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DIRECT LIQUID CHROMATOGRAPHIC RESOLUTION OF RACEMIC DRUGS BY MEANS OF α_1-ACID GLYCOPROTEIN AS THE CHIRAL COMPLEXING AGENT IN THE MOBILE PHASE

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SUMMARY

The plasma protein α_1-acid glycoprotein (orosomucoid) has been used as a chiral complexing agent in the mobile phase in combination with a non-chiral diol silica column for the resolution of racemic drugs (neuroleptics and tricyclic antidepressants). The retention behaviour of the solutes has been investigated and a model for the retention has been developed. From the retention data it has been possible to calculate the affinity constants for the binding of the enantiomers of promethazine, alimemazine and trimipramine to the protein.

The capacity factors of the solutes can be regulated by the protein concentration or by adding a tertiary amine to the mobile phase. Increasing the protein concentration had little influence on the separation factor but decreased the capacity factors. The addition of the tertiary amine to the mobile phase affected the stereoselectivity, and hydrophobic amines caused drastic effects.

INTRODUCTION

Direct liquid chromatographic resolution of racemates can be based on two different principles: the use of a chiral stationary phase or the use of a chiral mobile phase. Both types of chiral phase have been prepared in many different ways and have been reviewed recently[1]. However, many of the techniques described are only applicable to the resolution of amino acids and few studies have dealt with the resolution of racemic drugs.

The use of α_1-acid glycoprotein (α_1-AGP) as the chiral stationary phase for the resolution of racemic drugs has been reported recently[2–4]. These studies demonstrated that many different racemic drugs can be resolved by using α_1-AGP as the chiral stationary phase. The present paper describes the use of α_1-AGP as a chiral complexing agent in the mobile phase in combination with a non-chiral column. The possibility of regulating the capacity factors and the separation factors is investigated, as well as the retention behaviour of the solutes. It is also demonstrated that the retention of the solutes can be described by a simple equation, which enables the calculation of the affinity constants for the binding of the enantiomers of the drugs to α_1-AGP. This means that the technique described here can be an alternative ap-

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proach to the more time-consuming method of equilibrium dialysis for the determination of affinity constants of drugs to α_1-AGP. This technique, as well as the previously reported technique[2–4], also allow screening for stereoselective protein binding of enantiomers without radioactive isotopes being required.

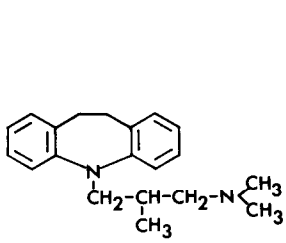
EXPERIMENTAL

Apparatus

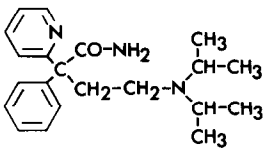
The high-performance liquid chromatography (HPLC) system consisted of a Waters M6000 A pump, a Waters U6K injector and a Shimadzu SPD-2A UV variable-wavelength detector. The pH was measured with an Orion Research Model 801A digital pH meter equipped with an Ingold combined electrode, Type 401.

Chemicals

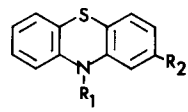
Racemic promethazine, propiomazine, alimemazine, thioridazine, trimipramine and disopyramide were of pharmacopeial grade and were obtained from drug manufacturers; (*R*)- and (*S*)-disopyramide were kindly supplied by Professor Wendel L. Nelson, School of Pharmacy, Department of Medicinal Chemistry, Seattle, WA, U.S.A.; (+)- and (−)-promethazine were prepared in our laboratory. The resolution and the determination of the absolute configuration of the promethazine enantiomers will be published elsewhere. The structures of the drugs are presented in Fig. 1.



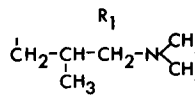
TRIMIPRAMINE



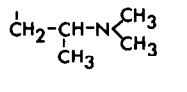
DISOPYRAMIDE



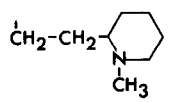
ALIMEMAZINE



PROMETHAZINE



PROPIOMAZINE



THIORIDAZINE

Fig. 1. The structures of the drugs.

