12-21-2012

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Published in final edited form as:
J Chromatogr A. 2012 December 21; 1269: 198–207. doi:10.1016/j.chroma.2012.09.009.

Optimization of human serum albumin monoliths for chiral separations and high-performance affinity chromatography

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Abstract

Various organic-based monoliths were prepared and optimized for immobilization of the protein human serum albumin (HSA) as a binding agent for chiral separations and high-performance affinity chromatography. These monoliths contained co-polymers based on glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) or GMA and trimethylolpropane trimethacrylate (TRIM). A mixture of cyclohexanol and 1-dodecanol was used as the porogen, with the ratio of these solvents being varied along with the polymerization temperature to generate a library of monoliths. These monoliths were used with both the Schiff base and epoxy immobilization methods and measured for their final content of HSA. Monoliths showing the highest protein content were further evaluated in chromatographic studies using *R*/*S*-warfarin and D/L-tryptophan as model chiral solutes. A 2.6–2.7-fold increase in HSA content was obtained in the final monoliths when compared to similar HSA monoliths prepared according to the literature. The increased protein content made it possible for the new monoliths to provide higher retention and/or two-fold faster separations for the tested solutes when using 4.6 mm i.d. × 50 mm columns. These monoliths were also used to create 4.6 mm i.d. × 10 mm HSA microcolumns that could separate the same chiral solutes in only 1.5–6.0 min. The approaches used in this study could be extended to the separation of other chiral solutes and to the optimization of organic monoliths for use with additional proteins as binding agents.

Keywords

Monolith columns; Human serum albumin; High-performance affinity chromatography; Chiral separations; Affinity monolith chromatography

1. Introduction

Monolithic columns have been of great recent interest for use in high-performance affinity chromatography (HPAC) because of their low back pressures, ease of preparation and good mass transfer properties [1–10]. The combination of affinity ligands with monolith columns is also known as affinity monolith chromatography (AMC) [4,11–14]. Both HPAC and AMC rely on the use of an immobilized binding agent (i.e., the affinity ligand) for the selective separation or analysis of substances in complex samples, including applications that involve chiral separations [3–6,13,14,16–23]. One protein that has been often used in

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Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma. 2012.09.009.

these methods for chiral separations and to study drug-protein interactions is human serum albumin (HSA), which can retain a variety of drugs and small organic solutes with moderately strong binding [5,16–22]. Alternative ligands that have been used in monoliths for chiral separations include bovine serum albumin and α_1-acid glycoprotein [3,18–22]. The advantages of using HPAC or AMC for chiral separations include their selectivity, ease of automation, high specificity, speed, and good reproducibility [1–8]. However, in preparing columns for this work it is important to consider the way in which the affinity ligand is attached to the support and the total amount of ligand that is present, especially if good retention and resolution are desired between the retained sample components [4,5].

Various types of support materials have been employed in AMC, with many reports using monoliths that contain co-polymers of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) (i.e., the functional monomer and cross-linking agent, respectively) [1–8]. The co-solvents utilized to prepare these monoliths are usually cyclohexanol and 1-dodecanol, which are responsible for pore formation during polymerization. It has been shown previously that GMA/EDMA monoliths can be used to immobilize proteins such as HSA for use in chiral separations or antibodies, lectins and protein A for use in HPAC [3–6,11,13]. An alternative cross-linking agent that is sometimes used in place of EDMA is trimethylolpropane trimethacrylate (TRIM), as has been used with GMA to prepare monoliths for normal-phase separations of *N*-glycans [15].

This study will use a combinatorial approach to optimize the total amount of protein that can be immobilized to monolithic supports for HPAC and AMC based on either GMA/EDMA or GMA/TRIM. HSA will be the affinity ligand and chiral stationary phase examined in this report. Factors to be considered will include the monomer and porogen composition of the polymerization mixture, as well as the temperature that is used for thermal-initiated polymerization. The resulting supports will be used with two immobilization techniques (i.e., the epoxy method and Schiff base method) and evaluated in terms of the total amount of HSA that can be placed within each monolith. Imaging techniques will be utilized to compare the overall morphology of these materials, and zonal elution studies will be used to compare the retention and stereoselectivity of these materials for model chiral solutes. The results should make it possible to create columns for HPAC and AMC that have higher retention or resolution for applications such as chiral separations.

2. Experimental

2.1. Reagents

The GMA (97% pure), EDMA (98%), cyclohexanol (>99%), 1-dodecanol (98%), 2,2′-azoisobutyronitrile (AIBN, 98%), TRIM (98%), 1-propanol (>99.5%), HSA (Cohn fraction V, essentially fatty acid free, ≥96%), sodium cyanoborohydride (94%, a mild reducing agent), sodium borohydride (98%, a strong reducing agent), periodic acid (>99%, an oxidizing agent), racemic warfarin (3-(α-acetonylbenzyl)-4-hydroxycoumarin, >98%) and D/L-tryptophan (>98%) were from Sigma–Aldrich (St. Louis, MO, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All buffers and aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and were filtered using 0.2 μm GNWP nylon filters from Millipore (Billerica, MA, USA).

2.2. Apparatus

Some of the monoliths were prepared in 4.6 mm i.d. × 50 mm or 10 mm long stainless steel columns with PEEK inner liners from All-tech (Deerfield, IL, USA). These columns included a special frit that could be used to compress the monoliths and avoid the formation

of gaps within the columns during and after their preparation. These columns were used with the final optimized HSA monoliths for chiral separations and to compare their results with similar monoliths prepared according to previous methods [5]. The remainder of the monoliths were prepared in 4.6 mm i.d. × 1 mm PEEK disks [3]. These disk columns were used during studies examining the effect of monolith preparation conditions on the amount of HSA that could be placed within such supports. The immobilization of HSA within the monoliths was accomplished through the use of a reciprocating Waters 501 HPLC pump from Millipore (Milford, MA, USA) [3,5].

The HPLC system used in the chromatographic studies consisted of a model 200 gradient pump and model 200 absorbance detector from Perkin Elmer (Waltham, MA, USA). Samples were injected using a Rheodyne Lab Pro valve (Cotati, CA, USA) and a 20 μL loop constructed from PEEK tubing. Chromatographic data were collected using LabView 5.1 (National Instruments, Austin, TX, USA) and processed using PeakFit 4.12 (SeaSolve Software, San Jose, CA, USA). Scanning electron microscopy (SEM) was performed in the Beadle Center at the University of Nebraska (Lincoln, NE, USA) by using a Hitachi S4700 field-emission scanning electron microscope with a W95/NT-based computerized operating system (Pleasanton, CA, USA).

