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Short Communication

Analysis of the Binding of Warfarin to Glyoxal- and Methylglyoxal-Modified Human Serum Albumin by Ultrafast Affinity Extraction

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Abstract

Ultrafast affinity extraction (UAE) and affinity microcolumns containing immobilized human serum albumin (HSA) were employed to evaluate the effect of advanced stage glycation on HSA and its binding to warfarin, a common site-specific probe for Sudlow site I of this protein. The modification of HSA by glyoxal (GO) and methylglyoxal (MGO) was considered, where GO and MGO are known to be important in the formation of many types of advanced glycation end products. Free drug fractions were measured by UAE for warfarin in solutions containing normal HSA or HSA that had been modified by GO or MGO at levels seen in serum during diabetes. The free fractions measured with the GO-modified HSA gave association equilibrium constants that ranged from 2.42-2.63 × 10^5 M^{-1} at pH 7.4 and 37 ºC. These values were not significantly different from a value of 2.33 (± 0.15) × 10^5 M^{-1} that was determined by the same method for warfarin with normal HSA. Similar studies using MGO-modified HSA gave association equilibrium constants for warfarin in the range of 3.07-3.31 × 10^5 M^{-1}, which were 1.32- to 1.42-fold higher than the value seen for normal HSA (differences that were significant at the 95% confidence level). These results will be valuable in future binding studies based on affinity chromatography or other methods that employ warfarin as a probe to examine drug interactions at Sudlow site I of HSA and modified forms of this protein. This work also illustrates how UAE can be used, with analysis times of only minutes, to detect and measure small changes in the binding by drugs with unmodified or modified forms of a soluble binding agent or protein.

Keywords: Ultrafast affinity extraction; Drug-protein binding; Human serum albumin; Advanced glycation end products; Warfarin

1. Introduction

Human serum albumin (HSA; molar mass, 66.7 kDa) is the most abundant transport protein in the serum, having a concentration in the range of 30-50 g/L [1,2]. Exogenous compounds such as drugs often interact with Sudlow sites I and/or II of HSA; this binding, in turn, affects the absorption, distribution, metabolism, and excretion of these drugs in the body [1–4]. High blood sugar levels during type II diabetes can result in the modification of HSA through its reaction with glucose or intermediate reactive dicarbonyl compounds such as methylglyoxal (MGO) and glyoxal (GO) [4–6]. MGO and GO are α-oxoaldehydes that are formed through the oxidation, dehydration, or cross-linking of sugar adducts on glycated HSA or free sugars.

Both GO and MGO can react with lysines, arginines, or the N-terminus of HSA and lead to the production of advanced stage glycation end products (AGEs) [4,7–9]. The modification of HSA by AGEs is governed by various competing reaction pathways, such as the oxidation of glycation adducts on HSA or reducing sugars and the counteracting elimination of GO and MGO by natural protein degradation or a glyoxalase pathway [4,10]. For instance, modification of HSA by GO and MGO may result in the formation of AGEs such as glyoxal-derived hydroimidazolone isomer 1 (G-H1) and methylglyoxal-derived hydroimidazolone isomer 1 (MG-H1), respectively, as shown in Figure 1 [4,7,11]. These structural alterations, along with those caused by early stage glycation, are of great current interest with regards to how they may affect the thermodynamics of drug-HSA interactions and the resulting free fraction of a drug in the circulation (i.e., the biologically active fraction for many pharmaceutical agents) [12–17].

Ultrafast affinity extraction (UAE) is a form of high performance affinity chromatography (HPAC) that has been recently employed to characterize the free fractions, thermodynamics, and kinetics of various drugs and hormones during their interactions with serum proteins such as HSA [17–21] and α_1-acid glycoprotein (AGP) [22–24]. In UAE, a sample containing a drug, either in

the presence or absence of a protein or soluble binding agent, is injected onto a small HPAC column containing a capture agent for the drug; these injections are made at a column residence time that minimizes drug dissociation from the soluble binding agent during passage of the sample through the microcolumn [20,25]. The conditions needed for this method can be produced by affinity microcolumns, which have volumes in low-to-mid μL range and can provide residence times in the millisecond-to-second range at typical HPLC flow rates [20,25]. At these short column residence times, the amount of drug that is captured and retained by the microcolumn can be used to determine the original free fraction of the drug in the sample at equilibrium, as shown in Figure 2. This free fraction can then, in turn, be used to estimate the equilibrium constant for binding of the drug with the protein/soluble binding agent in the sample [16,18,21–23].

In this study, UAE will be used to evaluate how the modification of HSA with clinically-relevant levels of MGO and GO alters the binding of this protein to the anticoagulant warfarin. Warfarin has well-characterized binding to Sudlow site I of normal, unmodified HSA [20,25,26] and is often used as a probe for this region on HSA in drug competition and displacement studies [15,27,28]. The information obtained from this report will be important in seeing if the modification of HSA by MGO or GO during type II diabetes has any effect on the binding of warfarin. The results will also provide valuable information on the use of UAE for studying changes in drug interactions with modified forms of a protein such as HSA, as well as on the use of warfarin as a site-specific probe for examining binding by other drugs with AGE-modified HSA.

