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BINDING OF DRUGS TO HUMAN SERUM ALBUMIN—XV

CHARACTERIZATION AND IDENTIFICATION OF THE BINDING SITES OF INDOMETHACIN*

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Abstract—The binding of indomethacin to human serum albumin (HSA)§ has been studied with HSA immobilized in microparticles of polyacrylamide, by equilibrim dialysis and by circular dichroism. Indomethacin is bound to three sites with high affinity. The association constants are 1.5–2.1 × 10^6, 3.5 × 10^5 and 5.5 × 10^4 M^{-1}, respectively, as found by spectropolarimetric titration and from Scatchard plots of data obtained from the studies with microparticles. The evaluation of the CD-data is simplified by the different extrinsic Cotton effects engendered when indomethacin binds to the different sites. With specific probes, binding exclusively to single sites, we have shown that indomethacin primarily binds to the warfarin-(azapropazone-) site and tertiarily to the diazepam-(flurbiprofen-) site on HSA. The identity of the secondary site has not been established. Probenecid does not affect the first site of indomethacin, but improves the binding of indomethacin to the second site, probably via allosteric effects from the third site.

Indomethacin is extensively bound to plasma proteins. Some papers report that the binding degree is over 96 per cent in plasma or serum [1–3] and with isolated human serum albumin (HSA) [4]. However, lower binding has also been reported [5] and conflicting results concerning the binding constants and number of binding sites have appeared [1, 4, 5]. In addition, the possibilities of other drugs interacting with the binding of indomethacin have not yet been fully clarified. Phenylbutazone, for instance, has been reported both to improve [1] and to inhibit [6] the binding of indomethacin. The contradictions seem to depend substantially on the fact that indomethacin is bound to several sites on albumin [1, 4, 7] with significant affinity. The specificity of these sites is certainly different, which means that the binding can be differently affected by different drugs depending on the number of sites which are occupied by indomethacin. The concentration and character of different endogenous substances inhibiting the binding [2, 8] can also vary considerably between serum samples used in the different studies. In addition, both competitive displacement and allosteric mechanisms can differently affect the binding to different sites [1, 6, 7, 9].

In order to be able to predict any drug interactions on the protein binding level involving indomethacin, it is necessary to know in some detail the sites on HSA having affinity for indomethacin and their binding characteristics. Such a study can conveniently be done with circular dichroism (CD) measurements and with HSA immobilized in microparticles of polyacrylamide. Indomethacin is known to give strong extrinsic Cotton effects when bound to HSA [10, 11], and in the present paper, CD measurements have been used to identify the binding sites on HSA. The HSA-microspheres have been used to determine quantitatively the binding to HSA [12]. We have earlier shown with such particles that indomethacin can bind to at least two sites, namely, the diazepam and warfarin sites [7]. The capacity of probenecid to interact sterically with the binding of indomethacin has also been studied.

MATERIALS AND METHODS

Human serum albumin. HSA was prepared from outdated blood as described by McMenamy *et al.* [13] or was bought from AB KABI, Stockholm, Sweden. Any bound small molecules were removed by treatment with active charcoal at pH 3 according to Chen [14]. The HSA gave only one band in polyacrylamide-gel electrophoresis at pH 8.3. The concentration was determined from the optical density at 280 nm ($A_{1\,cm}^{1\%}$ = 5.8), and 66,500 was used as the molecular weight.

Drugs. [^{14}C] Indomethacin (13.8 mCi/mmole or 509 MBq/mmole) was obtained from Merck, Sharp & Dohme, Rahway, NY. The radiochemical purity (>99 per cent) was checked by thin-layer chromatography. Unlabelled drugs were gifts from the different manufacturers or their Swedish representatives.

Microparticles. Microparticles with immobilized HSA were prepared according to the method of

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§ Abbreviations used: HSA, human serum albumin; CD, circular dichroism.

