Rapid determination of rate constants and binding constants for solution-phase drug-protein interactions by ultrafast affinity chromatography

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INTRODUCTION

Studies of the interactions between drugs and serum proteins are important in estimating the absorption, distribution, metabolism and excretion (ADME) of pharmaceutical agents within the body. High-performance affinity chromatography (HPAC) is a technique that has been increasingly used to characterize these drug-protein interactions. In an HPAC experiment, one of the components of such an interaction is immobilized as a binding agent and acts as the stationary phase. The other component is then injected onto the column or applied in the mobile phase. Recently, a new chromatographic-based approach has been developed to examine the degree of drug-protein interactions based on ultrafast affinity extraction and HPAC. In this technique, microcolumns containing immobilized antibodies or serum proteins are used for the rapid extraction of free drug or solute fractions as means to measure free drug fractions or estimate association equilibrium constants for drug-protein binding (see **Figure 1**) [1,2]. This approach has been found to be applicable to many drugs, such as warfarin, imipramine, ibuprofen and phenytoin [1]. Some advantages of this approach include its low-cost, speed, and good agreement with reference methods [1,2].

In this study, a new method was created in which ultrafast affinity extraction and free fraction analysis by HPAC were used to simultaneously measure the dissociation rate constants and association equilibrium constants for drug-protein interactions. Human serum albumin (HSA), which is the most abundant serum protein and is involved in the transport of several drugs and hormones within the body, was used as a model target for the binding studies. Various drugs were used to test this method and to compare the measured equilibrium and rate constants with literature values.

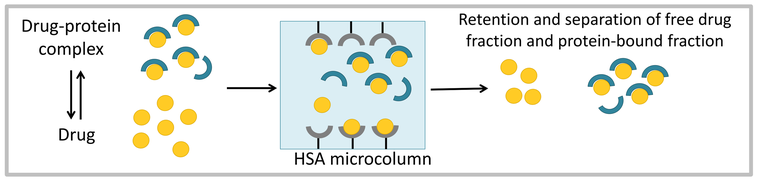

Figure 1. General scheme for the separation of the free and protein-bound fractions of a drug in a sample through the use of microcolumns that contain immobilized HSA

METHOD

▪ Free Drug Fraction Analysis

In the use of ultrafast affinity extraction for a free drug fraction analysis, a drug/solute in the presence or absence of a soluble binding protein is injected into an HPAC system which contains an immobilized binding agent. When the sample contains only the solute, most or all solute will be retained by the column (as illustrated in **Figure 2(a)**) On the other hand, if the sample contains a mixture of the solute and protein, the protein-bound fraction and excess protein in the injected mixture will not be retained and will elute from the column first, while the free fraction of the solute is extracted and retained by the column and elutes later, as shown in **Figure 2(b).** The areas of the peaks due to free solute fraction and pure solute are then measured and compared to determine the free solute fraction in the sample.

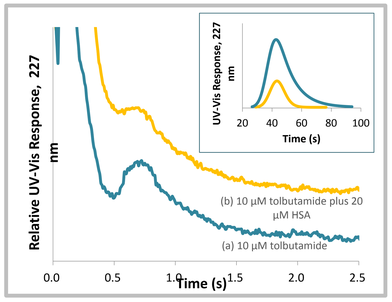

Figure 2. Chromatograms for injections of (a)10 µM tolbutamide or (b) 10 µM tolbutamide plus 20 µM HSA on a 5 mm x 2.1 mm i.d. HSA microcolumn at 2.0 mL/min. The inset shows the peaks obtained after fitting the data by using PeakFit v4.1.2.

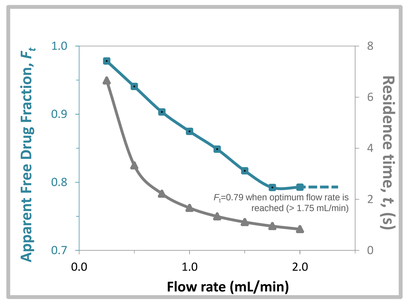

Figure 3. Effect of flow rate on column residence time (▲) and on the measurement of free fractions in samples containing 10 µM verapamil plus 20 µM HSA (■) using a 10 mm x 2.1 mm i.d. HSA microcolumn. The data were obtained at 37 °C.

THEORY

▪ Measurement of Dissociation Rate Constants (k_d)

The model that was used in this study was based on a set of two reactions that occur as the mixture of solute/analyte and protein (A and P) is applied to a microcolumn that contains an immobilized affinity ligand (L), which is also the same as P in the case of this study. The first of these reactions describes the equilibrium that is reached after mixing the analyte and protein in the sample before injection. The second reaction describes binding of the free fraction for the analyte to the immobilized ligand as the sample elutes through the microcolumn.

