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Short communication

Chromatographic analysis of carbamazepine binding to human serum albumin II. Comparison of the Schiff base and *N*-hydroxysuccinimide immobilization methods

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Abstract

Recent studies with carbamazepine on human serum albumin (HSA) columns have noted an appreciable degree of non-specific binding on supports prepared by the Schiff base immobilization method. This work examines an alternative immobilization method for HSA based on *N*-hydroxysuccinimide (NHS)-activated silica. This support was prepared by reacting HPLC-grade silica directly with disuccinimidyl carbonate. The resulting material was compared to an HSA support prepared by the Schiff base method in terms of its activity for carbamazepine and non-specific interactions with this drug. When examined by frontal analysis, both supports gave comparable association equilibrium constants for carbamazepine interactions with HSA ($(0.53–0.55) \times 10^4\ M^{-1}$ at 37 °C). However, columns prepared by the Schiff base method gave greater non-specific binding. These columns, as well as control columns prepared using the carbonyldiimidazole (CDI) immobilization method, were also evaluated for their non-specific binding to a variety of other solutes known to interact with HSA. From these results it was concluded that the NHS method was an attractive alternative to the Schiff base technique in the preparation of immobilized HSA for HPLC-based binding studies for carbamazepine. However, it was also noted that non-specific binding varies from one drug to the next in these immobilization methods, indicating that such properties should be evaluated on a case-by-case basis in the use and development of HSA columns for binding studies.

Keywords: Carbamazepine; Human serum albumin; High-performance affinity chromatography; Binding studies; Schiff base method; *N*-Hydroxysuccinimide method

1. Introduction

The interaction of a drug with a serum protein is important in determining the activity and fate of such a compound within the body. For instance, these interactions help determine the overall distribution, excretion, and toxicity of a drug [1–4]. One protein known to interact with many drugs is human serum albumin (HSA). HSA is the most abundant protein in serum, having a typical concentration of 50 g/l [3,4]. HSA has a molar mass of 66,438 g/mol and consists of a single chain of 585 amino acids held together by 17 disulfide bonds [3,4]. Many small organic compounds show reversible binding to HSA, including long-chain fatty acids, steroids, warfarin, tryptophan, ketoprofen, propranolol, and diazepam [5–8].

There are numerous techniques for examining solute binding to HSA. Examples include ultrafiltration, equilibrium dialysis, UV–vis spectroscopy, spectrofluorometry, crystallography, capillary isotachophoresis and affinity capillary electrophoresis, among others (see Refs. [3,5–17] and references cited therein). Another technique that has been used for such work is high-performance affinity chromatography (HPAC) [18–27]. This is typically performed by examining the retention and competition of solutes as they pass through an immobilized HSA column. Examples include reports in which HSA columns have been used to measure the binding strength of solutes with HSA, perform competition and displacement studies, generate structure-retention relationships, and locate binding regions on this protein [18,23–26]. Advantages of this approach include its speed, precision, and good correlation versus reference methods [18].

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It is important in such experiments to have a support that provides good activity for the immobilized HSA and low non-specific binding for the drug of interest. The Schiff base method has often been used to immobilize HSA for this purpose and meets these requirements for many drugs [19–23]. However, this is not the case for all drugs that have been tested. For instance, in one recent study it has been observed that carbamazepine has significant secondary interactions with silica supports prepared by the Schiff base method [27]. Thus, it is desirable in this case to find an alternative technique for immobilizing HSA within an HPLC column.

This study will examine the use of HSA columns that have been prepared using *N*-hydroxysuccinimide (NHS)-activated silica. Frontal analysis and zonal elution will be used to compare the behavior of these columns with those made by the Schiff base method. Carbamazepine will be used as the model drug for this report. This work will compare the equilibrium constants, binding capacities, and specific activities for the HSA columns, as well as their non-specific interactions with carbamazepine. The non-specific interactions of these columns with a variety of other drugs will also be considered and will be compared to those obtained with an alternative immobilization method for HSA (e.g., the carbonyldiimidazole or CDI method) [18]. From the results it will be possible to determine the advantages of each immobilization technique when it is used to prepare HSA columns for experiments with carbamazepine. In addition, these data should provide some useful insights into the testing and development of HSA columns for other compounds.

2. Experimental

2.1. Reagents

The carbamazepine (>98% pure), sodium nitrate, disuccinimidyl carbonate (DSC), digitoxin (>99% pure), lidocaine (>98% pure), propranolol hydrochloride (>99% pure), pindolol (97% pure), ibuprofen (>98% pure), tryptophan (>98% pure), verapamil hydrochloride (>99% pure), phenytoin (>99% pure), warfarin (>98% pure) and 1,1′-carbonyldimidazole were obtained from Sigma (St. Louis, MO, USA). Human serum albumin (Cohn fraction V, essentially fatty acid and globulin free) was purchased from Fluka (Milwaukee, WI, USA). The pyridine, triethylamine and 3-glycidoxypropyltrimethoxysilane (98% pure), and dimethylsulfoxide (DMSO, >99% pure) were from Aldrich (Milwaukee, WI, USA). Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was purchased from Macherey Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). Other chemicals used in this report were of the highest grades available. All buffers and aqueous solutions were prepared using water from a Barnstead Nanopure water system (Dubuque, IA, USA) and filtered through Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

