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Use of peak decay analysis and affinity microcolumns containing silica monoliths for rapid determination of drug–protein dissociation rates

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ABSTRACT

This report examined the use of silica monoliths in affinity microcolumns containing human serum albumin (HSA) to measure the dissociation rates for various drugs from this protein. Immobilized HSA and control monolith columns with dimensions of 1 mm × 4.6 mm i.d. were prepared for this work and used with a noncompetitive peak decay method. Several drugs known to bind HSA were examined, such as warfarin, diazepam, imipramine, acetohexamide, and tolbutamide. Items that were studied and optimized in this method included the sample volume, sample concentration, and elution flow rate. It was found that flow rates up to 10 mL/min could be used in this approach. Work with HSA silica monoliths at these high flow rates made it possible to provide dissociation rate constants for drugs such as warfarin in less than 40 s. The dissociation rate constants that were measured gave good agreement with values reported in the literature or that had been obtained with other solutes that had similar binding affinities for HSA. This approach is a general one that should be useful in examining the dissociation of other drugs from HSA and in providing a high-throughput method for screening drug–protein interactions.

1. Introduction

The information gathered from studying the interactions between drugs and serum proteins is important in describing the adsorption, distribution, metabolism, and excretion (ADME) of these drugs within the body [1]. One serum protein of interest in such work is human serum albumin (HSA). HSA is the most abundant serum protein and is involved in the transport of several drugs and hormones within the body. HSA is a single-peptide protein with a molecular weight of 66.5 kDa and a serum concentration of 30–50 g/L [2]. It is composed of 585 amino acids and has two major binding sites for drugs, which are known as Sudlow sites I and II [2,3]. Sudlow site I, or the warfarin-azapropazone site, is located in subdomain IIA of HSA and has a strong affinity for anticoagulants such as warfarin and non-steroidal anti-inflammatory drugs such as salicylates. Sudlow site II, or the indole-benzodiazepene site, is located in subdomain IIIA and binds strongly to tryptophan and profens such as ibuprofen [4,5].

The characterization of drug–protein interactions with serum proteins such as HSA has been traditionally performed by using the reference techniques of ultrafiltration and equilibrium dialysis [6–9]. However, these techniques usually require long analysis times (especially equilibrium dialysis) and relatively large amounts of sample. Other techniques that have been used to study drug–protein interactions include capillary electrophoresis [10–12], circular dichroism [6,7,9], surface plasmon resonance [13], solid-phase microextraction [14,15], fluorometry [6], and microcalorimetry [16]. High-performance affinity chromatography (HPAC) is another technique that has been of growing interest for examining the interactions between drugs and HSA. HPAC is a chromatographic technique that can be used to examine a biological interaction by using one of the components of this interaction as an immobilized binding agent and stationary phase [17]. The results obtained using HPAC with immobilized HSA columns have been shown to be comparable to those found when using soluble HSA, both in terms of the qualitative and quantitative behavior of this protein in its interactions with drugs and other solutes [1,17–19].

One factor that HPAC can be used to determine is the dissociation rate constants for solute–protein interactions. Approaches that have been used in HPAC to measure such dissociation rate constants have included band-broadening methods, such as techniques based on plate height and peak profiling measurements, and peak fitting methods [17,20–23]. Another method that can be used to measure the dissociation rate of an analyte from a protein is the peak decay method. In the peak decay method, a small amount of analyte is first injected onto a column containing the binding agent of interest. A high concentration of a displacing agent is later applied onto the column to elute the analyte and prevent re-association of this analyte as it is released from the binding agent. The resulting decay profile that is produced for the analyte is then used to determine the dissociation rate constant for the analyte from the binding agent [22,24,25].

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One recent report described a new, noncompetitive peak decay method [26]. This method was used with 2.5 mm × 2.1 mm i.d. columns containing silica particles to measure the dissociation rate constants for drugs with weak-to-moderate affinities for immobilized HSA. This approach eliminated the need for a displacing agent when using the peak decay method, thus reducing the number of steps involved in this technique and making it easier to employ with new analytes [26]. Compared with other chromatographic tools for measuring dissociation rate constants, this noncompetitive peak decay method did not require plate height measurements or linear elution conditions, as are needed in band-broadening methods, or changes in the flow rate and mobile phase between application of the analyte and the displacing agent, as is used in traditional peak decay methods [24,26]. Also, even though peak fitting methods do not require linear elution conditions, they do assume that kinetic processes such as stagnant mobile phase mass transfer have a negligible contribution to the results when compared with the analyte–ligand dissociation process. As a result, the dissociation rate constants obtained by peak fitting methods are often based on more than one kinetic process and can vary with the experimental conditions [24].

In another report, it was shown that small silica monoliths with lengths of 1–5 mm can be used with HSA in affinity microcolumns for zonal elution studies and to give reproducible estimates of retention factors and plate heights for injected solutes [27]. Using this type of support made it possible to obtain shorter retention times and lower back pressures than traditional HPLC affinity columns, with the added benefit of providing better mass transfer properties than particle-based supports [27]. However, the use of silica monoliths with methods for measuring dissociation rates has not yet been explored in affinity microcolumns.

This study will examine the use of silica monoliths in affinity microcolumns for the measurement of drug–protein dissociation rate constants in a high-throughput format. This work will use the noncompetitive peak decay method and HSA as a model protein. Various drugs will be examined in this work, including warfarin, diazepam, imipramine, acetohexamide, and tolbutamide. Warfarin is an anticoagulant that binds to Sudlow site I of HSA [28]. Diazepam, an anti-anxiety drug [14], and imipramine, an anti-depressant [29], are known to bind to Sudlow site II of HSA. Acetohexamide and tolbutamide are used to treat diabetes [30,31] and bind to both Sudlow sites I and II [32]. Various experimental factors will first be considered in the use of small HSA monoliths and the peak decay method for examining drug–protein dissociation, using warfarin as a test solute. The extension of this method to the other drugs will then be considered, and the results will be compared with those determined previously by other techniques. These studies should result in a general approach for characterizing the kinetics of drug–protein interactions that can be adapted for use with other drugs and analytes in high-throughput measurements.

