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Development and evaluation of *N*-hydroxysuccinimide-activated silica for immobilizing human serum albumin in liquid chromatography columns

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Abstract

A method was developed for preparing *N*-hydroxysuccinimide (NHS)-activated silica for use in immobilizing human serum albumin (HSA) within HPLC columns. This support was made by reacting aminopropyl silica with disuccinimidyl suberate (DSS). Solid state ^{13}C NMR was used to examine the steps in this synthesis. The number of NHS groups on the silica's surface was controlled by varying the amount of DSS or density of surface amine groups used in the preparing this material. Items considered in the use of this material for the immobilization of HSA included the amount of protein added to the support, the reaction time, and the pH of the coupling buffer. These supports were then evaluated in terms of their ability to perform chiral separations for R/S-warfarin and D/L-tryptophan. Advantages of this method compared to current immobilization techniques for HSA included its better long-term stability and the fact that it did not require the use of any reducing agents. The approaches developed in this work are not limited to HSA but can be used with other proteins or amine-containing ligands.

Keywords: Human serum albumin; *N*-Hydroxysuccinimide; Silica

1. Introduction

There has been growing interest in recent years in the use of high-performance affinity chromatography (HPAC) for the separation and analysis of chemicals [1–4]. This method makes use of an HPLC column that contains an immobilized ligand capable of specifically binding the analyte or group of analytes of interest. This approach has become popular in such areas as protein purification, chiral separations, and studies of biological interactions [5–7]. Some reasons for this popularity include the high specificity of this technique, as well as its speed, ease of automation, the ability to reuse the same ligand for multiple applications.

Human serum albumin (HSA) is the most abundant protein in blood and is known to bind a variety of biological and pharmaceutical compounds [8]. This binding generally occurs at two major sites on HSA (i.e., warfarin-azapropazone and indole-benzodiazepine sites) plus a number of minor binding regions [8–10]. To study these sites and their binding processes, HSA has frequently been immobilized to chromatographic supports for the measurement of its equilibrium constants and rate constants [11,12].

Most current HPAC methods utilize silica supports that immobilize biomolecules through free amine, sulfhydryl or carboxyl groups. One method often used to attach HSA to these supports is reductive amination (i.e., the Schiff base method). In this technique, aldehyde-activated silica is used to couple biomolecules through free amine groups to form an imine linkage, or Schiff base [13]. Due to the reversible nature of this bond, a reducing agent like sodium cyanoborohydride is employed to convert it to a more stable secondary amine. This approach is easy to perform but often requires immobilization times of several days. Also, strong reducing

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agents like sodium borohydride are often used in this technique for later reducing the unreacted aldehydes into inert alcohol groups. This can be detrimental to proteins that contain disulfide bonds, which can be reduced to thiols during this process and alter the protein's structure.

An approach that can avoid these problems is the *N*-hydroxysuccinimide (NHS) coupling method. This has several potential advantages compared to other common immobilization techniques, such as the Schiff base, cyanogen bromide, carbonyl diimidazole, and maleimide methods [14,15]. For instance, this is a relatively fast immobilization reaction for amine-containing ligands and involves a simple reaction scheme. In addition, it does not involve the use of any reducing agents or oxidizing agents that can alter a ligand's structure.

A previous report described the synthesis of NHS-activated silica by reacting aminopropyl silica with succinic anhydride, followed by reaction of the product with NHS [16]. This method can also be performed by reacting NHS with a commercially-available form of the aminopropyl silica/succinic anhydride product (e.g., Alltech Macrosphere WCX). In this study, an alternative approach will be developed by directly reacting disuccinimidyl suberate with aminopropyl silica, as shown in Fig. 1 This technique will be evaluated for use in the immobilization of HSA. Items considered in this study will include optimization of the active NHS groups on the final support and the reaction conditions for immobilization of HSA to this material. This material will be examined for its ability to bind and separate chiral compounds like *R*- or *S*-warfarin and D- or L-tryptophan. The results will then be compared to those obtained by the Schiff base method under similar experimental conditions.

2. Experimental

2.1. Materials

The disuccinimidyl suberate (DSS; FW, 368.3 g/mol) (3-aminopropyl)triethoxysilane (APTS; FW, 221.4 g/mol; density, 0.949 g/ml), and HSA (Cohn fraction V, essentially fatty acid and globulin free) were from Sigma (St. Louis, MO, USA). Anhydrous toluene, dimethylformamide (DMF), acetone, and NHS were from Aldrich (Milwaukee, WI, USA). The propylamine and deuterated chloroform ($CDCl_3$) used in the NMR studies were also from Aldrich. The Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was from Alltech (Deerfield, IL, USA). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). Other chemicals were of the purest grades available. All aqueous solutions were prepared using water from a NANOpure water system (Barnstead, Dubuque, IA, USA) and filtered using Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

2.2. Apparatus

The solid state NMR experiments were performed on a Bruker 600 MHz NMR spectrometer (Billerica, MA, USA). The cross polarization/magic angle spinning (CP/MAS) studies were conducted at 75.7 MHz using a spinning rate of 10 kHz, with NMR spectra being recorded through the use of a 90° pulse length of 4 μs and a contact time of 1 ms. A total of 12,000 scans were obtained for each sample in these measurements.

The solution NMR spectra were acquired on a GE Omega 300 MHz spectrometer (Fremont, CA, USA) at 75.7 MHz.

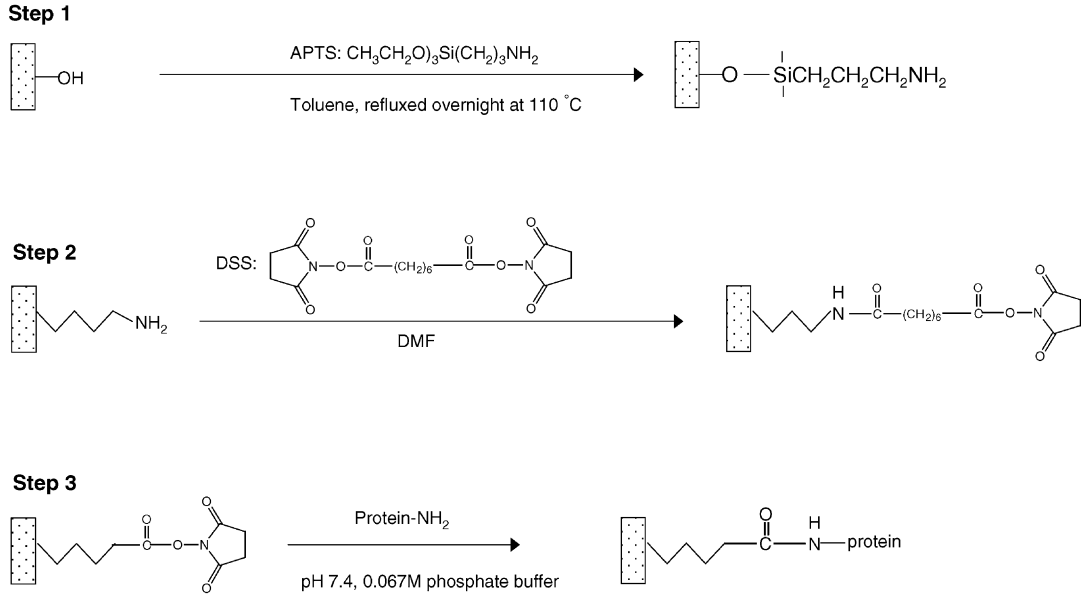

Fig. 1. Preparation of NHS-activated silica for protein immobilization.

