupled in series. The detectors were used for data acquisition and storage. The fluorescence detector was operated at 264 nm for excitation and 313 nm for emission, whereas the UV absorbance was monitored at 254 nm.

Reagents

All the reagents used were of analytical-reagent grade. Acetonitrile, ethyl acetate, hexane, propan-2-ol and methanol (Scharlau, Barcelona, Spain) were of HPLC grade. Water was distilled and de-ionized and filtered through 0.45 μm nylon membranes (Teknokroma, Barcelona, Spain). D,L-Amphetamine sulfate, D-amphetamine sulfate, (+)-pseudoephedrine hydrochloride, (−)-pseudoephedrine, (+)-ephedrine hydrochloride and (−)-ephedrine hydrochloride were obtained from Sigma (St. Louis, MO, USA). FMOC, FMOC-L-Pro (97% optical purity), dicyclohexylcarbodiimide (DCC) and triethylamine (TEA) were obtained from Aldrich (Steinheim, Germany), and hydrochloric acid, ammonium acetate, acetic acid and sodium hydroxide were purchased from Panreac (Barcelona, Spain). Sodium hydrogencarbonate (Probus, Badalona, Spain), and sodium dihydrogenphosphate monohydrate (Merck, Darmstadt, Germany) were also used.

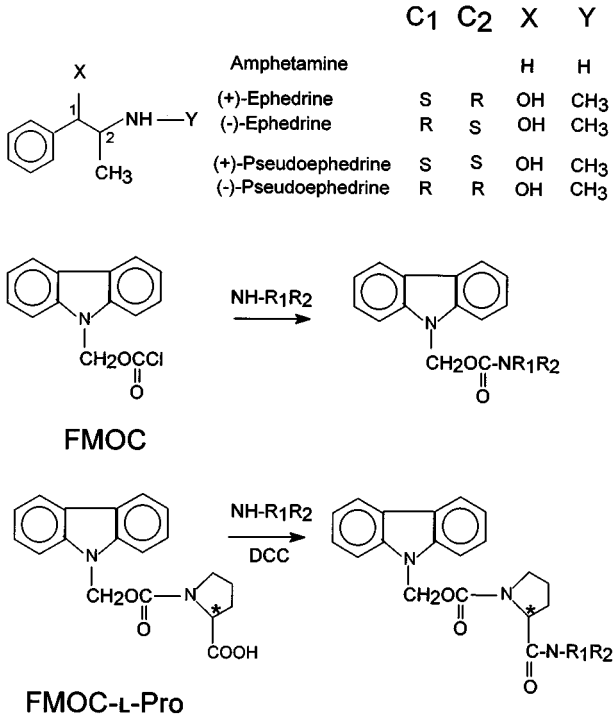

Fig. 1 Chemical structures of the amphetamines assayed and derivatization reactions with FMOC and FMOC-L-Pro. The asterisk indicates the chiral centre.

Pharmaceuticals

Difilina tablets labelled to contain 25 mg of ephedrine, and Mirazul solution, labelled to contain 0.50 mg mL^{-1} of ephedrine, were obtained from Laboratorio de Aplicaciones Farmacéuticas (Barcelona, Spain) and Laboratorio Liade (Alcalá de Henares, Spain), respectively.

Preparation of solutions

Stock standard solutions of each amphetamine (1000 μg mL^{-1}) were prepared in water. Working solutions of the amines were prepared by dilution of the stock solutions with water. Stock standard solutions of DCC (200 mM), FMOC (20 mM) and FMOC-L-Pro (20 mM) were prepared by dissolving the pure compounds in acetonitrile. The hydrogencarbonate buffer (4% m/v) was prepared by dissolving the appropriate amount of sodium hydrogencarbonate in water; after which the pH was adjusted to 10.0 with 1 M NaOH. The 0.05 M ammonium acetate buffer and the 0.01 M phosphate buffer were prepared by dissolving the appropriate amounts of ammonium acetate and sodium dihydrogenphosphate monohydrate, respectively, in water. In some instances, the buffers contained 1% TEA (v/v). The pH was then adjusted to appropriate values with 1 M NaOH or 1 M HCl. All solutions were stored in the dark at 2 °C.

