The *in Situ* Acetylation of an Immobilized Human Serum Albumin Chiral Stationary Phase for High-Performance Liquid Chromatography in the Examination of Drug–Protein Binding Phenomena

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The *in situ* modification of an immobilized human serum albumin (HSA) high-performance liquid chromatographic chiral stationary phase by *p*-nitrophenyl acetate is reported. This procedure, which is thought to affect primarily a single reactive tyrosine residue within the protein structure, influenced the chromatographic retention and enantioselectivity factors of a wide range of solutes. For certain solutes, increases in both capacity factor and chiral resolution were observed. Ultrafiltration studies on representative test solutes using free HSA, treated in a similar manner to the immobilized protein, gave similar results as the chromatographic observations, indicating that the latter effects are not artifactual results of immobilization. The effect of the modification of HSA on the binding behavior of drugs reportedly sharing the site predominantly affected by the derivatization, namely, the indole–benzodiazepine binding site, varied greatly. This observation suggests that the affected binding area is not a single, tightly structurally defined site.

KEY WORDS: chiral stationary phases; human serum albumin; protein binding; binding sites; high-performance liquid chromatography; immobilized proteins; chemical modification.

INTRODUCTION

Reversible attachment to serum proteins significantly modulates the pharmacokinetics and pharmacodynamics of many drugs (1). A clear understanding of the plasma protein binding behavior of a particular therapeutic agent is therefore fundamental to its safe and rational use. Of the various bloodborne components active in this respect, the most important is serum albumin.

Competitive binding experiments show that the majority of solutes which bind to human serum albumin (HSA) may be displaced by one of a very small number of "marker" compounds, implying that there are relatively few sites at which drugs bind. In fact, the binding of most drugs to HSA may be described in terms of attachment to one (or both) of two main loci, termed the warfarin–azapropazone binding area (also known as Site I) and the indole–benzodiazepine binding site (Site II) (2). A small number of compounds, such as digitoxin and tamoxifen, do not appear to bind to either of these sites, necessitating the postulation of further, minor binding sites (3).

While the site-oriented approach to binding is empirically successful in describing the majority of drug–HSA binding phenomena, enough contradictory data exist to suggest that it is perhaps dangerous blindly to assume the validity of this approach. Some authors, therefore, dispute the existence of preformed drug-binding sites on HSA (4).

Several studies have attempted to locate the regions of the HSA molecule in which the major binding sites lie and to determine the amino acid residues which constitute them (2,5,8). Two particularly susceptible amino acid residues have been the subject of the greatest attention: the lone tryptophan residue (Trp-214) of HSA, which appears to reside in the warfarin–azapropazone binding area (2), and a single reactive tyrosine residue (Tyr-411), which appears to be located within the indole-benzodiazepine binding site. Nitration of Tyr-411, which is 20 times more reactive than the remaining 17 tyrosines of HSA (6), reduced the binding of L-tryptophan and four achiral benzodiazepines to HSA by up to 90% (5). However, the range of ligands reported in this study was very limited, and no consideration was made of possible enantioselective aspects of drug-HSA binding.

Recently, this laboratory has been involved in the synthesis and characterization of a novel chiral stationary phase (CSP) based upon human serum albumin (HSA) immobilized within a commercially available diol high-performance liquid chromatography (HPLC) column (9–13). We have shown that the immobilized protein retains the binding characteristics (9,10,12), and conformational mobility (10,13), of the native form. The HSA-CSP therefore appears to be a useful tool for the rapid probing of solute–HSA interactions, HSA binding sites, and solute–solute cobinding interactions.

We describe here the effect of the limited acetylation of immobilized HSA, by *p*-nitrophenyl acetate, performed *in situ* within the HPLC column, on the capacity factors (k') and, where applicable, the enantioselectivity factors (α), of a series of test solutes.

