The Effect of Octanoic Acid on the Binding of the Enantiomers of Ibuprofen and Naproxen to Human Serum Albumin: A Chromatographic Implication

Vandana K. Cheruvallath,[1] Christopher M. Riley,[2] Sunanda R. Narayanan,[3] Siegfried Lindenbaum,[4] and John H. Perrin[5,6]

Received October 5, 1995; accepted October 10, 1995

Purpose. The heats of reaction between the enantiomers and racemates of ibuprofen and naproxen and human serum albumin (HSA) are to be measured with and without the addition of octanoic acid. The effects of octanoic acid on the free energies of interaction between the drugs and HSA is to be determined and compared to that estimated from theoretical equations.

Methods. The heats of reaction have been measured directly by flow microcalorimetry.

Results. The data showed that octanoic acid lowered the 1:1 binding constants for all the drug-HSA interactions investigated. The effect of octanoic acid was greater on the R than on the S forms of the drugs as shown by the differences in free energies of interaction in the presence and absence of octanoic acid.

Conclusions. The increased free energy differences for the binding of the enantiomers of both drugs to HSA in the presence of octanoic acid is closer to the value deemed to be necessary for the separation of enantiomers by Davenkov, and shows the importance of the addition of octanoic acid to the mobile phase in the separation of these enantiomers on immobilized albumin columns.

KEY WORDS: microcalorimetry; enantiomers; binding; albumin; octanoic acid; ibuprofen; naproxen.

Human serum albumin has two major binding sites for drugs (1-6). Warfarin can be regarded as a marker for site I and benzodiazepines for Site II. The coumarins, sulfonamides and the phenylbutazone all bind to site I. The tryptophan residue—Trp 214 in human serum albumin (HSA) and Lys 199 are both located in this site (7,11). Slight enantiomeric selectivity has been observed for the binding of the enantiomers of warfarin to site I (12, 18), the S enantiomer having a greater binding constant than the R isomer. The S enantiomers of phenprocoumon (19) and acenocoumarol (20) also bind to albumin more strongly than the R isomers. Drugs binding to site II, frequently called the diazepam site, include the benzodiazepines, tryptophan, ibuprofen, naproxen octanoic acid, clofibric acid and iopanic acid (22). This binding site is not as well defined as site I and seems to involve a larger area. Fragments A and C of the cyanogen bromide fragment of HSA (residues 124-585) contain the area of site II (23, 24). His 146, Lys 194 in fragment C (25), and the highly reactive tyrosine residue of fragment A-Tyr 411 (2) are all thought to play an active role in any binding to site II. There are many studies of stereospecific binding of drugs to this binding site, including tryptophan (26), oxazepam hemisuccinate (21), other benzodiazepines (27-29) and non steroidal antiinflammatories including ketoprofen (30) ibuprofen (31), pirprofen (32,33) and etodolac (34). It is therefore not surprising that immobilized albumins (human and bovine) can be used as chiral stationary phases in liquid chromatography (13,35) for the separation of appropriate enantiomers. Medium chain fatty acids, including octanoic acid (3,36,37) have been reported to bind to site II and so the addition of octanoic acid to a mixture of drug and albumin is likely to lower the binding constant for the drug albumin interaction. This competition whether direct or indirect, can be exploited in chromatography, because the addition of octanoic acid to the mobile phase with immobilized albumins as the stationary phase, can reduce the retention times of the enantiomers and alter the capacity factors (13,35). Davankov in 1980 (38) had predicted that, at room temperature, the difference in free energies of interaction of the chiral selector with the two enantiomers in a thermodynamically controlled equilibrium, must be at least 11 k J/mol to observe any enantioselective action. As microcalorimetry has been shown to be a useful tool in obtaining the thermodynamic parameters of drug—albumin interactions (39), it was decided to investigate the binding of the enantiomers of naproxen and ibuprofen to HSA, and also the effect of octanoic acid on the binding parameters by this technique.

EXPERIMENTAL

Materials

Essentially fatty acid free human serum albumin (lot no. 42H9313 prepared from Fraction V albumin) and octanoic acid were obtained from Sigma Chemicals (St. Louis, Missouri) and used without further treatment. S(+) and R(-) naproxen were gifts from Syntex Research, Palo Alto, California. S(+) ibuprofen was a gift from Sepracor, Inc., Marlborough, Massachusetts and R(-) ibuprofen was a gift from Research Biochemicals Incorporated, Natick, Massachusetts. All the gift chemicals, and diazepam, which was kindly supplied by Dr. Richard E. Tessel, Department of Pharmacology and Toxicology, University of Kansas, Missouri, were used as received. All other chemicals were of analytical grade. Deionized water, purified in a Milli-Q Water System (Millipore Corp., Bedford, Massachusetts) was used to prepare the sodium phosphate buffers which were used throughout. A Pierce BCA [R] protein assay kit (Pierce, Rockford, Illinois) was used to determine the human serum albumin concentrations in all the experiments.

