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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 820–830
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of an affinity silica monolith containing human serum albumin for chiral separations

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Received 23 January 2007; received in revised form 9 March 2007; accepted 19 March 2007
Available online 24 March 2007

Abstract

An affinity monolith based on silica and containing immobilized human serum albumin (HSA) was developed and evaluated in terms of its binding, efficiency and selectivity in chiral separations. The results were compared with data obtained for the same protein when used as a chiral stationary phase with HPLC-grade silica particles or a monolith based on a copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA). The surface coverage of HSA in the silica monolith was similar to values obtained with silica particles and a GMA/EDMA monolith. However, the higher surface area of the silica monolith gave a material that contained 1.3–2.2-times more immobilized HSA per unit volume when compared to silica particles or a GMA/EDMA monolith. The retention, efficiency and resolving power of the HSA silica monolith were evaluated using two chiral analytes: D/L-tryptophan and *R*/*S*-warfarin. The separation of *R*- and *S*-ibuprofen was also considered. The HSA silica monolith gave higher retention and higher or comparable resolution and efficiency when compared with HSA columns that contained silica particles or a GMA/EDMA monolith. The silica monolith also gave lower back pressures and separation impedances than these other materials. It was concluded that silica monoliths can be valuable alternatives to silica particles or GMA/EDMA monoliths when used with immobilized HSA as a chiral stationary phase.

Keywords: Affinity monolith; Silica monolith; Human serum albumin; Chiral separation; Schiff base method

1. Introduction

Affinity monolithic chromatography (AMC) is a type of liquid chromatography in which a biologically related ligand is used as the stationary phase inside a monolithic column [1–4]. AMC has seen growing interest in recent years due to several advantages of monoliths versus particle-based columns, including better mass transfer properties and an ability to perform faster separations [1–7]. Macroporous polymers based on glycidyl methacrylate (GMA) and ethylene dimethyacrylate (EMDA) have been employed in several previous studies to create affinity monoliths [3,4,8–18] and used in such applications as sample purification [3], chiral separations [4], and ultrafast immunoextractions [19].

In comparison to GMA/EDMA monoliths, silica monoliths have been examined in only a few applications involving immobilized biological agents. One example is the use of silica monoliths in immobilized enzyme reactors [20–24]. Silica monoliths containing immobilized penicillin G acylase have been utilized in chiral separations for ketoprofen, suprofen, fenoprofen [25], 2-aryloxyalkanoic acid methyl esters, 2-aryloxyalkanoic acids, and 2-arylpropionic acids [25–30]. A silica monolithic rod containing *t*-butyl-carbamoylquinine as a chiral anion-exchanger selector was used for the separation of *N*-derivatized amino acids and suprofen [31]. Finally, silica monolith capillaries containing 3,5-disubstituted phenylcarbamate derivatives of cellulose, amylose and amylose tris(3,5-dimethylphenylcarbamate) were examined for use in chiral separations [32,33].

The relatively small number of publications in which affinity ligands have been used with silica monoliths is surprising since these supports offer several potential advantages. One of these possible advantages is the high surface area of these materials [3,34], which would be expected to allow for a high level of immobilized ligand attachment. Another expected advantage of silica monoliths is their ability to use the same immobilization

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doi:10.1016/j.jpba.2007.03.017

methods with these supports that are employed when attaching affinity ligands to silica particles [3,6].

This study will focus on the development of an affinity silica monolith that contains immobilized human serum albumin (HSA) as a chiral stationary phase. HSA (MW, 66.5 kDa) is the most abundant protein in serum and binds to many drugs and small solutes. This protein has frequently been used in the past in HPLC columns based on silica particles to separate various chiral solutes [35–39,40–43] and to study drug–protein binding processes [3,6,38,44–50]. This report will first examine the preparation of a silica monolith for the immobilization of HSA. Previous work has used the epoxy immobilization method for attaching proteins to silica monoliths (e.g., see Ref. [21]). However, it is known that the epoxy method tends to give lower activities and lower protein coverages for HSA than other amine-based coupling methods [4]. Work in this study will instead use the Schiff base, an immobilization method which gives better results for HSA when used with other support materials [3,4]. The resulting support will be evaluated in terms of its activity, retention and chiral selectivity for two model compounds: D/L-tryptophan and *R*/*S*-warfarin. A comparison of the results for the silica monolith will then be made with those obtained for HSA immobilized to silica particles, as well as with data and chiral separations that have previously been obtained for HSA attached within a GMA/EDMA monolith [4]. The separation of racemic ibuprofen will also be tested with this column. These data should provide valuable information on the relative advantages and disadvantages of an HSA silica monolith versus these other supports when used in chiral separations or related applications, such as drug–protein binding studies.

2. Experimental

2.1. Reagents

The HSA (Cohn fraction V, essentially fatty acid free, >96% pure), carbamazepine (>98% pure), racemic tryptophan (>99% pure), L-tryptophan (>98% pure), racemic warfarin (>98% pure), racemic ibuprofen (>98% pure), periodic acid reagent (>99% pure; an oxidizing agent), sodium borohydride (98% pure; a strong reducing agent), sodium cyanoborohydride (94% pure; a mild reducing agent), and 3-glycidoxypropyltrimethoxysilane (97% pure) were from Sigma (St. Louis, MO, USA). GMA (97% pure), EDMA (98% pure), azobisisobutyronitrile (AIBN, 98% pure; an initiator for GMA/EDMA polymer preparation), D-tryptophan (>99% pure) and 1-dodecanol (98% pure) were purchased from Aldrich (Milwaukee, WI, USA). The cyclohexanol (>99% pure) was from Fluka (Milwaukee, WI, USA). The acetic acid (>99.7% pure; flammable) and sulfuric acid (95–98% pure; a corrosive, strong oxidizer, and carcinogenic agent) were from EMD chemicals (Gibbstown, NJ, USA). Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was obtained from Macherey Nagel (Dűren, Germany). All aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and filtered using Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA).

