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Liquid chromatographic separation of the enantiomers of diniconazole using a β-cyclodextrin-bonded column

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ABSTRACT

Enantiomers of the fungicide diniconazole were separated by reversed-phase high-performance liquid chromatography on a commercially available β-cyclodextrin (β-CD)-bonded column. The effects of organic modifier in the mobile phase, mobile phase pH and column temperature on the retention and resolution of the enantiomers were studied and optimum conditions were established. The retention behaviour of some structurally related compounds of diniconazole under the optimum conditions were examined to clarify the separation mechanism on the β-CD-bonded column. Optical separation of diniconazole using β-CD as a mobile phase modifier on an octadecylsilanized (ODS) silica gel column was also investigated for comparison with separation by the above method.

INTRODUCTION

Diniconazole (Fig.1), which has fungicidal activity, is an optically active compound and the enantiomers are very different in their activity, like many other bioactive reagents. In this instance the activity of the *R*-enantiomer is much higher than that of the *S*-enantiomer, and diniconazole-M, containing a high proportion of the *R*-enantiomer, was developed for the purpose of obtaining a high activity of the product [1]. Consequently, analytical methods for separating the enantiomers are very important for studies of biological action and activity, dynamics and kinetics and also for quality control of the compound. Although some normal-phase high-performance liquid chromatographic (HPLC) methods for diniconazole using chiral stationary phases based mainly on π–π interactions and hydrogen bonding have already been reported [2–4], no reversed-phase methods have been reported.

Cyclodextrin (CD)-bonded stationary phases have recently been developed for use in the reversed-phase mode for the separation of enantiomers [5–8]. CDs are torus-shaped cyclic oligosaccharides containing from six to twelve D-(+)-glucopyranose units, which are bonded through α-(1,4) linkages. The most common CDs are α-, β- and γ-CDs, containing six, seven and eight glucose units, respectively. The interior of the CD cavities is relatively hydrophobic, thus allowing them to form inclusion complexes with a variety of molecules.

The basic property of CDs that allows them to affect chiral separations is their ability to form enantioselective inclusion complexes with guest molecules. There are several requirements for chiral

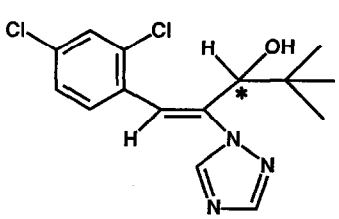

Fig. 1. Structure of diniconazole.

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recognition by CDs. The size of the CD cavity with respect to the size of the enantiomer to be complexed is of critical importance; tighter fitting molecules are preferable. Additionally, it is beneficial if the substituents attached to the chiral centre of the compound to be resolved interact with the secondary hydroxyl groups at the mouth of the CD cavity by hydrogen bonding [9].

We found that enantiomers of diniconazole were separated on a β-CD-bonded column, but not on an α-CD-bonded column, and were partly separated on a γ-CD-bonded column. In this study, the enantiomeric separation of diniconazole with respect to the chromatographic conditions and elution behaviour of structurally related compounds on the β-CD-bonded column were investigated and possible mechanisms are discussed.

EXPERIMENTAL

Apparatus

Chromatography was performed using a liquid chromatographic system which consisted of a Model L-6200 pump equipped with a Model L-4000 variable-wavelength spectrometric detector (Hitachi, Tokyo, Japan). The chromatograms were recorded on a Model C-R4A integrator (Shimadzu, Kyoto, Japan). The column temperature was controlled through a Model UC-55N water-bath (Tokyo Rikakikai, Tokyo, Japan).

Cyclobond I, II and III columns (250 mm × 4.6 mm I.D.) packed with 5-μm spherical silica gel with chemically bonded β-, γ- and α-CD, respectively. were purchased from Advanced Separation Technologies (Whippany, NJ, USA). A Sumipax ODS A-212 column (150 mm × 6 mm I.D., 5 μm) was purchased from Sumika Chemical Analysis Service (Osaka, Japan).

