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Resolution of the enantiomers of thiol compounds by reversed-phase liquid chromatography using chiral derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate

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ABSTRACT

A new derivatization procedure has been developed for converting enantiomeric thiol compounds into their diastereomers for resolution by reversed-phase liquid chromatography. The thiol compounds were derivatized with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate as chiral derivatization reagent and triethylamine as basic catalyst. The thiol group reacted smoothly with the isothiocyanate to form the dithiocarbamate derivative within 30 min at room temperature. The reaction mixture can be injected directly into the chromatograph without purification procedures. The ultraviolet detection wavelength was set at 250 nm, based on the absorption of the thiocarbonyl group. The resulting diastereomers were well separated on an octadecyl-bonded silica column with methanol–0.01 *M* phosphate buffer as the mobile phase.

INTRODUCTION

Various approaches for the chromatographic separation of enantiomers have been studied. Especially, high-performance liquid chromatography (HPLC) has been widely used in two ways: the direct resolution of enantiomers on a chiral stationary phase or with a chiral mobile phase, and derivatization with a chiral reagent followed by separation of diastereomers using a conventional column and mobile phase. Although the derivatization method has disadvantages in simplicity, this procedure is more favourable for the determination of racemic drugs in biological fluids with respect to sensitivity and versatility. A number of chiral derivatizing reagents have already been investigated for the enantiospecific determination of chiral amines [1–11], carboxylic acids [12–14], and alcohols [15,16]. However, there has been no study on the chiral derivatization of the thiol group to our knowledge.

We have studied a series of thiol compounds and developed three thiol compound, tiopronin [N-(2-mercaptopropionyl)glycine (**I**)] [17,18], rentiapril [(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid (**IIa**)] [19] and bucillamine [N-(2-mercapto-2-methylpropionyl)-L-cysteine (**IIIa**)] [20,21] as new drugs. They have a liver protective, an antihypertensive and an antirheumatic activity, respectively, and their molecular structures are shown in Fig. 1. Compound **I** is employed clinically as the racemic mixture, whereas other two drugs, **IIa** and **IIIa**, are the optically active compounds. Because the two enantiomers of a drug may have different pharmacological and/or pharmacokinetic characteristics, analytical methods are required to separate them.

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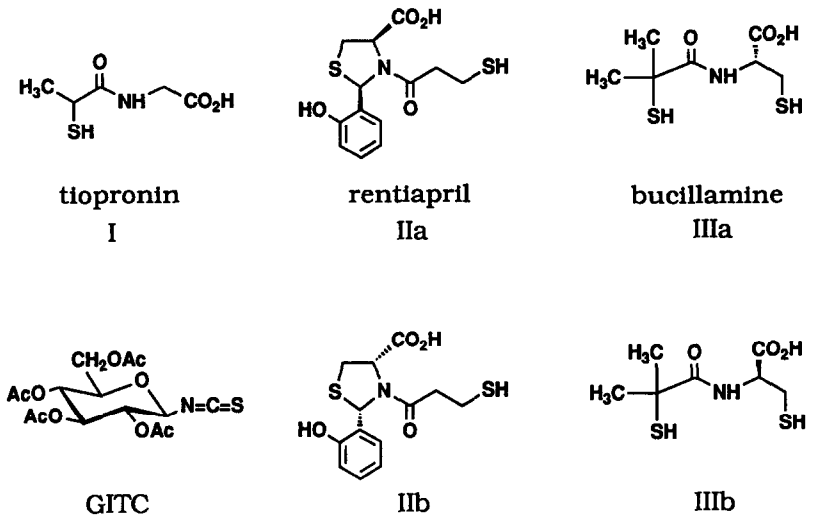

Fig. 1. Molecular structures of GITC and the thiol compounds used in this study.

These thiol molecules contain a carboxyl group. At first, we considered the chiral derivatization of the carboxyl group with the following chiral reagents: (−)-1-(4-dimethylamino-1-naphthyl)ethylamine [12] and (−)-1-(1-anthryl)ethylamine [14]. However, this method required concomitant protection of a free thiol residue not involved in the chiral derivatization reaction. Although we investigated the chiral derivatization of the thiol group, only poor separation was achieved with reversed-phase HPLC with the following derivatives: *l*-menthoxyacetyl, (*R*)-α-hydroxyphenylacetyl and N-benzyloxycarbonyl-L-prolyl.

One chiral reagent, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) is highly useful for the resolution of a variety of amino compounds [7,22–29]. It is known that the coupling reaction of the isothiocyanate group with the thiol group affords the corresponding dithiocarbamate derivative [30–32]. Because the isothiocyanate group of GITC reacts rapidly with thiol groups under mild conditions, we investigated the applicability of GITC to the resolution of enantiomeric thiol compounds.

