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LIQUID CHROMATOGRAPHIC RESOLUTION OF RACEMIC DRUGS USING A CHIRAL α_1-ACID GLYCOPROTEIN COLUMN

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SUMMARY

The plasma protein α_1-acid glycoprotein (α_1-AGP) was used as the chiral stationary phase for column liquid chromatographic resolution of racemic drugs. The influence of the solid phase protein loading on the retention, peak symmetry, enantioselectivity and the resolution are reported. The solutes were retained by a specific interaction with the protein and no indications were found for interactions between the solutes and the solid phase. Capacity factors could be regulated by the protein content of the solid phase, the concentration of 2-propanol or by adding N,N-dimethyloctylamine to the mobile phase. Twelve racemic drug substances from different pharmacological groups were resolved. The stability of the α_1-AGP column was also investigated. There was no tendency for degradation of the solid phase or denaturation of the protein, even after daily use of the column for more than 3.5 months.

INTRODUCTION

Many of the drug substances used today contain chiral carbon atoms or other chiral atoms such as phosphorus and sulphur. Most of these drugs are used as racemates. It is well known that enantiomers of a pharmacologically active compound often have different types of effects, and even if they have the same type of effect they show considerable differences in activities. The difficulty and cost of resolving racemates are commonly used arguments in support of their continued use. However, the use of racemates can be accepted only when it has been proved that the "inactive" isomer is harmless in all respects. A striking example of the drawback of racemates was recently reported for thalidomide, where only the *S*(−)-form is teratogenic in mice and rats[1].

Differences between enantiomers can also be observed in metabolism since the majority of the enzymatically catalyzed reactions are stereoselective[2]. Küpfer and co-workers[3,4] reported that aromatic hydroxylation is highly selective for the *S*(+)-form of mephenytoin; the *R*(−)-form undergoes N-demethylation. They concluded that the (*R*)-form of the demethylated metabolite was responsible for the major therapeutic benefit obtained from a chronic administration of racemic me-

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phenytoin[5]. They also speculated that the bone marrow toxicity observed during mephenytoin therapy may be due to an arene oxide formed as an intermediate in the aromatic hydroxylation of *S*-mephenytoin[4].

The above examples demonstrate the importance of studying the pharmacological effects and the disposition of enantiomers in order to minimize adverse reactions and optimize therapy. Important tools in such studies are analytical and preparative methods which enable the direct resolution of racemates. These methods have recently been reviewed[6,7]. However, the majority deal with the resolution of racemic amino acids; very few studies cover the resolution of racemic drugs.

The use of the plasma protein orosomucoid (α_1-AGP) as a chiral stationary phase for resolution of racemic drugs has previously been reported[8]. In those studies a maximum loading of 35 mg α_1-AGP per gram solid phase was used. The low protein content gave, for some compounds, poor resolution due to low separation factors, tailing peaks and low separation efficiency. In the present study the influence of the protein content per gram of solid phase on the retention, enantioselectivity, resolution and the chromatographic behaviour is examined.

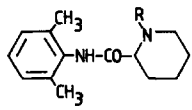
EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Waters M 6000 A pump, a Waters Model U6K injector and a Shimadzu SPD-2A variable wavelength UV detector. UV detection was carried out at 215 nm. An α_1-AGP silica column was used[8]. The preparation of such a column is reported elsewhere[8,9]. The α_1-AGP silica (mean particle diameter 13 μm) was packed in columns (100 × 3 mm) at 5000 p.s.i. using phosphate buffer, pH 7.0, as slurry medium and driving liquid in a Haskel pump. The pH was measured with an Orion Research Model 701 digital pH meter equipped with an Ingold combined electrode Type 401. All buffers were prepared at an ionic strength of 0.02.