2.2. Apparatus

The Chromolith Performance Si columns (4.6 mm i.d. × 10 cm) were donated by Merck KGaA (Darmstadt, Germany). These silica monoliths were created as described previously [34,51]. The GMA/EDMA monoliths were prepared in 4.6 mm i.d. × 5 cm PEEK-lined stainless steel columns from Alltech (Deerfield, IL, USA); these columns included a special frit that could be used to compress the monoliths and avoid gaps within the column. The columns containing silica particles (used for both chiral separations and as standards for estimating the protein content of the monoliths) had a diameter of 4.6 mm i.d. and lengths that ranged from 2 to 12 cm. Activating reagents for the monoliths were applied using a Pu980i pump from Jasco (Easton, MD, USA); the same pump was used to pass solutions of HSA through the activated monoliths for immobilization. The particle-based silica columns were packed using an Alltech column slurry packer. The chromatographic studies were performed using a Jasco Pu980i pump, along with a CM4100 gradient pump and UV100 absorbance detector from Thermoseparations (Riviera Beach, FL, USA). Samples were injected using a Rheodyne LabPro valve (Cotati, CA, USA) equipped with a 20 μL sample loop. Chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA).

2.3. Preparation of diol silica monolith

Conditions for preparing the diol silica monolith were adapted from methods described for diol silica particles [37,52–54]. All reactions performed in this method and in the various immobilization techniques used in this report were performed at room temperature, unless otherwise indicated. To prepare a diol silica monolith, an underivatized silica monolith was first washed with 20 mL of pH 5.5, 0.10 M sodium acetate buffer at 0.5 mL/min for 40 min. A 10 mL portion of pure 3-glycidoxypropyltrimethoxysilane was then passed through this monolith at 0.2 mL/min for 50 min. Both ends of the column containing the monolith were next sealed with PEEK column plugs from Alltech, and the column was placed in a water bath at 97 °C for 5 h. This column was later removed from the water bath and washed by applying 5 mL of pH 5.5, 0.10 M sodium acetate buffer at 0.1 mL/min for 50 min.

To assure maximum diol coverage for the silica monolith, another 5 mL of pure 3-glycidoxypropyltrimethoxysilane was passed through this monolith column at 0.1 mL/min for 50 min, with the column then being sealed at both ends and heated in a water bath at 97 °C for 5 h. At the end of this reaction, the column was washed with 50 mL of water applied at 0.2 mL/min for 4 h; this was followed by 10 mL of a dilute pH 3.0 sulfuric acid solution that was passed through the column at 0.2 mL/min for 50 min. The two ends of the column were again sealed and the column placed in a water bath at 70 °C for 3 h. The column

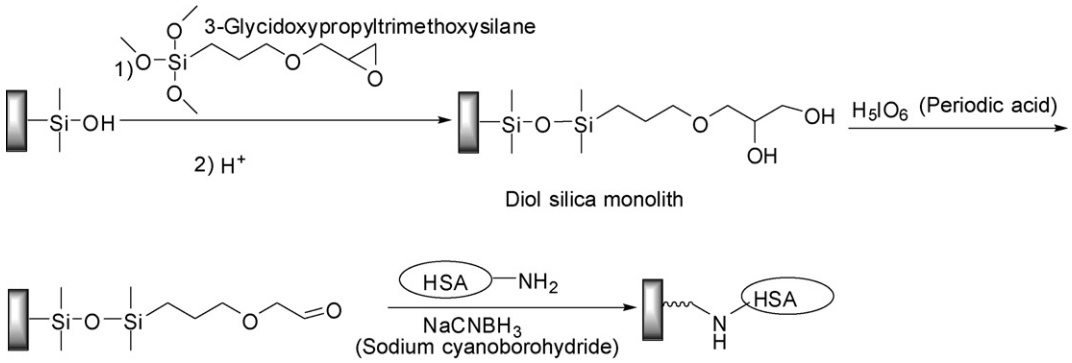

Fig. 1. Reactions involved in the preparation of a HSA silica monolith. Further details on these reactions can be found in the text. *Abbreviation*: HSA, human serum albumin.

was next washed with 50 mL of water at 0.2 mL/min for over 4 h and stored at room temperature until use.

2.4. Immobilization of HSA in silica monolith

The Schiff base method was used for the immobilization of HSA in a silica monolith, as shown in Fig. 1. This method was adapted from previous work with silica particles [37,52–55]. The Schiff base method was chosen for this study because this technique is commonly used for the immobilization of HSA and gives good activity for this protein when compared to other amine-based coupling methods [52,56]. In addition, this method has previously been used to immobilize HSA to both silica particles [52,53] and GMA/EDMA monoliths [4], allowing the results of this current study to be directly compared to work based on these other supports.

In this method, a diol silica monolith was first oxidized by using periodic acid to give aldehyde groups on the interior surface of the monolith. This was accomplished by passing through the monolith 50 mL of a 90% acetic acid solution in water, which was applied at 0.2 mL/min for approximately 4 h. An 80 mL portion of 0.05 g/mL periodic acid in the 90% acetic acid solution was then passed through the same monolith at 0.2 mL/min for 7 h in the dark. The monolith was next washed with 100 mL of water at 0.2 mL/min for 8 h.

The second step in this immobilization process involved allowing amine groups on HSA to react with aldehyde groups on the monolith to form a Schiff base. A mild reducing agent (sodium cyanoborohydride) was also present during this reaction to reduce the Schiff base to a more stable secondary amine linkage. This step was conducted by preparing a solution that contained 50 mg HSA and 25 mg sodium cyanoborohydride in 10 mL of pH 6.0, 1.4 M potassium phosphate buffer (KPB). This solution was circulated through the monolith for 24 h at 0.5 mL/min. This was followed by application of a 12 mL solution containing 60 mg HSA and 30 mg sodium cyanoborohydride in pH 6.0, 1.4 M KPB, which was circulated through the monolith for 60 h at 0.5 mL/min.

The monolith was next washed with 20 mL of pH 8.0, 0.10 M KPB at 0.5 mL/min for 40 min. Any remaining aldehyde groups on the support were reduced by using a syringe pump to pass through the monolith a 1 mg/mL sodium borohydride solution in pH 8.0, 0.10 M KPB, which was applied at 0.05 mL/min for 90 min. The monolith was washed at 0.2 mL/min for 50 min with 10 mL of pH 8.0, 0.10 M KPB containing 0.5 M sodium chloride. Finally, the monolith was washed with 50 mL of pH 7.4, 0.067 M KPB at 0.5 mL/min for 1.6 h. This monolith and all other columns and protein supports that were prepared in this report were stored in pH 7.4, 0.067 M KPB at 4 °C until use. The resulting HSA silica monolith had good long-term stability, with a decrease in separation factor of only 23% being observed for racemic tryptophan separation over the course of 4 months (i.e., approximately 400 column volumes). A diol silica monolith was used as the control column in later studies examining the behavior of the HSA silica monolith.

