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Evaluation of Affinity Microcolumns Containing Human Serum Albumin for Rapid Analysis of Drug-protein Binding

Michelle J. Yoo
University of Nebraska - Lincoln

John E. Schiel
University of Nebraska - Lincoln

David S. Hage
University of Nebraska - Lincoln, dhage1@unl.edu

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EVALUATION OF AFFINITY MICROCOLUMNS CONTAINING HUMAN SERUM ALBUMIN FOR RAPID ANALYSIS OF DRUG-PROTEIN BINDING

Michelle J. Yoo, **John E. Schiel**, and **David S. Hage***
Department of Chemistry University of Nebraska-Lincoln Lincoln, NE 68588-0304 (U.S.A.)

Abstract

This study examined the use of affinity microcolumns as tools for the rapid analysis and high-throughput screening of drug-protein binding. The protein used was immobilized human serum albumin (HSA) and the model analytes were warfarin and L-tryptophan, two solutes often used as site-specific probes for drug binding to Sudlow sites I and II of HSA, respectively. The use of HSA microcolumns in binding studies was examined by using both zonal elution and frontal analysis formats. The zonal elution studies were conducted by injecting the probe compounds onto HSA microcolumns of varying lengths while measuring the resulting retention factors, plate heights and peak asymmetries. A decrease in the retention factor was noted when moving from longer to shorter column lengths while using a constant amount of injected solute. However, this change could be corrected, in part, by determining the relative retention factor of a solute versus a reference compound injected onto the same microcolumn. The plate height values were relatively consistent for all column lengths and gave an expected increase at higher linear velocities. The peak asymmetries were similar for all columns up to 1 mL/min but shifted to larger values at higher flow rates and when using short microcolumns (e.g., 1 mm length). The association equilibrium constants and number of binding sites estimated by frontal analysis for warfarin with HSA were consistent at the various columns sizes that were tested and gave good agreement with previous literature values. These results confirmed affinity microcolumns provide comparable results to those obtained with longer columns and can be used in the rapid analysis of drug-protein binding and in the high-throughput screening of such interactions.

Keywords

High-performance affinity chromatography; Affinity microcolumn; Frontal affinity chromatography; Frontal analysis; Zonal elution; Human serum albumin; Tryptophan; Warfarin; High-throughput screening; Drug-protein binding

1. Introduction

Interactions between drugs and serum proteins are important in determining the transport, distribution, metabolism, excretion and activity of many pharmaceutical agents in the body

*Author for correspondence: Phone, 402-472-2744; Fax, 402-472-9402; dhage@unlserve.unl.edu.

[1–4]. The importance of these interactions has resulted in the development of numerous methods to examine such binding [1–7]. Human serum albumin (HSA) is the most abundant plasma protein and is involved in the transport of many drugs and small solutes [1,4]. Two solutes that are often used as site-selective probes in drug binding studies with HSA are warfarin and L-tryptophan [8,9]. Warfarin is an anticoagulant drug with two enantiomers, (*R*)- and (*S*)-warfarin, that both bind to Sudlow site I of HSA with an association equilibrium constant (K_a) on the order of 10^5–10^6 M^{-1} [4,5,7,10,11] The interaction between warfarin and HSA has been previously studied by several methods [6,12,13]. L-Tryptophan binds to Sudlow site II of HSA with a K_a between 10^4 and 10^5 M^{-1} and has also been examined by many techniques [4,14].

One method that has been used increasingly in recent years to study drug-protein interactions is high-performance affinity chromatography (HPAC) [10,15–22]. HPAC is a chromatographic technique that uses a biologically-related ligand (e.g., a protein such as HSA) as a stationary phase [23]. HPAC columns containing immobilized HSA have been shown in many studies to give comparable results to those obtained by methods that use soluble HSA, such as equilibrium dialysis and ultrafiltration [6,8,9,17,23]. Both frontal analysis (or frontal affinity chromatography, FAC) and zonal elution have been employed as means for conducting such experiments on traditional HPAC columns (i.e., columns with typical lengths of 5–10 cm and inner diameters of 2.1–4.6 mm) [15,16,23,24].

A few recent studies have examined drug-protein interactions by using smaller affinity columns [8,9,22,24,25]. One study utilized affinity columns with dimensions of 250 μm i.d. × 1 cm for work with FAC and mass spectrometry in the study of biomolecular interactions [22]. Another report employed affinity columns with dimensions of 250 μm i.d. × 3 cm for the high-throughput screening of mixtures of modified β-galactopyranosides by using FAC and electrospray mass spectrometry [25]. Smaller monolithic affinity columns have been used in related studies. One report used a 4.5 mm i.d. × 6 mm affinity monolith to capture *Eschericha coli* bacteria [26]. Another recent report explored the use of zonal elution studies with affinity silica monoliths having sizes of 4.6 mm i.d. and lengths of 3–5 mm for use with immobilized HSA and the high-throughput analysis of drug-protein interactions [27]. All of these previous reports suggest that small affinity columns can be employed in such work; however, no systematic comparison between the binding data obtained with these small columns and the data obtained with larger affinity columns has been made, particularly in the case of FAC. In addition, the range of column sizes that can be successfully used in such experiments in work with silica particles has not yet been determined for either FAC or zonal elution studies.

In this study, affinity microcolumns of various lengths and containing silica particles will be used to study drug-protein interactions. HSA will be used as the immobilized protein and warfarin and L-tryptophan will be the model solutes. The use of these columns in both zonal elution and frontal analysis will be considered and the results will be compared to those obtained with longer affinity columns. In the zonal elution studies, the effect of column length on the measured retention, efficiency and peak asymmetry for each injected analyte will be investigated. For frontal analysis, the effect of column size on the measured binding capacities and affinities will be considered. The results of this report should provide a better understanding of the size parameters that are required to study drug-protein interactions using affinity microcolumns, as well as the potential advantages and limitations of affinity microcolumns in such research.

2. Experimental

2.1 Reagents

The HSA (Cohn fraction V, essentially fatty acid free, ≥ 96% pure), racemic warfarin (98%), *R*-warfarin (≥ 97%), and L-tryptophan (> 98%) were from Sigma (St. Louis, MO, USA). Nucleosil Si-300 silica (300 Å pore size, 7 μm particle size) was purchased from Macherey Nagel (Düren, Germany). Reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). All buffers and aqueous solutions were prepared using water from a Nanopure system (Barnstead, Dubuque, IA, USA) and filtered using Osmonics 0.22 μm nylon filters from Fisher (Pittsburgh, PA, USA).

