ELSEVIER

Journal of Chromatography B, 724 (1999) 91–100

JOURNAL OF CHROMATOGRAPHY B

Characterisation of the binding of digitoxin and acetyldigitoxin to human serum albumin by high-performance affinity chromatography

David S. Hage*, Arundhati Sengupta

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

Received 7 July 1998; received in revised form 3 November 1998; accepted 15 December 1998

Abstract

Zonal elution and high-performance affinity chromatography were used to examine interactions of the drugs digitoxin and acetyldigitoxin with the protein human serum albumin (HSA). This was done by injecting small amounts of digitoxin and acetyldigitoxin onto an immobilized HSA column in the presence of mobile phases that contained various concentrations of digitoxin, acetyldigitoxin or other solutes as competing agents. A fixed concentration of β-cyclodextrin was also present in the mobile phase as a solubilising agent. It was found that digitoxin and acetyldigitoxin each had strong interactions at a single common binding site on HSA, but with slightly different equilibrium constants for this region. Neither compound showed any competition with warfarin or L-tryptophan, which were used as probes for binding at the warfarin-azapropazone and indole-benzodiazepine sites of HSA. These results confirmed the presence of a separate binding region on HSA for digitoxin-related compounds.

Keywords: Digitoxin; Acetyldigitoxin; Human serum albumin

1. Introduction

Digitoxin is a common cardiovascular drug used for the treatment of individuals with congestive heart failure or atrial fibrillation [1]. As shown in Fig. 1, the structure of digitoxin consists of a steroid backbone with a lactone ring at the C-17 position and a linear chain of three digitoxose sugar residues attached at the C-3 carbon [2]. A closely related molecule is acetyldigitoxin (or digitoxin monoacetate), in which an acetyl group replaces one of the hydroxyl groups on the digitoxose residues. Acetyldigitoxin is also used in pharmaceutical preparations as a cardiovascular drug [3].

Like many other drugs, both digitoxin and acetyldigitoxin are known to interact with carrier proteins in blood [4–6]. Such binding can play an important role in controlling the distribution, metabolism and excretion of drugs within the body [7]. One important carrier protein is human serum albumin (HSA). HSA is the most abundant protein in human blood plasma and is known to bind to a wide range of exogenous and endogenous compounds, including bilirubin, fatty acids, warfarin, tryptophan and benzodiazepines, among others [8].

Some previous studies have reported that both digitoxin and acetyldigitoxin bind to HSA [4,6,9]. This was noted in solution-phase radiolabel studies, in which acetyldigitoxin was found to displace digitoxin from HSA. In the same work, it was found that digitoxin was not displaced from HSA by other

*Corresponding author. Tel.: +1-402-472-2744; fax: +1-402-472-9402.

0378-4347/99/$ – see front matter
PII: S0378-4347(98)00589-1

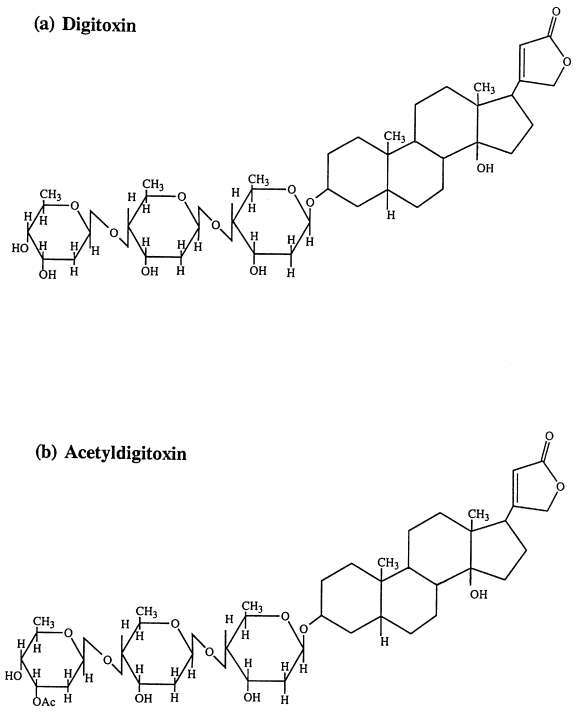

Fig. 1. The structures of (a) digitoxin and (b) acetyldigitoxin. The structure for acetyldigitoxin that is given, is for the α-form of this compound; the β-form has essentially the same structure but with the acetyl group (Ac) being located at the 4″ position instead of the 3″ position [33].

compounds that are known to interact with the major binding regions of HSA (i.e., the warfarin-azapropazone and indole-benzodiazepine binding sites) [6]. It was concluded from these results that a separate binding region exists on HSA, known as the digitoxin site, which is responsible for the interactions of digitoxin and related compounds with this protein [6]. However, the existence of this binding region still remains an area of controversy and little quantitative data is available on the overall strength of binding between digitoxin and acetyldigitoxin with HSA [4,6,9].

