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Separation of enantiomers of benzodiazepines on the Chiral-AGP column

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Abstract

The resolution of twenty-five 3-chiral and 5-chiral 1,4-benzodiazepines and related compounds was studied on a Chiral-AGP column. Relationship between the structure and enantioselective retention is discussed stressing the role of hydrophobic and hydrogen-bonding interactions as well as the importance of the conformation of the enantiomers. The majority of the benzodiazepines were separated with high separation factors and high resolution. The enantioselectivity was influenced by the nature and the concentration of the organic modifier in the mobile phase, as well as by the pH. Chiral chromatographic separation was compared with stereoselective binding on native AGP.

1. Introduction

Centrally chiral benzodiazepines belong to a special class of chiral compounds. Due to inversion of the boat-shaped diazepine ring the enantiomers have opposite chiral molecular conformation that determines the sign of their optical rotation [1].

Centrally chiral benzodiazepines can be conveniently resolved on various types of synthetic chromatographic chiral stationary phases [2,3]. Among protein phases immobilized human serum albumin (HSA) columns were successfully used for resolution of series of different 3-chiral 1,4-benzodiazepines [4–6], dihydrodiazepam [7], as well as tofisopam and its analogues [8,9]. The importance of these studies lies in the fact that these chromatographic results were useful to get information about the binding stereoselectivity on the native protein [10,11]. Since in plasma protein binding of benzodiazepines the contribution of albumin is dominating [12], binding stereoselectivity on α_1-acid glycoprotein (AGP) has not been studied to our knowledge, and even on the AGP column only very few benzodiazepine separations have been published [13].

In this work we present the HPLC separation on a Chiral-AGP column of a large number of racemic 3-substituted (hydroxy, alkoxy, acyloxy and alkyl) 1,4-benzodiazepines, 5-chiral 1,4-benzodiazepines and analogues as well as a 2,3-benzodiazepine drug, tofisopam. By studying the stereoselective retention of 25 benzodiazepine analogues we tried to get qualitative information of the nature of the chiral discrimination process.

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2. Experimental

2.1. Chromatography

A Chiral-AGP column (ChromTech AB, Hägersten, Sweden) (100 × 4.0 mm I.D.) with a guard column (10 × 3.0 mm I.D.) was used at ambient temperature (22–25°C).

The chromatographic system consisted of a Jasco Model PU-980 HPLC pump, a Rheodyne Model 7125 injector with a 20-μl loop, a Jasco Model UV-975 detector set at 220 nm and B.D.S. software (Barspec, Rehovot, Israel).

The mobile phase was 0.01 *M* phosphate buffer (pH 5.0, 6.0, 7.0) containing 5% or 10% (v/v) of isopropanol (IPA) or acetonitrile (ACN); the flow-rate was 0.9 ml/min.

Sample solutions were prepared by diluting (10 μl/1 ml eluent) the stock solutions (1 mg/2 ml ethanol).

Retention times (t_r) were determined in three parallel runs and corresponding capacity factors ($k' = [t_r - t_0]/t_0$) and separation factors ($\alpha = k'_2/k'_1$) were calculated.

2.2. Chemicals

3-Chiral 1,4-benzodiazepines (compounds 1–18; see Table 1) were synthesized as described previously [4,5,10]. 5-Chiral 1,4-benzodiazepines (compounds 19–22; Table 2) were obtained from Chemical Works of Gedeon Richter (Budapest, Hungary). Compounds 23–25 (Table 2) were kindly donated by Ciba-Geigy (Basle, Switzerland). Tofisopam enantiomers were provided by EGIS Pharmaceuticals (Budapest, Hungary).

2.3. Ultrafiltration

Ultrafiltration (two parallels) was carried out in an Amicon (Oosterhout, Netherlands) MPS-I system with YMT-membranes. Solutions containing racemic compounds 12 or 20 as well as native human AGP (Sigma, St. Louis, MO, USA) were prepared in 0.01 *M* phosphate buffer pH 7.0.

