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CIRCULAR DICHROISM STUDIES ON THE INTERACTION OF FOUR STRUCTURALLY RELATED LONG-ACTING SULFONAMIDES WITH HUMAN AND BOVINE SERUM ALBUMIN

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Abstract—The interaction of four sulfonamides with bovine and human serum albumin (BSA and HSA) was investigated by means of circular dichroism and u.v. absorbance measurements. In the case of all sulfonamides, extrinsic Cotton effects could be found for the interaction with BSA and HSA. The anisotropy factors of the electronic transitions in the *p*-amino benzenesulfonic acid and in the heterocyclic moieties of the drugs do not differ, therefore it is concluded that both parts of the sulfonamide molecules, the *p*-amino benzenesulfonic acid part and the heterocyclic ring are bound to the albumin surface. The circular dichroism measurements especially reveal some differences of the interaction of the sulfonamides with both albumins.

The binding of the sulfonamides to serum albumins, an important factor of the pharmacokinetic of these drugs, has been extensively studied by several workers [1–10], especially regarding the extent of binding, the stoichiometry, and the influence of the chemical structure on the binding. But only little information is available on the mechanism of the binding and on the nature of the sulfonamide–albumin complex [11].

Some workers have shown a correlation between the partition coefficients of the sulfonamides and the extent of the binding [8, 9] and concluded that the binding is mainly hydrophobic [5, 8, 9]. These observations do not sufficiently agree with the conclusions of Jardetzky and Wade-Jardetzky [11], who have suggested by NMR measurements with BSA that the *p*-amino benzenesulfonic acid moiety is the the main, probably the sole binding group of the sulfonamide molecule. All observed changes of the hydrophobic nature of the sulfonamides must be mainly due to the different heterocyclic moieties of the drugs, since the *p*-amino benzenesulfonic acid part remains unchanged. It seems very unlikely that changes of the hydrophobic nature of that part of the molecule, which should not take part in the binding mechanisms, has such strong effects on the extent of the binding. These considerations are supported by studies with benzodiazepines, indicating that the introduction of hydrophilic substituents in the benzodiazepine molecule influences the binding only—if the substituents are introduced in the main binding moiety of the benzodiazepine molecule [12].

We investigated, therefore, by circular dichroism measurements in which way the heterocyclic moieties participate in the binding mechanism. This method was shown to be very suitable for obtaining information on the binding moieties of ligand molecules [12–14]. The method based on the fact that the binding of optically inactive organic molecules to asymmetric macromolecules induces optical activity in the ligand molecule, so that in the regions of the absorbance bands of the ligands, Cotton effects can be observed [15, 16] which are called extrinsic Cotton effects [16].

A few studies are known on circular dichroism measurements of albumin bound sulfonamides [17–20]. Whereas Chignell has pointed out that the binding of sulfonamides to HSA does not generate extrinsic Cotton effects [15], Perrin and co-workers could show that the binding of sulfaethidole to BSA produces a biphasic extrinsic Cotton effect [17], but this observation was used only to get information on the extent of the binding and displacing phenomena [17–19]. Furthermore, Wood and Stewart have briefly reported that several sulfonamides show extrinsic Cotton effects when bound to BSA [20]. But in no case were conclusions regarding the mode of interaction of sulfonamides with albumins drawn from these experiments.

MATERIALS AND METHODS

Materials. Human and bovine serum albumin (HSA and BSA) were obtained from Behringwerke, Marburg (trocken, reinst: electrophoretic purity 100%). The sulfonamides were obtained from the manufactures: sulfadimethoxine (Madribon®), SDM, from Hoffmann–La Roche, Grenzach; sulfamethoxydiazine (Durenat®), SMD, from Bayer, Leverkusen; sulfamethoxypryridazine (Lederkyn®), SMPD, from Lederle–Cyanamid, München; and sulfamethoxypyrazine (Longum®), SMP, from Farmitalia, Freiburg. The chemical formulas of the sulfonamides are shown in Table 1. All other chemicals were of reagent grade. All solutions were prepared with deionized water.

