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Development of Enhanced Capacity Affinity Microcolumns by using a Hybrid of Protein Cross-linking/Modification and Immobilization

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Abstract

A hybrid method was examined for increasing the binding capacity and activity of protein-based affinity columns by using a combination of protein cross-linking/modification and covalent immobilization. Various applications of this approach in the study of drug-protein interactions and in use with affinity microcolumns were considered. Human serum albumin (HSA) was utilized as a model protein for this work. Bismaleimidohexane (BMH, a homobifunctional maleimide) was used to modify and/or cross-link HSA through the single free sulfhydryl group that is present on this protein. Up to a 75-113% increase in protein content was obtained when comparing affinity supports that were prepared with BMH versus reference supports that were made by using only covalent immobilization. Several drugs that are known to bind HSA (e.g., warfarin, verapamil and carbamazepine) were further found to have a significant increase in retention on HSA microcolumns that were treated with BMH (i.e., a 70-100% increase in protein-based retention). These BMH-treated HSA microcolumns were used in chiral separations and in ultrafast affinity extraction to measure free drug fractions in drug/protein mixtures, with the latter method giving association equilibrium constants that had good agreement with literature values. In addition, it was found that the reversible binding of HSA with ethacrynic acid, an agent that can combine irreversibly with the free sulfhydryl group on this protein, could be examined by using the BMH-treated HSA microcolumns. The same hybrid immobilization method could be extended to other proteins or alternative applications that may require protein-based affinity columns with enhanced binding capacities and activities.

Keywords

Protein immobilization; Human serum albumin; Drug-protein binding; Affinity microcolumn; Ultrafast affinity extraction

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1 Introduction

High-performance affinity chromatography (HPAC) is a type of high-performance liquid chromatography that uses biologically-related binding agents such as proteins or antibodies as the stationary phase [1-9]. This method has been widely used for chiral separations and the purification or analysis of biological agents, as well as for the characterization of biological interactions [1-9]. The retention and selectivity of this technique depend on the specific binding of the target analyte to the immobilized binding agent, and the amount of binding sites that are available in the column [4]. This latter factor is related, in turn, to the total amount of binding agent that is present in the column and its relative activity [2,4].

Covalent immobilization is often used in affinity chromatography to couple a binding agent such as a protein to a support. For example, this process might involve the use of amine, sulfhydryl, carboxyl, or carbonyl groups on a protein or glycoprotein [1,4,10-14]. This general approach can produce affinity columns that have high stabilities and that can be reused for many sample applications or experiments. However, the amount of protein that can be immobilized is generally limited by the size of protein and the surface area of the support [15]. In addition, effects such as improper orientation of the binding agent or steric hindrance may lead to a decrease in this agent's apparent activity [11,14]. As a result, it would be useful to have alternative strategies that could avoid such limitations and that can be used to increase the binding capacity and/or activity of columns that contain proteins as their stationary phases. A specific area in which this is of interest is in work with miniaturized affinity devices and affinity microcolumns, in which an increase in activity helps to provide higher retention and resolution for such devices [7,16-18].

This study will examine some possible routes for creating protein-based affinity microcolumns with increased binding capacities and activities. Human serum albumin (HSA), which is the most abundant protein in human serum [3,4,13], will be used as a model protein and binding agent for these experiments. HSA has been of interest for use in affinity chromatography as a chiral stationary phase [3,19,20] and in studies of the binding by this protein with drugs, long-chain fatty acids, and some hormones [13,21-22]. This protein has a single polypeptide chain consisting of 585 amino acid residues and with a mass of 66.5 kDa [13,21-22]. HSA has two major drug binding sites (i.e., Sudlow sites I and II) but only one free sulfhydryl group (Cys34), which is not located near either of these two sites [4,13,21-22]. Various methods have previously been developed to immobilize this protein to chromatographic supports, including both amine-based coupling methods [14,23-28] and the coupling of this protein through Cys34 to maleimide-activated supports [10,14]. However, these techniques are all inherently limited by the available surface area of the support and may be subject to at least some steric hindrance or other immobilization effects [11,14].

An alternative approach that will be considered in this study is to use the cross-linking or modification of a protein such as HSA to provide an increase in the amount of active protein that can be placed into small affinity columns. Such an approach may also allow a decrease in the size of affinity columns for high-throughput applications or use in microscale devices. Two possible schemes that will be examined are the use of modification/cross-linking of a protein after its immobilization, as illustrated in Fig. 1(a), or the use of this modification/

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cross-linking before immobilization, as shown in Fig. 1(b). Bismaleimidohexane (BMH), a homobifunctional cross-linker that reacts specifically with sulfhydryl groups [29-35], will be the reagent employed for this approach. The protein content and relative activity of HSA columns that are modified with BMH will be compared to those obtained by using only covalent immobilization. Affinity microcolumns that are made by this hybrid approach will then be examined for use in drug-binding studies and ultrafast affinity extraction. The possible use of such columns in chiral drug separations will also be considered. The results will be used to determine the advantages or possible limitations of this hybrid modification/ immobilization strategy, as well as its potential uses in areas such as rapid studies of drug-protein binding, biointeraction analysis, and microscale affinity-based separations.

2 Experimental

2.1 Reagents

The HSA (Cohn fraction V, essentially fatty acid free, 96% pure), carbamazepine, racemic verapamil, racemic warfarin, dimethyl sulfoxide (DMSO), sodium nitrate, sodium borohydride and sodium cyanoborohydride were obtained from Sigma (St. Louis, MO, USA). The ethacrynic acid was purchased from Fisher Scientific (Atlanta, GA, USA). The Zeba spin desalting columns (5 mL, 7 kDa cutoff), BMH, and reagents for the bicinchoninic acid (BCA) protein assay were from Thermo Scientific (Rockford, IL, USA). The Nucleosil Si 300 silica (7 μm particle diameter, 300 Å pore size) was purchased from Macherey Nagel (D ren, Germany). All buffers and aqueous solutions were prepared using water from a Milli-Q Advantage A 10 system (EDM Millipore Corporation, Billerica, MA, USA) and were passed through Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

2.2 Apparatus

The HSA solutions or silica slurries were mixed and allowed to react with added reagents by using a Labquake Shaker from Barnstead Thermolyne (Dubuque, IA, USA). The affinity microcolumns were packed using a Prep 24 pump from ChromTech (Apple Valley, MN, USA). The HPLC system consisted of a PU-2080 Plus pump, an AS-2057 autosampler, and a UV-2075 absorbance detector from Jasco (Easton, MD, USA). 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. ChromNAV v1.18.04 software and LCNet from Jasco were used to control the HPLC system. Chromatograms were analyzed through the use of PeakFit v4.12 software (Jandel Scientific, San Rafael, CA, USA).

