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Conformation selectivity in the binding of diazepam and analogues to α_1-acid glycoprotein

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Abstract—Diazepam, a 1,4-benzodiazepine lacking chiral centre, exists in an equimolar mixture of two chiral conformers. Induced circular dichroism spectra for the binding of diazepam and its 3,3-dimethyl substituted analogues to α_1-acid glycoprotein (AGP) revealed that opposite to human serum albumin, AGP preferably binds the P-conformers. Accordingly, slightly favoured binding of (*R*)-enantiomers of 3-alkyl derivatives having P-conformation was found. In case of 3-acyloxy derivatives, however, AGP preferably binds the (*S*)-enantiomers. Studies with the separated genetic variants of AGP proved similar binding affinities, but markedly different conformation selectivities. For diazepam bound by the F1-S variant, a P/M selectivity of about 2 could be estimated.

1. Introduction

The anxiolytic drug diazepam is a 1,4-benzodiazepine lacking chiral centre. Due to rapid inversion of the non-planar seven-membered ring the molecule is an equimolar mixture of two chiral 'P'- and 'M'-conformers[1] (Fig. 1). (*R*)- and (*S*)-enantiomers of 3-substituted derivatives for steric reason occupy opposite, sterically restricted P- and M-conformations, respectively.[2] The high stereoselectivities found in their central nervous system receptor activity in favour of the (*S*)-enantiomers suggested that diazepam adopts M-conformation in its receptor binding.[3,4] In the binding of 3-substituted 1,4-benzodiazepines to human serum albumin (HSA) the (*S*)-enantiomers are also preferred, while the binding affinities and the stereoselectivity values depend on the substitution.[5] The conformation selectivity for the binding of diazepam to HSA could be proved experimentally. Induced circular dichroism (ICD) spectrum of diazepam bound to HSA was found to be very similar to the CD spectrum of its (*S*)-3-methyl derivative.[6,7] The 3,3-dimethyl derivatives behaved analogously.[8] Since the free ligands are racemic, diazepam and 3,3-dimethyl derivatives bound to HSA are reasonably supposed to adopt exclusively M-conformation.

In this work, we investigated the conformation selectivity in the binding of diazepam and analogues (Fig. 2.) to human α_1-acid glycoprotein (AGP). AGP is a minor, acute phase serum component which also plays role in serum protein binding of drugs.[9] Earlier studies on the stereoselective binding of 1,4-benzodiazepines to AGP showed ambiguous results. A study on the binding of diazepam to AGP presented[10] an ICD spectrum from

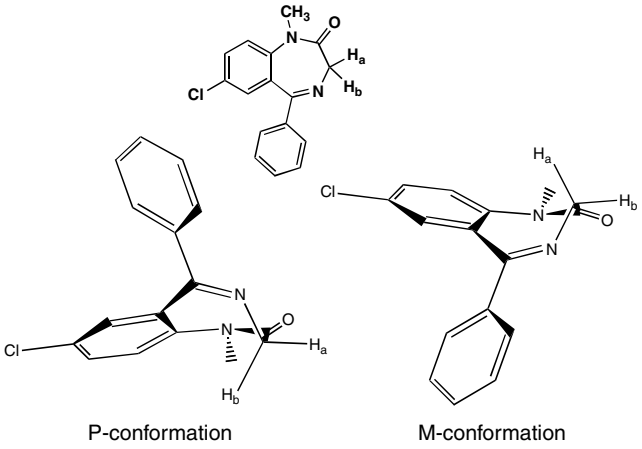

Figure 1. Chiral conformers of diazepam.