N,N-Dimethyloctylamine (DMOA) was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.); α_1-acid glycoprotein (α_1-AGP) was prepared as described previously[2]; LiChrosorb Diol, with a mean particle diameter of 5 μm, was obtained from E. Merck (Darmstadt, F.R.G.). All mobile phases contained 0.05% sodium azide to prevent the growth of microorganisms.

Column preparation

The LiChrosorb Diol column (150 × 3.2 mm I.D.) was packed by the balanced-density slurry technique described previously[5].

RESULTS AND DISCUSSION

Effect of α_1-AGP concentration on retention

The dependence of the capacity factors of the enantiomers of promethazine, alimemazine and trimipramine on the concentration of α_1-AGP was investigated with mobile phases containing 4.4–15.2 μM α_1-AGP in phosphate buffer, pH 7.55, μ = 0.05. The results of this study are demonstrated in Fig. 2. It can be noticed that the capacity factors decrease with increasing protein concentration in the mobile phase, which indicates formation of a complex between the solutes and α_1-AGP. It has been demonstrated previously that basic drugs are retained by a specific interaction with α_1-AGP when this protein is used as a chiral stationary phase for column chromatographic resolution of racemic drugs[2,3].

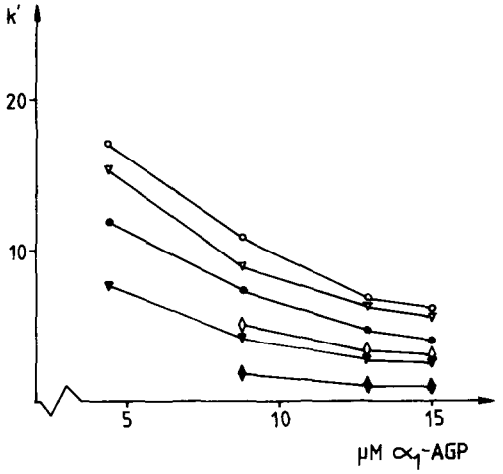


Fig. 2. Influence of α_1-AGP concentration on the capacity factors. Column, LiChrosorb Diol (150 × 3.2 mm I.D.); mobile phase, phosphate buffer, pH 7.55, μ = 0.05 with addition of α_1-AGP; flow-rate, 0.5 ml/min. Samples: ● = (−)-promethazine; ○ = (+)-promethazine; ▼ = alimemazine I; ▽ = alimemazine II; ◆ = trimipramine I; ◇ = trimipramine II.

The retention behaviour of the solutes was studied on the basis of the data presented in Fig. 2. The equilibria that are assumed to exist are summarized in Fig. 3. LH^+ is the predominant form of the solute in the mobile phase. This is supported by the fact that *ca.* 98% of the solute is ionized at pH 7.5, because the pK_a values of the solutes are *ca.* 9.1[6]. Therefore, it is reasonable to assume that it is the LH^+ form of the solute that is adsorbed (as ion pair) on the solid phase and forms a complex with the protein in the mobile phase.

The equilibrium constant for the distribution of the solutes, as ion pairs with phosphate, between the solid phase and the eluent is given by

$$K_{LHZA_s} = \frac{[LHZA]_s}{[LH^+]_m[Z^-]_m[A]_s} \quad (1)$$

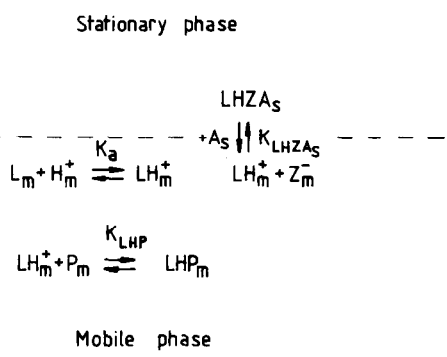


Fig. 3. Schematic illustration of the equilibria involved in the chromatographic process with α_1-AGP in the mobile phase. L_m = non-ionized solute; LH_m^+ = protonated solute; Z_m^- = phosphate ion; P_m = α_1-AGP; LHP_m = complex of α_1-AGP and protonated solute; $LHZA_s$ = solute ion pair with phosphate, adsorbed on the stationary phase; K_a = dissociation constant; K_{LHZA_s},K_{LHP} = formation constants.