2.5. Immobilization of HSA to other supports

The 300 Å pore size, 7 μm silica particles that were used in this study were chosen because they have been frequently used in previous work with HSA for chiral separations and drug binding studies [45,48,52,53]. HSA was immobilized onto these particles by the Schiff base method [52,55]. Diol silica particles for this method were prepared as described in previous reports [52], with the immobilization of HSA to this support following the same general scheme as shown in Fig. 1. The final protein content for these silica particles was determined in replicate by a BCA assay [57,58], using soluble HSA as the standard and diol silica as the blank. This HSA silica was packed into 4.6 mm i.d. columns with lengths that ranged from 2 to 12 cm. These columns were packed at 3500 psi (24 MPa) using pH 7.4, 0.067 M KPB as the packing solution. Control columns were prepared in the same manner by using silica which had been taken through the entire Schiff base method but with no HSA being added during the immobilization step.

The GMA/EDMA monolith used for the immobilization of HSA was the same as described in Ref. [4]. This monolith also contained HSA that was immobilized by the Schiff base method. The protein content for this type of monolith was determined by taking a small section of HSA GMA/EDMA and analyzing it in replicate by a BCA assay, using soluble HSA as the standard and a portion of a diol GMA/EMDA monolith as the blank [4]. The control column for the HSA GMA/EDMA column was a

GMA/EDMA monolith that had been taken through the entire Schiff base method but with no HSA being added.

2.6. Determination of protein content in silica monolith

The binding capacity and total amount of protein in the HSA silica monolith was originally estimated by employing frontal analysis. This was accomplished by using carbamazepine as the analyte, since carbamazepine is known to bind HSA [59] and is believed to have a single primary site on this protein [48]. Carbamazepine concentrations of 10–100 μM were applied in pH 7.4, 0.067 M KPB to columns containing the HSA monolith or a control monolith of identical size. This work was performed at 1.5 mL/min and 25 °C using an approach similar to that described for other affinity systems [60,61]. Elution of the carbamazepine was monitored at 214 nm. The results obtained with the control column were used to correct for the void time of the system and non-specific binding of carbamazepine (i.e., 55% of the binding capacity for carbamazepine in the HSA monolith). The total amount of HSA in the monolith was then calculated by using the corrected binding capacity along with specific activities that have been reported for carbamazepine with HSA when using the immobilization methods in Fig. 1 with silica particles [48]. An alternative approach can also be used in which the difference in protein concentration is measured before and after immobilization, but this method can be subject to errors due to nonspecific binding of a protein to the support [57,58,62]. The methods used in this current study did not suffer from this problem since the HSA columns were extensively washed with mobile phase before their protein content was determined.

A second estimate of the total protein content in the HSA silica monolith was obtained by injecting a 0.1% (w/v) solution of copper sulfate onto this column. This method used copper sulfate as a probe for the overall ion-exchange capability of the immobilized proteins. The retention factors measured for the injected sample at 258 nm on the HSA monolith was compared to those obtained for the same sample on columns packed with silica particles that had known amounts of immobilized HSA, as determined by a BCA protein assay. These experiments were performed at 25 °C using pH 4.3, 0.20 M sodium acetate buffer as the mobile phase. This particular mobile phase was selected for this work since it gave reasonable retention times for injected samples without causing irreversible damage to HSA. The nonspecific retention noted for copper sulfate on the control columns was less than 4% of the retention measured on HSA columns.

2.7. Chromatographic studies

The mobile phase used for the chiral separation of D/L-tryptophan or *R*/*S*-warfarin on the HSA columns was pH 7.4, 0.067 M KPB. The mobile phase for chiral separation of *R*/*S*-ibuprofen was pH 7.0, 0.067 M potassium phosphate buffer containing 5% isopropanol and 5 mM octanoic acid. This mobile phase was degassed under vacuum for at least 30 min prior to use. All chromatographic studies were performed at 25 °C. A 20 μM sample of D/L-tryptophan was prepared fresh daily in the given mobile phase and stored at 4 °C when not in use. Samples containing 20 μM *R*/*S*-warfarin or *R*/*S*-ibuprofen were prepared in a similar fashion and were used within 2 weeks, being stored at 4 °C between experiments. Three 20 μL injections were typically made of these samples under each given set of experimental conditions. No appreciable changes in retention times (i.e., random variations of less than 0.5%) were noted when using up to three-fold higher sample concentrations, indicating that linear elution conditions were present during this study. The maximum flow rate used in these studies was 3.0 mL/min. No measurable changes (i.e., random variations of less then 2%) were seen in the retention factors for all of the tested analytes when using lower flow rates (i.e., 0.2 mL/min), as has been noted in previous studies with HSA columns containing silica particles [45,48,63].

The following detection wavelengths were used in this study: tryptophan, 280 nm; warfarin, 310 nm; and ibuprofen, 225 nm. The system void time was determined by injecting 20 μL of 0.2 mM sodium nitrate onto the chromatographic system while monitoring the absorbance of the eluent at 205 nm. The extra-column void time was determined by injecting sodium nitrate onto the chromatographic system after replacing the column with a zero dead volume connector. All retention times were determined by using moment analysis or the $B/A_{0.5}$ method [64]. The widths of the chromatographic peaks were determined by these same methods and were used to calculate the plate numbers, plate heights, and peak resolutions for each column [56,65].

3. Results and discussion

3.1. General properties of affinity silica monolith

The pore volume of the silica monolith that was used as the starting material in this study was 1 mL/g monolith and its total surface area was 300 m^2/g. The total porosity for this type of monolith has been reported to be 80%, with 75% of this being due to macropores [66,67]. Macropores are defined in this report as being pores in the monolith with a diameter greater than 50 nm; the remaining pores are called mesopores, which have typical diameters between 2 and 50 nm. The silica monolith in this study had macropores with an average diameter of 2 μm and mesopores with an average diameter of 13 nm. The macropores help provide good permeability for a monoith, while the mesopores play an important role in determining the total surface area.

