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RESOLUTION OF AMINO ACID ENANTIOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED PRE-COLUMN DERIVATISATION WITH A CHIRAL REAGENT

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SUMMARY

A new and highly sensitive method for the determination of amino acid enantiomers is presented. Fluorescent diastereoisomers are formed by reaction with *o*-phthaldialdehyde and Boc-L-cysteine prior to chromatography. The reaction occurs rapidly at ambient temperatures and is performed by an automated pre-column derivatisation device integrated into the high-performance liquid chromatographic system. Separation of the diastereoisomers is carried out by reversed-phase chromatography with gradient elution and the derivatives are detected fluorimetrically. Complete resolutions were observed for the enantiomers of all 21 amino acids examined. The method allows the determination of individual amino acids, including the enantiomeric forms, in a single chromatographic run.

INTRODUCTION

The ability to resolve the enantiomers of amino acids has many applications in the areas of biochemical research and pharmaceutical chemistry. Because peptide drugs contain distinct enantiomers of amino acids, the amino acid composition as well as the enantiomeric purity are of great interest[1]. High-performance liquid chromatography (HPLC) resolution of amino acid enantiomers can be accomplished by using chiral stationary phases[2–4], chiral eluents[5–7] or derivatisation with chiral reagents prior to chromatography[8–10]. The diastereoisomers formed by the latter method can often be separated by conventional reversed-phase chromatography, and special chiral columns are not necessary. However, optically pure derivatisation agents are required, as are reaction conditions where no racemisation occurs.

In order to establish a simple and sensitive method for the determination of the enantiomers of different amino acids in a single chromatographic run, we modified the well-known derivatisation reagent for amino acids and primary amines, *i.e.* *o*-phthaldialdehyde–2-mercaptoethanol, by replacing the 2-mercaptoethanol with the chiral mercaptanes Boc-L-cysteine and N-acetyl-L-cysteine. The cysteine derivatives were expected to be ideal for this purpose because they are some of the few chiral

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mercaptanes available in an optically pure form. The derivatisation procedure was fully automated and integrated into the HPLC system in front of the column. By this means the manual sample handling was reduced to a minimum and pipetting errors were eliminated. The derivatisation process became very reproducible[11], and the external standard method could be used for all quantitative measurements.

With this method, we were able to determine the individual amino acids in peptide hydrolysates and check for the enantiomeric forms simultaneously. Analysis times ranged between 30 and 80 min, depending on the amino acids to be determined.

EXPERIMENTAL

Apparatus

The instrumentation consisted of two Altex Model 100 pumps, controlled by the microprocessor system 420 from Altex, an automatic sampling system ASI 45 from Kontron connected with a pneumatic twelve-port injection valve from Valco Instruments and a peristaltic pump. A Kontron SFM 23 LC spectrofluorimeter was used for detection and a Hewlett-Packard data system HP 3357 for data processing. The packed-bed reactor was made in the laboratory by filling glass beads of 0.28 mm mean diameter into a stainless steel tube 25 cm × 4.6 mm I.D.

Chemicals

Methanol (HPLC grade) and tetrahydrofuran (analytical grade) were from Rathburn (Walkerburn, U.K.) and the amino acids from Sigma (London, U.K.) or Fluka (Buchs, Switzerland). Boc-S-benzyl-L-cysteine and N-acetyl-L-cysteine were obtained from Fluka. *o*-Phthaldialdehyde (OPA) and all other chemicals were purchased from Merck (Darmstadt, F.R.G.). Water was distilled twice before use.

Boc-L-cysteine (N-*tert.*-butyloxycarbonyl-L-cysteine), a yellow oil, was prepared from Boc-S-benzyl-L-cysteine by reduction with sodium in liquid ammonia and extraction in acetic acid ethyl ester. It was stored in the refrigerator.

Derivatisation reagent

A 100-mg amount of OPA was dissolved in 3 ml of ethanol, and 220 ml of 0.4 *M* sodium borate buffer (pH 10) and *ca.* 300 mg of Boc-L-cysteine were added. This reagent was freshly prepared every other day.

Samples

For peak identification and standardisation, *ca.* 50 μmol of each amino acid enantiomer were dissolved in 1 l of water.

Protection of cysteine

If cysteine had to be determined, the following sample pre-treatment was carried out[13] 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 the 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 were added and the solution was allowed to stand for 10 min. Then 200 μl of 3 *M* hydrochloric acid were added and, after mixing, the flask was filled to 2 ml with 0.4 *M* sodium borate buffer (pH 10). The pH was checked to be between 7 and 10.