2.3. Preparation of monoliths

The general reaction scheme used for the preparation of the monolith columns has been described previously [3,5]. There were two types of monoliths prepared, as indicated in Fig. 1. The first type, shown at the top of Fig. 1, consisted of a mixture of GMA and EDMA that was prepared in either a 50:50 (v/v) ratio that was polymerized at 60 °C or in a 60:40 (v/v) ratio that was polymerized at 80 °C. These mixtures and temperatures were chosen because similar conditions have been used in prior work for the preparation of GMA/EDMA columns for the immobilization of immunoglobulin G (IgG), HSA, and trypsin [3,5,7]. The GMA and EDMA were combined with various amounts of cyclohexanol and 1-dodecanol (i.e., the porogenic solvents). The amount of GMA/EDMA compared to the porogen mixture was held constant at 40:60 (v/v). This meant the overall content of GMA and EDMA was 20% and 20% (v/v) in the mixture that was polymerized at 60 °C, or 24% and 16% in the mixture that was polymerized at 80 °C. AIBN was used as a thermal initiator and was present at a level equal to 1% (w/w) of the total amount of GMA and EDMA in the polymerization mixture.

The second type of monolith that was examined was based on GMA/TRIM, as illustrated at the bottom of Fig. 1. This type of monolith was prepared using a ratio of GMA to TRIM of 70:30 (v/v), as has been employed for other GMA/TRIM columns [8]. Cyclohexanol and 1-dodecanol were again used as the porogenic solvents, with the relative amount of these solvents being varied to alter the pore size and morphology of the corresponding monoliths. The amount of GMA/TRIM compared to the porogen was again held constant at 40:60 (v/v), giving 28% GMA and 12% TRIM (v/v) in the overall mixture. The amount of AIBN was 1% (w/w) of the total amount of GMA and EDMA in the polymerization mixture. The polymerization of these materials was carried out at either 60 °C or 80 °C.

Prior to polymerization, each mixture of reagents was combined in a flask and sonicated for 10 min. A stream of nitrogen was passed through the flask for 15 min to remove any air bubbles. A 1 mL syringe was used to place the polymerization mixture into a 4.6 mm i.d. × 50 mm or a 4.6 mm i.d. × 10 mm PEEK-lined column housing or into a vial containing four to six 4.6 mm i.d. × 1 mm PEEK disks. When using the PEEK-lined columns, one end of the column was sealed with a plug prior to the addition of reagents, with a second plug being added to the other end once the column had been filled. The 4.6 mm i.d. × 50 mm columns and the 4.6 mm i.d. × 10 mm columns were held upright in a sonicator for 5 min to remove

any air bubbles prior to placing the columns in a water bath at either 60 °C or 80 °C for 24 h. After polymerization of the monoliths in the 4.6 mm i.d. × 50 mm and the 4.6 mm i.d. × 10 mm columns, a special frit-insert was used to compress the monolith against the wall and to reduce the effects of polymer shrinkage. For the 4.6 mm i.d. × 1 mm disks, a Delrin housing and PEEK-lined frits were used to place each monolith into a column. After the columns were assembled, they were washed with acetonitrile for 2 h at 0.5 mL/min and room temperature, followed by a 1 h wash with water at the same flow rate.

2.4. Protein immobilization

The procedure employed for the immobilization of HSA by the Schiff base method is depicted in Fig. 2(a) and was carried out according to a previously-reported method [5]. This method involved the hydrolysis of the GMA/EDMA monolith epoxy groups to diol groups through the addition of a mild aqueous solution of sulfuric acid, followed by oxidation of the diol groups with periodic acid to form aldehydes. These aldehyde groups were then reacted with primary amine groups on HSA to form a reversible Schiff base. The Schiff base was converted upon formation to a more stable secondary amine through the use of a mild reducing agent such as sodium cyanoborohydride. Any unreacted aldehydes were later converted into alcohol groups through the addition of sodium borohydride.

To carry out the Schiff base method with the monolith columns, a 5 mL solution of 0.5 M sulfuric acid was passed through the column at 0.5 mL/min. The column was sealed and placed in a water bath at 60 °C for 4 h. The column was then removed from the water bath and washed with 100 mL water at 0.5 mL/min. A 40 mL solution containing 2 g periodic acid in a 90:10 (v/v) mixture of acetic acid and water was circulated and recycled through the column for approximately 4 h at 0.5 mL/min and room temperature, followed by a wash with 100 mL water at 0.5 mL/min. Next, for the disk columns a 10 mL solution of 5 mg/mL HSA in pH 6.0, 1.5 M potassium phosphate buffer, which also contained 25 mg sodium cyanoborohydride, was circulated and recycled through each disk at 0.5 mL/min for 3 days at room temperature. This step was followed by the application of a fresh 10 mL portion of the HSA and sodium cyanoborohydride solution, which was applied to the same column at 0.5 mL/min for an additional 3 days at room temperature. A 20 mL solution containing 2.5 mg/mL of sodium borohydride in pH 8.0, 0.1 M potassium phosphate buffer was applied to the column in a circulating manner for 2 h at 0.5 mL/min and room temperature. The column was then washed with pH 7.4, 0.067 M potassium phosphate buffer for 4 h at 0.5 mL/min and room temperature. The column was stored in the pH 7.4, 0.067 M phosphate buffer at 4 °C until use.

The procedure for the Schiff base immobilization of HSA onto the 4.6 mm i.d. × 50 mm columns and the 4.6 mm i.d. × 10 mm columns was carried out in the same manner as described for the disk columns but used a 20 mL solution of 10 mg/mL HSA in pH 6.0, 1.5 M potassium phosphate buffer, which also contained 100 mg of sodium cyanoborohydride and which was circulated and recycled through the column at 0.5 mL/min for 3 days at room temperature. This immobilization step was repeated for an additional 3 days with a fresh 20 mL portion of the HSA and sodium cyanoborohydride solution in pH 6.0, 1.5 M phosphate buffer. The remaining steps were the same as described for the disk columns.

The reactions for the epoxy immobilization method are shown in Fig. 2(b). This approach was also adapted from a previously-reported method [3,5]. This method involved the nucleophilic attack by a primary amine group present on HSA with an epoxy group on the monolith, leading to the base opening of these groups to generate a stable secondary amine linkage. In this study, this immobilization was carried out by circulating and recycling a 10 mL solution containing 6 mg/mL HSA in pH 8.0, 1.5 M potassium phosphate buffer through the monolith for 3 days at room temperature and 0.5 mL/min. After 3 days, a fresh portion

of the same type of HSA solution was passed through the column for an additional 3 days at room temperature. After the immobilization step, any remaining epoxy groups were blocked by passing through the column 60 mL of pH 8.0, 0.2 M Tris buffer at 0.5 mL/min for 2 h at room temperature [5]. The column was then washed for 4 h with pH 7.4, 0.067 M potassium phosphate buffer at 0.5 mL/min and stored in the same buffer at 4 °C until use.

