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Separation of the enantiomers of β-receptor blocking agents and other cationic drugs using a CHIRAL-AGP® column

Binding properties and characterization of immobilized α_1-acid glycoprotein

MÄRIT ENQUIST* and JÖRGEN HERMANSSON[a]
Apoteksbolaget AB, Central Laboratory, Department of Biomedicine, S 105 14 Stockholm (Sweden)
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ABSTRACT

The effect of the immobilization procedure on the conformation of α_1-acid glycoprotein (AGP) was investigated by recording the fluorescence spectra of native and immobilized AGP. A 20-nm red shift was obtained for the immobilized form of AGP compared with the emission maximum of 338 nm obtained for native AGP. This demonstrates that the tryptophan residues are exposed on the protein surface after immobilization, indicating that the immobilized form of AGP has a more unfolded structure than the native AGP. The effect of N,N-dimethyloctylamine on the enantioselectivity for some fentiazine derivatives, observed with immobilized AGP, was equal to that obtained with AGP as a chiral complexing agent in the mobile phase. This demonstrates that even though the immobilization procedure affects the conformation of the protein there still exist large similarities between native and immobilized AGP concerning chiral recognition.

The adsorption isotherm of (−)-terodiline was studied by use of the breakthrough technique. The adsorption isotherm indicates that (−)-terodiline is adsorbed to one site with high affinity and at least one more site with lower affinity. It was also observed that the enantiomers of amines, acids and non-protolytic compounds compete with the cationic compound, (−)-terodiline, for binding to the same sites.

The β-receptor blocking agents atenolol, metoprolol, pindolol, alprenolol, oxprenolol and propranolol were resolved on a CHIRAL-AGP® column. The retention and enantioselectivity are highly influenced by the structure of the solute and the nature of the uncharged mobile phase additives. Separation factors of 1.2–1.8 were obtained for the β-blockers under the studied conditions.

INTRODUCTION

An α_1-acid glycoprotein (AGP) column has been used for the resolution of a large number of chiral drugs [1]. The broad applicability of the chiral phases can be ascribed to the fact that the binding sites contain many different binding groups, giving the possibility of stereoselective interactions with a broad range of solutes, such as amines, acids and non-protolytes. Further, it has been demonstrated that it is possible to affect the chiral stationary phase reversibly with organic modifiers in such a way

[a] Present address: ChromTech AB, Box 512, 145 63 Norsborg, Sweden.

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that enantioselectivity can be induced. The effects of mobile phase additives of different character (hydrogen bonding properties, hydrophobicity and charge) and concentration and of the mobile phase pH and temperature on the retention and enantioselectivity have been discussed in several papers [2–6] and have also been reviewed recently [1]. This paper discusses the effects of uncharged modifiers, with different bonding properties, on the retention and enantioselectivity for a series of β-receptor blocking agents, with the purpose of establishing optimum separation conditions for these solutes.

It is well known that the conformations of proteins can be affected when they are bound to solid phases with different character [7]. Such conformational changes can be characterized using fluorescence spectroscopy. This technique was used in this study to determine differences in conformation between immobilized and non-immobilized AGP.

It has been suggested that amines, acids and non-protolytic compounds are bound to a single binding site on the AGP molecule [8,9]. Others [10,11], in contrast, have reported two different binding sites on AGP. In order to characterize the binding site(s) of immobilized AGP, adsorption studies were performed using (−)-terodiline as the model compound. Chromatographic experiments using (−)-terodiline as a mobile phase additive were also performed in order to obtain a deeper insight into the nature of the binding site(s).

EXPERIMENTAL

Apparatus

The liquid chromatographic system consisted of a Waters Assoc. M 6000 A pump, a Waters Assoc. U6K injector and a Shimadzu SPD-2A variable-wavelength UV detector operated at 215 nm. The chiral column was a commercially available CHIRAL-AGP® column from ChromTech (Norsborg, Sweden). Fluorescence spectr a of AGP were obtained with a Shimadzu RF-500 spectrofluorimeter.

Chemicals

(+)- and (−)-terodiline were kindly supplied by Kabi (Stockholm, Sweden). The other test compounds were obtained from their manufacturers. Analytical-reagent grade 1- and 2-propanol were obtained from E. Merck (Darmstadt, F.R.G.) and acetonitrile and methanol (UV grade) from FSA Laboratory Supplies (Loughborough, U.K.). Racemic 2-butanol and propionitrile were purchased from Fluka (Buchs, Switzerland), 1-butanol (Aristar) from BDH (Poole, U.K.), ethanol (95%) from Kemethyl (Stockholm, Sweden) and N,N-dimethyloctylamine (DMOA) from ICN Pharmaceuticals (Plainview, NY, U.S.A.).

