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# SEPARATION OF α-AMINO ACID ENANTIOMERS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE AND A SODIUM SALT OF 1-THIO-β-D-GLUCOSE

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## SUMMARY

A sensitive system for D,L-amino acid analysis has been developed, using fluorescence derivatization with *o*-phthaldialdehyde in the presence of sodium salt of 1-thio-β-D-glucose. The reagents rapidly form fluorescent diastereoisomeric derivatives with primary amino acids. These derivatives are efficiently separated on a conventional reversed-phase column with an analysis time of 60 min. Simultaneous determination of enantiomers of various amino acids was achieved by a simple binary gradient elution with methanol in 0.05 *M* aqueous sodium acetate.

## INTRODUCTION

During recent years a number of biologically active compounds of peptidic nature have been isolated [1,2]. Usually, only one of the enantiomers or stereoisomers possesses biological activity. Some of the peptidic compounds contain one or more D-amino acids in their structure, which is likely to influence the biological activity of the compounds. For example, cyclosporines, the cyclic undecapeptides, which are now widely used as immunosuppressive agents in human medicine, contain one D-alanine residue [3]. It is, therefore, of prime importance to develop analytical procedures for D,L-amino acid separation, or for control of the enantiomeric purity of the various isomers obtained either by enantioselective synthesis or separation techniques. The development of appropriate high-performance liquid chromatographic (HPLC) procedures for the separation of

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enantiomers has generated considerable interest and has been the subject of several recent papers [4]. The separation of D,L-amino acid enantiomers can be achieved with a mobile phase containing chiral metal complexes or other types of chiral discriminator, or with a chiral stationary phase. These methods allow the separation of most of the natural amino acids without derivatization.

Another possibility is derivatization of the sample with a chiral reagent to produce diastereoisomeric molecules that can be separated by a non-chiral chromatographic system. Moreover, with an adequate set of chiral reagents, the enantiomers with various functional groups can be separated on the same reversed-phase column. Recently, Boc-L-Leu-hydroxysuccinimide esters [5], acetylglycosyl isocyanates [6], cysteine derivatives with *o*-phthaldialdehyde (OPA) reagent [7] and 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside [8] have been described for the chiral derivatization of amino acids. The aim of this paper is to introduce a novel method for the separation of D,L-amino acids as their fluorigenic derivatives formed in the course of the reaction with OPA and the sodium salt of 1-thio-β-D-glucose (TG).

## EXPERIMENTAL

### *Instrumentation*

A Varian 5500 liquid chromatographic system equipped with a Rheodyne 7126 injection valve, a DS 604 data station and a Fluorichrom filter fluorescence detector was used. The excitation wavelength was 360 nm, while the emission wavelength was a bandpass above 420 nm. The analytical column was LiChrosorb RP-8, 5 μm (250 mm × 4 mm I.D.) from Merck (Darmstadt, F.R.G.). A back-pressure terminator, set at 0.6 MPa, was used to prevent formation of bubbles.

### *Chemicals*

The sodium salt of TG was prepared as previously described [9]; OPA (Calbiochem, Los Angeles, CA, U.S.A.), amino acids (Calbiochem or Sigma, St. Louis, MO, U.S.A.), methanol, boric acid, potassium hydroxide and sodium acetate (Lachema, Brno, Czechoslovakia) were used . 4-Fluoroglutamic acid was a generous gift of Dr. Tolman (Czechoslovak Academy of Sciences, Prague, Czechoslovakia).

### *Derivatization procedure*

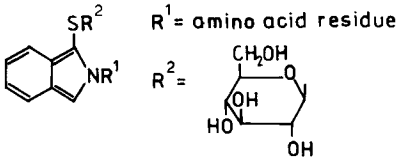
Stock solutions were prepared of 50 mg of TG in 1 ml of water, 50 mg of OPA in 1.25 ml of methanol, amino acids (4 m*M* in water), borate buffer prepared by dissolving 0.50 g of boric acid in 19 ml of water and adjusting the pH to 10.40 with potassium hydroxide solution (45 g potassium hydroxide in 100 ml of water). A 10-μl aliquot of the standard or of the serum, 30 μl of the borate buffer, 20 μl of the TG solution and 20 μl of the OPA solution (TG/OPA = 2.2) were introduced in a small glass vessel, and the mixture was thoroughly stirred. After 60 s, 120 μl of 0.05 *M* sodium acetate (pH 6.05) were added, and 10 μl of the sample were analysed.

*Chromatography*

Solvent A was 0.05 *M* sodium acetate adjusted to pH 6.05 with acetic acid and solvent B was a 1:9 (v/v) mixture of 0.1 *M* sodium acetate (pH 7.60) and methanol. Both solvents were carefully degasses prior to use. Isocratic elution with solvent A was carried out for 8 min, then a 47-min linear gradient to 45% A and 55% B was applied, followed by a column-wash with a linear gradient to 100% B within 5 min. A constant flow of 1.20 ml/min was maintained during the analysis.

