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Short Communication

Evaluation of Microcolumn Stability in Ultrafast Affinity Extraction for Binding and Rate Studies

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Abstract

Ultrafast affinity extraction (UAE) has recently been developed and employed for measuring non-bound (or free) fractions and binding or rate constants for drugs and other targets with soluble binding agents such as serum proteins. This study examined the long-term stability of 10 mm × 2.1 mm i.d. affinity microcolumns when used in UAE at both low and high flow rates (e.g., 0.5 and 3.5 mL/min) over an extended series of injections. This stability was investigated by using immobilized human serum albumin (HSA) and samples containing the drug warfarin with or without soluble HSA as a model system. The free warfarin fractions measured at 0.5 mL/min in the presence of soluble HSA were stable up to 150 injections and changed by < 10% at 3.5 mL/min. The association equilibrium constant for warfarin with HSA that was estimated by UAE at 3.5 mL/min had no significant change over 50 injections and a change of up to only ~18-22% over 100-150 injections. The dissociation rate constant for warfarin from HSA was found by combining UAE results at 0.5 and 3.5 mL/min and employing a new two-point approach, with no significant changes in this value being seen even after 200 injections. The effects of extended microcolumn use on the retention time, peak width, and peak asymmetry for warfarin, and on the backpressure of the microcolumn, were also considered. These results indicated that UAE and HSA microcolumns could be used to provide consistent values for free solute fractions, binding constants, and rate constants over a large series of injections. These results should be useful in future work by providing guidelines for the assessment, further development, and use of UAE in characterizing interactions involving other drugs and binding agents in solution-based samples.

Keywords: Ultrafast affinity extraction; Affinity microcolumn; Drug-protein binding; Free drug fraction; Human serum albumin; Warfarin

1. Introduction

Ultrafast affinity extraction (UAE) is a form of high-performance affinity chromatography that has recently been used to measure the non-bound (free) fractions of drugs and hormones in aqueous solutions and biological matrices [1–10]. This method has also been used to determine equilibrium and rate constants for the interactions of drugs and hormones with soluble binding proteins [1,3,9,10]. In UAE, a mixture in solution containing the solute of interest and a binding agent is injected onto an affinity microcolumn (i.e., a column with a volume in the mid-to-low microliter range); this microcolumn is designed to contain an immobilized agent that can quickly and selectively capture the solute in its free form [1-10]. The result is a separation of the free vs bound forms of the solute in the sample, as is illustrated in Figure 1 [4–10].

UAE has been employed in prior work with a variety of proteins and solutes. For example, this method has been used to investigate the binding of human serum albumin (HSA) with drugs that are anticoagulants, antihypertensives, antiarrhythmics, antidiabetics, and antipsychotics [7,8,10]. UAE has been utilized in the same manner to examine the interactions of α_1-acid glycoprotein (AGP) with various drugs, which have included antihypertensives, antiarrhythmics, anticoagulants, anticonvulsants, β-blockers, antipsychotics, antidepressants, kinase inhibitors, and anesthetics [4-6]. This approach has also been used to examine binding by HSA, or related proteins, and sex-hormone binding globulin with the hormone testosterone [1,3].

There are two sets of conditions under which UAE can be performed. If UAE is carried out at a relatively high flow rate and at a short column residence time for the non-retained sample components, it is possible to retain the free form of a solute without having significant release of the same target from its soluble binding agent [1–10]. These conditions make it possible to measure the true free fraction of the solute that was present at equilibrium with the binding agent in the original sample [1–10]. The same data can be used to determine the equilibrium constant(s)

for the interaction of the target solute with the soluble binding agent [1–10]. As an alternative, the use of a lower flow rate and longer column residence time can allow dissociation of the solute from the binding agent as this mixture passes through the microcolumn. These latter conditions will increase the amount of originally-bound solute that is captured and provide data on the dissociation rate of this solute from the soluble binding agent [1,3,7,9,10]. Some advantages of using UAE for these applications include its ability to work with systems that have low-to-strong affinities, its capability of measuring a free solute fraction in minutes, and its need for only a small volume of sample for each study (i.e., low μL range) [1–10].

One item that has not been examined in detail in prior work is the long-term stability of affinity microcolumns during UAE [1,3,9,10]. Such information is needed to assess how changes in this stability and microcolumn behavior may affect the measurement of solute free fractions and equilibrium or rate constants by this approach. This stability will be evaluated in this work by using HSA as both the immobilized binding agent and the soluble protein in injected samples. HSA is a major transport protein that binds to a variety of drugs, hormones, and fatty acids [11–17], leading to the creation of both protein-bound and free forms of these solutes. HSA has been used in UAE as a capture agent in microcolumns for many drugs and some hormones [1,2,7–10]. The model solute that will be used in this work is warfarin, a common anticoagulating agent with well-characterized binding to HSA (e.g., as examined in prior chromatographic studies and some reports using UAE) [9,10,18]. The stability of affinity microcolumns that are used to examine this model system will be assessed over an extended series of injections and at representative low and high flow rates that have been used in prior UAE studies [1,3,9,10]. The results should provide valuable information in extending affinity microcolumns and UAE to the measurement of free fractions, equilibrium constants, and rate constants for other solutes or binding agents.

2. Experimental

2.1. Reagents

The HSA (Cohn fraction V, essentially fatty acid-free, ≥ 96% pure), racemic warfarin (≥ 98%), and sodium nitrate (≥ 99%) were purchased from Sigma (St. Louis, MO, USA). Nucleosil Si-300 (7 μm particle diameter, 300 Å pore size) was obtained from Macherey Nagel (Dűren, Germany). All buffers and aqueous solutions were prepared using water from a Milli-Q system (Barnstead, Dubuque, IA, USA) and filtered using Osmonics 0.22 μM nylon filters obtained from Fisher Scientific (Pittsburgh, PA, USA). The reagents employed for the micro bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA).

2.2. Apparatus

The microcolumns were packed using a Prep 24 pump from ChromTech (Apple Valley, MN, USA). The chromatographic system consisted of a PU-2080 Plus pump, an AS-2057 autosampler, and a UV-2075 absorbance detector from Jasco (Easton, MD, USA), along with a six-port LabPro valve (Rheodyne, Cotati, CA, USA). A Jasco X-LC 3167CO column oven was used to maintain a temperature of 37.0 (± 0.1) °C during all experiments. ChromNAV v1.18.04 software and LCNet from Jasco were used to control the system. Chromatograms were analyzed and fitted using the progressive, linear, and exponentially-modified Gaussian (EMG) functions of PeakFit v4.12 software (Jandel Scientific, San Rafael, CA, USA). Data analysis was carried out using Excel for Office 365 (Microsoft, Redmond, WA, USA).

2.3. Methods

The stationary phase, which consisted of HSA immobilized to Nucleosil Si-300 diol-bonded silica, was prepared by the Schiff base method [9,19]. A control support was made in the

same way but without adding HSA during the immobilization step [9,19]. The protein content of the HSA support was determined by using a micro BCA assay that employed HSA as the standard and the control support as the blank. This assay gave an HSA content of 81.0 (± 4.0) mg HSA/g silica; the reproducibility of preparing such a support, as evaluated using the protein content of this support and two prior batches, was estimated to be ± 9% (1 R.S.D.). The HSA support was placed into several 10 mm long and 2.1 mm inner diameter (i.d.) stainless-steel microcolumns; three HSA microcolumns were prepared in this manner and used at a given flow rate or set of flow rates. The microcolumns were downward slurry-packed at 4000 psi (28 MPa) utilizing pH 7.4, 0.067 M potassium phosphate buffer as the packing solution; the average variation in packing the three microcolumns, as evaluated by comparing their initial retention for warfarin (i.e., measured according to methods given in the next paragraph) was ± 10% (range, ± 2.5-17%). The microcolumns and supports were stored at 4.0 °C when they were not being used.