Two items considered in the use of the silica monolith with HSA were the amounts of total and active protein that could be placed within this material. These values were estimated through a non-destructive method by measuring the binding capacity of the affinity monolith for carbamazepine, an achiral solute that interacts with HSA. Using these results and a density for the support in the silica monolith of 0.2 g/mL, the measured binding capacity was found to be 1.08 (±0.09) μmol carbamazepine/g of HSA monolith (where the number in parentheses represents ± 1S.D.). It has been found in previous work with silica particles that the same immobilization method and mobile phase conditions as used in this study result in a specific activity (i.e., moles of bound analyte per mole of protein)

for HSA of 0.56 (±0.03) or 56% for carbamazepine. This value made it possible to use the measured binding capacity of the HSA silica monolith to provide an estimate of its total protein content, giving a value of 1.94 (±0.18) μmol HSA/g monolith.

A second estimate of the total protein content for the HSA silica monolith was obtained by examining its retention for copper sulfate versus reference columns that contained known amounts of HSA. This approach gave a protein content of 1.72 (±0.09) μmol HSA/g monolith. This result differed by less than 11% from the estimate made using carbamazepine and frontal analysis. From these estimates of total protein content, the total mass of protein in the HSA silica monolith was determined to be 40 (±2) mg HSA. This amount was equal to 36% of the total HSA that had originally been passed through the silica monolith during the immobilization step. This result indicated that at least a two-fold excess of HSA had been used during the immobilization process.

Table 1 compares the HSA content of the silica monolith with those of silica particles that contained HSA and that were prepared using the same immobilization method. The total HSA content per unit mass of support in the silica monolith was 3.2-fold higher than that obtained with 300 Å pore size, 7 μm silica particles [45,48,63]. However, the silica particles and silica monolith in Table 1 had different surface areas (e.g., 100 m^2/g for the silica particles and 300 m^2/g for the silica monolith). When the amount of immobilized protein was expressed in terms of a surface coverage, the silica monolith and silica particles gave statistically equivalent values (i.e., 5.7 nmol/m^2). The silica monolith also gave 4.7-times more protein per unit mass of support than that reported for a GMA/EDMA monolith that contained HSA [4]. However, this GMA/EDMA monolith had a much lower surface area than the silica monolith (67.5 m^2/g versus 300 m^2/g) [19]. When the protein contents were normalized for this difference in surface area, the resulting surface coverage for HSA on the GMA/EDMA monolith (5.8 nmol/m^2) was statistically equivalent to the value obtained for the silica monolith.

Table 1
Properties of HSA immobilized to various supports[a]

Type of support	Protein content (nmol/g support)
Silica monolith	1820 (±90)[b]
Silica particles	570 (±40)
GMA/EDMA monolith	391 (±6)[c]
	Protein coverage (nmol/m^2)
Silica monolith	5.7 (±0.3)
Silica particles	5.7 (±0.4)
GMA/EDMA monolith	5.8 (± 0.1)
	Protein content per volume (nmol/mL)
Silica monolith	360 (±20)
Silica particles	270 (±20)
GMA/EDMA monolith	161 (±3)

[a] These results are for the specific types of silica particles and monoliths that were used in this work. Silica particles with different pore sizes or monoliths prepared under different polymerization conditions would be expected to give different absolute values for these parameters.

[b] The HSA content given for the silica monolith is the average of the estimates made by using frontal analysis and the copper sulfate assay, as described in the text.

[c] The results for the GMA/EDMA monolith are based on data given in Ref. [4].

Similar studies to those in Table 1 found that 100 Å pore size silica particles gave a protein content for HSA that was only 35–40% greater than that measured for the 300 Å pore size particles [68]. This occurs even though the 100 Å pore size particles had a 3.5-fold higher surface area (i.e., a value comparable to that for the silica monolith) [69]. The corresponding surface coverage of HSA on the 100 Å pore size particles was 2.2 nmol/m^2. This result indicated that the 300 Å pore size silica was a more comparable support (in terms of surface coverage for HSA) to the silica monolith and GMA/EDMA monolith. This difference is believed to be due to exclusion of HSA from part of the interior of the 100 Å pore size particles during the immobilization process, as has been noted for antibodies on such supports [69]. The consistency of the surface coverage results in Table 1 indicates that these exclusion effects were not significant (or were at least consistent) for the supports listed in this table.

Another way the supports in Table 1 differed was in their densities. The density of the silica monolith was 0.2 g/mL [70], while the packing density of the silica particles was 0.45–0.48 g/mL and the density of the GMA/EDMA monolith was 0.41 g/mL [4]. Table 1 shows the results that were obtained when the amount of immobilized protein was calculated per unit volume of support. It was found on a per volume basis that the amount of immobilized HSA in the silica monolith was 33% higher than that obtained with silica particles. When compared to the GMA/EDMA monolith, the silica monolith gave 2.2-fold more immobilized HSA per unit volume. Since all of these columns were prepared using the same immobilization method (i.e., the proteins should have had similar activities), this higher protein content per volume would be expected to create greater retention for analytes in the silica monolith. The impact of this effect will be examined further in the following sections.

3.2. Evaluation of HSA monolith using D- and L-tryptophan

The HSA silica monolith was first tested for use in chiral separations by examining its retention for D- and L-tryptophan. These analytes are of interest because of their biological and pharmaceutical properties, as well as their well-characterized binding with HSA [71]. For example, L-tryptophan has a single binding region on HSA (i.e., the indole-benzodiazepine site, or Sudlow site II) [72,73], with an association equilibrium constant for this interaction of 1.1–2.4 × 10^4 M^{-1} at 37 °C and pH 7.0 [60,73]. D-Tryptophan is also thought to bind at a single site on HSA (the location of which is not currently known) with an association equilibrium constant for this interaction of 3.6 × 10^3 M^{-1} at 37 °C and pH 7.0 [72]. These properties make D- and L-tryptophan useful as models for solutes that have weak-to-moderate interactions with HSA [4].