3. Results and discussion

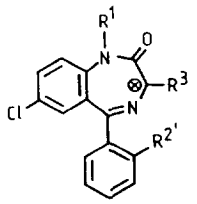
3.1. Separation of 3-chiral 1,4-benzodiazepines

Table 1 summarizes the capacity factors and separation factors for a series of 3-substituted 1,4-benzodiazepines. The mobile phase was 0.01 *M* phosphate buffer pH 7.0 with IPA (isopropanol) or ACN (acetonitrile) modifiers. Comparing the data obtained with 10% IPA and 10% ACN as mobile phase additives it can be observed that in the presence of ACN the enantioselectivity is highly improved. The retention is also somewhat higher. With the exception of compounds 2, 13, 17 and 18 all the racemates could be resolved. In case of the unresolved compounds having either hydroxy, acyloxy or alkyl substituent at the chiral centre, the common structural feature is $R^1 = CH_3$ and $R^{2'} = H$ substitution. When the detached aromatic ring has an ortho chlorine substituent, even the N(1)-methyl derivatives (4,14) could be resolved. Considering the series of oxazepam (1) as well as its methylether (5) and different esters (7–9) it can be seen that increasing hydrophobicity of the R^3 substituent enhances both the capacity and the separation factors. Oxazepam hemisuccinate (10) is a special case. This acidic ligand is bound with low affinity at pH 7 due to the net negative charge of AGP at this pH, nevertheless, its separation was very good (Fig. 1.). Comparing the 3-alkyl derivatives (15 and 16) it can also be observed that the more hydrophobic derivative has the higher retention and somewhat higher enantioselectivity.

Considering the elution orders established by using separated enantiomers, in most cases the (*S*)-enantiomer is the more retained one. It suggests that AGP similarly to other proteins [2,14,15] prefers the "M"-boat conformation (Fig. 2.). Nevertheless, the reversed elution order obtained for the 3-alkyl compounds when 5% IPA mobile phase modifier was used, calls attention to the fact that during chromatography the chiral recognition is a complex process and solvent-induced stereoselectivities may interfere [16].

Summarizing the results obtained for the sepa-

Table 1
Separation of racemic 3-chiral 1,4-benzodiazepines (pH 7.0)



No.	R^1	R^2	R^3	5% IPA			10% IPA		10% ACN		
				k'_1	α	Elution order	k'_1	α	k'_1	α	Elution order
1	H	H	OH	4.92	1		2.35	1	2.42	1.17	
2	CH_3	H	OH	4.35	1		2.00	1	2.56	1	
3	H	Cl	OH	6.85	1		2.93	1	3.64	1.41	
4	CH_3	Cl	OH	9.11	1.22				4.48	1.73	
5	H	H	OCH_3	5.67	1		2.08	1	3.15	1.22	
6	H	Cl	OCH_3	6.39	1.10		2.48	1	3.69	1.56	
7	H	H	$OCOCH_3$	7.69	1.18	*R,S*	2.88	1	4.03	2.15	*R,S*
8	H	H	$OCOCH_2CH_3$	11.50	1.58		4.03	1.21	7.18	2.55	
9	H	H	$OCO(CH_2)_2CH_3$	17.33	2.20		5.38	1.65	12.67	2.97	
10	H	H	$OCO(CH_2)_2COOH$	0.40	1.71	*R,S*			0.18	2.93	*R,S*
11	H	H	$OCO(CH_2)_2COOCH_3$	11.23	1.52				5.00	2.44	
12	H	Cl	$OCOCH_3$	9.39	1.33	*R,S*	3.33	1.19	6.69	2.32	*R,S*
13	CH_3	H	$OCOCH_3$	5.68	1		1.92	1	3.08	1	
14	CH_3	Cl	$OCOCH_3$	12.56	1.06		3.77	1	6.67	1.20	
15	H	H	CH_3	6.08	1.19	*S,R*	2.61	1.12	4.10	1.12	*R,S*
16	H	H	CH_2CH_3	11.50	1.29	*S,R*	3.45	1.21	8.20	1.40	*R,S*
17	CH_3	H	CH_3	6.50	1				4.30	1	
18	CH_3	H	CH_2CH_3	11.08	1				8.35	1	

