The Analyst

Indirect resolution of thiol enantiomers by high-performance liquid chromatography with a fluorescent chiral tagging reagent

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The fluorescent chiral tagging reagent, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS), was utilized for the resolution of thiol enantiomers as diastereomeric derivatives. The reagent reacts with thiol enantiomers in the presence of pyridine to produce the corresponding dithiocarbamate diastereomers under mild reaction conditions (50 °C for 40 min). The fluorescence properties (maximum wavelengths and intensities) of the derivatives were dependent on the solvents in the medium. Several thiols derivatized with the proposed reagent were efficiently resolved by an ODS column with water–acetonitrile containing 0.1% v/v trifluoroacetic acid as the mobile phase. The resolution (*Rs*) values of the thiols tested were in the range 1.05–3.33 for the diastereomers obtained with *R*-(−)-DBD-PyNCS. The detection limits (signal-to-noise ratio of 3) with the proposed HPLC separation and fluorescence detection were in the range 0.4–2.4 pmol. The dithiocarbamate of tiopronin resulting from the labelling reaction with *R*-(−)-DBD-PyNCS was fairly stable. However, the fluorophore moiety in the diastereomer produced from penicillamine transferred from the thiol functional group to the amino group (S → N conversion) with increasing reaction time. Finally, N,S-double labelled compounds were produced from the tagging reaction with excess amounts of DBD-PyNCS. These structures were elucidated from the fragments of molecular $(M + H)^+$ and product ions by on-line HPLC–electrospray ionization MS–MS.

Keywords: *Thiol enantiomers; pre-column derivatization; indirect resolution; diastereomer formation; fluorescence detection; reversed-phase liquid chromatography; electrospray ionization mass spectrometry*

Various attempts have been made to separate racemic mixtures of amino- and carboxyl-containing compounds by liquid chromatography.[1,2] The different pharmacokinetic effects of enantiomers of drugs have been monitored in this context.[3,4] Although many derivatization reagents have been developed for the detection of thiols by high-performance liquid chromatography (HPLC),[5–9] only a few reagents are able to resolve chiral thiols. This may be due to the low stabilities of analytes and the resulting derivatives. A category of promising reagents are isothiocyanate-bearing compounds such as 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[10] and (*R*,*R*)-*N*-[(2-isothiocyanato)cyclohexyl]-(3,5)-dinitrobenzoylamide (DDITC).[11] The isothiocyanate (–NCS) functional group in the reagent acts as an electrophile for the SH group in the analyte to produce a dithiocarbamate derivative. The resulting diastereomeric dithiocarbamates are well separated by reversed-phase liquid chromatography. Since the chiral isothiocyanate reagents reported previously are chromogenic labels, the detection sensitivity might not be sufficient for trace level analysis in real samples. However, the intrinsic reactivity, stability and handling of the isothiocyanate reagents are worthy of note. A sensitive functionality, such as a fluorophore is suitably induced in the reagents used. *o*-Phthalaldehyde (OPA) reacts with thiol compounds in the presence of an amine to produce the corresponding fluorescent isoindole derivatives. When the amine possesses chirality, such as in α-amino acids (L-valine, *etc*.), the racemic thiols are converted into a pair of diastereomeric isoindoles.[12] The idea is based on the resolution of the chiral amines by the reaction of OPA with a chiral thiol such as *N*-acetyl-L-cysteine. However, in some applications the isoindole derivatives generated from OPA–α-amino acids do not have adequate stability.

In a previous paper,[13] we reported the synthesis of a novel fluorescent isothiocyanate reagent, *i.e.*, enantiomers of 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*-(−)- and *S*-(+)-DBD-PyNCS]. The usefulness of the reagent was demonstrated for the resolution of some amines, amino acids[14–16] and amino-containing drugs.[17] Another functionality that can be derivatized with this isothiocyanate reagent, DBD-PyNCS, is the thiol group which leads to the corresponding dithiocarbamates.[18] In this paper we describe the optimization of the tagging reaction in detail, and the chiral separation of racemic mixtures of thiol compounds with *R*-(−)-DBD-PyNCS, based on diastereomer formation, by conventional reversed-phase liquid chromatography. The structures of the derivatives are also confirmed by on-line high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI MS).

Experimental

Materials and reagents

4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*-(−)-DBD-PyNCS] was synthesized as described previously.[13] The reagent is now commercially available from Tokyo Kasei (Tokyo, Japan). *R*-(+)/*S*-(−)-Tiopronin and *N*-(2-mercapto-2-methylpropionyl)-D/L-cysteine were kindly supplied by Santen Pharmaceutical (Osaka, Japan). D/L-Cysteine and D/L-penicillamine were obtained from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA), respectively. 2-Mercaptopropionic acid and 2-mercaptopropionic acid ethyl ester were purchased from Tokyo Kasei. The structures of the thiols tested are shown in Fig. 1. Quinuclidine hydrochloride and 1,8-diazabicyclo[5,4,0]-7-undecene (DBU) were obtained from Tokyo Kasei. Pyridine and triethylamine (TEA) were of special reagent grade (Wako, Osaka, Japan). Trifluoroacetic acid (TFA) and acetonitrile (CH_3CN) were of HPLC-grade (Wako). De-ionized, distilled water was used throughout. All other chemicals were of analytical-reagent grade and were used without further purification.

