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DIRECT LIQUID CHROMATOGRAPHIC RESOLUTION OF RACEMIC DRUGS USING α_1-ACID GLYCOPROTEIN AS THE CHIRAL STATIONARY PHASE

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SUMMARY

α_1-Acid glycoprotein (orosomucoid), a plasma protein, has been immobilized on silica microparticles and used as a chiral phase for liquid chromatographic resolutions of enantiomers of racemic drugs. The capacity factors of the solutes were easily regulated by changing the pH, or by adding 1-propanol to the mobile phase. The separation factor increases for some enantiomeric pairs, whereas it decreases for others with increasing pH of the mobile phase. Addition of 1-propanol to the mobile phase decreases the separation factor. Separation factors between 1.1 and 3.0 were obtained. Eight racemic drugs or drug metabolites were resolved.

INTRODUCTION

There are three basically different approaches for liquid chromatographic resolution of enantiomers: formation of diastereomeric derivatives followed by chromatography[1–5], addition of chiral complexing agents[6,7] or optically active ion-pairing agents[8] to the mobile phase or by the use of chemically bonded chiral phases.

Chemically bonded chiral phases have been prepared in two different ways: either by reaction with an optically active compound of low molecular weight[9–13] or by immobilization of a protein on a solid phase. In 1904, Willstätter pointed out the possibility that enantiomers might be adsorbed by proteins with different strengths[14]. Later it was demonstrated that wool and casein adsorb enantiomers of certain dyes, mandelic acid and naphtylglycolic acid with different affinities[15]. In 1958, McMenamy and Oncley[16] observed that the enantiomers of tryptophan were bound to albumin with different strengths. This observation was utilized some ten years ago by Stewart and Doherty[17], and later by another group[18], who used albumin as the chiral stationary phase for the separation of tryptophan and warfarin enantiomers. These results were recently reproduced using analytical columns[19].

The present study deals with the separation of enantiomers of basic drugs using α_1-acid glycoprotein (α_1-AGP) as the chiral chemically bonded phase (patents applied for). Such a chiral phase can be used for many different types of applications beside

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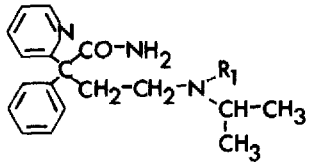
the analytical approach. It can be used to separate small amounts of isotope-labelled racemic drugs to be used as tracers in metabolic studies *in vitro*. Probably, it can also be used for the determination of binding constants of enantiomers or non-chiral drugs. Many racemic drugs can be screened, in short times, for stereoselective protein binding without the need for the pure enantiomers. Normally the protein-binding studies are performed using equilibrium dialysis. Such studies are time-consuming and often require labelled drugs.

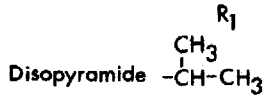
Preparation of diastereomeric derivatives is the most frequently used technique for the separation of enantiomers[1]. However, some solutes such as disopyramide, mepivacaine and bupivacaine cannot easily be derivatized. Therefore, the direct separation technique is the preferred method in such cases.

EXPERIMENTAL

Apparatus

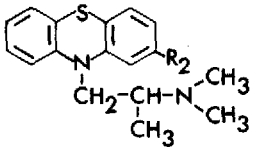
The liquid chromatographic system used was built from an Altex Model 100 solvent-delivery system and a Waters Model U6K injector. A Shimadzu SPD-2A UV detector with variable wavelength was used. UV detection was carried out at 215 nm. The pH was measured with an Orion Research Model 801 A digital pH meter equipped with an Ingold combined electrode type 401.





Disopyramide $-CH(CH_3)-CH_3$

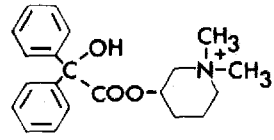
Desisopropyl-disopyramide $-H$



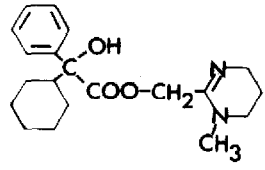
Promethazine $-H$

Propiomazine $-CO-CH_2-CH_3$

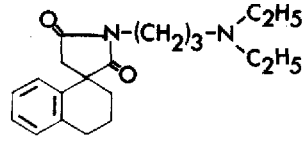
Fig. 1. Structures of samples.