ration of the enantiomers of 3-chiral 1,4-benzodiazepines the following conclusions can be drawn concerning the retention and chiral recognition mechanism: (1) The retention is mainly due to hydrophobic interaction; in this respect isopropanol competes with the solute molecules [16]. (2) The (*S*)-enantiomers having the "M"-conformation are more retained than the (*R*)-"P" enantiomers. The hydrophobic character of R^3 enhances the stereoselectivity. The solvent-induced reversal of the elution order, found for the 3-alkyl derivatives suggests a role of the oxygen atom in R^3 during chiral discrimination. (3) Hydrogen bonding via N(1)-H probably is involved in chiral interaction, since $R^1 = CH_3$ hinders separation. (4) Ortho chlorine substitution on the detached aromatic ring promotes chiral separation, outweighing the effect of $R^1 = CH_3$.

3.2. Separation of 5-chiral 1,4-benzodiazepines

The data in Table 2 present the capacity factors and separation factors obtained at pH 7.0 for 5-chiral 1,4-benzodiazepines as well as for related thiazepine and oxazepine compounds having sulphur or oxygen atom at position 4. It can be seen that compounds with this chiral structure can be easily resolved on Chiral-AGP, the only exception being the highly retained compound 25. Comparing effects of the mobile phase modifiers, the situation is not as simple as

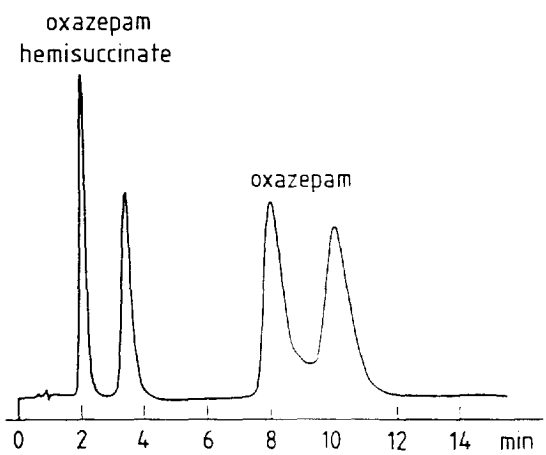


Fig. 1. Separation of *rac*-oxazepam (1) and *rac*-oxazepam hemisuccinate (10) on Chiral-AGP. Mobile phase: 0.01 *M* phosphate buffer pH 7.0 with 5% ACN.

found for the 3-chiral 1,4-benzodiazepines. In the presence of ACN the retention of the first eluted peaks is always higher than with the same concentration of IPA. The enantioselectivity was also affected by the nature of the organic modifier. Higher separation factors were obtained for compounds 21–24 using mobile phases containing ACN, whereas the enantioselectivity was reduced for compounds 19 and 20. Compared to the 3-chiral 1,4-benzodiazepines the effect of $R^1 = CH_3$ substitution on the enantioselectivity is also reversed. Furthermore, whereas for the 3-chiral 1,4-benzodiazepines the N(1)-H group was preferred in chiral discrimination, in this series the presence of an N(1)-methyl group is favoured. The high separation factor of dihydrodiazepam (20) compared to its desmethyl analogue (19) is striking, while the relation of compounds 21 and 22 is less pronounced. Considering the effect of the heteroatom in position

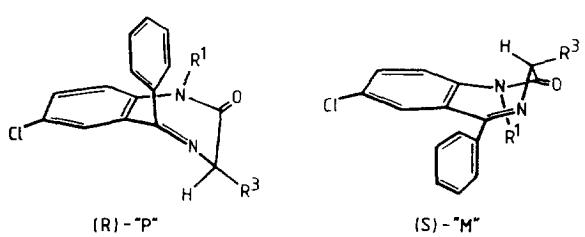


Fig. 2. Dominant conformers of 3-chiral 1,4-benzodiazepines.

