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DEVELOPMENT OF AN AFFINITY SILICA MONOLITH CONTAINING α$_1$- ACID GLYCOPROTEIN FOR CHIRAL SEPARATIONS

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Abstract

An affinity monolith based on silica and containing immobilized α$_1$-acid glycoprotein (AGP) 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 monoliths based on a copolymer of glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA). The surface coverage of AGP in the silica monolith was 18% higher than that obtained with silica particles and 61% higher than that measured for a GMA/EDMA monolith. The higher surface area of the silica monolith gave materials that contained 1.5- to 3.6-times more immobilized protein per unit volume when compared to silica particles or a GMA/EDMA monolith. The retention, efficiency and resolving power of the AGP silica monolith were evaluated by injecting two chiral analytes onto this column (i.e., *R/S*-warfarin and *R/S*-propranolol). In each case, the AGP silica monolith gave higher retention plus better resolution and efficiency than AGP columns containing silica particles or a GMA/EDMA monolith. The AGP 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 AGP as a chiral stationary phase.

1. INTRODUCTION

Monolithic columns have recently been shown to have several advantages over particle-based columns in high-performance affinity chromatography (HPAC) [1–6]. These advantages include better mass transfer properties, lower back pressures, and the ability to perform faster separations [1–6]. Organic polymers based on glycidyl methacrylate (GMA) and ethylene dimethyacrylate (EMDA) have been employed in several previous studies to create monoliths that contain affinity ligands [3,4,7–14]. These GMA/EDMA monoliths have been utilized for affinity purifications [3], chiral separations [4], and ultrafast immunoextractions [7]. The result is a method that has been referred to as affinity monolith chromatography (AMC) [3].

Silica monoliths have also been combined with affinity ligands in some previous studies [3, 8–10]. This includes the development of immobilized enzyme reactors based on trypsin or β-glucoronidase [8,10] and the use of immobilized penicillin G acylase for the chiral separation of ketoprofen enantiomers [9]. The potential advantages of using silica monoliths with affinity ligands include the high surface areas that are available on these materials for ligand attachment [11] and the ability to use the same immobilization methods that are employed with silica

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particles [9,12]. This study will investigate the development of a silica monolith that contains immobilized α$_1$-acid glycoprotein (AGP) as a chiral stationary phase. AGP (average MW, 41 kDa) is a serum protein that binds to various drugs [13–17]. AGP has been used in HPLC columns for both chiral separations [13,14,16–20] and for the study of drug-protein interactions [17,20].

This report will first examine the preparation of a silica monolith for the immobilization of AGP. In previous reports examining affinity silica monoliths, proteins have been immobilized onto epoxy-activated supports [9,10]. Although it is known that the epoxy method is easy to perform, this technique tends to give lower activities and lower protein coverages than other coupling methods [3,4,12]. Work in this current study will use the hydrazide immobilization method, a technique that has been shown to give site-selective coupling for glycoproteins like AGP, as demonstrated in previous work with silica particles [17,20]. The resulting AGP silica monolith will be evaluated in terms of its activity, retention and chiral selectivity for two chiral compounds: *R/S*-warfarin and *R/S*-propranolol. The results will be compared with those obtained for AGP immobilized to silica particles and a GMA/EDMA monolith that contains AGP. These experiments should provide information on the relative advantages and disadvantages of silica monoliths when they are used with immobilized AGP and other proteins for chiral separations.

2. Experimental

2.1. Reagents

The human AGP (Cohn fraction VI, 99% pure), carbamazepine (>98% pure), R/S-propranolol (hydrochloride, >99% pure), racemic warfarin (>98% pure), periodic acid reagent (>99% pure; an oxidizing agent), sodium borohydride (98% pure; a strong reducing agent), and 3-glycidoxypropyltrimethoxysilane (97% pure) were from Sigma (St. Louis, MO, USA). The GMA (97% pure), EDMA (98% pure), azobisisobutyronitrile (AIBN, 98% pure; used as an initiator for GMA/EDMA polymer preparation), 1-dodecanol (98% pure), and oxalic dihydrazide (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 (Dûren, 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).

2.2. Apparatus

The Chromolith Performance Si columns (10 cm × 4.6 mm I.D.) were donated by Merck (Darmstadt, Germany). These silica monolithic columns were created by the general approach shown in Figure 1 [11,21]. The GMA/EDMA monoliths were made in 5 cm × 4.6 mm I.D. PEEK (polyetheretherketone)-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. 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 AGP through the activated monoliths for immobilization. The silica particles were packed into a 5 cm × 4.6 mm or 4.1 mm I.D. stainless steel column using an Alltech slurry packer (Note: the estimated column-to-column variation in plate numbers and plate heights for the packed columns was 10–15%). 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 [22,23]. 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 97ºC 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 97ºC 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 70ºC for 3 h. The column 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 AGP in silica monolith

The scheme used to immobilize AGP in a silica monolith is shown in Figure 2. This approach was adapted from a recent method described for attaching AGP to hydrazide-activated silica particles [17]. This method was performed by first applying 50 mL of a 90% acetic acid solution in water to a diol silica monolith at 0.2 mL/min for 4 h. This monolith was then oxidized to an aldehyde-activated form by applying 80 mL of a 0.05 g/mL solution of periodic acid in 90% acetic acid at 0.2 mL/min for 6.7 h in the dark, followed by washing of the monolith with 50 mL of water at 0.2 mL/min for 4.2 h. A 50 mL solution containing 0.13 g oxalic dihydrazide in pH 7.0, 0.10 M potassium phosphate buffer (KPB) was next circulated through the aldehyde-activated monolith at 0.5 mL/min for 1.7 h. Any remaining aldehyde groups in the monolith were reduced by applying 1 mg/mL sodium borohydride in pH 8.0, 0.10 M KPB at 0.05 mL/min for 90 min using a syringe pump. This column was washed with 50 mL of water at 0.5 mL/min for 1.7 h.

