Reversible Binding Interactions Between the Tryptophan Enantiomers and Albumins of Different Animal Species as Determined by Novel High Performance Liquid Chromatographic Methods: An Attempt to Localize the D- and L-Tryptophan Binding Sites on the Human Serum Albumin Polypeptide Chain by Using Protein Fragments

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ABSTRACT

The stereoselectivity of the reversible binding interactions between the D- and L-tryptophan enantiomers and serum albumins of different animal species and fragments of human serum albumin (HSA) was investigated by applying three novel high performance liquid chromatographic (HPLC) arrangements. The separations were performed by means of 1) an achiral (diol-bond), 2) a chiral (bovine serum albumin-bond) silica gel sorbent, and 3) a column switching technique which uses both the diol- and HSA-bond HPLC stationary phases. A polarimetric detector and/or an ultraviolet (UV) spectrophotometer were used to monitor the separation process. HPLC arrangement 3 allowed the evaluation of enantioselective binding for D- and L-tryptophan to different albumins and albumin fragments. At present, column switching can be considered the technique of the broadest applicability for investigating the reversible binding interactions between a protein and drug enantiomers.


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KEY WORDS: protein-drug binding; Hummel and Dreyer method; column switching; high performance liquid chromatography; chiral separation; BSA/HSA-bond silica gel; polarimetric/chiroptical detection; enantioselectivity

In the homogenous liquid phase, the stoichiometry of weakly interacting components, e.g., a plasma protein (P) and a drug (D) yielding a protein-drug (PD) complex, has been most frequently investigated by using the method of equilibrium dialysis. Several high performance liquid chromatographic (HPLC) separation methods have been introduced,1,2 however, for the qualitative and/or quantitative analysis of the reversibly interacting drug-protein systems. The method of Hummel and Dreyer3 uses a single component (usually [D] = constant) added to the mobile phase for the HPLC determination of the instantaneous molar concentration (molar activity) of each component of a ternary mixture ([P], [D], [PD]) without disturbing the equilibrium. The fundamental requirement of the Hummel and Dreyer method for the applicability of a particular HPLC sorbent is its inertness against the injected protein sample. The rationale of the method is that the rate of stabilization of the composition of the PD complex, passing through the chromatographic column, is much higher than the speed of the separation process itself.4 Since, in practice, this is an intrinsic feature, the Hummel and Dreyer method can be considered an equilibrium chromatographic technique that can be used in investigating PD reversible binding interactions.5

It is well known that over 60% of the currently used drugs are chiroptically active and a majority of the synthetic chiral compounds are administered as racemates.6 Consequently, it is important to determine the reversible binding interactions between particular plasma proteins and individual drug enantiomers.7–9 This study describes some of the consequences which should be taken into account when the method of Hummel and Dreyer is used with two enantiomeric species run in the HPLC mobile phase.

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THEORY

The reversible binding interactions between the given protein and the two drug enantiomeric species (D and L) are governed by the following thermodynamic relationships:

\[ P + D \leftrightarrow PD \]
\[ P + L \leftrightarrow PL \]

When using the Hummel and Dreyer method, after the injection of a protein, a trough appears (as recorded by a chiroptically insensitive detector). The deeper the trough, the greater the total affinity between the interacting components, which can be expressed as

\[ \sum_{i=1}^{N} n_{iD}k_{DiD} + n_{iL}k_{LiL} \]

where \( n_i \) is the number of the binding sites for the D and L enantiomers available on the protein investigated and \( k_i \) represents the particular PD enantiomer association constant for the \( i \)th class of binding sites.

On the other hand, the greater the value of the term

\[ \sum_{i=1}^{N} |n_{iD}k_{DiD} - n_{iL}k_{LiL}| \]

the more pronounced the peak/trough at the drug retention time recorded by a chiroptically sensitive detector. Simultaneously, the recorded polarity orientation reveals which of the two enantiomers is being preferentially complexed by the assayed protein. Thus by exploiting the Hummel and Dreyer method, the enantioselectivity of a particular protein for the two drug enantiomeric species could be efficiently assayed.