2.5. Assessment of monoliths

The total amount of HSA that was present in each type of monolith after immobilization was measured by using a BCA assay [24]. For the BCA assay, each 4.6 mm i.d. × 1 mm disk monolith containing immobilized HSA was prepared in triplicate and washed with 100 mL water at 0.5 mL/min for 3 h at room temperature. The monolith was then removed from the column housing and ground to a fine powder through the use of a mortar and pestle. The powder was placed on a watch glass and placed in a vacuum oven overnight at room temperature to remove all water. The same procedure was used for control monoliths that were prepared under identical conditions to those used for the HSA supports but with no protein being added during the immobilization step. All samples were analyzed in triplicate by using the BCA assay, with soluble HSA being employed as the standard and the control support with no HSA present being utilized as the blank.

Some representative monoliths were also characterized through the use of SEM. The samples for these studies were polymerized and placed into column housings as described earlier. Each support was then washed with 100 mL acetonitrile at 0.5 mL/min for 3 h after polymerization. The monoliths were removed from their column housings and sliced into thin disks through the use of a razor blade. The samples were placed on a watch glass and dried under vacuum at 100 °C for 8 days. Prior to imaging, chromium was used to sputter coat the samples for a period of 5 min.

The chromatographic studies were performed at room temperature using pH 7.4, 0.067 M potassium phosphate buffer as the mobile phase. The mobile phase was degassed and sonicated for approximately 30 min prior to use. The sample consisted of 20 μM racemic warfarin or racemic tryptophan in pH 7.4, 0.067 M potassium phosphate buffer, which was injected in triplicate using a 20 μL loop and a flow rate of 0.1–1.0 mL/min. Sodium nitrate was used as a void marker, with a 20 μL injection being made of 0.2 mM sodium nitrate. The elution of *R*- and *S*-warfarin was monitored at 308 nm; the elution of D- and L-tryptophan was monitored at 280 nm; and the sodium nitrate was detected at 205 nm. No significant amount of non-specific binding was observed for *R/S*-warfarin or D/L-tryptophan to the monoliths in the absence of any immobilized HSA, as noted in previous studies with similar supports [5]. The extra-column void time was measured by injecting sodium nitrate onto the HPLC system with a zero dead volume connector being used in place of a column.

3. Results and discussion

3.1. Optimization of GMA/EDMA monoliths

The general procedure used in this report to make GMA/EDMA monoliths was modified from a previous method that had been optimized for IgG and later used directly for HSA [3,5]. To optimize this procedure for HSA, several parameters were considered, but an emphasis was placed on the composition of the porogen (i.e., the mixture of 1-dodecanol plus cyclohexanol). Altering the porogen composition was of interest because it has been shown to be a convenient route for varying the pore structure, morphology and surface area that is available for protein immobilization in GMA/EDMA monoliths [3].

Two types of GMA/EDMA monoliths were considered in this study, as based on previous work with various proteins [3,5,7]. The conditions used for making these monoliths are

summarized in Tables 1s and 2s in the Supplemental Materials. One set of thirteen monoliths, each made in triplicate, was prepared at 60 °C with the amount of monomers versus porogen being held at a 40:60 (v/v) ratio and the relative amounts of GMA and EDMA in this mixture being kept at 20:20 (v/v). A second set of thirteen monoliths was prepared at 80 °C and with the amount of monomers versus porogen again being kept at a 40:60 (v/v) ratio, but with the amount of GMA and EDMA now being 24:16 (v/v). For each set of monoliths, the ratio of 1-dodecanol to cyclohexanol was varied from 4:56 to 52:8 (v/v) of the total polymerization mixture (i.e., levels corresponding to a 1-dodecanol content of 6.7–86.7% in the porogen). Both the Schiff base and epoxy immobilization methods were used to attach HSA to these supports. A large excess of HSA (i.e., greater than 10-fold versus the final immobilized protein content) was applied to ensure that the maximum coverage was obtained.

Fig. 3 illustrates how the amount of HSA that could be immobilized to each type of monolith varied as the amount of 1-dodecanol versus cyclohexanol was altered in the polymerization mixture. The overall trend was similar to what has been seen in prior work for monoliths containing IgG, in which a maximum amount of immobilized protein is obtained at intermediate amounts of 1-dodecanol [3]. The reason for the lower protein content at large amounts of 1-dodecanol is that these conditions produce a monolith with large pores and a low surface area for ligand attachment. As the amount of 1-dodecanol versus cyclohexanol is decreased to intermediate levels, the overall pore size decreases and the surface area of the material increases, leading to an increase in the amount of protein that can be immobilized. However, a further decrease in the 1-dodecanol versus cyclohexanol levels eventually leads to the creation of pores that are too small for the protein to enter, thus leading to a smaller amount of immobilized protein [3].

The amount of 1-dodecanol needed to reach the maximum HSA content for the GMA/EDMA monoliths also depended on the polymerization temperature and monomer composition. As shown inFig. 3, the amount of 1-dodecanol that gave the highest amount of immobilized HSA for monoliths prepared at 60 °C and using 20:20 GMA/EDMA was roughly 17% of the porogen (i.e., 10% of the total polymerization mixture). For monoliths that were prepared at 80 °C and using 24:16 GMA/EDMA, this amount of 1-dodecanol was approximately 33% of the porogen (i.e., 20% of the total polymerization mixture). This shift could be explained based on the presence of possible differences in the pore structures and surface areas of these two sets of supports (see Fig. 4(a) and (b) and Ref. [3]). Similar trends in monoliths when changing the polymerization temperature and monomer composition have been noted in prior work with GMA/EDMA monoliths [3,7]. In addition, the monoliths prepared at 60 °C and using 20:20 GMA/EDMA resulted in a 53–57% lower maximum HSA content than the monoliths that were prepared at 80 °C using 24:16 GMA/EDMA.