2. Experimental

2.1. Materials

The racemic warfarin (≥ 98% pure) and HSA (Cohn fraction V, essentially fatty acid-free, ≥ 96%) were acquired from Sigma (St. Louis, MO, USA). Nucleosil Si-300 (300 Å pore size and

7 μm particle diameter) was purchased from Macherey Nagel (Dűren, Germany). The pH 7.4, 0.067 M potassium phosphate buffer was prepared using water from a Milli-Q system (EMD Millipore Sigma, Burlington, MA) and filtered using 0.22 μm GNWP nylon membrane filters acquired from Fisher Scientific (Pittsburgh, PA, USA). The reagents for the micro bicinchoninic acid (BCA) protein assay were purchased from Pierce (Rockford, IL, USA).

2.2. Instrumentation

A Prep 24 pump from ChromTech (Apple Valley, MN, USA) was employed for packing the affinity microcolumns. Chromatographic studies were performed using an HPLC system consisting of an AS-2057 autosampler, a UV-2075 absorbance detector, and a PU-2080 Plus pump from Jasco (Easton, MS, USA) with a six-port LabPro valve (Rheodyne, Cotati, CA, USA). The analysis was performed at a temperature of 37.0 (± 0.1) °C, as maintained by using a X-LC 3167CO column oven from Jasco. The chromatographic system was controlled using ChromNAV v1.18.04 software and LCNet from Jasco. Baseline correction and data smoothing of each chromatogram were performed using the progressive/linear and Savitzky-Golay smoothing functions, respectively, of PeakFit v4.12 (Jandel Scientific, San Rafael, CA, USA). The chromatographic peaks were used in fits based on the exponentially modified Gaussian (EMG) function of PeakFit v4.12. Each chromatogram was fitted five times and the average area of these chromatograms was determined. Additional data analysis was performed using Excel for Office 365 (Microsoft, Redmond, WA, USA).

2.3. Preparation of glyoxal- and methylglyoxal-modified HSA

The MGO- and GO-modified HSA samples were prepared as described previously [14,15]. For this process, a commercial sample of normal, unmodified HSA was dissolved under sterile

conditions in pH 7.4, 0.067 M potassium phosphate buffer containing 1 mM sodium azide as an additive. A 42 g/L solution of normal HSA was made in this manner to represent a typical physiological concentration of HSA [14,15]. Either 130 nM or 210 nM of GO was placed into a portion of this HSA solution to prepare the modified samples of HSA that will be referred to in this report as GO-HSA1 and GO-HSA2, respectively. The GO concentrations that were used to make these modified HSA samples were representative of typical serum concentrations seen for GO in healthy individuals or in patients with type II diabetes, respectively [10,11,14,15,29,30]. MGO levels of 40 nM or 120 nM were combined with the original normal, unmodified HSA solution in the same manner to prepare the samples of modified HSA that will be referred to in this work as MGO-HSA1 and MGO-HSA2; these samples were prepared to represent the typical serum concentrations of MGO that have been observed in healthy individuals and patients with prediabetes/type II diabetes, respectively [10,14,15,30]. These mixtures of HSA and GO or MGO were incubated for 4 weeks at 37 ℃ to mimic the exposure period of AGEs to HSA in the circulation [14,15]. Zeba spin desalting columns (0.5 mL, 7 kDa cutoff; Thermo Fisher Scientific, Rockford, IL, USA) were utilized to remove any excess or unreacted modifying agents from the GO- and MGO-HSA samples. The GO- and MGO-HSA samples were then lyophilized and stored at -80 ℃ [14,15].

Two fluorometric assays (i.e., the 9,10-phenanthrenequinone and *o*-phthalaldehyde, or OPA, assays) were employed to compare the levels of free arginine residues or primary amines/free lysines in the modified HSA samples vs the original preparation of unmodified and normal HSA that was used in this study [31,32]. The extent of modification for the arginines and lysines in the original preparation of normal HSA was < 0.1 mol/mol HSA. The levels of arginine modification for the samples of MGO-HSA1 and MGO-HSA2 were 0.24 (± 0.10) and 0.88 (±

0.11) mol/mol HSA, respectively; the extent of arginine modification in the samples of GO-HSA1 and GO-HSA2 were 0.20 (± 0.06) and 0.74 (± 0.05) mol/mol HSA, respectively. The modification levels of the primary amines or free lysines in MGO-HSA1 and MGO-HSA2 were 0.43 (± 0.14) and 1.12 (± 0.15) mol/mol HSA, respectively; for the GO-HSA1 and GO-HSA2, the extent of modification for the free lysines or primary amines was 0.15 (± 0.11) or 0.55 (± 0.12) mol/mol HSA, respectively [14,15].

2.4. Chromatographic conditions

The support that was utilized in the microcolumns for UAE was prepared by immobilizing normal HSA onto diol-bonded silica by the Schiff base method [25,33]. A control support was prepared in the same manner but without the addition of HSA during the immobilization process [25,33]. The protein content of the HSA support, as determined by using a micro BCA assay, was found to be 75 (± 4) mg HSA/g silica. This HSA support was packed into two 10 mm × 2.1 mm inner diameter (i.d.) affinity microcolumns. One of these microcolumns was used for injections of warfarin and mixtures of warfarin plus HSA, MGO-HSA1, or MGO-HSA2. The second microcolumn was used for injections of warfarin and warfarin plus GO-HSA1 or GO-HSA2. The microcolumns were downward slurry packed at 4000 psi (28 MPa) by employing pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. The HSA supports and microcolumns were stored in this pH 7.4 buffer at 4 ℃.