2.3 Preparation of affinity supports

The HSA supports that were used as reference materials in this work, as well as the starting materials for the scheme in Fig. 1(a), were prepared by immobilizing HSA onto silica through the Schiff base method, based on a procedure reported in Refs. [36,37]. In this method, the silica was prepared in a diol-bonded form and then oxidized with periodic acid to form aldehyde groups on the surface of this material. A 70 mg portion of HSA was dissolved in 1.0 mL of pH 6.0, 0.10 M phosphate buffer and mixed with 0.15 g of the aldehyde-activated silica. Sodium cyanoborohydride was also placed in this mixture during

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the immobilization step to reduce Schiff bases as they formed between the protein and support, resulting in the creation of a more stable secondary amine linkage. After this immobilization had been allowed to occur, the remaining aldehyde groups on the support were reduced by adding sodium borohydride [36,37]. A control support was prepared in the same manner but with no HSA being added during the immobilization step [37].

The BMH-treated HSA silica that was used in the chromatographic studies was prepared according to the strategy shown in Fig. 1(b). The final method involved dissolving 70 mg HSA in 1.0 mL of pH 7.0, 1.5 M potassium phosphate buffer and combining this solution with 30 μL of 0.072 M BMH in DMSO, giving a mixture with a final concentration of 2 mM BMH and 1 mM HSA (i.e., a 2:1 mol/mol ratio for BMH vs. HSA). This mixture was incubated for 8 h at room temperature, followed by additional mixing for 24 h at 4 °C. A desalting column (5.0 mL volume, 7 kDa cutoff) was used to remove excess reagents from the cross-linked or modified HSA. The pH of this protein solution was slowly adjusted to pH 6.0 by adding a small amount of 50% (*v/v*) hydrochloric acid, giving a final total volume of approximately 1 mL. This protein solution was then combined with 0.15 g silica, which had already been converted into an aldehyde-activated form and washed with pH 6.0, 0.10 M phosphate buffer for use in the Schiff base immobilization method [37]. Sodium cyanoborohydride was also added to this mixture to reduce Schiff bases upon their formation, and sodium borohydride was again added later to reduce and remove any aldehyde groups that remained after the immobilization process [37]. A control support was prepared by the same procedure but with no HSA being placed into the reaction buffer during the cross-linking/modification reaction.

All of HSA supports and control supports were washed overnight with pH 7.4, 0.067 M phosphate buffer at 0.10 mL/min prior to their use; the same supports were stored in pH 7.4, 0.067 M potassium phosphate buffer and at 4 °C when not in use. It was found through the periodic injection of compounds known to bind HSA that the BMH-treated HSA microcolumns and reference HSA microcolumns were stable for at least 400 injections and over 6 months of use.

A portion of each HSA support or BMH-treated HSA support was suspended in pH 7.4, 0.067 M phosphate buffer and used to measure its protein content by means of a BCA assay. This assay was carried out in triplicate using normal HSA as the standard and the control support as the blank. The same assay was also carried out using samples that contained 0.5 μM soluble BMH or a control support that was prepared from the reference HSA silica after treating this material with a two-fold mol excess of BMH, following protein immobilization. The soluble BMH did not have any noticeable effect on the results of the BCA assay, but the BMH-treated HSA control support gave a result that was 37.7% (± 2.1%) lower than that obtained for the same support before treatment with BMH. All of the protein assay results reported in this study and that involved BMH-treated HSA have been corrected for this BMH-induced decrease in response, based on the results of these control experiments.

2.4 Chromatographic studies

The supports that were used in the chromatographic studies were downward slurry packed into separate 10 mm × 2.1 mm i.d. microcolumns at 4000 psi (28 MPa) or into 5 mm × 2.1

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mm i.d. microcolumns at 3000 psi (20 MPa). The packing solution was pH 7.4, 0.067 M potassium phosphate buffer. The microcolumns were stored in the same buffer at 4 °C when not in use. The column-to-column variation in overall support content, as determined by making replicate injections of the tested drugs onto several new columns of the same size and packed with the same HSA support, was ± 0.4 to 6.8%. The mobile phase used in the chromatographic experiments was pH 7.4, 0.067 M potassium phosphate buffer, except where otherwise indicated. All of the sample injections were made at 37°C. The following wavelengths were employed for absorbance detection: warfarin, 308 nm; verapamil, 229 nm; carbamazepine, 284 nm; ethacrynic acid, 280 nm; and sodium nitrate, 205 nm.

The measurement of retention factors was made by injecting 5 μL samples that contained 10 μM of racemic warfarin, racemic verapamil, or carbamazepine dissolved in the mobile phase. All samples were injected in quadruplicate onto 5 mm or 10 mm × 2.1 mm i.d. HSA microcolumns at 0.10 to 1.5 mL/min. Because some non-specific binding has been reported for carbamazepine and verapamil on silica supports [25,38-40], all of the drugs that were examined in this study were also injected onto control columns containing no HSA to correct for these secondary interactions. Sodium nitrate, which had no retention on the HSA reference columns or control columns [11,19], was used as a void marker; this compound was injected onto each microcolumn under the same conditions as used for each drug. The extra-column void time was measured by injecting sodium nitrate onto the HPLC system with a zero dead volume connector being used in place of the column.

The retention due to interactions with BMH was measured by injecting each drug in quadruplicate onto a reference 10 mm × 2.1 mm i.d. HSA microcolumn before and after the microcolumn had been treated with BMH. The BMH solution that was used for this treatment contained 1.25 mM of BMH in pH 7.0, 1.5 M potassium phosphate buffer plus 3.5% DMSO. A syringe pump was used to apply this solution to the HSA microcolumn at 3 μL/min for 15 h at room temperature. The estimated ratio for BMH versus HSA was 20:1 mol/mol while the BMH solution was passing through the column. This column was then washed with pH 7.4, 0.067 M potassium phosphate buffer for 8 h at 0.25 mL/min and room temperature prior to its use in further retention studies.

The chiral separation of *R/S*-warfarin was carried out at 37 °C by injecting of 10 μM racemic warfarin at 1.0 to 1.5 mL/min onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn and using a mobile phase that consisted of pH 7.4, 0.067 M potassium phosphate buffer with 1.5% (v/v) 1-propanol. The injected sample volume was raised from 5 μL, as used in the previous paragraphs, to 10 μL to provide better detection for the broader peak of *S*-warfarin that was produced under these elution conditions. The reversible binding of ethacrynic acid with HSA was examined by injecting 20 μL of 10 μM ethacrynic acid onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn or reference HSA microcolumn at 1.5 mL/min.

In the ultrafast affinity extraction experiments, racemic warfarin, racemic verapamil, carbamazepine and HSA were injected onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn or reference HSA microcolumn. This was carried out by injecting 1 μL samples that contained 10 μM of warfarin or verapamil or a mixture containing 10 μM of the given drug plus 20 μM soluble HSA. In the work with carbamazepine, the drug and protein

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concentrations were 30 μM and 60 μM, respectively. These samples were incubated for at least 30 min prior to injection, with both the sample and mobile phase being preheated to 37 °C before passage through the affinity microcolumn. The flow rates used for ultrafast affinity extraction ranged from 0.25 to 2.5 mL/min for verapamil, 0.25 to 6.0 mL/min for warfarin and 0.05 to 1.0 mL/min for carbamazepine. Each free fraction was calculated by dividing the peak area for the free drug by the peak area for the total drug in the sample [23-25]. The peak areas were measured by using PeakFit 4.12, where the baseline of each chromatogram was normalized through the use of the autofit and subtract baseline options of this program.

