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CIRCULAR DICHROISM AND GEL FILTRATION STUDY OF BINDING OF PROCHIRAL AND CHIRAL 1,4-BENZODIAZEPIN-2-ONES TO HUMAN SERUM ALBUMIN

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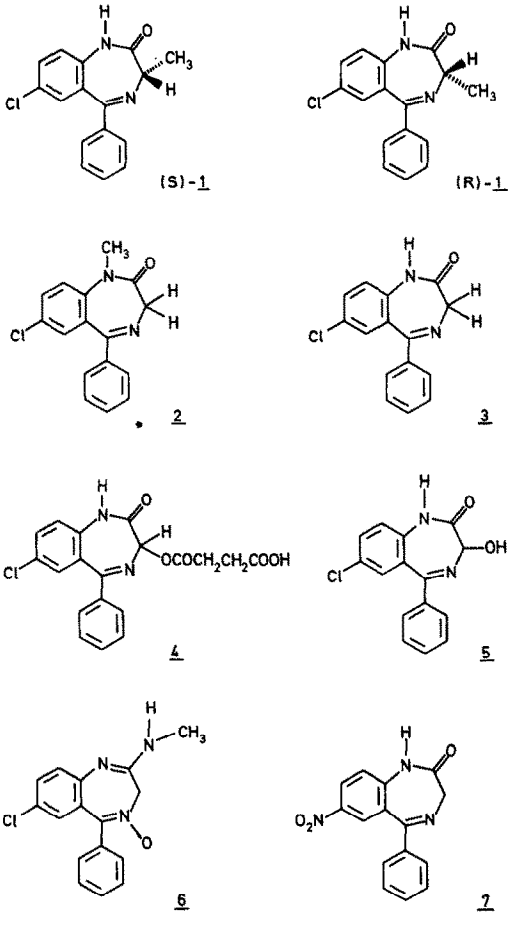
Abstract—Combining circular dichroism (c.d.) and gel filtration method in studying the binding of chiral ((*S*)/*R*)-1) and prochiral (2, diazepam and 3, desmethyldiazepam) benzodiazepines to human serum albumin (HSA), the following results were obtained: c.d. measurements revealed that both enantiomers of 1 are bound by HSA with different affinities. Gel filtration measurements revealed the following data on binding: (a) the HSA affinity for (*S*)-1 is about 40 times higher than for (*R*)-1, (b) for (*S*)-1 exist two independent and nonequivalent sites of high affinity and for (*R*)-1 two independent equivalent sites of low affinity, (c) at equimolar concentrations of 1 and HSA, (*S*)-enantiomer is bound up to 53 per cent, but (*R*)-enantiomer up to 18 per cent only; at the same ratio of ligand to protein prochiral 3 was bound up to 56 per cent.

The potential importance of protein binding of drugs with respect to their behaviour in organism was realized very early [1, 2]. One of the most important factors for the understanding of the pharmacodynamic properties of drugs is a knowledge of their binding properties to serum proteins [3–5]. Among many different methods used for studying serum protein–drug binding, e.g. ultrafiltration [6, 7], gel filtration [8], equilibrium dialysis [9, 10], ultracentrifugation [11], electrometry [12], polarography [13], pulse radiolysis [14], fluorescence spectroscopy [15, 15], differential spectrophotometry [17], electron spin resonance (e.s.r.) [18], microcalorimetry [19, 20], nuclear magnetic resonance (n.m.r.) [21, 22], and circular dichroism (c.d.), the last method seems to become of greater importance in this field [23]. Advantages and limitations of this method have been recently discussed [24, 25]. These methods are often combined, e.g. gel filtration and equilibrium dialysis [26, 27], u.v. spectroscopy and c.d. [28], ultracentrifugation and gel filtration [8].

Binding of 1,4-benzodiazepines, possibly the most important group of CNS active drugs today [29, 30], to the serum albumins has been studied by c.d. [25, 31–35] as well as by some other methods [36, 37].

Among numerous compounds studied, the main attention has been focused on the well known tranquillizing agents diazepam (2), its *N*(1)-demethyl derivative (3), oxazepam (5), chlorodiazepoxyde (6), nitrazepam (7), as well as on the resolved enantiomers of hemisuccinate (4) [37].