A pH 7.4, 0.067 M potassium phosphate buffer was utilized as the mobile phase for sample injection and elution during UAE and for preparing the warfarin and warfarin-protein solutions. This buffer was degassed for 25-30 min prior to use in HPLC. UAE was performed by injecting 20 μL samples containing 10 μM warfarin or a mixture of 10 μM warfarin plus 20 μM of the desired protein (i.e., normal HSA, MGO-HSA1, MGO-HSA2, GO-HSA1, or GO-HSA2). In the

final conditions selected for this work, these injections were made in replicate (n = 5-7) onto the respective HSA microcolumns at a flow rate of 4.75 mL/min (Note: flow rates of 3.5-4.75 mL/min were used in initial studies for method optimization, as shown in Figure 3). The warfarin and warfarin-protein samples were preheated to 37.0 (± 0.1) °C for 30-60 min in the autosampler of the HPLC system prior to injection; in addition, the microcolumn was equilibrated at 0.50 mL/min with the mobile phase at the same temperature and for the same amount of time prior to sample injection. The elution of warfarin was detected at 308 nm. The free fraction of warfarin was calculated by dividing the retained peak area of warfarin that was obtained during the injection of a warfarin-HSA mixture by the peak area measured when using samples containing warfarin alone at the same total drug concentration [16,17,20,21,25].

3. Results and discussion

UAE was carried out by injecting samples of warfarin in the absence and presence of normal HSA or HSA that had been modified with GO (i.e., GO-HSA1 and GO-HSA2) or MGO (MGO-HSA1 and MGO-HSA2). The association equilibrium constant (K_a) for each of these interactions was obtained from the free fraction that was present at equilibrium (F_0) between warfarin and soluble HSA in the original sample. This free fraction was measured by injecting the samples onto 10 mm × 2.1 mm i.d. UAE microcolumns containing immobilized normal HSA as a capture agent for warfarin. The value of K_a for each of the injected drug-protein mixtures was determined from the measured value of F_0 by using eq. (1) (see derivation given in the Supplementary Material).

$$K_a = \frac{(1-F_0)}{F_0(C_P-C_D+C_DF_0)} \quad (1)$$

This equation is for a system in which there is a 1:1 interaction between the drug and a soluble protein, where C_D and C_P are the total concentrations of the drug and the protein in their original sample mixture [16,17,20,21,25].

The initial UAE experiments in this report were conducted by using a set of flow rates ranging from 3.50 to 4.75 mL/min, as shown in Figure 3. These flow rates corresponded to column residence times of ~440 to 590 ms for the non-retained sample components. Both 3.50 and 4.00 mL/min gave apparent free fractions for warfarin that were higher than those observed at 4.50-4.75 mL/min. These differences, which were as large as 0.24 to 0.46 units in the measured free fraction at 3.50 mL/min vs 4.75 mL/min, were expected and due to dissociation of warfarin from soluble HSA within the samples at the longer column residence times that were present at the lower flow rates. However, consistent free fractions were seen at flow rates of 4.50 mL/min or higher, indicating that warfarin dissociation from HSA within the sample was minimal under these conditions.

A previous study with UAE has indicated that a good estimate for the true free fraction of warfarin at equilibrium and in presence of normal HSA can be obtained by using column residence times that are less than 550 ms [18]. When UAE was carried out with warfarin and normal HSA in this study at 4.75 mL/min, the value for F_0 that was acquired was statistically identical at the 95% confidence level to prior measurements of this value under the same sample composition, pH, temperature, and solution conditions [18]. Use of flow rates in the range of 4.50-4.75 mL/min (i.e., column residence times of 462-438 ms) for the modified forms of HSA also gave consistent measured free fractions and estimates for F_0. For instance, the F_0 values obtained for warfarin in presence of the GO- or MGO-modified HSA samples at 4.50 and 4.75 mL/min were statistically equivalent at the 95% confidence interval based on a Student's *t*-test. Based on these results, a

flow rate of 4.75 mL/min was used in all further work to determine F_0 and estimate values of K_a for warfarin with HSA and the modified forms of HSA.

Some typical chromatograms that were obtained for warfarin in the presence and absence of normal or modified HSA are given in Figure 4. The total analysis time for each of these samples by UAE was about 3 min. Table 1 shows the free fractions for warfarin that were obtained in presence of normal HSA and the MGO- or GO-modified HSA samples. The precision of these F_0 values ranged from ± 2.0-6.0% (average, ± 4.2%) and the standard error of the mean (SEM) for these values ranged from ± 0.8-2.7% (average, ± 1.8%) for 5-7 replicate injections. The value of F_0 gave a small apparent decrease of 2.3-7.8% for warfarin in going from the normal, unmodified HSA to the GO-modified samples of HSA; however, these apparent changes were not significant at the 95% confidence level. The same type of free fraction gave a decrease of 17-21% in going from normal HSA to MGO-modified HSA, which was significant at the 95% confidence level.