Method

Measurements were made in the LKB flow microcalo-

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66046.
[2] DuPont Merck Pharmaceutical Company, Wilmington, Delaware 19880.
[3] Drug Metabolism, Oread Laboratories, Lawrence, Kansas 66047.
[4] Deceased.
[5] J. Hillis Miller/Dept. of Med. Chem., University of Florida, Gainesville, Florida 32608.
[6] To whom correspondence should be addressed.

0724-8741/96/0100-0173$09.50/0

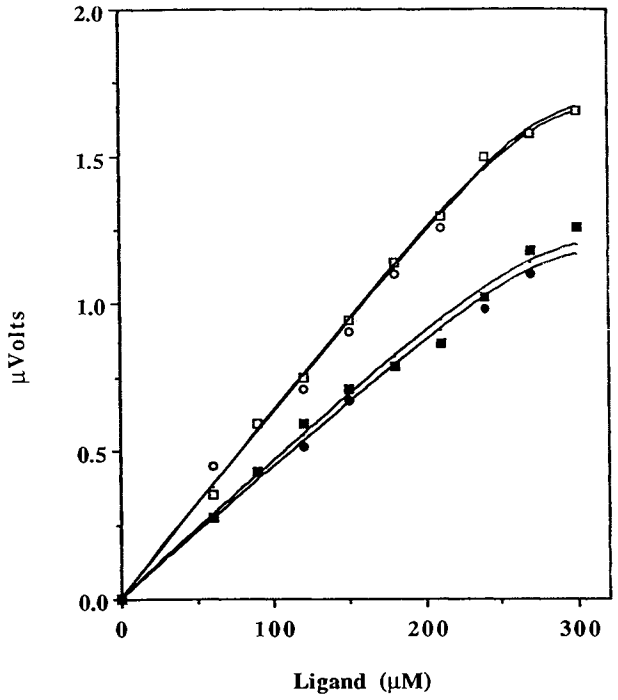

Fig. 1. Heat flux of the binding of ibuprofen enantiomers to human serum albumin as a function of ligand concentration. (○, □) S-ibuprofen, (●, ■) R-ibuprofen. Curves were generated by fitting the experimental data to equation (1) using Sigma plot and assuming a 1:1 stoichiometry. All data were collected in 0.1 M phosphate buffer, pH 7.4 at 25° C, with the protein concentration set at 300 µM.

rimeter model 2107-020 (LKB Produkter AB, Bromma, Sweden). The entire calorimeter was submerged in a water bath maintained at 25°C by Tronac PTC-40 temperature controller (Tronac Inc., Orem, Utah). A LKB Microperpex Dual Peristaltic pump, Model 2132 (LKB Produkter AB, Bromma, Sweden) was used to pump the protein and ligand solutions separately into the calorimeter. The combined flow rate was set at 25 ml hr^{-1}, so that the residence time in the mixing cell was approximately 1 minute. This was many orders of magnitude greater than the time for attainment of equilibrium between small molecules and proteins. The electrical heat of mixing was then amplified by a Keithley 150B Microvolt Ammeter (Keithley Instruments Inc. Cleveland, Ohio) and recorded on a chart recorder.

Before each run the calorimeter was electrically calibrated—the pen response to a known amount of electrically induced heat was recorded with buffer flowing through the mixing cell. Then, when the actual experiment was performed with the protein and ligand solutions, the pen response to the heat of mixing was compared to the electrical calibration performed previously. The heat of mixing of HCl and NaOH solutions was measured periodically to check the calibration. The measured heat of mixing, is a sum of the heat of reaction between the drug and protein and the heats of dilution of the drug solution and protein solution.

$$\text{Heat}_{(\text{Measured})} = \text{Heat (Drug-protein interaction)} + \text{Heat (Dilution of drug solution)} + \text{Heat (Dilution of HSA solution)}$$

Separate control experiments were therefore performed to determine the heats of dilution of drug and protein solutions (by mixing drug or protein with buffer) and these were subtracted from the total measured heat to yield the actual heat of mixing. All the experiments were conducted at 25°C in 0.1 M phosphate buffer, pH 7.4. The protein concentration was maintained constant at 300 µM. To study the effect of octanoic acid on the binding of non-steroidal anti inflammatory drugs, the acid was added to the protein solution. In all experiments, the octanoic acid concentration was half the albumin concentration. This solution was then titrated against the ligand solutions and new binding constants were obtained.

