CHIRALITY 11:675–679 (1999)

Site I on Human Albumin: Differences in the Binding of (R)- and (S)-Warfarin

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ABSTRACT The binding of drugs known to interact with area I on human serum albumin (HSA) was investigated using a chiral stationary phase obtained by anchoring HSA to a silica matrix. In particular, this high-pressure affinity chromatography selector was employed to study the binding properties of the individual enantiomers of warfarin. The pH and composition of the mobile phase modulate the enantioselective binding of warfarin. Displacement chromatography experiments evidenced significant differences in the binding of the warfarin enantiomers to site I. The (S)-enantiomer was shown to be a direct competitor for (R)-warfarin, while (R)-warfarin was an indirect competitor for the (S)-enantiomer. Salicylate directly competed with (R)-warfarin and indirectly with (S)-warfarin. This behavior was confirmed by difference CD experiments, carried out with the same [HSA]/[drug] system in solution. *Chirality 11:675–679, 1999.*

KEY WORDS: enantioselective binding; warfarin; salicylate; phenylbutazone; circular dichroism; protein binding; HSA; affinity chromatography

The binding of endogenous and exogenous compounds to human serum albumin (HSA) is characterized by high efficiency and selectivity.[1–7] A few specific binding areas have been characterized in the last 20 years. That identified as area I[4] is the most extensively investigated, mainly because of its fundamental role in the binding of many classes of therapeutically important drugs. Two binding sites exist in binding area I, the azapropazone and the warfarin binding sites.[8] Recently Yamasaki et al.[9] identified three ligands (dansyl-L-asparagine, n-butyl p-aminobenzoate, and acenocoumarol) which selectively bind to three sites of binding area I. The binding of these ligands was characterized by equilibrium dialysis experiments. Furthermore, the binding properties of warfarin and azapropazone were rationalized on the basis of this new model of the binding area. Area I was then proposed to consist of at least three partially overlapping regions. Warfarin, however, was considered a single compound, despite the differences reported for the binding of its single enantiomers.[10–15] This anticoagulant drug, used as a racemate, is extensively and enantioselectively bound to HSA. This enantioselectivity is species-specific.[16] The affinity constants have been determined by different methods and these show that the (S)-enantiomer has a higher affinity to the human protein. Furthermore, the (S)-enantiomer exhibits higher anticoagulant activity than the (R)-enantiomer in humans.[17]

We studied the binding of the single enantiomers of warfarin to HSA in order to better characterize the behavior of this drug in terms of enantioselectivity of binding and competition with other drugs. A more detailed picture of the binding of this drug to HSA, as well as of the interaction of each enantiomer with other drugs, has been obtained by affinity chromatography on an HSA-based HPLC column and by circular dichroism (CD) on the same system (drug/protein) in solution.

MATERIALS AND METHODS

Instruments

The HPLC system consisted of a Jasco 887-PU pump and a multiwavelength Jasco MULTI340 UV multichannel detector. A Rheodyne model 7125 injector with a 20 µL loop was used. The column temperature was maintained within ± 0.1°C with a Violet T-55 thermostat. Absorption and CD spectra were measured on a Perkin Elmer Lambda 9 spectrophotometer and a Jasco J-600 spectropolarimeter, respectively. All instruments were interfaced to personal computers to acquire and elaborate the data.

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Received for publication 5 January 1999; Accepted 29 April 1999

Chemicals

Warfarin [(R,S) 3-(α-acetonylbenzyl)-4-hydroxycoumarin] and human serum albumin (HSA), essentially fatty free fraction, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Salicylic acid (2-hydroxybenzoic acid) was supplied by Fluka (Buchs, Switzerland). The enantiomers of warfarin were kindly provided by Professor William Trager (University of Washington, Seattle, WA).

