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Displacement chromatography on cyclodextrin silicas

V. Separation of the enantiomers of 5,10-dideazatetrahydrofolic acid

Leif H. Irgens[☆], Gyula Farkas[☆☆] and Gyula Vigh*

Department of Chemistry, Texas A & M University, College Station, TX 77843-3255 (USA)

ABSTRACT

A displacement chromatographic method has been developed for the preparative separation of the enantiomers of 5,10-dideazatetrahydrofolic acid on a β-cyclodextrin silica column. The retention behavior of the chiral solute was studied in detail to find the separation conditions which provide the largest chiral selectivity values: the k' *vs.* acetonitrile modifier concentration, the k' *vs.* pH, the k' *vs.* citrate buffer concentration and the log k' *vs.* $1/T$ relationships; also, the α *vs.* acetonitrile concentration, the α *vs.* pH, the α *vs.* citrate buffer concentration and the log α *vs.* $1/T$ relationships have been determined. Cetyltrimethylammonium bromide, more retained and more strongly adsorbed than the chiral solute, was selected as displacer for the separation. With an α value of 1.14, good preparative chiral separations were observed both in the displacement mode and in the overloaded elution mode.

INTRODUCTION

In previous parts of this series [1–4] we reported that small molecules (geometric isomers, positional isomers and enantiomers) could be successfully separated on β-cyclodextrin silica columns [5,6] in the displacement mode of operation. Because the present paper is part of this series, neither the principles of displacement chromatography nor the properties of native β-cyclodextrin silica columns are reviewed here; instead, the reader is referred to refs. 1–9. The method development scheme first described in ref. 4 for the separation of the enantiomers of ibuprofen was applied here for the separation of the enantiomers of 5,10-dideazatetrahydrofolic acid (DDATHF), a compound with a much more complex structure than any of the enantiomers that were previously tackled by chiral displacement chromatography [1–4,7–9].

DDATHF (structure shown in Fig. 1), has been investigated at Eli Lilly Company as a potential pharmaceutical agent [10]. In order to provide sufficient quantities of the individual enantiomers for further studies, a preparative separation method was sought to augment the

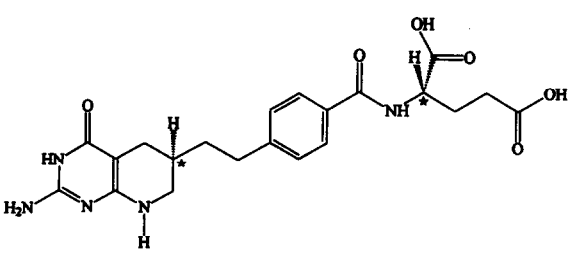

Fig. 1. The structure of DDATHF.

* Corresponding author.
☆ Present address: DuPont de Nemours, Belle, WV, USA.
☆☆ Present address: Chinoin Pharmaceutical Company, Budapest, Hungary.

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classical enantiomer separation methods applied [10]; this led us to attempt the preparative chiral separation by both displacement and overloaded elution mode chromatography using β-cyclodextrin silica as stationary phase. Considering that higher selectivity factors lead to higher production rates in preparative chromatography [11], maximization of the chiral selectivity is the most crucial step of the method development scheme. Separation selectivities on β-cyclodextrin silicas depend on the delicate balance between inclusion complex formation, hydrogen bond formation, and steric hindrance [12] which, in the case of ionic solutes, are influenced by the type and the concentration of the polar organic modifier in the eluent, the type, the concentration and the pH of the buffer, and the temperature of the eluent [13]. Therefore, the retention behavior of the enantiomers of DDATHF was investigated as a function of these parameters to find the chromatographic conditions which lead to maximized chiral selectivity and appropriate solute retention ($1 < k' < 20$). Next, the displacer was selected based on its relative retention with respect to the more retained enantiomer of DDATHF and its adsorption behavior [14], followed by the displacement chromatographic runs and the overloaded elution mode runs, and the analysis of the collected enantiomer fractions. Finally, the yields and the production rates were calculated as a function of the enantiomeric purity of the pooled fractions.

EXPERIMENTAL

The displacement chromatograph was built from commercial components as described in ref. 1. 5-μm native β-cyclodextrin silica, Cyclobond I (ASTEC, Whippany, NJ, USA), was slurry-packed into 250 mm × 4.6 mm I.D. stainless-steel columns (BST, Budapest, Hungary) using a Type 2HPAW air-amplifier pump (Haskel, Burbank, CA, USA). The column temperature was controlled within ± 0.5°C, using a water jacket, by a Type UF3 circulating water bath (Science/Electronics, Dayton, OH, USA).