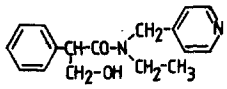
Chemicals

Racemic mepensolate bromide, ketamine, pentazocine, disopyramide, mepivacaine, bupivacaine hydrochloride. phenyramidol, chlorpheniramine, tropicamide and promethazine hydrochloride were of pharmacopoeial grade and obtained from drug manufacturers. (*R*)- and (*S*)-disopyramide were kindly supplied by Professor Wendel L. Nelson, School of Pharmacy, Department of Medicinal Chemistry, Seattle, Washington, U.S.A. (*R*)- and (*S*)-bupivacaine hydrochloride, (+)- and (−)-mepivacaine and (+)- and (−)-RAC 109 were gifts from Dr. R. Sandberg, Astra Läkemedel, Södertälje, Sweden. (+)- and (−)-promethazine were prepared within our laboratory. (+)- and (−)-3-PPP[10] and (+)- and (−)-8-OH-DPAT[11] were obtained from the Department of Organic Chemistry, Biomedical Centre, University of Uppsala, Uppsala, Sweden. When pure enantiomers were not available, they are referred to as I (lowest capacity factor) and II. The structures of the compounds are shown below:

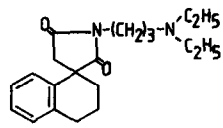


	R
BUPIVACAINE	CH_2-CH_2-CH_2-CH_3
MEPIVACAINE	CH_3

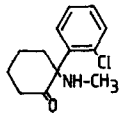
TROPICAMIDE



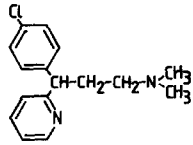
RAC 109



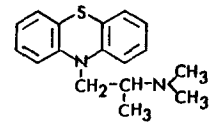
KETAMINE



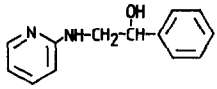
CHLORPHENIRAMINE



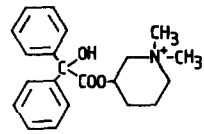
PROMETHAZINE



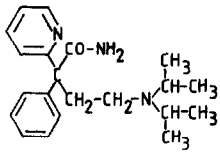
PHENYRAMIDOL



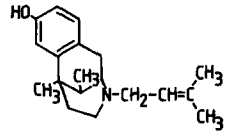
MEPENSOLATE BROMIDE



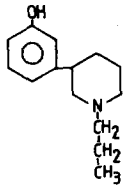
DISOPYRAMIDE



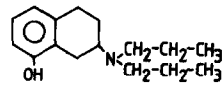
PENTAZOCINE



3-PPP



8-OH-DPAT



2-Propanol, analytical grade, was obtained from E. Merck (Darmstadt, F.R.G.).

RESULTS AND DISCUSSION

Variation of protein content on the solid phase

All samples except phenyramidol were not retained when chromatographed using a column without α_1-AGP bound to the solid phase (Fig. 1). However, the capacity factors increased with increasing protein content on the solid phase. This

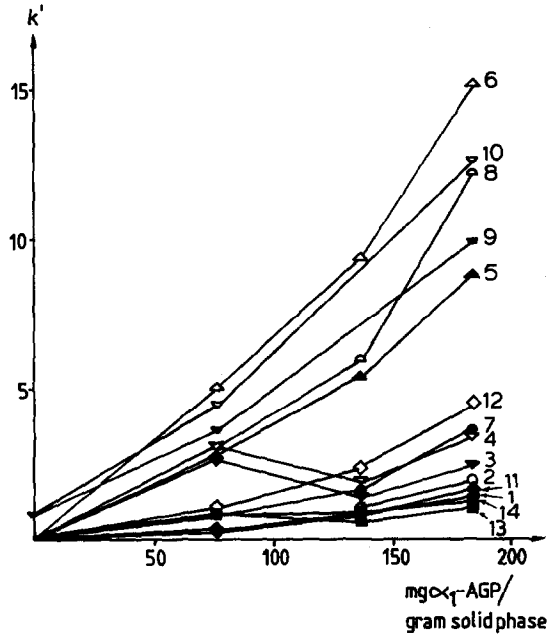


Fig. 1. Variation of the capacity factors (k') with the protein loading of the solid phase. Column: α_1-AGP (100 × 3.0 mm I.D.). Mobile phase: 6% (v/v) 2-propanol in phosphate buffer pH 7.1, μ = 0.02. Flow-rate: 0.5 ml/min. Samples: 1 = mepensolate bromide I; 2 = mepensolate bromide II; 3 = (*R*)-mepivacaine; 4 = (*S*)-mepivacaine; 5 = (*R*)-bupivacaine; 6 = (*S*)-bupivacaine; 7 = (*R*)-disopyramid; 8 = (*S*)-disopyramid; 9 = phenyramidol I; 10 = phenyramidol II; 11 = (+)-RAC 109; 12 = (−)-RAC 109; 13 = (−)-3-PPP; 14 = (+)-3-PPP.

