Separation of Amino Acid Enantiomers and Chiral Amines Using Precolumn Derivatization with (+)-1-(9-Fluorenyl)ethyl Chloroformate and Reversed-Phase Liquid Chromatography

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A new chiral reagent (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) was synthesized for separation of amino acid enantiomers and optically active amines. Highly fluorescent diastereomers of amino acids were obtained without racemization within 4 min at room temperature. The derivatives have favorable chromatographic properties, which is demonstrated by a reversed-phase separation of the D and L form of 17 primary amino acids in a single run. The secondary amino acids were resolved separately at a lower pH and can be determined without interferences from primary amino acids. The stability of the derivatives permitted confirmation studies with gas chromatography–mass spectrometry.

Amino acids and optically active amines form a large group of compounds of both biochemical and pharmaceutical interest (*1–3*). D-Amino acids are rare in nature compared with L-amino acids and require both sensitive and selective methods for their determination. The stereoisomers of racemic drugs often differ in pharmacological activity and the determination of these in biological systems is important, as is the assessment of optical purity. Much of the progress made in analyzing these substances is due to the rapid development of liquid chromatography and new derivatization procedures which enhance detectability.

Since the first report of chromatographic separation of optical isomers (*4*), a large number of methods have been introduced (*5, 6*). The methods can be divided into three groups: direct separation on chiral columns, separation on achiral columns with chiral mobile phases, and separation of diastereomers formed by precolumn derivatization with chiral reagents. Development of chiral columns has recently been reviewed (*7–9*), and a number of columns are commercially available (*10*). Large separation factors (α) are obtainable in certain cases, but many columns have only moderate efficiency and a number of compounds require derivatization prior to separation, e.g., primary amines (*11*). Chiral mobile phases have been made mainly by complexation of chiral ligands with a transition-metal ion (*12*) or with chiral ion-pairing agents (*13*). Detection is enhanced by precolumn or postcolumn derivatization (*14, 15*). Separation methods based on indirect separation of enantiomers by precolumn derivatization with chiral reagents have been reviewed recently (*9, 16*). The most commonly used reagents for amine compounds are based on isothiocyanate, which give UV-sensitive thiourea derivatives of both primary and secondary amines (*17*). The common protein amino acids have been resolved by using 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (*18*). The reagent has also been used for separation of amines (*19*). α-Methoxy-α-methyl-1-naphthaleneacetic acid is a fluorescent reagent that has been used for separation of amino acid methyl esters (*20*). More recently reported fluorescence reagents are based on *o*-phthalaldehyde (OPA) and cysteine, the amine function of cysteine being blocked with either an acetyl group (*21, 22*) or a *tert*-butyloxycarbonyl group (*23*). The reaction is selective for primary amines and proceeds under mild conditions. Because the products have limited stability, the period between derivatization and injection must be kept constant, which has promoted automation of the procedure (*23*).

The aim of this work was to develop a new reagent for chiral derivatization of primary and secondary amino acids, as well as amines, that would give stable, fluorescent, and easily separable products. It has been reported that 9-fluorenylmethyl chloroformate (FMOC-Cl) is a suitable reagent for precolumn derivatization of primary and secondary amino acids (*24–26*) and amines (*27*). The reaction conditions are mild and the products are fluorescent and stable.

This paper describes the synthesis of a new chiral reagent, related to FMOC-Cl. The reaction with amino acids was studied and compared with the FMOC-Cl reagent. The reagent was used for separation of amino acid enantiomers and chiral amines.

EXPERIMENTAL SECTION

Apparatus. The chromatographic system consisted of a Varian 5000 gradient delivery system and a Valco injection valve, fitted with a 8-μL loop. The derivatives were monitored with a Schoeffel Model FS 970 fluorescence detector, equipped with liquid filters (*24*). The excitation wavelength was set at 260 nm. The columns, 150 × 4.6 mm and 250 × 4.6 mm, were packed with 3- and 5-μm Spherisorb octyl materials, respectively. Gas chromatography–mass spectrometry analysis was performed with a Finnigan Mat 1020 instrument. The NMR spectra were obtained with a Bruker WP 80CW spectrometer.