2. Theory

The model used in the peak decay method is based on a set of two reversible reactions that occur as a drug is retained and released by a column that contains an immobilized binding agent such as HSA. The first of these reactions describes the mass transfer of the drug from the excluded volume of the column, or the flowing mobile phase region, to the stagnant mobile phase region within the pores, as shown in Eq. (1). The second reaction describes the binding and release of the drug from an immobilized protein or ligand, as described by Eq. (2).

$$A_e \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} A_p \tag{1}$$

$$A_p + L \underset{k_d}{\overset{K_a}{\rightleftarrows}} A - L \tag{2}$$

In these equations, A_e represents the analyte in the flowing mobile phase (or excluded volume), A_p is the analyte in the stagnant mobile phase (or pore volume), L is the immobilized protein or ligand, and $A-L$ is the analyte–ligand complex that forms on the support. The terms k_1 and k_{-1} are the forward and reverse mass transfer rate constants that describe the movement of analyte between the flowing and stagnant mobile phases, and k_a and k_d are the association and dissociation rate constants that describe the analyte–ligand interaction [25,26]. The association and dissociation rate constants in Eq. (2) can also be related to the association equilibrium constant (K_a) for the drug–ligand interaction, as shown in Eq. (3).

$$K_a = \frac{k_a}{k_d} \tag{3}$$

In the peak decay method it is desirable to select experimental conditions in which a retained analyte is immediately eluted from the column as it is released from the immobilized protein or ligand. This situation ideally requires that the mass transfer of the released analyte back into the stagnant mobile phase and rebinding of the analyte with the immobilized protein or ligand are negligible as dissociation of the analyte from the column is being observed. Under such conditions, the model in Eqs. (1) and (2) can be approximated by the following reactions that now describe the release of the analyte from the column [25,26].

$$A - L \xrightarrow{k_d} A_p + L \tag{4}$$

$$A_p \xrightarrow{K_{-1}} A_e \tag{5}$$

Under these conditions, the resulting elution profile for the analyte can be described by Eq. (6).

$$\frac{dm_{A_e}}{dt} = \frac{k_{-1}k_d m_{A_0}}{k_{-1} - k_d}[\exp(-k_d t) - \exp(-k_{-1} t)] \tag{6}$$

In this equation, m_{A_e} is the moles of analyte eluting at time t and m_{A_0} represents the moles of analyte that were initially bound to the column [25,26]. If experimental conditions are selected so that the value of k_d is much smaller than k_{-1} (i.e., dissociation of A from L is the rate-limiting step in analyte release from the column), Eq. (6) will be reduced to give Eq. (7) when expressed in a logarithmic form.

$$\ln \frac{dm_{A_e}}{dt} = \ln(k_d m_{A_0}) - k_d t \tag{7}$$

According to Eq. (7), a plot of the natural logarithm of the elution profile ($\ln dm_{A_e}/dt$) versus time should give a linear relationship with a slope that is now equal to $-k_d$. Thus, it is possible form the slope of such a plot to obtain an estimate of the dissociation rate constants, k_d, for the release of the analyte from the immobilized protein or ligand [25,26].

3. Materials and methods

3.1. Reagents

The HSA (Cohn fraction V, essentially fatty acid free, ≥96% pure), racemic warfarin (98%), diazepam, imipramine (≥98%), acetohexamide, and tolbutamide were from Sigma (St. Louis, MO, USA). All buffers and aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and were passed through Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

3.2. Apparatus

A Chromolith Performance Si column (4.6 mm i.d. × 100 mm) was donated by Merck KGaA (Darmstadt, Germany). Using a lathe, this column was cut into 1 mm long pieces to make shorter silica monolith columns. Reagents to activate the silica monoliths and to immobilize HSA were applied using a Beckman System Gold 118 Solvent Module pump (Fullerton, CA, USA).

The chromatographic system consisted of a PU-2080 Plus HPLC pump from Jasco (Easton, MD, USA), a six-port Lab Pro valve (Rheodyne, Cotati, CA, USA), and a UV-2075 Jasco detector. An Alltech water jacket (Deerfield, IL, USA) and an Isotemp 3013D circulating water bath from Fisher were used to maintain a temperature of 37.0 (±0.1) °C for the columns during all experiments described in this report. The chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA).

3.3. Methods

To immobilize HSA onto a silica monolith, a bare silica monolith was first converted into a diol-bonded form, as described in detail previously [27,33]. This process involved first placing a 1 mm long section of a bare silica monolith into a delrin housing for each desired column. The column was washed with pH 5.5, 0.1 M sodium acetate buffer, followed by the application of 3-glycidoxypropyltrimethoxysilane at room temperature. The ends of the column were sealed, and the column was placed in a water bath at 97 °C for 5 h. The column was then removed from the water bath and washed again with pH 5.5, 0.10 M, sodium acetate buffer and 3-glycidoxypropyltrimethoxysilane to ensure maximum diol coverage. The column ends were sealed, and the column was placed in a water bath at 97 °C for 5 h. After removing the column from the water bath, the column was washed with water and a pH 3.0 solution of dilute sulfuric acid in water. The column was then sealed at both ends and placed in a water bath at 70 °C for 3 h. All of the resulting diol silica monolith columns were washed with water prior to further use. Some of these diol monolith columns were used later for HSA immobilization, while others were used in their current form as control columns [27,33].