On-line HPLC–ESI MS–MS

A Hewlett-Packard 1100 HPLC Series instrument (Wilmington, DE, USA) coupled to a Finnigan-MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) fitted with an electrospray ionization (ESI) source was used. The separation of reaction products was carried out on an ULTRON VX-ODS column (150 × 4.6 mm id, 5 μm; Shinwa Chemicals, Kyoto, Japan) with water(A)–acetonitrile(B) containing 1% v/v acetic acid as the mobile phase at 1.0 ml min^{-1}. Linear gradient elution from A–B (85 + 15) to A–B (70 + 30) for 20 min, and from A–B (70 + 30) to A–B (60 + 40) for 30 min, and isocratic elution with A–B (60 + 40) for 15 min were used for the separation of penicillamine derivatives. The ESI capillary temperature and capillary voltage were 275 °C and 3.0 V, respectively. The source voltage and source current were 4.8 kV and 100 μA, respectively, and the tube lens offset was set at 20.0 V. All spectra were in the positive-ion mode, over the mass range *m/z* 200–900, at a rate of one scan every 2 s. The collision gas was helium (He), and the collision energy was 30.0%. Product ions were scanned between 100 and 550 *m/z*, and the spectra were collected in the form of continuous data.

HPLC

The HPLC system consisted of a CCPM pump and a PX-8010 controller (Tosoh, Tokyo, Japan). The sample solution was injected with a Rheodyne 7125 injector (Cotati, CA, USA). The analytical column used was an ULTRON VX-ODS (150 × 4.6 mm id, 5 μm). The column was maintained at 40 °C with a CO-8020 column oven (Tosoh). A Hitachi L-7480 fluorescence monitor equipped with a 12 μl flow cell (Tokyo, Japan) was used for the detection of the derivatives. The excitation and emission wavelengths were fixed at 455 and 568 nm, respectively. The peak areas obtained from the fluorescence monitor were calculated with a Chromatopac C-R7A Plus integrator (Shimadzu, Kyoto, Japan). The eluent consisted of various concentrations of aqueous acetonitrile solutions containing 0.1% v/v TFA. All mobile phases were de-gassed with an on-line de-gasser (SD-8022, Tosoh). The flow rate of the eluent was 1.0 ml min^{-1}.

The capacity factor (k'), separation factor (α) and the resolution value (R_s) were calculated according to the following equations: $k' = (t_R - t_0)/t_0$, $\alpha = k'_2/k'_1$ and $Rs = 2(t_{R2} - t_{R1})/(W_1 + W_2)$, where t_R, t_{R1} and t_{R2} are the peak retention times, t_0 is the void volume of the column (t_0 = 1.5 min) and W_1 and W_2 are the widths of the bases formed by triangulation of the peaks.

Recommended derivatization procedure for thiols

A 10 μl volume of the reagent [12 mM *R*-(−)-DBD-PyNCS] in acetonitrile was reacted with a 20 μl solution of thiol enantiomers (30 μM of each enantiomer) in 2 mM Na_2EDTA containing pyridine (final concentration, 1% v/v) at 50 °C for 60 min. After labelling, a 10 μl aliquot of the solution was injected onto the column for HPLC. The reagent blank without thiol was also treated in the same manner.

Structural elucidation of penicillamine derivatives

TEA, 1% v/v, was used for the tagging reaction of penicillamine instead of 1% v/v pyridine. The derivatization reaction with small amounts of R-(−)-DBD-PyNCS (0.5 equiv. against thiol) was also investigated as well as with large amounts of the chiral reagent (100 equiv. against thiol). The reaction vial of penicillamine was allowed to stand at room temperature for over 60 min. After labelling at fixed times, a 10 μl aliquot of the solution was subjected to on-line HPLC–ESI MS analysis. The structure of each peak detected by UV at 210 nm was analyzed using ESI MS and MS–MS.

Fluorescence properties of the derivatives

For the fluorescence spectra measurements, 10 μl of the reaction solution of 2-mercaptopropionic acid were injected onto the HPLC column, and the peak corresponding to the dithiocarbamate derivative was collected downstream at the outlet of the detector. The collection procedure was repeated a further four times (total volume, approximately 10 ml). The eluate collected in the tube was evaporated under reduced pressure, and then a small amount of acetonitrile was added to dissolve the residue. To an equal volume (10 μl) of the solution were added 3 ml of each of various solvents. The diluted solutions were used for the measurement of the fluorescence wavelengths (excitation and emission) and the fluorescence intensities with a 1 cm length quartz cell using a F-3010 fluorescence spectrophotometer (Hitachi).