Enantiomerically enriched DDATHF, containing about 64% (w/w) of the less retained enantiomer and 36% (w/w) of the more retained enantiomer, was a gift by Dr. J. Shih of Eli Lilly Company (Indianapolis, IN, USA) [10]. Citric acid, sodium hydroxide, triethylamine and cetyltrimethylammonium bromide were purchased from Aldrich (Milwaukee, WI, USA), HPLC grade acetonitrile from J.T. Baker (Philipsburg, NJ, USA), pH 4 and 7 aqueous pH standards from Fisher Scientific (Fair Lawn, NJ, USA). Water was produced by a Milli-Q unit (Millipore, Bedford, MA, USA). All chemicals were used as received, without further purification. The carrier and displacer solutions were freshly prepared using the weighing method described in Part I [1].

RESULTS

Retention studies of DDATHF

Because DDATHF can have both a net positive charge and a net negative charge as the pH is varied between 2 and 10 [10], its retention on β-cyclodextrin silica, and the value of the chiral selectivity factor, is influenced by the concentration of acetonitrile (the polar organic modifier), the pH, the buffer concentration, the concentration of the tailing-reducing additive (triethylamine) [6], and the temperature of the eluent.

First, the effects of the acetonitrile concentration were studied in 5 m*M* citrate–acetonitrile–water eluents. The apparent pH of the eluents was adjusted to 3.0 and 5.0, respectively, by adding a few μl of a concentrated sodium hydroxide solution to the hydroorganic eluent and monitoring its pH with a combination glass electrode, standardized against aqueous pH 4 and 7 standards [15].

The k'_2 values of the more strongly retained DDATHF enantiomer (both at low and high pH), are shown as solid lines, plotted against the left axis in Fig. 2, as a function of the % (v/v) acetonitrile (ACN) concentration. Unlike in regular reversed-phase HPLC with octadecylsilica stationary phases, where the log k' *vs.* % ACN relationship is linear [16], the log k'_2 values on β-cyclodextrin silicas tend to level off when the ACN concentration exceeds 50%. The k' values in the lower pH eluents are about half an order

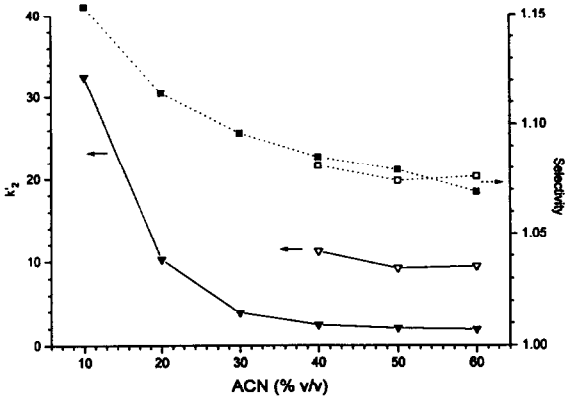

Fig. 2. The capacity factor of the more retained enantiomer of DDATHF (solid lines plotted against the left axis) and the chiral selectivity factor for the separation of the enantiomers of DDATHF (dotted lines plotted against the right axis) as a function of the acetonitrile concentration (%, v/v) of the eluent. Analytical citrate concentration, 5 m*M*. Full symbols = pH 3, open symbols = pH 5, triangles = k'_2 values, squares = selectivity values. Column temperature, 30°C; eluent flow rate, 1 ml/min.

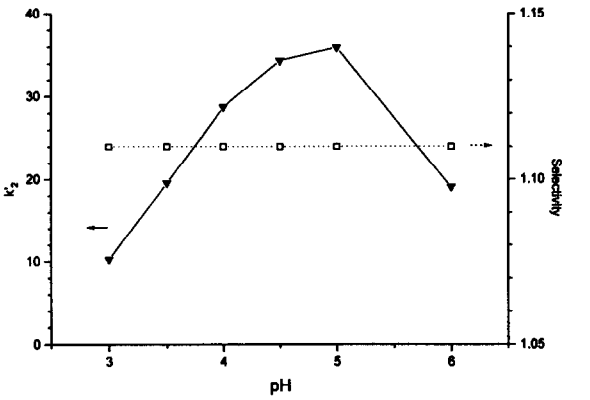

Fig. 3. The capacity factor of the more retained enantiomer of DDATHF (solid line plotted against the left axis) and the chiral selectivity factor for the separation of the enantiomers of DDATHF (dotted line plotted against the right axis) as a function of the pH of the eluent [ACN, 20% (v/v); analytical citrate concentration, 25 m*M*]. Symbols: ▼ = k'_2, □ = selectivity. Column temperature, 30°C; eluent flow rate, 1 ml/min.

of magnitude lower than in the high pH eluents indicating that pH is a very powerful retention controlling parameter.