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Gel filtration [8, 9, 32, 33, 37] and c.d.-spectroscopy [31, 33, 34, 37] data refer, however, often to different experimental conditions which makes a direct comparison of the results difficult or impossible. Having at hand many optically active 1,4-benzodiazepine derivatives [38] we chose the 3-methyl derivatives (*S*)-1 and (*R*)-1 for more detailed investigation under similar conditions to those used earlier [31, 32, 39] for prochiral 2 and 3. In this paper we describe the experimental results and conclusions drawn from the gel filtration data. A detailed and more general discussion of chiroptical data is presented in the following paper [40].

This study on the binding of chiral benzodiazepines to human serum albumin is a part of our general research program on chemical [38, 41], chiroptical [42, 43], pharmacological [44] and biotransformational [45, 46] properties of this group of CNS-active substances.

Moreover, we consider the investigation of the binding of the enantiomeric pair (*S*)- and (*R*)-1 of additional interest in the field of 1,4-benzodiazepines, because of the following reasons:

(a) Both enantiomers possess maximal structural similarity to the prochiral compounds 2 and 3 (diazepam and its *N*(1)-diemethyl derivative, respectively), which can be used as the reference compounds in such investigations. The pharmacological [47] and physicochemical aspects of compound 2 widely investigated, as were its binding properties to HSA [33] and BSA [34].

(b) Both enantiomers possess high chemical stability and do not racemize under a variety of conditions *in vitro* [38, 43].

(c) There are no special structural features in (*S*)/(*R*)-1 which would give them additional binding possibilities in comparison to those of 2 and 3 as our reference compounds.

(d) Absolute configurations of the chiral centre in the enantiomers of 1 are known, because they are prepared from *l*-(*S*) and *d*-(*R*) alanine, respectively [38].

(e) The (*S*)-1 enantiomer exhibited higher pharmacological (CNS) activity than (*R*)-1 [44].

Conditions (b) and (c) are not fulfilled by the earlier investigated enantiomeric pair of 4 [37].

MATERIALS AND METHODS

Compounds. 7-Chloro-1,3-dihydro-3(*S*)-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one, (*S*)-1, and its enantiomer (*R*)-1, were prepared according to previously described procedures [38, 48], and purified by crystallizations from an acetone–water mixture. Their rotations were: $[\alpha]_{578}$ + 178 (c = 2.0 in $CHCl_3$) for (*S*)-1; the *R*-enantiomer possessed equal absolute rotation. Diazepam (2) and its *N*(1)-demethyl derivative (3) were prepared by a hexamine method [49], and purified by repeated crystallization from ether.

Dry human serum albumin (HSA) was obtained from the Institute of Immunology, Zagreb, and was 100 per cent electrophoretically pure.

Chemicals. All other chemicals were p.a.; their solutions were prepared using deionized water.

Circular dichroism measurements were performed on a JASCO (Japan Spectroscopic Co., Tokyo) model J-20 automatic spectropolarimeter at ambient temperature using 10 mm cells. The instrument was calibrated with D-10-camphosulfonic acid. The results are expressed in differential absorbance $\Delta A = A_L - A_R$ or in $(\Delta A/d)$-units, where d is cell thickness, using 69000 as the molecular weight of HSA. The relation to the measured ellipticity $[\psi]$ (in degrees) and molar ellipticity $[\theta]$ is given by the equations: $\Delta A = [\psi]/33$ and $[\theta] = 3300\Delta\varepsilon$.

The mean deviation of the c.d.-values above 300 nm is approx. ±20%.

Preparation of solutions was performed by taking aliquots (2–10 μl) from the stock solution of the compound under investigation (0.14225 g/5 ml of 96% ethanol), and adding it into 10 ml of a solution of HSA (1 mg/ml in 0.067 M phosphate buffer, pH 7.4). All samples were shaken for 15 min before measurements. Final concentrations of the samples were as follows: 0.7×10^{-5}, 1.0×10^{-5}, 1.44×10^{-5}, 2.0×10^{-5}, 3.0×10^{-5}, 4.0×10^{-5} and 6.0×10^{-5} M, while HSA concentration was always 1.44×10^{-5} M. Each spectrum reported is the average of at least three measurements. Blanks were prepared in entirely the same way. The concentration of ethanol in the solutions in no case exceeded 0.064%.