2.2 Apparatus

The chromatographic system consisted of an isocratic HPLC pump (Jasco, Easton, MD, USA), a Dynamax solvent delivery system (Rainin, Woburn, MA, USA), a six-port Lab Pro valve (Rheodyne, Cotati, CA, USA), and a UV-2075 detector (Jasco, Easton, MD, USA). An Alltech water jacket (Deerfield, IL, USA) and a circulating water bath from Fisher (Pittsburgh, PA, USA) were used to maintain a temperature of 37.0 (± 0.1) °C for the chromatographic system during all experiments described in this report. The columns were packed using an Alltech slurry packer. Column lengths of 3 mm or less used a frit-in-column design, as described in Ref. [28]; longer columns were prepared using traditional stainless steel HPLC housings and end fittings. Chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA).

2.3 Methods

The stationary phase used in these studies consisted of silica particles containing immobilized HSA. To make this material, Nucleosil Si-300 silica was first converted into a diol form, as described previously [29]. HSA was then immobilized onto this diol silica by using the Schiff base method [10,30]. The final HSA silica was stored in pH 7.4, 0.067 M potassium phosphate buffer at 4 °C until use. A control support was prepared in the same method but with no HSA being added during the immobilization step.

Small portions of the HSA silica and control support were washed with water and dried under vacuum at room temperature. These dried samples were analyzed in triplicate using a BCA protein assay [31], using HSA as the standard and the control support as the blank. The final protein content of the HSA silica was found to be 51 (± 3) mg HSA per gram of silica. The remaining portions of the original HSA silica and control support were downward slurry packed at 4000 psi (28 MPa) for 40 min into their respective columns using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. The inner diameter of all the columns was 2.1 mm, but the column length was varied. The effective column lengths were 1, 2, 5, 10, and 20 mm (note: in preliminary studies with the given analytes, no significant changes in the measured binding parameters were noted when increasing the column length from 20 mm to 50–100 mm, indicating that it was not necessary to examine columns with these longer sizes in this present study).

Each column was placed in a water jacket and connected to a circulating water bath for temperature control. The columns were stored at 4 °C in pH 7.4, 0.067 M phosphate buffer when not in use. These columns were used within a period of 3 months and have been previously noted to be stable for up to one year under the storage and experimental conditions that were used in this study [32]. All mobile phases for the chromatographic studies were prepared from pH 7.4, 0.067 M phosphate buffer and were degassed for 25 min prior to use. The solutions of warfarin and L-tryptophan were also prepared in this buffer. Solutions of warfarin in pH 7.4, 0.067 M phosphate buffer are known to be stable for several days when stored at 4 °C and were

used within this period of time [11]. Solutions of L-tryptophan in this buffer were prepared fresh daily due to the limited stability of this analyte under these mobile phase conditions, as noted in previous studies [9].

The zonal elution studies were typically performed by injecting 5 μL of either 20 μM *R*-warfarin or 20 μM L-tryptophan at 0.1–5 mL/min onto the HSA and control columns. In preliminary studies, sample concentrations ranging from approximately 5–60 μM warfarin or L-tryptophan were also employed. The 20 μM sample concentration that was eventually selected for *R*-warfarin and L-tryptophan was chosen because of its ability to give easily detectable peaks for both these analytes and its creation of only small changes (i.e., less than 5–6% variation) in the measured retention factors on the 20 mm long column when compared when to work at lower sample concentrations. The wavelengths used for absorbance detection were 308 nm for *R*-warfarin, 280 nm for L-tryptophan, and 205 nm for sodium nitrate (i.e., a void volume marker). The void time of the system was determined by making 5 μL injections of 25 μM sodium nitrate in pH 7.4, 0.067 M phosphate buffer. The central moment, second moment, and asymmetry of each peaks was determined by using Peakfit 4.12 (Systat Software, San Jose, CA, USA). The retention factor and plate height measurements were corrected for the extra-column void time of the chromatographic system and extra-column band broadening by using results obtained for 5 μL injections of 25 μM sodium nitrate in pH 7.4, 0.067 M phosphate buffer with no column present.

Frontal analysis studies were conducted by using two pumps. The first pump was used to apply only buffer (i.e., pH 7.4, 0.067 M potassium phosphate buffer) to the desired column; the second pump was used to apply a solution containing a known concentration of the analyte (e.g., racemic warfarin) in this buffer. After a breakthrough curve had been obtained, only pH 7.4, 0.067 M potassium phosphate buffer was applied to the column to wash and elute the retained drug. Identical studies were carried out on a control column to correct for the void time of the system and for any secondary interactions between warfarin and the support, which were typically less than 5% of the total column binding capacity. In the frontal analysis studies, the concentrations of racemic warfarin that were used ranged from 1–10 μM and these solutions were applied at flow rates between 0.2 and 2 mL/min. The wavelength used for absorbance detection through the frontal analysis experiments was 308 nm. The mean breakthrough point of each frontal analysis curve was determined by the equal area method [23] using Labview.

3. Results and discussion

3.1. Zonal elution studies of analyte retention on HSA microcolumns

Zonal elution methods are often used to examine the binding of drugs with proteins such as HSA [21]. For instance, retention factors for injected analytes can be used under appropriate flow rate and elution conditions to estimate the percent of binding of an injected solute to an immobilized protein [24]. In addition, Eqn. (1) shows how the retention factor (k) is related to the global affinity constant for an injected solute with an immobilized protein [33].

$$k = \frac{(K_{A1} n_1 + \cdots K_{An} n_n)\, m_L}{V_M} \tag{1}$$

In this equation, K_{A1} through K_{An} are the association equilibrium constants for binding sites 1 through n, n_1 through n_n represent the number of each type of site in the column, m_L is the moles of binding sites in the column, and V_M is the column void volume (note: in this case, the global association equilibrium constant is the summation of the $K_A n$ terms). In addition, zonal elution studies and measurements of solute retention factors are often employed in

competition studies to determine the location of the binding sites for a solute on HSA through the use of site-selective probes such as warfarin and L-tryptophan [21,23,24].

