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A quantitative circular dichroic investigation of the binding of the enantiomers of ibuprofen and naproxen to human serum albumin

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Abstract

The binding constants for racemic, R and S naproxen and ibuprofen to human serum albumin have been determined by a circular dichroic technique. The ibuprofens and naproxens show no measurable extrinsic optical activity on interaction with the protein, and so the extrinsic Cotton effect shown following the diazepam–albumin interaction is used as a probe. The presence of the drugs reduce the amount of diazepam bound as shown by the reduced size of the induced ellipticity. The calculated primary binding constants show that the S form of both drugs bind to the albumin more tightly than the R form and that the racemic forms bind less tightly than either enantiomer.

Keywords: Enantiomers; Racemate; Ibuprofen; Naproxen; Extrinsic cotton effects; Finding constants; Diazepam

1. Introduction

Albumin drug interactions have been investigated by circular dichroism for over 25 years. If a chromophore in the drug molecule becomes optically active when it is perturbed by an asymmetric centre in the human serum albumin (HSA) molecule, as the perturbation is from a second molecule then the resultant Cotton effect is termed extrinsic. Early studies of this phenomenon were used in a qualitative manner to support quantitative data from dialysis and ultrafiltration studies [1–3]. Since the work of Rosen [4] many studies have estimated binding constants for the formation of drug HSA complexes using the proportionality between the intensity of the induced Cotton effect and the concentration of complex assuming a single binding phenomenon is involved [5–9]. Not all drug molecules with suitable chromophores give measurable Cotton effects following interaction with albumin. In 1973, Perrin and Nelson estimated binding constants of drug HSA complexes by

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using a probe giving a large extrinsic Cotton effect on binding to albumin and then measuring the amounts displaced by drugs competing for the same binding site but which produced no measurable circular dichroic signals following interaction with albumin [10]. Ibuprofen and naproxen have been reported to bind to the same binding site on HSA as diazepam [11,12]. Diazepam gives a quantifiable Cotton effect following binding to HSA with a maximum near 260 nm. On the other hand ibuprofen and naproxen display no measurable extrinsic Cotton effects under the experimental conditions found to give suitable ellipticities for measurement of the diazepam–HSA interaction. It was therefore decided to determine binding constants for the interaction between the enantiomers of ibuprofen and naproxen with HSA by measuring their ability to displace diazepam from HSA using the circular dichroic technique.

2. Experimental

2.1. Materials

Essentially fatty acid free HSA (lot no. 42H9313 prepared from Fraction V albumin) was obtained from Sigma (St. Louis, MO), and used without further treatment. S(+) and R(−) naproxen were gifts from Syntex Research (Palo Alto, CA). S(+) ibuprofen was a gift from Sepracor (Marlborough, MA) and R(−) ibuprofen was obtained as a gift from Research Biochemicals (Natick, MA). Diazepam was kindly supplied by Dr Richard E. Tessel, Dept. of Pharmacology and Toxicology, University of Kansas. The drugs were used as received. All other chemicals used were of analytical grade and sources. Deionized water, purified in a Milli-Q Water System, Millipore Corp. (Bedford, MA), was used to prepare the sodium phosphate buffer that was used throughout. The Pierce BCA® protein assay kit (Pierce, Rockford, IL) was used to determine the HSA concentrations in all the experiments.

2.2. Method

Measurements were made using an Aviv® circular dichroism spectrometer, model 60 DS (Aviv, Lakewood, NJ) using 10 mm cells at 25°C in thermostatic cell-holders. The slit width was programmed for a half band width of 1 nm and the dynode voltage never exceeded 0.3 kV. All solutions were scanned from a wavelength at which no induced ellipticity was observed, around 365–250 nm. Solutions were not scanned below 250 nm, in which region the ellipticity of the protein became more dominant and the rise in absorbance of the solution made good quantitation impossible. A scan of dilute solutions in shorter pathlength cells did indicate that there is little or no departure from the alpha helical structure associated with HSA at the lower wavelengths. The induced ellipticity was defined as the ellipticity of the drug albumin mixture minus the ellipticity of the HSA alone at the same wavelength and was expressed in degrees.

Induced ellipticity = ellipticity of drug
–HSA complex
– Intrinsic ellipticity of HSA.