The corresponding values of K_a that were obtained by UAE for warfarin with normal HSA and the GO- or MGO-modified forms of HSA are also provided in Table 1. The association equilibrium constants determined for warfarin in presence of normal HSA showed good agreement with previous values that have been obtained for this system by various methods and under the same pH and temperature conditions (i.e., reported range, 1.6-2.6 × 10^5 M^{-1}; best estimate for racemic warfarin, 2.3 × 10^5 M^{-1}) [20,25,34,35]. The precision of the K_a values that were estimated in this work for warfarin with normal HSA was ± 2.9-12.6% (± 1 SEM for n = 5-7; average, ± 7.5%). The K_a values for warfarin in the presence of GO-HSA1 and GO-HSA2 differed by only 1.04- to 1.13-fold compared to the association equilibrium constant that was obtained for normal HSA. However, these differences were not significant at the 95% confidence level. For the MGO-

modified samples, the K_a values increased by 1.32- to 1.42-fold compared to normal HSA and gave results that were significantly different at the 95% confidence interval.

Previous studies based on the zonal elution and frontal analysis formats of HPAC have found that no significant changes occur in the binding strength of warfarin when comparing glycated forms of HSA with normal, unmodified HSA [36,37]. This type of behavior, in which no significant changes in binding were detected, were also seen in this current study for warfarin in presence of GO-HSA that had been modified at levels representative of diabetes. However, modification of HSA by MGO at clinically-relevant levels did produce a significant change in the binding strength of warfarin at Sudlow site I. Studies with a number of anti-diabetic drugs have also shown a significant change in binding with HSA at Sudlow site I as a result of modification of HSA by MGO [14,15]. These changes may be due to the formation of MGO-related AGEs at or near Sudlow site I, as may occur at residues such as K199, K205, K233, K240, K276, K281, R186, R197, and R218 of HSA [4,15,38,39] (Note: see Ref. [39] for further details regarding the specific preparations of modified HSA that were used in this work).

4. Conclusion

UAE and affinity microcolumns were used to obtain the association equilibrium constants and free fractions for warfarin with HSA that had been modified with GO or MGO at clinically-relevant levels, such as those seen in type II diabetes. No significant change in K_a (using the value for normal HSA as a reference) was observed for warfarin in the presence of GO-modified HSA samples. However, an increase of 1.32- to 1.42-fold was noted in K_a for warfarin in the presence of MGO-modified HSA. These results indicate that modification of HSA by MGO, such as at lysine or arginine residues that are in the region of Sudlow site I, can significantly alter the binding of warfarin with this protein at modification levels that may occur in type II diabetes [14,15].

Similar conclusions have recently been reached in studies examining the binding of various antidiabetic drugs with GO- or MGO-modified HSA [14,15].

These results are important because warfarin is often used as a site-specific probe for Sudlow site I HSA in various analytical methods and in competition or displacement studies with other drugs [15,27,28]. The data that were obtained demonstrate the need in these applications to test whether the binding properties of a probe such as warfarin are changed as a protein is modified and, if present, whether a correction should be made for these changes (e.g., as can be accomplished in zonal elution-based competition studies in HPAC) [40,41]. The results of this report also indicate the extent to which binding by warfarin may change for GO- or MGO-modified HSA; this information can be used in future work with this probe and these modified forms of HSA. Finally, this study illustrates how UAE can be an analytical tool for rapidly investigating the interactions of drugs with small samples of modified proteins, as shown here for GO-HSA and MGO-HSA, and in detecting or evaluating the changes in binding strength that occur due to these modifications in diseases such as diabetes.

5. Declaration of competing interest

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

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Figure Legends

Figure 1. Examples of reactions that occur during the formation of advanced stage glycation end products (AGEs) due to modification of residues on HSA by glyoxal (GO) and methylglyoxal (MGO). This scheme is based on information from Refs. [4,14].

Figure 2. General scheme for use of ultrafast affinity extraction (UAE) in the measurement of a free drug fraction and estimation of the association equilibrium constant (K_a) for the binding of this drug in the presence of a soluble protein, as carried out at short column residence times for the sample and by using a microcolumn that contains an immobilized capture agent for the drug. The free fraction is measured by dividing the retained peak area of the drug in presence of the protein by the retained peak area of drug alone [18].

Figure 3. Effect of varying the injection flow rate, and corresponding column residence time, on the apparent free fraction that was measured for warfarin on 10 mm × 2.1 mm i.d. HSA microcolumns when 20 μL injections were made of samples that contained 10 μM warfarin (a) in the absence or presence of 20 μM normal HSA, GO-HSA1, and GO-HSA2; or (b) in the absence or presence of 20 μM normal HSA, MGO-HSA1, and MGO-HSA2. Conditions: mobile phase, pH 7.4, 0.067 M potassium phosphate buffer; temperature, 37.0 °C. The error bars in these plots represent a range of ± 1 S.E.M. (n = 5-7). The inset in (a) shows variation in column residence time as a function of the injection flow rate.