Mepensolate bromide



Oxyphencyclimine



RAC 109

Chemicals

Racemic mepensolate bromide, propiomazine maleate, disopyramide, promethazine hydrochloride, bupivacaine hydrochloride, mepivacaine, prilocaine hydrochloride, oxyphencyclimine were of pharmacopoeial grade and obtained from drug manufacturers. (*R*)- and (*S*)-disopyramide, *R*(−)-desisopropyl disopyramide oxalate and *S*(+)-desisopropyl disopyramide oxalate 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, (+)-prilocaine hydrochloride, (−)-prilocaine hydrochloride and (+)- and (−)-RAC 109 hydrochloride were kindly supplied by Dr. R. Sandberg, Astra Läkemedel, Södertälje, Sweden. The structures are presented in Fig. 1. When the optical rotation or the absolute configuration of the enantiomers is unknown, they are referred to as I (lowest capacity factor) and II.

The Cohn fraction V supernatant fraction was kindly supplied by Dr. M. Mikaelsson, KabiVitrum AB, Stockholm, Sweden. Coomassie blue G 250 and 3-glycidoxypropyltrimethoxysilane (GOPS) were obtained from Serva Fein Biochemica, Heidelberg, F.R.G., and LiChrosorb Si 300 (5 μm) was obtained from E. Merck, Darmstadt, F.R.G.

Preparation of CM-Sephadex column

CM-Sephadex (Pharmacia, Uppsala, Sweden) was swelled in 0.02 *M* phosphate buffer (pH 5.0), as recommended by the manufacturer. The swelled gel was packed into a column (250 × 50 mm) under gravity, but a pump was used for the application of the sample and elution of fractions. The column was equilibrated with 0.02 *M* phosphate buffer (pH 5.0).

Isolation of α_1-AGP

Isolation of α_1-AGP was carried out using a modification of a method described previously[20]. A 750-ml volume of Cohn V supernatant fraction[21,22] was dialysed for 65 h against 2 × 20 l of 0.02 *M* phosphate buffer (pH 5.0) at 4°C. The dialysed protein sample was applied onto the column using a flow-rate of 2.4 ml/min. Phosphate buffer (0.02 *M*; pH 5.0) was used as mobile phase. Fractions of 15 ml were collected. The absorbance of the fractions was measured at 280 nm. One single UV-adsorbing peak was eluted. The pooled fractions were dialysed at 4°C against 3 × 15 l of distilled water and then freeze-dried. A yield of *ca.* 15 g of dry powder was isolated (equal to *ca.* 13 g of pure protein after correction for salts).

Polyacrylamide disc electrophoresis of the isolated protein, according to the method of Davis[23], showed one single and homogeneous band when stained with Coomassie blue G 250.

Synthesis of epoxide-activated silica

The epoxide-activated silica was prepared mainly according to a procedure described by Herman *et al.*[24]. LiChrospher Si 100 or Si 300 (10 g) was dried for 24 h at 150°C. The dry silica was suspended in a solution of 5 ml of GOPS in 50 ml of dry toluene and refluxed for 4.5 h. The reflux condenser was kept at 65°C to remove the methanol formed from the reaction mixture[25]. After cooling and filtration of the reaction mixture the derivatized silica was washed with 300 ml of toluene, 250 ml of tetrahydrofuran and 500 ml of methanol, and then dried.

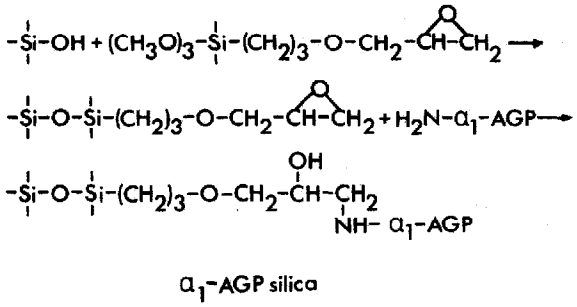


Fig. 2. Synthesis of α_1-AGP silica.