Ekman and Sjöholm [15] by emulsion polymerization of acrylamide and bisacrylamide together with albumin. The total concentration of monomers was 8 per cent and the crosslinking degree 25 per cent. The mean diameter of the microparticles was about 1 μm. The HSA content was determined by amino acid analysis after hydrolysis for 24 hr in 5.9 M HCl at 105°.

Binding studies. Binding studies with HSA-microparticles were carried out as described by Kober *et al.* [12]. Incubation with HSA-particles and drugs were performed in plastic tubes (5.5 × 1.1 cm) at room temperature (22–24°) in 0.1 M KCl with 0.005 M phosphate, pH 7.4. The maximal final concentration of ethanol, used to dissolve the drugs, was 0.33% in the samples. After equilibrium had been obtained (<15 min), the tubes were centrifuged in a table centrifuge at 3000 *g* for 20 min. Aliquots (100 μl in duplicates) were removed from the supernatant and the free drug concentration determined from the radioactivity as found by liquid scintillation counting. Protein binding data were analysed according to Scatchard [16]. The equation

$$\frac{r}{(D)} = n \cdot K_{app} - r \cdot K_{app}$$

was used, where r = moles of bound drug/moles of albumin, (D) = concentration of unbound drug, n = number of binding sites and K_{app} = the apparent association constant. In the Scatchard plots, linear regression was analysed using r as the independent and $r/(D)$ as the dependent variable. The experimental points selected for such analyses are defined in the Results section.

Circular dichroism (CD) spectra. These were obtained at room temperature using an automatic spectropolarimeter JASCO J-41 A, Japan Spectroscopic Co., Tokyo, Japan. The instrument was calibrated with D-10 camphorsulphonic acid. Rectangular cells with path lengths of 5–20 mm were used,

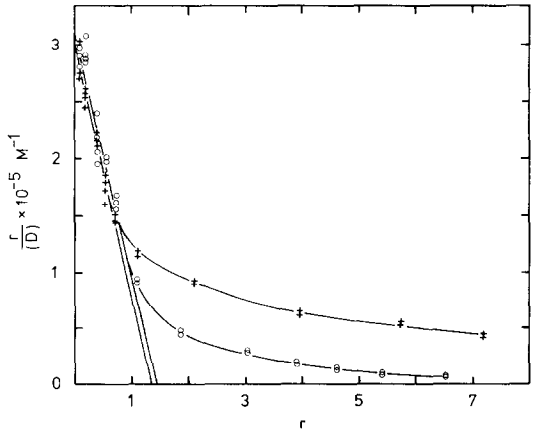

Fig. 1. Scatchard plots of the binding of indomethacin to human serum albumin immobilized in microparticles in 0.1 M KCl with 0.005 M phosphate buffer (pH 7.4) at 23°. The results are taken from several experiments with HSA concentration varying between 5.54 and 13.58 μM in the absence (–O–O–) and presence (–+–+–) of probenecid added in a molar ratio to HSA of 1.4:1.

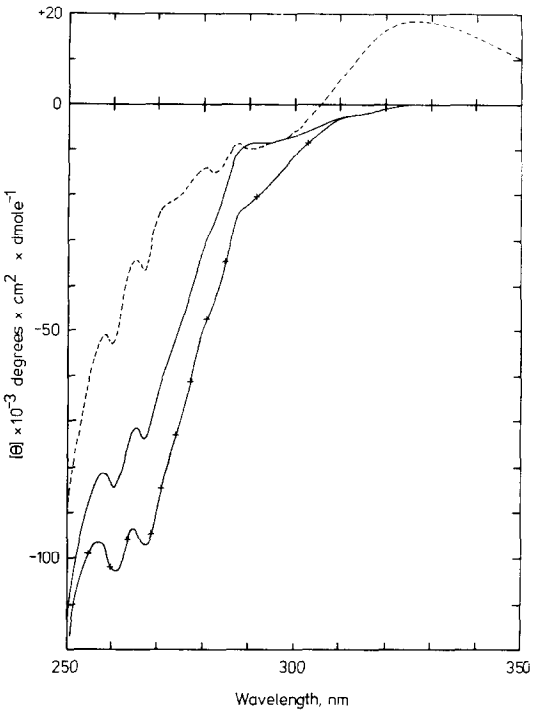

Fig. 2. Circular dichroism spectra of HSA (1.14 mg/ml, ——), of HSA: indomethacin in molar ratio 1:0.89 (——+——) and of HSA: indomethacin in molar ratio 1:4.45 (– – –) in 0.1 M KCl with 0.005 M phosphate buffer, pH 7.4.

