SUPPLEMENTARY INFORMATION

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

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EXPERIMENTAL SECTION

Materials and Reagents. The HSA (Cohn fraction V, essentially fatty acid free), human serum (from male AB plasma, H4522, lot 039K0728; sterile filtered and tested negative for HIV-1/HIV-2, hepatitis B and hepatitis C), and racemic warfarin (98% pure) were from Sigma (St. Louis, MO, USA). The reagents for the bicinchoninic acid (BCA) protein assay were from Pierce (Rockford, IL, USA). The Nucleosil Si-300 silica (7 μm particle diameter, 300 Å pore size) was purchased from Macherey Nagel (Dűren, 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 (Pittsburgh, PA, USA)

Apparatus. The affinity columns were packed using a Prep 24 preparative pump from ChromTech (Apple Valley, MN, USA). The chromatographic system consisted of a PU-2080 Plus HPLC pump from Jasco (Easton, MD, USA), two six-port Lab Pro valves (Rheodyne, Cotati, CA, USA), and a Shimadzu RF-10AXL fluorescence detector (Kyoto, Japan). An Alltech water jacket (Deerfield, IL, USA) and an Isotemp 3013D circulating water bath from Fisher were used to maintain a temperature of 37.0 (±0.1) °C for the columns during all experiments described in this report. The chromatographic data were collected and processed using in-house programs written in LabView 5.1 (National Instruments, Austin, TX, USA). The ultrafiltration studies were performed using a 5702RH centrifuge from Fisher and tubes containing Ultracel YM-T cellulose membranes (30 kDa cut-off), as obtained from Millipore (Billerica, MA, USA).

Column preparation. The stationary phase used in these studies consisted of HSA that was immobilized on Nucleosil Si-300 silica by the Schiff base, as performed according to the literature.[1] Control supports were prepared in the same manner but with no HSA being added during the immobilization step. The protein content of the final HSA support was determined in

triplicate by a BCA assay using HSA as the standard and the control support as the blank.

An HPLC column packer was used to place the supports into stainless steel columns with dimensions of 1 cm × 2.1 mm i.d. or 5 cm × 2.1 mm i.d. The columns with dimension of 3 mm × 2.1 mm i.d. used a frit-in-column design, as described in Ref. 2. The longer columns were prepared using traditional stainless steel HPLC housings and end fittings. The packing solution for all of these columns was pH 7.4, 0.067 M potassium phosphate buffer, and the packing pressure was 4000 psi (28 MPa).

Chromatographic studies. This work utilized a multi-dimensional system in which a 3 mm × 2.1 mm i.d. HSA microcolumn was used for the extraction of free drug fractions and a 5 cm × 2.1 mm i.d. HSA column was used for chiral separations of the retained free drug fractions. The sample loading, injection and column switching were controlled by two separate Rheodyne six port valves (Cotati, CA, USA), as illustrated in Fig. S1. The mobile phase was pH 7.4, 0.067 M potassium phosphate buffer in the free drug extractions and in the initial chiral separations. All samples containing racemic warfarin and HSA were prepared in this buffer and incubated for at least 1 h before injection to allow equilibrium to be established between the free and protein-bound fractions of the drug in the sample.[3]

The initial studies examining the free fraction extraction of *R*- and *S*-warfarin used a 1.0 μL sample of 10 μM racemic warfarin or a 10 μM racemic warfarin/20 μM HSA mixture that was injected onto a 3 mm × 2.1 mm i.d. HSA microcolumn at flow rates ranging from 0.5 mL/min to 6.0 mL/min. In the final method that was developed in this study, a 1.0 μL sample injection was made onto the 3 mm × 2.1 mm i.d. HSA microcolumn at a flow rate of 5.0 mL/min for extraction of the free drug fraction. Eighteen seconds later, a switching valve was used to transfer the eluting free drug fraction to a longer 5 cm × 2.1 mm i.d. HSA column for use in a

chiral separation at 0.5 mL/min. The aqueous samples used in these latter studies contained 5 μM racemic warfarin or a 5 μM warfarin/10 μM HSA mixture; 30 μM racemic warfarin or 30 μM warfarin/600 μM HSA, to examine the use of this method at clinically-relevant concentrations; and a mixture of 30 μM racemic warfarin and human serum (which contained approximately 600 μM HSA) to study the feasibility of using this system with human serum samples. The warfarin enantiomers were detected by monitoring their fluorescence at an excitation wavelength of 310 nm and an emission wavelength of 390 nm. The concentrations of *R*- and *S*-warfarin in each sample were determined by comparing the resulting peak areas to those obtained for warfarin standards.