2.2. Apparatus

The chromatographic system consisted of a PU-980i isocratic pump and P4000 gradient pump from Jasco (Tokyo, Japan), and one UV100 absorbance detector from ThermoSeparation Products (Riviera Beach, FL, USA). Samples were applied using a Rheodyne LabPro valve (Cotati, CA, USA) equipped with a 20 μl sample loop. The BCA protein assay and NHS assay were performed on a Shimadzu UV-160A spectrophotometer (Kyoto, Japan). An Isotemp 9100 circulating water bath from Fisher was used for temperature control of both the columns and mobile phases. All columns were downward slurry packed at 3500 psi (24 MPa) using an Alltech HPLC column slurry packer (Deerfield, IL, USA). Chromatographic data were collected and processed using programs written in Lab View 5.1 (National Instruments, Austin, TX, USA).

2.3. Preparation of NHS-activated silica

Nucleosil Si-300 was converted into an NHS-activated form by reacting this support with DSC in the presence of pyridine and triethylamine, as shown in Fig. 1 [28]. Prior to this reaction,

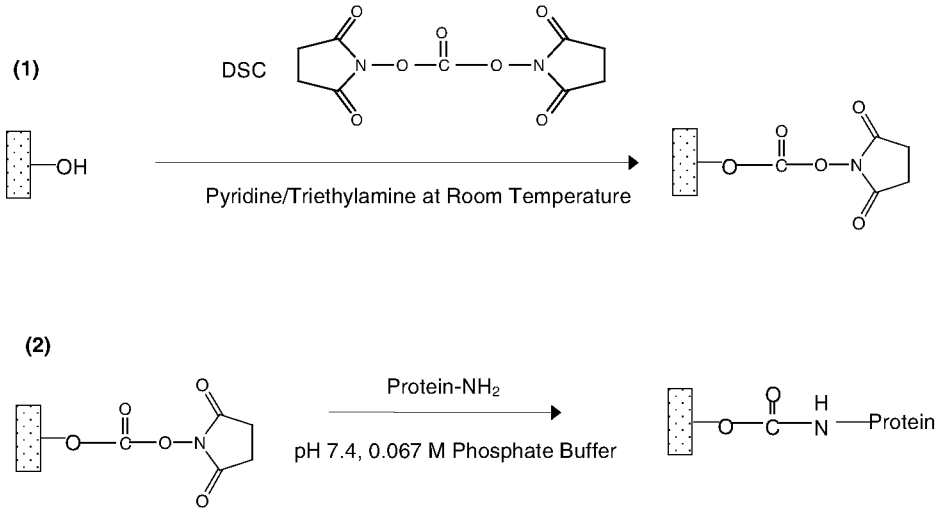

Fig. 1. Preparation of NHS-activated silica for protein immobilization.

the silica was pretreated by combining 3 g of this material with 16% (v/v) hydrochloric acid and refluxing at 110 °C overnight. This support was then washed several times with water and filtered using a 0.22 μm nylon filter. The recovered silica was dried overnight under vacuum at 150 °C.

The dried silica was next transferred to a 150 ml round bottom flask and combined with 50 mg DSC. Dry acetone (20 ml) was added to this flask while the silica suspension was gently agitated with a stirring bar. Triethylamine (2.5 ml) was mixed with dry pyridine (20 ml) and placed drop wise into the silica suspension under an argon atmosphere over the course of 30 min. This mixture was gently agitated for an additional 60 min, followed by six washes with acetone to remove any remaining DSC. The final support was dried under vacuum overnight at room temperature and stored in a dessicator at room temperature. In addition, a portion of this material was packed into a 2.1 mm i.d. × 3.5 cm or 5.0 cm column for use in non-specific binding studies.

Activated sites on the surface of this support were quantitated by measuring the NHS groups released into solution after hydrolysis [29]. This gave a value of 13.8 (±0.3) μmol activated groups/g silica (±1S.D.) for the support used in this study. With this same assay, it was determined that the NHS-activated silica was stable for over 6 months under the given storage conditions, with only a 10% decrease in active groups being noted over this period.

2.4. Preparation of Schiff base-activated support

In the Schiff base method, Nucleosil Si-300 silica was first converted into a diol-bonded form according to a previous procedure [30]. This was performed by placing 5 g Nucleosil Si-300 in 25 ml of pH 5.5, 0.1 M sodium acetate buffer (Note: The pH of this buffer was adjusted by adding a small amount of 1 M hydrochloric acid to a 0.1 M sodium acetate solution in water), with this mixture being degassed by sonication under vacuum for 30 min. Next, 3-glycidoxypropyltrimethoxysilane (1 ml) was added to this suspension. This mixture was shaken for 5 h at 90 °C. The epoxy silica formed by this reaction was washed several times with water and a pH 3.0 sulfuric acid solution. This support was then suspended in a pH 3.0 sulfuric acid solution (200 ml) and refluxed for 90 min. This produced diol-bonded silica. This support was washed several times with water, methanol, and ether and dried overnight under vacuum at room temperature. The diol coverage on this support was measured in triplicate using an iodometric capillary electrophoresis assay [31], giving a value of 336 (±4) μmol diol groups/g silica.

