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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ENANTIOMERIC AMINO ACIDS AND AMINO ALCOHOLS AFTER DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE AND VARIOUS CHIRAL MERCAPTANS

APPLICATION TO PEPTIDE HYDROLYSATES

R. H. BUCK* and K. KRUMMEN
Analytical Research and Development, Pharmaceutical Department, Sandoz Ltd., CH-4002 Basle (Switzerland)

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SUMMARY

o-Phthaldialdehyde in combination with a chiral mercaptan is a powerful chiral reagent for the pre-column derivatization of many enantiomeric compounds bearing primary amino groups. The diastereoisomers formed can efficiently be resolved on conventional reversed-phase columns. Simultaneous determination of the enantiomers of various amino acids, amino alcohols and biogenic amines was achieved by gradient elution and fluorescence detection. The resolution was optimized by varying the chiral mercaptan in the reagent, Boc-L-cysteine, N-acetyl-L-cysteine and N-acetyl-D-penicillamine being used for this purpose. The resolutions were calculated. Most of the enantiomers showed good resolution with each of the three chiral mercaptans, whereas some enantiomers were only separable by one or two of them. The method was applied to the analysis of peptide hydrolysates. The composition of peptides bearing L- and D-amino acids and an amino alcohol was determined.

INTRODUCTION

In recent years, the determination of the enantiomeric purity of chiral compounds by high-performance liquid chromatography (HPLC) has become of great importance in biochemical research and pharmaceutical chemistry. The increased interest in stereochemical aspects of pharmacological activity and drug design has led to the development of new specific and sensitive methods for the determination of enantiomers, including detection and quantification of one enantiomer in the presence of a large excess of the other. Two different chromatographic principles have been established for this purpose. The so-called direct chromatographic resolution uses either a chiral bonded stationary phase or chiral additives in combination with a non-chiral stationary phase. With the direct method the samples do not have to be derivatized, but for many applications derivatization with a non-chiral reagent is

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used to improve the chromatographic behaviour or the detection. The indirect enantiomeric separation method uses a derivatization step with a chiral reagent prior to chromatography. The diastereoisomers formed thereby can often be separated on standard achiral stationary phases. This method requires highly purified derivatization agents and reaction conditions such that no racemization occurs.

The type of reagent must be chosen according to the structure and the functional groups of the sample molecule, *e.g.*, various tartaric acid anhydrides have been used for derivatization of alkanolamines[1]. Norephedrine, norepinephrine and amphetamines were derivatized with acetylglycosyl isothiocyanates[2,3], 4-nitrophenylsulphonylprolyl chloride[4] or 4-nitrophenylsulphonylprolinamide[5]. Amino acids can be derivatized with Boc-L-Leu-hydroxysuccinimide esters[6], acetylglycosyl isothiocyanates[7] or, as in our laboratory, with the *o*-phthaldialdehyde (OPA) reagents OPA-Boc-L-cysteine or OPA-N-acetyl-L-cysteine[8]. The latter agents are modifications of the well known derivatization reagent OPA-2-mercaptoethanol. They react with the primary amino groups of amino acids to form highly fluorescent isoindole derivatives, as shown in Fig. 1. This derivatization principle is not limited to amino acids, but can also be applied to chiral amino alcohols or biogenic amines such as norephedrine and norepinephrine. Further, the derivatization product can be modified by using other chiral mercaptans such as N-acetyl-D-penicillamine.

Our interest in the determination of enantiomers of amino acids and various amino alcohols and in particular the confirmation of the stereochemical structure of pharmaceutical peptides has led us to investigate this method further.

EXPERIMENTAL

Apparatus

The instrumentation consisted of two Altex Model 100 A pumps, controlled by a 420 microprocessor system from Altex and a Kontron SFM 23 LC spectrofluorimeter for detection. A Hewlett-Packard HP 3357 data system was used for data processing.