in order to optimize the measuring conditions. CD is expressed as molar ellipticity, $\{\Theta\}$, in degrees × cm^2 × dmole^{-1}, calculated with reference to the HSA concentration, or as difference molar ellipticity, $\Delta\{\Theta\}$. The measurements were made in 0.1 M KCl with 0.005 M sodium phosphate, pH 7.4.

Spectropolarimetric titrations. These were performed at 270 nm as described earlier [17, 18] with HSA (13.6 μM) and indomethacin at pH 7.4 in 0.1 M KCl and 0.005 M phosphate buffer.

Equilibrium dialysis. The protein binding was also determined by equilibrium dialysis at room temperature in 0.1 M KCl with 0.005 M phosphate buffer, pH 7.4, using Technicon Type A standard membranes as described earlier [2]. After equilibration for 8 hr, radioactivity was determined in duplicates on both sides of the dialysis cells in 10 ml Instagel (Packard Instrument Co.) using a Beckman Scintillation Counter, LS 100-C.

RESULTS

Quantitative characterization of the binding of indomethacin. The binding of indomethacin to HSA was studied by the solid-phase technique with microparticles of polyacrylamide as described above. A Scatchard plot for the binding of indomethacin to HSA-microparticles is shown in Fig. 1. From this plot it can be concluded that HSA has one primary binding site for indomethacin with very high association constant, K_a, and at least two or three sec-

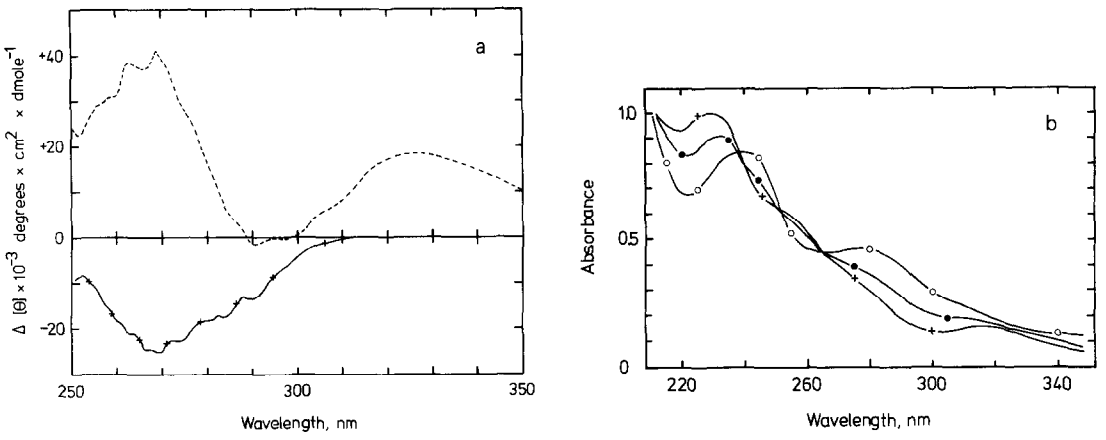

Fig. 3. Panel a: Difference CD spectra of indomethacin–HSA, (1.1:1, ——+——) and indomethacin–HSA (5.1:1, – – –) after subtraction of the HSA spectrum. Panel b: Absorption spectra of indomethacin (1.02 mg/100 ml of 0.1 M KCl with 10% ethanol) at pH 2.6 (——○——), pH 3.5 (——●——) and pH 4.7–9.7 (—+—).