This diol-bonded support is stable over several months when stored either under vacuum or in a neutral pH buffer. However, for the Schiff base method this material must next be converted into an aldehyde-activated form. This was accomplished by reacting the diol-bonded silica with periodic acid in the presence of a 90% (v/v) mixture of glacial acetic acid and water [30]. This aldehyde-activated support is known to have limited stability and was used for immobilization immediately after its preparation, as described in the next section. The epoxy silica, diol silica, aldehyde silica supports used for non-specific binding studies were prepared in the same fashion as described in this section, with separate 2.1 mm i.d. × 3.5 cm or 5.0 cm columns being packed with these materials.

2.5. Preparation of CDI control support

The CDI support for the non-specific binding studies was prepared as described in Ref. [32]. This was accomplished by combining 0.5 g of diol-bonded silica with 1 g of 1,1′-carbonyldiimidazole in 20 ml of dry acetonitrile and shaking at room temperature with a wrist action shaker for roughly 2 h. The resulting CDI-activated silica was then washed with approximately 200 ml of dry acetonitrile. This material was converted to an inactivated form (i.e., the form that would be produced after immobilization of a protein like HSA) by placing it into 0.5 M, pH 8 Tris buffer and reacting for 2 h at room temperature. This silica was then washed with 150 ml of pH 7.4, 0.067 M potassium phosphate buffer and packed into a 2.1 mm i.d. × 3.5 cm stainless steel column.

2.6. HSA immobilization

The immobilization of HSA to the NHS-activated silica was achieved by using a 20 mg/ml solution of HSA in pH 7.4, 0.067 M potassium phosphate buffer. (Note: This buffer was prepared by placing 1.865 g potassium dihydrogen phosphate and 9.432 g dipotassium hydrogen phosphate in 900 ml water, adjusting the pH to 7.4 with a small volume of concentrated hydrochloric acid or sodium hydroxide, and diluting the buffer to a total volume of 1.00 l with water.) A 3 ml aliquot of this solution was mixed with 0.2 g NHS-activated silica and reacted at room temperature for 2 h. After this reaction, the resulting HSA silica 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 control support (i.e., the NHS control) was similarly prepared by placing 0.2 g NHS-activated silica into 3 ml of pH 7.4, 0.067 M potassium phosphate buffer for 2 h in the absence of any protein. This control silica was washed several times with the pH 7.4 buffer and stored under the same conditions as the immobilized HSA silica.

HSA was immobilized to aldehyde-activated silica in the Schiff base method by combining 150 mg HSA with 70 mg sodium cyanoborohydride and 5 g of the aldehyde-activated support in the presence of 10 ml of pH 6.0, 0.1 M potassium phosphate coupling buffer. (Note: This buffer was prepared by placing 11.86 g potassium dihydrogen phosphate and 2.24 g dipotassium hydrogen phosphate in 900 ml water, adjusting the pH to 6.0 with a small volume of concentrated hydrochloric acid or sodium hydroxide, and diluting the buffer to a total volume of 1 l with water.) This mixture was allowed to react for 5 days at 4 °C. After immobilization, the resulting HSA silica was washed several times with pH 7.4, 0.1 M potassium phosphate buffer and treated with three 10 mg portions of sodium borohydride to reduce any remaining aldehydes on the support to alcohol groups. The HSA silica 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. A control support was similarly

prepared in the absence of protein and stored in the same manner as the immobilized HSA support.

The HSA coverage of each support was determined using a BCA protein assay. To perform this assay, a small portion (10–20 mg) of each support was washed several times with deionized water and dried under vacuum at room temperature. The amount of protein on this material was determined using HSA as the standard and the control support as the blank. With this procedure, the final HSA content of the NHS support was found through triplicate measurements to be 125 (±18) nmol HSA/g silica. The amount of HSA on the support prepared by the Schiff base method was 410 (±7) nmol HSA/g silica.

2.7. Chromatographic procedures

The immobilized HSA supports were packed into 2.1 mm i.d. × 5.0 cm stainless steel columns. The packing solution for these and all other supports used in this study was pH 7.4, 0.067 M potassium phosphate buffer. Each column was placed in a water jacket for temperature control. All mobile phases were degassed at least 15 min prior to use. The following wavelengths were used for detection: carbamazepine, 280 nm or 214 nm (the latter being used in the final set of non-specific binding studies); digitoxin and phenytoin, 205 nm; lidocaine, propranolol, pindolol, verapamil and ibuprofen, 225 nm; tryptophan, 280 nm; and warfarin, 309 nm. Column pressures less than 80 psi (0.55 MPa) were typically observed during the chromatographic studies, with no effects of pressure on the retention of carbamazepine being observed under these conditions.