	Before sample enters the microcolumn	After sample enters in and elutes from the microcolumn
Reaction	$A + P \underset{k_d}{\overset{k_{-d}}{\rightleftharpoons}} A-P$	$A + L(s) \xrightarrow{k_a} A-L(s)$ $A + P \underset{k_d}{\overset{k_{-d}}{\rightleftharpoons}} A-P$
Conc. of each component	$[A]_0$-$[D\text{-}P]_0$ *Analyte*; $[P]_0$-$[A\text{-}P]_0$ *Excess protein*; $[A\text{-}P]_0$ *Analyte-protein complex*	*0 Analyte*; $[P]_0$-$[A\text{-}P]_t$ *Excess protein*; $[A\text{-}P]_t$ *Analyte-protein complex*

A-P complex spend time t to pass through the column

The dissociation process is generally a first-order reaction for drug-protein interactions.

$$\frac{d[A-P]}{dt} = -k_d[A-P] + k_{-d}[A]([P]_0 - [A-P]) \overset{[A]=0}{\Longrightarrow} -k_d[A-P] \quad (1)$$

Integrate Eq. (1)

$$ln\frac{[A-P]_t}{[A-P]_0} = -k_d\, t \quad (2)$$

$[A\text{-}P]_t=(1\text{-}F_t)[A]_0$

$$ln\frac{(1-F_0)}{(1-F_t)} = k_d t \ (3) \quad \text{or} \quad ln\frac{1}{(1-F_t)} = k_d t - ln(1-F_0) \ (4)$$

t = Column residence time
F_0 = Free fraction of analyte obtained at or above the optimum flow rate
F_t = Free fraction of analyte obtained when the column residence time is t.

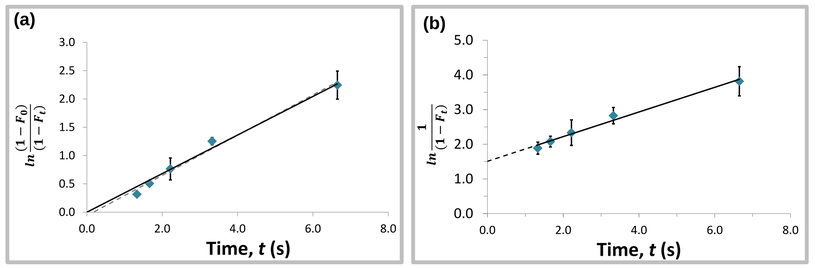

Figure 4. Measurement of dissociation rate constant for verapamil in samples containing 10 µM verapamil plus 20 µM HSA, as determined by using free fraction analysis according to (a) Eq. (3) or (b) Eq. (4).

▪ Measurement of Association Equilibrium Constants (K_a)

For a solute/drug and protein interaction that involves 1:1 binding, the free solute fraction (F_0) and association equilibrium constant (K_a) can be described by Eqs (5)-(6). Based on these equations, the value of K_a can also be calculated from the measured free solute fraction.

$$F_0 = \frac{[A]_0 - [A-P]_0}{[A]_0} \quad (5)$$

$$K_a = \frac{[A-P]_0}{([A]_0 - [A-P]_0)([P]_0 - [A-P]_0)} \quad (6)$$

$[A]_0$ = total conc. of drug in the sample
$[P]_0$ =total conc. of protein in the sample
$[[A\text{-}P]_0$ =conc. of drug-protein complex in the sample

RESULTS

- **Use of Free Fraction Analysis for Dissociation Rate Constant Measurements**

Table 1. Dissociation rate constant measured for various drugs with soluble HSA

Drug	HSA Microcolumn size	Measured k_d (s^{-1})	Literature k_d (s^{-1}) Refs. [3-5]
Warfarin	5 mm x 2.1 mm i.d.	0.72 (±0.05)	0.41-10
Tolbutamide	5 mm x 2.1 mm i.d.	0.58 (±0.06)	0.49 (±0.15)
Acetohexamide	5 mm x 2.1 mm i.d.	0.63 (±0.03)	0.58 (±0.02)
Verapamil	10 mm x 2.1 mm i.d.	0.36 (±0.02)	0.38 (±0.05)
Gliclazide	10 mm x 2.1 mm i.d.	0.59 (±0.04)	Not reported
Chlorpromazine	1mm x 2.1 mm i.d.	3.35 (±0.30)	Not reported

- **Use of Free Fraction Analysis for Association Equilibrium Constant Measurements**

Table 2. Association equilibrium constants measured for various drugs with soluble HSA.

Drug	Measured K_a (M^{-1}) Eqs (5)-(6)	Measured K_a (M^{-1}) Eqs (4)-(6)	Literature K_a (M^{-1}) Ref. [4-10]
Warfarin	2.4 (±0.4) x 10^5	1.6 (±0.2) x 10^5	(1.6-5) x 10^5
Tolbutamide	1.1 (±0.4) x 10^5	0.9 (±0.2) x 10^5	1.1(±0.3) x 10^5
Acetohexamide	1.8 (±0.5) x 10^5	1.3 (±0.1) x 10^5	1.7(±1.1) x 10^5
Verapamil	1.5 (±0.4) x 10^5	1.6 (±0.2) x 10^5	1.4(±0.1) x 10^4
Gliclazide	8.0 (±0.6) x 10^4	6.9 (±1.0) x 10^4	7.9(±0.5) x 10^4
Chlorpromazine	6.2 (±0.5) x 10^4	4.9 (±0.5) x 10^4	6.4x 10^4

CONCLUSIONS

A new method was used to measure dissociation rate constants for drug-protein interactions based on free fraction analysis by HPAC. It was found that the dissociation rate constants measured for various drugs with soluble HSA gave good agreement with literature values (see Table 1). Association equilibrium constants for the same drug-protein systems could also be determined by free fraction analysis, again giving results consistent with literature values (see Figure 2). These results demonstrate that free fraction analysis based on HPAC is a rapid approach for the study of drug-protein interactions that can provide information regarding both kinetic parameters and the affinity of a drug-protein interaction in the same experiment.

Acknowledgement

This work was supported by the National Institutes of Health under grant R01 GM044931.
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