experiment suggests that the solute retention is caused by an interaction between the protein and the solutes. Other forms of interaction with the solid phase would seem to be of minor importance under the conditions used. Comparatively high capacity factors were obtained when the column was loaded with 183 mg α_1-AGP per g solid phase in spite of the relatively high alcohol concentration in the mobile phase.

The relationship between the separation factor, α, and the protein content on the solid phase is shown in Fig. 2. The separation factor increased initially with increasing protein content on the solid phase. A further increase of the protein loading decreased the separation factor for RAC 109 and disopyramide, whereas the separation factor for the other solutes was almost unaffected. The resolution between the enantiomers was also increased with increasing protein loading (Fig. 3). This was a consequence of the increased capacity factors and an improvement both in peak symmetry and separation efficiency caused by increasing protein loading. With the use of a protein content of 183 mg per g solid phase, all peaks showed a good symmetry with asymmetry factors (asf) lower than 2 (Fig. 4). It can be concluded that it is sufficient to load the column with 75 mg α_1-AGP per g solid phase in order to obtain high separation factors and high resolution. However, there are two reasons for using a higher protein loading of the column: hydrophilic drugs can be retained and the symmetry of the peaks is improved.

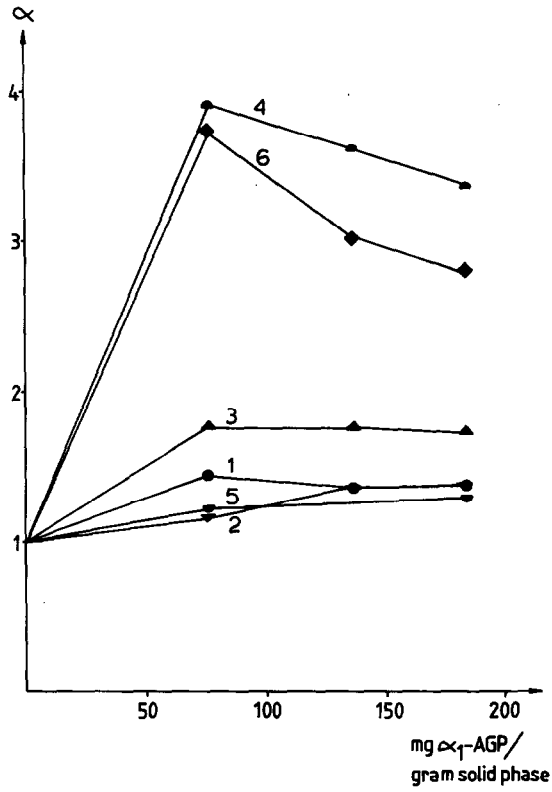


Fig. 2. Influence of protein content on the solid phase on the enantioselectivity. Conditions as in Fig. 1. Samples: 1 = mepensolate bromide; 2 = mepivacaine; 3 = bupivacaine; 4 = disopyramide; 5 = phenyramidol; 6 = RAC 109.

Regulation of the capacity factor and the separation selectivity

The addition of an organic modifier to the mobile phase is a convenient way to regulate the capacity factors of solutes over a wide range when using an α_1-AGP column. The influence of the concentration of 2-propanol in the mobile phase on the capacity factors and the separation factors was studied using the column with 183 mg α_1-AGP per g solid phase. The results are presented in Table I. Chromatography of solutes using a phosphate buffer pH 7.1 containing 1% 2-propanol as mobile phase gave very high capacity factors for some of the solutes. However, the capacity factors were significantly reduced when the alcohol concentration was increased to 2%.