Experiments were performed to check for intrinsic ellipticity of all ligands. No change was observed in the HSA spectrum on addition of ibuprofen or naproxen enantiomers. Diazepam, which is known to bind to the same site on HSA as the non-steroidal anti-inflammatory agents and which induces a signal in the HSA spectrum on binding, was therefore used as a probe to study the binding of ibuprofen and naproxen enantiomers to HSA. The Ibuprofen and naproxen give no measurable induced optical activity under these experimental conditions. The ratio of diazepam to HSA was kept constant at 2.4:1 and the concentration of the competing ligand was varied. HSA concentration was fixed at 1.5×10^{-5} M and all solutions were made in 0.1 M sodium phosphate buffer pH 7.4. The ellipticity of the solution in the cell was scanned from 365 to 250 nm, but only the values at 262 nm were used the calculation of binding constants. All the scans were adjusted to zero baseline at 365 nm. Every

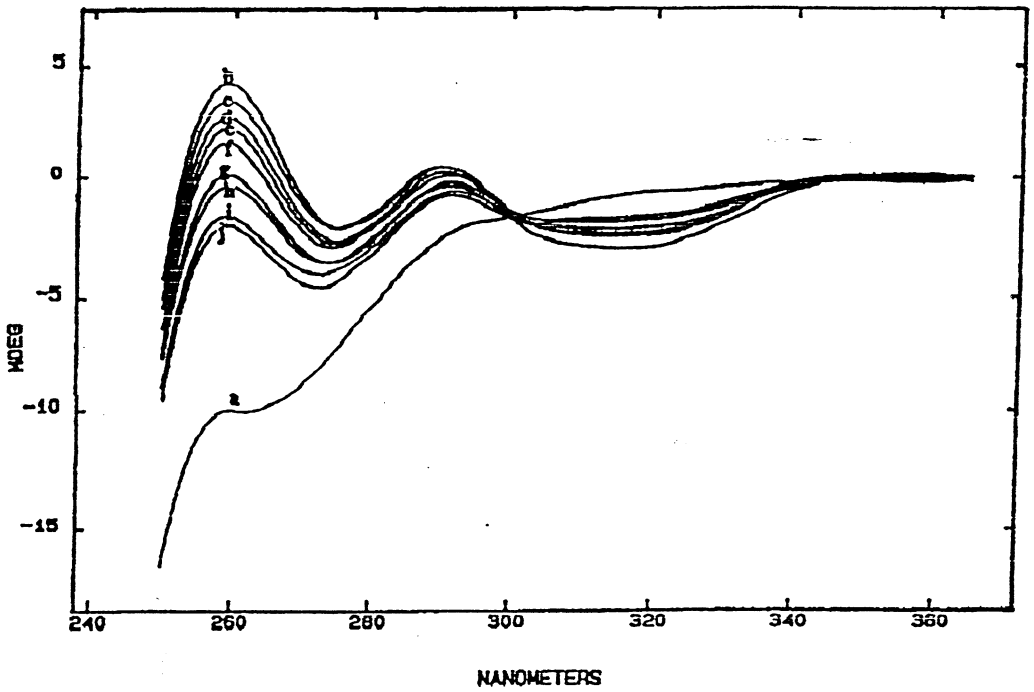

Fig. 1. CD spectra for diazepam binding to HSA, antagonised by racemic-ibuprofen in 0.1 M, phosphate buffer, pH 7.4 at 25°C. (a) HSA = 15 μM, diazepam = 0, ibuprofen = 0. (b) Diazepam/HSA = 2.4, ibuprofen = 0. R,S-ibuprofen/HSA concentration ratios (c) 0.45; (d) 0.68; (e) 0.90; (f) 1.13; (g) 1.35; (h) 1.79; (i) 2.23; (j) 2.67, all with the diazepam/HSA ratio held constant at 2.4. Each scan is the mean of three separate scans.

solution was scanned three times to yield an average value and all solutions were studied in duplicate.

3. Results and discussion

The enantiomers of ibuprofen and naproxen displayed no intrinsic ellipticity in the wavelength range 250–360 nm under the current experimental conditions, and so no corrections to the spectra were necessary. Both molecules have absorption maxima in the low UV range, where measurements in the presence of HSA are of little interest because of the relatively large ellipticity of the HSA molecule. Similarly at drug to albumin ratios of up to 10, no extrinsic Cotton effects following interaction of the naproxens and ibuprofens with albumin were observed at wavelengths above 250 nm. To use diazepam as a probe it was necessary to determine its binding constant with the same batch of HSA and under the same experimental conditions. For this estimation the ellipticity of diazepam–HSA complexes were measured at wavelengths between 250 and 360 nm at the fixed HSA concentration. Quantitative measurements were made at 262 nm and using an iterative least square technique a binding constant of 2.4×10^5 M^{-1} was obtained assuming a 1:1 interaction. This value compares to values of 1.8×10^5 M^{-1} [13] and 2.2×10^5 M^{-1} [14] in the literature.

