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Development of an On-line Immunoextraction/Entrapment System for Protein Capture and Use in Drug Binding Studies by High-Performance Affinity Chromatography

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Abstract

An on-line purification and entrapment system was developed that could extract a protein from a sample such as serum and entrap this protein within a small column for use in high-performance affinity chromatography. Human serum albumin (HSA) was employed as a model protein for this work. Immunoextraction columns containing polyclonal anti-HSA antibodies were developed to capture and isolate HSA from applied samples. This was followed by the use of a strong cation-exchange column to recapture and focus HSA as it eluted from the immunoextraction columns. The recaptured HSA was entrapped within 1.0 cm × 2.1 mm I.D. columns containing hydrazide-activated silica and in the presence of oxidized glycogen as a capping agent. The binding and elution properties of HSA on the various components of this system were examined and optimized. The entrapped columns produced by this system were then evaluated for their use in binding studies with several sulfonylurea drugs. The HSA columns created by this approach typically contained 0.3–0.6 nmol HSA and were stable over several weeks and more than 50–60 sample injections. Drug binding constants could be determined with these columns in 8 min or less by zonal elution and gave good agreement with literature values. The same system could be used for the capture and entrapment of other proteins by utilizing antibodies against the given target for immunoextraction.

Keywords

protein immobilization; entrapment; immunoextraction; human serum albumin; drug-protein binding; high-performance affinity chromatography

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COI statement

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1 Introduction

Human serum albumin (HSA) is the most abundant transport protein in serum, with an average physiological concentration of 42 g/L [1,2]. HSA has a fundamental role in the transportation of drugs and many other endogenous ligands in blood [1–3]. Binding to HSA can affect the free and active concentrations of drugs in the circulation, which can in turn impact a drug's absorption, distribution, metabolism, and excretion [1–4].

One approach that can be used to examine the binding of drugs with HSA is high-performance affinity chromatography (HPAC) [5,6]. HPAC is a technique which combines the use of an HPLC support with the selective and reversible interactions that can occur between a given analyte and an immobilized biologically-related binding agent for this target [6]. Columns for this method are often prepared through covalent immobilization of a protein such as HSA [7,8]. However, this type of immobilization can lead to multisite attachment or improper orientation of a protein and a loss of activity [7].

Many of these immobilization effects can be avoided by physically entrapping a binding agent such as a protein within a support [7,8]. For instance, it has been shown in recent years that this can be accomplished by using the reaction of aldehydes on mildly-oxidized glycogen with a hydrazide-activated support to entrap a soluble protein within HPLC-grade silica (see Figure 1) [8]. This method has previously been used to immobilize HSA and other serum proteins for use in HPAC and drug binding studies [8–12]. Advantages of this method include the ability of low-to-moderate mass drugs to access the entrapped protein and for protein to remain in a soluble and fully-active form [8,9].

In this study, an on-line immunoextraction and entrapment system will be developed that can isolate a protein such as HSA from samples like serum and entrap this protein within a small HPAC column (see Figure 2). This system will use polyclonal anti-HSA antibodies in an immunoextraction column to capture and isolate HSA from an applied sample. A strong cation-exchange (SCX) column will be used to recapture and focus the HSA as this protein elutes from the immunoextraction column. The recaptured HSA will then be entrapped within a small column containing hydrazide-activated silica and in the presence of oxidized glycogen as a capping agent. The binding and elution properties of HSA with the immunoextraction and SCX columns will be examined, along with the ability of the overall system to produce small columns that contain entrapped HSA. The prepared columns will then be evaluated in drug binding studies with the entrapped HSA, using several sulfonylurea drugs as models [13–16]. The results that are obtained with HSA as a model should provide valuable guidelines on the use of such a system and the creation of entrapment-based columns for future work with other proteins.

2 Materials & Methods

2.1 Materials

The polyclonal anti-HSA antibodies (goat, affinity-purified polyclonal antibodies, product 2080–01) were purchased from VWR (Radnor, PA, USA). The polyclonal anti-HSA goat serum (product A1151), protein G Sepharose 4B fast flow (recombinant protein expressed in

E. coli), immunoglobulin G (IgG; goat, ≥ 95% pure), HSA (essentially fatty acid free, ≥ 96%), glycogen (from bovine liver, ≥ 85% glucose), oxalic dihydrazide (> 99%), acetohexamide (99%), glibenclamide (≥ 99%), and racemic warfarin (≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Nucleosil Si-300 and Si-1000 silica (7 μm, particle diameter; pore size, 300 or 1000 Å, respectively) were acquired from Macherey-Nagel (Duren, Germany). All buffers and aqueous solutions were prepared using water from a Milli-Q Advantage 10 A Water system and were filtered using 0.2 μm nylon membranes (EMD Millipore Corporation, Billerica, MA, USA).

2.2 Instrumentation

The immunoextraction/entrapment system was developed using a Jasco 2000 series HPLC system (Tokyo, Japan) and two Harvard apparatus PHD Ultra syringe pumps (Hilston, MA, USA). The components of the Jasco system were as follows: three PU-2080 pumps, an AS-2057 autosampler, a DG-2080–53 degasser, a CO-2067 column oven, a LV-2080–03 solvent selector and two UV-2075 Plus absorbance detectors. This HPLC system also employed three Rheodyne Advantage PF six-port valves (Cotati, CA, USA), which were used to alternate the flow and application of buffers to the columns of this system (see Supplemental Material). The HPLC/syringe pump system was operated using LC-Net and ChromNav V 1.18 software from Jasco. Chromatograms were analyzed by using PeakFit 4.12 software from Jandel Scientific (San Rafael, CA, USA) and Microsoft Excel 2016 (Redmond, WA, USA). Ultrafiltration and buffer exchange were carried out by using VivaSpin 6 ultrafiltration spin columns (30 kDa) from Sartorious Stedim (Gottingen, Germany) and a 5702RH temperature-controlled centrifuge (Eppendorf, New York, NY, USA). The centrifuge was operated at 3000 rpm and 25°C in most cases except during the purification of oxidized glycogen, when the temperature was 37°C.

2.3 Development of immunoextraction columns

The anti-HSA immunoextraction columns were prepared by using affinity-purified polyclonal anti-HSA antibodies or anti-HSA goat serum. The affinity-purified anti-HSA antibodies were immobilized onto Nucleosil-1000 silica via the Schiff base method, as previously described [17]. A control support was developed in the same manner but in the absence of antibodies. Prior to immobilization, the buffer in which the anti-HSA antibodies were supplied was exchanged by ultrafiltration, with the antibodies being placed into pH 6.0, 0.10 M potassium phosphate buffer. The protein content of the anti-HSA support was determined in triplicate by utilizing a micro bicinchoninic acid (BCA) protein assay kit obtained from Thermo Fisher Scientific (Waltham, MA, USA). In this assay, goat IgG was used as the standard and the control support was employed as the blank.

