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ENANTIOMERIC RESOLUTION OF DANSYL AMINO ACIDS BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH β-CYCLODEXTRIN INCLUSION COMPLEXES

TOYOHIDE TAKEUCHI, HOMARE ASAI and DAIDO ISHII*

Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Chikusa-ku, Nagoya 464 (Japan)

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SUMMARY

Enantiomeric resolution of dansyl amino acids by micro high-performance liquid chromatography with β-cyclodextrin inclusion complexes was investigated. Chromatographic parameters which affect enantiomeric resolution were examined. Twelve pairs of dansyl amino acids were separated in a single chromatographic run.

INTRODUCTION

Chiral resolution has been achieved by high-performance liquid chromatography (HPLC) with either a chiral stationary phase or a mobile phase containing a chirality-recognizing reagent. Optical resolution by the latter method relies on chiral recognition by ligand exchange, ion-pair formation and inclusion complexing.

It is well known that the cyclodextrins show high stereoselectivity. They form inclusion complexes with a variety of molecules and ions. Debowski *et al.*[1,2] applied α-cyclodextrin and β-cyclodextrin as the mobile phase components for resolution of racemic mandelic acids and mandelic acid derivatives. Nobuhara *et al.*[3] resolved racemic 1-[2-(3-hydroxyphenyl)-1-phenylethyl]-4-(3-methyl-2-butenyl)piperazine with β-cyclodextrin as the mobile phase component.

Cyclodextrin-bonded phases for HPLC have recently been developed and their applications to the separation of optical, geometrical and structural isomers have been reported[4,5].

Enantiomeric resolution of dansyl amino acids by micro HPLC using β-cyclodextrin as the mobile phase component is described in this paper. The resolution of many pairs of racemic dansyl amino acids will be demonstrated after examination of the chromatographic parameters that affect the separation of enantiomers.

EXPERIMENTAL

Chromatography

Micro Feeder (Azumadenkikogyo, Tokyo, Japan) equipped with a 500-μl gas-

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tight syringe MS-GAN 050 (Terumo, Tokyo, Japan) or MPLC Micro Pump (Brownlee Labs., Santa Clara, CA, U.S.A.) was used. Most of the experiments were carried out with the former pump, although the latter was used for gradient separation. A UV spectrophotometer, UVIDEC-100II or 100V (Jasco: Japan spectroscopic, Tokyo, Japan), was equipped with a home-made flow cell (0.05–0.1 μl in volume) and used at 220 nm. An ML-422 micro valve injector (Jasco) was used to load the samples (19–20 nl). Fused-silica tubing (0.26 mm I.D.) or glass-lined stainless-steel tubing (0.3 mm I.D.) was selected as the material of the separation column. The former column was manually packed by a previously reported method[6], in which PTFE tubing was employed as the connecting tubing. The latter column could withstand high pressure and it was prepared by the high-pressure slurry-packing technique[7]; ODS-Hypersyl-3 (3 μm; Shandon, Runcorn, U.K.) was the stationary phase in this work.

Reagents

Twenty-one L-amino acids (Kit No. LAA-21), sixteen D-amino acids (Kit No. DAA-16), twenty-four D,L-amino acids (Kit No. DLAA-24) and fifteen dansyl-D,L-amino acids (Kit No. DAN-DL-15) were obtained from Sigma (St. Louis, MO, U.S.A.). β-Cyclodextrin was obtained from Tokyo Chemical Industry (Tokyo, Japan). Other reagents were from Wako (Osaka, Japan), unless otherwise noted. Dansylation of amino acids was also carried out in the laboratory at 40°C for 30 min. The pH of the mobile phase was adjusted with potassium phosphate and phosphoric acid (0.1 *M*).