This paper describes a new, simple and rapid procedure for the resolution of the enantiomers of thiol compounds. The method is based on derivatization with GITC and separation of the resulting diastereomeric dithiocarbamates by reversed-phase HPLC.

EXPERIMENTAL

Materials

Tiopronin (**I**), rentiapril (**IIa**) and its enantiomers (**IIb**), bucillamine (**IIIa**) and its enantiomer (**IIIb**), and 2-mercapto-2-methylpropionic acid were synthesized by the Central Research Laboratories of Santen Pharmaceutical (Osaka, Japan).

The chiral reagent, GITC, was prepared from α-acetobromoglucose and silver thiocyanate as described by Nimura *et al.* [22]. The yield was 72.9% and the melting temperature was 114–114.5°C (reported 113–115°C) [22]. The optical purity was confirmed to be sufficient to analyse the thiol compounds by optical rotation, HPLC and NMR analyses. Several recrystallizations of GITC gave no significant change in the optical rotation. We applied the GITC to DL-alanine and L-alanine (Fig. 2): the ratio of the two peak areas corresponding to D- and L-alanine was found to be 1.00, and no other peaks were found in the analysis (Fig. 3A). In addition, L-alanine derivatization with the GITC showed only one peak. GITC has five chiral centres and epimerization would be detectable by NMR, but no unexpected signal from GITC was observed.

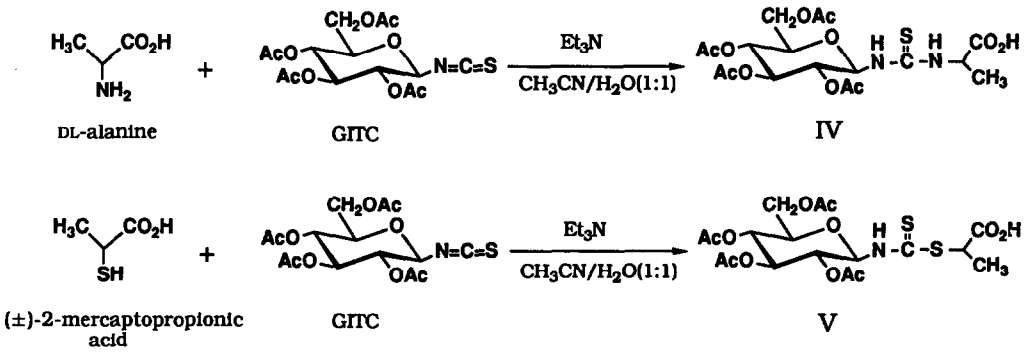

Fig. 2. Reaction schemes of DL-alanine and (±)-2-mercaptopropionic acid with GITC to give the thiourea derivative IV and the dithiocarbamate derivative V.

DL-Alanine, L-alanine, (±)-2-mercatopropionic acid, 2-mercaptoacetic acid, DL-homocysteine, DL-cysteine, DL-penicillamine, 2-mercaptoethanol, and triethylamine were obtained from Nakalai tesque (Kyoto, Japan). All other reagents and solvent were analytical grade from commercial sources and used without further purification.

Equipment and conditions

An L-6200 chromatographic pump (Hitachi, Tokyo, Japan) equipped with a variable-wavelength spectrophotometric detector (L-4000, Hitachi), a Chromatopac integrator (C-R3A, Shimadzu, Kyoto, Japan) and a column temperature control system (655A-52, Hitachi) were used. A reversed-phase column, TSKgel ODS-80TM (5 μm particle size, 150 × 4.6 mm I.D., Tosoh, Tokyo) was used at 40°C. The mobile phase (Table I) was delivered at 1.0 ml/min, and the column effluent was monitored at 250 nm.

^{1}H NMR spectra were measured on a JEOL GSX400 spectrometer (400 MHz) with tetramethylsilane as the internal standard in [$^2H_6$]dimethyl sulphoxide. For liquid chromatographic–atmospheric pressure chemical ionization mass spectrometry (LC–APCI-MS), a M-80B double-focusing mass spectrometer (Hitachi) equipped with an M-8093 APCI interface (Hitachi) was used. The conditions were as follows: mobile phase, methanol–0.1 *M* acetic acid (pH 2.8) (55:45, v/v); flow-rate, 1.0 ml/min; column temperature, 40°C; nebulizer temperature, 260°C; vaporizer temperature, 390°C; drift voltage, 180 V. IR spectra were recorded on a Perkin-Elmer 1640 FT infrared spectrophotometer (Yokohama, Japan). UV spectra were recorded on a UV-204 spectrophotometer (Shimadzu).