Chromatographic Conditions

The HSA column (15 × 0.4 cm, i.d.) was made as previously reported.[18] A solution of protein (10 mg/ml) in potassium phosphate buffer (0.05 M, pH 7.5) containing 2 M of ammonium sulphate was circulated through an epoxy-silica column in closed circuit for 24 h. The column was washed with 100 ml of potassium phosphate buffer (0.05 M, pH 7.5). The amount of the anchored protein was determined by UV absorbance at 280 nm of the protein solution before and after the immobilization procedure.[18] The mobile phases used in this study were based on phosphate buffer with different concentrations of HPLC grade 1-propanol as the organic modifier. The mobile phases were filtered using a 0.45 μm cellulose acetate filter (Sartorius, Germany) and degassed by ultrasonication immediately before use. A flow rate of 0.8 ml/min was used during the studies. For all the experiments, 1.7 μg of the compound, dissolved in 20 μL of mobile phase, was injected in duplicate or triplicate. When competitors were added to the mobile phase, the required amount was generally first dissolved in 1-propanol before addition of the buffer. The chromatographic retention of the solutes was followed at 240 nm and reported as capacity factor (k′), defined as $(t_{drug}\text{-}t_0)/t_0$ [t_{drug} = retention time of the fraction; t_0 = retention time of a nonretained solute]. The enantioselectivity ($\alpha = k'_2/k'_1$), was also calculated (k'_2 and k'_1, are the capacity factors of the second and the first eluted enantiomers).

Displacement Chromatography

The concentration of the displacer in the mobile phase was expressed relative to the concentration of the ligand in the column (*[comp]/[lig]*), to allow direct comparison of the chromatographic results with data obtained in solution with spectroscopic methods. The concentration ratio was estimated on the basis of an average concentration of 0.7 μM for the detected ligand. However, the precision of such evaluation, based on the ligand half-height peak width, does not affect the significance of the present work.

Difference CD Analysis

Standard solutions of the albumin were prepared in 0.05 M potassium phosphate buffer, pH 7.4, and their actual concentrations determined by measuring the absorbance at 279 nm ($\epsilon^{279} = 32180$[19]).

CD spectra of 1:1 [drug]/[HSA] complexes were recorded between 360 and 240 nm at room temperature, using both enantiomers of warfarin. The HSA concentration was 15 μM in phosphate buffer (50 mM, pH 7.4) solutions and the cell pathlength was 1 cm. The concentration of the competitor was expressed relative to the concentration of the warfarin (*[sal]/[war]*). Blank spectra were recorded with the same instrumental parameters (i.e., sensitivity, time constant, scan rate) and subtracted.

RESULTS AND DISCUSSION

A number of previous reports investigated binding to HSA using an immobilized HSA HPLC column.[10,20–30] Information on the affinity constants of the drugs and the interaction between ligands bound to the same site or to different regions was reliably obtained. The binding properties are directly related to the chromatographic parameters, retention being a measure of the affinity to the protein and the enantioselectivity factor, when the ligand is chiral, indicating enantioselective binding. This is possible because the immobilized protein retains the binding specificity[20,29] and the conformational mobility[30] of the native form. Displacement chromatography, i.e., the use of increasing amount of competing ligands added to the mobile phase, gave reliable information on the binding properties of many drugs. In particular, this methodology was useful in distinguishing between competitive, cooperative, noncooperative, and independent binding, following the definitions of Honoré.[31] As far as warfarin is concerned, enantioselective binding was demonstrated on HSA-based column[10–12] and chromatographic resolution investigated as a function of the experimental condition.[11] The chromatographic data obtained for (S)- and (R)-warfarin in the present investigation were compared with the data from the CD study on the same system (drug/protein) in solution. This allowed us to confirm that the immobilized HSA behaves like the protein in solution.

The resolution of *rac*-warfarin on HSA-based HPLC column has been investigated changing the experimental conditions adopted. The effects of mobile phase composition, pH, and temperature on the capacity factors (k′) of the single enantiomers and on enantioselectivity (α) are reported in Figure 1. The dependence of the chromatographic parameters on 1-propanol content was investigated using a phosphate buffer mobile phase (50 mM, pH 7.0) at 30°C. Increasing the 1-propanol content decreased the retention of both enantiomers and the enantioselectivity (Fig. 1a). When the concentration of the modifier was raised from 4% to 10%, enantioselectivity fell from 1.48 to 1.17 (21%). The effect of pH on enantioselectivity was investigated using a mobile phase composed of phosphate buffer (50 mM)/1-propanol (93/7, v/v) and 30°C (Fig. 1b). The retention of both enantiomers decreased with increasing pH, but enantioselectivity increased, with a maximum at about pH 7.0. When the pH of the mobile phase was raised from 6.5 to 7.4, enantioselectivity rose from 1.24 to 1.33 (7%). The retention of both enantiomers and enantioselectivity decreased on increasing the buffer concentration (Fig. 1c). In these studies, pH (7.0) and 1-propanol concentration (7%) were held constant at a temperature of 30°C. The molarity of the phosphate buffer was varied from 25 mM to 100 mM, which reduced enantioselectivity from 1.34 to 1.26 (6%). Finally the effect of temperature on enantioselectivity and retention factor is presented in Figure 1d. The mobile phase consisted of phosphate buffer (50

