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High-performance chiral displacement chromatographic separations in the normal-phase mode

II. Separation of the enantiomers of 1,2-O-dihexadecyl-*rac*-glycerol-3-O-(3,5-dinitrophenyl)carbamate using the Pirkle-type naphthylalanine silica stationary phase

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ABSTRACT

A normal-phase displacement chromatographic method has been developed for the preparative-scale separation of the enantiomers of 1,2-O-dihexadecyl-*rac*-glycerol-3-O-(3,5-dinitrophenyl)carbamate using the Pirkle-type covalently bonded naphthylalanine silica chiral stationary phase. The separation selectivity of the system has been maximized by varying the tetrahydrofuran concentration and the temperature of the tetrahydrofuran–hexane eluent within the range constrained by the solubility of the sample and the displacer. The 3,5-dinitrobenzoyl ester of *n*-heptanol, which is more retained under the selected separation conditions than any of the enantiomers of the glycerol ether derivative, was selected and used as displacer in a preparative displacement chromatographic separation of a 20-mg sample of the glycerol ether derivative. For comparison purposes, an analogous overloaded elution mode separation was also completed under identical separation conditions. A large number of product fractions were collected during both the displacement chromatographic run and the corresponding overloaded elution mode run; these were analyzed for enantiomeric purity such that the percent recoveries and the production rates for both separation methods could be calculated.

INTRODUCTION

Earlier, we published an HPLC method [1] for the analytical-scale separation of the enantiomers of platelet-aggregating factor analogues and 1,2-O-dihexadecyl-*rac*-glycerol ether derivatives. N-(2)-naphthyl-alaninate silica, a π-electron donor chiral stationary phase developed by Pirkle *et al.* [3–6] for resolvation of the enantiomers of solutes which contain a π-acid functional group (most often a 3,5-dinitrobenzoyl or 3,5-dinitrophenylcarbamoyl group) and an H-donor or H-acceptor group, was used for the separation.

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Since pure, individual glycerol ether enantiomers are needed for the determination of their respective physico-chemical and pharmacological properties, an attempt was made to develop a preparative displacement chromatographic HPLC method for the production, in 50–500-mg quantities, of these individual enantiomers. Both the chiral displacement chromatographic separations and the displacers used for them have recently been described in detail [7–11]. This paper describes the use of the retention and chiral selectivity data pertaining to the glycerol ether derivatives [1], and 3,5-dinitrobenzoyl ester displacer adsorption data [7] to develop an overloaded elution mode and a displacement mode preparative chiral separation.

EXPERIMENTAL

Materials

Three 250 mm × 4.6 mm I.D. Rexchrom columns, packed with a 5-μm D-naphthylalanine silica (DNAS) were obtained from Regis (Morton Grove, IL, USA). One column was used for the selectivity optimization studies and analysis of the fractions collected during the preparative chromatographic separations. Two columns, connected in series, were used for the preparative displacement chromatographic separations and the overloaded elution mode separations. All columns were equipped with water jackets and thermostatted by a UF-3 type recirculating water bath (Science/Electronics, Dayton, OH, USA). The temperature was maintained at 5, 10, 15, 20, 30 and 35°C, respectively, as indicated in the legends. Fractions were collected with a Cygnet fraction collector (ISCO, Lincoln, NE, USA).

The eluents, carrier solutions and displacer solutions were prepared by using HPLC-grade *n*-hexane (Baxter, Muskegon, MI, USA) and tetrahydrofuran (THF) (Fisher Scientific, Fair Lawn, NJ, USA). The displacers were synthesized using reagent-grade 3,5-dinitrobenzoyl chloride (DNB) and *n*-pentanol and *n*-heptanol, respectively (Aldrich, Milwaukee, WI, USA). The other chemicals used were obtained from Aldrich, Fluka, Eastman-Kodak (Rochester, NY, USA), Fisher Scientific, or Wiley Organic (Cochocton, OH, USA), and used without further purification.

Synthetic procedures

The details of displacer synthesis are described elsewhere [7,11]. Briefly, ester-type displacers were synthesized according to the general Schotten–Baumann reaction scheme and purified by repeated crystallization. Some of the displacers designed for chiral separations in the normal-phase mode are now commercially available from ASTEC (Whippany, NJ, USA).