RESULTS AND DISCUSSION

There are three cyclodextrins of different sizes available commercially, which differ in the number of glucose units in the ring. The cavity size of β-cyclodextrin is suitable to form inclusion complexes with compounds that have a naphthalene ring[4]. Dansyl derivatization has been widely employed for the analysis of amino acids because dansyl amino acids can be separated well by reversed-phase HPLC and detected sensitively by UV or fluorescent spectrophotometers. Enantiomers of dansyl amino acids have been successfully separated on a β-cyclodextrin-bonded phase with a mobile phase of methanol and water[4], when the L isomer of a dansyl amino acid elutes before its D isomer. This indicates that the inclusion complex with the D isomer is more stable than that with the L isomer. In the case of the separation with a mobile phase including β-cyclodextrin, the D isomer can be expected to elute before its L isomer because, as the stability of the isomer increases, the isomer is less retained on the stationary phase. In the latter case, the concentration of β-cyclodextrin in the mobile phase, the mobile phase composition and the pH of the mobile phase can affect the capacity factor and the separation factor.

Fig. 1 demonstrates the enantiomeric separation of dansyl serine with a mobile phase containing 10% (v/v) acetonitrile and different concentrations of β-cyclodextrin. As the concentration of β-cyclodextrin increases, the retention times of both isomers decrease, owing to the formation of inclusion complexes by β-cyclodextrin and the dansyl serine enantiomers. The difference in retention time between the enantiomeric isomers cannot be recognized on the chromatograms that correspond to β-cyclodextrin concentrations of less than 2.5 m*M*, while only a shoulder peak is observed for a β-cyclodextrin concentration of 5.0 m*M*.

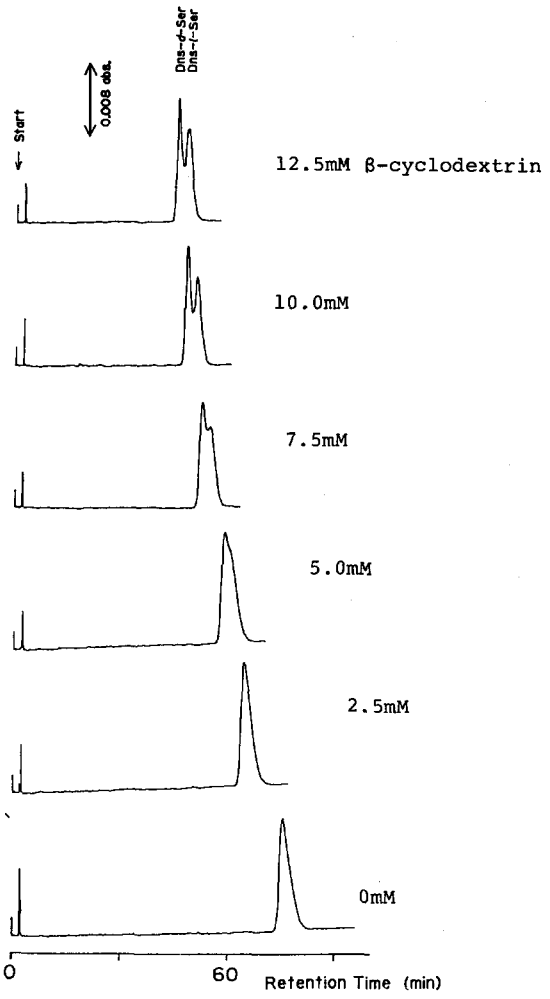
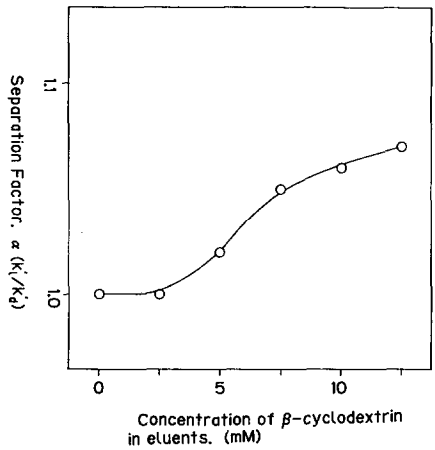


Fig. 1. Effect of the concentration of β-cyclodextrin on the enantiomeric separation of dansyl serine. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase; 10% (v/v) acetonitrile solution including different concentrations of β-cyclodextrin (pH 5.1); flow-rate, 2.1 μl/min; sample, dansyl-D,L-serine (100 pmol).