The relative amount of HSA that could be immobilized to these monoliths followed the same trend for the epoxy and Schiff base methods, as can be seen by comparing Fig. 3(a) and (c) with Fig. 3(b) and (d). The maximum amount of HSA that was immobilized by the Schiff base method for monoliths that were prepared at 80 °C and using 24:16 GMA/EDMA, as obtained with 33.3% 1-dodecanol in the porogen (i.e., 20% of the total polymerization mixture), was 51.6 (±0.6) mg HSA/g monolith. Using the same type of monolith with the epoxy immobilization method gave an HSA content of 32.2 (±1.8) mg HSA/g monolith. In comparison, the monoliths that were prepared with the Schiff base method, a polymerization temperature of 60 °C and using 20:20 GMA/EDMA with 16.7% of 1-dodecanol in the porogen (i.e., 10% of the total polymerization mixture) gave a protein content of 27.1 (±1.2) mg HSA/g monolith. The same monolith with the epoxy immobilization method resulted in an HSA content of 18.1 (±1.7) mg HSA/g monolith. Overall, the Schiff base method produced a 46–60% larger maximum amount of

immobilized HSA for these supports when compared to the epoxy immobilization method. A similar difference in the results for these immobilization methods was observed for the GMA/TRIM monoliths that were examined later in this report. This difference has been noted for these two coupling methods with other types of supports and is believed to be due to the different rates of these immobilization processes and the relative stabilities of their activated sites (e.g., hydrolytic loss of activated sites in the epoxy method over time)[5,7].

The polymerization and immobilization conditions that gave the highest content of HSA were also found to allow for the production of columns with relatively low back pressures. This observation agrees with previous studies that have examined similar GMA/EDMA supports [3,5,7]. The back pressure was typically less than 3.4 MPa (500 psi) at 0.5 mL/min for a 4.6 mm i.d. × 50 mm HSA column prepared under the final conditions selected for use with polymerization at 80 °C and using 24:16 GMA/EDMA, and was 1.4 MPa (200 psi) or less for columns prepared at 60 °C and using 20:20 GMA/EDMA. Similar trends were noted for these materials when they were utilized with 4.6 mm i.d. × 1 mm disk columns, giving back pressures at 0.5 mL/min that were less than 0.3 MPa (50 psi) and 0.2 MPa (30 psi), respectively. The difference in back pressure between these two sets of materials was probably related to the smaller average pore size of the monoliths that were prepared at 80 °C and using 24:16 GMA/EDMA. However, the measured back pressures indicated that all of these materials were suitable for use in HPLC systems for chiral separations or other applications of HPAC and AMC.

3.2. Optimization of GMA/TRIM monoliths

Similar studies to those described for the GMA/EDMA monoliths were conducted with GMA/TRIM monoliths. TRIM was of interest as an alternative monomer to EDMA in monolith preparation because it offered the potential to produce a higher degree of cross-linking, thus increasing the potential for functional groups to be present on the surface of the monolith [6,15]. Two sets of thirteen monoliths were prepared using 28:12 (v/v) GMA/TRIM at 60 °C or 80 °C and with various amounts of 1-dodecanol versus cyclohexanol being placed in the porogen. The results are summarized in Fig. 5 and in Tables 3s and 4s in the Supplemental Material. Some representative SEM images of these materials are provided in Fig. 4(c) and (d).

The overall trends seen for the GMA/TRIM monoliths in Fig. 5 were similar to those for the GMA/EDMA monoliths in Fig. 3. For instance, the largest amount of HSA that could be attached to these materials occurred at intermediate levels of 1-dodecanol versus cyclohexanol in the porogen. The location of this optimum was higher at 60 °C for the monoliths prepared with the 28:12 GMA/TRIM (i.e., occurring at roughly 22% 1-dodecanol in the porogen, or 13% in the total polymerization mixture) when compared to data for the 20:20 GMA/EDMA monoliths made at the same temperature in Fig. 3 (i.e., a 1-dodecanol content in the porogen of around 17%). However, the 1-dodecanol content that produced the largest HSA content did not show any noticeable change in going from a polymerization temperature of 60–80 °C for the GMA/TRIM monoliths.

The maximum HSA content for the GMA/TRIM monoliths was next determined. For the GMA/TRIM monoliths that were polymerized at 60 °C and using 21.7% 1-dodecanol in the porogen, the Schiff base method gave supports that contained 48.8 (±2.0) mg HSA/g monolith. Use of the epoxy method with the same type of support gave a 41% lower content of 28.8 (±0.8) mg HSA/g monolith. Similar results were found for the GMA/TRIM monoliths that were made at 80 °C and that also used 21.7% 1-dodecanol in the porogen. In this case, the Schiff base method gave 46.3 (±1.5) mg HSA/g monolith, and the epoxy method gave a 42% lower content of 27.2 (±1.3) mg HSA/g monolith. These protein contents were between those measured earlier for monoliths prepared at 60 °C using 20:20

GMA/EDMA and at 80 °C using 28:12 GMA/EDMA, the latter of which gave the highest overall HSA content in this study.

A set of 4.6 mm i.d. × 50 mm columns with HSA immobilized in GMA/TRIM monoliths polymerized at 60 °C and using 21.7% 1-dodecanol in the porogen gave an average back pressure of less than 2.0 MPa (300 psi) at 0.5 mL/min. Similar columns made with GMA/TRIM monoliths prepared at 80 °C and using 21.7% 1-dodecanol in the porogen also gave a back pressure of 2.0 MPa or less under these experimental conditions. Disk columns made with these materials and that were 4.6 mm i.d. × 1 mm in size gave an average back pressure less than 0.3 MPa (50 psi) at 0.5 mL/min. These properties indicated that such monoliths would be suitable for use in HPLC systems for chiral separations and HPAC.

3.3. Comparison with previous studies

Although the composition of GMA/EDMA or GMA/TRIM monoliths has not been optimized in prior work with HSA, a previous report has examined the preparation of GMA/EDMA monoliths for rabbit IgG [3]. Rabbit IgG has a larger mass and size than HSA (molar mass, 150 kDa versus 66.5 kDa). This size change would be expected to lead to some differences in the amount of surface area that is accessible to each of these proteins on a porous polymer. It has been determined for IgG that the optimum polymerization conditions for GMA/EDMA monoliths occur at a polymerization temperature of 80 °C when using 24:16 GMA/EDMA and roughly 30% 1-dodecanol in the porogen [3]. This resulted in GMA/EDMA monoliths that could contain 59 (±3) mg IgG/g monolith, or 390 (±20) nmol/g, when using the epoxy immobilization method. When the same conditions were applied to HSA without further optimization, the amount of protein that was immobilized by the Schiff base method was 282 (±19) nmol HSA/g monolith, or 19 (±1) mg/g [5].

As shown in Fig. 3, it was found there was a relatively sharp maximum in the amount of HSA that can be immobilized to GMA/EDMA monoliths as the amount of 1-dodecanol in the porogen was varied. Optimizing the level of 1-dodecanol in this region to 33% of the porogen at a polymerization temperature of 80 °C and using 24:16 GMA/EDMA resulted in 51.6 (±0.6) mg HSA/g monolith, or 776 (±9) nmol/g, now being obtained by the Schiff base method. This increase corresponded to over a 2.7-fold larger amount of HSA than could be placed in a GMA/EDMA monolith when compared to the conditions that were used in Ref. [5]. In a similar manner, the GMA/TRIM monoliths that were optimized in this report gave up to a 2.6-fold higher HSA content than previous GMA/EDMA monoliths that have been used for this protein [5].