The calorimetric data was fitted using Sigma plot 4.14 (Jandel Scientific, San Francisco, California) software package on a Macintosh SE (Apple computer, Inc., Cupertino, California) personal computer. Stateworks™ a statistical software package from Data Metrics, Inc. (Heyden and Son, Inc., Philadelphia, Pennsylvania) was used for any statistical analysis and a value of $p \leq 0.05$ was considered significant in all the studies.

RESULTS

The results are obtained as a microvolt output from the microcalorimeter as a function of ligand concentrations at the fixed concentration of 300µM HSA. The titration curves, so obtained for the enantiomers of ibuprofen are shown in figure 1, of the enantiomers of naproxen in figure 2, and of octanoic acid in figure 3. The effect of a fixed concentration, 150µM octanoic acid, on the binding of ibuprofen enantio-

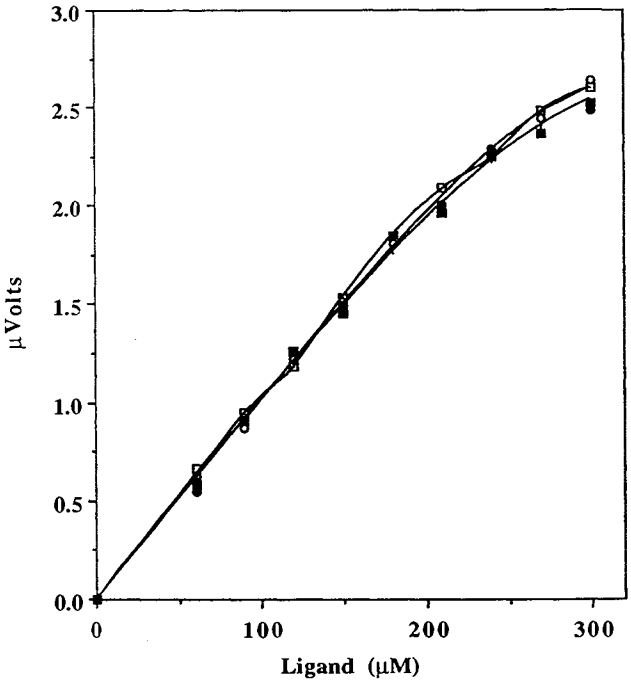

Fig. 2. Heat flux of the binding of naproxen enantiomers to human serum albumin as a function of ligand concentration. (○, □) S-naproxen, (●, ■) R-naproxen. Curves were generated by fitting the experimental data to equation (1) using Sigma plot and assuming a 1:1 stoichiometry. All data were collected in 0.1 M phosphate buffer, pH 7.4 at 25° C, with the protein concentration set at 300 µM.

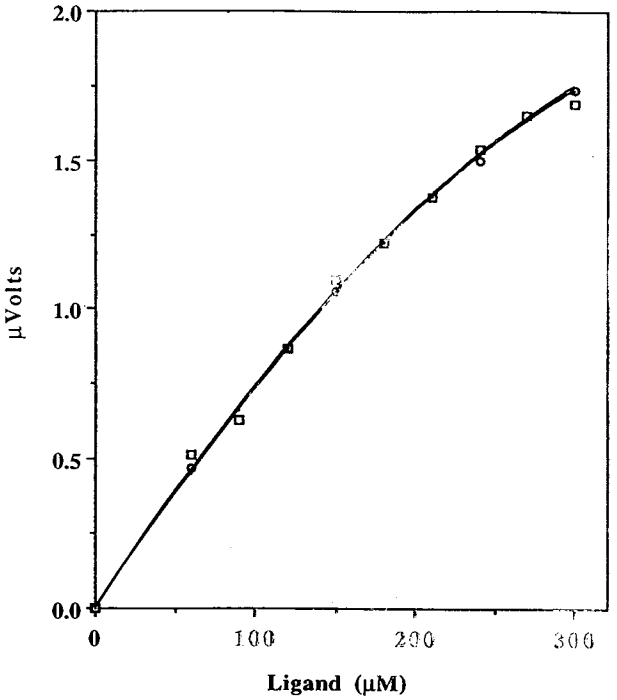

Fig. 3. Heat flux of the binding of octanoic acid to human serum albumin as a function of ligand concentration. (○, □) Octanoic acid. Curves were generated by fitting the experimental data to equation (1) using Sigma plot and assuming a 1:1 stoichiometry. All data were collected in 0.1 M phosphate buffer, pH 7.4 at 25° C, with the protein concentration set at 300 μM.

