Journal of Chromatography, 506 (1990) 481–493
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1677

Displacement chromatography on cyclodextrin–silicas

III. Enantiomer separations

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SUMMARY

The feasibility of preparative enantiomer separations by displacement chromatography on analytical-scale β-cyclodextrin–silica columns, operated in the reversed-phase mode, was demonstrated using the enantiomers of mephobarbital, hexobarbital, dansylleucine, dansylvaline and dansylphenylalanine as model solutes. The method developement scheme (which relies on the determination of the elution-mode retention behavior and the adsorption isotherms of the solutes and the candidate displacers) described in Parts I and II can be used to select the appropriate displacers and the conditions leading to a successful displacement chromatographic separation. The importance of the displacer (both type and concentration) to the success of the displacement chromatographic separation is demonstrated.

INTRODUCTION

Cyclodextrins are toroidally shaped molecules that contain 6–8 glucose units (α- to γ-cyclodextrins). The interior of the cyclodextrin cavity is hydrophobic and the exterior is hydrophilic owing to the presence of secondary hydroxyl groups at the larger lip of the cavity and primary hydroxyl groups at the smaller lip of the cavity[1–3]. Cyclodextrins can form inclusion complexes with molecules that penetrate their cavities[4,5]. The stability of the complex depends on the tightness of the fit between the solute and the cavity and the strength of their secondary intermolecular interactions (mostly hydrogen bonds)[6].

In liquid chromatography, cyclodextrins can be used either as mobile phase additives[7] or as stationary phases[3,8–12]. Cyclodextrins as mobile phase additives work well in analytical separations, but they are impractical in preparative separations, as they must be removed from the product. The cyclodextrin stationary phases are either cyclodextrin polymers[13,14] or cyclodextrin-silicas[10,15–21]. Currently, the hydrolytically stable cyclodextrin–silica stationary phases developed by Armstrong and co-workers[17–19] and commercially available from Astec (Whippany, NJ, U.S.A.)[20] are used for most separations.

0021-9673/90/$03.50

Cyclodextrins can effect the chromatographic separation of positional isomers[21–27], geometrical and *cis/trans* isomers[28–33] and, owing to the presence of chiral carbon atoms, enantiomers[34–44].

The resolution of enantiomers by cyclodextrin stationary phases has been extensively reviewed recently[9,10,12,37]. Chiral recognition is believed to be caused by the concerted action of inclusion complex formation between the cacity and the hydrophobic part of the solute, hydrogen bond formation between the polar functional groups of the solute in the vincinity of its chiral center and the hydroxyl groups of the cyclodextrin molecule and by the steric hindrance of substituents around the chiral center, which weaken the strength of hydrogen bonding for one of the enantiomers[38]. Separation selectivities for the enantiomeric pairs are generally low, 1.03–1.2[38–40]. Diastereomers occasionally show higher (2.0) separation factors[39].

Although there is a rich literature on the analytical-scale chromatographic application of cyclodextrin–silicas[12–44], papers on its preparative chromatographic use are scarce[20]. Cyclodextrin silicas have three major drawbacks in preparative elution-mode chromatographic separations: (i) strong non-selective solute retention, (ii) low chiral selectivity and (iii) low load capacity compared with that of the other silica-based stationary phases. Therefore, the conventional elution-mode preparative separation strategy cannot be applied very well with cyclodextrin–silicas. However, some of these problems can be eliminated when cyclodextrin–silicas are used for preparative chiral separations in the displacement mode.

The old, but unique, operating principles of displacement chromatography[45–51] can be combined with modern HPLC equipment to achieve efficient preparative separations, as demonstrated by Horváth and co-workers[52–61]. Currently, several research groups are pursuing the theoretical and practical aspects of displacement chromatography[52–70].

In displacement chromatography, the sample is adsorbed on the top of the column, which is pre-equilibrated with the carrier, which is a solvent so weak that it cannot elute the sample. The sample will start to move only when it is removed from the stationary phase by the more strongly adsorbing displacer, which is fed continuously into the column at a high concentration. As the sample and displacer fronts move down the column, the components become separated according to their adsorption strengths. Eventually, in the fully developed displacement train, all components move with the velocity of the displacer front, which, in turn, depends on the adsorption isotherm and concentration of the displacer. High solute concentrations and column loads can be achieved in the displacement mode.

