ELSEVIER

Journal of Chromatography B, 715 (1998) 183–190

JOURNAL OF CHROMATOGRAPHY B

Development of tryptophan-modified human serum albumin columns for site-specific studies of drug–protein interactions by high-performance affinity chromatography

Anuja Chattopadhyay, Tong Tian, Laurie Kortum, David S. Hage*

Chemistry Department, University of Nebraska, Lincoln, NE 68588-0304, USA

Abstract

Human serum albumin (HSA) is one of the main proteins involved in the binding of drugs and small solutes in blood or serum. This study examined the changes in chromatographic properties that occur for immobilized HSA following the chemical modification of HSA's lone tryptophan residue (Trp-214). Trp-214 was reacted with *o*-nitrophenylsulfenyl chloride, followed by immobilization of the modified protein and normal HSA onto separate silica-based HPLC supports. The binding properties of the modified and normal HSA were then analyzed and compared by using frontal analysis and zonal elution experiments employing *R*/*S*-warfarin and L-tryptophan as probe compounds for the warfarin and indole binding regions of HSA. The modified HSA was found to have the same number of binding sites as normal HSA for *R*-warfarin and L-tryptophan but lower association equilibrium constants for these test solutes. Zonal elution studies with *R*- and *S*-warfarin on the modified HSA column demonstrated the importance of Trp-214 in determining the stereoselective binding of HSA for these agents. These studies also indicated that tryptophan modification can alter HSA-based separations for chiral solutes.

Keywords: Drug–protein interactions; Tryptophan; Human serum albumin; Warfarin

1. Introduction

A knowledge of drug–protein binding is of fundamental importance when studying the biological activities of drugs since it influences the distribution, elimination and pharmacological effects of such agents [1–3]. Human serum albumin (HSA) is one of the main proteins involved in the binding of drugs and small solutes in blood or serum in humans [4–6]. This particular protein is also an excellent model to use in examining various molecular aspects of drug–protein interactions because it is a fairly homogeneous, well-defined and extensively-studied ligand [7,8]. Most compounds that interact with HSA appear to bind at a series of relatively well-defined sites on this protein. The two most important of these sites are the warfarin–azapropazone and indole–benzodiazepine binding regions (i.e., Sudlow sites I and II, respectively) [4]. As their names imply, these sites are characterized by their ability to bind certain solutes, such as warfarin or indole-containing compounds.

Recent crystallographic studies have identified the warfarin–azapropazone binding region as being in the IIA domain of HSA [9]. The crystal structure of HSA shows that the lone tryptophan residue (Trp-214) of HSA is located in the hydrophobic binding pocket of subdomain IIA and plays an important

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00140-6

structural role by limiting solvent accessibility at this site. This amino acid residue also participates in an additional hydrophobic packing interaction between the IIA and IIIA subdomains of HSA, the latter region being the location of the indole–benzodiazepine site [9]. A number of previous solution–phase studies have examined the role of Trp-214 in solute binding by selectively modifying this residue with various reagents (e.g., *o*-nitrophenylsulfenyl chloride, as shown in Fig. 1) [10]. In fact, the observation that modification of Trp-214 reduces the binding of warfarin to HSA was an early clue that Trp-214 was located at the warfarin–azapropazone binding region [11,12].

This work will examine the development and use of high-performance affinity chromatography (HPAC) columns which contain HSA that has been chemically-modified at its Trp-214 residue. It has been shown in a number of previous studies with normal HSA that HPAC columns provide effective qualitative and quantitative models for examining the interactions of small solutes with HSA [13–24]. The goal behind this particular project is to extend the applicability of HPAC for such studies by obtaining a modified HSA column that can be used to specifically determine the relative importance of solute binding at a particular region on HSA, namely the warfarin–azapropazone region. To do this, the Trp-214 residue of HSA will first be reacted with *o*-nitrophenylsulfenyl chloride, followed by immobilization of the modified protein onto an appropriate support. The binding properties of the modified HSA support will then be characterized by frontal analysis or zonal elution and compared to those properties obtained for a support containing normal HSA. Two groups of model solutes will be used for this comparison: *R*- or *S*-warfarin, which act as probes for the warfarin–azapropazone site of HSA, and L-tryptophan, which is a common probe used to examine interactions at the indole–benzodiazepine region of HSA [11,12,20,22–24].