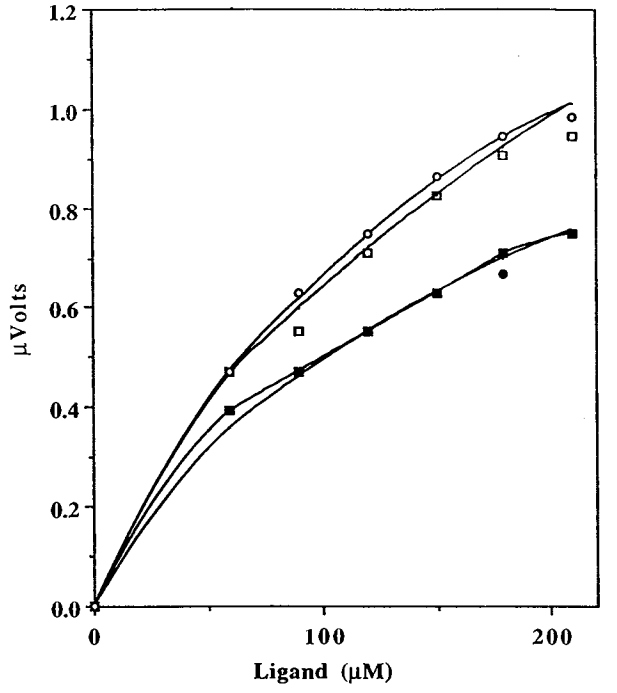

Fig. 4. Heat flux of the binding of ibuprofen enantiomers to human serum albumin in the presence of octanoic acid, as a function of ligand concentration. (○, □) S-ibuprofen, (●, ■) R-ibuprofen. Curves were generated by fitting the experimental data to equation (1) using Sigma plot and assuming a 1:1 stoichiometry. All data were collected in 0.1 M phosphate buffer, pH 7.4 at 25° C, with the protein concentration set at 300 μM, the octanoic acid concentration being 150 μM.

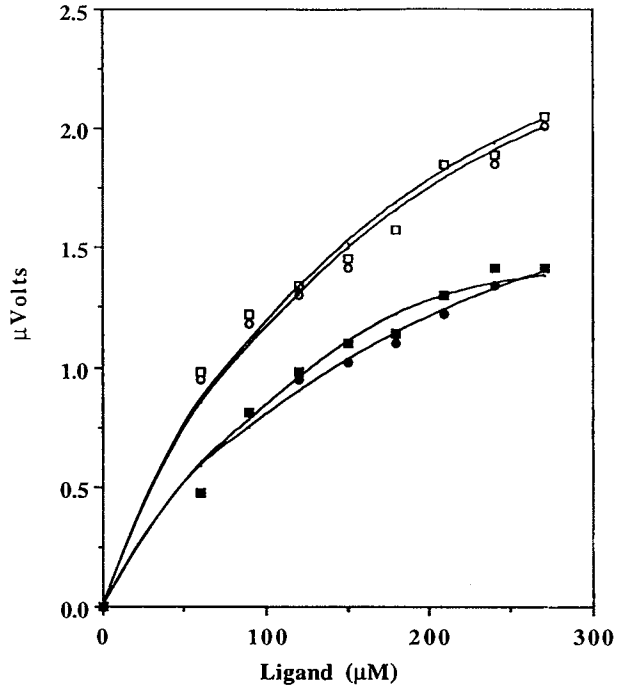

Fig. 5. Heat flux of the binding of naproxen enantiomers to human serum albumin in the presence of octanoic acid, as a function of ligand concentration. (○, □) S-naproxen, (●, ■) R-naproxen. Curves were generated by fitting the experimental data to equation (1) using Sigma plot and assuming a 1:1 stoichiometry. All data were collected in 0.1 M phosphate buffer, pH 7.4 at 25° C, with the protein concentration set at 300 μM, the octanoic acid concentration being 150 μM.