Chemicals. Acetonitrile, tetrahydrofuran, and acetone were purchased from Rathburn (Walkerburn, U.K.). The amino acid standards and the FMOC-Cl reagent were obtained from Sigma (St. Louis, MO). Fluorene, BuLi (1.6 M in hexane), D-camphor-10-sulfonyl chloride, $LiAlH_4$, and phosgene were from Fluka (Bucks, Switzerland). (+)-1-(9-Fluorenyl)ethyl chloroformate is available from Eka Nobel AB (Sweden).

Synthesis of 1-(9-Fluorenyl)ethanol. To a solution of 8.3 g of fluorene in 100 mL of dry ether, 31 mL of BuLi (1.6 M in hexane) was added. The mixture was refluxed for 30 min, and then cooled in an ice bath. To the resulting mixture a solution of 2.8 mL of acetaldehyde in 40 mL of dry ether was added over a 15-min period and then refluxed for 1 h. Water (100 mL) was added and the ether layer collected in a separatory funnel, dried ($MgSO_4$), and evaporated. The product was further purified by flash chromatography and recrystallized from ligroin (bp 80–100 °C) to give white needles: mp 101–103 °C, mass spectrum M^+ m/z 210.15; ^1H NMR ($CDCl_3$) 0.87 (d, 3 H), 1.70 (s, 1 H), 4.09 (d, 1 H), 4.35–4.65 (m, 1 H), 7.15–7.85 (m, 8 H).

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Optical Resolution of 1-(9-Fluorenyl)ethanol. To a solution of 4.0 g of 1-(9-fluorenyl)ethanol in 25 mL of anhydrous pyridine, an equimolar amount of D-camphor-10-sulfonyl chloride (4.12 g) was added, and the mixture was stirred at room temperature for 3 h. The solution was poured into ice water and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with dilute hydrochloric acid, dried ($MgSO_4$), and evaporated to dryness. The crude ester was recrystallized several times from EtOH to give the less soluble diastereoisomeric ester in pure form. The optical purity of the diastereomeric ester was checked on a chiral stationary phase ((−)-dinitrobenzoylphenylglycine coupled to aminopropylsilica, Pirkle's phase) and was found to be >99%.

Hydrolysis of the Ester. To a solution of the optically pure diastereoisomeric ester (1.0 g) in 50 mL of dry ether, 0.8 g of $LiAlH_4$ was added. The mixture was stirred at room temperature for 1 h giving the alcohol after work up, mp 91–93 °C dec.

(+)-1-(9-Fluorenyl)ethyl Chloroformate (FLEC). To a solution of phosgene (0.8, 8.1 mmol) in 15 mL of dry toluene cooled to 0 °C, a solution of optically pure alcohol (0.44 g, 2.1 mmol) and triethylamine (0.30 mL, 2.1 mmol) in 20 mL of dry toluene was added dropwise. After addition was completed, stirring was continued for 2 h at 0 °C. The triethylamine hydrochloride was then removed by filtration and the filtrate was concentrated at reduced pressure to give an oil. $[\alpha]_D^{25} = +67.9°$ (CH_2Cl_2, C = 1); $[\alpha]_{578}^{25} = +70.5°$ (CH_2Cl_2, C = 1). ^1H NMR ($CDCl_3$): 0.76 (d, 3 H), 4.30 (d, 1 H), 5.47–5.75 (m, 1 H), 7.18–7.75 (m, 8 H). Anal. Calcd for $C_{16}H_{13}O_2Cl$: C, 70.46; H, 4.80; Cl, 13.00. Found: C, 70.64; H, 4.80; Cl, 13.13.

Derivatization Procedures. For derivatization of amino acids, 0.4 mL of sample was mixed with 0.1 mL of borate buffer (1 M, pH 6.85) in a 3-mL reaction vial. Then 0.5 mL of the FLEC reagent (15 mM in acetone/acetonitrile, 3/1) was added and after 4 min the reaction mixture was extracted with pentane to remove excess reagent. The extraction was repeated twice, and then the aqueous phase was ready for injection.

For selective derivatization of secondary amino acids 0.9 mL of sample and 0.1 mL of borate buffer (0.8 M, pH 9.5) were mixed with 0.1 mL of the OPA reagent (50 mg of OPA and 25 μL of mercaptoethanol per mL, in acetonitrile) and allowed to react for 30 s. Then 0.1 mL of iodoacetamide (140 mg/mL in acetonitrile) was added and 30 s later 0.3 mL of the FLEC reagent was added (5 mM in acetone). After 2 min the solution was extracted with pentane. The extraction was repeated twice and the pentane phases were discarded. The sample was then injected on the column.