Although considerable information is available on the selection of the operation parameters in displacement chromatography[52–65], little is known about the rules of displacer selection and selectivity control. The greatest hindrance to the wider use and acceptance of displacement chromatography is the paucity of well characterized displacers and the lack of solute adsorption isotherms. Displacer selection is still done by trial and error. Most modern displacement chromatographic separations rely on a reversed-phase system to separate small polar molecules, antibiotics, oligopeptides and small proteins[52–65].

In Parts I[66] and II[67] we described the first displacement chromatographic separations that were realized with cyclodextrin–silicas. We combined the unique chromatographic selectivity of cyclodextrins with the preparative efficiency of displace-

ment chromatography and successfully separated several geometrical, positional[66] and *cis/trans* isomers[67]. Samples as large as 60 mg could be loaded onto and separated with 4.6 mm I.D. analytical columns, operated in the reversed-phase mode. In this paper we describe the use of the same phase system for the displacement chromatographic separation of enantiomers.

EXPERIMENTAL

A computer-controlled displacement chromatograph was built for these studies, as described in Part I[66]. Separations were carried out with commercially available 5-μm β-cyclodextrin–silica, Cyclobond I (Astec, Whippany, NJ, U.S.A.). The columns were slurry packed in our laboratory into 4.6 mm I.D. stainless-steel tubes of different lengths. All measurements were carried out at 30°C, maintained by thermostated water-jackets. The solute and displacer adsorption isotherms were determined by the breakthrough method, as described in Part I[66].

The barbitals and dansylamino acids were from Sigma (St. Louis, MO, U.S.A.) and the displacers from Aldrich (Milwaukee, WI, U.S.A.). All substances were used without further purification. The carrier and displacer solutions were prepared from HPLC-grade methanol and acetonitrile (Fisher, Fair Lawn, NJ, U.S.A.) and water produced by a Milli-Q unit (Millipore, Bedford, MA, U.S.A.).

The solutions were freshly prepared immediately before use by the weighing method described in Part I[66].

RESULTS

The purpose of these studies was to determine whether efficient, preparative-scale enantiomer separations are possible when cyclodextrin–silica columns are strongly overloaded and operated according to the principles of non-linear chromatography in the displacment mode. Members of two characteristic, very different substance families, barbiturates and dansylated amino acids, were used as model substances.

Preparative displacement chromatographic separation of barbiturate enantiomers

The analytical separation of the enantiomers of mephobarbital and hexobarbital was achieved with methanol–buffer eluents on Cyclobond I β-cyclodextrin–silica[10]. As discussed in Parts I and II[66,67], the first step in the development of a displacement chromatographic separation is the determination of the composition of the carrier solution. The analytical separations are often good starting points for the development of a displacement chromatographic separation.

In order to find the carrier composition that provides $k' > 5$–10 for the least retained solute[66,67], the log k' *vs.* methanol concentration relationship had to be determined. Fig. 1 shows the log k' *vs.* methanol concentration relationship for the mephobarbital enantiomers. As it was known from our previous work[66] that 1-naphthol is a good displacer, it was also included in the retention studies. Fig. 1 shows that the k' of (*S*)-mephobarbital exceeds 10 when the methanol concentration of the eluent becomes less than 20% (v/v). Therefore, 20% (v/v) aqueous methanol was selected as the carrier solvent.