Chemicals

Diniconazole and structurally related compounds were synthesized by Sumitomo Chemical (Osaka, Japan). β-CD was purchased from Kanto (Tokyo, Japan). Organic solvents and other reagents were of analytical-reagent grade from Kanto or Wako (Osaka, Japan). Water was processed through an RO/NANOpure II system (Barnstead, Dubuque, IA, USA).

Chiral stationary phase system

Experiments were carried out with Cyclobond I unless specified otherwise. The mobile phase was prepared by mixing an organic solvent with water or 0.1% triethylammonium acetate (TEAA) buffer. Sample solutions were prepared by dissolving each compound in methanol to give a concentration of 0.3 mg/ml. A 5-μl portion of the sample solutions was injected. The column temperature was kept at 20°C unless specified otherwise. The flow-rate was 0.5 ml/min and the detector was set at 254 nm. The void volume was determined by injecting methanol.

Chiral mobile phase system

The column used was Sumipax ODS A-212. For the mobile phase, β-CD was dissolved in 4.0 or 4.5 *M* aqueous urea, the pH of the solution was adjusted to 6.0 with diluted phosphoric acid and then acetonitrile was added. Sample solutions were 0.03 mg/ml in methanol. Separations were performed at controlled room temperature (*ca.* 25°C) with a flow-rate of 1.0 ml/min; the other conditions were the same as above.

RESULTS AND DISCUSSION

Chiral recognition of CDs

The effect of the type of CDs on the chiral recognition of diniconazole was investigated by using α-, β- and γ-CD-bonded columns.

The enantiomers of diniconazole were separated completely only on the β-CD-bonded column; they were not separated at all on the α-CD-bonded column and only partly separated on the γ-CD-bonded column (Table I). This shows that the formation of an inclusion complex depends on the size of the CD

TABLE I

ENANTIOMERIC SEPARATION OF DINICONAZOLE ON CYCLODEXTRIN-BONDED COLUMNS

Column	k'_1[a]	α[b]	Mobile phase[c]
Cyclobond I	6.47	1.19	80:20
Cyclobond II	4.96	1.04	87:13
Cyclobond III	4.99	1.00	90:10

[a] Capacity factor of first-eluted enantiomers.
[b] Separation factor.
[c] Mobile phase, acetonitrile–water (v/v).

cavity. The molecule of diniconazole can fit in the cavity of β-CD. Although complexation is also possible with γ-CD, the large cavity probably includes the molecule loosely, not differentiating between the enantiomers. The cavity of α-CD is too small for the molecule to enter. The β-CD bonded column was used in all subsequent experiments.

Effect of the structure of organic modifier

Because CDs form inclusion complexes with various hydrophobic compounds, organic solvent molecules in the mobile phase compete with the solute in occupying the CD cavity. The effects of the hydrophobicity and bulkiness of the organic modifier in the mobile phase on the separation of diniconazole were investigated using series of primary and secondary alcohols, and also acetonitrile. Each organic solvent concentration was adjusted so that the capacity factor of the first-eluted enantiomer was about 7.

As shown in Table II, the separation factors and resolutions decreased with increase in the number of carbon atoms. In a comparison of 1-propanol and 2-propanol, or 1-butanol, 2-methyl-1-propanol and 2-methyl-2-propanol, when the branched-chain isomers were used the peaks tended to tail and smaller resolution values were obtained, although the separation factors were almost the same as when the corresponding straight-chain alcohol was used. This result could be explained as follows; an increase in the bulkiness and hydrophobicity of the alcohol would increase the interaction between the alcohol and the CD cavity, shown as association constants, log K_a [10] in Table II , and then the alcohol would compete more strongly with the solute.

Acetonitrile–water was found to provide much better resolution than alcohol–water systems, probably because of its low interaction with hydroxyl groups of CD, and was therefore chosen as the mobile phase in subsequent experiments.

TABLE II

EFFECT OF ORGANIC MODIFIERS ON SEPARATION FACTORS, α, AND RESOLUTION, R_s

Column, Cyclobond I.