MATERIALS AND METHODS

Materials

Racemic ibuprofen, and its enantiomers were obtained from Sepracor Inc. (Marlborough, MA). The remaining solutes were purchased from the Sigma Chemical Company (St. Louis, MO) and used without further purification. *p*-Nitrophenyl acetate, triethanolamine, and human serum albumin (essentially fatty acid free) were obtained from Sigma. The Centrifree micropartition cartridges used in the ultrafiltration studies were obtained from Amicon (Danvers, MA).

Chromatography

The modular liquid chromatograph employed consisted of a Spectroflow 400 pump, 480 injector module (20-μl loop), and 1000S diode array detector (all from Applied Biosystems, Ramsey, NJ, USA) and a DataJet integrator (Spectra-Physics, San Jose, CA). The column temperature was maintained at 25 ± 0.1°C, using a CH-30 temperature regulating

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jacket (FIAtron Laboratory Systems Inc., Oconomowoc, WI).

The HSA-CSP [150 × 4.6 (i.d.) mm] was prepared as described previously (9), by Société Française Chromato Colonne (Neuilly-Plaisance, France).

All chromatography was carried out isocratically, with a mobile phase of sodium phosphate (50 m*M*, pH 6.90):propan-1-ol (90:10, v/v), delivered at a flow rate of 0.9 ml · min^{-1}.

The amount injected of each solute was 0.5 μg for the racemates or 0.25 μg for the nonchiral solutes. The chromatographic parameters measured were capacity factor [k', which is defined as $(t_R - t_0)/t_0$, where t_R is the retention time of the solute of interest, and t_0 is that of an unretained solute (water)] and α (which is the ratio of the capacity factors of the second- and the first-eluting enantiomers), where relevant.

In Situ Acetylation of Immobilized HSA

The *in situ* acetylation of the HSA-CSP was effected by a modification of the method of Means and Bender (6) for HSA in free solution. A mobile phase containing *p*-nitrophenyl acetate (167 μ*M*), triethanolamine (5 m*M*), acetonitrile (10%, v/v) and propan-1-ol (5%, v/v) in sodium phosphate (50 m*M*, pH 6.90), at 23°C, was passed through the HPLC column at a flow rate of 0.9 ml · min^{-1} for 1 hr. The course of the reaction was monitored by following the generation of *p*-nitrophenolate in the column eluent, by absorbance measurement at 430 nm. After reaction (approximately 1 hr), the column was washed with sodium phosphate (50 m*M*, pH 7.5):acetonitrile (85:15, v/v) for 2 hr, then with water:propan-1-ol (90:10, v/v) overnight.

Reaction of Free HSA with *p*-Nitrophenyl Acetate

Reaction conditions, broadly similar to those described above for the acetylation of immobilized HSA were employed. A solution of *p*-nitrophenyl acetate (0.1%, w/v), triethanolamine (5 m*M*), acetonitrile (10%), and HSA (5 g) in sodium phosphate (50 m*M*, pH 6.90) was prepared. Reaction was allowed to proceed for 1 hr, after which time the solution was dialyzed against 0.02 *M* acetic acid for 24 hr, followed by dialysis against water for a further 72 hr. The solution of modified protein from within the dialysis membrane was finally adjusted to volume, to yield a solution of approximately 500 μ*M* acetyl-HSA, a final buffer strength of approximately 50 m*M*, and a final pH of 6.9.

Ultrafiltration Studies of Drug Binding to Acetyl-HSA

The binding of representative solutes to modified and unmodified HSA was studied by ultrafiltration. The percentages of binding of benoxaprofen enantiomers, salicylic acid, chlordiazepoxide, (*R*)- and (*S*)-ibuprofen, and (*R*)- and (*S*)-oxazepam hemisuccinate (OXH) to native, underivatized HSA were initially determined. The degree of binding of these solutes to acetyl-HSA was then measured.

The solutes were incubated at 25°C, with either unmodified or acetylated HSA, at molar drug:protein ratios ranging from 0.25:1 to 8:1. The incubation medium was sodium phosphate (50 m*M*, pH 6.90):propan-1-ol (95:5, v/v), the alcohol being added in an attempt to parallel the conditions employed for chromatography. Incubation was allowed to proceed for at least 2 hr, before ultrafiltration. The solutes did not bind, to any determinable extent, to the ultrafiltration membrane or cartridge assembly.