Amine derivatives were prepared by mixing 50 μL of sample with 10 μL of borate buffer (1 M, pH 7.85) and 50 μL of the FLEC reagent (1 mM in acetone). After 30 min of reaction time, 20 μL of 12 mM hydroxyproline was added and 2 min later the solution was ready for injection.

For methylation of FLEC–amino acid derivatives, the aqueous sample was acidified with hydrochloric acid, and then the derivative was extracted into ether. Methyl esters were prepared by reaction with diazomethane generated from *N*-methyl-*N*-nitroso-*N*-nitroguanidine as described by Fales et al. (28).

Reaction Rate Measurements. The rate of the reaction of the amino acids with the FLEC reagent was determined by derivatization of the amino acids (one at a time) at pH 8.0 (sample plus buffer). After a certain time interval the reaction was stopped by adding acetic acid, and the excess of the FLEC reagent was removed by pentane extractions. The amount that remained unreacted was determined by precolumn derivatization with *o*-phthaldehyde/mercaptoethanol, followed by liquid chromatography. The results were compared with a solution treated in the same way, but without the FLEC reagent (original amount of amino acid), from which the conversion to a FLEC-derivative could be calculated. Each value is the mean of two measurements.

The relative reaction rates of the FLEC and the FMOC-Cl reagents were determined for valine, glutamic acid, proline and lysine. The yield obtained when a standard amino acid solution was derivatized with each of the reagents was compared with the results when a mixed FLEC/FMOC reagent was employed. The derivatives were separated by chromatography and the peak areas compared.

Chromatography Procedures. For the separation of the prolines (Figure 2), a 250 × 4.6 mm column packed with 5-μm Spherisorb octyl material was used. The elution was carried out with acetonitrile and phosphoric acid (0.1 M). The eluent was varied linearly from 37% to 45% acetonitrile over 2 min, with an initial delay for 6 min at 37% acetonitrile. The flow rate was 1.5 mL/min.

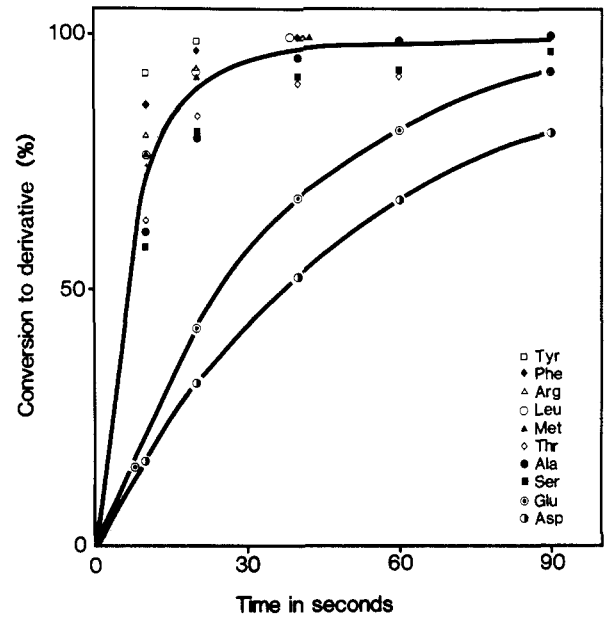


Figure 1. Reaction rate of alanine, arginine, aspartic acid, glutamic acid, leucine, methionine, phenylalanine, serine, threonine, and tyrosine with the FLEC reagent. The uppermost curve is drawn through a mean for the amino acids (except aspartic and glutamic acid).

(*S*)- and (*R*)-Metoprolol (Figure 3) were separated on the same column as the prolines, under isocratic conditions; 60% acetonitrile and 40% water. The flow rate was 2 mL/min.

