Supplementary Material

Preparation of immunoaffinity column containing anti-AGP antibodies

The stationary phase used in the immunoaffinity column consisted of anti-AGP antibodies that were immobilized to Nucleosil Si-1000 silica by the Schiff base method [S1]. The polyclonal goat anti-human AGP antibodies (affinity purified, 1 mg/mL in pH 7.2 phosphate buffered saline) were obtained from Lee Biosolutions (St. Louis, MO, USA), and the Nucleosil Si-1000 silica (7 μm particle diameter, 1000 Å pore size) was obtained from Macherey Nagel (Düren, Germany). A 1 mL portion of a 1 mg/mL anti-AGP antibody solution, which had previously been exchanged into pH 6.0, 0.10 M potassium phosphate buffer by using a Zeba desalt spin column (Thermo Scientific, Rockford, IL, USA), was added to 25 mg silica that had been converted into an aldehyde form. The antibodies were added to this support in a ratio of 1 mg antibodies per 25 mg silica. This was followed by the addition of 3 mg sodium cyanoborohydride, with this mixture then being allowed to react at 4°C for 6 days. The silica was then washed with pH 8.0, 0.10 M potassium phosphate buffer, followed by the addition of 0.425 mg sodium borohydride that was dissolved in 2 mL of the same pH 8.0 buffer. This slurry was then mixed for 90 min to remove any remaining aldehyde groups on the support. The final antibody support was then washed several times with 0.5 M sodium chloride in pH 8.0, 0.1 M potassium phosphate buffer, followed additional washing steps with pH 7.4, 0.067 M phosphate buffer. This support was packed into a 5 mm × 2.1 mm i.d. stainless steel column at 3000 psi (20.7 MPa) using pH 7.4, 0.067 M potassium phosphate buffer as the packing solution. This column and the remaining support were stored in the same pH 7.4 buffer at 4°C when not in use.

Measurement of AGP in serum

According to Eqs. (3)-(7) in the main body of the text, the determination of $K_{a,A}$ is based on the assumption that the initial concentrations are known for AGP, HSA and the drug in a sample. The concentration of the spiked drug was known from the preparation conditions for the sample, and the levels of HSA that were present in the serum samples were obtained from the supplier of these materials. However, the level of AGP in the serum samples was not known in advance. To determine the concentration of AGP in the serum samples, an anti-AGP antibody column was prepared and used to specifically bind and measure AGP.

For this analysis, the serum samples were filtrated twice using Acrodisc 13 mm syringe filters with 0.2 μm nylon membranes (Pall Corporation, Ann Arbor, MI, USA) prior to the addition of any drugs. A series of AGP standards with concentrations ranging from 0.50 to 1.00 mg/mL were prepared in pH 7.4, 0.067 M phosphate buffer for creating a calibration curve. A 5 mm × 2.1 mm i.d. anti-AGP antibody column was placed into the HPLC system to bind and retain AGP from the serum samples. These chromatographic experiments were carried out at 37˚C and 0.10 mL/min. Triplicate injections were made for each serum sample or standard. For each injection, 5 μL of the sample was applied to the anti-AGP column in the presence of pH 7.4, 0.067 M phosphate buffer, which was also used to wash away any non-retained components in the sample. When 20 min had elapsed since sample injection, the AGP that was bound to the anti-AGP column was eluted by passing through this column a pH 2.5, 0.067 M potassium phosphate buffer for 20 min; this was followed by regeneration and re-equilibration of the column with the pH 7.4, 0.067 M phosphate buffer for 20 min. The elution of AGP was monitored at 280 nm. A calibration curve was made by plotting the

retained peak area for AGP peak versus the concentration of the standard AGP solutions, which was then used to determine the concentration of AGP in the serum samples.

Theoretical prediction of free drug fractions

For a sample containing only the drug and AGP, the free drug fraction can be predicted using the quadratic expression that is shown in Eq. (S1). This equation is converted from Eq. (2), as given in the main body of the text.

$$K_{a,A}[D]_0F^2 + \left(K_{a,A}[A]_0 - K_{a,A}[D]_0 + 1\right)F - 1 = 0 \qquad \text{(S1)}$$

In Eq. (S1), F is the free drug fraction, $K_{a,\mathrm{A}}$ is the association equilibrium constant for a drug D in its interactions with protein A (e.g., AGP), and $[\mathrm{D}]_0$ and $[\mathrm{A}]_0$ are the initial concentrations of D and A in the original sample. Eq. (S2), which is the positive solution to the quadratic relationship in Eq. (S1), can then be used to obtain the theoretical free drug fraction.

$$F = \frac{-\left(K_{a,\mathrm{A}}[\mathrm{A}]_0 - K_{a,\mathrm{A}}[\mathrm{D}]_0 + 1\right) + \sqrt{\left(K_{a,\mathrm{A}}[\mathrm{A}]_0 - K_{a,\mathrm{A}}[D]_0 + 1\right)^2 + 4K_{a,A}[\mathrm{D}]_0}}{2K_{a,\mathrm{A}}[\mathrm{D}]_0} \qquad \text{(S2)}$$

For a sample that contains a drug D and the proteins A and H (or AGP and HSA), The solver tool of Excel 2010 (Microsoft, Redmond, WA) was used to first calculate the values of $[\mathrm{D\text{-}A}]_{eq}$ and $[\mathrm{D\text{-}H}]_{eq}$ (i.e., the equilibrium concentrations of the complexes for drug D with A and H, respectively). This was accomplished based on Eqs. (S3) and (S4), which were derived by using the following information.