Chromatographic conditions

Chromatography was performed at room temperature and the flow-rate was 0.9 ml/min. The mobile phases used were phosphate buffers containing different concentrations of uncharged modifiers, DMOA or (−)-terodiline. The concentration of phosphate was 0.01 *M* in the mobile phases containing uncharged modifiers and 0.02 *M* in those phases containing charged modifiers. The pH was adjusted to 7.0 with sodium hydroxide. The mobile phases were degassed in an ultrasonic bath before being

used. The hold-up volume of the column (V_m) was determined by injection of water or mobile phase with a slight difference in composition.

Fluorescence spectra of AGP

AGP in solution. AGP was dissolved in 0.01 *M* phosphate buffer (pH 7.0) at a concentration of 100 μg/ml ($2.5 \cdot 10^{-6}$ *M*). An excitation wavelength of 295 nm was used. Emission spectra were recorded between 300 and 400 nm.

Immobilized AGP. Packing material from the CHIRAL-AGP column was packed into a 1-mm flow cell and the material was equilibrated with 0.01 *M* phosphate buffer (pH 7.0) [7]. The fluorescence was measured as above.

Adsorption of (−)-terodiline on the CHIRAL-AGP column

The amounts of (−)-terodiline adsorbed by the chiral stationary phase from the mobile phase were determined by the breakthrough technique [12]. The column was first equilibrated with a mobile phase of phosphate buffer (pH 7.0) (0.02 *M* phosphate). A second mobile phase (with the same phosphate concentration and pH) containing (−)-terodiline was then pumped onto the column. The loading of the amine was followed by UV detection at 215 nm. The amount of (−)-terodiline, $[T]_s$, in moles adsorbed on the column was calculated with the equation

$$[T]_s = (V_R - V_m)C_m \quad (1)$$

where the breakthrough volume (V_R) is the net retention volume corresponding to the front. V_R is measured as the volume that has been pumped through the column from the moment the mobile phase containing (−)-terodiline is introduced onto the column to the inflection point of the breakthrough curve. C_m is the concentration of (−)-terodiline in the mobile phase. After each loading occasion the column was washed with 50 ml of 20% (v/v) 1-propanol in water and reconditioned with 50 ml of phosphate buffer (pH 7.0).

RESULTS AND DISCUSSION

Characterization of AGP

AGP is a human plasma protein which consists of a single peptide chain containing 181 amino acids. Five carbohydrate units are linked to the peptide chain via the asparagine residues. AGP contains many binding groups of different character, acidic and basic groups and hydrogen-bonding groups. The protein has a moderate hydrophobic character as it contains many hydrophobic amino acid residues such as tryptophan, lysine and phenylalanine [13].

In the CHIRAL-AGP column, AGP is immobilized onto silica particles by a covalent linkage and cross-linking of adjacent protein molecules [1]. Some of the binding groups which are free for interaction in native AGP are utilized in the immobilization and cross-linking procedures. The conformation of both native and immobilized AGP was studied using fluorescence spectroscopy as described under Experimental.

The fluorescence spectra of native AGP in solution and of AGP in the immobilized form are given in Fig. 1a and b. The idea behind this experiment was to

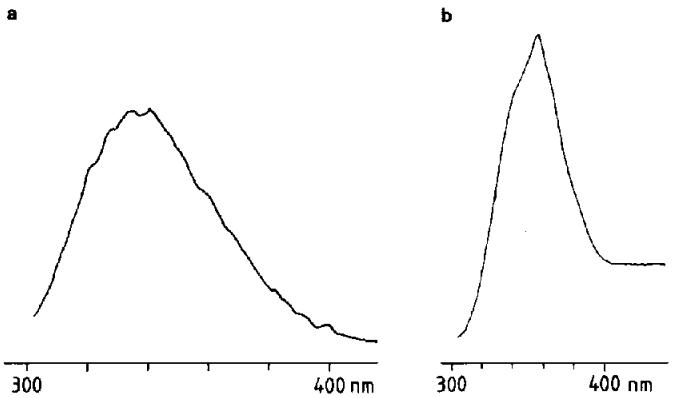


Fig. 1. (a) Emission spectrum of AGP dissolved in a 0.01 *M* phosphate buffer (pH 7.0). AGP concentration, 100 μg/ml; excitation, 295 nm; band width, 1.5 nm; scan speed, low. (b) Emission spectrum of AGP immobilized on silica in 0.01 *M* phosphate buffer (pH 7.0). Conditions as in (a).

study the emission of the three tryptophan residues in the AGP molecule, as differences in the emission maximum of the tryptophans reflect differences in the protein conformation. A buried tryptophan residue has another emission maximum compared with the one that is exposed on the surface of the protein molecule [7]. An emission maximum of 338 nm was obtained for AGP in solution, indicating that the tryptophans in native AGP are buried within the hydrophobic microenvironment of the molecule. However, a 20-nm red shift was obtained for the immobilized form of AGP. Obviously, the immobilization of AGP onto the silica surface influences the protein conformation in such a way that one, two or all three tryptophans are exposed on the surface of the protein molecule. In order to simulate a surface-exposed tryptophan residue, a fluorescence spectrum was also recorded for N-acetyltryptophanamide, which gave an emission maximum of 360 nm, which is in good agreement with the value obtained for tryptophan of immobilized AGP.