The 1,2-O-dihexadecyl glycerol ether-3-O-(3,5-dinitrophenyl)carbamate derivative (HGC) was synthesized as described in detail in refs. 1 and 12. Briefly, triflic acid-catalyzed opening of the epoxy ring of glycidol 3-nitrobenzenesulfonate ester in the presence of excess *n*-hexadecanol was followed by alkylation with excess *n*-hexadecyl triflate in the presence of equimolar 1,8-bis(dimethylamino)naphthalene, deprotection with tetrabutylammonium hydroxide, isolation of the diether derivative and finally, reaction of the remaining hydroxyl group with 3,5-dinitrophenylisocyanate, generated *in situ* by thermal decomposition of the corresponding azide [6].

Apparatus

A liquid chromatograph consisting of a Type 2020 pump, a Type 2050 variable wavelength UV detector (all from Varian, Walnut Creek, CA, USA), a pneumatically activated, computer-controlled Type 7125 injection valve (Rheodyne, Cotati, CA, USA) equipped with 10 and 50-μl sample loops, and a Maxima 820 Chromatographic Work Station (Millipore, Bedford, MA, USA) was used for the selectivity optimization and the fraction analysis work. All preparative separations were carried out with a custom-built displacement chromatograph consisting of two Type 2020 pumps, a Type 2050 variable-wavelength UV detector (set at 390 nm) and a Type RI-3 differential refractive index (RI) detector (all from Varian) [8]. The samples were injected by a pneumatically activated, computer-controlled Type 7125 injection valve (Rheodyne) equipped with 100-μl to 5-ml sample loops, and a Type 7010 switching valve (Rheodyne). The dead volume of each individual column was determined at the respective separation temperature by injecting an *n*-heptane:THF sample into the *n*-hexane:THF eluent and recording the signal of the differential refractive index detector. At 15°C, the dead volume of the preparative separation column was 5.91 ± 0.02 ml. A Type 4270 integrator (Varian), connected to a NEC Powermate I AT computer (Computer Access, College Station, TX, USA) and running the Chromplot-1 program developed in our laboratory [8], was used for system control, data collection and analysis.

Displacement chromatographic separations

The operational sequence described in ref. 8 was followed here. Briefly, at first, the columns were equilibrated with the carrier solvent, while the displacer solution was pumped into a dummy column to precompress it to the operating pressure. Next, the sample, dissolved in the carrier solvent and loaded into the sample loop, was injected by activating the Rheodyne 7125 valve. After an experimentally determined period of time, the Rheodyne 7125 injection valve was turned back to the load position, and the Rheodyne 7010 switching valve was activated to introduce the displacer solution into the analytical column and re-route the carrier solvent into the dummy column. This injection sequence prevented the displacer from passing through the loop behind the sample and losing some of the sharpness of its rear boundary. Both the UV detector signals and the RI detector signals were recorded simultaneously. As soon as the first enantiomer began to emerge from the column, 32.5-μl sample fractions were collected with the Cygnet fraction collector. The data collection and the fraction collection processes were terminated when the RI detector signal reached its plateau corresponding to the feed concentration of the displacer.

Column regeneration

Following the displacement chromatographic runs, the displacer was removed from the columns by pumping THF through them at flow-rates as high as permitted by the pressure limit of the system. After the absorbance of the effluent decreased back to that of the THF wash, the carrier solvent pump was turned on to re-equilibrate the column for the next run.

Overloaded elution mode separations

For a simple, direct comparison, overloaded elution mode separations were completed under the same (non-optimized) conditions as the displacement mode separations, except that no displacer was used and no column regeneration step was necessary.

Fraction analysis

Once the preparative separation was completed, the solvent was evaporated from each collected fraction. The residues were redissolved in identical volumes of the eluent that contained an achiral internal standard as well, and analyzed for chiral purity. The analysis conditions were optimized to achieve complete separation of both enantiomers and the displacer in the shortest period of time. Peak areas were converted to concentrations using individual calibration curves. The reconstructed displacement chromatograms and the reconstructed overloaded elution mode chromatograms were derived from these concentrations and from the fraction volume data.

RESULTS AND DISCUSSION

Retention studies of the HGC enantiomers

The retention behavior of the HGC enantiomers was studied at 35°C using various THF–*n*-hexane eluents. The capacity factor values, k', and the separation selectivity values, α, as a function of the % (v/v) THF concentration are shown in Figs. 1 and 2, respectively. Both the capacity factors and the separation selectivity increase monotonously with decreasing THF content in the eluent.

