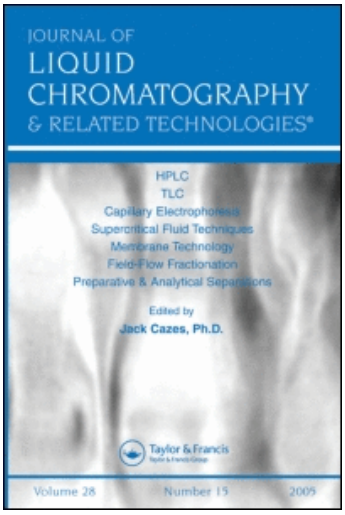

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SEPARATION OF AMINO ACID ENANTIOMERS USING PRECOLUMN DERIVATIZATION WITH o-PHTHALALDEHYDE AND 2,3,4,6-TETRA-O-ACETYL-1-THIO-β-GLUCOPYRANOSIDE

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ABSTRACT

A method is described for resolution of amino acid enantiomers. The D- and L-amino acids were reacted with o-phthalaldehyde (OPA) and the optically active 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG). The reaction was complete in a few minutes at room temperature and the derivatives were quite stable. The formed diastereomers were separated by reversed phase chromatography and the selectivity was generally good, except for lysine and ornithine. A mean separation factor (α) of 1.27 was obtained with acetonitrile as an organic modifier. The fluorescence excitation and emission maxima were at 342 nm and 410 nm respectively, and the electrochemical half-wave potential $E_{1/2}$ ≈ 0.65 - 0.75 V. The detection limits (for L-leucine) were 23 pmol and 1 pmol (S/N 3:1) in the respective detection modes. With laser-induced fluorescence detection (He-Cd laser, 325 nm) and microcolumns, a detection limit in the fmol range is obtainable.

INTRODUCTION

The resolution of optical isomers has recieved much interest in recent years. Both free (1) and derivatized (2) amino acid enantiomers have been separated by reversed-phase chromatography with optically active metal chelate additives. Alternatively, the amino acids have been derivatized with chiral reagents, and the formed diastereomers separated on non-chiral columns (3).

The reaction of o-phthalaldehyde (OPA) and thiols with amino acids is widely used, both for pre- and post-column derivatization, because of the simple reaction conditions and the intense fluorescence of the derivatives (4). The derivatives are also amenable for electrochemical detection (5,6). The work of Simons and Johnson (7) showed that the reaction is general for thiols and amines. A number of different thiol structures have been employed, mainly in search of derivatives that are more stable than the most common mercaptoethanol-based derivatives (6). The possibility of using chiral thiols for resolution of amino acid enantiomers was recently recognized by Aswad (8), who employed OPA and N-acetyl-L-cysteine (NAC) for separation of D- and L-aspartic acid. The reagent was later utilized by Lam (9), who applied the abilities of the OPA-NAC derivatives to form complexes with copper for resolution of amino acid enantiomers in a copper-proline system, and Nimura and Kinoshita (10) who developed a single-run separation of the enantiomers of 13 amino acids. An alternative to NAC is a BOC-protected cysteine as shown by Buck and Krummen (11). A few years ago the chiral reagent, 2,3,4,6-tetra-O-acetyl-β-glucopyranosyl isothiocyanate (GITC) was introduced for resolution of D- and L-amino acids (12). Good enantioselectivity was obtained in reversed phase systems (13), which allowed resolution of the enantiomers of the protein amino acids in a single run (14). The derivatives were detected by UV-absorption at 250 nm.

Miniaturized LC systems are attractive when high separation efficiencies are required (15,16). An example is the analysis of complex samples such as physiological fluids which contain a large number of amino acids in widely varying concentrations. Laser induced fluorescence and electrochemical detection techniques have been demonstrated to be well suited for high sensitivity detection in sub-microliter cell volumes and are thus compatible with microcolumns (15,17,18).

In the present study, the commercially available thiol analogue of GITC, 2,3,4,6-tetra-O-acetyl-1-thio-β-glucopyranoside (TATG), was employed in a reaction with OPA for precolumn conversion of amino acid enantiomers to diastereomers. The aim was to obtain easily separable diastereomers that are detectable by fluorescence and electrochemistry.