Absorption spectra were taken with an automatic Varian UV-Vis Techtron M 635 double-beam spectrophotometer.

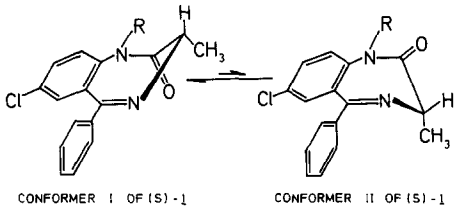
Determination of binding to HSA by gel filtration was performed using a column 30 × 0.9 cm (Pharmacia, Uppsala) filled with Sephadex G-25 Fine (Pharmacia, Uppsala), and equilibrated with 0.067 M phosphate buffer, pH 7.4; sample volumes were regularly 20 ml. A constant flow rate (24 ml/hr) was maintained by means of a peristaltic pump P-3 (Pharmacia, Uppsala). All measurements were performed at ambient temperature (23°). Collected fractions, 3 ml each, were monitored by u.v. absorption measurements; fractions containing protein were monitored at 280 nm, while protein free fractions were monitored at 229 nm.

Calculations of the results. Results are presented and calculated according to Scholtan [8], and Scatchard [51]. The experimental values for the binding of (*S*)- and (*R*)-1 were measured at 229 nm and the calibrating straight lines were constructed for starting concentration of ligands and for the maxima of the elution curves without HSA.

RESULTS

Investigation of the binding of chiral and prochiral benzodiazepines on HSA by c.d. measurements

Chiroptical studies were done by determination of differential c.d. curves for HSA-bound benzodiazepines (*S*)- and (*R*)-1, and prochiral 2 and 3 (Figs. 1, 2.). The molar ratio of ligand to protein in solution was 1:1 (Fig. 1), and 3.47:1, respectively (Fig. 2) Differential curves were obtained by graphical subtraction of c.d. spectra of HSA alone from c.d. spectra of benzodiazepine obtained in the presence of HSA. It is interesting to note that the curves of free enantiomers, without the presence of protein in solution, (curves a and b, respectively) and those obtained after binding to HSA (curves c and d) do not differ much in shape nor in intensity. We determined chiroptical properties of bound prochiral compounds 2 (diazepam) and 3 (*N*(1)-demethylated



Scheme 1. Stable (I) and unstable (II) conformer of (*S*)-1, R = H.

diazepam), as well, although previously studied [33, 34], in order to get maximal accuracy for comparison with the corresponding data of chiral derivatives. Differential spectra of 2 and its *N*(1)-demethyl derivative 3 at the same molar ratio of ligand to protein as for chiral derivatives (Fig. 1 and Fig. 2, curves f and e) exhibited qualitative similarity to those of the chiral compound (*S*)-1, i.e. a typical negative Cotton effect at 312–315 nm, and another two positive ones at shorter wavelength. The magnitude of the c.d. of 3 is, however, only approx. 50 per cent of that of (*S*)-1 (Fig. 1). This correlates very well with the results obtained by gel filtration, that both compounds at the same concentrations as above are bound to 56 per cent and 53 per cent, respectively, to HSA (Table 4). NMR studies have shown* that the seven-membered ring of chiral 1,4-benzodiazepines is quite rigid in a number of solvents at 35°, and present practically only in one conformation (Scheme 1), while prochiral 1,4-benzodiazepines possess different degrees of conformational mobility depending on *N*(1) substitution and the solvent [55–57]. The complexed molecule of prochiral 1,4-benzodiazepin-2-ones could, therefore, always adopt the same absolute conformation. Thus, from the c.d. spectra presented in Figs. 1 and 2, it can be concluded that the greatest part of 3 is bound to HSA in energetically preferable conformation of the (*S*)-1 (conformer I, Scheme 1).