The general goal of this study is to perform a detailed examination of the binding between digitoxin and acetyldigitoxin with HSA by using high-performance affinity chromatography (HPAC) and zonal elution competitive binding experiments. In this approach, HSA will be immobilized onto a HPLC-based silica support and packed into a column. The immobilized HSA will then be used as a stationary phase to examine the binding of drugs or other solutes that are applied to the chromatographic system. In previous work, it has been repeatedly shown that there is good correlation between the behaviour observed with these immobilized HSA supports and that seen for solution-phase HSA [10–19]. However, the chromatographic approach has the advantages of being faster, easier to use and more precise than traditional solution-phase methods for the measurement of drug-protein interactions [19].

The immobilized HSA columns in this work will be used to examine the retention of digitoxin and acetyldigitoxin in the presence of various competing agents. The competition of each drug with itself will be used to determine the total number of binding regions for these solutes, while the competition of acetyldigitoxin with digitoxin will be used to examine the number of their common binding sites. Other competing agents, such as warfarin and L-tryptophan, will be employed to determine whether or not digitoxin and acetyldigitoxin really do have sites on HSA that are separate from the major binding regions of this protein. From these studies, data will be obtained that should allow a more complete picture to be developed for the binding of HSA to digitoxin and related compounds.

2. Theory

The binding of digitoxin and acetyldigitoxin to HSA was studied by using the technique of zonal elution [19]. In this method, a known concentration of a competing agent (I) is continuously applied to a column that contains an immobilized ligand (L) (e.g., HSA) while injections of a small amount of analyte (A) are made. If I and A compete for a single site on L, and A binds to no other type of site on the support, then the following reactions can be used to describe the binding events that take place in the column.

$$A + L \overset{K_{AL}}{\rightleftharpoons} A - L \quad (1)$$

$$I + L \overset{K_{IL}}{\rightleftharpoons} I - L \quad (2)$$

In these equations K_{AL} and K_{IL} are the association

equilibrium constants for the formation of the complexes A−L and I−L, respectively.

It was necessary in this study to also use β-cyclodextrin as a mobile phase additive to help complex and dissolve the digitoxin and acetyldigitoxin in solution. If such a solubilising agent (S) has 1:1 interactions with A and/or I, then the following additional reactions will occur within the mobile phase during the zonal elution study.

$$A + S \overset{K_{AS}}{\rightleftharpoons} A - S \quad (3)$$

$$I + S \overset{K_{IS}}{\rightleftharpoons} I - S \quad (4)$$

In these reactions, K_{AS} and K_{IS} are the association equilibrium constants for the formation of the complexes A−S and I−S, respectively.

For the reaction scheme shown in Eqs. (1)–(4), the following relationship has been previously derived to describe the change in retention for A as the mobile phase concentration of the competing agent is varied [20].

$$1/k'_A = \frac{(1 + K_{AS}\, C_S)(1 + K_{IS}\, C_S)}{K_{AL}\, C_L\, (1 + K_{IL}\, C_S)} + \frac{(1 + K_{AS}\, C_S)(K_{IL}\, C_I)}{K_{AL}\, C_L\, (1 + K_{IL}\, C_S)} \quad (5)$$

In Eq. (5), k'_A is the capacity factor for the injected solute, or $k'_A = (t_r/t_m - 1)$, where t_r is the measured retention time of the analyte and t_m is the column void time. The term C_L is the total concentration of all active ligand sites in the column, C_S is the total mobile phase concentration of the solubilising agent, and C_I is the total concentration of competing agent in the mobile phase. It is assumed in Eq. (5) that the amount of A that is injected is much smaller than the amount of competing agent or immobilized ligand in the column (i.e., linear elution conditions are present). It is also assumed that the amount of solubilising agent is much larger than the amount of A or I that is complexed by this agent in the mobile phase.

For the 1:1 direct competition of A and I for L, Eq. (5) predicts that a plot of $1/k_A$ versus C_I should yield a linear relationship, with the ratio of the intercept to the slope for this graph giving a value equal to $(1+K_{IS}C_S)/K_{IL}$. The values for some of the equilibrium constants in this system can be determined by preparing a second plot of the intercept/slope ratio versus the concentration of solubilising agent (C_S) that was used in each study. If the test system follows the reaction model shown in Eqs. (1)–(4), then this second plot should give a linear relationship with an intercept equal to $1/K_{IL}$ and a slope equal to K_{IS}/K_{IL}. By taking the reciprocal of the intercept obtained for this plot, the association equilibrium constant K_{IL} for the binding of I to L can be determined at the site of competition between A and I on the immobilized ligand [20].

3. Experimental

3.1. Reagents

Digitoxin and acetyldigitoxin (2:1 ratio of α- and β-forms), β-cyclodextrin, HSA (Cohn fraction V, 99% globulin free, fatty acid free), racemic warfarin and L-tryptophan were obtained from Sigma (St. Louis, MO, USA). The HSA and diol-bonded silica supports were prepared using Nucleosil Si-300 silica (7μm particle diameter, 300 Å pore-size) from Macherey-Nagel (Duren, Germany). Other chemicals and biochemicals used were of the purest grades available. All aqueous solutions were prepared using water purified by a Nanopure water system (Barnstead, Dubuque, IA, USA).