For the chiral solutes, enantioselective chromatographic analyses were employed to determine the free fractions of the individual enantiomers. For benoxaprofen, a column based on ovomucoid (Ultron ES-OVM, 15 cm × 4.6-mm i.d., Mac-Mod, Chadds Ford, PA) was used, with an eluent of sodium phosphate (0.015 *M*, pH 6.00):propan-1-ol (93:7, v/v), at a flow rate of 1.0 ml · min^{-1}. Under these conditions, the enantiomers of benoxaprofen gave k's of 8.0 and 10.2. The remaining solutes were analyzed on the HSA-CSP. For oxazepam hemisuccinate, chlordiazepoxide, and salicylic acid, a 15-cm column was used, with a mobile phase composed of sodium phosphate (50 m*M*, pH 6.9):acetonitrile:propan-1-ol (85:10:5, v/v/v), at a flow rate of 0.8 ml · min^{-1}. Under these conditions the observed capacity factors were as follows: (*R*)-oxazepam hemisuccinate, 7.9; (*S*)-oxazepam hemisuccinate, 12.4; chlordiazepoxide, 2.9; and salicylic acid, 3.1. For the enantiomers of ibuprofen, a 5-cm HSA column was used, with a mobile phase of sodium phosphate (50 m*M*, pH 6.9):acetonitrile (90:10, v/v), with 5 m*M* octanoic acid, which resulted in k's of 6.7 for (*S*)-ibuprofen and 8.2 for the (*R*)-enantiomer.

RESULTS

After *in situ* acetylation of HSA within an HPLC column, a series of test solutes was chromatographed. The k's and α's of these solutes had been determined previously on the same column, before derivatization (Table I).

The acetylation of the HSA-CSP had a significant effect on the retention behavior of the majority of the solutes examined in this study, with the exception of salicylic acid, and the first-eluting enantiomers of temazepam and lorazepam. The more retarded enantiomer of temazepam, both enantiomers of benoxaprofen and (*S*)-oxazepam hemisuccinate all showed large increases in k', on derivatization of the CSP, leading to substantial enhancements in enantioselectivity (Fig. 1). The remaining solutes experienced significant reductions in k' and α. In the case of lorazepam, acetylation of the HSA-CSP resulted in complete loss of chiral recognition.

The results of the ultrafiltration studies are in concordance with the chromatographic observations. The effect of acetylation of HSA on the free fractions of the test solutes examined is presented in Table II.

Chlordiazepoxide underwent a decrease in k' of 11% upon acetylation of the CSP. Ultrafiltration studies demonstrated that at drug:protein molar ratios of less than unity, the binding of chlordiazepoxide to HSA was actually enhanced by acetylation. Scatchard analysis of the data revealed that binding occurs to at least two other classes of binding site and that the binding at these sites was reduced (Fig. 2) by acetylation of HSA.

The retention of salicylic acid on the HSA-CSP was not significantly altered by acetylation of the stationary phase. Scatchard analysis of the data from the ultrafiltration studies on unmodified HSA (Fig. 3) showed that salicylic acid binds

Table 1. Effect of Acetylation of Immobilized Human Serum Albumin on Chromatographic Parameters of Selected Solutes