Figure 4. Chromatograms for UAE showing the non-retained and retained peaks obtained with 10 mm × 2.1 mm i.d. HSA microcolumns for 20 μL sample injections of 10

μM warfarin (a) in the absence or presence of 20 μM normal HSA, GO-HSA1, and GO-HSA2; or (b) in the absence or presence of 20 μM normal HSA, MGO-HSA1, and MGO-HSA2. The insets show an expanded view of the retained peaks in these chromatograms. The *y*-axis in each plot is given in relative absorbance units, as measured at 308 nm. Conditions: mobile phase, pH 7.4, 0.067 M potassium phosphate buffer; temperature, 37.0 °C; flow rate, 4.75 mL/min.

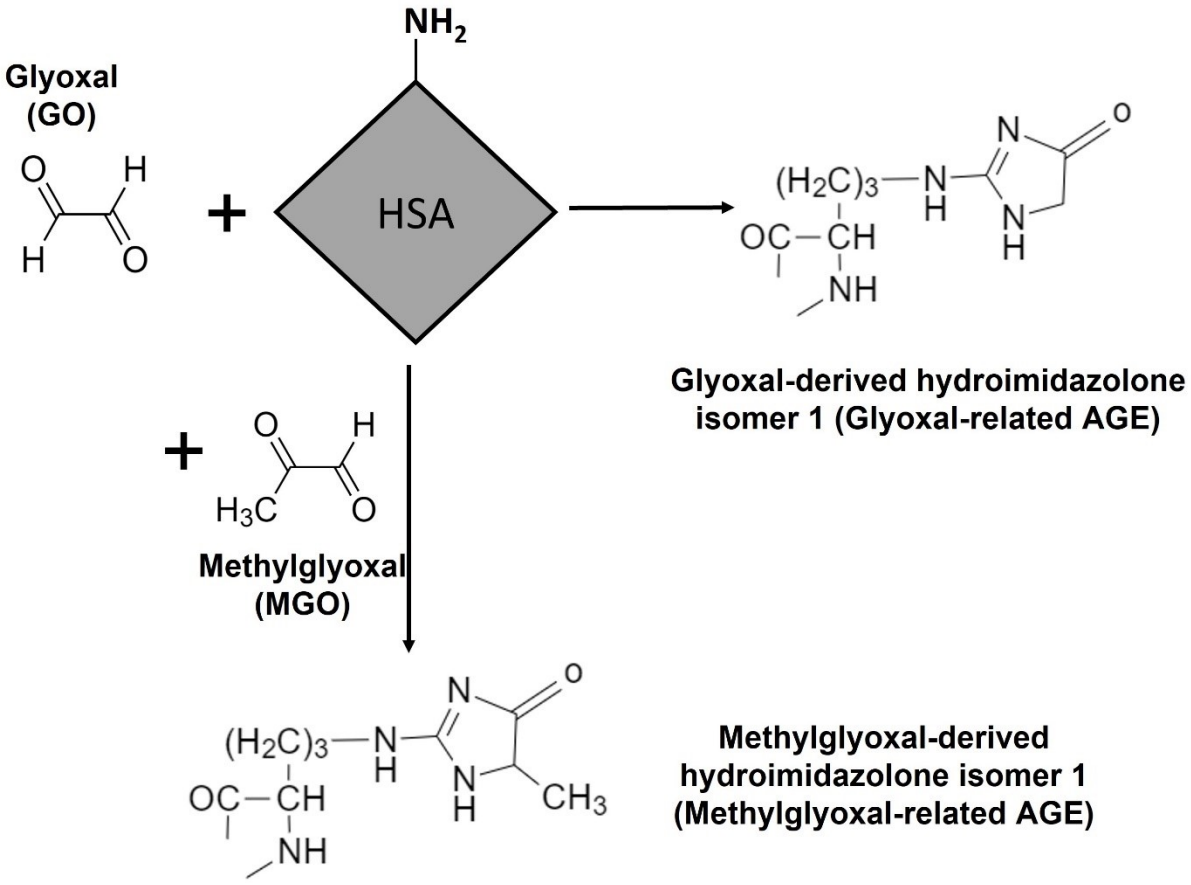
Glyoxal
(GO)
NH2
HSA
Glyoxal-derived hydroimidazolone
isomer 1 (Glyoxal-related AGE)
Methylglyoxal
(MGO)
Methylglyoxal-derived
hydroimidazolone isomer 1
(Methylglyoxal-related AGE)