A pH 7.4, 0.067 M potassium phosphate buffer was employed for the preparation of solutions and acted as the mobile phase for applying and eluting sample components during the chromatographic studies. The mobile phase was degassed for 25-30 min before use. Free fraction analysis by UAE was performed by injecting 20 µL samples containing 10 µM warfarin without HSA or a mixture of 10 µM warfarin plus 20 µM HSA. These injections were made in replicate (n = 5) onto HSA microcolumns at flow rates ranging from 0.5 mL/min to 4.0 mL/min. The samples were preheated to 37.0 (± 0.1) °C for 30-60 min in the autosampler; the microcolumn was equilibrated with the mobile phase at the same temperature using a column oven. The detection wavelength for warfarin was 308 nm. The column void time was determined at each flow rate by making 20 µL injections of 10 µM sodium nitrate, which was monitored at 205 nm. The behavior of a microcolumn over a large series of injections was typically monitored at 50 injection intervals,

with results being combined for a set of five sequential injections. The free fraction was calculated by dividing the baseline-corrected retained peak area of warfarin for the warfarin-HSA mixture by the peak area of this drug in samples containing the same total amount of warfarin but no HSA [2,7–10]. Other parameters that were calculated or measured during this process (e.g., equilibrium constants, rate constants, and retention times or retention factors) are described in Section 3.

3. Results and discussion

3.1. General effect of flow rate on free fraction measurements by UAE

Figure 2 shows the general effect on the measured free fraction for warfarin in UAE as the flow rate used for sample injection was changed over the course of a moderate number of injections (i.e., a total of 70 injections over the set of flow rates and replicates used in this example). This effect was examined by using samples that contained mixtures of warfarin and soluble HSA vs solutions that contained warfarin alone, which was used as a reference. The term F_t (as given on the *y*-axis of Figure 2) was used to represent the apparent free fraction for the captured solute (i.e., warfarin) as different amounts of time were allowed in the microcolumn for dissociation of warfarin from its binding agent in the sample. The residence time for non-retained components in the microcolumn (t) was determined from the microcolumn void volume and the injection flow rate [7,9,10].

As is illustrated in Figure 2, the relative size of the free fraction that was measured by UAE depends, to some extent, on the flow rate at which the sample was injected. In this example, the injection of a warfarin-HSA mixture (vs a warfarin standard) at a moderate-to-low flow rate (< 3.0 mL/min) gave an apparent free fraction for warfarin that increased with a decrease in flow rate. This occurred because the relatively long sample residence times present at these flow rates (i.e., >550 ms up to 3.3 s) allowed partial dissociation of warfarin from the complex of this drug with

HSA. This effect led to an increase in the observed value for F_t, as noted in previous studies [7,9,10]. However, the measured free fraction for warfarin approached a constant value once this dissociation was minimized. In this example, this condition occurred at a flow rate of 3.0 mL/min or higher (i.e., when the residence time decreased below ~550 ms).

The results obtained at short residence times in UAE are known to provide conditions that allow minimal dissociation of a solute from its complex with a binding agent in a sample (e.g., warfarin-HSA); such conditions, in turn, can allow the measured free fraction of the solute at equilibrium (F_0) to be used to estimate the affinity for the solute with the binding agent [1,2,7–10]. For instance, eq. (1) shows how the association equilibrium constant (K_a) can be obtained from the value of F_0 for a solute and binding agent that have a 1:1 interaction,

$$K_a = \frac{1-F_0}{F_0([P]-[D]+[D]F_0)} \quad (1)$$

where [D] and [P] are the total concentrations of the solute and binding agent (e.g., a drug and protein) in the injected sample [9,10].

For the data shown in Figure 2, an estimate of K_a for warfarin with soluble HSA was obtained by using free fraction measurements that were made at a flow rate of 3.0 mL/min or higher. It was found that free fractions for warfarin that were measured over this range (i.e., from 3.0 to 4.0 mL/min in Figure 2) gave values that were equivalent at the 95% confidence level (Student's *t*-test). The average value obtained for F_0 over this upper range of flow rates, when expressed as a percent, was 25.2 (± 3.2)%. The value of K_a that was determined from this result and by using eq. (1) was 2.4 (± 0.5) × 10^5 M^{-1} at pH 7.4 and 37 ºC. This equilibrium constant was within the range of previous values obtained by various techniques at the same temperature and pH (1.6 × 10^5 – 2.6 × 10^5 M^{-1}) [9,10,20,21].

The data acquired at a lower flow rate or over a range of flow rates in UAE can be used to examine the rate of dissociation of a solute from its binding agent. This can be done by using eq. (2), where k_d is the dissociation rate constant of the solute from the binding agent, and other terms are the same as defined earlier in this report [7,9,10].

$$\ln \frac{1}{(1-F_t)} = k_d\, t - \ln(1 - F_o) \qquad (2)$$

A plot that was prepared according to eq. (2) and for the system examined in this report is given in the inset of Figure 2. A linear response was obtained with a correlation coefficient of 0.998 (*n* = 7) and a slope that provided a k_d value of 0.59 (± 0.02) s^{-1} [7,9,10]. This dissociation rate constant was within the overall range of previously-reported k_d values for HSA and warfarin at pH 7.4 and 37 ºC (i.e., 0.35 – 0.8 s^{-1}) [9,10,22,23].

3.2. Change in solute peak area and measured free fraction over long-term microcolumn use

The next set of studies examined the stability of HSA microcolumns when used over many cycles of sample injections in UAE for either equilibrium or kinetic measurements. Flow rates of 3.5 mL/min and 0.5 mL/min were selected as representing typical sample injection conditions and residence times that have been used in UAE for estimating association equilibrium constants and dissociation rate constants, respectively. For instance, the column residence time of 475 msec at 3.5 mL/min that was present for the microcolumns used in this report was in the range of 100-950 ms that has been used in UAE with other drug/protein systems for estimating K_a or F_0 [7,9,10]. The column residence time of 3.3 s at 0.5 mL/min for the microcolumns employed here was in the general range of times that has been used in prior work for examining the dissociation kinetics of drugs and solutes with HSA or other serum proteins (e.g., 1.7-6.7 s) [7,9,10].

Figure 3 shows how the amount of warfarin captured by HSA microcolumns changed as the number of sample injections and corresponding volume of applied mobile phase (i.e., pH 7.4, 0.067 M phosphate buffer) increased. These data were obtained for samples containing only warfarin or warfarin in the presence of soluble HSA, as shown in Figures 3(a) and 3(b), respectively. The results that were acquired at 0.5 mL/min indicated good stability for a microcolumn over at least 150 injections, or 2.1 L of applied mobile phase (i.e., >60,000 times the void volume of the HSA microcolumn). At this flow rate, there was no significant change at the 95% confidence level, based on a Student's *t*-test, in the area of captured warfarin for most injected samples containing warfarin plus HSA; the single exception was an 8% decrease after 200 injections at 0.5 mL/min. There was also no significant change, at the 95% confidence level, in the peak area of the captured solute for samples containing only warfarin over up to 200 injections and 2.8 L of applied buffer (~81,000 microcolumn volumes).

A larger change in area for the captured warfarin, both in the presence and absence of soluble HSA, was seen when an HSA microcolumn was used at 3.5 mL/min. In this case, the peak area had a decrease of 34-35% after 50 injections and the application of 1.1 L (~32,000 microcolumn volumes) of the mobile phase. The extent of this change increased slightly over the next 100 injections, giving a decrease of 40-45% from the original value, and over a total of 200 injections gave a net decrease of 59-69% from the original value. These changes were all significant at the 95% confidence level.

Figure 3(c) shows the change in the free fraction of warfarin that was measured at the two selected flow rates and as a function of the applied volume of mobile phase. The free fraction obtained at 0.5 mL/min did not show any significant change over 150 injections, with a decrease of only ~8%, over 200 injections and 2.8 L of applied buffer. At 3.5 mL/min, the area of captured

warfarin in the absence or presence of soluble HSA resulted in a change of less than 10% in the measured free fraction after 150 injections and 3.3 L of applied buffer, as well as a modest decrease of ~24% after 200 injections and 4.4 L of applied mobile phase. These results indicated that UAE could provide reasonably stable free fraction measurements even at high flow rates. This occurred because long-term changes in the microcolumn tended to create similar relative changes in the amount of captured solute whether it was in the absence or presence of the soluble binding agent.