4 (cf. 19 vs. 23 and 24) it can be observed that while in the case of the thiazepine (24) the retention of both enantiomers is highly increased, the oxazepine compound (23) has the higher enantioselectivity. Chlorine substitution in the 2′-position provoked very strong retention for both enantiomers of compound 25, and resolution is lost.

The reversed elution order found for dihydrodiazepam (20) and for its N(4)-carbamoyl derivative (22) is not surprising, because carbamoylation exerts inversion of the seven-membered ring (Fig. 3.), even reversing the sign of the optical rotation, as proved by X-ray [17] and chiroptical studies [18]. Thus, the higher affinity of (+)-(*S*)-20 and (+)-(*R*)-22 on Chiral-AGP suggests that discrimination is due to the chiral conformation of the molecule rather than to the absolute configuration at the C(5) centre.

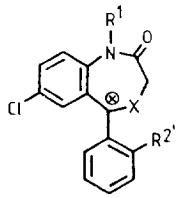
3.3. Separation of tofisopam

Fig. 5 shows the chromatograms of *rac*-tofisopam and its enantiomers obtained at pH 7.0 with 5% IPA and 5% ACN modifiers.

Tofisopam is a 2,3-benzodiazepine having a chiral centre at the C(5) position. It was proved by NMR and chiroptical investigations [19,20] that tofisopam in solution exists in two conformations and conformers of the same enantiomer show opposite optical rotation (Fig. 4.). In crystalline form the molecules are predominantly in the thermodynamically more stable conformation in which the C(5)-ethyl group is pseudo-equatorial with respect to the diazepine ring, while in solution equilibrium is achieved within a few hours [8]. The conformers as diastereomers can be separated even by non-chiral reversed-phase HPLC [21]. On an HSA-Sepharose column it is possible to separate the major conformers of the two enantiomers, as well as the conformers of the (*S*)-enantiomer [8].

The chromatogram obtained on Chiral-AGP with IPA as modifier also indicates the separation of the conformers of (*S*)-absolute configuration (Fig. 5a). The first peak on the chromatogram of the racemate (Fig. 5c) involves the total amount of (*R*)-enantiomer as well as about 20%

Table 2
Separation of racemic 5-chiral 1,4-benzodiazepines and related compounds (pH 7.0)



No.	X	R^1	R^2	10% IPA			10% ACN		
				k'_1	α	Elution order	k'_1	α	Elution order
19	NH	H	H	2.39	1.46		4.00	1	
20	NH	CH_3	H	2.42	5.03	*R,S*	4.58	2.06	*R,S*
21	N-$CONH_2$	H	H	1.43	1.53		1.65	1.65	
22	N-$CONH_2$	CH_3	H	1.67	2.05	*S,R*	3.88	2.14	*S,R*
23	O	H	H	4.83	2.73		6.80	3.09	
24	S	H	H	7.92	1.36		10.60	1.86	
25	S	H	Cl	18	1		27	1	

of the amount of the other enantiomer corresponding to the (+)-(*S*)-conformer. When ACN modifier was used the conformers of both enantiomers could be separated. It is interesting that in the case of (*S*)-tofisopam the minor conformer is more retained (Fig. 5d), which is opposite to the elution order found with IPA modifier. For the (*R*)-enantiomer, however, the first eluted peak is the minor conformer (Fig. 5e). The chromatogram of the racemate (Fig. 5f) indicates that while the minor conformers are nicely separated, the two major conformers show identical retention. Such variance in elution orders is presumably due to specific solvation effects.

