Published in final edited form as:

J Chromatogr A. 2014 December 5; 0: 82–89. doi:10.1016/j.chroma.2014.10.092.

Analysis of Free Drug Fractions by Ultrafast Affinity Extraction: Interactions of Sulfonylurea Drugs with Normal or Glycated Human Serum Albumin

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Abstract

Ultrafast affinity extraction and a multi-dimensional affinity system were developed for measuring free drug fractions at therapeutic levels. This approach was used to compare the free fractions and global affinity constants of several sulfonylurea drugs in the presence of normal human serum albumin (HSA) or glycated forms of this protein, as are produced during diabetes. Affinity microcolumns containing immobilized HSA were first used to extract the free drug fractions in injected drug/protein mixtures. As the retained drug eluted from the HSA microcolumn, it was passed through a second HSA column for further separation and measurement. Items that were considered during the optimization of this approach included the column sizes and flow rates that were used, and the time at which the second column was placed on-line with the HSA microcolumn. This method required only 1.0 μ L of a sample per injection and was able to measure free drug fractions as small as 0.09–2.58% with an absolute precision of $\pm$ 0.02–0.5%. The results that were obtained indicated that glycation can affect the free fractions of sulfonylurea drugs at typical therapeutic levels and that the size of this effect varies with the level of HSA glycation. Global affinity constants that were estimated from these free drug fractions gave good agreement with those predicted from previous binding studies or determined through a reference method. The same approach could be utilized with other drugs and proteins or modified binding agents of clinical or pharmaceutical interest.

Keywords

Ultrafast affinity extraction; Affinity chromatography; Drug-protein binding; Sulfonylurea drugs; Human serum albumin; Protein glycation

1. Introduction

Diabetes is a disease that is associated with insulin deficiency or glucose intolerance, which can both result in elevated levels of glucose in the bloodstream [1]. Recent reports by the

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International Diabetes Federation and the American Diabetes Association have indicated that diabetes affects 366 million people in the world and 25.8 million people in the U.S. [1,2]. Type II diabetes accounts for about 90–95% of the confirmed cases of diabetes and results from insulin resistance [1,2]. Sulfonylurea drugs are commonly used to treat type II diabetes by stimulating the release of insulin, thereby decreasing the level of glucose in blood [3]. Figure 1 shows the general structure of a sulfonylurea drug [4,5]. Examples of common first-generation sulfonylurea drugs are acetohexamide and tolbutamide; second-generation drugs include gliclazide and glibenclamide, which tend to be more easily excreted and effective than the first-generation sulfonylurea drugs [6].

Sulfonylurea drugs are highly bound to serum proteins and, in particular, to human serum albumin (HSA) [7,8]. HSA is the most abundant serum protein, accounting for 60% of the total serum protein content and having a normal concentration of 35–50 g/L (526–752 μ M) [9,10]. HSA has a mass of 66.5 kDa and is composed of 585 amino acids. HSA functions as a transport protein for many low mass hormones, fatty acids, and drugs in the bloodstream [9]. There are two major drug binding sites on HSA, which are often known as Sudlow sites I and II [9,11,12].

The elevated levels of glucose in blood during diabetes can result in the non-enzymatic glycation of proteins [13–16]. Early stage glycation involves the nucleophilic addition of a reducing sugar (e.g., glucose) to a free amine group on a protein; advanced glycation products can also form through further reactions [7,18–20]. It has been found that there can be a 2- to 5-fold increase in the amount of HSA that is glycated in diabetic patients when compared with healthy individuals [7,21]. Structural investigations of glycated HSA have further found that glycation-related modifications can often occur at or near Sudlow sites I and II [22–25]. In addition, glycation has been shown to cause changes in the affinity of various sulfonylurea drugs for HSA [26–34]. Such changes are of concern because it has been proposed that they may affect the free, or non-protein bound, and active fraction of these drugs in the bloodstream [7,26, 32,34].