Fig. 2(a) provides some typical chromatograms for D- and L-tryptophan on a 4.6 mm i.d. × 10 cm HSA silica monolith. This column gave average retention times of 1.12 and 7.64 min at 3.0 mL/min. The retention factors, separation factor and

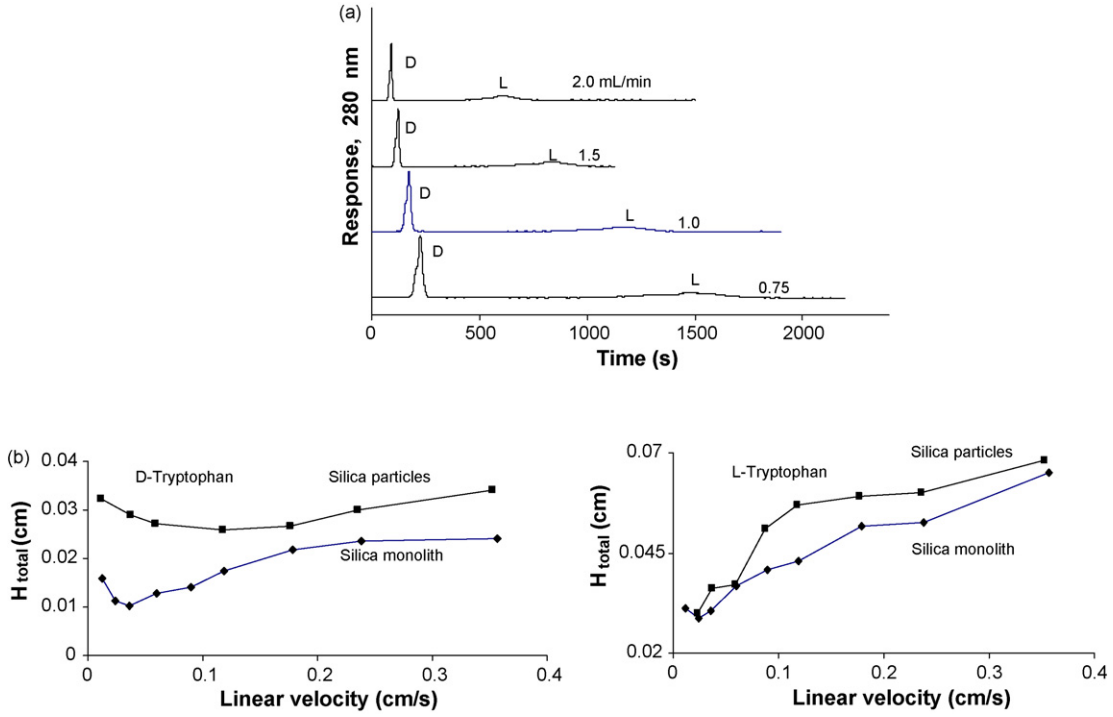

Fig. 2. (a) Representative chromatograms obtained for the injection of racemic tryptophan onto an HSA silica monolith, and (b) plots of total plate height (H_{total}) vs. linear velocity for D- and L-tryptophan when using the HSA silica monolith (♦) or a column containing HSA immobilized to 300 Å pore size, 7 μm silica particles (■). The mobile phase was pH 7.4, 0.067 M KPB. Other conditions are given in the text. The average retention factors for D-tryptophan in (b) were 1.16 for the silica monolith and 0.82 for the column containing silica particles; the average retention factors for L-tryptophan on these same columns were 13.80 and 8.65, respectively.

resolution that were obtained are summarized in Table 2. A baseline separation was achieved for D- and L-tryptophan even at 3.0 mL/min, providing a resolution (R_s) of 4.5 and a separation factor (α) of 14.4 at this flow rate. The resolution increased to 6.5 (± 0.2) when the flow rate was lowered to 0.2 mL/min, while the separation factor showed only random variations between values of 13 and 15 at 0.2–3.0 mL/min. A control monolith with no HSA present gave a retention factor of only 0.06 (±0.01) for both D- and L-tryptophan. Fig. 2(b) shows the van Deemter plots for these analytes on the HSA silica monolith. These plots

Table 2
Chromatographic parameters for D/L-tryptophan and *R*/*S*-warfarin on various HSA columns[a]

Analyte/support	Retention factor, k	Optimum plate height, H_{opt} (cm)[b]	Separation factor, α[c]	Resolution, R_s[c]
D/L-Tryptophan				
Silica monolith	1.16 (±0.06) (D) 13.80 (±0.04) (L)	0.010 (±0.001) (D) 0.028 (±0.003) (L)	14.4 (±0.3)	4.5 (±0.1)[d]
Silica particles	0.82 (±0.02) (D) 8.65 (±0.06) (L)	0.026 (±0.001)(D) 0.030 (±0.005) (L)	11.9 (±0.2)	3.5 (±0.1)
GMA/EDMA monolith	0.59 (±0.01) (D) 2.55 (±0.02) (L)	0.023 (±0.001) (D) 0.041 (±0.003) (L)	4.32 (±0.02)	1.02 (±0.02)
R/S-Warfarin				
Silica monolith	119 (±1) (*R*) 163 (±1) (*S*)	0.043 (±0.002) (*R*) 0.042 (±0.001) (*S*)	1.37 (±0.01)	1.04 (±0.02)[d]
Silica particles	56 (±1) (*R*) 73 (±1) (*S*)	0.056 (±0.002) (*R*) 0.043 (±0.002) (*S*)	1.30 (±0.03)	0.92 (±0.06)
GMA/EDMA monolith	34 (±1) (*R*) 53 (±1) (*S*)	0.043 (±0.002) (*R*) 0.052 (±0.001) (*S*)	1.52 (±0.01)	0.82 (±0.30)

[a] These results in this table are for the specific types of silica particles and monoliths that were used in this work.
[b] The standard deviations given for the optimum plate heights are the typical precisions observed for the plate heights of a given analyte.
[c] The resolution and separation factors given are for a flow rate of 3.0 mL/min. The values given for the silica monolith were obtained on a 10 cm long column; the values for the GMA/EDMA column and column packed with silica particles were obtained on a 5 cm long column, as discussed in Ref. [4].
[d] The corresponding resolution calculated for a 5 cm long HSA silica monolith was 3.2 (±0.1) for D/L-tryptophan and 0.74 (±0.2) for *R*/*S*-warfarin. The results shown for *R*/*S*-warfarin on the silica monolith were obtained after several months of column use, while the results for the other analytes and columns used in this study were obtained nearer to the beginning of the column lifetime.

gave an optimum (minimum) plate height (H_{opt}) of 0.010 cm at 0.036 cm/s for D-tryptophan and 0.028 cm at 0.024 cm/s for L-tryptophan.

The results obtained on the HSA silica monolith for D- and L-tryptophan were compared with those for a packed 4.6 mm i.d. × 5 cm HSA column that contained 300 Å pore size, 7 μm silica particles [53,72]. As expected based on their differences in protein content, the packed HSA column produced lower retention factors than the HSA silica monolith (see Table 2). Nonspecific binding by D- and L-tryptophan on the packed column again was again low, giving a retention factor of only 0.06 (±0.01) for both D- and L-tryptophan. The retention times measured on the packed HSA column were only about one-fourth of those seen on the HSA silica monolith. This was due to both the larger volume of the silica monolith and the higher protein content of this monolith.