Prior to immobilization, the carbohydrate residues on AGP were oxidized under mild conditions to generate aldehyde groups [17]. This was accomplished by preparing a solution containing 10 mL of 5 mg/mL AGP in pH 7.0, 20 mM sodium acetate buffer containing 0.15 M sodium chloride plus 10 mL of 20 mM periodic acid prepared in the same buffer. This mixture was stirred for 30 min at 4ºC. Under these conditions, approximately five reactive aldehyde groups were generated per AGP molecule [17,24]. The resulting oxidation reaction was quenched after 10 min by adding 5 mL of ethylene glycol. Oxidized AGP was purified by passing this mixture through a Econo-Pac 10 DG desalting column (6 kDa exclusion limit, 10 mL volume; Bio-Rad, Hercules, CA, USA), using pH 7.0, 0.10 M KPB as the elution buffer. The collected fractions gave 20 mL of a solution containing approximately 2.5 mg/mL oxidized AGP.

The hydrazide-activated silica monolith was washed with 30 mL of pH 7.0, 0.10 M KPB at 0.5 mL/min for 1 h. A 12 mL portion of the 2.5 mg/mL oxidized AGP solution in pH 7.0, 0.10 M KPB was circulated through this monolith at 0.5 mL/min for 48 h. Another 8 mL of the oxidized AGP solution was combined with the first oxidized AGP solution and circulated through the same monolith for 48 h at 0.5 mL/min to obtain maximum protein coverage of the support. This monolith was then washed with pH 7.4, 0.067 M KPB at 0.5 mL/min for 2 h. The resulting column showed good stability, with less than a 7% change in separation factor being observed for racemic warfarin over at least two months. A hydrazide-activated silica monolith was used as the control column in studies examining the behavior of this AGP silica monolith.

2.5. Immobilization of AGP to other supports

A packed silica column containing immobilized AGP was prepared according to a previous procedure [17] and followed the general reaction scheme given in Figure 2. A 300 Å pore size, 7 μm particle size silica support was employed since this material has previously been examined for use with AGP and the immobilization method in Figure 2 for chiral separations and drug-protein binding studies [17,20]. In addition, a recent study using the protein human serum albumin (HSA; MW, 66.5 kDA) has found that 300 Å pore size silica gives a better fit than 100 Å pore size silica when compared in terms of protein coverage to the same silica and GMA/EDMA monoliths that were used in this current study [25]. Besides being tested for use in 8 chiral separations, AGP silica was also used in this study as a secondary standard for determining the protein content of the AGP silica monolith. A pH 7.4, 0.067 M KPB solution was used to pack the AGP silica particles at 3500 psi (24 MPa) into a 5 cm × 4.6 mm I.D. column (for work with R/S-warfarin) or a 5 cm × 4.1 mm I.D. column (for R/S-propranolol). The final protein content of the AGP silica particles was determined by comparing the final and initial concentration of AGP in the reaction slurry by using absorbance measurement sat 280 nm [20]. The control columns used with this support were prepared using hydrazide-activated silica particles (with no AGP present) [17,20].

A GMA/EDMA monolith containing immobilized AGP was also prepared by the general scheme shown in Figure 2 [4,7,17]. This monolith was synthesized by using a 60:40 (v/v) mixture of GMA and EDMA. This GMA/EDMA mixture was combined in a 40:60 (v/v) ratio with a 80:20 (v/v) mixture of cyclohexanol and 1-dodecanol as the porogen. AIBN was added as an initiator at a 1% (w/w) level versus GMA and EDMA. These conditions were chosen since they have previously been utilized to prepare GMA/EDMA monoliths that have been compared with silica particles and silica monoliths for use with the another immobilized protein (HSA) in chiral separations and drug-protein binding studies [4,25]. After sonicating for 10 min, argon gas was passed through this mixture for 10 min. The mixture was introduced into a 5 cm × 4.6 mm I.D. column with one end sealed with a plug; the other end of the column was then sealed and the column placed in a water bath at 80ºC for 24 h for polymerization. After polymerization, the column was washed with 100 mL of acetonitrile at 0.5 mL/min for 3 h. The average diameter of the throughpores in this monolith was 1–2 μm, as estimated by scanning electron microscopy. Further information on this type of monolith can be found in Refs. [4] and [7].

AGP was oxidized for immobilization in the GMA/EDMA monolith by using the same conditions as employed for the AGP silica monolith and silica particles [17]. A hydrazide-activated form of the GMA/EDMA monolith was prepared in a similar manner to that used for the silica monolith. First, 5 mL of a 0.5 M sulfuric acid solution was passed through the GMA/EDMA monolith at 0.2 mL/min for 25 min, followed by heating of the sealed column at 60°C for 4 h in a water bath. This column was next washed with 100 mL of water at 0.5 mL/min for ~ 3 h. This was followed by the application of a 40 mL solution of 2 g periodic acid in 90%

acetic acid at 0.5 mL/min for 4 h. A 50 mL solution containing 0.13 g oxalic dihydrazide in pH 7.0, 0.10 M KPB was next circulated through the monolith at 0.5 mL/min for 1.7 h. Any remaining aldehyde groups in the monolith were reduced by applying a 1 mg/mL solution of sodium borohydride in pH 8.0, 0.10 M KPB at 0.05 mL/min for 90 min using a syringe pump. This column was washed with 50 mL of water at 0.5 mL/min for 1.7 h. A solution of oxidized AGP was immobilized in this column using the same approach as described for the AGP silica monolith. The amount of protein in the AGP GMA/EDMA column was estimated by using a copper sulfate assay (see next section). The control column for this support was prepared by using a hydrazide-activated GMA/EDMA monolith to which no AGP had been added.