HSA was immobilized onto a diol monolith by using the Schiff base method, which was also carried out as reported in detail previously [27,33]. In this method a 90% (v/v) solution of acetic acid in water was first passed through a diol silica monolith column, followed by application of a solution of 0.5 g/mL periodic acid in 90% acetic acid in water. The column was then washed with water. A 10 mL solution containing 50 mg HSA and 25 mg sodium cyanoborohydride in pH 6.0, 1.5 M potassium phosphate buffer was circulated through the column for 24 h. This step was followed by the application of a fresh 12 mL solution of 60 mg HSA and 30 mg sodium cyanoborohydride in the same pH 6.0 buffer, which was circulated through the column for 65 h. A 5 mL solution of pH 8.0, 0.10 M potassium phosphate buffer containing 1 mg/mL sodium borohydride was then applied to each column. The column was washed with pH 8.0, 0.10 M potassium phosphate buffer containing 0.5 M sodium chloride. The column was also washed with pH 7.4, 0.067 M potassium phosphate buffer. The HSA and control monoliths were stored in pH 7.4, 0.067 M potassium phosphate buffer at 4 °C until use and were stable for over one year.

The protein content of the HSA silica monolith was estimated as described in a previous report [27,33] by measuring the retention for 5 μL injections of 30 μM carbamazepine, a solute with a known affinity for immobilized HSA, on both the HSA and control columns while using pH 7.4, 0.067 M phosphate buffer as the mobile phase. The estimated protein content of the HSA monoliths was 1.8 (±0.1) μmol HSA/g support. Previous work has been shown that the immobilization of HSA onto silica monoliths like those used in this study can result in a high protein coverage [33]. For instance, under the same immobilization conditions as used in this report, the amount of HSA that can be immobilized per unit area to such a silica monolith is equivalent to the coverage that is seen for HSA on 300 Å pore size silica particles. This similarity indicates that HSA was able to reach a significant amount of surface area that was present in the mesopores of the silica monolith, as well as the surface that was located about the larger through pores of the monolith [33].

The mobile phase used for the chromatographic studies was pH 7.4, 0.067 M potassium phosphate buffer. All mobile phases were degassed for 25 min prior to use. Injection volumes of 5 or 100 μL were used for the warfarin samples. The diazepam, imipramine, acetohexamide, and tolbutamide were injected using a 100 μL sample volume. These samples were injected in triplicate under all tested conditions. Each sample was prepared in pH 7.4, 0.067 M potassium phosphate buffer and stored at 4 °C when not in use. The solutions of warfarin were used within one week of preparation, and the solutions of all other drugs were used within 2–3 weeks of preparation. All of these sample solutions were stable under such conditions.

The concentrations of warfarin that were applied to the HSA and control columns ranged from 5 to 30 μM. A concentration of 20 μM was used in the studies with diazepam, imipramine, acetohexamide, or tolbutamide. The following detection wavelengths were employed: warfarin, 308 nm; diazepam, 236 nm, imipramine, 251 nm; acetohexamide and tolbutamide, 250 nm. Flow rates ranging from 0.25 to 10 mL/min were used in these studies. A baseline correction was performed on the collected data, and these corrected results were then used to prepare a plot of the natural logarithm of the response versus time, as shown in Fig. 1. The slope of the linear range of natural logarithm of the elution profile was used to determine the dissociation rate constant for the drug–protein interaction. The linear range was selected by comparing the profiles for the control column and HSA column and choosing a region that did not include any significant overlap in their elution profiles for a given analyte.

4. Results and discussion

4.1. General behavior of peak decay method

For the noncompetitive peak decay method that was used in this study, a plug of the desired analyte solution was injected onto the HSA and control columns at various flow rates. The subsequent elution profiles were then monitored at 37 °C. During this process, the analyte was able to bind and partially saturate the immobilized HSA as the injected sample passed through the column. After the sample plug had exited the column, the unbound analyte in the stagnant mobile phase was quickly washed away, along with any analyte that was non-specifically and weakly retained by the support. The analyte that had been bound to the immobilized HSA was then allowed to dissociate from the column as the elution profile was monitored. Fig. 1(a) shows some typical elution profiles that were obtained for warfarin on the HSA and control monolith columns. Similar results were seen for the other analytes examined in this report.

Fig. 1(b) gives examples of plots of the natural logarithm for the elution profiles that were obtained for warfarin on the HSA and control monolith columns. Similar plots were again found for the other analytes used in this study. As expected, the washing away of non-bound analyte from the column and the release of any non-specifically bound analyte occurred much more quickly than the release of analyte from the immobilized HSA. The decay