Preparation of α_1-AGP silica

A 100-mg sample of α_1-AGP was dissolved in 4.0 ml of 0.1 *M* borate buffer (pH 8.5) containing 0.2 *M* sodium chloride. Epoxide silica (1 g) was washed in the coupling buffer and added to the α_1-AGP solution. The mixture was allowed to react at room temperature by slow agitation in an extraction apparatus. After 48 h the reaction mixture was centrifuged and the α_1-AGP concentration in the supernatant was determined using a UV spectrophotometer at 280 nm. The amount of α_1-AGP bound to the epoxide silica was calculated by comparing the amounts of α_1-AGP in

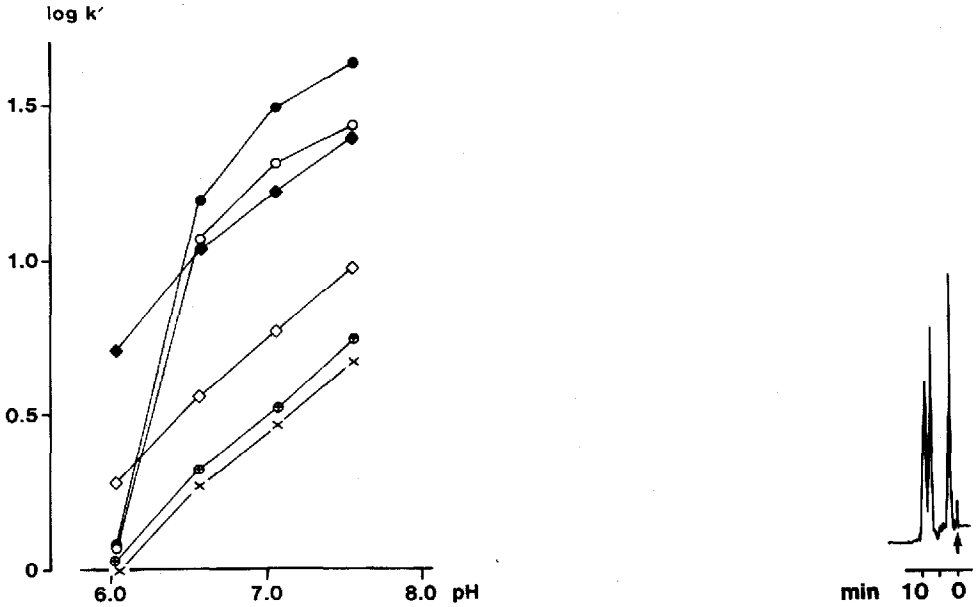


Fig. 3. Regulation of the capacity factor with pH. Column: α_1-AGP silica (150 × 3.2 mm). The protein content of this silica was not determined. Mobile phase: phosphate buffer. Flow-rate: 0.5 ml/min. Samples: ○ = oxyphencyclimine I; ● = oxyphencyclimine II; ◆ = *S*(+)-disopyramide; ◇ = *R*(−)-disopyramide; × = mepensolate bromide I; ⊕ = mepensolate bromide II.

Fig. 4. Separation of the mepensolate bromide enantiomers. Mobile phase: phosphate buffer (pH 7.01; μ = 0.05). Flow-rate: 0.5 ml/min. Column: α_1-AGP silica (150 × 3.2 mm). The protein content was 23 mg α_1-AGP/g silica. Sample amount: 0.19 nmol.

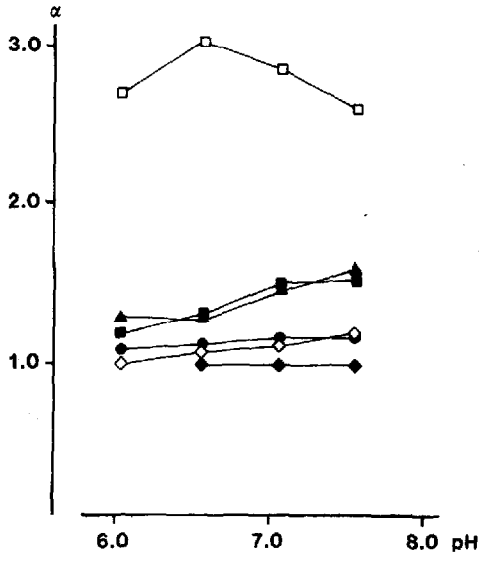


Fig. 5. Variation of the separation factor with pH. Conditions as in Fig. 3. Samples: □ = disopyramide; ▲ = oxyphencyclimine; ■ = bupivacaine; ● = mepensolate bromide; ◇ = mepivacaine; ◆ = prilocaine.

the buffer before and after the reaction. The α_1-AGP silica was carefully washed with coupling buffer and stored at 4°C until used.