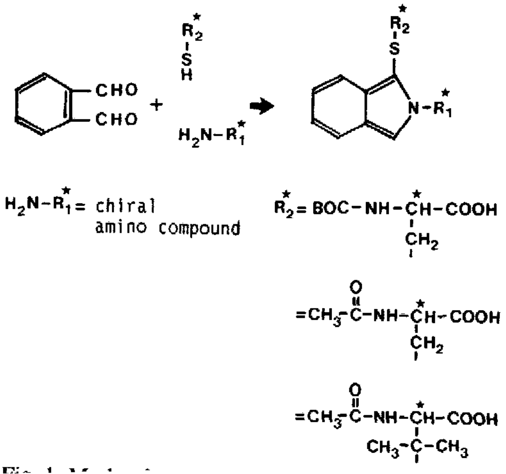

Fig. 1. Mode of reaction of OPA with a chiral mercaptan and a chiral amino compound.

Chemicals

Methanol (HPLC grade) and tetrahydrofuran (analytical-reagent grade) were obtained from Rathburn (Walkerburn, U.K.), the amino acids from Sigma (London, U.K.) or Fluka (Buchs, Switzerland) and L- and D-phenylalaninol (Phe-ol), L- and DL-alaninol (Ala-ol), L- and D-valinol (Val-ol), (*S*)- and (*R*)-1-amino-2-propanol (1-AP-ol), (*S*)- and (*R*)-2-amino-1-butanol (2-AB-ol), (±)-2-aminopentane, Boc-S-benzyl-L-cysteine, N-acetyl-D-penicillamine (NAP) and N-acetyl-L-cysteine (NAC) from Fluka. *o*-Phthaldialdehyde (OPA) and all other chemicals were purchased from Merck (Darmstadt, F.R.G.). Water was distilled twice before use. L- and D-threoninol (Thr-ol) and L- and D-*allo*-threoninol (*allo*-Thr-ol) were synthesized by reduction of L- and D-threonine or L- and D-allo-threonine respectively. Boc-L-cysteine (N-*tert.*-butyloxycarbonyl-L-cysteine) (BocC) was prepared from Boc-S-benzyl-L-cysteine by reduction with sodium in liquid ammonia and extraction in ethyl acetate according to ref. 9, and was kept in a deep-freezer. Octreotide and [D-Cys^2]-octreotide were synthesized in the research and development laboratories of Sandoz Ltd.; octreotide is an octapeptide with pharmaceutical activity[10].

Derivatization reagents

A 30-mg amount of OPA was dissolved in 1 ml of ethanol and 22 ml of 0.4 *M* sodium borate buffer (pH 10). About 30 mg of the chiral mercaptan were added to this solution. The reagents were freshly prepared every other day.

Preparation of amino acid and amino alcohol standards

Stock solutions of the individual enantiomers of the amino acids and amino alcohols were prepared in water at a concentration of 40 μmol/ml. As D-Ala-ol was not available in an optically pure form, the racemic mixture was used at a concentration of 80 μmol/ml. Standard mixtures used for calibration during the quantitative analysis were prepared by combining the appropriate stock solutions followed by dilution with water to yield a final concentration of 3.3 μmol/ml for each individual component.

Protection of cysteine

If cysteine had to be determined, the following sample pre-treatment was carried out to protect the thiol group of cysteine. A 1-ml volume of sample or standard solution was treated with 100 μl of 20 m*M* dithioerythritol to reduce any cystine to cysteine. Then 100 μl of 400 m*M* methyl iodide solution in water–methanol (50:50, v/v) and 200 μl of 3 *M* aqueous sodium hydroxide solution were added and the solution was allowed to stand for 10 min, followed by the addition of 200 μl of 3 *M* hydrochloric acid and, after briefly mixing, 400 μl of 0.4 *M* sodium borate buffer (pH 10). The pH was checked to ensure it was *ca.* 10.

Derivatization procedure

A 100-μl volume of the standard solution or the peptide hydrolysate was mixed with 400 μl of the chosen derivatization reagent. After exactly 10 min, the solution was injected into the HPLC system. In order to determine the change in fluorescence response with time, the derivatization time was varied from 0.5 to 30 min.