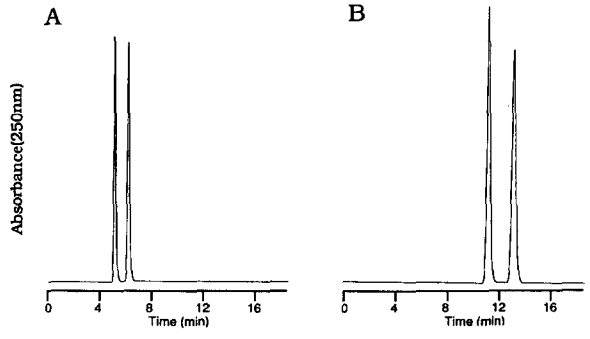

Fig. 3. HPLC resolution of (A) DL-alanine and (B) (±)-2-mercaptopropionic acid, after reaction with GITC. Chromatographic conditions are given in Experimental and in Table I.

Derivatization procedure

The following procedure was generally used. GITC (0.105–0.3 m*M*) was added to a solution of the thiol compound (0.1 m*M*) in acetonitrile–water (1:1, v/v; 10 ml), containing triethylamine (0.1–0.3 m*M*). After a brief vortexing, the resulting solution was allowed to stand at room temperature for 30 min. The reaction mixture was then diluted with a mobile phase, and then an aliquot (20 μl) of the mixture was injected directly into the chromatograph. In some cases, 2-mercaptoethanol (50 μl) was added to the reaction mixture at the end of the

TABLE I

SEPARATION OF ENANTIOMERIC THIOLS AFTER DERIVATIZATION WITH GITC

Capacity (k'), separation (α), and resolution (R_s) factors were determined as follows: $k'_1 = (t_1 - t_0)/t_0$, $k'_2 = (t_2 - t_0)/t_0$, $\alpha = k'_2/k'_1$, $R_s = 2(t_2 - t_1)/(W_1 + W_2)$, where t and W are retention time and bandwidth, respectively. Subscripts 0, 1, and 2 refer to the solvent peak, and the first- and second-eluting isomers, respectively.

Compound	Mobile phase[a]	k'_1	k'_2	α	R_s
DL-Alanine	M:A (50:50)	1.41 (L)	1.88 (D)	1.34	3.24
(±)-2-Mercaptopropionic acid	M:A (50:50)	4.23	5.14	1.22	3.55
DL-Homocysteine	M:A (60:40)	3.25	3.81	1.17	2.42
DL-Cysteine	M:A (60:40)	3.02 (L)	4.54 (D)	1.50	5.97
DL-Penicillamine	M:A (60:40)	3.99 (L)	5.24 (D)	1.32	4.63
Tiopronin (I)	M:A (50:50)	2.12 (*S*)	2.63 (*R*)	1.24	3.14
Rentiapril (IIa) + (IIb)	M:B (58:42)	7.83 (*R,R*)	8.55 (*S,S*)	1.09	1.57
Bucillamine (IIIa) + (IIIb)	M:A (53:47)	3.50 (*S*)	4.07 (*R*)	1.16	1.31

[a] Volumes of the two components were mixed in the ratios indicated. M = Methanol; A = 0.01 *M* phosphate (KH_2PO_4 – H_3PO_4) buffer (pH 2.8); B = 0.01 *M* phosphate (KH_2PO_4 – Na_2HPO_4) buffer (pH 5.0). Other chromatographic conditions are given in Experimental.

30-min derivatization period in order to destroy any excess GITC. Chromatographic analysis was performed after 10 min.

Characterization of the dithiocarbamate derivative (V)

To a solution of (±)-2-mercaptopropionic acid (5 m*M*) in dry tetrahydrofuran (20 ml), GITC (5 m*M*) and triethylamine (5 m*M*) were added, and the resulting solution was allowed to stand at room temperature for 30 min. After removal of the solvent, the residue was dissolved in ethyl acetate (50 ml), and washed successively with 2 *M* HCl (2 × 20 ml), water (20 ml) and brine (20 ml). The organic layer was dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness under vacuum. The residue obtained was purified through a silica gel column (50 g) with chloroform–methanol (50:1, v/v). The amorphous powder was obtained with a 93.2% yield and identified by ^1H NMR, UV, IR and LC–APCI-MS analyses.

Derivatization time-course study

GITC (0.105 m*M*) was added to a solution of a thiol compound (0.1 m*M*) in solvent (10 ml) containing triethylamine (0.1 or 0.3 m*M*). After a brief vortex-mixing, the resulting solution was allowed to stand at room temperature. At appropriate times, 1-ml samples were withdrawn in a test-tube. 2-Mercaptoethanol (50 μl) and the mobile phase (5.0 ml) were added to the test-tube, and an aliquot (20 μl) of the resulting solution was injected directly into the chromatograph.

