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Analysis of Free Fractions for Chiral Drugs Using Ultrafast Extraction and Multi-Dimensional High-Performance Affinity Chromatography†

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Abstract

A multi-dimensional chromatographic approach was developed to measure the free fractions of drug enantiomers in samples that also contained a binding protein or serum. This method, which combined ultrafast affinity extraction with a chiral stationary phase, was demonstrated using the drug warfarin and the protein human serum albumin.

Many drugs and small solutes exist in multiple forms in biological systems. Drugs are often reversibly bound to carrier agents such as serum proteins in the circulatory system, causing these drugs to exist in two forms: a free fraction and bound fraction.^{1–4} Because the free drug fraction is generally thought to be the biologically-active form,^{3,4} there has been ongoing interest in the creation of improved tools for measuring free drug fractions and for studying drug-protein interactions in clinical and pharmaceutical samples.^{1,2,5} The methods of equilibrium dialysis and ultrafiltration are often used for this work but can have long analysis times and large sample requirements (e.g., equilibrium dialysis) or can introduce errors due to nonspecific adsorption to a membrane (e.g., ultrafiltration or equilibrium dialysis).⁶

Chromatographic approaches based on high-performance affinity chromatography (HPAC) and ultrafast affinity extraction have recently been proposed as an alternative means for measuring free drug or free hormone fractions.^{1,2,7–9} In this type of approach, small columns containing immobilized antibodies^{1,2,7,8} or other binding agents, such as immobilized transport proteins,⁹ are employed to extract the free form of a target drug or solute on a time scale that minimizes release of the target from its protein-bound form in a sample. Potential advantages of this approach include its speed, small sample requirements, good precision and low detection limits, especially when used with detection based on chemiluminescence or near-infrared fluorescence.^{1,7,8} However, previous systems based on this method have been designed to look at only a specific drug or solute (e.g., warfarin, phenytoin and thyroxine)^{1,2,7–9} rather than samples that may contain multiple or related forms of the same target (e.g., a mixture of drug enantiomers).

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This report describes a multi-dimensional HPAC system that used ultrafast affinity extraction and chiral chromatography to simultaneously examine the free forms of drug enantiomers in complex samples (e.g., serum) and to study the binding of such drugs with proteins. *R/S*-Warfarin and its binding protein human serum albumin (HSA) were used as models to develop and evaluate this approach. Warfarin is an anticoagulant that is often used as a racemic mixture for the treatment of thromboembolic diseases, with the *R*- and *S*-enantiomers having noticeable differences in their pharmacokinetics and protein binding properties.^{3,10,11} HSA (molar mass, 66.5 kDa) is the main binding protein for warfarin and many other drugs in serum and is known to have strong interactions with both *R*- and *S*-warfarin at a region on this protein known as Sudlow site I.^{10,12,13}

Fig. 1 shows the general separation and analysis strategy that was used in this study to examine the free fractions of *R*- and *S*-warfarin (see Supplementary Information for experimental details and system configuration). Ultrafast extraction based on an immobilized HSA microcolumn was first used to separate the free and protein-bound fractions of *R*- and *S*-warfarin in the presence of a sample that contained soluble HSA. In this process, the protein-bound drug and proteins in the sample were eluted in a non-retained peak from the microcolumn, while the free drug fraction in the sample was extracted and retained. This retained free drug fraction was later eluted from the microcolumn under isocratic conditions and delivered to a second, larger HSA column, which was utilized as a chiral stationary phase for the separation and measurement of the captured drug enantiomers.¹⁰

Studies were first performed with this system to find the optimum flow rate conditions for extraction of the free fraction of *R/S*-warfarin without creating significant interferences from the portion of the drug that was originally bound to sample proteins but was released as the initial free drug fraction was removed. Fig. 2 shows the apparent free drug fractions that were obtained for warfarin/HSA mixtures at various flow rates and at 37 °C in pH 7.4, 0.067 M phosphate buffer when using a 3 mm × 2.1 mm i.d. HSA microcolumn for ultrafast affinity extraction. Based on a measured protein content of 57 (± 1) mg HSA/g silica, the microcolumn was determined to contain 4.0 nmol HSA. The amount of warfarin applied per injection in Fig. 2 was 0.25% of the estimated binding capacity. Experiments with injections of only *R/S*-warfarin indicated that more than 99% extraction occurred for this drug's enantiomers under these injection conditions and at all of the flow rates tested in this study.

In Fig. 2, the apparent free drug fractions that were measured for a mixture of warfarin with soluble HSA was elevated at lower flow rates because the longer extraction period provided in the microcolumn under these conditions allowed for some dissociation of this drug from its protein-bound fraction in the sample, as noted previously.^{1,9} This process was decreased at higher flow rates and had no significant effect on the measured free fractions at a flow rate of 5.0 mL/min or higher, which corresponded to a residence time of 100 ms or less for the sample on the HSA microcolumn.

