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Evaluation of a chiral stationary phase based on mixed immobilized proteins

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Abstract

The preparation of a new chiral stationary phase (CSP) based on mixed immobilized human serum albumin (HSA) and α_1-acid glycoprotein (AGP) is reported. Enantiomeric separations of basic, acidic and neutral compounds on this AGP–HSA CSP were compared to those obtained on a HSA CSP and on a AGP CSP. The results show that the new CSP has a wider range of applications than the initial CSPs. For two compounds, the enantioselectivity on the mixed CSP was higher than on the HSA CSP.

1. Introduction

In the past few years there has been a rapid development of high-performance liquid chromatographic (HPLC) chiral stationary phases (CSPs) for the analytical and preparative separation of enantiomeric compounds [1]. At present there are over a hundred commercially available CSPs based on a variety of mechanistic approaches. One of the most successful approaches has employed protein stationary phases (PSPs) created by the immobilization of various proteins on silica supports. PSPs based upon α_1-acid glycoprotein (AGP) [2,3], ovomucoid [4], cellulase [5], bovine serum albumin [2] and human serum albumin (HSA) [6–9] have been reported. Proteins can interact with small molecules in a variety of ways. These multiple recognition mechanisms allow protein phases to separate a large number of solutes with very different structural characteristics [1,9].

Here, we report the preparation of a mixed CSP based on immobilized proteins. The term "mixed" stationary phase refers to phases prepared by a combination of two existing phases and which retain characteristics of both phases. The most notable example in chiral HPLC is the production of π-donor/π-acceptor Pirkle-type CSPs. Several of these mixed phases were synthesized by grafting a π-donor and a π-acceptor chiral moiety on the same silica [10], by mixing a π-donor and a π-acceptor bonded silica [10] or by bonding to the silica one moiety which contains both a π-donor and a π-acceptor group [11–13]. The objective was to prepare a new CSP with an extended range of application in

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comparison with CSPs containing only one group [10–13].

Considering the already wide scope of protein based CSPs, a mixed protein CSP is expected to have an extremely broad applicability. The choice of HSA and AGP as the immobilized proteins was justified by the fact that their applications were quite complementary: HSA is most useful for acidic compounds while AGP is more favourable for cationic compounds [2]. Both are plasmatic proteins and the major carrier proteins for drug compounds.

A column was packed with a 50:50 mixture of HSA CSP and AGP CSP. We report the preliminary chromatographic results on this column and compare them to those obtained on columns packed with pure HSA CSP or pure AGP CSP.

2. Experimental

2.1. Chemicals

The compounds and proteins used in this study were obtained from the following suppliers: HSA (fatty acid free fraction V) and human AGP, (*R*,*S*)-temazepam, (*R*,*S*)-oxazepam, (*R*,*S*)-oxprenolol, (*R*,*S*)-propranolol, (*R*,*S*)-suprofen, (*R*,*S*)-ketoprofen, (*R*,*S*)-disopyramide, were from Sigma (St. Louis, MO, USA); (*R*,*S*)-promethazine, (*R*,*S*)-hexobarbital, (*R*,*S*)-pentobarbital, (*R*,*S*)-benzoin, *S*-benzoin and octanoic acid were obtained from Aldrich (Milwaukee, WI, USA); (*R*,*S*)-warfarin, *R*-warfarin and *S*-warfarin were gifts of DuPont–Merck (Wilmington, DE, USA); (*R*,*S*)-ketorolac, *R*-ketorolac and *S*-ketorolac were gifts from Syntex (Palo Alto, CA, USA); (*R*,*S*)-verapamil was a gift from GD Searle (Skokie, IL, USA). (Rac)-Mefloquine was a gift from Hoffman–La Roche (Basel, Switzerland); (Rac)-enproline was a gift from the Walter Reed Army Institute of Research (Washington, DC, USA).

The silica Kromasil 200 Å, 10 μm was a gift from Eka-Nobel (Sweden). (3-glycidoxypropyl)trimethyloxysilane was obtained from Aldrich. Other chemicals were of analytical grade and were obtained from local suppliers.