Carbamazepine samples were prepared by dissolving this drug in pH 7.4, 0.067 M potassium phosphate buffer at concentrations of 0–50 μM. These solutions were stored at 4 °C until use, with carbamazepine being stable for several months under such conditions [27]. All other sample solutions were also prepared in pH 7.4, 0.067 M potassium phosphate buffer. The tryptophan samples were used within 12 h of preparation. All other solutions were prepared and used over periods of less than 1 week.

Frontal analysis was performed by applying solutions of 0–50 μM carbamazepine to the HSA or control columns at 0.1 ml/min. This flow rate was well within the range needed to establish a local equilibrium in the HSA column, as determined in earlier studies [18,20]. The retained carbamazepine was eluted by washing the column with pH 7.4, 0.067 M potassium phosphate buffer. The amount of carbamazepine required to saturate the HSA or control columns was determined by integration to find the mean position of the resulting breakthrough curves [33]. The results obtained for the control columns were subtracted from those obtained for HSA columns to correct for the void time and to adjust for any non-specific interactions between carbamazepine and the support's surface. The void times for the HSA columns and control columns were estimated by injecting sodium nitrate as a non-retained solute.

The stability of each HSA column was checked periodically over the course of this study (i.e., 6 months and approximately 500 column volumes). This was accomplished by making periodic injections of carbamazepine under a set of standard conditions (i.e., pH 7.4, 0.067 M phosphate buffer applied at 25 °C and 0.1 ml/min). Less than a 3% change in retention was observed for both the Schiff base and NHS columns during the work described in this report. The non-specific binding studies based on zonal elution experiments were performed using 20 μl sample injections of the following solutions in pH 7.4, 0.067 M phosphate buffer: 10 μM digitoxin, propranolol, tryptophan or pindolol; 20 μM lidocaine, pindolol, warfarin, ibuprofen, verapamil or phenytoin; and 5 μM carbamazepine. All of these non-specific binding studies were performed in at least triplicate at 37 °C and 1.0 ml/min. The void time in the zonal elution studies was determined using DMSO as a non-retained solute.

3. Results and discussion

3.1. General retention properties of the HSA columns

The first item studied was the relative degree of retention of carbamazepine on HSA columns prepared by the NHS and Schiff base methods. Fig. 2 gives typical chromatograms obtained for carbamazepine on these columns as well as on the corresponding control columns. It can be seen from these results that both types of HSA columns gave good retention for this drug, with total retention factors of 3.36 and 5.40 being measured on columns prepared by the NHS and Schiff base methods, respectively.

However, one significant difference in these immobilization methods was the amount of non-specific adsorption that occurred between carbamazepine and the control columns. This

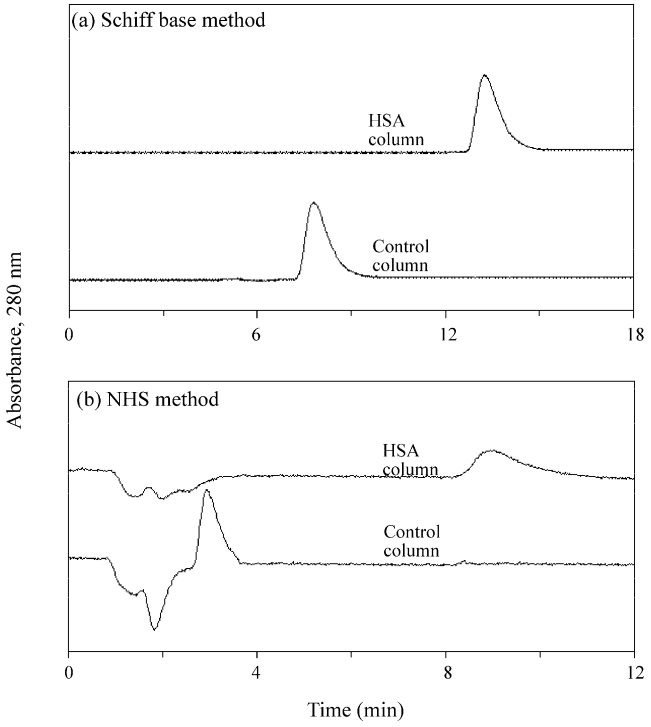

Fig. 2. Typical chromatograms for carbamazepine on 2.1 mm i.d. × 5.0 cm HSA columns and control columns prepared by the (a) Schiff base method or (b) NHS method.