Various methods have been developed in the past to measure free drug fractions [35–44]. Two common methods used for free drug analysis are equilibrium dialysis and ultrafiltration. However, these techniques often require long separation or analysis times (ranging from 15–30 min to hours) and relatively large sample volumes (i.e., typically in the milliliter range). In addition, the non-specific adsorption of drugs to the membranes or components of these methods can introduce errors in the free fraction measurements [35,37–41]. Ultrafast affinity extraction is an alternative technique that has been used to measure the free and protein-bound forms of drugs and hormones in clinical and pharmaceutical samples [37–40,45–47]. In this method, an affinity microcolumn that contains an immobilized binding agent with relatively fast and strong binding for the drug or hormone of interest is used to extract the free form of this analyte on a time scale that minimizes dissociation of the same analyte from its protein-bound form in the sample. The advantages of this approach include its speed, ease of automation, good correlation with reference methods, and need for only a small amount of the analyte and protein [35–45]. This method has been used to measure the free fractions of thyroxine, warfarin and phenytoin in clinical samples [38–40,45,46]. This technique has also been used to estimate the association equilibrium

constants for various drugs with normal HSA [37,47]. In addition, a multidimensional system combining ultrafast affinity extraction with a chiral stationary phase has recently been used to simultaneously measure the free fractions of warfarin enantiomers in complex samples [38].

This study will examine the development and use of ultrafast affinity extraction in a multi-dimensional system to measure and compare the free fractions of various sulfonylurea drugs in the presence of normal HSA or glycated HSA. The HSA will be glycated *in vitro* at levels similar to those found in patients with prediabetes or diabetes. Various factors, such as column size and flow rate, will be considered in the optimization of this system for determining the free fractions of sulfonylurea drugs. The system will then be used to measure the free fractions and global affinity constants for these drugs with normal HSA or glycated HSA by using samples that have been prepared at typical therapeutic or physiological concentrations of these drugs and proteins. The results will provide insight concerning how glycation may alter the free fractions of sulfonylurea drugs in the circulatory system. The same results will also provide valuable information as to how ultrafast affinity extraction can be modified and developed for use in the study of drug-protein interactions at clinically-relevant concentrations.

2. Experimental

2.1. Reagents

The HSA (Cohn fraction V, essentially fatty acid free, 96% pure), tolbutamide, acetohexamide, gliclazide, and glibenclamide were from Sigma (St. Louis, MO, USA). The reagents for the bicinchoninic acid (BCA) protein assay were obtained from Pierce (Rockford, IL, USA). The Nucleosil Si-300 silica (7 μm particle diameter, 300 Å pore size) was from Macherey Nagel (Dren, Germany). All buffers and aqueous solutions were prepared using water from a NANOpure system (Barnstead, Dubuque, IA, USA) and were passed through Osmonics 0.22 μm nylon filters from Fisher Scientific (Pittsburgh, PA, USA).

2.1 Apparatus

The microcolumns used in this study were packed using a Prep 24 pump from ChromTech (Apple Valley, MN, USA). The HPLC system was comprised of a PU-2080 Plus pump, AS-2057 autosampler, and UV-2075 absorbance detector from Jasco (Easton, MD, USA), plus a six-port Lab Pro valve (Rheodyne, Cotati, CA, USA). An Alltech water jacket (Deerfield, IL, USA) and an Isotemp 3013D circulating water bath from Fisher Scientific were used to maintain a temperature of $37.0 (\pm 0.1) ^\circ\text{C}$ for the columns during all experiments in this report. ChromNAV v1.18.04 software and LCNet from Jasco were used to control the system. Chromatograms were analyzed through the use of PeakFit v4.12 software (Jandel Scientific, San Rafael, CA, USA).

2.2. Methods

2.2.1. Column preparation and protein glycation—Two batches of glycated HSA with different levels of glycation were prepared *in vitro* as described previously [29,31].

These glycosylated HSA samples (referred to later in this paper as gHSA1 and gHSA2) were made by incubating normal HSA at 37 °C and over four weeks with either moderate or high levels of D-glucose (i.e., 5 or 10 mM) in sterile pH 7.4, 0.067 M phosphate buffer. The resulting protein solutions were lyophilized and stored at –80 °C until use. The glycosylation levels of these modified proteins were determined by using a fructosamine assay from Diazyme Laboratories (San Diego, CA, USA), as described in Refs. [29,31]. The glycosylation levels measured for the normal HSA, gHSA1 and gHSA2 samples were 0.24 ($\pm$ 0.15), 1.39 ($\pm$ 0.28) and 3.20 ($\pm$ 0.13) mol hexose/mol HSA, respectively.

Normal HSA was immobilized to Nucleosil Si-300 silica for use as a stationary phase in ultrafast affinity extraction and the multi-dimensional affinity system. This immobilization was carried out by using the Schiff base method, as described in Ref. [48]. A control support, in which no HSA was added during the immobilization step, was prepared by the same process. A BCA assay was used, according to instructions provided by the manufacturer of this assay, to determine the protein content of the final HSA support by using HSA as the standard and the control support as the blank [29–34]. This assay gave a protein content of 65 ($\pm$ 2) mg HSA/g silica.