3 Results and discussion

3.1 Optimization of HSA modification

Two possible cross-linking and modification strategies were explored for increasing the HSA content of affinity supports that contain this immobilized protein. This modification and cross-linking could be performed either after protein immobilization, as shown in Fig. 1(a), or before immobilization, as illustrated in Fig. 1(b).

In the approach represented by Fig. 1(a), HSA was first immobilized onto silica, as achieved in this particular study by using the Schiff base method. Soluble HSA and BMH were then incubated with this immobilized HSA silica for cross-linking and modification of the soluble and immobilized forms of this protein. A support prepared by using only the Schiff base immobilization method had a protein content of 61.4 (± 0.7) mg HSA/g silica; this content was representative of the typical range of 55-65 HSA/g silica that has been reported for the same method and type of support in previous studies [24,25]. Initial experiments with the strategy shown in Fig. 1(a) were carried out by placing 40 mg of this HSA support (with 2.4 mg immobilized HSA) into a 0.25 mL slurry containing 10 mg/mL soluble HSA and 0.60 mM BMH in pH 7.0, 0.10 M phosphate buffer and 0.15 M NaCl. These conditions gave a 15.3 (± 2.2)% increase in the final protein content of the support, with no appreciable increase in this content being noted as the amount of added soluble HSA was increased by 4-fold. The soluble HSA and BMH were also added in three portions to the HSA silica in 5 h intervals, which only gave an increase in protein content of up to 3.7 (± 2.0)% when compared to the original support.

These small or negligible changes in the immobilized protein content when using the strategy in Fig. 1(a) were probably the result of steric hindrance that prevented the soluble HSA from reaching the immobilized HSA and/or orientation effects that made it difficult for the immobilized and soluble HSA to align properly for cross-linking through their modified or free sulfhydryl groups [14]. It is also likely that the BMH had cross-linked some of the soluble HSA with other molecules of soluble HSA, which would then not have been available for reacting with the free sulfhydryl groups on the immobilized HSA.

In a second approach, as illustrated in Fig. 1(b), the HSA was first dissolved in the reaction buffer and then incubated with BMH for cross-linking and modification. After removing the excess BMH, the cross-linked or modified proteins were immobilized by the Schiff base method. This method was performed by dissolving 5-20 mg of HSA in 1.0 mL of the BMH

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reaction buffer. This mixture initially contained 0.00, 0.15 mM or 0.75 mM BMH for a 5 mg HSA sample; or 0.00, 0.60 or 3.01 mM BMH for a 20 mg HSA sample, providing a 0:1, 2:1, or 10:1 mol/mol ratio for BMH versus HSA. When the concentration of BMH was 10-fold higher than that of the soluble HSA, the protein content of the final support was increased by 64 (± 4)% or 65 (± 3)% for solutions containing 5.0 mg/mL or 20.0 mg/mL of HSA and when compared to an immobilization scheme in which no BMH had been added. When the concentration of BMH was only 2-fold higher than the concentration of soluble HSA, the protein content of the final support increased by 75 (± 4)% when using 20.0 mg/mL of soluble HSA. However, decreasing the BMH content further (e.g., down to a 1:2 mol/mol ratio of BMH versus HSA) did not provide any further increase in the protein content.

The concentration of the reaction buffer that was used in both of the strategies that are shown in Fig. 1 was also considered. This was done by increasing the concentration of the pH 7.0 phosphate buffer to 1.5 M [10,19,41]. It has been suggested that increasing the ionic strength of the buffer for this reaction can inhibit charge repulsion of adjacent proteins and increase the rate of nucleophilic addition of a sulfhydryl group to maleimide [41]. For the immobilization/modification strategy in Fig. 1(a), no apparent increase in protein content was found when a higher ionic strength for the buffer was used during the modification reaction. However, an increase as high as 113 (± 2)% was observed when this same buffer was used for the modification/immobilization strategy in Fig. 1(b). This modification/ immobilization strategy and set of conditions were later employed in the following sections of this study.

3.2 Effects of protein modification on retention

The next set of studies sought to characterize the effects of combining protein modification and immobilization on the retention that was observed for various drugs on HSA columns. One drug that was used in these experiments was warfarin, which is an anticoagulant known to bind to HSA at Sudlow site I [26-28,36]. Two other drugs that were tested were verapamil, a calcium channel blocking agent that has a primary binding site at Sudlow site I [38], and carbamazepine, an anticonvulsant that binds to Sudlow site II [39]. These drugs represented binding strengths that spanned roughly a 100-fold range in affinities for HSA, with reported association equilibrium constants (K_a) at 37 °C and pH 7.4 of 2.1-2.6 × 10^5 M^{-1} for racemic warfarin, 1.4 (± 0.1)× 10^4 M^{-1} for racemic verapamil, and 5.2-5.5 × 10^3 M^{-1} for carbamazepine [26-28,36,38-40].

Fig. 2 shows some typical chromatograms that were obtained for these drugs on BMH-treated HSA microcolumns and reference HSA microcolumns. As is suggested by these results, the BMH-treated HSA columns gave a significant increase in retention for each of the tested drugs. In the case of verapamil, a partial chiral separation with a resolution (R_s) of 0.61 (± 0.06) could be achieved by the BMH-treated HSA microcolumns in the presence of only pH 7.4 phosphate buffer at some of the flow rates that were used; however, no chiral separation was observed at the same flow rates when using the reference HSA microcolumn, as indicated in Fig. 2(a). Both types of columns gave at least a partial separation for racemic warfarin in the presence of only pH 7.4, 0.067 M phosphate buffer, with R_s = 0.59 (± 0.02) on the reference HSA microcolumn and 0.74 (± 0.02) on the BMH-treated HSA

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microcolumn, as shown in Fig. 2(b) (Note: the elution order of the warfarin enantiomers under similar operating conditions and on other HSA columns has been determined previously) [19,20,26].

It was possible to increase the extent of the chiral separation for warfarin to nearly baseline resolution, with R_s = 1.39 (± 0.01) at 1.0 mL/min and R_s = 1.32 (± 0.05) at 1.5 mL/min, for the BMH-treated HSA microcolumn by including a small amount of 1.5% 1-propanol in the mobile phase. An example of such a separation is provided in Fig. 2(c). Prior work under similar conditions and with the same type of column as used here for the reference HSA microcolumn gave a resolution of 1.12 (± 0.03) at 1.0 mL/min [24]. Along with the changes in retention that occurred due to the increase in protein content, part of the increase in the chiral selectivity for the BMH-treated HSA microcolumns may have been a result of the modification of Cys34. For instance, an 11.4% increase in the separation factor, or enantioselectivity, for racemic warfarin that has been observed on HSA columns before and after Cys34 was covalently modified with ethacrynic acid [20].