Despite the fact that the conformation of AGP is affected by the immobilization procedure, there are still large similarities between native and immobilized AGP with respect to chiral recognition. This was demonstrated by comparing the effect of DMOA on the enantioselectivity for some fentiazine derivatives (Fig. 2) on a column with immobilized AGP (CHIRAL-AGP) (Fig. 3) and with AGP as a chiral complexing agent in the mobile phase (Fig. 4, data from ref. 14). The enantioselectivity increased for propiomazine and promethazine whereas it decreased for trimipramine and alimemazine on increasing the DMOA concentration in the mobile phase. The fentiazines were affected in the same way by DMOA both when using the protein as an immobilized chiral selector and when using AGP as a chiral complexing agent in the mobile phase.

Adsorption of (−)-terodiline on the CHIRAL-AGP column

In order to study the binding of enantiomers to the chiral stationary phase, the adsorption isotherm of the cationic compound (−)-terodiline was determined using the breakthrough technique. In Fig. 5 the amount of (−)-terodiline adsorbed to a CHIRAL-AGP column is plotted against the concentration of (−)-terodiline in the

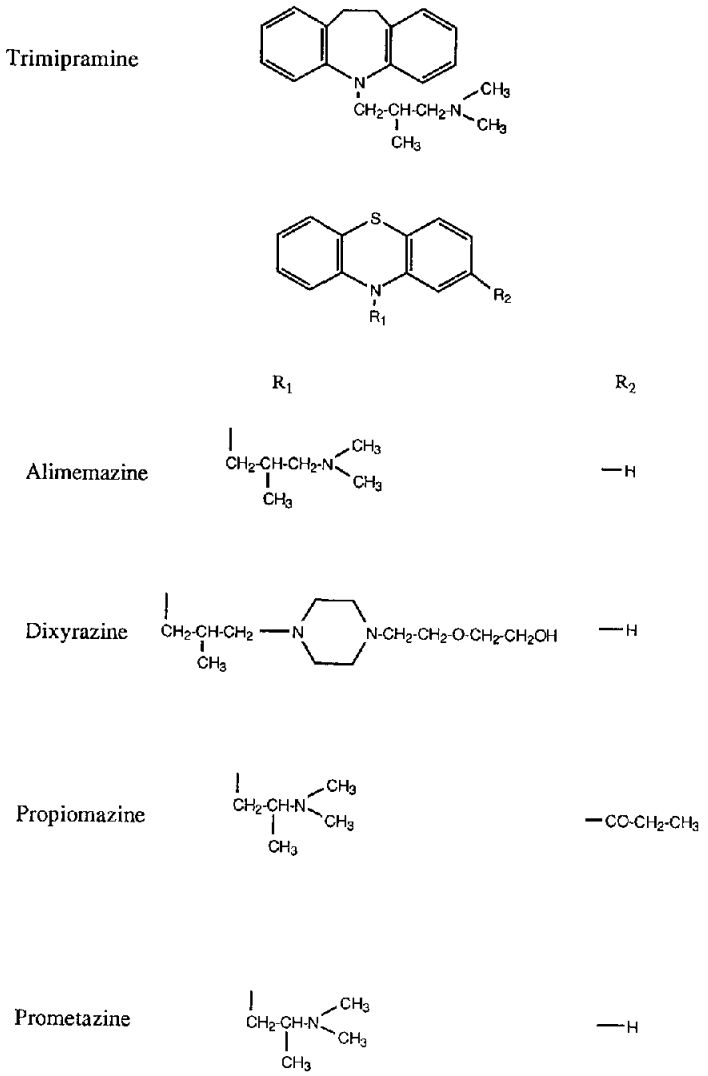


Fig. 2. Structures of fentiazine derivatives and trimipramine.

mobile phase (0 79.5 μM). At low concentrations of (−)-terodiline in the mobile phase the amount of adsorbed (−)-terodiline increases dramatically with increasing concentration. At concentrations above 15 μM the curve levels off and the slope is changed. No saturation of the stationary phase was observed in the concentration range studied. The shape of the curve indicates that (−)-terodiline is adsorbed to more than one site, one site with high affinity and at least one more site with lower affinity. It has been demonstrated previously [6,15] that the charged compound sodium dodecyl sulphate changes the secondary structure of AGP. Thus, an alternative explanation can be that (−)-terodiline at a certain concentration affects the protein in such a way