Chromatography

Mobile phase A was 0.05 *M* sodium phosphate buffer (pH 7.0) with 1% tetrahydrofuran, mobile phase B was methanol. A reversed-phase HPLC cartridge column, Spheri 5, RP-8, 5 μm, 22 cm × 4.6 mm I.D. (Brownlee Labs, Santa Clara, U.S.A.) was used. The flow-rate was 1.4 ml/min, and the column pressure was *ca.* 150 bar. Derivatisation was carried out in 100% A, and the separation of the enantiomers was accomplished with a linear gradient from 0 to 64% B in 40 min. Derivatisation and chromatography were carried out at room temperature. The derivatives were monitored by fluorescence detection with excitation at 344 nm and emission at 443 nm.

Derivatisation procedure

Fig. 1 shows the schematic arrangement of the automated pre-column derivatisation device combined with the reversed-phase HPLC system. The mobile phase flow from the gradient system is split by a mixing T and led to a pneumatic twelve-port injection valve equipped with a 25-μl loop for the sample and a 100-μl loop for the reagent solution. Behind the valve the eluent streams rejoin at the second mixing T. The loop volumes and the lengths of the capillaries are chosen in such a way that with each injection the sample segment is embedded in the reagent segment at the mixing T.

The combined segments flow through the packed-bed reactor where complete

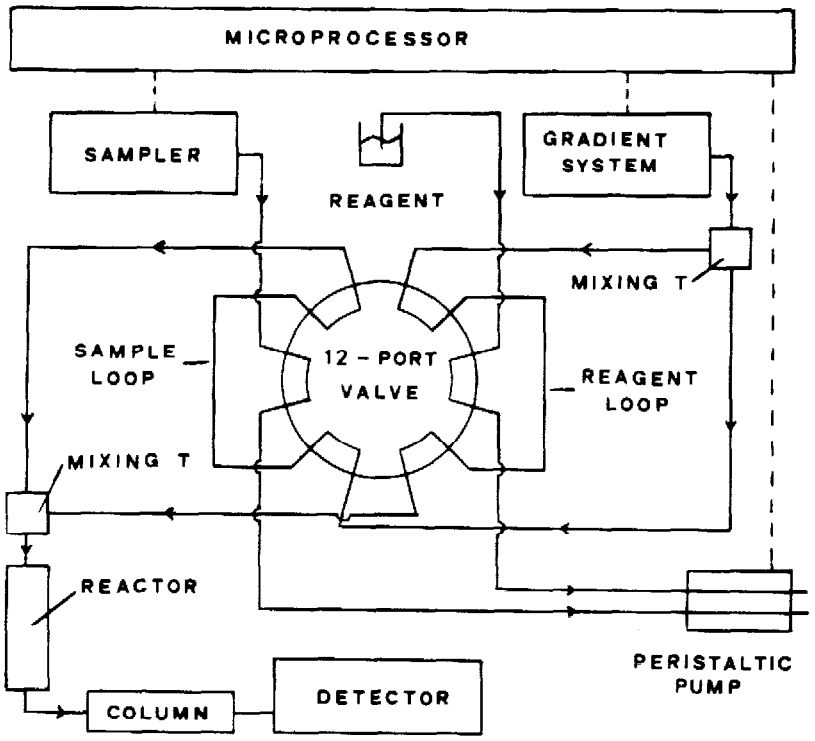

Fig. 1. Automated pre-column derivatisation device; twelve-port valve shown in "inject" position.

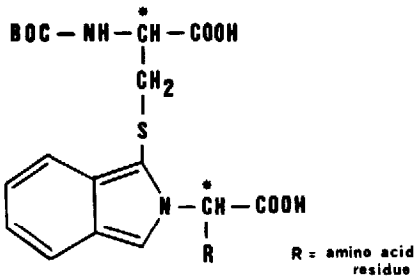

Fig. 2. Suggested structure of the amino acid derivatives formed with *o*-phthaldialdehyde–Boc-L-cysteine. The asterisks mark the chiral centres of the molecule.

mixing and reaction occur. The reaction time is controlled by the flow-rate and the size of the packed-bed reactor. Under the conditions described the reaction time is *ca.* 75 sec. When the chromatographic column is reached, the reaction is stopped by the separation of sample and reagent. The derivatives are then separated by gradient elution and monitored by fluorescence detection.

