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Characterization of the protein binding of chiral drugs by high-performance affinity chromatography Interactions of *R*- and *S*-ibuprofen with human serum albumin

David S. Hage[a,*], Terence A.G. Noctor[b], Irving W. Wainer[b]

[a]*Department of Chemistry, University of Nebraska, Lincoln, NE 68588-0304, USA*
[b]*Department of Oncology, McGill University, Montreal, Quebec H3G 1A4, Canada*

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Abstract

Zonal elution and high-performance affinity chromatography were used to study the different binding characteristics of *R*- and *S*-ibuprofen with the protein human serum albumin (HSA). This was done by injecting small amounts of *R*- and *S*-ibuprofen onto an immobilized HSA column in the presence of a mobile phase that contained a known concentration of *R*- or *S*-ibuprofen as a competing agent. These studies indicated that *R*- and *S*-ibuprofen had one common binding site on the immobilized HSA column. In addition, *S*-ibuprofen had at least one other major binding region. The association equilibrium constant for *R*-ibuprofen with HSA was found to be $5.3 \cdot 10^5\ M^{-1}$ at pH 6.9 and 25°C. Under the same conditions, the association constants for *S*-ibuprofen at its two sites were $1.1 \cdot 10^5\ M^{-1}$ and $1.2 \cdot 10^5\ M^{-1}$. The *S*-ibuprofen sites were present in about a 1:1 ratio and appeared to exhibit some allosteric interactions at high *S*-ibuprofen concentrations. The chromatographic technique used in this work is a general one which can be adapted for use in studying the interactions of other chiral compounds with either HSA or additional proteins.

1. Introduction

Protein binding in blood is a significant factor in the transport and release of many drugs and hormones. These interactions can influence the biological distribution of these compounds as well as their excretion, therapeutic activity and toxicity [1]. One protein that is involved in such binding processes is human serum albumin (HSA). HSA is the major protein in serum and is known to bind to a wide range of small organic and inorganic compounds [2]. This binding is believed to occur at two major sites on HSA (i.e., the warfarin–azapropazone site and the indole–benzodiazepine site) plus a number of minor binding regions [3–6].

In characterizing the interactions of a chiral compound with HSA, it is important to consider the behavior of each individual form of the solute since the binding of HSA can be stereoselective in nature [7–14]. One way in which this stereoselectivity can be produced is by the chiral forms of a compound having different binding strengths for HSA. For example, *R*- and *S*-warfarin bind to the same site on HSA but have significantly different association constants

* Corresponding author.

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for this region [10–12]. In addition, stereoselectivity may be produced by the various forms of a chiral compound having different binding regions. Examples of this latter case include the binding of HSA with D- and L-tryptophan [13] or *R*- and *S*-oxazepam hemisuccinate [14].

Ibuprofen is one chiral drug which may exhibit different binding to HSA for its individual enantiomeric forms. Ibuprofen [i.e., 2-(4-isobutylphenyl)-propionic acid] is a common nonsteroidal, anti-inflammatory agent used in the treatment of rheumatoid arthritis and related conditions [15]. This compound exists in two forms, *R*- and *S*-ibuprofen, which differ in their therapeutic and pharmacological properties [16]. Racemic ibuprofen is 99% protein bound in human plasma at normal therapeutic levels [17]. Most or all of this binding is believed to occur with HSA.

A large number of studies have been performed examining the binding of ibuprofen to HSA; however, the actual strength of this binding and number of sites involved in this interaction are still areas of controversy. Most studies agree that ibuprofen has a primary binding site on HSA with an association equilibrium constant in the range of 10^5 to 10^6 M^{-1} [17–25]. However, it is not yet clear whether there is only one ibuprofen binding region on HSA [20–23] or additional, weaker binding sites [17,19,24,25]. One potential problem with most of these earlier studies is that the experiments were performed using racemic ibuprofen mixtures [17–25]. Recent work using the individual enantiomers of ibuprofen suggests that there are different numbers of sites and/or affinities involved in the interactions of HSA with *R*- and *S*-ibuprofen [26,27].