The retention of *R*-warfarin and L-tryptophan on HSA microcolumns of various lengths was studied by performing zonal elution studies. The observed retention times for *R*-warfarin were 10.3 min, 2.4 min, and 46 s for the 2 cm, 5 mm, and 1 mm long columns at 0.5 mL/min, respectively. The observed retention times for L-tryptophan were 77, 33, and 22 s for the 2 cm, 5 mm, and 1 mm long columns at 0.5 mL/min, respectively. This information demonstrated the relative range of elution times that would be expected on these microcolumns for analytes such as L-tryptophan and warfarin with K_a values for HSA in the range of 10^4–10^5 M^{-1} or 10^5–10^6 M^{-1}, respectively.

Figures 1 and 2 show how varying the flow rate affected the retention factor measured for *R*-warfarin and L-tryptophan on the microcolumns with lengths of 2 cm, 5 mm and 1 mm (note: similar trends were noted for the other columns examined in this study). For *R*-warfarin, the average retention factors obtained at all flow rates on the 2 cm, 5 mm, and 1 mm long columns were 82 (± 4), 57 (± 6), and 38 (± 8), respectively (note: the numbers in parentheses represent 1 S.D.). For L-tryptophan on the 2 cm, 5 mm, and 1 mm long columns, the average retention factors at all samples flow rates were 7.4 (± 0.7), 4.6 (± 1.4), and 1.6 (± 0.8). The decrease in the measured retention factors noted for both these analytes as the column length decreased was found through sample loading studies to be related to the increase in sample loading that occurred as a fixed amount of solute was injected onto each of these columns, thus introducing non-linear elution conditions.

Changing the amount of injected sample in proportion to the column size would be one approach for minimizing the change that was noted in retention when using smaller HSA microcolumns. However, one difficulty with this approach is that the analytes will also be more difficult to detect as their injected quantities are decreased in proportion to column size. The use of a relative comparison of retention factors, such as is achieved by using the selectivity factor (α), was considered as an alternative means for adjusting for changes in analyte retention with column size. In this approach, the retention factor for the analyte of interest can be compared to the retention factor for a reference substance that is injected onto the same column under the same flow rate conditions. An example of this approach is given in Figure 3, in which the relative retention of *R*-warfarin versus L-tryptophan was found by taking the ratio of their apparent retention factors and calculating the selectivity factor, where $\alpha = (k_{R\text{-Warfarin}})/(k_{\text{L-Tryptophan}})$. As shown in Figure 3, the selectivity factor for these two solutes showed no apparent changes in going from a column length of 2 cm to 5 mm, but did show a slight increase in going from a length of 5 mm to 2 mm and a larger increase in going to a length of 1 mm. These results indicated that the use of a retention factor ratio for an analyte versus a reference compound injected onto the same column could be used, at least in part, to help correct for column overloading effects that occur as smaller affinity columns are used for binding studies. This data also indicates that a retention factor ratio could be used with small affinity columns to quickly determine the relative affinity of an unknown compound by comparing it to a reference solute with a known affinity for the ligand within the column.

The results in Figures 1–3 all demonstrate that there will be a loss in precision for the measurement of either absolute retention factors or relative retention factors as the size of an HSA microcolumn is decreased. As an example, the precision of the average measured retention factors for *R*-warfarin changed from ± 1% to ± 5% when comparing the 2 cm and 1 mm long columns. The precision for the measured retention factors for L-tryptophan decreased from ± 3% to ± 12% when going from the 2 cm to 1 mm long columns. Similar trends have been noted in work with carbamazepine and *R*-warfarin on silica monolith columns containing immobilized HSA [27]. The precision in the retention factor ratio (i.e., selectivity factor) for

R-warfarin versus L-tryptophan changed from 3% to 15% in going from the 2 cm to 1 mm long columns. The loss of precision in these cases is related to the shorter times that are allowed for data collection with the smaller columns; however, the decrease in analysis time is still an advantage as long as the measured retention or selectivity still has sufficient precision for the desired application. In the case of HSA, high precision work would require the use of 2 cm or longer columns, while high-throughput screening of binding properties could still make use of the data that are obtained with shorter HSA columns.

3.2. Band-broadening and peak asymmetry for analytes on HSA microcolumns

Band-broadening and peak asymmetry are two other parameters that can be used in zonal elution studies with HPAC to obtain information on biological interactions. Such measurements can be used to examine the rate at which a solute interacts with an immobilized ligand or as part of scheme for determining both rate constants and equilibrium constants for a biomolecular interaction [23,33]. This work has included the use of HSA columns in such measurements [33]. As a result, the effect of using smaller lengths in HSA columns on band-broadening and peak asymmetry measurements was also considered in this report. For instance, Eqn. (2) shows how plate height measurements can be related to the dissociation rate constant k_d for an analyte from an immobilized ligand [33],

$$H_k = \frac{2uk}{k_d(1+k)^2} \tag{2}$$

where H_k is the plate height contribution due to stationary phase mass transfer, u is the linear velocity of the mobile phase in the column, and k is the retention factor for the injected solute.

Figure 4 shows some plate height plots that were obtained for injections of *R*-warfarin for the 2 cm, 5 mm, and 1 mm long columns. Similar results were noted for L-tryptophan and for the other columns used in this work. For linear velocities that ranged from 0.027 to 0.16 cm/s, the plate height varied from 0.027 to 0.23 cm, 0.020 to 0.20 cm, and 0.031 to 0.13 cm for the 2 cm, 5 mm, and 1 mm long HSA columns, respectively, when using *R*-warfarin as the injected analyte. These values compared well with a previously-reported plate height of 0.02 cm that was measured for 3 mm × 4.6 mm i.d. silica monolith columns containing immobilized HSA [27]. As expected, the silica particle-based columns used in this current study shifted to higher plate height values as linear velocity increased. For the same range of linear velocity values as listed earlier, the plate height values measured when using L-tryptophan were 0.018 to 0.81 cm, 0.008 to 0.39 cm, and 0.005 to 0.031 cm. The corresponding number of theoretical plates on the 2 cm, 5 mm, and 1 mm long columns ranged from 9 to 73, 3 to 25, and 1 to 3 for *R*-warfarin and 25 to 113, 12 to 62, and 1 to 21 for L-tryptophan over the given range of linear velocities.