The capacity factor for (*S*)-bupivacaine was 31.6 using a mobile phase of 4% 2-propanol in phosphate buffer pH 7.2 (Table I). The capacity factor for the analogue (*S*)-mepivacaine was 5.9, *i.e.*, the additional three methylene groups at the piperidine ring nitrogen of bupivacaine caused an approximately five-fold increase in the capacity factor. This suggests that hydrophobic interactions are important in solute retention on the α_1-AGP column. It is also obvious that this column has properties which are comparable with ordinary reversed-phase columns. The separation factors for some of the solutes decreased with increasing 2-propanol concentration, whereas

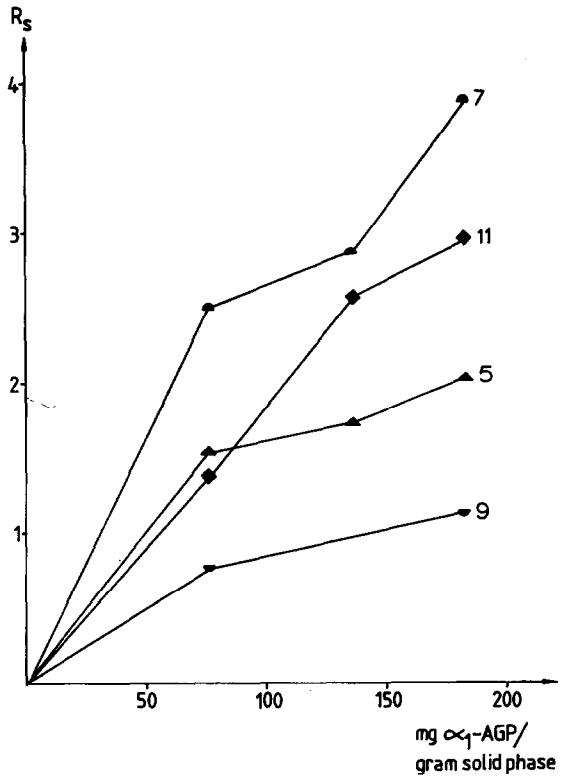
TABLE I

EFFECT OF 2-PROPANOL CONTENT IN MOBILE PHASE ON RETENTION AND ENANTIOSELECTIVITY

Conditions: column 100 × 3.0 mm I.D., 183 mg α_1-AGP per gram solid phase; mobile phase, phosphate buffer pH 7.20, μ = 0.02 with addition of 2-propanol; flow-rate 0.5 ml/min.

Solute	*1% C_3H_7OH*			*2% C_3H_7OH*			*4% C_3H_7OH*			*6% C_3H_7OH*			*8% C_3H_7OH*		
	k'_R	k'_S	α	k'_R	k'_S	α	k'_R	k'_S	α	k'_R	k'_S	α	k'_R	k'_S	α
Disopyramide	–	–	–	–	–	–	8.51	31.48	3.70	3.62	12.19	3.37	1.77	5.66	3.20
Phenyramidol*	–	–	–	26.65	32.18	1.21	5.38	6.86	1.28	9.91	12.95	1.28	–	–	–
Chlorpheniramine*	11.16	26.15	2.34	7.35	12.54	1.71	4.59	6.32	1.38	–	–	–	–	–	–
Tropicamide*	10.40	12.59	1.21	6.67	8.16	1.22	3.02	3.70	1.22	1.89	1.89	1.00	–	–	–
Mepensolate bromide*	–	–	–	6.35	9.79	1.54	2.65	3.72	1.40	1.42	1.96	1.38	1.00	1.21	1.21
Mepivacaine	26.00	35.43	1.36	10.67	13.96	1.31	4.42	5.87	1.33	2.48	3.39	1.36	1.58	2.13	1.35
Bupivacaine	–	–	–	–	–	–	18.55	31.60	1.70	8.84	15.22	1.72	5.01	8.72	1.74
3-PPP	4.85	8.55	1.76	–	–	–	1.43	2.27	1.59	1.03	1.44	1.39	0.90	1.06	1.17
8-OH-DPAT**	–	–	–	–	–	–	2.49	3.54	1.42	1.50	1.96	1.30	1.24	1.24	1.00
RAC 109***	–	–	–	–	–	–	3.09	9.60	3.11	1.61	4.50	2.80	1.05	2.56	2.44