This work will use an immobilized HSA column and high-performance affinity chromatography (HPAC) to examine the binding of HSA to the *R*- and *S*-enantiomers of ibuprofen. In this case, a column that contains HSA immobilized to a silica-based support will be used as the basis for the experiment. Previous studies have shown that the properties of this type of column show good correlation with the properties of HSA in solution [12–14,27–31]. For example, equilibrium constant measurements performed on HSA columns with *R*- and *S*-warfarin or D- and L-tryptophan have resulted in binding affinities that differ by less than 20% from solution values measured by equilibrium dialysis [12,13,29]. Displacement phenomena and allosteric interactions observed on the same types of HSA columns have also been shown to agree with the behavior of HSA in solution [14,27–31]. Similar conclusions have been reached by other workers, who have used columns containing HSA (or other types of serum albumin) immobilized to agarose-based supports [8,10].

The binding of *R*- and *S*-ibuprofen to HSA will be examined in this work by injecting small amounts of each enantiomer onto an HSA column while a known concentration of *R*- or *S*-ibuprofen is applied to the column in the mobile phase. By examining how the mobile phase concentration of the ibuprofen additive affects the retention of the injected solute, information will be gained on the type of competition which is occurring between these two species. This, in turn, will be used to determine the number of binding regions for each enantiomer and the corresponding association constants for these sites. These results will then be compared with those of previous studies which have examined the binding of ibuprofen with HSA.

2. Theory

The binding of *R*- and *S*-ibuprofen to HSA was examined by using the technique of zonal elution. In this method, a known concentration of a competing agent (I) is continuously applied to a column that contains an immobilized ligand (L) while injections of a small amount of analyte (A) are made. If I and A compete at a single site on L, then the following equation can be used to describe the retention of A on the column [28]:

$$\frac{1}{(k' - X)} = \frac{V_m K_I [I]}{K_A m_L} + \frac{V_m}{K_A m_L} \quad (1)$$

In the above equation, V_m is the void volume of the column (i.e., the elution volume of a non-retained solute), m_L is the moles of binding sites in the column involved in the competition

of A with L, and [I] is the concentration of the competing agent in the mobile phase. K_A is the association equilibrium constant for the binding of A to L at the site of competition and K_I is the association equilibrium constant for the interaction of I at the same site. The term k' is the capacity factor for the injected solute, or $k' = (t_R/t_m - 1)$, where t_R is the measured retention time of the solute and t_m is the void time of the column. The term X is a constant that represents the portion of k' due to the binding of A to sites at which I does not compete or due to sites for which the contribution to k' is already known through independent measurements. If no additional binding sites are present in the column (i.e., $X = 0$), then Eq. 1 reduces to the same form as derived in other previous studies [29,32,33].

If the amount of injected analyte is sufficiently small (i.e., linear elution conditions are present), then Eq. 1 predicts that a plot of $1/(k' - X)$ versus [I] for a system with single-site competition will give a linear relationship with a slope of $V_m K_I/K_A m_L$ and an intercept of $V_m/K_A m_L$. The value of K_I can be determined directly from this plot by calculating the ratio of the slope to the intercept. If the concentration of binding sites in the column is known (i.e., m_L/V_m), then the value of K_A can also be obtained from the intercept.

If multisite binding or allosteric interactions are present, then deviations from the linear behavior predicted by Eq. 1 will be seen. This makes plots of $1/(k' - X)$ versus [I] useful in detecting such interactions [12,13,28,29]. Eq. 1 can also be used to monitor competition or allosteric interactions at specific sites on a ligand, even when one of the agents (A or I) has multisite binding with L. This is possible since the value of $1/(k' - X)$ is affected only by changes in those sites at which both A and I bind. By using an analyte or a competing agent that is known to have single-site interactions with the ligand, the binding of other compounds at the same site can be examined [29,34].

In the situation where single-site binding is present and the same compound is used as both the competing agent and the injected analyte, Eq. 1 reduces to the following form:

$$\frac{1}{k'} = \frac{V_m[I]}{m_L} + \frac{V_m}{K_A m_L} \quad (2)$$

This expression is obtained by setting X equal to zero (i.e., by assuming that there are no sites that do not interact with both A and I) and by setting K_A equal to K_I (i.e., by assuming that the binding strengths for A and I are identical at each site). This new relationship predicts that a plot of $1/k'$ vs. [I] will yield a linear relationship for a system with single-site competition. In this case, the ratio of the slope to the intercept gives K_A and the inverse of the slope gives m_L/V_m, or the effective concentration of binding sites in the column. As noted with Eq. 1, non-linear behavior in a plot made according to Eq. 2 might be observed if the interactions of A and L take place at more than one type of site. However, if the contributions to k' are known for these additional sites, then a linear plot can still be obtained by using Eq. 1 and a value for X that corresponds to the portion of k' that is due to these regions.

