Journal of Chromatography, 202 (1980) 375–379
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,253

REVERSED-PHASE LIQUID CHROMATOGRAPHIC RESOLUTION OF AMINO ACID ENANTIOMERS BY DERIVATIZATION WITH 2,3,4,6-TETRA-O-ACETYL-β-D-GLUCOPYRANOSYL ISOTHIOCYANATE

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(Received August 14th, 1980)

SUMMARY

A novel method for reversed-phase high-performance liquid chromatographic resolution of amino acid enantiomers by the formation of diastereomers using a new chiral reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), is described. GITC reacts readily with enantiomeric amino acids at room temperature and the reaction mixture can directly be injected into the chromatograph. The derivatives were detected spectrophotometrically at 250 nm. Complete resolutions were observed for all enantiomers examined on a reversed-phase column eluted with aqueous methanol.

INTRODUCTION

Chiral derivatization, *i.e.*, the conversion of enantiomers into the corresponding diastereomers, has been used for the liquid chromatographic resolution of optical isomers[1–3]. Recent advances[4–6] in high-performance liquid chromatography (HPLC) prompted the development of chiral reagents having functional groups which react readily and selectively with enantiomers, and appropriate chromophores or fluorophores which may readily be detected after chromatographic separation. Among the reagents so far developed, the terpene isothiocyanates synthesized by Nambara *et al.*[5] have been shown to be suitable for HPLC; the isothiocyanate group is selective toward primary and secondary amines under mild conditions, and the thiourea derivative produced is very sensitive to UV detection. The diastereomers prepared using these reagents have been resolved on normal phase or ion-exchange columns[7]. However, reversed-phase chromatography of these derivatives gave only poor resolution.

The present paper describes the derivatization of enantiomeric amino acids using a new chiral reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[8]. Glycosyl isothiocyanates have extensively been investigated as a starting material for the N-glycoside synthesis by Ukita *et al.*[9] and Ogura and co-workers[10–14]. The resolution of GITC derivatives of enantiomeric amino acids on a reversed-phase column was better than that obtained with conventional chiral reagents.

0021-9673/80/0000–0000/$02.25

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. Amino acid ethyl esters were prepared by treatment with absolute ethanol and hydrogen chloride in the usual manner. Ultraviolet (UV) spectra were taken with a Model 200-20 spectrophotometer (Hitachi Ltd., Tokyo, Japan).

Preparation of GITC

α-Acetobromoglucose (4.1 g) was heated at about 100°C in dry xylene (20 ml) with thoroughly dried silver thiocyanate (5.0 g) under efficient stirring. After 1 h, silver thiocyanate (3.4 g) was added in two equal portions at intervals of 30 min, and the heating and stirring were continued for another 30 min. The reaction mixture was then filtered while still hot and the precipitate was washed twice with 10-ml portions of hot xylene. To the combined filtrates was added light petroleum (60 ml), and the crystalline isothiocyanate deposited almost instantaneously. The product was collected after 16 h and dried over P_2O_5 under reduced pressure. Yield 2.9 g (75%), m.p. 113–115°C.

Chromatography

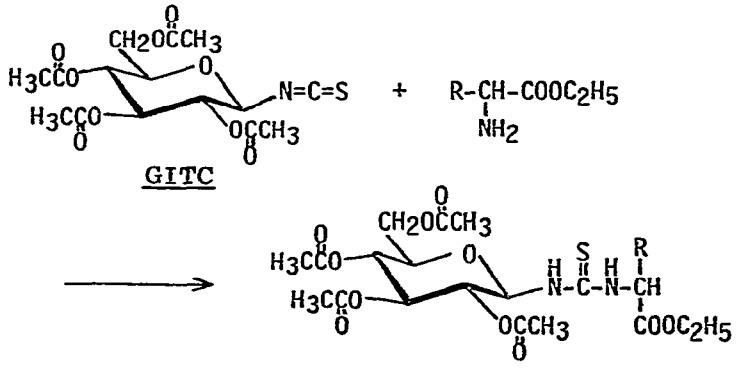
The modular liquid chromatographic system consisted of a NSP-800-9DX pump equipped with NV1-3001 valve injector and NPG-800L pressure gauge (Sanuki Industry Co., Tokyo, Japan), and SPD-2A spectrophotometric detector (Shimadzu, Kyoto, Japan). A stainless-steel column (250 × 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size 5 μm; E. Merck, Darmstadt, G.F.R.) by a slurry packing method[15].