Affinity columns with lengths of 5 to 25 mm and 2.1 mm i.d. were packed into standard stainless steel housings by using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. The affinity columns with a length of 1 mm and 2.1 mm i.d. were packed in a similar manner but used a frit-in-column design, as described in Ref. [49]. The packing pressure was 4000 psi (28 MPa) for the 10 or 25 mm long columns and 3000 psi (20 MPa) for the 1 mm or 5 mm long columns. These columns were stored in pH 7.4, 0.067 M potassium phosphate buffer at 4 °C when not in use.

2.2.2. Chromatographic studies—The multi-dimensional affinity system had two HSA columns that could be connected in series through the use of a six-port valve (see Supplementary Material for more details) [38]. In this system, which is illustrated in Figure 2, an HSA microcolumn was first used to extract a free drug fraction from a sample. A second HSA column was then placed on-line with the first column to further separate the extracted fraction from other sample components (e.g., drug that had dissociated from proteins in the sample during passage through the first column). The mobile phase for both columns was pH 7.4, 0.067 M potassium phosphate buffer. All samples containing sulfonylurea drugs were dissolved in this buffer. The mixtures of the drugs and normal HSA or glycosylated HSA were prepared by dissolving each protein in the corresponding drug solutions. These drug/protein mixtures were incubated for at least 30 min at 37 °C before injection to allow equilibrium to be established between the free and protein-bound fractions of the drug in the sample [37]. Replicate injections ($n = 4$) were made for all samples and standards onto the system. The concentrations of the drugs in the tested samples were representative of the therapeutic ranges for these agents (i.e., 184–370 μ M for tolbutamide, 61–216 μ M for acetohexamide, 15–31 μ M for gliclazide, and 0.08–0.4 μ M for glibenclamide) [50,51]. The samples also contained concentrations of normal HSA or glycosylated HSA that were representative of the physiological levels of this protein (i.e., 526–752 μ M) [50].

The column sizes and flow rates that were used for ultrafast affinity extraction were determined as described in Section 3.1. The HSA microcolumns that were used for this purpose in the final system had the following dimensions: 5 mm × 2.1 mm i.d. for tolbutamide and acetohexamide, 10 mm × 2.1 mm i.d. for gliclazide, and 1 mm × 2.1 mm i.d. for glibenclamide. A 1.0 µL sample injection was made onto each of these HSA microcolumns at an initial flow rate of 2.25 mL/min for tolbutamide, 2.5 mL/min for acetohexamide, 2.5 mL/min for gliclazide, and 0.35 mL/min for glibenclamide during extraction of the free drug fractions. The valve to which these microcolumns were connected was then placed on-line with the second HSA column at 1.5 min after injection for tolbutamide, 2.2 min for acetohexamide, 0.85 for gliclazide, and 5.0 min for glibenclamide. The flow rate was changed at the same time to 0.50, 0.75, 0.50 or 0.25 mL/min, respectively, for the second portion of the separation. The size of the second HSA column was 10 mm × 2.1 mm i.d. for tolbutamide and acetohexamide, 25 mm × 2.1 mm i.d. for gliclazide, and 5 mm × 2.1 mm i.d. for glibenclamide. The following wavelengths were employed for absorbance detection: 227 nm for tolbutamide, 248 nm for acetohexamide, 226 nm for gliclazide, and 302 nm for glibenclamide. The free drug concentration was determined by comparing the resulting peak area to those obtained for standards containing only the drug (see Supplementary Material for more details). The free fraction was calculated by dividing the free drug concentration by the total concentration of the drug in the sample [37,38,47].

3. Results and discussion

3.1. Optimization of conditions for ultrafast affinity extraction

The residence time for the sample in the extraction column is one factor to consider when using ultrafast affinity extraction to isolate a free drug fraction. One way this factor can be adjusted is by altering the column size that is used for the extraction process [37,38,47]. A relatively short column will provide a smaller column residence time than a longer column operated at the same flow rate. A shorter column will also have a lower backpressure, allowing work at higher flow rates, while a longer column size should provide higher retention and a better separation of the free drug fraction from other sample components [37,47].