Keywords: α_1-Acid glycoprotein; Genetic variants; Diazepam; Benzodiazepine conformation; Protein binding; Induced circular dichroism.
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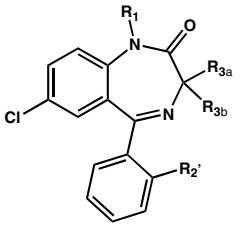

Compound	No.	R_1	R_{3a}	R_{3b}	$R_{2'}$
Diazepam	**1**	CH_3	H	H	H
Desmethyl diazepam	**2**	H	H	H	H
3,3-dimethyl diazepam	**3**	CH_3	CH_3	CH_3	H
3,3-dimethyl desmethyl diazepam	**4**	H	CH_3	CH_3	H
3-methyl desmethyl diazepam	**5**	H	H	CH_3	H
Lorazepam acetate	**6**	H	H	$OCOCH_3$	Cl

Figure 2. Structure of 1,4-benzodiazepines investigated.

which preferred P-conformation could be presumed. A systematic study on the resolution of 3-substituted benzodiazepines on immobilized AGP (Chiral-AGP) HPLC column indicated[11] preferred retention of (*S*)-3-acyloxy derivatives, while in cases of 3-alkyl-substituted analogues reversed, solvent dependent elution orders were found. Since native AGP is a mixture of two main genetic variants (about 70% 'F1-S' variant and 30% 'A' variant) which have different drug binding characteristics,[12–17] the benzodiazepine conformation selectivities of the two separated variants were investigated, as well. ICD spectra were recorded in HSA and AGP solutions. Enantioselectivities for the binding of 3-alkyl and 3-acyloxy derivatives to the genetic variants were also studied.

2. Results

2.1. Comparison of conformation selectivities of HSA and AGP

ICD spectra detected for the interaction of benzodiazepines **1**–**4** with HSA and AGP are shown in Figure 3. It can be observed that the CD bands are of opposite signs for the two proteins in all cases. While HSA preferably binds the M-conformers and thus the ICD spectra are very similar to those of the (*S*)-3-substituted analogues,[6] AGP binding prefers the opposite P-conformers, the ICD spectra resemble those of the (*R*)-3-substituted analogues. Dimethyl substitution in C3 position results in red shift in both protein solutions. In fact a shift of about 8 nm can be observed even when UV spectra of the free ligand solutions of **1** and **3** (310 and 318 nm) as well as **2** and **4** (312 and 321 nm) are compared.