ondary binding sites with moderate affinity. Figure 1 also includes the Scatchard plot with indomethacin in the presence of probenecid (at a drug–HSA ratio of 1.4:1). As can be seen probenecid does not interfere with the binding of indomethacin to its primary site. Regression analyses of the data with $r < 0.8$ gave $K_a = 2.12$ and $2.29 \times 10^6\ M^{-1}$, respectively, which are within the experimental errors. The secondary binding of indomethacin was, on the other hand, significantly improved. This means that the association constants are higher in the presence of probenecid. However, no calculation of the values has been done, since the number of binding sites is not known, and the curves do not contain sufficient information for a significant estimation of n. Equilibrium dialysis studies performed under similar conditions confirmed that probenecid has no influence on the binding of indomethacin to its primary binding site and increases the binding to secondary sites.

Spectropolarimetric characterization of the binding of indomethacin to albumin. The binding of indomethacin to HSA was also followed qualitatively in the spectropolarimeter in the wavelength region 250–350 nm. When a drug binds to a protein, new extrinsic Cotton effects are obtained at wavelengths where the drug has absorption bands. Figure 2 shows the CD spectra for HSA alone, and with indomethacin at two different concentrations. At the lower concentration, indomethacin is essentially bound only to the first site and the ellipticity was decreased over all the wavelength region. When the concentration was increased to almost a 5-fold excess, the ellipticity was increased.

Indomethacin has no ellipticity of its own and the changes seen can be fully ascribed to the extrinsic Cotton effects obtained when indomethacin was bound to the protein surface. The changes produced can be better seen in the difference spectra presented in Fig. 3a. Here the HSA spectrum was subtracted from the spectra obtained with the drug present. The resulting extrinsic Cotton effects are directly proportional to the concentration of the drug bound at the respective sites. Evidently, indomethacin gives a negative Cotton effect with maximum at 270 nm when bound to the primary binding site on HSA. However, at higher concentration, indomethacin will give positive effects, which have positive maxima at 270 and 325 nm.

In Fig. 3b the extrinsic Cotton effects can be compared with absorption spectra of indomethacin at different pH in 10% ethanol. Indomethacin is an acid with pK_a around 4 (depending on the solvent) and the salt has an absorption maximum at 319 nm (probably originating from the indol chromophore) and an inflection at 270 nm. These two bands seem to be responsible for the extrinsic Cotton effects noted when the drug binds to HSA.

Obviously, the 270-band may be used for spectro-

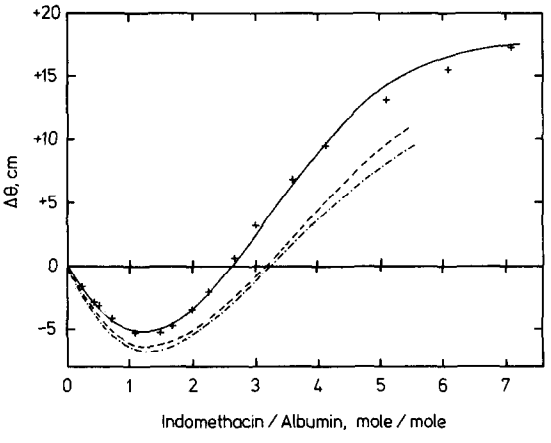

Fig. 4. Spectropolarimetric titration of HSA with indomethacin at 270 nm (——). Probenecid was added in separate experiments to molar ratio 0.99:1 (– – –) and 4.93:1 (–·–·–). The concentration of HSA was 0.903 mg/ml. The experimental conditions were the same as in Fig. 2. Theoretical ellipticity values (+) were calculated for the binding of indomethacin to HSA with the association constants and $\Delta\Theta_{max}$ -values given in the text.