Spectral widths of 18 kHz with a 16 K data size were used. A total of 256 scans (acquisition time, 0.91 s/scan) were obtained for propylamine and DSS. $CDCl_3$ was used as a chemical shift marker in these studies.

The NHS measurements and BCA protein assay were performed using a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). The system used in the chromatographic studies consisted of a PU-980 pump from Jasco (Tokyo, Japan), a P4000 gradient pump, and a UV100 absorbance detector from ThermoSeparation Products (Riviera Beach, FL, USA). Samples were injected using a Rheodyne Lab Pro valve (Cotati, CA, USA) with a 20 μl sample loop. An Isotemp 9100 circulating water bath from Fisher was used for temperature control of both the column and mobile phase. All columns were packed using an Alltech HPLC column slurry packer. Chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA).

2.3. Methods

The following procedures are described for Nucleosil Si-300, which has a surface area of 100 m^2/g. However, the same synthetic methods can be adapted for other types of silica by altering the amounts of reagents in proportion to the surface area of the desired support versus Nucleosil Si-300.

2.3.1. Synthesis of aminopropyl silica

The first step in preparing NHS-activated silica involved obtaining an amine-containing support. This can be accomplished by using one of the various commercial aminopropyl silicas or, as performed in this study, by reacting bare silica with APTS through a condensation method [17] (see Step 1 in Fig. 1). In this latter method, 2 g of Nucleosil Si-300 silica was suspended in a 10% (v/v) solution of HCl in water and stirred gently for 1 h at room temperature. This support was then washed several times with water and passed through a 0.22 μm nylon filter. The recovered silica was dried overnight under vacuum at 110 °C, followed by its immediate use. The purpose of this initial set of steps was to cleave siloxane bonds on the surface of the silica, maximizing the number of silanol groups that could later be reacted with APTS.

The dried silica was next combined with 20 ml toluene and a stir bar in a 150 ml two-neck round bottom flask. This suspension was placed under an argon stream while APTS was added dropwise with stirring. A 2 ml volume of APTS was typically added, although 0.1–2 ml were used in some experiments to examine the effects of varying the surface amine group density on the properties of the NHS-activated silica. The temperature of this mixture was next raised to 110 °C. It was then gently stirred and refluxed overnight under argon. The resulting aminopropyl silica was rinsed six times each with anhydrous toluene and acetone. It was dried under vacuum overnight at room temperature and stored in a dessicator at room temperature until further use. According to manufacturers of similar supports, this material should be stable for several years under these storage conditions.

2.3.2. Synthesis of NHS-activated silica

The NHS-activated silica was prepared by reacting aminopropyl silica with DSS, as shown in Step 2 of Fig. 1 To do this, 1 g of aminopropyl silica was placed into a 100 ml round bottom flask and combined with DSS (typically 0.5 g, or 1.36 mmol). A stir bar was added and these solids were placed under an argon atmosphere. While this mixture was stirred, anhydrous DMF (20 ml) was added to the flask. The resulting slurry was stirred gently under argon for 8 h at room temperature. The product, NHS-activated silica, was washed three times each with anhydrous DMF and acetone to remove excess DSS. The final support was dried under vacuum overnight at room temperature and stored in a dessicator at room temperature until further use. This support was stable for over nine months under these storage conditions, losing only 15% of its active NHS groups over this period of time.

2.3.3. Measurement of NHS groups

The reactive groups on NHS-activated silica were measured by hydrolysis and measuring the released NHS [18]. The rate of this hydrolysis is known to increase under basic conditions [14,16]; however, in this study only slightly alkaline conditions were used (pH 7.4) to avoid the dissolution of silica that can occur at a higher pH. This allowed the use of simple filtration for removal of the released NHS groups from the treated silica.

In this assay, the sample was prepared by placing a known mass of dried NHS-activated silica (typically 20 mg) in 2 ml of pH 7.4, 0.067 M potassium phosphate buffer. This suspension was mixed frequently on a vortex mixer and reacted for at least 1 h at room temperature to allow hydrolysis of the NHS groups. Under similar conditions, it has been reported in [16] that a reaction time of 1 h corresponds to approximately two half-lives for NHS groups on succinic anhydride-activated silica. However, it was found in this current study that the use of reaction times up to 3 h did not give any noticeable increase versus a 1 h assay in the measured levels of NHS; this indicated that a shorter half-life for the coupled NHS groups was probably present for the support developed in this current report.

After the hydrolysis step, the silica was filtered using a 0.22 μm syringe filter, with the filtrate being collected and examined for its NHS content by measuring the absorbance at 260 nm. Standard solutions were prepared from a stock solution that contained 1 mM NHS in pH 7.4, 0.067 M phosphate buffer. The working standards had NHS concentrations of 0–100 μM and were prepared by diluting the stock solution in pH 7.4, 0.067 M phosphate buffer. When measured in triplicate at 260 nm, these standards gave a linear relationship with a lower limit of detection of 2.5 μM NHS at a signal-to-noise ratio of 2.5 and an upper linear limit of at least 100 μM. The correlation coefficient over this range was 0.9999 (n =

8). The typical concentrations of NHS observed in the filtrate samples were 80–100 μM.

2.3.4. Immobilization of HSA

HSA was immobilized on the NHS-activated silica as shown in Step 3 of Fig. 1. This process began by preparing a 20 mg/ml solution of HSA in 7.4, 0.067 M phosphate buffer. A 3 ml volume of this solution was mixed with 0.2 g of the NHS-activated silica, with the resulting suspension being shaken at room temperature for 2 h. The HSA silica that was produced was washed several times with pH 7.4, 0.067 M potassium phosphate buffer and stored in this buffer at 4 °C prior to use. A BCA assay of this material indicated it had no measurable change in its protein content when stored for over nine months under these conditions.

A control support was prepared in a similar manner by taking 0.2 g of the original NHS-activated silica and mixing this with 3 ml of pH 7.4, 0.067 M potassium phosphate buffer. This was also allowed to react for 2 h at room temperature. At the end of this period, the control silica was washed several times with pH 7.4, 0.067 M potassium phosphate buffer and stored under the same conditions as used for the HSA silica.