Fig. 2 shows the relationship between the separation factor and the concentration of β-cyclodextrin. The separation factor increases with increasing concentration of β-cyclodextrin up to 12.5 m*M*. Higher concentrations of β-cyclodextrin could not be achieved owing to solubility problems. The value of the separation factor is relatively low, but this can be compensated by the larger theoretical plate number, which can be easily attained by octadecylsilica columns. In addition, the elution order of dansyl amino acids strongly depends on their solvophobicity, and the low value of the separation factor (*ca.* 1.1) facilitates the separation of multiple pairs of enantiomers in a single chromatographic run.

Fig. 3 illustrates the effect of the concentration of acetonitrile in the mobile



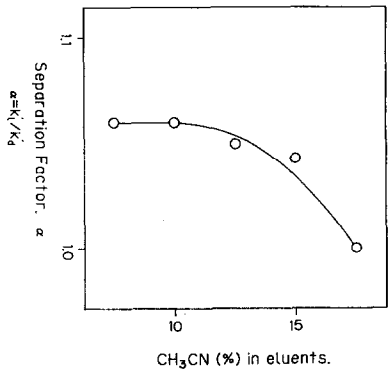


Fig. 2. Relationship between the separation factor and the concentration of β-cyclodextrin. Operating conditions as in Fig. 1.

Fig. 3. Effect of the concentration of acetonitrile on the separation factor. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer including 10 m*M* β-cyclodextrin (pH 5.0–5.1); flow-rate, 2.1 μl/min; sample, dansyl-D,L-serine (100 pmol).

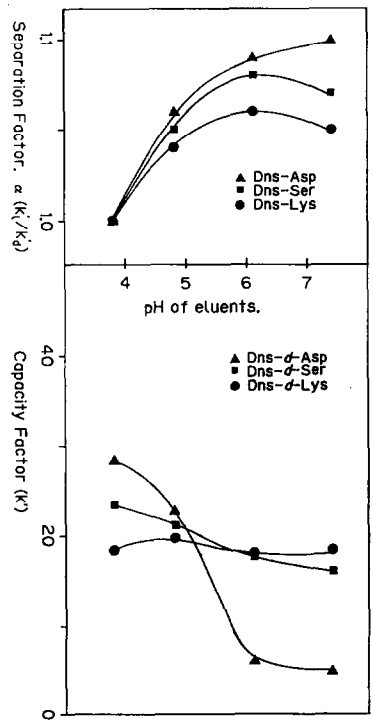


Fig. 4. Effect of the pH of the mobile phase on the separation and the capacity factor. Column, ODS-Hypersil-3 (108 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer (10:90) containing 12.5 m*M* β-cyclodextrin; sample, dansyl-D,L-Ser (100 pmol), dansyl-D,L-Asp (100 pmol), and dansyl-D,L-Lys (*ca.* 50 pmol).

TABLE I

THE CAPACITY FACTOR AND THE SEPARATION FACTOR

Concentration of β-cyclodextrins, 12.5 m*M*.

Dansyl amino acid	*Mobile phase*		*Capacity factor*		*Separation factor*
	Acetonitrile (%)	*pH*	*D*	*L*	
Ala	10	6.1	28.9	30.4	1.05
Asp	10	6.1	6.4	7.1	1.10
Glu	10	6.1	7.1	7.7	1.09
Lys	10	6.1	17.6	18.7	1.06
Ser	10	6.1	18.1	19.5	1.07
Thr	10	6.1	21.7	24.3	1.12
Arg	10	6.5	21.2	22.6	1.07
Asn	10	6.5	12.5	13.2	1.06
Gln	10	6.5	17.5	18.5	1.05
α-AB	15	6.5	19.5	20.9	1.07
Met	15	6.5	83.9	87.9	1.05
Pro	15	6.5	21.0	22.1	1.05
Ile	20	6.1	18.7	20.0	1.07
Leu	20	6.1	21.7	23.2	1.07
Nle	20	6.1	30.3	31.7	1.05
Nval	20	6.1	13.5	14.1	1.05
Phe	20	6.1	30.7	32.4	1.06
Trp	20	6.1	29.6	29.6	1.00
Val	20	6.1	10.1	10.8	1.07