* The enantiomers are not available. In this case k'_R and k'_S are for the enantiomer with the lowest and highest capacity factors, respectively.
** The enantiomer with the lowest capacity factors is the (−)-form. The absolute configuration is not known.
*** The enantiomer with the lowest capacity factor is the (+)-form. The absolute configuration is not known.



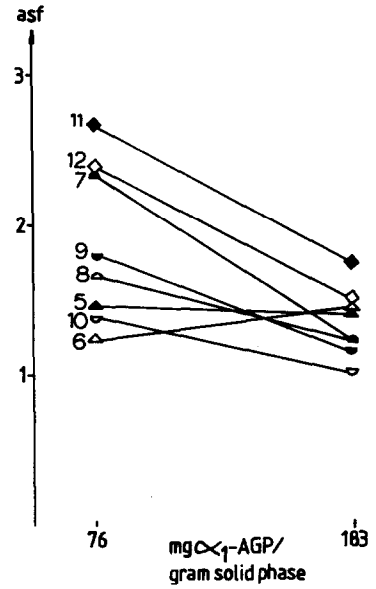


Fig. 3. Influence of solid phase protein content on the resolution factor (R_s). Conditions as in Fig. 1. Samples: 5 = bupivacaine; 7 = disopyramide; 9 = phenyramidol; 11 = RAC 109.

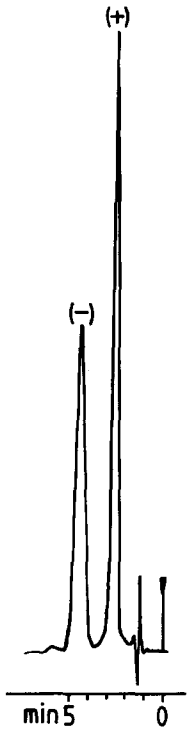
Fig. 4. Peak symmetry with different solid phase protein contents. Conditions and samples as in Fig. 1.

those for the enantiomers of mepivacaine and bupivacaine were almost unaffected.

From Table I it can be seen that the separation factors for the enantiomers of RAC 109 and disopyramide were 2.45 and 3.20 respectively, despite the fact that a comparatively high 2-propanol concentration (8%, v/v) was used. The separation of the enantiomers of RAC 109 using this alcohol concentration is demonstrated in Fig. 5.

Addition of N,N-dimethyloctylamine (DMOA) to the mobile phase

α_1-AGP is the most glycosylated plasma protein known. It comprises 45% carbohydrate including fourteen sialic acid residues per molecule[12]. This means that α_1-AGP is a very acidic protein, which is also indicated by the isoelectric point at pH $\approx$ 2[10]. Thus, the molecule has a net negative charge at pH $\approx$ 7, *i.e.*, the pH of the mobile phase used in this study. The capacity factors and the separation factors can be regulated by addition of the tertiary amine N,N-dimethyloctylamine (DMOA) to the mobile phase, as demonstrated in Figs. 6 and 7. From Fig. 6 it is seen that an increase of the DMOA concentration from 0.5 to 1.95 m*M* causes a decrease of about 55% in the capacity factors of (*R*)- and (*S*)-disopyramide. A likely cause of this effect is that the tertiary amine DMOA will compete with the solutes for ionic binding to