3. Experimental

3.1. Reagents

The individual enantiomers of *R*- and *S*-ibuprofen were kindly supplied by Upjohn Labs. (Kalamazoo, MI, USA). Racemic ibuprofen (>98% pure) was obtained from Sigma (St. Louis, MO, USA). The immobilized HSA column was obtained from Shandon Scientific (Runcorn, UK). Other chemicals and solvents used were of the purest grades available. All buffers and aqueous solutions in this study were prepared using water from a Milli-Q water system (Millipore, Milford, MA, USA).

3.2. Apparatus

The chromatographic system consisted of one Spectroflow Model 400 pump, a Model 480 injector and a Model 783 programmable absorbance detector from ABI Analytical (Ramsey, NJ, USA). The immobilized HSA column was 15 cm × 4.6 mm I.D. and was thermostated

at 25 ± 0.1°C using a CH-30 temperature-regulated jacket from FIAtron Laboratory Systems (Oconomowoc, WI, USA).

3.3. Chromatography

The immobilized HSA column was prepared using diol-bonded Nucleosil 300-7 (Machery-Nagel, Düren, Germany) activated with 1,1'-carbonyldiimidazole (CDI) [30]. The final protein content of this support was estimated to be 95 ± 2 mg HSA per gram of support, as determined by the supplier [30].

All mobile phases were prepared by adding 0 to 30 μM of the desired competing agent to 0.05 M sodium phosphate buffer (pH 6.90) containing 15% (v/v) acetonitrile. All mobile phases containing ibuprofen were prepared using the individual enantiomers of this compound. Prior to their use, the mobile phases were degassed by ultrasonication and passed through 0.45-μm Millipore HV filters. Mobile phases were applied to the immobilized HSA column at a flow-rate of 0.8 ml/min. In each experiment, a 20-μl sample was injected which contained 0.5 μg of analyte dissolved in 1-propanol–water (50:50, v/v). The sample solution generally contained only *R*- or *S*-ibuprofen. However, injections of samples containing racemic ibuprofen could also be made when the chromatographic conditions were sufficient to produce baseline resolution of the individual ibuprofen enantiomers.

Elution of the injected ibuprofen was detected by monitoring the absorbance of the column eluent at 265 nm. Because this study used an ibuprofen enantiomer as both the injected solute and mobile phase additive, there was an increase in the background signal due to the mobile phase as higher concentrations of competing agent were used. This did not create any significant problems under the range of ibuprofen concentrations studied in this work (i.e., 0–30 μM). However, a decrease in the sensitivity and precision of the absorbance measurements was noted at higher mobile phase levels of ibuprofen.

The capacity factor (k') was determined in duplicate or triplicate for each injected compound by comparing the analyte's mean retention time (t_R) to the retention time of water (t_m). The mean retention time of each peak was determined by the modified B/A half-height method [35]. In plots of $1/(k' - X)$ versus [I], the value of X for sites with no competition between I and A was assigned a value of zero when A and I represented the same compound. When A and I were not identical, the value of X was determined by iterative testing, as described previously [28].

Data on the second binding site for *S*-ibuprofen were obtained by using the calculated capacity factor for *S*-ibuprofen at its first binding site (i.e., the site shared by *R*- and *S*-ibuprofen) as the value of X in a plot of $1/(k' - X)$ for the *S*-/*S*-ibuprofen competitive binding studies. The capacity factor for the first site was determined by using the relationship $X = (Km_L/V_m)/(K[I] + 1)$, where m_L/V_m and K represent the concentration and association constant of the first *S*-ibuprofen site, as measured in *R*-/*S*-ibuprofen competitive binding experiments. After calculating the value of X at each level of competing agent used, the results obtained were subtracted from the overall experimentally measured capacity factors. A plot of $1/(k' - X)$ versus [I] was then made and analyzed according to Eq. 1, as discussed earlier.