Table 1 lists the average retention factors that were obtained at 37 °C and pH 7.4 for each drug on 10 mm × 2.1 mm i.d. microcolumns containing the BMH-treated HSA support or the reference HSA support. For each drug and tested flow rate, there was a consistent and significant increase in the total retention factor that was measured on the BMH-treated HSA microcolumns compared to the reference HSA microcolumns. This overall increase ranged from 88% for warfarin to 100-105% for carbamazepine and 157-168% for verapamil. Part of this increase was found to be the result of non-specific interactions by some of the drugs with BHM residues, as determined through control studies that compared retention on a reference HSA column vs. the same column after treatment with BMH, but with no cross-linking to soluble HSA. The increase in retention due to these non-specific interactions was found to be only 4.3 (± 6.1)% for carbamazepine and 18.1 (± 6.3)% for warfarin; however, this effect lead to 73 (± 21)% of the increase in retention that was noted for verapamil. This trend agrees with previous reports in which verapamil has been noted to have much higher non-specific interactions on other types of affinity microcolumns than warfarin, carbamazepine or other drugs [25,38].

The retention increase due to the immobilized HSA was determined by correcting the overall change in retention for the increase due to the non-specific interactions with BMH. These corrected values are also included in Table 1. The size of this corrected increase in retention was now in the general range of 70-100% for all of the tested drugs, regardless of whether these drugs were known to bind at Sudlow site I (i.e., warfarin and verapamil) or at Sudlow site II (carbamazepine) [26-28,36,38,39]. As noted in the previous section, the use of BMH as a cross-linker and modifying agent gave a 75-113% increase in the overall protein content of the modified HSA supports when they were compared to the reference HSA supports that were prepared by using only covalent immobilization. The observed increase in protein-based retention in Table 1 was in the same general range as this increase in protein content. The similarity in these values indicates that the increase in retention was directly related to the higher protein content of the BMH-treated HSA supports. This increase was probably also aided by the site-selective nature of the modification process that

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was used in this study and the fact that Cys34, the modification site, is distant from both Sudlow sites I and II of HSA [4,13,21-22].

3.3 Use of BMH-treated HSA microcolumns with a sulfhydryl-reactive drug

One unique application that was explored for the BMH-treated HSA microcolumns was their use in studying the reversible binding of a drug that can also undergo a covalent linkage with HSA at Cys34. Ethacrynic acid was used as the model drug for this work. Ethacrynic acid is a diuretic drug that can rapidly form a covalent bond with Cys34 at a neutral pH (see Fig. 3) [20,42-44]. In prior work, this drug has been used to modify HSA columns to distinguish between reversible and covalent interactions by other solutes with this protein [20,43-44]. However, this drug is also known to be able to reversibly bind at major two sites on HSA (i.e., Sudlow sites I and II) [20,42-45]. Isothermal titration microcalorimetry and circular dichroism spectroscopy have previously been used to study these latter processes, resulting in estimated binding constants on the order of 10^5-10^6 M^{-1} for these reversible interactions [42,44,46,47].

Fig. 3(b) shows some chromatograms that were obtained when ethacrynic acid was injected at 1.5 mL/min onto a 10 mm × 2.1 mm i.d. reference HSA microcolumn or a BMH-treated HSA microcolumn. On the BMH-treated HSA column, a peak for ethacrynic acid was observed with a retention time of about 12 min. This value corresponded to a retention factor of 502 (± 2) at 37 °C and pH 7.4 and is the type of behavior that would be expected for a strong but reversible drug-protein interaction. However, no peak was seen when the same drug sample was injected onto the reference HSA microcolumn, as would occur if this drug were quickly and covalently binding to HSA. Injections of ethacrynic acid onto an inert control column with no protein present gave a peak area for ethacrynic acid that differed by only 3.0% from that observed on the BMH-treated HSA microcolumn, indicating that all the injected ethacrynic acid was being eluted and recovered from this microcolumn. In addition, the retention factor for ethacrynic acid on the control column, 0.56 (± 0.12), was negligible compared to the overall retention of this solute on the BMH-treated HSA column. Neither the area or position of this peak was affected by including up to 100 μM BMH as a competing agent in the injected sample, confirming that this retention was due to the interaction of ethacrynic acid with HSA rather than with the groups added by BMH to HSA.

The retention factor that was measured for ethacrynic acid on the BMH-treated HSA microcolumn was used to estimate the global affinity constant (nK'_a) for this drug at its reversible binding sites on HSA. This value was determined by using the relationships given in Eqs. (1-2) [4,18,21].

$$k=\frac{(n_1K_{a1}+n_2K_{a2}+\cdots+n_nK_{an})\,m_L}{V_M} \quad (1)$$

$$k=\frac{\left(nK'_a\right)m_L}{V_M} \quad (2)$$

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In these equations, the terms K_{a1} through K_{an} represent the association equilibrium constants for ethacrynic acid at each of its reversible binding sites with HSA, and n_1 through n_n are the mole fractions for each type of site in the column, where nK'_a is the sum of n_1K_{a1} through n_nK_{an}. The term m_L is the total moles of all binding sites for ethacrynic acid in the column, and V_M is the void volume of the column.

The interactions of ethacrynic acid at its two major and reversible binding sites on HSA (i.e., Sudlow sites I and II) are known to have binding constants that are roughly two orders of magnitude higher than those for any weak binding sites that are present for this drug on HSA [42,44,46,47]. Under these conditions, Eq. (1) can be simplified to the two-site form in Eq. (3),

$$k=\frac{(n_1K_{a1}+n_2K_{a2})\,m_L}{V_M}=\frac{nK'_a\,(m_{L1}+m_{L2})}{V_M} \quad (3)$$

where n_1 and n_2 are the relative number of sites 1 and 2 (e.g., Sudlow sites I and II on HSA), and m_{L1} and m_{L2} are the corresponding moles of these sites in the column. It was possible in this study to estimate the values of m_{L1} or m_{L2} by using Eq. (2) along with the void volume of the column, the corrected retention factors that had been measured on the BMH-treated HSA microcolumns for warfarin and carbamazepine (i.e., probes for Sudlow sites I and II), and the known association equilibrium constants of these drugs with HSA (see Table 2). By then placing these values for m_{L1} and m_{L2} into Eq. (3), along with the column void volume and measured retention factor for ethacrynic acid, the global affinity constant for the reversible interactions of ethacrynic acid with HSA could be obtained. This approach gave a global affinity constant for ethacrynic acid with HSA of 3.2 (± 0.1) × 10^5 M^{-1} at 37 °C and pH 7.4. This value was in good agreement with previous literature values of 1.65 × 10^5 M^{-1} to 1.2 × 10^6 M^{-1} that have been obtained under similar conditions by using techniques such as circular dichroism or isothermal titration microcalorimetry [42,45-47].

3.4 Use of BMH-treated HSA microcolumns in ultrafast affinity extraction

Another application considered for the BMH-treated HSA microcolumns was their use in ultrafast affinity extraction and free drug fraction measurements. These experiments were carried out by using samples that contained a drug in the presence or absence of soluble HSA, with small amounts of these samples being injected onto a BMH-treated HSA microcolumn or a reference HSA microcolumn [23-25]. These injections were made under column size and flow rate conditions that allowed part or all of the non-bound (or free) drug fraction in the sample to be extracted by the immobilized HSA without allowing sufficient time for dissociation of a significant portion of the protein-bound form of the drug in the sample. As shown in Fig. 4(a), the excess protein and original drug-protein complex eluted as a non-retained peak in this experiment, while the extracted free form of the drug was retained and eluted later from the column. This separation made it possible to detect and measure the free drug fraction without interference from the protein or drug-protein complex in the sample. By comparing the retained peak area for the free drug and the total peak area for the same drug in the absence of any soluble HSA, the free drug fraction was then determined for the original sample [23-25]. This approach has recently been used to measure

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free drug fractions in clinical samples or drug/protein samples at typical therapeutic and clinical concentrations, as based on the reference HSA columns that were employed in this current study. This method has been found in such experiments to give good correlation with reference methods, such as ultrafiltration [24].