Hydrolysis of peptides

A 0.5–5-mg amount of the peptide hydrolysate was transferred into a hydrolysis tube and dissolved in 1 ml 6 *M* hydrochloric acid containing *ca.* 1% of indolylpropionic acid to avoid oxidation of Trp and Cys. The tube was sealed under vacuum and heated at 115°C for 4 h. After hydrolysis, the hydrochloric acid was removed by evaporation and the resulting residue dissolved in 10–100 ml of water. From these solutions aliquots were used for the enantiomer analysis.

Chromatography

The following reversed-phase HPLC columns were used for chromatographic separations: Spherisorb ODS II, 3 μm (12 cm × 4.0 mm I.D.) from Knauer (Berlin, F.R.G.), Ultrasphere, 3 μm (7 cm × 4.6 mm I.D.) with an Ultrasphere (3 μm) guard column in the XL cartridge system from Beckman (Basle, Switzerland) or Spheri 5 RP 8, 5 μm (22 cm × 4.6 mm I.D.) in the cartridge system from Brownlee Labs. (Santa Clara, CA, U.S.A.).

For gradient elution, two mobile phase systems were used: mobile phase system I consisted of 0.05 *M* sodium phosphate buffer (pH 6.5) (solvent A) and 0.05 *M* sodium phosphate buffer (pH 6.5)–methanol–tetrahydrofuran (350:650:10, v/v) (solvent B); mobile phase system II consisted of 0.05 *M* sodium phosphate buffer (pH 6.5) (solvent A) and 0.05 *M* sodium phosphate buffer (pH 6.5)–methanol (350:650, v/v) (solvent B).

The injection volume was 25 μl and the flow-rate was set at 1.4 ml/min. The gradient profile was chosen individually for each sample. The derivatives were monitored by fluorescence detection with excitation at 344 nm and emission at 443 nm.

RESULTS AND DISCUSSION

Substances bearing primary amino groups form highly fluorescent compounds after derivatization with OPA in the presence of a mercaptan. This type of derivatization can possibly be applied to all kinds of primary amines and to a wide range of mercaptans. If both the amino compound and the mercaptan show chirality, diastereoisomers will result. The derivatization is assumed to occur as shown in Fig. 1 by analogy with the reaction with OPA-mercaptoethanol. The diastereomeric isoindoles so formed can often be resolved by conventional reversed-phase chromatography with gradient elution. Such determinations of amino acid enantiomers derivatized with OPA-BocC and OPA-NAC were reported recently[8,11,13]. In the same way, OPA-NAP can be used as a reagent; Fig. 2 shows the resolution of seven amino acid enantiomers derivatized with OPA-NAP. The enantiomers of Gln, Tyr, Val and Trp are baseline resolved, whereas those of Ser, Ala and Ile are only partially separated. The resolutions and elution orders are given in Table I, in comparison with those obtained with OPA-BocC and OPA-NAC.

Most of the amino acids show sufficient resolution with each of the three derivatization reagents. OPA-NAC may have some preference for the aromatic amino acids Tyr, Trp and DOPA, whereas aliphatic amino acids such as Ala, Val and Ile are resolved best with OPA-BocC. The Lys enantiomers can only be separated with OPA-BocC and no resolution could be achieved with OPA-NAC and OPA-NAP. Regarding the elution order, we found that with OPA-BocC all L-enantiomers elute