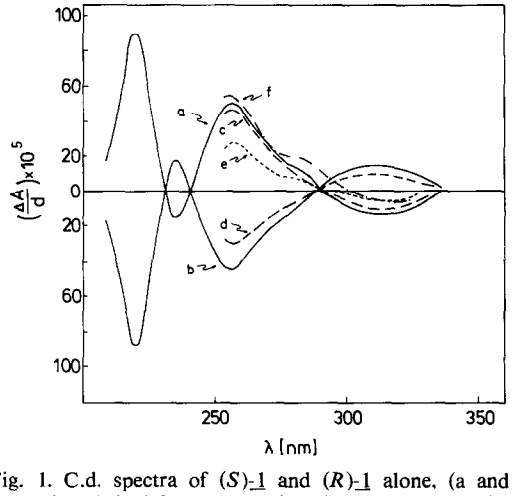


Fig. 1. C.d. spectra of (*S*)-1 and (*R*)-1 alone, (a and b respectively), in 0.067 M phosphate buffer pH 7.4; and c.d. spectra of (*S*)-1, (*R*)-1, 3, 2 (c, d, e, and f respectively) in the presence of HSA, after subtraction of the spectrum of HSA alone. The concentration of compounds and of HSA was 1.44×10^{-5} M; scale 0.005°; 10 mm cells.

* Unpublished results from Department of Biomedical and Biochemical Research, CRC.

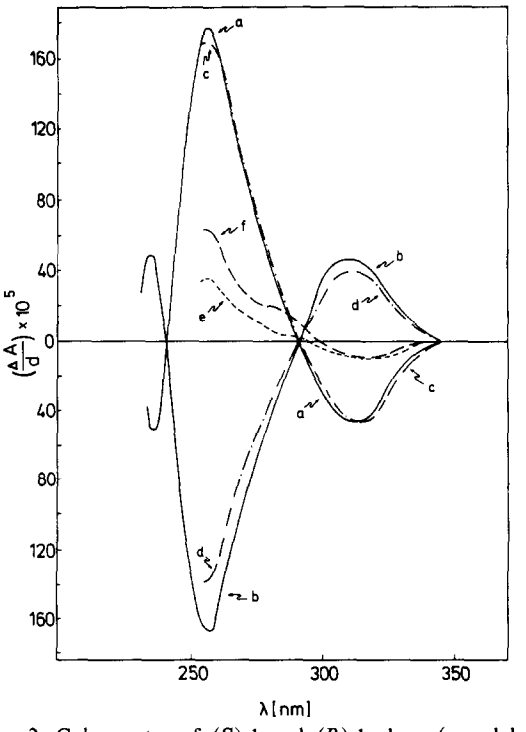


Fig. 2. C.d. spectra of (*S*)-1 and (*R*)-1 alone (a and b respectively), in 0.067 M phosphate buffer pH 7.4; and c.d. spectra of (*S*)-1, (*R*)-1, 3 and 2 (c, d, e, and f respectively) in the presence of HSA after subtraction of the spectrum of HSA alone. The concentration of compounds was 5.0×10^{-5} M and of HSA 1.44×10^{-5} M; scale 0.005°; 10 mm cells.

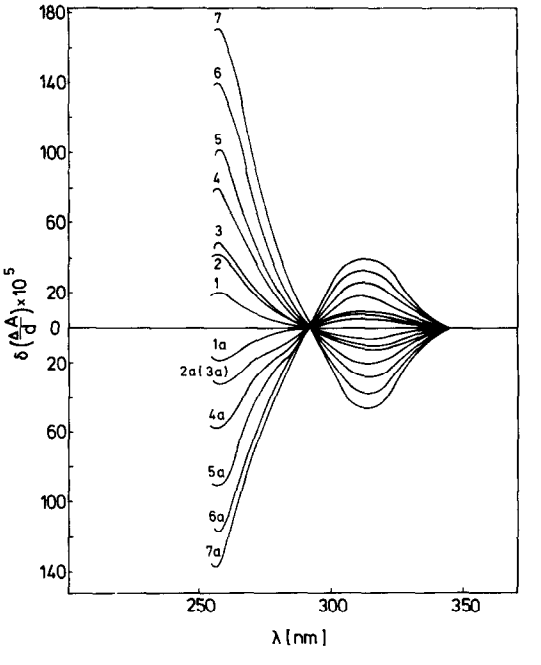


Fig. 3. C.d. spectra of (*S*)-1 (1–7) and (*R*)-1 (1a–7a), at different concentrations, in the presence of HSA after subtraction of c.d. spectrum of HSA alone. Concentrations of compounds were as follows: 0.7×10^{-5} M (1, 1a); 1.0×10^{-5} M (2, 2a); 1.44×10^{-5} M (3, 3a); 2.0×10^{-5} M (4, 4a); 3.0×10^{-5} M (5, 5a); 4.0×10^{-5} M (6, 6a); 5.0×10^{-5} M (6, 6a). Concentration of HSA was always 1.44×10^{-5} M. Measurements were made in 0.067 M phosphate buffer pH 7.4; scale 0.005°; 10 mm cells.