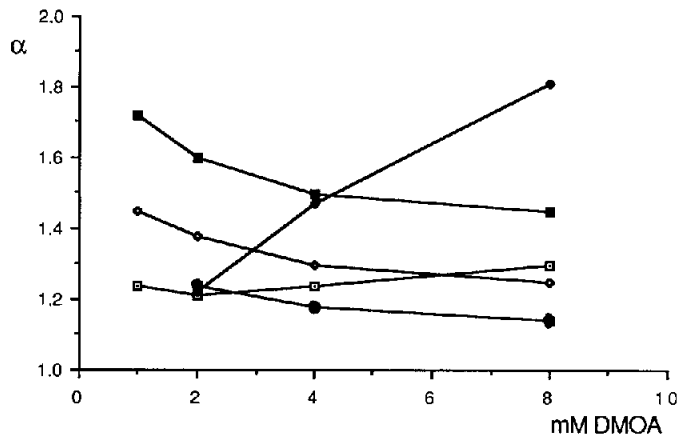


Fig. 3. Effects of the concentration of DMOA on the enantioselectivity of fentiazine derivatives and trimipramine using immobilized AGP as chiral stationary phase. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.0) (0.01 *M* phosphate) containing 2% (v/v) 2-propanol and different concentrations of DMOA; flow-rate, 0.9 ml/min. ◆, Propiomazine; ■, trimipramine; ◇, alimemazine; □, prometazine; ●, dixyrazine.

that new binding groups in the binding site(s) are exposed and become accessible for binding of solute molecules. The resolution of terodiline on the CHIRAL-AGP column using a mobile phase of phosphate buffer (pH 7.2) containing 15% (v/v) 2-propanol is demonstrated in Fig. 6.

Effects of (−)-terodiline on the retention and enantioselectivity

The effects of uncharged modifiers on the enantioselectivity and retention have been discussed recently [1,6]. It was suggested that the enantiomers compete with the

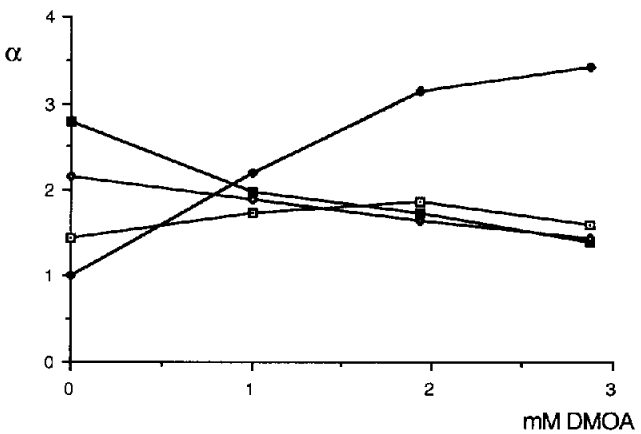


Fig. 4. Effects of the concentration of DMOA on the enantioselectivity of fentiazine derivatives and trimipramine using AGP as chiral complexing agent in the mobile phase. Column, LiChrosorb diol (150 × 3.2 mm I.D.); mobile phase, phosphate buffer (pH 7.55) (ionic strength, $\mu = 0.05$) containing 8.8 μM AGP and different concentrations of DMOA; flow-rate, 0.5 ml/min. Symbols as in Fig. 3. Data from ref. 14.

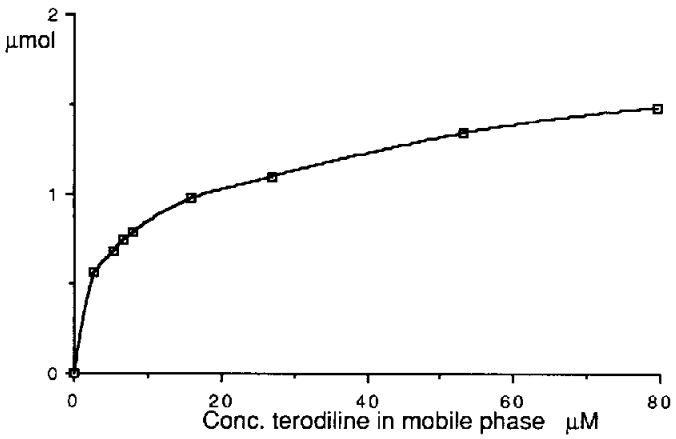


Fig. 5. Adsorption isotherm of (−)-terodiline. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.0) (0.02 *M* phosphate).