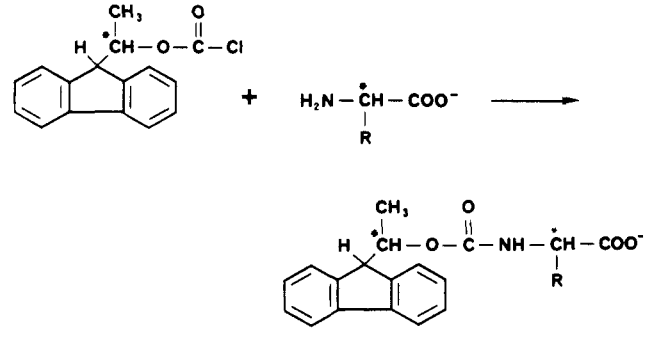
For separation of amino acids (Figure 5) a 150 × 4.6 mm column packed with 3-μm Spherisorb octyl material was employed. The mobile phase consisted of acetonitrile (ACN), tetrahydrofuran (THF), and an acetic acid buffer (1.8 mL of glacial acetic acid in 1 L of water; pH adjusted to 4.35 with NaOH): gradient, 0–8 min, 8% ACN, 17% THF, 75% buffer (8/17/75); 8–22 min, 8/17/75 to 0/30/70; 22–70 min, 0/30/70 to 0/50/50. Flow rate was 0.8 mL/min.

The capacity factors (k') and the separation factors (α) of the amino acid derivatives were evaluated under isocratic conditions (Table I), using the same column as for the prolines.

The compositions of the eluents were (A) 35% THF and 65% acetic acid buffer (3 mL of glacial acetic acid in 1 L of water, pH 4.35), (B) 40% THF and 60% acetic acid buffer, (C) 45% THF and 55% acetic acid buffer, and (D) 37% acetonitrile and 63% phosphoric acid (0.1 M).

RESULTS AND DISCUSSION

Derivatization Procedures. The reaction of the FLEC reagent with amino acids occurs at room temperature under basic conditions in an aqueous solution. When the reaction is complete, the excess reagent and the hydrolysis byproduct are removed from the aqueous phase by pentane extractions and the sample is then ready for separation. In order to



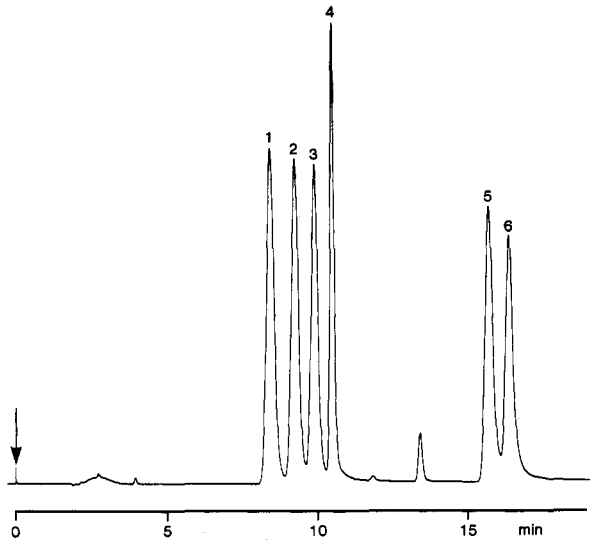


Figure 2. Separation of (1) *trans*-4-hydroxy-L-proline, (2) *trans*-4-hydroxy-D-proline, (3) *cis*-4-hydroxy-L-proline, (4) *cis*-4-hydroxy-D-proline, (5) L-proline, and (6) D-proline in an equimolar mixture of 15 primary amino acids. The derivatization was carried out on *trans*-4-hydroxy-L-proline, *cis*-4-hydroxy-L-proline, and L-proline with a racemic FLEC reagent.

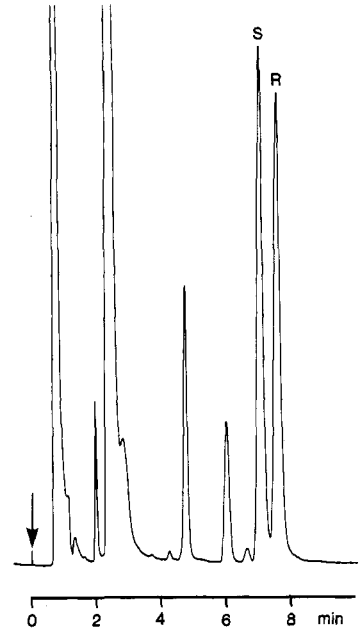


Figure 3. Separation of (*S*)- and (*R*)-metoprolol.