The HSA microcolumns that were used in this report for ultrafast affinity extraction had sizes of 1–10 mm × 2.1 mm i.d. These microcolumns were used at flow rates up to 3.5 mL/min, giving column backpressures of 1.9–3.2 MPa or less under these conditions. The ultrafast affinity extractions that were conducted with gliclazide, which has relatively weak binding to HSA [33,47], used 10 mm × 2.1 mm i.d. microcolumns. The other drugs that were considered, which have stronger binding to HSA [30–34], were examined by using 5 mm × 2.1 mm i.d. microcolumns (e.g., for acetohexamide and tolbutamide) or even 1 mm × 2.1 mm i.d. microcolumns (for glibenclamide).

The flow rate that was used for ultrafast affinity extraction was also adjusted to control the sample residence time in the affinity microcolumns (e.g., see Figure 3). Low-to-moderate injection flow rates can result in some dissociation of a drug from its complexes with proteins in the sample, giving an apparent free fraction that is higher than what was present

in the original sample [37–39,46,47]. However, this effect can be minimized or made negligible when the flow rate is raised above a certain threshold level, which typically occurs when the sample residence time in the column is on the order of a few hundred milliseconds (ms) [37–39,47]. The size of this effect will vary from one drug to the next, as is shown in Figure 3, and depends on such factors as the rate of dissociation of the drug from the soluble proteins and the degree of drug-protein binding that was present in the original sample [47].

Experiments were conducted early in this work to identify the flow rate conditions that could be used during ultrafast affinity extraction to measure the free fractions of sulfonylurea drugs in the presence of soluble HSA. These initial studies were carried out by injecting 1.0 μ L samples that contained 5–10 μ M of the desired drug or a mixture containing 5–10 μ M of this drug and 5–20 μ M of normal HSA. Some typical results are given in Figure 3. It was found for tolbutamide that a consistent free drug fraction was obtained when using a minimum flow rate of 2.0 mL/min for sample injections on a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn (i.e., a column residence time of 415 ms or less). Similar experiments with the other sulfonylurea drugs provided the following conditions for their ultrafast affinity extraction: acetohexamide, 2.5 mL/min or greater when using a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn; gliclazide, 2.5 mL/min or greater when using a 10 mm $\times$ 2.1 mm i.d. HSA microcolumn; and glibenclamide, 0.30 mL/min or greater when using a 1 mm $\times$ 2.1 mm i.d. HSA microcolumn. These conditions for acetohexamide, gliclazide and glibenclamide corresponded to maximum column residence times during the extraction process of 333 ms, 665 ms and 554 ms, respectively. This range of residence times was in good agreement with previous kinetic studies or extraction experiments that have used some of the same analytes or drugs with similar affinities for HSA [37–39,47].

Figure 4(a) shows some typical chromatograms that were obtained when using a HSA microcolumn for the ultrafast affinity extraction of tolbutamide. These results were generated by using samples that contained a therapeutic level of tolbutamide, in the absence or presence of a physiological concentration of HSA, and which were injected onto a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn at 2.25 mL/min. In this example, the non-retained peak for the soluble protein and drug-protein complex appeared within 30–40 sec (s) of injection (Note: injections of only HSA or glycated HSA produced similar non-retained peaks). The peak for the retained, free fraction of tolbutamide had a maximum that appeared at 50–55 s and eluted within 2 min from the HSA microcolumn. Similar chromatograms were generated for gliclazide, acetohexamide, and glibenclamide at therapeutically-relevant concentrations. The non-retained peaks for these other drugs occurred within 0.3–0.6 min (at 2.5 mL/min) or within 3.0 min (at 0.35 mL/min), respectively. The retained peaks for these other drugs had maxima occurring at 40–45 s, 65–70 s, or 8.0–8.3 min and eluted within 1.5–2.0 min, 3.5–4.0 min or 13–15 min under the final conditions that were employed for ultrafast affinity extraction.

3.2. Optimization of conditions for multi-dimensional affinity system

Baseline resolution was not obtained between the non-retained and retained peaks for most of the sulfonylurea drugs when using only a single HSA microcolumn. This issue was

overcome by using a multi-dimensional system with a second HSA column that was placed online after the free drug fraction had begun to elute from the first column. Figure 4(b) shows some chromatograms that were generated during the second part of this separation. The additional column helped to improve resolution of the retained free drug fraction from other sample components, including any drug that had been released from drug-protein complexes in the sample during passage through the first column.