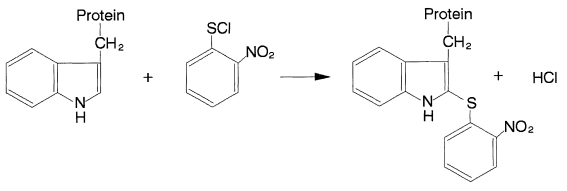

Fig. 1. Modification of tryptophan residues on a protein with the reagent *o*-nitrophenylsulfenyl chloride [10].

2. Experimental

2.1. Reagents

The HSA (Cohn fraction V, 99% globulin-free), *o*-nitrophenylsulfenyl chloride, L-tryptophan and D-tryptophan were from Sigma (St. Louis, MO, USA). The *R*- and *S*-warfarin were generously provided by DuPont (Wilmington, DE, USA). Nucleosil Si-300 (7 μm diameter, 300 Å pore size) was from Alltech (Deerfield, IL, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockfield, IL, USA). All other chemicals were of the purest grades available. All solutions were prepared using water from a Nanopure purification system (Barnstead, Dubuque, IA, USA).

2.2. Apparatus

The chromatographic system consisted of one CM3000 isocratic pump, one CM4000 gradient pump and one SM3100 UV–Vis variable wavelength absorbance detector from LDC/Thermoseparations (Riviera Beach, FL, USA). Samples were injected using a Rheodyne 7010 injection valve (Cotati, CA, USA). Data were collected using a Thermochrom interface and software from LDC. Chromatograms were processed by programs written in Microsoft QuickBASIC (Redmond, WA, USA) using double-precision logic. Columns and mobile phases were temperature-controlled using an Isotemp 9100 circulating water bath from Fisher (Pittsburgh, PA, USA). Columns were prepared using an HPLC column slurry packer from Alltech.

2.3. Methods

The tryptophan residue of human serum albumin was modified with *o*-nitrophenylsulfenyl chloride according to a previously-published procedure [25]. A total of 30 mg *o*-nitrophenylsulfenyl chloride was

dissolved in 0.5 ml glacial acetic acid and added to 0.5 g HSA in 15 ml of a 20% acetic acid solution while stirring. The reaction time was 5 h at 0°C, followed by dialysis of the reaction mixture against water for 60 h at 4°C. A small portion of the final solution was lyophilized to determine the extent of protein modification, while the remainder was dialyzed overnight against 0.067 *M* phosphate buffer (pH 7.4) at 4°C for use in circular dichroism studies and for later immobilization onto an HPAC support.

Circular dichroism measurements were made using a Jasco J600 spectropolarimeter (Easton, MD, USA). A 0.067 *M* phosphate buffer (pH 7.4) was used in this study along with a control solution that contained 2 mg/ml of normal HSA in the pH 7.4 phosphate buffer. The optical pathlength used was 0.1 cm and the wavelength range was 185–260 nm.

Diol-bonded Nucleosil Si-300 silica was prepared as described previously [15]. The diol coverage of the Nucleosil prior to activation was 230±1 (1 S.D.) μmol of diol groups per gram of silica, as determined in triplicate by a capillary electrophoresis assay [26]. The immobilized HSA support was prepared using the Schiff-base method, as described earlier [27]. The amount of immobilized HSA (see Table 1) was determined by a BCA protein assay [28], using HSA as the standard and diol-bonded silica as the blank.