Columns and mobile phases

For the direct chiral separation of the FMOC derivatives, a 250 × 4 mm id column pre-packed with immobilized β-CD, d_p 5 μm (LiChroCART ChiraDex, Merck), was used. For the separation of the FMOC-L-Pro diastereomeric derivatives, a LiChrospher 100 RP_{18}, 5 μm, 125 × 4 mm id column (Merck) or a Hypersil ODS-C_{18}, 5 μm, 250 × 4 mm id column (Merck) was used.

For elution under reversed-phase conditions, the mobile phase consisted of different mixtures of acetonitrile or methanol, and water, 0.01 M phosphate or 0.05 M ammonium acetate buffers. The pH of the buffers was adjusted to between 3.5 and 7.0, as specified. For elution under normal-phase conditions, different mixtures of acetonitrile–acetic acid–TEA, hexane–propan-2-ol and acetonitrile–ethyl acetate were tested. Several isocratic and gradient elution programs were tested, the flow rates ranging between 0.25 and 1.0 mL min^{-1}. In all instances, solvents were filtered through 0.45 μm nylon membranes (Teknokroma) and de-gassed with helium before use.

Derivatization

Derivatization with FMOC. For the derivatization with FMOC, 0.25 mL of the samples and 0.25 mL of the carbonate buffer were placed in 2 mL injection glass vials. Next, 0.50 mL of 20 mM FMOC solution was added. After a reaction time of 2 min, 20 μL of the resulting solution were injected onto the analytical column.

Derivatization with FMOC-L-Pro. The derivatizing solutions were prepared daily by mixing appropriate volumes of 200 mM DCC and 20 mM FMOC-L-Pro; if required, these mixtures were further diluted with acetonitrile. For derivatization of the amphetamines, 0.25 mL of sample and 0.25 mL of buffer were placed in 2 mL injection glass vials (in some instances no buffer was used when derivatizing amphetamine). Next, 0.50 mL (0.25 mL for amphetamine) of the derivatizing solution was added and the resulting solution was allowed to react for a given time. Finally, 20 μL of the solution were injected onto the analytical column.

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

Preparation of Standards for calibration

Calibration graphs were obtained by derivatizing standard solutions of the amphetamines with FMOC-L-Pro, the concentration of each isomer being in the range 0.5–20.0 μg mL^{-1}. For amphetamine, 0.25 mL of derivatizing solution was added to 0.25 ml of the sample. After a reaction time of 2 min, 20 μL of the resulting solution were injected onto the analytical column. For ephedrine and pseudoephedrine, 0.25 ml of the sample was mixed with 0.25 mL of the carbonate buffer solution and 0.50 mL of the derivatization reagent. The resulting mixture was allowed to react, and then 20 μL were injected onto the analytical column. The chromatographic conditions used are summarized in Table 1. Calibration graphs were obtained by plotting peak area (or peak height) *vs.* analyte concentration. Each concentration was assayed in duplicate.

Analysis of pharmaceutical samples

Three Difilina tablets were weighed and powdered, and the required amount was suspended in distilled water and filtered. For the Mirazul preparation, 0.25 mL of the sample was diluted to 5 mL with distilled water. In both cases, aliquots of 0.25 mL of the resulting solutions were analysed as described above.

Results and discussion

Direct separation on the β-CD column

According to the literature, the resolution of the enantiomers of FMOC functionalized amino acids and peptides on β-CD stationary phases can be achieved in either the conventional reversed-phase mode or with a polar organic solvent (acetonitrile and small amounts of glacial acetic acid or TEA modifier).[14,15] Consequently, both approaches were investigated for the enantiomeric resolution of the FMOC-amphetamines. For the reversed-phase mode, methanol or acetonitrile was used as the organic component of the eluent, whereas water, 0.01 M phosphate buffer or 0.05 M ammonium acetate buffer (at different pHs between 3 and 7) was the aqueous component of the mobile phase. In some instances, the ammonium acetate buffer contained 1% TEA. Several polar organic solvent compositions, as well as different mixtures of hexane–propan-2-ol or acetonitrile–ethyl acetate, were also tested. In each case, different isocratic and gradient elution profiles were studied, with flow rates ranging from 0.25 to 1.0 mL min^{-1}. Conditions used for derivatization with FMOC were those established previously.[13]

The optimum resolution of amphetamine enantiomers was obtained with a 50 + 50 (v/v) methanol–ammonium acetate buffer containing 1% TEA (pH 7) mobile phase, pumped at a flow rate of 0.25 mL min^{-1}. Under such conditions, near baseline separation was achieved for this amine (Fig. 2). However, partial overlap between the first eluting D-amphetamine isomer and the peak corresponding to the hydrolysed reagent was observed. Mobile phases with lower acetonitrile contents did not provide a significant improvement in resolution, while giving longer analysis times. Although the separation might be satisfactory for most applications, elimination of the excess of reagent may be required, particularly in the quantification of D-amphetamine at very low concentrations.