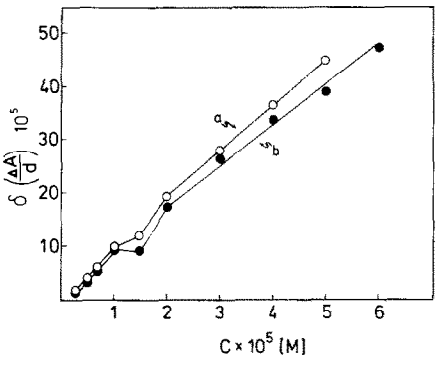


Fig. 4. The dependence of $\delta(\Delta A/d)$-values of (*S*)-1 and (*R*)-1 in the presence of HSA at 312–315 nm on the concentration of (*S*)-1 (curve a) and (*R*)-1 (curve b). The concentration of HSA was 1.44×10^{-5} M. Measurements were made in 0.067 M phosphate buffer pH 7.4; scale 0.005°; 10 mm cells.

A plot of $\Delta\varepsilon$-values at approx. 313 nm vs concentration (Fig. 4), calculated from the titration curves presented in Fig. 3, reveals for (*S*)-1 and (*R*)-1 two regions of linear correlation, which are connected by a pronounced inflexion. This occurs at a concentration of ligand of approx. 1:1. The plot done in the same manner for prochiral 3 approaches maximal value at ligand concentrations higher than about 2×10^{-5} M, thus indicating a saturation of "c.d.-active" binding site. The curve obtained was similar to that published for prochiral compound 2 [33].

Investigation of the binding of chiral and prochiral benzodiazepines on HSA by gel filtration

Binding of the compounds (*S*)-1 and (*R*)-1 at different concentrations is expressed as a percentage of bound compound. Furthermore, binding is characterized by the apparent binding constant k^*, and by the slope coefficient m, i.e. by parameters from Scholtan's

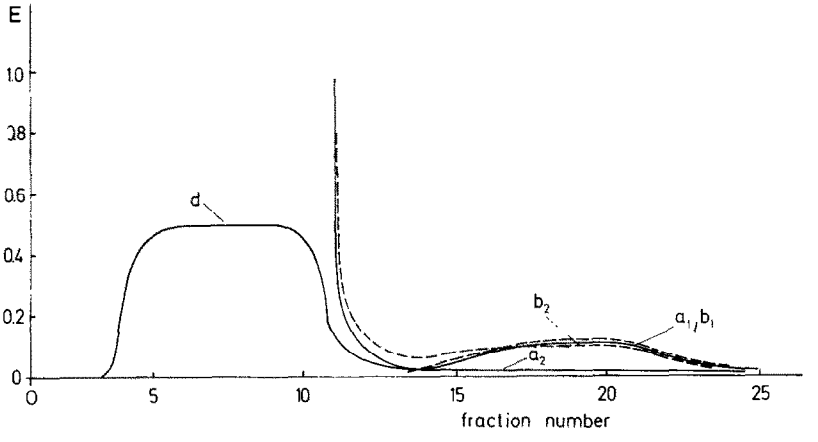


Fig. 5. Elution curves of (*S*)-1 and (*R*)-1 in 0.067 M phosphate buffer pH 7.4, in the absence and in the presence of HSA. The concentration of both compounds was 3.0×10^{-6} M and of HSA 1.44×10^{-5} M. Elution curves: (*S*)-1 and (*R*)-1 alone in buffer.—a_1 and b_1, respectively; (*S*)-1 and (*R*)-1 unbound fractions in the presence of HSA—a_2 and b_2, respectively; HSA alone and HSA in the complex—d; (the absorbance for free compounds was measured at 229 nm and for free HSA and HSA in the complex at 260 nm).