Compound[a]	k' (premodification)	k' (postmodification)	% $\Delta k'$[b]	% $\Delta\alpha$[c]
Compounds binding at Site I				
(*R*)-Warfarin	11.58	9.17	−21	—
(*S*)-Warfarin	16.35	10.76	−34	−59
Phenylbutazone	27.75	20.06	−28	n/a[d]
Salicylic acid[e]	1.99	2.06	+4	n/a
Tolbutamide[e]	6.00	5.15	−14	n/a
Compound binding at digitoxin binding site				
Digitoxin	2.54	2.08	−18	n/a
Compounds binding at Site II				
Ketoprofen A	9.96	5.01	−50	—
Ketoprofen B	13.27	6.11	−54	−33
Fenoprofen A	18.62	12.51	−33	—
Fenoprofen B	34.54	18.22	−47	−47
(*S*)-Ibuprofen	24.31	12.13	−50	—
(*R*)-Ibuprofen	87.76	42.14	−52	−5
Suprofen A	12.07	7.81	−35	—
Suprofen B	33.59	15.68	−53	−43
Benoxaprofen A	28.67	33.23	+16	—
Benoxaprofen B	31.89	44.54	+40	+209
Chlordiazepoxide	1.51	1.34	−11	n/a
Temazepam A	1.33	1.32	−1	—
Temazepam B	1.98	3.08	+56	+63
(*R*)-Oxazepam hemisuccinate	2.77	2.34	−16	—
(*S*)-Oxazepam hemisuccinate	4.14	8.55	+106	+443
Lorazepam A	2.41	2.20	−9	—
Lorazepam B	2.63	2.20	−16	−100

[a] A and B designate the first- and second-eluting enantiomers, respectively.
[b] Percentage change in capacity factor.
[c] Percentage change in enantioselectivity factor.
[d] Not applicable—achiral solutes.
[e] These compounds also bind approximately equally to other sites.

at two classes of binding site, with fairly similar affinity constants (5.0×10^4 and 2.5×10^4 M^{-1}). Acetylation of the protein resulted in a loss of the second class of site and in a small increase in the affinity of binding to the first class.

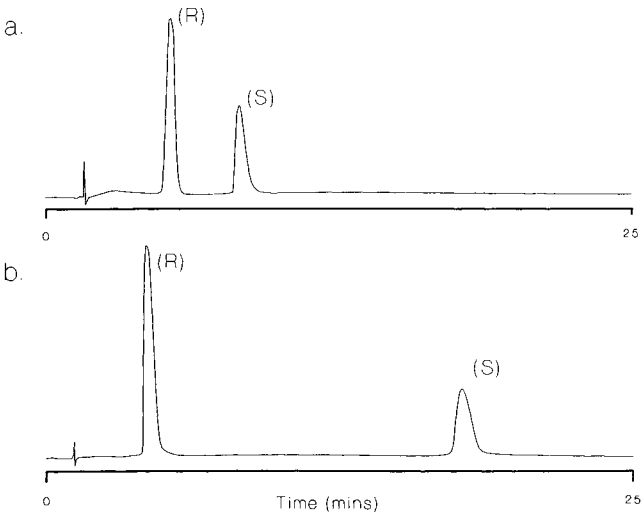

Fig. 1. The separation of the enantiomers of oxazepam hemisuccinate on (a) unmodified HSA and (b) acetyl-HSA. Mobile phase, sodium phosphate (50 m*M*, pH 6.90):propan-1-ol (90:10, v/v), at 0.9 ml · min^{-1}; detection, 230 nm; 25°C.

The (*S*)-enantiomer of oxazepam hemisuccinate, which is the more retarded enantiomer on the HSA-CSP, experienced an increase in k' of approximately 100% when immobilized HSA was acetylated (Fig. 1). Scatchard analysis of the ultrafiltration data of (*S*)-OXH did not provide helpful information, since at low drug:protein ratios, which provide the greatest information on specific binding affinities, the levels of free drug present fell below the limits of detection of the assay method. This was also found to be the case for the other highly bound solutes studied. However, the fraction of free drug, at drug:protein ratios of 0.5:1, decreased by approximately 80%, going from unmodified to modified protein, which is entirely consistent with the chromatographic findings.

The ultrafiltration studies on (R)-OXH did not reveal any specific binding to either native or modified HSA, therefore, in this case it was not possible to correlate the binding studies with the chromatographic observations.