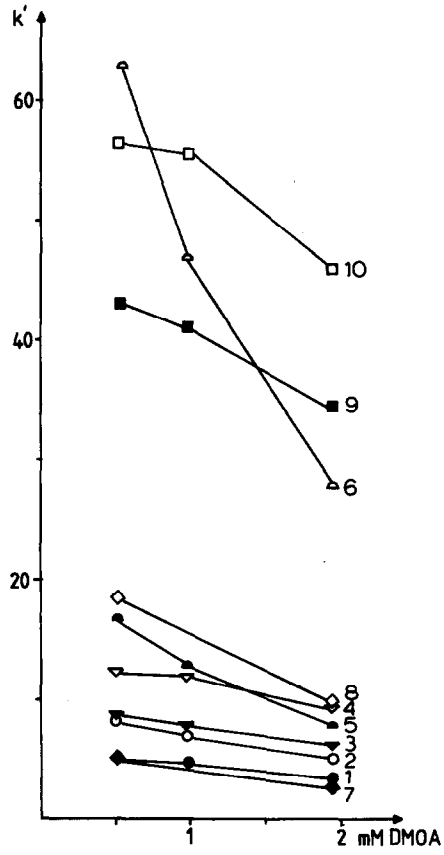


Fig. 5. Resolution of racemic RAC 109. Column: 100 × 3.0 mm I.D., 183 mg α_1-AGP per gram solid phase. Mobile phase: 8% (v/v) 2-propanol in phosphate buffer pH 7.20, μ = 0.02. Flow-rate: 0.5 ml/min.

Fig. 6. Regulation of the capacity factors with DMOA. Column: as in Fig. 5. Mobile phase: 2% (v/v) 2-propanol in phosphate buffer pH 7.15 with addition of DMOA. Flow-rate: 0.5 ml/min. Samples: 1 = mepensolate bromide I; 2 = mepensolate bromide II; 3 = (*R*)-mepivacaine; 4 = (*S*)-mepivacaine; 5 = (*R*)-disopyramide; 6 = (*S*)-disopyramide; 7 = (+)-RAC 109; 8 = (−)-RAC 109; 9 = (+) = promethazine; 10 = (−)-promethazine.

the negatively charged groups of the protein, *i.e.*, sialic acid residues or other negatively charged groups of the binding site of the protein. It was reported previously[13] that the binding affinity for propanolol decreased from $8.4 \cdot 10^5$ to $6 \cdot 10^5$ M^{-1} when the sialic acid was removed from α_1-AGP, whereas the binding affinity for the neutral molecule progesterone was not affected. These data may indicate that sialic acid is involved in ionic binding of DMOA and basic solutes. It is also reasonable to assume that DMOA will compete with the solutes for binding to the hydrophobic part of the binding site. In a previous study, indications were found for both ionic and hydrophobic interactions between the solutes and the binding site of the protein[8].

Addition of DMOA to the mobile phase also affected the separation factor (Fig. 7). This effect was more limited than that obtained when using α_1-AGP as a chiral complexing agent in the mobile phase, in combination with a non-chiral col-