The residence time of the drug/protein sample in the affinity extraction column is an important factor to consider in this type of free fraction analysis. The use of a relatively long sample residence time may cause dissociation of the protein-bound form of a drug, resulting in a high value for the apparent free fraction. This effect can be minimized by using a short column and/or by increasing the flow rate that is used for sample injection [23-25]. It is also necessary to use a column size and flow rate conditions that allow sufficient resolution to be obtained between the non-retained and retained drug fractions to avoid having high free fraction measurements [23-25].

Fig. 4(b) shows the results of free fraction measurements for verapamil/HSA mixtures that were injected onto both a BMH-treated HSA microcolumn and a reference HSA microcolumn at various flow rates. The apparent free fraction decreased as the flow rate was increased until a consistent free fraction was obtained when the flow rate was at or above 1.25 mL/min on the BMH-treated HSA column. However, the same measurements on the reference HSA microcolumn required a flow rate of at least 1.75 mL/min for consistent free fractions to be obtained (Note: typical chromatograms for verapamil at these flow rates are provided in Fig. 4(a). When used at or above these minimum flow rates, both the BMH-treated HSA microcolumn and reference HSA microcolumn provided statistically-equivalent values (at the 95% confidence level) for the measured free drug fractions. The differences in the flow rate requirements for these measurements probably reflect the higher retention and better resolution that was between the free and bound drug peaks on the BMH-treated HSA microcolumns, which also helped minimize positive errors in the apparent free drug fraction [3,10]. The same overall trends were observed for warfarin and carbamazepine (see Fig. 5), in which the two types of columns gave essentially identical free drug fractions at high flow rates, but with lower or equivalent flow rates being needed for these measurements on the BHM-treated HSA columns.

The free drug fractions that were measured at or above the optimum flow rates were used to estimate the association equilibrium constants for each drug with soluble HSA. These values were calculated by using Eq. (4) [23,24].

$$K_{\mathrm{a}}=\frac{1-F_0}{F_0\left([\mathrm{P}]_{tot}-[\mathrm{D}]_{tot}+[\mathrm{D}]_{tot}F_0\right)} \quad (4)$$

In Eq. (4), F_0 is the free drug fraction that is measured at equilibrium (i.e., at or above a flow rate that minimizes drug-protein dissociation in the sample), while $[D]_{tot}$ and $[P]_{tot}$ are the total concentrations of the drug and soluble protein in the original sample. The value of K_a in this equation is the association equilibrium constant for a drug and protein with a single-site interaction, or the global affinity constant for a drug that has multiple but independent binding sites on the protein [23,24].

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Table 2 shows the K_a values that were obtained by using Eq. (4) and free drug fractions that were measured on the BMH-treated HSA microcolumns or reference HSA microcolumns. The values that were obtained in this report had relative precisions in the range of ± 7-8% and were based on peak area measurements that could be made within 1 to 6.5 min of sample injection. In each case, there was good agreement between the K_a values that were measured on these columns and with reference values that have been determined under the same pH and temperature conditions. These results confirmed that the BMH-treated HSA microcolumns could be successfully used to quickly estimate K_a values for drug-protein binding based on free drug fraction measurements.

4 Conclusions

This study developed a new hybrid method to produce enhanced capacity affinity microcolumns containing HSA by combining BMH as a reagent for protein modification or cross-linking with a covalent immobilization method. Various factors were considered in determining the optimum conditions for this method, including the use of BMH modification before or after protein immobilization. The supports that were obtained in the final method had up to a 75-113% increase in protein content when compared to supports that were prepared by using only covalent immobilization. These BMH-modified HSA supports also gave a large increase in protein-based retention (i.e., 70-100%) versus a reference HSA support when both types of materials were used in microcolumns to bind drugs known to interact at Sudlow sites I or II of HSA.

The BMH-treated HSA microcolumns were tested for use in estimating free drug fractions and association equilibrium constants for drug-protein interactions. These columns gave results comparable with those determined by reference HSA microcolumns or reported in literature. In addition, the BMH-treated HSA microcolumns were used in chiral separations and to investigate the reversible interactions between HSA and ethacyrnic acid, a drug which can also covalently bind to HSA through its free sulfhydryl group. This type of hybrid modification/immobilization scheme is not limited to HSA but should be useful in future work with other proteins and in the study of alternative drug-protein systems (e.g., the binding of HSA with benzodiazepines, nonsteroidal anti-inflammatory drugs, or platinum-contained drugs) [13,20,22,44]. Possible applications for such columns include the high-throughput analysis of drug-protein binding, protein-based chiral separations, the analysis of free drug fractions in clinical or pharmaceutical samples, the screening of drug candidates for their protein interactions, and the creation of affinity-based microscale separation devices [23-25].

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Highlights

- A hybrid modification and immobilization method was developed for proteins.
- This method was used to make enhanced capacity affinity supports and microcolumns.
- This approach was optimized by using human serum albumin as a model protein.
- The final supports were used in drug-binding studies and ultrafast affinity extraction.

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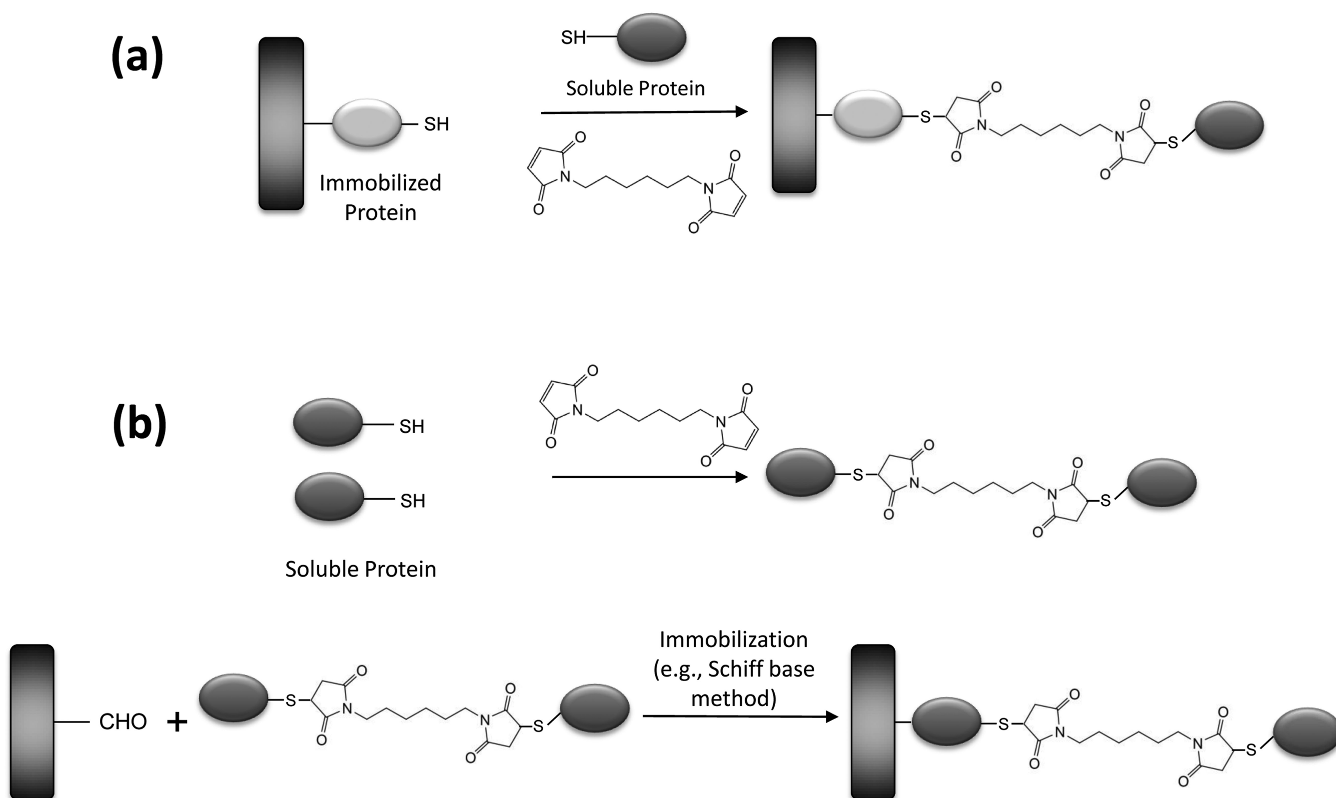