2.2. Synthesis of the stationary phases

The silica gel was heated for 15 h at 180°C and 1 mmHg (1 mmHg = 133.322 Pa). To 5.17 g of silica in 100 ml of dry xylene 2 g of distilled (3-glycidoxypropyl)trimethoxysilane in 5 ml of dry xylene were added dropwise and the mixture was refluxed for 36 h. After filtration on a fritted disc of porosity 4, the product was washed twice with dry xylene and dry acetone. The bonded silica was dried for 18 h at 40°C under vacuum. The activated silica was then packed into 150 × 4.6 mm I.D. HPLC columns. The column was washed with 100 ml of acetone and dried at 70°C under helium. A solution of protein (10 mg/ml) in potassium phosphate buffer (0.05 *M*, pH 7.5) containing 2 *M* of ammonium sulphate was circulated through the column, in closed circuit for 24 h. The column was washed with 100 ml of potassium phosphate buffer (0.05 *M*, pH 7.5). The amount of protein immobilized was determined by UV absorbance at 280 nm of the protein solution before and after the immobilization procedure. Two HSA and two AGP CSP columns were prepared. One of each was used directly, the other was emptied. The HSA and AGP CSPs were mixed in equal proportion and repacked in an analytical column to produce the AGP–HSA CSP.

The amount of protein immobilized on each of the analytical columns was as follow: HSA CSP: 85 mg HSA per g silica; AGP CSP: 105 mg AGP per g of silica; AGP–HSA column: HSA CSP 80 mg HSA per g of silica and AGP CSP 100 mg per g of silica.

2.3. Chromatography

Three chromatographic systems were used in this study. They consisted of a SpectraSystem P1000 isocratic pump, a Spectra 100 variable-wavelength UV detector and a Data Jet integrator, all from Thermo Separation Products (San Jose, CA, USA). All columns were 150 × 4.6 mm I.D. The injection volume was 20 μl and the flow-rate was maintained at 0.8 ml/min. The separations were carried out at room temperature. Mobile phases composition: (1) phosphate buffer (pH 7, 0.02 *M*); (2) phosphate buffer (pH

7, 0.02 *M*)–acetonitrile (90:10, v/v); (3) phosphate buffer (pH 5, 0.02 *M*)–acetonitrile (85:15, v/v); (4) phosphate buffer (pH 7, 0.02 *M*)–*n*-propanol (94:6, v/v); (5) phosphate buffer (pH 5.5, 0.02 *M*)–*n*-propanol (94:6, v/v) containing 2 m*M* octanoic acid.

3. Results and discussion

The immobilization procedure was different to those used to prepare commercial HSA- or AGP-based stationary phases. In particular, the proteins did not undergo any further modifications after immobilization and it is expected that their binding characteristics should be closer to the native proteins.

In theory, if there is no special effect due to the proximity of the two proteins, the capacity factor on the AGP–HSA CSP can be expressed by:

$$k'_{\text{AGP-HSA}} = (0.95k'_{\text{AGP}} + 0.94k'_{\text{HSA}})/2 \qquad (1)$$

The factors 0.95 and 0.94 were introduced in this expression to take into account the lower amount of protein immobilized on the AGP–HSA column (80 mg/g silica vs. 85 for HSA and 100 mg/g vs. 105 for AGP). The same factors should be used in the calculation of the enantioselectivity but in this case their effect is negligible. Therefore, the enantioselectivity can be expressed by:

$$\alpha = \frac{k'_{\text{B,AGP}} + k'_{\text{B,HSA}}}{k'_{\text{A,AGP}} + k'_{\text{A,HSA}}}$$

where A and B are the first and the second eluted enantiomers on the AGP–HSA CSP, respectively. These expressions indicate that α and k' on the mixed phase are expected to take intermediate values between those obtained on the HSA and AGP CSPs. The maximum α is obtained when a compound is only retained on one of the initial CSPs. In that case α is equal to the α obtained on the CSP on which the compound separated.