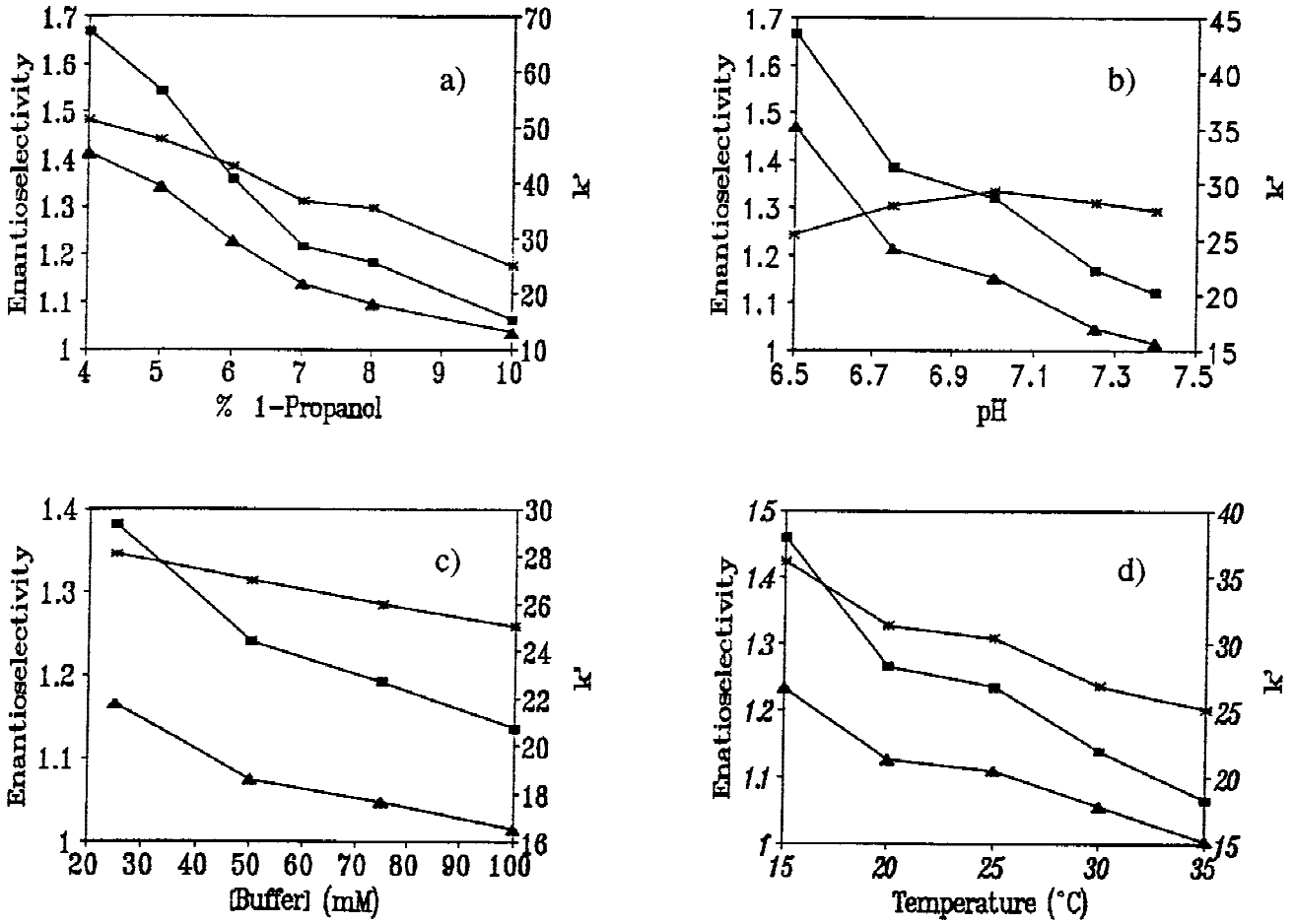

Fig. 1. The dependence of the capacity factor and enantioselectivity on the 1-propanol concentration [phosphate buffer, 50 mM, pH 7 / 1-propanol, at 30°C] **(a),** the pH [phosphate buffer, 50 mM / 1-propanol, 93/7, v/v, at 30°C] **(b),** the buffer concentration [phosphate buffer, pH 7 / 1-propanol, 93/7, v/v, at 30°C] **(c),** and the temperature [phosphate buffer pH 7 / 1-propanol, 93/7, v/v] **(d).** Enantioselectivity (α) *-*-*-*-*; k′(R)-WAR ▲-▲-▲-▲-▲; k′(S)-WAR ■-■-■-■.

mM, pH 7.0)/1-propanol (93/7, v/v). The temperature was varied from 15°C to 35°C in 5°C steps. When the temperature increased, the enantioselectivity fell from 1.42 to 1.20 (15%).