RESULTS

Derivatization procedure and chromatographic separation

In preliminary experiments, DL-alanine and (±)-2-mercaptopropionic acid were derivatized with GITC (Fig. 2). (±)-2-Mercaptopropionic acid has a thiol group instead of the amino group of DL-alanine. These two racemates (0.1 m*M*) were treated with GITC (0.105 m*M*) in acetonitrile–water (1:1, v/v; 10 ml), containing triethylamine (0.1 m*M*) at room temperature for 30 min. The resulting mixture was diluted with a mobile phase, and then an aliquot (20 μl) was injected directly into the chromatograph. Each diastereomer was well resolved under the same HPLC conditions (Fig. 3). The column and mobile phase used were ODS and methanol–0.01 *M* phosphate buffer (pH 2.8) (Table I), respectively, under the similar conditions to those used by Kinoshita *et al.* [23].

Characterization of (±)-2-mercaptopropionic acid derivatized with GITC

The dithiocarbamate derivative (V) formed from

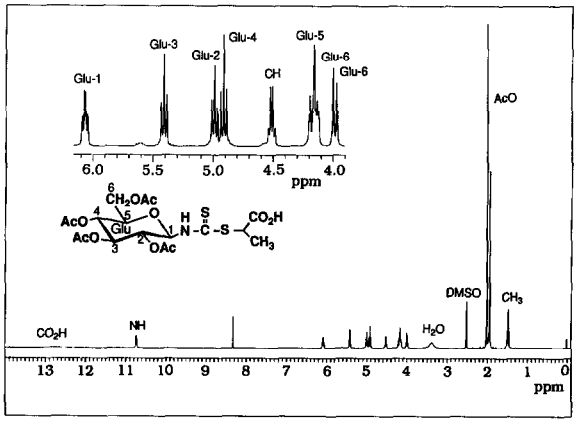

Fig. 4. ^{1}H NMR spectrum of the reaction product of (±)-2-mercaptopropionic acid with GITC, with tetramethylsilane (TMS) as the internal standard in [$^2H_6$]dimethyl sulphoxide (DMSO).

(±)-2-mercaptopropionic acid with GITC was obtained with a good yield (93.2%). This structure was identified by ^{1}H NMR, UV, IR and LC–APCI-MS analyses.

The structure of compound V was borne out by ^{1}H NMR spectrum, and all the peaks were assigned (Fig. 4). The UV absorption spectrum of compound V ($1 \cdot 10^{-4}$ *M*/methanol) is shown in Fig. 5: the absorption maximum at 248 nm is due to the thiocarbonyl group (ε = 12 610). The IR spectrum of compound V is shown in Fig. 6. The absorption bands centered at 3274, 1751, 1537 and 1224 cm^{-1}

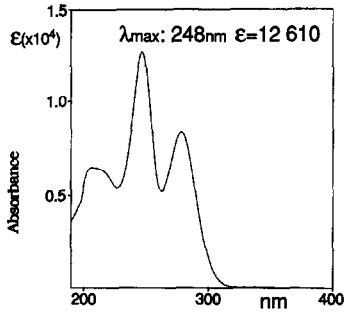

Fig. 5. UV absorption spectrum of the reaction product of (±)-2-mercaptopropionic acid with GITC ($1 \cdot 10^{-4}$ *M*/methanol solution).

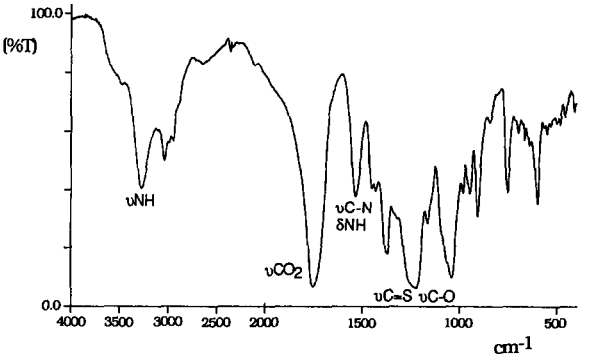

Fig. 6. Infrared spectrum of the reaction product of (±)-2-mercaptopropionic acid with GITC, obtained by the potassium bromide disk method.

were assigned to νNH, νCO_2H, νC–N and δNH, νC=S, and νC–O, respectively. The LC–APCI-MS spectra of compound V are shown in Fig. 7. The diastereomers of compound V were separated by HPLC. The total ion chromatogram showed two peaks (a) and (b), which gave the same spectra (B) and (C). The protonated molecule, $(M + H)^+$, appeared at *m/z* 496 as expected. The fragment ion at *m/z* 331 was assigned to the tetra O-acetylglucopyranosyl moiety.