3.4. Chromatographic studies of monoliths

The next stage of this study employed the HSA monoliths that were optimized in this report and explored their use in chiral separations. *R/S*-Warfarin was one chiral analyte that was considered. Warfarin is an important anti-coagulant that is known to bind in a stereoselective manner to Sudlow site I of HSA. This binding has led to the frequent use of *R*- and *S*-warfarin as probes to examine and model the interactions of other drugs and solutes at Sudlow site I and to evaluate HSA columns for chiral separations [18,25,26].

The chromatographic experiments were first conducted by utilizing a 4.6 mm i.d. × 50 mm GMA/EDMA monolith that was made using 24:16 GMA/EDMA, 33% 1-dodecanol in the porogen, and a polymerization temperature of 80 °C, with HSA being immobilized to the resulting support by the Schiff base method. These were the conditions found in Figs. 3 and 5 to give the largest final protein content, with a value of around 52 mg HSA/g monolith. A 20 μL portion of 20 μM *R/S*-warfarin was then injected onto this column in the presence of pH 7.4, 0.067 M potassium phosphate buffer at various flow rates. An example of a typical

chromatogram that was obtained at 0.75 mL/min is shown in Fig. 6. The retention factor for *R*-warfarin (k_R) on the optimized column under these conditions was 81 (±1), and the retention factor for *S*-warfarin (k_S) was 121 (±2). The plate numbers measured for these two peaks were both around 420–425, which corresponded to a total plate height of 0.12 mm. Greater than baseline resolution (R_s = 1.71 (±0.04)) was produced between the warfarin enantiomers in this example even though no organic modifiers were used to sharpen the peaks or to modify the separation in this particular experiment (i.e., a relatively common practice in protein-based chiral separations) [21–23,26]. The separation factor (α) for these peaks was 1.49 (±0.03), where α represented the ratio of the retention factors ($\alpha = k_S/k_R$).

For comparison, the same separation conditions and samples were used with an HSA GMA/EDMA monolith that was prepared as described in Ref. [5]. The results are also shown in Fig. 6. The retention factors for *R*- and *S*-warfarin for this second, reference column were 37 (±2) and 55 (±1). The separation factor in this case was essentially the same as for the optimized HSA monolith, giving a value for α of 1.48 (±0.03); however, the resolution was now 1.49 (±0.03). The higher retention and resolution of the optimized HSA monolith were both directly related to the 2.7-fold higher protein content of this column when compared to the column based on Ref. [5]. This also explains why similar separation factors were observed for the two columns, because the value of α would have normalized for any actual or apparent differences in protein content when using analytes such as *R*- and *S*-warfarin that bind to the same region of HSA [5,26].

Both columns in Fig. 6 allowed for essentially baseline resolution (i.e., a resolution of 1.5 or greater) when using only an aqueous mobile phase with no organic additives. However, the HSA monolith that was prepared according to Ref. [5] could not be used at higher flow rates without leading to some overlap in the peaks and less than baseline resolution. This was not the case for the newer, optimized HSA monolith. As shown in Fig. 7(a), this new type of HSA support could be used at flow rates up to 1.5 mL/min and still provided essentially baseline resolution between *R*- and *S*-warfarin. Thus, one result of having a higher content of HSA in this monolith was that a chiral separation for *R*- and *S*-warfarin could be obtained that was two times faster than seen for the same column in Fig. 6.

It was also possible to increase the resolution and efficiency of the newer, optimized HSA monolith by placing a small amount of an organic modifier into the mobile phase. For instance, the addition of 0.5% 1-propanol to the mobile phase and the use of a flow rate of 0.75 mL/min gave retention factors on this column of 91 (±2) and 185 (±1) for *R*- and *S*-warfarin, with a resolution and separation factor that increased to 2.91 (±0.04) and 2.02 (±0.02), respectively. This increase in resolution was due to both a shift in the relative retention of the enantiomers as well as the creation of narrower peaks, which now gave plate numbers of 530–615 and plate heights of 0.08–0.09 mm.

A second experiment was completed in which D/L-tryptophan was used as a model analyte. L-Tryptophan is often used as a probe for the Sudlow site II of HSA [18], and D- and L-tryptophan are known to bind in a stereoselective manner to HSA [27], making these enantiomers useful models in the initial evaluation of HSA columns for chiral separations. The same HSA monolith as used in Fig 7(a) for *R*- and *S*-warfarin was tested for use in the separation of D- and L-tryptophan. The results are provided in Fig. 7(b). At 1.5 mL/min, the retention factor for D-tryptophan was 1.49 (±0.05) and for L-tryptophan it was 5.98 (±0.04). The plate numbers that were obtained for these peaks were 430 and 270, respectively, which gave total plate heights of 0.11 and 0.18 mm. The corresponding separation occurred in less than 4 min, giving a resolution of 4.52 (±0.05) and a separation factor of 4.01 (±0.02). These results indicated that the same type of optimized HSA monolith could be used in even

smaller columns and/or at higher flow rates to further reduce the time of this separation while still providing baseline resolution between the tryptophan enantiomers.

As was noted for R/S-warfarin, an improvement in the resolution and efficiency of the new HSA monoliths could be obtained for D- and L-tryptophan when adding a small amount of organic modifier to the mobile phase. For instance, at 0.75 mL/min the use of 0.5% 1-propanol in the mobile phase gave retention factors of 2.02 (±0.03) for D-tryptophan and 8.51 (±0.05) for L-tryptophan. The separation of these enantiomers again took place in less than 4 min but now gave a resolution of 5.62 (±0.02) and a separation factor of 4.21 (±0.01). The number of plates was 695 or 380 for D- and L-tryptophan under these conditions, which corresponded to a total plate height of 0.07 or 0.13 mm.

3.5. Use of monoliths in affinity microcolumns

The final stage of this report examined the use of the improved HSA monoliths in affinity microcolumns for chiral separations or for the rapid screening of drug–protein binding. These studies were carried out by using the same polymerization and immobilization conditions as employed in the previous section (i.e., an HSA monolith made using 24:16 GMA/EDMA, 33% 1-dodecanol in the porogen, and a polymerization temperature of 80 °C, with HSA being immobilized by the Schiff base method). However, the final monolith in this case now had a size of 4.6 mm i.d. × 10 mm. This column exhibited a back pressure of roughly 0.6 MPa (90 psi) at 0.5 mL/min when using pH 7.4, 0.067 M phosphate buffer as the mobile phase.