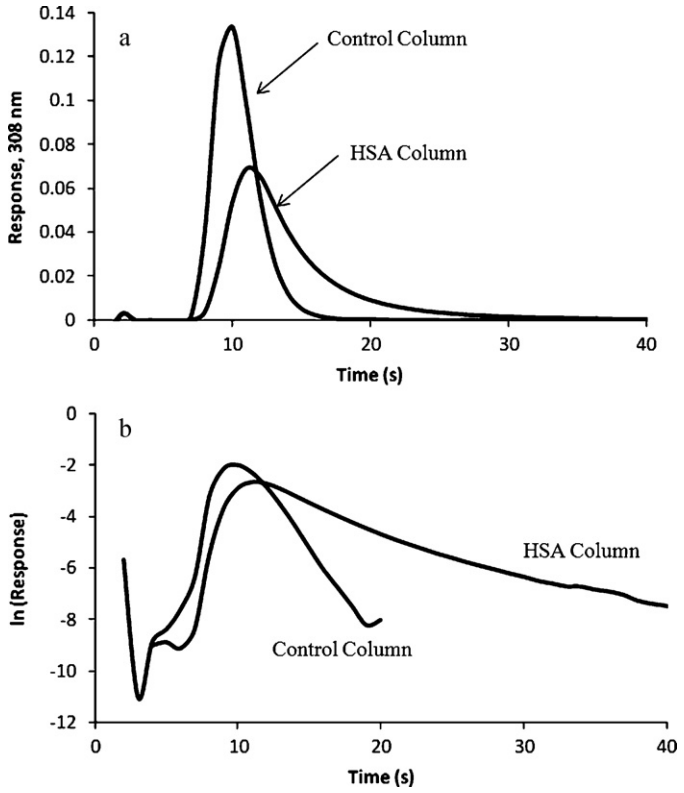

Fig. 1. (a) Elution profiles and (b) natural logarithm of the elution profiles for 100 μL injections of 10 μM racemic warfarin made at 4 mL/min onto 1 mm × 4.6 mm i.d. silica monolith columns containing immobilized HSA or a control support. These results and those shown in all later figures were obtained at 37 °C and using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase.

portion for each type of elution profile gave an essentially linear decrease in the natural logarithm of the elution profile once the original sample plug has passed through the column. When this linear fit was made for the HSA columns, this was done by using a region in the decay curve in which release of the same analyte from the control column no longer gave any significant response. Plots prepared using this linear range gave correlation coefficients of 0.9885–0.9999 (n = 4–83) for the various analytes and experimental conditions that were used in this report.

4.2. Optimization of peak decay method

Warfarin was the analyte used to initially optimize the experimental parameters for the HSA silica monoliths when using the noncompetitive peak decay method. This drug was useful as a model for this work because it has known equilibrium and rate constants for its interactions with immobilized HSA [23,28]. This solute has also been used in previous work with the peak decay method and HSA columns containing silica particles [26], thus making it possible to directly compare this earlier report with the results that were obtained in this current study using silica monoliths. Warfarin was utilized to examine the effects of sample volume, sample concentration, and flow rate as part of the optimization process for the silica monoliths.

The size of the sample volume versus column volume is an important parameter to consider in the determination of dissociation of rate constants by the peak decay method. Previous work has shown that columns with small sizes and a small number of theoretical plates are useful in obtaining the conditions needed to generate a good peak decay profile [26]. In this study, 1 mm long and 4.6 mm i.d. silica monolith columns were used for this purpose. Sample volume effects were studied by using 5 μL and 100 μL sample loops. Previous work with HSA columns containing silica particles has shown that the use of a sample volume that is much larger than the column void volume will allow more time for the analyte to initially interact with the stationary phase [26]. This effect allows more analyte to be placed onto the column before the decay curve is generated, making it easier to detect the analyte and to more accurately monitor analyte–protein dissociation. In this current study, the column void volume of the silica monoliths was approximately 13.3 μL. It was found that the use of a 5 μL sample, which had a volume equivalent to 37% of the column void volume, gave an apparent dissociation rate constant that was 25% lower than the value measured when using a 100 μL sample, which had a volume that was 7.5-fold larger than the column void volume. The value found with the 100 μL sample was also closer to previous dissociation rate constants that have been reported for this system (see Section 4.4). These results agreed with the general observations and conditions that were selected when using the noncompetitive peak decay method for warfarin with a 2.5 mm × 2.1 mm i.d. HSA column containing silica particles [26]. From these results, a 100 μL sample size was selected for use in all later studies with warfarin and the other drugs tested in this report.

In using the peak decay method with other column dimensions, using a longer column with the same inner diameter as the monoliths used in this study would have led to a smaller sample-to-column volume ratio and a larger number of theoretical plates. Neither of these conditions would be expected to improve the quality of the peak decay results, as based on prior work with packed columns [26]. The use of a column with the same length as employed in this study but with a smaller inner diameter might be useful in future work with the peak decay method by allowing the efficiency to remain the same while providing a smaller column volume and larger sample-to-column volume ratio for any given volume of an applied sample.

The concentration of warfarin was varied from 5 to 30 μM to determine the effect of sample concentration on dissociation rate constant measurements made using HSA silica monoliths. Fig. 2 shows some of the elution profiles and logarithmic plots that were generated during these experiments. It was found in these experiments that a change in analyte concentration did not have a significant effect on the slopes that were obtained from the logarithmic elution profiles over the range of analyte concentrations that were tested. This is demonstrated by Table 1, which summarizes the dissociation rate constants that were obtained for warfarin. In this case, all dissociation rate constants measured for warfarin were in the range of 0.36–0.49 s^{-1}, with essentially only random variations being seen in these rate constants as the sample concentration was altered. Based on these results, sample concentrations of 20–30 μM were used for warfarin and the other drugs studied in most of the remainder of this report.

Table 1
Apparent dissociation rate constants measured using various sample concentrations of warfarin[a].

Warfarin conc. (μM)	Apparent dissociation rate constant, k_d (s^{-1})
5	0.381 (±0.013)
10	0.486 (±0.002)
20	0.393 (±0.021)
30	0.360 (±0.005)

[a] These results were obtained at pH 7.4 and 37 °C. The values in parentheses represent a range of ±1 SD, as determined by using error propagation and the slopes of plots prepared according to Eq. (7). The results at 10 μM warfarin were obtained at a different time from the other results and may reflect some small variations in protein activity over time.