The pH and the concentration of the organic modifier were the most important parameters affecting the resolution of *rac*-warfarin.

The resolution of *rac*-warfarin on an HSA-based HPLC column has also been reported by Loun and Hage[11] to obtain information on the binding properties of this anticoagulant drug to the serum carrier. Different results, however, were obtained; actually, almost a reverse behavior was observed by Loun and Hage[11] upon increasing the ionic strength. Furthermore, we did not observe the reversal of the elution order at 15°C. This last result is clearly in contrast with the affinity constants of the single enantiomers, since (S)-warfarin is more tightly bound to protein under physiological conditions (37°C). The same authors report a higher affinity constant for the (S)-enantiomer over the entire range of temperature used in their study, i.e., 4–45°C.[11] As far as the influence of pH is concerned, again Loun and Hage[11] observed different behavior, as the retention of both enantiomers decreased upon lowering the pH. It is worth mentioning that different immobilization procedures were employed to anchor the protein to the silica matrix and this could be the reason for the different behavior observed. One possible explanation is that the maintainance of the binding properties of the protein in solution depends on the anchoring procedure. In particular, cross-linked albumin supports may give good chromatographic performances in chiral separation, but they do not provide information on the binding of drugs. We then carried out CD experiments on the [drug]/[HSA] system in solution, to obtain independent information on the binding of warfarin to the protein.

Differences in the binding of the single enantiomers of warfarin to HSA have been demonstrated. Besides a higher affinity constant, the (S)-enantiomer was shown to have a peculiar behavior, giving a specific cooperative interaction with the (S)-lorazepam hemisuccinate.[23,32] Loun and Hage[11,33] suggested different binding for (R)- and (S)-warfarin on the basis of thermodynamic studies, even though displacement chromatography data carried out using the complementary enantiomer as the competitor in the mobile phase indicated a common binding site for the two enantiomers. However, different binding mechanisms

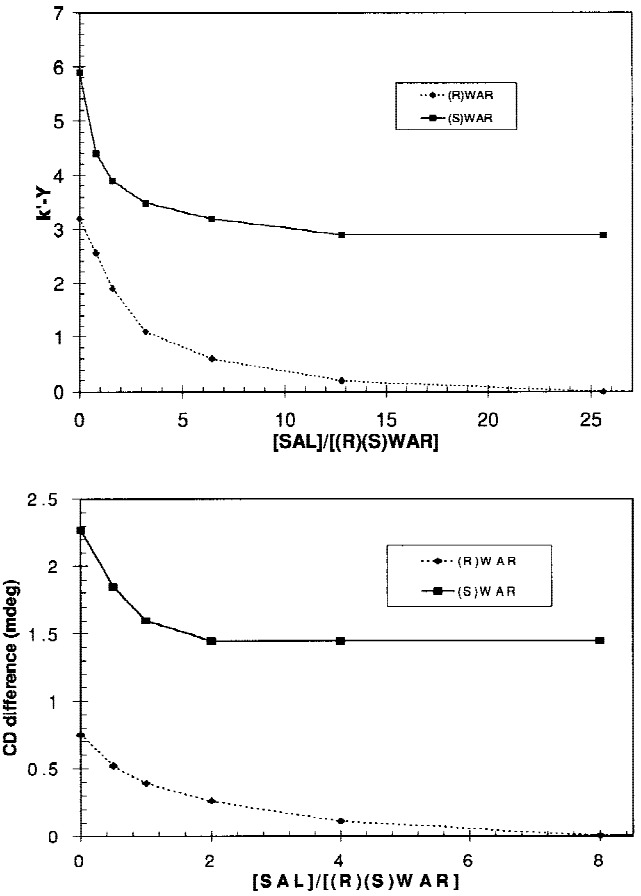

Fig. 2. Competition of salicylate (SAL) vs. (S)- and (R)-warfarin (WAR) (square and rhomboid, respectively) followed by affinity HPLC (**a,** top) and difference CD (**b,** bottom). The term Y represents the nonsaturable contribution of the capacity factor, due to the portion of nonspecific interaction of the ligand with the stationary phase (other than the HSA binding sites).[29] Monitoring of CD at 310 nm.