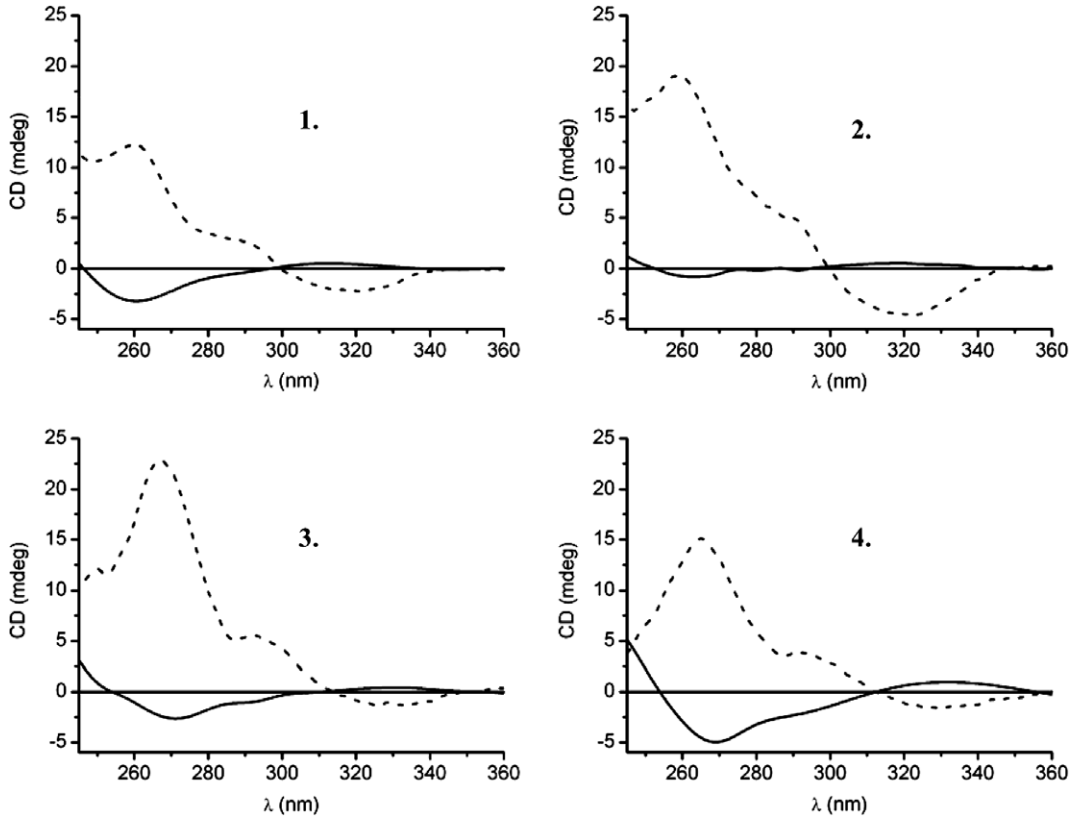

Figure 3. Induced CD spectra of diazepam (**1**) and desmethyl diazepam (**2**) and their 3,3-dimethyl analogues (**3**,**4**) in HSA (- - -) and AGP (—) solutions ($c_{ligand} = c_{protein} = 30$ μM; 15 μM in case of diazepam–HSA interaction).

Table 1. Molar CD absorption coefficients ($\Delta\varepsilon$) for two peaks calculated for benzodiazepines (**1**–**4**) bound to HSA and AGP

Ligand	Protein	Bound ligand (μM)	$\Delta\varepsilon$ (M^{-1} cm^{-1})	Peak ratio[b]
15 μM **1**	15 μM HSA	8 ± 1	46.5 (260 nm) −8.3 (321 nm)	−5.6
30 μM **1**	30 μM AGP	10 ± 1	−9.6 (261 nm) 1.6 (313 nm)[a]	−6.0
30 μM **2**	30 μM HSA	17 ± 2	33.9 (258 nm) −8.1 (321 nm)	−4.2
30 μM **2**	30 μM AGP	9 ± 2	−2.7[a] (263 nm) 2.0[a] (319 nm)	−1.4[a]
30 μM **3**	30 μM HSA	17 ± 2	41.0 (267 nm) −3.4 (332 nm)	−12.1
30 μM **3**	30 μM AGP	14 ± 2	−5.8 (271 nm) 0.9 (331 nm)	−6.4
30 μM **4**	30 μM HSA	13 ± 2	35.3 (265 nm) −3.7 (330 nm)	−9.5
30 μM **4**	30 μM AGP	13 ± 2	−11.5 (269 nm) 2.3 (332 nm)	−5.0

Bound ligand concentrations belonging to the induced CD spectra in Figure 2 were determined by ultrafiltration.
[a] Uncertain value due to weak CD signal.
[b] $\Delta\varepsilon$ (260–270 nm)/$\Delta\varepsilon$ (320–330 nm).

The binding to both proteins provokes about 10 nm red shift for all benzodiazepines, indicating hydrophobic environment of the bound ligands.[7,8] In case of AGP the ICD signals are less intense. Ultrafiltration results proved that the weaker signals cannot be explained by weaker binding. The molar CD absorption coefficients ($\Delta\varepsilon$) in Table 1 calculated for the corresponding bound ligand concentrations are markedly smaller for the AGP bound benzodiazepines. In case of **1** and **3** binding the $\Delta\varepsilon$ values are about five and seven times smaller with AGP than with HSA, **2** bound to AGP has very weak CD activity, while **4** shows the least difference between the two proteins. The ratio of the two dominant CD peaks (at 260–270 and 320–330 nm) is similar for diazepam when bound to HSA or AGP, in cases of **3** and **4** these ratios are much higher with HSA. It suggests that in cases of the 3,3-dimethyl derivatives the bound ligand conformations on HSA and AGP are not exactly mirror images. Binding data in Table 1 indicate that 3,3-dimethyl substitution improves the binding on AGP, while opposite effect is valid for HSA.[8] Due to $R_1 = CH_3$ substitution the binding to HSA is enhanced, in case of AGP significant changes were not found.