Results and discussion

Fig. 2 shows the derivatization reaction of thiols with the chiral fluorescent reagent, *R*-(−)-DBD-PyNCS. Since the derivatiza-

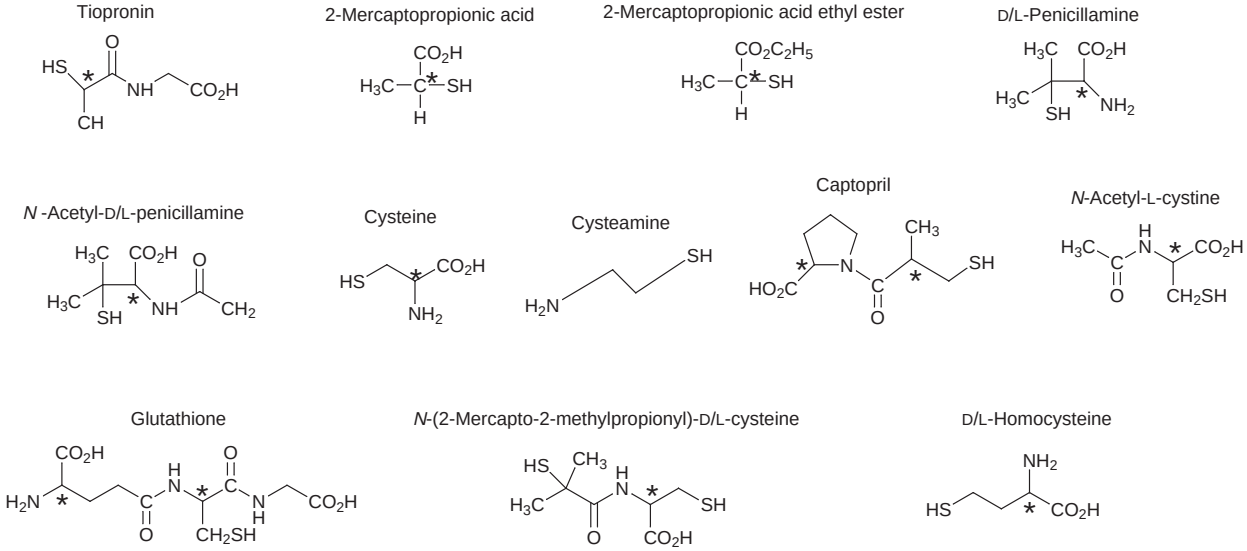

Fig. 1 Structures of the thiol compounds tested.

tion reaction is affected by various parameters, such as the species of catalyst, the concentration of the catalyst and the tagging reagent, and the reaction temperature, the derivatization conditions were optimized using tiopronin which was selected as a representative thiol drug. The derivatization reaction effectively proceeded in a basic medium. Therefore, the effect of the catalysts, *i.e.*, pyridine, TEA, DBU and quinuclidine, on the tagging reaction was studied first. Amongst the catalysts tested, the highest yield of tiopronin derivative was obtained using pyridine. Although TEA was also applicable to the labelling reaction as the catalyst, some impurities in TEA interfered with the peak separation of the resulting derivatives. Thus, pyridine was selected as the derivatization catalyst for the tagging of thiol-containing compounds. The concentration of the catalyst was also an important factor for the labelling. As shown in Fig. 3, comparable yields of the tiopronin derivative were obtained in the range 0.5–2% v/v, and the peak slightly decreased with higher concentrations of pyridine. Therefore, the racemic thiols as well as the achiral thiols tested were derivatized with *R*-(−)-DBD-PyNCS in a 1% v/v pyridine-containing medium.

The effect of the concentration of tagging reagent on the derivatization was studied next. The peak areas of the *S*-tiopronin derivative gradually increased with the addition of increasing amounts of reagent to the thiols. A plateau was reached with the use of a 100-fold molar excess of *R*-(−)-DBD-PyNCS (6 mM) in the reaction of tiopronin (Fig. 4). Since the reagent seemed to be consumed by various materials usually present in real samples, a high concentration, *viz.*, a 200-fold molar excess of *R*-(−)-DBD-PyNCS (12 mM), was subsequently used in the reaction medium. The time course of the labelling reaction of tiopronin was studied at 50 °C under the conditions mentioned above. Fig. 5 shows the peak areas of the derivative at each reaction time. The labelling of tiopronin was almost complete after 40 min at 50 °C. In addition, the reaction rates were almost comparable for both enantiomers of DBD-PyNCS, because similar results were obtained with *S*-(+)-DBD-PyNCS (data not shown). Based on these observations, the derivatization conditions at 50 °C for 60 min in 1% v/v pyridine in water–acetonitrile solution were used for the preparation of diastereomers of tiopronin and the other thiols tested [cysteine, *N*-(2-mercapto-2-methylpropionyl)-D/L-cysteine and 2-mercaptopropionic acid, *etc*.]. The tiopronin derivatives in the reaction mixture of water–acetonitrile containing pyridine were stable for at least 48 h in a refrigerator and/or 1 h at 50 °C without any significant change. The good stability of *R*-(−)-DBD-PyNCS[13] and the derivatives is an important property for the determination of trace amounts of thiols.

For the reaction with penicillamine in the presence of 1% v/v pyridine, the yield of the derivatives was relatively low, as compared with tiopronin. The formation of the dithiocarbamate diastereomers was increased with use of TEA, instead of pyridine. However, the peaks corresponding to the derivatives of D/L-penicillamine rapidly decreased with reaction time at room temperature with the concurrent appearance of new peaks.