Table 1. Values for the concentration of unbound (c_f) and bound (c_b and β) (*S*)-1 to HSA*

Concentration of (*S*)-1 investigated $c \times 10^5$ (M)	Unbound (*S*)-1 $c_f \times 10^5$ (M)	Bound (*S*)-1 $c_b \times 10^5$ (M)	Per cent of bound (*S*)-1 β (%)
6.4	5.184	1.216	18.99
5	3.691	1.309	26.18
4	2.791	1.209	30.22
3	1.943	1.057	35.23
2	1.080	0.920	46.00
1.45	0.676	0.774	53.36
1	0.330	0.670	67.03
0.8	0.209	0.591	73.89
0.5	0.085	0.415	82.93
0.3	0.33	0.267	89.02
0.1	0.003	0.097	97.38

* The concentration of HSA was kept 1.44×10^{-5} M.

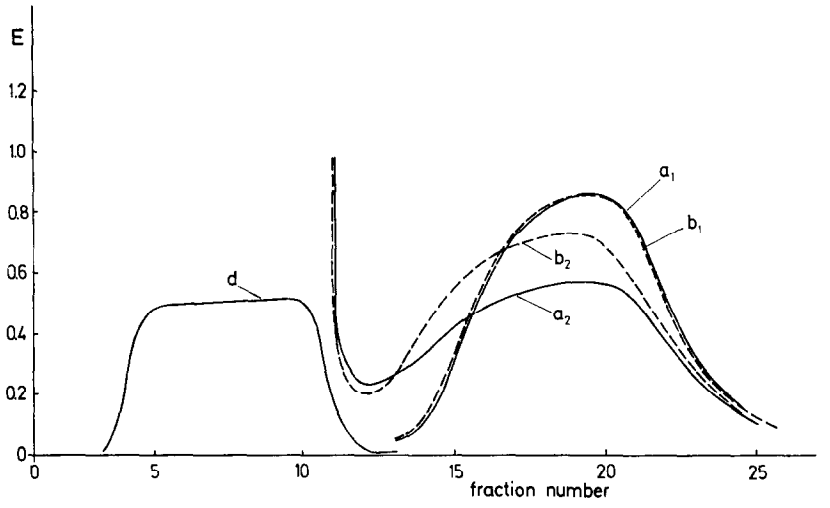


Fig. 6. Elution curves of (*S*)-1 and (*R*)-1 in 0.067 M phosphate buffer pH 7.4, in the absence and in the presence of HSA. The concentration of both compounds was 4.0×10^{-5} M and of HSA 1.44×10^{-5} M. Elution curves: (*S*)-1 and (*R*)-1 alone in buffer—a_1 and b_1, respectively; (*S*)-1 and (*R*)-1 unbound fractions in the presence of HSA—a_2 and b_2, respectively; HSA alone and HSA in the complex—d; (the absorbance for free compounds was measured at 229 nm and for free HSA and HSA in the complex at 280 nm).

logarithmic plot [8, 50], as well as by the total binding constant K_1, number of binding sites n, and free energy of binding $-\Delta F°$.

Figures 5 and 6 show elution profiles for the compounds (*S*)-1 and (*R*)-1, both dissolved in 0.067 M phosphate buffer, with and without HSA. HSA itself and the complex HSA–benzodiazepine were eluted in fractions 3–12, while unbound benzodiazepine appears in fractions 12–28. The curve for eluted protein and complex has a plateau, as is usually observed. On the contrary, the elution curves for the free ligands deviate slightly from the theoretical bell-shaped form, the highest concentration being consistently found in fraction 19.

In general, when the binding of 1,4-benzodiazepines has been followed by c.d. a concentration of 1.44×10^{-5} M (0.1%) of HSA was used. On the other hand, gel filtration experiments were mainly performed at 10–40 times higher concentrations of the protein (1–4%), following Scholtan [8, 50] or Kriegelstein and Kuschinsky [52]. We did our gel filtration experiments at the same concentrations of HSA (1.44×10^{-5} M) and of the benzodiazepines ($0.1–6.4 \times 10^{-5}$ M) as used for the c.d. measurements.