Since the $\Delta\varepsilon$ values determined for the HSA bound benzodiazepines can be rendered totally to the M-conformers[6–8] it would be possible to estimate the P-conformer excess and the corresponding P/M conformation selectivity for AGP binding. Nevertheless, since native AGP is a mixture, the conformation selectivities were determined for the separated two main genetic variants.

2.2. Comparison of conformation selectivities of AGP genetic variants

ICD spectra obtained by the addition of **1** or **4** to solutions of F1-S and A genetic variants of AGP are shown in Figure 4. In case of diazepam binding ICD spectra with the A variant were practically absent, the dimethyl derivative binding, however, produced about three times more intense ICD spectra with the A variant compared to the F1-S variant. The binding degrees of the benzodiazepines to the two genetic variants were compared by ultrafiltration and results in Table 2 indicate slightly stronger binding of both ligands on the A variant. Since the bound ligand concentrations with the two AGP variants do not differ much, the large intensity differences in the ICD spectra reveal different conformation selectivities in favour of the P-conformation. Actual conformer excess values for the bound ligand concentrations belonging to the ICD spectra were calculated by using the molar CD parameters of bound M-conformers which could be obtained from HSA binding experiments (cf. Table 1). The resulting conformer excess values and the corresponding conformer compositions for the bound ligands (Table 2) show that for diazepam molecules bound to the F1-S variant the population of the P-conformer is about 1.7 times higher compared to the M-conformer, while A variant has no conformation selectivity. In case of compound **4** this quantitative approach is not quite straightforward. In the ICD spectra obtained with HSA and AGP the intensity ratios of the two characteristic bands are different (cf. Table 1). If the most intense band at about 270 nm is considered, the resulting P/M conformation selectivity value of 1.6 on the F1-S variant is equal to that of diazepam, while on the A variant this value is 3.8. On the other hand, if the relatively low molar CD value for HSA bound **4** at 330 nm is applied, the evaluated conformation selectivity values for AGP can be as high as 3 and 11 for F1-S and A variants, respectively. Probably, the higher intensity band around 270 nm is a more reliable basis of conformation selectivity calculation. Interactions of derivatives **2** and **3** with AGP variants produced weak ICD signals (not shown), and their conformation selectivities were similar to those obtained for **1** and **4**, respectively.

2.3. Comparison of enantioselectivity of AGP genetic variants

Enantioselectivities for the binding of a 3-alkyl (**5**) and a 3-acyloxy (**6**) benzodiazepines to the AGP genetic