The peak of unreacted FMOC can easily be separated from those corresponding to the FMOC-ephedrine or FMOC-pseudoephedrine derivatives. However, the enantiomers of these amphetamines were insufficiently resolved under all conditions tested, probably due to the difficulties encountered by the more bulky compounds (such as the secondary amphetamines) in penetrating the β-CD cavities.[2] It should be mentioned that since the addition of a buffer is necessary in order to keep the analytes in a suitable form for reaction,[13] aqueous solutions were directly injected into the chromatographic system. This may result in a slight loss of resolution when working in the normal-phase mode or with polar organic solvents. Injection of non-aqueous samples would involve the

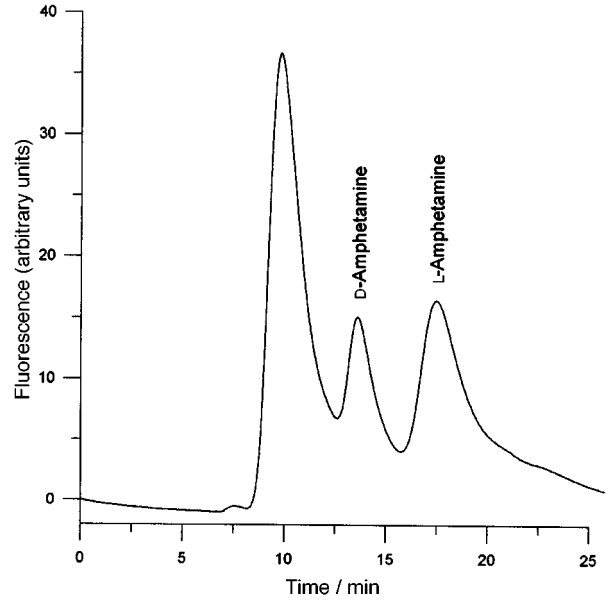

Fig. 2 Chromatogram obtained for racemic mixture of amphetamine derivatized with FMOC and chromatographed on a β-CD column. Concentration of each enantiomer, 10 μg mL^{-1}. For other experimental details, see text.

Table 1 Enantioseparation of the FMOC and FMOC-L-Pro derivatized amphetamines

Compound	Method	Chromatographic conditions		t_1/min	t_2/min	α	R
Amphetamine	FMOC	Column	β-CD	13.6	16.5	1.34	0.97
		Mobile phase	50 + 50 methanol–0.05 M ammonium acetate buffer (pH 7.0, 1% TEA) at 0.25 mL min^{-1}				
	FMOC-L-Pro	Column	C_{18} LiChrospher	17.2	18.2	1.06	0.96
		Mobile phase	60 + 40 acetonitrile–0.01 phosphate buffer (pH 7.0), at 1.0 mL min^{-1}				
Ephedrine	FMOC	Column	β-CD	—	—	—	—
	FMOC-L-Pro	Column	C_{18} Hypersil	10.7	13.8	1.20	1.65
		Mobile phase	60 + 40 acetonitrile–water at 0.50 mL min^{-1}				
Pseudoephedrine	FMOC	Column	β-CD	—	—	—	—
	FMOC-L-Pro	Column	C_{18} LiChrospher	26.2	26.9	1.03	1.08
		Mobile phase	60 + 40 acetonitrile–water at 0.40 mL min^{-1}				

extraction of the FMOC-amphetamine derivatives into an organic solvent, and, therefore, a more laborious procedure.