Isolation of polyclonal anti-HSA antibodies from anti-HSA goat serum was performed by using HSA silica [17] or protein G Sepharose [18]. A 200 mg portion of lyophilized anti-HSA goat serum was dissolved in 2.0 mL of pH 7.4, 0.067 M potassium phosphate buffer and added to 4 mg of protein G Sepharose or 0.50 g of HSA silica, with the latter being prepared using Nucleosil Si-1000 silica and the Schiff base method [17]. The serum and support slurry were allowed to mix at room temperature for 2 h. The slurry was centrifuged at 7500 rpm for 3 min and room temperature to separate the retained anti-HSA antibodies

from other serum components. The supernatant was decanted, and the support washed with 2.0 mL of the pH 7.4 buffer prior to another centrifugation and decanting step. This step was repeated a total of three times. The retained anti-HSA antibodies were eluted from the protein G Sepharose or HSA silica by washing the support with three 1.0 mL portions of pH 2.5, 0.10 M potassium phosphate buffer, followed by a centrifugation step and recollection of the supernatant after each step. The pH of the collected anti-HSA antibodies was adjusted to pH 6.0 by adding pH 8.0, 0.10 M potassium phosphate buffer. These antibodies were immobilized onto 1.0 g of Nucleosil 1000–7 by the Schiff base method [17]. A control support was prepared in the same fashion but without any antibodies being included in the reaction mixture. The protein content of the anti-HSA support was measured by using a BCA assay, as described earlier.

The support made with the affinity-purified anti-HSA antibodies was packed into 1.0 cm × 2.1 mm I.D. stainless steel columns at 4000 psi (27.6 MPa), with pH 7.4, 0.067 M potassium phosphate buffer being used as the packing solution. The anti-HSA support made from anti-HSA goat serum was packed in the same manner into a 2.0 cm and 5.0 cm × 2.1 mm I.D. columns (combined effective length, 7.0 cm). Control columns of the same sizes were also prepared in this manner. The columns were stored all in pH 7.4, 0.067 M potassium phosphate buffer at 4.0 °C. The binding capacity of each anti-HSA column was measured in triplicate by frontal analysis at 0.10 mL/min and by using a 5.0 μM solution of HSA in pH 7.4, 0.067 M potassium phosphate buffer at 37°C. In this measurement, the active antibody content was determined by utilizing the difference in the midpoints of the breakthrough curves that were obtained on the anti-HSA column and a control column [18].

2.4 Preparation of hydrazide-activated silica and oxidized glycogen

Hydrazide-activated silica was prepared by using Nucleosil-300 silica as the starting material, which was first converted into a diol-bonded form [8,19]. The diol-bonded silica was oxidized into an aldehyde form by using periodic acid, and hydrazide groups were incorporated by combining the aldehyde-activated silica with oxalic dihydrazide, as described previously [8,9]. Any remaining aldehyde groups on the support were reduced by using sodium borohydride [8,19]. The hydrazide-activated silica was stored at 4°C in pH 7.0, 0.10 M phosphate buffer, conditions under which this support is stable for up to 6 weeks [19]. This support was packed into 1.0 cm × 2.1 mm I.D columns in the same manner as described in Section 2.3 for the anti-HSA supports. These columns were used within 24 h of being packed to minimize any loss of hydrazide groups on the support prior to entrapment.

Oxidation of glycogen was carried out by reacting 17 mg of bovine glycogen with 135 mg of periodic acid in 4.0 mL of pH 5.0, 20 mM acetate buffer containing 15 mM sodium chloride [9]. The glycogen solution was vortexed for 5 min, degassed for 5 min, and placed on a wrist shaker (set at 300 oscillations per min) for 16 h at room temperature. The oxidized glycogen was purified by ultrafiltration, in which the oxidized glycogen solution was washed three times with water and three more times with pH 6.0, 0.10 M potassium phosphate buffer. Each wash was followed by a centrifugation step that was performed at 3000 rpm and 37 °C. The oxidized glycogen solution was removed from the ultrafiltration device with 4.0 mL of

pH 6.0, 0.10 M potassium phosphate buffer and used immediately in the entrapment of HSA.

2.5 Construction of immunoextraction/entrapment system

The final immunoextraction/entrapment system used an effective anti-HSA column size of 8.0 cm × 2.1 mm I.D. (i.e., one 1.0 cm long column based on affinity-purified anti-HSA antibodies, plus 2.0 cm and 5.0 cm × 2.1 I.D. columns using anti-HSA antibodies from goat serum), along with a 1.0 cm × 2.1 mm I.D. strong cation exchange (SCX) Biobasic guard column (Sigma-Aldrich, St. Louis, MO, USA). The SCX column was used to recapture HSA that was retained and eluted from the immunoextraction columns and to place this protein into a buffer that could be used for entrapment. The elution of HSA during the development and utilization of this system was monitored at 280 nm.

In this final system, the anti-HSA immunoextraction columns were first equilibrated with pH 7.4, 0.067 M potassium phosphate buffer at 0.10 mL/min. The SCX column was equilibrated with pH 2.5, 0.10 M potassium phosphate buffer at 0.50 mL/min. All of these columns were kept at 37°C. A 20.0 μL sample containing HSA was injected at 0.10 mL/min onto the immunoextraction columns in the presence of pH 7.4, 0.067 M potassium phosphate buffer. After 10 min, the retained HSA was eluted at 0.10 mL/min by applying pH 2.5, 0.10 M potassium phosphate buffer. This elution was carried out for 25 min and was done with the immunoextraction columns connected on-line with the SCX column. The HSA that was captured by the SCX column was later eluted by using pH 6.0, 0.10 M potassium phosphate buffer at 0.50 mL/min. The HSA that was eluted from the SCX column was collected over a period of 55 s in an on-line 500 μL loop.

The HSA collected in the loop was next entrapped within a 1.0 cm × 2.1 mm I.D. column that contained hydrazide-activated silica. This step was done by first passing the captured protein repeatedly through the column for 4 h at 20 μL/min by using two syringe pumps that were synchronized to withdraw and infuse the protein solution through the column [9,12]. The HSA solution was then combined manually with an equal volume of a solution containing 4.25 mg/mL of oxidized glycogen; this mixture was circulated through the column for another 16–20 h. In the final step, 450 μL of pH 6.0, 0.10 M potassium phosphate buffer containing 1.0 mg/mL oxalic dihydrazide was applied for 2 h to the column to cap any remaining active aldehyde groups on the oxidized glycogen. A control column was made the same way but with pH 6.0, 0.10 M phosphate buffer being used in place of the HSA solution. The columns were then washed with 30.0 mL of pH 7.4, 0.067 M potassium phosphate buffer at 0.50 mL/min and stored in the same buffer at 4°C.