Ultrafiltration studies. Before sample introduction, each ultrafiltration device was washed three times with 1 mL water and spun at 1500 × g for 5 min. The devices were then washed three times in the same manner with 1 mL of pH 7.4, 0.067 M potassium phosphate buffer. Any remaining buffer in the device was removed by spinning the filtration device at 1500 × g for 15 min. Immediately after these washing and pretreatment steps, a 1 mL sample containing warfarin or warfarin plus HSA, as prepared in pH 7.4, 0.067 M potassium phosphate buffer or human serum, was introduced into three ultrafiltration devices and spun at 1500 × g and 37ºC for 2.5 min or 6.0 min, respectively (Note: different spinning periods were used to make sure that no more than 0.5 mL of the sample passed into the filtrate vial, thus allowing for accurate free drug fraction measurements).[4]

The resulting filtrates were collected for the measurement of their warfarin concentrations by using an HPLC-based chiral separation. This was accomplished by making a 5 μL injection of each filtrate sample at 1.0 mL/min onto a 1 cm × 2.1 mm i.d. HSA column. The mobile phase in this case consisted of pH 7.4, 0.067 M potassium phosphate buffer containing

1.5% (v/v) 1-propanol. The elution of warfarin enantiomers from this column was again monitored by using a fluorescence detector, as described in the previous section, and the concentrations of *R*- and *S*-warfarin in each filtrate were determined by comparing the resulting peaks areas to those that were obtained by the same approach when using warfarin standards.

Prediction of Free Fractions from Association Equilibrium Constants. For a drug and protein interaction that involves 1:1 binding, the relationship between the theoretical free fraction (F) and the association equilibrium constant (K_a) for this interaction can be described by using eqs (1) and (2),

$$F = \frac{C_d - [D-P]}{C_d} \quad (1)$$

$$K_a = \frac{[D-P]}{(C_d - [D-P])(C_p - [D-P])} \quad (2)$$

in which C_d is the total concentration of drug in the original sample, C_p is the total concentration of protein in the sample, [*D*-*P*] is the concentration of the drug-protein complex in the original sample.[3]

In this study, both *R*- and *S*-warfarin were present in a sample containing racemic warfarin and both enantiomers were able to interact with any HSA that was present. Thus, the free fractions for these two enantiomers (F_R and F_S) and their association equilibrium constants ($K_{a,R}$ and $K_{a,S}$) were calculated separately, as described by eqs (3)-(6),

$$F_R = \frac{C_R - [R-P]}{C_R} \quad (3)$$

$$K_{a,R} = \frac{[R-P]}{(C_R - [R-P])(C_p - [R-P] - [S-P])} \quad (4)$$

$$F_S = \frac{C_S - [S-P]}{C_S} \quad (5)$$

$$K_{a,S} = \frac{[S-P]}{(C_S - [S-P])(C_p - [R-P] - [S-P])} \quad (6)$$

where C_R and C_S represent the concentrations of *R*- and *S*-warfarin in the original sample. According to the information provided by their supplier, the *R*- and *S*-warfarin were present in identical amounts in their original racemic mixture. Under these conditions, the relationship of their concentrations with C_d can be described by eq (7).

$$2\ C_R = 2\ C_S = C_d \qquad (7)$$

In the initial studies examining use of the multi-dimensional HPAC method to measure free fractions, the sample consisted of 2.5 μM *R*-warfarin (C_R), 2.5 μM *S*-warfarin (C_S) and 10 μM HSA (C_P). The association equilibrium constants ($K_{a,R}$ and $K_{a,S}$) of *R*- and *S*-warfarin with HSA have been reported to be 2.1 (± 0.2) $\times 10^5$ and 2.6 (± 0.4) $\times 10^5$ M^{-1} under the same pH and temperature conditions as used in this current study.[5] Substituting these values into eqs (3)-(7), gave predicted free fractions for *R*- and *S*-warfarin of 0.41 (± 0.02) and 0.36 (± 0.03), respectively, as found by using the Solver function in Microsoft Excel. A similar process was used to estimate free fractions for the other samples that were examined in this study.