The immobilized HSA and diol-bonded silica matrices were downward slurry-packed at 24.1 MPa (3500 psi) into separate 4 cm×4 mm I.D. columns of a previously-published design [29]. These columns were then enclosed in water jackets for temperature control. All chromatographic studies, except where otherwise stated, were performed at 37±0.1°C. All mobile phases and packing solvents used in this work were prepared using 0.067 *M* potassium phosphate buffer (pH 7.4). Prior to use, the mobile phases were filtered through a 0.45 μm cellulose acetate filter (Gelman, Ann Arbor, MI, USA) and degassed under vacuum for 10 min. The elution of L-tryptophan and *R*/*S*-warfarin was detected by monitoring the absorbance of the mobile phase at 290 nm or 310 nm, respectively.

The binding activity of each immobilized HSA column was measured using frontal analysis. In this procedure, solutions containing L-tryptophan or *R*-warfarin were continuously applied to the HSA columns at flow-rates of 0.25 to 0.5 ml/min. The concentrations of applied L-tryptophan or *R*-warfarin ranged from $1.25 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ *M* and $2 \cdot 10^{-6}$ to $1.2 \cdot 10^{-5}$ *M*, respectively. The retained *R*-warfarin and L-tryptophan were eluted by later applying 0.067 *M* potassium phosphate buffer (pH 7.4) alone to the column. The breakthrough times were determined from the resulting saturation curves after correcting for the void time of the system, as measured on a diol-bonded silica column made from the same initial support as used in the immobilized HSA column [15]. From the resulting breakthrough times, the apparent association equilibrium constants and binding capacities for each analyte were determined, as

Table 1
Protein content and binding properties of the normal and tryptophan-modified HSA supports[a]

Test solute and property	Normal HSA support	Modified HSA support
Total protein content (nmol HSA/g silica)	600 (± 30)	560 (±20)
R-Warfarin		
Binding capacity (nmol solute/g silica)	170 (± 10)	180 (± 20)
Specific activity (mol solute/mol HSA)	0.28 (± 0.02)	0.31 (±0.03)
Association equilibrium constant, K_a (M^{-1})	2.2 (± 0.2)·10^5	1.3 (± 0.1)·10^5
L-Tryptophan		
Binding capacity (nmol solute/g silica)	77 (± 7)	65 (± 6)
Specific activity (mol solute/mol HSA)	0.13 (± 0.01)	0.12 (± 0.01)
Association equilibrium constant, K_a (M^{-1})	1.3 (± 0.1)·10^4	0.8 (± 0.1)·10^4

[a] All numbers in parentheses represent a range of 1 S.D. The binding capacities, specific activities and association equilibrium constants were all determined at 37°C in 0.067 *M* phosphate buffer (pH 7.4).

described previously for other affinity chromatographic systems [15,30].

3. Results and discussion

After the HSA had been treated with *o*-nitrophenylsulfenyl chloride, the resulting degree of protein modification was initially examined by taking advantage of the absorbance of light at 365 nm due to the 2-thioaryl moiety of the modified tryptophan residues [31]. This provided an estimated degree of modification of 1.6 ± 0.5 mol thioaryl groups per mol of HSA, a value in good agreement with those reported in earlier studies with HSA and this same type of modification [12,32,33]. The overall secondary structure of the modified HSA was also compared to that of normal HSA by using circular dichroism. The circular dichroism spectra that were obtained (see Fig. 2) did not show any significant differences between the modified and normal HSA, thus indicating that the treatment of HSA with *o*-nitrophenylsulfenyl chloride did not result in any gross changes in the secondary structure of this protein.