Because the column temperature effects both the retention of the solutes and the separation selectivity, the k'_2 and the α values of HGC were also determined at 5, 10, 15, 20 and 30°C using the THF–*n*-hexane (7.5:92.5, v/v) eluent. The results are shown in Figs. 3 and 4; the expected van 't Hoff-type behavior is observed. Though the chiral selectivity is the highest at

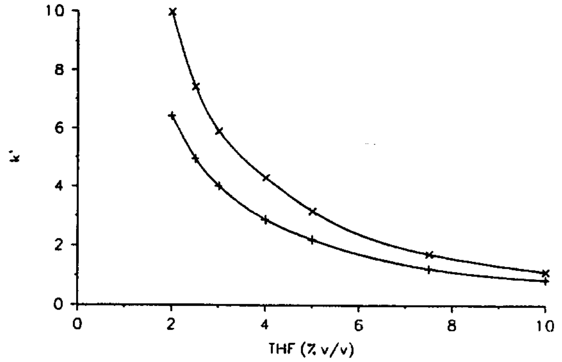

Fig. 1. k' values of the R- and the S-enantiomers of HGC as a function of the % (v/v) THF concentration in the THF–n-hexane eluent at 35°C. Symbols: + = S-HGC; × = R-HGC.

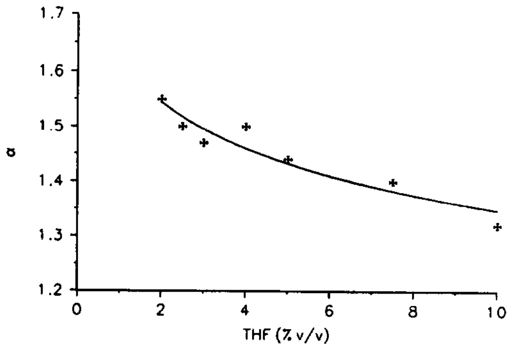

Fig. 2. α values for the separation of the R- and the S-enantiomers of HGC as a function of the % (v/v) THF concentration in the THF–n-hexane eluent at 35°C.

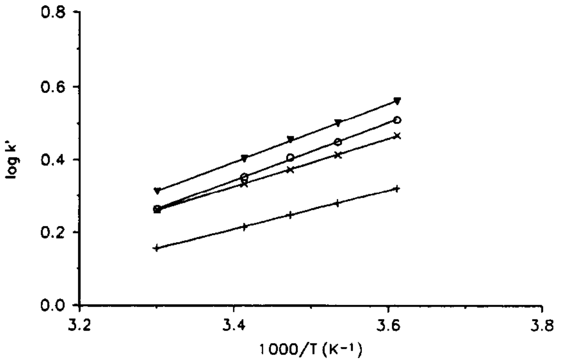

Fig. 3. Log k' of the R- and S-enantiomers of HGC, and the DNB esters of n-pentanol and n-heptanol as a function of the inverse absolute column temperature. Eluent: 7.5% (v/v) THF in n-hexane. Symbols: + = S-HGC; × = R-HGC; ○ = C_7DNB; ▼ = C_5DNB.

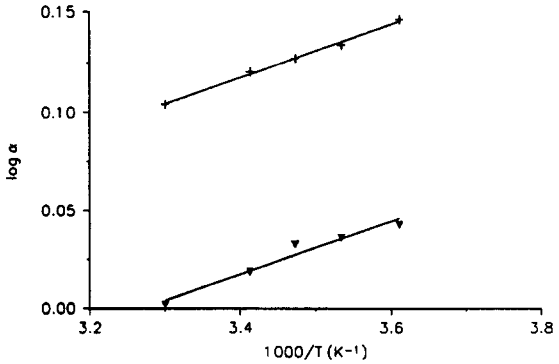

Fig. 4. α values for the separation of the enantiomers of HGC, and the R-enantiomer of HGC and the DNB ester of n-heptanol as a function of the inverse absolute column temperature. Eluent: 7.5% (v/v) THF in hexane. Symbols: + = R-HGC/S-HGC; ▼ = C_7DNB/R-HGC.

5°C, a highly desirable feature for preparative work, the solubility of the HGC enantiomers is much lower there than at 15°C. Therefore, as a practical compromise, all preparative separations were completed at 15°C.