3.3. Change in estimates of equilibrium or rate constants over long-term microcolumn use

The data in Figure 3 were next used to examine how estimates of the association equilibrium constant, K_a, or dissociation rate constant, k_d, varied upon long-term use of a microcolumn in UAE. The results are shown in Figure 4. The estimates that were obtained for K_a in Figure 4(a) were generated by using eq. (1) and the data acquired at 3.5 mL/min. The relative standard deviations for the K_a values, as determined through error propagation (see derivation and equations provided in the Supplementary Material) and precisions given in Figure 3, were in the range of ± 4-19% (average precision, ± 10%). No significant change at the 95% confidence level was seen in the estimated value of K_a over at least the first 50 sample injections. After 100-150 injection cycles there was a ~18-22% increase in the value of K_a, which was significant at the 95% confidence level, and after 200 injections the apparent value of K_a rose by 69% vs the initial result obtained with a new microcolumn. In each case, a decrease in the measured free fraction for warfarin, as shown in Figure 3(c), resulted in an increase in the estimate that was acquired for K_a.

The change that might be acceptable in such measurements will depend on the precision that is present and degree of accuracy that is desired for the estimate of K_a. For instance, at the levels of precision that are present in Figure 4(a), an experiment that is designed to obtain a maximum deviation in the measured value for K_a of 10% or less would ideally require a

microcolumn that has been used for 50 injections or less at high flow rates (e.g., 3.0-4.0 mL/min in this current study). Similarly, an experiment with an allowed deviation 20% or less in an estimate for K_a could be obtained by using a microcolumn over up to 150 injection cycles under the same flow rate conditions.

The estimates that were obtained for the dissociation rate constant k_d in Figure 4 were found by revising eq. (2) for use in a two-point method. This new, simplified approach was then employed with the results provided at both 0.5 and 3.5 mL/min in Figure 3. This method used free fractions for warfarin that were measured at low and high flow rates within the linear range of the response for eq. (2) (e.g., as shown within the inset of Figure 2). An advantage of the two-point method was that, because it could use data acquired at only two representative flow rates, it allowed much shorter analysis times and higher sample throughput than the multi-point method that has previously been utilized with eq. (2) [7,9,10].

Eq. (3) shows the modified form of eq. (2) that was used in the two-point method,

$$k_d = \frac{\ln(1/[1-F_{t_1}]) - \ln(1/[1-F_{t_2}])}{t_1 - t_2} \quad (3)$$

where F_{t_1} is the apparent free fraction observed for the solute (i.e., warfarin) in the presence of a soluble binding agent (HSA) and at the lower flow rate examined in the linear range of eq. (2) (e.g., 0.5 mL/min in this study). The term F_{t_2} is the solute's free fraction that is observed at a higher flow rate in the same range (e.g., 3.5 mL/min). The terms t_1 and t_2 are the residence times of the non-retained components in the microcolumn at the same set of flow rates.

Figure 4(b) shows the values of k_d that were obtained by the two-point approach. The precisions of these values were determined through error propagation (see Supplementary Material for equation and details) and were in the range of ± 11-16% (average, ± 13%). It was found that there was only a small apparent change of ~3-7% in the estimated value of k_d over 50-150

injections, followed by a small apparent drop of ~9% after 200 injections. However, none of these small changes in k_d were statistically significant at the 95% confidence interval. The greater consistency in the estimates for k_d vs K_a was probably due to the use of data from multiple flow rates to obtain k_d, instead of a single flow rate for determining K_a, which more effectively compensated for changes in microcolumn behavior over time.

3.4. Evaluation of other potential measures of microcolumn stability in UAE

Several other potential ways of monitoring microcolumn stability were evaluated for use in UAE. One such measure was the retention time for the retained peak of the captured solute. Some representative chromatograms that were obtained in this study are provided in Figure 5(a). At low flow rates (e.g., 0.5 mL/min), there was sufficient resolution to get a partial separation of the two chiral forms of *R/S*-warfarin, while at higher flow rates these two forms gave a single broad peak. However, in both situations it was possible to use the average retention time (i.e., central moment) of the elution profile(s) for the two forms to monitor and look for any change in retention over extended periods of microcolumn use.

Figure 5(b) shows how the retention time for warfarin varied during the extended use of HSA microcolumns at 0.5 and 3.5 mL/min. The relative precisions for these retention measurements were in the range of ± 0.6-4% at both 0.5 mL/min (average, ± 1.2%) and 3.5 mL/min (average, ± 1.9%). A decrease in retention time was seen over extended use of HSA microcolumns at both these flow rates, but with a larger change occurring at 3.5 mL/min. For instance, a 21-42% decrease in retention time was seen for warfarin in the presence or absence of soluble HSA at 3.5 mL/min and over 150 injections (3.3 L applied buffer), while the same number of injections (and 2.1 L applied buffer) at 0.5 mL/min gave a 7-17% decrease in retention time.

Further experiments were conducted to determine the reasons for this loss in retention. For instance, it was known from previous simulations that the shift in retention seen in Figure 5(b) followed the trend expected for a gradual loss of active, immobilized binding agent within the microcolumn [24]. One possible source of this shift was loss of the immobilized binding agent from the support. However, the measured protein content for supports that had been removed from the microcolumns after extended use did not show any detectable changes in this content under the conditions employed in this study (data not shown). The immobilized binding agent may also have been lost over time due to slow dissolution of the support. The presence of this effect was confirmed by an increase in the void volume of 15% or more at 0.50 or 3.5 mL/min over the course of 200 injections on control microcolumns. Some ways this dissolution of the silica-based support could be reduced in the future is by including a guard/saturator column in the system or by employing an alternative buffer to potassium phosphate as the mobile phase [8,25,26].

The shift in retention time seen at 3.5 mL/min showed a good correlation ($|r|$ = 0.963-0.976, n = 5) with the decrease in warfarin peak area under the same conditions (see Supplementary Material). A connection between these parameters was expected because both the retention properties and capture efficiency of a microcolumn are related to the amount of active, immobilized binding agent [24,27]. The small changes seen for retention times and relative peak areas at 0.5 mL/min had no apparent correlation ($|r|$ = 0.338-0.601 for n = 5). The changes in retention time shown in Figure 5 occurred even under conditions where consistent free fractions and values for K_a or k_d were obtained (Section 3.2-3.3). Hence, monitoring the retention time, or peak area, of a solute could be an early indicator of changes in microcolumn behavior; however, it was also found that only a small or modest change in these factors did not prevent a microcolumn

from being employed over long periods of use for free fraction measurements and equilibrium or kinetic studies.

Other parameters that were considered for evaluating microcolumn stability included the column backpressure, the width of the retained peak, and asymmetry of this peak (see Supplementary Material). The microcolumns used in this study had stable backpressures in the range of 1.1-1.9 MPa (160-276 psi) at 0.5 mL/min and 8.8-10.5 MPa (1276-1523 psi) at 3.5 mL/min. The width of the retained peak was evaluated by using the number of theoretical plates (*N*), as obtained by using the peak width at half maximum [28]. The value of *N* showed a net, overall small decrease of 6-13% at 0.5 mL/min and 5-21% at 3.5 mL/min over the course of 200 injections, with most of these changes being significant at the 95% confidence interval. The shape of the retained peak was examined by using the asymmetry factor that was measured at 10% of the peak height (AS_{10}) [29]. In general, no significant changes in this value were seen (at the 95% confidence level) under any of the tested conditions up to 150 injections. Even after 200 injections a slight change (16%, significant at the 95% confidence level) was only seen at 0.5 mL/min for the sample containing warfarin plus soluble HSA. Thus, even though backpressure, *N*, and AS_{10} may play a role as general indicators of system performance, they were not as useful as the free fraction, K_a, k_d, and retention time for evaluating microcolumn stability in UAE.

4. Conclusion

This report examined the stability of affinity microcolumns during their use in UAE for measuring free solute fractions, equilibrium constants, and rate constants over an extended series of sample injections and at both low and high flow rates. This study used 10 mm × 2.1 mm i.d. affinity microcolumns containing immobilized HSA and samples containing the drug warfarin with or without soluble HSA present. Stable free fractions for warfarin were obtained over at least

150 injections at both low and high flow rates (i.e., 0.5 and 3.5 mL/min), and only a small-to-moderate decrease (~8-24%) was seen after 200 injections. The association equilibrium constant for warfarin with HSA, as estimated by using free fractions measured at 3.5 mL/min, was found to be consistent and have no significant changes over 50 injections and only a modest change (~18-22%) after 100-150 injections. A new and simplified two-point method, based on data collected at both 0.5 and 3.5 mL/min, was employed for determining the dissociation rate constant for warfarin from HSA; no significant change in estimates of this dissociation rate constant were seen even after 200 injections. These results represent the first time that the potential variations in these values have been measured and compared during the extended use of affinity microcolumns in UAE.

