# Analysis of drug binding with soluble proteins by ultrafast affinity chromatography using immobilized alpha$_1$-acid glycoprotein

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## INTRODUCTION

The overall extent of drug binding to serum proteins as reflected by the drug's non-bound or "free" fraction, and the rate of drug-protein dissociation in serum can have a significant effect on the pharmacokinetics and pharmacodynamics of a drug. Equilibrium dialysis has often been used to study extent of drug-protein binding and is well established reference method for such work, but suffers from long analysis times and large sample requirements. Many others methods has been examined to overcome these problems, including the use of high-performance affinity chromatography (HPAC). HPAC is based on the immobilization of biological ligand, like serum protein, to an HPLC support and injecting a solute like a drug into the column. Some advantages which make this approach of interest for drug binding studies are its speed and its ease of automation.

The kinetics of drug-protein interactions have been studied by chromatographic techniques such as the peak decay method or peak profiling [1] and techniques like surface plasmon resonance. But all these current techniques measure the interactions of drugs or targets with immobilized proteins. In this study, ultrafast affinity chromatography was explored as an alternative method to measure the kinetics and binding parameters of drugs with proteins directly in solution.

## GOALS OF STUDY

This study sought to access information on both the binding and dissociation kinetics of drugs with soluble serum proteins, by using ultrafast affinity chromatography. Ultrafast affinity chromatography uses miniaturized columns containing immobilized biological agent to quantitatively extract analytes such as drugs in sub-second time domain (**Figure 1**) [2].

Alpha$_1$-acid glycoprotein (AGP) was used as both the immobilized binding agent and protein of interest in this study. AGP is an important acute phase protein and is the principal binding protein for basic and neutral drugs in blood. Drugs that are known to have significant binding to AGP and that were considered in these binding studies included propranolol, imipramine, lidocaine, verapamil, disopyramide and mifeprestone.

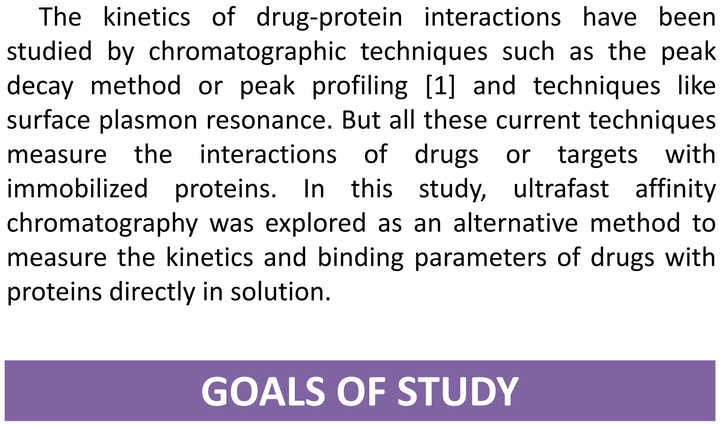
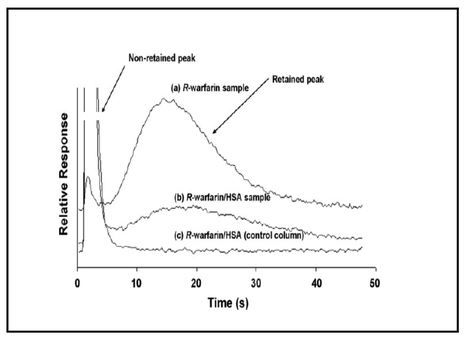



**Figure 1**. General scheme for separation of free and protein-bound fractions of a drug by using an affinity microcolumn that contains immobilized protein or binding agent (in this example, HSA, with AGP being used in this current study)

## METHODS

### ❖ Determination of free drug fractions in samples

To determine the free fraction of a drug by using ultrafast affinity chromatography, the drug/solute was incubated with a soluble protein prior to the injection of this mixture onto an affinity microcolumn. Under appropriate flow rate conditions, the free fraction of the drug was retained by the column and eluted after the non-retained peak due to the protein-bound form of the drug in the sample, as illustrated in **Figure 2**.




$$\text{Free drug fraction} = \frac{\text{Retained peak area of free drug fraction}}{\text{Total peak area for the drug without HSA}}$$

**Figure 2.** Typical chromatograms obtained at 4.5 mL/min for injections of a) *R*-warfarin on an HSA microcolumn b) *R*-warfarin + soluble HSA on an HSA micro column, or c) *R*-warfarin + soluble HSA on a control column [2].

### ❖ Optimization of chromatographic parameters

The column size and flow rate were both optimized to provide sample residence times of a few hundred milliseconds to prevent the dissociation of drugs that were bound to soluble proteins in a sample, as illustrated in **Figure 3**. The re-association of this target to the soluble proteins was prevented by using a large excess of protein immobilized on the column compared to the amount of soluble protein, which made this technique valuable for studying the kinetics of drug-protein interactions in the sample.