The chiral selectivity (α) values which were observed for the DDATHF enantiomers are shown as dotted lines, plotted against the right axis of Fig. 2, as a function of the % (v/v) ACN concentration. The chiral selectivity values are very similar in both the pH 3 and pH 5 eluents, and they do vary strongly with the acetonitrile concentration. This behavior is opposite to what was observed for ibuprofen [4], an anionic solute with a structure much simpler than that of DDATHF. Since the gain in α with decreasing % ACN concentration is significant, the organic modifier concentration of the carrier solution will play a major role in the success of the preparative separation.

Due to the strong dependence of k'_2 on the pH of the eluent, a more detailed pH study was completed using 20% (v/v) ACN eluents. As before, the k'_2 values of the more strongly retained DDATHF enantiomer are shown as a solid line, plotted against the left axis in Fig. 3, as a function of the pH of the eluent. The chiral selectivity (α) values are shown as a dotted line, plotted against the right axis of Fig. 3. It can be seen that the retention of DDATHF varies greatly between pH 3 and 6, corresponding to the changes in its state of protonation. Remarkably, chiral selectivity remains the same, providing us with yet another tool that can be used to increase or decrease the retention of DDATHF at will, without compromising the chiral selectivity of the system. Because the solubility of DDATHF is much higher in the higher pH eluents, a clear benefit in preparative separations, pH 6 is the preferred eluent pH.

As DDATHF is ionic at pH 6, it can be expected that its retention, and perhaps the chiral selectivity, will be influenced by the concentration of the buffer. Therefore, the analytical citrate concentration was systematically varied in the 20% (v/v) acetonitrile:water eluents, while the apparent pH was maintained at 6.0. The k'_2 values of the more retained DDATHF enantiomer, plotted as a solid line against the left axis in Fig. 4, decrease significantly as the analytical citrate concentration is increased from 5 m*M* to 25 m*M*. On the other hand, chiral selectivity, plotted as a dotted line against the right axis in Fig. 4, remains constant as the analytical citrate concentration is varied. Thus, the concentration of the buffer affords yet another powerful tool for the control of the

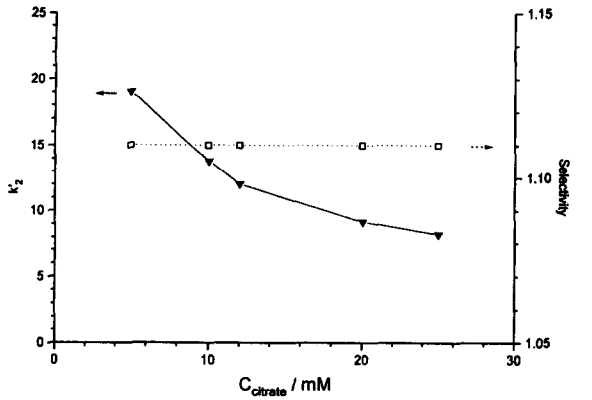

Fig. 4. The capacity factor of the more retained enantiomer of DDATHF (solid line plotted against the left axis) and the chiral selectivity factor for the separation of the enantiomers of DDATHF (dotted line plotted against the right axis) as a function of the analytical citrate concentration of the eluent [ACN, 20% (v/v), pH = 6.0]. Symbols: ▼ = k'_2, □ = selectivity. Column temperature, 30°C; eluent flow rate, 1 ml/min.

separation of DDATHF: it can be used to greatly vary the retention without compromising the chiral selectivity.

Triethylamine (TEA) is a widely used masking agent in β-cyclodextrin silica-based separations [6]. Therefore, the influence of the TEA concentration on the separation of the DDATHF enantiomers was studied as well. Increasing amounts of TEA were used to produce pH 6.0, 12 m*M* citrate, 20% (v/v) ACN eluents with 1, 5 and 10 m*M* TEA concentrations. The final pH of the eluents was adjusted by adding a few μl of a concentrated sodium hydroxide solution, as before. Even TEA concentrations as high as 10 m*M* resulted only in a minute decrease of the k'_2, but no change in the α values. However, the symmetry of the DDATHF peaks improved up to a TEA concentration of 10 m*M*. Therefore, 10 m*M* TEA was incorporated into each eluent in the subsequent experiments.