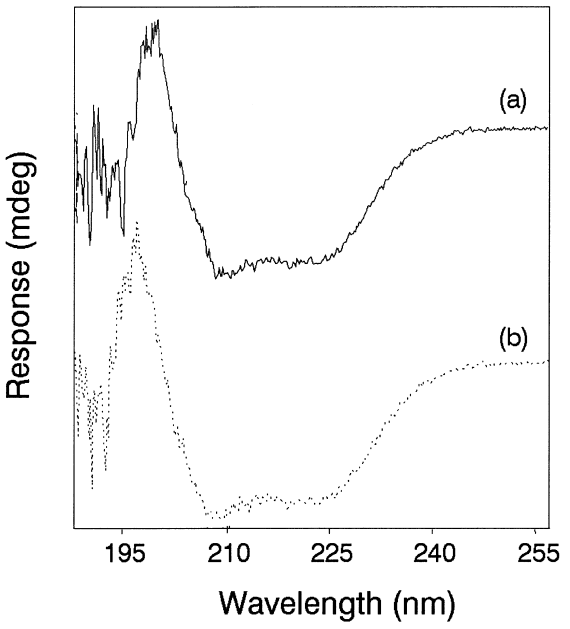

Fig. 2. Circular dichroism spectra of normal (a) and modified (b) HSA in 0.067 *M* phosphate buffer, pH 7.4. Each spectrum represents the mean of four scans.

After the modified HSA had been prepared in solution, it was purified and immobilized onto diol-bonded silica by using the Schiff base method; an identical amount of normal, unmodified HSA was immobilized by this method to the same diol-bonded support. These two HSA matrixes were then evaluated for their protein content and were packed into separate columns for binding capacity measurements. The results are summarized in Table 1. As shown in this table, essentially the same amount of protein was present in both the normal HSA and modified HSA columns. The specific activities of these columns for L-tryptophan and *R*-warfarin were also statistically identical and were in the same general range as reported in other papers that have previously used the Schiff base immobilization method for HSA [15,19,20]. The fact that these specific activities are less than one simply reflects the fact that not all of the immobilized HSA retains its binding activity because of such factors as steric hindrance, improper orientation or denaturation of the protein during the coupling process [15,19].

The binding capacities and association equilibrium constant values shown for L-tryptophan and *R*-warfarin in Table 1 were determined by the method of frontal analysis. In this method, a solution containing a known concentration of the test analyte is continuously applied to a column that has a fixed amount of immobilized ligand. As the ligand becomes saturated, the amount of analyte eluting from the column increases, forming a characteristic breakthrough curve. The mean position of this curve is related to the concentration of applied analyte ($[A]$), the amount of ligand present in the column and the association equilibrium constant (K_a) for the system. For a solute–ligand system with 1:1 binding, the following relationship can be used to relate the true moles of active ligand sites present in the column (m_L) to the apparent moles of solute required to reach the mean position of the breakthrough curve (m_{Lapp}) [15,19,20].

$$1/m_{Lapp} = 1/(K_a m_L [A]) + 1/m_L \quad (1)$$

This equation predicts that a plot of $1/m_{Lapp}$ versus $1/[A]$ for a system with single-site binding

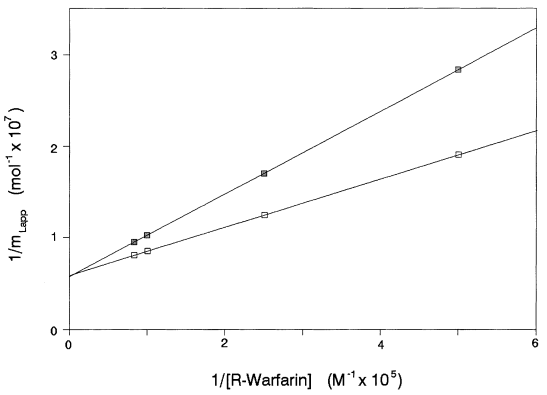

Fig. 3. Double-reciprocal frontal analysis plots for the application of *R*-warfarin to columns containing normal HSA (■) and modified HSA (□) at 37°C and pH 7.4. Other experimental conditions are given in the text.

will give a straight line with a slope of $1/(K_a m_L)$ and an intercept of $1/m_L$. The value of K_a can be determined by calculating the ratio of the intercept to the slope. The value of m_L (i.e., the binding capacity of the column) can be obtained from the inverse of the intercept.