3.4. Effect of pH

Table 3 summarizes the capacity and separation factors measured for several neutral 3-chiral and 5-chiral 1,4-benzodiazepines and analogues at pH 5.0, 6.0 and 7.0, both with ACN and IPA modifiers. The retention of these compounds was only slightly affected by the pH. The fluctuation of the k' values found in some cases is not significant enough to be caused by structural characteristics. By increasing the pH from 5 to 7 using mobile phases containing ACN, the enantioselectivity increases for nine of the ten compounds. For some compounds the effect was more pronounced, i.e. for compounds 6, 7, 10 and 16. A very strong increase of the separation factor was also observed for compound 20, using mobile phases with 5 and 10% IPA. For compound 20, contrary to the other compounds presented in Table 3, it can be noted that a higher enantioselectivity was observed when IPA was used as modifier compared to ACN over the pH range studied. These findings are in accordance with previous observations for neutral compounds made by Hermansson et al. [22,23]. The charge of these compounds can not be affected by pH [24]. This means that the effects on the enantioselectivity could be assigned to changes of the chiral bonding properties of the protein, induced by the charge of the proteolytic amino acid residues.

The acidic compound oxazepam hemisuccinate (10), behaved in a different way with respect to both the retention and the enantioselectivity,

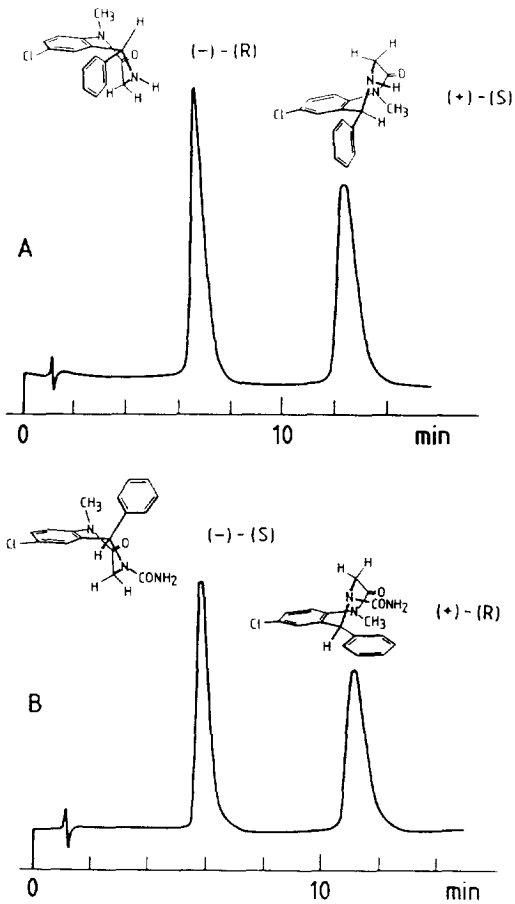


Fig. 3. Separation of (A) *rac*-dihydrodiazepam (20) and (B) *rac*-uxepam (22) on Chiral-AGP. Mobile phase: 0.01 *M* phosphate buffer pH 7.0 with 10% ACN.

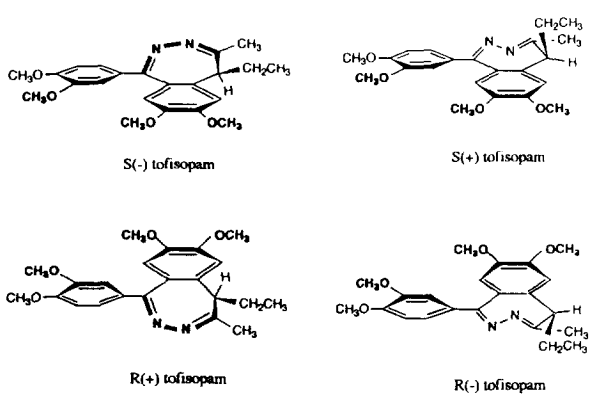


Fig. 4. The four species present in solution of *rac*-tofisopam.