The results in Figure 4(b) were obtained for samples containing 185 μM tolbutamide/526 μM HSA and using various times for switching the second column on-line with the first HSA microcolumn. A single retained peak was recovered and observed for tolbutamide on this multidimensional affinity system. This peak had a maximum that appeared at 7.8–8.4 min after sample injection, as acquired at a flow rate of 0.50 mL/min when the second HSA column was placed on-line and this column that had a size of 10 mm $\times$ 2.1 mm i.d. Similar chromatograms were observed for acetohexamide, gliclazide and glibenclamide, which gave peaks with maxima at 10.0–10.5 min (final flow rate, 0.75 mL/min; second HSA column size, 10 mm $\times$ 2.1 mm i.d.), 8.4–8.6 min (0.50 mL/min; 25 mm $\times$ 2.1 mm i.d.), and 27.2–27.7 min (0.25 mL/min; 5 mm $\times$ 2.1 mm i.d.), respectively. The flow rate and column conditions that were utilized for the second part of this multi-dimensional system were again selected based on the retention of each drug on the HSA columns, the backpressures of these columns, and the overall time of analysis.

The time at which the second column was placed on-line with the first column after sample injection was an important factor to consider when measuring small free drug fractions on the multi-dimensional affinity system. Figure 5 shows how the peak area and apparent free drug fraction changed for acetohexamide as the time of this switch was varied. Similar trends were noted for the other drugs that were examined in this report. The time of this switching event occurred within the time frame that the free drug peak eluted from the first column. As this switching time was increased, the total peak area (i.e., a measure of the recovery) seen for the drug on the second column decreased, as demonstrated in Figure 5(a). This effect occurred because less of the retained peak from the first column was passed onto the second column at longer switching times. A correction for this effect was made by comparing the peak areas for the free drugs that were captured from samples to the peak areas that were obtained on the same system for standards with known concentrations of these drugs.

Less contamination due to other sample components, including drugs that had dissociated from proteins in the sample during ultrafast affinity extraction, also occurred as the switching time was increased. This effect resulted in the apparent free fraction dropping and approaching a constant value as the switching time was increased. In Figure 5(b), the measured free fraction became a consistent value when a switching time of 2.2 min or longer was used for acetohexamide. However, there was also a loss of precision in the measured free fraction if the switching time was too long because only a small amount of the free drug was then being passed onto the second column. This latter effect occurred in Figure 5(b) at switching times longer than 2.3 min. The combination of these effects meant that there was a relatively well-defined window of times for the switching event that could be used to provide both reasonable accuracy and precision for free fraction measurements in

the multi-dimensional affinity system. Based on these criteria and similar plots to those Figure 5(b) for the other drugs that were examined, the switching times that were used in all later parts of this study were 2.2 min for acetohexamide, 1.5 min for tolbutamide, 0.85 min for gliclazide, and 5.0 min for glibenclamide.

3.3. Measurement of free fractions for sulfonylurea drugs

The next part of this project examined the use of ultrafast affinity extraction and the multi-dimensional system to measure the free fractions of various sulfonylurea drugs in the presence of normal HSA or glycated HSA at typical therapeutic or physiological levels for these agents. Table 1 summarizes the results of these measurements. This approach was found to be useful in reliably measuring small free drug fractions (e.g., values as small as 0.09% to 2.58%). This method had a good absolute precision for these values (± 0.02 –0.5%) and a reasonable relative precision (± 3.6 –30%). These free drug fractions also agreed with predicted values based on previously-measured binding constants for the same drugs with similar protein preparations (see Table 2 and Supplementary Material) [30–34]. In addition, these results gave good correlation with free fractions that were determined for some of the same samples when using ultrafiltration as a reference method (see Supplementary Material).

The sulfonylurea drugs that were used in this study are known to have strong binding to HSA and to have only small free fractions in serum [7,8]. The free fractions in Table 1 show how this biologically-active fraction can be only a few percent of the total drug content in serum at therapeutic concentrations. These results further show how the size of this fraction can vary with the type of sulfonylurea drug that is being administered. These differences are directly related to the affinities of these drugs for HSA (see Table 2) and their total therapeutic concentrations (e.g., see Supplementary Material).

The results in Table 1 made it further possible to compare the free fractions that were seen for each drug in the presence of normal HSA or HSA with glycation levels similar to those seen in prediabetes (gHSA1) or diabetes (gHSA2). It was found that the level of this glycation and the type of drug that was being examined both affected the change that was noted in the free fraction. For example, going from normal HSA to gHSA1 (which had only a modest level of glycation) produced a possible 0.85- to 1.30-fold change in the free fractions for these sulfonylurea drugs. This level of variation was not significant at the 95% confidence level and was only significant at the 90% confidence level for tolbutamide. However, going from normal HSA to gHSA2 gave changes of 0.59- to 1.56-fold for tolbutamide, glibenclamide, and gliclazide, which were all significant at the 95% confidence level. Acetohexamide was the only exception, which gave only a 1.19-fold change between normal HSA and gHSA2 that was not significant. Similar trends were seen when comparing the free fractions measured in the presence of gHSA1 versus gHSA2.