where $[LHZA]_s$ is the ion pair adsorbed on the adsorption sites, A_s, of the diol column. $[LH^+]_m$ and $[Z^-]_m$ are the concentrations in the mobile phase of the solute in its ionized form and of phosphate, respectively. If it is assumed that the solutes are bound to one binding site of the protein (P), the equilibrium constant for the complex formation, K_{LHP}, can be expressed by

$$K_{LHP} = \frac{[LHP]_m}{[LH^+]_m[P]_m} \tag{2}$$

It can be assumed that the drug–protein complex, $[LHP]_m$, is only adsorbed on the outside of the diol silica particles, owing to the small pore size of this silica. Therefore, the adsorption of the drug–protein complex is neglected in the following discussion, because the outer surface of the silica particles is very small compared with the surface within the pores. This assumption is supported by the results obtained in a previous study, when α_1-AGP was immobilized on epoxide-activated silica with pore diameters of 100 Å and 300 Å in the underivatized form[2]. In that study it was demonstrated that α_1-AGP was preferentially bound to the outside of the silica particles of the 100-Å silica. The commercial LiChrosorb Diol silica has the same pore diameter as the epoxide form of the 100-Å silica.

If the above assumptions are valid and the solute ion pairs are adsorbed on only one type of binding site (A_s) of the solid phase, the capacity factor, k', is given by

$$k' = q\frac{[LHZA]_s}{[LH^+]_m + [LHP]_m} \tag{3}$$

where q (g/l) is the phase ratio and $[LHZA]_s([LH^+]_m + [LHP]_m)^{-1}$ is the distribution ratio of the solute between the solid phase and the eluent.

The adsorption capacities of the solid phase and the protein are limited and can be expressed by

$$K_0 = [A]_s + [LHZA]_s \tag{4}$$

$$NC_p = [P]_m + [LHP]_m \tag{5}$$

where $[A]_s$ and $[P]_m$ are the concentration of free binding sites on the solid phase and on the protein, respectively, C_p is the total concentration of α_1-AGP, and N is the number of sites per mole of protein. If it is assumed that the solutes are bound to only one site of the protein, *i.e.* $N = 1$, a combination of eqns. 1–5 gives

$$k' = \frac{qK_0K_{LHZA_s}[Z^-]_m}{(1 + K_{LHZA_s}[LH^+]_m[Z^-]_m)\left(1 + \frac{K_{LHP}C_p}{1 + K_{LHP}[LH^+]_m}\right)} \tag{6}$$

If a small amount of solute is injected, it can be assumed that the solute concentration in the sample zone on the column is low enough to obtain linear adsorption isotherms. This means that

$$K_{LHZA_s}[LH^+]_m[Z^-]_m \ll 1$$

and $K_{LHP}\,[LH^+]_m \ll 1$

and that eqn. 6 can be reduced to

$$k' = \frac{qK_0K_{LHZA_s}[Z^-]_m}{1 + K_{LHP}C_p} \tag{7}$$

Inversion of eqn. 7 gives

$$\frac{1}{k'} = \frac{1}{qK_0K_{LHZA_s}[Z^-]_m} + \frac{K_{LHP}C_p}{qK_0K_{LHZA_s}[Z^-]_m} \tag{8}$$

The experiments were performed at constant phosphate concentration and pH, which means that $qK_0K_{LHZA_s}[Z^-]_m$ is constant. A plot of $1/k'$ *versus* the α_1-AGP concentration, in accordance with eqn. 8, gave a linear relationship in the protein concentration range 4.4–15.2 μM, which is demonstrated in Fig. 4. The affinity constants for the binding of the enantiomers to the chiral protein were calculated from the slopes and the intercepts and are given in Table I. The affinity constant for chlorpromazine, obtained by equilibrium dialysis, is also included in Table I for comparison[7,8]. From the plots in Fig. 4 it can be seen that straight lines were obtained; this is a good indication of the validity of eqn. 8. A further indication of the validity of this equation is the agreement between the affinity constant for chlorpromazine and the affinity constants calculated from the experimental results for the analogues promethazine and alimemazine. The only difference between the molecular structures of chlorpromazine and promethazine is the branched side-chain of promethazine and the 2-Cl atom in the phentiazine ring of chlorpromazine. However, alimemazine has one