Several chromatographic parameters were examined during these studies as potential measures of microcolumn stability. The retention time for captured warfarin was found to decrease over a large series of injections made at both 0.5 and 3.5 mL/min, including conditions under which consistent values were obtained for the measured free fractions and binding or rate constants. This change in retention was mostly likely linked to a slow loss of the support, as suggested by prior simulation studies [24] and void time measurements of the microcolumns. The backpressure of the microcolumns and the width or symmetry of the retained peaks for the captured warfarin were also examined; however, these parameters either showed only small variations in their values or no significant changes over the course of the UAE studies.

The results obtained in this study should be useful in future work with UAE and affinity microcolumns by providing guidelines for the assessment, further development, and use of these tools for measuring free solute fractions and in examining solution-phase interactions. For example, these results indicate that affinity microcolumns can be used in UAE to provide

consistent values for equilibrium constants and rate constants even over a large series of sample injections. In addition, the trends seen in this work show what types of deviations may be expected in these values and provide ways to detect changes in microcolumn performance (e.g., by monitoring retention factors, void times, and peak areas), as can be employed to monitor and avoid these effects. Potential sources for changes in these microcolumn properties (e.g., loss of the binding agent and/or support) were also investigated, as can be used to aid in minimizing these effects. Future studies can apply these results in the extension of UAE to alternative systems, such as the binding of other drugs or hormones with additional serum proteins or soluble binding agents [30].

5. Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6. Acknowledgment

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Figure Legends

Figure 1. General scheme for measuring free solute or drug fractions by ultrafast affinity extraction. Terms: K_a, association equilibrium constant (or "binding constant"); k_d, dissociation rate constant.

Figure 2. Effect of injection flow rate on the apparent free fraction (F_t, solid line) and column residence (t, dotted line) for 20 µL injections of 10 µM warfarin plus 20 µM HSA (vs samples containing 10 µM warfarin alone) on a 10 mm × 2.1 mm i.d. HSA microcolumn at pH 7.4 and 37.0 °C for flow rates ranging from 0.5 to 4.0 mL/min. The residence time was calculated by using the column void volume and the flow rate. The inset shows the results that were obtained when the same data were plotted according to eq. (2). The error bars represent a range of ± 1 S.D. (n = 3-5) and in some cases were comparable to the size of the data symbols.

Figure 3. Effect of increasing the volume of applied mobile phase or number of sample injections on the relative peak areas measured for captured warfarin when using UAE and HSA microcolumns in (a) the absence and (b) presence of soluble HSA and at 0.5 mL/min (solid line) or 3.5 mL/min (dotted line). The plots in (c) show the relative free fractions that were obtained for warfarin in the presence of soluble HSA when combining the results in (a) and (b) at the two tested flow rates. The inset in (c) shows the relationship between the number of injections and the volume of applied mobile phase. All values on the y-axes were normalized to a value of 1.0 for the results that were initially acquired for the given samples on a new affinity microcolumn. These data were obtained for 20 µL injections of 10 µM warfarin or 10 µM warfarin plus 20 µM HSA made onto 10 mm × 2.1 mm i.d. HSA

microcolumns at pH 7.4 and 37.0 °C. The error bars represent a range of ± 1 S.D. for five sequential injections and in some cases were comparable to the size of the data symbols.

Figure 4. Relative change in the estimates made for the (a) association equilibrium constant, K_a and (b) the dissociation rate constant, k_d, for warfarin with soluble HSA with an increase in the number of injections that were made onto HSA microcolumns for HSA. These results are based on eqs. (1-2) and the data provided in Figure 3(c) at (a) 3.5 mL/min and (b) a combination of the results at 0.5 and 3.5 mL/min. All *y*-axis values are normalized to a value of 1.0 for the K_a or k_d values that initially acquired on a new HSA microcolumn. The error bars represent a range of ± 1 S.D., as obtained for five sequential injections; in some cases, these bars were comparable to the size of the data symbols. The horizontal dashed lines are provided for reference and show the levels at which the measured results deviate from the original values by ± 10% or ± 20%, as well as the point at which a result would be identical to the initial value.

Figure 5. (a) Chromatograms obtained for 20 µL samples of 10 µM warfarin (black lines) or a mixture of 10 µM warfarin and 20 µM soluble HSA (gray lines) injected onto 10 mm × 2.1 mm i.d. HSA microcolumns at pH 7.4, 37.0 °C, and (top) 0.5 mL/min or (bottom) 3.5 mL/min. (b) Change in the average retention time measured for *R/S*-warfarin under the conditions given in (a) and over various volumes of applied buffer for samples containing (top) only warfarin or (bottom) warfarin plus soluble HSA and at 0.5 mL/min (solid line) or 3.5 mL/min (dashed line). The error bars in

(b) represent a range of ± 1 S.D. for five sequential injections and in some cases were comparable to the size of the data symbols.

CRediT Author Statement

Sazia Iftekhar: Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, **David S. Hage:** Conceptualization, Formal Analysis, Writing – Review & Editing, Visualization, Supervision, Project Administration, Funding Acquisition