The chromatogram of a very dilute sample of the racemic mixture of HGC, obtained using THF–n-hexane (7.5:92.5, v/v) eluent at a 15°C and a 0.5 ml/min flow-rate, is shown in Fig. 5. This eluent represents a promising carrier solution composition for preparative displacement chromatography given the resolution characteristics observed.

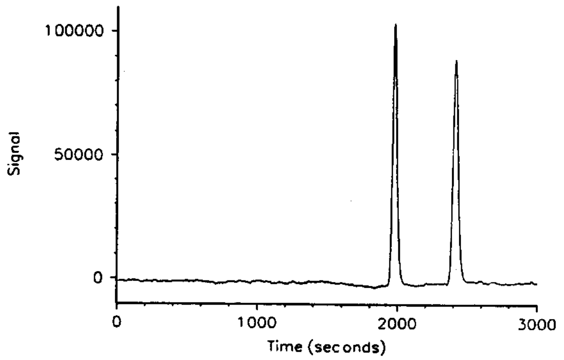

Fig. 5. Chromatogram of a very dilute sample of the racemic mixture of HGC. Eluent: 7.5% (v/v) THF in n-hexane at 15°C and 0.5 ml/min flow-rate. First peak: S-HGC, second peak: R-HGC.

Selection of the displacer

The success of a displacement chromatographic separation critically depends not only on the value of the selectivity factor, but also, on the availability of a suitable displacer. The displacer must meet contradictory criteria: its chromatographic characteristics should, preferably, be similar to those of the solute, have a convex adsorption isotherm, be adsorbed more strongly on the stationary phase than the more retained of the enantiomers, and be highly soluble in the carrier solvent to allow the preparation of concentrated displacer solutions. For Pirkle-type stationary phases, a successful generic displacer structure has been determined: the displacer should contain a π-acid (*e.g.* a 3,5-dinitrophenyl) or π-base (*e.g.* a naphthylamine–) anchor group, a hydrogen-donor/acceptor group (*e.g.* an amide group), and a hydrophobic, solubility adjusting group (*e.g.* a long-chain alkyl group) [7]. Preferably, these functional groups should be attachable to a common core structure using inexpensive reagents and simple preparative methods. Recently, we synthesized a homologous series of 3,5-dinitrobenzoyl esters of alcohols (DNB esters) and determined their retention and adsorption characteristics on Pirkle-type stationary phases [7]. These data indicate that at 35°C, the 3,5-dinitrobenzoyl esters of *n*-heptanol (C_7DNB ester) and *n*-pentanol (C_5DNB ester) are more retained than the *R*-enantiomer of HGC, and could be considered as potential displacers. k' values of the C_5DNB esters and C_7DNB esters, determined as a function of temperature, are shown in Fig. 3. The selectivity factor calculated for the separation of the *R*-enantiomer of HGC and the less retained displacer, the C_7DNB ester, is shown in Fig. 4. The *n*-heptyl derivative was selected as displacer, since both the separation selectivity and the solubility of the C_7DNB ester are sufficiently high in the carrier solution at 15°C.

The displacement chromatographic separation

The displacement chromatogram of a 20.1 mg sample of crude HGC racemate using two 250 mm × 4.6 mm I.D. Rexchrom DNAS columns at 15°C, 7.5% (v/v) THF in *n*-hexane carrier, 150 m*M* *n*-heptyl DNB displacer, and a flow-rate of 0.5 ml/min is shown in Fig. 6. The reconstructed chromatogram (determined from the analysis of the collected fractions), is shown in Fig. 7. (An impurity, the DNB carbamate of *n*-hexadecanol, a by-product in the synthesis of the HGC sample, is more retained than the *R*-enantiomer of HGC, but less than the C_7DNB ester displacer.) The bands of the enantiomers are clearly separated and sharp indicating that the 20 mg sample does not exhaust the loading capacity of the column.

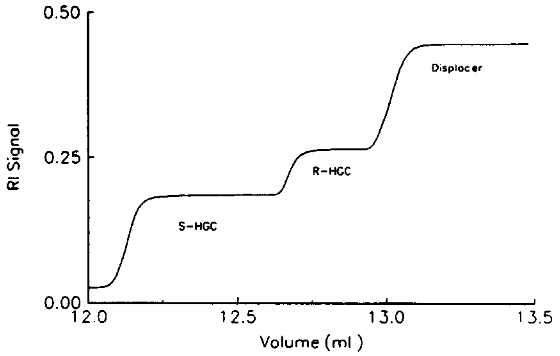

Fig. 6. Displacement chromatogram of a 20.1-mg sample of crude racemic HGC. For conditions see text.