Estimation of Association Equilibrium Constants from Measured Free Fractions. Based on the free drug fractions that were measured in this study, it was possible to estimate the overall association equilibrium constants for each drug with HSA. This was accomplished by using the free fraction data and the equations introduced in the previous section. For instance, by using eq (3) the concentration of the *R*-warfarin/HSA complex ([*R*-*P*]) could be calculated from the measured free fraction of *R*-warfarin (F_R). Substituting the value of [*R*-*P*] into eq (4) then made it possible to obtain $K_{a,R}$. [3] The same process was employed for the calculation of $K_{a,S}$ by using eqs (5) and (6). The results that were obtained by this process are shown in both Table 2 in the main body of the paper and in Table 1S in this Supplementary Information.

Use of Alternative Conditions for the Chiral Separation of *R*- and *S*-Warfarin. The

results shown in Fig. 3(b) in the main body of this paper were based on the use of pH 7.4, 0.067 M phosphate buffer as a mobile phase for both of the HSA columns in the multi-dimensional HPAC system. This was done for the sake of simplicity in the initial design of this system and resulted in the type of separation that is shown in Fig. S2(a) and Fig. 3(b). However, it was also possible to conduct chiral separations by using small amounts of organic modifiers in the mobile phase to further improve this separation. For instance, Fig. S2(b) shows the effects of adding 1.5% (v/v) 1-propanol to the mobile phase while keeping all of the other conditions the same as in Fig. S2(a). This change lead to a large increase in resolution but lowering the retention factor for *R*-warfarin and increasing the retention factor for *S*-warfarin, as noted previously for similar HSA columns.[5]

Other conditions could also be used to improve this chiral separation. As an example, using the same flow rate and amount of 1-propanol as in Fig. S2(b) but decreasing the HSA column size to 1 cm × 2.1 mm i.d. still gave baseline resolution between *R*- and *S*-warfarin but decreased the separation time to around 18 min at 0.50 mL/min, as shown in Fig. S2(c). Using 1.5% 1-propanol in the mobile phase and increasing the flow rate to 1.0 mL/min with the 1 cm × 2.1 mm i.d. HSA column also gave baseline resolution, but with the separation now being complete in roughly 9 min (see Fig. S2(d)). Another way to improve this separation would be to use an alternative support, such as a monolith column. For instance, a separation of *R*- and *S*-warfarin in 6 min was recently reported when using a 1 cm × 4.6 mm i.d. HSA monolith column based on a co-polymer of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) that was operated at 2.0 mL/min and that used 0.5% 1-propanol in a pH 7.4, 0.067 M phosphate buffer as the mobile phase.[6]

References

1 C. M. Ohnmacht, J. E. Schiel, D. S. Hage, *Anal. Chem.* **2006,** *78,* 7547-7556.

2 J.E. Schiel, Ph.D. Thesis, University of Nebraska-Lincoln, Lincoln, NE, 2009.

3 R. Mallik, M. J. Yoo, C. J. Briscoe, D. S. Hage, *J. Chromatogr. A,* **2010,** *1217,* 2796-2803.

4 J.B. Whitlam, K.F. Brown, *J. Pharmaceut. Sci.* **1980**, *70,* 146-150.

5 B. Loun, D. S. Hage, *Anal. Chem.* **1994,** *66,* 3814-3822.

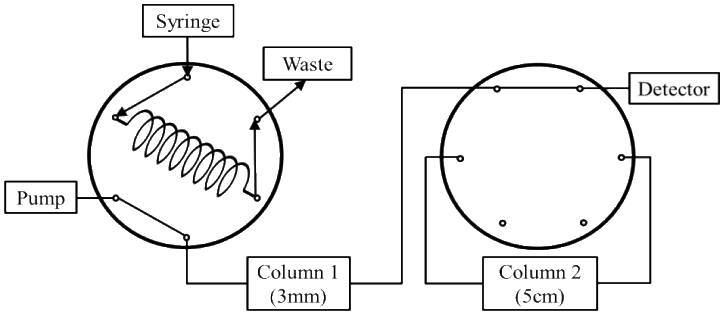
6 E. L. Pfaunmiller, M. Hartmann, C. M. Dupper, S. Soman, D. S. Hage, *J. Chromatogr. A*, 2012, **1269**, 198-207.