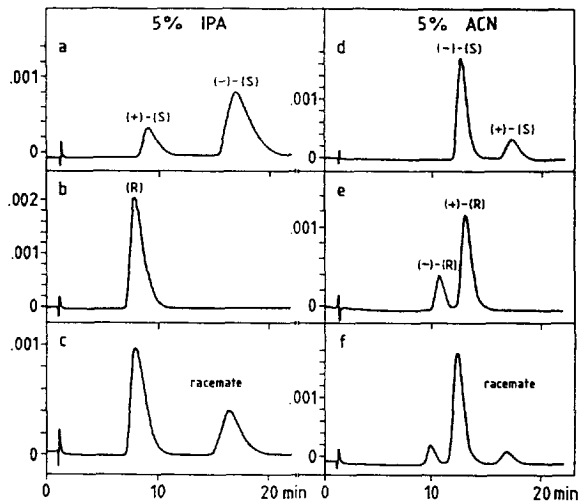


Fig. 5. Chromatograms of (*S*)-, (*R*)- and *rac*-tofisopam obtained on Chiral-AGP. Mobile phase: 0.01 *M* phosphate buffer pH 7.0 with 5% IPA (a,b,c) and 5% ACN (d,e,f).

Table 3
Influence of pH on the capacity factors and separation factors of chiral benzodiazepines

No.		pH 5.0		pH 6.0		pH 7.0	
		k'_1	α	k'_1	α	k'_1	α
1	5% ACN	5.9	1.24	6.8	1.29	6.8	1.29
	5% IPA	3.8	1	4.0	1	4.2	1
3	5% ACN	7.8	1.54	8.1	1.62		
	5% IPA	5.2	1	5.6	1	5.9	1
6	5% ACN	12.3	1.26	10.3	1.60	11.9	1.93
	5% IPA	5.6	1	5.5	1	5.0	1.21
7	10% ACN	4.2	1.62	4.8	1.94	3.8	2.29
	5% IPA	6.0	1.08	6.5	1.16	6.9	1.26
12	10% ACN	5.6	2.34	6.2	2.68	6.4	2.56
	5% IPA	8.0	1.35	8.2	1.46	8.5	1.49
16	10% ACN	9.1	1	7.4	1.22	8.2	1.40
	10% IPA	3.5 (*S*)	1.23	3.4	1.20	3.5	1.21
20	10% ACN	4.8	1.71	4.6	1.72	4.9	1.90
	10% IPA	1.9	2.86	2.2	3.55	2.5	4.23
	5% IPA	4.3	4.53	5.3	5.91	6.2	6.70
23	10% ACN	7.4	2.35	6.8	2.62	6.8	3.09
	10% IPA	4.2	2.01	4.3	1.88	4.7	2.60
24	10% ACN	12.3	1.72	11.1	1.69	10.6	1.68
	10% IPA	6.6	1.54	6.5	1.56	7.2	1.48
25	10% ACN	29	1	27	1	27	1
	10% IPA	16	1	16	1	16	1

compared to the neutral compounds, as demonstrated in Figs. 6A,B. The enantioselectivity was strongly improved by increasing the pH from 5 to 7. It can be noted that the separation factor is 1.0 at pH 5 with ACN as modifier, however, increasing pH to 7 gives a separation factor of