Determination of the apparent binding constants and regression coefficients

These parameters were obtained according to Scholtan [8], i.e. assuming that the relation between the concentrations of unbound and bound ligand could be represented by the following equation:

$$\log c_b = \log k^* + m \log c_f$$

where c_b is the concentration of bound ligand, c_f the

Table 2. Values for the concentrations of unbound (c_f) and bound (c_b and β) (*R*)-1 to HSA*

Concentration of (*R*)-1 investigated $c \times 10^5$ (M)	Unbound (*R*)-1 $c_f \times 10^5$ (M)	Bound (*R*)-1 $c_b \times 10^5$ (M)	Per cent of bound (*R*)-1 β (%)
6.4	5.563	0.837	13.08
5	4.215	0.785	15.70
4	3.262	0.738	18.44
3	2.491	0.509	16.97
2	1.652	0.348	17.41
1.45	1.195	0.255	17.60
1	0.807	0.193	19.28
0.8	0.635	0.165	20.61
0.5	0.394	0.106	21.24
0.3	0.239	0.061	20.37
0.1	0.064	0.036	35.91

* The concentration of HSA was kept 1.44×10^{-5} M.

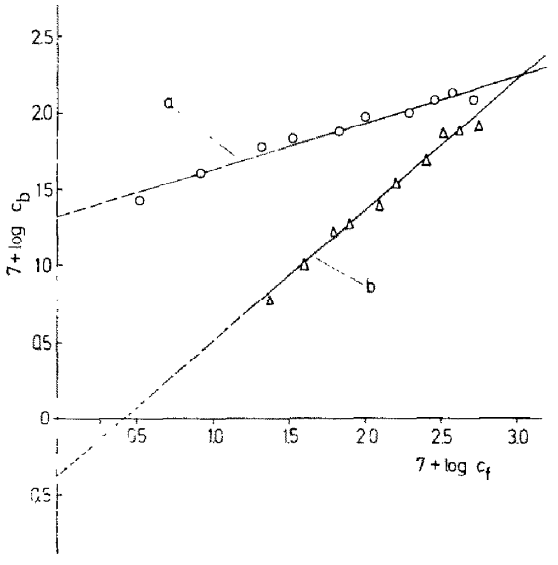


Fig. 7. Scoltan's plot calculated by computer for (*S*)-1, (a) and (*R*)-1, (b).

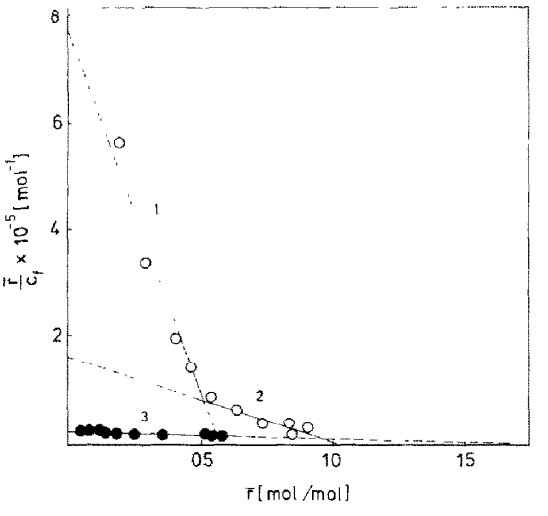


Fig. 8. Scatchard plots for binding of (*S*)-1 (1 and 2) and (*R*)-1 (3) to HSA. Concentration of HSA was 1.44×10^{-5} M.

concentration of unbound (free) ligand, k^* the apparent binding constant, and m the regression coefficient.

Values for c_f, c_b, and percentage of bound ligand (β) for the compounds (*S*)-1 and (*R*)-1 are given in Tables 1 and 2, respectively.

Figure 7 presents Scholtan's plot from which one obtains the apparent binding constants for (*S*)-1 (log $k^* = 1.33$) and (*R*)-1 (log $k^* = 0.35$) (cf. also Table 3).

Determination of the total binding constant, number of binding sites, and free energy of binding

According to the method of Scatchard [51, 53] the number of moles of ligand bound per mole of HSA ($\bar{r}$), the concentrations of the free ligand (c_f), the number of binding sites (n), and k_1/n have been calculated. Figure 8 represents Scatchard's plot for the enantiomeric pair (*S*)-1/(R)-1 . From this it follows that for (*S*)-1 there exist at least two different binding sites on the HSA, whereas for (*R*)-1 it cannot unequivocally be decided whether there is only one or are two such binding sites (cf. Table 3).