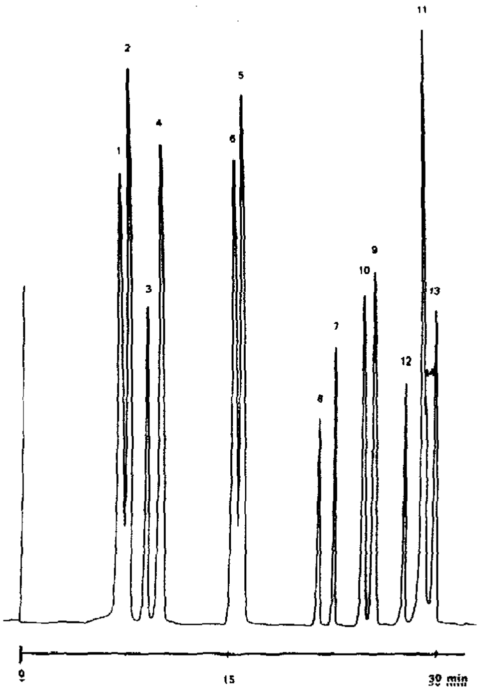

Fig. 2. Separation of amino acid enantiomers after pre-column derivatization with OPA-NAP. Column: Knauer Hypersil ODS, 3 μm (120 × 4.0 mm I.D.). Mobile phase: A, 0.05 *M* sodium phosphate buffer (pH 7.0); B, 0.05 *M* sodium phosphate buffer (pH 7.0)–methanol–tetrahydrofuran (350:650:10, v/v); gradient, 30–100% B in 48 min. Peaks: 1,2 = L-, D-Ser; 3,4 = L-, D-Gln; 5,6 = L-, D-Ala; 7,8 = L-, D-Tyr; 9,10 = L-, D-Val; 11,12 = L-, D-Trp; 13,14 = L-, D-Ile.

before the corresponding D-enantiomers. With OPA-NAC and OPA-NAP the elution order changes with the amino acid hydrophilicity. With OPA-NAC the hydrophilic amino acids including Ala elute with the D- before the corresponding L-enantiomers, whereas the hydrophobic amino acids elute with the L- before the D-enantiomers. With OPA-NAP, owing to the D-form of the penicillamine, the opposite elution characteristic is given. Here the hydrophilic amino acids elute with the L- before the D-enantiomers whereas the hydrophobic amino acids elute with the D- before the L-enantiomers.

Apart from the amino acid enantiomers, the same principle can be applied to the determination of amino alcohol enantiomers. Several chiral amino alcohols, including norepinephrine and norephedrine, were derivatized with Boc-L-cysteine, N-acetyl-L-cysteine and N-acetyl-D-penicillamine and the diastereoisomers were chromatographed on conventional reversed-phase columns. Chromatograms of the enantiomers of six amino alcohols are shown in Fig. 3a–c; the resolution and elution orders are listed in Table I. The chiral mercaptan used for derivatization greatly influences the resolution. Several of the enantiomers can be resolved by all three derivatization reagents whereas some compounds are only separable with one or two of them. However, owing the possibility of varying the type of chiral mercaptan, resolution could be achieved for all amino alcohol enantiomers tested.

TABLE I

RESOLUTION AND ELUTION ORDER OF VARIOUS CHIRAL OPA-MERCAPTAN DERIVATIVES

For chromatographic conditions see text.

Enantiomer	*Derivatization reagent*					
	OPA-BocC		*OPA-NAC*		*OPA-NAP*	
	Resolution	*Elution order*	*Resolution*	*Elution order*	*Resolution*	*Elution order*
Ser	2.1	L,D	0.6	D,L	1.0	L,D
Gln	1.8	L,D	–	–	1.8	L,D
Thr	2.6	L,D	1.4	D,L	–	–
Ala	3.7	L,D	1.1	D,L	0.9	D,L
Tyr	2.0	L,D	3.5	L,D	1.8	D,L
Val	5.2	L,D	3.8	L,D	1.6	D,L
Trp	3.6	L,D	5.4	L,D	2.5	D,L
Ile	4.3	L,D	3.7	L,D	1.4	D,L
Lys	1.0	L,D	0	–	0	–
DOPA	1.8	L,D	1.9	L,D	1.0	D,L
allo-Ile	0	–	2.5	L,D	0.8	D,L
Thr-ol	1.5	L,D	0	–	1.9	L,D
allo-Thr-ol	3.8	L,D	2.0	L,D	2.8	L,D
Ala-ol	2.9	L,D	2.0	L,D	1.1	L,D
2-Amino-1-butanol	3.4	*S,R*	1.7	*S,R*	2.9	*S,R*
Val-ol	2.6	L,D	0	–	5.0	L,D
Phe-ol	1.6	L,D	1.2	D,L	4.1	L,D
1-Amino-2-propanol	0	–	1.1	*R,S*	0	–
Norepinephrine	0	–	1.5	L,D	1.0	D,L
Norephedrine	1.2	L,D	2.7	L,D	2.1	D,L
2-Aminopentane	0	–	0	–	0	–