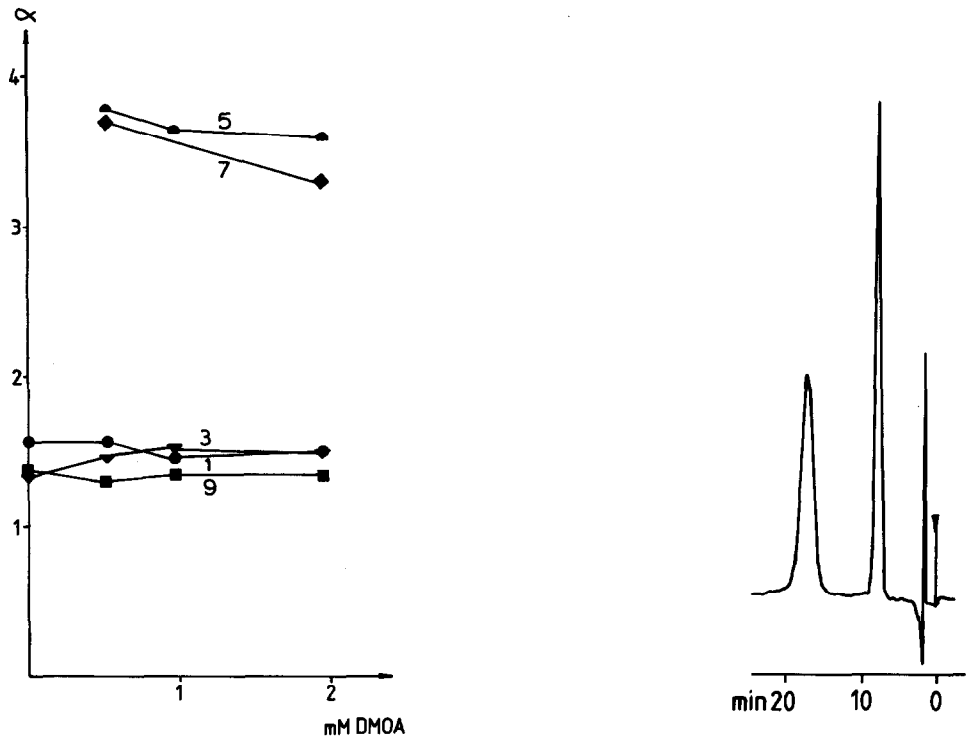


Fig. 7. Influence of the DMOA concentration on the enantioselectivity. Conditions as in Fig. 6. Samples: 1 = mepensolate bromide; 3 = mepivacaine; 5 = disopyramide; 7 = RAC 109; 9 = promethazine. The separation factor of promethazine is obtained using a mobile phase of 8% (v/v) 2-propanol in phosphate buffer pH 7.10.

Fig. 8. Resolution of racemic ketamine. The enantiomers are not available in pure form. Column: 100 × 3.0 mm I.D., 136 mg α_1-AGP per gram solid phase. Mobile phase: phosphate buffer pH 7.0 with addition of 1.95 m*M* DMOA. Flow-rate: 0.5 ml/min.

Fig. 9. Resolution of racemic pentazocine. Conditions as in Fig. 8. The enantiomers are not available in pure form.

Fig. 10. Resolution of the enantiomers of 3-PPP. Column: 100 × 3.0 mm I.D., 194 mg α_1-AGP per gram solid phase. Mobile phase: 2% (v/v) 2-propanol in phosphate buffer pH 7.10. Flow-rate: 0.5 ml/min.

umn[14]. Addition of DMOA to a mobile phase containing α_1-AGP destroys the enantioselectivity for some solutes and is essential to obtain enantioselectivity of other solutes[14]. As mentioned above, it may be speculated that these drastic effects are due to a strong binding of DMOA to the protein with a subsequent alteration in the conformation of the protein. It has been demonstrated, by use of circular dichroism, that sodium dodecyl sulphate (0.005–0.05 *M*) has a significant effect on the conformation of α_1-AGP[15]. It can be assumed that the limited effect of DMOA on the separation factor using α_1-AGP as chiral stationary phase is due to a more stable conformation of α_1-AGP in the immobilized form.

Resolution of racemic drugs

The alternative approach to the direct resolution of enantiomers is to prepare diastereomeric derivatives followed by chromatography on a non-chiral column. This procedure is time-consuming and the derivatization procedure must be carefully controlled to ensure that the enantiomers react to the same extent with the chiral (asymmetric) reagent. It has been demonstrated that the enantiomers of ketamine react at different rates with N-trifluoroacetyl-(*S*)-prolyl chloride or (*S,S*)-N-trifluoroacetylproline anhydride[16]. The same authors also observed considerable racemization during the derivatization procedure. This means that the direct resolution procedures are preferred, especially in cases when the enantiomers of a compound react at different rates (*e.g.*, ketamine), or when the compound lacks a suitable group to deri-