Derivatization and separation procedure

A 5-mg amount of amino acid ethyl ester was dissolved in acetonitrile containing 0.2% (w/v) triethylamine to give a volume of 5 ml. To a 50-μl aliquot of this amino acid solution were added 50 μl of 0.5% (w/v) GITC in acetonitrile. The resulting mixture was allowed to stand at room temperature for 60 min, and a 5-μl aliquot was injected directly into the chromatograph and eluted with either 50% or 60% (v/v) aqueous methanol at a constant flow-rate of 0.4 ml/min, at room temperature.

RESULTS AND DISCUSSION

GITC reacts readily with amino acids in the presence of base[11] as follows:



The resulting mixture can directly be injected into the chromatograph. Both GITC and thiourea derivatives absorb light at 250 nm. However, the molar extinction coefficient of GITC is 1000 whereas those of GITC-amino acids are around 12,000, *i.e.*, the reagent shows far lower absorbance than those of the diastereomers. The excess of reagent used for the derivatization gives a single peak after D-alanine when eluted with 50% aqueous methanol and it does not interfere with the analysis of any diastereomer. On the other hand, the reagent peak appears shortly before L-tyrosine when eluted with 60% aqueous methanol. Nevertheless, the reagent peak does not affect the detection of L-tyrosine, although slight increase in the peak height of the amino acid is observed. The limit of detection of each diastereomer monitored at 250 nm is 5 ng per sample injected.

The retention and resolution values of the diastereomeric GITC derivatives are listed in Table I. Nambara *et al.*[5] reported that the diastereomers derived from enantiomeric amino acid methyl esters using terpene isothiocyanate were partially separated on a μPorasil column, whereas separation on a μBondapak C_{18} column was not attained. The diastereomeric GITC derivatives of amino acid ethyl esters showed excellent resolution on an ODS column, LiChrosorb RP-18. This indicates that GITC derivatization is suitable for reversed-phase chromatographic resolution of amino acids.

Excellent resolutions of the amino acid derivatives were accomplished by the present method. All the amino acids listed in Table I were resolved using aqueous 60% methanol, but serine, alanine, proline, aspartic acid and glutamic acid gave

TABLE I

SEPARATION OF DIASTEREOMERIC THIOUREA DERIVATIVES FORMED FROM AMINO ACID ETHYL ESTERS WITH GITC

t_0 = 4.0 min. Column, LiChrosorb RP-18 (25 cm × 0.4 mm I.D.). Mobile phase, 50% aqueous methanol (A), 60% aqueous methanol (B); flow-rate, 0.4 ml/min. k', α and R refer to the capacity ratio, separation factor and resolution value for a pair of diastereomers, respectively.

Amino acid	k'		α	R	*Mobile phase*
	L	*D*			
Serine	1.70	1.50	1.13	1.12	A
	0.95	0.83	1.13	0.71	B
Alanine	2.69	2.93	1.09	1.63	A
	1.50	1.62	1.08	1.16	B
Proline	4.28	4.67	1.21	1.21	A
	1.58	1.61	1.02	0.38	B
Aspartic acid	5.38	5.71	1.06	1.01	A
	2.67	2.83	1.06	0.62	B
Glutamic acid	5.90	6.26	1.06	0.95	A
	2.85	2.97	1.04	0.47	B
Tyrosine	2.57	2.90	1.13	1.56	B
Valine	3.50	4.13	1.18	2.48	B
Phenylglycine	4.31	4.81	1.12	1.78	B
Tryptophan	5.66	6.64	1.17	3.09	B
Isoleucine	5.67	6.71	1.18	2.90	B
Leucine	5.70	6.73	1.18	3.00	B
Phenylalanine	6.85	8.65	1.26	4.28	B

resolution values less than unity. The resolution values for these amino acids became greater than unity when aqueous 50% methanol was used as the eluent. Enantiomeric pairs were always eluted in the order of L before D, with the exception of serine for which the D-amino acid exhibited a larger retention value than the corresponding L-enantiomer. Figs. 1 and 2 depict the chromatograms of several isomeric amino acids showing good resolutions.