Fig. 6(*a*) and (*b*) shows the chromatograms of the reaction solution at 5 and 60 min under reagent-rich conditions [*i.e.*, 100-fold molar excess of *R*-(−)-DBD-PyNCS], respectively. On the other hand, Fig. 6(*c*) represents the chromatogram of the reaction solution after 20 min at a lower concentration of *R*-(−)-DBD-PyNCS (*i.e.*, 0.5-fold molar excess). At high concentrations of the tagging reagent, two peaks at around 7–9 min ($\mathbf{I}_D$ and $\mathbf{I}_L$) were converted into two pairs of peaks at around 22–24 min ($\mathbf{II}_D$ and $\mathbf{II}_L$) and 43–45 min ($\mathbf{III}_D$ and $\mathbf{III}_L$) [Fig. 6(*a*) and (*b*)], but only two peaks at 22–24 min ($\mathbf{II}_D$ and $\mathbf{II}_L$) gradually appeared with time at low concentrations of the reagent [Fig. 6(*c*)]. The time courses of the change of each peak are shown in Figs. 7 and 8.

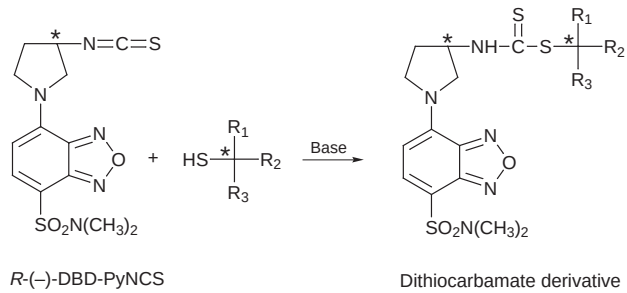

Fig. 2 Reaction of a chiral thiol with *R*-(−)-DBD-PyNCS.

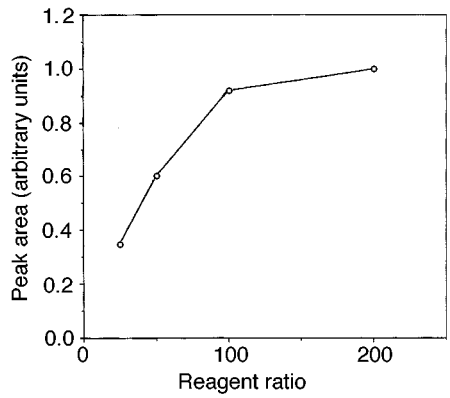

Fig. 4 Effect of *R*-(−)-DBD-PyNCS concentration on the derivatization of *S*-tiopronin.

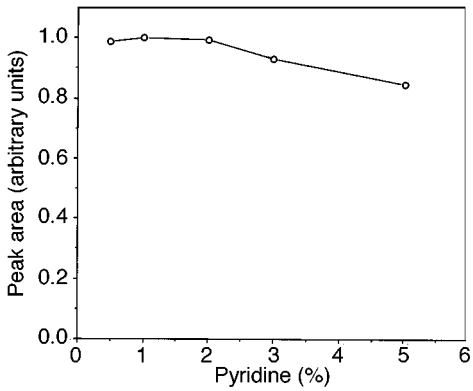

Fig. 3 Effect of pyridine concentration on the derivatization of *S*-tiopronin with *R*-(−)-DBD-PyNCS.

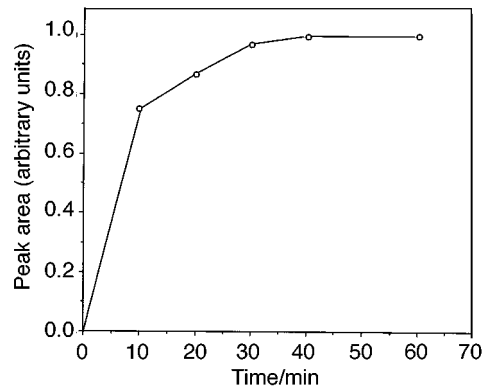

Fig. 5 Time course of the derivatization reaction of *S*-tiopronin with *R*-(−)-DBD-PyNCS at 50 °C.