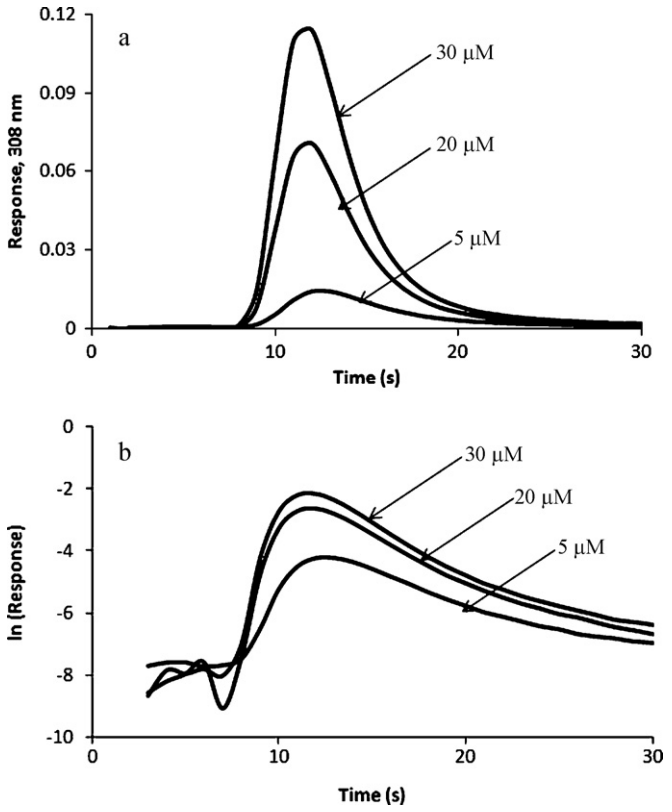

Fig. 2. (a) Elution profiles and (b) natural logarithm of the elution profiles for 100 μL injections of (from top-to-bottom) 30 μM, 20 μM or 5 μM racemic warfarin made at 4 mL/min onto a 1 mm × 4.6 mm i.d. silica monolith column containing immobilized HSA.

The effect of flow rate was examined by using flow rates of 0.25–9 mL/min to monitor the dissociation of warfarin from the HSA and control monolith columns. Fig. 3 shows the slopes that were measured at these flow rates in the linear range of the logarithmic elution profiles for warfarin on both the HSA and control monolith columns. The measured dissociation rate constants showed a steady increase when going from low-to-moderate flow rates and then started to level off at high flow rates. This behavior agrees with earlier results obtained with HSA columns containing silica particles, in which the use of high flow rates was found to prevent re-association of warfarin with HSA and to minimize the effects of movement by the released analyte from the flowing mobile phase back into the stagnant mobile phase. The overall result of such conditions is that it becomes easier to distinguish between analyte dissociation and mass transfer effects at high flow rates, with the slope of the logarithmic elution profile then providing a more accurate estimate of k_d [26]. Similar flow rate plots were obtained for the other drugs in this study, with the high flow rate data being used in all final k_d measurements.

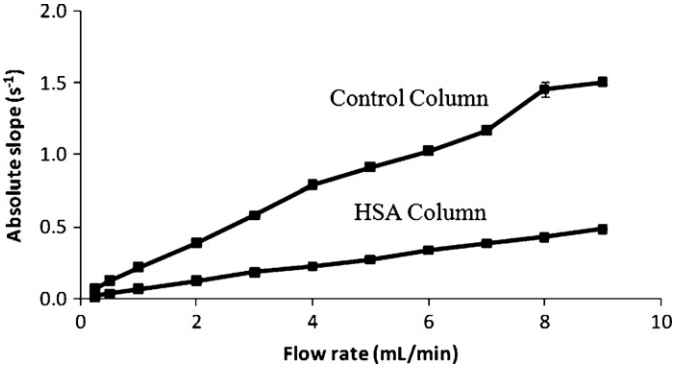

Fig. 3. Absolute values of the slopes measured at 0.25–9 mL/min in the natural logarithm plots of elution profiles obtained for 100 μL injections of 10 μM racemic warfarin made onto 1 mm × 4.6 mm i.d. silica monolith columns containing immobilized HSA or a control support.

4.3. Use of silica monoliths in peak decay studies

In the last section it was found that HSA microcolumns based on silica monoliths gave similar trends to those seen previously for silica particles (as used in Ref. [26]) in peak decay analysis. However, several differences were noted in these experiments when comparing these two types of supports. These differences were related to the mass transfer properties and flow properties of these two materials. For instance, it has been well-established in work by others that the structure of monolithic columns can lead to better mass transfer properties and lower back pressures than traditional particle-based supports, making monoliths appealing for use in fast separations or high-throughput analysis [34]. These same features made monolithic supports attractive for peak decay analysis in this study by providing conditions that should have allowed more reliable estimates to be made of drug–protein dissociation rate constants.

The low back pressures of silica monoliths made it relatively easy to use microcolumns based on these materials at high flow rates. As shown earlier in Fig. 3, the use of a high flow rate in the peak decay method provided for more reliable estimates of solute–protein dissociation rate constants by minimizing the effects of analyte re-association and movement by the analyte from the flowing mobile phase into the stagnant mobile phase. In this study, the back pressures that were measured for 1 mm long HSA and control monoliths ranged from only 73 psi (0.5 MPa) at 0.25 mL/min to 972 psi (6.7 MPa) at 10 mL/min. In contrast to this, earlier peak decay studies with HSA microcolumns containing silica particles were carried out using flow rates that extended up to only 5 mL/min [26]. This ability to use higher flow rates with the silica monoliths meant that it was easier to extend the use of the peak decay method with these supports to new drugs and solutes (e.g., see Section 4.4) and to more easily obtain conditions that provided accurate estimates of drug–protein dissociation rate constants.