A second set of studies examined the ability of the multi-dimensional HPAC system to separate warfarin enantiomers in the retained free drug fraction (see Fig. 3). Samples containing 1.0 µL of 5 µM warfarin or a 5 µM warfarin/10 µM HSA mixture were applied to

the multi-dimensional system. The upper peak in Fig. 3(a) was obtained by injecting only warfarin onto the HSA microcolumn at 5 mL/min. The free fraction of warfarin that was extracted by this microcolumn eluted with a retention time of approximately 17–20 s. This figure also shows a chromatogram that was obtained when the same amount of warfarin and a two-fold mol excess of soluble HSA were injected onto the HSA microcolumn under identical conditions. In this case, a large non-retained peak was now observed at 5–7 s due to the elution of the soluble HSA and protein-bound fraction of warfarin. The second retained peak in this case represented the free fraction of warfarin that was extracted by, and later eluted from, the HSA microcolumn.

The free warfarin fraction, or a representative portion of this fraction, that was extracted by the HSA microcolumn was then eluted in the presence of the pH 7.4 phosphate buffer onto a 5 cm × 2.1 mm i.d. HSA column at 0.5 mL/min for a chiral separation. The chromatograms in Fig. 3(b) show the peaks that were obtained for *R*- and *S*-warfarin in samples that contained only racemic warfarin or racemic warfarin plus soluble HSA. With this combined approach, it was possible to simultaneously study the free fractions for both *R*- and *S*-warfarin in the original sample. Although the chiral separation obtained with the pH 7.4 buffer in Fig. 3(b) provided baseline resolution for the warfarin enantiomers and was sufficient for these exploratory studies, the speed of this latter step could be reduced to less than 10 min by using a more efficient HSA monolith column and/or by adding an organic modifier to the mobile phase (see Supplementary Information for examples).^{10,14,15}

The free fraction of *R*- or *S*-warfarin in a mixture of racemic warfarin and soluble HSA was calculated by dividing the concentration of the enantiomer's free fraction, as represented by lower peaks in Fig. 3(b), by the concentration measured for the enantiomer and at the same total sample concentration but with no soluble protein present, as represented by the upper peaks in Fig. 3(b). The concentrations of the *R*- and *S*-warfarin fractions were determined by comparing the sizes of these peaks to those obtained with the same chromatographic system and using warfarin standards. The free fractions for *R*- and *S*-warfarin in various samples were measured by this approach based on multi-dimensional HPAC and by employing a reference method that made use of ultrafiltration followed by a chiral separation using an HSA column (see Supplementary Information for experimental details). The results that were obtained by each method are summarized in Table 1.

Table 1 shows that there was good agreement between the free fractions that were measured in Fig. 3 by the multi-dimensional HPAC method and by ultrafiltration followed with a chiral separation. For a sample that contained 5 μ M racemic warfarin and 10 μ M soluble HSA, the absolute difference in these free fractions was 1–6%, with the results showing no significant difference at the 95% confidence level. In addition, these measured free fractions agreed with the range of 33–43% that was predicted for *R*- and *S*-warfarin based on the known binding constants of this system.^{9,10}

The multi-dimensional HPAC method was also used to examine other samples. One of these samples contained 30 μ M racemic warfarin and 600 μ M HSA, representing a clinically-relevant concentration of HSA and a typical therapeutic concentration for warfarin.¹⁶ Good agreement between the multi-dimensional HPAC system and reference method was again

obtained, with an absolute difference in the measured free fractions of 0.3–0.5% and no significant differences at the 95% confidence level. In addition, the measured free fractions were consistent with a range of approximately 0.5–2% that was estimated from the reported binding constants for the warfarin-HSA interaction.^{9,10} The free fractions of the warfarin enantiomers were also examined in human serum that was spiked with a therapeutic level of this drug. The results for the multi-dimensional HPAC system and reference method were again comparable, with an absolute difference of 0.2–0.5% and no significant differences being noted at the 95% confidence level.

The multi-dimensional HPAC system was next used as a screening tool to determine the association equilibrium constants for *R*- and *S*-warfarin with HSA. This was accomplished by using the free fraction data along with a single-site binding model (see Supplementary Information for details). Table 2 shows the results that were obtained for the 5 μ M warfarin/10 μ M HSA mixture. This table also lists previous binding constants that have been reported for *R*- and *S*-warfarin at Sudlow site I of HSA under the same pH and temperature conditions.¹⁰ Under these conditions, the association equilibrium constants that were estimated by multi-dimensional HPAC and the reference methods continued to show good agreement at the 95% confidence level with one another and with the literature values.