MATERIALS

Chemicals

Acetonitrile, methanol and tetrahydrofuran were obtained from Rathburn (Walkerburn U.K.). Amino acid standards, o-phthalaldehyde (OPA) and 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (TATG) were from Sigma (St. Louis, MO). The elution buffers were made of acetic acid (50 mM) in double-distilled water. The pH was adjusted with sodium hydroxide.

Apparatus

The chromatographic system (Figure 1) is identical with that previously described for the separation of amino acid derivatives on packed fused silica columns (16), except that the spectro-fluorimeter was replaced by a laser-based fluorescence detector. A He-Cd laser, model 4210 NB from Liconix with an output power

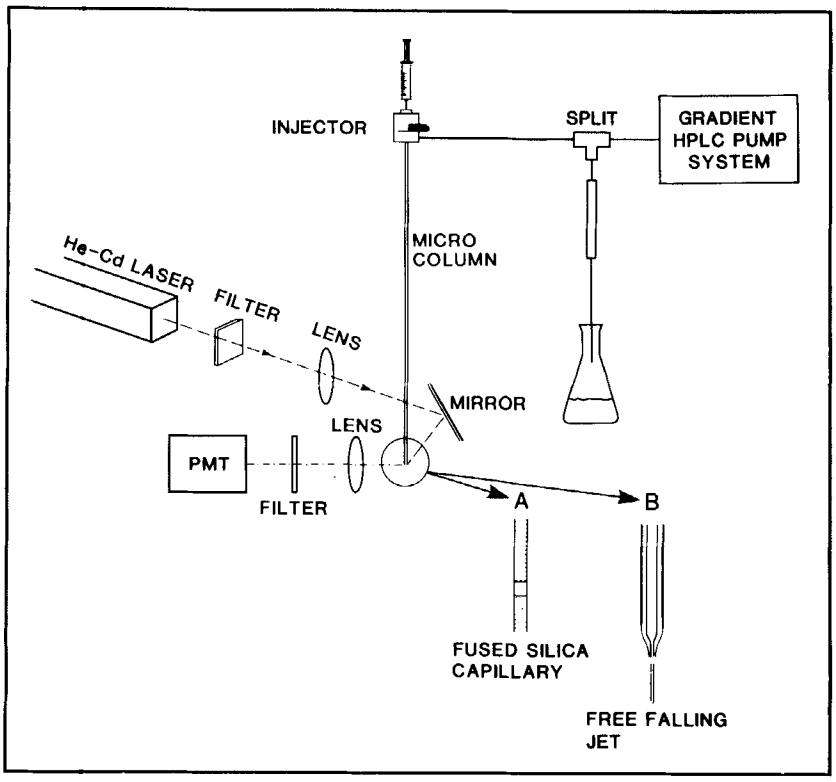

Figure 1. Instrumental set-up:
A) Fused silica capillary flow cell for flow rates <20 µl/min (on- or off-column detection)
B) Free falling jet flow cell for flow rates >20 µl/min.
Other details in text.

of 1.5 mW at the UV-line 325 nm was used. In contrast to the layout of the optical system previously described (17,19), the collecting optics were placed in the horizontal plane, perpendicular to the flow cell (20). The laser beam was focused onto the detector cell after reflection in a front surface mirror positioned out of the horizontal plane. The beam waist at the flow cell was 35 µm. A free falling jet detector cell (B in Figure 1) was appropriate for volumetric flow rates down to 20 µl/min (19), whereas for lower flow rates, a fused silica capillary (SGE,

Australia) was used (A in Figure 1). The fused silica cell was either a part of the column (on-column mode)(16), or the outlet capillary of the column (off-column mode).

For comparative studies of the fluorescence and the electrochemical detectability, a Schoeffel FS 970 LC fluorimeter and a Waters 460 electrochemical detector were used.

METHODS

Derivatization procedure

The sample (250 µl) and the borate buffer (50 µl, 0.4 M, pH 9.5) were mixed in a reaction vial. Then 50 µl of the reagent (8 mg OPA and 44 mg TGTA dissolved in 1 ml acetonitrile) was added and allowed to react for 6 minutes whereafter the derivatized sample was injected on the column.