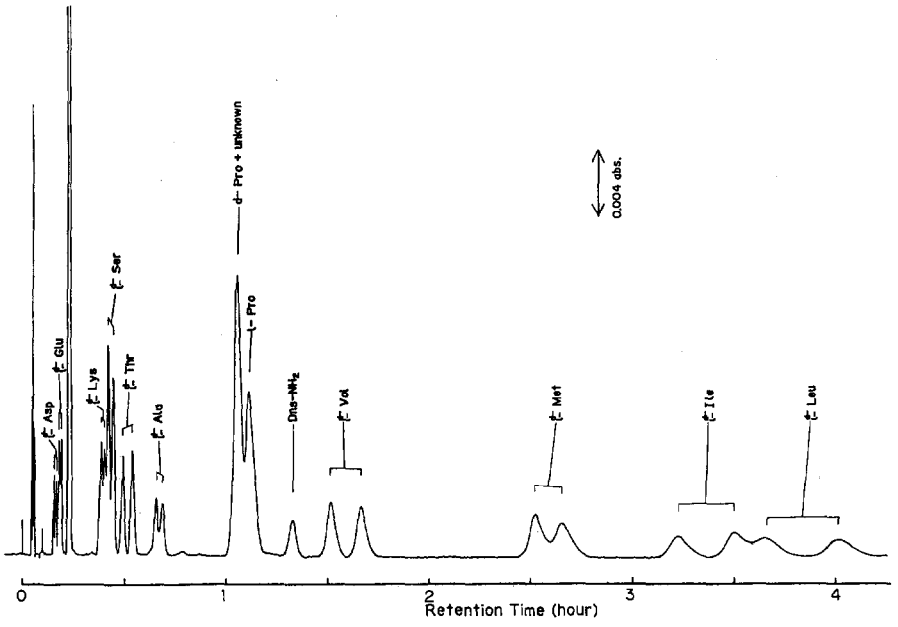


Fig. 5. Isocratic separation of racemic dansyl amino acids. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer (20:80) containing 12.5 m*M* β-cyclodextrin; flow-rate, 2.1 μl/min; sample, *ca.* 20 pmol each.