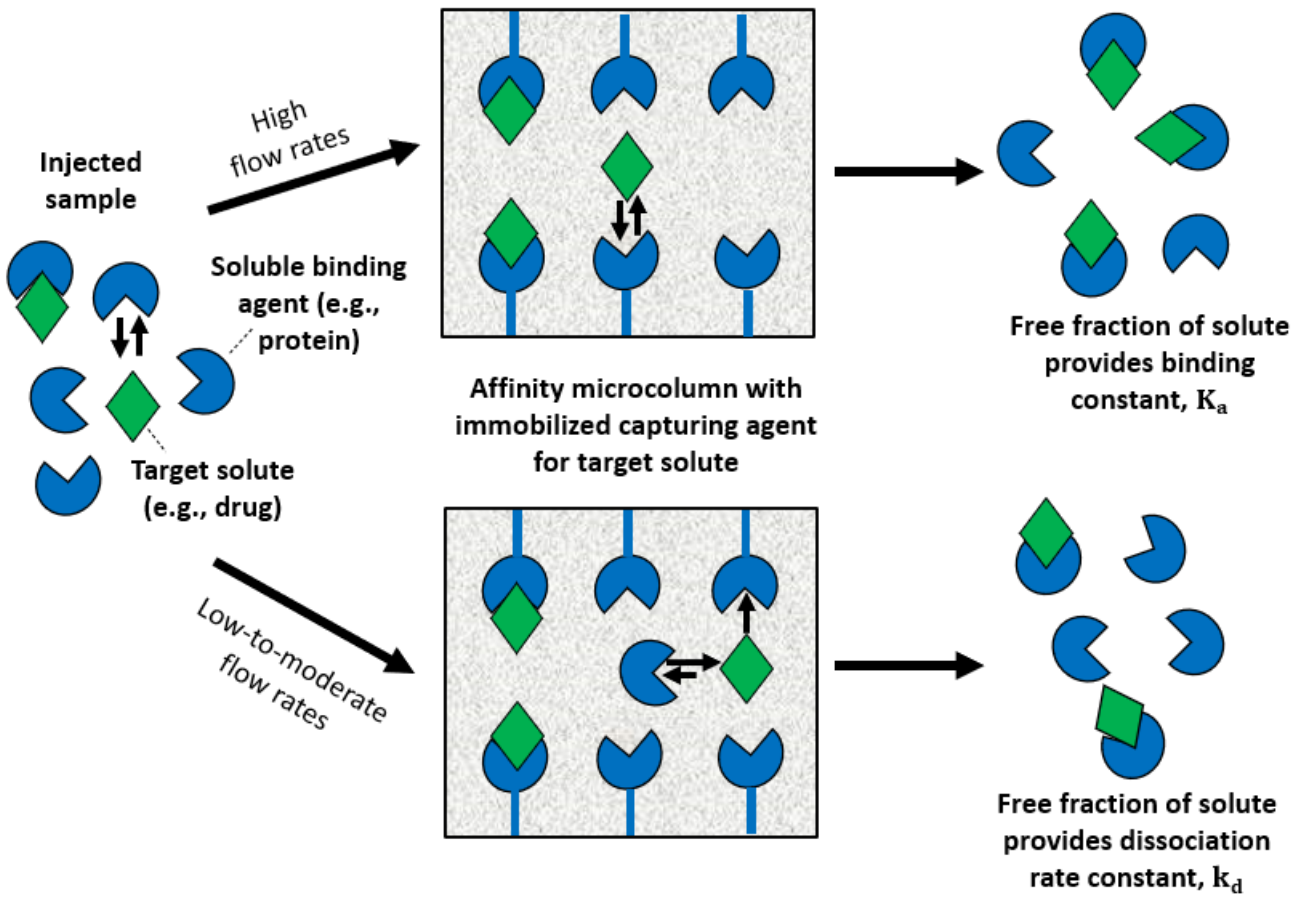

Injected sample
High flow rates
Soluble binding agent (e.g., protein)
Target solute (e.g., drug)
Affinity microcolumn with immobilized capturing agent for target solute
Free fraction of solute provides binding constant, K_a
Low-to-moderate flow rates
Free fraction of solute provides dissociation rate constant, k_d

Figure 1

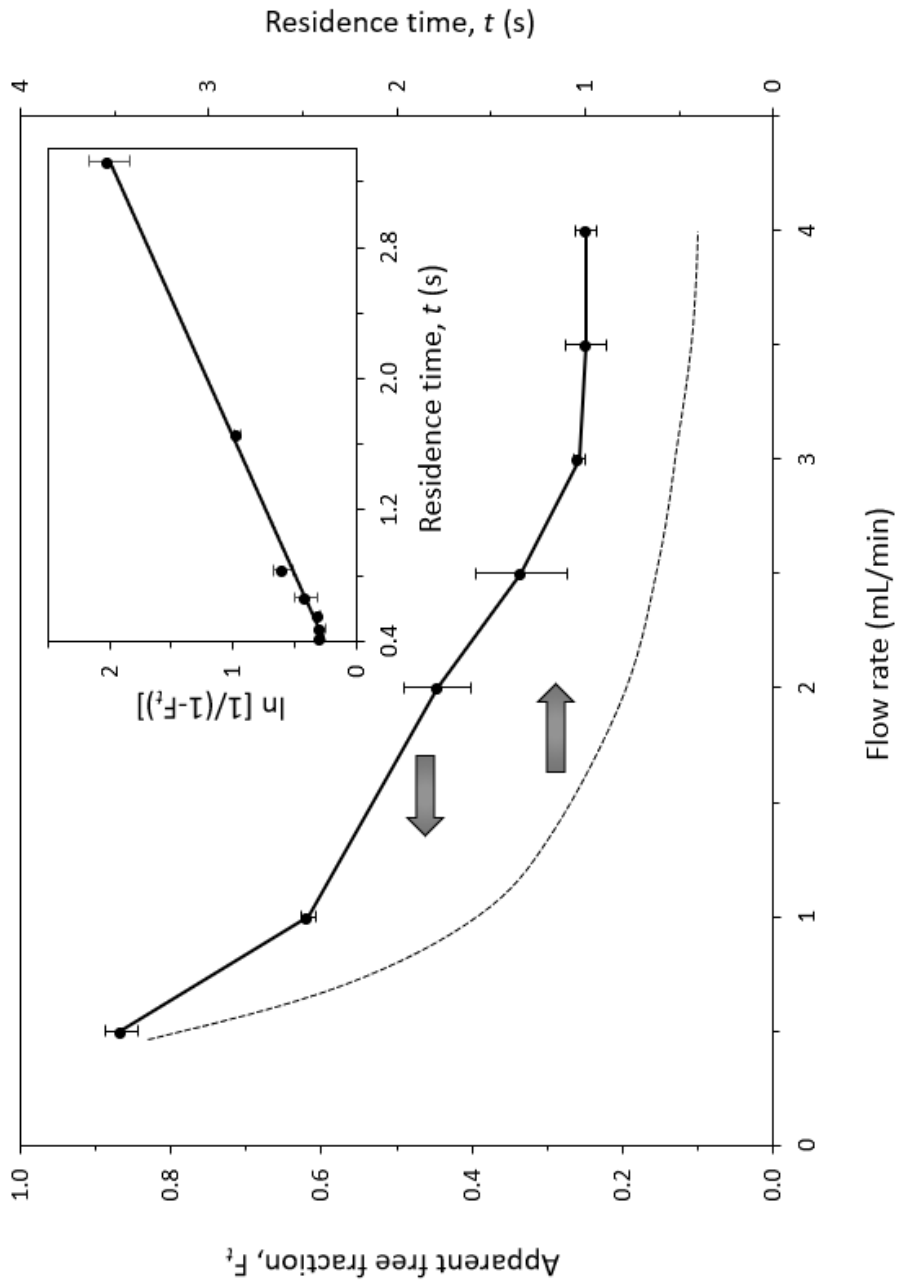
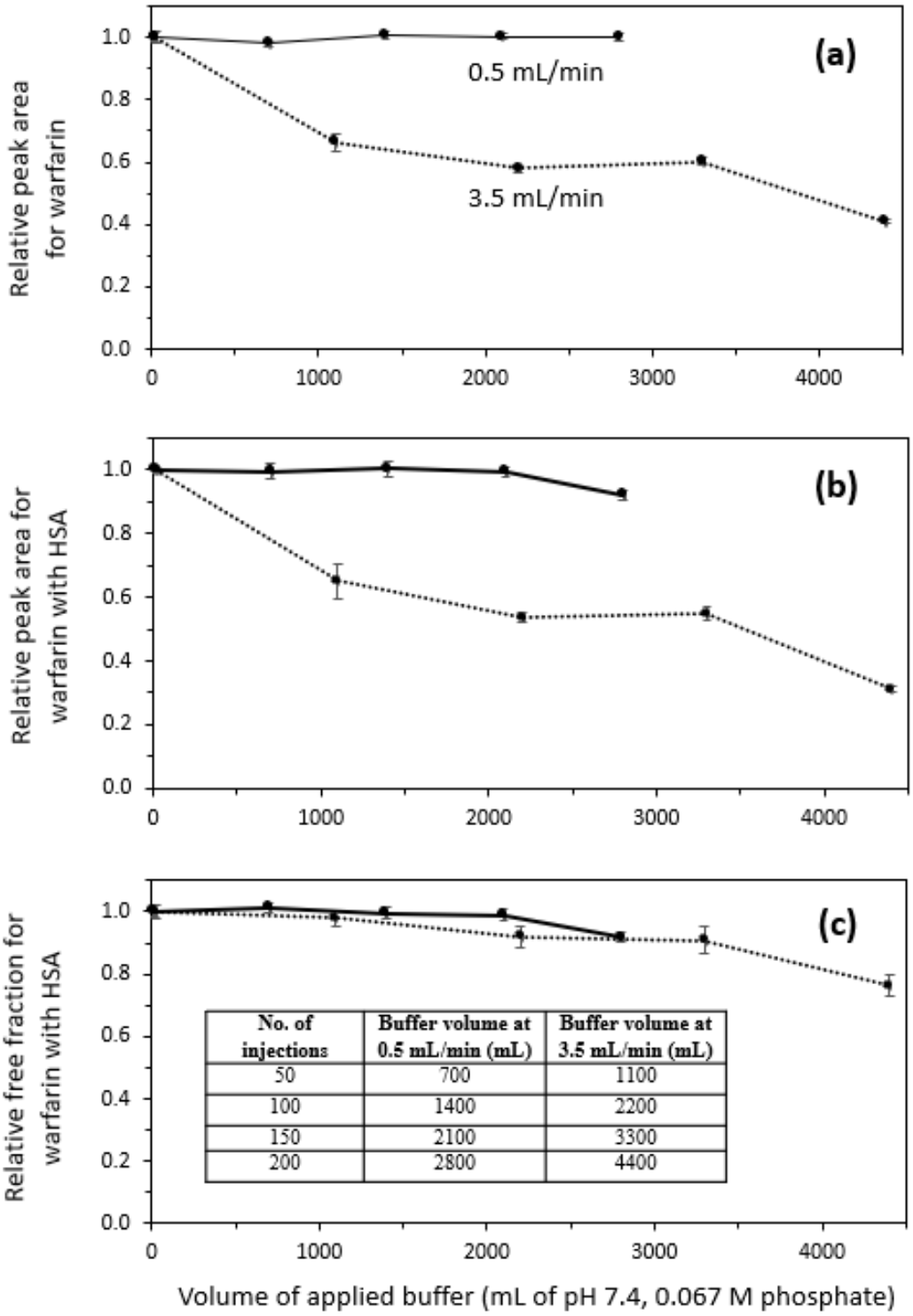