Preparation of α_1-AGP silica column

The α_1-AGP silica was suspended in phosphate buffer (pH 7.0) and poured into the packing column, which was filled up with the same buffer. The column was packed at 2000 p.s.i. using phosphate buffer (pH 7.0) as the driving liquid. An Altex Model 100 pump was used.

RESULTS AND DISCUSSION

During the last ten years evidence has been demonstrated that α_1-AGP constitutes the major binding protein in human serum for several basic drugs, such as

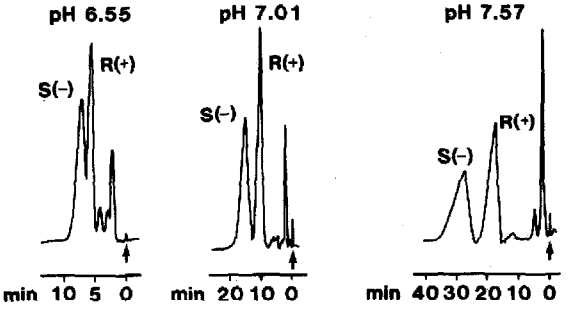


Fig. 6. Separation of bupivacaine enantiomers at different pHs. Mobile phase: phosphate buffer (μ = 0.05). Other conditions as in Fig. 4. Sample amount: 0.22 nmol.

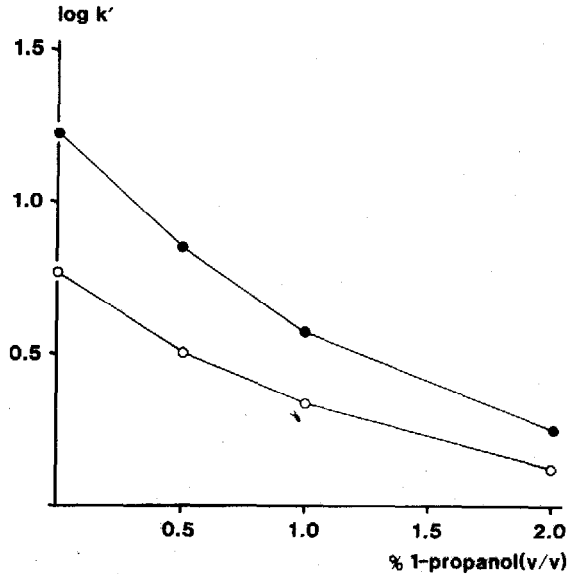


Fig. 7. Regulation of the retention of disopyramide enantiomers with 1-propanol. Mobile phase: phosphate buffer (pH 7.06; μ = 0.05) containing 1-propanol. Other conditions as in Fig. 4. Samples: ○ = *R*(−)-disopyramide; ● = *S*(+)-disopyramide.

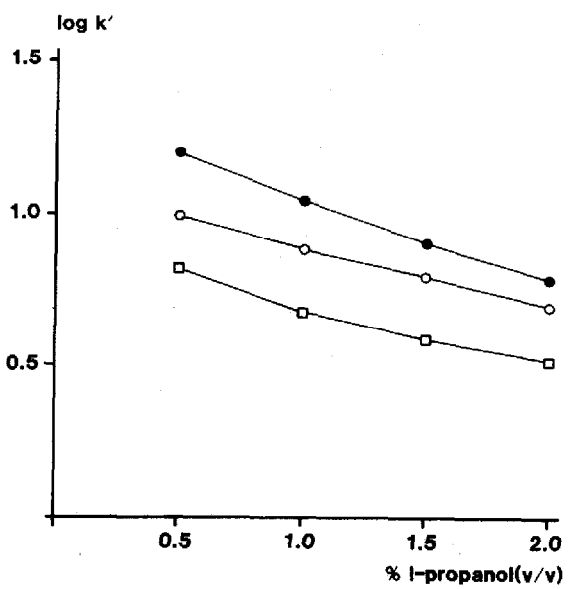


Fig. 8. Regulation of the retention of prometazine and the enantiomers of propiomazine. Mobile phase: phosphate buffer (pH 6.52; μ = 0.05) containing 1-propanol. Other conditions as in Fig. 4. Samples: ○ = propiomazine I; ● = propiomazine II; □ = promethazine.