modifiers for binding to the same site(s) on the protein and that the hydrophobicity and hydrogen-bonding properties are of great importance for the effects on the enantioselectivity. The influence of charged modifiers on the retention and enantioselectivity is more complicated. The modifiers might affect the conformation of the protein as discussed above and it can also compete with the enantiomers for the binding groups of the protein. In this study the effect of the cationic compound (−)-terodiline on the retention of amines, non-protolytic compounds and strong and weak acids was examined. It was observed that the retentions of the amines metoprolol and 2-amino-5-methoxytetralin and of bendroflumethiazide and ethyl mandelate (a weak acid uncharged at pH 7 and a non-protolyte, respectively) decreased with

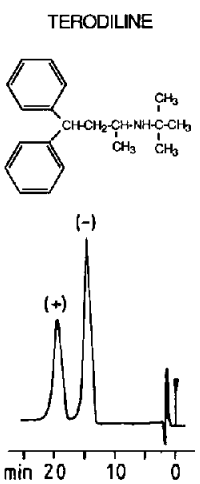


Fig. 6. Resolution of the enantiomers of terodiline. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.4) (0.01 *M* phosphate) containing 15% 2-propanol; flow-rate, 0.6 ml/min.

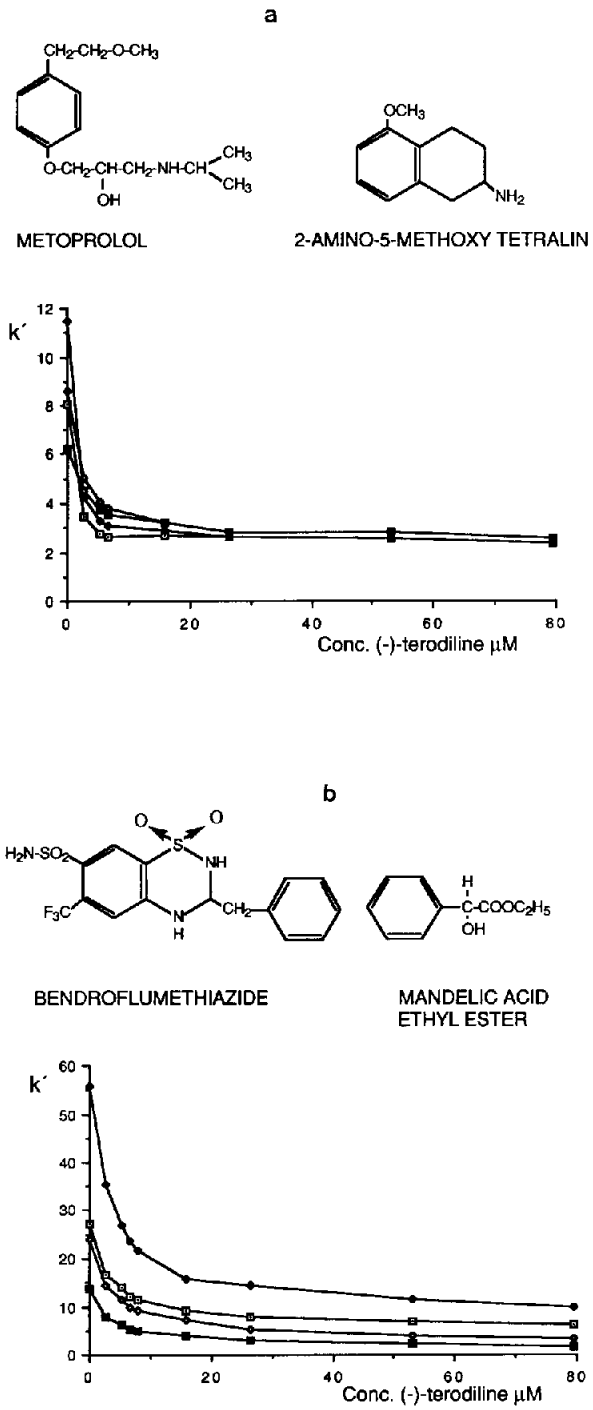

a
METOPROLOL
2-AMINO-5-METHOXY TETRALIN
k´
Conc. (-)-terodiline μM
b
BENDROFLUMETHIAZIDE
MANDELIC ACID
ETHYL ESTER
k´
Conc. (-)-terodiline μM

Fig. 7.