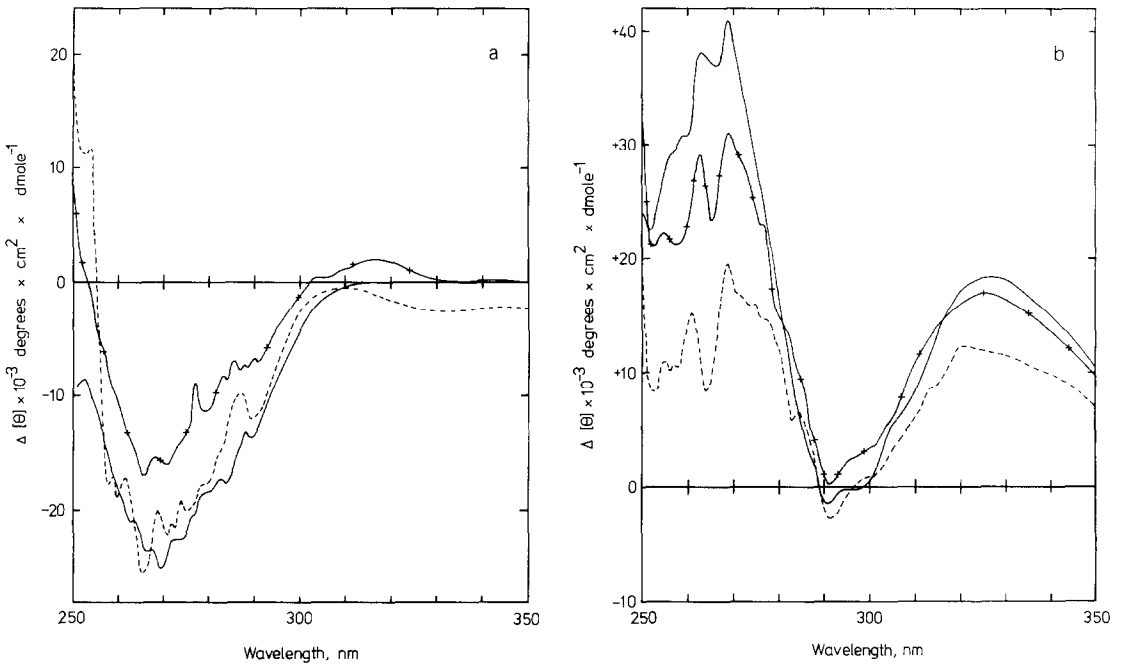

Fig. 5. Panel a: The effect of probenecid on the CD difference spectrum of indomethacin. The concentration of HSA was 1 mg/ml and the molar ratio of indomethacin–probenecid–HSA was 1.1:0:1 (——), 0.8:1:1 (—+—) and 0.8:10:1 (- - -). Panel b: The effect of probenecid on the CD difference spectrum of indomethacin. The concentration of HSA was 1 mg/ml and the molar ratio of indomethacin–probenecid–HSA was 5.1:0:1 (——), 3.9:1:1 (—+—) and 3.9:10:1 (- - -).

polarimetric titration of the binding of indomethacin to HSA [17, 18]. Such a titration is shown in Fig. 4. As expected, a negative ellipticity is obtained initially, but when the first site is saturated the positive Cotton effects from secondary sites will neutralize the negative one and eventually produce a strong positive increase.

The titration curve shown with indomethacin in Fig. 4 suggests that at least two binding sites can produce Cotton effects when indomethacin binds to HSA. However, no reasonable results were obtained in a numerical analysis of the curve when a 2-sites program was used [18]. Computer simulation with a 3-site program was then tried, starting with the binding constants obtained from the Scatchard plots of the results with the HSA-particles. As seen in Fig. 4, a good fit to the experimental curve with well-discriminating variables was obtained with this model, when the $K_a : s$ 1.5×10^6, 3.5×10^5, $5.5 \times 10^4 M^{-1}$ and $\Delta\Theta_{max} : s$ -66, -52 and $+310 \times 10^6$ degrees $\times$ cm^2 $\times$ dmole^{-1}, respectively, were used. At higher indomethacin–HSA ratios, small deviations from the curve can be seen, suggesting that interactions between the sites or binding to weaker sites may occur. However, the experimental conditions do not allow estimation of the binding characteristics of these sites.