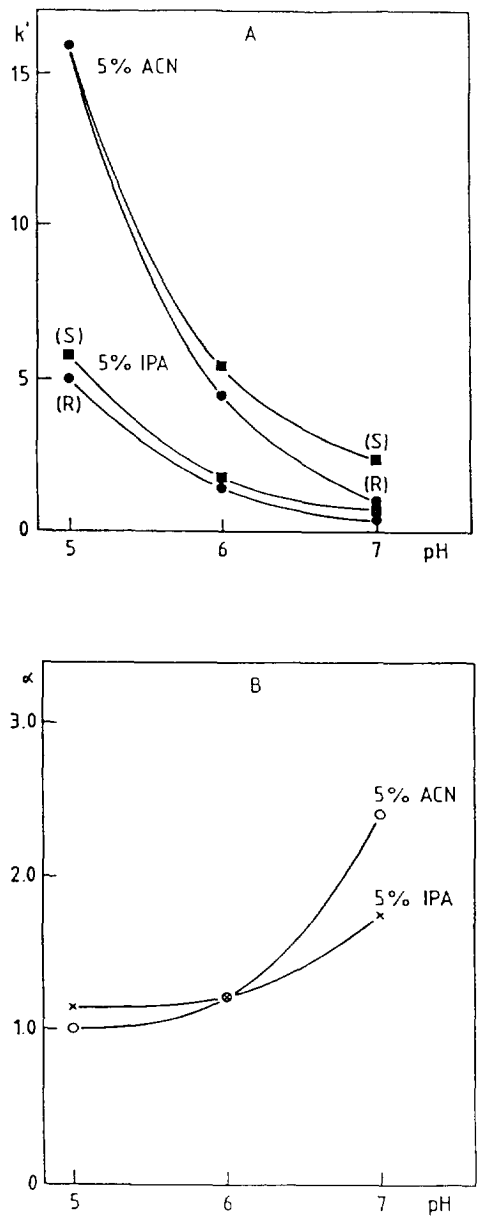


Fig. 6. Effect of pH on the capacity factors (A) and the separation factors (B) of oxazepam hemisuccinate (10) on Chiral-AGP.

2.4. This behaviour has not been reported previously for acidic compounds. Normally the separation factor increases for acidic compounds by reducing the pH [22,23].

3.5. Chromatographic resolution and binding stereoselectivity

Since the majority of the investigated benzodiazepines could be conveniently resolved on Chiral-AGP, it was of interest to check whether these compounds show any stereoselective binding on native AGP. Binding studies were performed with representatives of 5- and 3-chiral compounds, i.e. dihydrodiazepam (20) and lorazepam acetate (12). In case of dihydrodiazepam the chiral separation was very good with both modifiers, exceptionally high with IPA (see Table 2), while the separation of lorazepam acetate enantiomers was better in the presence of ACN (see Table 1). Ultrafiltration of solutions containing the racemic ligand and native AGP were performed, and the free fractions for both enantiomers were determined by comparing the chromatograms obtained on Chiral-AGP for the racemic ligand solution as well as for the ultrafiltrate. The results can be seen in Table 4. In accordance with the chromatographic behaviour, in both cases the more retained (*S*)-enantiomers indicated stronger binding, though to different extents. In the case of dihydrodiazepam the enantiomeric distribution was $R/S = 76/24$, while the chromatogram of lorazepam acetate filtrate indicated only slight excess of the first peak ($R/S = 54/46$). The binding stereoselectivities, i.e. the ratio of enantiomeric binding constants (K^S/K^R) which can be calculated [25] from these data, are 7.7 and 1.4 for compounds 20 and 12, respectively. These enantioselectivities show correlation with the chromatographic results obtained with IPA modifier, but differ from those measured with ACN modifier. Thus, the high chromatographic selectivity of dihydrodiazepam enantiomers on Chiral-AGP is in agreement with its remarkable native binding stereoselectivity. The case of lorazepam acetate, however, indicates that good chromatographic separation on Chiral-AGP does not necessarily

Table 4
Stereoselective binding of dihydrodiazepam (20) and lorazepam acetate (12) studied by chromatographic analysis of ultrafiltrates on Chiral-AGP (eluent: phosphate buffer pH 7.0, 10% ACN)

	Dihydrodiazepam	Lorazepam acetate
C_{rac} (μM)	29	22
C_{AGP} (μM)	50	50
Free fractions (%)	(*R*): 64 ± 2 (*S*): 20 ± 2	(*R*): 47 ± 2 (*S*): 39 ± 2
Binding constants (M^{-1})	$K^R = 1.3 \times 10^4$ $K^S = 1.0 \times 10^5$	$K^R = 2.6 \times 10^4$ $K^S = 3.6 \times 10^4$

require highly stereoselective binding to the native protein.

4. Conclusion

Chiral-AGP can separate the enantiomers of benzodiazepines with a broad range of structures. The effects of substituent on retention and enantioselectivity within homologous series indicate the role of hydrophobic and hydrogen bonding interactions. Exceedingly high chiral separation was found for 4,5-dihydrodiazepam, for which the existence of a remarkably high native binding stereoselectivity favouring the (*S*)-enantiomer was revealed. Discrimination according to chiral molecular conformations, which is the special structural feature of benzodiazepines, plays an important role in the separation mechanism.

Acknowledgements

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