2.6. Determination of protein content in silica monoliths

The binding capacity and total amount of protein in the AGP silica monolith were originally estimated by employing frontal analysis. This was accomplished by using carbamazepine, an analyte that has a single primary site on AGP. Carbamazepine concentrations of 10–50 μM were applied in pH 7.4, 0.067 M KPB to columns containing the AGP 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 [26,27]. Elution of the carbamazepine was monitored at 214 nm. The results obtained with the control columns were used to correct for the void time of the system and non-specific binding of carbamazepine to the column (i.e., 35% of the binding capacity in the AGP monolith). The total amount of AGP in the monolith was then calculated by using the corrected binding capacity along with specific activities that have been reported for carbamazepine with AGP when using the immobilization method in Figure 2 with silica particles [24,28].

A second estimate of the total protein content in each monolith was obtained by injecting a 0.1% (w/v) solution of copper sulfate onto the AGP columns. This method used copper sulfate as a probe for the overall ion-exchange capability of the immobilized proteins [25]. The retention factor measured for the injected sample at 258 nm on the AGP monolith was compared to those obtained for the same sample on columns packed with silica particles that had known amounts of immobilized AGP. This experiment was performed on the AGP columns at 37°C using pH 3.0, 0.5 M 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 AGP. The nonspecific retention noted for copper sulfate on the control columns was less than 4% of the retention measured on AGP column.

2.7. Chromatographic studies

The mobile phase utilized for the chiral separation of *R/S*-propranolol on the AGP columns was pH 7.4, 0.067 M KPB containing 3% isopropanol. The mobile phase for *R/S*-warfarin was pH 7.0, 0.067 M KPB containing 5% isopropanol. These conditions were used to help reduce analyte retention and minimize band-broadening and were selected based on previous work with silica particles containing AGP. These mobile phases were degassed under vacuum for at least 30 min prior to use. The chromatographic studies using warfarin and propranolol were performed at 25°C and 37°C, respectively.

The injected samples were prepared in the given mobile phase and contained 20 μM of the desired analyte. These samples were stored at 4°C and used within two weeks of preparation. 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 in this study. Three 20 μL injections were typically made of the samples under each given set of experimental conditions. 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 AGP columns containing silica particles [17,20].

A detection wavelength of 225 nm was used for propranolol and 310 nm was used for warfarin. The system void time was determined by injecting 20 μL of 0.2 mM sodium nitrate onto the chromatographic system while monitoring 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 [29]. 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 [30,31].

3. Results and Discussion

3.1. General properties of affinity silica monolith

The structure of a monolith is often described by classifying its pores into two categories: macropores, with widths exceeding 50 nm, and mesopores, with widths more than 2 nm but less than 50 nm. The silica monoliths used in this report have macropores with an average diameter of 2 μm and mesopores with an average diameter of 13 nm. The macropores in these materials help to increase their permeability, while the mesopores play an important role in determining the total surface area. The total porosity of these monoliths is more than 80%, with 75% of this being due to the macropores [32,33]. The pore volume of the silica monoliths used in this study was 1 mL/g monolith and their total surface area was 300 m^2/g.

Two items considered early in this work were the amounts of total and active AGP that could be placed within a silica monolith. These values were first estimated through a nondestructive method by measuring the binding capacity of the affinity monolith for carbamazepine, an achiral solute that interacts with AGP. Using these results and a density for the support in the silica monoliths of 0.2 g/mL, the measured binding capacity was found to be 1.21 (± 0.07) μmol carbamazepine/g of AGP monolith (where the number in parentheses represents ± 1 standard deviation). 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 AGP of 0.67 (± 0.01) or 67% for carbamazepine. This value made it possible to use the measured binding capacity of the monolith to also provide an estimate of its total protein content, giving 1.81 (± 0.06) μmol AGP/g monolith.

A second estimate of the total protein content for the AGP silica monolith was obtained by examining its retention for copper sulfate versus reference columns that contained known amounts of AGP. This approach gave a protein content of 1.79 (± 0.06) μmol AGP/g monolith. This result differed by less than 2% from the estimate made using carbamazepine and frontal analysis. From these total protein contents, the total mass of immobilized protein in the silica monolith was determined to be 24.3 (± 0.6) mg AGP. This amount was equal to 49% of the total AGP that had originally been passed through the silica monolith during the immobilization step, indicating that a two-fold excess of AGP had been used in this process.

Table 1 compares the AGP content of the silica monolith with those of silica particles that contained AGP and that were prepared using the same immobilization method. The total protein content per unit mass of support in the silica monolith was 3.7-fold higher than that obtained with the 300 Å pore size, 7 μm silica particles employed in this work. However, the silica particles and silica monolith had different surface areas (e.g., 100 m^2/g for the silica particles and 300 m^2/g for the silica monolith) [20,34,35]. When the amount of immobilized protein

was expressed in terms of a surface coverage, the silica monolith gave an 18% higher value than the silica particles (i.e., 5.8 versus 4.9 nmol AGP/m^2).

One reason for selecting the silica particles and GMA/EDMA monolith that were used in this comparison is that they have been found to give equivalent protein coverages to a silica monolith in a previous study examining the protein HSA [25]. Using silica particles with a smaller pore size of 100 Å will give a 3.5-fold greater surface area than 300 Å pore size silica particles (i.e., a surface area similar to that for the silica monolith in this study); however, changing to the former support gives only a 35–40% increase in immobilized HSA and a 61% decrease in protein coverage per unit area [25,36]. This previous result indicated that the 300 Å pore size silica was a more comparable support (in terms of protein surface coverage) to the silica monolith and GMA/EDMA monolith. Although AGP has a lower mass than HSA, it has a comparable or slightly larger size due to its many carbohydrate chains [37,38]. This helps explain why similar protein coverages were obtained for the various supports in Table 1. The small differences in protein coverage that were noted in Table 1 are probably due to the different pore size distribution of these supports and the exclusion of AGP from some of these pores during the immobilization process, as has been noted for antibodies on such materials [7,39].

The silica monolith also gave a 7.2-times higher AGP content per unit mass of support than the GMA/EDMA monolith. However, the GMA/EDMA monolith had a much lower surface area than the silica monolith (67.5 m^2/g versus 300 m^2/g) [7]. When the protein contents were normalized for this difference in surface area, the resulting surface coverage for AGP in the silica monolith was 61% higher than in the GMA/EDMA monolith. This latter observation may reflect differences in the pore structure and distribution of pores in these two types of supports, which may have created some differences in the total surface area that was accessible to AGP for its immobilization.