2.6 Chromatographic studies

All injections onto the entrapped protein columns were made in triplicate at 0.25 or 0.50 mL/min and 37°C in the presence of pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. The injected volume of the probe or drug solutions was 20 μL. A sample concentration of 10.0 μM warfarin or L-tryptophan was used during optimization of the entrapment conditions in Section 3.4; a warfarin concentration of 1.0 μM was used in Sections 3.5 and 3.6. The void volume was measured by injecting 5.0 μM sodium nitrate,

which is a non-retained solute for the types of columns and supports that were used in this study [20]. All sulfonylurea drugs were injected at a sample concentration of 1.0 μM except for glibenclamide, for which the sample concentration was 5.0 μM (Note: this was done to allow for good detection of the broad and strongly-retained peak for glibenclamide). The following wavelengths were used for detection: acetohexamide, 252 nm; glibenclamide, 290 nm; tolbutamide or glipizide, 226 nm; sodium nitrate, 205 nm; L-tryptophan, 280 nm; and warfarin, 308 nm. Serum was filtered prior to injection by using an Acrodisc PVDF syringe filter (0.2 μm pore size) from Waters (Milford, MA, USA).

3 Results and Discussion

3.1 Characterization of immunoextraction columns

The total antibody content for each immunoextraction support was measured by using a protein assay. The protein content when using the affinity-purified anti-HSA antibodies was 22.1 (± 1.2) mg antibodies/g silica, and the protein content when using anti-HSA antibodies from goat serum was 18.0 (± 0.1) mg antibodies/g silica when using HSA silica for purification and 30.5 (± 1.4) mg antibodies/g silica when using protein G. The support made with the affinity-purified anti-HSA antibodies was packed into a 1.0 cm × 2.1 mm I.D. column, while the support made using the anti-HSA antibodies from goat serum was packed into 2.0 cm and 5.0 cm × 2.1 mm I.D. columns (combined length, 7.0 cm; using HSA silica and protein G for antibody purification, respectively). The total antibody content of these columns, based on a molar mass for the antibodies of 150 kDa and a known packing density for the supports of 0.45 g/cm^3, was 2.30 (± 0.12) nmol or 35.2 (± 1.2) nmol, respectively.

The binding capacity of each column was determined by using frontal analysis and solutions with known concentrations of HSA that were applied at 0.10 mL/min, pH 7.4 and 37°C. The binding capacity for the 1.0 cm × 2.1 mm column was 0.27 (± 0.02) nmol HSA, while the binding capacities for the 2.0 cm and 5.0 cm × 2.1 mm columns were 1.38 (± 0.05) nmol HSA and 1.50 (± 0.05) nmol HSA, respectively. By combining the data from the protein assays and frontal analysis, it was determined that 11.9 (± 1.1)%, 36.9 (± 0.1)% and 9.5 (± 0.1)% of the immobilized antibodies were active and able to bind HSA within the 1.0 cm, 2.0 cm, and 5.0 cm long immunoextraction columns, respectively. The 1.0 cm × 2.1 mm column containing affinity-purified anti-HSA antibodies was used in most of the following studies but was combined with the 2.0 cm and 5.0 × 2.1 mm anti-HSA columns to increase the overall binding capacity in the final immunoextraction/entrapment system (see Sections 2.5 and 3.5).

3.2 Optimization of conditions for immunoextraction

A 1.0 cm × 2.1 mm I.D. immunoextraction column was used to study the capture efficiency of HSA while varying the injection flow rate. This was done by injecting 20 μL of 10.0 μM of HSA in pH 7.4, 0.067 M potassium phosphate buffer onto the column at 37°C. The retained HSA was eluted at 37°C using pH 2.5, 0.10 M potassium phosphate buffer. Elution was performed at pH 2.5 because this pH is known to effectively dissociate HSA from polyclonal antibodies without irreversibly affecting the structure of this protein or the antibodies [21–23]. This elution buffer was also chosen because it could act as a weak

mobile phase for an SCX column, thus allowing use of this latter column for the retention and re-concentration of HSA that was eluted from the immunoextraction column (see Section 3.3).

In these experiments, the HSA was injected at flow rates ranging from 0.10 to 0.50 mL/min into the pH 7.4 application buffer of the immunoextraction column. After 10 min, the retained HSA was eluted at pH 2.5 and 0.10 mL/min, and the area of the eluted peak was measured. Injections using the same volume and concentration of HSA, but now in the presence of pH 2.5, 0.10 M phosphate buffer as the sample buffer and mobile phase, were made onto an inert control column to provide the total expected peak area.

The results are summarized in Table 1. Almost 94% of the applied HSA was captured by a 1.0 cm × 2.1 mm I.D. immunoextraction column at 0.10 mL/min. The protein sample in this experiment contained 0.20 nmol HSA, which represented 74% of the measured column binding capacity. For the same amount of sample, there was a small decrease of 6.6–7.3% (i.e., a difference significant at the 95% confidence level) when going from 0.10 to 0.25–0.50 mL/min. Thus, an application flow rate of 0.10 mL/min was used in all further work with the immunoextraction columns. Similar or higher capture efficiencies would be expected for smaller amounts of applied HSA or columns with a higher binding capacity, such as the 2.0 cm plus 5.0 cm × 2.1 mm immunoextraction columns that were used later in Sections 3.5–3.6 as part of the final immunoextraction/entrapment system [21,22].

The backpressure and stability of the immunoextraction columns were also considered, as well as their ability to release the captured HSA. For a 1.0 cm × 2.1 mm I.D. immunoextraction column, the backpressure increased from 0.3 MPa (44 psi) at 0.10 mL/min to 1.1 MPa (160 psi) at 0.50 mL/min. A larger proportional change was noted for a 2.0 cm plus 5.0 cm × 2.1 mm I.D. immunoextraction columns, which gave an increase in backpressure of 1.7 MPa (247 psi) at 0.10 mL/min to 3.6 MPa (522 psi) at 0.25 mL/min.

The stability of the combined immunoextraction columns and their release of captured HSA were monitored by comparing the peak area of the retained HSA after each injection for a standard sample of this protein (i.e., 20 μL of 10.0 μM HSA). After 48 injections there was a decrease of only 10% in the retained peak area (or ~0.2% per application of HSA). This small decrease may have been due to a slow loss of activity in the anti-HSA antibodies over time, or a small level of carryover of non-eluted HSA. The gradual loss of column binding capacity was not a major issue in this study because the retained peak area was used to monitor and correct for the amount of HSA that was passed on to the rest of the system for capture by SCX or for use in entrapment.

The time needed to elute the captured HSA from an immunoextraction column at pH 2.5 was next studied. This was done by using a 20 μL sample of 630 μM HSA (i.e., 42 g/L HSA, a typical serum concentration of this protein; total injected HSA, 12.6 nmol) to saturate a set of coupled immunoextraction columns (i.e., the 1.0 cm and 2.0 cm plus 5.0 cm × 2.1 mm I.D. columns) with a combined binding capacity for HSA of 3.15 (± 0.07) nmol. The HSA sample was injected at 0.10 mL/min and 37°C in the presence of pH 7.4, 0.067 M potassium phosphate buffer, followed by a 10 min wash at pH 7.4 and elution of the retained HSA at

0.10 mL/min using pH 2.5, 0.10 M phosphate buffer. A similar study was also carried out by applying 20 μL of 10 μM HSA at 0.10 mL/min to a 1.0 cm × 2.1 mm I.D. anti-HSA column to examine the elution profile when an immunoextraction column was not fully saturated with HSA (i.e., conditions using 74% of the available binding capacity).