If there exist two then from the straight line 3 (Fig. 8) one can conclude that they are independent and equivalent [9]. The two binding sites for the enantiomer (*S*)-1, however, must be nonequivalent by the same arguments [9]. However, the Scholtan representation (Fig. 7) reveals only one binding process and this probably arises from the lack of sensitivity of the first method. It should be also noted that in the presence of two binding processes, the limiting slopes at low and high r values give the true binding constants only in limiting cases. If k_2 is sufficiently larger than k_1, at low $\bar{r}$, the slope is true k_1 value, but the slope at high $\bar{r}$ is $(n_1 + n_2)k_2/n_2$ [54].

Table 3. Computer derived data on the binding of (*S*)-1 and (*R*)-1 to HSA as determined by the gel filtration method*

Compound	(*S*)-1		(*R*)-1	3
β (%) at $c = 1.45 \times 10^{-5}$ M	53		18	56
and at $c = 5.0 \times 10^{-5}$ M	26		16	26
m	0.30 ± 0.01		0.86 ± 0.01	
k^* $(10^{-7}\ \text{M})^{1-m}$	21.40 ± 0.64		0.45 ± 0.01	
Concentration range $\times 10^5$ M	0.3 – 1.45	1.45 – 6.4	0.3 – 6.4	
$k_1 \times 10^{-5}$ (mol^{-1})	7.7 ± 0.3	1.6 ± 0.1	0.2 ± 0.1	
$k_1/n \times 10^{-5}$ (mol^{-1})	13.5 ± 0.5	1.6 ± 0.1	0.1 ± 0.03	
n	0.6 ± 0.1	1.0 ± 0.1	1.7 ± 0.5	
$-\Delta F°$ (kJ mol^{-1})	33.3 ± 1.3	29.5 ± 2.7	24.1 ± 7.2	
$-\Delta F°$ (kcal mol^{-1})	8.0 ± 0.3	7.0 ± 0.6	5.8 ± 1.7	

* β, Percentage of bound compound; m, regression coefficient for Scholtan's log plot; k^*, apparent binding constant; k_1, total binding constant; n, number of binding sites per one HSA molecule; $-\Delta F°$, free energy of binding.

DISCUSSION

Investigation of binding of chiral benzodiazepines 1 to HSA at a 1:1 and 3.5:1 molar ratio of ligand to protein revealed only a slight difference between differential c.d. curves for both enantiomers compared to the c.d. curves of the free ligands between 350 and 250 nm (Figs. 1–3). The ratio 3.5:1 was chosen because this is already known to lead to complete saturation [34, 37]. Both enantiomers are bound by HSA but with different affinities (Fig. 4). Gel filtration in addition to this qualitative result gives also quantitative values for the binding; e.g. the HSA affinity for (*S*)-1 is about 40 times larger than for (*R*)-1. This is in good agreement with earlier investigations on the binding of the enantiomers of 4, which revealed also about 40 times higher affinity for one of the two enantiomers (RV 1208) than for the other (RV 1210) [37]. Furthermore, the number of binding sites for RV 1210 was determined to be 1.80, and for RV 1208 1.28. For each enantiomer only one binding constant was found, however. From their differential c.d. spectra the authors [37] concluded on a stereospecific binding, i.e. that both enantiomers bind in the same absolute conformation, equal to that which is adopted by achiral 2 in the complexed form with HSA. The knowledge of the absolute configurations and c.d. curves of our compounds 1 and their *N*(1)-methyl derivatives as well as of the c.d. curves of the enantiomers of 4 allows us to determine the absolute configuration of 4: the isomer RV 1208, called "*d*-isomer" [37] possesses (*S*)-configuration at C(3), while RV 1210, the "*l*-isomer", possesses (*R*)-configuration. Thus also in this case the (*S*)-enantiomer binds more strongly than the (*R*)-enantiomer. We disagree, however, with these authors [37] in the conclusions drawn from the experimental c.d. data about the binding site on the benzodiazepine molecules. A more detailed discussion of chiroptical properties of binding processes of prochiral, as well as of chiral 1,4-benzodiazepin-2-ones, will be given in the following paper [40].

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