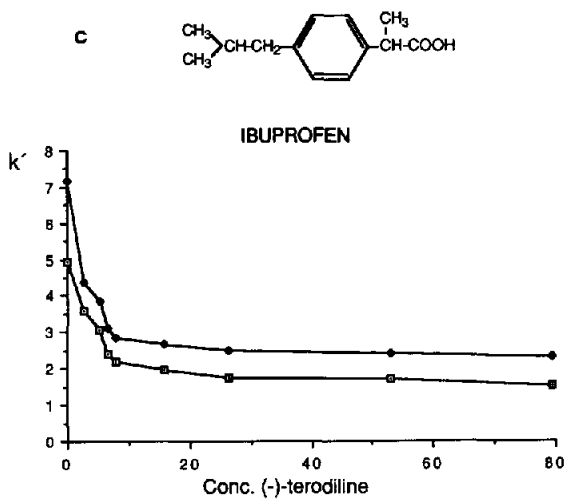


Fig. 7. Effect of (−)-terodiline content in the mobile phase on retention. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.0) (0.02 *M* phosphate) containing different concentrations of (−)-terodiline; flow-rate, 0.9 ml/min. (a) □, (*R*)-Metoprolol; ◆, (*S*)-metoprolol; ■, (1) 2-amino-5-methoxytetralin; ◇, (2) 2-amino-5-methoxytetralin. (b) □, (1) Bendroflumethiazide; ◆, (2) bendroflumethiazide; ■, (1) ethyl mandelate; ◇, (2) ethyl mandelate. (c) □, (1) Ibuprofen; ◆, (2) ibuprofen. [(1) and (2) refer to the two enantiomers.]

increasing concentration of (−)-terodiline (Fig. 7a and b). Fig. 7c shows that the retention of the enantiomers of the anionic compound ibuprofen also decreased with increasing concentration of (−)-terodiline. At (−)-terodiline concentrations below 10 μM the capacity factors for the enantiomers decreased dramatically, but at higher concentrations (10–20 μM) the decrease levelled off. The enantiomers of the test solutes are still retained with capacity factors of 1.5–9.9 at a concentration of 79.5 μM(−)-terodiline. It can be concluded that anionic, cationic and also non-protolytic compounds are bound to and compete for the same sites on the protein. The above data also suggest that the solutes, independently of their nature, are bound to one high-affinity and one low-affinity site. The high-affinity site is described by the steep part of the capacity factor curves obtained at low concentrations of (−)-terodiline demonstrated in Fig. 7a–c. The second site can be an unspecific binding area on the protein or a site created by the binding of (−)-terodiline to the protein, as discussed above.

It is interesting that the enantioselectivity of the amines metoprolol and 2-amino-5-methoxytetralin disappeared at a concentration of *ca.* 20 μM(−)-terodiline but the chiral selectivity for the uncharged solutes decreased only slightly. The separation factor for the enantiomers of ibuprofen increased with increasing concentration of (−)-terodiline, as demonstrated in Fig. 8. Similar results have been observed previously for the arylpropionic acid derivatives ibuprofen, naproxen and ketoprofen on adding another cation, DMOA, to the mobile phase [2,3].

The above effects of (−)-terodiline on the retention and the enantioselectivity may indicate that the solutes of different character interact with different binding groups in the same binding sites.

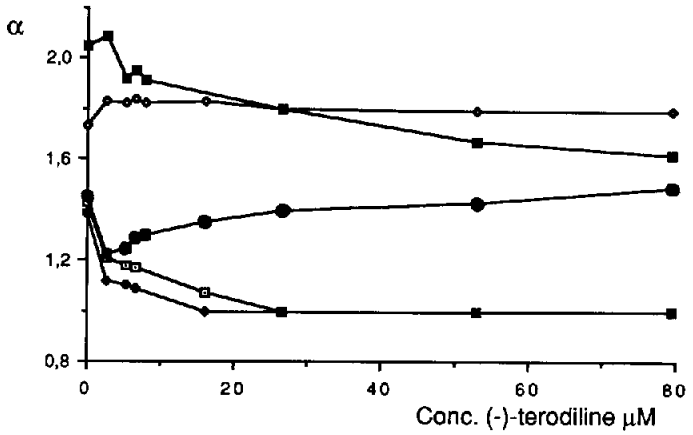
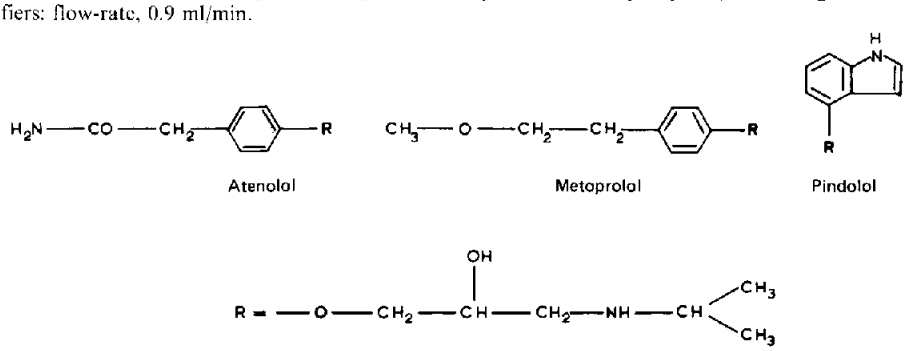


Fig. 8. Effect of (−)-terodiline on enantioselectivity. Conditions as in Fig. 7. □, Metoprolol; ◆, 2-amino-5-methoxytetralin; ■, bendroflumethiazide; ◇, ethyl mandelate; ●, ibuprofen.