β-blockers[26], tricyclic antidepressants and neuroleptics[27]. Some of the drug substances in these groups contain chiral carbon atoms. However, the ability of α_1-AGP to bind the enantiomers with different strength has not been investigated. Only one report dealing with the stereoselective binding of enantiomers to α_1-AGP has appeared in the literature[28]. The binding of the enantiomers of methadone was determined using equilibrium dialysis. In the present study, α_1-AGP was isolated from human blood and used for the preparation of a chemically bonded chiral phase.

Synthesis of α_1-AGP silica

Activation of silica was performed by reaction with 3-glycidoxypropyltrimethoxysilane giving silica containing reactive epoxide groups on the surface as demonstrated in Fig. 2. Coupling of α_1-AGP to the activated silica was performed at room temperature in buffers of different pH. The amount of α_1-AGP bound to silica increases with increasing pH, as was expected. However, owing to the sensitivity of silica to aqueous solutions of high pH, 8.5 was the highest pH used.

Two different silica supports were used in this study, *viz.*, LiChrospher Si 100 and Si 300, with pore diameters of 100 and 300 Å, respectively, and a specific surface area of *ca.* 250 m^2/g in the underivatized form (data from the manufacturer). There was a significant difference between the amount of α_1-AGP bound to the 100- and 300-Å silicas. A very small amount of α_1-AGP was bound using the 100-Å silica, indicating that the α_1-AGP molecules are excluded from the pores and react preferentially with epoxide groups on the outside of the silica particles. Silica particles with larger pore diameters than 300 Å have not been tested, but the 300-Å pores are apparently large enough to allow the protein to penetrate. This is supported by the

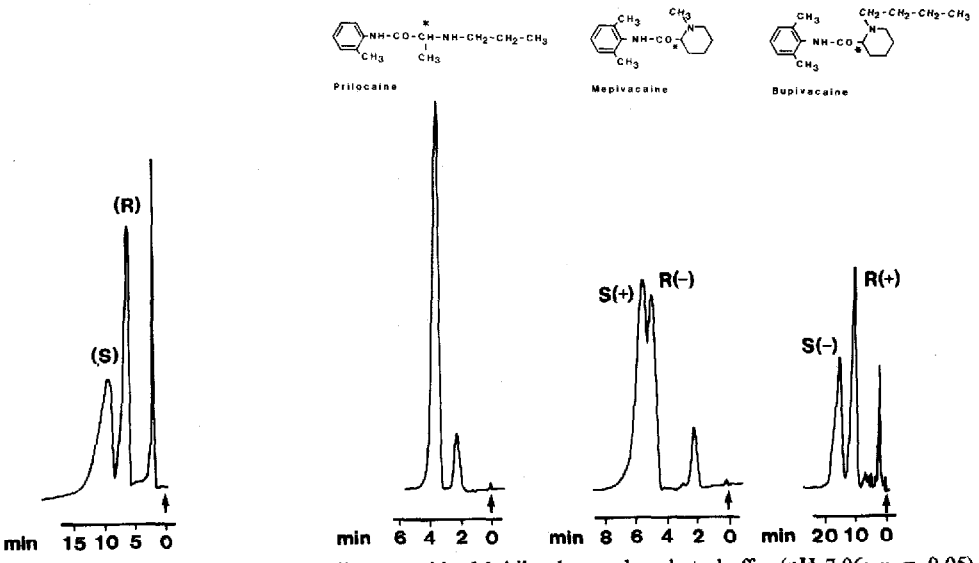


Fig. 9. Separation of (*R*)- and (*S*)-disopyramide. Mobile phase: phosphate buffer (pH 7.06; μ = 0.05) containing 1% 1-propanol. Other conditions as in Fig. 4. Sample amount: 0.43 nmol.