Table 1
Properties of HSA columns prepared by the Schiff base and NHS methods

Property[a]	Measured or calculated value[b]	
	NHS column	Schiff base column
Amount of immobilized HSA	125 (±18) nmol/g silica	410 (±7) nmol/g silica
Total retention factor	3.36 (±0.02)	5.40 (±0.01)
Non-specific retention factor	0.40 (±0.01)	3.01 (±0.01)
Specific retention factor	2.96 (±0.03)	2.39 (±0.02)

[a] The total retention factor is the retention factor measured for carbamazepine on a given HSA column. The non-specific retention factor is that obtained on the control column, and the specific retention factor is the difference between total and non-specific retention factors.
[b] All values in parentheses represent a range of ±1S.D.

is demonstrated in Table 1. For instance, although the overall retention for carbamazepine on the Schiff base HSA column was 1.6-fold higher than on the NHS-prepared column, more than half of the retention seen for the Schiff base column (55.7%) was also noted on its control support ($k = 3.01$). On the other hand, the control column for the NHS method gave retention for carbamazepine that was only 11.9% of that seen on the NHS immobilized HSA column ($k = 0.40$). Thus, it was found that substantially less non-specific binding occurred for carbamazepine when using the NHS method.

In the Schiff base method, 3-glycidoxypropyltrimethoxysilane was reacted with the silica to place epoxy groups, and later aldehyde groups, on the support's surface. This resulting in a material with a propyl group on the support as a spacer between the surface and amine-reactive sites; alcohol groups were also present at the end of this support after it had been used in immobilization or inactivated. However, no such spacer or groups were present in support prepared by the NHS method. In the NHS method, DSC was reacted directly with silanols to form an active NHS ester. After these NHS groups had been used in immobilization, any remaining active sites were removed by hydrolysis [28]. This explains why the NHS control column gave similar results to a bare silica column. A further consideration of the sources of this non-specific binding is given later in Section 3.5.

3.2. Binding capacity measurements

The binding of carbamazepine to the HSA columns was examined in more detail by using frontal analysis [34–36]. This method involved applying a known concentration of the test solute continuously to a column containing the immobilized ligand of interest. As the amount of solute that was bound by the column increased, this formed a breakthrough curve, where the mean point of this curve was related to the concentration of applied solute and the amount of immobilized ligand.

If an applied solute (S) binds only to a single type of site on the ligand (L) and this binding has relatively fast association/dissociation kinetics, the following relationship can be used to relate the true number of active ligand binding sites on the column (m_L) to the apparent moles of solute (m_{Lapp}) required to reach the mean point of the breakthrough curve [18,36]. This is shown in Eq. (1),

$$\frac{1}{m_{Lapp}} = \frac{1}{K_a m_L [S]} + \frac{1}{m_L} \quad (1)$$

where K_a is the concentration-dependent association equilibrium constant for the binding of S to L, and [S] is the concentration of solute applied to the column. Eq. (1) predicts that a plot of $1/m_{Lapp}$ versus 1/[S] will give a straight line for a system with 1:1 binding. Furthermore, this line will have a slope equal to $1/(K_a m_L)$ and an intercept of $1/m_L$. This makes it possible to obtain the association equilibrium constant K_a by taking the ratio of the intercept to the slope. In addition, the true number of binding sites (m_L) in the column can be determined from the inverse of the intercept [18].

In correcting for non-specific adsorption, it has been shown that subtracting the breakthrough times for a control column from that obtained for an immobilized HSA column gives a good estimate for the specific interactions between an applied drug and HSA [27]. In this particular study, it was found with the Schiff base columns that this correction gave association equilibrium constants at all tested temperatures that were within 5–15% of those determined using a more complex multi-site model. An even better correlation would be expected for the NHS results due to the lower amount of non-specific binding that was observed for carbamazepine with the NHS support.

The frontal analysis results obtained in this study are summarized in Fig. 3. For both the NHS and Schiff base methods, plots of $1/m_{Lapp}$ versus 1/[carbamazepine] gave linear relationships over the entire concentration range studied at 37 °C. The correlation coefficients for these plots were 0.9997 (Schiff base data) and 0.9999 (NHS data) for six data points. According to Eq. (1), this linear behavior indicated that carbamazepine was binding to a single type of site on the immobilized HSA. This same conclusion has been reached in previous work with carbamazepine and HSA in solution phase studies [27].

From the intercepts of the plots shown in Fig. 3, it was possible to estimate the moles of active binding sites for carbamazepine on each HSA column. Table 2 shows the values

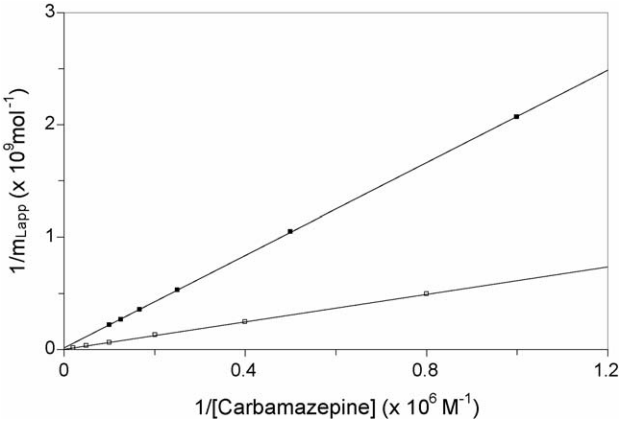

Fig. 3. Double-reciprocal plots obtained for frontal analysis studies with carbamazepine at pH 7.4 and 37 °C using HSA immobilized by the NHS method (■) or Schiff base method (□).