The acetylation of the HSA-CSP resulted in a large (approximately 50%) reduction in the capacity factors of both enantiomers of ibuprofen but had only a slight effect on enantioselectivity (a decrease of 5%). The free protein studies on ibuprofen were again consistent with the chromatographic findings. The free fractions of both isomers of ibuprofen substantially increased upon acetylation of HSA, the effect being very similar for both enantiomers. As was the case for OXH, rigorous Scatchard analysis of the ultrafiltra-

Table II. The Binding of Solutes to Unmodified and Acetylated HSA, as Determined by Ultrafiltration

Compound	% bound to unmodified HSA at drug:protein molar ratio			% bound to modified HSA at drug:protein molar ratio		
	0.5:1	1:1	2:1	0.5:1	1:1	2:1
(*R*)-Oxazepam hemisuccinate	17.6	17.4	18.4	15.8	18.2	16.8
(*S*)-Oxazepam hemisuccinate	72.2	69.1	55.6	95.0	77.8	44.9
Chlordiazepoxide	63.3	51.6	35.2	86.3	65.1	17.92
Salicylic acid	71.4	65.0	52.9	85.6	61.24	42.5
(*R*)-Ibuprofen	98.0	93.3	84.5	93.8	86.5	58.5
(*S*)-Ibuprofen	97.4	93.0	83.1	92.4	79.3	52.8
Benoxaprofen A	96.1[a]	93.3	90.6	96.8[a]	93.0	84.2
Benoxaprofen B	97.7[a]	94.1	92.0	98.0[a]	94.0	86.5

[a] Indicates a drug:protein ratio of 0.67:1. The binding of the enantiomers of benoxaprofen to both native and acetylated HSA was such that no free drug could be detected at a molar drug-to-protein ratio of 0.5:1.

tion data of ibuprofen was not feasible, due to the very high degree of binding of these solutes.

Both enantiomers of benoxaprofen underwent an increase in k' on the acetyl-HSA-CSP, relative to the unmodified column. This effect was mirrored in the studies on the free proteins; decreases in the free fractions of both isomers on derivatization of HSA with *p*-nitrophenyl acetate were observed, at low drug:protein molar ratios. Due to the very high degree of binding of the enantiomers of benoxaprofen; however, it was not possible to detect either of the free enantiomers at ratios of less than 0.67:1. At higher drug:protein ratios, i.e., those which reflect binding at secondary sites, there appears to be a decrease in the bound fraction of both enantiomers upon derivatization of HSA.

DISCUSSION

The results presented on the binding of solutes to HSA could be interpreted according to the concept of site-orientated attachment. Until now, the effect of the acetylation of HSA on sites other than Site II has not been examined. Phenylbutazone and the enantiomers of warfarin are thought to bind primarily at Sjöholm's Site I (3). All three solutes experienced a similar decrease in k' on modification of the HSA-CSP. Digitoxin is proposed to bind at a specific site on HSA which is discrete from the other, major drug-binding sites (3). The binding of digitoxin was diminished upon acetylation of the HSA-CSP. The acetylation of HSA, under mild conditions such as those described here, affects primarily a single tyrosine residue, Tyr-411, which has been proposed (5,8) as being fundamental to the binding of solutes to Site II. If the sole effect of acetylation of Tyr-411 is "blocking" of Site II, then these observations are unexpected.

The remaining solutes considered in the present study were selected because they are thought to bind, either exclusively or predominantly, to Site II (the indole–benzodiazepine site). Compounds considered to bind at Site II include the benzodiazepines (by definition) and the 2-aryl propionic acid antiinflammatory drugs (the "profens") (14). Several examples of these important (at least in terms of prescription volume) groups were studied, revealing at least three discernable types of behavior: those solutes which experienced a decrease in k' on the acetyl-HSA-CSP, relative to the unmodified column; those for which binding increased; and those which were not significantly affected by the modification procedure. Clearly, such a spread of results is at odds with the supposition that each of these solutes binds at the same site.