RESULTS AND DISCUSSION

Derivatization

The reaction of the amino acids with OPA/TATG (Figure 2) is proposed to be in analogy with the OPA/mercaptoethanol reaction (7). A study of the reaction rate, including L-alanine, L-lysine, L-methionine, L-aspartic acid, L-histidine, L-serine and L- and D-threonine, showed that the reaction was complete in less than 3 minutes for all the amino acids except threonine which needed 6 minutes to react. No significant difference was found in the reaction rate of L-threonine compared with D-threonine. A comparative study of TATG with other thiols was not made. The rapid reaction of OPA and the bulky TATG molecule with the amino acids is in agreement with Simons and Johnson (7), who observed that an increase in the steric bulk α to the thiol had no obvious effect on the overall reaction rate.

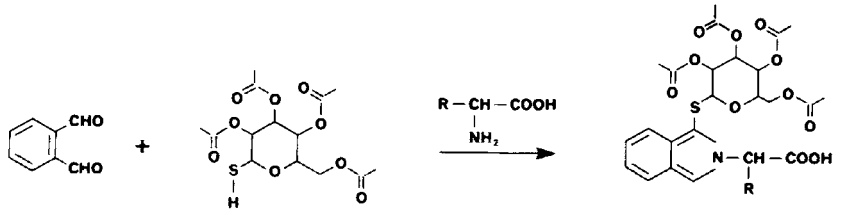

Figure 2. Reaction formula.

In the use of the OPA reagent for amino acid analysis, derivative-instability is generally a serious problem. Allison and coworkers (6) reported that less than 25% of the fluoresence response of the OPA/mercaptoethanol derivatives of glycine, ornithine and lysine remained after a 40 minutes period. Contrary to this, the derivatives of OPA/TATG were found to be quite stable. The alanine, arginine, glycine and serine derivatives showed no breakdown after 1ı/2 hour. After 10 hour storage at room temperature, 68% and 37% remained of the fluorescence response of the ornithine and lysine derivatives respectively.

The chemical purity of the commercial TATG reagent was checked chromatographically by separation under the same mobile phase conditions as in the initial isocratic part of the standard separation (Figure 4). No fluorescent impurities were observed. However, with electrochemical detection impurities more hydrophilic than the reagent were observed. These impurities may be thiols and responsible for the ghost peaks visible in the chromatography of the amino acid derivatives. These peaks are also proportional to the amount of the amino acids and elute in the first part of the chromatogram.

The optical purity of the OPA/TATG reagent was checked by derivatization of L-tyrosine. Using standard addition the peak corresponding to D-tyrosine was found to be 0.1% of the L-tyrosine. This results indicates not only that the reagent is of

high purity, but also that racemization during the reaction is negligible.

Detection

The fluorescence spectra of the OPA/TATG amino acid derivatives are similar to those of the OPA/mercaptoethanol derivatives, with a shift towards lower emission wavelengths. Excitation maximum is at 342 nm and emission maximum at 410 nm. The derivatives are therefore compatible for excitation with the 325 nm wavelength of the He-Cd laser, and thus suited for microcolumn liquid chromatography. The electrochemical response of the derivatives is shown in the voltammogram in Figure 3. Compared with the half-wave potential for OPA/mercaptoethanol derivatives, $E_{1/2} \approx 0.45$–0.6 V vs. Ag/AgCl as reported by Allison and coworkers (6), the half-wave potential for the OPA/TGTA derivatives is higher, $E_{1/2} \approx 0.65$–0.75 V.

Fluorescence detection has the advantage of being non-responsive to the reagents in the OPA reaction. With electrochemical detection the thiol component must either be separated in the chromatographic run or removed prior to the injection. The excess of TATG interferes with the most hydrophilic derivatives in the first part of the chromatogram. Addition of iodoacetamide (21) and subsequent extraction with pentane removed a major part of the TATG peak.

The fluorescence and electrochemical response of the OPA/TATG derivatives were evaluated for the leucine derivative using the same chromatographic conditions as in Figure 3. With a capacity factor (k') 8.7, injection volume 4.5 µl, detector time constant 0.5 sec and signal to noise ratio 3:1, the detection limits were 23 pmol and 1 pmol for the fluorescence and the electrochemical detectors respectively.