Organic modifier[a]	α	R_s	Log K_a[b]
Acetonitrile (20:80)	1.19	2.75	–
Methanol (36:64)	1.19	2.23	−0.49
Ethanol (25:75)	1.20	2.18	−0.03
1-Propanol (14:86)	1.16	1.93	0.57
2-Propanol (15:85)	1.17	1.76	0.58
1-Butanol (7:93)	1.13	1.70	1.22
2-Butanol (9:91)	1.16	1.78	1.19
2-Methyl-1-propanol (6:94)	1.13	1.59	1.62
2-Methyl-2-propanol (10:90)	1.14	1.06	1.68

[a] Mobile phase, organic modifier–water(v/v).

[b] Logarithm of association constant for β-CD–alcohol complex [10].

Effect of acetonitrile content

The effects of the acetonitrile content on the retention and resolution were investigated by changing the acetonitrile–water ratio in the mobile phase. Fig. 2 shows plots of the capacity factors of the first-eluted enantiomers and separation factors *versus* acetonitrile content. Both the retention time and selectivity decreased with increase in the acetonitrile content. A 20% acetonitrile content was adopted in subsequent experiments because a good separation was achieved in a reasonable time.

Effect of pH

The influence of pH on the capacity factors and separation factors of diniconazole was investigated by changing the pH of the mobile phase using 0.1% TEAA buffer. pH had no effect on the separation factors, as expected for this non-ionic compound,

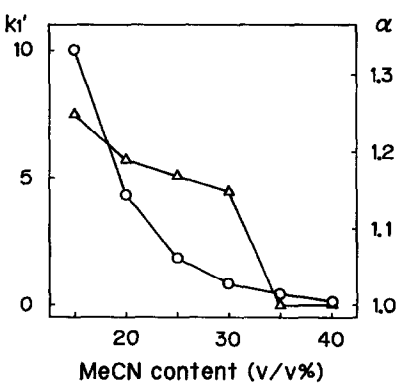


Fig. 2. Effect of acetonitrile content in the mobile phase on (○) capacity factors, k'_1 (capacity factors of the first-eluted enantiomers), and (△) separation factors, α. Column, Cyclobond I; mobile phase, acetonitrile–water.

TABLE III

EFFECT OF pH ON CAPACITY FACTORS, k'_1[a], AND SEPARATION FACTORS, α

Column, Cyclobond I; mobile phase, acetonitrile-0.1% TEAA buffer or water (20:80, v/v).

pH	k'_1[a]	α
4.0	4.39	1.18
5.0	4.56	1.18
6.0	4.98	1.19
7.0	4.48	1.19
Water	6.47	1.19

[a] Capacity factor of first-eluted enantiomer.

although the capacity factors were smaller when TEAA buffer was used than that when water was used, as shown in Table III.

Effect of column temperature

The effect of column temperature on the retention and resolution was examined over the range 0–60°C. The retention and resolution were significantly influenced by the column temperature, as can be seen in Fig.3. The capacity factors and the separation factors decreased with increase in the column temperature, and the enantiomers were not resolved at 60°C (k'_1 = 1.14). It is interesting to compare this result with that shown in Fig. 2 at the lower temperature, in which optical resolution was still achieved when k'_1 was 0.86 (at a 30% acetonitrile content). These results indicate that inclusion complex formation is prevented at temperatures higher than 60°C, as reported previously [11].

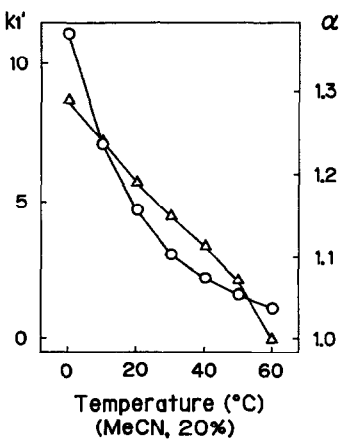


Fig. 3. Effect of column temperature on capacity factors, k'_1 and separation factors, α. Symbols as in Fig. 2; column, Cyclobond I; mobile phase, acetonitrile–water (20:80, v/v).