mers is shown in figure 4 and on the naproxen enantiomers in figure 5. These curves can be converted to the usual thermodynamic parameters assuming binding occurs at only one binding site (ie 1:1 interaction), from the following equations (40)

$$K = \frac{c}{(a - c)(b - c)} \tag{1}$$

c = bound drug
a = initial concentration of drug
b = initial concentration of HSA als given

c rearranges to a quadratic, however C is also given at any drug concentration by

$$c = \frac{\mu V}{\nu V_{max}} \tag{2}$$

where μV is the measured heat flux and μV_{max} is the heat flux associated with the formation of one mole of drug-HSA complex. Eliminating C gives

$$\mu V = \mu V_{max} \left(\frac{1 + K(a + b) \pm \sqrt{K^2(a + b)^2 + 1 + 2K(a + b) - 4K^2ab}}{2K} \right) \tag{3}$$

This equation has two unknowns, in μV_{max} and K. An iterative least squares technique was used to fit the experi-

Table I. Thermodynamic Parameters[a] of Binding Interaction of NSAID Enantiomers to Human Serum Albumin—as Determined by Microcalorimetry

Ligand	ΔH (kJ/mol)	K (M^{-1})	Δg° (kJ/mol)	ΔS° (J/mol °K)
S-Ibuprofen	−15.8 ± 0.1	4.4 ± 0.3 × 10^5	−32.2 ± 0.2	55 ± 1
R-Ibuprofen	−11.5 ± 0.4	2.9 ± 0.9 × 10^5	−31.1 ± 0.8	66 ± 3
Racemic Ibuprofen	−16.1 ± 1.2	1.6 ± 0.3 × 10^5	−29.7 ± 0.5	46 ± 4
S-Naproxen	−26.3 ± 0.7	1.5 ± 0.5 × 10^5	−29.5 ± 0.8	11 ± 4
R-Naproxen	−26.6 ± 0.4	1.0 ± 0.2 × 10^5	−28.4 ± 0.5	6 ± 2
Racemic Naproxen	−27.2 ± 0.1	0.9 ± 0.1 × 10^5	−28.3 ± 0.3	4 ± 1

[a] Each value in the table represents mean ± standard error from two separate sets of measurements.

mental data to the negative root. The heat of reaction between drug and HSA (ΔH) was calculated by the method described by Hardee et al (40).

$$\Delta H = \mu V_{max} \; . \; . \; x \; . \; . \; calibration \; . \; . \; constant \; . \; . \; x \; . \; . \; \frac{1}{total \; . \; . \; flow \; . \; . \; rate} \quad (4)$$

The calibration constant relates volts to watts and is obtained electrically (40). ΔG° and ΔS° are obtained from the derived K and AH values in the usual way. The values for the enantiomers and racemates are shown in Table I. The value of K obtained for octanoic acid was 1.7 X $10^4 M^{-1}$. This is an order of magnitude lower than that of the drugs.

The effect of the octanoic acid on the thermodynamic parameters of the binding of the enantiomers of naproxen and ibuprofen is shown in Table II. Table III compares the binding constant and free energies of the enantiomers in the presence and absence of octanoic acid, the δΔG's are the differences in free energy of binding of the two enantiomers, in the presence and absence of octanoic acid.

DISCUSSION

Figures 1-3, show the expected binding curves following evolution of heat. The upper limit of drug concentration was limited by concern that secondary binding sites would become involved at higher drug to albumin ratios. The S forms of both drugs bind more strongly than the R form with the racemic form binding less tightly. That there is some stereospecificity in the binding of the enantiomers suggests that the enantiomers either occupy a slightly different area of the site II with considerable overlapping, or that the two enantiomers cause a different allosteric effect on the protein. The enantiomers act as two drugs competing for the same binding area on albumin. The naproxen drugs evolve more heat on reaction with HSA, entropy contributing more to the reaction in the case of the ibuprofens. The increased area of the naphthyl rings seem to have reduced the entropy of interaction, although this may also be related to the different effects of the drugs on the conformation of albumin at pH 7.4 (41,42). It is apparent that hydrogen bonds between drug-water HSA-water, water-water, complex-water, drug-HSA make significant contributions to the thermodynamics of the total process of complex formation. The binding constant of 1.7 x 10^4 M^{-1} obtained for the octanoic-HSA interaction can be compared to the values of 3.36 x 10^4 M^{-1} (43) and 62.5 x 10^4 M^{-1} (44) in the literature.