3.1. Chromatographic results

The separation of a series of chiral compounds including anionic, cationic and neutral compounds was studied on the HSA, the AGP and the AGP–HSA CSPs under various conditions. The separations were not systematically optimized but the retention and enantioselectivity of test compounds were compared on the three columns under the same conditions. The three columns were used on three separate chromatographic systems so that all the results were collected simultaneously. The mobile phases were used in the same order on each system. The capacity factor of the first eluted enantiomer and the enantioselectivity on all three CSPs for 16 compounds are summarized in Table 1. This table also includes the values calculated from the expressions above for $k'_{\text{AGP-HSA}}$ and $\alpha_{\text{AGP-HSA}}$.

As the eluting conditions on the AGP and HSA CSPs for a given compound can be quite different, a large number of solutes tested could not be separated on both AGP and HSA in the same conditions even though the enantiomeric separation was possible on both columns. The separations reported here were carried out in conditions where the solute was eluted from all three columns.

Compounds which could not be separated on the HSA CSP in the conditions necessary for their elution from the AGP CSP, included verapamil, promethazine, propranolol, oxprenolol, disopyramide, enpiroline and mefloquine. For all of them, the observed k' and α where within 20% of the calculated values which suggest that the AGP–HSA CSP is performing as the sum of the two initial phases. As expected, oxprenolol which had a very low retention on HSA ($k' = 1.6$), was separated on the AGP–HSA CSP with an α value of 1,17, equal to the α observed on the AGP CSP. Examples of chromatograms are given on Fig. 1 for disopyramide.

Acidic compounds such as non steroidal anti-inflammatory drugs and warfarin had extremely low retentions on the AGP CSP in the conditions used to elute them on the HSA CSP. The chromatographic parameters obtained for warfarin were in relatively good accordance with calculated values. However, for ketoprofen, sup-

Table 1
Capacity factor of the first eluted enantiomer (k'_1) and enantioselectivity (α) on the AGP, HSA and mixed AGP–HSA CSPs and calculated k'_1 and α for the mixed stationary phase

Compound	MP	AGP		HSA		AGP–HSA observed		AGP–HSA calculated	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
Compounds separated on AGP–HSA CSP									
Hexobarbital	1	5.5	1.44	3.8	1.34	2.8	1.00	4.4	1.10
Pentobarbital	1	7.7	1.33	5.2	1.08	3.8	1.1	6.1	1.16
Benzoin	1	14.8	1.06	13.9	1.21	12.5	1.12	13.5	1.13
Temazepam	2	6.7	1.77	2.8	6.05	5.6	1.23	6.9	1.62
Oxazepam	2	5.5	2.15	3.5	3.35	3.6	2.40	4.2	2.60
Compounds separated on AGP CSP									
Verapamil	2	12.8	1.33	6.04	1.00	7.3	1.23	8.9	1.22
Promethazine	2	6.8	1.35	5.33	1.00	5.8	1.14	5.7	1.20
Propranolol	2	40.3	1.30	4.9	1.00	19.8	1.2	21.4	1.26
Oxprenolol	2	24.3	1.17	1.6	1.00	11.1	1.17	12.3	1.16
Disopyramide	2	10.0	1.23	1.81	1.00	4.63	1.10	5.6	1.19
Enpiroline	3	15.6	2.09	2.5	1.00	12.2	1.65	9.1	1.94
Mefloquine	3	6.0	1.30	3.1	1.00	4.0	1.20	4.3	1.20
Compounds separated on HSA CSP									
Ketorolac	2	0.15	1.00	24.8	2.65	6.5	4.4	12.5	2.64
Warfarin	2	2.5	1.00	28.0	1.64	12.2	1.45	15.2	1.59
Suprofen	5	3.3	1.00	9.1	1.19	6.2	1.33	10.5	1.16
Ketoprofen	4	3.6	1.00	18.1	1.64	6.7	1.10	10.2	1.11

Italics indicate solutes which are retained by one CSP only (k' on the other CSP is less than 2). Mobile phase (MP) numbers are given in the Experimental section.

rofen and ketorolac, the observed capacity factors were 40% lower on average than their expected value. Moreover, for ketorolac and suprofen, α values greater than the expected values were obtained on the mixed stationary phase.

