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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF NON-ESTERIFIED ENANTIOMERIC AMINO ACIDS BY DERIVATIZATION WITH 2,3,4,6-TETRA-O-ACETYL-β-D-GLUCOPYRANOSYL ISOTHIOCYANATE AND 2,3,4-TRI-O-ACETYL-α-D-ARABINOPYRANOSYL ISOTHIOCYANATE

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SUMMARY

Novel methods for reversed-phase high-performance liquid chromatographic resolution of non-esterified amino acid enantiomers by the formation of diastereomers using two chiral reagents, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate, are described. These compounds react readily with enantiomeric free amino acids at room temperature and the reaction mixture can be injected directly into the chromatograph. The separation of the enantiomers was monitored spectrophotometrically at 250 nm. Complete resolutions were observed for all enantiomers examined on a reversed-phase column eluted with methanol–10 m*M* potassium phosphate (pH 2.8).

INTRODUCTION

Many attempts have been made to resolve optical isomers by liquid chromatographic techniques, using chiral derivatization reagents[1–3], chiral eluents or chiral stationary phases[4]. In particular, various pre-column chiral derivatization methods[5–8] have recently been developed for the resolution of enantiomeric amino acids by high-performance liquid chromatography (HPLC). However, these methods require concomitant protection of free amino or carboxyl residues not involved in the chiral derivatization reaction.

The present paper describes the chiral derivatization of amino acid enantiomers using either 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) or 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). The derivatives thus prepared could effectively be resolved by reversed-phase chromatography without further protection of the free carboxyl residue. Simple and rapid separation of enantiomeric amino acids was accomplished.

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MATERIALS AND METHODS

Amino acids and other reagents were obtained from Wako (Osaka, Japan) and Tokyo Chemical Industry Co. (Tokyo, Japan). All the reagents were of analytical reagent grade. Methanol and water were distilled before use. GITC and AITC were prepared by treatment of α-acetobromoglucose and β-acetobromoarabinose with silver thiocyanate as described previously[9]. The 10 m*M* phosphate buffer was prepared from monobasic potassium phosphate and was adjusted to pH 2.8 with perchloric acid.

Equipment

The chromatographic system consisted of a high pressure pump equipped with a universal valve injector (Sanuki Industry Co., Tokyo, Japan), a Develosil ODS-5 column (15 cm × 4.6 mm I.D., particle size 5 μm; Nomura Chemical, Seto, Japan) and an SPD-2A spectrophotometric detector (Shimadzu Seisakusho, Kyoto, Japan).

Derivatization and separation

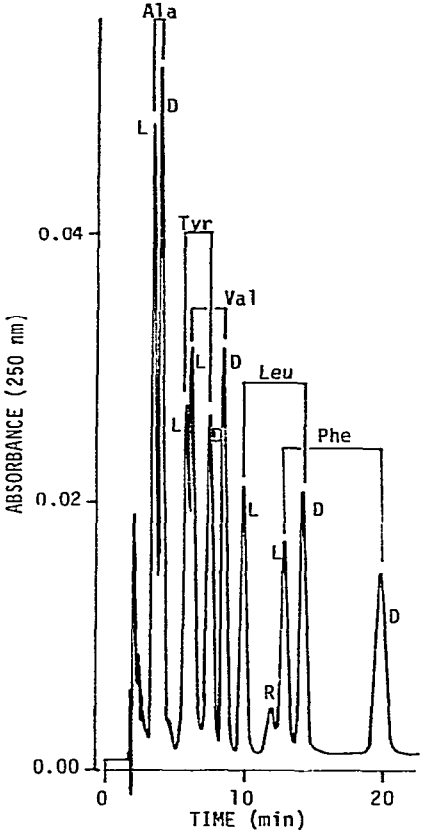
A 5-mg amount of each amino acid was dissolved in 50% (v/v) aqueous acetonitrile containing 0.4% (w/v) triethylamine to give a final volume of 10 ml. To a 50-μl aliquot of this stock solution were added 50 μl of 0.2% (w/v) chiral reagent, either GITC or AITC, in acetonitrile. The resulting mixture was allowed to stand at room temperature for 30 min and a 2-μl aliquot was injected directly into the chromatograph. The column was eluted at room temperature and at a flow-rate of 0.9 ml/min with a mobile phase prepared by mixing methanol and 10 m*M* phosphate buffer, pH 2.8, in an appropriate ratio.