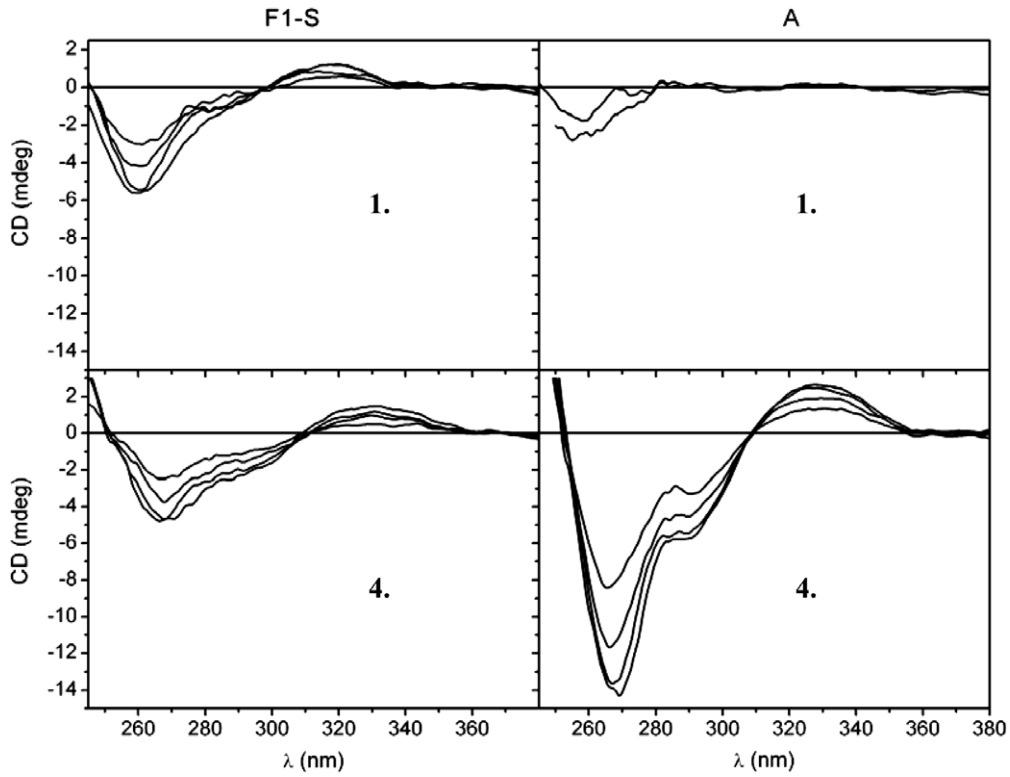

Figure 4. Induced CD spectra of diazepam (**1**) and 3,3-dimethyl desmethyl diazepam (**4**) ligands (30, 60, 90 and 120 μM) in solutions of the separated F1-S or A genetic variants of AGP (60 μM). In case of diazepam binding to the A variant ligand concentrations were 60 and 120 μM.

Table 2. Conformation selectivities estimated for diazepam (**1**) and 3,3-dimethyl desmethyl diazepam (**4**) bound to the F1-S and A genetic variants of AGP

Ligand	Protein	Bound ligand (μM)	Conformer excess (μM) [P] – [M]	Conformation selectivity [P]/[M]
30 μM **1**	60 μM AGP/F1-S	9 ± 1	2	1.6
60 μM **1**	60 μM AGP/F1-S	12 ± 1	3	1.7
30 μM **1**	60 μM AGP/A	12 ± 1	0	1
60 μM **1**	60 μM AGP/A	17 ± 1	0	1
30 μM **4**	60 μM AGP/F1-S	9 ± 2	2 (270 nm)	1.6
			4 (330 nm)	2.6
30 μM **4**	60 μM AGP/A	12 ± 2	7 (270 nm)	3.8
			10 (330 nm)	11.0

Bound ligand concentrations belonging to the corresponding CD spectra in Figure 4 were determined by ultrafiltration. Molar CD parameters belonging to the bound M-conformations of the benzodiazepines were taken from the HSA binding results.

variants were investigated by chiral analysis of the ultrafiltrates of solutions containing the racemic ligands and the protein. CD/UV spectra in Figure 5. show that in both cases the free ligand concentrations were practically identical for both genetic variants, but the enantiomeric compositions were very different. In case of **5**, (*S*)-enantiomer excess could be detected in the ultrafiltrate with the F1-S variant, the filtrate from the A variant had substantially lower optical activity. In case of **6**, however, both the enantioselectivity and the variant selectivity were opposite; (*R*)-enantiomer excess could be detected in the ultrafiltrate with the A variant, while the low optical activity with the F1-S variant was not characteristic. The enantiomeric composition of the ultrafiltrates was also analysed by chiral HPLC method, the results of which were in accordance with the CD results. The *R*:*S* enantiomeric compositions in case of **5** were 47/53 and 50/50, in case of **6** these values were 52/48 and 61/39 for the F1-S and A variants, respectively. The considerable enantioselectivity in the binding of **6** to the A variant allowed quantitative evaluation of the binding enantioselectivity. The measured free fraction values for the lorazepam acetate enantiomers ($\alpha_R = 0.69$ and $\alpha_S = 0.45$) correspond to $K_S/K_R = 2.7$ for the ratio of the enantiomeric binding constants.