For the Schiff base immobilization method, Nucleosil Si-300 silica was first converted into a diol-bonded form according to a previously described procedure [13]. This diol-bonded silica was then reacted with a periodic acid solution and converted into an aldehyde form [13]. A total of 5 g of this aldehyde-activated support was placed into pH 6.0, 0.10 M potassium phosphate buffer along with 150 mg HSA and 70 mg sodium cyanoborohydride. This mixture was allowed to react five days at 4 °C. After immobilization, the HSA silica was washed with pH 7.4, 0.10 M potassium phosphate buffer and treated with three 10 mg portions of sodium borohydride to reduce the excess aldehyde groups. The final HSA silica support was then washed several times with pH 7.4, 0.067 M potassium phosphate buffer and stored in this buffer at 4 °C prior to use. Previous studies have shown that this material is stable over six months under these storage conditions [7,12]. A control support was prepared and stored in the same manner, but with no HSA being added to this material during the immobilization process.

The amount of immobilized protein on the HSA supports was determined by a BCA assay. In this assay, a small portion of each support was washed several times with deionized water and dried under vacuum at room temperature. These samples were then assayed in triplicate by the BCA method, using HSA as the standard and the appropriate control silica as the blank. With this procedure, the final protein content was found to be 222 (±15) nmol HSA/g silica (±1 S.D.) for the NHS method and 410 (±6) nmol HSA/g silica for the Schiff base method.

2.3.5. Chromatographic studies

Each final support was downward slurry packed at 3500 psi (214 bar) into a 5.0 cm × 2.1 mm i.d. stainless steel column using pH 7.4, 0.067 M potassium phosphate buffer as the packing solvent. These columns were stored in this buffer at 4 °C. The mobile phase for all chromatographic studies was pH 7.4, 0.067 M potassium phosphate buffer, which was degassed under vacuum at least 15 min prior to use. All chromatographic studies were performed at room temperature.

The samples used in the chromatographic studies were prepared fresh daily and contained 10 μM *R*/*S*-warfarin or 1 μM D/L-tryptophan. These concentrations were relatively small compared to the amount of immobilized HSA and were sufficient to produce linear elution conditions. The elution of warfarin and tryptophan was monitored at 307 or 214 nm, respectively. The flow rate was 0.1 ml/min for tryptophan and 0.5 ml/min for warfarin. The column backpressure was less than 600 psi (36.7 bar) under these conditions. No measurable changes in the retention factors were observed when slight variations were made in these flow rates, as observed in previous studies with similar HSA columns [7].

3. Results and discussion

3.1. NMR spectra for modified silicas

The various steps in the synthesis of NHS-activated silica were examined qualitatively by using solid state ^{13}C CP/MAS NMR, as has been used to examine other chemically bonded phases on silica [19,20]. Fig. 2(a) shows a typical spectrum obtained for the aminopropyl silica. Three major peaks were observed at +43.8, +27.0, and +10.4 ppm. These were determined to represent the C_1, C_2, and C_3 carbons in the aminopropyl silica, respectively. The identities of these peaks were confirmed by acquiring the solution ^{13}C NMR spectrum for free propylamine, as shown in Fig. 2(b). In addition, the solid state spectrum in Fig. 2(a) is similar to earlier results reported in the literature [19], confirming the successful preparation of the aminopropyl silica.

A careful measurement of the peak intensities for the C_1, C_2, and C_3 peaks in Fig. 2(a) indicated that these all were about the same size. This would be expected if these groups had the same relative response in the solid state ^{13}C NMR spectrum, since they occur in a 1:1:1 ratio in the aminopropyl group on the silica's surface.

Fig. 3(a) shows the solid state ^{13}C NMR spectrum for the NHS-activated silica. Three major peaks were again seen in this spectrum, which appeared at +42.0, +25.7, and +10.2 ppm. Although this spectrum is similar to that seen in Fig. 2(a) for aminopropyl silica, the positions of these peaks differed by 1.9–4.8% from those in Fig. 2(a), which is a significant shift in such an experiment. It is also interesting to note that the relative intensities of these three peaks no longer occurred in the 1:1:1 ratio seen for the aminopropyl silica. Instead, these peaks had an approximate ratio of 1:2:1.

Although the shift in peaks between Figs. 2(a) and 3(a) can be explained by the different chemical environments experienced by the C_1–C_3 carbons in the aminopropyl and NHS-activated silica, this does not explain why the ratio of these

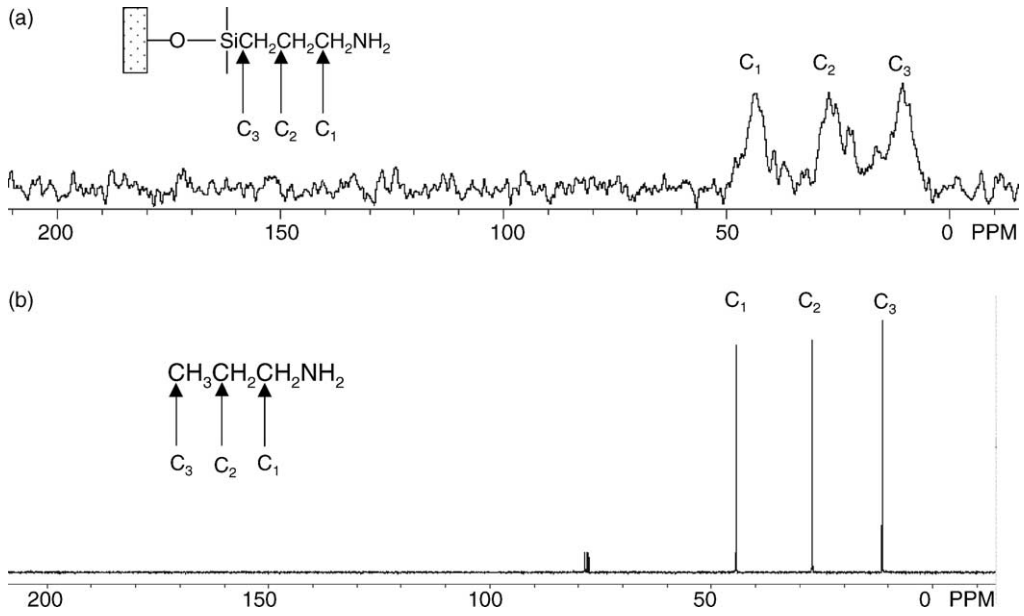

Fig. 2. (a) Solid state ^{13}C CP/MAS NMR spectrum for aminopropyl silica and (b) a solution ^{13}C NMR spectrum for propylamine.

peaks was also altered. In addition, it is necessary to consider the role played by the other carbons in the NHS-activated silica (i.e., carbons C_4–C_{10}). These later carbons were examined by acquiring a solution ^{13}C NMR spectrum for DSS, which was used to prepare the NHS-activated silica and contains similar functional groups.