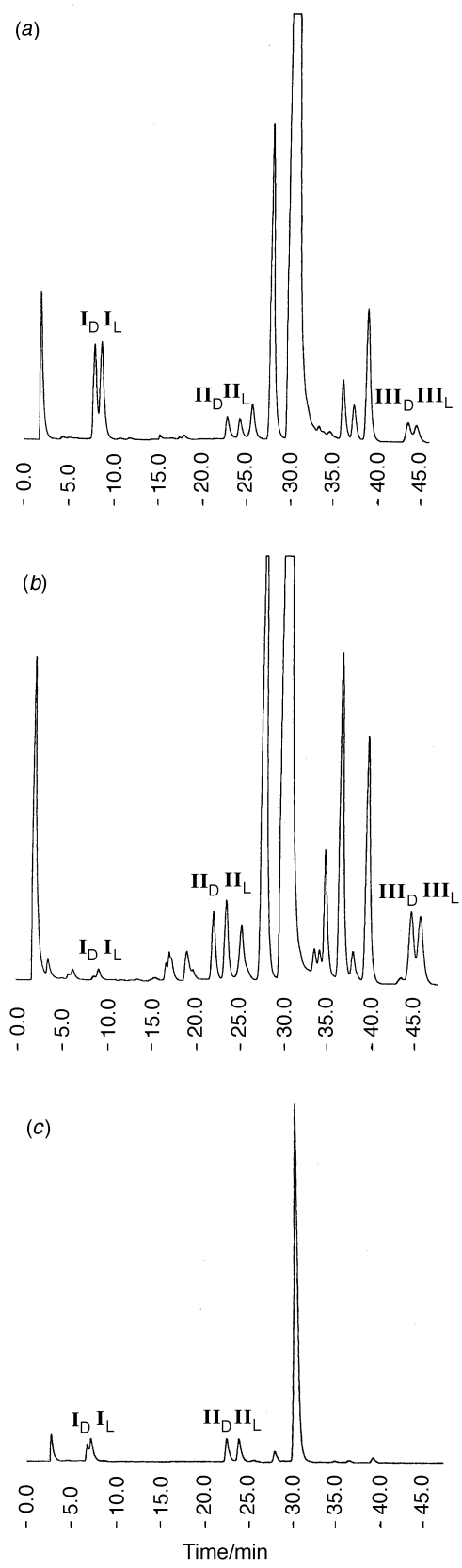

Fig. 6 Chromatograms obtained from the reaction of D/L-penicillamine with *R*-(−)-DBD-PyNCS at room temperature. Chromatogram: (*a*) after 5 min (100-fold molar excess of reagent); (*b*) after 60 min (100-fold molar excess of reagent); (*c*) after 20 min (0.5-fold molar excess of reagent). Peaks: $\mathbf{I}_D$, D-penicillamine-S-label; $\mathbf{I}_L$, L-penicillamine-S-label; $\mathbf{II}_D$, D-penicillamine-N-label; $\mathbf{II}_L$, L-penicillamine-N-label; $\mathbf{III}_D$, D-penicillamine-S,N-label; $\mathbf{III}_L$, L-penicillamine-S,N-label.

The products appearing on the chromatograms were analyzed with an on-line HPLC–ESI MS–MS system. Judging from the fragment ions (*m/z*) with MS analysis, the molecular weights of compounds $\mathbf{I}_{D/L}$, $\mathbf{II}_{D/L}$ and $\mathbf{III}_{D/L}$ appear to be 502, 502 and 855, respectively. The possible structures of compounds $\mathbf{I}_{D/L}$ and $\mathbf{II}_{D/L}$ are penicillamine-S-DBD-PyNCS (structure **I** in Fig. 9) and penicillamine-N-DBD-PyNCS (structure **II** in Fig. 9). From the product ions at *m/z* 388, 354, 312 and 267 with MS–MS analysis, compound $\mathbf{I}_{D/L}$ gave the characteristic fragmentation pattern of structure **I** in Fig. 9, whereas compound $\mathbf{II}_{D/L}$ indicated the structure **II**, judging from the product ions at *m/z* 371, *etc*. On the other hand, compound $\mathbf{III}_{D/L}$ was identified as penicillamine-S,N-di-DBD-PyNCS (structure **III** in Fig. 9). Since the fragmentation patterns are the same in the three paired peaks $\mathbf{I}_D$ and $\mathbf{I}_L$, $\mathbf{II}_D$ and $\mathbf{II}_L$, $\mathbf{III}_D$ and $\mathbf{III}_L$), the products are the diastereomeric isomers. These results suggest that the formation of the S-labelled derivative of penicillamine occurs at an early stage of the reaction, after which the DBD-PyNCS moiety transfers to the amino group (S → N) to form the N-labelled penicillamine derivative. Finally, $-S^-$ produced from the transfer of the fluorophore probably attacks another one equivalent of reagent; as a result, an N and S double-labelled derivative (structure **III** in Fig. 9) might be produced. The structures of the derivatives resulting from *R/S*-tiopronin were also confirmed as dithiocarbamate diastereomers [$(M + H)^+$ = 517 (*m/z*)] with on-line HPLC–MS. The good stability of tiopronin seems to be due to the absence of an amino group in the structure, which is essential for the transfer of the fluorescent moiety.

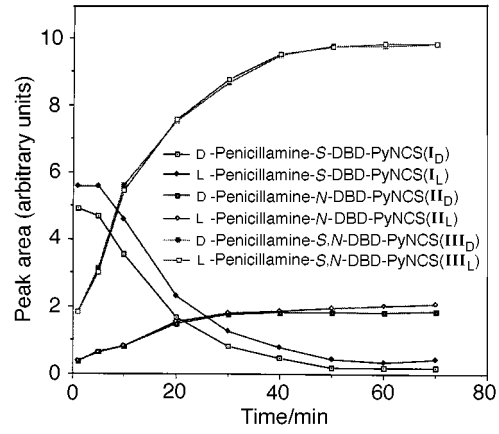

Fig. 7 Time courses of conversion of each peak under reagent-rich conditions.