## RESULTS AND DISCUSSION

Recent studies have demonstrated that amino acid mixtures derivatized with OPA–2-mercaptoethanol can be successfully separated by HPLC [10]. By analogy with this reaction [11], 1-isoindolyl-(1-thio-β-D-glucoside) derivatives are assumed to be formed in the course of the reaction with TG.




If both the amino acid and the thio-sugar are chiral, diastereoisomers are formed, which can be separated on a non-chiral column. Fig. 1 shows the separation of nine amino acid enantiomers derivatized with OPA–TG, and the resolution and elution orders are given in Table I. Although a multi-step gradient is often necessary for the resolution of all derivatives, as in the case of OPA–2-mercaptoethanol derivatives [10], in a real sample, e.g. hydrolysate of peptide, all amino acids are never present.

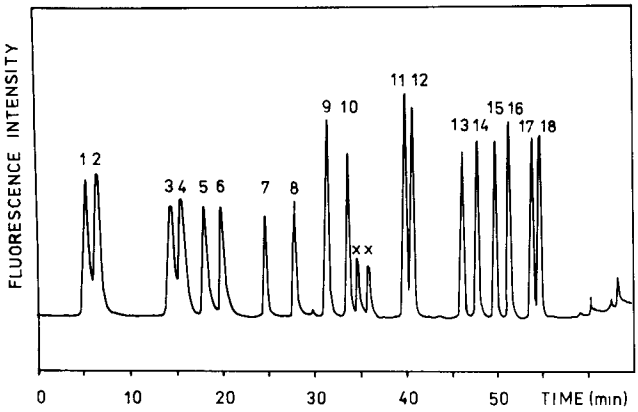



Fig. 1. Separation of amino acid enantiomers after derivatization with the OPA-TG reagent. Column, Merck LiChrosorb RP-8, 5 μm (250 mm × 4 mm I.D.); mobile phase A, 0.05 *M* sodium acetate buffer (pH 6.04); mobile phase B, 0.1 *M* sodium acetate buffer (pH 7.60)–methanol (1:9, v/v); for chromatographic conditions see Experimental. Peaks: 1, 2 = D-, L-Asp; 3, 4 = L-, D-Glu; 5, 6 = L-, D-Ser; 7, 8 = L-, D-Thr; 9, 10 = L-, D-Ala; 11, 12 = L-, D-α-amino-*n*-butyric acid; 13, 14 = D-, L-Val; 15, 16 = D-, L-Phe; 17, 18 = L-, D-Leu. Each peak represents 2 nmol.

TABLE I

RESOLUTION AND RETENTION TIMES OF VARIOUS CHIRAL OPA-TG DERIVATIVES

For chromatographic conditions see text; $t_0$ = 1.15 min; resolution = 1.177 $(t_2-t_1)/(w_{1/2(1)}+w_{1/2(2)})$.

| Amino acid | Retention time (min) | | Resolution |
|---|---|---|---|
| | L | D | |
| Asp | 6.4 | 5.2 | 1.0 |
| Glu | 14.4 | 15.6 | 0.8 |
| Ser | 18.2 | 20.1 | 2.0 |
| Thr | 25.0 | 28.1 | 6.7 |
| Arg | 29.0 | 30.3 | 0.6 |
| Ala | 31.6 | 34.0 | 3.8 |
| Tyr | 37.0 | 37.3 | 0.4 |
| α-Abu | 40.1 | 41.0 | 1.4 |
| Val | 48.0 | 46.4 | 2.7 |
| NVal | 46.1 | 47.2 | 1.5 |
| Trp | 47.9 | 48.7 | 2.5 |
| Phe | 51.5 | 50.0 | 2.7 |
| Ileu | 53.9 | 52.8 | 2.2 |
| Leu | 54.0 | 54.9 | 1.6 |
| NLeu | 54.3 | 55.2 | 1.6 |

The derivatization time was varied from 20 s to 180 min in order to determine the change in fluorescence response with time. At pH 10.4 95% of maximum fluorescence occurs within 40 s for most amino acids. Accordingly, a 60-s period was allowed for derivatization, and the derivatized sample was injected at exactly 60 s. The coefficients of variation (C.V.) showed that the present system is highly reproducible. The average between-day C.V. of the fluorescence constant, calculated from twelve measurements over three days, was ca. 3.5% and the within-day C.V. was less than 2%. By injecting serially diluted standard mixtures until a zero standard signal, the lower detection limit was found to be less than 1 pmol. However, the sensitivity may be further increased by changing the detector settings.