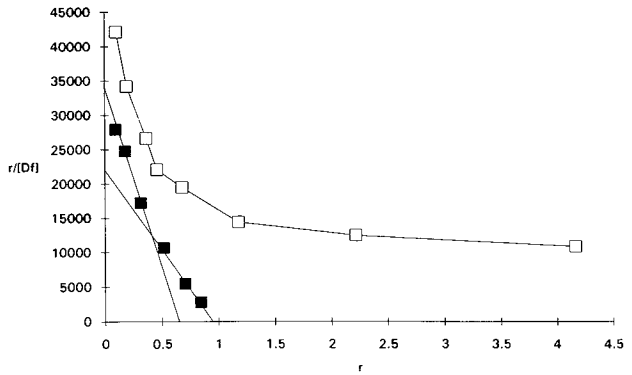

Fig. 2. Scatchard analysis of the ultrafiltration data obtained from incubation of chlordiazepoxide with unmodified and acetylated HSA. Filled squares, unmodified HSA; open squares, acetyl-HSA. r is the number of moles of drug bound per mole of protein, and [Df] is the concentration of free drug.

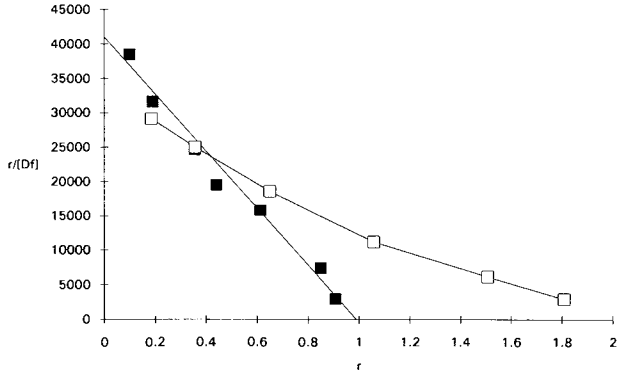

Fig. 3. Scatchard analysis of the ultrafiltration data obtained from incubation of salicylic acid with unmodified and acetylated HSA. Open squares, unmodified HSA; filled squares, acetyl-HSA. r is the number of moles of drug bound per mole of protein, and [Df] is the concentration of free (unbound) drug.

The modification of Tyr-411 should decrease the binding of "Site II" solutes, at least according to Fehske *et al.* (5). For the majority of the solutes in Table I which bind at Site II, this hypothesis is, in fact, consistent with the experimental findings. However, the binding of several of these solutes was actually enhanced by the acetylation of HSA.

The answer may be that the indole–benzodiazepine binding site of HSA, rather than being a closely defined, receptor-like site, may be better described as a larger, flexible area, the precise topography of which is determined not only by solutes binding there, but also by the presence of other molecules binding at other, perhaps remote, sites (15). It is possible that the acetylation of Tyr-411 may disrupt an important intramolecular hydrogen bond, responsible for the tertiary structure of HSA in the microenvironment of Site II, revealing previously occluded residues to which, for instance, the enantiomers of benoxaprofen are able to bind more efficiently.

Although the enantioselective binding of solutes to biopolymers is sometimes taken as being evidence of receptor-like specificity, the existence of a receptor-like site is not necessarily implied. Cellulose and its derivatives are eminently able to effect the resolution of enantiomers, despite the lack of anything which would be conventionally described as a "receptor site" (16). The differential binding of enantiomers by a protein molecule does not therefore implicitly require the existence of a highly structurally defined, tight-fitting binding site. All that is required is that at least three interactions occur between the solute and the chiral selector, which are directly influenced by the stereochemistry of the solute. We therefore view the indole–benzodiazepine binding site, despite its high enantioselectivity, as a rather large area, comprised of perhaps several overlapping subsites, similar to the presently accepted conception of the warfarin–azapropazone binding area (17).

The influence of the acetylation of Tyr-411 on other "drug binding sites," as reported here, is not really unexpected. HSA is known to be a very flexible protein, which has led to great difficulty in obtaining an accurate picture of its tertiary structure (18). It may be anticipated, therefore, that modification of key residues within the primary structure may possibly result in effects in remote regions of the protein.

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