Only 2-aminopentane could not be resolved with any of the three derivatization reagents. As 2-aminopentane is the only one of all the compounds tested that bears no carboxy or hydroxy group, we can speculate that these functional groups lead to the differences in the retention behaviour of the diastereoisomers by forming hydrogen bonds between the residues of the amino compounds and the mercaptans. Three-dimensional structures of the isoindole derivatives are thus fixed, which lead to different lipophilicities of the diastereoisomers on the stationary phase.

The fluorescence intensities and the stabilities of the derivatives with respect to the different enantiomeric forms were studied for Trp and Phe-ol with the derivatization reagent OPA-BocC. The measured fluorescence signals as a function of reaction time are shown in Fig. 4. The maximum fluorescence occurs after *ca.* 1 min, whereas after 30 min a decrease in fluorescence of about 10% is observed. This is much less than that with OPA-2-mercaptoethanol or OPA-ethanethiol. With L-Trp the specific fluorescence is about 10% below that of D-Trp whereas the specific fluorescence of L-Phe-ol is about 10% higher than that of D-Phe-ol. These differences are not caused by any inaccuracy in the concentration of the sample solutions. The

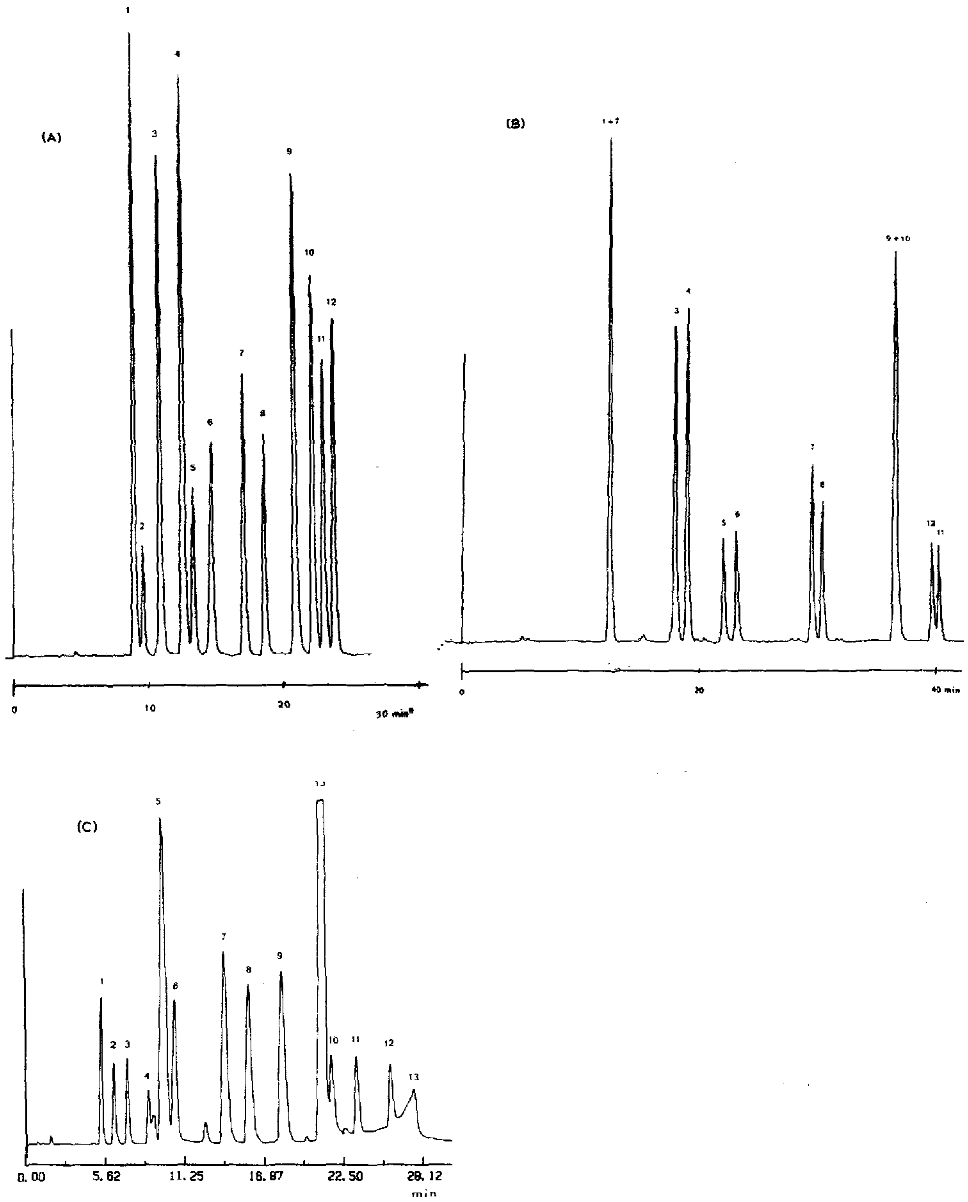