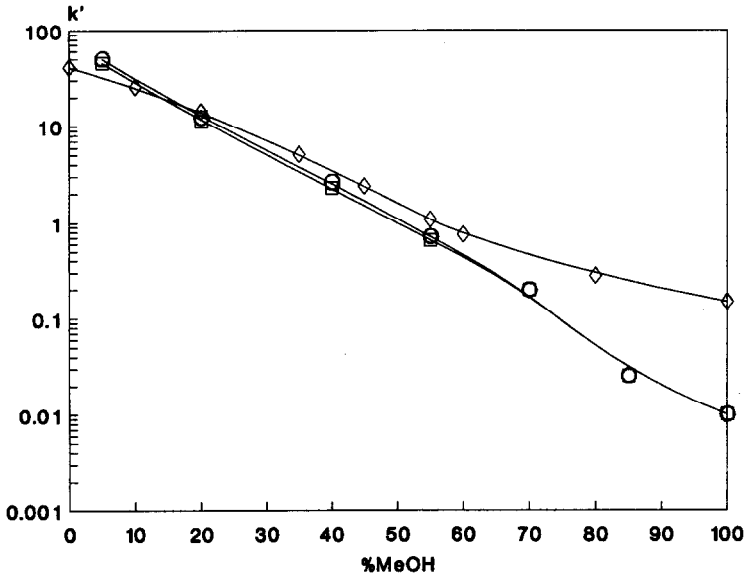

Fig. 1. Retention of the mephobarbital enantiomers and 1-naphthol on β-cyclodextrin–silica as a function of the methanol concentration of the eluent. For chromatographic conditions, see text. ◇ = 1-Naphthol; □ = (*S*)-mephobarbital; ○ = (*R*)-mephobarbital. MeOH = Methanol.

The log k' of the barbital enantiomers varies more steeply than that of 1-naphthol with methanol concentration and the retention curves cross at *ca.* 13% (v/v) methanol. However, in 20% (v/v) aqueous methanol, which was selected as the carrier solution, the k' of 1-naphthol (14.2) is larger than that of the more retained enantiomer, (*R*)-mephobarbital (k' = 12.1). Therefore, 1-naphthol was tried as a potential displacer.

The adsorption isotherms of 1-naphthol and the breakthrough volumes corresponding to each point on the isotherm were known from our previous work on the displacement chromatographic separation of positional and geometrical isomers[66]. As the concentration of the displacer increases, the corresponding breakthrough volume decreases. Therefore, the highest safe displacer concentration, at which the breakthrough volume of the displacer is equal to (or greater than) the elution volume of the last-eluted (*R*)-mephobarbital, can be selected (5 m*M*). This value represents a conservative estimate ensuring that the displacer front will not overrun the solute, even if the concentration of the solute is very low. In reality, higher displacer concentrations are still permissible, because the partition coefficent of the solute also decreases as the sample concentration increases.

The displacement chromatogram of a 6.1 μmol sample of racemic mephobarbital is shown in Fig. 2. The displacer was a 5 m*M* solution of 1-naphthol in 20% (v/v) aqueous methanol. The flow-rate of the displacer was 0.2 ml/min and the temperature was 30°C. Effluent fractions of 0.2 ml were collected during the separation and subsequently analysed in the elution mode. The analytical results are plotted in Fig. 3, yielding the reconstructed displacement chromatogram of mephobarbital. It can be seen that, although the elution-mode separation selectivity for the mephobarbital

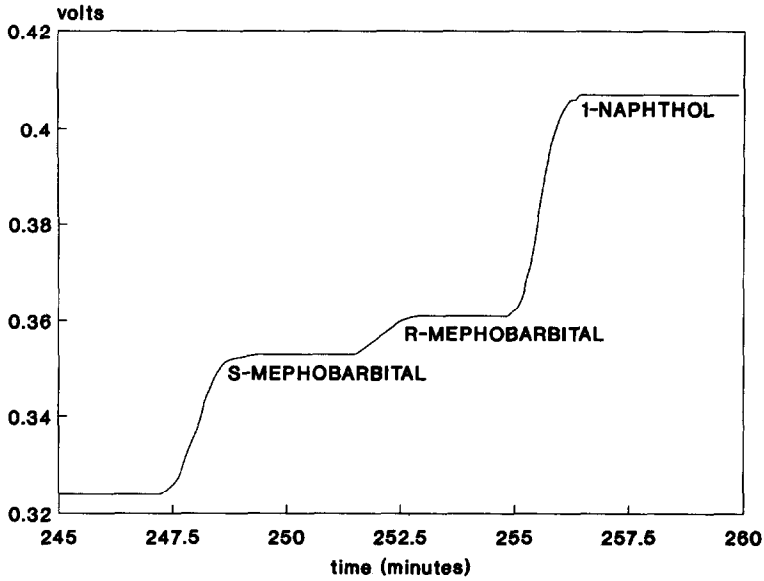

Fig. 2. Displacement chromatogram of a 6.1-μmol racemic mephobarbital sample on two 250 × 4.6 mm I.D. analytical β-cyclodextrin–silica columns, with a 5-mM solution of 1-naphthol in 20% (v/v) aqueous methanol as displacer; flow-rate, 0.2 ml/min; 30°C.

enantiomers is only 1.08, good separation of the two enantiomers is achieved in the preparative displacement mode.