This small monolith was evaluated by again using *R/S*-warfarin and D/L-tryptophan as model chiral solutes. Flow rates up to 3 mL/min were used with D/L-tryptophan and up to 2.0 mL/min were used with *R/S*-warfarin, as made possible by the lower back pressure of the 4.6 mm i.d. × 10 mm monolith compared to the 4.6 mm i.d. × 50 mm monoliths in the previous section. A mobile phase consisting of pH 7.4, 0.067 M phosphate buffer was used with this column, with 0.5% 1-propanol being added as an organic modifier in the initial studies.

Some examples of chiral separations that were obtained with this HSA microcolumn are shown in Fig. 8. In the separation that is shown in Fig. 8(a), the retention factor for *R*-warfarin on the HSA microcolumn was 71 (±1) and for *S*-warfarin the retention factor was 128 (±2). The separation factor that was produced for these enantiomers was 1.80 (±0.01) and greater than baseline resolution was obtained in less than 6.0 min (R_S = 1.55 (±0.03)). The efficiencies noted for *R*-warfarin and *S*-warfarin were similar in this system, giving a total plate height of 0.05 mm and plate numbers of 190–195 at 2.0 mL/min. All of these results indicated that 4.6 mm i.d. × 10 mm HSA affinity microcolumns could be successfully used to separate the warfarin enantiomers. In addition, these columns could do so in a total separation time that was 5–13-times faster than was obtained with the 4.6 mm i.d. × 50 mm HSA columns used in Figs. 6 and 7. This decrease in separation time was a result of the shorter retention times of the HSA microcolumns and their lower back pressures, the latter of which allowed higher flow rates to be used in this separation.

A similar separation was carried out for D/L-tryptophan on the 4.6 mm i.d. × 10 mm HSA microcolumn. Fig. 8(b) shows some typical results that were acquired at 3.0 mL/min when using a mobile phase that consisted of pH 7.4, 0.067 M phosphate buffer plus 0.5% 1-propanol as an organic modifier. Under these conditions, the retention factors for D-tryptophan and L-tryptophan were 1.75 (±0.05) and 7.90 (±0.03). The separation factor for these enantiomers was 4.51 (±0.01). The total plate heights measured for D-tryptophan and L-tryptophan were 0.05 and 0.12 mm, which corresponded to plate numbers of 200 and 85 at 3.0 mL/min. Greater than baseline resolution of these enantiomers was obtained in less than

1.5 min, with an estimated resolution of 3.10 (±0.01). These results confirmed that the HSA microcolumn could also be used for the rapid separation of these chiral solutes.

It was further found that the HSA microcolumn gave good separations for these compounds in the presence of only pH 7.4, 0.067 M phosphate buffer and no organic modifier, as might be used in drug–protein binding studies. The separation that was obtained under these conditions at 2.0 mL/min gave retention factors for *R*- and *S*-warfarin of 44 (±1) and 78 (±1), with a separation factor of 1.76 (±0.02) and a resolution of 1.33 (±0.02). The total plate height for both warfarin enantiomers was 0.007 cm, which corresponded to 150 theoretical plates. The retention factors measured for D- and L-tryptophan at 3.0 mL/min under the same mobile phase conditions were 1.10 (±0.03) and 3.50 (±0.01), resulting in a separation factor of 3.20 (±0.03) and a resolution of 2.90 (±0.02). The total plate heights for D-tryptophan and L-tryptophan were 0.08 mm and 0.13 mm, respectively, with plate numbers of 135 and 75.

4. Conclusions

This study examined and optimized GMA/EDMA and GMA/TRIM monoliths for the immobilization of HSA as a chiral binding agent. The polymerization conditions used for these monoliths were varied, with particular emphasis being given to the effects of changing the composition of the porogen. Use of both the Schiff base and epoxy immobilization methods with these monoliths was also considered. A 2.6–2.7-fold increase in HSA content was obtained in the final monoliths when compared to similar HSA monoliths prepared according to the literature. The increased protein content made it possible for the new monoliths to provide fast separations with good retention and resolution for chiral agents such as *R/S*-warfarin and D/L-tryptophan. It was further demonstrated that the final optimized monoliths could be used in 4.6 mm i.d. × 10 mm HSA microcolumns to separate these chiral solutes in only 1.5–6.0 min. Areas in which these monoliths should be useful include not only fast chiral separations but also rapid HPAC studies of drug–protein interactions involving HSA, as could be used for high-throughput drug screening [18,28–31]. In addition, the approaches used in this study could be extended to the separation of other chiral solutes and to the optimization of organic monoliths for use with other proteins as binding agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported, in part, by the National Institutes of Health under grant R01 GM044931 and by the National Science Foundation/EPSCoR program under grant EPS-1004094. Support for the remodeled facilities that were used to perform these experiments was provided under NIH grant RR015468-001. We thank Dr. Han Chen for performing the SEM imaging of the monolith samples.

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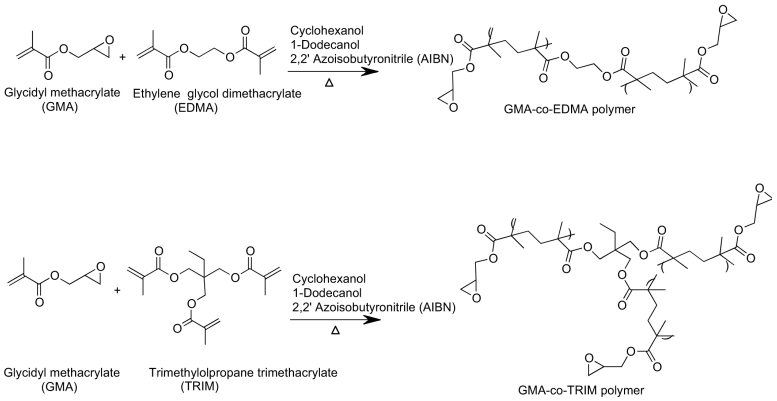

Fig. 1.

General scheme for the preparation of (top) GMA/EDMA monoliths and (bottom) GMA/TRIM monoliths.