Figure 1.

General schemes considered for the preparation of high capacity HSA supports, such as (a) immobilization of HSA followed by modification/cross-linking of the immobilized protein with soluble protein or (b) modifying/cross-linking of soluble HSA, followed by immobilization of the resulting protein products.

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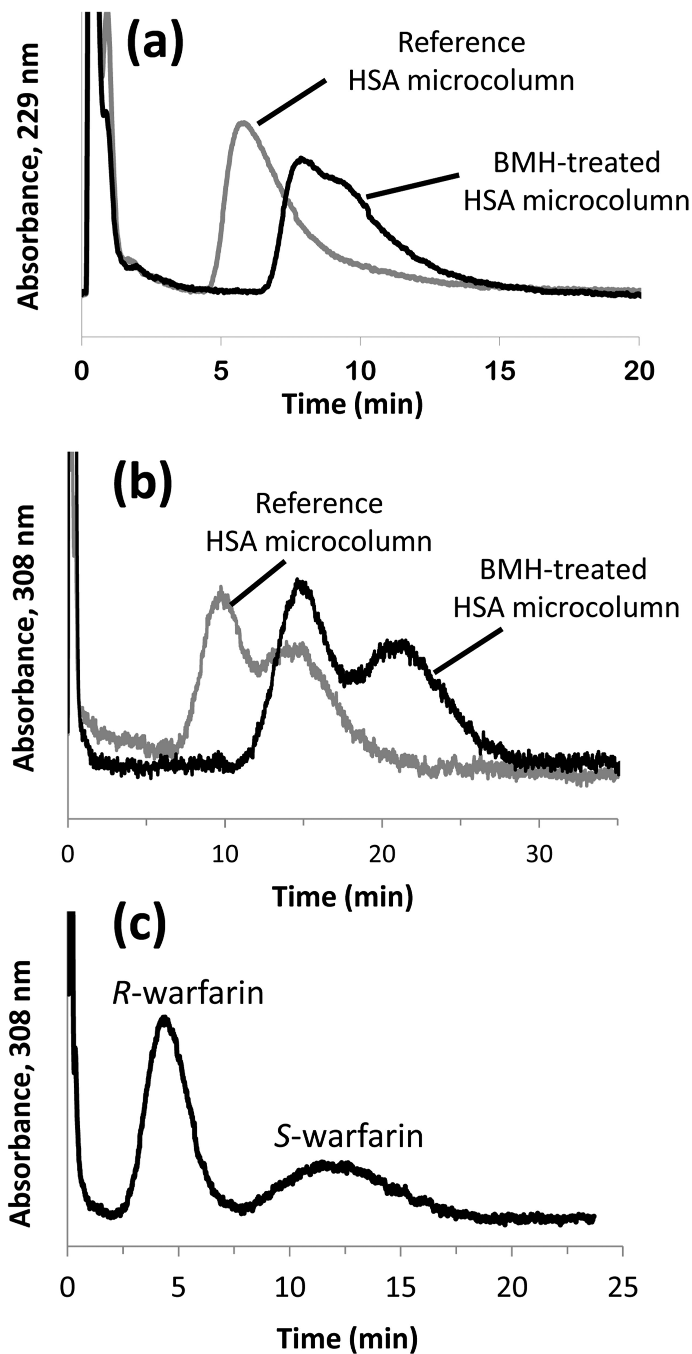

Figure 2.

Chromatograms obtained at pH 7.4 for the injection of (a) racemic verapamil onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn or a reference HSA microcolumn at 0.25 mL/min; (b) racemic warfarin onto the same columns at 0.5 mL/min; or (c) racemic warfarin onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn at 1.5 mL/min in the presence of 1.5% (v/v) 1-propanol. All of these separations were carried out using BMH-treated HSA columns that were prepared with the scheme described in *Section 3.1*.