Another way these supports differed was in their densities. The density of the silica monolith was 0.2 g/mL [40], 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 AGP in the silica monolith was 1.5-fold higher than that obtained with silica particles. When compared to the GMA/EDMA monolith, the silica monolith gave 3.6-fold more immobilized AGP 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 AGP monolith using *R/S*-warfarin

One chiral drug that binds to AGP is *R/S*-warfarin. Racemic warfarin binds to AGP with an average association equilibrium constant of $2–7 \times 10^5$ M^{-1} at 37ºC and pH 7.4 [41,42]. It has been reported that the main binding site for this interaction is the same as that involved in the binding of *R/S*-propranolol (i.e., the other model analyte examined in this report) [41].

Chromatograms showing the separation of *R/S*-warfarin by an AGP silica monolith are given in Figure 3(a). The results of these separations are summarized in Table 2. The average retention factors for *S*-warfarin and *R*-warfarin on the AGP silica monolith were 8.54 and 11.94, which corresponded to retention times of 4.85 min and 6.58 min at 3.0 mL/min (note: a control column with no AGP present gave a retention factor of 0.11 ($\pm$ 0.01) for both warfarin enantiomers). The separation factor for *R/S*-warfarin on the AGP silica monolith ranged from 1.35–1.45 between 0.1 and 3.0 mL/min. The resolution of these enantiomers was 3.08 at 3.0 mL/min and gave a maximum value of $R_s = 3.9$ at 0.5 mL/min. Figure 3(b) shows the corresponding van

Deemter plots. An optimum plate height of roughly 0.04 cm was observed in these plots at 0.024–0.036 cm/s for *R*- and *S*-warfarin.

Table 3 and Figure 3 compare the results obtained on the AGP silica monolith for *R*- and *S*-warfarin with those found when using a 5 cm × 4.6 mm I.D. packed AGP column that contained 7 μm silica particles. The lower protein content of the packed AGP column produced smaller average retention factors than those seen on the AGP silica monolith, giving $k = 3.42$ for *S*-warfarin and 4.37 for *R*-warfarin (note: a control column containing silica particles and no AGP gave $k = 0.08$ (± 0.01) for *R*- and *S*-warfarin). In addition, the retention times measured on the packed AGP column (0.89 min and 1.07 min for *S*-warfarin and *R*-warfarin, respectively, at 3.0 mL/min) were about one-fifth of those seen on the AGP silica monolith. The separation factors for *R*- and *S*-warfarin on the packed AGP column (range 1.2–1.3 between 0.08 and 3.0 mL/min) were similar to those on the AGP silica monolith. However, the resolution on the packed AGP column (0.51–0.67 between 0.08 and 3.0 mL/min) was much lower than that observed on the 10 cm long AGP silica monolith (see Table 3) or that calculated for a 5 cm long AGP silica monolith, with the latter giving a value of 2.2 (± 0.1). This lower resolution was partly due to the lower retention of the packed AGP column but was also a result of the lower efficiency of this column versus the AGP silica monolith. This latter feature is illustrated in Figure 3(b), where *R*- and *S*-warfarin gave optimum plate heights on the packed AGP column that were 1.5–2.6 times higher than on the AGP silica monolith.

A comparison was also made between the AGP silica monolith and a 5 cm × 4.6 mm I.D. GMA/EDMA monolith containing AGP. The lower content of AGP in the GMA/EDMA monolith (see Table 1) again resulted in lower average retention factors, giving $k = 2.23$ for *S*-warfarin and 2.78 for *R*-warfarin (note: nonspecific binding by *R*- and *S*-warfarin on the GMA/EDMA column gave a retention factor of 0.12 (± 0.01) for both enantiomers). The retention times noted on the AGP GMA/EDMA monolith (0.62 min and 0.73 min for *R*- and *S*-warfarin at 3.0 mL/min) were also much lower than those seen for the AGP silica monolith. The separation factor for *R*- and *S*-warfarin on AGP GMA/EDMA monolith varied from 1.2–1.3 between 0.1 and 3.0 mL/min, which was similar to the results for the AGP silica monolith. However, *R*- and *S*-warfarin had a much lower resolution on the AGP GMA/EDMA column (i.e., a maximum R_s of only 0.52 between 0.1 and 3.0 mL/min). The optimum plate heights determined for the AGP GMA/EDMA monolith were 0.017 cm for *R*-warfarin and 0.011 cm for *S*-warfarin at 0.12–0.15 cm/s. These plate heights were about two times higher than those for the AGP silica monolith and comparable to the results for the packed AGP column.

3.3. Evaluation of AGP monolith using *R/S*-propranolol

The second chiral drug that was used as a model in this study was *R/S*-propranolol. This drug has strong binding with AGP and has been previously separated by AGP columns into its separate enantiomers [13,17–19,43–49]. AGP is known to bind *R*- and *S*-propranolol with association equilibrium constants of 0.8×10^6 M^{-1} and 1.04×10^6 M^{-1} at 37ºC and pH 7.4, with these two enantiomers binding to the same primary site on AGP [20].

Chromatograms that were generated for *R/S*-propranolol on the 10 cm × 4.6 mm I.D. AGP silica monolith are provided in Figure 4(a); the results of these separations are summarized in Table 2. The average retention factors for *R*- and *S*-propranolol on this column were 31.1 and 37.2, giving retention times of 16.4 min and 19.5 min at 3.0 mL/min (note: these same solutes had a retention factor of 0.11 (± 0.01) on a control monolith with no AGP present). The separation factor for *R/S*-propranolol on the AGP silica monolith ranged from 1.16–1.21 between 0.3 and 2.5 mL/min, with a maximum resolution of 1.25 being observed under these conditions. Figure 4(b) gives the van Deemter plots for these solutes on the AGP silica monolith. Both *R*- and *S*-propranolol showed only small changes in plate height on this column

over the linear velocities that were examined in this report, with a minimum plate height of approximately 0.008 cm being found for both enantiomers.