One consequence of being able to use higher flow rates on silica monoliths for more accurate k_d estimates is that the analysis times were also decreased. For example, the peak decay experiments shown in Fig. 4 were carried out within 40 s or less when using flow rates of 4–8 mL/min. As was noted earlier, the rate of warfarin dissociation from the immobilized HSA approached a constant response as drug–protein dissociation became the rate-limiting step in drug release from the column. However, the washing away of excess and non-bound drug from the column, as illustrated by the results for the control support in Fig. 4, occurred on a much shorter time scale as higher flow rates were utilized. This effect was further aided by the greater efficiency that has been noted at high flow rates for HSA silica monoliths versus HSA columns containing silica particles [27,33]. The overall result was that it became easier to distinguish between the decay curves for the control column and HSA column as higher flow rates were used. This, in turn, meant that linear fits could be made to the decay curves for the HSA column over shorter elution times. The result was that less time was needed for estimating the value of k_d for the drug–protein interaction.

4.4. Measurement of drug dissociation rates from HSA

After the use of peak decay analysis had been optimized on the HSA silica monoliths, these columns were used to estimate the dissociation rate constants for warfarin and other drugs with HSA.

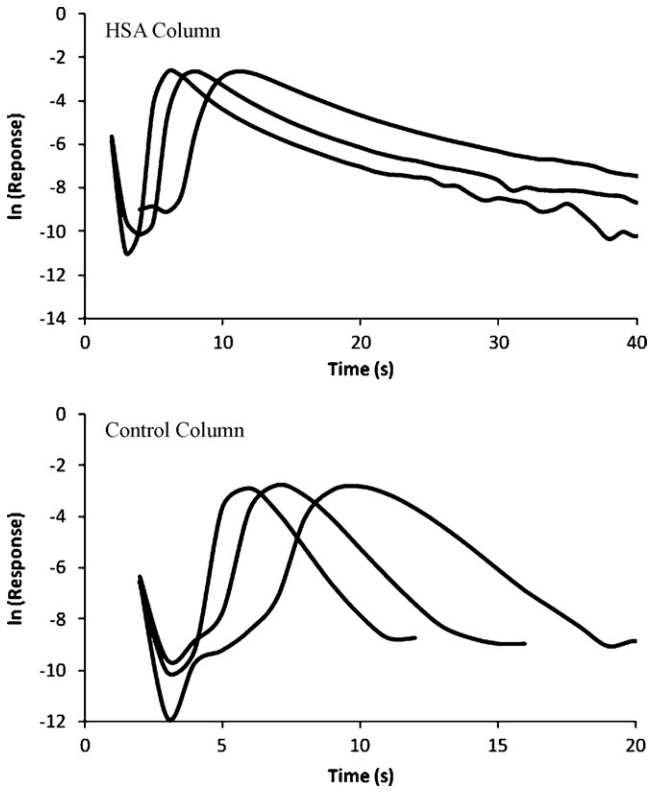

Fig. 4. Natural logarithm of the elution profiles obtained for 100 μL injections of 10 μM racemic warfarin made at 4, 6 or 8 mL/min (from right-to-left) on 1 mm × 4.6 mm i.d. silica monolith columns containing (a) immobilized HSA or (b) a control support.

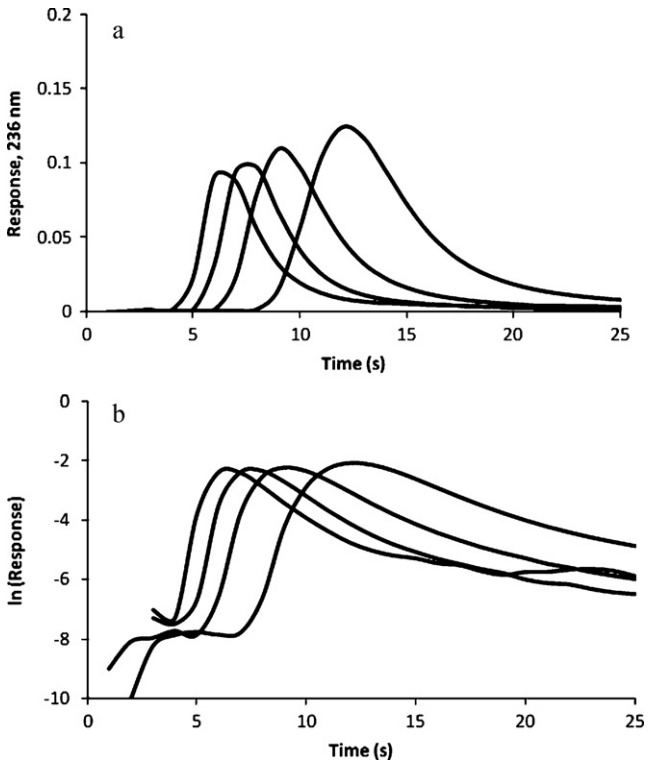

Fig. 5. (a) Elution profiles and (b) natural logarithm of the elution profiles for 100 μL injections of 20 μM diazepam made at 4, 6, 8, or 10 mL/min (from right-to-left) onto a 1 mm × 4.6 mm i.d. silica monolith column containing immobilized HSA.

The results, which were all obtained at pH 7.4 and 37 °C, are summarized in Table 2. In this list, racemic warfarin was used as an example of a drug that has selective binding to Sudlow site I of HSA. The average rate constant of 0.41 (±0.06) s^{-1} that was measured in this study for racemic warfarin with HSA was in good agreement with previous values of 0.35–0.66 s^{-1} that have been reported at 25–37 °C based on work with band-broadening measurements and peak decay studies on HSA columns containing silica particles [27,33].