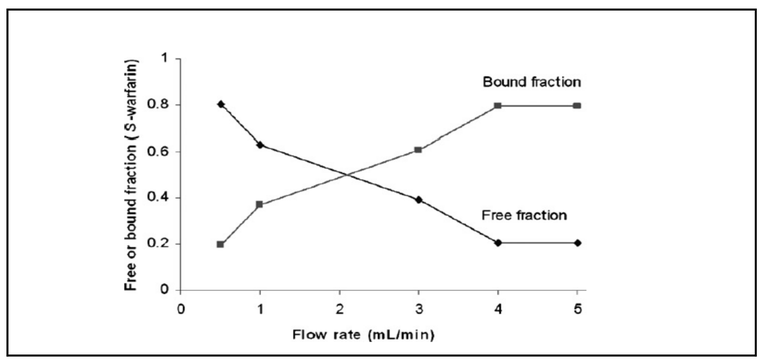



**Figure 3**. Effect of flow rate on apparent free fractions measured in samples Containing warfarin and soluble HSA on HSA microcolumns

### ❖ Determination of Association Equilibrium Constant, $K_a$

The association equilibrium constant, $K_a$, for a drug with a soluble protein can be determined from the measured free fraction by using equations (1) and (2) that describe a drug-protein interaction that involves 1:1 binding.

$$F_0 = \frac{[D]_0 - [D\text{-}P]}{[D]_0} \qquad (1)$$

$$Ka = \frac{[D\text{-}P]}{([D]_0 - [D\text{-}P])([P]_0 - [D\text{-}P])} \qquad (2)$$

$F_0$ = measured free fraction
$[D]_o$ = total concentration of drug in the sample including both free and protein-bound fractions
[D-P] = concentration of drug-protein complex in the sample
$[P]_o$ = total concentration of protein in the sample

### ❖ Determination of Dissociation Rate Constant, $k_d$

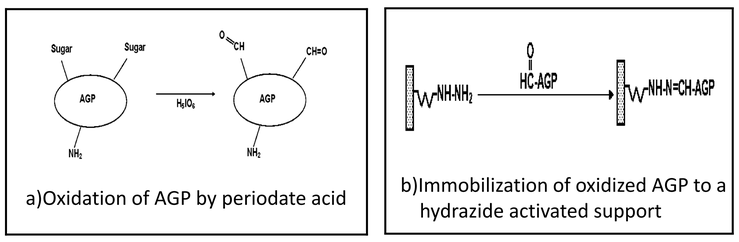
A model was developed for ultrafast affinity chromatographic studies [3] in which the dissociation rate constant for a drug from a soluble protein can be determined based on equation (3)

$$\ln \frac{[1-F_0]}{[1-F_t]} = k_d t \qquad (3)$$

$F_0$ = Free fraction at optimum flow rate
$F_t$ = Free fraction at residence time, t
t = residence time for the sample in the column

### ❖ Preparing AGP affinity microcolumns

The immobilization of AGP to silica has been previously optimized to make covalently coupled AGP columns which can model the binding of drugs to soluble AGP (see **Figure 4**) [4]. Another alternative route to prepare AGP microcolumns would be entrapment, as illustrated in **Figure 5** [5].




a)Oxidation of AGP by periodate acid

b)Immobilization of oxidized AGP to a hydrazide activated support

**Figure 4**. Covalent immobilization of AGP to hydrazide-activated silica

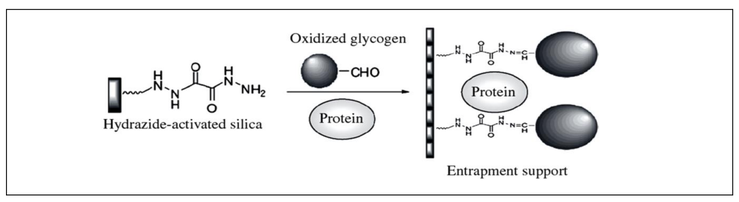



**Figure 5**. Non-covalent immobilization of AGP through entrapment

## RESULTS

**Table 1.** Association equilibrium constants measured for various drugs with soluble AGP using ultrafast affinity chromatography

| Drug | Association equilibrium constant ($K_a$) Experimental value | Association equilibrium constant ($K_a$) Reference/Theoretical value [Ref] |
|---|---|---|
| Imipramine | 4.2 (± 0.4) × $10^5$ $M^{-1}$ | 4.1 × $10^5$ $M^{-1}$ [6] |
| Lidocaine | 1.1 (± 0.7) × $10^5$ $M^{-1}$ | 1.1 – 1.7 × $10^5$ $M^{-1}$ [7] |
| Verapamil | 1.16 (± 0.44) × $10^5$ $M^{-1}$ | 1.2 × $10^5$ $M^{-1}$ [8] |

**Table 2**. Dissociation rate constants measured for various drugs with soluble AGP using ultrafast affinity chromatography

| Drug | Dissociation rate constant ($k_d$) Experimental value | Dissociation rate constant ($k_d$) Reference/Theoretical value [Ref] |
|---|---|---|
| Imipramine | 0.89 (± 0.1) $s^{-1}$ | Not reported |
| Lidocaine | 0.52 (± 0.06) $s^{-1}$ | 0.73 (± 0.07) $s^{-1}$ [1] |
| Verapamil | 1.45 (± 0.06) $s^{-1}$ | Not reported |

## CONCLUSIONS

Ultrafast affinity extraction based on AGP microcolumns was optimized and used to measure the extent of binding and rate of dissociation for several drugs with soluble AGP. The association equilibrium constants determined by this approach closely agreed with the reference values. Though reference values for the dissociation rate constants were known for only one of these drugs, all of the results were comparable to those that have been reported for drugs with HSA (another serum protein) in systems that have similar association equilibrium constants. This approach is not limited to the drugs examined in this study, or to AGP, but could also be extended to the screening and rapid analysis of other solute-protein interactions of biomedical interest.

## ACKNOWLEDGEMENTS

This work was supported by the NIH under Grant R01 GM044931.

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