TABLE I

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR β-RECEPTOR BLOCKING AGENTS

Column, CHIRAL-AGP; mobile phase, phosphate buffer (pH 7.2) (0.01 *M* phosphate) containing different modifiers; flow-rate, 0.9 ml/min.

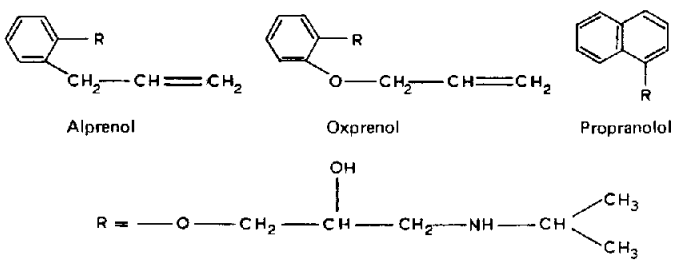


Modifier	Concentration (*M*)	Atenolol		Metoprolol		Pindolol	
		k'_1	α	k'_1	α	k'_1	α
–	–	4.58	1.31	37.1	1.36		
Methanol	1.48	3.08	1.11	8.64	1.37	–	–
	2.47	2.91	1.05	6.09	1.25	32.3	1.20
Ethanol	0.88	3.21	1.0	5.55	1.15	22.5	1.21
	2.11	2.84	1.0	3.73	1.0	8.40	1.29
1-Propanol	0.66	3.09	1.0	4.14	1.0	8.98	1.0
	1.06	2.88	1.0	3.43	1.0	6.66	1.0
1-Butanol	0.22	3.32	1.0	4.68	1.0	9.01	1.0
	0.44	4.13	1.0	3.85	1.0	7.30	1.0
2-Propanol	0.13	3.19	1.05	7.93	1.22	41.1	1.08
	1.06	2.53	1.0	3.13	1.0	6.85	1.04
Acetonitrile	0.95	3.59	1.0	5.93	1.11	28.6	1.53
	2.85	–	–	–		6.82	1.23

TABLE II

INFLUENCE OF UNCHARGED MODIFIERS ON k' AND α FOR β-RECEPTOR BLOCKING AGENTS

Conditions as in Table I.



Modifier	Concentration (M)	Alprenol		Oxprenol		Propranolol	
		k'_1	α	k'_1	α	k'_1	α
—		—	—	—	—	—	—
Ethanol	1.76	49.7	1.49	33.9	1.28	—	—
	2.11	36.3	1.47	23.1	1.25	—	—
1-Propanol	0.665	22.8	1.20	15.4	1.04	41.9	1.11
	1.06	12.9	1.0	8.15	1.0	25.41	1.0
1-Butanol	0.218	25.3	1.16	17.1	1.08	—	—
	0.436	12.21	1.0	7.77	1.0	23.4	1.0
2-Propanol	1.06	19.6	1.21	15.7	1.12	34.6	1.24
	1.60	12.8	1.07	8.7	1.05	21.5	1.03
2-Butanol	0.436	18.3	1.13	14.9	1.04	33.0	1.06
Acetonitrile	0.951	—	—	—	—	—	—
	2.85	30.6	1.83	15.6	1.11	33.9	1.11

Direct resolution of β-receptor blocking agents

Atenolol, metoprolol, pindolol, alprenolol, oxprenolol and propranolol were resolved on a CHIRAL-AGP column using a mobile phase of phosphate buffer (pH 7.2) with and without uncharged modifiers. Tables I and II summarize the capacity factors (k') and separation factors (α) for these compounds. Practolol and acebutolol are not included as no enantioselectivity was obtained under the described conditions.

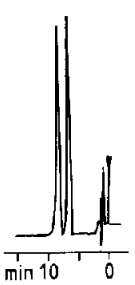


Fig. 9. Separation of the enantiomers of atenolol. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (pH 7.2) (0.01 M phosphate).

The enantiomers of atenolol have previously been reported to separate on an AGP column after preparation of an enantiomeric acetyl derivative [16]. It was observed that the retention of the first-eluted enantiomer decreased and that of the last-eluted enantiomer increased when atenolol was acetylated. The enantioselectivity thereby became dramatically improved. However, atenolol, can also be resolved in the underivatized form on the CHIRAL-AGP column using a mobile phase of phosphate buffer (pH 7.2) without modifier, as demonstrated in Fig. 9.