Fig. 3. Separation of amino alcohol enantiomers after pre-column derivatization with various chiral OPA-mercaptan reagents. (A) OPA-BocC; (B) OPA-NAC; (C) OPA-NAP. Column: Knauer Hypersil ODS, 3 μm (120 × 4.0 mm I.D.). Mobile phase: A, 0.05 *M* sodium phosphate buffer (pH 7.0); B, 0.05 *M* sodium phosphate buffer (pH 7.0)–methanol (350:650, v/v); gradient, (A) 62–100% B in 28 min, (B) 20–85% B in 47 min and (C) 50–95% B in 33 min. Peaks: 1,2 = L-, D-threoninol; 3,4 = L-, D-allo-threoninol; 5,6 = L-, D-alaninol; 7,8 = (*S*)-, (*R*)-2-amino-1-butanol; 9,10 = L-, D-valinol; 11,12 = L-, D-phenylalaninol; 13 = impurity.

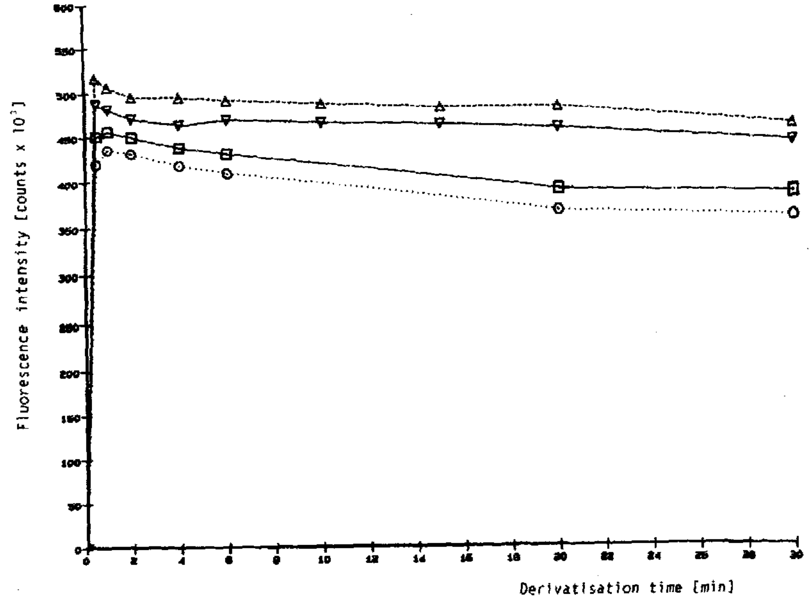

Fig. 4. Fluorescence response of the OPA-BocC derivatization as a function of reaction time for L-Phe-ol (△), D-Phe-ol (▽), D-Trp (□) and L-Trp (○). The reaction time was varied between 0.5 and 30 min. Chromatographic conditions as in Fig. 3.

concentrations were checked by measuring the UV absorbance at 280 nm for TRP and at 270 nm for Phe-ol. The same phenomenon has been reported by Aswad[11] for D- and L-aspartate derivatized with OPA-NAC. These results indicate that the diastereomeric OPA derivatives exhibit different specific fluorescence intensities. This means that quantitative measurements can only be made by comparing the signals of compounds of the same enantiomeric form.