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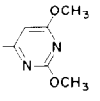
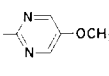
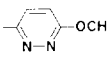
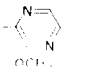
Table 1. Physico-chemical data of the sulfonamides

Substance	H_2N—⟨benzene⟩—SO_2—NH—R — R	p^*	pK_a	Buffer λ_{max} (nm)	Buffer $\epsilon \times 10^{-4}$	90% Ethanol λ_{max} (nm)	90% Ethanol $\epsilon \times 10^{-4}$
Sulfadimethoxine (SDM)		9.00	5.90* 6.10†	258 268	2.32 2.73	260 271	2.20 2.52
Sulfamethoxydiazine (SMD)		0.62	6.65* 7.00†	245 253 315	2.46 2.41 0.29	230 270	1.33 1.90
Sulfamethoxypyridazine (SMPD)		1.27	6.70* 7.20†	245 257 324	1.81 1.89 0.26	268 325	2.22 0.27
Sulfamethoxypyrazine (SMP)		0.37	6.70* 6.10†	250 313	2.08 0.84	267 297	1.88 1.02

The chemical formulas, the partition coefficients between isobutanol and buffer P, the pK_a-values, and the molar extinction coefficients of the sulfonamides at the wavelength of the u.v. maxima λ_{max} in buffer pH 7.40 and in 90% ethanol.

* Taken from reference [8];

† Taken from reference [27].

Circular dichroism measurements. CD measurements were made at 27° with a Cary 61 CD spectropolarimeter calibrated with *d*-10-camphorsulfonic acid. All spectra were recorded in cylindrical cells with 10-mm path length, using a full-scale deflection of 0.02° or 0.05° θ and a spectral bandwidth of 2 nm. Results are expressed as molar ellipticities. $[\theta]$ (deg × cm^{-2} × $dmole^{-1}$), calculated with reference to the BSA or HSA concentration, using a molecular weight of 69,000 for both albumins. Molar ellipticities of the sulfonamides bound to BSA or HSA represent difference values, using the molar ellipticities of the albumins at the same wavelengths as blank. The solutions for the CD measurements were prepared as described elsewhere [12]. The final BSA or HSA concentrations were 13.1 μM (0.09%). The sulfonamide concentrations were varied. All solutions were made with M 15 phosphate buffer and adjusted to the desired pH with 1 M HCl or 1 M NaOH. Each CD spectrum reported is the average of three individual observations.

The anisotropy factors (*g*-values) [14] were calculated following the equation [14]

$$g = \frac{[\theta]_\lambda}{3300\epsilon_\lambda}$$

were $[\theta]_\lambda$ = the molar ellipticity of the sulfonamides (50 μM) in the presence of BSA or HSA 13.1 μM at the wavelength of the CD maximum, calculated with reference to the concentration of the bound sulfonamides, and ϵ_λ = the molar extinction coefficient of the sulfonamides (10 μM) in presence of BSA or HSA (13.1 μM) at the wavelength of the CD maximum.

Ultraviolet measurements. Ultraviolet measurements were made with a Gilford spectrophotometer, model 2400. Molar extinction coefficients of the sulfonamides were determined using a sulfonamide concentration of 10 μM dissolved in M 15 phosphate buffer pH 7.40, 90% ethanol or BSA or HSA 13.1 μM in buffer, pH 7.40, using an albumin solution without sulfonamide as blank.

Albumin binding measurements. The binding of the sulfonamides to BSA or HSA (13.1 μM) was determined using the ultracentrifugation method of Scholtan [21]. Pollyallomer tubes were used instead of cellulose nitrate tubes. The sulfonamide concentration in the supernatant was determined by the method of Bratton and Marshall [22].