measure the rate of derivative formation, the reaction was stopped after certain time intervals by lowering the pH. The excess FLEC reagent was then removed from the reaction solution by extractions, and the amount of the amino acid that remained unreacted was determined by precolumn derivatization with OPA/mercaptoethanol. The advantage of this approach, used previously by Ahnfelt and Hartvig (*29*), is that the absolute yield in the reaction is measured. The reaction rates for 10 amino acids are shown in Figure 1. The measurements were made at pH 8.0 (sample plus buffer). Most of the amino acids were fully derivatized in 60 s. The fastest reaction was with the aromatic amino acids, but the acid amino acids, aspartic and glutamic acid, reacted significantly more slowly. A comparison was made of the reaction rate of the FLEC reagent and the FMOC-Cl reagent. The comparison included four amino acids: glutamic acid, lysine, proline, and valine. The FMOC-Cl reagent was found on the average to react faster by a factor of 2.8. The difference in reactivity is probably due to steric hindrance, caused by the additional methyl group. No significant difference in fluorescence response was found between the derivatives of the two reagents.

A method has previously been described for selective determination of secondary amino acids by reaction of primary amino acids with OPA/mercaptoethanol and subsequent derivatization of secondary amino acids with FMOC-Cl (*25*). This method was modified and used with the FLEC reagent: the reaction time was increased, and the ether which is used in the extraction step was replaced by pentane. Ether removes the hydrolysis byproduct more efficiently, but loss of proline was observed, due to higher lipophilicity of the FLEC derivatives. The chromatogram in Figure 2 illustrates the imino acids derivatized in an equimolar solution of 15 primary amino acids. The selectivity of the method has the advantage that low concentrations of imino acids can be determined in the presence of high concentrations of primary amino acids. The imino acids in Figure 2 were derivatized with a racemic FLEC reagent since 4-*trans*-D-hydroxyproline was not available. The elution order was confirmed by separate runs of the other isomers, using an optically pure reagent.

The method used for analyzing amino acids is quite selective for compounds having an ionizable group as well as the amine functional group, because hydrophobic amine derivatives are removed in the extraction step. In order to determine these amines, other methods for removing the reagent excess have to be employed. The problem can be overcome by subsequent reaction with an excess of a hydrophilic amino acid, which consumes the reagent. Figure 3 illustrates a separation of metoprolol, where the reagent excess has been derivatized with hydroxyproline. The hydroxyproline derivative elutes in the beginning of the run, followed by the hydrolysis byproduct. There are no traces of the reagent left, which would otherwise interfere with the metoprolol derivatives.

Optical Purity. The risks involved in using chiral reagents to determine optical isomers, as opposed to direct resolution methods, include the possibility of racemization during the derivatization and limited optical purity of the reagent. Incomplete yield in the reaction combined with different reaction rates of the isomers can also cause errors. It is therefore crucial that the reaction proceeds quantitatively under mild conditions, and that the reagent is obtainable in high optical purity. The combined effect of these factors was evaluated by derivatizing an L-glutamine standard and measuring the concentration of D-glutamine obtained. The results are shown in Figure 4. The injected amount of L-glutamine was 7.9 nmol. The D-glutamine peak was calculated to be 4.8 pmol, by standard addition of D-glutamine. This amounts to 0.06% of the L-glutamine and is similar to the proportion of D-glutamine in a commercial L-glutamine standard (0.09% of D-Gln) reported by Takaya et al. (*30*). It can therefore be concluded that racemization during the derivatization is negliglible and the FLEC reagent is of high optical purity.

Chromatography. The advantages of using reversed-phase chromatography for separation of amino acid derivatives are that water samples can be directly injected and the carboxylic group does not have to be derivatized. The retention and the separation factors (α) of the amino acid enantiomers are listed in Table I. Good separation was generally obtained for all the enantiomers. The separation factor was found to increase with increased hydrophobicity of the derivatives. All amino acid enantiomers except the imino acids were separated with THF and an acetic acid buffer at pH 4.35. The D enantiomer eluted consistently before the L enantiomer, which is an advantage because the L enantiomer is the dominant component in most samples. This is illustrated in Figure 4 for D- and L-glutamine. The chromatographic behavior of carbamate diastereomers in a normal phase system has been studied by Pirkle and Hauske (*31*). Such studies have not been done on

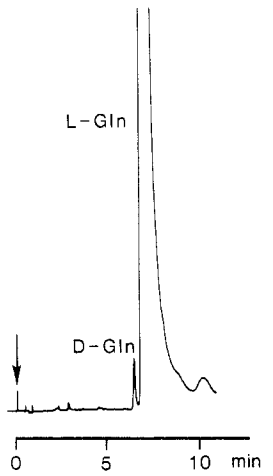


Figure 4. Separation of an L-glutamine standard. The D-glutamine peak amounts to 0.06% of the L-glutamine.