The stability of OPA–TG derivatives is much higher than that found for the OPA–2-mercaptoethanol derivatives. The decrease of the measured fluorescence signals of derivatized samples after 180 min at 25 °C on air depends on the nature of the amino acid used. For example, the values 5.8% for L-Asp, 19.4% for L-Glu, 11.7% for L-Ser, 2.2% for L-Val and 13.4% for L-Lys were found, expressed as the percentage decrease of the fluorescence compared with the sample injected 60 s after derivatization. The stability of a mixed OPA–TG solution itself is, however, limited. Therefore the use of separate stock solutions, which are stable for months, is recommended.

Buffers with pH values from 2.0 to 9.0 were used for elution. Generally, more acidic buffers caused fluorescence quenching. The capacity ratios and resolution of some derivatives are pH-dependent; therefore, some separations might be fur-

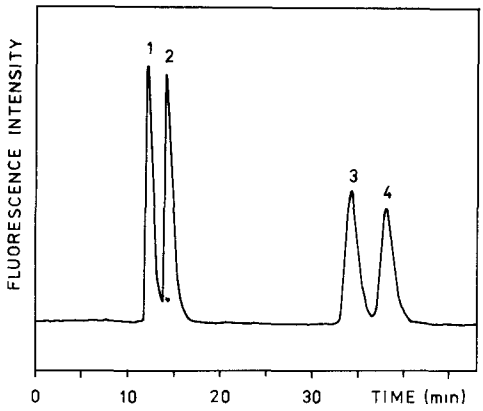



Fig. 2. Separation of D,L-aspartic acid and D,L-glutamic acid enantiomers after derivatization with the OPA–TG reagent. Column, Merck LiChrosorb RP-8, 5 μm (250 mm × 4 mm I.D.); mobile phase, 0.05 *M* sodium acetate buffer (pH 5.0); flow-rate 1.20 ml/min. Peaks: 1, 2 = D-, L-Asp; 3, 4 = L-, D-Glu. Each peak represents 2 nmol.

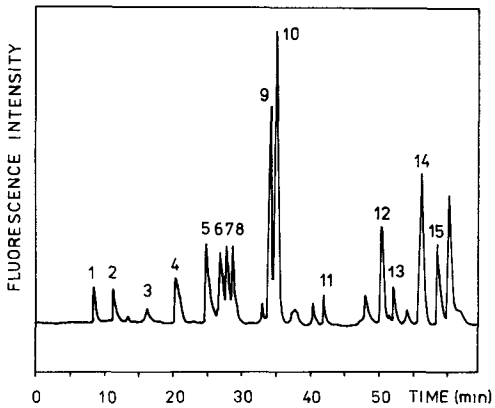



Fig. 3. Elution profile of OPA–TG-derivatized free amino acids from serum of a mouse treated with 4-fluoroglutamic acid. Column, Merck LiChrosorb RP-8, 5 μm (250 mm × 4 mm I.D.); mobile phase A, 0.05 *M* sodium acetate buffer (pH 5.50); mobile phase B, 0.1 *M* sodium acetate buffer (pH 7.60)–methanol (1:9, v/v); isocratic elution with A for 12 min, then a 43-min linear gradient to 45% A and 55% B was applied; flow-rate 1.20 ml/min. Peaks: 1 = (*S,R*)-4-fluoroglutamic acid; 2 = (*R,S*)-4-fluoroglutamic acid; 3 = L-Asp; 4 = L-Glu; 5 = L-Ser; 6 = L-Gln; 7 = L-Thr; 8 = Gly; 9 = L-Arg; 10 = L-Ala; 11 = L-Tyr; 12 = L-Val; 13 = L-Phe; 14 = L-Leu; 15 = L-Lys.

ther improved by changing the pH of the buffer. For example, the capacity ratios of aspartic and glutamic acid derivatives decrease with the buffer pH, and also the D,L derivatives themselves are not sufficiently resolved in a buffer with a pH above 6.2. Moreover, the chromatographic peaks were split, probably owing to the interaction of one carboxylate group with hydroxyl groups of the sugar moiety. The determination of aspartic acid racemization is of interest with respect to its role in biological ageing, and also as a geochronological tool for dating fossils [12,13]. For this particular case, the use of isocratic conditions is recommended (see Fig. 2).

The enantiomers of most primary amino acids are baseline-resolved, with the

exception of tyrosine and lysine, which are only partially resolved. Hydroxylysine presents a specific problem because its derivatives give several chromatographic peaks. There is no simple rule to predict the elution order of L and D derivatives. With the exception of valine, isoleucine, phenylalanine and aspartic acid, all other L enantiomers elute before the corresponding D enantiomers. Besides common amino acids, which the D forms are not typically present in physiological fluids, a number of their derivatives are used in a medical research as selective enzyme inhibitors [14,15]. In this particular case, D,L enantiomers exhibit not only different biological activity, but sometimes also different metabolic pathways. The analysis of *erythro*-D,L-4-fluoroglutamic acid illustrates the utility of the present method for this interesting approach (see Fig. 3).

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