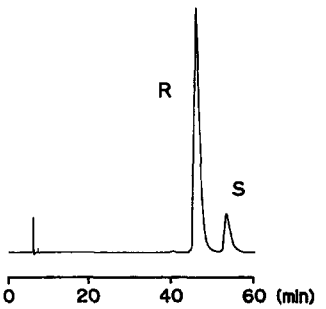


Fig. 4. Enantiomeric separation of diniconazole-M on Cyclobond I. Mobile phase, acetonitrile–water (20:80, v/v); temperature, 20°C.

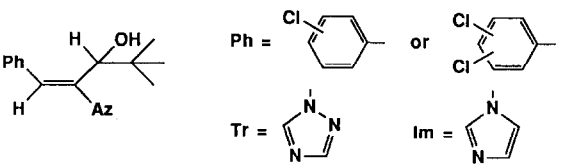
Retention behavior of structurally related compounds

Fig. 4 shows the optical resolution of diniconazole-M under the optimum conditions and Table IV

TABLE IV

RETENTION BEHAVIOUR OF STRUCTURALLY RELATED COMPOUNDS OF DINICONAZOLE ON CYCLOBOND I

Chromatographic conditions as in Fig. 4.



Ph	Az	k'_1[a]	α[b]
2-Cl	Tr	2.77	1.08
3-Cl	Tr	2.64	1.00
4-Cl	Tr	3.23	1.00
4-Cl	Im	4.95	1.00
2,3-Cl_2	Tr	2.56	1.00
2,4-Cl_2	Tr[c]	3.20	1.18
2,4-Cl_2	Im	6.71	1.17
3,4-Cl_2	Tr	4.23	1.10
3,5-Cl_2	Tr	1.81	1.00

[a] Capacity factor of first-eluted enantiomer.
[b] Separation factor.
[c] Diniconazole.

lists the capacity factors and separation factors of structurally related compounds of diniconazole under the same conditions. Among the triazoles, the 2-chlorophenyl and 3,4-dichlorophenyl analogues were resolved into the enantiomers, in addition to diniconazole, and the other substituents were not resolved. Imidazoles were retained longer than triazoles because of their higher hydrophobicity [12], but the enantioselectivity was not affected by the triazole or imidazole ring in the molecule.

From these results, it was concluded that the benzene ring of the solute inserted into the CD cavity and its substituent(s) played an important role from a steric point of view, a tight fit being important. The hydroxyl group at the chiral centre and a nitrogen atom in the triazole ring could interact with the hydroxyl groups at the mouth of the CD cavity by hydrogen bonding at the same time.

β-CD as a chiral additive

The method using β-CD as a chiral mobile phase additive was applied to the separations of diniconazole enantiomers. The enantiomers were slightly resolved on the Sumipax ODS A-212 column with 30 m*M* β-CD in 4.5 *M* aqueous urea (pH 6.0)–acetonitrile (60:40, v/v). Higher concentrations of β-CD seem to be necessary for complete separation, but it could not be achieved owing to the limited solubility of β-CD.

In this method, the *S*-enantiomer eluted first, in contrast to the above method using the chiral stationary phase. This phenomenon is explained as follows: the enantiomer forming a more stable complex with the CD elutes first in the chiral mobile phase method because CDs interact little with the hydrophobic stationary phase owing to the hydrophilic nature of the external faces of them; on the other hand, this enantiomer elutes later in the chiral stationary phase method because it is retained longer on the CD-bonded phase.

CONCLUSIONS

It has been demonstrated that a β-CD-bonded column exhibits a high enantioselectivity for diniconazole and some analogues. The investigation of the effects of the size of CDs, the mobile phase composition, column temperature and the small changes in the structure of the solute on the retention and resolution suggested that inclusion complex formation between the benzene ring of the solute and the CD cavity is the most important factor in the chiral recognition.

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