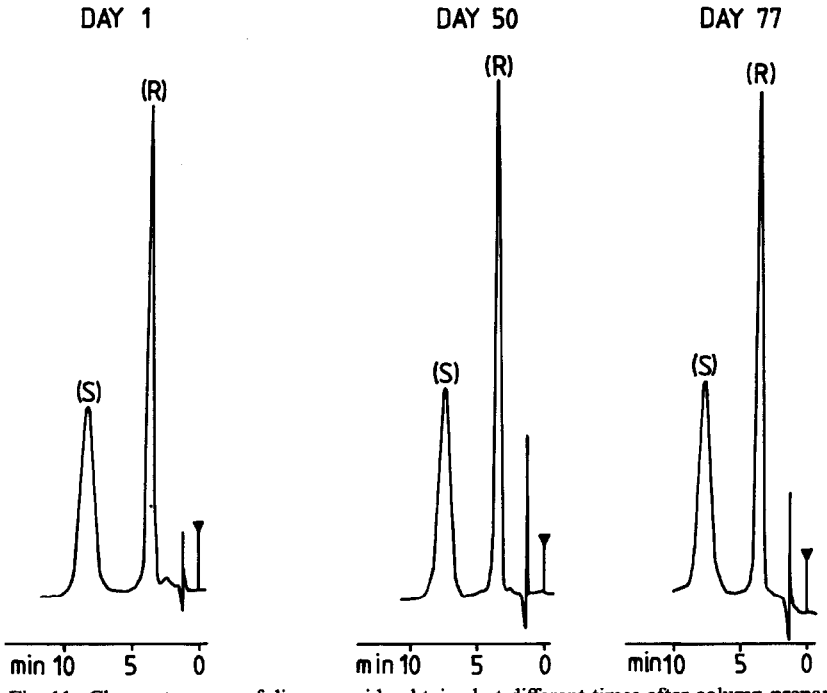


Fig. 11. Chromatograms of disopyramide obtained at different times after column preparation. Column as in Fig. 5. Mobile phase: 8% (v/v) 2-propanol in phosphate buffer pH 7.20. Flow-rate: 0.5 ml/min.

vatize. The utility of the α_1-AGP column as a powerful tool for direct resolution of racemates is demonstrated in Figs. 8–10 where the enantiomers of ketamine (α = 2.51), pentazocine (α = 1.52) and 3-PPP (α = 1.78) are separated. Table I summarizes the separation factors obtained for ten test solutes under different conditions.

Stability of the α_1-AGP column

The stability of the α_1-AGP column was studied using a column (100 × 3.0 mm) with 183 mg α_1-AGP per gram solid phase. Racemic disopyramide was used as test solute. Disopyramide was injected on the first day and after 50 and 77 days. The chromatograms are presented in Fig. 11. The column has been in daily use and 60 different mobile phases with pH between 6 and 7.5 have been used during this period. Six different organic modifiers, *i.e.*, ethanol, propanol, butanol, ethylmethyl ketone, diisobutyl ketone and tetrahydrofuran, have been used and many of the mobile phases also contained DMOA. The use of a mobile phase with a pH > 7, a low content of organic modifier and with addition of DMOA is known to be very "aggressive" to reversed-phase columns. However, the α_1-AGP column has shown a very good stability. Fig. 12 demonstrates the capacity factors obtained for the disopyramide enantiomers after different times.

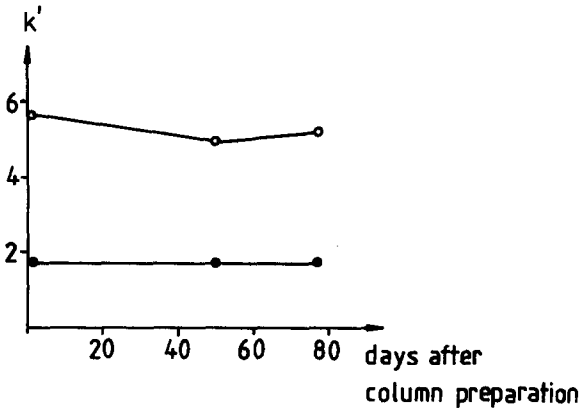


Fig. 12. Capacity factors for the enantiomers of disopyramide obtained at different times after column preparation. Conditions as in Fig. 11. Samples: ●, (*R*)-disopyramide; ○, (*S*)-disopyramide.

The column is still in daily use 3.5 months after its preparation and there is no tendency for degradation of the solid phase or denaturation of the protein.

In conclusion, the α_1-AGP column with a high protein loading shows enantioselectivity for many different amines and high separation factors and resolution factors can be obtained. It has also shown a remarkable stability despite the fact that very "aggressive" mobile phases have been used over a long period.

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