The profile of the eluting peak was examined by peak decay analysis [24–27]. This was done by employing Eqn. (1) to determine the dissociation rate constant (*kd*) for HSA as it was released from an immunoextraction column [25].

$$\ln\left(\frac{dm_{Ae}}{dt}\right) = \ln\left(k_d m_{Ae}\right) - k_d t \quad (1)$$

In this equation, *mAe* represents the moles of HSA (or analyte, A) that were initially bound to the column, and *t* represents the elution time. This equation indicates that a plot of the natural logarithm of the elution peak versus time for a process that follows pseudo-first order dissociation (i.e., as is often present during the elution of analytes from immobilized antibodies) should result in a linear response with a slope equal to -*kd* [25].

Figure 3 shows some typical results that were obtained when Eqn. (1) was fit to the elution profile for HSA when the combined 1.0 cm and 2.0 cm plus 5.0 cm × 2.1 mm I.D. immunoextraction columns were saturated with this protein. A relatively broad peak was seen for HSA from these columns, as shown in Figure 3(a) and has been noted for other systems based on immunoextraction [22,27]. The integrated form of the peak, as shown in Figure 3(b), indicated that >99% of the HSA eluted in 15.2 min at 0.10 mL/min and >99.9% eluted in 18.0 min. Based on these results, elution was allowed to occur for at least 18 min and up to 25 min in all later experiments in this study. The logarithmic transform of this peak profile gave a reasonably good fit with Eqn. (1) and a first-order decay model at times of 1.7 min or greater at 0.10 mL/min, as shown by the inset of Figure 3. The slope for this fit gave an estimated dissociation rate constant between HSA and the anti-HSA antibodies of 4.2 (± 0.1) × 10^{-3} s^{-1} at pH 2.5 and 37°C. Similar dissociation rate constants of 3.8–4.4 × 10^{-3} s^{-1} were obtained over comparable elution times and at 0.10 or 0.25 mL/min when a non-saturating amount of HSA was applied to a 1.0 cm × 2.1 mm I.D. immunoextraction column (see Table 1).

3.3 Optimization of conditions for strong cation-exchange

The flow rate and pH conditions to be used with a small SCX column to capture and elute HSA were next considered. In these experiments, 20 μL of 2.5 μM HSA (or 0.05 nmol) in pH 2.5, 0.10 M potassium phosphate buffer was injected onto a 1.0 cm × 2.1 mm I.D. SCX column. These injections were made in the presence of pH 2.5, 0.10 M potassium phosphate buffer at 0.10 mL/min and 37°C (i.e., the same conditions selected in the previous section for eluting HSA from the immunoextraction columns). Under these injection and application conditions, an average of 95.5 (± 1.4)% HSA was retained by the SCX column.

The effect of altering the pH of the SCX elution buffer was next considered. The isoelectric point (pI) of HSA is 4.7. This means the overall charge of HSA at a pH higher than 4.7 will

be negative [1,21], with elution of this protein becoming easier from a negatively-charged SCX column as the pH is increased to 5.0 or higher. In addition to the pH of 5.0 that was initially employed, elution buffers with a pH of 6.0 or 7.0 were also examined. This work was done at an elution flow rate of 0.25 mL/min and 37°C. As shown in Figure 4(a), pH 5.0 gave the broadest elution profile for HSA from the SCX column, with narrower elution profiles being obtained at pH 6.0 and 7.0. For instance, increasing the mobile phase pH from 5.0 to 6.0 or 7.0 at 0.25 mL/min resulted in 2.0-to 2.5-faster elution of HSA from the SCX column. An elution pH of 6.0 was selected for later use in this report due to its ability to quickly elute HSA from the SCX column and its compatibility with the conditions that were needed for entrapment, as discussed in Section 3.4.

The elution flow rates and times that could be used to remove and collect HSA from the SCX column were examined. The backpressure of the SCX column at pH 6.0 ranged from only 0.4 MPa (58 psi) at 0.10 mL/min to 1.3 MPa (188 psi) at 0.50 mL/min. All of these backpressures were acceptable for use in this report. Figure 4(b) shows the elution profile of HSA from the SCX column that was obtained at 0.50 mL/min and pH 6.0, following application of a 20 μL solution containing 42 g/L HSA onto the immunoextraction columns, and capture of the retained HSA by the SCX column, as described in the previous section. The resulting elution peak was similar in shape to those shown in Figure 4(a) at 0.25 mL/min, but with the peak maximum at 0.50 mL/min appearing at a proportionately shorter time. As shown in Figure 4(b), 95% of this peak eluted within 25 s at 0.50 mL/min and 99% eluted within 46 s (i.e., elution volumes of ~208 and 380 μL, respectively). As this peak eluted, it was collected by passing the SCX column eluent through an on-line 500 μL loop. This collection occurred over the first min of elution from the SCX column, or between 35 and 36 min after the original injection of the HSA sample onto the immunoextraction column (i.e., a time over which >99.7% of the HSA was eluted). Based on this information and the results in Figure 4, an elution pH of 6.0 and flow rate of 0.50 mL/min for the SCX column were used in all further studies in this report.

3.4 Optimization of conditions for entrapment

Protein entrapment by the method shown in Figure 1 is normally performed at pH 5.0 to allow hydrazide groups on the support to react with oxidized glycogen and entrap a protein such as HSA [8–12]. However, other pH values that are above or below 5.0 may also be used for this process [19]. In the previous section it was determined that pH 6.0, 0.10 M phosphate buffer was effective in releasing HSA from the SCX column. Because this pH was higher than the value of 5.0 that is often used for entrapment [8–12], the effect of using pH 6.0 for the entrapment of HSA was next examined. A 300 μL portion of a solution containing 100 mg/mL HSA in pH 6.0, 0.10 M phosphate buffer was first infused and withdrawn through a 1.0 cm × 2.1 mm I.D. column containing hydrazide-activated silica, according to conditions given in Section 2.5 and used previously at pH 5.0 [9]. This was followed by the infusion and withdrawal of an equal volume mixture of 4.25 mg/mL oxidized glycogen and 100 mg/mL HSA in pH 6.0 buffer, 0.10 M phosphate buffer, and then capping of any remaining aldehyde groups (i.e., as described in Section 2.5) [9]. For comparison, a column containing entrapped HSA was prepared in the same manner but using pH 5.0, 20 mM acetate buffer containing 15 mM sodium chloride. Control columns

were also prepared at pH 6.0 and pH 5.0 with no HSA being applied during the entrapment process.