The data obtained on the AGP silica monolith for *R*- and *S*-propranolol were compared with those for the 5 cm × 4.6 mm I.D. AGP column packed with 7 μm silica particles. The packed AGP column gave average retention factors of *k* = 11.6 for *R*-propranolol and 13.7 for *S*-propranolol, while a control column with no AGP present had a retention factor of 0.09 (± 0.01). The retention times measured on the packed AGP column were about one-seventh of those seen on the AGP silica monolith (i.e., retention times of 2.45 min and 2.86 min for *R*- and *S*-propranolol at 3.0 mL/min). The separation factor for *R*- and *S*-propranolol on the packed AGP column (*α* = 1.1–1.2 at 0.25 to 3.0 mL/min) was equivalent to that seen on the AGP silica monolith. However, the resolution ranged from only 0.55–0.68 between 0.25 and 3.0 mL/min. This latter value was much lower than the resolution that was seen for the 10 cm AGP silica monolith and was lower than that predicted for a 5 cm AGP silica monolith (i.e., R_s = 0.78 (± 0.02) for a 5 cm × 4.6 mm I.D. monolith). This lower resolution was due to both the weaker retention and lower efficiency of the packed AGP column, where the plate heights for the packed AGP column were 1.8–2.5 times higher than those for the AGP silica monolith, as shown in Figure 4(b).

A 5 cm × 4.6 mm I.D. AGP GMA/EDMA monolith gave average retention factors for *R*- and *S*-propranolol of 4.53 and 6.86. Nonspecific binding of *R*- and *S*-propranolol to this column gave a retention factor of only 0.11 (± 0.01) for *R*- and *S*-propranolol. The retention times measured on the AGP GMA/EDMA monolith were 1.00 min and 1.44 min for *R*- and *S*-propranolol at 3.0 mL/min. The lower retention factors for AGP in the GMA/EDMA monolith versus the silica monolith were expected due to the lower protein content of the GMA/EDMA monolith that was measured previously. The AGP GMA/EDMA monolith gave a separation factor for *R*- and *S*-propranolol that ranged from 1.45–1.53 between 0.1 and 3.0 mL/min. The resolution at 3.0 mL/min was 0.79 and the maximum resolution between 0.1 and 3.0 mL/min was 1.03. These values were lower than those seen for the 10 cm AGP silica monolith but were comparable to those for an AGP silica monolith of equivalent size. The plate heights determined for *R*- and *S*-propranolol on the AGP GMA/EDMA monolith were 0.02 cm and 0.015 cm at 0.045 cm/s, and were about two-fold higher than the plate heights found for *R*-and *S*-propranolol on the AGP silica monolith at the same linear velocity.

3.4. Column back pressure and permeability

The AGP silica monolith was further compared to the other support materials in terms of its back pressure and permeability. 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 (see Figure 5). The silica monolith also gave lower back pressures than the GMA/EDMA monolith. For instance, at 0.5 mL/min the typical back pressure was 7 psi/cm (48 kPa/cm) for the 10 cm × 4.6 mm I.D. silica monolith, 15 psi/cm (103 kPa/cm) for a 5 cm × 4.6 mm I.D. column containing silica particles, and 12 psi/cm (83 kPa/cm) for a 5 cm × 4.6 mm I.D. GMA/EDMA monolith. The back pressures measured at 3.0 mL/min (i.e., the highest flow rate used in this study) were as follows: 10 cm × 4.6 mm I.D. silica monolith, 600–640 psi (4.1–4.4 MPa); 5 cm × 4.6 mm I.D. column containing silica particles, 940–995 psi (6.5–6.9 MPa); 5 cm × 4.6 mm I.D. GMA/EMDA monolith, 420–450 psi (2.9–3.1 MPa).

These supports were also compared in terms of their separation impedance (*E*) [11,21],

$$E = (\Delta P \cdot t_M) \big/ (N^2 \cdot \eta) \qquad (1)$$

where ΔP is the pressure drop across the column, t_M is the column void time, N is 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 pressure drop with the change in efficiency that occurs in a column at different linear velocities. As shown in Figure 6, the AGP silica monolith gave separation impedances for warfarin and propranolol that were consistently better that those for a packed column containing silica particles. 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 AGP silica monolith and packed AGP column). Similar results have been noted in a previous comparison of C_{18} columns based on silica monoliths and 3.5 μm silica particles [11]. When the GMA/EDMA monolith was also considered, it was found that this material gave a separation impedance that was 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.

4. CONCLUSIONS

In this study, a silica monolith containing immobilized AGP was developed and evaluated for use in chiral separations. The results were compared to those obtained when using the same protein and immobilization method with 300 Å pore size, 7 μm silica particles or a GMA/EDMA monolith. The amount of protein that could be immobilized per unit area was 18% to 61% higher for the silica monolith versus silica particles or an GMA/EDMA monolith, respectively. However, the amount of AGP that was immobilized per unit volume was found to be 1.5- to 3.6-times higher for the silica monolith versus these other supports. The differences noted here between the GMA/EDMA and silica monoliths are consistent with a previous observation that GMA/EDMA monoliths tends to work well for large analytes (e.g., proteins and nucleic acids) while silica monoliths are better for separating small analytes, partly due to the larger surface area of the latter type of support [50].

One consequence of this higher protein content was that the AGP silica monolith gave higher retention for both chiral analytes that were tested in this study. An AGP column containing silica particles gave the second highest retention, followed by the GMA/EDMA monolith. This higher retention also contributed to the greater resolution that was noted when using AGP in the silica monolith. This should make affinity silica monoliths that contain AGP useful in separating chiral agents that have weak retention on other types of supports. A high protein content should also help minimize column overloading effects, as can often occur on protein-based chiral stationary phases. However, the presence of high protein content can lead to long retention times for solutes that have strong binding to the immobilized protein, as was observed for *R/S*-propranolol on the AGP column. This issue could be dealt with by placing a smaller amount of protein in the silica monolith or by altering the composition of the mobile phase to give lower retention (e.g., adding an organic modifier such as isopropanol) [20].