For testing the stability of the L-alanine derivative, derivatisation was done by mixing 0.5 ml of the L-alanine sample with 2 ml of reagent solution in a sample vial. This solution was chromatographed every 2 h.

TABLE I

RETENTION TIME AND RESOLUTION OF AMINO ACID ENANTIOMERS

For chromatographic conditions see text.

Amino acid	*Retention time (min)*		*Resolution factor*
	L-*Enantiomer*	D-*Enantiomer*	
Asp	11.6	12.2	1.3
Glu	15.0	15.5	1.1
Asn	19.9	20.9	2.2
Ser	21.0	22.0	2.1
Gln	22.5	23.3	1.8
Thr	23.7	24.9	2.6
His	25.6	26.2	1.1
Ala	25.7	27.4	3.7
Arg	27.0	28.0	2.2
Tyr	28.2	28.9	2.0
Me-Cys	28.6	29.8	2.4
Val	31.6	33.6	5.2
Trp	32.0	33.6	3.6
Met	32.6	33.9	3.2
Phe	34.3	35.1	2.8
Ile	34.6	36.3	4.3
Leu	35.8	37.3	3.3
Lys	39.2	39.5	1.0
Cit	23.7	24.9	2.4
Orn	38.5	39.1	1.4
Dopa	26.4	27.3	1.8

RESULTS AND DISCUSSION

The derivatisation reagent OPA–Boc-L-cysteine forms highly fluorescent derivatives with amino acids and primary amines at a pH between 8 and 10. The reaction occurs rapidly and quantitatively at ambient temperatures. The fluorescence intensity of the derivatives is similar to that obtained with OPA–2-mercaptoethanol. The detection limit is in the lower picomole or in the femtomole range. The limiting factor is not the signal-to-noise ratio but ghost peaks caused by impurities either in the chemicals or introduced during sample preparation. The wavelength maxima of excitation and emission were determined for the L-alanine derivative and were found to be 344 nm and 443 nm. The stability of the isoindole formed was tested over 12 h. The loss in fluorescence intensity was 37% within that time, which is much less than with OPA–2-mercaptoethanol[12] or other mercaptanes[13]. The common structure of the derivatisation products is assumed to be as shown in Fig. 2, in analogy to the derivatives formed with OPA–2-mercaptoethanol[14].

As Boc-L-cysteine is a chiral substance, diastereoisomers are formed by reaction with enantiomers. In the case of amino acids, the diastereoisomers of 21 amino acids can be resolved by reversed-phase chromatography with a linear gradient. Table I shows the retention times and resolution of the enantiomers of all