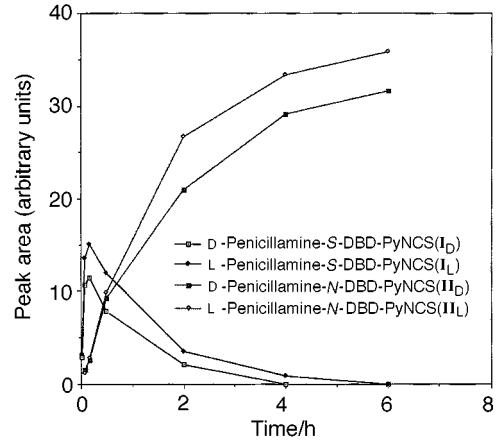

Fig. 8 Time courses of conversion of each peak under insufficient reagent conditions.

Table 1 shows the maximum fluorescence wavelengths (excitation and emission) and the relative fluorescence intensity (RFI) of the dithiocarbamate derivative of 2-mercaptopropionic acid in various solvents. The maximum wavelengths and intensity appear to be dependent on the hydrophobicity of the solvent. Although the excitation maxima were almost comparable in each solvent, the emission maxima shifted towards shorter wavelengths with hydrophobic solvents. Furthermore, the fluorescence intensities increased with increasing hydrophobicity of the medium. There were no significant changes in the fluorescence wavelengths and the intensity with and without 0.1% v/v TFA in the water–acetonitrile (1 + 1, v/v) mixture, which was used as the mobile phase for the separation of dithiocarbamate derivatives of thiol compounds in subsequent experiments. Since these characteristics were essentially the same as those of DBD-PyNCS itself (data not shown), the fluorescence seems to be due to the fluorophore of the benzofurazan reagent.

The separation of the racemic thiols was studied by reversed-phase HPLC after derivatization with *R*-(−)-DBD-PyNCS. In these experiments, a column temperature of 40 °C was selected to obtain reproducible results for the retention times of the derivatives. The resulting derivatives were well resolved by reversed-phase HPLC with water–acetonitrile containing 0.1% v/v TFA as the eluent. The relationship between capacity factor (k') and acetonitrile concentration in the mobile phase is depicted in Fig. 10. The capacity factors (k'), separation factors (α) and resolution values (Rs) for each pair of derivatives derived from *R*-(−)-DBD-PyNCS are listed in Table 2. The thiol enantiomers possessing an asymmetric carbon atom at the α-position to an SH group, such as tiopronin and 2-mercaptopropionic acid, were well separated under the proposed elution conditions (Table 2). However, the resolution (Rs) of cysteine and bucillamine, which have a β-asymmetric carbon atom, was smaller than that of tiopronin and 2-mercaptopropionic acid. Since the optical resolution was known to depend on the proximity of the two chiral centers of the diastereomers, it is probably due to the large distance between the two stereogenic centers (five bonds *versus* four bonds). In addition, D- and L-homocysteine, in which there is a longer distance between the asymmetric carbon atom and the SH group than with cysteine (homocysteine, γ-position *versus* cysteine, β-position), were