Finally, the effects of the eluent temperature on both solute retention and chiral selectivity were investigated. The log k' *vs.* $1/T$ and the log α *vs.* $1/T$ relationships were linear as usual [4]. The k'_2 and the chiral selectivity values increased from 12 to 27 and 1.08 to 1.14, respectively, as the column temperature was decreased from 30°C to 4°C.

In conclusion one can state that there are two types of eluent parameters which can be changed to maximize the separation selectivity for the DDATHF enantiomers on β-cyclodextrin silicas. The first type of parameter (the concentration of ACN and the temperature of the eluent) will change both the k'_2 and the α values; the second type of parameter (the pH and the buffer concentration of the eluent) will only change k', without compromising the value of α. Therefore, α is maximized first using the eluent parameters which belong to the first group, followed by the adjustment of k'_2 to the desired level using the parameters which belong to the second group.

Preparative chromatographic separation of the DDATHF enantiomers in the displacement mode and in the overloaded elution mode of operation

Using the results of the retention and the selectivity studies, an eluent containing 10 m*M* TEA, 25 m*M* citrate and 25% (v/v) acetonitrile in a pH 6.0 solution at 4°C was selected as a compromise between separation selectivity (α = 1.14) and the time required to complete the separation (k'_2 = 17.4). The displacement chromatographic separations were completed with this carrier solution, at a flow rate of 0.3 ml/min, using two 250 mm × 4.6 mm I.D. Cyclobond-I columns connected in series. Because it was known from previous studies [14] that cetyltrimethylammonium bromide (cetrimide) had favorable retention and Langmuirian adsorption characteristics on the Cyclobond I columns, it was selected as a possible displacer for the separation of the DDATHF enantiomers.

Since the objective of this work was to demonstrate the feasibility of a chiral displacement chromatographic separation for a complicated solute, no attempts were made to optimize the preparative scale separations in terms of production rates. Initially, several displacement chromatographic separations of a large sample (about 1 mg) were completed at increasing cetrimide concentrations in order to find the displacer concentration at which the moderately soluble, more retained enantiomer of DDATHF began to precipitate. Once this limiting value was determined, the cetrimide concentration in

the displacer solution was decreased by 10%, to 1.33 m*M*, and several separations were completed by doubling the DDATHF sample load from 50 μg/injection upwards, until the amount of the less retained DDATHF enantiomer produced (at 95% enantiomeric purity) began to decrease. 60-μl fractions were collected throughout the separations and analyzed off-line for enantiomeric purity using another Cyclobond-I column.

The reconstructed displacement chromatogram of the last sample where production still improved with the load (a 962 μg sample) is shown in Fig. 5. With the 1.33 m*M* cetrimide displacer, the bands of the two enantiomers reach the 0.22 m*M* (for the less retained DDATHF enantiomer) and the 0.27 m*M* (for the more retained DDATHF enantiomer) concentration levels. There is a slight, gradual increase in the concentration of the first enantiomer, from 0.18 m*M* at the beginning of the band to 0.22 m*M* at the end of the band, indicating that for the 962 μg load and the 1.33 m*M* displacer concentration the isotachic train was not completely formed.

The reconstructed chromatogram for the overloaded elution mode separation of a similar

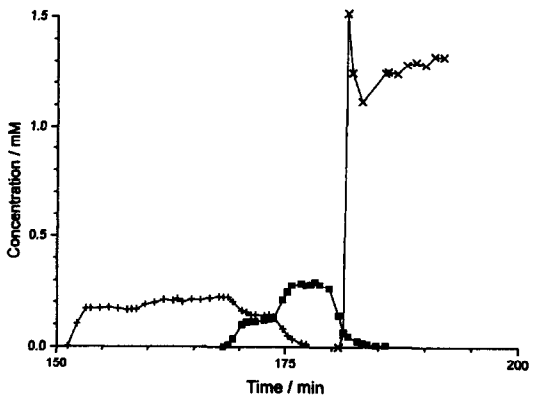

Fig. 5. The reconstructed displacement chromatogram of a 962 μg DDATHF sample using two Cyclobond I columns, connected in series. The displacer is a 1.33 m*M* solution of cetrimide, dissolved in the carrier solution [ACN, 25% (v/v); analytical citrate concentration, 25 m*M*, analytical TEA concentration, 10 m*M*, pH 6.0, temperature, 4°C], delivered at a flow rate of 0.3 ml/min. Symbols: + = less retained enantiomer of DDATHF, ■ = more retained enantiomer of DDATHF, × = cetrimide displacer.