Separation of the FMOC-L-Pro derivatives

As for the separation of the FMOC derivatives, different elution conditions were evaluated for the eventual resolution of the diastereomers derived from FMOC-L-Pro on conventional (achiral) C_{18} columns. The results showed that the FMOC-L-Pro derivatives of the amphetamines assayed can be at least partially resolved under reversed-phase elution conditions. Moreover, unreacted and/or hydrolyzed FMOC-L-Pro is rapidly eluted from the column, thus making the elimination of the excess of reagent unnecessary. A suitable selection of the working conditions even allowed the separation of the three amphetamines in a single run within a reasonable analysis time. This is illustrated in Fig. 3a, which shows the chromatogram obtained for a mixture of the compounds of interest, using the Hypersil C_{18} column and a mobile phase of water–acetonitrile (40 + 60) pumped at a flow rate of 0.4 mL min^{-1}. As can be seen, baseline separation is achieved for ephedrine, whereas norephedrine and amphetamine are fairly well separated. Indeed, the resolution for a single amphetamine can be improved and/or the analysis time can be reduced. For example, the time required to achieve a comparable resolution of amphetamine derivatives can be reduced when using the LiChrospher C_{18} column (Fig. 3b), because the flow rate can be increased to 1.0 mL min^{-1} (lower flow rates did not improve the separation while giving very long retention times). This column also provides a complete resolution of pseudoephedrine enantiomers (Fig. 3c). In the latter instance, the resolution was improved by decreasing the mobile phase flow rate. As a compromise, a flow rate of 0.4 mL min^{-1} was selected as the best option for the enantioseparation of this drug. The optimum elution conditions for each amphetamine are summarized in Table 1. which also shows the capacity factors and selectivities achieved under the proposed conditions.

It should be noted that, for a particular concentration of analyte, peak heights (and thus sensitivity) obtained when using a C_{18} analytical column were much higher than those observed when working with the β-CD column. On the basis of the above results, the FMOC-L-Pro approach can be considered the method of choice for the chiral determination of both primary and secondary amphetamines.

Derivatization with FMOC-L-Pro

Conditions for the solution derivatization of the amphetamines with FMOC-L-Pro were investigated, including the reaction time, amount of derivatizing reagent and pH. In this study, the chromatographic conditions used are summarized in Table 1.

As expected, significant reaction between the analytes and FMOC-L-Pro was not observed in the absence of the coupling agent DCC, even when using a large excess of FMOC-L-Pro. In addition, a proper activation of the FMOC-L-Pro reagent was found to be important in order to obtain maximum analyte conversion. This is illustrated for amphetamine isomers in Fig. 4a, which shows the effect of the time elapsed after the mixing of the FMOC-L-Pro and DCC solutions until the addition of the sample (0.25 mL of amphetamine plus 0.25 mL of carbonate buffer) on the analytical response. As can be deduced, derivatizations have to be carried out at least 5 min after the FMOC-L-Pro and DCC solutions have been mixed. For times longer than 5 min, the response remains approximately constant for several hours. However, a decrease of about 8% in the analyte response was observed 24 h after preparation of the reagent, so the derivatizing solution should be prepared daily. On the other hand, the DCC to FMOC-L-Pro concentration ratio must be in the range 8–30 in order to achieve the best conversion yields (Fig. 4b). According to these results, in subsequent experiments the reaction mixture was prepared by mixing equal volumes of DCC and FMOC-L-Pro standard solutions (giving DCC : FMOC-L-Pro ratios of 10). Derivatiza-