Time-course for derivatization

The time-courses of the derivatization of DL-alanine and (±)-2-mercaptopropionic acid with GITC are shown in Fig. 8A. The reactions were complete within 15 min. The thiol group reacted faster than the amino group with GITC. Fig. 8B shows the rate of formation of the dithiocarbamate derivatives from (±)-2-mercaptopropionic acid with GITC in the following solvents: acetonitrile–water (1:1, v/v), acetonitrile and dimethylformamide. These solvents have been used for the derivatization of amino group with GITC [22–29]. In any solvent used, the reaction was completed within 15 min. Time-courses of derivatization of mercaptoacetic acid, 2-mercaptopropionic acid and 2-mercapto-2-methylpropionic acid with GITC are shown in Fig. 8C. The reaction was rapidly with primary and secondary thiol compounds, but slow with tertiary thiol groups. As shown in Fig. 8C, excess triethylamine was necessary to obtain rapidly the maximum yield of dithiocarbamate derivatives.

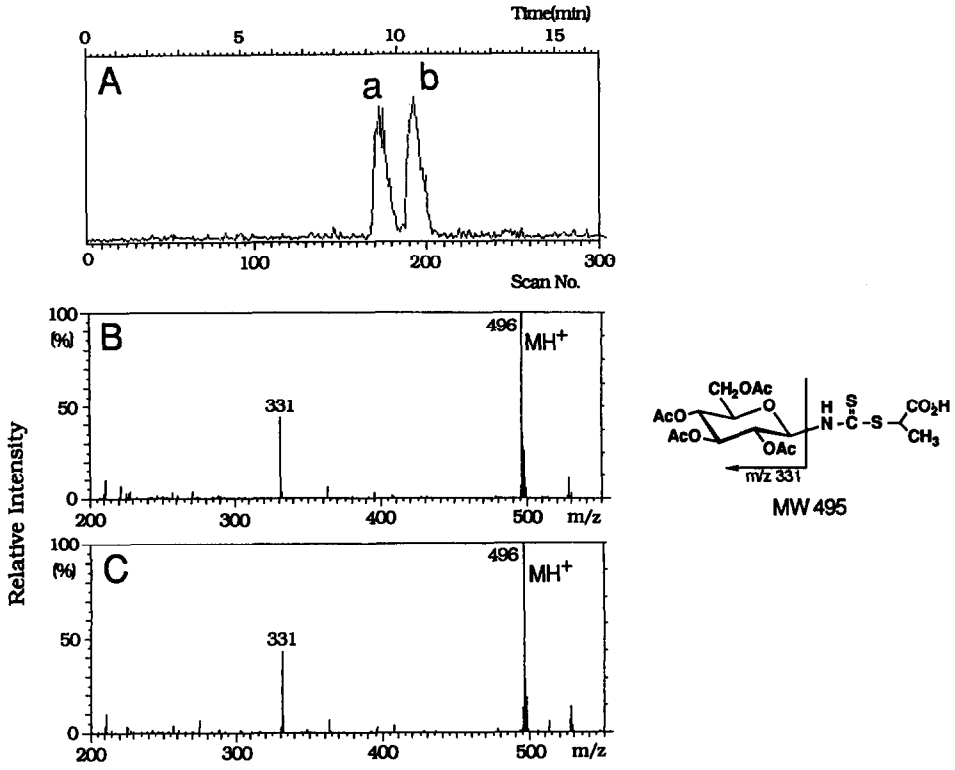

Fig. 7. LC–APCI-MS spectra of the reaction product of (±)-2-mercaptopropionic acid with GITC. Conditions are given in Experimental. (A) Total ion chromatogram (*m/z* 200–600); (B) mass spectrum of peak a; (C) mass spectrum of peak b.

Chromatographic separation of GITC derivatives formed from DL*-homocysteine,* DL*-cysteine and* DL*-penicillamine*

These molecules contain thiol and amino groups. GITC reacted with both functional groups, and then formed the corresponding thiourea and dithiocarbamate derivatives. The structures of these products were confirmed by ^{1}H NMR and MS analyses (data not shown). Fig. 9 shows the HPLC chromatograms of these products. The resolution parameters are summarized in Table I. Each pair of diastereoisomers was well resolved.

Excess GITC in the reaction mixture eluted at a retention time (t_R) that depended on the composition of the mobile phase. Under the chromatographic conditions employed for the derivatives of DL-homocysteine and DL-cysteine, GITC had a t_R of 8.2 min (peak a, Fig. 9A). The peaks of GITC and the dithiocarbamate derivatives overlapped. Excess GITC in the reaction mixture was converted into a derivative with a short t_R by a reaction with 2-mercaptoethanol at the end of the derivatization, and it was allowed to stand for 10 min. In the resolution of DL-homocysteine, DL-cysteine and DL-penicillamine, the reaction product of 2-mercaptoethanol with GITC had a t_R of 4.0 min (peak b, Fig. 9B–D).