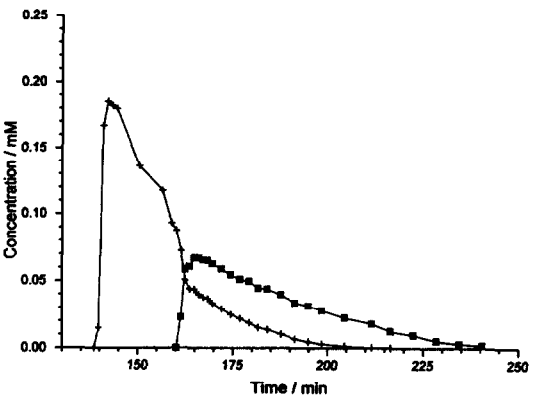

Fig. 6. The reconstructed overloaded elution mode chromatogram of a 807 μg DDATHF sample using two Cyclobond I columns connected in series. The eluent is the same as the carrier solution in the displacement separation in Fig. 5. Symbols: + = less retained enantiomer of DDATHF, ■ = more retained enantiomer of DDATHF.

sample, obtained under the same conditions as the displacement chromatogram, is shown in Fig. 6 (807 μg sample). The peak concentrations of the enantiomers are lower than in the displacement mode; they are somewhat lower for the less retained enantiomer (0.19 m*M*), much lower for the more retained enantiomer (0.07 m*M*). There is a strong decrease in the concentration of the less retained enantiomer as soon as the elution of the more retained enantiomer begins, in agreement with the predictions of the ideal model of overloaded elution mode chromatography [17].

The enantiomer productions (μg) and the % recoveries as a function of the % enantiomeric purity of the collected fractions were calculated from the reconstructed chromatograms. The productions are shown in Fig. 7 for the displacement mode separation: the solid line is for the less retained enantiomer, the dotted line for the more retained enantiomer. The % recoveries are shown in Fig. 8, again for the less retained enantiomer (solid line) and the more retained enantiomer (dotted line). Since the purpose of this separation is chiral resolution, the enantiomeric purities rather than the chemical purities are used in the figures. In this case, for the less retained enantiomer, the enantiomeric purity and the chemical purity are identical. For the more retained enantiomer, the chemical purity is

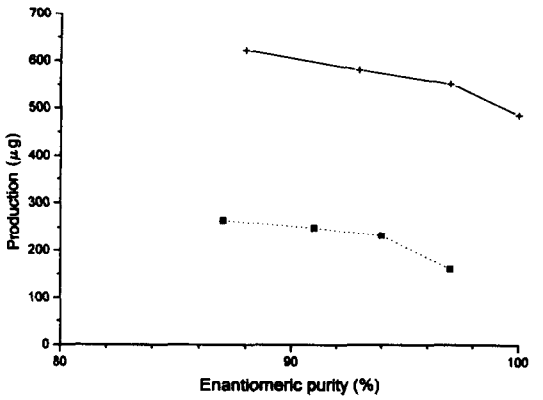

Fig. 7. Production (μg) of the DDATHF enantiomers as a function of the % enantiomeric purity of the pooled fractions obtained from the displacement chromatographic separation in Fig. 5. Symbols: + = less retained enantiomer of DDATHF, ■ = more retained enantiomer of DDATHF.

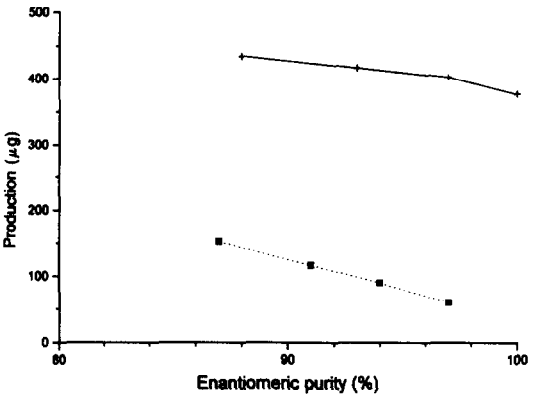

Fig. 9. Production (μg) of the DDATHF enantiomers as a function of the % enantiomeric purity of the pooled fractions obtained from the overloaded elution mode separation in Fig. 6. Symbols: + = less retained enantiomer of DDATHF, ■ = more retained enantiomer of DDATHF.

less than the enantiomeric purity, because the tail end of the band of the more retained enantiomer is contaminated by the displacer. However, because the cetrimide displacer is cationic, its traces can easily be removed at high pH from the anionic DDATHF by an additional ion exchange step.