Similar calculations to those used in Table 2 were carried out for the 30 μ M warfarin/600 μ M HSA mixture and for the spiked serum samples (see Supplementary Information, Table S1). The association equilibrium constants that were obtained by multi-dimensional HPAC and the reference approach were again consistent with each other; however, the estimated binding constants were 38–71% smaller than the literature values. This latter difference is probably due to the greater uncertainty that was present for these samples in the measurement of their relatively small free fractions. It is also possible there were some deviations from a single-site binding model due to greater nonspecific interactions by warfarin with the much larger amounts of HSA, and other proteins in the case of the serum, in this second group of samples.

In summary, it was shown that a multi-dimensional HPAC system that used ultrafast affinity extraction in combination with a chiral separation could be used to simultaneously measure the free fractions of *R*- and *S*-warfarin in serum or drug-protein mixtures. This approach was also used to estimate the binding constants for these enantiomers with HSA. The results of this method gave good agreement with a reference method that was based on ultrafiltration plus a chiral separation. However, the multi-dimensional HPAC method had several potential advantages over ultrafiltration. For instance, this method required only 1 μ L of sample per injection and could isolate the free warfarin fractions within 20–30 s of injection. In comparison, a 1 mL sample was needed for ultrafiltration and 1 h was required for the separation of a free drug fraction by this approach. It was also possible to directly couple the ultrafast extraction with a chiral separation to automate and complete both steps using a single system. This approach is not limited to warfarin or HSA but could easily be extended to other chiral drugs, or drug mixtures, and their binding proteins through the use of similar affinity microcolumns and chiral stationary phases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. General scheme for separation of the free and protein-bound fractions of a drug and resolution of the enantiomers in free drug fraction through the use of ultrafast extraction and multi-dimensional HPAC.

Fig. 2. Effect of flow rate on the measurement of free fractions in mixtures of racemic warfarin and HSA when using ultrafast affinity extraction. Conditions: 1.0 μ L of 10 μ M warfarin or 10 μ M warfarin/20 μ M HSA injected onto a 3 mm $\times$ 2.1 mm i.d. HSA microcolumn.

Fig. 3. Chromatograms for 1.0 μL injections of 5 μM racemic warfarin or 5 μM racemic warfarin plus 10 μM HSA on (a) an HSA microcolumn at 5.0 mL/min or (b) a chiral HSA column at 0.5 mL/min after sample passage through the HSA microcolumn. The arrow in (a) shows the time at which a valve was switched to pass the retained components from the HSA microcolumn onto the larger HSA column that was used for the chiral separation. These results were obtained at 37 $^{\circ}\text{C}$ using pH 7.4, 0.067 M phosphate buffer as the mobile phase for both columns.

Table 1Free drug fractions measured for *R*- and *S*-warfarin in samples containing soluble HSA or human serum

Sample & Analyte	Measured free fraction ^a	
	Multi-dimensional HPAC	Ultrafiltration + chiral separation
<i>Racemic warfarin (5 μM) + HSA (10 μM)</i>		
<i>R</i> -Warfarin	42 (± 4)%	41 (± 4)%
<i>S</i> -Warfarin	38 (± 5)%	32 (± 3)%
<i>Racemic warfarin (30 μM) + HSA (600 μM)</i>		
<i>R</i> -Warfarin	1.8 (± 0.8)%	1.5 (± 0.5)%
<i>S</i> -Warfarin	1.3 (± 0.2)%	1.8 (± 0.2)%
<i>Racemic warfarin (30 μM) + human serum^b</i>		
<i>R</i> -Warfarin	2.7 (± 1.7)%	2.5 (± 0.2)%
<i>S</i> -Warfarin	1.1 (± 0.3)%	1.6 (± 0.7)%

^aThese values were obtained at 37 °C in pH 7.4, 0.0067 M potassium phosphate buffer. The numbers in parentheses represent ± 1 S.D. (*n* = 3).

^bThe human serum contained approximately 600 μM HSA.

Table 2

Association equilibrium constants estimated for *R*- and *S*-warfarin with soluble HSA based on a single-site binding model

Sample & Analyte	Association equilibrium constant, K_a (M^{-1}) ^a		
	Multi-dimensional HPAC	Ultrafiltration + chiral separation	Literature value [Ref.10]
<i>Racemic warfarin (5 μM) + HSA (10 μM)</i>			
<i>R</i> -Warfarin	$2.0 (\pm 0.4) \times 10^5$	$2.1 (\pm 0.4) \times 10^5$	$2.1 (\pm 0.2) \times 10^5$
<i>S</i> -Warfarin	$2.4 (\pm 0.6) \times 10^5$	$3.2 (\pm 0.4) \times 10^5$	$2.6 (\pm 0.4) \times 10^5$

^aThese values were obtained at 37 °C in pH 7.4, 0.0067 M potassium phosphate buffer. The numbers in parentheses represent ± 1 S.D. ($n = 3$).