The spectropolarimetric titration thus shows that the first two sites on HSA for indomethacin give negative Cotton effects, when the drug is bound, and that the third site gives rise to a positive Cotton effect at 270 nm.

The effect from probenecid on the spectropolarimetric titration curve at 270 nm is also seen in Fig. 4, where the results with probenecid present in two different concentrations are included. Probenecid

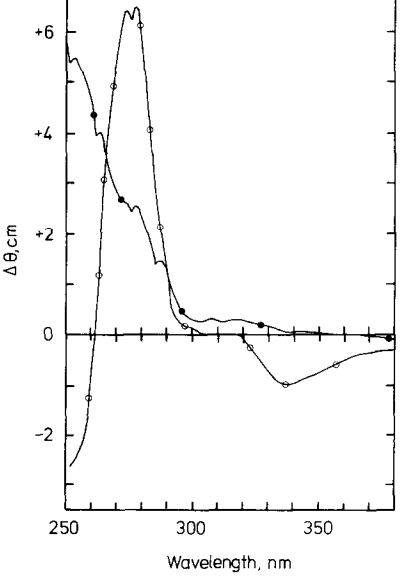

Fig. 6. The CD difference spectra of azapropazone–HSA (——○——) and flurbiprofen–HSA (——●——) obtained after subtraction of the HSA contribution. The concentration of HSA was 1.24 mg/ml and the drugs were added in a molar ratio of 0.97:1. The experimental conditions were the same as in Fig. 2.

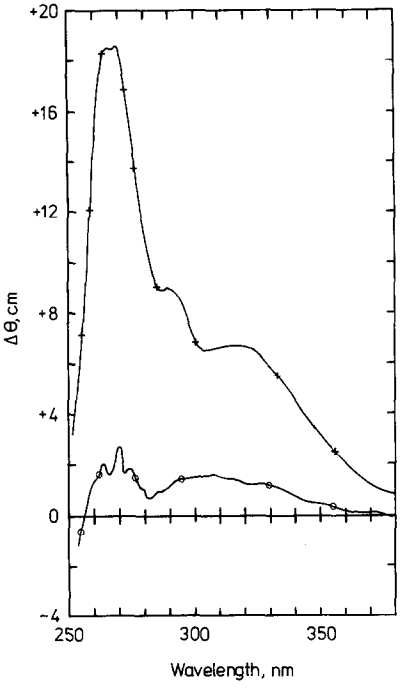

Fig. 7. The CD difference spectra obtained with indomethacin–HSA in the presence of azapropazone, after subtraction of the contribution from the azapropazone–HSA spectrum. The molar ratio of indomethacin–azapropazone–HSA was 0.97:0.97:1.0 (——○——) and 4.84:0.97:1.0 (——+——). The experimental conditions were the same as in Fig. 2.

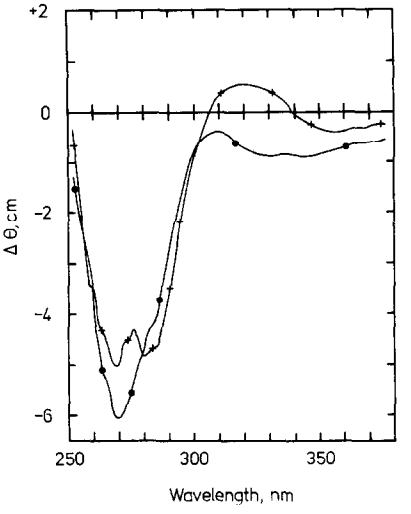

Fig. 8. The CD difference spectra obtained with indomethacin–HSA in the presence of flurbiprofen, after subtraction of the contribution from the flurbiprofen–HSA spectrum. The molar ratio of indomethacin–flurbiprofen–HSA was 0.97:0.97:1.0 (——●——) and 4.84:0.97:1.0 (—+—). The experimental conditions were the same as in Fig. 2.