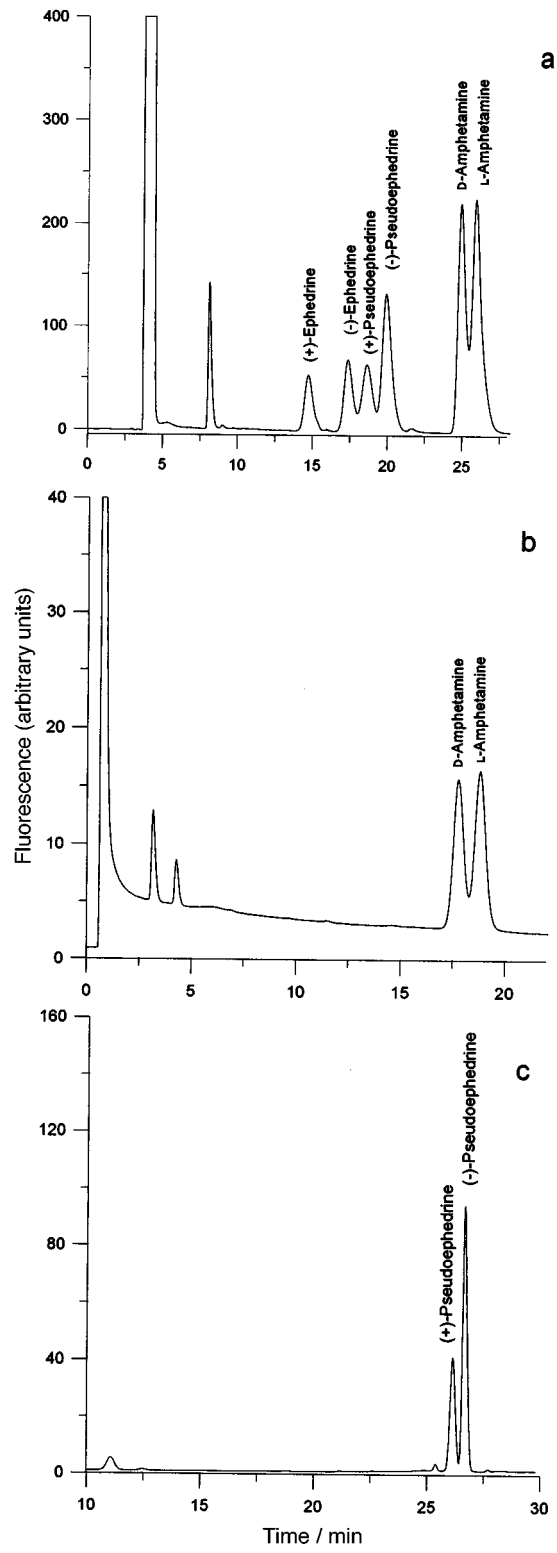

Fig. 3 Chromatogram obtained for (a) a mixture of the amphetamines (100 μg mL^{-1} of each enantiomer) after derivatization with FMOC-L-Pro and separation on a Hypersil C_{18} column; mobile phase: water–acetonitrile (40 + 60) at 0.5 mL min^{-1}. And chromatograms obtained using the LiChrospher C_{18} column for (b) amphetamine [mobile phase: acetonitrile–0.01 phosphate buffer, pH 7.0 (60 + 40) at 1 mL min^{-1}; 10 μg mL^{-1} of each enantiomer], and (c) pseudoephedrine [mobile phase: acetonitrile–water (60 + 40) at 0.4 mL min^{-1}; 50 μg/mL of each enantiomer]. Conditions used for derivatization: FMOC-L-Pro, 10 mM; reaction time, 10 min.

tions were performed at least 10 min after the preparation of the derivatization mixture.

Derivatization of amphetamine. The effect of the mole ratio of FMOC-L-Pro to amphetamine on analyte conversion was investigated in the range 1.5–37.5, for a total concentration of amphetamine of 20 μg mL^{-1} (the highest concentration assayed). The results of this study are shown in Fig. 5a. The derivatization was independent of the mole ratio of FMOC-L-Pro to amphetamine, when this ratio was higher than 7 (equivalent to a FMOC-L-Pro concentration of about 1 mM). The effect of the reaction time was tested in the range 0–10 min (Fig. 5b). For a FMOC-L-Pro concentration of 1 mM, the reaction time necessary to achieve the maximum analytical response was 2 min. On the other hand, it was observed that the pH of the medium has no significant effect on analyte conversion. In fact, the reaction proceeds rapidly in a water–acetonitrile medium, even in the absence of the buffer solution. Compared with the FMOC method (conversion yields of about 100% are achieved in a few seconds when derivatizing with FMOC), the reaction between FMOC-L-Pro and amphetamine is slightly slower, but still very rapid.

Derivatization of ephedrine and pseudoephedrine. As for amphetamine, constant conversion yields were obtained for ephedrine enantiomers (total concentration of 20 μg mL^{-1}) under the same derivatization conditions, but the addition of the buffer was found to be necessary in order to obtain the maximum response. No significant differences in derivatization yields were observed for different pHs within the range 9.5–10.1 (data not shown).