These variations agree with a previous estimate that HSA glycation may lead to a 0.6- to 1.7-fold change in the free drug fractions for these sulfonylurea drugs (i.e., as based on prior binding studies using both *in vitro* and *in vivo* glycated HSA) [7,26]. Such changes in binding have been attributed to variations in the amount and types of glycation-related modifications that are formed at or near specific regions on HSA (e.g., Sudlow sites I and II)

as the overall level of glycation for this protein is increased [22–25]. These variations in the free fractions of sulfonylurea drugs are of concern in that they represent a large difference in the actual versus expected dosage of such drugs, with the possible result being either inadequate control of elevated glucose levels (i.e., hyperglycemia) or the production of low glucose levels (hypoglycemia) in patients [7].

3.4. Estimation of affinity for sulfonylurea drugs with normal HSA or glyated HSA

The free fraction results that were obtained by ultrafast affinity extraction and the multidimensional affinity system were also used to estimate overall affinities of the sulfonylurea drugs with normal HSA or glyated HSA. It is known that these drugs each have at least two major binding regions on HSA [7,27,30–34], so in this case the free fractions were used to calculate a global affinity constant (nK_a') for these interactions. These global affinity constants were determined by using Eqn. (1), which can provide either the association equilibrium constant (K_a) for a system with single-site binding or the global affinity constant for a system involving multiple and independent binding regions [37,38,47].

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

In this equation, [D] and [P] are the total concentrations of the drug and soluble protein in the original sample, while F_0 is the free fraction measured for the drug in this sample.

Table 2 shows the values of nK_a' that were found by using Eqn. (1) and the free fractions from Table 1 for the sulfonylurea drugs with normal HSA or glyated HSA. No significant changes in these values were noted when varying the drug or protein concentration within the therapeutic or physiological range of these agents. As an example, the use of samples that contained 185 μM tolbutamide and 526 μM HSA, 185 μM tolbutamide and 752 μM HSA, 370 μM tolbutamide and 526 μM HSA, or 370 μM tolbutamide and 752 μM HSA all gave estimates for nK_a' that were in the range of $0.95\text{--}1.08 \times 10^5 \text{ M}^{-1}$. This indicated there was a negligible effect of sample concentration on the global affinity constants that were estimated through ultrafast affinity extraction. A similar conclusion has been reached in the use of this approach with other drug/protein systems at non-therapeutic concentrations [47].

The values in Table 2 agreed well with global affinity constants that were determined through previous binding data acquired for the same drugs and using comparable preparations of glyated HSA [30–34]. The values estimated by ultrafast affinity extraction and the multidimensional affinity system had a relative precision of $\pm 6\text{--}29\%$ and differed from these literature results by only $0\text{--}22\%$ (average difference, 7%). These global affinity constants also agreed with values that were determined for some of the same samples by using ultrafiltration (see Supplementary Material). For instance, the nK_a' values obtained by ultrafiltration for acetohexamide with the same preparations of HSA and glyated HSA differed by only $0\text{--}7.7\%$ (average difference, 4.5%) from the results shown in Table 2 based on ultrafast affinity extraction.

4. Conclusions

This study examined the use and development of ultrafast affinity extraction and a multidimensional affinity system for measuring free drug fractions at therapeutic levels. This approach was then used to compare the free fractions and global affinity constants of several sulfonylurea drugs in the presence of normal HSA or glycated HSA. Factors that were considered in the optimization of this approach included the column size, flow rate and column residence times that could be used with sulfonylurea drugs during ultrafast affinity extraction with an HSA microcolumn. The flow rate and column size used for the second affinity column in the system were also considered, as well as the time at which this second column was placed on-line with the HSA microcolumn. This system was a general one that could also be adapted for use in coupling an HSA microcolumn with other types of analytical columns, such as those containing reversed-phase, ion-exchange or size-exclusion supports. These latter types of columns will be considered in future work as this approach is further optimized or extended to more complex mixtures of free solutes (e.g., mixtures of drugs or drugs and their metabolites).