All experiments in this study were performed on a single HSA column over the course of three weeks and approximately 50 injections. The binding properties of the immobilized HSA column were stable throughout this time period, as indicated by a total variation of only 4–14% in the measured retention of *R*- and *S*-ibuprofen when no competing agent was present. Similar stability has been noted in previous work with other HSA columns [12,13,29].

4. Results and discussion

4.1. Competitive binding studies using R-ibuprofen as a mobile phase additive

Initial studies were performed by injecting small amounts of *R*- or *S*-ibuprofen onto the immobilized HSA column in the presence of

Table 1
Influence of the mobile phase concentration of *R*-ibuprofen on the capacity factors of injected *R*-ibuprofen (k'_R) and *S*-ibuprofen (k'_S)

[*R*-Ibuprofen] (μM)	k'_R	k'_S
0.0	75.66	23.20
0.5	56.86	20.11
1.0	43.37	17.74
2.0	31.73	15.70
3.0	24.79	14.80
4.0	20.94	13.89
5.0	19.55	12.65
6.0	15.52	12.49
8.0	11.96	11.49
10.0	10.92	11.09

The values shown for k'_R and k'_S represent the mean results of duplicate or triplicate injections. The quantity [*R*-ibuprofen] represents the mobile phase concentration of *R*-ibuprofen that was applied to the column during each experiment.

mobile phases containing different concentrations of *R*-ibuprofen as a competing agent. For both enantiomers, a decrease in retention was observed as increasing amounts of *R*-ibuprofen were added to the mobile phase. The capacity factors measured for the injected solutes are shown in Table 1. The corresponding graphs of $1/(k' - X)$ versus [*R*-ibuprofen] are given in Fig. 1.

The plot shown in Fig. 1 for the injections of *R*-ibuprofen was obtained by using a value of zero for X in Eq. 1. This X value was chosen since the analyte and competing agent were identical, and thus had no sites on the column for which they did not compete. The *R*-ibuprofen plot in Fig. 1 was linear over the entire concentration range studied. The correlation coefficient was 0.9956 for the 10 data points shown. This linear relationship indicated that *R*-ibuprofen had one major class of binding sites on the column. By taking the ratio of the slope to the intercept for this plot, an association equilibrium constant of 5.5 $(\pm 1.0) \cdot 10^5$ M^{-1} was calculated for *R*-ibuprofen at its binding site, where the number in parentheses represents a range of ± 1 S.D. Although the exact location of this binding region was not examined in this work, data from earlier competition studies indicate that this is probably located at the indole–benzodiazepine site of HSA [27].

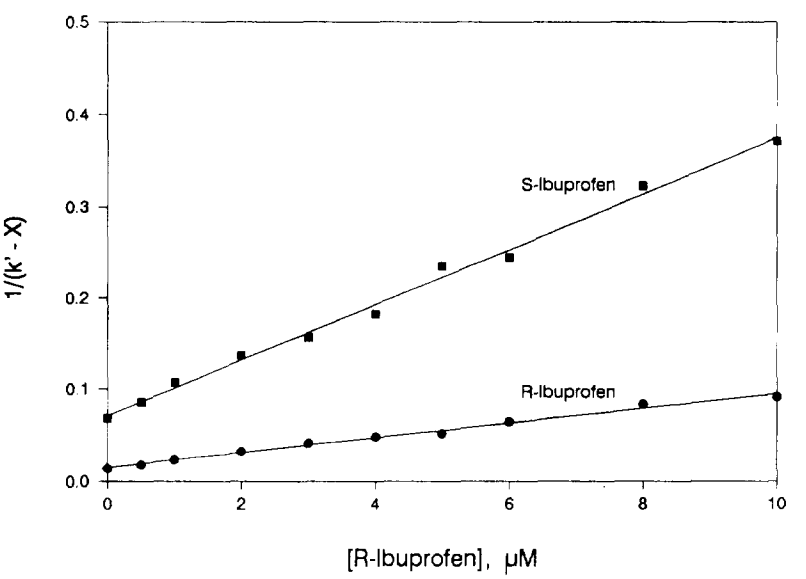

Fig. 1. Change in $1/(k' - X)$ with mobile phase concentration of applied *R*-ibuprofen as injections of *R*-ibuprofen (●) and *S*-ibuprofen (■) were made onto an immobilized HSA column. The equations of the best-fit lines were $y = (8100 \pm 300\ M^{-1})x + (0.015 \pm 0.003)$ for *R*-ibuprofen and $y = (30\,500 \pm 800\ M^{-1})x + (0.071 \pm 0.008)$ for *S*-ibuprofen. The values of X used for the *R*- and *S*-ibuprofen plots were 0.00 and 8.40, respectively.