Table 2
Binding capacities and activities for carbamazepine on HSA columns prepared by the Schiff base and NHS methods[a]

Temperature (°C)	Binding capacity ($\times 10^{-7}$ mol)		Specific activity (% mol carbamazepine/mol HSA)	
	NHS column	Schiff base column	NHS column	Schiff base column
4	0.25 (±0.01)	1.60 (±0.05)	20 (±3)	39 (±1)
15	0.29 (±0.01)	2.09 (±0.07)	24 (±3)	51 (±1)
25	0.45 (±0.01)	2.30 (±0.12)	37 (±5)	56 (±1)
37	0.88 (±0.01)	3.06 (±0.05)	72 (±10)	75 (±1)
45	1.39 (±0.04)	3.17 (±0.09)	113 (±16)	77 (±1)

[a] The values in parentheses represent a range of ±1S.D.

obtained at several temperatures. Both columns gave a similar trend in the binding capacity as the temperature increased. The HSA column made by the Schiff base method consistently gave the highest binding capacity (14–43% larger than the NHS prepared column), but this column also contained more immobilized protein.

Using the known protein content of each support and the measured binding capacities, it was possible to determine the specific activity for each type of immobilized HSA column. These results are given in Table 2. It was found that the NHS and Schiff base methods gave comparable activities for the immobilized HSA when measured at 37 °C, with values of 72–75%. However, this activity changed with temperature, with HSA immobilized by the NHS method showing a larger variation than that prepared by the Schiff base technique. The reason for this is not yet clear, but it may indicate that different degrees of multi-site attachment occur between HSA and the support in these two methods. This, in turn, would affect the ability of the HSA to alter its conformation with temperature. If this is the case, HSA immobilized by the Schiff base method appears to be less flexible in its structure since its activity is more stable with regards to changes in temperature.

3.3. Association equilibrium constant measurements

In addition to the binding capacity, the association equilibrium constant for carbamazepine with HSA was obtained from the plots in Fig. 3. Table 3 shows the values obtained over 4–45 °C for the NHS and Schiff base columns. Both columns gave similar trends in the association equilibrium constants as the temperature was varied, with a decrease of five- to six-fold in K_a as the temperature was raised from 4 to 45 °C. This same trend has been seen for other compounds, including L-tryptophan, *R*-warfarin, *S*-warfarin and L-thyroxine [19,36].

Table 3
Association equilibrium constants for carbamazepine on HSA columns prepared by the Schiff base and NHS methods

Temperature (°C)	Association equilibrium constant ($\times 10^4$ M^{-1})[a]	
	NHS column	Schiff base column
4	2.2 (±0.1)	2.1 (±0.1)
15	1.8 (±0.1)	1.2 (±0.1)
25	1.1 (±0.1)	1.1 (±0.2)
37	0.55 (±0.01)	0.53 (±0.08)
45	0.33 (±0.01)	0.46 (±0.12)

[a] The values in parentheses represent a range of ±1S.D.

The association equilibrium constant for carbamazepine with immobilized HSA prepared by either method was $(5.3–5.5) \times 10^3$ M^{-1} at 37 °C. This is similar to earlier results obtained in solution phase studies using microdialysis or HPLC, in which association equilibrium constants of $(0.7–1.0) \times 10^4$ M^{-1} have been reported [37]. The similarity of these values indicates that the immobilized HSA was a good model for the binding of HSA in solution. The fact that the Schiff base and NHS methods gave essentially identical association equilibrium constants at all of the temperatures studied again indicates that it was the relative amount of active protein, rather than the equilibrium constants for HSA, which gave rise to the differences in specific activity observed in Table 2 for these two methods.

3.4. Thermodynamic studies

Thermodynamic parameters for the HSA columns were determined by using the concentration-dependent association equilibrium constants in Table 3 to prepare van't Hoff plots. This was accomplished by using Eq. (2),

$$\ln K_a = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (2)$$

where K_a is the association equilibrium constant for the binding of solute to ligand, T the absolute temperature, and R is the gas law constant [36]. Other terms in Eq. (2) include the changes in entropy (ΔS) and enthalpy (ΔH) for the solute-protein interaction. Eq. (2) indicates that a system with 1:1 interactions will give a plot for $\ln K_a$ versus $1/T$ that produces a straight line with a slope equal to $-\Delta H/R$ and an intercept of $\Delta S/R$. The changes of enthalpy and entropy can be directly calculated from the slope and intercept of this plot, respectively. In addition, the total change in Gibbs free energy (ΔG) can be obtained by using Eq. (3) along with measured values of T and K_a.

$$\Delta G = -RT \ln(K_a) \quad (3)$$

The resulting graphs of $\ln K_a$ versus $1/T$ obtained for the NHS and Schiff base immobilized HSA columns are shown in Fig. 4. Both plots gave linear behavior with correlation coefficients of 0.977 and 0.971 ($n = 5$) for the NHS and Schiff base data, respectively. This linearity further confirmed there was a single type of binding site for carbamazepine on the HSA immobilized by either the NHS or Schiff base method.