For the corresponding overloaded elution mode separation the calculated enantiomer productions (μg) and % recoveries are shown in Figs. 9 and 10, respectively, for the less retained enantiomer (solid line) and the more retained enantiomer (dotted line).

It can be seen by comparing Figs. 7 and 9 that for the less retained enantiomer a little more material can be produced at the 95% enantiomeric purity level in the displacement mode (about 600 μg), than in the overloaded elution mode (about 400 μg). For the more retained enantiomer the difference is a little more pronounced (about 200 μg *vs.* about 100 μg). This means that under the given conditions both the

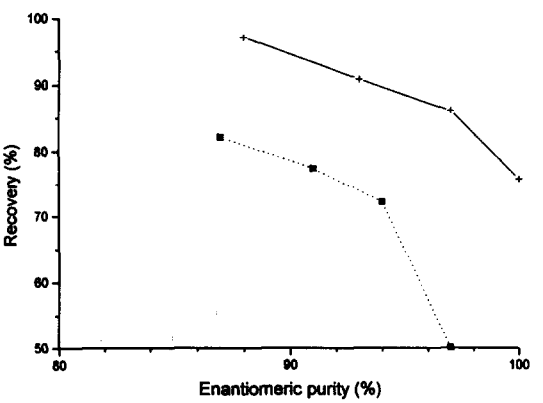

Fig. 8. Recovery (%) of the DDATHF enantiomers as a function of the % enantiomeric purity of the pooled fractions obtained from the displacement chromatographic separation in Fig. 5. Symbols: + = less retained enantiomer of DDATHF, ■ = more retained enantiomer of DDATHF.

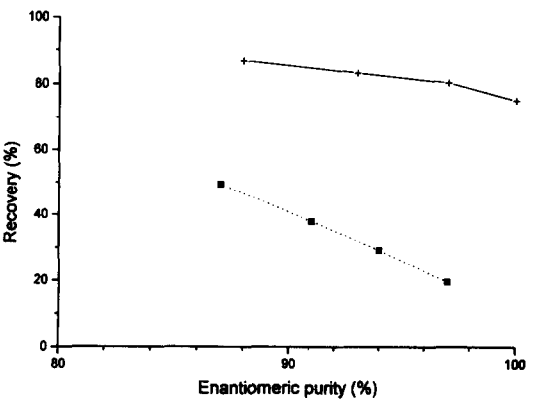

Fig. 10. Recovery (%) of the DDATHF enantiomers as a function of the % enantiomeric purity of the pooled fractions obtained from the overloaded elution mode separation in Fig. 6. Symbols: + = less retained enantiomer of DDATHF, ■ = more retained enantiomer of DDATHF.

displacement mode and the overloaded elution mode separations yield commensurable amounts of pure material for the less retained enantiomer, but the overloaded elution mode yields less of the second enantiomer than does the displacement mode. The same conclusions apply to the % recoveries (Figs. 8 and 10): at the 95% enantiomeric purity level less material is recovered in the elution mode than in the displacement mode, but the difference is much more pronounced for the more retained enantiomer than for the less retained enantiomer.

CONCLUSIONS

A displacement chromatographic method has been developed for the separation of the enantiomers of DDATHF on a β-cyclodextrin silica column. The k' *vs.* ACN concentration, the k' *vs.* pH, the k' *vs.* citrate concentration and the log k' *vs.* 1/T relationships have been determined, along with the α *vs.* ACN concentration, the α *vs.* pH, the α *vs.* citrate concentration and the log α *vs.* $1/T$ relationships in order to select the carrier solution composition which results in maximum chiral selectivity and sufficient retention for the less retained enantiomer ($1 < k'_1$), but not excessive retention for the more retained enantiomer ($k'_2 < 20$). Cetrimide, which is more retained than the more strongly adsorbed enantiomer of DDATHF, and has a suitable, Langmuirian adsorption isotherm [14], was used as displacer for the separation. A comparison of the production (μg) and % recovery values at different levels of % enantiomeric purity indicated that under the given conditions the displacement mode and the overloaded elution mode separations perform comparably for the less retained enantiomer, but the displacement mode performs better for the more retained enantiomer.

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