In spite of their structural similarities (see Fig. 1), ephedrine and pseudoephedrine exhibit a different kinetic behaviour. The FMOC-L-Pro to pseudoephedrine concentrations and reaction times required to obtain constant responses for the diastereomers are clearly higher than those required for the ephedrine isomers. This indicates that the reaction between FMOC-L-Pro and pseudoephedrine is more difficult and thus slower. In addition, signals obtained for the (−)-pseudoephedrine derivative were always higher than those observed for the (+)-pseudoephedrine isomer for either UV or fluorescence chromatograms (see Fig. 3). As can be seen in Fig. 6, differences in analytical signals are higher as the FMOC-L-Pro concentration or the reaction time are lower. This suggests that the diastereomers exhibit different reaction constants (due to steric factors), rather than different spectral properties, which agrees with the fact that the UV spectral profiles are identical for both diastereomers.[21]

According to these findings, the FMOC-L-Pro concentration and reaction time must be high enough in order to ensure the maximum analyte conversion yields. In this instance, a FMOC-L-Pro concentration of 10 mM was selected (higher concentrations cause solubility problems) and the reaction time was extended to 5 min. Under such conditions, linear responses for each isomer were obtained in the range 0.5–20 μg mL^{-1}. Moreover, the (−)-pseudoephedrine to (+)-pseudoephedrine peak height ratios obtained for racemic mixtures were approximately constant within the calibration interval (1.47 ± 0.09, n = 10), and no significant differences in the analytical signals were observed between samples containing a single isomer and those containing racemic mixtures of the analyte. This would indicate that, in spite of kinetic resolution, under the selected conditions quantification of pseudoephedrine enantiomers is possible, at least in the concentration interval assayed.

The final conditions used for derivatization of the amphetamines under investigation are summarized in Table 2, which also gives relevant data for linearity, reproducibility and sensitivity obtained under the selected derivatization conditions. As can be deduced, linearity and reproducibility are satisfactory in the concentration interval tested and comparable to those given by most HPLC assays. On the other hand, the limits of detection observed for UV detection are higher than those reported for other UV reagents used in chiral analysis,[7,8] but the sensitivity is comparable to (and sometimes better than) that achieved by other HPLC methods which employ fluorogenic reagents.[11,21,22] This indicates that, under the proposed conditions, no racemization occurs during the derivatization.

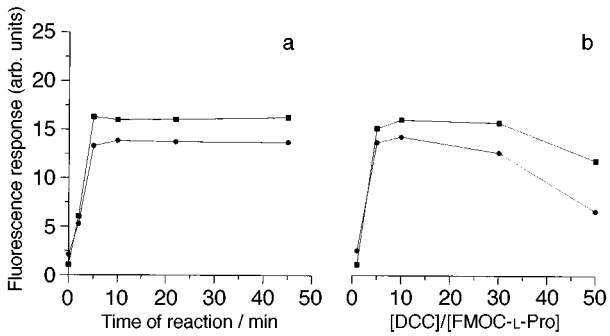

Fig. 4 Effect of (a) the time elapsed after the mixing of the DCC and FMOC solutions before derivatization and (b) the concentration ratio of DCC to FMOC-L-Pro on the analytical response obtained for amphetamine. Concentration of each enantiomer, 10 μg mL^{-1}; reaction time, 2 min; concentration of FMOC-L-Pro, 1 mM; [(■), D-amphetamine; (●), L-amphetamine]. For other experimental details, see text.

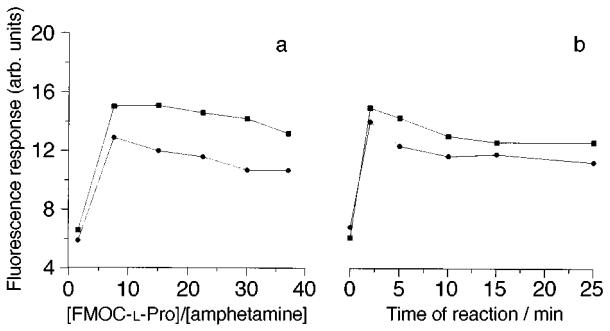

Fig. 5 Effect of (a) concentration ratio of FMOC-L-Pro to amphetamine (reaction time, 2 min) and (b) reaction time on the analytical response obtained for amphetamine (concentration of FMOC-L-Pro, 0.5 mM). Concentration of each enantiomer, 10 μg mL^{-1}; [(■), D-amphetamine; (●) L-amphetamine]. For other experimental details, see text.