The purpose of the present study was to validate the above presented thesis. The system comprising serum albumin and the two tryptophan enantiomers was used as a working model. The selection of this system is supported by at least two facts: tryptophan (L-enantiomer) binding to human plasma proteins (mostly to albumin) is rather extensive (>80%) and highly enantioselective. This is evident from the reported association constants \( k_D = 1.1 \times 10^4 \), \( k_O = 1.3 \times 10^5 \), \( k_L = 2.7 \pm 0.3 \times 10^4 \), \( k_P = 4 \pm 1 \times 10^3 \) (pH 7.4, 25°C), determined by affinity chromatography with immobilized human serum albumin (HSA).

MATERIALS AND METHODS

Reagents

The following products of the Sigma Chemical Co. (St. Louis, MO) were used in this study: DL-, D-, and L-tryptophan; HSA (A 8763 essentially globulin free, purity grade >99%; molecular weight 66.21 kDa); bovine serum albumin (BSA; A 0281 essentially fatty acid free, purity grade >99%, as well as A 1900 containing >98% of the monomeric BSA of molecular weight 66,267 Da); horse serum albumin (ESA; A 9888); chicken serum albumin (GSA; A 3014); pig serum albumin (PSA; A 2764); sheep serum albumin (SSA; A 3264); guinea pig serum albumin (GPSA; A 2639); dog serum albumin (DSA; A 9263); and rabbit serum albumin (OSA; A 0639)—the abbreviations used were taken from a report by Allenmark.

The albumin polypeptidic fragments A299-585, B1-123, C124-298, D1-298, and Bp1-307 were kindly donated by Drs. Doyen and Lapresle. The A299-585 (molecular weight ≈37 kDa) and D1-298 fragments represent the C- and N-terminal halves of the albumin molecule, whereas fragments B1-123 (molecular weight ≈18 kDa) and C124-298 (molecular weight ≈18 kDa) are the N- and C-terminal halves of the fragment D1-298. Fragment Bp1-307 is the N-terminal half of the albumin molecule.

KH2PO4 and Na2HPO4·12H2O, both of p.a. purity grade were supplied by Merck (Darmstadt, Germany). Water was of MILLI-Q grade quality (Water Purification System, Millipore Corporation, Bedford, MA). The phosphate buffer solution (0.067 mol/l, pH 7.4) was cleansed immediately before use by its filtration through an activated (5 ml) of CH3OH, 5 ml of H2O C18 silica gel packed cartridge (Bond Elut C18 Cartridge, 500 mg; Varian, Sunnyvale, CA), 1 l of the buffer per one cartridge.

Methods

All chromatographic separations were performed at ambient temperature using three different HPLC arrangements:

1. The first system consisted of a mobile phase delivery pump (Model 422, Kontron Instruments S.A., Yvelines, France), a sample injection valve equipped with a 10 µl loop (Model 7125, Rheodyne, Cotati, CA), and a variable-wavelength photometric detector (Model Spectra 100, Spectra-Physics, Inc., Autolab Division, San Jose, CA) (Fig. 1A). On working with this arrangement the outlet port of the spectrophotometer (UV 1) was connected to the inlet port of an HPLC polarimeter detector (CHIRALYSER®, IBZ Messtechnik GmbH, Hannover, Germany). A stainless-steel “precolumn” (4.6 mm × 5 cm) packed with LiChroSph Si 300 (mean particle size 10 µm; Merck) was inserted between the HPLC pump and the sample injection valve to saturate the mobile phase with SiO2. The separations were done in an analytical stainless-steel column (4.6 mm × 15 cm) “in-home” packed with LiChrosorb Diol (mean particle size 10 µm; Merck).

2. The second system was built from the same modules as the above described HPLC equipment 1, operated only with the UV spectrophotometric detector. In this part of the study, the separations were performed by a tandem of...
a guard column (4.0 mm × 3 cm), packed with Resolvosil® (Macherey Nagel, Düren, Germany) and a Resolvosil® BSA-7PX chiral column (4.0 mm × 15 cm; Macherey Nagel).

3. The third HPLC system is depicted in Figure 1A,B. In this case, a 20-µl aliquot of the eluate, manifesting a drug deficit, was directed by a switching valve (Model 7000, Rheodyne) onto a stainless-steel column (4.6 mm × 15 cm) packed with HSA-bond silica gel (mean particle size 10 µm, porosity 200 Å; Kromasil, Eka Nobel AB, Bohus, Sweden); the content of the in situ bound protein = 95.8 mg HSA/1 g of the silica gel18. The mobile phase, a bicomponent mixture of the phosphate buffer and 1-propanol = 97.0:3.0 (v/v), was delivered by a second high pressure pump (Model 422). A stainless-steel “precolumn” (4.6 mm × 5 cm) packed with LiChrospher Si 300 (mean particle size 10 µm; Merck) was inserted between the pump and the switching valve to saturate the slightly alkaline (pH 7.4) highly aqueous mobile phase with SiO₂. The eluate leaving the HSA-bond chiral column was monitored by using a second HPLC spectrophotometer (Model Spectra 100).