The protein content of each column was determined by means of zonal elution and using warfarin and L-tryptophan as probe compounds [17,28]. Warfarin is known to bind at Sudlow site I of HSA and L-tryptophan binds at Sudlow site II with association equilibrium constants of 2.4×10^5 M^{-1} [17] and 1.1×10^4 M^{-1} [28], respectively, at pH 7.4 and 37°C. The moles of active protein (mL) in each entrapped HSA column was estimated by using Eqn. (2) and the measured retention factor (k) for a given probe compound [29].

$$k = \frac{K_a m_L}{V_m} \tag{2}$$

In this equation, K_a is the association equilibrium constant for the probe with HSA, and V_m is the column void volume (i.e., as determined by injecting sodium nitrate as a non-retained solute). Based on this relationship, the HSA contents of the columns made at pH 5.0 and 6.0 were determined to be 19.5 (± 1.3) nmol and 14.6 (±0.7) nmol, respectively. A decrease of about 25% in HSA content occurred when using pH 6.0 instead of 5.0 for entrapment, a difference that was significant at the 95% confidence level; however, these results did indicate that pH 6.0 could still be effectively used for the entrapment process. Thus, pH 6.0 was used as both the elution pH for the SCX column and as the pH for the entrapment process in the remainder of this report.

3.5 Preparation of HSA columns using immunoextraction/entrapment system

The final immunoextraction/entrapment system, as based on the conditions selected in Sections 3.1–3.4, was first tested for use in preparing small columns for drug binding studies by using a sample that contained a commercial sample of purified HSA. This sample was prepared in pH 7.4, 0.067 M phosphate buffer at a physiological concentration of HSA (42 g/L). A 20 μL injection of this HSA solution was made onto the system, and the captured HSA was entrapped within a 1.0 cm × 2.1 mm I.D. hydrazide-activated silica column in the presence of oxidized glycogen. A control column was prepared in a similar manner off-line and with no HSA being applied. The protein content of the entrapped HSA column was again determined by using Eqn. (2) and injecting warfarin as a probe compound with known binding properties for HSA [17]. The protein content of the column was found in this manner to be 0.56 (± 0.10) nmol HSA.

The entrapped HSA column was employed to measure the global affinity constants (nK_a) [10–13] for HSA with several sulfonylurea drugs: acetohexamide, glibenclamide, glipizide and tolbutamide. These drugs have been used in the management of glucose levels in blood in diabetic patients and have global affinity constants for HSA that have been measured in prior studies [13–16]. All of these drugs were injected in triplicate at 0.25 mL/min and 37 °C using pH 7.4, 0.067 M phosphate buffer as the mobile phase (i.e., the same temperature, pH and buffer conditions as used in prior binding studies in Refs. [13–16]). The same drugs and conditions were used on a control column to detect and correct for any non-specific interactions of these drugs with the support. Figure 5 shows some typical chromatograms

that were obtained in these studies, with results being obtained for most of the drugs within 3.0 min of injection at 0.25 mL/min. The only exception was glibenclamide, which had the strongest binding and highest retention on the HSA column and which eluted within about 8 min.

The retention factors measured for the sulfonylurea drugs on the HSA and control columns are summarized in Table 2. The specific retention factor for each drug due to HSA was obtained by subtracting the retention factor for the drug on the control column from that measured on the HSA column. The non-specific interactions of these drugs with the support, as determined by comparing their retention on the HSA and control columns, accounted for only 1–21% (average, 10%) of the total retention factors measured on the HSA column. The specific retention factors measured for these drugs had precisions of ± 9.5–13% (average, ± 11%).

The global affinity constants that were calculated from these results are shown in Table 3. The precision of these values ranged from ± 16–18% (average, ± 17%). The corresponding global affinity constants that were measured for the HSA that was applied in buffer spanned from 1.22–19.8 × 10^5 M^{-1}. These global affinities were consistent with those that have been previously reported for the same drugs with HSA under equivalent pH and temperature conditions [13–16]. These values were all equivalent to those from the literature at the 95% confidence level. The results indicated that the immunoextraction/entrapment system could be used to make HSA columns for binding studies and that these columns could be effectively used in the rapid determination of a drug's overall affinity for HSA.

3.6 Preparation of entrapped HSA columns from serum

The system was also used to entrap HSA from serum. This was performed by filtering the serum and mixing it 1:1 with pH 7.4, 0.067 M phosphate buffer. This mixture was then applied to the immunoextraction/entrapment system and used to entrap the captured HSA in a 1.0 cm × 2.1 mm I.D. column, as described in Section 3.5. It was determined by using Eqn. (2) and injecting warfarin as a probe that 0.35 (± 0.08) nmol of HSA from the diluted serum was entrapped by this method.

The global affinity constants for acetohexamide, glibenclamide, glipizide, and tolbutamide were again determined, but now using the entrapped HSA column that was prepared from serum. The results are included in Table 3. These results were again equivalent at the 95% confidence level to those reported in the literature [13–16]. Similar agreement was seen with the values obtained in Section 3.5 when using an entrapped HSA column that was prepared from commercial, purified HSA. The precisions of the global affinities obtained when using entrapped HSA from serum were ± 9.7–13.6% (average, ± 11.6%), which were similar to the precisions seen in Section 3.5 for columns made through the entrapment of purified HSA.

The stabilities of both the columns made from serum and purified HSA were also examined. No significant variations, at a 95% confidence level, were observed in the retention times of drugs that were injected over the course of two weeks on either type of column. This level of stability was seen over at least 60 injections on a column containing purified HSA (as

observed over several applications of fresh portions of HSA) and a similar value of over 54 injections on a column made from serum. The combined results indicated that the immunoextraction/entrapment system could be used to capture and isolate a protein like HSA from a solution or a sample such as serum and to place this protein into a column for use in HPAC and multiple drug binding studies.

4. Conclusion

In this study a system was developed that could purify, extract and entrap HSA within small affinity columns. The system utilized an immunoextraction column containing polyclonal anti-HSA antibodies that could extract HSA from either aqueous samples or serum. The immunoextraction column was used in tandem with a SCX column to concentrate the captured HSA and place it into a buffer that had a suitable pH for entrapment. The entrapment of HSA was performed on this system by using a hydrazide-activated column and oxidized glycogen as a capping agent. The 1.0 cm × 2.1 mm I.D. HSA columns that were created by this system had a typical protein content of 0.3–0.6 nmol. These columns were stable over several weeks and more than 50–60 sample injections when used in drug binding studies.

The HSA columns created by using this system were used to measure the global affinity constants for HSA with various sulfonylurea drugs. These binding constants could be determined in a matter of minutes, had precisions of around ± 10–18%, and gave good agreement with literature values. A potential future application of this system is in the field of personalized medicine. For instance, small HSA columns could be developed by using individual serum samples to examine drug interactions with the entrapped protein to aid in patient treatment [30]. This would include work with both normal HSA and glycated forms of HSA, which have been shown to have equivalent binding to the types of polyclonal anti-HSA antibodies that were employed in this study [30]. Given the strong and selective binding of antibodies when used in affinity columns [31,32], the same type of system as used here for HSA could also be adapted in future work to capture and entrap other, lower-abundance proteins for binding studies or related applications by employing alternative antibodies against the given target for immunoextraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- An on-line purification and entrapment system was developed to prepare affinity columns.
- Human serum albumin (HSA) was employed as a model protein for entrapment.
- The binding and elution properties of HSA on this system were examined and optimized.
- The entrapped HSA columns were evaluated in binding studies with sulfonylurea drugs.
- The columns were stable over several weeks and gave binding constants in 8 min or less.
- The same non-covalent immobilization system can be adapted for used with other proteins.