Several other drugs were also examined through peak decay studies conducted on the HSA and control monolith columns. Fig. 5 shows some typical chromatograms and logarithmic elution profiles that were obtained in these studies for diazepam. Fig. 6 shows the slopes that were measured for this drug on the HSA and control monolith columns at various flow rates. The behavior seen in Figs. 5 and 6 was similar to that noted for warfarin on the same columns. In addition, the same general behavior and types of plots

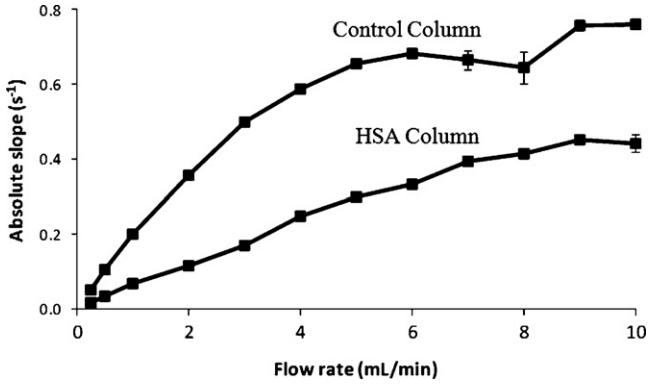

Fig. 6. Absolute values of the slopes measured at 0.25–10 mL/min in the natural logarithm plots of the elution profiles obtained for 100 μL injections of 20 μM diazepam made onto 1 mm × 4.6 mm i.d. silica monolith columns containing immobilized HSA or a control support.

Table 2
Dissociation rate constants measured for various drugs on HSA microcolumns.

Drug	Association equilibrium constant(s) [Ref.]	Binding site on HSA[a]	Dissociation rate constant, k_d (s^{-1})	
			Value from current study[b]	Literature value [Ref.]
Warfarin	2.1–2.6 × 10^5 [23]	I	0.41 (±0.06)	0.35–0.66 [26][c]
Imipramine	1.6 × 10^5 [35][d]	II	0.29 (±0.11)	0.41–0.67 [36][d]
Diazepam	2.2 × 10^5 [9]	II	0.44 (±0.02)	–
Acetohexamide	0.43–1.3 × 10^5 [32]	I, II	0.58 (±0.02)	–
Tolbutamide	5.3–5.5 × 10^4 [32]	I, II	0.49 (±0.15)	–

[a] These binding regions refer to Sudlow sites I and II of HSA.
[b] All of the results from this current report were measured at pH 7.4 and 37 °C. The values in parentheses represent a range of ±1 SD, as determined by using error propagation and the slopes of plots prepared according to Eq. (7).
[c] These results are a collection of values obtained from 25 to 37 °C for immobilized HSA in Refs. [23] and [26].
[d] The association equilibrium constant is for the high affinity site of imipramine on HSA. The dissociation rate constant was determined at 37 °C.

were seen for the other drugs that were examined. The overall agreement in these trends with those found for warfarin suggested that the peak decay method and the silica monolith columns could be successfully utilized in examining the dissociation rates for other drugs from HSA.

In Table 2, diazepam and imipramine were used as examples of drugs that are known to bind to Sudlow site II of HSA. Both of these drugs have similar affinities to that for the binding of warfarin with HSA, with association equilibrium constants of $1.6–2.2 \times 10^5\ M^{-1}$ at pH 7.4 and 37 °C [9,35]. No previous dissociation rate constants have been reported for diazepam with HSA, but the k_d of 0.44 ($\pm$0.02) s^{-1} that is listed in Table 2 for this drug does agree with the k_d values that have been reported for warfarin. Imipramine gave a slightly lower k_d value of 0.29 ($\pm$0.11) s^{-1}, but this result still agreed with the range of dissociation rate constants that have been noted for warfarin. In addition, this result gave reasonable agreement with recent estimates of 0.41–0.67 s^{-1} that have been made for imipramine–HSA dissociation by using a separate approach (i.e., peak profiling) [36].

Acetohexamide and tolbutamide were used as examples of drugs that are known to have strong binding at both Sudlow sites I and II of HSA [32]. The association equilibrium constants for these drugs with HSA were similar to those for warfarin and the other solutes examined in this study, with values of $0.43–1.3 \times 10^5\ M^{-1}$ for acetohexamide and $5.3–5.5 \times 10^4\ M^{-1}$ for tolbutamide at pH 7.4 and 37 °C [32]. In this situation, the apparent dissociation rate constants measured for these drugs would be expected to be a function of the dissociation rate constants for acetohexamide and tolbutamide at each of their binding sites on HSA. However, even in these multi-site systems, the peak decay experiments gave an overall response and plots that were similar to those for warfarin and the other tested solutes. The apparent dissociation rate constants of 0.49–0.58 s^{-1} that were measured for tolbutamide and acetohexamide in these experiments were in agreement with the values listed in Table 2 for the other drugs that had similar binding affinities with HSA.

5. Conclusions

This study examined the use of small silica monoliths and the noncompetitive peak decay method to measure the dissociation rates for various drugs from HSA. It was found in initial work with racemic warfarin that 1 mm × 4.6 mm i.d. silica monoliths containing immobilized HSA or a control support could be used to carry out dissociation rate constant measurements at flow rates up to 10 mL/min. The effects of sample volume, sample concentration and flow rate were all considered and optimized in this work. The trends noted in the optimization of these parameters were similar to those seen in prior work with HSA columns containing silica particles. However, several advantages were also noted in the use of silica monoliths for such experiments. These advantages included the better mass transfer properties, higher efficiencies and, lower back pressures of the silica monoliths when compared with columns containing silica particles. Such features made it possible to carry out the peak decay studies at higher flow rates that can easily be obtained with silica particles and made it easier to detect and examine drug dissociation from the immobilized HSA. These effects, in turn, lead to a decrease in the overall analysis times (e.g., analysis times of 40 s or less in studies with warfarin) and made it easier with the monolith columns to obtain accurate estimates of dissociation rate constants in the systems that were examined.