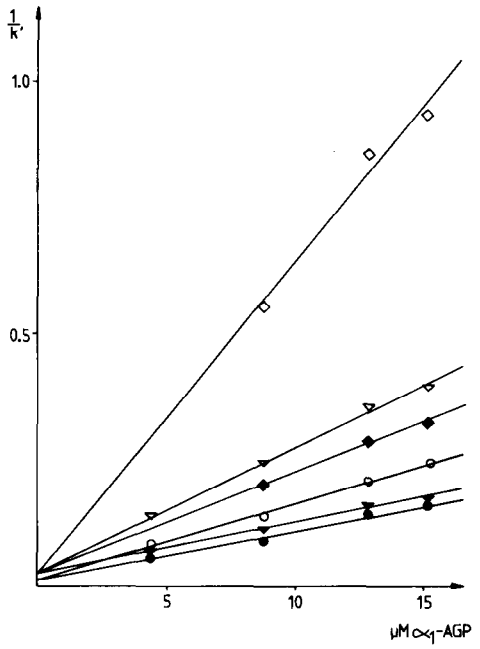


Fig. 4. Plot of $1/k'$ *versus* the α_1-AGP concentration P_m according to eqn. 8. Conditions as in Fig. 1. Samples: ◇ = trimipramine I; ◆ = trimipramine II; ▽ = alimemazine I; ▼ = alimemazine II; ○ = (−)-promethazine; ● = (+)-promethazine.

TABLE I

AFFINITY CONSTANTS CALCULATED FROM THE PLOTS IN FIG. 3

	$K_{LHP} \cdot 10^{-6}$ (M^{-1})
(−)-Promethazine	1.11
(+)-Promethazine	0.71
Alimemazine I	0.87
Alimemazine II	0.36
Trimipramine I	2.15
Tripipramine II	0.69
Chlorpromazine	1 and 3*

* The two affinity constants for chlorpromazine are taken from refs. 7 and 8, respectively.

extra carbon in the branched side-chain (*cf.* Table I). These results also indicate that the initial assumptions seem to be valid under the experimental conditions described.

Effect of α_1-AGP concentration on the separation factor

The influence of the protein concentration in the mobile phase on the separation factor, α, was studied at protein concentrations between 4.4 and 15.2 μM in phosphate buffer, pH 7.55. The results, presented in Fig. 5, show that the separation factor increases for alimemazine and trimipramine with increasing protein concentration, whereas the separation factor for the promethazine enantiomers is only

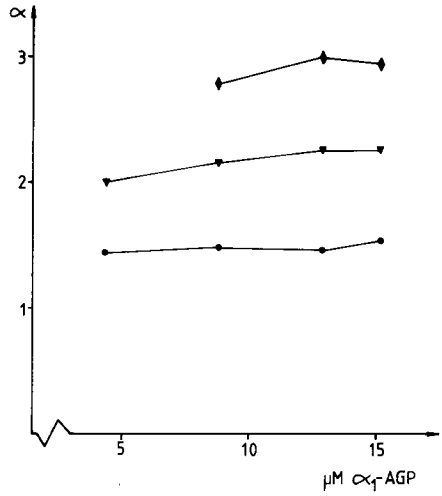


Fig. 5. Influence of α_1-AGP concentration on the separation factor. Conditions as in Fig. 1. Samples: ◆ = trimipramine; ▼ = alimemazine; ● = promethazine.

slightly affected. Rather high separation factors, between 2 and 3, were obtained for the enantiomers of alimemazine and trimipramine. It is interesting to note the difference in the α-values obtained for the enantiomers of alimemazine and promethazine, respectively. The only difference between the molecular structures of alimemazine and promethazine is one extra methylene carbon in the side-chain of alimemazine, which obviously gives this molecule better structural prerequisites for obtaining a different binding affinity for the enantiomers.