For the sake of comparison, an overloaded elution mode separation of a 24-mg aliquot of the HGC sample was also completed using the same experimental conditions as in the displacement mode. The recorded and reconstructed chromatograms are shown in Figs. 8 and 9,

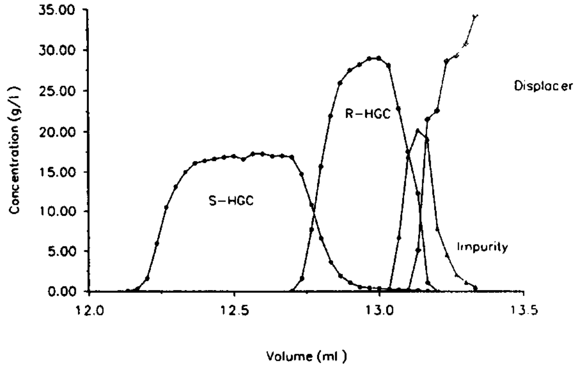

Fig. 7. Reconstructed displacement chromatogram of a 20.1-mg sample of crude racemic HGC. For conditions see text.

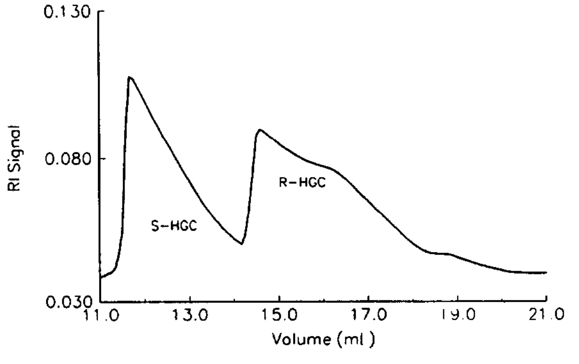

Fig. 8. Overloaded elution mode chromatogram of a 24-mg sample of crude racemic HGC. For conditions see text.

respectively. These chromatograms display a prominent tag-along effect, first described by Golshan-Shirazi and Guiochon [13], and the splitting of the *R*-HGC band into two parts by the impurity. The concentration maxima are about three-to-four times lower than in the displacement mode.

Purity calculations

Enantiomeric purity calculations. The reconstructed chromatograms can be unambiguously charted from the well defined purities of the individually collected fractions. However, the collected fractions can be pooled in a number of ways resulting in products of different enantiomeric purity. For example, in forward pooling, the pooling for the less retained enantiomer starts with the first fraction and proceeds toward increasing fraction numbers; the enantiomeric purity remains high, up to the point where the more retained enantiomer first appears in the individual fractions. At this point, the enantiomeric purity of the pooled material begins to decrease rapidly. Therefore, a certain target level of purity for the pooled product can be specified and the pooling process can be stopped there. To forward pool the more retained enantiomer, one can start where pooling of the less retained enantiomer was stopped and proceed towards increasing fraction numbers. In this case, the enantiomeric purity for the more retained enantiomer increases from an initial low value towards a high, but certainly less than 100%, value. Naturally, the purity level for the pooled, more retained enantiomer will be constrained by the amount of the less retained enantiomer contamination introduced in the first few fractions. Alternatively, backward pooling can be used for the more retained enantiomer; here pooling starts with the last collected fraction and proceeds towards decreasing fraction numbers. The enantiomeric purity of the pooled material then decreases from 100% (*i.e.*, no less retained enantiomer tailing into the last fractions of the more retained enantiomer), or from a high, but less than 100% value, towards a prespecified enantiomeric purity level for the pooled (more retained) product. Pooling can be stopped once this target purity level is reached. A number of other pooling schemes are also possible. Pooling then becomes an involved optimization problem, but once optimized, it will yield the highest amount of material at a specified level of enantiomeric purity. Often, though, the latter schemes are not practical due to the amount of labor involved. Therefore, only the simple schemes were used in the subsequent calculations: forward pooling for the less retained enantiomer and backward pooling for the more retained enantiomer.