As mentioned in the Theory section, one unique feature of using the same compound as both the injected analyte and the competing agent, as was done in this study with *R*-ibuprofen, is that this causes the association constants for the two agents to be identical (i.e., $K_I = K_A$). As shown in Eq. 2, the slope obtained for a plot of $1/k'$ vs. [I] should then be equal to V_m/m_L, from which the concentration of binding sites in the column (m_L/V_m) can be easily obtained. Based on this approach, the concentration of *R*-ibuprofen sites in the HSA column was calculated to be $1.24\ (\pm 0.04) \cdot 10^{-4}\ M$.

From the protein content and packing density of the immobilized HSA support, it was possible to estimate the total amount of HSA that was present in the column. A final value of 1.6 μmol HSA, or an effective concentration of $8.0 \cdot 10^{-4}$ M, was obtained by this approach. If it is assumed, as indicated by Fig. 1, that *R*-ibuprofen has only one binding site on HSA, then a comparison of this amount of HSA with the measured concentration of *R*-ibuprofen sites indicates that only about 15% of these sites were active in the column. Similar values have been reported for the binding of immobilized HSA to other solutes [12,13,29]. This relatively low activity is probably due to denaturation, steric hindrance or improper orientation of the HSA as a result of the immobilization process [12,13,29]. Fortunately, the approach used in this work to study ibuprofen–HSA interactions was not affected to any major extent by these non-binding regions, because only those sites that showed competition between the injected and applied ibuprofen were monitored [29].

A comparison of the concentration of *R*-ibuprofen sites in the column with the applied concentrations of *R*-ibuprofen, as given in Table 1, shows that the binding sites were present in a large excess during these studies. In this case, the mobile phase levels of *R*-ibuprofen ranged from 0 to 8% of the effective binding site concentration. This represents a very different situation from that present in other methods used to study solute–protein binding (e.g., ultrafiltration or equilibrium dialysis), in which solute concentrations often approach or exceed the amount of binding protein. Although the lower solute levels used here may make the detection of weak binding sites difficult, these same conditions would also tend to make the chromatographic approach more selective in the analysis of higher affinity sites.

As was found for injections of *R*-ibuprofen, the graph shown in Fig. 1 for the competition of injected *S*-ibuprofen with *R*-ibuprofen in the mobile phase also gave a linear relationship. The best-fit line had a correlation coefficient of 0.9971 over the ten data points shown. This behavior suggested that *R*- and *S*-ibuprofen had a single class of sites for which they competed in the column. Protein binding interactions between *R*- and *S*-ibuprofen have been suggested in earlier studies [36], but the number of sites involved in these interactions has been examined only recently [27].

From the slope and intercept of the plot for *S*-ibuprofen in Fig. 1, the association equilibrium constant for *R*-ibuprofen at the site of competition was determined to be $4.3\ (\pm 0.5) \cdot 10^5\ M^{-1}$. This value was statistically identical to the association constant obtained in the *R*-ibuprofen/*R*-ibuprofen study. This indicated that the same binding site for *R*-ibuprofen was being sampled in both experiments.

By using the concentration of *R*-ibuprofen binding sites that was determined earlier and by assuming that *R*- and *S*-ibuprofen had equal access to this site [12], it was possible to use the slope of the *S*-ibuprofen curve in Fig. 1 to calculate the association constant for *S*-ibuprofen at the site of competition. An association constant of $1.1\ (\pm 0.1) \cdot 10^5\ M^{-1}$ was obtained for *S*-ibuprofen by this procedure.