The frontal analysis plots that were obtained for *R*-warfarin and L-tryptophan are shown in Figs. 3 and 4. For each test solute and protein column a linear relationship was observed for plots of $1/m_{Lapp}$ versus $1/[A]$ over the entire concentration range that was sampled. The correlation coefficients for the *R*-warfarin graphs in Fig. 3 were 0.9967 to 0.9977

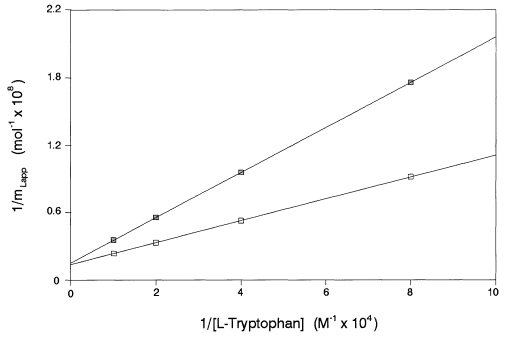

Fig. 4. Double-reciprocal frontal analysis plots for the application of L-tryptophan to columns containing normal HSA (■) and modified HSA (□) at 37°C and pH 7.4. Other experimental conditions are given in the text.

over the four concentrations studied, and the correlation coefficients for the L-tryptophan data in Fig. 4 ranged from 0.9988 to 0.9996. As stated earlier, such behavior is suggestive of 1:1 solute–ligand interactions [34]. This type of behavior is in agreement with previous studies that have examined the binding of *R*-warfarin and L-tryptophan to normal HSA in solution [35–37] or to normal HSA immobilized in columns similar to those used in this work [15,19,20].

It is interesting to note in Figs. 3 and 4 that the intercepts of the plots were essentially the same for the modified and normal HSA columns for each test analyte; this similarity is also apparent when comparing the binding capacity values provided in Table 1, which were directly determined from the intercepts of Figs. 3 and 4. This similarity in binding capacities indicates that the modification of Trp-214 on HSA did not appreciably affect the binding stoichiometry of the test analytes at either the warfarin or indole binding regions of HSA. Such a result is in agreement with the circular dichroism data shown earlier in Fig. 2, in which no noticeable changes in the secondary structure of HSA were observed due to the modification reaction. The association equilibrium constants that were determined from the slopes and intercepts of the best-fit lines in Figs. 3 and 4 are given in Table 1. The association constants determined for *R*-warfarin and L-tryptophan on the normal HSA column were statistically identical to those reported previously for these same solutes with normal HSA immobilized to HPAC columns that were equivalent to those used in this work [15,19,20].

For *R*-warfarin, the modification of Trp-214 with *o*-nitrophenylsulfenyl chloride reduced the association constant obtained at 37°C by about 40% even though the binding stoichiometry was unaffected. This result is in excellent agreement with previous solution–phase studies, which demonstrated that the modification of this tryptophan residue by *o*-nitrophenylsulfenyl chloride only reduces the affinity of warfarin but does not affect the number of binding sites [12]. The reason that has been proposed for this is that the 2-thioaryl substituent that is placed on Trp-214 can freely rotate inside the warfarin binding pocket of HSA. As a result, it is thought that warfarin can still fit into at least part of this binding

region, thereby giving it the same binding stoichiometry to HSA, but will now do so at a reduced affinity [12].

The L-tryptophan results in Table 1 indicate that the association constant for this test solute was also lowered in the presence of the modified HSA, with a decrease in binding affinity of 40% again being seen at 37°C. This result again agrees with previous observations made in solution–phase studies [32,38]. Although Trp-214 is not located near the region on HSA that interacts with L-tryptophan on HSA (i.e., the indole binding site), crystallographic studies have shown that the Trp-214 residue does play an important role in a hydrophobic packing interaction that occurs at the interface between the IIA and IIIA subdomains of HSA (i.e., the locations of the warfarin and indole binding regions, respectively) [9]. Because of this, it is possible that the tryptophan modification may slightly alter the nature of this packing interaction and thereby change the local environment of the indole binding site through an allosteric mechanism [38].