*Chromatographic separation of GITC derivatives formed from tiopronin (**I**), rentiapril (**IIa**) and bucillamine (**IIIa**)*

Fig. 10A shows the chromatogram of dithiocarbamate derivatives of compound **I**. Fig. 10B1 shows the chromatogram of the dithiocarbamate mixture formed from compound IIa and its enantiomer IIb, and Fig. 10C1 the chromatogram of compound **IIIa** and its enantiomer **IIIb**. The chromatographic parameters are summarized in Table I. Each pair of diastereoisomers was well resolved.

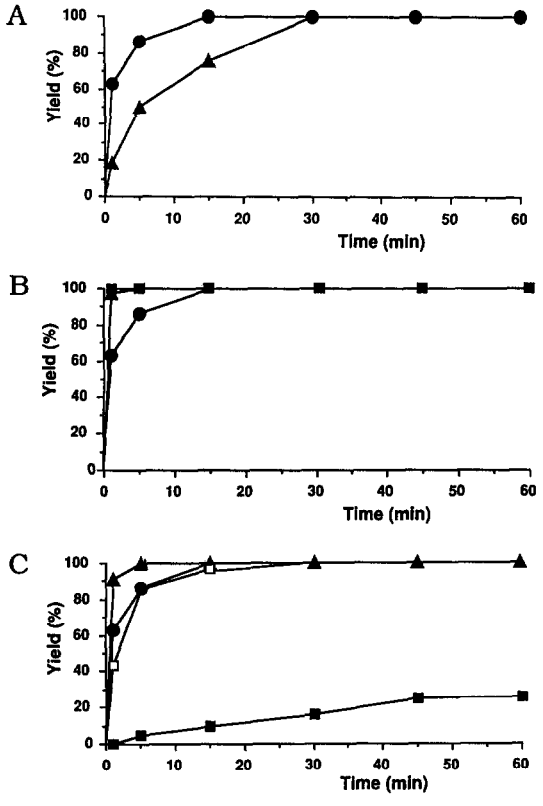

Fig. 8. Time-course of the derivatization of thiol compounds with GITC: (A) (▲) DL-alanine and (●) (±)-2-mercaptopropionic acid at room temperature; (B) (±)-2-mercaptopropionic acid in different solvents: ● = acetonitrile–water (1:1), ▲ = acetonitrile, and ■ = dimethylformamide; (C) mercaptoacetic acid, (±)-2-mercaptopropionic acid and 2-mercapto-2-methylpropionic acid at room temperature: ▲ = mercaptoacetic acid [triethylamine (0.1 m*M*)], ● = (±)-2-mercaptopropionic acid [triethylamine (0.1 m*M*], ■ = 2-mercapto-2-methylpropionic acid [triethylamine (0.1 m*M*)], □ = 2-mercapto-2-methylpropionic acid [triethylamine (0.3 m*M*)].

Fig. 10B2 and C2 show the chromatograms of the derivatives from compounds **IIa** and **IIIa**, respectively. Neither **IIb** nor **IIIb** was detected. The limits of detection for the enantiomers of **IIa** and **IIIa** were 0.1 and 1.0%, respectively. The limit of detection for thiol compounds was 5 ng (absolute).

DISCUSSION

A number of chiral derivatizing reagents have already been developed for the liquid chromatographic resolution of enantiomers [1–16]. However, there has been no study on the chiral derivatization of the thiol group to our knowledge. In this study we have demonstrated the enantiomeric resolution of thiol compounds.

The chiral reagent GITC is highly useful for the resolution of a variety of amino compounds. The isothiocyanate group reacts rapidly and selectively with primary and secondary amino groups under mild conditions to form the corresponding thiourea derivatives, and the resulting diastereomers are resolved under reversed-phase HPLC conditions. GITC has been used for the HPLC resolution of enantiomers of various amino acids [22,23], several β-adrenergic antagonists [24,25], catechol amines [26], and other amines [7,27–29]. The coupling reaction of isothiocyanate with thiol group to afford the dithiocarbamate derivative is also well established [30–32], so we considered that the isothiocyanate group of GITC reacts rapidly with the thiol group under mild conditions and investigated the applicability of GITC to the liquid chromatographic resolution of enantiomeric thiol compounds.

As expected, the isothiocyanate group of GITC reacted rapidly with thiol group under conditions similar to those for the amines, *i.e.* in the presence of triethylamine in the solvents acetonitrile–water (1:1, v/v), acetonitrile and dimethylformamide at room temperature within 30 min. The reaction was rapid with primary and secondary thiol groups, but slow with tertiary thiol (Fig. 8C). Addition of excess triethylamine takes the reaction to completion. The structures of the resulting derivatives were confirmed by ^{1}H NMR, IR, and LC–APCI-MS analyses, indicating that the reaction of isothiocyanate group of GITC with thiol group gives the corresponding dithiocarbamate derivatives (Fig. 2).