Table II shows the binding constants of the enantiomers of both drugs were greatly reduced in the presence of octanoic acid, the effect on the naproxens being greater than on the ibuprofens. In both cases the octanoic acid has a bigger effect on the R than the S form of the drugs. It is unlikely that the effect of the octanoic acid is by direct competition for the binding site in the large area of site II, more likely, the two ligands bind to overlapping areas and that a change in shape of the binding site is induced by the octanoic acid. It is interesting to note that the binding of the naproxens proceeded with a significant decrease in entropy. Table III shows that the presence of octanoic acid clearly increases the discrimination by HSA between the R and S enantiomers, the ratio of binding constants (S/R) for ibuprofen increases from 1.5 to 3.4, and for naproxen from 1.5 to 3.0 in the presence of octanoic acid. This is also shown in the increase in δΔG's, note that these values are far from the 11kJ $mole^{-1}$ at 25°C predicted by Davankov for the separation of enantiomers. His value can be obtained by accepting a one percent contamination by the other enantiomer and so δΔG = RTln 99/1 = -11.4 kJ$mole^{-1}$ at 25°C. In 1989, Davankov (45) predicted that chromatography would reduce δΔG values to near 0.3 kJ $mole^{-1}$ for chiral selectivity. This value can be obtained from the usual resolution equation (41) namely

Table II. Thermodynamic Parameters[a] of the Binding Interaction of NSAID Enantiomers to Human Serum Albumin in the Presence of Octanoic Acid—as Determined by Microcalorimetry

Ligand	ΔH (kJ/mol)	K (M^{-1})	ΔG° (kJ/mol)	ΔS° (J/mol °K)
S-Ibuprofen	−14.1 ± 0.8	6.2 ± 0.4 × 10^4	−27.3 ± 0.2	44 ± 3
R-Ibuprofen	−13.7 ± 0.2	1.8 ± 0.1 × 10^4	−24.3 ± 0.1	36 ± 1
S-Naproxen	−32.6 ± 0.2	1.8 ± 0.1 × 10^4	−24.3 ± 0.1	−28 ± 1
R-Naproxen	−34.1 ± 1.4	6.3 ± 0.3 × 10^3	−21.7 ± 0.1	−42 ± 5

[a] Each value in the table represents mean ± standard error from two separate sets of measurements.

Table III. Effect of Octanoic Acid on the Binding Interaction Between NSAID Enantiomers and Human Serum Albumin—as Determined by Microcalorimetry

Ligand	K (M^{-1})	K[a] (M^{-1})	δG (kJ/mol)	|dΔG°| (kJ/mol)	ΔG°[a] (kJ/mol)	|dΔG°| (kJ/mol)
S-Ibuprofen	4.4×10^5	6.2×10^4	−32.2		−27.4	
				1.1		3.1
R-Ibuprofen	2.9×10^5	1.8×10^4	−31.1		−24.3	
S-Naproxen	1.5×10^5	1.8×10^4	−29.5		−24.3	
				1.1		2.6
R-Naproxen	1.0×10^5	0.6×10^4	−28.4		−21.7	

[a] Indicates values calculated in the presence of octanoic acid.

$$Rs = 0.25 \sqrt{N} \frac{k'}{k' + 1} (\alpha_{min} - 1) \quad (5)$$

where δ min = K_2/K_1 = smallest difference to allow separation, and the Ks are the binding constant for the enantiomer—HSA interaction, and where for example N the number of theoretical plates is 5000 and the k^1 the capacity factor is for example 5. If the resolution Rs, is taken as 1.0, to give a partial but detectable separation, then a value of δ min of 1.101 is obtained. Substitution, in the $\delta\Delta G = -RT\ln \alpha$ gives a δΔG value of -0.24 kJ $mole^{-1}$, close to Davankov's estimate of near 0.3 $kJmole^{-1}$. Differences in the free energies of interaction in the presence of octanoic acid between drug and HSA for the R and S ibuprofen and R and S naproxen are 3.1 and 2.6 respectively as shown in Table III; clearly above the values deemed necessary by Davankov (45). The above data suggests that the addition of octanoic acid to the mobile phase for the separation of the enantiomers of ibuprofen and naproxen using an immobilized HSA column can be expected to reduce the retention time because the binding constants between the drugs and HSA are lowered and to enhance the separation because the differences in free energies of interaction between the two enantiomers and HSA is increased.