In terms of efficiency, the silica monolith containing AGP gave lower optimum plate heights than columns containing silica particles, as well as a smaller change in plate height with flow rate. The AGP silica monolith also gave lower optimum plate heights than a GMA/EDMA monolith containing AGP, but part of this difference was due to the much larger retention factors that were noted on the silica monolith. In addition, the AGP silica monolith gave the lowest back pressures and best separation impedances for the tested analytes, followed by the GMA/EDMA monolith and silica particles. The trends noted here for the efficiencies of the silica monolith, silica particles and GMA/EDMA monoliths agree with those noted for columns containing immobilized HSA [25].

There are some similarities in the results of this study and previous work that has used silica monoliths in other separation modes [32,51–58]. For example, a silica monolith containing a cation-exchange based chiral selector was found to give comparable efficiency to a column containing 3.5 μm silica particles but better efficiency than a polymethacrylate monolith for the separation of basic chiral drugs [53]. A comparison of reversed-phase columns indicated that a silica monolith had a comparable efficiency to a column containing 3–5 μm silica particles but higher efficiency than a butyl methacrylate-co-ethylene dimethacrylate monolith [51]. Another study using surface molecularly imprinted polymers found that a silica monolith had better efficiency than a column containing a methacrylic acid-co-ethylene dimethacrylate copolymer [53]. It has been suggested in such work that the lower efficiencies noted for organic polymer monoliths versus silica monoliths for small molecules could be a result of differences in pore size distribution or due to greater surface heterogeneity in the organic polymers [52].

It was concluded from the results of this study that affinity silica monoliths with immobilized AGP can be used as alternatives to columns that contain silica particles or GMA/EDMA monoliths for chiral separations. This is particularly true for work that is required at high flow rates, where the silica monoliths can provide good efficiencies along with reasonable back pressures. The advantages noted here for the affinity silica monoliths (e.g., high protein content and good efficiency) also make these materials attractive for use in the study of drug-protein binding. Such columns should be particularly valuable when examining solutes that have weak protein binding and only low-to-moderate retention on other AGP columns.

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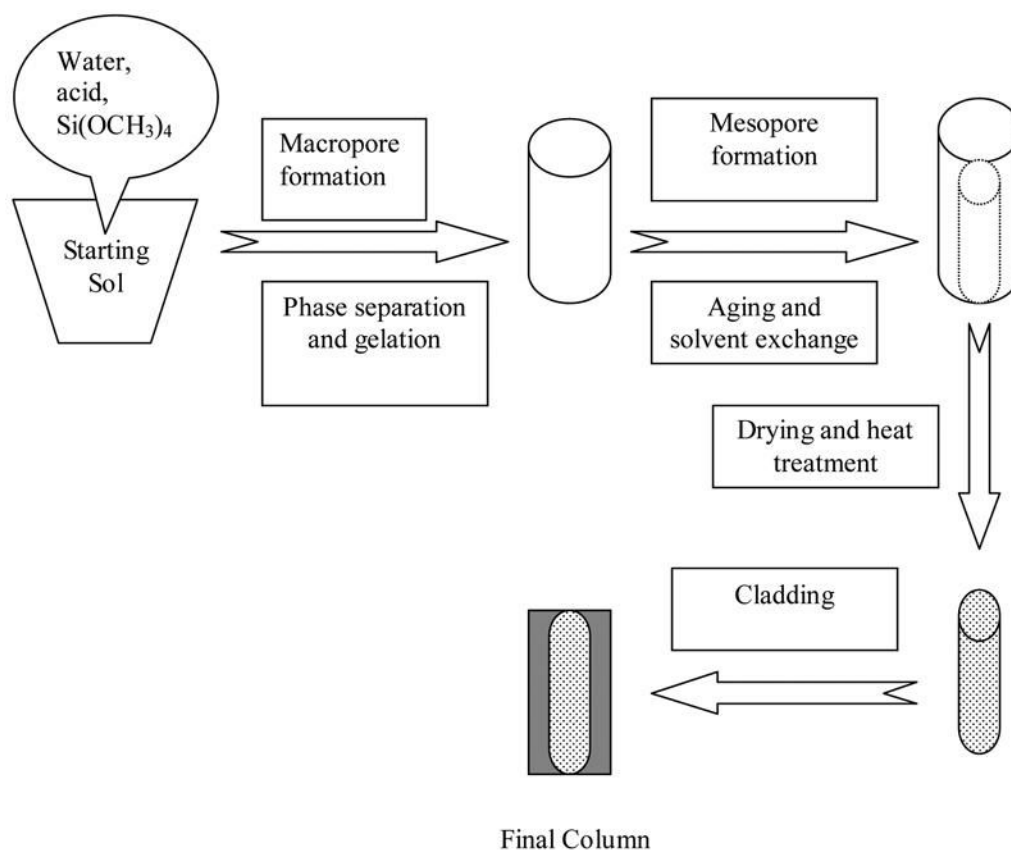
Figure 1.
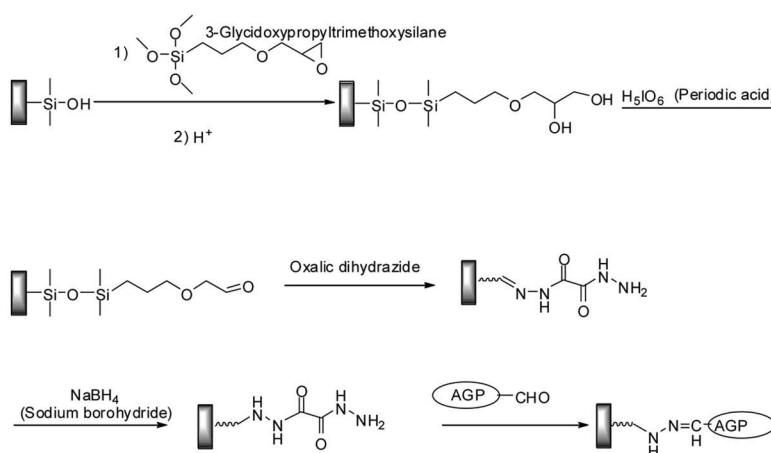General approach for the preparation of a silica monolith.