Regulation of the capacity factor and enantioselectivity with tertiary amines

The capacity factor of the solutes can be regulated by addition of N,N-dimethyloctylamine (DMOA) to the mobile phase. Fig. 6 shows that the capacity factors increase when DMOA is added to the mobile phase. The strongest effect of DMOA was obtained at concentrations up to 1 m*M*. Higher concentrations influenced the capacity factors to a lesser extent. The fact that the capacity factors increase with increasing DMOA concentration may indicate that DMOA competes with the solutes for the binding site of the protein. Other tertiary amines with different hydrophobicity, such as dimethylethylamine, triethylamine and tripropylamine, were also tested as mobile phase additives. The results (Table II) show that the addition of the less hydrophobic amines, dimethylethylamine and triethylamine, decreased the capacity factors, whereas the addition of tripropylamine and DMOA increased the capacity factors, compared with those obtained with only α_1-AGP in the mobile phase. It is reasonable to assume that the decrease in the capacity factors obtained by adding dimethylethylamine and triethylamine is caused by competition with the solutes, predominantly for binding to the hydrophilic solid phase rather than to the protein. This may be because these amines are more hydrophilic than tripropylamine and dimethyloctylamine.

The enantioselectivity is also significantly affected by the addition of the ter-

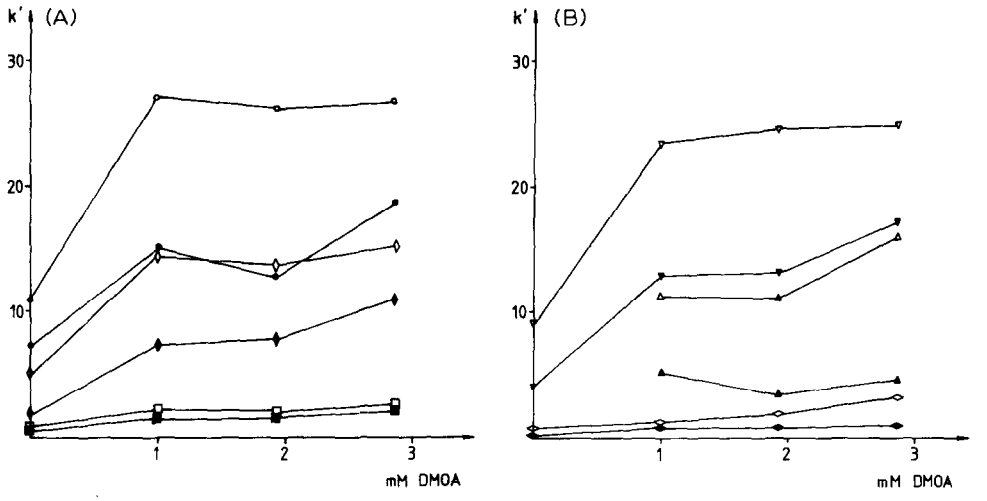


Fig. 6. Regulation of the capacity factor with DMOA. Column as in Fig. 1. Mobile phases, phosphate buffer, pH 7.55, μ = 0.05, containing 8.8 μM α_1-AGP and with addition of DMOA; flow-rate, 0.5 ml/min. Samples: (A) ○ = (+)-promethazine; ● = (−)-promethazine; ◇ = trimipramine II; ◆ = trimipramine I; □ = (*R*)-disopyramide; ■ = (*S*)-disopyramide; (B) ▽ = alimemazine II; ▼ = alimemazine I; △ = propiomazine II; ▲ = propiomazine I; ◇ = thioridazine II; ◆ = thioridazine I.

tiary amines to the mobile phase. Fig. 7 demonstrates the influence of the concentration of triethylamine on the separation factor for different solutes. It can be seen that the separation factors for trimipramine, alimemazine and promethazine are only slightly affected, whereas drastic changes resulted for disopyramide and propioma-

TABLE II

INFLUENCE OF DIFFERENT TERTIARY AMINES ON THE CAPACITY FACTORS AND THE SEPARATION FACTORS

Conditions: column, LiChrosorb Diol (150 × 3.2 mm I.D.); mobile phase, phosphate buffer, pH 7.55, containing 8.8 μM α_1-AGP and a tertiary amine. DMEA = dimethylethylamine, TEA = triethylamine, TPA = tripropylamine, DMOA = dimethyloctylamine; I and II = the enantiomer with the lowest and highest capacity factors, respectively.