Overall Reaction:	D	+	A	+	H	⇌	D-A	+ D-H
Initial Conc.:	$[\mathrm{D}]_0$		$[\mathrm{A}]_0$		$[\mathrm{H}]_0$		0	0
Conc. Change:	$-[\mathrm{D\text{-}A}]_{eq}$ $-[\mathrm{D\text{-}H}]_{eq}$		$-[\mathrm{D\text{-}A}]_{eq}$		$-[\mathrm{D\text{-}H}]_{eq}$		$[\mathrm{D\text{-}A}]_{eq}$	$[\mathrm{D\text{-}H}]_{eq}$
Equilibrium:	$[\mathrm{D}]_0-[\mathrm{D\text{-}A}]_{eq}$ $-[\mathrm{D\text{-}H}]_{eq}$		$[\mathrm{A}]_0-[\mathrm{D\text{-}A}]_{eq}$		$[\mathrm{H}]_0-[\mathrm{D\text{-}H}]_{eq}$		$[\mathrm{D\text{-}A}]_{eq}$	$[\mathrm{D\text{-}H}]_{eq}$

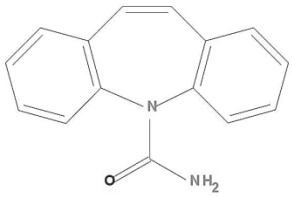
$$K_{a,\mathrm{A}} = \frac{[\mathrm{D-A}]_{\mathrm{eq}}}{([\mathrm{D}]_0 - [\mathrm{D-A}]_{\mathrm{eq}} - [\mathrm{D-H}]_{\mathrm{eq}})([\mathrm{A}]_0 - [\mathrm{D-A}]_{\mathrm{eq}})} \quad \text{(S3)}$$

$$K_{a,\mathrm{H}} = \frac{[\mathrm{D-H}]_{\mathrm{eq}}}{([\mathrm{D}]_0 - [\mathrm{D-A}]_{\mathrm{eq}} - [\mathrm{D-H}]_{\mathrm{eq}})([\mathrm{H}]_0 - [\mathrm{D-H}]_{\mathrm{eq}})} \quad \text{(S4)}$$

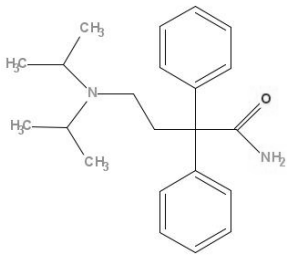
In these equations, $[H]_0$ is the initial concentration of protein H (HSA) in the original sample. After the predicted values of $[D\text{-}A]_{eq}$ and $[D\text{-}H]_{eq}$ have been determined through this process, the free drug fraction can then be calculated according to Eq. (4), as given in the main body of the text.

Model drugs used in free fraction measurements

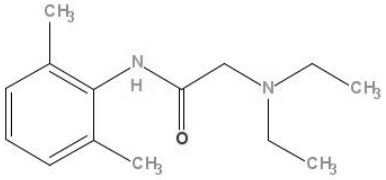
Figure S1 shows the structures of the model drugs that were used to develop and evaluate the AGP microcolumns and ultrafast affinity extraction method. Additional details on each of these drugs can be found in the main body of the text.



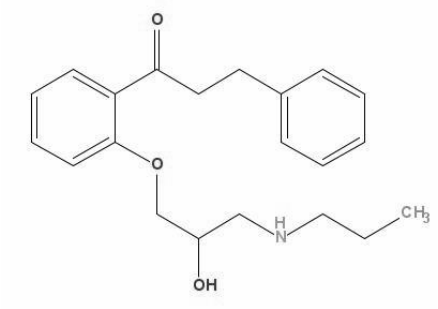
Carbamazepine



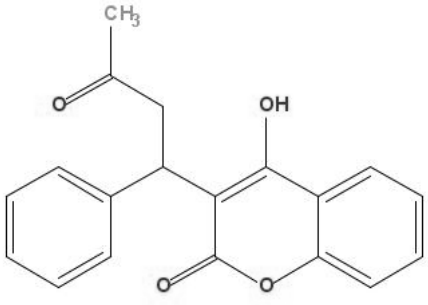
Disopyramide



Lidocaine



Propafenone



Warfarin

Figure S1. Structures of the model drugs that were examined in this study.

References

[S1] M.A. Nelson, A. Moser, D.S. Hage, Biointeraction analysis by high-performance affinity chromatography: kinetic studies of immobilized antibodies, J. Chromatogr. B 878 (2010) 165-171.