Handling of DL-Tryptophan Containing Mobile Phases and of Protein Samples

We observed that the exact application of the Hummel and Dreyer method operating with a phosphate-buffered DL-tryptophan solution as the mobile phase can be strongly influenced by the presence of ubiquitous microorganisms. Cell colonies present in the reservoir bottle contaminated the eluent despite ultrafiltration of the mobile phase through a cellulose acetate membrane with 0.45-µm pores (Type DE 67, Schleicher & Schuell, Dasel, Germany) performed immediately prior to the HPLC work. To suppress the growth of the microorganisms, and their preferential catabolism of the biogenic L-tryptophan amino acid, the eluent bottle was kept in a water-ice bath (+5°C) and the mobile phase reservoir as well as the teflon connecting tubes were wrapped in aluminum foil. Using these precautions a constant 50:50 ratio of the two tryptophan enantiomers was obtained in the mobile phase for at least one working day period. Similar storage conditions seem mandatory on working with any (phosphate-buffered) D-, DL-, or L-tryptophan containing stock solutions.

In light of the above described phenomena, it is evident that on working with a phosphate-buffered DL-tryptophan solution containing a protein, the problem of sample contamination by microorganisms is significant. That is why fresh aqueous albumin/albumin fragment solutions were preferably injected into the HPLC system whenever feasible. Due to their poor solubility, the aqueous solutions of the albumin fragments Bα1,123, D1,298, and Bp1,307 were cleansed prior to loading through a syringe filter with 0.20-µm pores (3 mm i.d.; Sun International Trading, Ltd., Wilmington, NC). This applied particularly to fragment D1-298, which even at a concentration of 2.0 mg in 1 ml of H₂O yielded a turbid opalescent sample.

RESULTS AND DISCUSSION
Chromatographic Analysis

HPLC arrangement 1. A highly concentrated phosphate-buffered DL-tryptophan solution (1.0 × 10⁻³ mol/l) run at a flow rate of 2.0 ml/min was applied for ~1 hr to saturate the HPLC column packing of LiChrosorb Diol. During the “measurement” stage of the study, the HPLC system operated with a phosphate-buffered DL-tryptophan solution of 1.0 × 10⁻⁴ mol/l at a flow rate of 0.5 ml/min. Under these experimental conditions, the spectrophotometric detector manifested a very stable baseline at the 280-nm setting (Fig. 2, curves A, B, E, H). Its stability was much better than the output signal of the polarimetric detector CHIRALYSER®, which was effectively operative only at the standard mode, since at a higher sensitivity setting the noise and the drift of the recorded baseline became markedly pronounced. The polarity orientation of the record, in the plus or minus directions, was revealed by analyzing the aqueous and phosphate-buffered solutions of either the D- or L-tryptophan enantiomer (Fig. 2, curves C, D). The CHIRALYSER® detection limit found was about 0.5 µg of the on-column loaded D- or L-tryptophan. Nevertheless, despite the rather long polarized light-cell path
length (200 mm), the CHIRALYSER® sensitivity to the analyte (microgram amounts of a single tryptophan enantiomer) is much poorer than that of the conventional HPLC spectrophotometric detector (cf. curves B and D in Fig. 2).

Chromatographic curves E and H in Figure 2 represent typical peak and trough pairs registered by the UV detector (280 nm) when an aqueous albumin solution is being analyzed. The relatively deep trough observed resulted from the graphic superposition of the negative absorbance signal of both the loaded water (10 µl) and the deficiency in D- as well as L-tryptophan in the sample run. In spite of a great albumin on-column load (10 µg), the resolution observed between the high- and low-molecular-weight sample components was adequate (Fig. 2, curves E, H). On the other hand, at the retention time of the drug, no positively oriented peak corresponding to a D-tryptophan excess was observed using the polarimetric detector (Fig. 2, curves F, J). Even at the highest assayed albumin load of 40 µg (i.e., [protein]/[drug] = 0.6:1.0), the CHIRALYSER® sensitivity was not sufficient to provide evidence for a preferential complexation of L-tryptophan by the protein.