An example of how the modified HSA column might be used in studying the binding mechanisms of chiral drugs to HSA is shown in Fig. 5. In this case, a racemic mixture of *R*- and *S*-warfarin was injected onto HPAC columns of the same size that contained equivalent amounts of active normal HSA or HSA

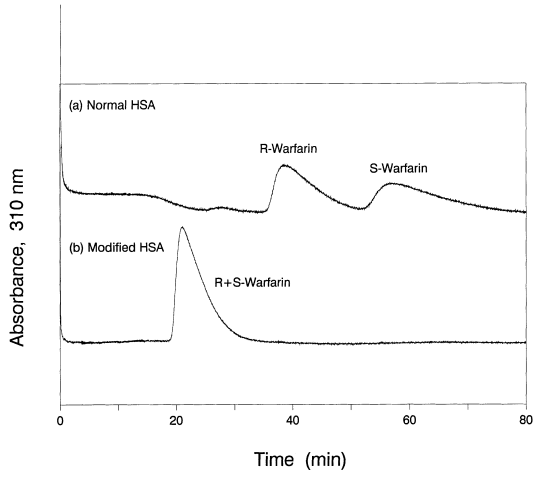

Fig. 5. Chromatograms for the injection of *R*/*S*-warfarin onto columns containing (a) normal HSA or (b) modified HSA. Conditions: flow-rate, 1.35 ml/min; sample, $9.7 \cdot 10^{-5}$ *M* racemic warfarin, 20 μl; temperature, 25°C. All other conditions are provided in Section 2.

that had been modified at Trp-214. On the normal HSA column, a baseline separation of the two enantiomers was obtained at room temperature, with the *R*-warfarin eluting first from the column. This result was expected since the binding constant of *S*-warfarin for HSA is known to be about 1.3-times larger than the binding constant for *R*-warfarin under these elution conditions [20]. However, injection of the same racemic mixture onto the modified HSA column resulted in only one peak that appeared at a shorter elution time than observed for either *R*- or *S*-warfarin on the normal HSA column. Further studies with pure *R*- or *S*-warfarin samples indicated that this peak was identical in position and width to that obtained for either single enantiomer. The same type of behavior was noted at 37°C, in which *R*- and *S*-warfarin gave different retention times on the normal HSA column but identical peak shapes and retention times when applied to the modified HSA support.

The results in Fig. 5 offer some interesting insights into the nature of the binding between *R*- or *S*-warfarin and HSA. In earlier thermodynamic and kinetic studies it has been proposed that these two enantiomers both bind to the warfarin region of HSA but interact with different locations within this binding region. For example, it has been suggested that *S*-warfarin interacts more with amino acids near the surface of the warfarin binding region while *R*-warfarin penetrates deeper into the warfarin binding pocket [20,22]. The data in Fig. 5 allow this model to be further refined by illustrating the importance of Trp-214 (or the general vicinity of this amino acid) in the chiral recognition of *R*- and *S*-warfarin by HSA. The fact that both enantiomers have a large decrease in retention following the modification of Trp-214 indicates that this residue or those in its near vicinity play an important role in the binding of both *R*- and *S*-warfarin to HSA. Also, the fact that identical retention behavior is seen for *R*- and *S*-warfarin after Trp-214 has been modified shows that the region about this residue is crucial in establishing the stereoselective binding of HSA for the warfarin enantiomers.