Because the thiocarbonyl group of the dithiocarbamate derivatives has a strong UV absorbance, HPLC detection was performed by monitoring the absorbance at 250 nm. The diastereoisomers were resolved on an ODS column, with methanol–0.01 *M* phosphate buffer as the mobile phase. When the GITC peak overlapped that of the desired dithiocarbamate derivative, 2-mercaptoethanol was added to the reaction mixture to prevent the interference: it reacted with the excess GITC to give a product that eluted before the peak of the desired derivative (Fig. 9).

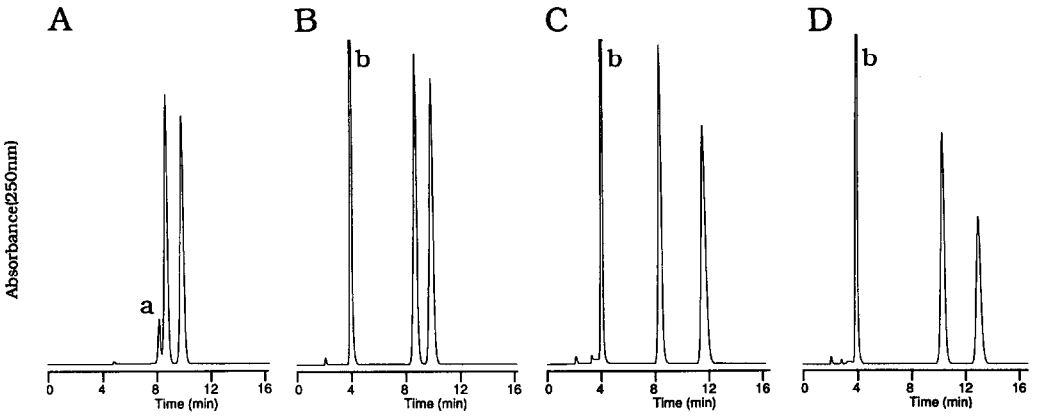

Fig. 9. HPLC resolution of DL-homocysteine, DL-cysteine and DL-penicillamine after reaction with GITC. Chromatographic conditions are given in Experimental and in Table I. (A) DL-Homocysteine derivatized with GITC. (B) DL-Homocysteine derivatized with GITC; after treatment with 2-mercaptoethanol. (C) DL-Cysteine derivatized with GITC; after treatment with 2-mercaptoethanol. (D) DL-Penicillamine derivatized with GITC; after treatment with 2-mercaptoethanol. Peaks: a = excess GITC; (b) 2-mercaptoethanol derivatized with GITC.