3.2. Apparatus

The chromatographic system consisted of a CM3000 solvent delivery system from Thermoseparations (Riviera Beach, FL, USA), a Rheodyne 7010 injection valve (Cotati, CA, USA) equipped with a 20 μL sample loop, and a SM3100 UV–Vis variable wavelength detector from Thermoseparations. A Winner-on-Windows interface from Thermoseparations was used for data collection. Chromatograms were processed by programs written in Microsoft QuickBASIC (Redmond, WA, USA). All columns and mobile phases were maintained at 37±0.2°C by using an Isotemp 9100 water bath (Fisher Scientific, Pittsburgh, PA, USA). The columns were downward slurry-packed using an HPLC column slurry packer from Alltech (Deerfield, IL, USA).

3.3. Methods

Digitoxin and acetyldigitoxin were placed into pH 7.4, 0.067 *M* phosphate buffer by using β-cyclodextrin as a solubilising agent. The concentration of this solubilising agent was varied from 1.0–2.6 m*M* during the zonal elution studies. Both digitoxin and acetyldigitoxin were found to be stable in this solution for over two weeks at room temperature.

Diol-bonded silica was prepared as described previously [21]. The diol coverage of the final Nucleosil support was 230±3μmol (±1 SD) per gram of silica, as determined in duplicate by an iodometric capillary electrophoresis assay [22]. The Schiff base immobilisation method was used to couple HSA to this diol support [23,24]. A bicinchoninic acid (BCA) protein assay was performed in triplicate on the HSA support using HSA as the standard and diol-bonded silica as the blank [25], according to previous methods [12]. The final protein content of the HSA support was found to be 426±2 nmol HSA per gram of silica.

A 3 cm×2 mm I.D. stainless steel column was downward slurry-packed at 3500 p.s.i. with the immobilized HSA silica and enclosed in a water jacket for temperature control. A pH 7.4, 0.067 *M* potassium phosphate buffer containing 1.0–2.6 m*M* β-cyclodextrin was used to prepare all mobile phases. To each of these solutions was added 0–10 μ*M* of digitoxin, acetyldigitoxin or warfarin, or 0–50 μ*M* of L-tryptophan as a competing agent. Linear behaviour in plots made according to Eq. (5) was observed when using mobile phase concentrations of 0–0.5 μ*M* digitoxin or acetyldigitoxin; as discussed later, these were the final conditions selected for use in all equilibrium constant measurements. These concentrations were approximately ten times higher than the therapeutic range of digitoxin (i.e., 26–46 n*M*) [26], and thus should have provided sufficient amounts of these drugs for the detection of any significant interactions between these compounds and HSA under conditions of normal use. After preparation, each mobile phase was filtered through a 0.22 μm nylon filter and degassed under vacuum for 15 min. The samples of acetyldigitoxin or digitoxin to be used as the injected analyte were then prepared in the same mobile phases as employed in each individual zonal elution study.

The zonal elution experiments were performed at flow-rates between 0.2 and 0.5 ml/min, with the desired analyte being applied in replicate injections. The column back-pressures under these conditions ranged from 200–500 p.s.i. The elution of the injected acetyldigitoxin and digitoxin was monitored at 221 nm. The retention time of each probe was calculated by using the first statistical moment of its corresponding peak [27]. The void time of the column was determined by making similar injections with sodium nitrate, a non-retained compound. Tests for non-specific binding between digitoxin and acetyldigitoxin and the support were performed by injecting these compounds onto a diol-bonded silica column; in each case, only negligible interactions were noted. The capacity factors for the injected probe compounds were determined over sample concentrations of 0.1–100 μ*M*, with 0.1 μ*M* being the typical level employed. There were no noticeable shifts in the capacity factors over this range of sample concentrations, indicating that linear elution conditions were present, as assumed in Eq. (5).

4. Results and discussion

4.1. General design of zonal elution studies.

The binding studies performed in this work were based on the change in retention observed for a small amount of an injected analyte that was passed through an immobilized protein column in the presence or absence of a fixed concentration of a competing agent. If direct competition occurs between the analyte and competing agent, then a decrease in retention should be obtained for the analyte as the competing agent's concentration is increased. If indirect (or allosteric) competition is present, then either an increase or decrease in analyte retention may be seen at higher concentrations of the competing agent, depending on whether the indirect competition is positive or negative in nature. Examples of typical chromatograms and shifts in retention that are obtained in such studies are provided in [10–19]. By analysing the data of these experiments according to equations derived for specific reaction models (e.g., see Eqs. (1)–(5) for direct 1:1 competition), it is possible to not only identify the particular

type of interaction that is occurring but also to determine the equilibrium constants that are involved in these binding processes.