The separation factor for D- and L-tryptophan on the packed HSA column (α = 11.9 at 3.0 mL/min or 11.5–12.3 from 0.2 to 3.0 mL/min) was comparable to that for the HSA silica monolith. Although the resolution on the 4.6 mm i.d. × 5 cm packed HSA column (3.5 at 3.0 mL/min) was lower than that measured with the 4.6 mm i.d. × 10 cm HSA silica monolith, this resolution was similar to the value of 3.2 (±0.1) predicted for a 5 cm long HSA silica monolith. These data indicated that the silica monolith and silica particles gave HSA columns with similar performance in the separation of D- and L-tryptophan. As shown in Fig. 2(b), the HSA silica monolith did give a moderate improvement in plate height versus the packed HSA column for both D- and L-tryptophan. The lower plate heights seen for the silica monolith fit with the better mass transfer properties that are often noted for these materials when compared to particulate supports [34]. However, part of this improvement in plate height was a result of the larger retention noted on the HSA silica monolith versus packed HSA column since some band broadening processes are affected by the extent of analyte retention (i.e., stationary phase mass transfer and stagnant mobile phase mass transfer) [56,65].

The results for D- and L-tryptophan on the HSA silica monolith were further compared with previous data obtained on a 4.6 mm i.d. × 5 cm GMA/EDMA monolith that contained HSA (see summary included in Table 2). As expected from their differences in their protein content, HSA GMA/EDMA monolith gave much lower retention factors for D- and L-tryptophan than the HSA silica monolith. Nonspecific binding of D- and L-tryptophan on the GMA/EDMA column was similar to that on the silica monolith, giving a retention factor of 0.04 (±0.01) for both D- and L-tryptophan. The retention times observed on the HSA GMA/EDMA monolith (e.g., 0.30 and 0.68 min for D- and L-tryptophan at 3.0 mL/min) were also much lower than those for the HSA silica monolith (e.g., calculated values of 0.6 and 3.82 min at 3.0 mL/min for a 4.6 mm × 5 cm HSA silica monolith). One consequence of the lower retention for D- and L-tryptophan on the HSA GMA/EDMA monolith was that this column gave lower resolution than the HSA silica monolith or packed HSA column. The plate heights for the HSA GMA/EDMA monolith (H_{opt} = 0.02 cm for D-tryptophan and 0.04 cm for L-tryptophan at 0.05 cm/s) were higher than those for the HSA silica monolith and comparable to values for the packed HSA column at an equivalent degree of retention [4].

3.3. Evaluation of HSA monolith using R/S-warfarin

A second chiral solute that was examined for separation on a HSA silica monolith was *R*/*S*-warfarin. The enantiomers of warfarin have been shown in previous studies to be resolved by columns containing HSA [52,56]. Warfarin is also commonly utilized in drug binding studies as a probe for the warfarin-azapropazone site of HSA (i.e., Sudlow site I) [52]. Both *R*- and *S*-warfarin have been reported to bind to this site, with association equilibrium constants of 2.1×10^5 and 2.6×10^5 M^{-1}, respectively, at 37 °C and pH 7.4 [52,56]. These properties make *R*- and *S*-warfarin useful as models for solutes that have strong interactions with HSA.

Fig. 3(a) shows some typical chromatograms for *R*/*S*-warfarin on a 4.6 mm i.d. × 10 cm HSA silica monolith, with the results of these separations being summarized in Table 2. This column gave average retention times of 62.2 and 85.1 min for *R*- and *S*-warfarin at 3.0 mL/min. A control monolith column with no HSA gave retention factors of only 0.13 (±0.01) for both warfarin enantiomers. The separation factor for *R*- and *S*-warfarin on the HSA silica monolith at 3.0 mL/min was reasonable (α = 1.37) but the resolution was only 1.04; this latter value reflects the broad nature of the peaks that are often obtained for *R*- and *S*-warfarin on HSA columns due to the slow dissociation of this system under the given pH and mobile phase conditions [4,52,56]; a reduction in both retention and this peak broadening can be achieved by adding small amounts of an organic modifier such as 1-propanol to the mobile phase [60,74]. The maximum resolution found for *R*- and *S*-warfarin was 1.49 (±0.02) at 1.0 mL/min, with the separation factor showing only random variations (α = 1.3–1.4) over flow rates of 1.0–3.0 mL/min. As shown in Fig. 3(b), the smallest plate height measured for both *R*-warfarin and *S*-warfarin was 0.04 cm at 0.12 cm/s, with only a small increase being noted at higher linear velocities. Linear velocities below 0.12 cm/s were not examined in this study due to the long retention times of *R*- and *S*-warfarin under these conditions (e.g., a retention time of ~4 h at 1.0 mL/min and 0.12 cm/s).

As was done with D- and L-tryptophan, the results for *R*- and *S*-warfarin on the HSA silica monolith were compared with data obtained on a 4.6 mm i.d. × 5 cm packed HSA column. This packed HSA column again produced lower retention factors than the HSA silica monolith, giving retention times of 10.9 and 14.2 min at 3.0 mL/min (*note*: the nonspecific binding of *R*- and *S*-warfarin on a packed control column gave a retention factor of only 0.10 (±0.01) for *R*- and *S*-warfarin). The separation factor ranged from α = 1.26–1.33 between 0.8 and 3.0 mL/min, giving values equivalent to those seen with the HSA silica monolith. The resolution for *R*/*S*-warfarin at 3.0 mL/min on the packed HSA column was 0.92 and gave a maximum value of 1.36 (±0.04) over the same flow rate range. The resolution obtained at 3.0 mL/min and the maximum resolution of the packed HSA column were lower than those values obtained for the 4.6 mm i.d. × 10 cm long HSA silica monolith at the same