Figure 1

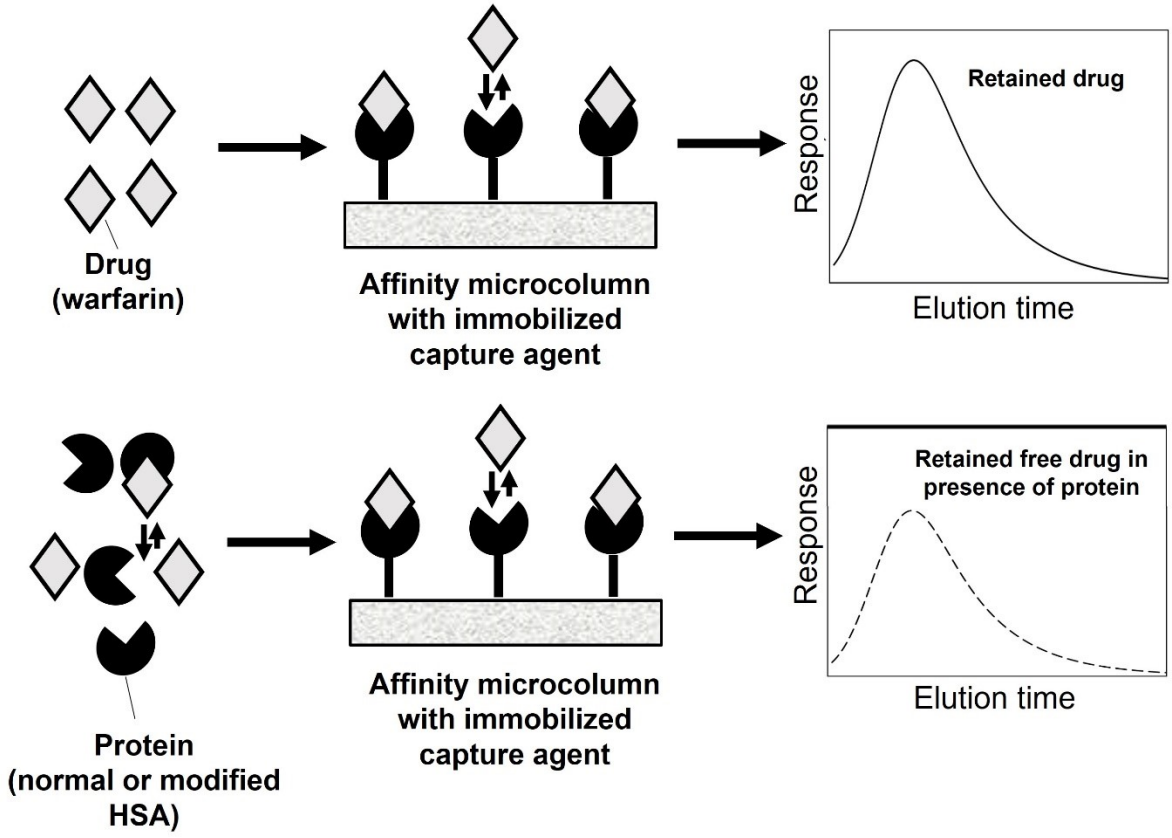
Drug
(warfarin)
Affinity microcolumn
with immobilized
capture agent
Response
Retained drug
Elution time
Protein
(normal or modified
HSA)
Affinity microcolumn
with immobilized
capture agent
Response
Retained free drug in
presence of protein
Elution time

Figure 2

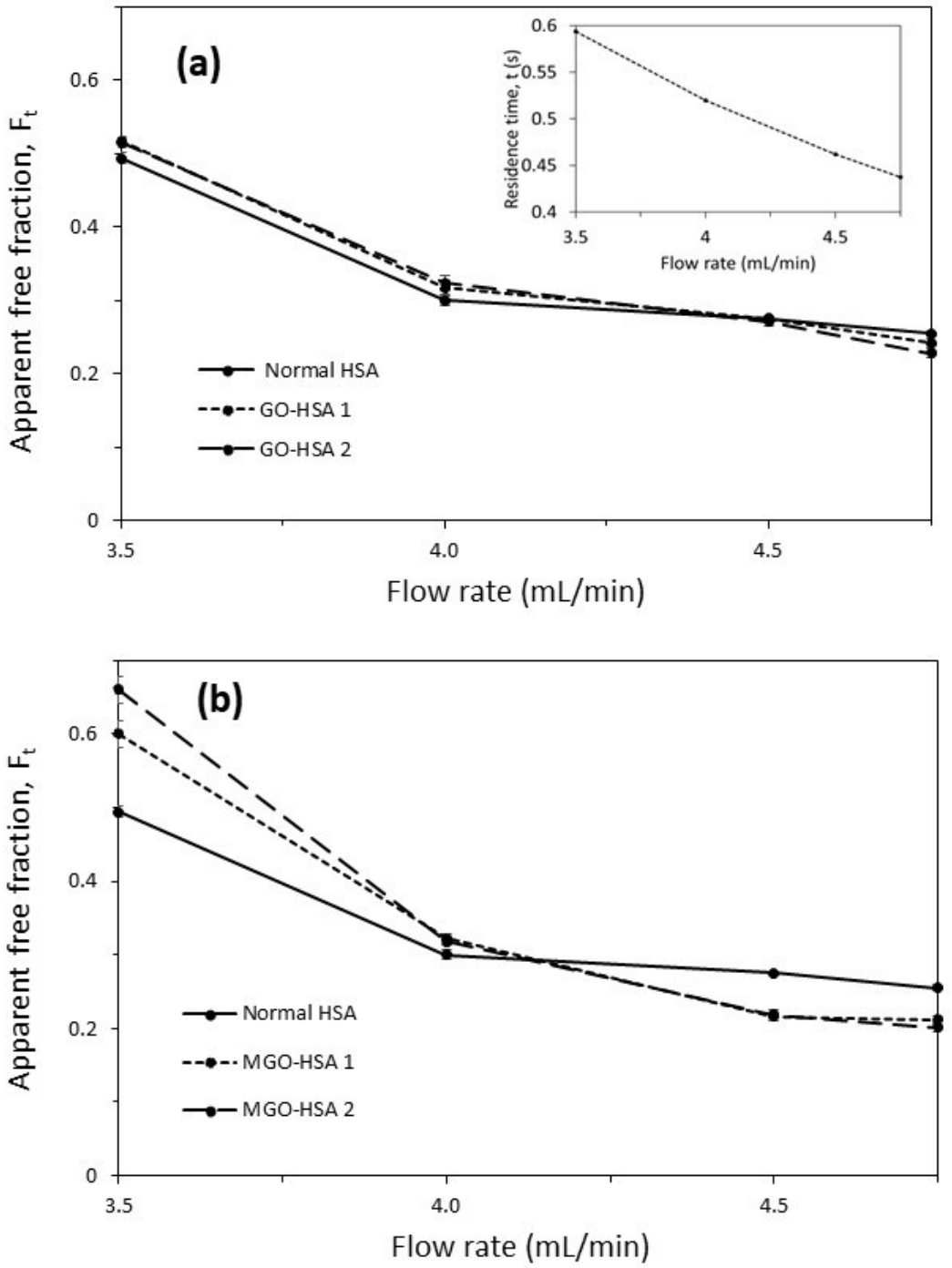
(a)
Apparent free fraction, F_t
0.6
0.4
0.2
0
3.5
4.0
4.5
Flow rate (mL/min)
Normal HSA
GO-HSA 1
GO-HSA 2
Residence time, t (s)
0.6
0.55
0.5
0.45
0.4
3.5
4
4.5
Flow rate (mL/min)
(b)
Apparent free fraction, F_t
0.6
0.4
0.2
0
3.5
4.0
4.5
Flow rate (mL/min)
Normal HSA
MGO-HSA 1
MGO-HSA 2