Figure 2.
Reactions involved in the preparation of a AGP silica monolith. Further details on these reactions can be found in the text. Abbreviation: AGP, α_1-acid glycoprotein.
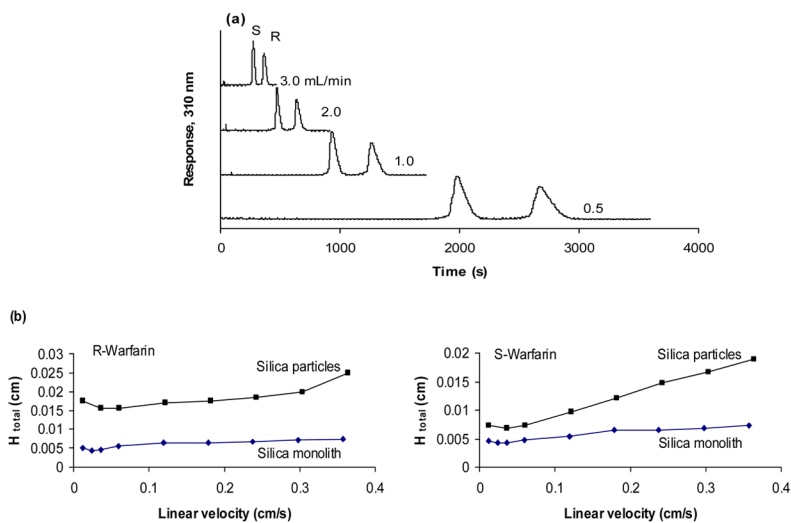
Figure 3.
(a) Representative chromatograms obtained for the injection of racemic warfarin onto an AGP silica monolith, and (b) plots of total plate height versus linear velocity for *R*- and *S*-warfarin when using the AGP silica monolith (◆) or a column containing AGP immobilized to 300 Å pore size, 7 μm silica particles (■). The mobile phase was pH 7.0, 0.067 M KPB containing 5% isopropanol. Other conditions are given in the text. The average retention factors for *R*-warfarin in (b) were 11.94 for the silica monolith and 4.37 for the column containing silica particles; the average retention factors for *S*-warfarin on these same columns were 8.54 and 3.42, respectively.
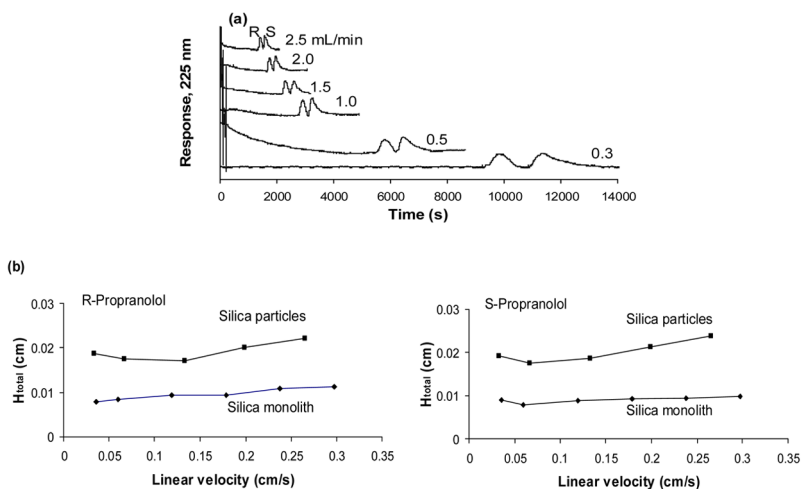
Figure 4.
(a) Representative chromatograms obtained for the injection of racemic propranolol on an AGP silica monolith, and (b) plots of total plate height versus linear velocity for *R*- and *S*-propranolol when using the AGP silica monolith (◆) or a column containing AGP immobilized to 300 Å pore size, 7 μm silica particles (■). The mobile phase was pH 7.4, 0.067 M KPB containing 3% isopropanol. Other conditions are given in the text. The average retention factors for *R*-propranolol in (b) were 31.1 for the silica monolith and 11.6 for the column containing silica particles; the average retention factors for *S*-propranolol on these same columns were 37.2 and 13.7, respectively.
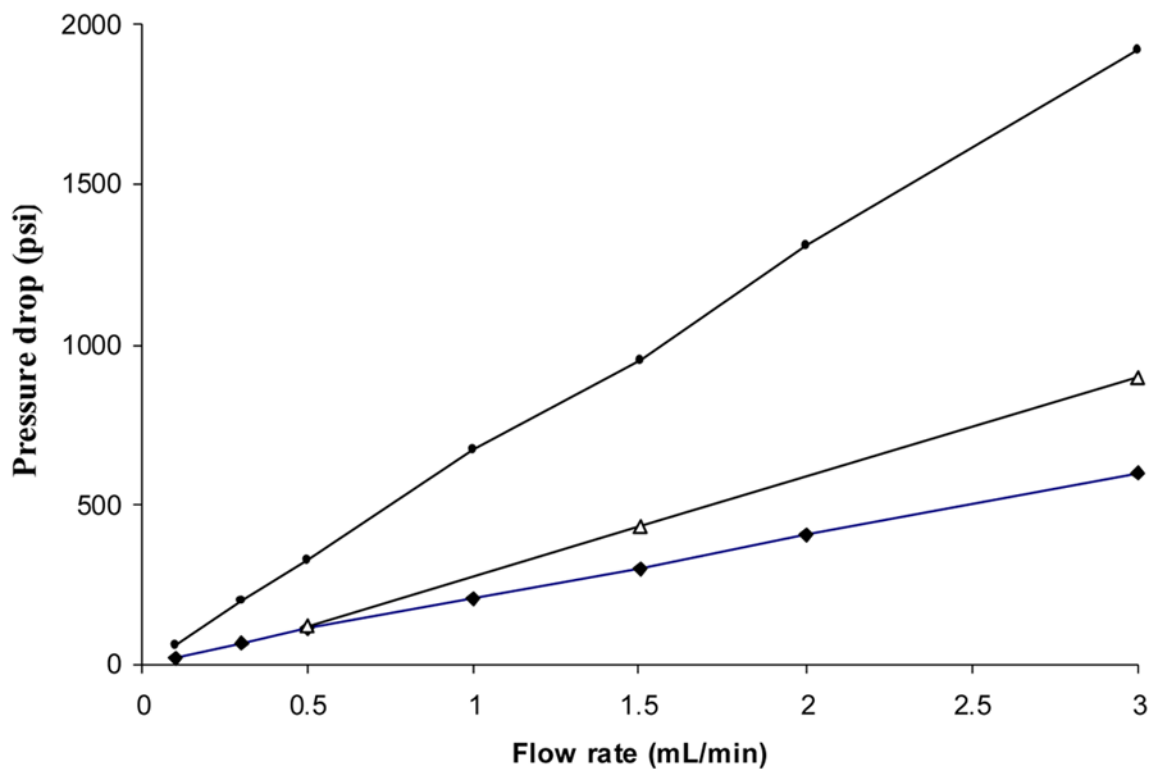
Figure 5.
Pressure drop (psi) versus flow rate (mL/min) plot for AGP columns based on a silica monolith (♦), packed silica (300 Å pore diameter, 7 μm particle diameter) (●) or a GMA/EDMA monolith (△). The pressure drops given in this graph for the packed silica column and GMA/EDMA monolith (both 5 cm × 4.6 mm I.D.) were multiplied by a factor of two to allow these values to be directly compared with those obtained for the longer silica monolith (10 cm × 4.6 mm I.D.).
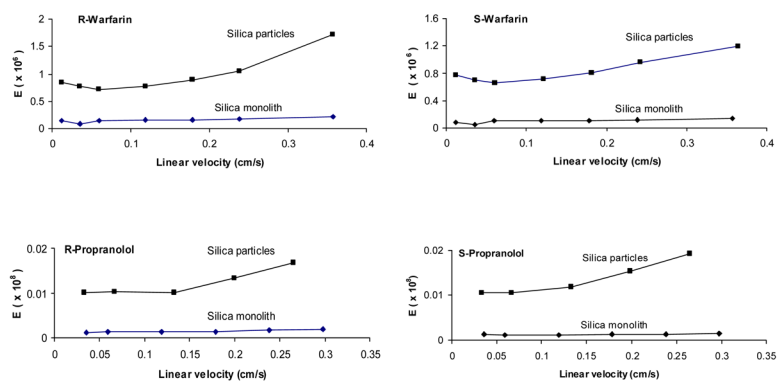
Figure 6.
Separation impedance (*E*) versus linear velocity for AGP columns using R/S-warfarin or *R/S*-propranolol as the analyes. The supports used in case were (♦) a silica monolith or (■) 300 Å pore size, 7 μm silica particles. Other experimental conditions are given in the text.