Several practical factors had to be considered when using these types of zonal elution experiments to examine the interactions of digitoxin and acetyldigitoxin with immobilized HSA. The first problem encountered was the relatively low water solubility of these drugs, which made it difficult to reliably put enough of these compounds in the mobile phase for detection by a standard HPLC absorbance detector or for use as a competing agent in the zonal elution studies. One possible way of enhancing the solubility of these agents would be to add an organic solvent as a modifier to the mobile phase. However, this would also be likely to cause a change in the binding properties and/or structure of the immobilized HSA [28,29].

An alternative approach has recently been developed in which β-cyclodextrin, or a related compound like α- or γ-cyclodextrin, is used as a mobile phase additive to help solubilise non-polar compounds during HPLC-based binding studies [20]. In this present study it was found that a solution of 1–2.6 m*M* β-cyclodextrin could easily dissolve acetyldigitoxin and digitoxin in pH 7.4, 0.067 phosphate buffer at concentrations that were compatible with zonal elution experiments (i.e., 0.1–100 μ*M*). Absorbance and light-scattering measurements indicated that were no apparent changes in the solubility of these acetyldigitoxin or digitoxin solutions when stored for several weeks at 4–37°C, thus indicating that they were sufficiently stable for use as mobile phases in HPLC studies.

β-Cyclodextrin was selected as a solubilising agent for acetyldigitoxin and digitoxin since it is known to bind to many small, non-polar compounds and yet it has no measurable binding to HSA [20,30]. This means that the presence of β-cyclodextrin in the mobile phase should not alter the nature or strength of the binding between immobilized HSA and solutes like acetyldigitoxin and digitoxin. Instead, β-cyclodextrin and HSA would be expected to act as independent ligands for these drugs, with the β-cyclodextrin being used to merely help keep acetyldigitoxin and digitoxin in the mobile phase through the formation of soluble host: guest complexes. This type of behaviour has been confirmed in earlier HPLC experiments that have examined the interactions of *cis*- and *trans*-clomiphene with immobilized HSA in the presence of soluble β-cyclodextrin [20]. A second advantage of using β-cyclodextrin as a complexing agent is that it has no absorbance under the UV/Vis detection conditions that are commonly employed to monitor injected solutes in HPLC studies of drug-protein interactions.

Although the presence of β-cyclodextrin did help produce sufficient mobile phase concentrations of acetyldigitoxin and digitoxin for HPLC studies, the presence of this secondary ligand did add some complexity to these measurements. This was true even though the β-cyclodextrin did not directly affect the interactions of these compounds with HSA. For example, it was now necessary to consider how the elution of acetyldigitoxin or digitoxin changed as a function of the concentrations of both the solubilising agent (β-cyclodextrin) and any competing agent that was present in the mobile phase (e.g., acetyldigitoxin, digitoxin, L-tryptophan or R/S-warfarin). The equations and experiments that were used for this purpose are described in detail in [20] as well as in the Theory and Sections 4.2–4.4 of this current report.

A second complication created by the presence of β-cyclodextrin was the relatively small retention and small changes in retention that now occurred during the zonal elution studies. This was caused by the increased time that the acetyldigitoxin and digitoxin spent in the mobile phase because of their complexation to β-cyclodextrin, which in turn led to a decrease in their observed retention times and capacity factors. Most of the capacity factors measured in this work were in the range of 0.10–0.25, with some experimental conditions giving k' values that were even as low as 0.03. Non-specific binding did not create a problem at these small retention values because neither acetyldigitoxin or digitoxin were found to have any significant interactions with the diol-bonded support that was used for the immobilisation of HSA. But these small levels of retention did require that several precautions be taken in order to obtain reliable estimates of shifts in k' values as the experimental conditions were changed. For instance, a low injection flow rate (down to 0.2 ml/min) and a fast data acquisition rate (usually 60 points per second) were used to obtain

good time resolution in the chromatographic data that were generated. This, combined with the use of replicate moments analysis for determining the true center of each peak, provided an estimated precision of ±0.001–0.002 min (or about ±0.05–0.10 s) for the retention time measurements performed on any given peak. Replicate injections under each set of experimental conditions gave a typical observed run-to-run variation of ±0.002–0.004 min (± 1 SD). When the retention times from these replicate runs were averaged, the resulting precision for the mean retention time became a value of $\pm 0.002/\sqrt{N}$ to $0.004/\sqrt{N}$ (or ±0.001–0.002 min for N=3). This level of precision was found to be sufficient for examining most of the shifts in k' that occurred throughout this work. In those cases that needed even higher precision (e.g., the experiments performed at $k'=0.03$), a larger of number of replicate injections were used to further decrease the standard deviations of the mean retention times until the precision of the average capacity factors was at an acceptable level.