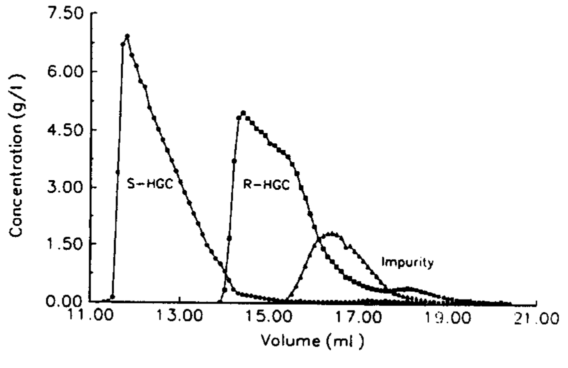

Fig. 9. Reconstructed overloaded elution mode chromatogram of a 24-mg sample of crude racemic HGC. For conditions see text.

The calculated enantiomeric purity of the pooled fractions as a function of the amount of the pure enantiomer recovered is shown in Fig. 10 for the displacement mode separation, and in Fig. 11, for the overloaded elution mode separation. In the displacement mode, if one wants to recover 7.5 mg of each enantiomer, it can be

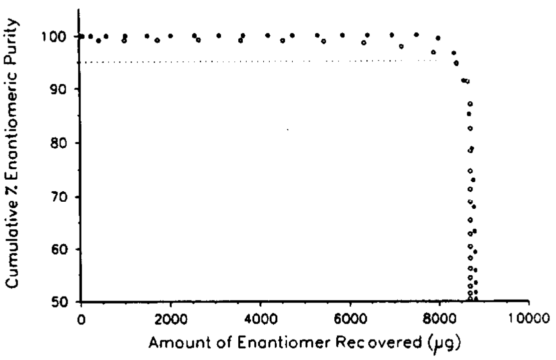

Fig. 10. Cumulative % enantiomeric purity as a function of the amount of HGC enantiomer recovered in the displacement mode separation. Symbols as in Fig. 7.

done at 100% enantiomeric purity for the less retained enantiomer (*S*-HGC) and at 97.5% enantiomeric purity for the more retained enantiomer (*R*-HGC). However, if one wants recover more of the enantiomers, the optical purity of the pooled products decreases very rapidly. In the overloaded elution mode, 10 mg of the *S*-enantiomer of HGC can be recovered at a 100% enantiomeric purity, however, due to the steady presence of the less retained enantiomer in the band of the more retained one (tag-along effect), enantiomeric purity of the *R*-enantiomer of HGC is never higher than 95%, regardless of the quantity recovered.

Component purities. In addition to the enantiomeric purities, the component purities of the pooled fractions were also calculated, because a contaminant of different chemical composition was also present in the sample. In displacement mode, the percent component purities for *S*-HGC (forward pooling) are the same as the enantiomeric purities, because the additional contaminant does not interfere with the band of *S*-HGC. However, because *R*-HGC is flanked on both sides by an interfering component (*S*-HGC at the front, and the contaminant at the rear), the amount of material that can be recovered at the 97.5% component purity level is only 5.4 mg. If the purity requirement for the pooled material is relaxed to 95%, 6.3 mg of *R*-HGC (63% of the loaded amount) can be recovered.

In overloaded elution mode for *S*-HGC, the % component purities are the same as the enantiomeric purities, because the contaminant does not interfere with the *S*-HGC band. For the more retained enantiomer the component purities are lower due to the presence of the reaction by-product; 2.8 mg of *R*-HGC can be recovered at the 97.5% component purity level (28% of the loaded material), and 5.6 mg at the 95% purity level (56% of the loaded material).

% Recoveries. When the pooled amounts of each enantiomer are divided by the total amount of the respective enantiomer loaded and plotted against the enantiomeric purity of the pooled fraction, the cumulative recovery curves for the enantiomers are obtained for the displacement (Fig. 12) and overloaded elution modes (Fig. 13). For *S*-HGC, the % recoveries are similar in

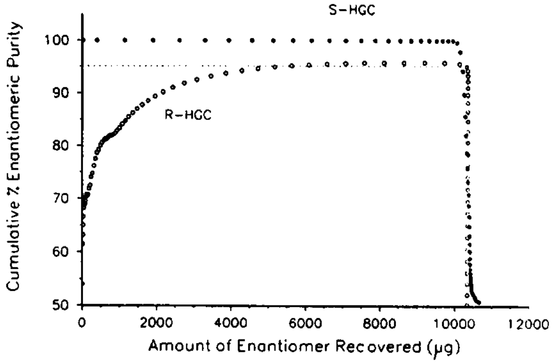

Fig. 11. Cumulative % enantiomeric purity as a function of the amount of HGC enantiomer recovered in the overloaded elution mode separation. Symbols as in Fig. 7.