The two plots shown in Fig. 4 were statistically identical over the range of temperatures given. From the slopes and inter-

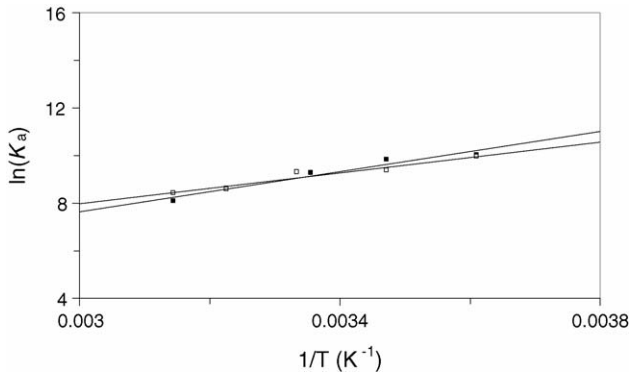

Fig. 4. A van't Hoff plot for the interactions of carbamazepine with HSA immobilized by the NHS method (■) or Schiff base method (□). The best-fit slope and intercept for the NHS column were 4.21 (±0.07) × 10^3 and −5.14 (±0.39), respectively. The best-fit slope and intercept for Schiff base column were 3.24 (±0.02) × 10^3 and −1.76 (±0.01). The correlation coefficient was 0.977 (n = 5) for the NHS column and 0.971 (n = 5) for the Schiff base column.

cepts of these plots, it was possible to estimate the changes in the total Gibbs free energy, enthalpy, and entropy for the binding of carbamazepine with the immobilized HSA columns. The results indicated that the binding of carbamazepine to HSA gave a large change in enthalpy of −27 to −35 kJ/mol, or −6.5 to −8.4 kcal/mol. This was accompanied by a decrease in entropy for the system, as represented by changes in entropy of −15.0 to −42.7 J/(mol K), or −3.6 to −10.2 cal/(mol K). The total changes in Gibbs free energy measured for the binding of carbamazepine on HSA with these columns was −21.7 to −22.4 kJ/mol at 37 °C (i.e., −5.2 to −5.35 kcal/mol).

These results indicate that a change in enthalpy is the main driving force for carbamazepine–HSA binding, with this working in opposition to a decrease in entropy to produce a stable complex. The decrease in entropy seen upon the binding of carbamazepine to HSA is somewhat unusual in that most drugs show an increase in entropy when binding to this protein [38]. However, a decrease in entropy has been noted in some previous cases, such as binding of benzodiazepines or heptacarboxyl porphyrin to HSA [39–41].

3.5. Sources of non-specific binding by carbamazepine and other solutes

The last section of this study compared the possible sources of non-specific binding for carbamazapine in the HSA columns that were prepared by the Schiff base and NHS-activation methods. This was done by taking samples of these supports at each stage of their synthesis, placing these materials into columns and comparing their retention for carbamazepine under the same mobile phase conditions as used in the work with immobilized HSA. The results are summarized in Table 4.

First, a comparison was made between the retention factors measured for carbamazepine on the Schiff base control column versus bare silica and supports collected at various stages of the Schiff base immobilization process. For both carbamazepine and the other tested solutes, a close correlation was noted between the non-specific binding seen on the Schiff base columns and diol silica. This was not surprising since the surfaces of these two supports differ by only one alcohol group at the end of their organosilane chains (i.e., two in the diol silica and one in the Schiff base control). Although the aldehyde silica gave higher non-specific binding for carbamazepine than the Schiff base control, aldehyde groups should not have been present in the final Schiff base support due to the use of sodium borohydride as a reducing agent to remove these groups at the end of this immobilization process. Some non-specific binding due to the propyl backbone of organosilane chains may also have been present (as suggested by the epoxy silica results), but the increase in retention between the epoxy and diol silica results indicates that the terminal alcohol groups on these chains were the main source of non-specific binding for carbamazepine in these particular materials.