3. Discussion

The binding affinity of diazepam to AGP is almost as high as to HSA,[18] and there is no remarkable difference between the AGP genetic variants,[12] the conformation

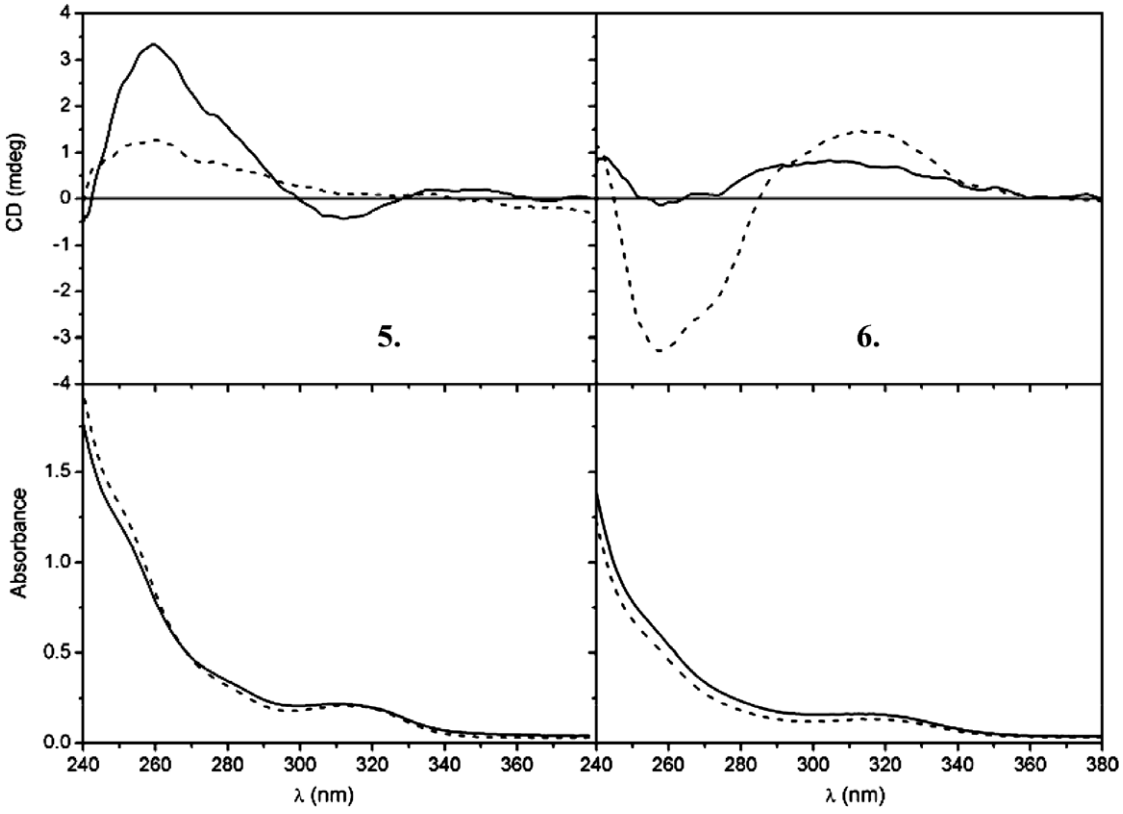

Figure 5. CD/UV spectra of the ultrafiltrates of solutions containing *rac*-3-methyl desmethyl diazepam (**5**) or *rac*-lorazepam acetate (**6**) and separated F1-S (—) or A (- - -) genetic variants of AGP ($c_{ligand} = c_{protein} = 60$ μM).