Residence time, t (s)
Apparent free fraction, F_t
Flow rate (mL/min)
ln [1/(1-F_t)]
Residence time, t (s)

Figure 2



No. of injections	Buffer volume at 0.5 mL/min (mL)	Buffer volume at 3.5 mL/min (mL)
50	700	1100
100	1400	2200
150	2100	3300
200	2800	4400

Figure 3

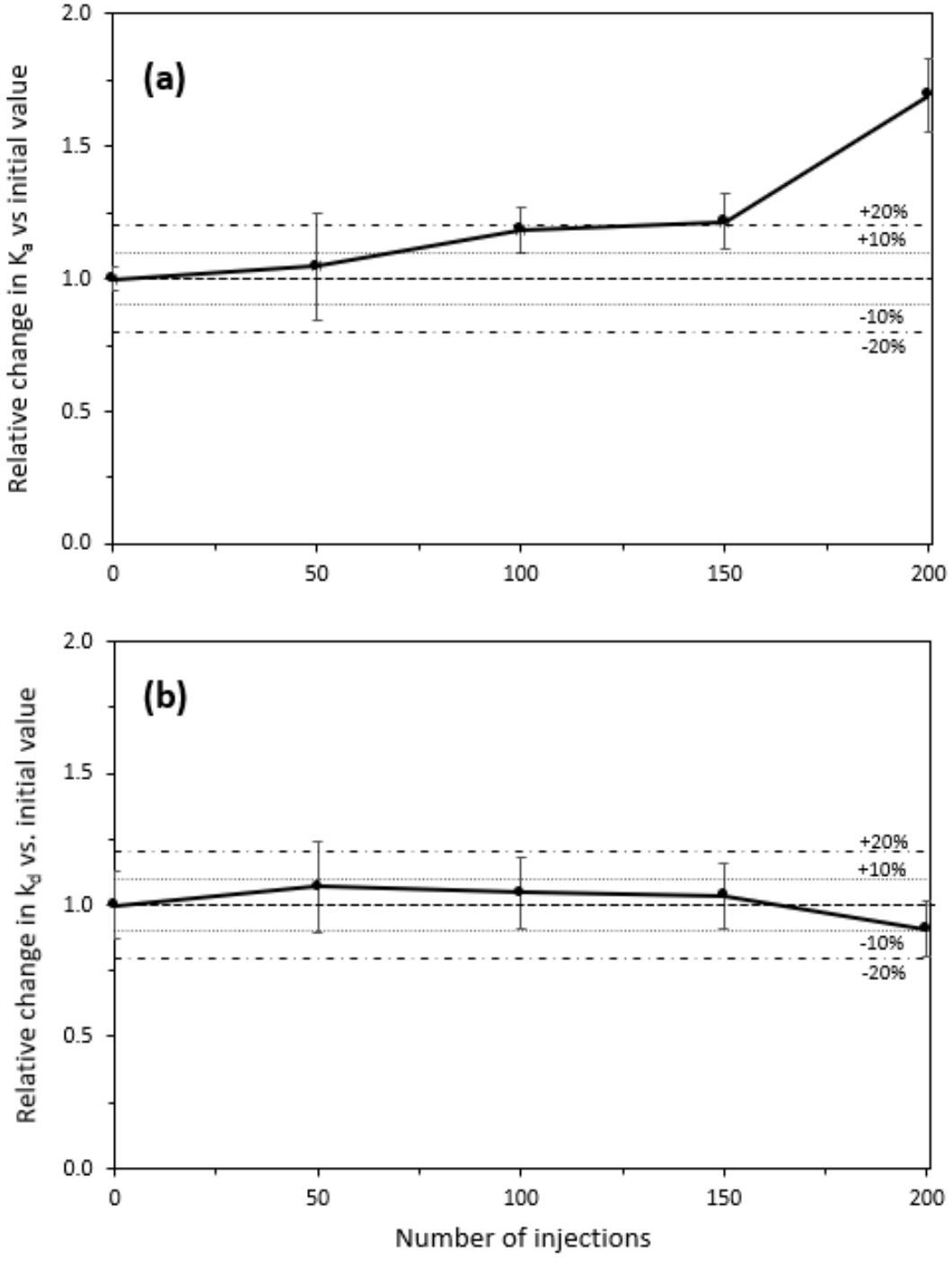

(a)
Relative change in K_a vs initial value
2.0
1.5
1.0
0.5
0.0
0
50
100
150
200
+20%
+10%
-10%
-20%
(b)
Relative change in k_d vs. initial value
Number of injections