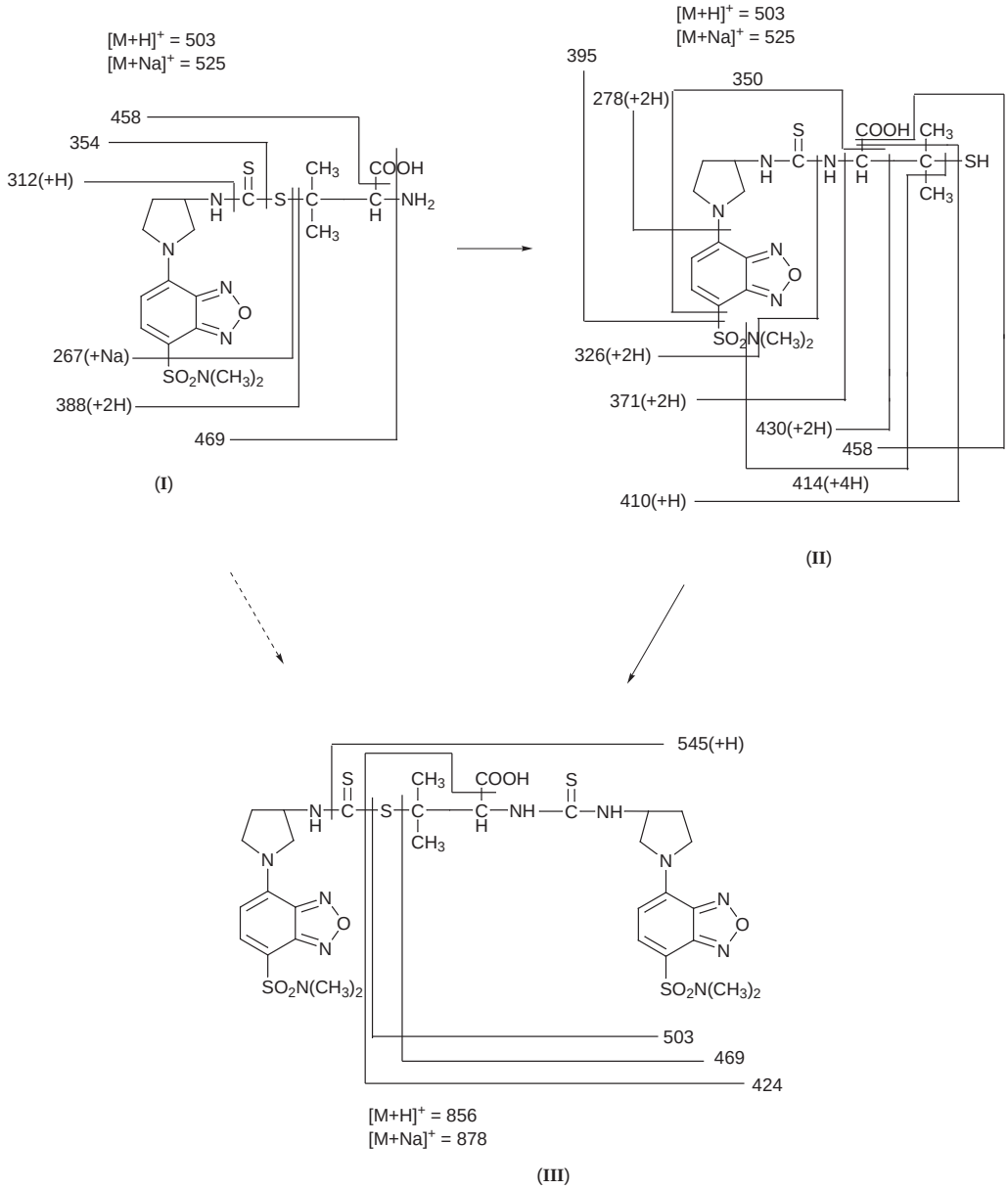

Fig. 9 Proposed structures of penicillamine derivatives.

not separated under the chromatographic conditions. The diastereomers derived from D (or *R*)-enantiomers with use of *R*-(−)-DBD-PyNCS eluted faster than those of L (or *S*)-isomers. Of course, it is possible to change the elution order by using the opposite enantiomer of the tagging reagent, *S*-(+)-DBD-PyNCS. Achiral thiols such as cysteamine were also labelled and detected sensitively with the proposed method.

A chromatographic separation of some thiols including achiral thiols was carried out with a linear gradient elution on a reversed-phase column. The sample was prepared by derivatizing a solution containing four racemic thiols (60 nmol each) according to the recommended procedure. Fig. 11 shows the separation of the derivatized thiol compounds [30 pmol each of D/L-cysteine, *R/S*-tiopronin, *N*-(2-mercapto-2-methylpropionyl)-D/L-cysteine), and D/L-2-mercaptopropionic acid (9 pmol)]. The derivatives produced from each pair of thiol enantiomers were clearly separated by linear gradient elution with water–acetonitrile containing 0.1% v/v TFA. The peak eluted at around 7.5 min seems to be a hydrolysis product, 4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-APy), because this peak was also identified from the chromatogram of a blank solution without thiol. The largest peak (*ca*. 60 min) observed in the chromatogram is that of the unreacted excess of reagent, while other small peaks seem to be caused by impurities in the analytes. The limits of detection (signal-to-noise ratio of 3) of *R/S*-tiopronin, *R/S*-2-mercaptopropionic acid, *R/S*-2-mercaptopropionic acid ethyl ester, D/L-penicillamine, D/L-cysteine, D/L-homocysteine, *N*-acetyl-L-cysteine, captopril, glutathione, cysteamine and *N*-(2-mercaptopropionyl)-D/L-cysteine on the chromatogram were 0.40, 0.62, 0.70, 2.4, 1.1, 1.1, 2.1, 1.7, 0.82, 1.1 and 1.0 pmol, respectively. The sensitivity is higher than that obtained with other fluorescent and UV labels such as OPA–chiral amines,[12] GITC[10] and DDITC.[11]

Table 1 Fluorescence properties of 2-mercaptopropionic acid labelled with *R*-(−)-DBD-PyNCS in various solvents

Solvent	Excitation/ nm	Emission/ nm	RFI*
Acetonitrile–water (1 + 1, v/v)	452	560	12.2
Acetonitrile†	450	545	100
Methanol	450	549	30.7
Ethanol	450	545	53.5
Dimethylformamide	457	545	90.6
Acetone	450	539	135
Ethyl acetate	448	532	138
Dichloromethane	445	533	149

* RFI = Relative fluorescence intensity. † Fluorescence intensity in acetonitrile was tentatively taken as 100.