Fig. 4 shows the log k' *vs.* methanol concentration relationship for the hexobarbital enantiomers and 1-naphthol. Again, sufficient retention is observed with 20%

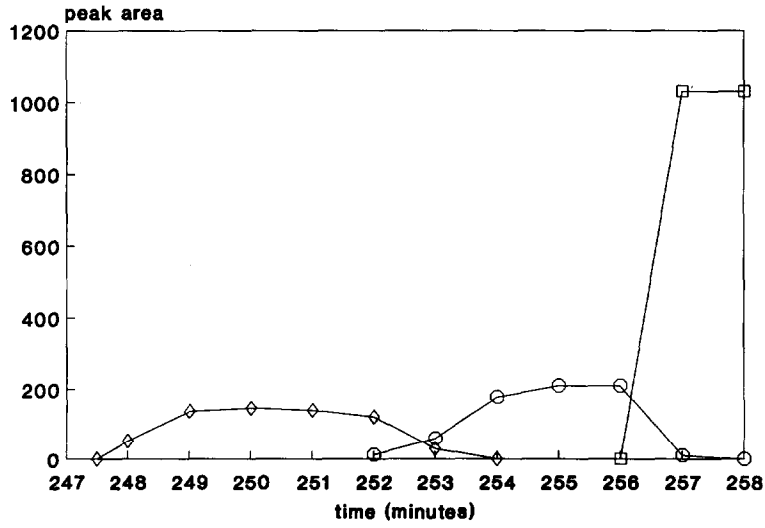

Fig. 3. Reconstructed displacement chromatogram of the separation shown in Fig. 2. Fraction size, 200 μl. ◇ = (*S*)-Mephobarbital; △ = (*R*)-mephobarbital; □ = 1-naphthol. Peak area × 10^{-3} m V · s.

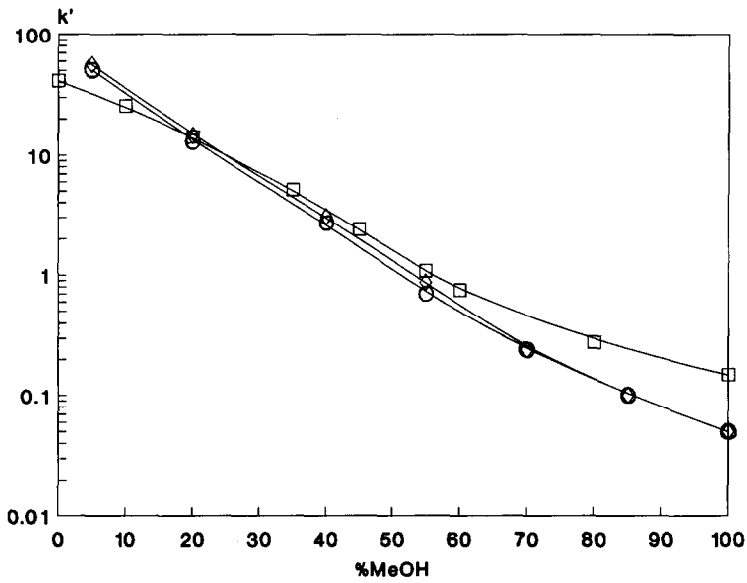

Fig. 4. Retention of the hexobarbital enantiomers and 1-naphthol on β-cyclodextrin–silica as a function of the methanol concentration of the eluent. □ = 1-Naphthol; ○ = (*S*)-hexobarbital; △ = (*R*)-hexobarbital.

(v/v) aqueous methanol carrier solutions. However, the hexobarbital enantiomers are more retained than the mephobarbital enantiomers, and 1-naphthol is eluted together with them by 20% (v/v) methanol. This indicates that 1-naphthol probably cannot be used as a displacer.

However, it was known from our previous work on the displacement chromatographic separation of positional isomers that cetrimide is more retained than 1-naphthol on β-cyclodextrin–silica. Cetrimide also proved to be an appropriate displacer for the separation of the naphthol isomers (see Figs. 10 and 11 in ref. 66). Therefore, a 1.5 m*M* cetrimide solution was prepared with the carrier solvent and used as displacer.