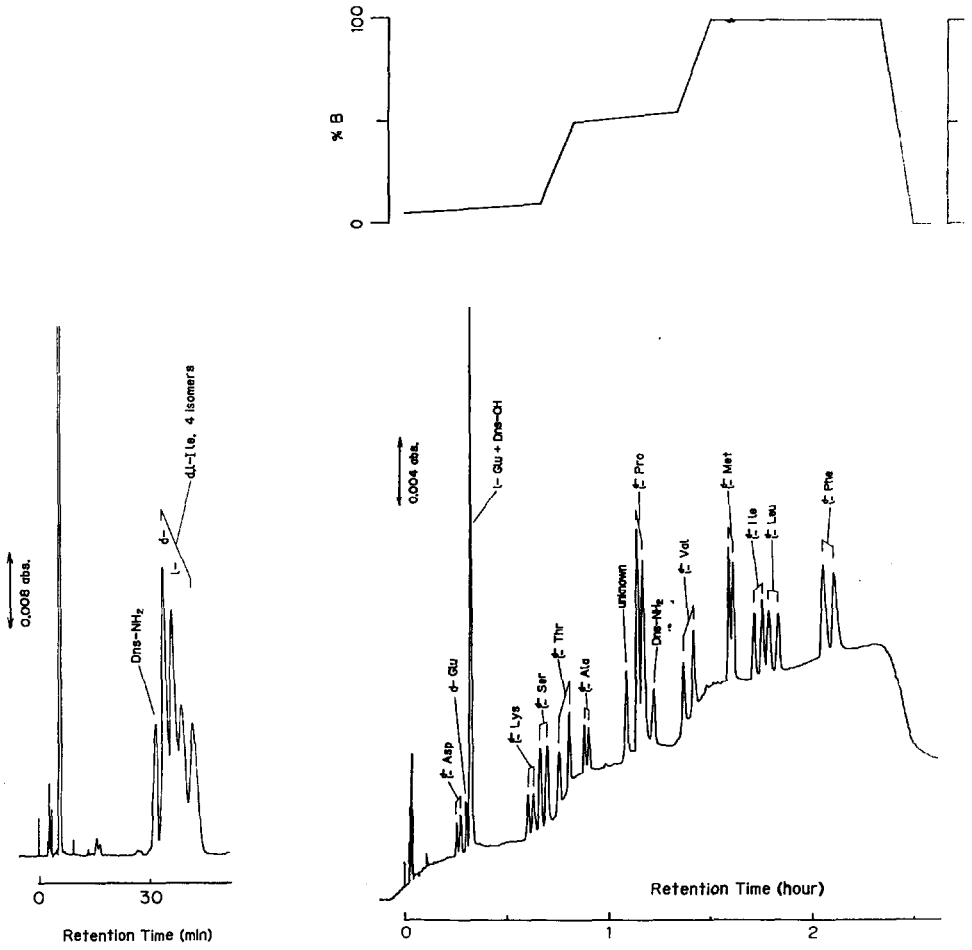


Fig. 6. Separation of dansyl isoleucine diastereomers. Column, ODS-Hypersil-3 (106 × 0.26 mm I.D.); mobile phase, acetonitrile–phosphate buffer (20:80) containing 12.5 m*M* β-cyclodextrin; flow-rate, 2.1 μl/min.

Fig. 7. Gradient separation of racemic dansyl amino acids. Column, ODS-Hypersil-3 (144 × 0.3 mm I.D.); mobile phase, (A) acetonitrile–phosphate buffer (10:90) containing 12.5 m*M* β-cyclodextrin (pH 6.4), (B) acetonitrile–phosphate buffer (20:80) containing 12.6 m*M* β-cyclodextrin (pH 6.4) with the gradient profile as indicated; flow-rate, 5.0 μl/min; sample, *ca.* 20 pmol each.

phase on the separation factor when using dansyl-D,L-serine as test solutes. The separation factor decreases with increasing concentrations of acetonitrile. The enantiomeric isomers could not be distinguished when the concentration of acetonitrile exceeded 17.5%, even if they were retarded on the stationary phase.

The effects of the pH of the mobile phase on the separation factor and the capacity factor are illustrated in Fig. 4. The separation factor shows a maximum around pH 6 for dansyl serine and dansyl lysine, whereas the separation factor of dansyl aspartic acid increases with increasing pH. Thus, the following experiments were carried out at pH 6–7. The variation of the capacity factor with pH depends on

the acidity of the tested solutes. Thus, the curve corresponding to dansyl aspartic acid is characteristic of acidic compounds.

Table I shows the results of enantiomeric separations of dansyl amino acids with 12.5 m*M* β-cyclodextrin. Enantiomers of all examined dansyl amino acids except tryptophan could be resolved with a separation factor of 1.05–1.12. The indole group as well as the dansyl group of tryptophan may form inclusion complexes with β-cyclodextrin. This prevents the resolution of the enantiomers. Amino acids with an aliphatic branched-chain substitution group show a larger separation factor, than those with a straight-chain group. The largest separation factor is observed for dansyl threonine.

Fig. 5 demonstrates the isocratic separation of eleven pairs of enantiomers of dansyl amino acids. The D isomer of each dansyl amino acid elutes before the L isomer, owing to the difference in the stabilities of the inclusion complexes. The elution time can be decreased by gradient separation.

Fig. 6 shows the separation of diastereomers of dansyl isoleucine. Isoleucine has two chiral centres, and the four peaks due to diastereomers are separated (Kit No. DLAA-24). Dansyl D and dansyl L isomers prepared in our laboratory gave a single peak, represented by "d" or "l".

Gradient separation of twelve pairs of dansyl amino acids is demonstrated. The separability and separation time are improved by gradient elution.

CONCLUSION

Enantiomeric resolution of dansyl amino acids by micro HPLC using β-cyclodextrin as the mobile phase additive was successfully demonstrated. Twelve pairs of enantiomers were separated in a single chromatographic run. The use of β-cyclodextrin as well as α- and γ-cyclodextrin as the mobile phase additive will extend the applicability of this technique to the chiral separation in HPLC.

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