There were several attractive features to this approach. First, the use of affinity microcolumns containing HSA, which has both fast and relatively strong binding to sulfonylurea drugs, made it possible to isolate the free fractions of these drugs under time conditions that minimized dissociation of the protein-bound fractions of the same drugs as the samples passed through the microcolumns [37–40,45–47]. Given the relatively fast rate of these dissociation processes, such a separation would be difficult to obtain by using a more conventional reversed-phase or size-exclusion column [36,37,40,46,47,52]. Another advantage of using an HSA microcolumn for these free drug measurements is that the same immobilized agent, which binds to many pharmaceuticals, could be used for all of the drugs of interest in this study. In addition, the mobile phase for this column was an aqueous buffer with a physiological pH, which should not have significantly affected the drug-protein interactions in the original samples. Finally, the use of these microcolumns for free drug measurements required only 1.0 μL of a sample per injection and was able to measure free drug fractions as small as 0.09–2.58% with an absolute precision of ± 0.02 –0.5%.

The free drug fractions and global affinity constants that were determined by this approach showed good agreement with those predicted from previous binding studies or determined through a reference method. This technique was used to examine the possible effects of glycation on the binding of sulfonylurea drugs to HSA. It was found that glycation could affect the free fractions of sulfonylurea drugs at typical therapeutic levels and that the size of this effect varied with the level of HSA glycation. The same multi-dimensional approach could be utilized to examine other drug-protein systems. Possible future applications include the clinical analysis of free drug or hormone levels for disease diagnosis or treatment [38–40, 45, 46] and the high-throughput screening of drug-protein interactions [36–38, 47].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health under grants R01 GM044931 and R01 DK069629. Additional support for R. Matsuda was obtained through a fellowship from the Molecular Mechanisms of Disease Program at the University of Nebraska-Lincoln.

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Highlights

- Multi-column ultrafast affinity extraction was used to measure free drug fractions.
- This system was used with sulfonylurea drugs and human serum albumin (HSA).
- The effects of HSA glycation on the free drug fractions were examined.
- Results were obtained within minutes and gave good agreement with predicted values.

Figure 1.
Structures of common sulfonylurea drugs. The portion within the dashed box represents the core structure of these drugs.

Figure 2.

General scheme for the separation of the free and protein-bound fractions of a drug in a sample and measurement of the free drug fraction through the use of ultrafast affinity extraction and a multi-dimensional affinity system.

Figure 3.

Effect of injection flow rate on ultrafast affinity extraction of the free fractions for various sulfonylurea drugs. The results were obtained at pH 7.4 and 37 °C for 1.0 μ L injections of (a) 10 μ M tolbutamide in the presence of 20 μ M HSA and injected onto a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn, (b) 10 μ M acetohexamide in the presence of 20 μ M HSA and injected onto a 5 mm $\times$ 2.1 mm i.d. HSA microcolumn, (c) 10 μ M gliclazide in the presence of 20 μ M HSA and injected onto a 10 mm $\times$ 2.1 mm i.d. HSA microcolumn, or (d) 5 μ M glibenclamide in the presence of 5 μ M HSA and injected onto a 1 mm $\times$ 2.1 mm i.d. HSA microcolumn. The values shown for the free fraction on the y-axis have no units, because they represent the ratio of the free drug concentration vs. the total concentration of the same drug in each sample.

Figure 4.

Typical chromatograms obtained at pH 7.4 and 37 °C on (a) a 5 mm × 2.1 mm i.d. HSA microcolumn at 2.25 mL/min for 1 µL injections of 185 µM tolbutamide in the absence or presence of 526 µM HSA, or (b) the retained peak obtained on a 10 mm × 2.1 mm i.d. HSA column at 0.5 mL/min and that was put in line with the first 5 mm × 2.1 mm i.d. HSA microcolumn after various times following injection of a 1 µL sample 185 µM tolbutamide/526 µM HSA onto the first column at 2.25 mL/min. The change in signal at 5–10 s for the chromatogram in (a) for only tolbutamide was due to a temporary change in pressure that occurred when the sample was injected onto the system.

Figure 5.

Effect of valve switching time on (a) the area of the final peak observed for a 138 μM acetohexamide sample that was applied to the multi-dimensional affinity system, and (b) the apparent free fractions that were measured by this system for a sample that contained 138 μM acetohexamide and 640 μM HSA. These measurements were all made at pH 7.4 and 37 $^{\circ}\text{C}$ using a 5 mm $\times$ 2.1 mm i.d. HSA column operated at 2.5 mL/min, followed by the on-line addition of a 10 mm $\times$ 2.1 mm i.d. HSA column and the use of a flow rate of 0.75 mL/min at the given switching times. The error bars represent a range of ± 1 standard error of the mean ($n = 4$). The times shown for the valve switching event represent the time that has elapsed since sample injection, and were selected to be beyond the elution time for the non-retained sample components leaving the first, affinity microcolumn in this system.