The displacement chromatogram of a 4-μmol sample of racemic hexobarbital is shown in Fig. 5. The separation conditions were the same as for the mephobarbitals. Again, a clear separation of the enantiomers is obtained in the displacement mode, even though the selectivity factor for the two enantiomers in the elution mode is only 1.1.

Preparative displacement chromatographic separation of the enantiomers of dansylated amino acids

Elution-mode, analytical separations of the enantiomers of dansylated leucine, valine and phenylalanine have been carried out with β-cyclodextrin–silica and methanol–buffer eluents[10,17,18,38]. Based on this information, a methanol concentration range leading to k' values between 5 and 20 was selected, and the capacity factors of the individual amino acid enantiomers were determined. The results are given in Table I. It can be seen that the dansylamino acids are much more strongly retained

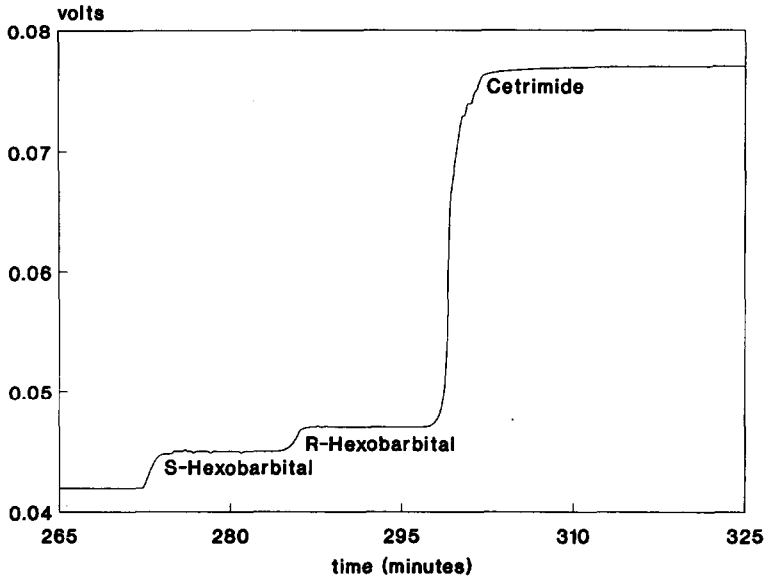

Fig. 5. Displacement chromatogram of a 4-μmol racemic hexobarbital sample on two 250 × 4.6 mm I.D. analytical β-cyclodextrin–silica columns, with a 1.5-mM solution of cetrimide in 20% (v/v) aqueous methanol as displacer; flow-rate, 0.2 ml/min; 30°C.

than the previously studied barbiturates. The displacers described in Parts I and II[66,67] are too weak to be used with the dansylamino acids. Therefore, a detailed search was initiated to design and synthesize a series of displacers with tailor-made retention and adsorption characteristics. These displacers consists of three main parts: an anchor group that forms an inclusion complex with the cyclodextrin cavity, a middle section that can interact via hydrogen bonding with the secondary hydroxyl groups at the larger opening of the cavity and a solubility-adjusting section that prefers the mobile phase. The results of this work will be reported in future papers[68,69].

TABLE I

CAPACITY FACTORS (k') AND SEPARATION SELECTIVITY FACTORS (α) OF DANSYLAMINO ACID ENANTIOMERS ON A β-CYCLODEXTRIN–SILICA COLUMN, WITH TWO METHANOL–BUFFER [0.01% (w/w) TRIETHYLAMMONIUM ACETATE, pH 4.1] ELUENTS

Solute	*Enantiomer*	*50% (v/v) methanol*		*40% (v/v) methanol*	
		k'	α	k'	α
Dansylleucine	D	6.57	1.05	14.61	1.07
	L	6.23		13.62	
Dansylvaline	D	6.69	1.06	14.88	1.08
	L	6.29		13.78	
Dansylphenylalanine	D	12.04	1.06	25.32	1.07
	L	11.41		23.58	