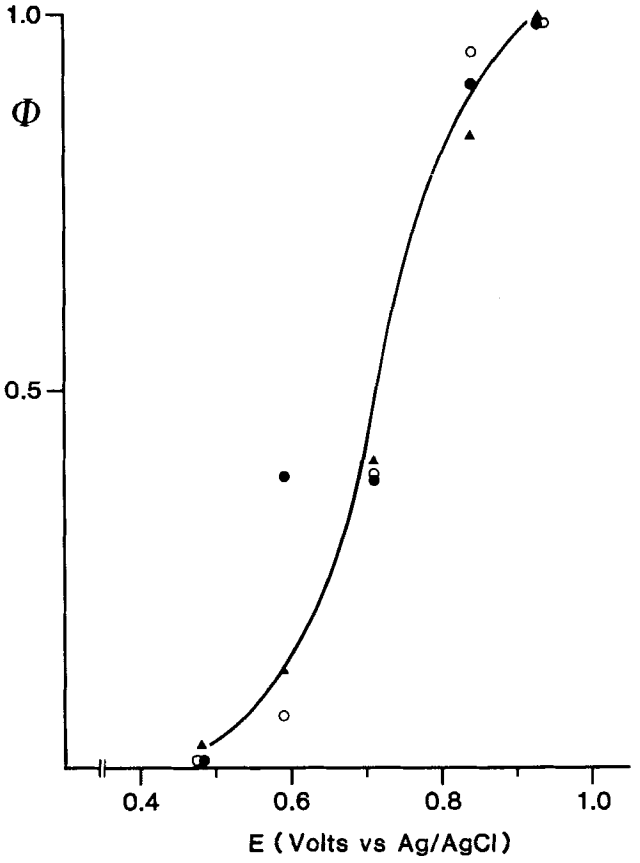

Figure 3. Voltammogram for TATG/OPA derivatives of tryptophane (x), methionine (o) and leucine (Δ). Φ is defined as the ratio of the peak current to the limiting current.
Mobile phase: methanol/THF/buffer (50 mM acetic acid, 1 mM Na_2EDTA, pH 7.03) (50:2:48);
Column: 5 x 0.46 cm, Spherisorb ODS2, 3 μm;
Flow rate: 1.0 ml/min.

In Table I a comparison is made of the concentration detection limits for the different fluorescence detectors. In spite of the relatively low output power of the He-Cd laser, the concentration detection limit was about the same for all three detectors. This favours the use of small bore packed columns for high sensitivity measurements since the lower dispersion in these columns allows smaller amounts to be detected. The free falling jet cell, which works with volumetric flow rates down to 20 µl/min, is suitable for columns with inner diameters down to about 0.5 mm. For columns with smaller inner diameters, a fused silica capillary cell is required.

Separation

The OPA/TATG derivatives of the enantiomers of 16 amino acids were separated under isocratic conditions with three different organic solvents; acetonitrile, methanol and tetrahydrofuran (THF), and an acetic acid buffer (Table II).

TABLE 1

Fluorescence Detection Limits

Concentration detection limits for the OPA/TATG L-leucine derivative. Signal to noise ratio 3:1.

Detector*	Cell volume**	Detection limit
Schoeffel FS 970	5 µl	16 nM
Laser, 100 µm I.D. fused silica capillary	0.3 nl	19 nM
Laser, free falling jet (orifice ≈ 15 µm)	3 pl	10 nM

*) Detector time constant: Schoeffel 0.5 sec.; Laser 0.25 sec.
**) Laser: illuminated volume

TABLE 2

Capacity Factors and α-Values in Three Solvent Systems

	Methanol			THF			Acetonitrile		
Amino acid	k'(L)	k'(D)	α	k'(L)	k'(D)	α	k'(L)	k'(D)	α
Aspartic acid	2.79	3.63	1.30	1.03	1.34	1.31	2.71	3.44	1.27
Glutamic acid	4.65	5.86	1.26	1.40	1.66	1.18	3.95	5.10	1.29
Serine	1.65	1.99	1.21	2.20	2.45	1.11	2.19	2.71	1.24
Histidine	1.67	1.99	1.19	1.51	1.74	1.15	1.95	2.43	1.24
Tyrosine	2.05	3.11	1.52	3.80	4.60	1.21	3.29	4.76	1.45
Threonine	3.23	3.51	1.09	3.46	3.46	1.00	3.38	4.00	1.18
Alanine	3.31	4.26	1.29	3.11	3.40	1.09	3.57	4.62	1.29
Arginine	4.26	4.26	1.00	3.97	4.14	1.04	5.24	6.14	1.17
Methionine	2.12	2.31	1.09	3.91	3.91	1.00	2.62	3.05	1.16
Valine	2.20	2.66	1.21	3.80	4.14	1.09	2.43	3.10	1.27
Phenylalanine	2.08	2.91	1.39	5.13	6.12	1.19	3.76	5.29	1.41
Tryptophane	1.00	1.33	1.33	4.69	6.23	1.33	2.67	3.90	1.46
Isoleucine	3.34	4.00	1.19	5.46	5.86	1.07	3.62	4.71	1.30
Leucine	4.54	4.54	1.00	5.91	5.91	1.00	4.62	5.24	1.13
Ornithine	9.23	8.60	1.07	7.97	7.80	1.02	3.43	3.43	1.00
Lysine	12.5	12.5	1.00	10.3	10.3	1.00	4.29	4.29	1.00