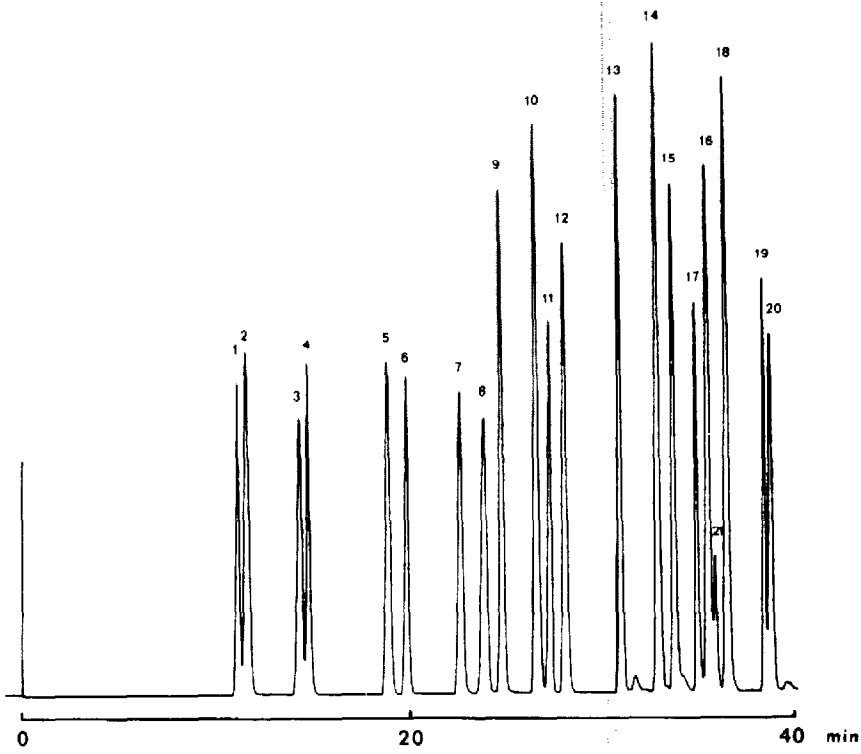

Fig. 3. Standard separation of amino acid enantiomers after automated pre-column derivatisation with OPA–Boc-L-cysteine. Peaks: 1,2 = L-, D-Asp; 3, 4 = L-, D-Glu; 5, 6 = L-, D-Asn; 7, 8 = L-, D-Thr; 9, 10 = L-, D-Ala; 11, 12 = L-, D-Tyr; 13, 14 = L-, D-Val; 15, 16 = L-, D-Ile; 17, 18 = L-, D-Leu; 19, 20 = L-, D-Lys; 21 = impurity. For conditions see Experimental.

21 amino acids. For most of the isomers base line separation can be achieved. The minimum resolution factor found is 1.0. With all amino acids, the L-enantiomer elutes before the corresponding D-antipode. As an example, a chromatogram of the separation of ten amino acid enantiomers in a single run of 40 min is shown in Fig. 3. For many amino acid mixtures of unknown composition, the identification of the amino acids and the determination of the optical isomer ratio can be done in a single chromatogram.

The chiral reagent Boc-L-cysteine was found to be at least 99.8% optically pure. In standard mixtures of L-amino acids no D-enantiomers could be detected.

Not detectable with this method are proline and cysteine. Proline does not react with the reagent owing to the lack of a primary amino group, and cysteine forms only a weak fluorescent derivative with OPA. After methylation of the thiol group, D- and L-cysteine can easily be determined as their S-methyl-cysteines (Me-Cys).

Similar determinations of amino acid enantioners can be obtained by the use of other chiral mercaptanes, such as N-acetyl-L-cysteine[9]. Under the same chromatographic conditions, separations of several enantiomers were obtained. Fig. 4 shows a separation of the enantiomers of five amino acids. With hydrophilic amino acids, the D-enantiomers elute before the corresponding L-antipodes, whereas with hydrophobic amino acids L elute before D. The resolution obtained was less than

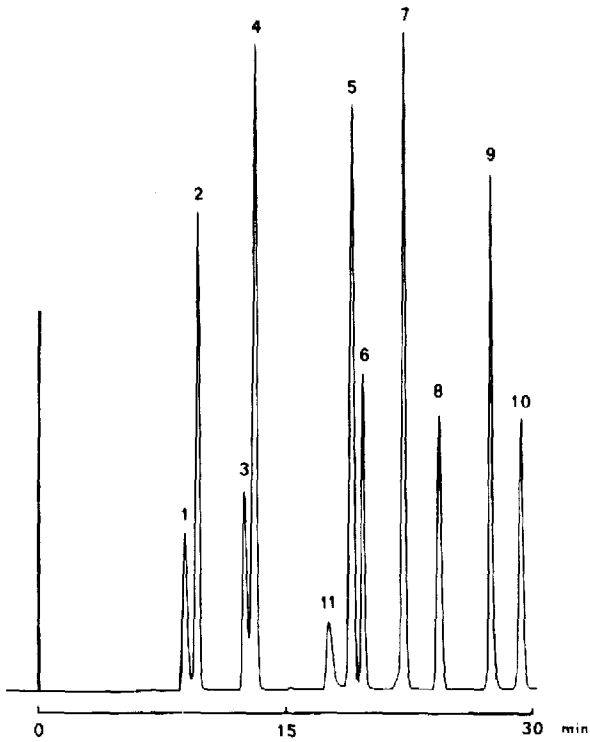

Fig. 4. Separation of amino acid enantiomers after pre-column derivatisation with OPA–N-acetyl-L-cysteine. Peaks: 1, 2 = D-, L-Thr; 3, 4 = D-, L-Ala; 5, 6 = L-, D-Tyr; 7, 8 = L-, D-Val; 9, 10 = L-, D-Ile; 11 = impurity. For conditions, see Experimental.

with the corresponding Boc-L-cysteine derivatives. However, N-acetyl-L-cysteine has the advantage of being commercially available. Based on the reaction principle, similar results can be expected from other chiral mercaptanes. However, only a few of these can be purchased optically pure.

The method described is suitable for the identification and the quantification of the amino acid enantiomers in peptide hydrolysates because of its simplicity and high sensitivity.

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