RESULTS

Circular dichroism measurements. The binding of all four sulfonamides investigated to BSA and HSA generates extrinsic Cotton effects (Figs. 1, 2 and 3, and Table 2). These induced Cotton effects superpose the intrinsic circular dichroism spectra of the albumins (Fig. 1). By subtracting the ellipticities of the albumins alone at each wavelength, difference spectra were obtained (Figs. 2 and 3). In most cases the induced Cotton effects of the sulfonamides in the presence of both albumins have three ellipticity bands (Figs. 2 and 3). All four drugs have, in presence of both albumins, a positive extrinsic CD band near 250 nm, which maximum can not be determined for some substances because of the high ellipticity values of the albumins alone at wavelengths shorter than 250 nm (Fig. 1). Except SMD and SMP when bound to BSA, the drugs investigated have a negative CD band near

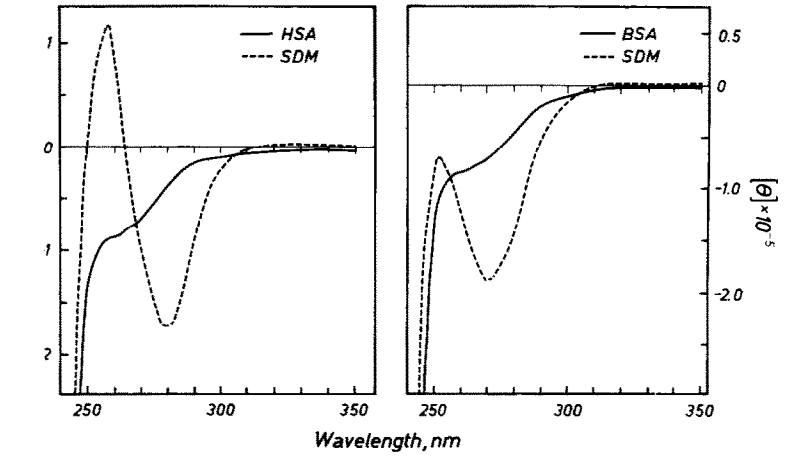


Fig. 1. CD spectra of BSA and HSA (0.09%) alone and of SDM (50 μM) in presence of BSA or HSA (0.09%) at pH 7.40. Ordinate: molar ellipticity calculated with respect to the albumin concentration. Abscissa: wavelength in nm.

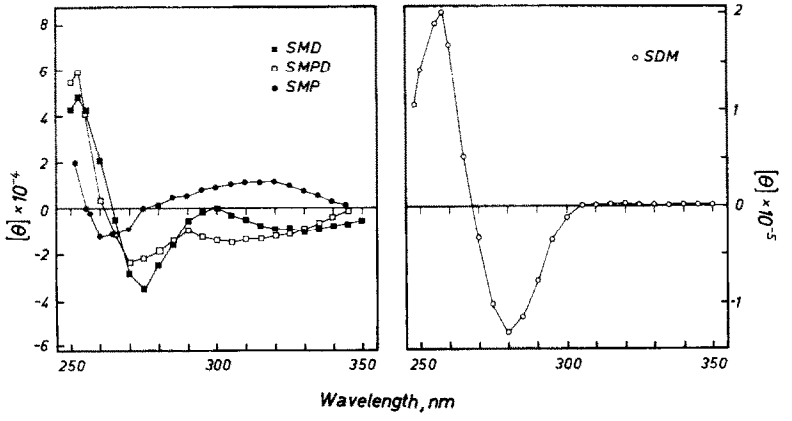


Fig. 2. Extrinsic Cotton effects of the sulfonamides (50 μM) in the presence of HSA (0.09%) at pH 7.40. At each point of the curves the effects of HSA alone are subtracted. Each point represents the mean of three determinations. Ordinate: molar ellipticity calculated with respect to the HSA concentration. Abscissa: wavelength in nm.