The plots in Fig. 1 for both *R*- and *S*-ibuprofen gave good agreement between the experimental intercepts (i.e., k' when no competing agent was present) and the intercepts which were determined by linear regression of the entire data set. For example, the experimental and best-fit intercepts for *R*-ibuprofen were 0.013 and 0.015 (± 0.003), respectively. For *S*-ibuprofen, these values were 0.067 and 0.070 (± 0.008). The agreement between these values is significant since it indicates that the displacement of either

Table 2
Influence of the mobile phase concentration of *S*-ibuprofen on the capacity factors of injected *R*-ibuprofen (k'_R) and *S*-ibuprofen (k'_S)

[*S*-Ibuprofen] (μM)	k'_R	k'_S
0.0	80.86	27.37
2.0	72.97	22.22
4.0	70.73	16.56
6.0	61.94	16.07
8.0	49.05	13.91
10.0	45.21	12.45
15.0	41.51	11.81
20.0	34.60	10.88
30.0	Not done	10.86

The values shown for k'_R and k'_S represent the mean results of duplicate or triplicate injections. The quantity [*S*-ibuprofen] represents the mobile phase concentration of *S*-ibuprofen that was applied to the column during each experiment.

R- or *S*-ibuprofen by *R*-ibuprofen was through a direct, rather than an allosteric, mechanism of competition [29].

4.2. Competitive binding studies using *S*-ibuprofen as a mobile phase additive

Studies similar to those described for *R*-ibuprofen were also performed using *S*-ibuprofen as a mobile phase additive. A decrease in the retention of *R*- and *S*-ibuprofen was seen as higher mobile phase concentrations of *S*-ibuprofen were applied to the column. Table 2 lists the capacity factors that were obtained for *R*- and *S*-ibuprofen during this study. The corresponding plots of $1/(k' - X)$ versus [*S*-ibuprofen] are given in Fig. 2.

The data in Fig. 2 for the injections of *R*-ibuprofen gave a linear relationship with a correlation coefficient of 0.9850 over the eight data points shown. This confirmed that *S*- and *R*-ibuprofen had single-site competition on HSA, as indicated earlier in Fig. 1. One difference between these results and those in Fig. 1 was that the slope/intercept ratio of Fig. 2 gave the association constant for *S*-ibuprofen, rather than

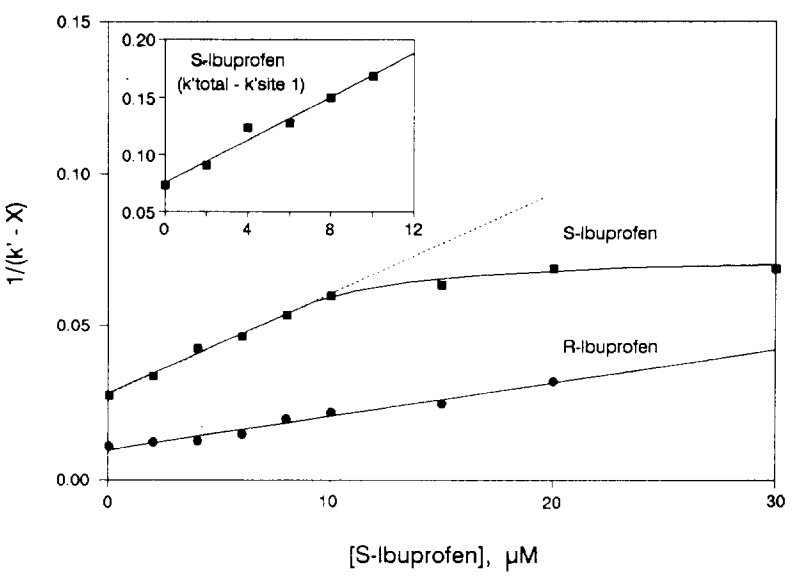

Fig. 2. Change in $1/(k' - X)$ with mobile phase concentration of applied *S*-ibuprofen as injections of *R*-ibuprofen (●) and *S*-ibuprofen (■) were made onto an immobilized HSA column. The equation of the best-fit line shown for *R*-ibuprofen was $y = (1400 \pm 100\ M^{-1})x + (0.013 \pm 0.002)$. The best-fit line shown for the first five points of the *S*-ibuprofen data was $y = (4400 \pm 300\ M^{-1})x + (0.037 \pm 0.002)$. The values of X used for the *R*- and *S*-ibuprofen plots were 11.04 and 0.00, respectively. The insert shows the *S*-ibuprofen data after subtracting out the calculated contribution to k' from the *S*-/*R*-ibuprofen common binding site. The best-fit line for the five points in the insert was $y = (9400 \pm 700\ M^{-1})x + (0.076 \pm 0.006)$ and the correlation coefficient was 0.9873.