This method was next used to measure the dissociation rate constants for warfarin and a number of other drugs from HSA. It was found that the peak decay method could be used for a variety of drugs that had interactions with Sudlow sites I and/or II of HSA. Good agreement was also seen in the rate constants that were measured by the peak decay method and literature values or rate constants that were measured for drugs with similar affinities with HSA. These results indicated that silica monoliths could be used to prepare small HSA columns for peak decay studies of drug–protein dissociation. The ability of this method to be used at high flow rates should make it useful as a tool in the high-throughput screening of drug interactions with HSA. Furthermore, it should be possible to extend this method to other drugs and proteins.

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References

[1] D.S. Hage, A. Jackson, M.R. Sobansky, J.E. Schiel, M.J. Yoo, K.S. Joseph, J. Sep. Sci. 32 (2009) 835.
[2] T. Peters Jr., All About Album: Biochemistry, Genetics, and Medical Applications, Academic Press, San Diego, CA, 1996.
[3] M. Otagiri, Drug Metab. Pharmacokinet. 20 (2005) 309.
[4] P. Ascenzi, A. Bocedi, S. Notari, G. Fanali, R. Fesce, M. Fasano, Mini-Rev. Med. Chem. 6 (2006) 483.
[5] C. Bertucci, E. Domenici, Curr. Med. Chem. 9 (2002) 1463.
[6] K. Matsuyama, A.C. Sen, J.H. Perrin, J. Pharm. Pharmacol. 39 (1987) 190.
[7] G.A. Ascoli, E. Domenici, C. Bertucci, Chirality 18 (2006) 667.
[8] M.J. Banker, T.H. Clark, Curr. Drug Metab. 9 (2008) 854.
[9] J. Wilting, B.J.T. Hart, J.J. De Gier, Biochim. Biophys. Acta 626 (1980) 291.
[10] D. El-Hady, S. Kuehne, N. El-Maali, H. Waetzig, J. Pharm. Biomed. Anal. 52 (2010) 232.
[11] H. Wan, A. Oestlund, S. Joensson, W. Lindberg, Rapid Commun. Mass Spectrom. 19 (2005) 1603.
[12] N.H.H. Heegaard, C. Schou, in: D.S. Hage (Ed.), Handbook of Affinity Chromatography, CRC Press/Taylor & Francis, New York, 2006, Chapter 26.
[13] R.L. Rich, Y.S.N. Day, T.A. Morton, D.G. Myszka, Anal. Biochem. 296 (2001) 197.
[14] H. Yuan, J. Pawliszyn, Anal. Chem. 73 (2001) 4410.
[15] D. Vuckovic, J. Pawliszyn, J. Pharm. Biomed. Anal. 50 (2009) 550.
[16] P. Coassolo, M. Sarrazin, J.C. Sari, C. Briand, Biochem. Pharmacol. 27 (1978) 2787.
[17] D.S. Hage, J. Chromatogr. B 768 (2002) 3.
[18] S.S. Singh, J. Mehta, J. Chromatogr. B 834 (2006) 108.
[19] H.S. Kim, I.W. Wainer, J. Chromatogr. B 870 (2008) 22.
[20] A.M. Talbert, G.E. Tranter, E. Holmes, P.L. Francis, Anal. Chem. 74 (2002) 446.
[21] J.E. Schiel, C.M. Ohnmacht, D.S. Hage, Anal. Chem. 81 (2009) 4320.
[22] R.M. Moore, R.R. Walters, J. Chromatogr. 384 (1987) 91.
[23] B. Loun, D.S. Hage, Anal. Chem. 68 (1996) 1218.
[24] J.E. Schiel, D.S. Hage, J. Sep. Sci. 32 (2009) 1507.
[25] R.R. Walters, in: I.M. Chaiken (Ed.), Analytical Affinity Chromatography, CRC Press, Boca Raton, FL, 1987, Chapter 3.
[26] J. Chen, J.E. Schiel, D.S. Hage, J. Sep. Sci. 32 (2009) 1632.
[27] M.J. Yoo, D.S. Hage, J. Sep. Sci. 32 (2009) 2776.
[28] K.S. Joseph, A.C. Moser, S.B.G. Basiaga, D.S. Hage, J. Chromatogr. A 1216 (2009) 3492.
[29] S.H. Preskorn, R.C. Dorey, G.S. Jerkovich, Clin. Chem. 34 (1988) 822.
[30] S. Neelam, M. Kanojia, Chem. Biol. Drug Des. 72 (2008) 290.
[31] T.G. Skillman, J.M. Feldman, Am. J. Med. 70 (1981) 361.
[32] K.S. Joseph, D.S. Hage, J. Chromatogr. B 878 (2010) 1590.
[33] R. Mallik, D.S. Hage, J. Pharm. Biomed. Anal. 46 (2008) 820.
[34] F. Svec, J. Sep. Sci. 27 (2004) 747.
[35] M.J. Yoo, Q.R. Smith, D.S. Hage, J. Chromatogr. B 877 (2009) 1149.
[36] J.E. Schiel, Ph.D. Dissertation, University of Nebraska, Lincoln, NE, 2009.