For the resolution of phenylalanine diastereomers, the reagent peak was removed as follows. To the reaction mixture described above were added 10 μl of 0.25% (w/v) monoethanolamine in acetonitrile and the resulting mixture was allowed to stand for 10 min at room temperature prior to the injection.

RESULTS

GITC and AITC react readily with free amino acids under mild conditions without the formation of by-products. The resulting mixture can be injected directly into the chromatograph. The diastereomers resolved were detected spectrophotometrically at 250 nm; the molar extinction coefficients of the thiourea derivatives were around 12,000 $l \cdot mol^{-1} \cdot cm^{-1}$, whereas those of the reagents were around 1000 $l \cdot mol^{-1} \cdot cm^{-1}$.

Figs. 1 and 2 show the chromatograms of several sets of isomeric amino acids when GITC and AITC respectively are used for the derivatization. The reagent peaks were well separated from those of most of the amino acid derivatives and do not interfere with the detection. The limit of the detection for amino acids was 5 ng. However, a slight overlapping was observed between the peaks of the reagent(s) and phenylalanine diastereomers, 50 ng phenylalanine being required for unambiguous detection. This interference could be avoided by addition of monoethanolamine to the reaction mixture, since monoethanolamine reacts with the excess of reagent and the reaction products are eluted faster than any of the amino acid



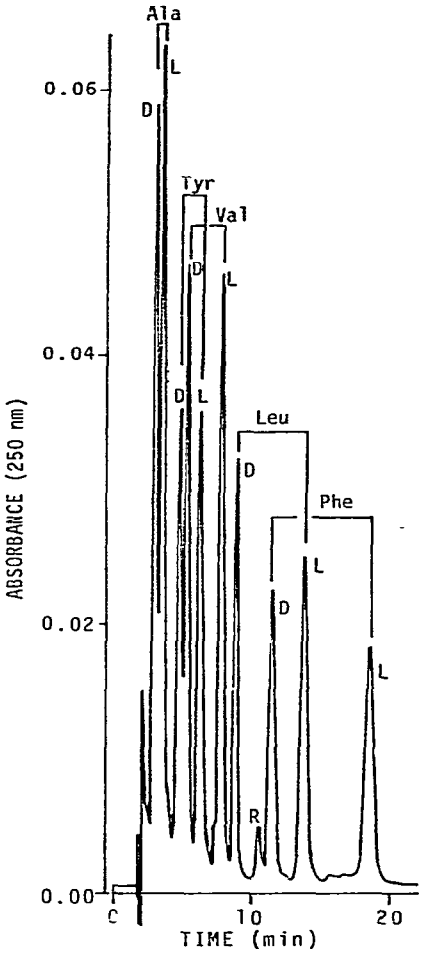


Fig. 1. Separation of diastereomeric thiourea derivatives formed from amino acids with GITC. Mobile phase: methanol–10 m*M* phosphate buffer (pH 2.8) (55:45); flow-rate 0.9 ml/min. About 250 ng of each derivative were injected. R = Reagent (GITC).

Fig. 2. Separation of diastereomeric thiourea derivatives formed from amino acids with AITC. Mobile phase: methanol–10 m*M* phosphate buffer (pH 2.8) (50:50); flow-rate 0.9 ml/min. About 250 ng of each derivative were injected. R = Reagent (AITC).

derivatives. This procedure did not affect the detection of other amino acids and 5 ng phenylalanine could be detected.

The retentions and resolutions of the diastereomeric GITC and AITC derivatives are listed in Table I; k', α and R_s refer to the capacity ratio, separation factor and resolution respectively for a pair of diastereomers. The resolution of diastereomeric GITC and AITC derivatives of non-esterified amino acids on the reversed-phase column, Develosil ODS, with methanol–10 m*M* phosphate buffer (pH 2.8) as mobile phase, was excellent.

TABLE I

SEPARATION OF DIASTEREOMERIC THIOUREA DERIVATIVES FORMED FROM FREE AMINO ACIDS WITH GITC AND AITC

t_0 = 2.0 min. Column, Develosil ODS-5 (15 cm × 4.6 mm I.D.). Mobile phase: methanol–10 m*M* phosphate buffer (pH 2.8) (40:60) (A), (45:55) (B), (50:50) (C), (55:45) (D); flow-rate 0.9 ml/min. k', α and R_s are defined in the text.