REFERENCES

1. W.E. Müller and U. Wollert. Human serum albumin as a "silent receptor" for drugs and endogenous substances. *Pharmacology*. 19:59-67 (1979).
2. K.J. Fehske, W.E. Müller, and U. Wollert. A highly reactive tyrosine residue as part of the indole and benzodiazepine binding site of human serum albumin. *Biochim. Biophys. Acta*. 577:346-359 (1979).
3. K.J. Fehske, W.E. Müller, and U. Wollert. The location of drug binding sites in human serum albumin. *Biochem. Pharmacol.* 30(7):687-692 (1981).
4. R. Brodersen, T. Sjödin, and I. Sjöholm, Independent binding of ligands to human serum albumin. *J. Biol. Chem.* 252:5067-5072 (1977).
5. G. Sudlow, D.J. Birkett, and D.N. Wade. The characterization of two specific drug binding sites on human serum albumin. *Mol. Pharmacol.* 11:824-832 (1975).
6. G. Sudlow, D.J. Birkett, and D.N. Wade. Further characterization of specific drug binding sites on human serum albumin. *Mol. Pharmacol.* 12:1052–1061 (1976).
7. P.Q., Behrens, A.M. Spiekerman, and J.R. Brown. Structure of human serum albumin. *Fed. Proc.* 34:591 (1975).
8. B. Meloun, L. Moravek, and V. Kostka. Complete amino acid sequence of human serum albumin. *FEBS Lett* 58:134-137 (1975).
9. J.B. Swaney and I.M. Klotz. Amino acid sequence adjoining the lone tryptophan of human serum albumin. A binding site of the protein. *Biochemistry* 9:2570-2574 (1970).
10. D. Hawkins, R.N. Pinckard, and R.S. Farr. Acetylation of human serum albumin by acetylsalicylic acid. *Science* 160:780-781 (1968).
11. J.E. Walker. Lysine residue 199 of human serum albumin is modified by acetylsalicylic acid. *FEBS Lett.* 66:173-175 (1976)
12. C. Lagercrantz, T. Larsson and I. Denfors. Stereoselective binding of the enantiomers of warfarin and tryptophan to serum albumin from some different species studied by affinity chromatography on columns of immobilized serum albumin. *Comp. Biochem. Physiol.* 69C:375-378. (1981).
13. T.A.G. Noctor, G. Felix, and I.W. Wainer. Stereochemical resolution of enantiomeric 2-aryl propionic acid non-steroidal anti-inflammatory drugs on a human serum albumin based high-performance liquid chromatographic chiral stationary phase. *Chromatographia*. 31:55-59 (1991).
14. E. Domenici, C. Bertucci, P. Salvadori, S. Motellier and I.W. Wainer. Immobilized serum albumin—rapid HPLC probe of stereoselective protein-binding interactions. *Chirality* 2:263-268 (1990).
15. E.M. Sellers and J. Koch-Weser. Interaction of warfarin stereoisomers with human albumin. *Pharmacol. Res. Commun.* 7:331-336 (1975).
16. J.H.M. Miller and G.A. Smail. Interaction of enantiomers of warfarin with human serum albumin, peptides and amino acids. *J. Pharm. Pharmacol.* 29:33P (1977).
17. A. Yacobi and G. Levy. Protein binding of warfarin enantiomers in serum of humans and rats. *J. Pharmacokinet. Biopharm.* 5:123-131 (1977).
18. K. Veronich, G. White and A. Kapoor. Effects of phenylbutazone, tolbutamide and clofibric acid on binding of racemic warfarin and its enantiomers to human serum albumin. *J. Pharm. Sci.* 68:1515-1518.
19. N.A. Brown, E. Jähnchen, W.E. Müller and U. Wollert. Optical studies on the mechanisms of the interaction of the enantiomers of the anticoagulant drugs phenprocoumon and warfarin with human serum albumin. *Mol. Pharmacol.* 13:70-79 (1977).
20. I. Fitos, J. Visy, A. Magyar, J. Kaftar and M. Simanyi. Inverse stereoselectivity in the binding of acenocoumarol to human serum albumin and alpha-acid glycoprotein. *Biochem. Pharmacol.* 38:2259-2262 (1989).
21. W.E. Muller and U. Wollert. Influence of various drugs on the binding of L-tryptophan to human serum albumin. Res. Commun. Chem. Path. Pharmac. 10:565-568 (1975).
22. G.H. Mudge, G.R. Stibitz, M.S. Robinson and W. Gemborys. Competition for binding to multiple sites of human serum albumin for cholecystographic agents and sulfobromophthalein. Drug Metab. Dispos. 6:440-451 (1978).
23. I. Sjöholm and I. Ljungstedt. Studies on the tryptophan and drug-binding properties of human serum albumin fragments by affinity chromatography and circular dichroism measurements. J. Biol. Chem. 248:8431-8441 (1973).
24. K.K. Gambhir and R.H. McMenamy. Location of the indole binding site in human serum albumin. Characterization of major cyanogen bromide fragments with respect to affinity labeling positions. *J. Biol. Chem.* 248:1956-1960 (1973).