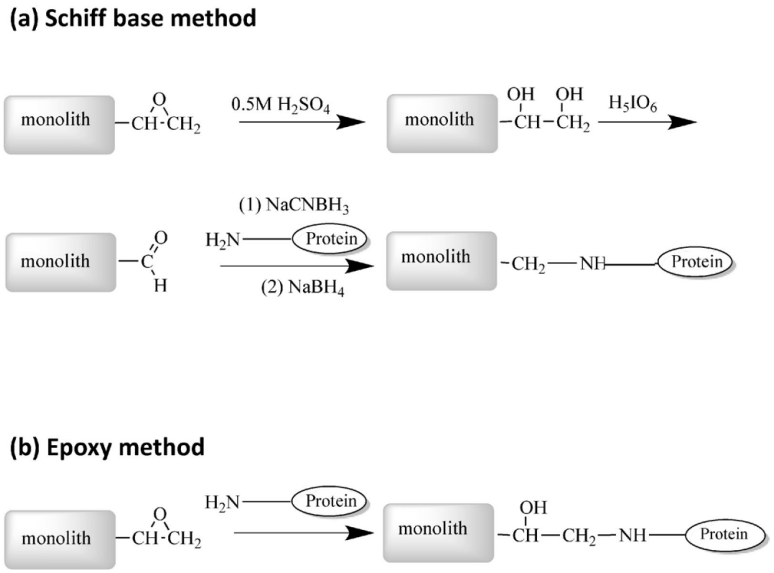

Fig. 2.
The (a) Schiff base and (b) epoxy immobilization methods.

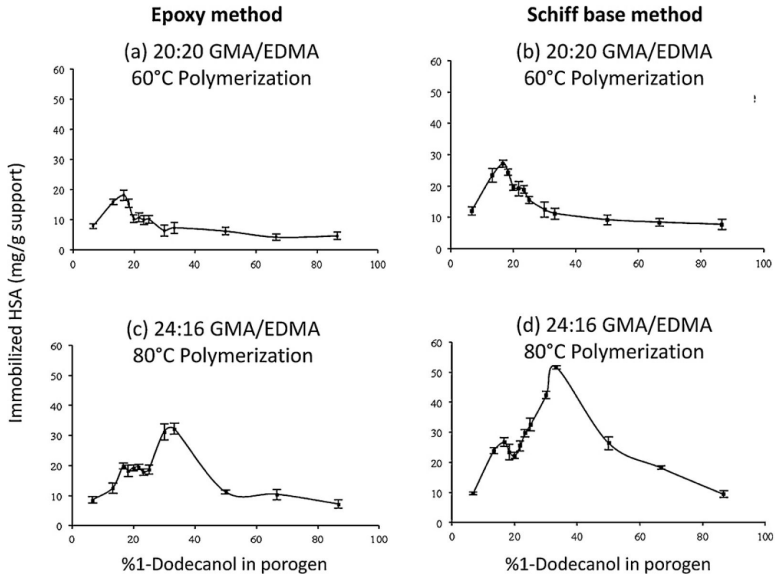

Fig. 3.

Effects of varying the porogen composition on the amount of HSA that could be attached to GMA/EDMA monoliths prepared at 60 °C and utilizing (a) the epoxy method or (b) Schiff base method for immobilization; and to GMA/EDMA monoliths prepared at 80 °C and using (c) the epoxy method or (d) Schiff base method for immobilization. The error bars represent a range of ±1 standard deviation of the mean for triplicate measurements.

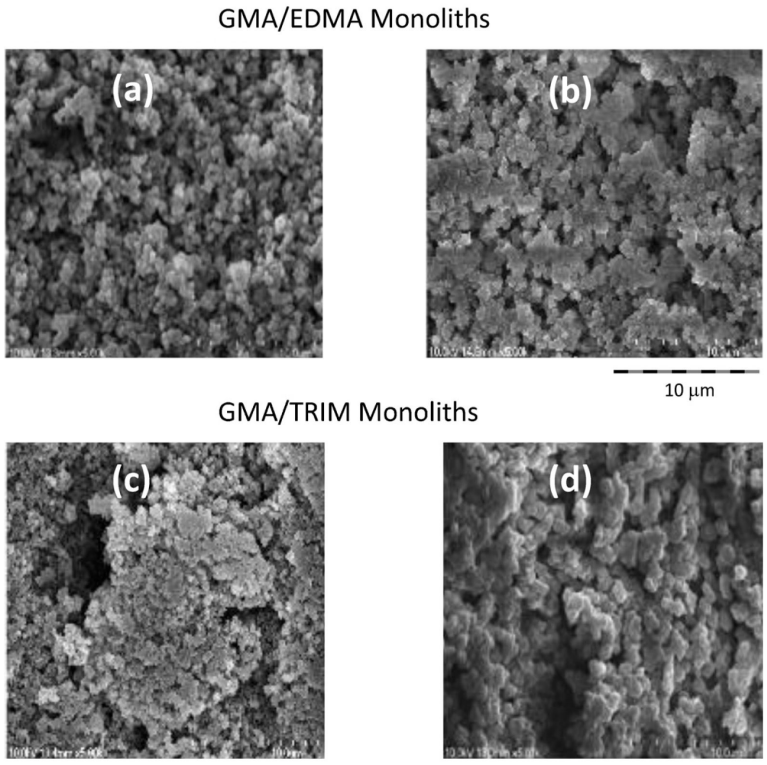

Fig. 4.

Scanning electron micrographs for monoliths polymerized (a) at 80 °C using 24:16 GMA/EDMA and 40:20 cyclohexanol:1-dodecanol in the polymerization mixture; (b) at 60 °C using 20:20 GMA/EDMA and 50:10 cyclohexanol:1-dodecanol in the polymerization mixture; (c) at 80 °C using 28:12 GMA/TRIM and 47:13 cyclohexanol:1-dodecanol in the polymerization mixture; or (d) at 60 °C using GMA/TRIM and 47:13 cyclohexanol:1-dodecanol in the polymerization mixture. Other conditions are given in the text.

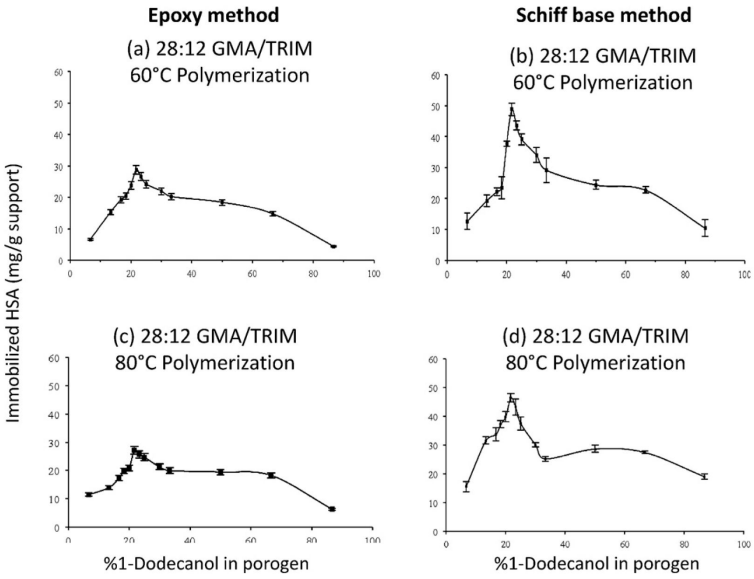

Fig. 5.