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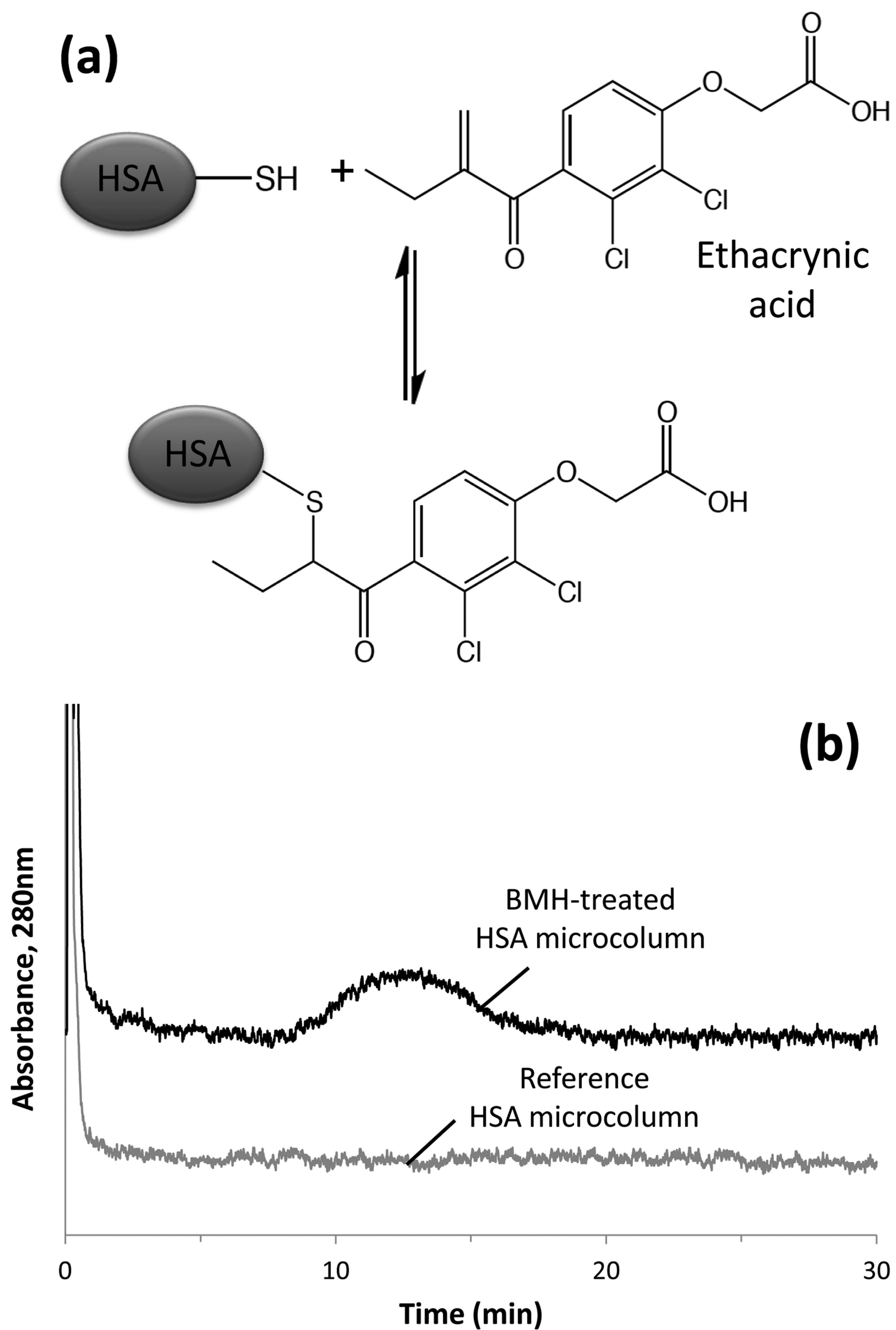

Figure 3.

(a) The reaction between ethacrynic acid and Cys34 on HSA and (b) chromatograms obtained at 1.5 mL/min for injections of ethacrynic acid onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn or a reference HSA microcolumn.

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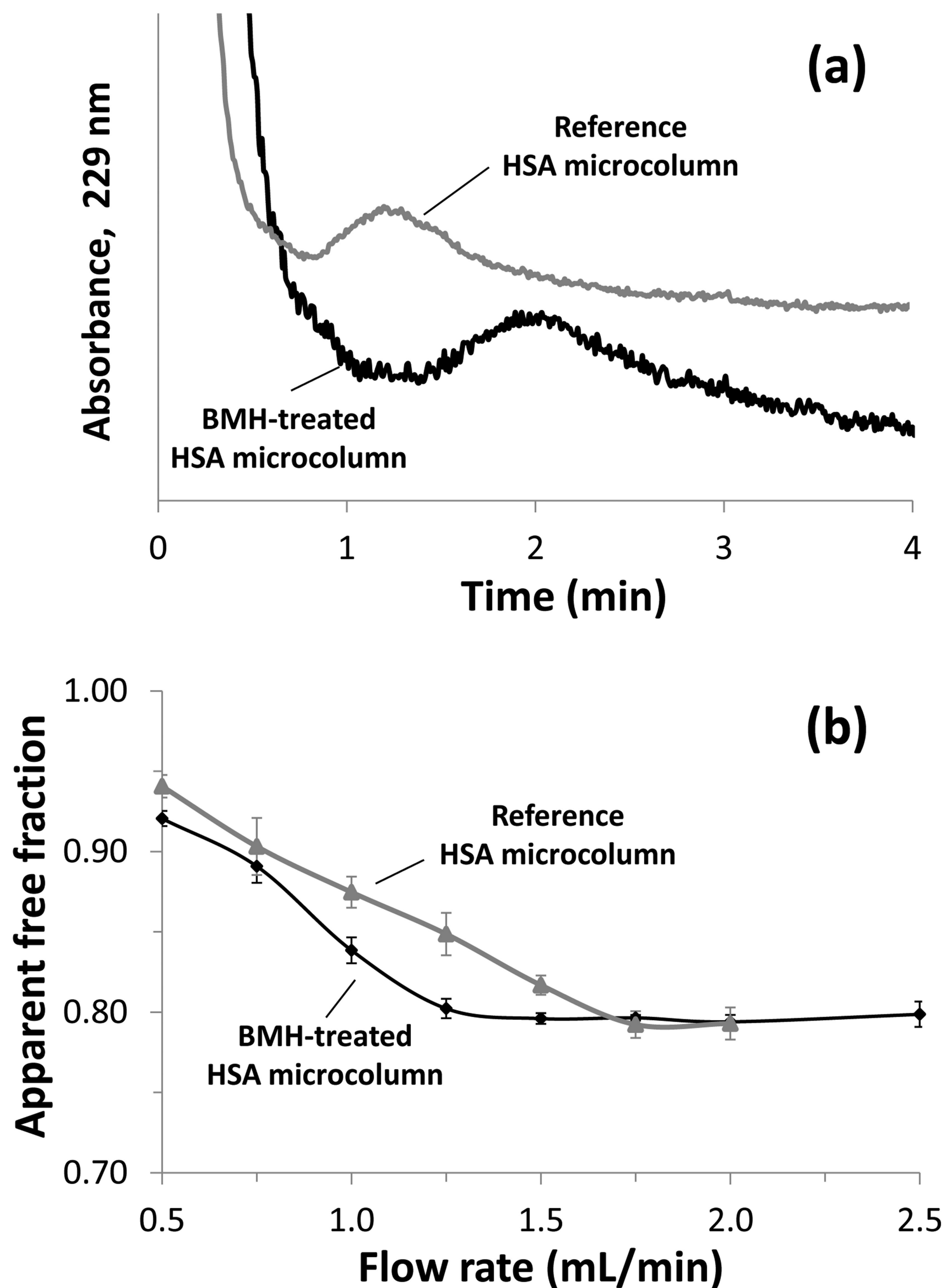

Figure 4.

(a) Chromatograms obtained for injections of racemic verapamil/HSA made at 1.25 mL/min onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn (black) or at 1.75 mL/min on a reference HSA microcolumn of the same size (gray), and (b) the effect of flow rate on the apparent free drug fractions that were measured for the same samples on the BMH-treated HSA microcolumn (modification/immobilization strategy; black line, ◆) or the reference HSA microcolumn (gray, ▲). The error bars represent a range of ± 1 S.D. of mean (n = 4).