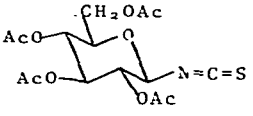
Amino acid	*GITC*					*AITC*				
	k'		α	R_s	*Mobile phase*	k'		α	R_s	*Mobile phase*
	L	D				D	L			
Glutamic acid	2.70	3.15	1.17	1.80	B	1.20	1.40	1.17	1.14	A
Aspartic acid	3.80	4.35	1.14	1.69	B	1.40	1.65	1.18	1.43	A
Proline	2.80	3.70	1.32	3.00	B	1.55	2.15	1.39	3.00	A
Alanine	3.85	5.50	1.43	4.40	B	1.60	2.45	1.53	3.78	A
Tyrosine	1.75	2.55	1.46	3.20	D	1.65	2.45	1.48	3.56	C
Valine	2.00	3.10	1.55	4.00	D	1.95	3.45	1.77	4.96	C
Phenylglycine	2.10	2.80	1.33	2.80	D	2.15	3.25	1.51	4.40	C
Isoleucine	3.55	5.50	1.55	5.01	D	4.05	7.25	1.79	6.94	C
Leucine	3.75	5.65	1.51	5.43	D	4.25	7.35	1.73	7.75	C
Tryptophan	4.95	7.25	1.46	5.11	D	6.55	9.85	1.50	6.00	C
Phenylalanine	5.00	8.10	1.62	6.89	D	6.25	11.10	1.78	8.43	C

DISCUSSION

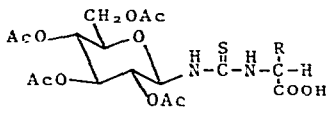
Chiral reagents for pre-column derivatization have been developed for the liquid chromatographic resolution of enantiomers[1–8]. Among these reagents, the terpene isothiocyanates which have recently been synthesized by Nambara *et al.*[6] were suitable for HPLC separation of enantiomeric amino acids on normal phases but not on reversed phases. Scott *et al.*[1] and Nambara and co-workers[6,7] have pointed out that the degree of separation of diastereomers on a normal-phase column should be dependent on the rigidity of the conformation. Introduction of a bulky group into the amino acids increases the conformational rigidity. Nambara *et al.*[6] reported that *tert.*-butyldimethylsilyl groups improve the resolution through their stereochemical effect.

We have recently reported[9] the reversed-phase chromatographic resolution of enantiomeric amino acid ethyl esters using GITC for the pre-column derivatization. The GITC derivatives were well resolved compared with terpene thiourea derivatives of *tert.*-butyldimethylsilyl esters. This fact was assumed to be due to the bulkiness of the tetraacetylglucosyl moiety[9].

The present study has demonstrated that esterification of the carboxyl group of GITC or AITC is not necessary for the reversed-phase resolution. The excellent resolution obtained may be due to the lipophilic nature of the acetylglycosyl residues as well as their conformational rigidity. This greatly simplifies the pre-column derivatization. An examination using the Corey–Pauling Koltun molecular model (Ealing Corp., MA, U.S.A.) suggests that the conformations of the GITC- and

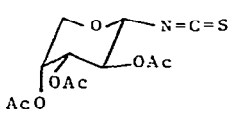


GITC

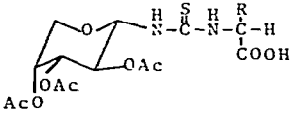


GITC-Amino acid

Ac = $-COCH_3$



AITC



AITC-Amino acid

AITC-amino acids are rigidly fixed owing to the bulky acetylglycosyl residues. Interestingly, ennantiomeric pairs were generally eluted in the order L before D when GITC was used, but in the opposite order when AITC was used. The model revealed that both amino acid derivatives have hydrophobic surfaces which may interact with the hydrophobic ODS residues and thus account for the resolution observed. The conformational difference between the two acetylglycosyl residues, especially at the anomeric carbon atom which affects the surface structure, may be responsible for the difference in the order of elution. This also suggests that pre-column labelling using sugar derivatives may result in specific separation in liquid chromatography.

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