The chiral reagents used were at least 99.9% optically pure. The purity was determined by measuring the minimum amount of D-enantiomer detectable in pure L-amino acids. As the derivative of L-sample/D-reagent and D-sample/L-reagent give diastereoisomers with the same retention time, this procedure was used for determining the optical purity of the chiral reagents.

The methodology described was applied to the determination of amino acid and amino alcohol enantiomers in hydrolysates of the peptides octreotide and [D-Cys²]-octreotide. Octreotide is a recently developed pharmaceutically active compound. It is a somatostatin analogue that consists of five L-amino acids, two D-amino acids and one L-amino alcohol in the following sequence:

D-Phe–L-Cys–L-Phe–D-Trp–L-Lys–L-Thr–L-Cys–L-Thr-ol

As evidence for the enantiomeric correctness of the structures, octreotide and [D-Cys²]-octreotide were hydrolysed with hydrochloric acid and the resulting amino acids and the amino alcohol were identified by HPLC after pre-column derivatization with OPA-BocC. The two cysteines were methylated prior to derivatization in order to avoid intramolecular reaction[12]. Fig. 5 shows the chromatograms of the deriva-

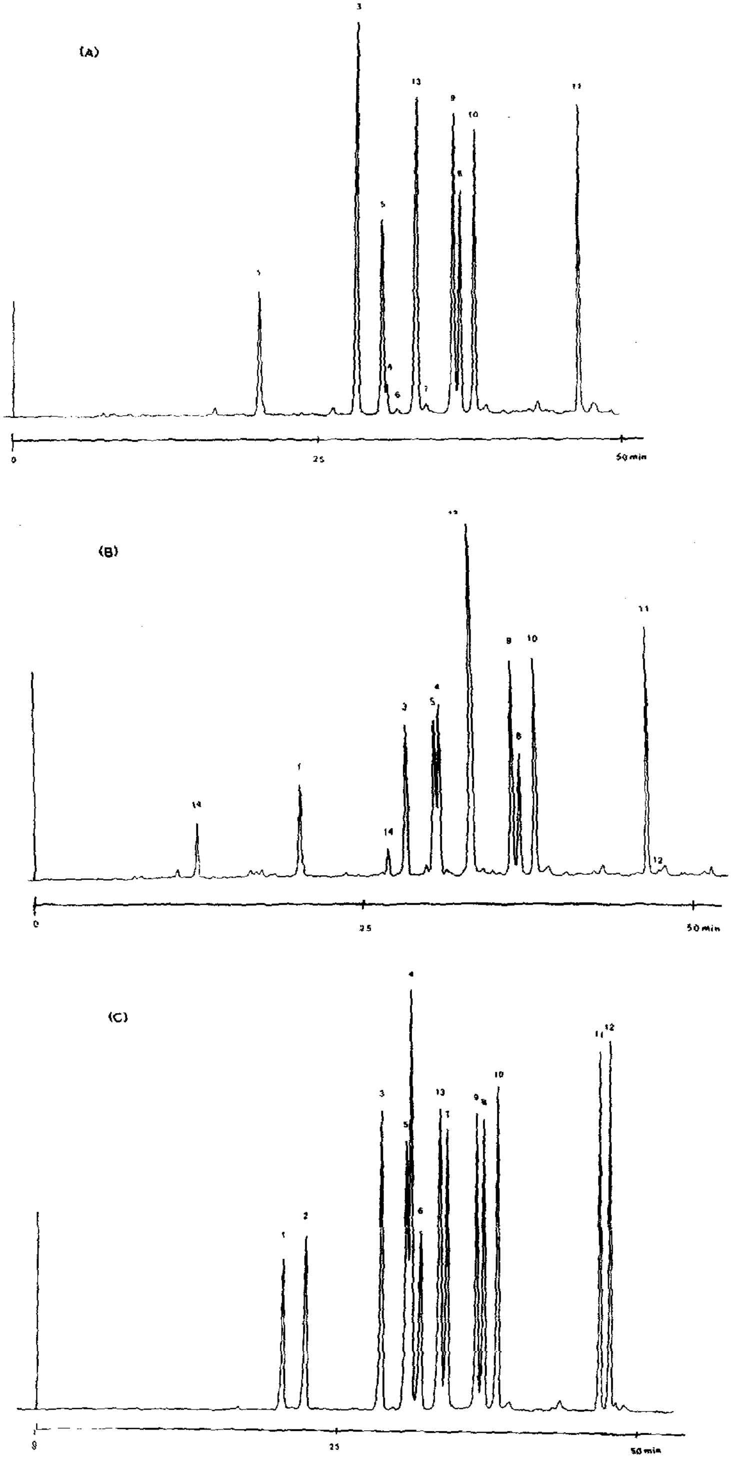

Fig. 5. Elution profiles of peptide hydrolysates of (A) octreotide, (B) [D-Cys^2]-octreotide and (C) a standard mixture of the enantiomers of the amino acids and the amino alcohol present in octreotide, after derivatization with OPA-BocC. Experimental conditions as in Fig. 2. Peaks: 1,2 = L-, D-Thr; 3,4 = L-, D-Cys (determined as S-methyl derivative); 5,6 = L-, D-Thr-ol; 7,8 = L-, D-Trp; 9,10 = L-, D-Phe; 11,12 = L-, D-Lys; 13 = impurity in the water; 14 = unknown.

TABLE II

AMINO ACID COMPOSITIONS OF TWO DIFFERENT OCTREOTIDES

