SUPPORTING INFORMATION

Determination of Rate Constants and Equilibrium Constants for Solution-Phase Drug-Protein Interactions by Ultrafast Affinity Extraction

Xiwei Zheng, Zhao Li, Maria I. Podariu, and David S. Hage*

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588, USA

*Corresponding author. Phone: +1-402-472-2744; Fax: +1-402-472-9402; E-mail: dhage1@unl.edu

ABSTRACT

This Supporting Information contains additional discussion on the chromatographic conditions that were used in this study, including some examples of typical chromatograms. Other items that are included are the derivation of key equations that were used in this report, data that were acquired at various sample concentrations, and a summary of results that were obtained when the measured dissociation rate constants and association equilibrium constants were employed to estimate association rate constants.

Chromatographic conditions and results. Samples containing 10 μM of the desired drug or a mixture of 10 μM drug and 20 μM soluble HSA were injected at the following flow rates, as determined by the degree of retention for each drug on the microcolumns and their rate of dissociation from soluble HSA: acetohexamide, 0.5-2.75 mL/min; chlorpromazine, 0.5-2.0 mL/min; gliclazide, 0.25-3.0 mL/min; tolbutamide, 0.25-2.5 mL/min; verapamil, 0.25-2.5 mL/min; and warfarin, 0.5-3.5 mL/min. The following wavelengths were employed for absorbance detection: acetohexamide, 248 nm; chlorpromazine, 254 nm; gliclazide, 226 nm; tolbutamide, 227 nm; verapamil, 229 nm; and warfarin, 308 nm.

Some typical chromatograms that were obtained in this study are shown in Figure 1S. Similar chromatograms were acquired for all of the drugs that were examined in this report. To generate these chromatograms, each drug was injected in either the presence or absence of excess soluble HSA onto an HSA microcolumn. As the sample passed through the microcolumn at a moderate-to-high flow rate, the protein-bound fraction of the drug and the excess soluble protein eluted as a non-retained peak while the free fraction of the drug was extracted, retained and later eluted from the column. Depending on the application flow rate, the non-retained peak eluted in 2 min or less. The retained peak for the free drug fraction eluted within 2-10 min of sample injection for all of the drugs that were studied, depending on the column size, degree of retention for the drug on the column and the injection flow rate. For drugs with low-to-moderate affinities for HSA, results were obtained within only 2-6 min.

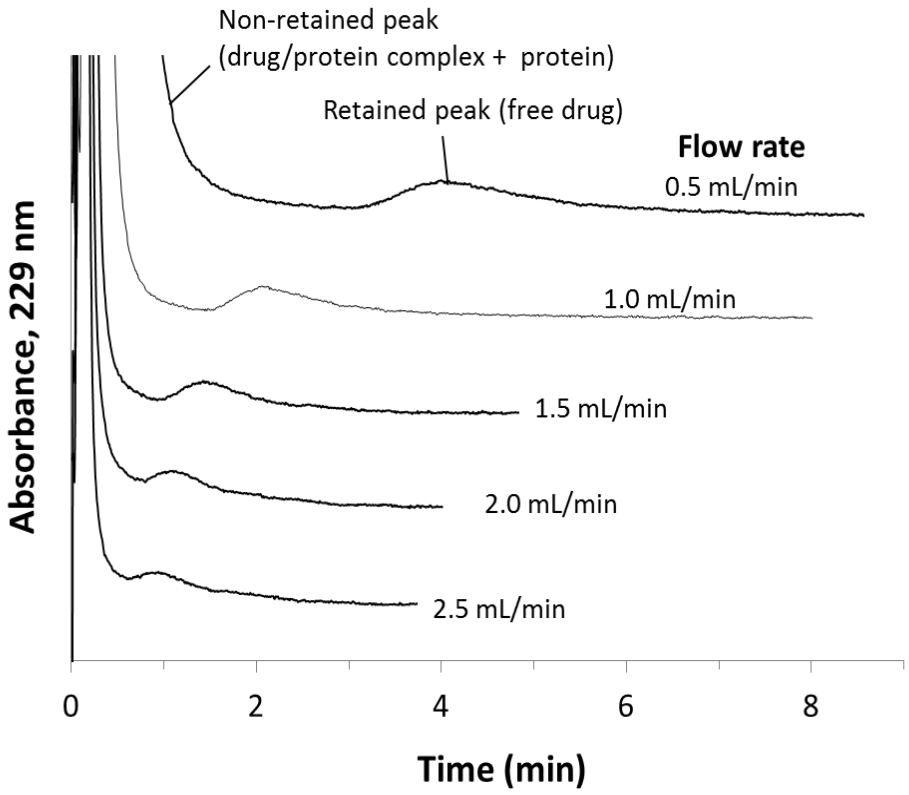

Figure S1. Chromatograms obtained at various flow rates for 1 μL injections of 10 μM verapamil and 20 μM HSA onto a 10 mm × 2.1 mm i.d. HSA microcolumn at pH 7.4 and 37 °C.