4.2. Competitive binding studies using digitoxin as a mobile phase additive.

The first set of zonal elution studies in this study examined the competitive binding of injected digitoxin and acetyldigitoxin in the presence of digitoxin as a mobile phase additive. Plots of $1/k'_A$ versus C_I were initially generated in these experiments over a wide range of competing agent concentrations in the mobile phase. When using 1.0–2.6 m*M* β-cyclodextrin as a solubilising agent, a linear region in these plots was generally observed whenever the concentration of digitoxin or acetyldigitoxin in the mobile phase was below 0.5 μ*M* (see Fig. 2). Similar results have been observed in zonal elution studies of clomiphene binding to HSA in the presence of β-cyclodextrin [20]. Since Eq. (5) assumes that the solubilising agent is present in a large excess versus the competing agent, 0–0.5 μ*M* digitoxin or acetyldigitoxin was the mobile phase concentration range used for all later zonal elution studies in this work.

When using less than 0.5 μ*M* digitoxin as a competing agent and digitoxin as the injected probe, plots of $1/k'_A$ versus [digitoxin] gave linear relationships, as predicted by Eq. (5) for systems with 1:1 competition. The graphs that were obtained are shown in Figs. 2(a) and 3(a–c). The correlation coefficients for all these plots were 0.9999 over the four data points generated at 1.0–2.6 m*M* β-cyclodextrin. A second graph (Fig. 4) was made for the digitoxin/digitoxin data by taking the ratio of the intercepts and slopes of the best-fit lines and plotting these values versus the total, or analytical, concentration of β-cyclodextrin that was present in the mobile phase. This graph also gave linear behaviour, with a correlation coefficient of 0.9974 over the four concentrations of β-cyclodextrin that were used.

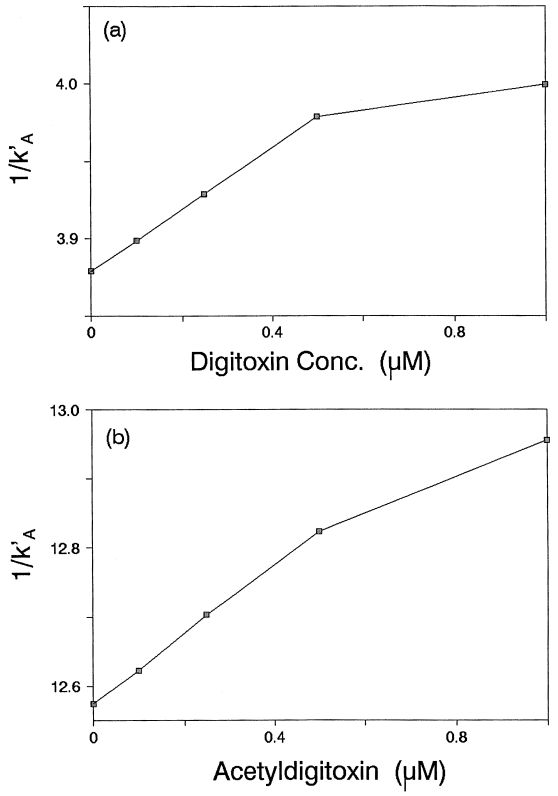

Fig. 2. Plots of $1/k'_A$ versus competing agent concentration for digitoxin injected into the presence of either (a) digitoxin or (b) acetyldigitoxin in the mobile phase. The mobile phase concentration of β-cyclodextrin was 1.0 m*M* in both (a) and (b).

From the reciprocal of the intercept for the digitoxin/digitoxin data in Fig. 4, it was possible to obtain the association equilibrium constant for the competition of digitoxin with itself at its binding regions on HSA. This gave a K_{IL} value for this

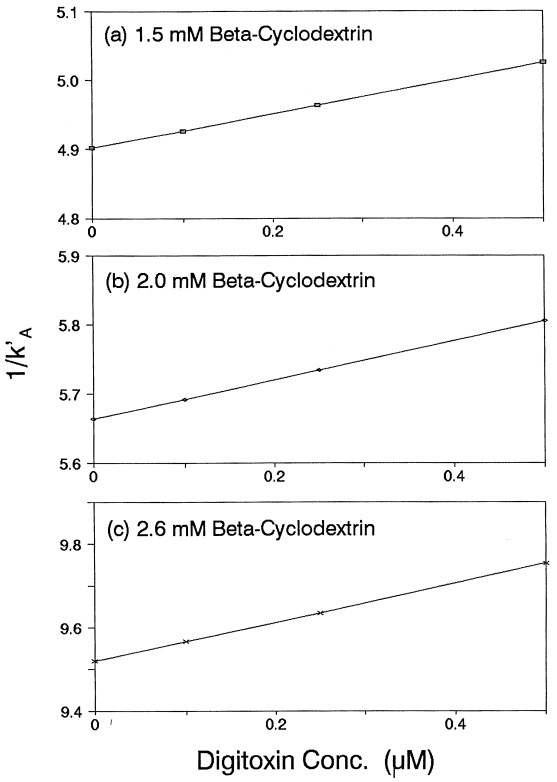

Fig. 3. Representative plots of $1/k'_A$ versus [digitoxin] in the mobile phase for the injection of digitoxin at several different mobile phase concentrations of β-cyclodextrin.