Figure 4

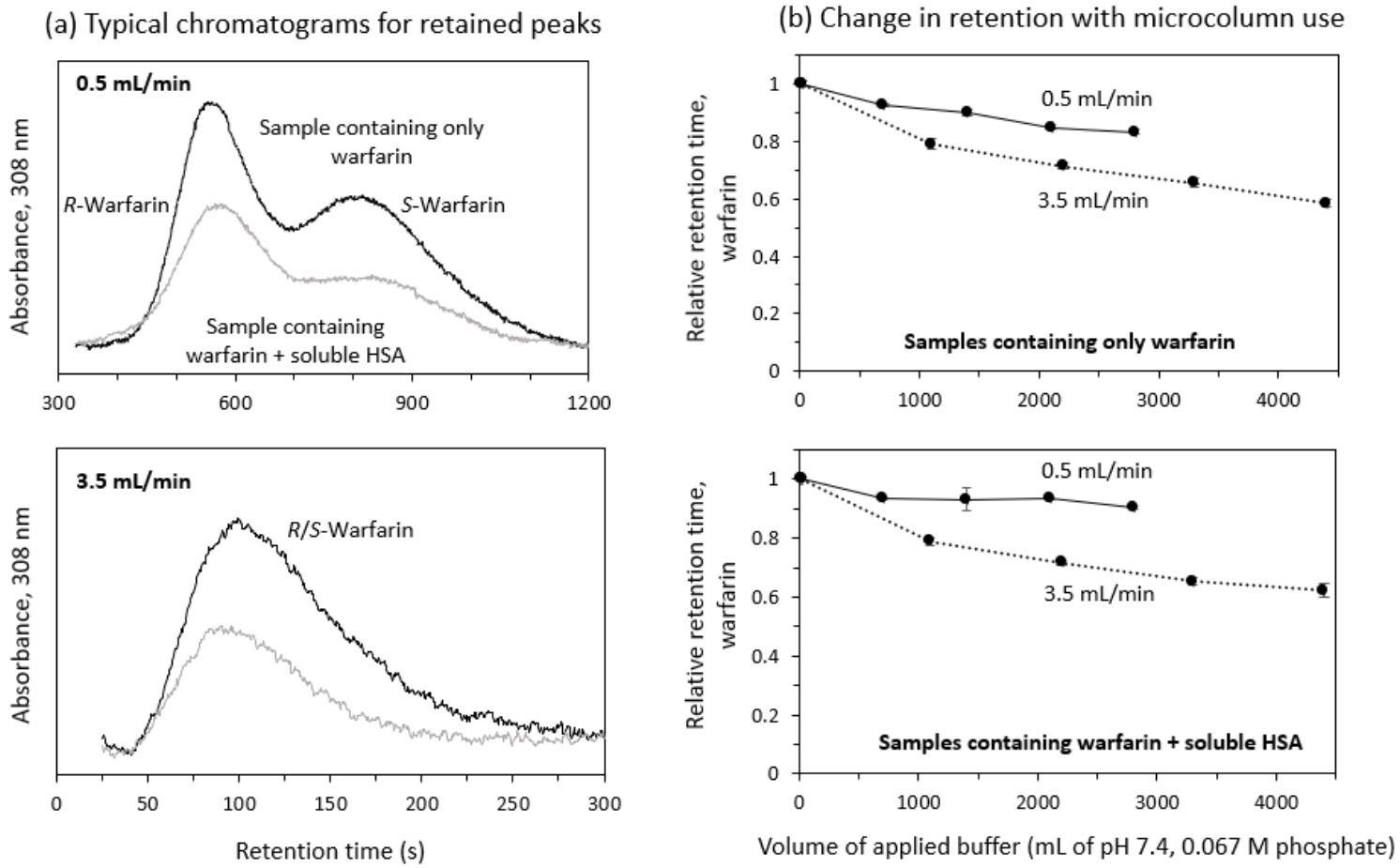

(a) Typical chromatograms for retained peaks
(b) Change in retention with microcolumn use
0.5 mL/min
Sample containing only warfarin
R-Warfarin
S-Warfarin
Sample containing warfarin + soluble HSA
Absorbance, 308 nm
300
600
900
1200
3.5 mL/min
R/S-Warfarin
Absorbance, 308 nm
0
50
100
150
200
250
300
Retention time (s)
Relative retention time, warfarin
0.5 mL/min
3.5 mL/min
Samples containing only warfarin
0
1000
2000
3000
4000
Samples containing warfarin + soluble HSA
Volume of applied buffer (mL of pH 7.4, 0.067 M phosphate)

Figure 5

Supplementary Material

Error propagation during estimation of an association equilibrium constant by UAE

Eq. (1) in the main body of this paper shows how the association equilibrium constant (K_a) can be obtained in ultrafast affinity extraction (UAE) from the value of original free fraction (F_0) for a solute and binding agent with a 1:1 interaction, where is [D] and [P] are the total concentrations of the solute and binding agent (or drug and protein, in this case) that were in the original sample [1,2]. The standard deviation of K_a (S_{K_a}) can be found through error propagation by first taking the derivative of K_a with respect to F_0 as shown in eq. (1S) and then substituting the derivative in the error propagation formula given in eq. (2S).

$$\frac{\delta_{K_a}}{\delta_{F_0}} = \frac{(-F_0)([P]-[D]+[D](F_0))-(1-F_0)([P]-[D]+[D](2F_0))}{(F_0([P]-[D]+[D]F_0))^2} \quad (1S)$$

$$S_{K_a} = \sqrt{\left(\frac{\delta_{K_a}}{\delta_{F_0}}\right)^2 \left(S_{F_0}\right)^2} \quad (2S)$$

In this equation, S_{F_0} is the standard deviation that is obtained experimentally for F_0.

Error propagation during estimation of a dissociation rate constant by UAE

As stated in the main body of this paper, data that are acquired at low-to-moderate flow rates in UAE can be used to examine the dissociation rate of a solute from its binding agent in a sample. One way this can be accomplished is by using eq. (2) in the main body of the text, where k_d is the dissociation rate constant for solute with the binding agent, F_t is the apparent free fraction of a target solute in presence of a soluble binding agent at residence time t for non-retained components in the microcolumn, and other terms are the same as defined previously [1–3]. In this relationship, the standard deviation of the term $\ln \frac{1}{(1-F_t)}$, as represented by $S_{\ln \frac{1}{(1-F_t)}}$, can be found through error propagation by using (3S).

$$S_{\ln\frac{1}{(1-\mathrm{F}_t)}} = \left(\frac{1}{(1-\mathrm{F}_t)}\right)\left(S_{\mathrm{F}_t}\right) \quad (3S)$$

In this equation, S_{F_t} is the standard deviation that is obtained experimentally for $\mathrm{F_t}$.

Error propagation in the two-point method for determining a dissociation rate constant

The dissociation rate constant (k_d) shown for warfarin with soluble human serum albumin (HSA) in Figure 4(b) were estimated by using UAE and a two-point method. This approach employed free fractions for warfarin that were measured at low and high flow rates and within the linear range in response obtained over a broader set of flow rates. The relationship that was used for this approach is given by eq. (3) in the main body of the text. In this equation, the term F_{t_1} represents the apparent free fraction observed for the solute in the presence of soluble binding agent at the first flow rate in the range examined, while F_{t_2} represents the free fraction observed at the second and higher flow rate. The terms t_1 and t_2 are the residence times of the non-retained sample components in the microcolumn at the same set of flow rates.

The precision of the estimate for k_d that is obtained by the two-point method can be represented by the standard deviation term ($S_{\mathrm{k_d}}$), as can be found through error propagation by using eq. (4S).

$$S_{\mathrm{k_d}} = \sqrt{\left(\left(1/[(t_1-t_2)(1-\mathrm{F}_{t_1})]\right)^2\left(S_{\mathrm{F}_{t_1}}\right)^2 + \left(1/[(t_1-t_2)(1-\mathrm{F}_{t_2})]\right)^2\left(S_{\mathrm{F}_{t_2}}\right)^2\right)} \quad (4S)$$

In this equation, $S_{\mathrm{F}_{t_1}}$ and $S_{\mathrm{F}_{t_2}}$ are the standard deviations that are obtained experimentally for F_{t_1} and F_{t_2}, with all other terms being the same as described for eq. (3).

Change in backpressure during using of a microcolumn in UAE

Figure S1 shows the backpressures that were measured for 10 mm × 2.1 mm i.d. HSA microcolumns during their extended use in UAE studies. The backpressure went from an initial value of 1.1 MPa to a final value of 1.9 MPa at 0.5 mL/min over 200 injections (i.e., 2.8 L of applied buffer). The backpressure at 3.5 mL/min went from 8.8 MPa to 10.5 MPa over 200 injections (4.4 L of applied buffer).

Effect on efficiency and peak asymmetry during use of a microcolumn in UAE

Figure S2 shows how the measured efficiency of 10 mm × 2.1 mm i.d. HSA microcolumns and the asymmetry of their retained peaks were affected during the extended use of such microcolumns in UAE. The efficiency is represented here by the number of theoretical plates (N) [4,5], as measured here by using the retention times and peak widths for warfarin that was captured from samples containing warfarin only or warfarin plus soluble HSA. The asymmetry of these peaks were measured by using the asymmetry factor that was measured at 10% of their total height (AS_{10}) [4]. The general trends that were observed are summarized in the main body of the paper.

Change in retention time vs retained peak area during extended use of UAE microcolumns

Figure S3 shows the relationship between the retention time that was measured for a captured solute (warfarin) and the retained peak area for this solute, as obtained during the extended use of 10 mm × 2.1 mm i.d. HSA microcolumns in UAE. The best-fit line for each combination of sample and flow rate is provided, as well as the absolute value of the correlation coefficient for the fit. These results are discussed and summarized in the main body of the paper.

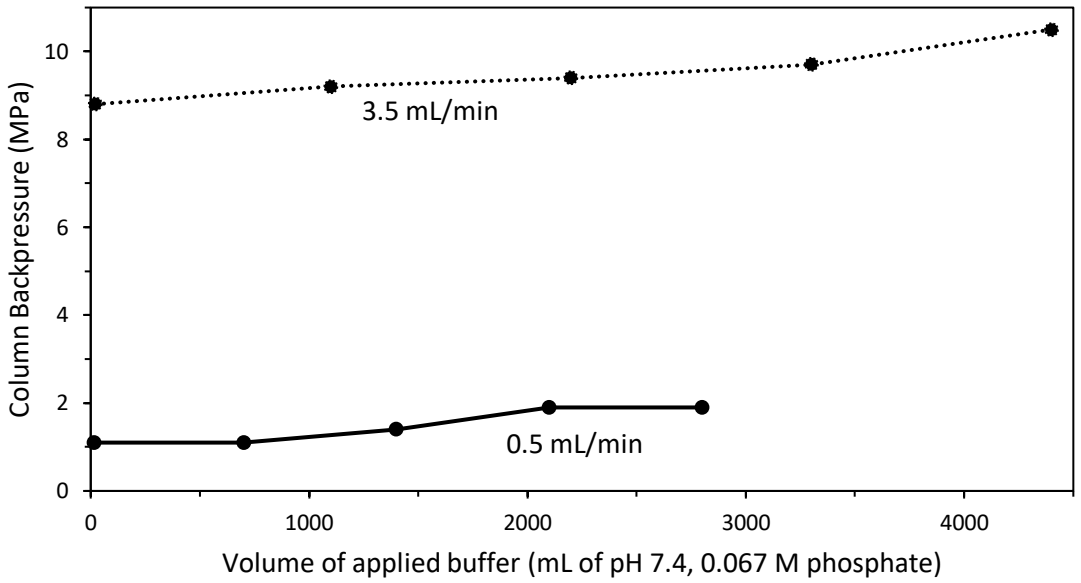


Figure S1. Change in backpressure of 10 mm × 2.1 mm i.d. HSA microcolumns with an increase in the volume of applied pH 7.4, 0.067 M phosphate buffer at 37.0 ºC and 0.5 mL/min (solid line) or 3.5 mL/min (dotted line). Other conditions are given in the main body of the text.