A similar comparison was made between the retention factors on bare silica and the NHS control support. The results were quite similar for carbamazepine, indicating that the silica itself

Table 4
Non-specific binding by carbamazepine and other solutes to various silica-based supports

Analyte	Bare silica	Epoxy silica	Diol silica	Aldehyde silica	Schiff base control	NHS control	CDI control
Measured retention factor, k[a]							
Carbamazepine	0.26 (±0.01)	1.55 (±0.03)	2.33 (±0.03)	3.37 (±0.06)	2.18 (±0.02)	0.21 (±0.01)	4.00 (±0.18)
Tryptophan	0.10 (±0.01)	0.04 (±0.03)	0.15 (±0.01)	0.28 (±0.02)	0.12 (±0.02)	0.02 (±0.01)	2.40 (±0.06)
Warfarin	0.00 (±0.01)	0.51 (±0.02)	0.52 (±0.01)	0.51 (±0.01)	0.50 (±0.01)	−0.02 (±0.02)	6.55 (±0.29)
Digitoxin	0.75 (±0.02)	0.01 (±0.01)	0.13 (±0.01)	0.09 (±0.02)	0.12 (±0.04)	0.91 (±0.04)	0.65 (±0.08)
Lidocaine	3.38 (±0.01)	0.41 (±0.01)	1.13 (±0.11)	0.76 (±0.11)	1.09 (±0.03)	3.03 (±0.04)	0.82 (±0.08)
Propranolol	4.76 (±0.02)	2.47 (±0.05)	6.75 (±0.04)	9.07 (±0.52)	6.33 (±0.01)	4.77 (±0.01)	7.01 (±0.22)
Pindolol	2.02 (±0.04)	0.52 (±0.01)	1.39 (±0.02)	1.27 (±0.10)	1.34 (±0.02)	1.50 (±0.01)	1.58 (±0.13)
Ibuprofen	−0.04 (±0.01)[b]	0.05 (±0.04)	0.12 (±0.03)	0.20 (±0.05)	0.11 (±0.02)	−0.13 (±0.03)[b]	0.24 (±0.08)
Phenytoin	0.11 (±0.02)	0.99 (±0.09)	0.93 (±0.08)	4.58 (± 0.14)	0.77 (±0.06)	0.01 (±0.01)	2.22 (±0.17)
Verapamil	57.4 (±2.8)	8.93 (±0.10)	39.4 (±1.0)	31.5 (±1.2)	38.6 (±0.1)	58.4 (±3.6)	28.1 (±1.1)

[a] The numbers in parentheses represent a range of ±1S.D. All of these retention factors were measured at 37 °C and in the presence of pH 7.4, 0.067 M phosphate buffer.

[b] The slightly negative retention factors seen for ibuprofen on the bare silica and NHS control supports are believed to be due to the ionic repulsion of ibuprofen from the silica's surface at pH 7.4.

was the source of the relatively small amount of non-specific binding seen when using carbamazepine with HSA that had been immobilized by this method. This was not surprising since the conditions used in this study for the NHS method gave rise to only a small amount of activation for this support, leaving the rest of the surface available for possible interactions with the injected solutes.

The non-specific binding by other solutes were also compared in the Schiff base and NHS supports. These solutes included several compounds that are commonly used as site-selective probes for HSA (i.e., tryptophan, warfarin, and digitoxin) and a variety of drugs that are known to have significant binding to this protein (e.g., lidocaine, propranolol, pindolol, ibuprofen, phenytoin, and verapamil) [3–5,18]. As was seen for carbamazepine, some of these solutes gave lower non-specific binding on the NHS support than on the Schiff base support; these solutes included tryptophan, warfarin, propranolol, ibuprofen, and phenytoin. However, there were also several of these compounds that gave lower non-specific binding when using the Schiff base method (e.g., digitoxin, pindolol, and verapamil).

One additional immobilization method that was considered in this comparison of non-specific binding was the CDI method. This was examined since it has also been used in many studies for the immobilization of HSA to silica, although it has been observed to give lower activity for this protein than the Schiff base method [18,42]. For carbamazepine, the CDI method gave greater non-specific binding than either the Schiff base or NHS methods. This was also the case for tryptophan, warfarin, propranolol, pindolol, ibuprofen, and phenytoin. The CDI method gave lower non-specific binding than these other immobilization techniques for only two of the tested compounds (lidocaine and verapamil) and gave non-specific binding between that of these other methods for one solute (digitoxin).

4. Conclusions

This study examined the binding between HSA and carbamazepine by using HSA columns prepared by two immobilization techniques: the Schiff base and the NHS method. The NHS column was prepared by immobilizing HSA to silica that had been directly modified with DSC. It was found that the Schiff base and NHS columns gave comparable equilibrium constants, activities, and thermodynamic parameters for carbamazepine–HSA binding, making them both useful in creating columns for studies of this interaction. However, the NHS method gave a support with much less non-specific binding for carbamazepine than supports prepared by the Schiff base method.

The results obtained in this study indicate that the NHS method is a useful alternative to the Schiff base method for work with carbamazepine. However, as is demonstrated in Table 4, the selection of an immobilization method for HSA still should be considered on a case-by-case basis in drug–protein interaction studies by HPAC. This is agreement with recent work with related NHS-activated supports (created by treating aminopropyl silica with disuccinimidyl suberate), which gave comparable non-specific binding to Schiff base-activated supports for D- and L-tryptophan but higher non-specific binding for *R*- and *S*-warfarin [43]. Thus, it is recommended that several immobilization techniques (e.g., the Schiff base, NHS, and CDI methods), be considered in initial experiments with other solutes in drug binding studies based on HPAC and HSA columns.

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