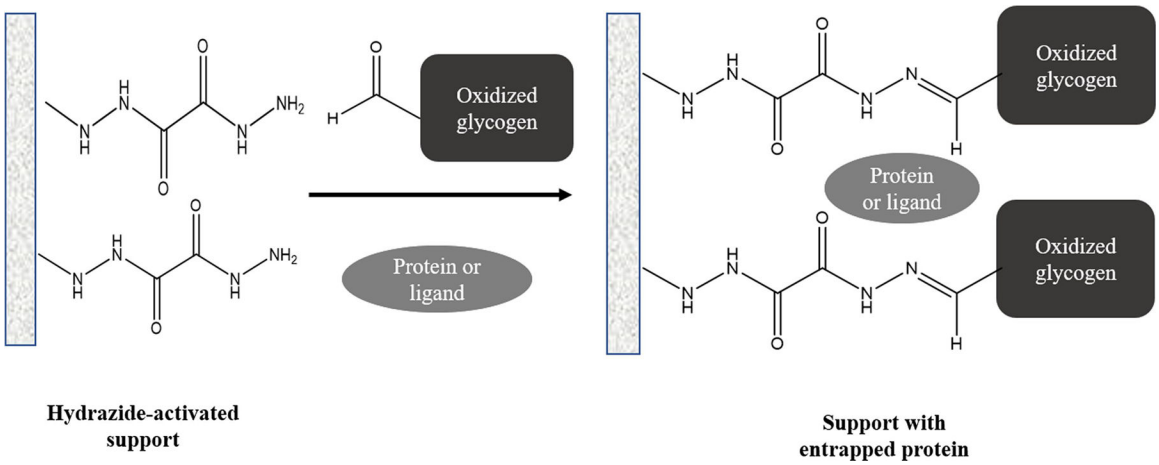

Figure 1.

Entrapment of a protein by using the coupling of oxidized glycogen with hydrazide-activated silica.

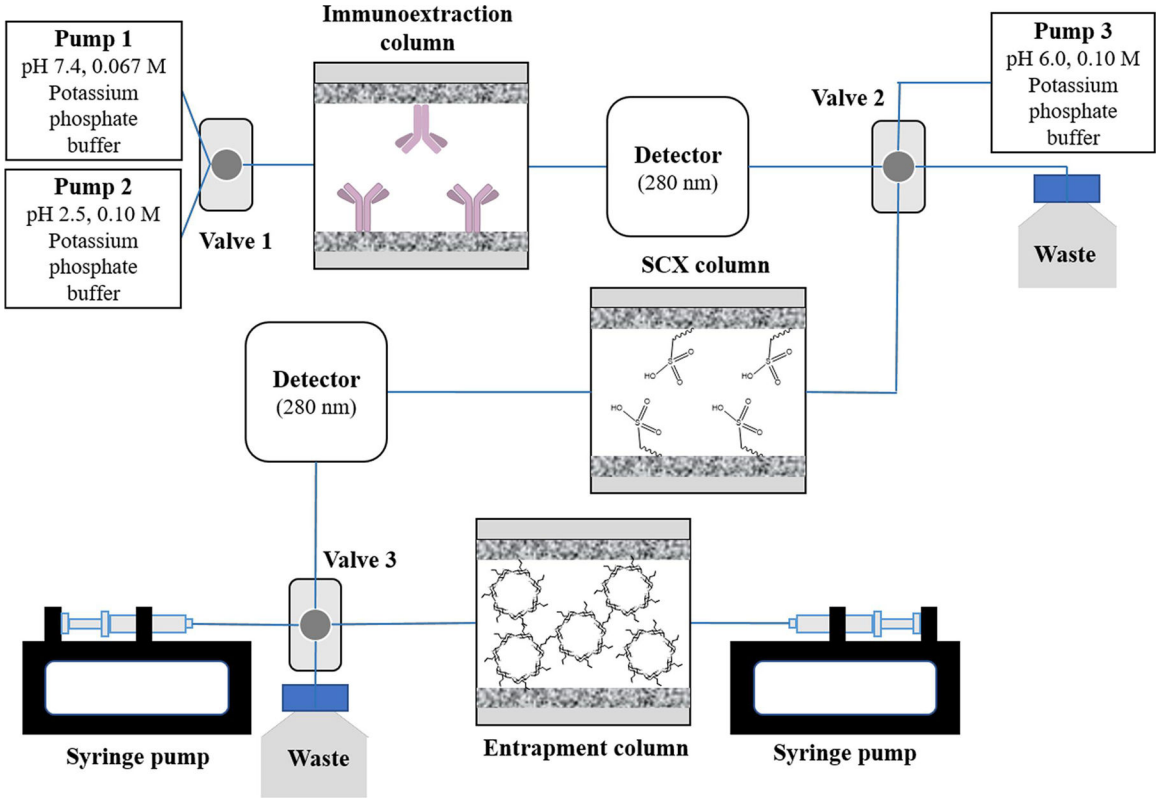

Figure 2.

General design of the on-line immunoextraction/entrapment system. The injection buffer used for the immunoextraction column was pH 7.4, 0.067 M potassium phosphate buffer, and the elution buffer was pH 2.5, 0.10 M potassium phosphate buffer. For the strong cation exchange (SCX) column, the elution buffer was pH 6.0, 0.10 M potassium phosphate buffer. The elution of HSA from each column was monitored at 280 nm. The isolated HSA was passed through a 1.0 cm × 2.1 mm I.D. hydrazide-activated silica column at pH 6.0 and entrapped in the presence of oxidized glycogen.