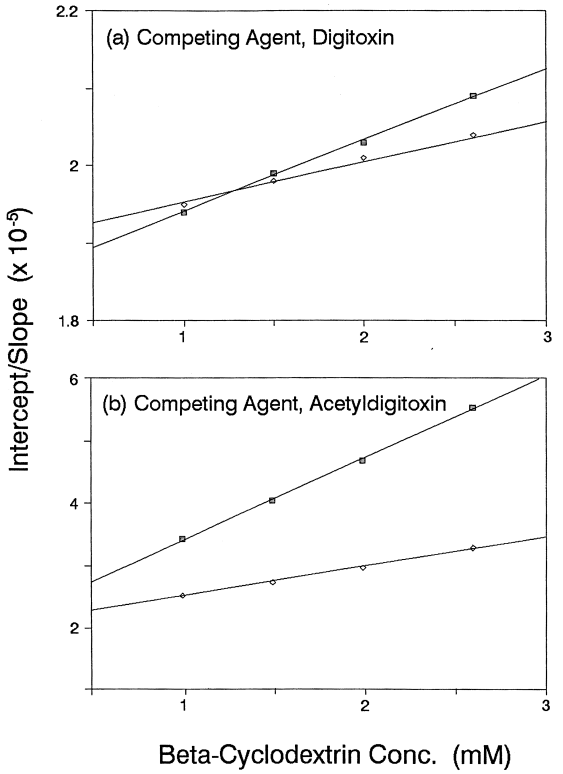

Fig. 4. Plots of intercept/slope ratios versus the total mobile phase concentration of β-cyclodextrin for the injection of (◇) digitoxin or (□) acetyldigitoxin into the presence of (a) digitoxin or (b) acetyldigitoxin as a competing agent.

interaction of 5.2 ($\pm$0.2)$\times 10^4$ M^{-1}. The linearity of the graphs in Figs. 2–4 for the competition of digitoxin with itself supports a model in which digitoxin is interacting at only one type of site on HSA. The size of this association constant is also in good agreement with previous estimates of 4–7$\times 10^4$ M^{-1} that have been made for the binding of digitoxin to HSA in solution [31].

Similar competitive binding studies were performed between acetyldigitoxin and digitoxin by injecting small amounts of acetyldigitoxin into the presence of digitoxin as a mobile phase additive. Plots of $1/k'_A$ versus [digitoxin] for these experiments again gave linear relationships at competing agent concentrations below 0.5 μ*M* (see Fig. 5), with correlation coefficients ranging from 0.9994–0.9999 over four data points obtained at each level of β-cyclodextrin. The ratio of the intercepts and slopes from these graphs were then plotted versus the total concentration of β-cyclodextrin in the mobile phase. The result was a linear relationship with a correlation coefficient of 0.9988 over four levels of β-cyclodextrin (Fig. 4). The reciprocal of the intercept from this plot gave an association equilibrium constant of 5.4 ($\pm$0.2)$\times 10^{-4}$ M^{-1} for the interaction of digitoxin at the site at which it competes with acetyldigitoxin for HSA binding. This value was statistically identical to that obtained in the previous experiment for the competition of digitoxin with itself, indicating that the same site on HSA was involved in both binding processes. Furthermore, the linearity of the acetyldigitoxin/digitoxin plots in Figs. 4–5 indicates that digitoxin and acetyldigitoxin had only one region on HSA at which they had competitive binding, as observed for the digitoxin/digitoxin system in Figs. 2–4.

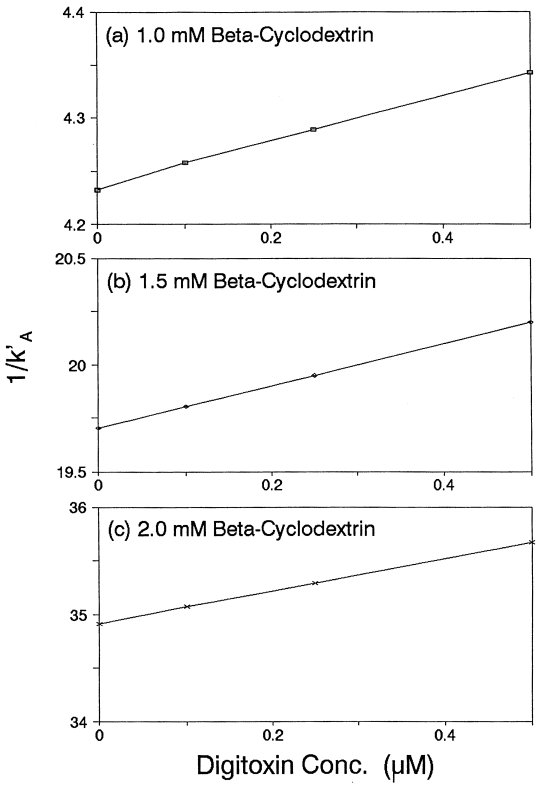

Fig. 5. Representative plots of $1/k'_A$ versus [digitoxin] in the mobile phase for the injection of acetyldigitoxin at several different mobile phase concentrations of β-cyclodextrin.