for *R*-ibuprofen, at their common binding site. The equilibrium constant calculated from Fig. 2 for *S*-ibuprofen at the site of competition was 1.1 (± 0.2)$\cdot 10^5$ M^{-1}. This value was the same as the previous estimate made from Fig. 1.

The association constant for *R*-ibuprofen with HSA was determined from Fig. 2 by using the slope of the *S*-ibuprofen plot and the concentration of *R*-ibuprofen binding sites measured earlier. A result of 6.2 (± 0.9)$\cdot 10^5$ M^{-1} was obtained. This value was in good agreement with the association constant values that were determined for *R*-ibuprofen from Fig. 1.

The competition of injected *S*-ibuprofen with *S*-ibuprofen in the mobile phase was the only case in this work which produced any type of non-linear behavior. The *S*-ibuprofen plot in Fig. 2 gave a linear response up to a competing agent concentration of 8 μM. This was followed by a leveling off of the *S*-ibuprofen curve at higher competing agent concentrations. This behavior indicated that at least two separate sites were involved in the binding of *S*-ibuprofen to the immobilized HSA column. Multisite binding was also indicated by the inverse slope of this plot (i.e., m_L/V_m), which was twice the value predicted by the number of *R*-ibuprofen sites that were measured on the column. The properties of these additional sites were studied by correcting the overall capacity factor for the contribution due to the interactions of *S*-ibuprofen at the shared *R*-/*S*-ibuprofen binding site. The resulting graph is shown in the inset of Fig. 2.

The plot obtained for the adjusted capacity factors was similar in shape to the original graph shown in Fig. 2, but with higher values for $1/(k' - X)$. By applying Eq. 1 to the linear portion of this graph, the concentration of the additional *S*-ibuprofen binding sites was estimated to be 1.06 (± 0.09)$\cdot 10^{-4}$ M and the apparent association constant for these sites was determined to be 1.2 (± 0.1)$\cdot 10^5$ M^{-1}. The ratio of the concentration of these additional sites vs. the concentration of sites shared by *S*-ibuprofen and *R*-ibuprofen was 0.9:1.0. From this data the contribution to the capacity factor for *S*-ibuprofen that was due to the additional sites, where $k' = K_A m_L/V_m$, was estimated to be 13 (± 2). This agreed with the X value of 11.04 that was determined in Fig. 1 for the competition of injected *S*-ibuprofen with *R*-ibuprofen in the mobile phase. All of this information indicated that there were probably only two major classes of sites involved in the binding of *S*-ibuprofen to the immobilized HSA column. The exact location of these sites currently remains unknown.

The similarity of the association constants measured for *S*-ibuprofen at the two binding sites explains why a linear region was initially observed for the *S*-ibuprofen data in Fig. 2. The non-linearity of this data at high *S*-ibuprofen levels indicates that there is probably some allosteric or indirect competition between the *S*-ibuprofen sites at high concentrations. One reason that similar behavior was not noted for the *R*-ibuprofen results in Fig. 2 may be that the stronger binding of *R*-ibuprofen minimized or prevented allosteric effects from being seen under the experimental conditions used. Alternatively, only the second site for *S*-ibuprofen may have been altered or the binding of *R*- and *S*-ibuprofen at the first site may have been sufficiently different that only the *S*-enantiomer was affected by the interactions of *S*-ibuprofen at the second site. A similar difference in allosteric effects for enantiomers binding to the same region on HSA has been reported for *R*- and *S*-warfarin [31].

4.3. Comparison of data with previous models for ibuprofen–HSA binding

The results presented in this work indicate that there is one major binding site on HSA for *R*-ibuprofen and two or more major sites on HSA for *S*-ibuprofen. The average association constants measured for these sites are summarized in Table 3. These averages are based on the three estimates obtained for the association constant of *R*-ibuprofen at its major binding region, (site A), the two estimates obtained for *S*-ibuprofen at the same site, and a single measurement made for the binding of *S*-ibuprofen at its second site (site B).