Utility of FMOC-L-Pro method

The utility of the described approach in chiral analysis was evaluated by determining ephedrine enantiomers in pharmaceutical preparations. As an example, Fig. 7 shows the chromato-

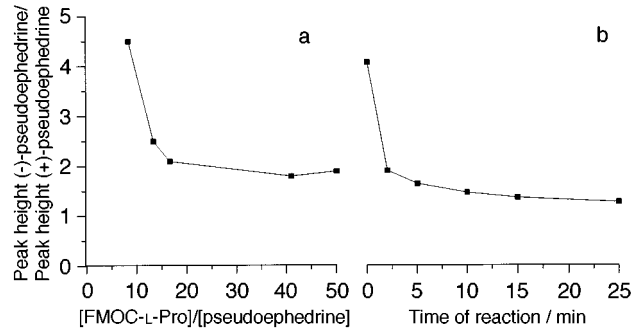

Fig. 6 Effect of (a) concentration ratio of FMOC-L-Pro to pseudoephedrine (for a reaction time, 2 min) on (−)-pseudoephedrine to (+)-pseudoephedrine peak height ratio and (b) effect of the reaction time (for a FMOC-L-Pro concentration of 1 mM) on (−)-pseudoephedrine to (+)-pseudoephedrine peak height ratio. Concentration of each enantiomer, 10 μg mL^{-1}. For other experimental details, see text.

grams obtained for a blank and for one of the formulations analysed, which corresponds to a concentration of (5.05 ± 0.07) and (4.9 ± 0.1) μg mL^{-1} of the (+)-ephedrine and (−)-ephedrine isomers, respectively (n = 3). Compared with the declared amount of drug, the method provided differences of +1.2% (n = 6) and −7% (n = 5) for the two samples analysed. In the latter sample, the amount of ephedrine found was significantly lower than the declared amount (probably due to the age of the pharmaceutical sample analysed), but the results can be considered satisfactory for most applications. Since ephedrine enantiomers are completely resolved, the assay can also be used for enantiomeric purity studies.

Resolution of pseudoephedrine is also satisfactory, whereas for enantiomeric purity studies of amphetamine, calibration graphs with different ratios of enantiomers should be prepared, because a large amount of first-eluting diastereomer could disturb the measurement of the second-eluting diastereomer. In this instance, calibration by using peak heights instead of peak areas is preferable.

Conclusions

Derivatization with chloroformates is a valid alternative for the chiral chromatographic determination of amphetamine and amphetamine-type compounds. Formation of the FMOC derivatives and subsequent separation of the enantiomers on a β-CD chiral stationary phase provides successful resolution of the primary amine amphetamine. However, for the enantiomeric determination of more bulky secondary amines, an indirect method *via* the formation of diastereomers with FMOC-L-Pro is preferable. The FMOC-L-Pro method provides suitable resolution for most applications within fairly short times using conventional C_{18} columns. Indeed, the choice of the chromatographic conditions depends on the amphetamine to be determined.

For amphetamine, the resolution achieved with the FMOC-L-Pro approach is worse than that reported for other options. Therefore, the described procedure may be inadequate for enantiomeric purity studies. However, calibration by using peak heights allows the analysis of samples containing similar concentrations of each isomer (racemic samples, for example). Moreover, the proposed procedure offers some advantages over previously reported procedures. For example, compared with assays involving derivatization with OPA and *N*-acetyl-L-cysteine,[11] the present method provides better sensitivity in the determination of amphetamine isomers. Moreover, FMOC-L-Pro also reacts with secondary amphetamines (which cannot be determined with OPA in pre-column mode). In addition, since the FMOC-L-Pro tag is highly fluorescent, the proposed method provides better sensitivity and selectivity than those achieved with other reagents commonly used for the determination of primary and secondary amines (for example, methods using 3,5-dinitrobenzoyl chloride for derivatization and a Pirkle-type column for separation,[7,8] because the derivatives formed are not fluorescent). Hence, the FMOC-L-Pro method combines the