Effect of varying the porogen composition on the amount of HSA that could be attached to GMA/TRIM monoliths prepared at 60 °C and utilizing (a) the epoxy method or (b) Schiff base method for immobilization; and to GMA/TRIM monoliths prepared at 80 °C and utilizing (c) the epoxy method or (d) Schiff base method for immobilization. The error bars represent a range of ±1 standard deviation of the mean for triplicate measurements.

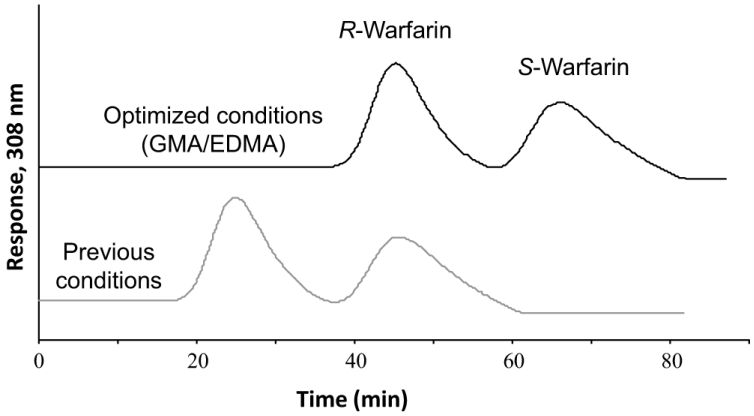

Fig. 6.

Chiral separation of *R*- and *S*-warfarin on 4.6 mm i.d. × 50 mm GMA/EDMA monolith columns. The lower chromatogram was obtained for a column that was prepared under the same conditions as used in Ref. [5] but using conditions which had optimized for IgG rather than HSA. The upper chromatogram shows the results that were obtained using the conditions that were optimized in this current study for HSA, which involved the use of a mixture of 24:16 GMA:EDMA and 40:20 cyclohexanol:1-dodecanol that was polymerized at 80 °C, followed by use of the Schiff base immobilization method. The mobile phase in each case was pH 7.4, 0.067 M phosphate buffer and the flow rate was 0.75 mL/min. Other conditions are given in the text.

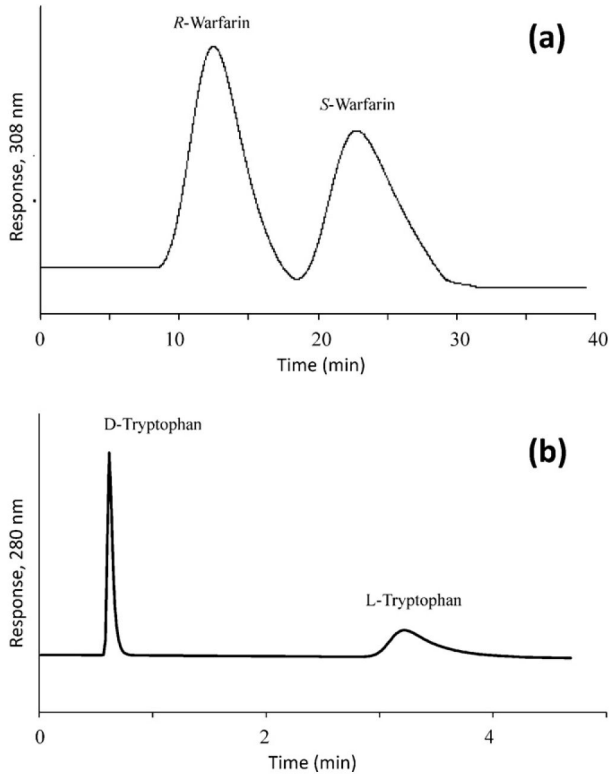

Fig. 7.

Chiral separations for (a) *R*- and *S*-warfarin and (b) D- and L-tryptophan on a 4.6 mm i.d. × 50 mm GMA/EDMA monolith column, as prepared using the polymerization and immobilization conditions that were optimized in this study for HSA (Note: see conditions given in Fig. 6). The mobile phase was pH 7.4, 0.067 M phosphate buffer and the flow rate was 1.50 mL/min. Other conditions are given in the text.

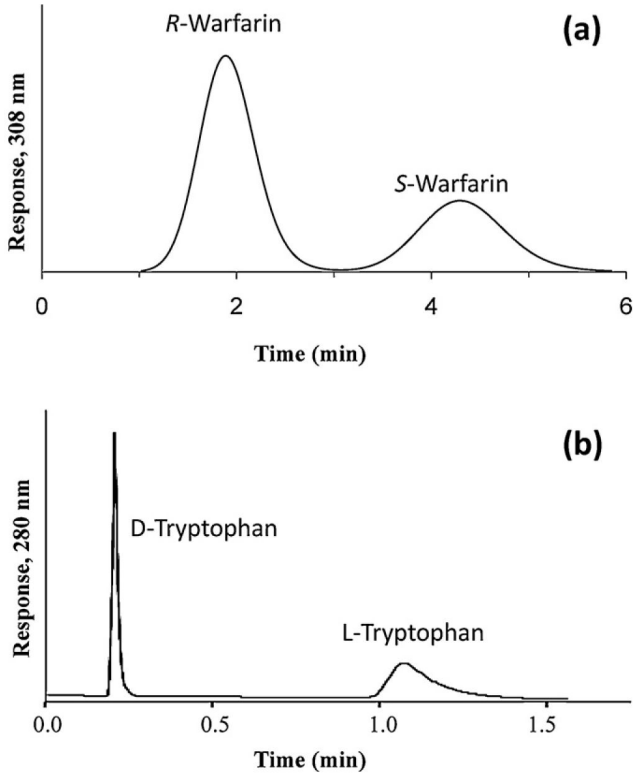

Fig. 8.

Chiral separations for (a) *R*- and *S*-warfarin and (b) D- and L-tryptophan on a 4.6 mm i.d. × 10 mm GMA/EDMA monolith column, as prepared using the polymerization and immobilization conditions that were optimized in this study for HSA. The mobile phase was pH 7.4, 0.067 M phosphate buffer that contained 0.5% 1-propanol. The flow rate in (a) was 2.0 mL/min and the flow rate in (b) was 3.0 mL/min. Other conditions are given in the text.