itself does not produce any significant extrinsic Cotton effects at 270 nm, when bound to HSA. The changes in the titration curve can therefore be ascribed to changes in the indomethacin binding. Figure 4 shows that the largest effect (a negative one) is seen after an equimolar concentration of indomethacin is reached. It was earlier concluded (from the Scatchard plots in Fig. 1) that probenecid promotes an increased secondary binding of indomethacin. This conclusion is confirmed by the increased negative ellipticity engendered by increased indomethacin binding to its secondary site. At higher indomethacin concentrations the ellipticity was further decreased, which may be due to a decreased binding to the third site or to a decreased $\Delta\Theta_{max}$ for the binding of indomethacin to this site.

The probenecid effect on the extrinsic Cotton effects from indomethacin is further shown in Fig. 5. In Fig. 5a the primary site for indomethacin is studied. As seen, the effect from probenecid is negligible. On the other hand, probenecid considerably decreases the positive CD-band from the third binding site obtained at higher indomethacin concentration (Fig. 5b). The decreased ellipticity seen when probenecid is present may be due to at least three phenomena: probenecid increases $\Delta\Theta_{max}$ of the second binding site for indomethacin, it displaces indomethacin from the third site, and/or it decreases $\Delta\Theta_{max}$ of this site. The effects detected are most evident for the main band at 270 nm, but can also be seen at 320 nm.

Identification of the indomethacin binding sites. To identify the binding sites of indomethacin, two drugs, azapropazone and flurbiprofen, were used as probes. It has been known earlier that azapropazone strongly binds to the warfarin/bilirubin binding site on HSA, while flurbiprofen binds to the diazepam site [7]. Their primary association constants are high, about $1 \times 10^6 M^{-1}$. When bound to HSA these drugs give distinct Cotton effects between 250 and 380 nm, as shown in Fig. 6. Both the drugs affect the CD-effects obtained from the binding of indomethacin to HSA (Figs. 7 and 8).

As is evident from Fig. 7, indomethacin does not give any negative Cotton effects when added to HSA in equimolar amounts, if azapropazone is present. As is obvious from above (compare Figs. 3a and 4), binding of indomethacin to the primary site of HSA induces negative Cotton effects, which thus are blocked by azapropazone. The small positive Cotton effects detected in Fig. 7 originate from the fraction of the added indomethacin, available in this situation to bind to site 3, which produces strong positive ellipticities. These positive effects dominate over those produced by the fraction of indomethacin displacing the bound azapropazone at site 1 and from indomethacin at site 2. At higher concentrations of indomethacin (5-fold molar excess) the positive Cotton effects from the third site will dominate completely. Thus, the results indicate that azapropazone binds to the primary indomethacin binding site.

Figure 8, correspondingly, shows that flurbiprofen blocks the tertiary binding site of indomethacin. Only the negative Cotton effects from the primary binding site of indomethacin, shown in Fig. 3a, are obtained at equimolar concentration of indomethacin. At higher concentration (5-fold molar excess), the strong positive effects originating from the tertiary binding are abolished.

DISCUSSION

In the present study, three binding sites for indomethacin on HSA have been detected. The binding constant at the primary binding site is high. With HSA-microparticles $K_a = 2.1 \times 10^6 M^{-1}$ was obtained from the Scatchard plot and $K_a = 1.5 \times 10^6 M^{-1}$ from the curve-fitting procedure of the CD-data. This value approaches the K_a ($1 \times 10^6 M^{-1}$) found by Hultmark *et al.* with a commercial HSA preparation [4]. The binding constant satisfactorily explains the high binding degree of indomethacin in serum and the small apparent distribution volume in man (6–10 l. ref. 19).