	No amine		*1 mM DMEA*		*1 mM TEA*		*1.3 mM TPA*		*1 mM DMOA*	
	k'	α	*k'*	α	*k'*	α	*k'*	α	*k'*	α
(−)-Promethazine	7.23	1.49	4.89	1.57	4.38	1.60	9.03	1.49	15.6	1.73
(+)-Promethazine	10.75		7,67		7.02		13.44		27.1	
Alimemazine I	4.11	2.16	3.21	2.14	3.24	2.01	4.70	2.12	12.3	1.90
Alimemazine II	8.80		6.87		6.48		11.0		23.4	
Propiomazine I	2.70*	1.0	3.25	1.0	2.35	1.40	4.82	1.0	5.10	2.20
Propiomazine II					3.28				11.2	
Trimipramine I	1.80	2.79	1.54	2.65	1.20	2.80	2.41	2.74	7.42	1.97
Trimipramine II	5.02		4.08		3.79		6.60		14.6	
(*S*)-Disopyramide	0.34	2.36	0.89	1.0	0.68	1.0	0.70	1.0	1.30	1.71
(*R*)-Disopyramide	0.80								2.22	

* Mobile phase, phosphate buffer, pH 7.56, containing 12.1 μM α_1-AGP. The enantiomers are not available.

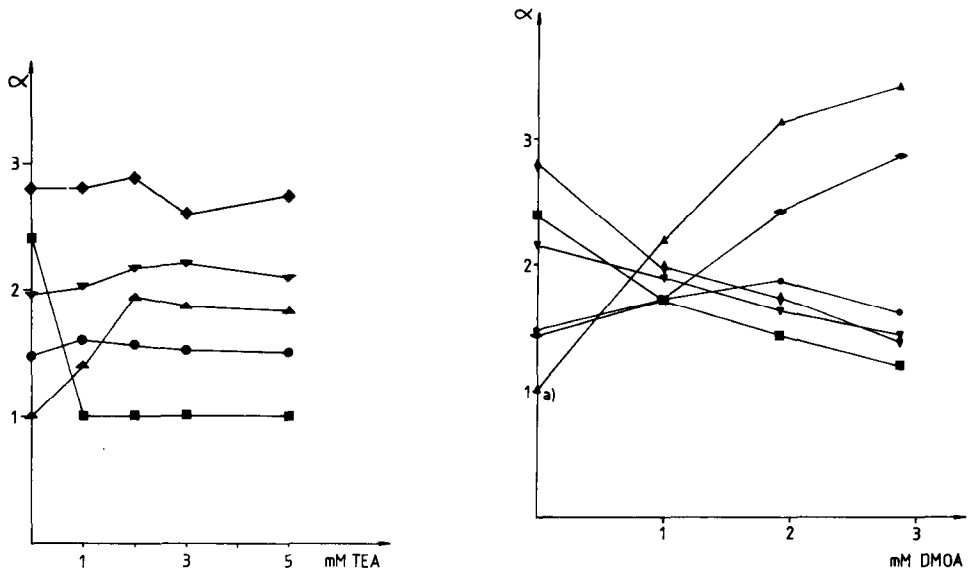


Fig. 7. Influence of triethylamine concentration on the separation factor. Mobile phases, phosphate buffer, pH 7.55, containing 8.8 μM α_1-AGP and with addition of triethylamine (TEA). Other conditions as in Fig. 1. Samples: ◆ = trimipramine; ■ = disopyramide; ▼ = alimemazine; ● = promethazine; ▲ = propiomazine.

Fig. 8. Influence of the DMOA concentration on the separation factor. Conditions as in Fig. 5; samples as in Fig. 6. (a) Mobile phase, phosphate buffer, pH 7.56, μ = 0.05, containing 12.8 μM α_1-AGP.