As shown in Fig. 3(b), DSS gave several peaks for its CH_2 carbons in the region of 25–32 ppm. This also produced a number of peaks for its carbonyl carbons at approximately 170 ppm. The peaks between 25 and 32 ppm are similar to those expected for the C_4 and C_7–C_9 carbons in NHS-activated silica. The fact that these overlap with the peak for

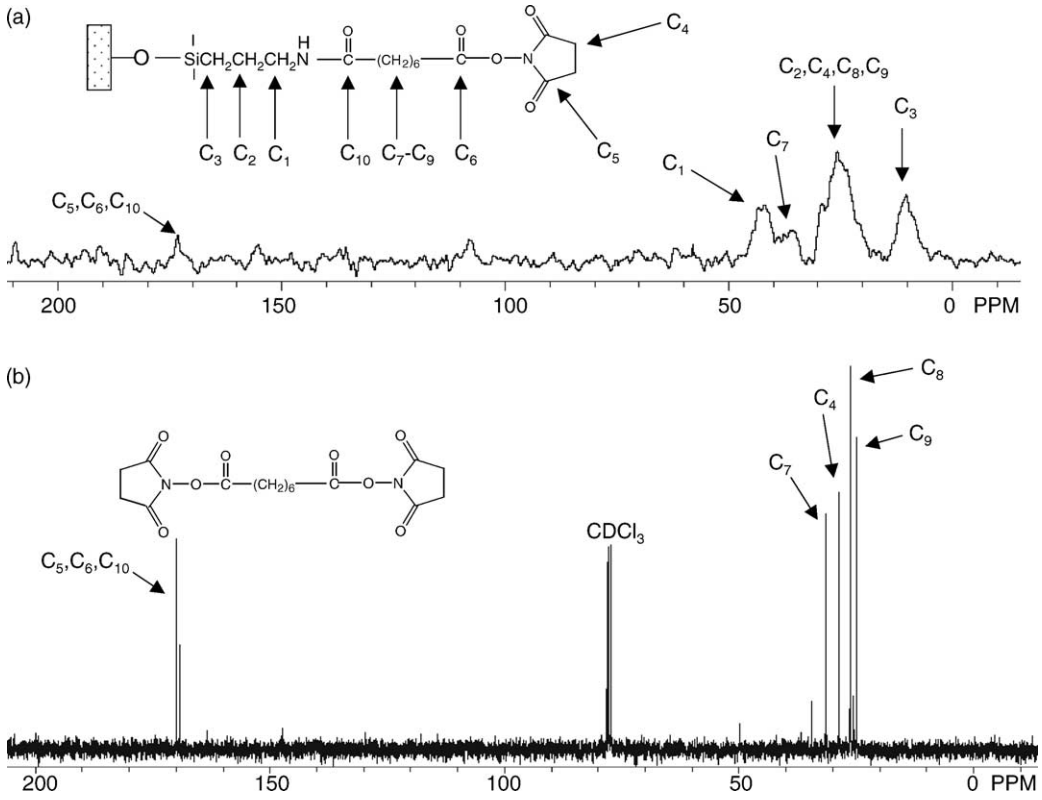

Fig. 3. (a) Solid state ^{13}C CP/MAS NMR spectrum for NHS-activated silica and (b) a solution ^{13}C NMR spectrum for free DSS.

C_2 explains why Fig. 3(a) has a higher relative size for this peak versus those for C_1 and C_3. This confirmed that the support in Fig. 3(a) had been converted from aminopropyl silica into NHS-activated silica. Although some signal may have been present at 170 ppm for the C_5, C_6 and C_{10} carbonyl carbons of the NHS-activated silica, these peaks were too small to give quantitative information. This low response was probably due to the lack of hydrogens on these carbons and the use of cross-polarization to enhance the other peaks in this spectrum.

3.2. Control of DSS attachment

One factor examined in optimizing the preparation of NHS-activated silica was the reaction between DSS and aminopropyl silica. This was of concern since it is possible for a homobifunctional crosslinking agent like DSS to have both its ends react with the support, leaving no free NHS groups for ligand attachment. This effect has been demonstrated previously in the reaction of dihydrazides with aldehyde-activated silica, in which a high degree of such coupling was observed and only a small fraction of hydrazide sites was available for ligand immobilization [21].

There are several ways to reduce this problem. For instance, it was found in the work with dihydrazide supports that a low density of aldehyde groups results in a greater amount of hydrazide sites on the final support [21]. By analogy, if aminopropyl silica could be prepared with a low density of amine groups on its surface, the distance between these groups would make it less likely that both ends of DSS could react with this surface. Another approach explored in the dihydrazide studies was to use a large excess of crosslinking agent, making its soluble form a more effective competitor with its immobilized form for any reactive sites on the support [21].

The first option considered in this work was control of the surface amine groups. This was accomplished by preparing aminopropyl silica through the use of various amounts of APTS. Table 1 gives the results of this study, in which the amount of aminopropyl groups was determined by measuring the nitrogen content of the support. As the amount of APTS in the reaction mixture was increased from 0 to 2.5% (v/v), there was a large increase in the nitrogen content of the aminopropyl silica. Only a small increase beyond this point was noted when using larger amounts of APTS, indicating that these levels had essentially saturated the reactive groups on the silica's surface. Based on the last three entries in Table 1, the maximum amount of aminopropyl groups obtained was estimated to be 720 (±30) μmol/g silica, or 7.2 μmol/m^2. This compares favorably with the original silanol content for this support of approximately 500 μmol/g silica, as reported by the manufacturer. The larger value obtained for the aminopropyl groups versus initial silanol groups may be due to the pretreatment process used in this study to convert siloxane groups into additional silanols that could react with APTS. Polymerization of the aminopropyl silane on the support's surface may have been another possible factor. Although it is possible that some unreacted aminopropylsilane may also have been present, this amount should have been minimal due to the extensive washing performed with the aminopropyl support after its preparation.

The various supports shown in Table 1 were next reacted with a constant amount of DSS (i.e., 0.1 g aminopropyl silica plus 0.01 M or 25 μmol DSS in 2.5 ml DMF) to prepare NHS-activated silica. Fig. 4(a) shows the amount of NHS that was determined to be on these activated supports. As the number of aminopropyl groups on the silica increased, the amount of NHS that could be placed onto these supports also increased. This was especially true when employing up to 2.5% (v/v) APTS in the previous reaction. As noted earlier, using larger amounts of APTS gave only a small increase in the amine group density and, as show in Fig. 4(a), only a small change (1.4%) in the amount of NHS that could later be placed onto this support.