Fig. 10. Influence of the molecular structure on the resolution. Mobile phase: phosphate buffer (pH 7.01; μ = 0.05). Other conditions as in Fig. 4. Sample amounts: bupivacaine, 0.22 nmol; mepivacaine, 0.28 nmol; prilocaine, 0.10 nmol.

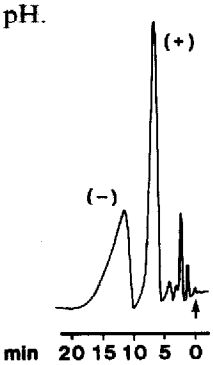
fact that a drastic increase in the amount of bound α_1-AGP was observed using the 300-Å silica compared to the 100-Å silica. The 300-Å silica has a maximum binding capacity of *ca.* 35 mg of α_1-AGP per g of silica using the procedure described under Experimental, whereas the 100-Å silica bound *ca.* 2.5 mg/g. Studies on silicas with a larger pore diameter, and a more detailed study of the influence of the amount of bound protein as a function of the pore diameter, are in progress.

Regulation of the retention and the separation selectivity

The retention of the samples can be regulated in three ways, either by varying the pH or the ionic strength of the mobile phase, or by addition of an organic modifier to the mobile phase. Fig. 3 shows some examples to demonstrate the influence of the pH of the mobile phase on the capacity factors. As can be seen from Fig. 3, the capacity factors are affected to a large degree by a small change in pH. All solutes, except oxyphencyclimine, have almost the same slope. A likely cause of the deviating behaviour of this solute is ionization of a second nitrogen in the pyrimidinyl ring.

Fig. 4 demonstrates a separation of the enantiomers of mepensolate bromide. The separation factor for the enantiomers of this solute increases slightly with increasing pH (Fig. 5), whereas the capacity factors are highly affected (*cf.* Fig. 3). Obviously this effect is caused only by a change in the properties of the stationary phase with pH. This is supported by the fact that mepensolate bromide contains a quaternary nitrogen which is ionized independently of the pH of the mobile phase. The isoelectric point of α_1-AGP is very low. Depending on the buffer chosen for the determination, its value ranges between 1.8 and 2.7 (ref. 29), which means that the protein has a net negative charge under the described conditions. The fact that the capacity factor of mepensolate bromide increases with increasing negative charge of the protein, *i.e.*, increasing pH, indicates that ionic binding is involved in the interaction with the solute.

The separation factor is also affected by the pH of the mobile phase; this is demonstrated in Fig. 5. The highest separation factor (α) obtained for the enantiomers of disopyramide was 3.0. It can also be seen from Fig. 5 that the separation factor for the disopyramide enantiomers increases with decreasing pH and reaches a maximum at *ca.* pH 6.5, whereas the α-values for the other samples presented in Fig. 5 increase with increasing pH. An example of this effect is illustrated in Fig. 6, where the enantiomers of the local anaesthetic drug bupivacaine are separated at three different pH values. The bupivacaine enantiomers are better resolved at pH 7.5 compared to lower pH values owing to the higher α-values obtained with increasing pH.



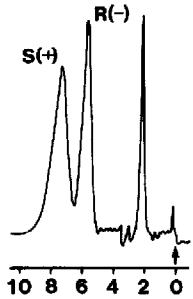


Fig. 11. Separation of (+)- and (−)-RAC 109. Mobile phase: phosphate buffer (pH 6.55; μ = 0.05). Other conditions as in Fig. 4. Sample amount: 0.46 nmol.

Fig. 12. Resolution of (*R*)- and (*S*)-desisopropyl disopyramide. Mobile phase: phosphate buffer (pH 7.01; μ = 0.05). Other conditions as in Fig. 4.

The fact that the separation factor increases for some solutes and decreases for others with decreasing pH may indicate either that different binding sites are involved in the interaction with different solutes or that different functional groups of the same site are involved in the three-point interaction with different solutes. Despite the fact that the binding of basic drugs to α_1-AGP has been known for many years, little information is available on the molecular aspects of the binding of drugs to α_1-AGP. However, the existence of several binding sites of α_1-AGP has been demonstrated[30].