Table 1

Properties of AGP immobilized to various supports[a]

Type of support	Protein content (nmol/g support)	Protein coverage (nmol/m^2)	Protein content per volume (nmol/mL)
Silica monolith	1800 ($\pm$ 40)[b]	5.8 ($\pm$ 0.2)	356 ($\pm$ 9)
Silica particles	490 ($\pm$ 20)	4.9 ($\pm$ 0.2)	236 ($\pm$ 2)
GMA/EDMA monolith	250 ($\pm$ 10)	3.6 ($\pm$ 0.1)	100 ($\pm$ 5)

[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 the listed parameters.

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

Table 2

Chromatographic parameters for *R/S*-warfarin and *R/S*-propranolol on various AGP columns[a]

Analyte/Support	Retention factor, k	Optimum plate height, H_{opt} (cm)[b]	Separation factor, α[c]	Resolution, R_s[c]
R/S-Warfarin				
Silica monolith	8.54 (± 0.04) (*S*)	0.004 (± 0.001) (*S*)	1.39 (± 0.01)	3.08 (± 0.10)[d]
	11.94 (± 0.07) (*R*)	0.004 (± 0.001) (*R*)		
Silica particles	3.42 (± 0.08) (*S*)	0.007 (± 0.001) (*S*)	1.28 (± 0.05)	0.51 (± 0.02)
	4.37 (± 0.06) (*R*)	0.015 (± 0.002) (*R*)		
GMA/EDMA monolith	2.23 (± 0.03) (*S*)	0.011 (± 0.001) (*S*)	1.25 (± 0.04)	0.40 (± 0.02)
	2.78 (± 0.02) (*R*)	0.017 (± 0.002) (*R*)		
R/S-Propranolol				
Silica monolith	31.1 (± 0.4) (*R*)	0.008 (± 0.001) (*R*)	1.20 (± 0.01)	1.11 (± 0.03)[d]
	37.2 (± 0.4) (*S*)	0.008 (± 0.001) (*S*)		
Silica particles	11.6 (± 0.2) (*R*)	0.017 (± 0.001) (*R*)	1.18 (± 0.05)	0.55 (± 0.03)
	13.7 (± 0.3) (*S*)	0.018 (± 0.001) (*S*)		
GMA/EDMA monolith	4.53 (± 0.06) (*R*)	0.020 (± 0.001) (*R*)	1.51 (± 0.06)	0.79 (± 0.03)
	6.86 (± 0.05) (*S*)	0.015 (± 0.001) (*S*)		

[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.

[d]The corresponding resolution calculated for a 5 cm long AGP silica monolith was 2.2 (± 0.1) for *R/S*-warfarin and 0.78 (± 0.02) for *R/S*-propranolol.