To further test this, 0.1 g of a silica support with a high amine group density (i.e., 750 μmol/g silica, as prepared using 2 ml APTS in Table 1) was reacted with various amounts of DSS. The amount of NHS found on these activated supports is shown in Fig. 4(b). The maximum concentration of DSS used in this synthesis was 0.05 M in 2.5 ml DMF. This gave a 1.7-fold mol excess of this reagent versus aminopropyl groups on the silica. It was found that the available NHS

Table 1
Nitrogen and aminopropyl content of silica supports reacted with various amounts of APTS

APTS added to reaction mixture (ml)[a]	Content of APTS in reaction mixture (%, v/v)	Nitrogen content of support (%, w/w)[b]	Aminopropyl content of support (μmol/g)[c]
0	0	≤0.01	≤10
0.1	0.5	0.34	240
0.3	1.5	0.65	460
0.5	2.5	0.84	600
0.7	3.5	0.96	680
1.0	5.0	1.02	730
2.0	10.0	1.05	750

[a] The total volume of the reaction mixture was 20 ml.
[b] The nitrogen content was determined by combustion analysis (Desert Analytic Services, Tucson, AZ). The absolute precision of each nitrogen measurement was estimated to be ±0.02% (w/w).
[c] The aminopropyl content was calculated from the measured nitrogen content.

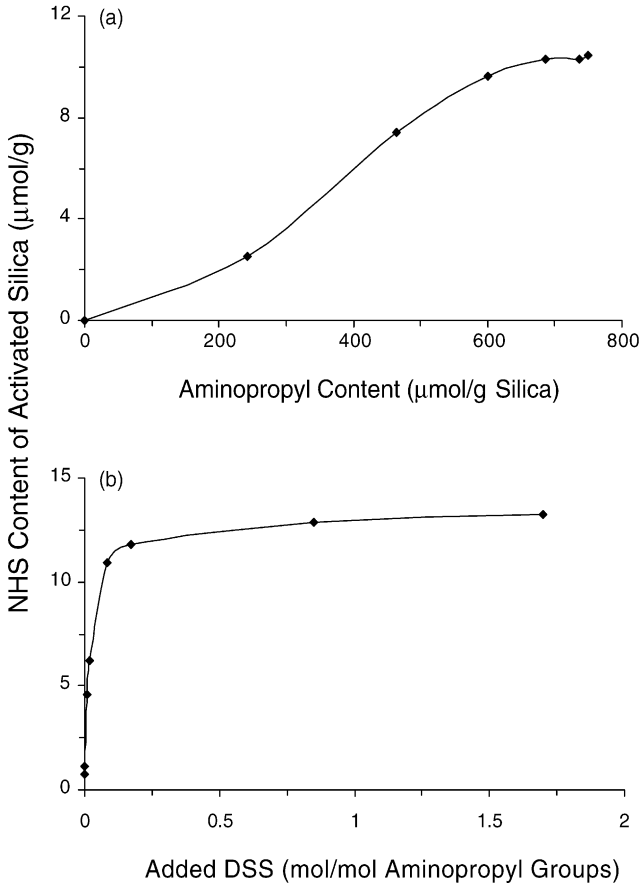

Fig. 4. NHS groups on silica prepared using (a) a constant amount of DSS reacted with silica samples with different amine group densities and (b) a variable amount added to silica with a constant amine group density. The experimental conditions are given in the text.

groups increased as the amount of DSS increased up to a 0.2-fold mol ratio; however, this leveled off as larger amounts of DSS were added. This indicated that DSS was beginning to saturate the available reaction sites on the support under these conditions. This behavior and the maximum amount of activation observed in Fig. 4(b) agree with the results given in Fig. 4(a).

A control sample using unmodified silica was combined with DSS to see if the NHS detected in Fig. 4 was simply due to incomplete washing of the supports. However, no detectable NHS was noted with this control even at the highest concentrations of DSS used in Fig. 4. This indicated that the measured NHS was actually due to activation of the aminopropyl silica rather than the presence of DSS.

It is interesting to note in Fig. 4 that although the maximum amount of aminopropyl groups was an average of 730 μmol/g silica, the maximum amount of NHS that could be released from the final activated support was about 12 μmol/g. This was 1.3% of the level originally noted for the aminopropyl groups on the silica's surface and is lower than the roughly 150 μmol NHS/g reported for succinic anhydride-activated 300-Å pore silica in [16]. There are several possible explanations for this low degree of activation. One is that some of the DSS underwent hydrolysis during the activation process, preventing its reaction with aminopropyl groups. However, this does not explain why the use of larger amounts of DSS in Fig. 4(b) gave rise to an apparent saturation of the aminopropyl sites. It is possible that some of the DSS was only partially hydrolyzed, giving it one NHS group for coupling to the support but not a second group for ligand immobilization. Another possible scenario is some DSS molecules had both ends react with aminopropyl groups. As mentioned earlier, similar behavior has been noted in the reaction of aldehyde silica with dihydrazides [21].

From these experiments, conditions were selected for the preparation of NHS-activated silica in later parts of this study. This involved the use of at least 2.5% (v/v) APTS during synthesis of the aminopropyl silica and roughly a 2-fold excess of DSS versus these aminopropyl groups in the activation step. As shown in Table 1 and Fig. 4, this gave the highest yield of active NHS groups on the silica for ligand attachment.

3.3. Optimization of ligand coupling to NHS-activated silica

Several items were considered in the immobilization of proteins onto the NHS-activated silica. In this study these factors were considered specifically for HSA, although the general trends observed should hold for many other proteins. Items considered included the amount of HSA added to the support for immobilization, the reaction time, and the pH of the reaction medium.

Fig. 5(a) shows how the amount of immobilized HSA varied when changes were made in the concentration of HSA added to a fixed amount of NHS-activated silica. This experiment was performed by placing HSA in 3 ml of pH 7.4, 0.067 M potassium phosphate buffer and reacting this with 0.2 g NHS-activated silica for 2 h at room temperature. As the concentration of added HSA increased from 1 to 50 mg/ml, the amount of immobilized protein increased from 1.7 to 6.3 mg/g silica. At higher concentrations (i.e., 75 and 100 mg/ml HSA), less than a 2% increase was noted in the final protein content of the silica. This was expected since the amount of HSA used at this point far exceeded the amount of NHS groups available for coupling. For instance, an approximately equal number of moles of HSA and NHS groups were present when using 25 mg/ml HSA, with an excess of this protein being present at higher concentrations. Unless stated otherwise, all further studies made use of a 50 mg/ml HSA solution combined in 3 ml of buffer with 0.2 g of NHS-activated silica.