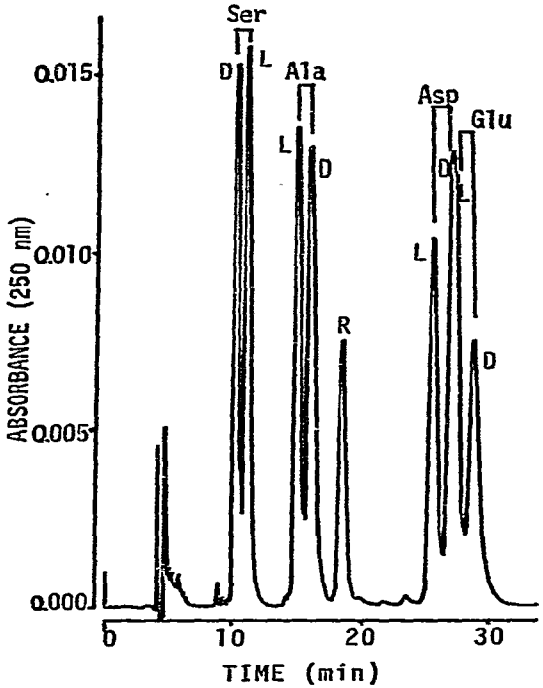


Fig. 1. Separation of diastereometic thiourea derivatives formed from amino acid ethyl esters with GITC. Mobile phase: 50% aqueous methanol; flow-rate, 0.4 ml/min. About 250 ng of each derivative were injected. R = Peak of excess of reagent (GITC).

Some workers[1,5,6] have found that the degree of separation for diastereomers on a normal phase column is largely dependent on the rigidity of their conformation. Introduction of a bulky group into the ester moiety makes the conformation more rigid. For example, Nambara *et al.*[5] have reported that the neomenthylthiourea derivatives of amino acid *tert.*-butyldimethylsilyl esters were completely resolved, whereas the corresponding methyl ester derivatives could not perfectly be resolved.

In the present study, reversed-phase chromatography coupled with GITC derivatization facilitated complete resolution of amino acid ethyl esters. An examination using the Corey-Pauling-Koltun model suggests that the conformations of GITC-amino acid ethyl esters are rigidly fixed owing to the bulky acetylglucosyl residue of GITC. This bulkiness seems to favour the separation. On the other hand, the model revealed that GITC-amino acids have hydrophobic surfaces as a result of the conformational rigidity. This surface is assumed to interact with hydrophobic

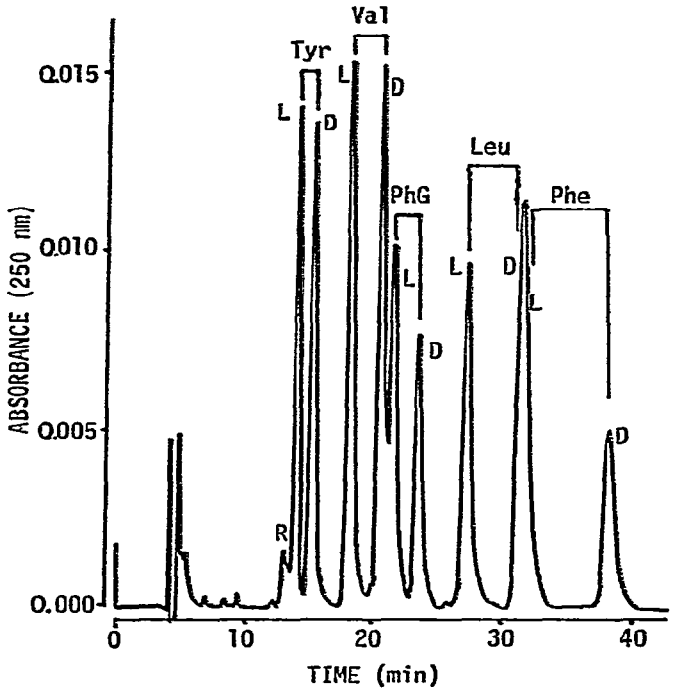


Fig. 2. Separation as in Fig. 1 except with 60% aqueous methanol as mobile phase. PhG = Phenylglycine.

ODS residues and amplify the resolution effect. Liquid chromatographic resolution of enantiomeric amino acids conjugated with various substituted glycosyl isothiocyanates other than GITC is expected to elucidate the relation between the reagent structure and the resolution.

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