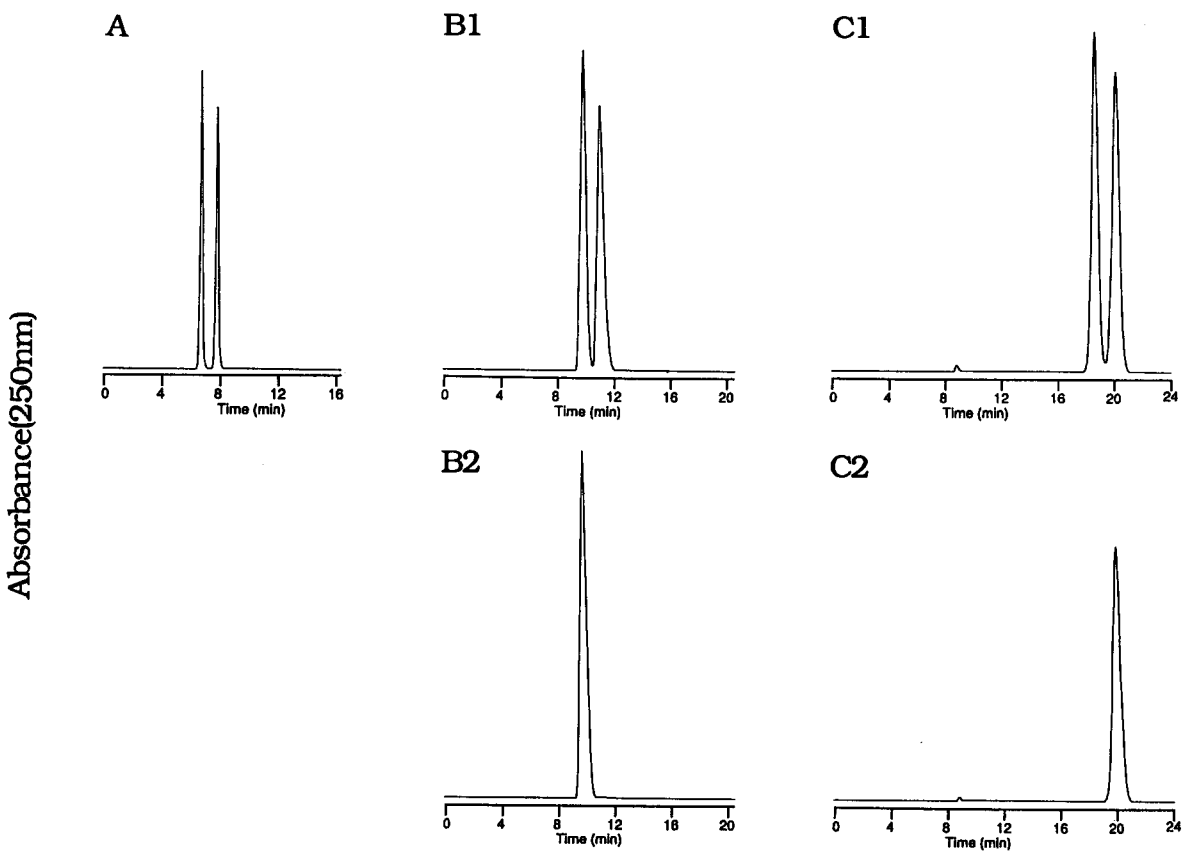

Fig. 10. HPLC resolution of tiopronin (I), rentiapril (IIa) and its enantiomer (IIb) and bucillamine (IIIa) and its enantiomer (IIIb) after reaction with GITC. Chromatographic conditions are given in Experimental and in Table I. (A) Tiopronin (**I**); (B1) rentiapril (**IIa**) and (**IIb**); (B2) rentiapril (**IIa**); (C1) bucillamine (**IIIa**) and (**IIIb**); (C2) bucillamine (**IIIa**).

The resolution of seven thiol racemates was studied (Table I). Because three of these racemates, homocysteine, cysteine and penicillamine, have both thiol and amino groups in the molecule, N,S-bis-GITC derivatives were produced. The separation of the diastereomeric derivatives was excellent, as judged by the value of the resolution factor (R_S, Table I). The resolution factor of homocysteine (R_s = 2.42) was lower than that of cysteine (R_s = 5.97), because the distance between the chiral carbon and thiol group in homocysteine is longer than that in cysteine. The GITC derivatives of the L-enantiomers of cysteine and penicillamine elute before the corresponding D-enantiomers.

The separation depends on the rigidity of the conformation around the chiral centres and the proximity between the two chiral centres of the diastereoisomer [2,3]. The chiral centre of compound **I** is located at the α-position from thiol group, and the distance between the chiral carbon of compound **I** and anomeric carbon of GITC is the same (three atoms) as in the GITC derivatives of usual amino acids [23]. So the derivative of compound **I** was well resolved, presumably owing to the bulkiness of the GITC residue.

Although thiol groups of compounds **IIa** and **IIb** are located at the ε-position from the chiral centres, and the distance between the chiral carbons of compounds **IIa** and **IIb** and anomeric carbon of GITC is therefore longer than those of usual amino acids and β-adrenergic antagonists, the rentiapril derivatives were considerably resolved, presumably owing to the rigid five-membered ring structure of 2-phenyl-4-thiazolidinecarboxylic acid. It was possible to detect 0.1% of **IIb** in the presence of 99.9% of **IIa**.

Compound **IIIa** possesses two thiol groups. The mono-GITC derivative with the thiol group of the cysteine residue was obtained (52%) when 1.1 *M* GITC was used in dimethylformamide solution without triethylamine. Although this derivative was not completely resolved, the bis-dithiocarbamate derivative was separated nearly completely, presumably owing to the bulkiness of two GITC residues. It was obtained in good yield when at least 2.1 mole of GITC was used in acetonitrile or acetonitrile–water in the presence of triethylamine. A 1.0% contamination of optical impurity (**IIIb**) was detectable by this method.

The optical purity of tiopronin (**I**) was determined by this method. Three lots of raw materials of **I** were used. We confirmed that **I** was the racemic compound in Fig. 10A. It was shown that the ratio of the peak area of the first to the last eluted diastereomer was 1.00. The optical purities of the raw materials, rentiapril (**IIa**) and bucillamine (**IIIa**), were measured in a similar manner. Neither opposite enantiomer was detected in Fig. 10B2 and C2. The enantiomeric purities of **IIa** and **IIIa** were shown to be at least 99.9% and 99.0%, respectively.

In conclusion, GITC is a useful reagent for the resolution of the enantiomers of thiol compounds by reversed-phase HPLC. The thiol group reacts with GITC to form the dithiocarbamate derivative within 30 min at room temperature. The reaction mixture can be injected direclty into the chromatograph without purification. The formed diastereomers are well separated on a reversed-phase ODS column with methanol–0.01 *M* phosphate buffer as the mobile phase. The present method may be generally applicable to the resolution of optical isomers, even if the thiol group is distant from the chiral centre.

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