The time necessary to complete the immobilization of HSA was also examined. To test this, the same amount of NHS-activated silica (0.1 g) was combined in 5 ml pH 7.4, 0.067 M potassium phosphate buffer with 20 mg/ml HSA and reacted for up to 60 min at room temperature. Based on the protein content of the final supports, this immobilization process was found to be quite rapid, with most of the HSA being immobilized within only a few minutes. Similar results have

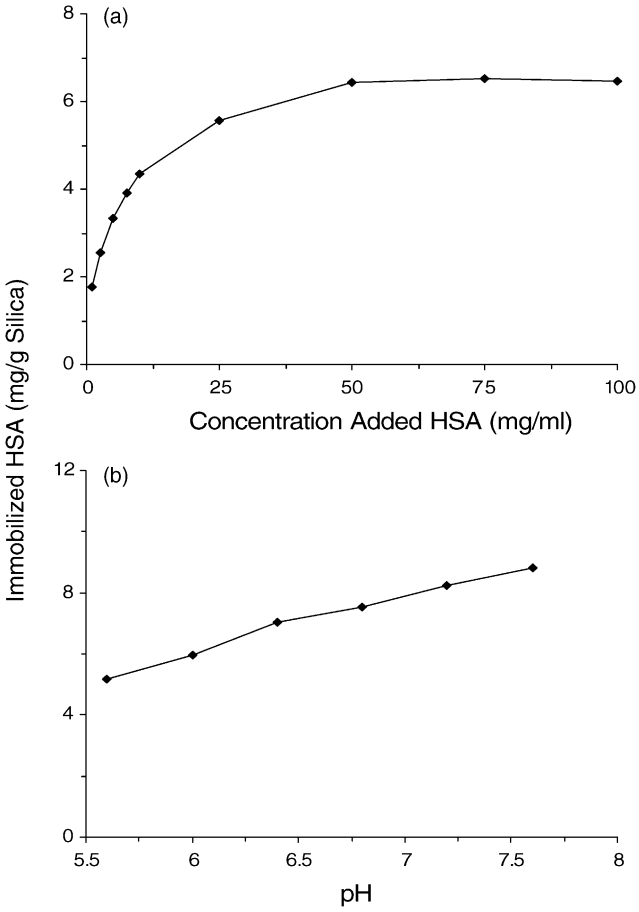

Fig. 5. Amount of immobilized HSA as a function of (a) the amount of HSA added to the reaction slurry for immobilization and (b) the immobilization pH. The experimental conditions are given in the text.

been noted in the reaction of protein A with NHS-activated supports [16]. This makes this approach appealing as a rapid means for protein attachment. Another advantage of working under these short periods of time is that it minimizes the effect that NHS hydrolysis will have during the immobilization process.

Fig. 5(b) shows the amount of HSA that was coupled to NHS-activated silica at several pH values. These experiments were performed using a 3 ml potassium phosphate buffer that contained 50 mg/ml HSA and 0.2 g NHS-activated silica and that was allowed to react for 2 h at room temperature. As the pH increased from 5.6 to 7.6, the amount of immobilized HSA increased by about 41%. This follows the general pattern seen for the titration curve of HSA, where the number of non-protonated amine groups grows with an increase in pH [22,23]. Based on these results, a pH of 7.4 was selected for use in further work. This not only gave rise to a high level of coupling for HSA, but it also mimicked the natural pH of this protein within the body. The use of a higher pH was avoided since silica is less stable under such conditions [24]. Also, the rate of NHS hydrolysis increases at higher pH values. For instance, it has been estimated that the half-life for NHS-ester compounds in an aqueous environment free of primary amines is 4–5 h at pH 7.0 and 0 °C [25] but decreases to 29 min at pH 7.5 and room temperature [16] and to 10 min at pH 8.6 and 4 °C [26].

3.4. Enantiomeric separation of warfarin and tryptophan

One application for immobilized HSA columns has been their use in separating and analyzing chiral drugs. In this study, the HSA columns were tested by using them to resolve two sets of enantiomers: *R*/*S*-warfarin and D/L-tryptophan. These compounds were selected for this work since their behavior on other HSA columns (e.g., those prepared through the Schiff base immobilization method) has been well-characterized [7,27]. In addition, *R*/*S*-warfarin and L-tryptophan are often used as probe compounds for the two major binding regions of HSA, the warfarin-azapropazone and indole-benzodiazepine sites [11,28,29].

As shown in Tables 2 and 3, neither *R*- and *S*-warfarin nor D- and L-tryptophan were separated on a control column that contained NHS-activated silica with no HSA present (*note*: these supports were used after the NHS groups had been allowed to fully hydrolyze). However, a small amount of non-specific binding to this control support was noted for each solute. This interaction may involve the spacer arms placed by APTS or DSS on the silica [30], carboxylate groups produced after the hydrolysis of NHS, or interactions with silanols or free aminopropyl groups remaining on the support. The non-specific binding seen for the control column was 9.0 and 7.2% of the total retention noted for *R*- and *S*-warfarin on the HSA column. For D- and L-tryptophan, the retention on the control column was 43.3 and 3.3%, respectively, of the total retention observed on the HSA column.

Chiral separations for *R*/*S*-warfarin and D/L-tryptophan mixtures were seen on the NHS-activated HSA column, as illustrated in Fig. 6. Although the peak widths shown here are broader than those typical of traditional HPLC methods such as reversed-phase liquid chromatography, they are similar to results obtained for these same analytes in previous studies with HSA columns [31,32]. This large degree of peak broadening is believed to be related to the relatively strong binding and slow dissociation kinetics of such systems under the elution conditions used in this work [33]. However, even with these peak widths, baseline separation was achieved for both sets of compounds under the given elution conditions. This resulted in mean elution times of 23 and 34 min for *R*- and *S*-warfarin, and 1.5 and 5.9 min for D- and L-tryptophan.

Some typical results obtained in these separations are shown in Tables 2 and 3. These tables also give data obtained under the same chromatographic conditions for an HSA column prepared using the Schiff base method, which has been commonly used to make such columns in past studies. In this case, the same batch of HSA and silica were used for both immobilization methods and all columns were identical in size.

Table 2
Chromatographic parameters for the separation of *R*- and *S*-warfarin by immobilized HSA[a]

Column	*R*-Warfarin		*S*-Warfarin		Selectivity factor	Resolution
	Retention factor (*k*)	*k*/(amount HSA)	Retention factor (*k*)	*k*/(amount HSA)		
HSA (NHS method)	60.4 (± 0.1)	4.08 (± 0.01)	75.1 (± 0.4)	5.07 (± 0.01)	1.24 (± 0.02)	1.16 (± 0.03)
HSA (Schiff base method)	62.3 (±0.2)	2.00 (±0.01)	98.2 (±0.2)	3.16 (±0.01)	1.58 (±0.03)	1.42 (±0.04)
Control (NHS method)	5.42 (±0.01)	N/A	5.42 (±0.01)	N/A	N/A	N/A
Control (Schiff base method)	0.13 (±0.01)	N/A	0.13 (±0.01)	N/A	N/A	N/A

[a] The values in parentheses represent a range of ±1 S.D. The amount of immobilized HSA is in units of nmol HSA/g silica.