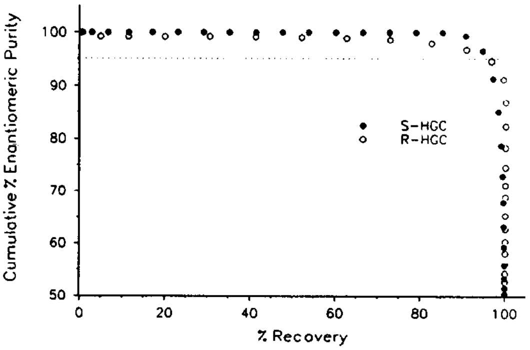

Fig. 12. Cumulative % enantiomeric purity as a function of the % recovery rate of the HGC enantiomers in the displacement mode separation. Symbols as in Fig. 7.

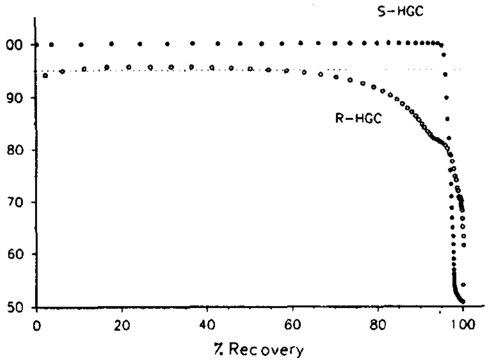

Fig. 13. Cumulative % enantiomeric purity as a function of the % recovery rate of the HGC enantiomers in the overloaded elution mode separation. Symbols as in Fig. 7.

both the displacement and the overloaded elution modes. For *R*-HGC, however, the % recoveries are slightly better in the displacement mode.

CONCLUSIONS

It has been found that the selectivity factor could be maximized by varying the THF concentration and eluent temperature over the range constrained by the solubility limits of the sample; capacity factors of the more retained glycerol ether enantiomer could be adjusted to the $2 < k' < 10$ range, as desired for the displacement chromatographic separation. Using *n*-heptyl DNB ester as displacer, 20 mg of a crude racemic HGC sample could be separated on 4.6 mm I.D. analytical columns at good enantiomeric purity and yield. By injecting 24 mg of the same crude racemic HGC sample, broadly comparable purity and yield values were observed in the complementary overloaded elution mode separation.

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REFERENCES

1 P.L. Camacho, E. Geiger, Gy. Vigh, R. Webster and D.H. Thompson, *J. Chromatogr.*, 506 (1990) 611.
2 W.H. Pirkle, J.M. Finn, J. L Schreiner and B.C. Hamper, *J. Am. Chem. Soc.*, 103 (1981) 3964.
3 W.H. Pirkle and T.C. Pochapsky, *J. Org. Chem.*, 51 (1986) 102.
4 W.H. Pirkle and T.C. Pochapsky, *J. Am. Chem. Soc.*, 108 (1986) 352.
5 W.H. Pirkle and T.C. Pochapsky, *J. Am. Chem. Soc.*, 108 (1986) 5267.
6 W.H. Pirkle, T.C. Pochapsky, G.S. Mahler, D.E. Corey, D.S. Reno and D.M. Alessi, *J. Org. Chem.*, 51 (1986) 102.
7 P.L. Camacho, Gy. Vigh, and D.H. Thompson, *J. Chromatogr.*, 641 (1993) 31.
8 Gy. Vigh, G. Quintero and Gy. Farkas, *J. Chromatogr.*, 484 (1989) 251.
9 Gy. Vigh, G. Quintero and Gy. Farkas, *J. Chromatogr.*, 484 (1989) 256.
10 Gy. Vigh, L.H. Irgens and Gy. Farkas, *J. Chromatogr.*, 502 (1990) 11.
11 P.L. Camacho, *Dissertation*, Texas A&M University, College Station, TX, 1991.
12 D.H. Thompson, C.B. Svendsen, C. Di Meglio, V.C. Anderson, *J. Org. Chem.*, submitted for publication.
13 S. Golshan-Shirazi and G. Guiochon, *Anal. Chem.*, 62 (1990) 220.