Some test solutes could be separated on both the AGP and the HSA CSPs under the same experimental conditions. They included hexobarbital, pentobarbital, benzoin, temazepam and oxazepam. For all these compounds, the final separation factor on the mixed phase depended on the elution order on the initial phases. For oxazepam, the elution order was the same on AGP and HSA CSPs and the α value was 2.40, intermediate between the α values on both initial phases. For all the four other compounds, the elution order was different on AGP and HSA and the resulting α was lower on the mixed phase than on either original phases. As shown in Fig. 2, hexobarbital separated on both the AGP and the HSA CSPs with α values of 1.44 and 1.34, respectively. When chromatographed on the mixed phase there was a complete loss of enantioselectivity. The separations induced by both CSP compensate each other so that the enantioselectivity is lost. Among the five solutes in this category, two (hexobarbital and pentobarbital) had significantly lower retention on the mixed CSP than expected. These two compounds were acidic compounds but as opposed to ketoprofen, suprofen and ketorolac were not ionized in the conditions of the assay.

The reason for this unexpected chromatographic behavior of acidic compounds is not clear. It does not seem to be due to a partial loss or inactivation of the HSA since the few cationic compounds which had a quite high retention on

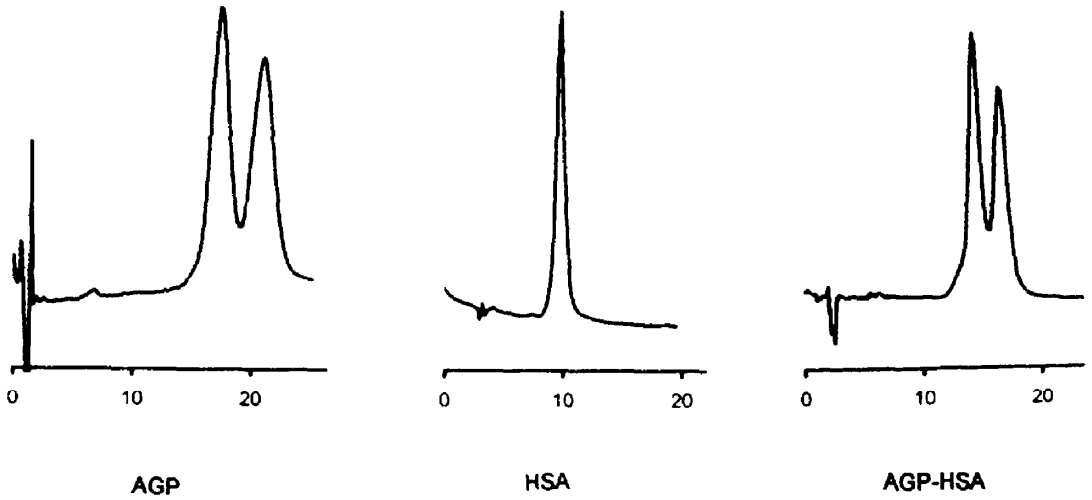

Fig. 1. Enantiomeric separation of disopyramide on (A) AGP, (B) HSA and (C) AGP–HSA CSPs. Column dimensions: 150 × 4.6 mm I.D., mobile phase: phosphate buffer (pH 7, 0.02 *M*)–acetonitrile (90:10, v/v), flow-rate: 0.8 ml/min, detection UV absorbance at 254 nm, injection volume: 20 μl.

the HSA CSP were not affected to the same extent. Column-to-column variability is possible but would certainly affect all the compounds, not just the acidic solutes. Another possibility is that the presence of the AGP creates an obstacle to the interaction of acidic drugs with the HSA. It is unlikely that the proteins could physically interact with each other since they were immobilized on different particles of silica. When the AGP and the HSA columns were coupled on-line with no possible interaction between the proteins, the results for ketorolac were within 2% of the expected values of k' and α.