Figure 5 shows the effect of flow rate on the measured peak asymmetry at 10% of the maximum for *R*-warfarin on the 2 cm and 1 mm long columns. Similar results were noted for L-tryptophan and for the other columns used in this work. It was found that the asymmetry was relatively consistent across all tested flow rates for the 2 cm long column, with a variation of only 12% from 0.25–5 mL/min. Similar peak asymmetry values were obtained even on the 1 mm long column at moderate flow rates (i.e., 0.25–1.0 mL/min); however, this value did increase by roughly two-fold as the flow rate was increased to 5 mL/min. The other HSA microcolumns that were examined followed the same trend in asymmetry values; similar results were also seen when using L-tryptophan as the injected analyte. These results demonstrate that affinity microcolumns can be used at even moderate flow rates without creating any appreciable compromise in peak shape for HSA microcolumns.

3.3 Frontal analysis studies of solute binding on HSA microcolumns

Frontal analysis is a second tool that is commonly used with HSA columns for binding studies. One advantage of frontal analysis is that it can use, and actually requires, analyte concentrations that are sufficient to eventually saturate the column. Frontal analysis is also useful in that it can directly provide information on both the equilibrium constants and binding capacities for biomolecular interactions [15,23,33].

This section of this report used frontal analysis to examine the binding of racemic warfarin to HSA columns of varying lengths and using flow rates that ranged from 0.2–2 mL/min. Figure 6 shows typical frontal analysis curves that were obtained using 2 cm, 5 mm, and 1 mm long HSA columns. Figures 6(a) and 6(b) show the results that were obtained at a single flow rate (i.e., 0.5 mL/min), at which the breakthrough times noted for warfarin varied from 4.2–9.5 min and 30–45 s for the 2 cm and 1 mm long columns. Compared to the 2 cm column, the breakthrough times obtained for the 1 mm column were 8 to 13-fold lower for each respective applied concentration at the given flow rate. The difference in times can be attributed to the difference in protein content across all column lengths. The 2 cm long column contained 24 (± 2) nmol HSA compared to 1.2 (± 0.1) nmol HSA for the 1 mm long column, which is roughly 20-fold less protein.

There was also a noted difference in sample volume between column lengths. For example, 0.53 – 0.63 mL of warfarin was needed to form a breakthrough curve for the 5 mm long HSA column. In comparison, the 2 cm long HSA column needed 2.8 – 2.9 mL of warfarin. This four- to five-fold decrease in sample volume shows that a lower amount of volume was required to saturate the column with decreasing column length, which led to a decrease in the amount of sample needed to obtain the similar results in frontal analysis studies. The average breakthrough volumes for 5 μM warfarin were 2.8 (± 0.1), 1.30 (± 0.03), 0.69 (± 0.06), 0.43 (± 0.04), and 0.29 (± 0.03) mL for the 2 cm, 1 cm, 5 mm, 2 mm, and 1 mm long HSA columns at various flow rates.

Figure 6(c) illustrates the effect of changing only the flow rate during frontal analysis studies on an HSA microcolumn. In this case, a 5 μM solution of warfarin was applied to the 1 mm long HSA microcolumn at flow rates ranging from 0.2–2 mL/min. Similar results were obtained on the other columns used in this report. As would be expected, an increase in flow rate caused a decrease in the breakthrough time obtained for each curve, as demonstrated in Figure 6(c) by the shift from right-to-left as the flow rate was increased. In this situation, an increase in flow rate from 0.2 to 2 mL/min have a decrease in breakthrough time from 159 s to 19 s (i.e., an eight-fold decrease in overall breakthrough time under the conditions used in this particular study).

Figure 7 shows double-reciprocal plots that were obtained when the data for warfarin on the 2 cm and 1 mm long HSA microcolumns at 0.5 mL/min were analyzed according to Eqn. (3), which is based on a 1:1 binding model [23].

$$\frac{1}{m_{Lapp}} = \frac{1}{(K_a m_L [A])} + \frac{1}{m_L} \tag{3}$$

In Eqn. (3), m_{Lapp} is the apparent moles of analyte required to saturate the column, [A] is the concentration of applied analyte, and m_L is the moles of binding sites in the column. Eqn. (3) predicts that a plot of $1/m_{Lapp}$ versus 1/[A] with linear response will give a slope equal to $1/(K_a m_L)$ and an intercept of $1/m_L$ for a system with one-site binding. This type of relationship makes it possible to calculate the association equilibrium constant for this interaction and the total moles of binding sites in the column. Both Eqn. (3) and its corresponding binding model

have been used successfully in past work in examining binding of warfarin to HSA on longer HPAC columns than those used in this current report [8,23,33]. Similar agreement between Eqn. (3) and frontal analysis results that were obtained with HSA microcolumns was noted under the conditions utilized in this current study.

Using the slopes and intercepts measured for plots like those in Figure 7, the binding capacity (m_L) and association equilibrium constant (K_a) were determined for *R*-warfarin on each HSA microcolumn at various flow rates. The results are summarized in Table 1. As expected, the binding capacity decreased in proportion to column size. For example, the measured binding capacity for the 1 mm long HSA column was around twenty-fold less than it was for the 2 cm long HSA column. However, the precision of binding capacity measurements decreased as column length decreased. For the 2 cm long HSA column, the precision ranged from ± 4.3% to ± 4.7% at the flow rates that were examined, with an average precision of ± 4.4%. The 1 mm long column gave a precision that ranged from ± 15% to ± 25% at the same flow rates, with an average of ± 19%. This decrease in precision is again thought to be due to the decrease in amount of time and stationary phase that is available for drug binding when using the affinity microcolumns.

It was noted in Table 1 that there was relatively good agreement in the average K_a values that were obtained for each HSA microcolumn. All of these results also agreed with literature values of 2.1×10^5 to 2.6×10^5 M^{-1} for *R*- and *S*-warfarin, or an average of approximately 2.4×10^5 M^{-1} for racemic warfarin [6,8,11]. The K_a values obtained from all columns at flow rates of 0.2–2 mL/min were between 2.0 and 3.4×10^5 M^{-1}, with the precision ranging from ± 5% to ± 42%. The average precision of K_a values obtained at individual flow rates for the 2 cm long column was ± 5%, while the 1 mm column had ± 25% precision. From these results, it was concluded that reliable estimates of m_L and K_a by frontal analysis can be obtained at flow rates as high as 2 mL/min and with columns as short as 1 mm.