4.3. Competitive binding studies using acetyldigitoxin as a mobile phase additive.

The next group of zonal elution studies examined the binding of acetyldigitoxin in the presence of acetyldigitoxin as a mobile phase additive. As seen earlier in the digitoxin studies, linear behaviour was obtained for plots of $1/k'_A$ versus [acetyldigitoxin] at competing agent concentrations below 0.5 μ*M*. The linear region of these curves gave correlation coefficients of 0.9980–0.9999 over the four data points in this range. The ratio of the intercepts and slopes of these results were then plotted against the concentration of β-cyclodextrin. The result was a linear relationship (correlation coefficient, 0.9995), indicating the presence of single-site binding between acetyldigitoxin and HSA (see Fig. 4). From the reciprocal of the intercept in this graph, an association equilibrium constant of 4.8 $(\pm 0.2)\times 10^{-4}$ M^{-1} was obtained for the competition of acetyldigitoxin with itself at its binding regions on HSA.

Another zonal elution study examined the competition of injected digitoxin in the presence of acetyldigitoxin as a mobile phase additive. The plots of $1/k'_A$ versus various acetyldigitoxin concentrations below 0.5 μ*M* gave linear relationships with correlation coefficients of 0.9995–0.9999 over the four data points obtained at each concentration of β-cyclodextrin. The ratio of the intercepts and slopes were plotted against the concentration of β-cyclodextrin, which gave a linear relationship with a correlation coefficient of 0.9991 (see Fig. 4). From the reciprocal of the intercept in this plot, an association equilibrium constant of 4.9 $(\pm 0.2)\times 10^{-4}$ M^{-1} was found for acetyldigitoxin at the site where both it and digitoxin were binding to HSA. This association equilibrium constant was statistically identical to that seen for acetyldigitoxin at its site of competition with itself, again indicating that these two agents each had just one common binding region on HSA.

4.4. Competitive binding studies using warfarin or L-tryptophan as mobile phase additives.

It has previously been proposed in the literature that digitoxin has a distinct site on HSA that is separate from the warfarin and indole binding regions [6]. It has also been reported that digitoxin does not compete with warfarin or diazepam (a marker for the indole site of HSA) in the presence of rat α-fetoprotein, which is closely-related to HSA in its structure [32]. The binding (or lack of binding) of digitoxin and acetyldigitoxin at the warfarin and indole sites of HSA was examined in this study by performing competitive binding zonal elution studies between these drugs and warfarin or L-tryptophan, which are often used as probes for the warfarin and indole sites of HSA.

The first zonal elution studies in this section involved the injection of digitoxin or acetyldigitoxin into the presence of warfarin as a mobile phase additive. In these experiments, only random variations of ±2–3% were noted in k'_A for injections of acetyldigitoxin and digitoxin when 0–10 μ*M* warfarin was present in the mobile phase. Furthermore, both acetyldigitoxin and digitoxin gave graphs of $1/k'_A$ versus [warfarin] that had small correlation

coefficients (0.1865–0.1942 over seven data points) and best-fit slopes that were statistically equal to zero (i.e., slopes that overlapped with zero within a range of ±1 SD). These results indicated that no competition existed between these compounds and warfarin for binding sites on HSA, in agreement with previous observations [6]. This confirmed that digitoxin and acetyldigitoxin were not binding at the warfarin site of HSA.

Competitive binding studies between digitoxin or acetyldigitoxin and L-tryptophan produced similar negative results. For these experiments, random variations of ±0.3–0.4% were noted in k'_A for injections of acetyldigitoxin and digitoxin when 0–50 μ*M* L-tryptophan was used as a mobile phase additive. The correlation coefficients for plots of $1/k'_A$ versus [L-tryptophan] gave values of only 0.0081–0.0417 over seven data points, and the best-fit slopes were again statistically equal to zero within a range of ±1 SD. As in the previous solution-phase studies [6], this indicated that digitoxin and acetyldigitoxin were not competing with L-tryptophan and did not have any detectable interactions at the indole site of HSA.

5. Conclusion

In this report, HPAC and competitive binding zonal elution studies were used to characterize the binding of digitoxin and acetyldigitoxin to immobilized HSA, with β-cyclodextrin being used in the mobile phase as a solubilising agent. These experiments indicated that digitoxin and acetyldigitoxin each had a single common binding site on HSA, but with slightly different equilibrium constants for this region. The association constants that were measured (see Table 1) were in good agreement with earlier solution-phase estimates [31]. Neither digitoxin or acetyldigitoxin showed any competition with warfarin or L-tryptophan, which were used as probes for binding at the warfarin-azapropazone and indole-benzodiazepine sites of HSA. Thus, these data support a model in which HSA has a separate binding region for digitoxin-related compounds, as proposed previously [6]. Similar competitive binding studies could be used to examine the binding of other compounds to HSA, including digitoxin-related cardiovascular drugs or other therapeutic agents that may be co-administered with digitoxin or acetyldigitoxin.