Eluent conditions:

I	Asp, Glu:	35% MeOH, 18% THF, 18% ACN
II	Ser, His, Tyr, Thr, Ala, Arg:	50% MeOH, 25% THF, 28% ACN
III	Met, Val, Phe, Trp, Ile, Leu:	60% MeOH, 28% THF, 33% ACN
IV	Orn Lys:	60% MeOH, 32% THF, 40% ACN

Buffer: Acetic acid 3 ml/l, adjusted to pH 7.0 with NaOH
Column: 25 x 0.46 cm packed with 5 μm Spherisorb octyl

The best resolution was generally obtained with acetonitrile, but even methanol gave satisfactory α-values for most amino acids. All enantiomers were successfully resolved with the exception of the doubly derivatized ornithine and lysine. The reason for the poor resolution of the bis-derivatives is not clear, but similar results have been obtained with other reported chiral thiols, e.g. N-protected cysteine (10,11). The amino acids eluted in groups with fairly similar k' values (Table II). For resolution of the amino acids within each group, an advantage can be taken of the selectivity differences of the three organic solvents.

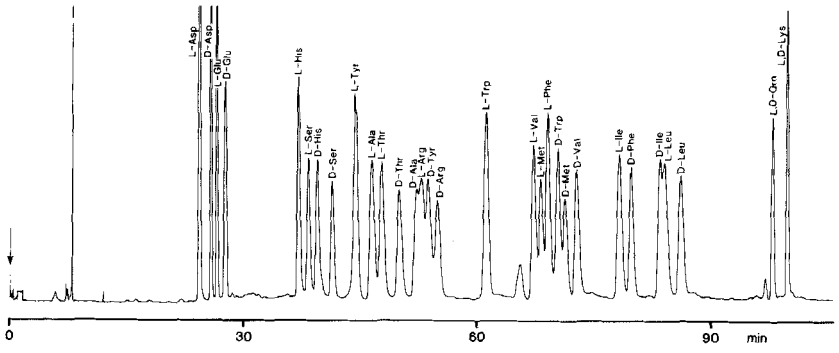

Figure 4. Gradient separation of a standard solution of L- and D-amino acids. Column: 50 x 0.02 cm, fused silica packed with Spherisorb octyl, 5μm; flow rate: 1.5 μl/min. Detector: laser, on-column.
Gradient: 0 min, 20% MeOH, 10% THF, 70% buffer
10 min, 20% MeOH, 10% THF, 70% buffer
20 min, 35% MeOH, 10% THF, 55% buffer
50 min, 60% MeOH, 15% THF, 25% buffer
Buffer: Acetic acid, 2 ml/l, adjusted to pH 7 with sodium hydroxide.

A gradient separation of a mixture of D- and L-amino acids is shown in Figure 4. The elution was carried out with methanol and THF. Methanol was chosen rather than acetonitrile, because it gave a better overall selectivity of the different amino acid derivatives. Mixing methanol with THF retarded the elution of tyrosine, phenylalanine and tryptophan relative to other amino acid derivatives, which was useful in tuning the separation. With these two solvents the majority of the common protein amino acids were resolved. Besides the organic solvents, both the ionic strength and the pH of the buffer can be used for selectivity control. The pH was kept constant in the present study, but the elution of the arginine pair was selectively effected by the ionic strength of the elution buffer. A decrease in ionic strength resulted in an increase in the retention of the arginine pair relative to the other amino acids.

In many applications only one or a few amino acids are of interest for resolution of the D- and L-forms. From the results shown in Table II and Figure 4 the separation can be optimized for the desired components for obtaining a separation free of interferences from other amino acids.

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