Retention and selectivity was also highly influenced by the content of organic modifier in the mobile phase, as illustrated in Figs. 7 and 8 for disopyramide, propiomazine and promethazine. From Fig. 7 it can be seen that the separation selectivity for the enantiomers of disopyramide decreases by *ca.* 70% with an increase in 1-propanol concentration from 0 to 2% (v/v). There are indications for the existence of a hydrophobic core of α_1-AGP[31]. A 0.6% solution of α_1-AGP dissolved 1.28 g of benzene per 100 ml, whereas the corresponding amount for a 1% albumin solution is 0.061 g[31]. It is reasonable to assume that hydrophobic interaction is one type of interaction involved in the retention of the rather hydrophobic solutes promethazine and propiomazine, and that competition exists between the solutes and propanol for adsorption to such a hydrophobic part of the protein. Adsorption of alcohols to hydrophobic supports is well known[32,33].

Fig. 9 illustrates the separation of (*R*)- and (*S*)-disopyramide using a mobile phase of phosphate buffer (pH 7.06) containing 1% 1-propanol.

Influence of molecular structure on selectivity

Fig. 8 shows that there is no separation between the enantiomers of promethazine, whereas the enantiomers of propiomazine give a separation factor of 1.6 on using a mobile phase of phosphate buffer (pH 6.52) containing 0.5% propanol. The difference between these molecules is the acetyl group in the 2-position (see Fig. 1), which obviously gives the propiomazine molecule the structural prerequisites for obtaining a three-point interaction with the stationary phase (protein and the solid phase) necessary for resolution to occur[34]. It is likely that the additional carbonyl function present in propiomazine forms a hydrogen bond with a hydrogen-donating group at the binding site of the protein.

The local anaesthetic drugs mepivacaine, bupivacaine and prilocaine are other examples which demonstrate clearly that small changes in the molecular structure substantially influence the separation factor (Fig. 10). The enantiomers of bupivacaine are almost baseline-separated using phosphate buffer (pH 7.06) as mobile phase and a separation factor of 1.6 is obtained. Chromatography of the analogue mepivacaine, which has three carbon atoms less at the piperidine ring nitrogen, markedly reduces the separation factor to 1.2. It is obvious that this carbon chain is one of the structural elements which in some way contribute to the three-point attachment. From this experiment it can also be concluded that a limited mobility of the chiral carbon is another important property in order to obtain high separation factors. The chiral carbon of mepivacaine and bupivacaine has a limited mobility compared to prilocaine, since it is located in the piperidine ring. This property minimizes the number of possible interactions of the solute (as hydrogen bonding, hydrophobic interaction) with the "active groups" in the asymmetric binding site of α_1-AGP, which give the prerequisites necessary for obtaining different binding strength of the enantiomers.

RAC 109, another local anaesthetic drug, is an additional example indicating that a rigid structure around the chiral carbon is one important property of a solute in order to achieve enantioselectivity. A separation of the RAC 109 enantiomers is demonstrated in Fig. 11. An α value of 2.1 was obtained using a mobile phase of pH

6.55, whereas an increase of the pH to 7.01 increases α to 2.6.

A separation factor of 3.0 was obtained for the disopyramide enantiomers. N-Dealkylation of disopyramide with loss of one N-isopropyl group drastically reduces the separation factor to 1.3. However, the enantiomers of this disopyramide metabolite are well resolved on the 150-mm α_1-AGP silica column, as is demonstrated in Fig. 12.

As shown in Fig. 1, oxyphencyclimine is an ester, and the molecule also contains a pyrimidinyl ring. Rather high α values were obtained for this compound, whereas separation selectivity and retention were completely lost if the ester was hydrolysed to the carboxylic acid compound. Obviously the pyrimidinyl ring plays a crucial role in the interaction with the binding site of the protein. Hence, it can be hypothesized that the ring nitrogens are involved in ionic interactions with the protein, which obviously are crucial for this solute to achieve a three-point interaction with the stationary phase.

In conclusion, it is noted that rather high separation factors can be obtained for the enantiomers of basic drugs, using α_1-AGP as the chiral bonded phase. The experiments indicate that ionic and hydrophobic interactions as well as hydrogen bonding may be involved in the retention of the solutes. However, further studies must be carried out in order that a deeper understanding of the mechanisms involved in the retention of the solutes can be obtained.

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