Contrary to the single positive peak detected by the HPLC spectrophotometer at 280 nm, even in the far UV wavelength range (Fig. 2, curve G), the CHIRALYSER® detector indicated the presence of another sample component which rotated plane polarized light in the minus direction. As also indicated by curve I in Figure 2, this additional negative peak was not an experimental artifact (e.g., due to a greater amount of the albumin injected) since the two negative peaks were clearly evident when only 1 µg of the monomeric BSA was loaded on-column. At the present stage of this study, however, any interpretation of the origin of the additional chiral component(s) found in the assayed albumin samples could be only speculative. Nevertheless, these observations advocate the use of an on-line polarimetric or circular dichroic (CD) detector along with the common HPLC spectrophotometric/spectrofluorometers. As the optical rotation of the polarized light is a unique property of the chiral compounds, an appropriately sensitive CD spectropolarimeter could provide additional information during HPLC analysis.19

**HPLC arrangement 2.** As a starting step, the highly concentrated phosphate-buffered DL-tryptophan solution of $1.0 \times 10^{-3}$ mol/l was again applied for fast saturation of the chiral stationary phase (CSP) of the Resolvosil® BSA-7PX column. The chromatographic system, run at a flow rate of 0.5 ml/min, indicated attainment of the equilibrium state within less than 1 h. The aliquots of the mobile phase, slightly enriched with the individual drug enantiomers and the “blank” phosphate buffer, served as samples for determining the D- and L-tryptophan retention times. Under the experimental conditions described above, the D- and L-tryptophan rich samples were eluted at 3.9 and 4.0 min, i.e., there was only a trace resolution between the two enantiomers (Fig. 3). However, on utilizing the less concentrated phosphate-buffered DL-tryptophan solutions as eluents, the enantioselectivity factor ($\alpha$) exceeded the value of 1.0. As evident from Figure 3, the rise of the $\alpha$ values is predominantly the consequence of the remarkable preferential attraction of the given CSP (silica gel bound BSA crosslinked by glutaraldehyde) for the L-tryptophan enantiomer. While the capacity factor ($k'$) of the D-isomer changes only minutely, the $k'$ value of the L-tryptophan species increases dramatically on weakening the eluting/“displacing” property of the mobile phase, carrying the chemically identical “competitive” drug molecules (Fig. 3).

When the DL-tryptophan concentration in the eluent was $7.0 \times 10^{-5}$ mol/l, two negative peaks for the D- and L-tryptophan enantiomers (with the $\alpha$ value of 3.5) were obtained after injecting the blank phosphate buffer sample (Fig. 4, curve a). For such a blank sample the ratio between the areas under curves (AUCs) of the two enantiomers should be 1.0. On the other hand, injection of albumin dissolved in the mobile phase yielded a ratio of AUC$_D$/AUC$_L > 1.0$ (AUC$_D$/AUC$_L < 1.0$) and thus corroborated the expectation of preferential L-tryptophan complexation by the assayed protein. Yet, as the baseline of the two negatively oriented enantiomer peaks cannot be exactly recognized (Fig. 4, curves b or c), the variance in the calculated AUC$_D$/AUC$_L$ (AUC$_D$/AUC$_L$) parameters reached unacceptably high values. Moreover, since the high- and low-molecular-weight sample components were evidently inseparable, the main requirement for the applicability of the Hummel and Dreyer assay method concerning the column packing non-adsorptive properties to the protein injected failed to be fulfilled.

Although some advantages of this HPLC arrangement were demonstrated on analyzing the D-/L-tryptophan complexation by cyclodextrin oligosaccharides, design 2 has limited application potential. In fact, there were only a few chiral drugs/analytes separable up to baseline by commercially marketed CSPs when aqueous (buffered) solutions of the ligand racemate were assayed as the mobile phase.