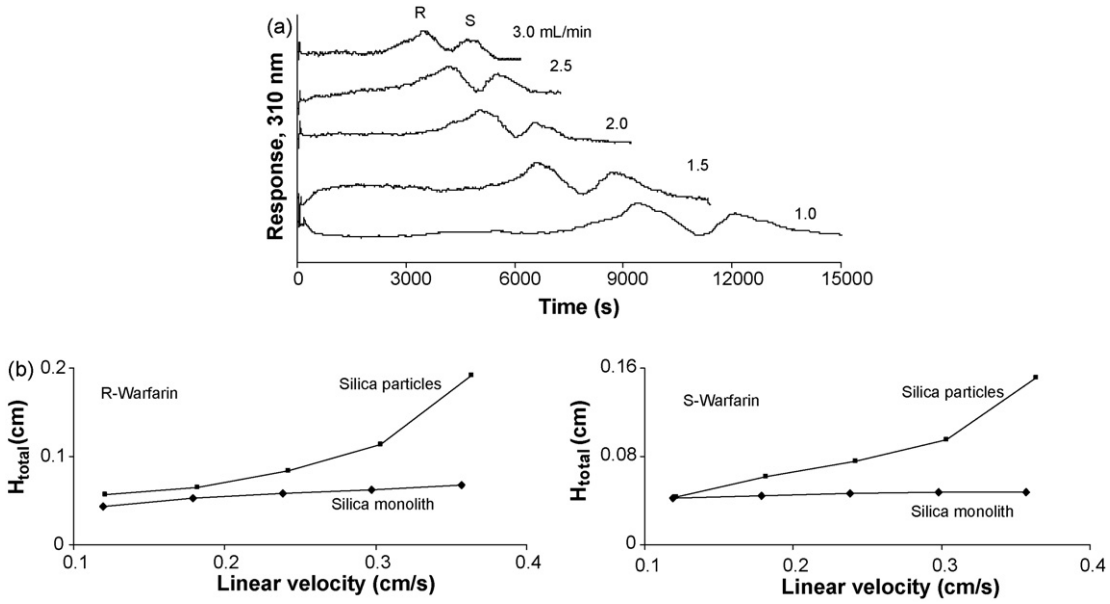

Fig. 3. (a) Representative chromatograms obtained for the injection of racemic warfarin onto an HSA silica monolith, and (b) plots of total plate height vs. linear velocity for *R*- and *S*-warfarin when using the HSA silica monolith (♦) or a column containing HSA immobilized to 300 Å pore size, 7 μm silica particles (■). The mobile phase was pH 7.4, 0.067 M KPB. Other conditions are given in the text. The average retention factors for *R*-warfarin in (b) were 119 for the silica monolith and 56 for the column containing silica particles; the average retention factors for *S*-warfarin on these same columns were 163 and 73, respectively.

flow rate but were higher than the resolution predicted for a 4.6 mm i.d. × 5 cm HSA silica monolith (*note*: part of this difference occurred because the packed column results were obtained early in the lifetime of the given column, while the warfarin results for the HSA silica monolith were obtained after several months of column use and a loss of 10–15% in binding capacity).

Over the range of linear velocities that were examined in Fig. 3(b), the packed HSA column gave larger plate heights for both *R*- and *S*-warfarin versus the HSA silica monolith and showed a sharper increase in these plate heights with an increase in linear velocity. This difference suggests that silica monoliths might be particularly useful for work at higher flow rates in the separation of chiral solutes that are strongly retained by HSA. This difference in efficiency between the silica monolith and silica particles, especially at the higher linear velocities, was much larger than that found in Fig. 2 for D- and L-tryptophan. This trend agrees with a previous study where chiral analytes with high retention (e.g., *R*/*S*-warfarin) were found to give the greatest improvement in separation on HSA columns containing GMA/EDMA monoliths versus silica particles [4]. Such an effect is believed to be due to the lower relative contribution to band-broadening that is made by stationary phase mass transfer versus stagnant mobile phase mass transfer for solutes with high retention on affinity columns [4].

The results for *R*- and *S*-warfarin obtained on the HSA silica monolith were also compared with previous data obtained for these analytes on the 4.6 mm i.d. × 5 cm GMA/EDMA monolith containing HSA [4]. As expected from their protein contents, the GMA/EDMA monolith gave lower retention factors for *R*- and *S*-warfarin than the HSA silica monolith (*note*: nonspecific binding on the GMA/EDMA control column gave a retention factor of 0.25 (±0.01) for both *R*- and *S*-warfarin). The separation factor (α = 1.46–1.51 between 1.0 and 3.0 mL/min) was similar to that seen on the HSA silica monolith, but the resolution was lower (0.82 at 3.0 mL/min, with a maximum of 1.31 (±0.05) between 1.0 and 3.0 mL/min). These resolutions were lower than those found for the 10 cm long HSA silica monolith but were comparable to those predicted for a 5 cm HSA silica monolith (see values given in previous paragraph). The plate heights measured for *R*- and *S*-warfarin on the HSA GMA/EDMA monolith were also similar to those seen on the HSA silica monolith (i.e., 0.04–0.06 cm at linear velocities of 0.1–0.3 cm/s).

3.4. Column back pressure and permeability

The HSA silica monolith was also compared to the other support materials in terms of their back pressures and permeabilities. In all the experiments conducted in this study, the change in back pressure per unit length for the silica monolith was approximately half that of the packed columns. In addition, the silica monolith gave lower back pressures than those that have been observed for GMA/EDMA monoliths [4]. For instance, at 3.0 mL/min some typical back pressures were as follows: 4.6 mm i.d. × 10 cm silica monolith, 600–640 psi (4.1–4.4 MPa); 4.6 mm i.d. × 5 cm columns containing silica particles, 940–995 psi (6.5–6.9 MPa); and 4.6 mm i.d. × 5 cm GMA/EMDA monolith, 420–450 psi (2.9–3.1 MPa).

These supports were also compared in terms of their separation impedance (*E*), which is determined as follows [51].

$$E = \frac{(\Delta P \cdot t_M)}{(N^2 \cdot \eta)} \quad (1)$$

In this relationship, ΔP is the pressure drop across the column, t_M the column void time, N the plate number, and η is the viscosity of the mobile phase. The separation impedance is useful in comparing different supports since it combines the effect of pres-