Table 3
Chromatographic parameters for the separation of D- and L-tryptophan by immobilized HSA[a]

Column	D-Tryptophan		L-Tryptophan		Selectivity factor	Resolution
	Retention factor (*k*)	*k*/(amount HSA)	Retention factor (*k*)	*k*/(amount HSA)		
HSA (NHS method)	0.48 (±0.01)	0.032 (±0.01)	6.42 (±0.01)	0.434 (±0.01)	12.6 (±0.1)	3.83 (±0.01)
HSA (Schiff base method)	0.51 (±0.01)	0.016 (±0.01)	8.98 (±0.01)	0.288 (±0.01)	17.6 (±0.1)	4.67 (±0.01)
Control (NHS method)	0.21 (±0.01)	N/A	0.21 (±0.01)	N/A	N/A	N/A
Control (Schiff base method)	0.16 (±0.01)	N/A	0.16 (±0.01)	N/A	N/A	N/A

[a] The values in parentheses represent a range of ±1 S.D. The amount of immobilized HSA is in units of nmol HSA/g silica.

As shown in Tables 2 and 3, both the Schiff base and NHS columns containing HSA were able to separate racemic warfarin and tryptophan mixtures with resolutions of 1.2 or greater. A higher retention, resolution and separation factor for each pair of solutes was noted for the Schiff base column, but this was found to be mainly due to the larger amount of HSA present in this column (i.e., over two times that in the NHS column). This occurred since the retention factor in such a separation is directly proportional to the amount of active ligand (i.e., HSA, in this case) within the affinity column.

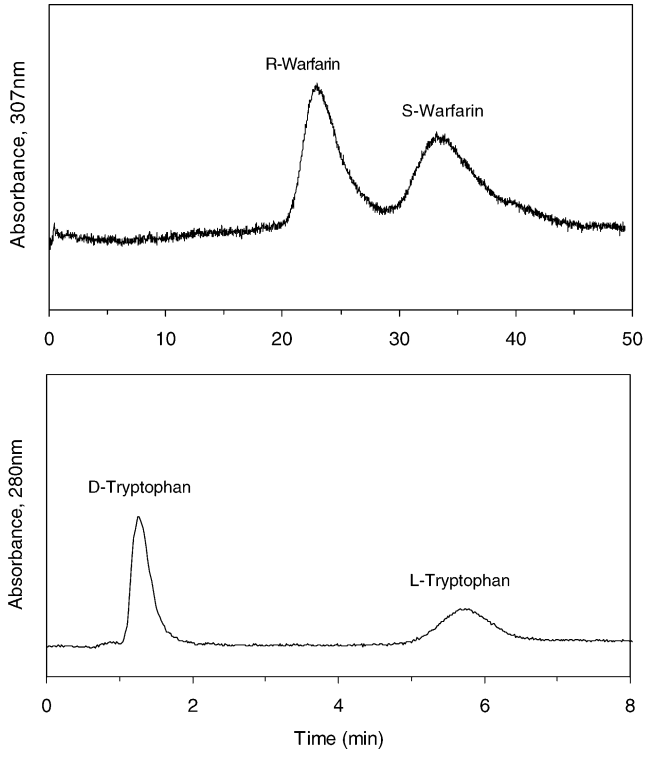

Fig. 6. Chiral separations of (a) *R*/*S*-warfarin and (b) D/L-tryptophan on an immobilized HSA column prepared using NHS-activated silica. The separation conditions are given in Section 2.3.5.

To adjust for this difference in protein content, all of the retention factors in Tables 2 and 3 were divided by the amount of immobilized HSA to give a normalized indicator of ligand activity. This is represented by the term "*k*/(amount HSA)" in these tables. This correction is based on the following relationship between the retention factor for a solute on an affinity column (*k*), the association equilibrium constant for solute-ligand binding (K_a), and the effective concentration of active ligand (m_L/V_M), where m_L is the moles of active ligand and V_M is the column void volume [33].

$$k = K_a \left(\frac{m_L}{V_M} \right) \quad (1)$$

In Eq. (1), the term (m_L/V_M) can be related to the total amount of ligand per unit mass of support (referred to here as "amount ligand") by Eq. (2)

$$\frac{m_L}{V_M} = \frac{\alpha_L \rho_s V_{tot}(\text{amount ligand})}{V_M} \quad (2)$$

where "amount ligand" is in units of mol ligand/g support, α_L is the fraction of active ligand, ρ_s is the packing density of the support (in g/L), and V_{tot} is the total column volume (in L). When comparing affinity columns made with the same initial support, ρ_s and the ratio V_M/V_{tot} should be constant. When these terms are combined into a single constant (*C*), Eq. (1) can be rewritten as follows.

$$\frac{k}{(\text{amount ligand})} = K_a \alpha_L C \quad (3)$$

This new expression indicates that *k*/(amount ligand) is affected by changes in both the binding affinity (K_a) and

the relative amount of active ligand (α_L), making this ratio useful in comparing the overall effects of immobilization on a ligand that is coupled by various means to a given support.

When Eq. (3) was used to examine the data in Tables 2 and 3, it was found that HSA immobilized by the NHS method had a 2.0–2.3-fold higher binding activity for *R*- and *S*-warfarin than HSA immobilized by Schiff base technique. For D- and L-tryptophan the same trend was observed, with HSA immobilized by the NHS method giving a 1.5–2.0-fold higher activity. Part of the reason for this difference may be that the NHS method was performed under mild physiological conditions, while the Schiff base method required the use of reducing agents (i.e., $NaCHBH_3$ and $NaBH_4$) that may have reduced some of the disulfide groups in HSA and altered its activity.

Further studies were conducted to examine the stability of both types of HSA columns. Fig. 7 shows the change in retention factor that was observed for *R*- and *S*-warfarin over time on these columns. Both types of HSA columns begin to lose some chiral selectivity after extensive use (i.e., approximately 2000 column volumes, or 40 h of operation at 0.1 ml/min for a 5 cm × 2.1 mm i.d. column). However, the degree and nature of this change varied between the two immobilization methods. For *R*- and *S*-warfarin, a parallel decrease in retention factor was noted over time for the NHS