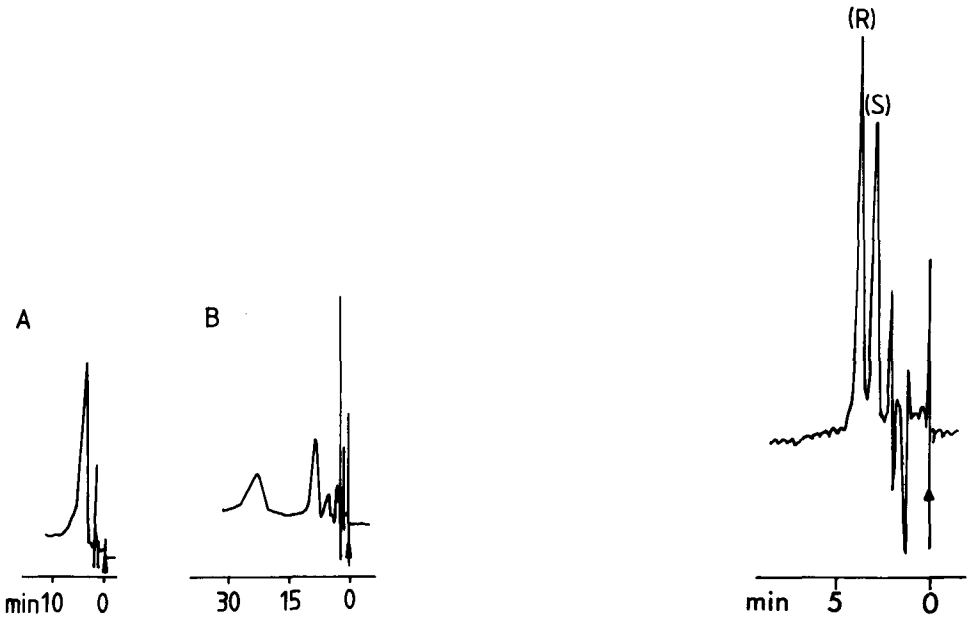


Fig. 9. Influence of DMOA on the enantioselectivity of propiomazine. Column as in Fig. 1. (A) Mobile phase, phosphate buffer, pH 7.56, μ = 0.05, containing 12.8 μM α_1-AGP; flow-rate, 0.5 ml/min. (B) Mobile phase, phosphate buffer, pH 7.54, μ = 0.05, containing 8.8 μM α_1-AGP and 2.9 mM DMOA; flow-rate, 0.5 ml/min.

Fig. 10. Resolution of racemic disopyramide. Column as in Fig. 1. Mobile phase, phosphate buffer, pH 7.57, μ = 0.05, containing 8.8 μM α_1-AGP and 1.93 mM DMOA. flow-rate, 0.5 ml/min.

zine. Chromatography of propiomazine with a mobile phase containing α_1-AGP but no tertiary amine gave no resolution of the enantiomers. On increasing the triethylamine concentration to 2 m*M*, a separation factor of 1.94 was obtained. The opposite effect was obtained for disopyramide. A separation factor of 2.4 was obtained by using only α_1-AGP in the mobile phase. However, when triethylamine was added, the enantioselectivity was completely lost. A more drastic effect on the enantioselectivity was obtained by adding DMOA to a mobile phase containing 8.8 μM α_1-AGP in phosphate buffer, pH 7.56. The results of this study are shown in Fig. 8. The separation factors decreased drastically for disopyramide, trimipramine and alimemazine, whereas a large increase of the separation factors resulted for the propiomazine and thioridazine enantiomers. The separation factor for the promethazine enantiomers is influenced to a minor extent.

It is very interesting to note the remarkable change of the separation factor for the propiomazine enantiomers from 1.0 to 3.4, produced by an increase of the DMOA concentration from 0 to 2.9 m*M*. The chromatograms obtained without and with DMOA are shown in Fig. 9. A reasonable explanation may be that the tertiary amines affect the conformation of the protein. It has been demonstrated that addition of sodium dodecylsulphate to an α_1-AGP solution influences the conformation of α_1-AGP[9].

The usefulness of the chromatographic system described is shown by the resolution of the enantiomers of disopyramide (Fig. 10).

ACKNOWLEDGEMENT

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