The association constants obtained in this study show good agreement with previous values

Table 3
Binding of *R*- and *S*-ibuprofen to immobilized HSA

Binding region	Association equilibrium constant (M^{-1})	
	R-Ibuprofen	*S*-Ibuprofen
Site A	$5.3 (\pm 0.9) \cdot 10^5$	$1.1 (\pm 0.1) \cdot 10^5$
Site B	–	$1.2 (\pm 0.1) \cdot 10^5$

The given values were measured at 25°C in 0.05 *M* sodium phosphate buffer containing 15% (v/v) acetonitrile. The numbers in parentheses represent ± 1 S.D.

reported for the primary binding regions of ibuprofen on HSA. For example, Sollene and Means [20] measured an association constant of $1.4 \cdot 10^5\ M^{-1}$ at 25°C and pH 8.0 for the main binding site of racemic ibuprofen on HSA. Kober and Sjöholm [21] determined a primary binding constant of $1 \cdot 10^6$–$1.3 \cdot 10^6\ M^{-1}$ at 20–22°C and pH 7.4 for racemic ibuprofen mixed with human serum, HSA in solution and HSA immobilized onto microparticles. Similar association constants, ranging from $1 \cdot 10^5$ to $2.7 \cdot 10^6\ M^{-1}$, have been reported for racemic ibuprofen in studies performed at pH 7.4 and 37°C [17,18,22–25]. The individual association constants given in Table 3 for *R*- and *S*-ibuprofen are larger than those determined recently by microcalorimetry [37]. However, the binding constant for *R*-ibuprofen does agree with the results of earlier chromatographic experiments that examined the competition of this enantiomer with various benzodiazepines [27].

The presence of at least one common binding site for *R*- and *S*-ibuprofen, as shown in Table 3, has been suggested by Lee et al. [36]. Furthermore, the existence of at least two ibuprofen binding regions on HSA has been proposed in a recent report by Noctor et al. [27]. In this latter study, it was found that *R*- and *S*-ibuprofen gave different types of behavior when allowed to compete with benzodiazepines on an immobilized HSA column. It was concluded that two stereoselective binding regions were present, each having preferential binding to a different ibuprofen enantiomer [27]. The same trend was observed in the present study, with site A binding most tightly to *R*-ibuprofen and site B having interactions with only *S*-ibuprofen. From these results, it is proposed that sites A and B are the same as the IBU_R and IBU_S binding regions noted in the earlier report [27].

The fact that *R*- and *S*-ibuprofen share one binding site plus the fact that sites A and B have similar binding constants for *S*-ibuprofen may explain why there have been apparent discrepancies in past studies concerning ibuprofen–HSA binding. For example, the use of a racemic ibuprofen mixture would make it impossible to detect the different affinities of *R*- and *S*-ibuprofen for site A. Work based on racemic ibuprofen would also make it difficult to detect the presence of an additional site for one of the enantiomers, unless a large number of replicates and a wide range of experimental conditions are used. Even with the use of the pure enantiomeric samples, the similarity of the association constants for *S*-ibuprofen at sites A and B would make it hard to distinguish between these two sites unless competitive binding studies are performed, such as described in this work, that use agents with different binding affinities to these regions (e.g., *R*- or *S*-ibuprofen).

The different association constants and number of sites observed in this work for *R*- and *S*-ibuprofen indicate the importance of considering individual enantiomers in developing an accurate picture of drug–protein binding. Similar conclusions have been reached in previous studies which have examined the protein binding of other chiral compounds [12,13,27,28]. It was also found in this work that HPAC was a useful tool in examining the protein binding of the ibuprofen enantiomers. For example, by examin-

ing the changes in the capacity factors for *R*- and *S*-ibuprofen during the competitive binding studies, it was possible to determine whether or not the two forms of the drug had any common binding sites with the competing agent present in the mobile phase. By treating the data further, it was possible to determine the number of sites on HSA which were binding to each enantiomer and to estimate the association constants for these sites. The technique used in this work is not limited to ibuprofen or HSA, but can also be applied to the study of other chiral drugs or binding proteins.

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