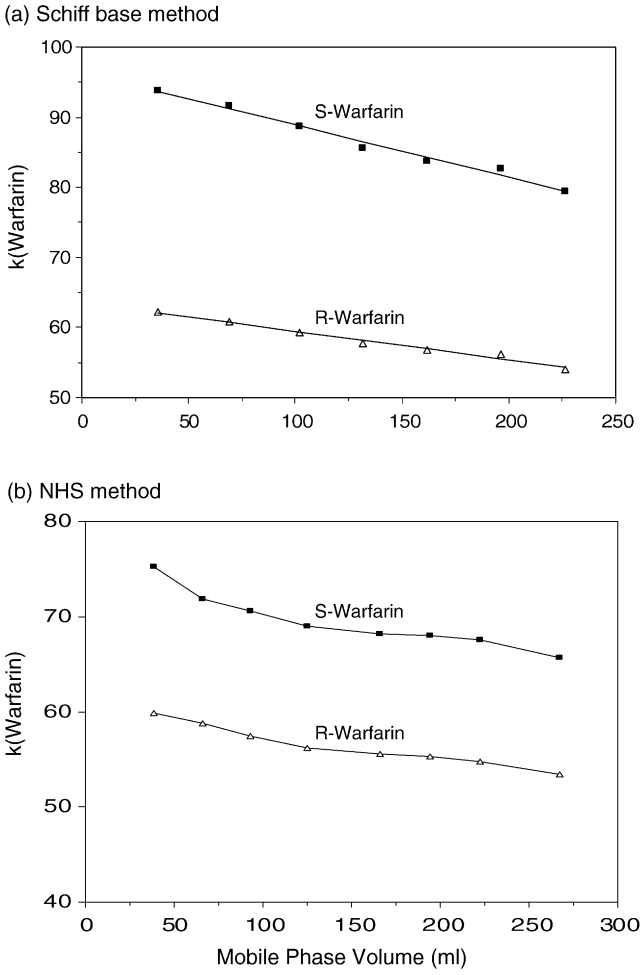

Fig. 7. Change in the retention factors for *R*- and *S*-warfarin as function of the volume of mobile phase passed through HSA columns prepared by (a) the Schiff base method and (b) the NHS method. In these studies, 100 mL is equal to approximately 725 column void volumes. The slopes of the best-fit lines in (a) for *S*- and *R*-warfarin were −0.075 (±0.001) and −0.041 (±0.001) with correlation coefficients of 0.992 and 0.993, respectively. The best-fit intercepts for these lines were 96.3 (±1.2) and 63.5 (±0.6). The data in (b) for *S*- and *R*-warfarin gave slopes of −0.0035 (±0.0001) and −0.0026 (±0.0001), intercepts of 74.9 (±0.1) and 60.3 (±0.1), and correlation coefficients of 0.938 and 0.976, respectively.

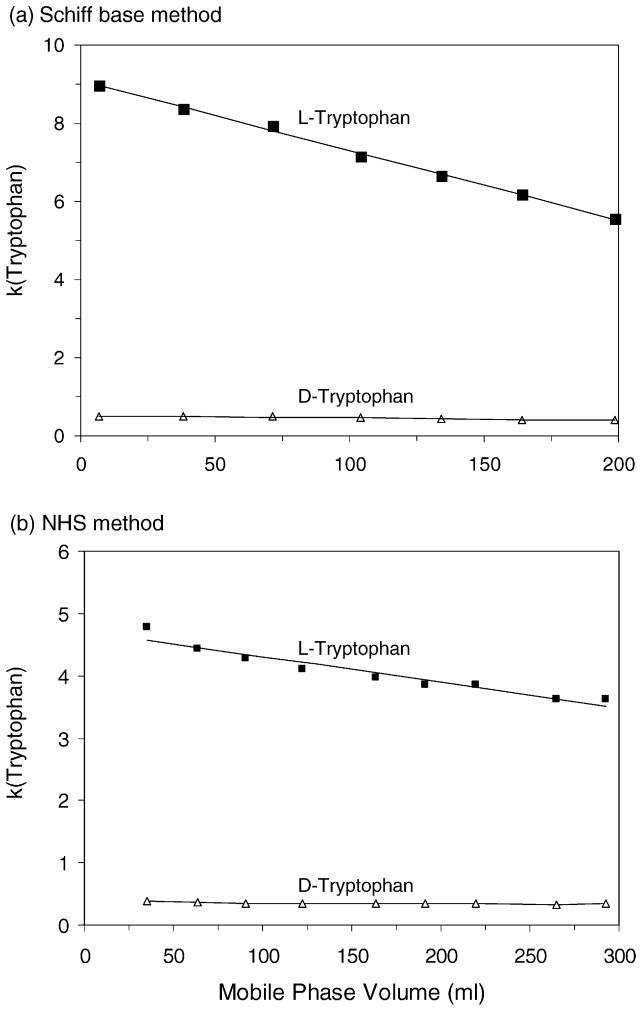

Fig. 8. Change in the retention factors for D- and L-tryptophan as a function of the amount of mobile phase passed through HSA columns prepared by (a) the Schiff base method and (b) the NHS method. In these studies, 100 mL is equal to approximately 725 column void volumes. The slopes of the L-tryptophan plots in (a) and (b) were −0.018 (±0.01) and −0.016 (±0.001), with best-fit intercepts of 9.09 (±0.01) and 3.55 (±0.02) and correlation coefficients of 0.999. For the Schiff base method, D-tryptophan gave a best-fit line with a slope of −0.0006 (±0.0001), an intercept of 0.52 (±0.01) and a correlation coefficient equal to 0.986. The results for D-tryptophan in the NHS method gave a slope of −0.0007 (±0.0001), an intercept of 0.30 (±0.01) and a correlation coefficient of 0.985.

column. However, for the Schiff base column the retention factor for *S*-warfarin decreased slightly more rapidly than it did for *R*-warfarin. This indicated that the local regions for *R*- and *S*-warfarin interactions on HSA had different stabilities in the Schiff base method, but similar stabilities in the NHS immobilization technique. This fits a previous model in which *R*- and *S*-warfarin have been proposed to have a common binding site on HSA but to interact at different local regions within this site [27].

A different trend in stability was observed for D/L-tryptophan. As shown in Fig. 8, the retention factor for L-tryptophan on both the NHS and Schiff base columns gave a linear decrease with time with similar slopes (-0.016 ± 0.01 versus -0.018 ± 0.001). This suggested that the L-tryptophan binding site on HSA was affected to the same degree over time in each of these coupling methods. In addition, both types of columns gave no appreciable change in the retention factor for D-tryptophan. This difference from the behavior seen for L-tryptophan was not surprising because it has been suggested that these enantiomers have very different binding regions on HSA [7].

4. Conclusions

This paper described the preparation of NHS-activated silica for the immobilization of HSA and other proteins. In this approach, silica was first reacted with APTS to produce aminopropyl groups on its surface, with these groups then being reacted with DSS to produce NHS-activated silica. Solid state ^{13}C NMR, combustion analysis, and NHS measurements were used to confirm and examine the various steps in this process. It was found that the amount of NHS groups on the activated support could be controlled by varying the amount of aminopropyl groups or DSS used in the synthesis. The immobilization of HSA onto the NHS-activated silica could be performed under physiological conditions and required only a short period of time. The resulting HSA support was successfully used to separate racemic *R*/*S*-warfarin and D/L-tryptophan, giving improved activity and stability versus HSA immobilized by the Schiff base method. These features make this approach attractive for the preparation of HSA as a chiral stationary phase or as a tool in the study of drug-protein interactions. Similar advantages may be seen when this coupling technique is used with other proteins.

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