Another potential use of the modified HSA column involves its use in chiral separations. This is illustrated in Fig. 6 using the separation of D- and L-tryptophan as an example. For normal HSA there is

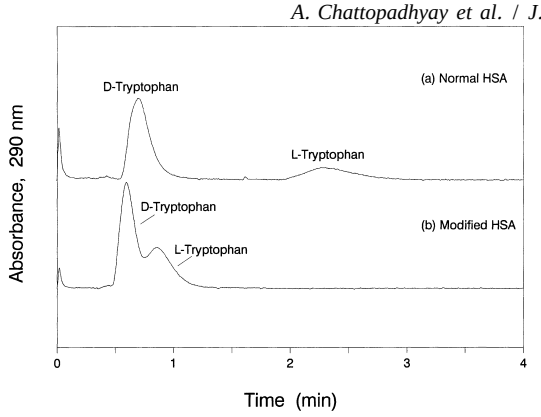

Fig. 6. Chromatograms for the injection of D/L-tryptophan onto columns containing (a) normal HSA or (b) modified HSA. Conditions: flow-rate, 1.35 ml/min; sample, $1.3 \cdot 10^{-4}$ *M* racemic warfarin, 20 μl; temperature, 25°C. All other conditions are provided in Section 2.

a large difference in retention for D- and L-tryptophan, with the L-enantiomer peak being much broader than the peak for the D-enantiomer. This agrees with previous observations that L-tryptophan binds about tentimes more strongly to HSA than D-tryptophan and has slower dissociation kinetics for these interactions [19,23,24]. On the modified HSA column, there was a large change in both the degree of retention and width of the L-tryptophan peak. The decrease in L-tryptophan binding was expected based on the association constant data given earlier in Table 1. In addition, the smaller peak widths shown in Fig. 6 indicate that the kinetics of the L-tryptophan–HSA interactions were enhanced by the modification of Trp-214. A small change in the retention and peak shape for D-tryptophan was also observed in going from normal HSA to the modified HSA column; this is most likely due to the allosteric interactions that have been reported to be present between the D-tryptophan binding site and the warfarin region of HSA [19]. Although the overall change in retention for D- and L-tryptophan did cause some loss of resolution when using the modified HSA column, the accompanying decrease in peak widths gave a separation that was still adequate for quantitation based on the peak heights. This indicates that modification of Trp-214 on HSA can alter the stereoselective recognition and separation of some chiral agents by HSA.

4. Conclusion

In summary, this study examined the development and use of modified HSA in the study and control of the binding of chiral solutes by HPAC columns. It was found that HSA which had been chemically-modified at its Trp-214 residue gave the same number of binding sites as normal HSA for *R*-warfarin and L-tryptophan (i.e., probes for the warfarin and indole sites of HSA) but lower association equilibrium constants for both of these test solutes. The results for *R*-warfarin are believed to be due to a direct blocking of HSA's warfarin binding region by the modified tryptophan residue, while the decrease in L-tryptophan retention is thought to be due to an allosteric-induced change in the indole binding region of HSA.

Data obtained for *R*/*S*-warfarin and D/L-tryptophan indicated some potential applications for columns containing the modified HSA. For instance, in work with *R*- and *S*-warfarin such a column was used to study the importance of Trp-214 in determining the stereoselective binding of HSA for these chiral agents. Studies with D- and L-tryptophan showed that modification of Trp-214 also caused changes in both the binding strength and interaction kinetics of these solutes with HSA. Further work with more test solutes is needed to fully explore the possible use of these effects for HSA-based chiral separations, but these results already indicate the valuable role that modified HSA supports may play in HPAC studies of drug–protein interactions.

Acknowledgements

This work was supported by the National Institutes of Health under grant no. GM44931.

References

[1] W.J. Jusko, M. Gretch, Drug Metab. Rev. 5 (1976) 43.
[2] W.E. Lindup, in: J.W. Bridges, L.F. Chasseaud, G.G. Gibson (Eds.), Progress in Drug Metabolism, Vol. 10, Taylor and Francis, New York, 1987, Chapter 4.
[3] R.H. Levy, D. Schmidt, Epilepsia 26 (1985) 199.