selectivities, however, are being shown to be different and dependent on the substitution of the benzodiazepine. The nature of substitution in C3 position plays decisive role in the direction of conformation selectivity. Opposite to HSA, AGP preferably binds the P-conformers of diazepam and its 3-alkyl or 3,3-dimethyl derivatives. The conformation selectivity values, however, are rather low and depend on the substitution of the benzodiazepine. The conformation selectivities of the two main genetic variants also show significant differences. For these ligands bound to the F1-S variant the population of the P-conformers is about twice as large as compared to the M-conformers. The binding of diazepam and a 3-alkyl analogue to the A variant is slightly stronger, but there is no conformation selectivity. The A variant, however, shows exceedingly high P/M $\geqslant$4 conformation selectivity with 3,3-dimethyl desmethyl diazepam. The opposite conformation selectivity between HSA and AGP is not valid for the binding of 3-acyloxy benzodiazepines, where the (*S*)-enantiomers are preferred by both serum proteins. It follows, that there must be a specific interaction between the acyloxy group and the binding site of AGP, and the M-conformation of the molecule assures a favoured accommodation. In case of lorazepam acetate the stereoselective binding could be attributed to the A variant. The measured $K_S/K_R = 2.7$ value on the A variant is in accordance with the value of 1.4 determined with native AGP.[11]

Diazepam is a characteristic ligand of one of the main drug binding sites on HSA,[19] located in domain III.[20] Its ICD spectrum can even be used to label this stereoselective binding site.[21] Binding studies with HSA fragments indicated[22,23] that low-affinity binding of diazepam in domain I provoked an inverse ICD spectrum. AGP binding shows analogy with this secondary binding of diazepam on HSA. The benzodiazepine binding site on AGP was characterized by photolabelling with flunitrazepam.[24] The exact three-dimensional structure of AGP is not yet known.

4. Conclusions

Opposite stereoselectivities in the binding of chiral drugs to the two main serum proteins may be of pharmacological relevance.[25] In the strong plasma protein binding of diazepam the abundant HSA is the decisive component, AGP binding is not of practical importance. The presented various conformational preferences provide some further evidences that beside configuration of chiral drugs, discrimination according to chiral molecular conformations plays role in their interactions with biological macromolecules.[26] CD spectroscopy is a very sensitive and useful technique to reveal chiral aspects of the complexes.[27–29]

5. Experimental

5.1. Chemicals

Benzodiazepines were synthesized as described previously,[5,8] their purities were checked by HPLC and

UV/CD spectroscopy. Human AGP and HSA (fatty acid free) were purchased from Sigma–Aldrich (St. Louis, MO). The two main genetic variants of AGP were separated following the method of Hervé et al.[30] as described previously.[15–17] AGP concentration was calculated by using a molecular mass value of 40,000. AGP variant solutions of the same concentration were checked by UV absorbance. Binding studies were performed in physiological Ringer buffer, pH 7.4.

5.2. CD measurements

CD and UV spectra were recorded on a Jasco J-715 spectropolarimeter at 20 °C, in a rectangular cell with 10 mm pathlength, equipped with magnetic stirring. The spectra were accumulated three times with a bandwidth of 1.0 nm. ICD spectra were obtained as the difference of spectra of ligand(s)–protein mixture and of protein solution alone and ellipticities were expressed in millidegrees. Ligands were added in small aliquots of 6 mM ethanolic stock solutions to 2 mL protein solutions.

5.3. Ultrafiltration

Ultrafiltration was performed with an Amicon MPS-1 system using YMT 30 membranes. Non-specific loss values were checked by the filtration of protein-free solutions and were used for correction. The free fraction values of **1**–**6** were measured by HPLC analysis on Chiral-AGP column (ChromTech Ltd.), using mobile phase of 0.01 M phosphate buffer, pH 7.0, containing 7–12% acetonitrile. The system was composed of a Jasco PU-980 pump with a Rheodyne 7125 injector (20 μL loop), a Jasco 975 UV detector (225 nm) and Borwin software.

Acknowledgments

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