reversed-phase systems but the resolution of the diastereomers is generally considered to depend on the proximity of the chiral centers and the rigidity of their conformation. Even if the chiral centers in amides are separated by two atoms compared with three in the carbamates, diastereomeric amide derivatives of amino acid methyl esters separate poorly with reversed-phase chromatography (*20*). Although esters are not strictly comparable with free carboxylic acids, the bulky, planar fluorene moiety seems to have an important role in the resolvability of the FLEC diastereomers. Good selectivity was obtained in the separation of different amino acids. Figure 5 shows a separation of 17 primary amino acids in D and L form, on a conventional 15-cm column packed with 3-μm reversed phase material. A simple linear gradient with tetrahydrofuran was employed, mixed with acetonitrile in the beginning to facilitate the separation of asparagine and glutamine. Improved peak shape and increased quantum yield of the bis(histidine) derivative was obtained when THF was used, compared with separations using acetonitrile (*24*).

Different separation conditions are required for separation of the imino acid enantiomers. Excessive band broadening of FMOC–prolines compared with other FMOC amino acid derivatives has been noted before (*25*). The same phenomenon has been reported for proline-containing dipeptides, with proline at the carboxy terminal (*32*). There it was explained as being caused by slow kinetics of interconversion between the cis and trans forms of the peptide bond, which led in the case of alanyl–proline to the separation of the two forms. By protonation of the carboxylate group the relaxation rate of

Table I. Capacity Factors (k') and Separation Factors (α) of Amino Acids Derivatized with FLEC

amino acid	k'(D)	k'(L)	α	mobile phase[a]
asparagine	1.34	1.46	1.09	A
glutamine	1.29	1.46	1.13	A
serine	2.01	2.09	1.04	A
aspartic acid	2.37	2.54	1.07	A
threonine	2.94	3.31	1.13	A
glutamic acid	3.26	3.69	1.13	A
arginine	3.54	4.03	1.14	A
alanine	5.63	6.20	1.10	A
proline	5.51	5.51	1.00	A
tyrosine	3.29	3.91	1.19	B
methionine	3.57	4.29	1.20	B
phenylalanine	4.83	6.09	1.26	B
valine	5.00	5.91	1.18	B
tryptophan	6.00	6.71	1.12	B
cysteine	7.90	11.53	1.46	B
isoleucine	8.17	9.69	1.19	B
leucine	8.46	10.20	1.21	B
histidine	3.37	4.77	1.42	C
ornithine	5.37	6.69	1.25	C
lysine	7.23	8.37	1.16	C
4-*trans*-hydroxyproline	5.51	4.94	1.12	D
4-*cis*-hydroxyproline	6.50	5.94	1.09	D
proline	19.57	17.71	1.10	D

[a] For eluent composition see Experimental Section.

isomerization increases (*33*), and the cis:trans ratio for acetyl–proline changes from 50% to 20% of the cis conformer (*34*). With the pH of the mobile phase lowered, a good separation of the imino acid enantiomers was achieved. The separation shown in Figure 2 was obtained with 0.1 M phosphoric acid and acetonitrile. The α values are comparable with the primary amino acids (Table I), but the elution order is reversed; the L isomer is first to elute.

An important area of application is the separation of pharmaceuticals in synthetic mixtures and biological fluids. Many drugs are primary or secondary amines that can be derivatized with the FLEC reagent. The following cardiovascular drugs were separated in a reversed-phase system with acetonitrile as an organic modifier (the separation factor given in parentheses): atenolol (1.1), propanolol (1.06), α-hydroxymetoprolol (1.09), tocainide (1.06), and metoprolol (1.1). The separation of metoprolol is depicted in Figure 3. This work is being continued in our laboratory and will be reported later.