4. Conclusions

This work studied the effect of column length on zonal elution and frontal analysis studies to examine drug-protein interactions. Zonal elution studies of *R*-warfarin and L-tryptophan showed that the retention factor of each analyte shifted to smaller values as column length decreased, but stayed fairly consistent across various flow rates. The ratio of the retention factors, or selectivity factor, for *R*-warfarin versus L-tryptophan also appeared to be fairly consistent for most HSA microcolumns versus a reference 2 cm long HSA column, although a larger increase was seen for 1 mm long columns. Band-broadening studies showed that as column length increased, the plate height values obtained at various flow rates were similar for the various columns that were studied. In addition, the peak asymmetries obtained with short and long microcolumns were consistent at low to moderate flow rates, but an increase in peak asymmetry was noted at high flow rates. For each of these measured parameters, the precision decreased as shorter column lengths were used.

This work also considered the effect of column length on frontal analysis studies of racemic warfarin using HPAC. It was found that all column lengths (1 mm to 2 cm) could be used to obtain reliable estimates of association equilibrium constants. The best estimate for the association equilibrium constant of warfarin at Sudlow site I of HSA was 2.6 (± 0.6) × 10^5 M^{-1} at pH 7.4 and 37 °C, which compares well with previous results obtained with longer HSA columns (e.g., see Ref. [6]). From these studies, there is now a clear indication that using microaffinity columns in place of traditional longer columns does not compromise the accuracy of frontal analysis measurements for faster analysis times. These results show that the faster analysis times and slight decrease in precision obtained with HSA microcolumns make these

attractive tools for use in rapid studies of drug-protein binding or high-throughput screening of drug candidates with proteins such HSA.

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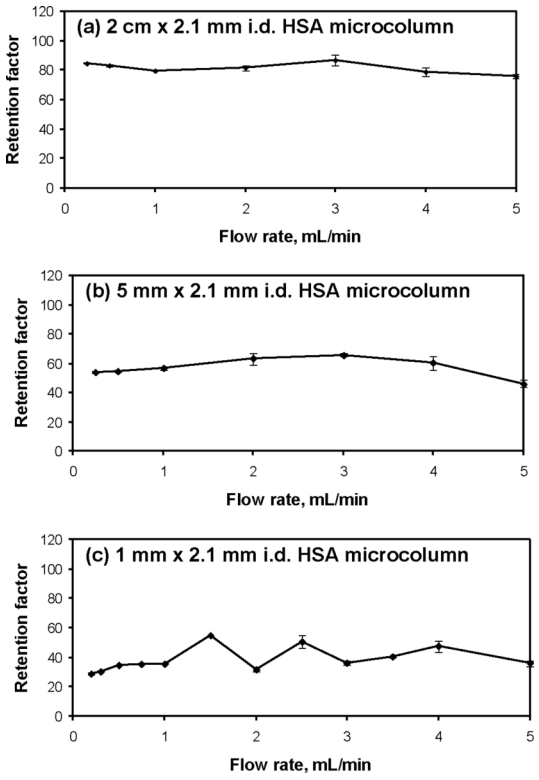

Figure 1.

Retention factors measured for *R*-warfarin at various flow rates for 5 μL injections of 20 μM *R*-warfarin onto HSA microcolumns with lengths of (a) 2 cm, (b) 5 mm, or (c) 1 mm and an inner diameter of 2.1 mm. Other experimental conditions are given in the text. The error bars represent a range of ± 1 S.D. of the mean.

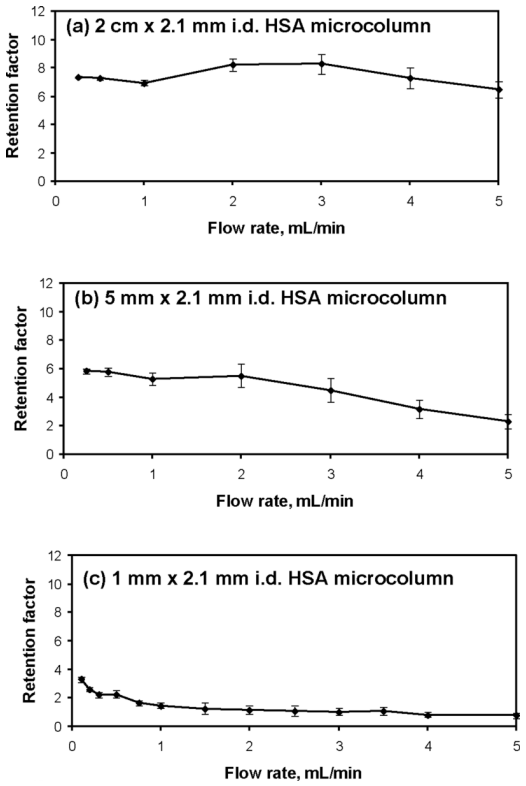

Figure 2.

Retention factors measured for L-tryptophan at various flow rates for 5 μL injections of 20 μM L-tryptophan onto HSA microcolumns with lengths of (a) 2 cm, (b) 5 mm, or (c) 1 mm and an inner diameter of 2.1 mm. Other experimental conditions are given in the text. The error bars represent a range of ± 1 S.D. of the mean.