**HPLC arrangement 3.** To use HPLC arrangement 3, i.e., the column switching approach, the conditions used for separating the enantiomers (Fig. 1B) should first be optimized. The experiments running the pure phosphate buffer as the eluent showed that while the D-tryptophan enantiomer was eluted from the chiral HSA-bond column...
at a relatively low $k'$ value (Fig. 5), the L-isomer was too strongly retained. For effective liberation/displacement of the L-tryptophan enantiomer from the (immobilized) HSA binding site(s), addition of even a small percentage of 1-propanol into the mobile phase was found to be very effective.\(^{12}\)

It is obvious from the data in Figure 5 that a decrease in the DL-tryptophan sample load resulted in an increase in the enantioselectivity factor $\alpha$, due to the increased $k'$ value of the later eluted L-tryptophan. The higher $\alpha$ value obtained allows one to use a higher content of organic modifier in the mobile phase. This results in sharper and more symmetrical D- and L-tryptophan peaks. Thus at a total DL-tryptophan sample load of 0.4 µg and a 3% 1-propanol content in the mobile phase was found to be very effective.\(^{12}\)

![Fig. 4. Chromatograms recorded using HPLC arrangement 2 when (curve a) blank phosphate buffer, (curve b) HSA—10 µg, and (curve c) BSA—10 µg samples were injected into the eluent stream of phosphate-buffered DL-tryptophan solution (1.0 × 10^{-4} mol/l). The albumins were dissolved in the mobile phase used. The UV records were traced at 280 nm detector setting. The HPLC column is Resolvosil\(^{18}\) BSA-7PX.](image)

HPLC METHODS FOR PROTEIN-DRUG BINDING STUDIES 377

The injected protein samples were aqueous albumins from different animal species (≥2.0 mg/ml, i.e., ≥ca. 30 µmol/l) and aqueous solutions of the HSA fragments (2.0 mg/ml). At the recorded minimum of the negative peak, indicated at 280 nm by the UV 1 detector (Fig. 2, curves E, H), a 20-µl aliquot of the eluate was switched into the chiral HSA-bond column. The D- and L-tryptophan content in the eluate was determined at 280 nm by means of the second HPLC spectrophotometer. The $AUC_D/AUC_L$ ratios were evaluated as the parameter scored (Fig. 6). The coefficient of variance values of the $AUC_D/AUC_L$ parameters determined from the quadruplicate analysis of the HSA, BSA, and monomeric BSA samples expressed the within-day reproducibility, which was 3.7%, 4.6%, and 2.9%, respectively.

Reversible Binding Interactions Between the Tryptophan Enantiomers and Albumins/HSA Fragments

As shown in Figure 6, all of the albumin samples analyzed (by exploiting the proposed column switching HPLC design 3) demonstrate a preference for L-tryptophan. The values of the scored $AUC_D/AUC_L$ parameter, however, differ for each of the individual animal species studied and for particular protein lots. While the monomeric BSA sample demonstrates the highest enantioselectivity for the L-tryptophan enantiomer, some other albumins, with lower $AUC_D/AUC_L$ values, behave comparably. This finding may be accounted for by the high percentages of amino acid sequence identities noted, e.g., between the samples of HSA and BSA (76%), HSA and ESA (76%), BSA and ESA (73%).\(^{20}\)

Also seen in Figure 6, all of the assayed HSA fragments...
The observation of the preferential attraction of the 1-298 HSA fragment for the D-tryptophan enantiomer as well as the preference of the 299-585 HSA fragment for the L-enantiomer appears to support the Yang and Hage 12, 21 statement that separate regions exist for the binding of the L- and D-tryptophan enantiomers in the immobilized HSA molecule. Upon analyzing in detail the data in Figure 6, the commentary reported by Wang et al. 17 seems to be relevant and plausible. They investigated the ligand-HSA fragment(s) reversible binding interactions in the homogenous liquid phase by spectrofluorometric technique, concluding: “It could be inferred that the drug-binding mechanism in the native HSA molecule is probably a result of a combination of several types of forces, such as electrostatic, hydrogen bonding, and hydrophobic interactions. Most likely, the combination of these forces is destroyed during fragmentation. Alternatively, the binding center is totally destroyed during the fragmentation process, and the fragments probably rearrange to form a new binding site that does not exist in the native HSA molecule.”

CONCLUSIONS

Three different HPLC instrumental arrangements were evaluated by studying the enantioselectivity of the albumin and albumin fragments that associate with tryptophan enantiomers. Although column switching technique 3 displayed the broadest current applicability, future progress in the detection of chiral compounds by sensitive CD spectropolarimeters could change the preference to that of the more universal design 1.

On utilizing HPLC arrangement 3, differences in the stereoselectivity of albumins from different animal species for D- and L-tryptophan enantiomers can be conveniently determined. The sensitivity of HPLC method 3 allows one to assess binding to HSA fragments and potentially other rare proteins available only in trace quantities.

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