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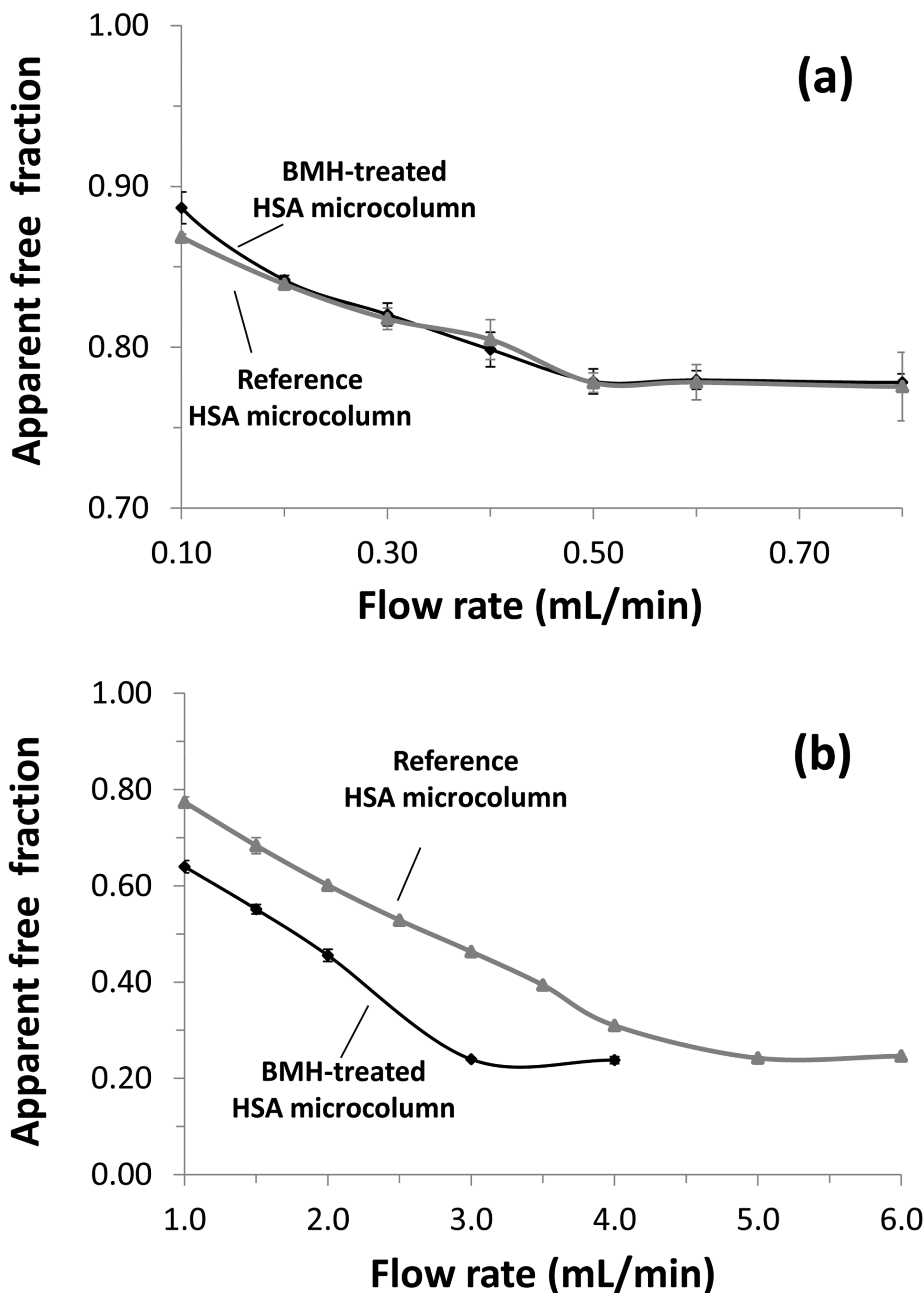

Figure 5.

Effect of flow rate on the apparent free drug fractions measured for (a) carbamazepine/HSA (b) or warfarin/HSA samples injected onto a 10 mm × 2.1 mm i.d. BMH-treated HSA microcolumn (modification/immobilization strategy; black line, ◆) or a reference HSA microcolumn of the same size (gray, ▲). The error bars represent a range of ± 1 S.D. of mean ($n = 4$), where this range is often comparable to the size of the data markers that are used in these plots.

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Table 1

Retention factors (k) measured on BMH-treated HSA supports and standard, reference HSA supports[a]

Drug	Flow rate (mL/min)	k, Reference HSA support	k, BMH-treated HSA support	Total change in k (%)	Specific change in k due to HSA (%)[b]
Warfarin[c]	1.50	116 (± 7)	218 (± 3)	88 (± 7)	70 (± 9)
	0.50	124 (± 3)	233 (± 5)	88 (± 5)	70 (± 8)
Verapamil	1.00	13.3 (± 2.2)	35.7 (± 1.5)	168 (± 20)	95 (± 29)
	0.50	14.4 (± 1.2)	37.3 (± 1.5)	159 (± 13)	86 (± 24)
	0.25	13.7 (± 1.6)	35.2 (± 2.2)	157 (± 20)	84 (± 29)
Carbamazepine	0.50	2.3 (± 0.1)	4.6 (± 0.3)	100 (± 14)	96 (± 15)
	0.25	2.1 (± 0.1)	4.2 (± 0.2)	100 (± 11)	96 (± 13)
	0.10	2.1 (± 0.2)	4.3 (± 0.1)	105 (± 11)	100 (± 12)

[a]The retention factors were determined at pH 7.4 and at 37°C for 10 mm × 2.1 mm i.d. columns. These values were obtained after subtracting out the retention factors measured on the control columns, which had the following values: warfarin, 0.8 (± 0.6); verapamil, 28.8 (± 0.7); and carbamazepine, 2.1 (± 0.5). The values in parentheses represent a range of ± 1 S.D., as determined by error propagation.

[b]The estimated retention due to interactions with BMH were 18.1 (± 6.3)% for warfarin, 73 (± 21)% for verapamil, and 4.3 (± 6.1)% for carbamazepine.

[c]The results provided for warfarin and verapamil are based on the average retention factors for R- and S-enantiomers. The separate enantiomers would have slightly lower or higher retention factors than these values.

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Table 2

Association equilibrium constants measured for various drugs with soluble HSA by using free fraction analysis on a BMH-treated HSA microcolumn or reference HSA microcolumn[a]

Drug	Reference HSA microcolumn	BMH-treated HSA microcolumn	Literature value [Ref.]
Warfarin	2.5 (± 0.2) × 10^5 M^{-1}	2.6 (± 0.2) × 10^5 M^{-1}	2.1-2.6 × 10^5 M^{-1} [26]
Verapamil	1.5 (± 0.4) × 10^4 M^{-1}	1.4 (± 0.1) × 10^4 M^{-1}	1.4 (± 0.1) × 10^4 M^{-1} [38]
Carbamazepine	5.3 (± 0.4) × 10^3 M^{-1}	5.3 (± 0.4) × 10^3 M^{-1}	5.2-5.5 × 10^3 M^{-1} [39,40]

[a]These results obtained in this study were measured at pH 7.4 and at 37°C. The values in parentheses represent a range of ± 1 S.D., as determined by error propagation. The measured values for warfarin and verapamil are for racemic mixtures and represent the average values for the enantiomers. The reference value given for warfarin is the range of association equilibrium constants for the *R*- and *S*-enantiomers; the value given for verapamil is average result for the enantiomers at the high affinity site of this drug on HSA.