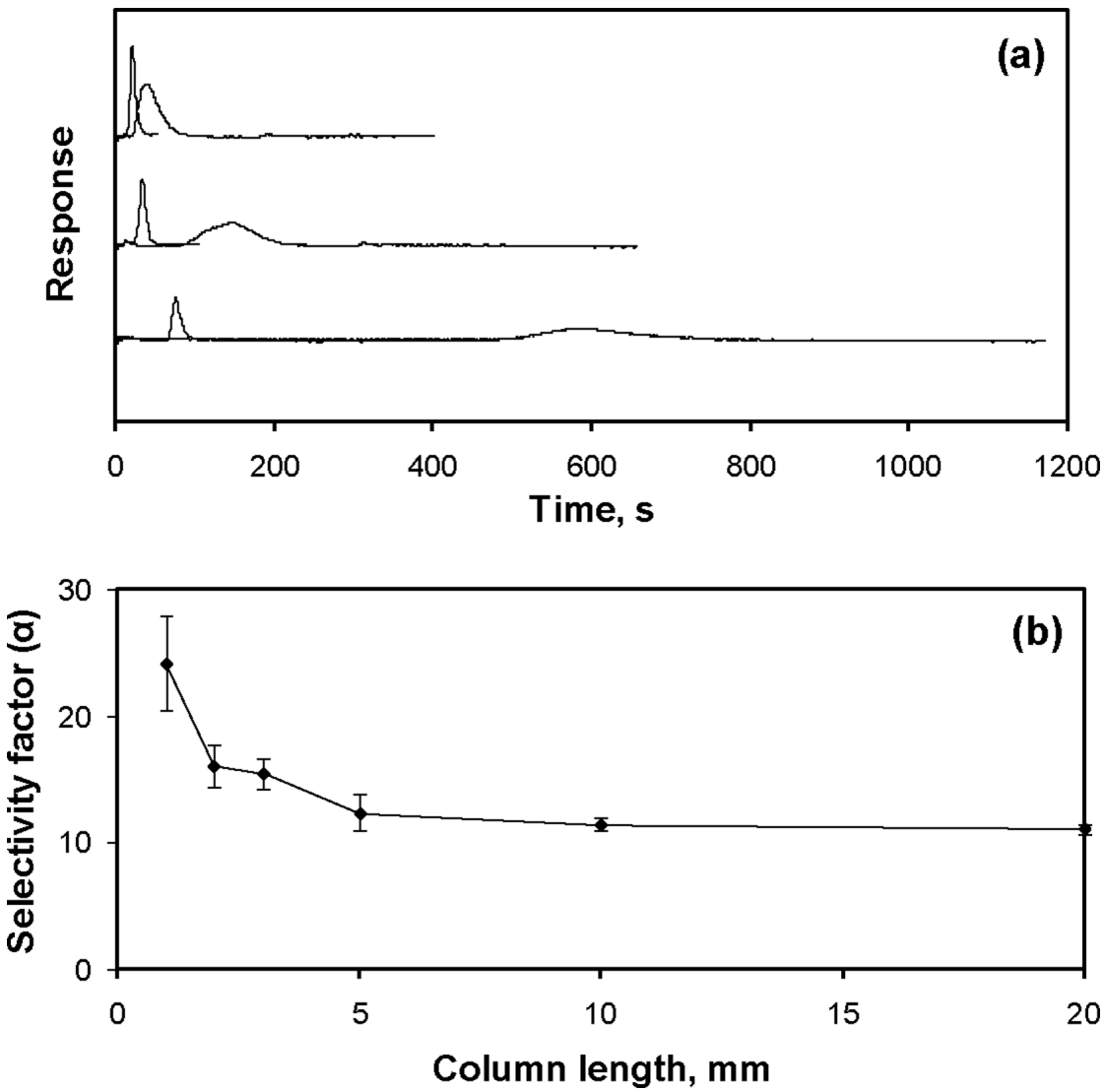

Figure 3.

(a) Typical chromatograms obtained for *R*-warfarin and L-tryptophan and (b) the resulting selectivity factors determined *R*-warfarin versus L-tryptophan for HSA microcolumns. The results in (a) were generated using HSA microcolumns with lengths of 20, 5 or 1 mm and an inner diameter of 2.1 mm. Other experimental conditions are given in the text. The error bars represent a range of ± 1 S.D. of the mean.

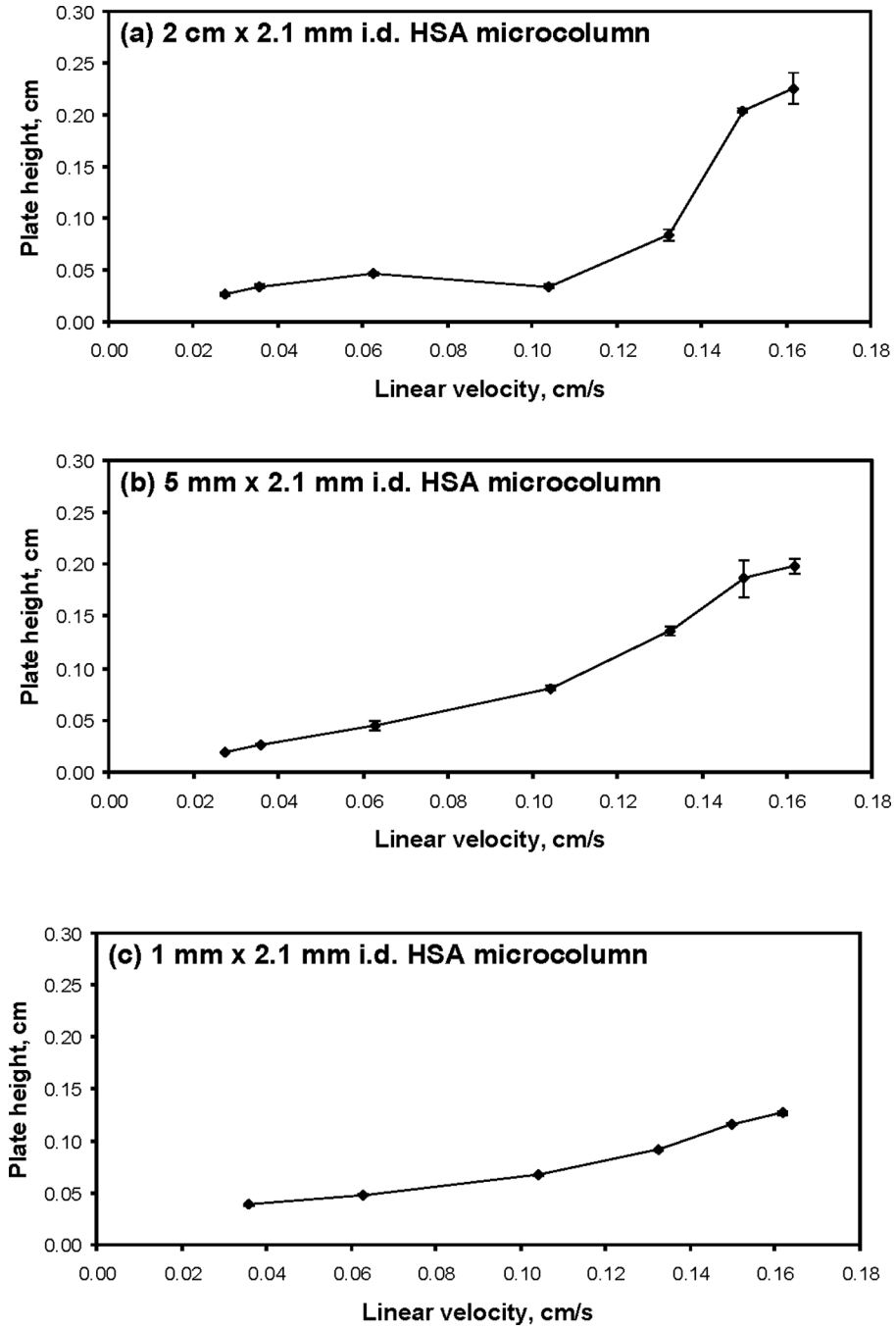

Figure 4.