A concentration ratio of 2.4 diazepam to HSA was found to give a suitable signal for displacement measurements. At these concentrations of drug and HSA 78% of the diazepam is bound. This probe was then displaced by known concentration of S,R and racemic and naproxen. In the absence of a competing ligand a diazepam–HSA complex gives circular dichroic

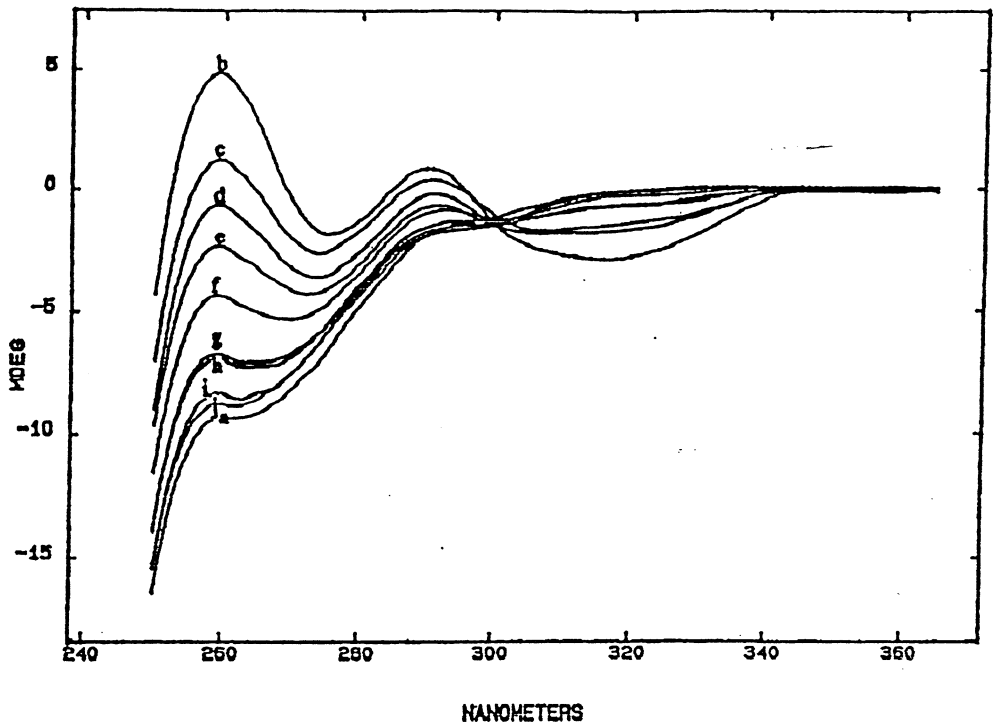

Fig. 2. CD spectra for diazepam binding to HSA, antagonised by S-naproxin, in 0.1 M, phosphate buffer, pH 7.4 at 25°C. (a) HSA = 15 μm, diazepam = 0, S-naproxon = 0. (b) Diazepam/HSA = 2.4, S-naproxon = 0. S-naproxon/HSA concentration ratios (c) 0.40; (d) 0.61; (e) 0.81; (f) 1.21; (g) 1.60; (h) 2.00; (i) 2.39; (j) 2.78 all with the diazepam/HSA ratio held constant at 2,4. Each scan is the mean of three separate scans.

spectra with maximum at 259 and 290 nm and a minimum at 318 nm (Fig. 1 and Fig. 2). The general shape of these spectra were not changed following the addition of the competing drugs, however the magnitude was reduced in an apparently quantitative manner (Fig. 1 and Fig. 2). Quantitative measurements were made at 262 nm. Fig. 3 shows the changes in the induced ellipticity at 262 nm of the diazepam–HSA complex as a function of the concentration of the ibuprofens. Naproxen gives a similar set of curves. It is immediately obvious that the racemic ibuprofen (and naproxen) have less ability to displace the diazepam than the enantiomers. In both cases the S enantiomer appears to have a greater displacing ability. The binding constant for the enantiomer and racemic ibuprofen and naproxen can be calculated from the circular dichroic data assuming a 1:1 binding phenomenon by the following equations [10].

$$\frac{K_{\text{ligand}}}{K_{\text{Diaz}}} = \frac{[\text{LP}][\text{D}]}{[\text{DP}][\text{L}]} \tag{1}$$

[LP] is the concentration of bound ligand and [L] the concentration of free ligand. [DP] is the concentration of bound diazepam and [D] the concentration of free diazepam.

$$\text{DP} = [\text{DP}]_{\text{L}=0} \times \frac{D_{\text{L}}}{D_{\text{L}=0}} \tag{2}$$

D_{L} and $D_{\text{L}=0}$ are observed ellipticities in the presence and absence of competing ligand $[\text{DP}]_{\text{L}=0}$ is the amount of diazepam bound at the fixed HSA concentration in the absence of competing ligand and is obtained using the binding constant already derived for the diazepam complex.