Amino acid enantiomer	*Octreotide*		*[D-Cys²]-Octreotide*	
	Theoretical	*Found*	*Theoretical*	*Found*
L-Thr	1	0.9	1	0.9
D-Thr	0	<0.1	0	<0.1
L-Cys*	2	1.7	1	0.9
D-Cys*	0	0.1	1	0.8
L-Thr-ol	1	0.7	1	0.8
D-Thr-ol	0	<0.1	0	<0.1
L-Trp	0	<0.1	0	<0.1
D-Trp	1	0.4	1	0.6
L-Phe	1	1.0	1	1.0
D-Phe	1	1.0	1	1.0
L-Lys	1	0.9	1	0.9
D-Lys	0	<0.1	0	<0.1

* Determined as S-methylcysteine.

tized peptide hydrolysates of octreotide, [D-Cys²]-octreotide and a standard mixture of the enantiomers of all the amino acids and the amino alcohol present in octreotide. A quantitative evaluation of the results is summarized in Table II. The amino acid ratios, including the ratios of the enantiomers, are in good agreement with the expected values. The figures for Trp are below the theoretical values because of decomposition of this amino acid during hydrolysis. Also, the D-Cys detectable in the hydrolysate of octreotide is formed by racemization of L-Cys under the conditions of hydrolysis.

The method described is suitable for the determination of the enantiomers of nearly all naturally occuring amino acids (the most important exceptions being proline and hydroxyproline), many amino alcohols and certain catecholamines. By using the various chiral mercaptans, an optimal resolution can be found for almost every pair of enantiomers.

N-Acetyl-L-cysteine or N-acetyl-D-penicillamine may be given some preference as they are commercially available. On the other hand, Boc-L-cysteine has the major advantage that it can also be used for the determination of Lys enantiomers.

The method is also adequate for the determination of enantiomers in peptide hydrolysates. In this way the composition of peptides can be proved and error peptides due to racemization during synthesis can be identified.

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