[4] W.E. Muller, K.J. Fehske, S.A.C. Schlafer, in: M.M. Reidenberg, S. Erill (Eds.), Drug–Protein Binding, Praeger, New York, 1986, Chapter 2.
[5] T. Peters Jr., Adv. Protein Chem. 37 (1985) 161.
[6] U. Kragh-Hansen, Pharmacol. Rev. 33 (1981) 17.
[7] W.E. Muller, U. Wollert, Pharmacology 19 (1979) 59.
[8] W.E. Muller, K.J. Fehske, U. Wollert, in: G. Blauer, H. Sund (Eds.), Transport by Proteins, Walter de Gruyter Verlag, Berlin, 1978, p. 341.
[9] X.M. He, D.C. Carter, Nature 358 (1992) 209.
[10] R.L. Lundblad, Chemical Reagents for Protein Modification, second ed., CRC Press, Boca Raton, FL, 1991.
[11] K.J. Fehske, W.E. Muller, U. Wollert, Biochem. Pharmacol. 30 (1981) 687.
[12] K.J. Fehske, W.E. Muller, U. Wollert, Mol. Pharmacol. 16 (1979) 778.
[13] E. Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagne, S. Motellier, I.W. Wainer, Chromatographia 29 (1990) 170.
[14] E. Domenici, C. Bertucci, P. Salvadori, I.W. Wainer, J. Pharm. Sci. 80 (1991) 164.
[15] B. Loun, D.S. Hage, J. Chromatogr. 579 (1992) 225.
[16] E. Domenici, C. Bertucci, P. Salvadori, S. Motellier, I.W. Wainer, Chirality 2 (1992) 263.
[17] T.A.G. Noctor, C.D. Pham, R. Kaliszan, I.W. Wainer, Mol. Pharmacol. 42 (1992) 506.
[18] T.A.G. Noctor, I.W. Wainer, D.S. Hage, J. Chromatogr. 577 (1992) 305.
[19] J. Yang, D.S. Hage, J. Chromatogr. A 645 (1993) 241.
[20] B. Loun, D.S. Hage, Anal. Chem. 66 (1994) 3814.
[21] D.S. Hage, T.A.G. Noctor, I.W. Wainer, J. Chromatogr. A 693 (1995) 23.
[22] B. Loun, D.S. Hage, Anal. Chem. 68 (1996) 1218.
[23] J. Yang, D.S. Hage, J. Chromatogr. A 725 (1996) 273.
[24] J. Yang, D.S. Hage, J. Chromatogr. A 766 (1997) 15.
[25] C. Jacobsen, Eur. J. Biochem. 27 (1972) 513.
[26] A. Chattopadhyay, D.S. Hage, J. Chromatogr. A 758 (1997) 255.
[27] P.-O. Larsson, Methods Enzymol. 104 (1984) 212.
[28] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[29] R.R. Walters, Anal. Chem. 55 (1983) 591.
[30] I.M. Chaiken (Ed.), Analytical Affinity Chromatography, CRC Press, Boca Raton, 1987.
[31] E. Boccu, F. Veronese, A. Fontana, C.A. Benassi, Eur. J. Biochem. 13 (1970) 188.
[32] K.J. Fehske, W.E. Muller, U. Wollert, Biochim. Biophys. Acta 577 (1979) 346.
[33] K.J. Fehske, U. Schlafer, U. Wollert, W.E. Muller, Mol. Pharmacol. 21 (1981) 387.
[34] S.A. Tweed, B. Loun, D.S. Hage, Anal. Chem. 69 (1997) 4790.
[35] J.H.M. Miller, G.A. Smail, J. Pharm. Pharmacol. 29 (1977) 33.
[36] Y.T. Oester, S. Keresztes-Nagy, R.F. Mais, J. Becktel, J.F. Zaroslinski, J. Pharm. Sci. 65 (1976) 1673.
[37] R.H. Mcmenamy, R.H. Seder, J. Biol. Chem. 238 (1963) 3241.
[38] K.J. Fehske, W.E. Muller, U. Wollert, Hoppe-Seyler's Z. Physiol. Chem. 359 (1978) 709.