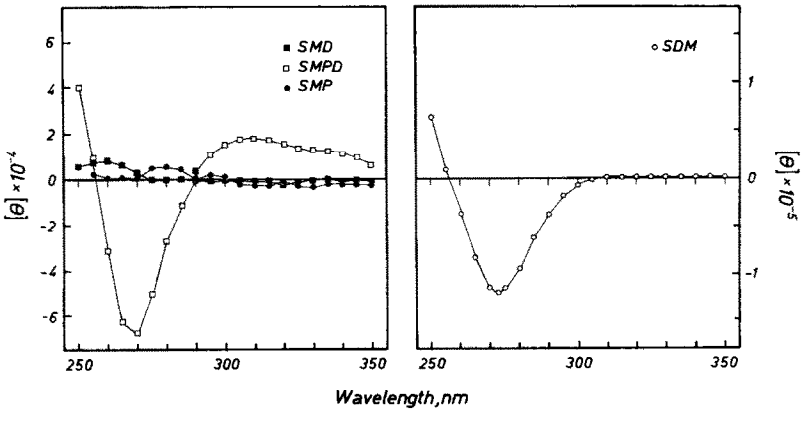


Fig. 3. Extrinsic Cotton effects of the sulfonamides (50 μM) in the presence of BSA (0.09%) at pH 7.40. All other conditions are similar to the conditions of Fig. 2.

Table 2. Spectroscopic data of the albumin–sulfonamide complexes

Albumin	Substance	λ_{max}CD (nm)	$[\theta] \times 10^{-4}$ $\bar{x} \pm s_{\bar{x}}(n)$	$g \times 10^4$	λ_{max}u.v. (nm)	$\epsilon \times 10^{-4}$
BSA	SDM				259	2.37
		273	−11.21 ± 0.20 (9)	−7.61	271	3.18
	SMD				246	2.51
		260	+0.51 ± 0.14 (6)	+1.58	255	2.42
		320	−0.21 ± 0.08 (6)	−5.30	314	0.30
	SMPD				242	0.94
		270	−6.36 ± 0.14 (9)	−16.71	258	1.55
		310	+1.17 ± 0.20 (9)	+9.28	324	0.26
	SMP				250	1.82
		280	+0.65 ± 0.17 (6)	+10.85		
		330	−0.37 ± 0.07 (6)	5.17	314	0.89
HSA	SDM	258	+19.83 ± 0.15 (9)	+12.88	261	2.44
		280	−13.82 ± 0.39 (9)	−7.71	273	3.00
	SMD	252	+5.80 ± 0.17 (9)	+5.64	246	2.80
		275	−3.16 ± 0.30 (9)	−4.68	256	2.53
		325	−1.12 ± 0.21 (9)	−10.17	319	0.29
	SMPD	253	+4.53 ± 0.17 (9)	+5.65	240	0.81
		270	−2.56 ± 0.18 (9)	−4.26	261	1.50
		305	−1.89 ± 0.24 (9)	−11.24	321	0.24
	SMP				249	1.80
		260	−1.25 ± 0.10 (6)	−4.14		
		315	+0.93 ± 0.05 (6)	+5.18	314	0.86

The molar ellipticities of the sulfonamides bound $[\theta]$ at the wavelengths of the CD maxima λ_{max}CD, calculated with reference to the albumin concentration, the corresponding anisotropy factors g, and the molar extinction coefficients ϵ at the wavelengths of the u.v. maxima λ_{max}u.v. of the sulfonamides bound to BSA or HSA.

270 nm, when bound to both albumins (Figs 2 and 3, and Table 2). SMD and SMP bound to BSA have a positive band at this wavelength (Fig. 3, and Table 2). In contrast, the signs of the induced CD bands of the sulfonamides bound to BSA and HSA near 310 nm differ (Figs. 2 and 3 and Table 2). This band is absent in the case of the interaction of SDM with both albumins (Figs 2 and 3). The intensities of the induced Cotton effects of the sulfonamides in presence of BSA and HSA differ greatly, as shown by the molar ellipticities in Table 2. Similar differences were found for the anisotropy factors (g-values), which give an information on the degree of the optical perturbation of the electronic transitions of the drugs [14]. The g-values (Table 2) are corrected for the amount of drug bound and for binding-induced changes of the u.v. absorbance of the sulfonamides.