FIGURE LEGENDS

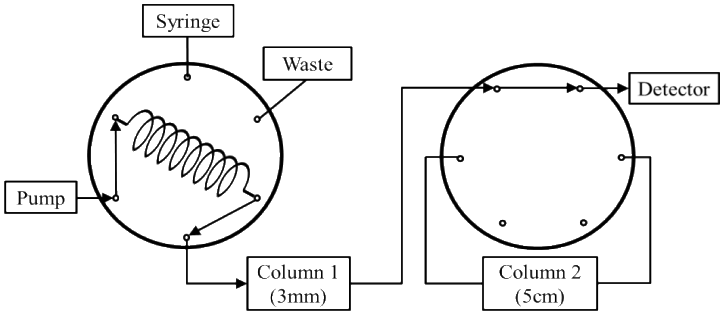
Fig. S1 Valve configurations used in the multi-dimensional HPAC system. The first valve was used for (a) loading and (b) injecting a sample onto an HSA microcolumn for a free fraction separation. (c) The second valve was switched when the protein-bound drug complex and excess protein had been passed through the HSA microcolumn and the free drug fraction had just begun to exit this microcolumn. The free drug fraction, or a representative portion, was then passed on to a second and longer HSA column for a chiral separation.

Fig. S2 Chromatograms for injections of racemic warfarin solution onto HSA columns for chiral separation under different conditions. The results shown in (a) were obtained at 0.5 mL/min by injecting 5 μL of 30 μM racemic warfarin onto 5 cm × 2.1 mm i.d. HSA column in the mobile phase of pH 7.4, 0.067 M potassium phosphate buffer. The results shown in (b) were obtained for the same sample and column with (a) in the mobile phase containing 1.5% 1-propanol as modifier. The results shown in (c) were obtained for the same sample and mobile phase with (b) on to a 1 cm × 2.1 mm i.d. HSA column. The results shown in (d) were obtained for the same sample, mobile phase and column with (c) when the flow rate is 1.0 mL/min. The HSA columns were prepared by the Schiff base method. And these chromatographic studies were finished at the temperature of 37 ℃.