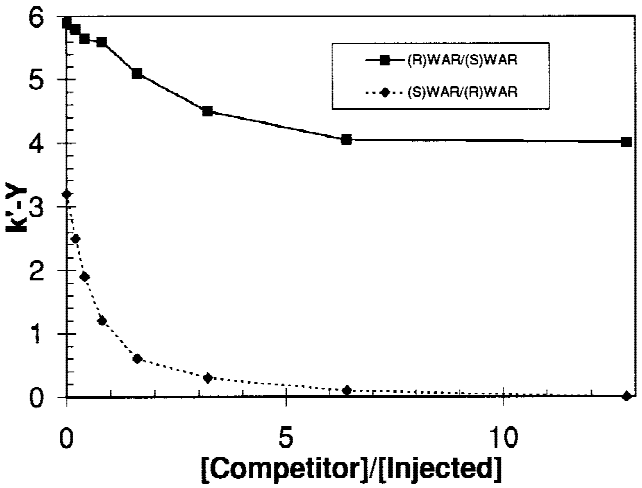

Fig. 3. Reciprocal competition of the enantiomers of warfarin (WAR) by affinity HPLC: displacement of (S)-War by (R)-War (square) and of (R)-War by (S)-War (rhomboid). The term Y represents the nonsaturable contribution of the capacity factor, due to the portion of nonspecific interaction of the ligand with the stationary phase (other than the HSA binding sites).[29]

can be distinguished for the two enantiomers of warfarin, if the nonsaturable interactions of the ligand with the stationary phase are taken into account in the displacement experiments. The residual retention time of the ligand can be determined by same-drug competition experiments.[29] The term Y (Fig. 2, 3) represents the nonsaturable contribution of the capacity factor, due to the fraction of nonspecific interactions of the ligand with the stationary phase (other than the HSA binding sites). This value can be obtained experimentally by measuring the plateau reached by k′ at a high concentration of the same-drug displacer.[29] The term Y is therefore characteristic of each analyte under constant chromatographic conditions. The determination of the residual retention time of the ligands allows us to study pharmacologically relevant saturable interactions and, among these, to distinguish between direct and indirect competition when co-binding of two drugs to the protein occurs.[29] The displacement of (R)- and (S)-warfarin using salicylate as the competitor is reported in Figure 2. The chromatographic experiments showed a complete displacement of (R)-warfarin at [salicylate]/[(R)-warfarin] molar ratios higher than six (Fig. 2a). On the contrary, only a reduction of the retention time was observed in the case of (S)-warfarin (Fig. 2a). These results were confirmed by difference circular dichroism experiments. Indeed, the induced CD signal of the (R)-warfarin bound to the protein decreased to zero by increasing the concentration of salicylate (Fig. 2b), indicating a competitive interaction. On the contrary, while a significant decrease of the induced CD signal was observed in the case of (S)-warfarin/HSA complex, a constant value, different from zero, was obtained even at relatively high [salicylate]/[warfarin] molar ratios (Fig. 2b), suggesting an indirect competition mechanism. Phenylbutazone was also used as a displacer and it was a direct competitor for both (R)- and (S)-warfarin.[29]

Further evidence of the different binding mechanism of the two enantiomers of warfarin was obtained by reciprocal competition experiments. The retention of (R)- and (S)-warfarin decreased with increasing concentration of the antipode in the mobile phase (Fig. 3). However, while (R)-warfarin was completely displaced by (S)-warfarin, the (S)-enantiomer was only partially displaced by the (R)-antipode. Two reciprocal cases of competitions were thus shown, (S)-warfarin being a direct competitor for (R)-warfarin while the opposite interaction involved indirect competition.[29]

CONCLUSIONS

Biochromatography using an HSA-based HPLC column was efficient in characterizing the binding of the enantiomers of warfarin to the protein. The pH of the mobile phase and the concentration of 1-propanol as organic modifier were the most effective parameters to modulate the enantioselective binding of warfarin to immobilized HSA. The results obtained, when compared to literature data, suggest that the anchoring method used for the protein affects its binding properties. Evidence has been obtained for the different binding of (R)- and (S)-warfarin. In particular, (S)-warfarin is a direct competitor for both (R)- and (S)-warfarin, while the (R)-enantiomer showed a noncoopera-

tive binding in the presence of its antipode. This behavior has been confirmed by CD analysis on the protein/drug system in solution. These results give deeper insights into the binding properties of site I of HSA.

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