In the presence or absence of competing ligand

$$[\text{P}] = \frac{[\text{DP}]}{K_{\text{Diaz}}[\text{D}]} \tag{3}$$

where [P] is the free protein concentration. [D] is the difference between the total diazepam concentration and [DP].

$$[LP] = [P_{total}] - [DP] - [P] \quad (4)$$

where $[P_{total}]$ is the total HSA concentration. Finally

$$[L] - [L_{total}] - [LP] \quad (5)$$

Substituting Eqs. (2)–(4) and Eq. (5) into Eq. (1) with the already obtained K_{Diaz} allows K_{Ligand} to be estimated.

Table 1, gives the values from the ibuprofens and naproxens, respectively. The values are in excellent agreement with those obtained from microcalorimetry [15] in spite of the fact that in the calorimetry studies a 20 times higher concentration range was used in these studies. Unfortunately adding octanoic acid to the system caused a marked decrease in the signal to noise ratios in the circular dichroic studies and so no measurements were made to compare with those observed in the microcalorimetric investigation. Octanoic acid was shown to increase the difference in the affinities to HSA of the enantiomers of both drugs, confirming the use of octanoic acid to improve the separation of these enantiomers on immobilized albumin columns [16,17].

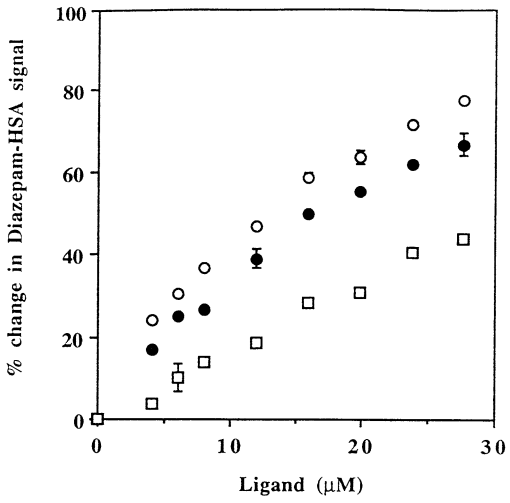

Fig. 3. Per cent change in the induced signal of the diazepam-H complex at 262 nm with increasing concentrations of (●) R; (○) S and (□) racemic ibuprofen. The HSA concentration was 15 μm in 0.1 M phosphate buffer, pH 7.4 at 25°C and the diazepam/HSA concentration was 2.4. Each data point represents an average of two readings with the error bar representing the standard error of the mean.

Table 1
Apparent binding constants of naproxens and ibuprofens enantiomers to HSA, determined by circular dichroism in 0.1 M phosphate buffer, pH 7.4 at 25°

Ligand	K_{app} (M^{-1})
S-Ibuprofen	$8.0 \pm 0.11 \times 10^5$
R-Ibuprofen	$4.8 \pm 0.07 \times 10^5$
Racemic ibuprofen	$1.4 \pm 0.04 \times 10^5$
S-Naproxen	$3.1 \pm 0.66 \times 10^6$
R-Naproxen	$1.6 \pm 0.32 \times 10^6$
Racemic naproxen	$5.5 \pm 0.08 \times 10^5$

The concentration of HSA was 15 μM l^{-1}, the diazepam/HSA ratio was set at 2.4 and the concentration of the NSAID enantiomers was varied from 0 to 28 μM l^{-1}. The changes in the induced ellipticity at 262 nm was varied from 0 to 28 μM l^{-1}. The changes in the induced ellipticity at 262 nm were used in the calculation of the *K* values. Each value represents the mean ± S.E. of 147 estimates.

For both drugs the S form binds more tightly to the albumin than the R form, apparently the S has a better fit into the binding site of diazepam. If all were ideal, i.e. both drugs bound to the same area in the binding site with no resultant conformation change, then the racemic forms of the drugs should bind with a binding constant between those of the R and S enantiomers [18]. The binding constants for the racemic ibuprofen and racemic naproxen is below that of both the component enantiomers. A possible explanation is that the drugs do not bind to exactly the same fraction of the total binding area on HSA. It is well accepted that the so called diazepam binding site is not as well defined as the warfarin binding site, a larger area being involved. It is also possible that the R and S enantiomers alter the conformation of albumin to differing extents, with the result that the binding of one or probably both is reduced in a mixture of enantiomers. The results show that measurement of the reduction of the ellipticity induced into a drug following the binding to HSA by ligands which do not have induced activity following the binding to HSA is a very sensitive method of obtaining the binding con-

stant for the ligands at the binding site of the reference drug and is a suitable technique for quickly showing differences in binding affinities of closely related molecules. Much less material is needed than is necessary for ultrafiltration, dialysis and microcalorimetry. There are very few studies which investigate the binding of racemic as well as enantiomeric forms of drugs to HSA, however the racemic form of flurbuprofen binds at a value between those of the enantiomers [19] whereas racemic ketoprofen has a lower affinity for HSA than the enantiomers at certain concentrations [20].

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