(a) Sample loading



(b) Sample injection and free drug/protein-bound drug separation



(c) Chiral separation of free drug fraction

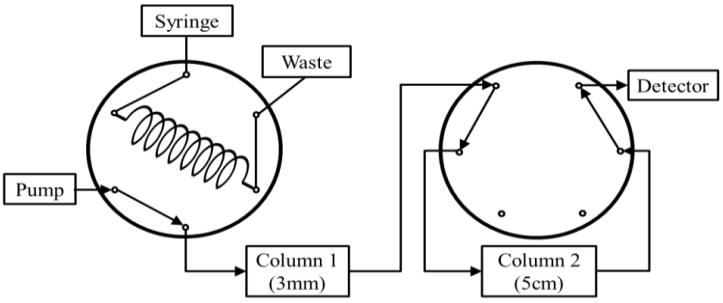


Fig. S1

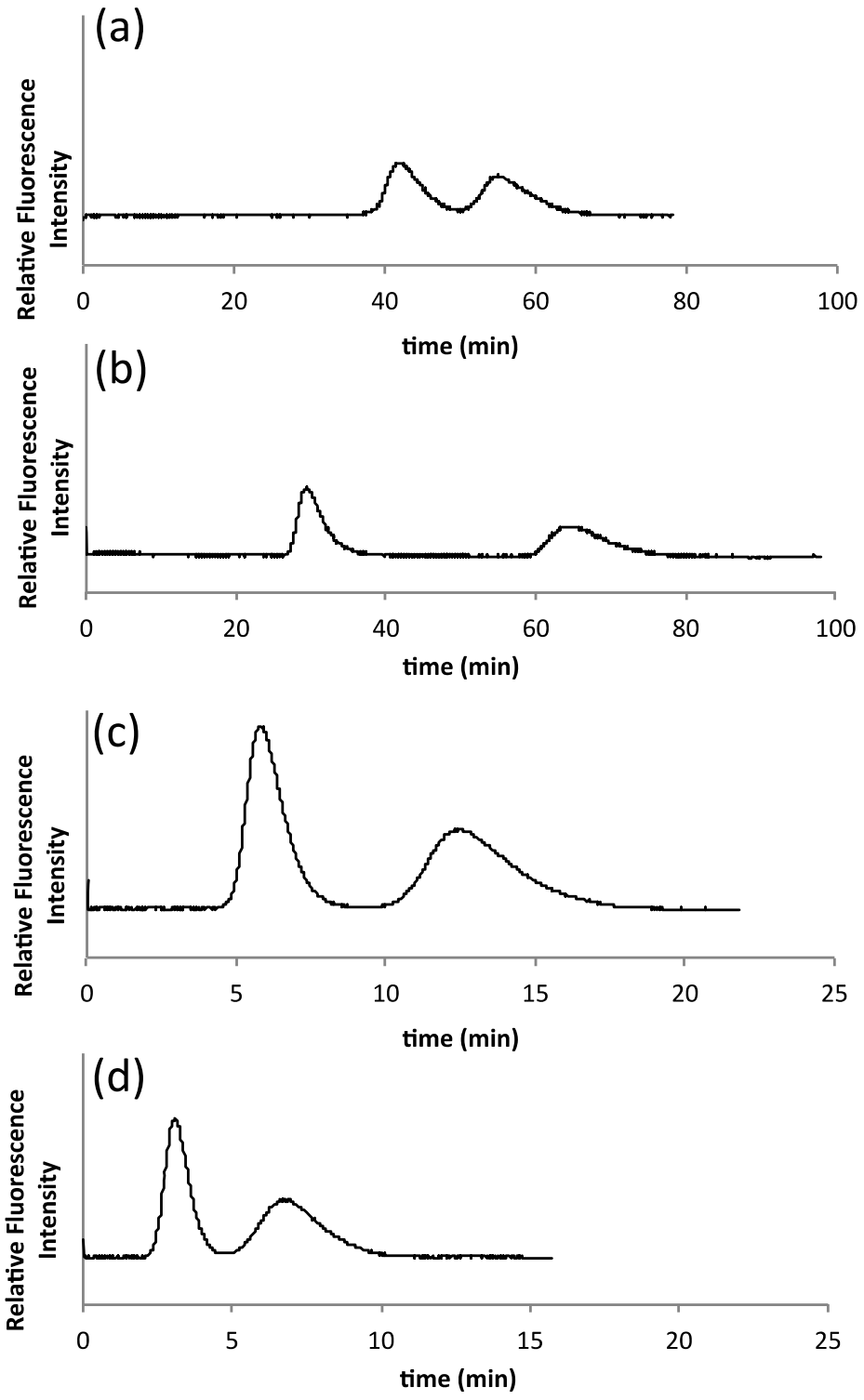


Fig. S2.

Table S1. Association equilibrium constants for *R*- and *S*-warfarin with soluble HSA

Sample & Analyte	Association equilibrium constant, K_a (M^{-1})[a]		
	Multi-dimensional HPAC	**Ultrafiltration + chiral separation**	**Literature value [Ref. 5]**
Racemic warfarin (30 μM) + HSA (600 μM)			
R-Warfarin	1.0 (± 0.4) ×10^5	1.2 (± 0.4) ×10^5	2.1 (± 0.2) ×10^5
S-Warfarin	1.3 (± 0.2) ×10^5	0.9 (± 0.1) ×10^5	2.6 (± 0.4) ×10^5
Racemic warfarin (30 μM) + Human serum[b]			
R-Warfarin	0.6 (± 0.4) ×10^5	0.7 (± 0.1) ×10^5	2.1 (± 0.2) ×10^5
S-Warfarin	1.6 (± 0.5) ×10^5	1.1 (± 0.5) ×10^5	2.6 (± 0.4) ×10^5

[a]These values were measured for the given samples at 37 °C in pH 7.4, 0.067 M potassium phosphate buffer. The numbers in parentheses represent a range of ±1 S.D. Values from Ref. 5 were measured under the same conditions by using frontal analysis.

[b]The human serum contained approximately 600 μM HSA.