Figure 3

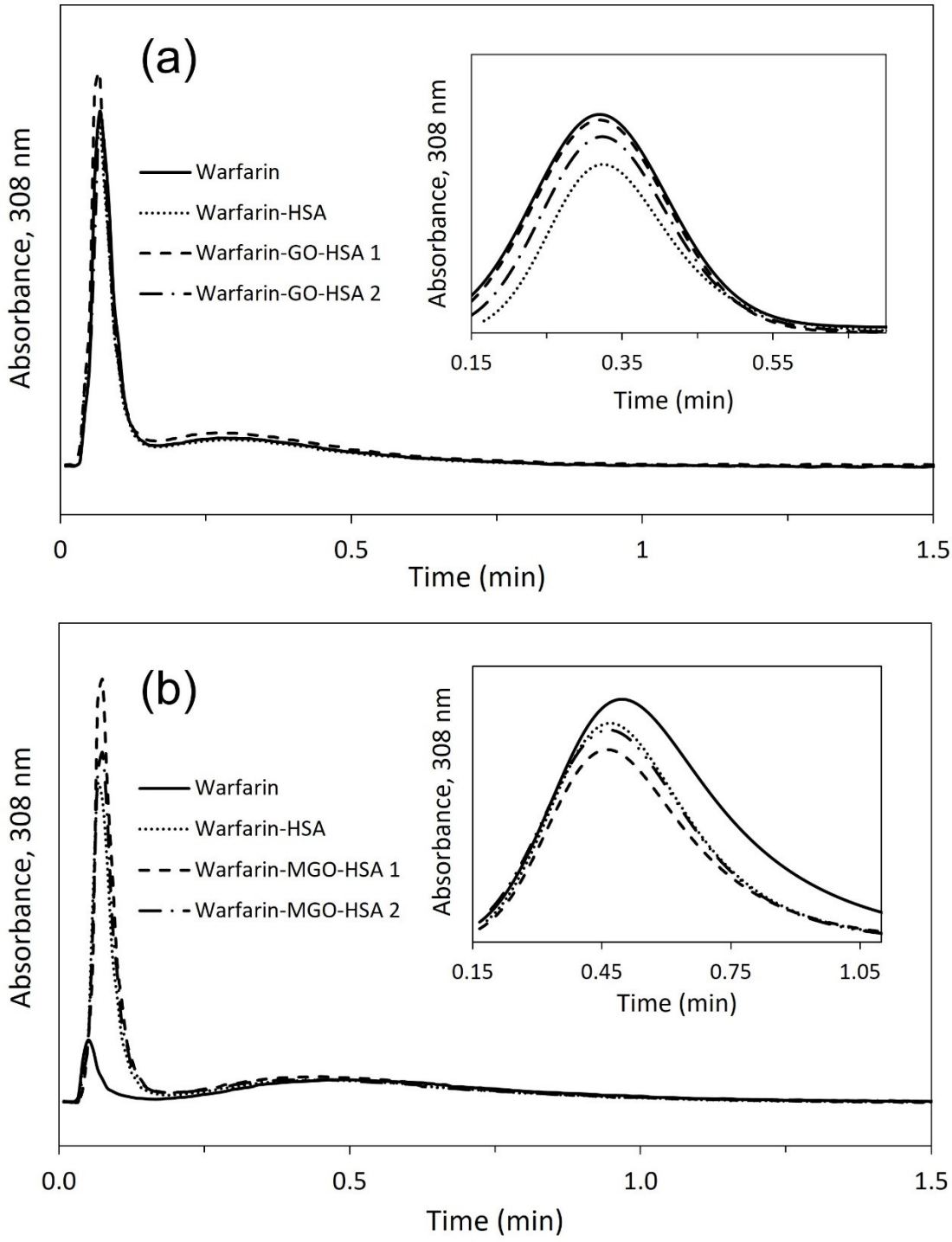
(a)
Absorbance, 308 nm
Warfarin
Warfarin-HSA
Warfarin-GO-HSA 1
Warfarin-GO-HSA 2
Absorbance, 308 nm
0.15
0.35
0.55
Time (min)
0
0.5
1
1.5
Time (min)
(b)
Absorbance, 308 nm
Warfarin
Warfarin-HSA
Warfarin-MGO-HSA 1
Warfarin-MGO-HSA 2
Absorbance, 308 nm
0.15
0.45
0.75
1.05
Time (min)
0.0
0.5
1.0
1.5
Time (min)