Table 1

Free drug fractions measured for various sulfonylurea drugs in the presence of normal HSA or glycated HSA (gHSA)

Drug and sample	Measured free fraction ^a		
	Normal HSA	gHSA1	gHSA2
Tolbutamide (275 μ M) + HSA (640 μ M)	2.58 ($\pm$ 0.31)%	2.20 ($\pm$ 0.08)% ^b	1.58 ($\pm$ 0.09)% ^c
Acetohexamide (138 μ M) + HSA (640 μ M)	1.18 ($\pm$ 0.35)%	1.53 ($\pm$ 0.27)%	1.41 ($\pm$ 0.16)%
Gliclazide (23 μ M) + HSA (640 μ M)	2.17 ($\pm$ 0.29)%	2.48 ($\pm$ 0.51)%	1.27 ($\pm$ 0.31)% ^c
Glibenclamide (0.4 μ M) + HSA (526 μ M)	0.09 ($\pm$ 0.02)%	0.11 ($\pm$ 0.03)%	0.14 ($\pm$ 0.02)% ^d

^a These free drug fraction values were determined at pH 7.4 and 37 °C by using ultrafast affinity extraction and a multi-dimensional affinity system. The values in parentheses represent a range of $\pm$ 1 S.D. ($n = 4$), as determined by error propagation.

^b This value was significantly different from the result for normal HSA at the 90% confidence level but not the 95% confidence level.

^c This value was significantly different from the results for normal HSA and gHSA1 at the 95% confidence level.

^d This value was significantly different from the results for normal HSA at the 95% confidence level and from gHSA1 at the 90% but not the 95% confidence level.

Table 2
Global equilibrium constants estimated for sulfonylurea drugs with normal HSA or glycosylated HSA (gHSA)^a

Drug & Sample	Global affinity constant, nK_a ($\times 10^5$ M ⁻¹)				
	Normal HSA	Literature value ^b	gHSA1 ^c	Literature value ^b	gHSA2 ^d
Tolbutamide (275 μ M) + HSA (640 μ M)	1.0 ($\pm$ 0.1)	1.1 ($\pm$ 0.1)	1.2 ($\pm$ 0.1)	1.3 ($\pm$ 0.1)	1.7 ($\pm$ 0.1)
Acetohexamide (138 μ M) + HSA (640 μ M)	1.7 ($\pm$ 0.5)	1.7 ($\pm$ 0.1)	1.3 ($\pm$ 0.2)	1.4 ($\pm$ 0.1)	1.4 ($\pm$ 0.1)
Glitclazide (23 μ M) + HSA (640 μ M)	0.73 ($\pm$ 0.10)	0.79 ($\pm$ 0.05)	0.64 ($\pm$ 0.13)	0.64 ($\pm$ 0.05)	1.26 ($\pm$ 0.31)
Glibenclamide (0.4 μ M) + HSA (526 μ M)	21.1 ($\pm$ 4.7)	21.6 ($\pm$ 8.0)	17.3 ($\pm$ 4.7)	18.9 ($\pm$ 8.0)	13.6 ($\pm$ 1.9)
					13.7 ($\pm$ 4.0)

^aThe global affinity constants from this study are based on the free drug fractions listed in Table 1. All of the values in this table are for drug-protein binding that occurs at pH 7.4 and 37 °C. The values in parentheses represent a range of $\pm$ 1 S.D. ($n = 4$), as determined by error propagation.

^bThe global affinity constants from the literature were calculated by using the association equilibrium constants and binding stoichiometries that had been measured for these drugs at Sudlow sites I and II [30–34], as well as at the digitoxin site in the case of glibenclamide [34]. A similar range of values has been reported in Ref. [28].

^cThe level of glycosylation for gHSA1 was similar to that of an *in vitro* glycosylated HSA sample that was used in the previous binding studies and that contained 1.31 ($\pm$ 0.05) mol hexose/mol HSA [29,31–34].

^dThe glycosylation level for gHSA2 was between the glycosylation levels for two *in vitro* glycosylated HSA samples that were used in previous binding studies and that contained 2.34 ($\pm$ 0.13) or 3.34 ($\pm$ 0.14) mol hexose/mol HSA [29,31–34].