3.2. Stability of the stationary phases

Pentobarbital and benzoin were chromatographed with mobile phase 1 at the beginning of

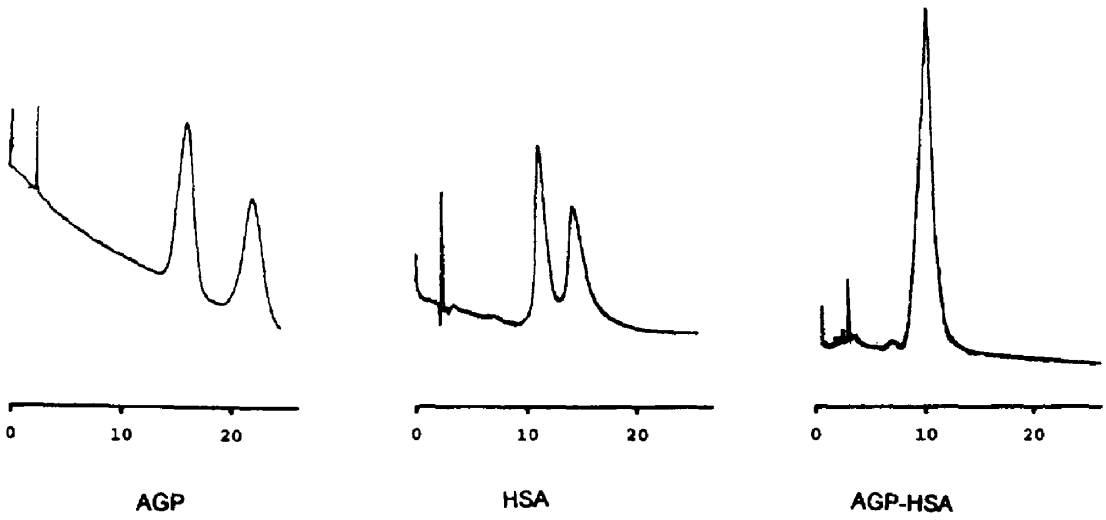

Fig. 2. Enantiomeric separation of hexobarbital on (A) AGP, (B) HSA and (C) AGP–HSA CSPs. Mobile phase: phosphate buffer (pH 7, 0.02 *M*), other conditions as in Fig. 1.

the column life and after seven different mobile phases were passed through the column including solutions containing ionized modifiers such as octanoic acid and dimethyloctylamine and about 200 injections were made. No apparent column degradation (peak broadening or peak splitting) was observed. A decrease in enantioselectivity was observed for benzoin on the HSA CSP (α decreased from 1.12 to 1.07) and on the AGP–HSA CSP (1.21 to 1.16) but an increase in α from 1.06 to 1.18 was observed on the AGP CSP. Capacity factors where almost unchanged. For pentobarbital, very little change in k' and α was observed on the AGP–HSA and HSA CSPs but a large decrease in k' from 7.7 to 3.9 for the first eluted enantiomer and from 10.3 to 5.5 for the second eluted one on the AGP CSP. The observed changes in retention and enantioselectivity could be due to insufficient conditioning of the stationary phases prior to the first use. Another explanation can be a modification of the protein structure due to insufficient washing of the mobile phase modifiers.

4. Conclusions

The new AGP–HSA CSP has an even wider range of applications than either initial CSP; only one out of 16 compounds tested could not be separated on the mixed protein column. The mixed protein phase combines the useful enantioselectivity of both individual proteins except for the small number of samples for which both proteins show enantioselectivity but with enantioselectivity in opposing senses. When both proteins were involved in the retention, not only chiral but also non-chiral interactions were increased; the resulting enantioselectivity was generally intermediate between the values obtained on the two initial CSPs. The best α values were obtained in conditions where only one of the original CSPs participated in the retention and separation.

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