Ultraviolet absorbance measurements. The binding of the sulfonamides to both albumins has only a small influence on the u.v. absorbance of the drugs. This can be seen by comparing the wavelengths of the u.v. maxima and the molar extinction coefficients of the drugs in buffer (Table 1) and when bound to BSA or HSA (Table 2). In most cases, the binding to BSA or HSA produces a small red-shift of the absorbance maxima, accompanied by a small increase of the extinction coefficients. Higher effects on the u.v. absorbance can be found by changing the solvent from buffer to 90% ethanol (Table 2). Whereas the u.v. absorbance of SDM and SMP is influenced in a smaller degree, the absorbance of SDM and SMPD is highly changed, as regards the wavelength positions of the maxima and the extinction coefficients.

Albumin binding measurements. Using concentration conditions similar to the CD measurements, the binding of the sulfonamides to both albumins was determined by ultracentrifugation. The percentages of free and bound drug (α- and β-values) and the quotient between α- and β-values are given in Table 3.

DISCUSSION

The quotient of the percentage of bound and free drug can be used to characterize the binding of drugs to albumins [23]. In the case of the four sulfonamides bound to BSA and HSA, the extent of the binding seems to depend on the lipophilic nature of the drugs (Fig. 4, left). A similar relation was reported by Scholtan [8]. All sulfonamides are higher bound to HSA than to BSA (Fig. 4, left). Note the large difference of the binding of SMD to BSA and HSA (Fig. 4, left). This difference was also found for the interaction of SMD with bovine and human serum [5]. It is concluded from our results that this different binding behaviour must be due to specific differences of both serum albumins.

All investigated sulfonamides show a positive CD band near 250 nm when bound to BSA (Fig. 3) and HSA (Fig. 2), but in the latter case the intensities are smaller. The wavelength position of this CD band corresponds with a maximum of the u.v. spectra of the drugs bound (Table 2). The wavelength position of this u.v. band is relatively similar in the case of all four sulfonamides investigated as well as the position and the signs of the corresponding CD bands. It is known that the wavelength position of the ^1L_b band of the three diazines is influenced to a greater extent by the position of the two nitrogen atoms [24] and in the case of the methoxy-substituted diazines is largely located at wavelengths higher than 250 nm

Table 3. The albumin binding of the sulfonamides

Albumin	Substance	α $\bar{x} \pm s_{\bar{x}}(n=6)$	β	β/α
BSA	SDM	62.76 ± 1.60	37.24	0.59
	SMD	88.81 ± 1.12	11.19	0.13
	SMPD	68.81 ± 2.53	31.19	0.45
	SMP	90.68 ± 1.31	9.32	0.10
HSA	SDM	47.16 ± 0.75	52.84	1.12
	SMD	68.73 ± 1.53	31.27	0.46
	SMPD	57.24 ± 1.43	42.76	0.75
	SMP	83.75 ± 1.54	16.25	0.19

α = the percentage of free drug; β = the percentage of bound drug,

β/α = the ratio of the percentages of bound and free drug.

[24]. We suggest, therefore, that this u.v. band and the corresponding CD band are due to the ^1L_b transitions of the *p*-amino benzenesulfonic acid moieties of the molecules. This is supported by the findings that this band is located in sulfanilamide at 258 nm [25]. In the sulfanilamide anion, which is the present form of the investigated drugs at pH 7.40 [4, 27], this band is located at 251 nm [26].

The CD band near 270 nm (negative in nearly all cases) does not correspond to a u.v. maximum of the substance bound in the case SMPD and SMP (Table 2). But, as regards the u.v. maxima of these substances in ethanol (Table 1), it can be seen that there are u.v. bands at this wavelength, which seem to be superposed in aqueous solution by the high band at 250 nm. The wavelength position of this u.v. band and of the corresponding CD band as well as the signs of these CD bands are different for the investigated sulfonamides. We think that this CD band is due to a perturbation of the ^1L_b transition of the different substituted diazine moieties of the sulfonamides, which is located in the case of the three diazines near 270 nm [24] and is largely influenced by the position of the two nitrogen atoms as well as by the substituents [24].