Derivation of eq 4 and eq 5. The derivation of eqs 4 and 5 is based on the reaction model that is given in eq 3.

$$\mathrm{AP} \xrightarrow{k_\mathrm{d}} \mathrm{A} + \mathrm{P} \tag{3}$$

In this model, the observed rate of dissociation for the soluble complex AP is described by the rate expression in eq 1S and by the equivalent integrated forms of this expression in eqs 2S and 3S.

$$\frac{d[\mathrm{AP}]}{dt} = -k_\mathrm{d}[\mathrm{AP}] \tag{1S}$$

$$\ln\frac{[\mathrm{AP}]_t}{[\mathrm{AP}]_0} = -k_\mathrm{d}t \tag{2S}$$

$$\ln\frac{[\mathrm{AP}]_0}{[\mathrm{AP}]_t} = k_\mathrm{d}t \tag{3S}$$

The term $[\mathrm{AP}]_0$ in these equations represents the initial concentration of soluble complex AP, and $[\mathrm{AP}]_t$ is the concentration of this complex after it has been allowed to dissociate for time *t*. It is possible to relate the values of $[\mathrm{AP}]_0$ and $[\mathrm{AP}]_t$ to the original free fraction (F_0) of A in the sample and the apparent free fraction (F_t) after AP has been allowed to dissociate for time *t*. This is accomplished by using eqs 4S and 5S, in which $[\mathrm{A}]_{\mathrm{tot}}$ represents the total concentration of A in the original sample.

$$[\mathrm{AP}]_0 = (1 - F_0)[\mathrm{A}]_{\mathrm{tot}} \tag{4S}$$

$$[\mathrm{AP}]_t = (1 - F_t)[\mathrm{A}]_{\mathrm{tot}} \tag{5S}$$

Substitution of eqs 4S and 5S into eqs 2S and 3S leads to the equivalent expressions in eqs 4 and 5 that can be used with ultrafast affinity extraction to provide information on the dissociation rate of A from P in the solution-phase sample.

$$\ln\frac{(1-F_0)}{(1-F_t)} = k_\mathrm{d}t \tag{4}$$

$$\ln\frac{1}{(1-F_t)} = k_\mathrm{d}t - \ln(1 - F_0) \tag{5}$$

Measurement of K_a and k_d at various sample concentrations. Samples with various drug/protein concentrations were used to further test the validity and robustness of this method. Table 1S provides examples of some combinations of drugs and HSA that were used, as well as the values of K_a (or nK_a') and k_d that were measured. The k_d that was measured for warfarin with HSA was consistently in the range of 0.72-0.94 s^{-1} under all of the sample conditions that were examined, giving good agreement with values of 0.41-2 s^{-1} that have been reported for this system in the literature.[S1,S2,S3] The k_d values for tolbutamide and acetohexamide varied from only 0.58 to 0.64 s^{-1} and 0.63 to 0.67 s^{-1}, respectively, under each of the tested conditions, also giving good agreement with previous literature values.[S1,S2,S3,S4] Similar consistency in the value of k_d was noted for chlorpromazine as the sample concentrations were altered. These results all indicated that the method used in this report for dissociation rate constant measurements was robust and not affected significantly by changes in the sample concentrations under the conditions that were used in this study.

A similar conclusion was made when comparing the association equilibrium constants that were measured using different sample concentrations, as demonstrated in Table 1S. For instance, the K_a that was estimated for warfarin was found in the range of 1.7-2.4 × 10^5 M^{-1}, under all of the tested sample conditions, in good agreement with reference values.[S5,S6,S7] The K_a for chlorpromazine that was in the range of 0.62-0.65 × 10^5 M^{-1}, also agreeing with prior values in the literature.[S8] The nK_a' values for tolbutamide and acetohexamide were consistently in the range of 1.1 × 10^5 M^{-1} and 1.7-1.8 × 10^5 M^{-1}, respectively, as noted in prior work.[S9,S10]

Table S1 also lists some of the free drug fractions that were measured or estimated to be present at equilibrium in each of the samples that were examined. These values, and those used in studying the other drugs that were considered in this work, covered a range of 25% to almost

80%. Although the usable free fraction range of this method has not yet been fully explored, these results already indicate that a relatively broad range of free drug fractions can also be examined by this method, which further minimizes the effect a change in sample concentration may have on the final results of this approach.