Figure 4

Table 1. Association equilibrium constants measured by UAE for warfarin with normal HSA and GO- or MGO-modified HSA[a]

Value	Normal HSA	GO-HSA1	GO-HSA2	MGO-HSA1	MGO-HSA2
F_0	0.255 (± 0.004)	0.249 (± 0.002)	0.235 (± 0.003)	0.212 (± 0.004)	0.201 (± 0.005)
K_a ($\times 10^5$ M^{-1})	2.33 (± 0.15)	2.42 (± 0.07)	2.63 (± 0.15)	3.07 (± 0.30)	3.31 (± 0.42)
Relative K_a vs normal HSA	------	1.04 (± 0.07)[b]	1.13 (± 0.10)[b]	1.32 (± 0.15)	1.42 (± 0.20)

[a]The F_0 and K_a were determined at pH 7.4 and 37.0 °C and the number in parentheses represent a range of ± 1 S.E.M. (n = 5-7).

[b]These results are not significantly different from those for normal HSA at the 95% confidence level.

Supplementary Material

Derivation of the eq. (1) relating the free fraction and association equilibrium constant

The following reaction describes the 1:1 saturable and reversible binding interaction between a solute or drug (D) and a serum protein or any binding agent (P) [S1,S2].

$$D + P \leftrightarrow DP \tag{S1}$$

The association equilibrium constant for this system (K_a) is described by the following relationship [S1,S2].

$$K_a = \frac{[DP]}{[D][P]} \tag{S2}$$

In this relationship, [D] and [P] are the concentrations of solute/drug, and protein/binding agent at equilibrium, while [DP] is the concentration of the complex formed between the drug and protein. This reaction can also be described by the following mass balance equations.

$$[D] = C_D - [DP] \tag{S3}$$

$$[P] = C_P - [DP] \tag{S4}$$

$$F_0 = \frac{C_D - [DP]}{C_D} \tag{S5}$$

The term C_D in eqs. (S3) and (S5) represents the total concentration of solute/drug in the original sample. The term C_P in eq. (S4) represents the total concentration of protein/binding agent in the original sample. Finally, the term F_0 in eq. (S5) represents the original free fraction of the drug or the solute in the original sample.

The term [DP] can be determined from the measured value of F_0 and the known value of C_D as shown in eq. (S6).

$$[DP] = C_D(1 - F_0) \tag{S6}$$

Substituting eqs. (S3) and (S4) into eq. (S2) results in the combined relationship for K_a that is provided in eq. (S7).

$$K_a = \frac{[DP]}{(C_D - [DP])(C_P - [DP])} \tag{S7}$$

Eq. (S8) is obtained when the terms in the denominator of eq. (S7) are combined through multiplication.

$$K_a = \frac{[DP]}{C_D C_P - C_D[DP] - C_P[DP] + [DP]^2} \tag{S8}$$

The term [DP] in eq. (S8) can also be expressed in terms of F_0 and C_D, as shown previously in eq. (S6). Combining eqs. (S6) and (S8) results in eq. (S9).

$$K_a = \frac{C_D(1 - F_0)}{C_D C_P - C_D(C_D - C_D F_0) - C_P(C_D - C_D F_0) + (C_D - C_D F_0)^2} \tag{S9}$$

Eq. (S9) can be simplified through multiplication and combination of common terms in the denominator, which produces eq. (S10).

$$K_a = \frac{C_D(1 - F_0)}{{C_D}^2 {F_0}^2 - {C_D}^2 F_0 + C_D C_P F_0} \tag{S10}$$

Eq. (S10) can be further simplified by factoring out the term C_D from the numerator and denominator, as shown in eq. (S11).

$$K_a = \frac{C_D(1 - F_0)}{C_D({C_D} {F_0}^2 - C_D F_0 + C_P F_0)} \tag{S11}$$

It is now possible to eliminate the term C_D from the numerator and denominator by division. This produces the final expression for K_a in terms of C_D, C_P, and F_0 that is given in the main body of the text as eq. (1) [S3].

$$K_a = \frac{(1 - F_0)}{F_0(C_P - C_D + C_D F_0)} \tag{1}$$

References (Supplementary Material)

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