The CD band at the longest wavelength seems to be due to electronic transitions of the heterocyclic moieties of the sulfonamides bound, probably to the

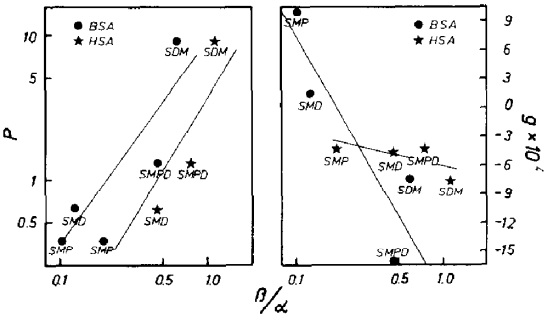


Fig. 4. Left: The relationship between the partition coefficient of the sulfonamides (ordinate) and the quotient between the percentage of bound and free drug (β/α) (abscissa). Right: The relationship between the anisotropy factors of the CD band near 270 nm (ordinate) and the quotient β/α (abscissa).

$n \rightarrow \pi^*$ transition of the diazines, which is located near 300 nm [24]. But at present, a contribution of the secondary benzene band of the *p*-amino benzenesulfonic acid moieties can not be excluded. Because of the sufficient agreement of the wavelength positions of the CD bands and the u.v. maxima of the sulfonamides investigated, we conclude with other authors [17, 19, 20] that the Cotton effects observed for the complexation of sulfonamides with albumins are extrinsic in the origin.

Based on these considerations, it must be concluded for the binding of the four sulfonamides to HSA that (1). The sulfonamides form a rigid and well defined complex with HSA, which is a condition for the generation of extrinsic Cotton effects [14, 15] (2). These complexes of the four sulfonamides with HSA must be similar, which is obvious from the similar signs and wavelength positions of the CD bands near 250 nm and 270 nm. (3) Both moieties of the sulfonamide molecule are rigidly fixed at the albumin surface, near or at an asymmetric locus of the albumin molecule, since it is known that the perturbation of electronic transitions decreases when the distance between the asymmetric locus and the perturbed chromophore increases [14, 28] and because the anisotropy factors of both CD bands, due to the *p*-amino benzenesulfonic acid moiety and to the heterocyclic moiety of the molecule, are of the same order of magnitude. A binding of the sulfonamide molecule only by the *p*-amino benzenesulfonic acid moiety would not hinder the heterocyclic ring from rotating freely, and therefore only perturbations of the electronic transitions of this part of the molecule lower than those observed (Table 2), would be expected [12, 14].

This is partially in contradiction to the conclusions of Jardetzky and Wade-Jardetzky [11], who have assumed that the *p*-amino benzenesulfonic acid moiety represents the main, or probably the sole, binding group of the sulfonamide molecule. It must be noted that Jardetzky and Wade-Jardetzky were working with BSA [11]. Our results with BSA reveal differences in the interaction of the sulfonamides with both albumins, especially regarding the different signs of the heterocyclic band near 270 nm (Table 2). This indicates differences in the interaction of the diazine rings with BSA. But despite these differences, it must be concluded from the partially high anisotropy factors of this band that the heterocyclic moiety is attached to the BSA molecule when the drugs are bound. The optical perturbation of the heterocyclic band near 270 nm depends only partially on similar factors as the extent of the binding (Fig. 4, right). In the case of HSA, the *g*-values of the four derivatives differ only slightly and, in the case of BSA, the signs of this band are positive for the weakly-bound derivatives SMP and SMD and are negative for the strongly-bound derivatives SMPD and SDM (Fig. 4, right). It seems, therefore, that there are in addition some other factors by which the heterocyclic moieties influence the type of the sulfonamide–albumin complex.

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