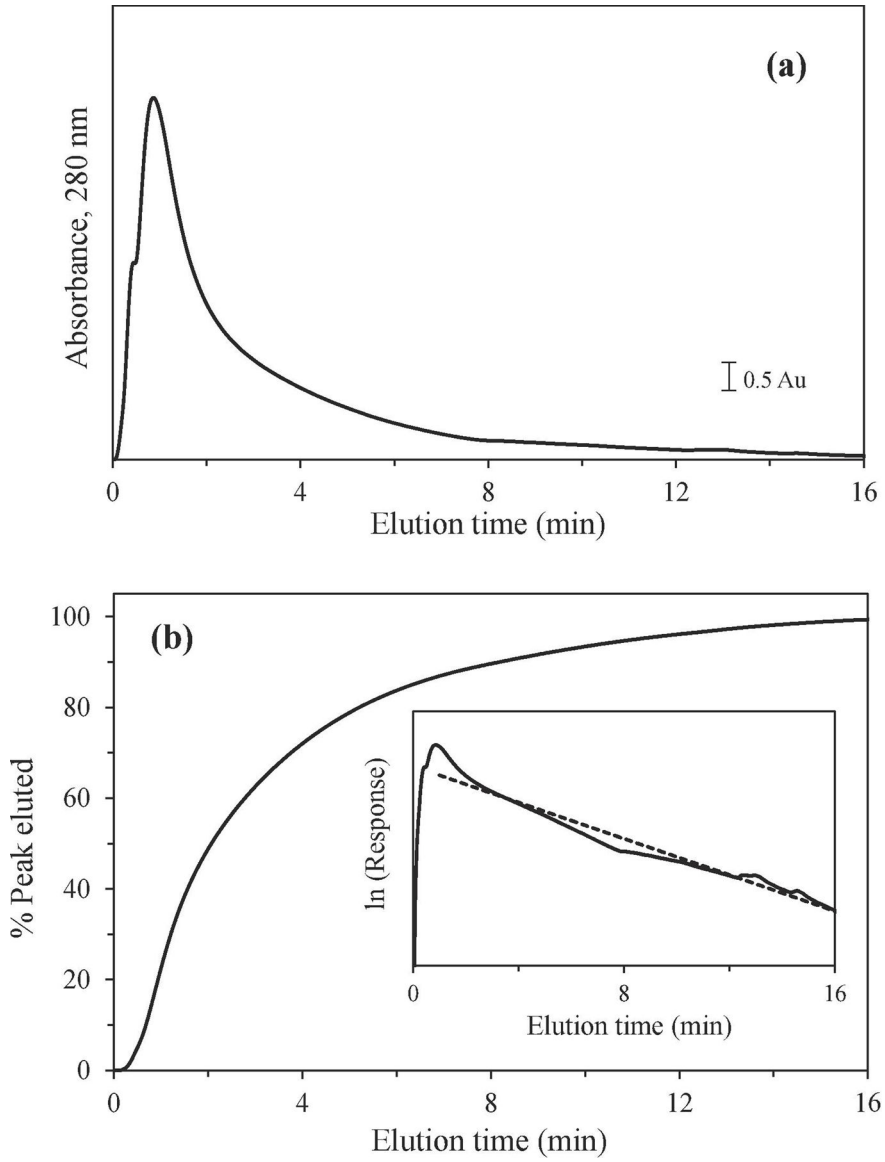

Figure 3.

(a) Elution of HSA from a set of immunoextraction columns (1.0 cm and 2.0 cm plus 5.0 × 2.1 mm I.D. anti-HSA columns connected in tandem) at 0.10 mL/min and 37°C in the presence of pH 2.5, 0.10 M phosphate buffer and (b) the relative amount of HSA eluted versus time under these conditions. The time scale begins at the point at which the elution buffer was applied to the immunoextraction columns (i.e., 10 min after the initial injection of HSA). The chromatograms have been corrected for the background response obtained when no HSA was present. The inset in (b) shows the logarithmic transform of the elution peak and the linear region of this plot, as determined according to Eqn. (1) and described by the best-fit line $y = -0.2494\ x + 12.49$ (correlation coefficient, 0.9814). Abbreviations: Au, absorbance units.

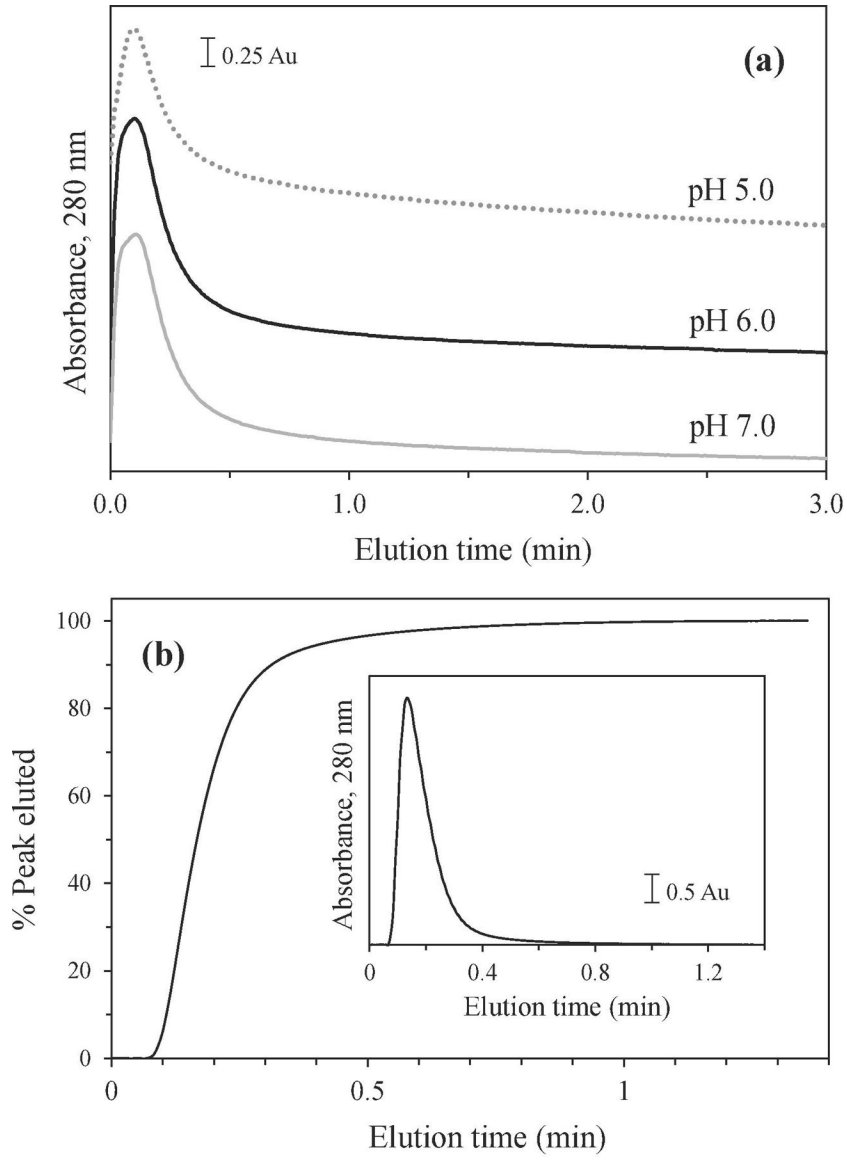

Figure 4.

(a) Elution of HSA from a 1.0 cm × 2.1 mm I.D. SCX column at 0.25 mL/min and at pH 5.0, 6.0 or 7.0 and (b) the relative amount of HSA eluted versus time from the SCX column at pH 6.0 and 0.50 mL/min, where the inset shows the original elution profile. The time scale begins at the point at which the elution buffer was applied to the SCX column (i.e., 35 min after injection of HSA onto the immunoextraction columns). The chromatograms have been corrected for the background response obtained when no HSA was present. Abbreviations: Au, absorbance units.