Table 1S. Association equilibrium constants and dissociation rate constants measured at various sample concentrations for drugs with soluble HSA by using ultrafast affinity extraction on HSA microcolumns[a]

Sample	F_0 (%)	k_d (s^{-1})	K_a or nK_a' ($\times 10^5\ M^{-1}$)
5 μM Warfarin/10 μM HAS	44.7 (± 4.1)[b]	0.94 (± 0.10)	1.7 (± 0.3)[b]
10 μM Tolbutamide/10 μM HAS	59.8 (± 5.3)	0.64 (± 0.01)	1.1 (± 0.3)
10 μM Acetohexamide/10 μM HSA	53.1 (± 1.1)	0.63 (± 0.09)	1.7 (± 0.1)
10 μM Chlorpromazine/10 μM HSA	69.0 (± 2.6)	3.40 (± 0.28)	0.65 (± 0.10)
10 μM Warfarin/20 μM HAS	25.0 (± 2.7)[b]	0.72 (± 0.05)	2.4 (± 0.4)
10 μM Tolbutamide/20 μM HAS	39.5 (± 6.6)	0.58 (± 0.04)	1.1 (± 0.4)
10 μM Acetohexamide/20 μM HSA	29.5 (± 4.9)	0.63 (± 0.03)	1.8 (± 0.5)
10 μM Chlorpromazine/20 μM HSA	51.7 (± 1.9)	3.35 (± 0.30)	0.62 (± 0.05)

[a]These values were measured at pH 7.4 and 37°C. The values in the parentheses represent a range of ± 1 SD. The F_0 were measured experimentally, except were otherwise indicated by footnote (b). The k_d and K_a values were determined by using eqs 5 and 6, except where indicated otherwise by footnote (b).

[b]These values for F_0 were determined from the intercept of eq 5. The K_a values in these cases were calculated based on eq 6 and these F_0 values.

Estimation of association rate constants. The K_a and k_d values that were determined in this study were used to estimate the second-order association rate constant (k_a) for each drug with soluble HSA, as based on the relationship $k_a = k_d K_a$. The values that were used in these calculations and the resulting estimates of k_a that were obtained are summarized in Table 2S. The results for warfarin gave good agreement with previous k_a values that have been reported for this drug with HSA at pH 7.4 and 37ºC.[S1,S2,S3,S11] The k_a values estimated for acetohexamide, tolbutamide, and racemic verapamil with HSA were similar to those obtained when using previously-reported k_d and K_a or nK_a' values for these same systems.[S2,S4,S9,S10,S12] In addition, k_a values found for gliclazide and chlorpromazine agreed with the range of k_a value that have been noted in prior work for other drugs with similar association equilibrium constants and dissociation rate constants for HSA.[S2,S4,S13,S14,S15]

Table 2S. Association rate constants calculated for various drugs with soluble HSA by using ultrafast affinity extraction on HSA microcolumns[a]

Drug	k_d **(s^{-1})**	K_a **or** nK_a**' (M^{-1})**	k_a **($M^{-1}s^{-1}$)**
Warfarin	0.72 (± 0.05)	2.4 (± 0.4) × 10^5	1.7 (± 0.3) × 10^5
Tolbutamide	0.58 (± 0.04)	1.1 (± 0.4) × 10^5	6.4 (± 2.4) × 10^4
Acetohexamide	0.63 (± 0.03)	1.8 (± 0.5) × 10^5	1.1(± 0.3) × 10^5
Verapamil	0.36 (± 0.02)	1.5 (± 0.4) × 10^4	5.4 (± 1.5) × 10^3
Gliclazide	0.59 (± 0.04)	8.0 (± 0.6) × 10^4	4.7 (± 0.5) × 10^4
Chlorpromazine	3.35 (± 0.30)	6.2 (± 0.5) × 10^4	2.1 (± 0.3) × 10^5

[a]The k_a values were calculated based on binding and dissociation data that were measured at pH 7.4 and at 37^{o}C for samples containing 10 µM of the given drug and 20 µM of HSA. The values in the parentheses represent a range of ± 1 SD. The k_d and K_a values in this table were determined by using experimentally measured free fraction values and eqs 5 and 6. The k_a values were calculated by using the formula $k_d = k_d K_a$.

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