Plate height plots obtained for 5 μL injections of 20 μM *R*-warfarin onto HSA microcolumns with lengths of (a) 2 cm, (b) 5 mm, or (c) 1 mm and an inner diameter of 2.1 mm. Other experimental conditions are given in the text. The error bars represent a range of ± 1 S.D. of the mean.

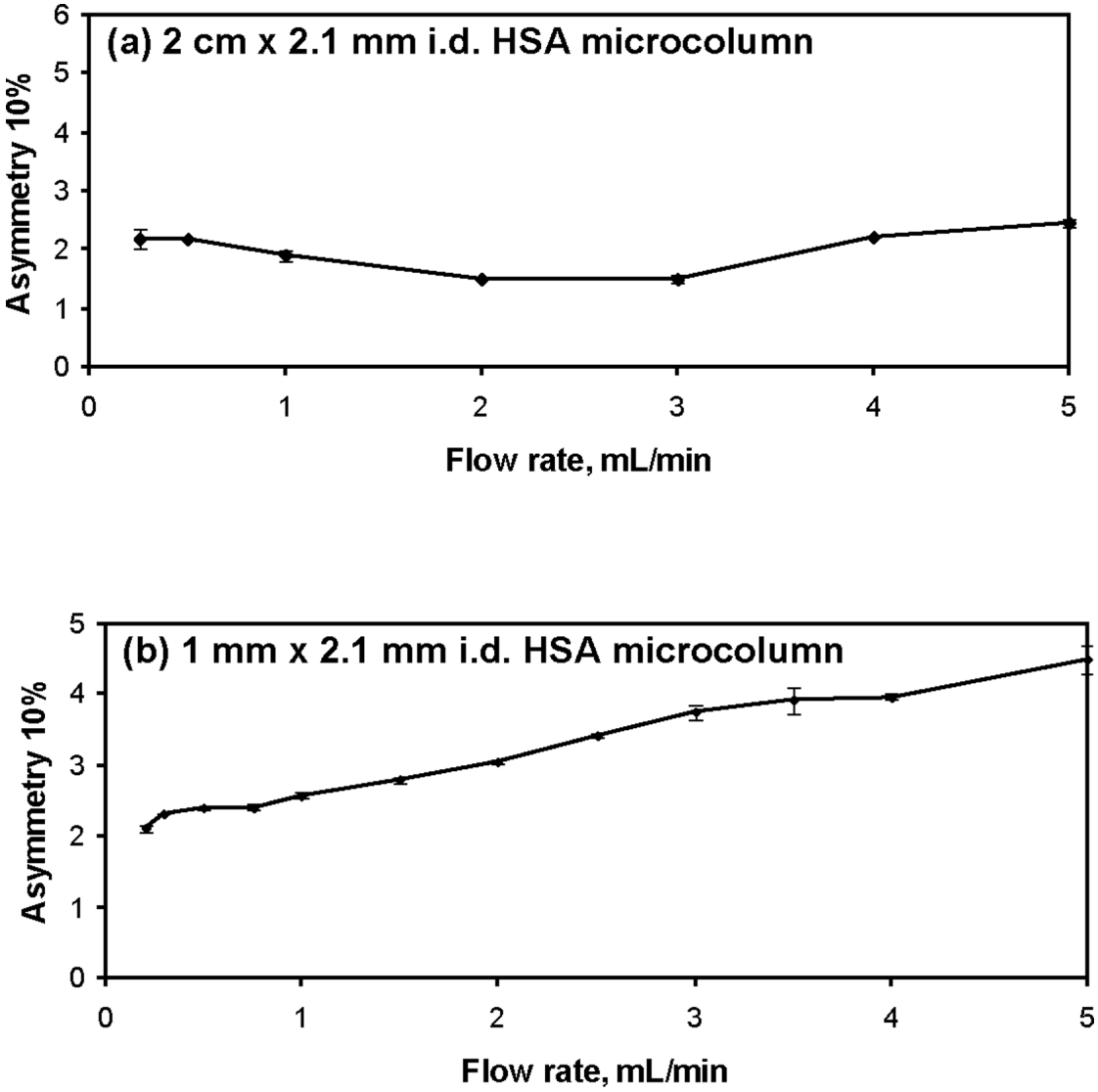

Figure 5.

Measured peak asymmetry at tenth height width for 5 μL injections of 20 μM *R*-warfarin onto HSA microcolumns with lengths of (a) 2 cm or (b) 1 mm and an inner diameter of 2.1 mm. Other experimental conditions are given in the text. The error bars represent a range of ± 1 S.D. of the mean.

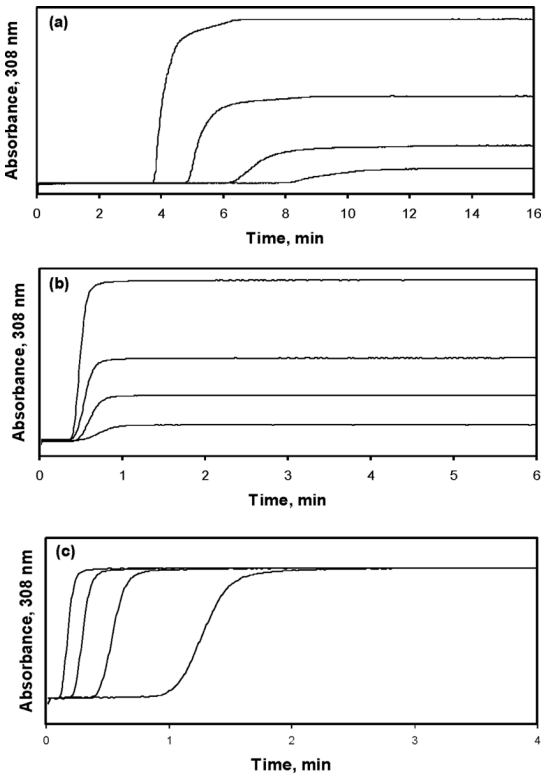

Figure 6.