Confirmation Studies with GC–MS. High thermal stability of carbamates has been demonstrated by the use of gas chromatography for separation of amines derivatized with

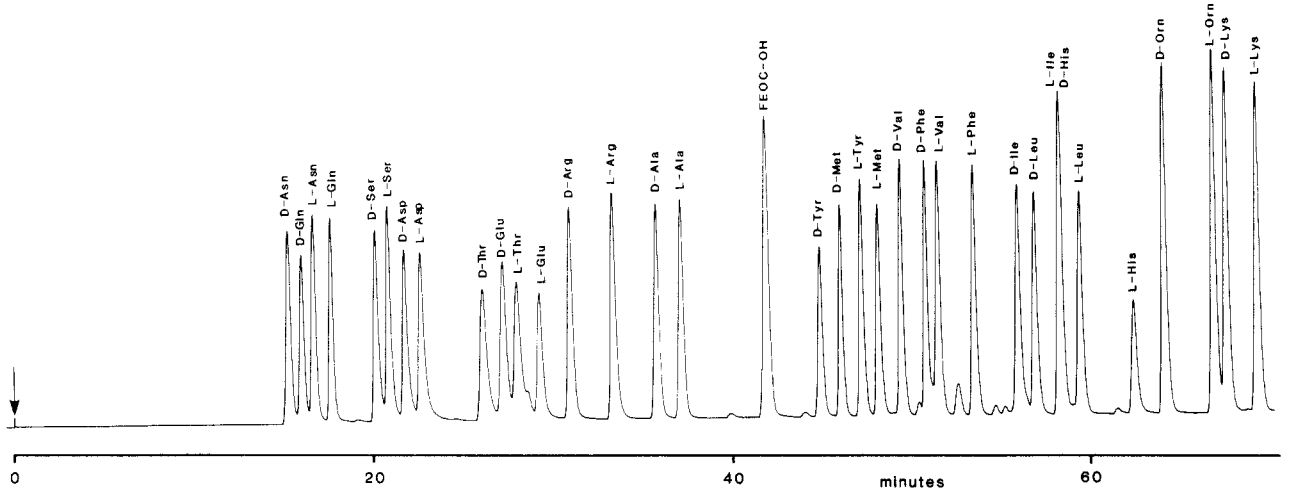


Figure 5. Separation of a standard solution of D- and L-amino acids.

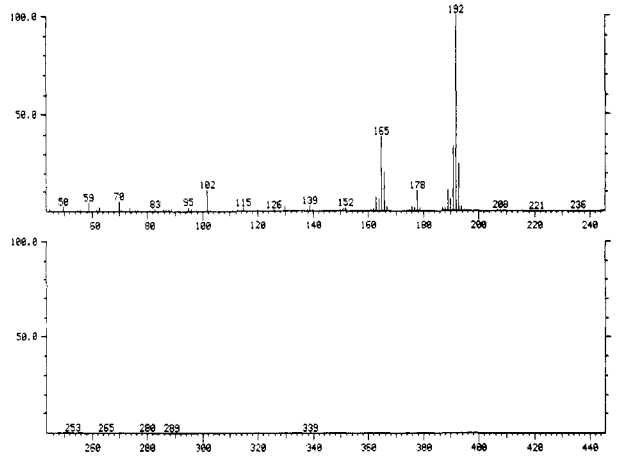


Figure 6. Mass spectrum of a methylated FLEC–alanine derivative.

chloroformate reagents (*35*, *36*). Westley and Halpern (*37*) used (−)-menthyl chloroformate for making diastereomeric carbamate derivatives of amino acid methyl esters which then were separated by gas chromatography at about 200 °C. FLEC derivatives of amino acids could be separated by gas chromatography after methylation of the carboxyl moiety with diazomethane. Electron impact ionization (EI) mass spectra were obtained to confirm the expected structure of the derivatives. The mass spectrum of FLEC–Ala–Me with molecular ion m/z 339 is shown in Figure 6. The spectrum is dominated by 9-fluorenylethylene m/z 192 which stems from the labeling agent.

Mass spectrometry is an important technique for confirming the identity of unknown compounds. Because FLEC derivatives are amenable to GC–MS analysis, it is possible to collect unknown compounds after separation by HPLC and then use GC–MS for identification.

CONCLUSIONS

The determination of amino acid enantiomers requires both adequate resolution of the enantiomers and good selectivity in the separation of different amino acids. The separations in Figures 2 and 5 show the potential of the FLEC reagent for solving such problems. Application of the reagent in combination with a high-resolution chromatography system would enable screening of D-amino acids in complex matrices such as biological fluids. This would be an interesting research project as the rather limited distribution of D-amino acids in nature inevitably reflects the screening methods (*38*).

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