TABLE II

CAPACITY FACTORS k' OF TWO DINITROPHENYL FUNCTIONAL-GROUP-BASED DISPLACERS ON A β-CYCLODEXTRIN–SILICA COLUMN WITH TWO DIFFERENT METHANOL–BUFFER [0.01% (w/w) TRIETHYLAMMONIUM ACETATE, pH 4.1] ELUENTS

Compound	k'	
	50% (v/v) methanol	*40% (v/v) methanol*
2,4-Dinitrophenol	10.66	19.30
3,5-Dinitrobenzoic acid	13.27	18.67

Two of these potential displacers, both with a dinitrophenyl functional group, are more retained (Table II) than the dansylated amino acids (Table I), and have favorable solubilities in 40% (v/v) methanol–buffer solutions [0.01% (w/w) triethylammonium acetate, pH 4.1]. Both of them can be considered as potential displacers for the separation of the enantiomers of the dansylated amino acids. To test this hypothesis, their adsorption isotherms were determined in this solvent. Regular Langmuirian adsorption behavior was observed, as shown in Fig. 6.

Using the isotherms as guidelines, the displacement chromatogram of a 5.6-mg sample of racemic dansylleucine was obtained with a 10 m*M* solution of 2,4-dinitrophenol in the carrier solvent as displacer. The displacement chromatogram is shown in Fig. 7. The steps observed in the chromatogram have very similar heights,

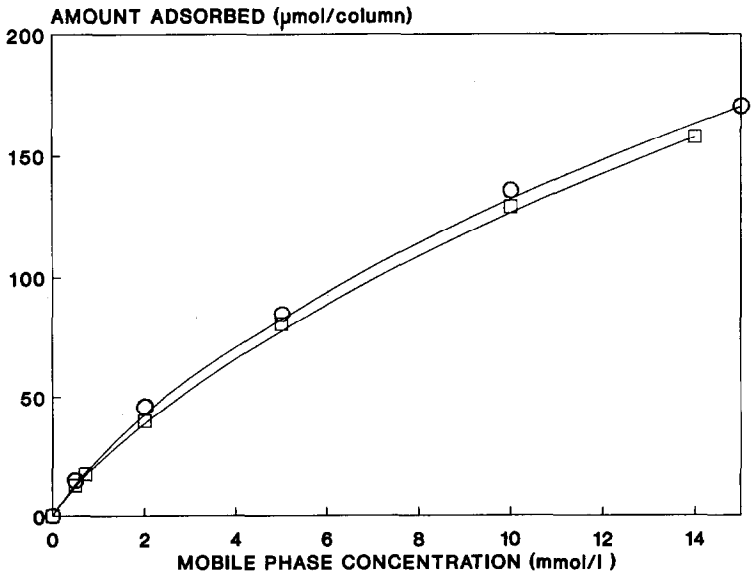

Fig. 6. Adsorption isotherms of (□) 2,4-dinitrophenol and (○) 3,5-dinitrobenzoic acid from 40% v/v methanol–buffer [0.01% (w/w) triethylammonium acetate, pH 4.1] on a β-cyclodextrin–silica column.

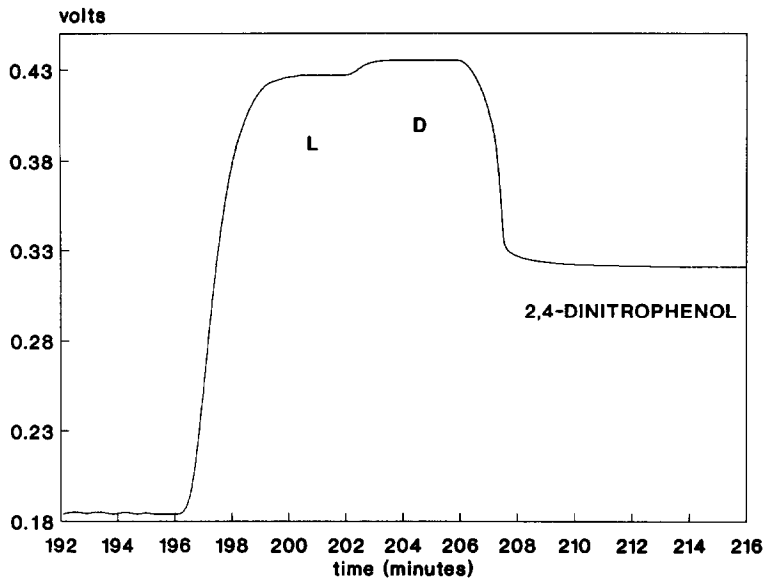

Fig. 7. Displacement chromatogram of a 5.6-mg racemic dansylleucine sample on two 250 × 4.6 mm I.D. analytical β-cyclodextrin–silica columns, with a 10-m*M* solution of 2,4-dinitrophenol in 40% (v/v) methanol–buffer [0.01% (w/w) triethylammonium acetate, pH 4.1] as displacer; flow-rate, 0.2 ml/min; 30°C.