Typical breakthrough curves obtained for racemic warfarin on HSA microcolumns. The results in (a) and (b) were obtained at 0.5 mL/min on 2 cm and 1 mm long HSA microcolumns with inner diameters of 2.1 mm and using warfarin concentrations (from left-to-right and top-to-bottom) of 10, 5, 2.5, and 1 μM. The results in (c) were obtained for a 5 μM sample of warfarin applied to a 1 mm × 2.1 mm i.d. HSA microcolumn at flow rates of 2.0, 1.0, 0.5, or 0.2 mL/min. All of these experiments were conducted at pH 7.4 and 37 °C. Other experimental conditions are given in the text.

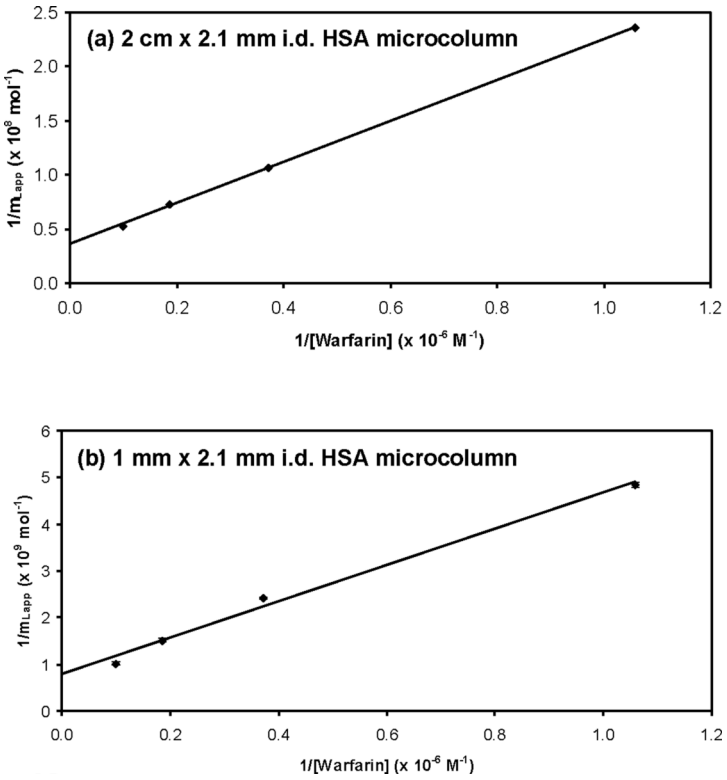

Figure 7.

Double-reciprocal frontal analysis plots prepared according to Eqn. (3) for the application of warfarin at 0.5 mL/min to (a) a 2 cm or (b) a 1 mm long HSA microcolumn and an inner diameter of 2.1 mm for samples with warfarin concentrations that ranged from 1 to 10 μM. The equation for the best-fit lines were (a) $y = 189\ (\pm 3)\ x + 3.6\ (\pm 0.2) \times 10^7$ ($r = 0.9998$) and (b) $y = 3895\ (\pm 230)\ x + 8.8\ (\pm 1.1) \times 10^8$ ($r = 0.9965$). The error bars represent a range of ± 1 S.D. of the mean.

Table 1

Effect of column length and flow rate on frontal analysis results

Column length (mm)	Flow rate (mL/min)	m_L(nmol)	K_a(x 10^5 M^{-1})	Relative Activity (vs. 20 mm column)[a]
20	0.5	27.5 (± 1.3)	1.9 (± 0.1)	
	1	27.8 (± 1.2)	1.9 (± 0.1)	
	2	27.6 (± 1.2)	2.0 (± 0.1)	
	Average [b]	**27.6 (± 0.2)**	**2.0 (± 0.1)**	**1.0 (± 0.01)**
10	0.5	10.0 (± 0.5)	2.8 (± 0.2)	
	1	9.5 (± 0.8)	3.1 (± 0.3)	
	2	9.7 (± 1.0)	3.0 (± 0.4)	
	Average [b]	**9.7 (± 0.2)**	**3.0 (± 0.1)**	**0.68 (± 0.02)**
5	0.5	3.9 (± 1.0)	3.7 (± 1.1)	
	1	4.3 (± 0.8)	3.0 (± 0.6)	
	2	4.2 (± 0.9)	3.5 (± 0.9)	
	Average [b]	**4.1 (± 0.3)**	**3.4 (± 0.3)**	**0.58 (± 0.05)**
3	0.2	3.6 (± 0.4)	2.5 (± 0.3)	
	0.5	3.7 (± 0.4)	2.4 (± 0.3)	
	1	3.8 (± 0.3)	2.3 (± 0.2)	
	2	4.2 (± 0.7)	2.2 (± 0.4)	
	Average [b]	**3.8 (± 0.3)**	**2.3 (± 0.1)**	**0.92 (± 0.08)**
2	0.2	2.3 (± 0.4)	2.3 (± 0.4)	
	0.5	2.5 (± 0.4)	2.1 (± 0.3)	
	1	2.5 (± 0.4)	2.2 (± 0.3)	
	2	2.9 (± 0.4)	1.9 (± 0.3)	
	Average [b]	**2.6 (± 0.3)**	**2.1 (± 0.2)**	**0.92 (± 0.08)**
1	0.2	1.2 (± 0.3)	2.3 (± 0.6)	
	0.5	1.3 (± 0.2)	2.1 (± 0.4)	
	1	1.2 (± 0.2)	2.2 (± 0.3)	
	2	1.0 (± 0.2)	3.7 (± 0.8)	
	Average [b]	**1.2 (± 0.1)**	**2.6 (± 0.8)**	**0.83 (± 0.08)**

[a] These values were determined by comparing the specific activity measured for each column to the specific activity measured for the 2 cm long column.

[b] These values were determined by using the average for the various flow rates that were examined.