The spectrapolarimetric titration, moreover, showed that the secondary binding constant is also high, $3.5 \times 10^5 M^{-1}$, which is higher than K_a for the binding of e.g. warfarin and diazepam to HSA [12, 17]. The third site gave a K_a-value of about $5.5 \times 10^4 M^{-1}$, which is significantly higher than those of e.g. salicylic acid [12] or phenytoin*. In patients, indomethacin will be bound to all the sites. The distribution between the sites will of course depend on the blood level and the relation between the K_a-values, but also on the presence of competing drugs or endogenous inhibitors [2, 8]. The partition of bound drug between the sites means that the displacement of indomethacin at one site can to some extent be compensated for by binding to another site. It is therefore hardly possible, in spite of the small distribution volume of indomethacin in man, that displacement phenomena will have any significant pharmacokinetic consequences.

Spectropolarimetry is an efficient technique to study drug–protein interactions. The organization of different binding sites of a protein varies from site to site, which means that a drug bound to the different sites will be bound differently. The asymmetric conformation around the chromophore will be different, which theoretically can lead to different extrinsic Cotton effects. Indomethacin gave rise to negative Cotton effects when bound to the first two sites on HSA, while the third site gave a strong positive Cotton effect. Similar findings have been reported by Rosen for the binding of oxyphenbutazone to HSA, yielding Cotton effects of different signs when the concentration of the drug changes [20]. The different Cotton effects make it possible to study specifically the events taking place at a certain site.

Earlier, we have characterized the specificity of three binding sites on HSA, the diazepam, digitoxin and warfarin sites, named after the drug probes used in the study [7]. There are also indications that a fourth site exists, to which the drug tamoxifen binds [7]. To the warfarin site, azapropazone is bound with high affinity, producing strong positive Cotton effects, and to the diazepam site flurbiprofen is similarily bound. The drugs are essentially bound to just their primary sites, when present in equimolar amounts relative to HSA. In the present study these drugs have been shown to block specifically the formation of the specific extrinsic Cotton effects from indomethacin and thus inhibit the binding to the different sites. Thus, we can conclude that the primary binding site of indomethacin is the azapropazone–warfarin site, and that the tertiary site on HSA is the flubiprofen–diazepam site. The identity of the secondary site is not yet established.

The binding of indomethacin to the second and third sites is specifically affected by probenecid, as detected by both the CD measurements and the HSA microparticles. The findings can be best interpreted as improved binding of indomethacin to the second site, which was shown both with the microparticles and in the CD-studies, while the binding to the third site seems to be impaired. It seems reasonable to assume that probenecid primarily binds to this site and thereby competitively inhibits the indomethacin binding. Such binding has earlier been detected [7]. Concomitantly, the secondary binding site may be allosterically modified so that the affinity for indomethacin is improved. Our results can be summarized as in Table 1.

Our results are in accordance with the findings of Solomon *et al.* [6], who showed that indomethacin, as expected, displaced warfarin. Moreover, Mason and Queen [1] found that ibuprofen only weakly displaced indomethacin *in vitro*. It is now known that ibuprofen binds primarily to the diazepam site of HSA with very high affinity [21]. The small displacement of indomethacin seen was thus due to the fact that only a small fraction of the indomethacin was bound to the diazepam site from which it could be displaced and which is the third site of indomethacin. Mason and Queen [1], however, were not able to detect any displacement of indomethacin by phenylbutazone, which binds to the primary binding site of indomethacin, i.e. the warfarin site [7].

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Table 1. Binding of indomethacin to human serum albumin

	Association constant, K_a (M^{-1})	Difference molar ellipticity, (degrees × cm^2 × $dmole^{-1}$)	Inhibition by azapropazone	Inhibition by flurbiprofen	Effect of probenecid on binding
Site 1	$1.5–2.1 \times 10^6$	-66×10^6	+	−	None
Site 2	3.5×10^5	-52×10^6	−	−	Increase
Site 3	5.5×10^4	$+310 \times 10^6$	−	+	Decrease

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