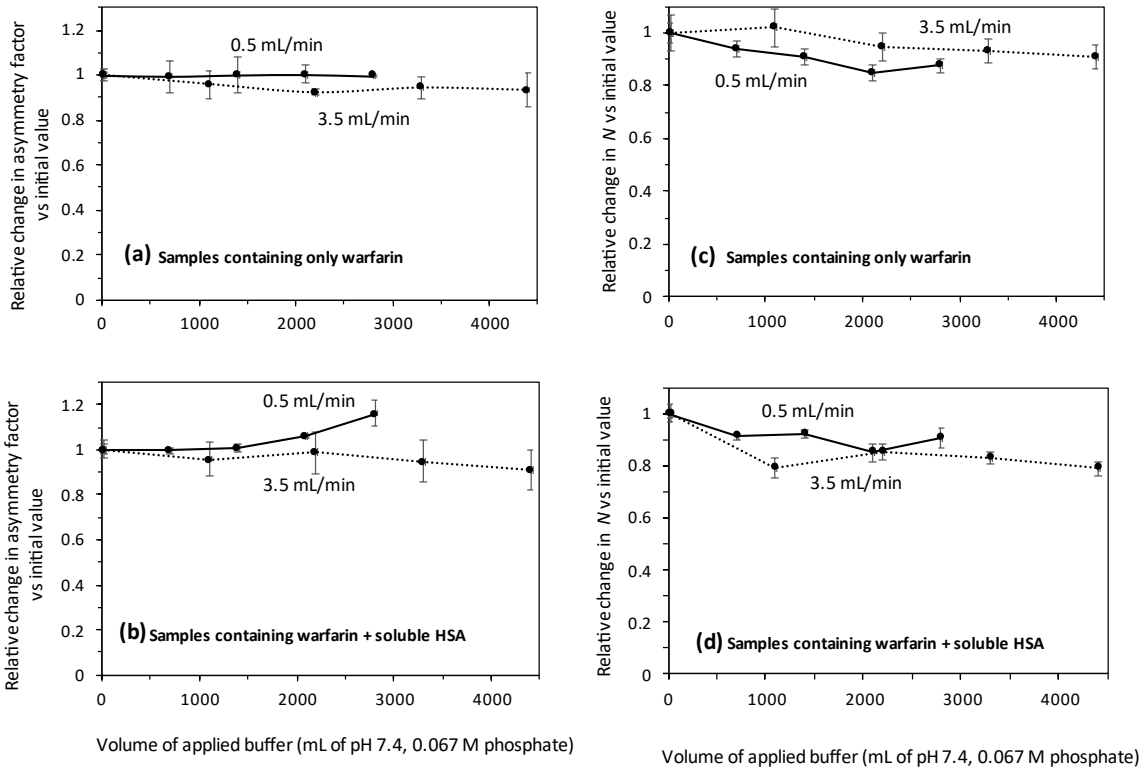


Figure S2. Effect of extended use of HSA microcolumns in UAE on peak asymmetry, as measured by the asymmetry factor at 10% peak height (AS_{10}) (a-b), and system efficiency, as measured by the number of theoretical plates (N) (c-d). These results were obtained fort the indicated flow rates and samples on 10 mm × 2.1 mm i.d. HSA microcolumns at pH 7.4 and 37.0 °C. Other conditions are provided in the main body of the text. The error bars represent a range of ± 1 S.D. (n = 5) and in some cases, were comparable to the size of the data symbols.

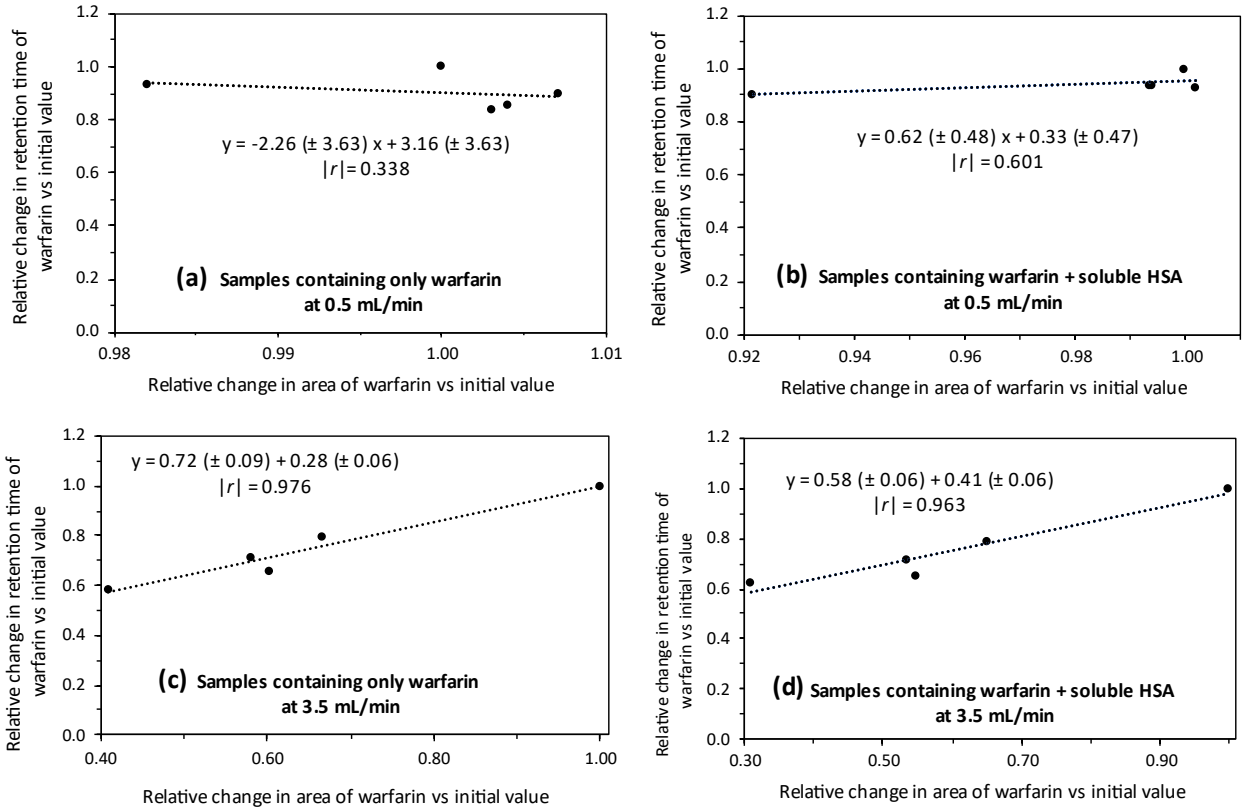


Figure S3. Relation between retention time and retained peak area for captured warfarin at 0.5 (a-b) or 3.5 mL/min (c-d) and samples containing only warfarin or warfarin plus soluble HSA. The best-fit lines and absolute values for each fit are shown within the plots. These results were obtained for 20 µL sample injections made over up to 200 total injections on 10 mm × 2.1 mm i.d. HSA microcolumns. Other conditions are provided in the main body of the text.

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