25. K.K. Gambhir, R.H. McMenamy and F. Watson. Positions in human serum albumin which involve the indole binding site. Sequence of 107-residue fragment. *J. Biol. Chem.* 250:6711-6719 (1975)
26. R.H. McMenamy and J.L. Oncley. The specific binding of L-tryptophan to serum albumin. *J. Biol. Chem.* 233:1436-1447 (1958).
27. L. Aarons, D.M. Grennan and M. Siddiqui. The binding of ibuprofen to plasma proteins. *Eur. J. Clin. Pharmacol.* 25:815-818 (1983).
28. M. Simonyi, I. Fitos, J. Kajtar and M. Kajtar. Application of ultrafiltration and CD spectroscopy for studying stereoselective binding of racemic ligands. *Biochem. Biophys. Res. Commun.* 109:851-857 (1982).
29. I. Fitos, M. Simonyi, Z. Tegyey, L. Otvos, J. Kajtar and M. Kajtar. Resolution by affinity chromatography: Stereoselective binding of racemic oxazepam esters to human serum albumin. *J. Chromatogr.* 259:494-498 (1983).
30. S. Rendic, T. Alebic-Kolbah, F. Kajfez and V. Sunjiv. Stereoselective binding of (+) and (-)-alpha-(benzoylphenyl)propionic acid (ketoprofen) to human serum albumin. II Framaco. Ed. Sc. 35:51-59 (1979).
31. A.M. Evans, R.L. Nation, L.N. Sansom, F. Bochner and A.A. Somogyi. Stereoselective plasma protein binding of ibuprofen enantiomers. Eur. Clin. Pharmacol. 36:283-290 (1989).
32. M. Otagiri, K. Masuda, T. Imai, Y. Imamura and M. Yamasaki. Binding of piprofen to human serum albumin studied by dialysis and spectroscopy techniques. Biochem. Pharmacol. 38:1-7 (1989).
33. J. Oravcova, V. Mylnarik, S. Bystricky, L. Soltes, P. Szalay, L. Bohacik and T. Trnovec. Interaction of pirprofen enantiomers and human serum albumin. Chirality 3:412-417 (1991).
34. N. Muller, F. Lapicque, C. Monot, E. Payan, R. Dropsy and P. Netter. Stereoselective binding of etodolac to human serum albumin. Chirality 4:240-246 (1992).
35. S. Allenmark and S. Andersson. Optical resolution of some biologically active compounds by chiral liquid chromatography on BSA-silica (Resolvosil) columns. Chirality 1:154-160 (1989)
36. N.P. Sollene and G.E. Means. Characterization of a specific drug binding site of human serum albumin. Mol. Pharmacol. 15:754-757 (1979).
37. S.W.M. Koh and G.E. Means. Characterization of a small apolar anion binding site of human serum albumin. Archs. Biochem. Biophys. 192:73-79 (1979).
38. V.A. Davankov. Resolution of racemates by ligand-exchange chromatography. Advances of chromatography. New York, Marcel Dekker (1980).
39. J.H. Perrin. Bonds Between Drugs and Plasma Component in Protein and Drug Transport p115-127, Symposia Medica Hoechst 20. F.K. Schaultauer Verlag. Stuttgart-New York (1985).
40. G.E. Hardee, M. Otagiri and J.H. Perrin. Microcalorimetric Investigations of Pharmaceutical Complexes 1. Drugs and β cyclodextin. Acta Pharm. Suecia a 15:188-199 (1978)
41. J. Wilting, W.F. van Giesen, L.H.M. Janssen, M.M. Weideman, M. Otagiri and J.H. Perrin. The Effect of Albumin Conformation on the Binding of Warfarin to Human Serum Albumin. J. Biol. Chem. 255:3032-3027 (1980).
42. J. Wilting, B.J. E'Hart and J. J. de Gier. The role of albumin conformation in the binding of diazepam to human serum albumin Biochem Biophys Acta 626:291-298 (1980).
43. J.D. Ashbrook, A.A. Spector and J.E. Fletcher. Medium chain fatty acid binding to human plasma albumin. J. Biol. Chem. 247:7038-7042 (1972).
44. S.W.M. Koh and G.E. Means. Characterization of a small apolar anion binding site of human serum albumin. Arch. Biochem. biophys. 192:73-79 (1979).
45. V.A. Davankov. Introduction to chromatographic resolution of enantiomers. Chiral separations by HPLC. Applications to pharmaceutical compounds. Chichester, Ellis Horwood Limited (1989).
46. L.R. Snyder and J.J. Kirkland. Introduction to Modern Liquid Chromatography. 2nd Edition J. Wiley New York (1979).