Atenolol and metoprolol are relatively hydrophilic compounds and the highest separation factors, 1.31 and 1.36, respectively, are obtained in pure phosphate buffer (pH 7.2). Addition of uncharged modifier decreases the retention and the enantioselectivity dramatically. However, low concentrations of methanol as mobile phase additive reduced the retention without destroying the chiral selectivity. A separation factor of 1.25 and a baseline separation were obtained within 8 min for the enantiomers of metoprolol when the concentration of methanol in the mobile phase was 10% (2.47 *M*). It is very important to study the effects on the enantioselectivity of modifiers with different bonding properties in order to obtain chromatographic systems giving high enantioselectivity and low retention. This is of special importance when determining drugs present in, for example, plasma samples at low concentrations.

Bioanalytical methods for studies of the enantiomers of metoprolol in plasma, using a CHIRAL-AGP column and mobile phases containing 2-propanol or acetonitrile, have recently been developed [17–19]. In order to improve the sensitivity in those studies the gradient technique [17,18] and the column enrichment technique [19] had to be used.

Another example of modifier effects is shown in Fig. 10, where the effects of 1-propanol and acetonitrile on the enantioselectivity of pindolol are compared. No enantioselectivity was observed for the enantiomers of pindolol, using a mobile phase containing a low concentration of 1-propanol. However, if 1-propanol, with both hydrogen-accepting and -donating properties, was replaced with 10% (2.85 *M*) acetonitrile, with only hydrogen-accepting properties, the enantiomers were baseline resolved with a separation factor of 1.36. Similar effects have been observed previously [1,5]. This demonstrates that the hydrogen-bonding properties of the uncharged modifiers can sometimes be crucial for the chiral recognition of a certain solute.

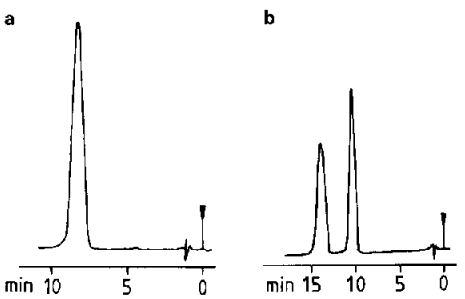


Fig. 10. Resolution of the enantiomers of pindolol. Column, CHIRAL-AGP (100 × 4 mm I.D.); mobile phase, phosphate buffer (0.01 *M* phosphate), (a) pH 7.4 containing 5% 1-propanol and (b) pH 7.3 containing 10% acetonitrile; flow-rate, 0.9 ml/min.

The retention and the enantioselectivity obtained for alprenolol are higher than those for oxprenolol, independently of the mobile phase modifier used (see Table II). However, when the solutes were chromatographed on the AGP column as oxazolidone derivatives both the capacity factors and separation factors were higher for oxprenolol [20]. Chromatography of alprenolol and oxprenolol using a mobile phase of phosphate buffer (pH 7.2) without an uncharged organic modifier gives a very high retention for these solutes. In order to decrease the retention, uncharged modifiers must be added to the mobile phase. However, the enantioselectivity also decreases with increasing concentration and hydrophobicity of the added modifiers. Ethanol and acetonitrile can be recommended as mobile phase additives for the resolution of alprenolol and oxprenolol. Much higher concentrations of the most hydrophilic modifiers, acetonitrile and methanol, can be used with maintained enantioselectivity, but such data are not included here. For example, alprenolol was separated with a separation factor of 1.25 using a mobile phase containing 7.61 *M* (40%, v/v) acetonitrile [6].

Propranolol is a relatively hydrophobic solute and the retention on the CHIRAL-AGP column is therefore high. In Table II it is demonstrated that the best chiral selectivity for propranolol is obtained using 2-propanol as mobile phase additive.

CONCLUSIONS

Fluorescence studies have demonstrated that the immobilized form of AGP has a more unfolded structure than native AGP. However, despite this, there are large similarities between native and immobilized AGP concerning chiral recognition. The chromatographic studies using (−)-terodiline in the mobile phase suggest that chiral solutes of different character, such as amines, acids and non-protolytic compounds are bound to the same sites on the protein. Adsorption experiments with (−)-terodiline indicate that one high-affinity site and at least one more site with lower affinity are involved in the binding of the solutes.

Six β-receptor blocking agents were separated on the CHIRAL-AGP column with a separation factor between 1.2 and 1.8 using a phosphate buffer (pH 7) with or without uncharged modifiers as the mobile phase. It was observed that the hydrogen-bonding properties of the modifier to a large extent affected the enantioselectivity.

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