because the adsorption isotherms of the enantiomers are very close to each other, and the equilibrium concentrations of the enantiomers in the fully developed displacement train (determined by the intersection of the operational line and the adsorption isotherms) are very similar. As their molar refractive indices are identical, the observed signals are almost the same. However, this does not mean that their separation is not complete: the quality of the separation is reflected by the ratio of the width of the pure band to the width of the transition band (information on the horizontal axis), and not by the ratio of the band heights (information of the vertical axis).

The displacement chromatogram of a 6.3-mg sample of racemic dansyl valine was obtained with a 10.5 m*M* solution of 2,4-dinitrophenol in the carrier solvent as displacer. The displacement chromatogram is shown in Fig. 8. As in the previous instance, the steps observed in the chromatogram have similar heights. For both amino acids good enantiomer separation was obtained in the displacement mode, even though the selectivity factors in the elution mode are as small as 1.07 and 1.08 (Table I).

As the retention of dansylphenylalanine in 40% (v/v) methanol–buffer solution is too large (k' = 25.3, Table I), 50% (v/v) methanol–buffer carrier solution had to be selected for the analysis. In this carrier, one of the potential displacers, 3,5-dinitrobenzoic acid, is more retained than the D-enantiomer of dansylphenylalanine and it was therefore selected as a displacer.

However, when the displacement chromatographic separation was attempted as before, analysis of the second step, the band of the dansyl-*d*-phenylalanine enantiomer showed that it contained a mixture of dansyl-*d*-phenylalanine and 3,5-

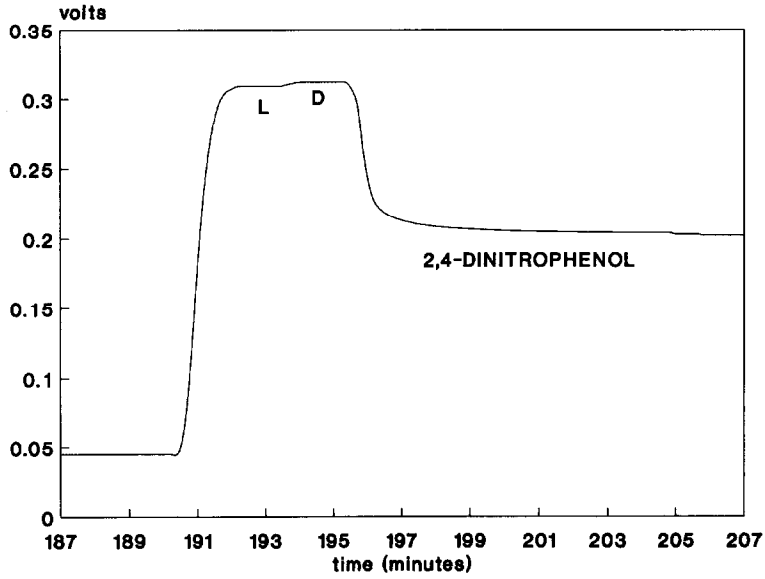

Fig. 8. Displacement chromatogram of a 6.3-mg racemic dansylvaline sample on two 250 × 4.6 mm I.D. analytical β-cyclodextrin–silica columns, with a 10.5-m*M* solution of 2,4-dinitrophenol in 40% (v/v) methanol–buffer [0.01% (w/w) triethylammonium acetate, pH 4.1] as displacer; flow-rate, 0.2 ml/min; 30°C.