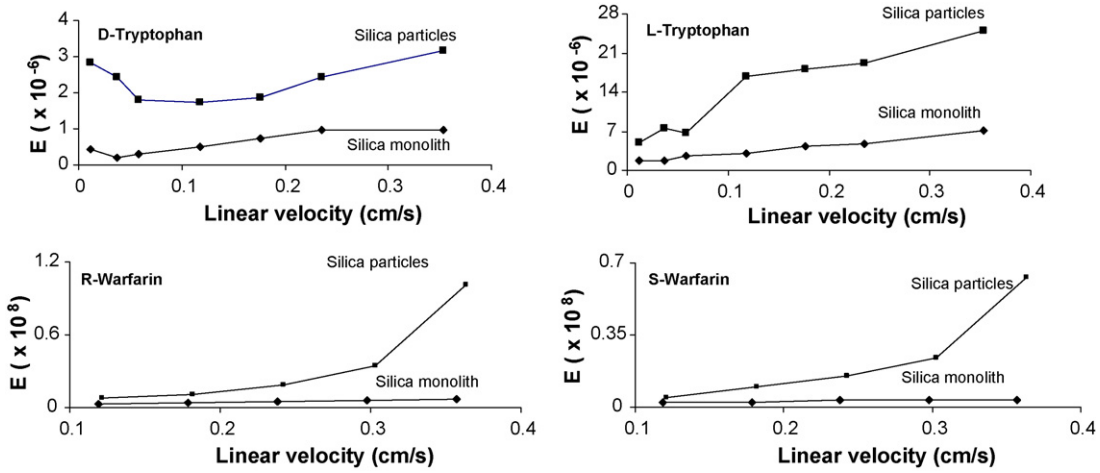

Fig. 4. Separation impedance (E) vs. linear velocity for HSA columns using D/L-tryptophan or *R*/*S*-warfarin as the analytes. The supports used in case were (♦) a silica monolith or (■) 300 Å pore size, 7 μm silica particles. Other experimental conditions are given in the text.

sure drop with the change in efficiency that occurs in a column at different linear velocities. As shown in Fig. 4, the HSA silica monolith gave separation impedances for D-tryptophan and L-tryptophan that were consistently better that those for packed columns containing silica particles. This was also observed for *R*- and *S*-warfarin, where the largest differences in separation impedances were noted at high linear velocities (i.e., conditions that gave rise to a larger difference in efficiency between the HSA silica monolith and packed HSA column). These results are similar to those found by Cabrera for C_{18} silica monoliths when compared to a column that contained 3.5 μm diameter silica particles [34]. The GMA/EDMA monolith was found to give separation impedances for HSA that were intermediate between those for the silica monolith and column containing silica particles. Both the separation impedances and back pressure results indicated that the silica monolith gave better performance and lower resistance to solvent flow than the other tested materials, especially when used at high flow rates.

3.5. Chiral separation of racemic ibuprofen

Once the HSA silica monolith had been evaluated using *R*/*S*-warfarin and D/L-tryptophan, the extension of this column to other chiral analytes was also considered. *R*/*S*-Ibuprofen was the specific drug that was examined. Several studies have suggested that *R*- and *S*-ibuprofen have one common binding site on the HSA [35,44,75]. In addition, *S*-ibuprofen has at least one other major binding region [35,44,75]. The association equilibrium constant for *R*-ibuprofen with HSA has been estimated to be $5.3 \times 10^5\ M^{-1}$ at pH 6.9 and 25 °C. Under the same conditions, the association equilibrium constants for *S*-ibuprofen at its two sites have been found to be 1.1×10^5 and $1.2 \times 10^5\ M^{-1}$ [76].

Retention factors of 3.49 (±0.05) and 6.29 (±0.04) were observed for *S*- and *R*-ibuprofen on the HSA silica monolith. The control monolith gave only 1.6 and 0.88% of the total retention noted for these analytes on the HSA silica monolith. Fig. 5 shows a typical chromatogram obtained for injections of *R*/*S*-ibuprofen on the HSA monolith at 2.5 mL/min. A baseline separation was achieved in approximately 13 min, giving a resolution and selectivity factor equal to 2.4 (±0.1) and 1.80 (±0.04), respectively. These results indicated that silica monoliths could also be employed in separating other chiral solutes that are known to bind to HSA.

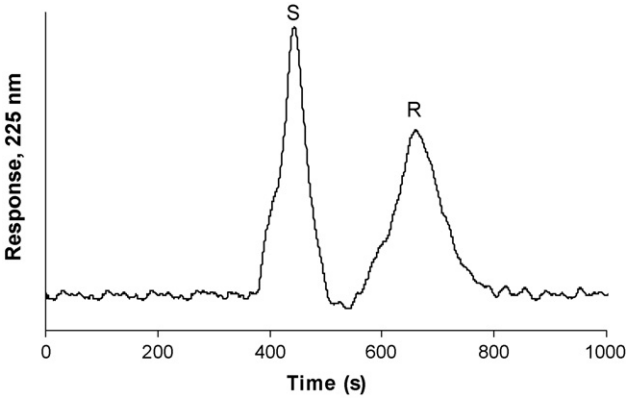

Fig. 5. Representative chromatogram obtained for the injection of racemic ibuprofen onto an HSA silica monolith at 2.5 mL/min. The mobile phase for this separation was pH 7.0, 0.067 M potassium phosphate buffer containing 5% isopropanol and 5 mM octanoic acid. Other experimental conditions are given in the text.

4. Conclusions

In this study, an HSA silica monolith was developed and evaluated for use in chiral separations. Although the amount of HSA per unit area of silica monolith was similar to that of the 300 Å pore size, 7 μm silica particles or GMA/EDMA monolith, the amount of HSA per unit volume was 1.3–3.6 times higher for the silica monolith. This higher coverage of HSA in the silica monolith gave higher retention for the tested analytes, which in turn contributed to the greater resolution of this monolith compared to silica particles or the GMA/EDMA monolith. The optimum plate heights of the HSA silica monolith were lower than those for columns containing silica particles. The silica monolith also gave lower optimum plate heights than the HSA

GMA/EDMA monolith, but part of this difference was due to the much larger retention factors noted on the silica monolith. The silica monolith also gave the lowest back pressures and best separation impedances for the tested analytes.

In conclusion, a silica monolith containing immobilized HSA was found to be a useful alternative to HSA columns containing silica particles or a GMA/EDMA monolith for chiral separations. This type of column should be particularly valuable in applications requiring high flow rates, where the silica monolith can provide good efficiencies along with reasonable back pressures. The advantages noted here for the HSA silica monolith (e.g., high protein content and good efficiency) also make this type of column attractive for use in the study of drug–protein binding, especially for solutes that have only low-to-moderate retention on other types of HSA columns [48,53,72]. The low back pressures and good permeabilities of silica monoliths should make them useful in other affinity applications that require fast flow rates, such as ultrafast immunoextractions or rapid chromatographic immunoassays [19].

Acknowledgment

This work was supported by the National Institutes of Health under grant R01 GM044931 and was conducted in facilities renovated under NIH grant RR015468.

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