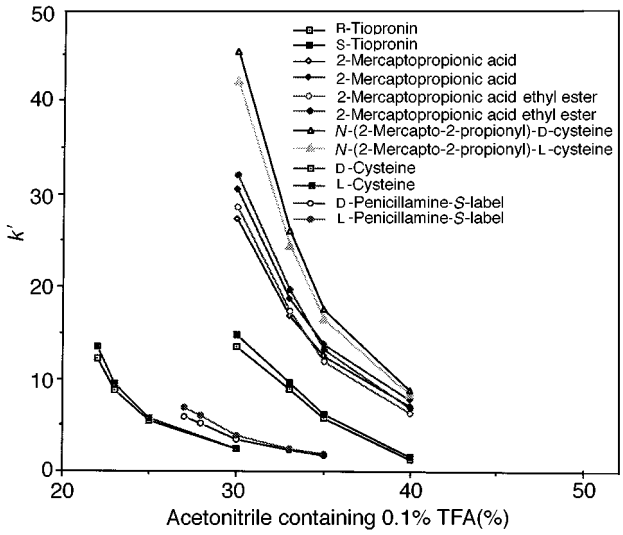

Fig. 10 Correlation between *k*′ and acetonitrile concentration in mobile phase.

Table 2 Separation of thiols after derivatization with *R*-(−)-DBD-PyNCS

Thiol	*k*′	α	*Rs*	Eluent*
Tiopronin†	13.54(*R*), 14.74(*S*)	1.09	1.50	b
2-Mercaptopropionic acid†	12.59, 13.89	1.10	1.33	c
2-Mercaptopropionic acid ethyl ester‡	22.25, 32.65	1.12	2.40	b
D/L-Penicillamine‡	4.00(D), 4.62(L)	1.17	3.33	b
N-(2-Mercapto-2-methyl-propionyl)-D/L-cysteine†	16.55(D), 17.59(L)	1.06	1.05	c
D/L-Cysteine†	12.30(D), 13.46(L)	1.09	1.15	a
D/L-Homocysteine†	2.94			b
Captopril†	7.12			d
Glutathione†	2.27			b
N-Acetyl-L-cysteine†	20.85			b
Cysteamine†	1.10			c

* Eluent: acetonitrile–water containing 0.1% v/v TFA. Acetonitrile content: a = 22; b = 30; c = 35; d = 40% v/v. † 1% v/v pyridine as the base. ‡ 1% v/v TEA as the base.

Conclusion

The chiral reagent, DBD-PyNCS, is a promising reagent for the resolution of thiol enantiomers by reversed-phase chromatography. The resulting dithiocarbamate derivatives exhibit good stability and strong fluorescence in the long wavelength region. However, care should be taken in the derivatization of analytes containing both thiol and amino groups in their structure because it is possible to transfer the fluorescence moiety from S to N. Furthermore, double-labelled derivatives might be

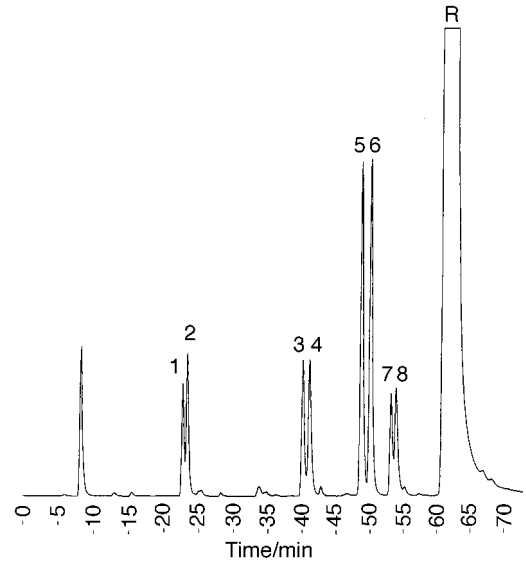

Fig. 11 Reversed-phase chromatogram obtained for some racemic thiols after derivatization with *R*-(−)-DBD-PyNCS. Peaks: 1, D-cysteine; 2, L-cysteine; 3, *R*-tiopronin; 4, *S*-tiopronin; 5 and 6, (+)/(−)-2-mercaptopropionic acid; 7, *N*-(2-mercapto-2-propionyl)-D-cysteine; 8, *N*-(2-mercapto-2-propionyl)-L-cysteine; R, *R*-(−)-DBD-PyNCS. Eluents: (A), water containing 0.1% v/v TFA; (B), acetonitrile containing 0.1% v/v TFA; linear gradient elution from A–B (85 + 15, v/v) to A–B (70 + 30, v/v) for 20 min, and from A–B (70 + 30, v/v) to A–B (60 + 40, v/v) for 30 min, and isocratic elution with A–B (60 + 40, v/v) for 15 min. Other HPLC conditions are given under Experimental.

produced with some types of analyte. Since the proposed method combined with reversed-phase chromatography and fluorescence detection provides satisfactory separation and sensitivity, it is adaptable to the determination of chiral and achiral thiols in real samples such as plasma and urine. Of course, the fluorescent tagging reagent used reacts with primary and secondary amines under similar conditions. Consequently, care should be taken in the resolution of real samples containing amines and thiols.

The authors thank Dr. C. K. Lim, MRC Toxicology Unit, University of Leicester, for reviewing the manuscript. Thanks are also due to Michiko Kanai, Demo Laboratory Manager of ThermoQuest Co. (Tokyo, Japan), for technical assistance with the mass spectra measurements.

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Paper 8/00712H
Received January 26, 1998
Accepted March 24, 1998