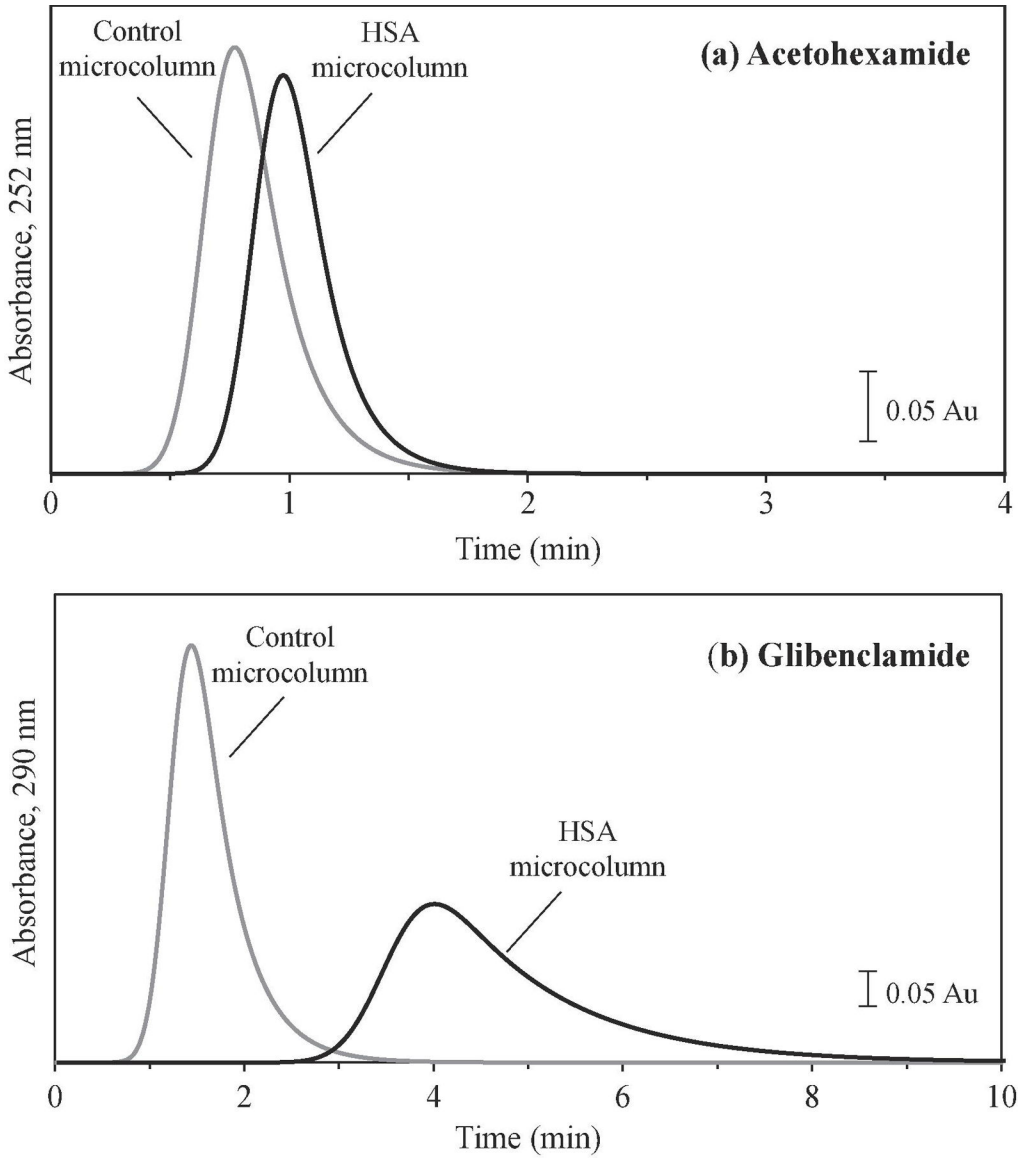

Figure 5.

Retention noted for samples of (a) 1.0 μM acetohexamide or (b) 5.0 μM glibenclamide that were injected onto a 1.0 cm × 2.1 mm I.D. entrapped HSA column or control column in the presence of pH 7.4, 0.067 M potassium phosphate buffer at 0.25 mL/min and 37°C. Abbreviations: Au, absorbance units.

Table 1.

Degree of capture and rate of elution of retained HSA at various flow rates when using a 1.0 cm × 2.1 mm I.D. anti-HSA immunoextraction column[a]

Flow rate (mL/min)	Retained HSA	Dissociation rate constant at pH 2.5 ($\times 10^{-3}$ s^{-1})
0.10	93.8 (± 1.4)%	3.8 (± 0.1)
0.25	86.5 (± 1.0)%	4.4 (± 0.1)
0.50	87.2 (± 1.0)%	5.9 (± 0.2)

[a]The values in parentheses represent a range of ± 1 S.D. ($n = 3$). The dissociation rate constants were obtained by fitting the natural logarithm of the elution peak at pH 2.5 and the given flow rate to Eqn. (1), as described in the text.

Table 2.

Retention factors measured for various sulfonylurea drugs on a 1.0 cm × 2.1 mm I.D. entrapped HSA column and control column[a]

Drug	Retention factor on control column	Retention factor on HSA column	Specific retention factor due to HSA[b]
Acetohexamide	0.60 (± 0.07)	3.47 (± 0.36)	2.87 (± 0.37)
Glibenclamide	8.71 (± 0.99)	41.2 (± 2.9)	32.4 (± 3.1)
Glipizide	0.02 (± 0.02)	3.35 (± 0.35)	3.33 (± 0.35)
Tolbutamide	0.02 (± 0.01)	2.01 (± 0.82)	1.99 (± 0.26)

[a]The values in parentheses represent a range of ± 1 S.D. (n = 3).

[b]The specific retention factor due to HSA represents the difference between the retention factors that were measured for a drug in the presence of entrapped HSA and on the control column in the absence of any HSA. The values in parentheses for these results were determined by error propagation.

Table 3.

Global affinity constants obtained for representative sulfonylurea drugs using 1.0 cm × 2.1 mm I.D. columns containing entrapped HSA[a]

Drug	Global affinity constant (M^{-1})[a]		
	Entrapment using purified HSA	Entrapment using human serum	Literature value [Ref.]
Acetohexamide	1.75 (± 0.31) × 10^5	1.67 (± 0.22) × 10^5	1.7 (± 0.1) × 10^5 [13]
Glibenclamide	1.98 (± 0.35) × 10^6	2.56 (± 0.29) × 10^6	2.11 (± 0.47) × 10^6 [14]
Glipizide	2.03 (± 0.33) × 10^5	2.27 (± 0.22) × 10^5	2.57 (± 0.80) × 10^5 [15]
Tolbutamide	1.22 (± 0.22) × 10^5	1.27 (± 0.15) × 10^5	1.08 (± 0.03) × 10^5 [16]

[a]The values shown in parentheses represent a range of ± 1 S.D. (n = 3). All global affinity constants were measured at pH 7.4 and 37°C.