Acknowledgements

This work was supported by the National Institutes of Health under grant R01 GM44931.

References

[1] Y. Fujil, Y. Ikeda, M. Fujil, M. Yamazaki, Biol. Pharm. Bull. 17 (1994) 467.
[2] R. Stenzel, B. Reckmann, Clin. Chem. 38 (1992) 2228.
[3] M. Windholz, S. Budavari (Eds.), Merck Index, 10th Ed, Merck, Rahway, NJ, 1983.
[4] O. Bros, D. Fremstad, C. Poulsson, Pharmacol. Toxicol. 72 (1993) 310.
[5] J. Doucet, J. Fresel, G. Hue, N. Moore, Eur. J. Clin. Pharmacol. 45 (1993) 577.
[6] I. Sjoholm, in: M. Reidenberg, S. Erill (Eds.), Drug-protein binding, Praeger Publishers, New York, 1986, Chap. 4.
[7] J. Koch-Weser, E.M. Sellers, New Engl. J. Med. 294 (1976) 311.

Table 1
Association equilibrium constants for the competitive binding of digitoxin and acetyldigitoxin to HSA at pH 7.4 and 37°C

	Competing agent and its association equilibrium constant for HSA its site of competition with the injected probe[a]	
	Digitoxin	Acetyldigitoxin
Injected probe		
Digitoxin	$5.2(\pm0.2)\times10^4\ M^{-1}$	$4.9(\pm0.2)\times10^4\ M^{-1}$
Acetyldigitoxin	$5.4(\pm0.2)\times10^4\ M^{-1}$	$4.8(\pm0.2)\times10^4\ M^{-1}$
Average association constant for competing agent	$5.3(\pm0.2)\times10^4\ M^{-1}$	$4.8(\pm0.2)\times10^4\ M^{-1}$

[a] The numbers in parentheses represent a range of 1 SD. All values were measured in pH 7.4, 0.067 *M* phosphate buffer.

[8] U. Kragh-Hansen, Pharmacol. Rev. 33 (1981) 17.
[9] A. Brock, Acta Pharmacol. Toxicol. 36 (1975) 13.
[10] E. Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagne, S. Motellier, I.W. Wainer, Chromatographia 29 (1990) 170.
[11] E. Domenici, C. Bertucci, P. Salvadori, I.W. Wainer, J. Pharm. Sci. 80 (1991) 164.
[12] B. Loun, D.S. Hage, J. Chromatogr. 579 (1992) 225.
[13] E. Domenici, C. Bertucci, P. Salvadori, S. Motellier, I.W. Wainer, Chirality 2 (1992) 263.
[14] T.A.G. Noctor, C.D. Pharm, R. Kaliszan, I.W. Wainer, Mol. Pharmacol. 42 (1992) 506.
[15] T.A.G. Noctor, I.W. Wainer, D.S. Hage, J. Chromatogr. 577 (1992) 305.
[16] J. Yang, D.S. Hage, J. Chromatogr. 645 (1993) 241.
[17] B. Loun, D.S. Hage, Anal Chem. 66 (1994) 3814.
[18] D.S. Hage, T.A.G. Noctor, I.W. Wainer, J. Chromatogr. A 693 (1995) 23.
[19] D.S. Hage, S.A. Tweed, J. Chromatogr. B 699 (1997) 499.
[20] D.S. Hage, A. Sengupta, Anal. Chem. 70 (1998) 4602.
[21] P.F. Ruhn, S. Garver, D.S. Hage, J. Chromatogr. A 669 (1994) 9.
[22] A. Chattopadhyay, D.S. Hage, J. Chromatogr. A 758 (1997) 255.
[23] P.O. Larsson, Methods Enzymol. 104 (1984) 212.
[24] B. Loun, D.S. Hage, J. Chromatogr. 579 (1992) 225.
[25] 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.
[26] N.W. Tietz (Ed.), Clinical Guide To Laboratory Tests, 2nd Ed, Saunders, Philadelphia, 1990, p. 708.
[27] E. Grushka, M.N. Myers, P.D. Schettlez, J.C. Giddings, Anal. Chem. 41 (1969) 881.
[28] J. Yang, D.S. Hage, J. Chromatogr. A 725 (1996) 273.
[29] J. Yang, D.S. Hage, J. Chromatogr. A 766 (1997) 15.
[30] J. Haginaka, J. Wakai, Anal. Chem. 62 (1990) 997.
[31] D.C. Carter, J.X. Ho, Adv. Prot. Chem. 45 (1994) 153.
[32] F. Herve, K. Rajkowski, M.T. Martin, P. Dessen, N. Cittanova, Biochem. J. 221 (1984) 401.
[33] D. Satoh, J. Morita, Chem. Pharm. Bull. 17 (1969) 1456.