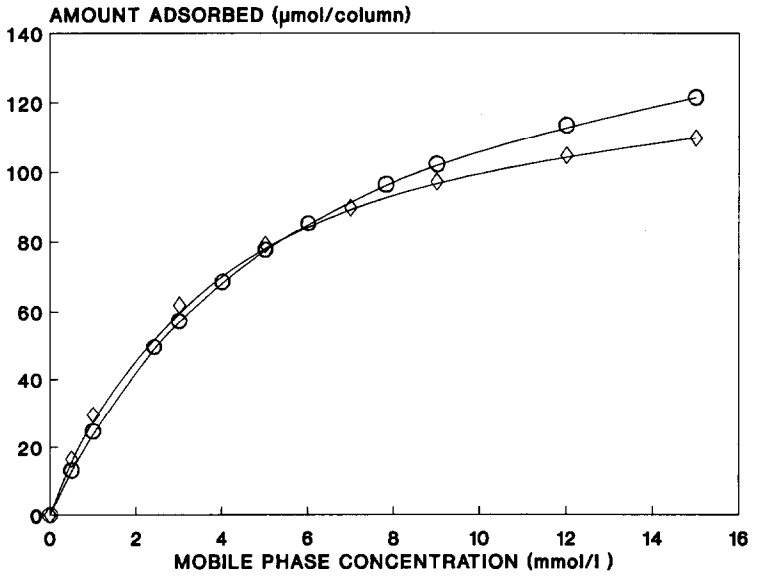

Fig. 9. Adsorption isotherms of (○) dansyl-*d*-phenylalanine and (◇) 3,5-dinitrobenzoic acid from 50% (v/v) methanol–buffer [0.01% (w/w) triethylammonium acetate, pH 4.1] on a β-cyclodextrin–silica column.

dinitrobenzoic acid. The concentration ratio of the two components remained almost constant along the entire band.

In order to find the reasons for the failed separation, the adsorption isotherms of both 3,5-dinitrobenzoic acid and dansyl-*d*-phenylalanine were determined in 50% (v/v) methanol carrier solvent. The results are shown in Fig. 9. It can be seen that dansyl-*d*-phenylalanine is more strongly adsorbed at low concentrations than is 3,5-dinitrobenzoic acid, but the two isotherms cross at higher concentrations. This means that an adsorption azeotrope[70] is formed, and separation is impossible with this displacer. This again indicates that, even though the knowledge of the elution-mode retention behaviour of the solutes and the displacers is important for the design of a displacement chromatographic separation, it is by no means sufficient in certain instances. Knowledge of the non-linear behavior of the system is indispensable for the solution of these separation problems.

CONCLUSIONS

This work has shown that the simple method development guidelines described in Parts I and II[66,67] for the separation of positional, geometrical and *cis/trans* isomers can also be used to develop preparative, displacement chromatographic separations of enantiomers on cyclodextrin–silica stationary phases.

In most instances, the elution-mode log k' *vs.* organic modifier concentration, k' *vs.* pH and k' *vs.* ionic strength relationships must first be determined in order to select the composition of the carrier solution that will ensure sufficient initial sample retention for the least retained enantiomer ($5 < k' < 10$). Potential displacers can then be selected by comparing their initial breakthrough volumes and the elution-mode capacity factor of the most retained solute.

The adsorption isotherms of the selected displacer (and, preferably, of the most retained solute) must be determined when a separation has to be designed for a more complex situation. Knowledge of the adsorption isotherms takes the guesswork out of the design of the initial displacement chromatographic separation and accounts for the solubility limitations and peculiar adsorption characteristics of both the solutes and the displacer.

The first displacement chromatographic separations of a number of racemic model substances (barbiturates and amino acids) were accomplished using analytical-scale β-cyclodextrin–silica columns, methanol–buffer and acetonitrile–buffer carrier solutions and several non-chiral displacers. Further work is in progress in our laboratory to design, synthesize and characterize a number of displacers with tailor-made adsorption and solubility characteristics and to solve several isomer and enantiomer separation problems that are of current interest to the pharmaceutical industry and to the life sciences.

ACKNOWLEDGEMENTS

Financial support by the Texas Coordination Board of Higher Education TATR program (Grant Number 3376) and the Minority Access for Research Careers, National Institute of Health program (Grant Number 5F31GM11689) is acknowledged. The authors are grateful to Dr. Thomas Beesley of Astec for the β-cyclodextrin-silica stationary phase sample used.

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