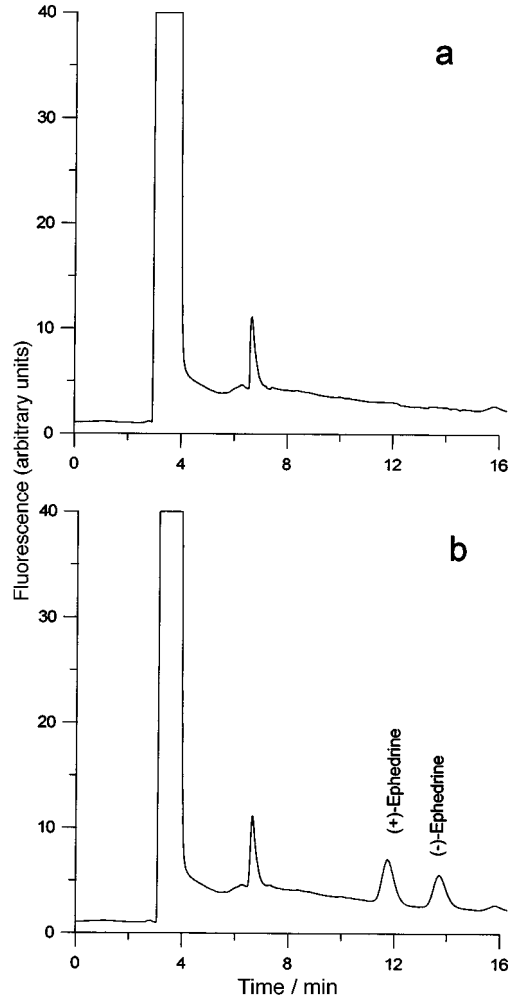

Fig. 7 Chromatograms obtained for (a) a blank (water) and (b) a pharmaceutical sample containing ephedrine. Conditions used for derivatization and separation are summarized in Table 2.

Table 2 Derivatization conditions and analytical data for the determination of amphetamines

Compound	Derivatization conditions[a]		Linearity[b] (n = 10)			Limit of detection/ ng mL^{-1}	
	Concentration of FMOC-L-Pro/mM	Reaction time/ min	$y = ax + b$	R^2	Within-day precision[c] (%) (n = 6)	UV	Fluorescence
D-Amphetamine	1	2	a = 7.93 ± 0.01 b = 10 ± 14	0.98	3	100	10
L-Amphetamine			a = 7.27 ± 0.01 b = −2 ± 8	0.990	3	100	10
(−)-Ephedrine	1	2	a = 5.69 ± 0.07 b = 21 ± 13	0.9990	5	100	25
(+)-Ephedrine			a = 6.4 ± 0.1 b = 18 ± 6	0.993	4	100	25
(+)-Pseudoephedrine	10	5	a = 4.48 ± 0.04 b = 0.6 ± 0.4	0.995	7	500	50
(−)-Pseudoephedrine			a = 4.55 ± 0.04 b = 1.3 ± 0.9	0.990	9	250	25

[a] 0.50 mL of sample + 0.50 mL of derivatizing reagent for amphetamine, and 0.25 mL of sample + 0.25 mL of buffer + 0.50 mL of derivatizing reagent for ephedrine and pseudoephedrine. [b] Five data points in duplicate. [c] Determined at half the highest concentration in the range tested.

high sensitivity provided by chloroformates with the possibility of resolving the isomers of secondary amphetamines.

Compared with the derivatization with FMOC, the FMOC-L-Pro method requires longer reaction times in order to obtain maximum analyte conversion yields, especially for pseudoephedrine. However, no significant differences in analyte sensitivity are observed between the FMOC and FMOC-L-Pro methods, when working under optimized conditions. Compared with other chiral chloroformates such as (+)-1-(fluorenyl)ethyl chloroformate, FMOC-L-Pro involves shorter reaction times;[20] theoretically, FMOC-L-Pro should be superior to the reagent (−)-menthyl chloroformate because its bulky and rigid fluorenyl group should produce better enantioselectivity and detectability.[23]

In spite of the kinetic resolution encountered for pseudoephedrine, a proper selection of the derivatization conditions leads to a linear response in the concentration interval studied. Therefore, the described assay can be used for quantitative purposes.

Acknowledgements

The authors are grateful to the CICyT for financial support received for the realisation of Project SAF 95-0586.

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