A Rapid Spectrofluorimetric Technique for Determining Drug-Serum Protein Binding Suitable for High-Throughput Screening

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Purpose. To develop and validate a rapid method for determining the dissociation constants with which pharmaceutical candidates and drugs bind to serum albumin and to α_1-acid glycoprotein with the goal of deducing the extent of binding.

Methods. The quenching of the intrinsic tryptophan fluorescence of serum albumin and α_1-acid glycoprotein was monitored by spectrofluorimetry and the data were used to calculate the apparent dissociation constant. Sodium warfarin was used to probe the warfarin-binding site of serum albumin and diazepam was used to probe the benzodiazepine binding site. Additionally, the binding of sodium salicylate, phenylbutazone, sulfinpyrazone, iophenoxic acid, theophylline, chloramphenicol, acetaminophen, lithium chloride and ampicillin were also investigated. Chlorpromazine hydrochloride and imipramine hydrochloride were used as probes for α_1-acid glycoprotein. The assays were also extended to the multiwell format. The quenching curves were fitted to the quadratic binding equation to determine the dissociation constants.

Results. Intrinsic fluorescence measurements are an excellent predictor of the drug binding to human serum albumin and to α_1-acid glycoprotein. These measurements detect binding to the warfarin and benzodiazepine binding sites of human serum albumin. The dissociation constants estimated using the method compare favorably to the dissociation constants previously reported by Epps *et al.* using extrinsic fluorescence methodology, and the results correlate well with equilibrium dialysis using drug displacement endpoints.

Conclusions. These measurements can be carried out with small samples and do not require separation of the bound and unbound species. Additionally, the proposed methods eliminate membrane separations, are not compound specific and do not require analytical chromatography or mass spectrometry for quantitation. Spectrofluorimetry may prove to be a useful method for rapidly determining the protein binding of combinatorial libraries.

KEY WORDS: serum albumin; α_1-acid glycoprotein; fraction bound; intrinsic fluorescence; high throughput screen; combinatorial pharmaceutics.

INTRODUCTION

Potent, pharmacologically active new drug candidates can be effective *in vivo* only if they are able to achieve and maintain therapeutic concentrations at the site of action. Pharmaceutical properties such as solubility, partition coefficient, permeability and protein binding contribute to *in vivo* disposition and frequently, these properties are important determinants of clinical outcome. The recent successes of combinatorial chemistry in accelerating drug discovery have also increased the interest in rapid, resource-sparing approaches to determining pharmaceutical properties.

The binding of drugs to serum proteins is particularly important because it affects both the activity of drugs and their disposition (1,2). According to the "free drug" hypothesis only unbound drug exerts pharmacological activity (3) and disposition is often altered by drug binding (4,5).

Here, we focus on a method for the rapid determination of the binding of drugs to human serum albumin (HSA) and α_1-acid glycoprotein (AAG) that may potentially prove useful for screening combinatorial libraries. The screen uses spectrofluorimetry, a technique that has been used widely to study biomolecular interactions. The method proposed is rapid, requires small sample volumes and is amenable to automation.

A variety of techniques have been proposed for protein binding measurements including dialysis, ultrafiltration (6), circular dichroism (7), and extrinsic fluorescence (8–11). Surprisingly, little drug-serum protein binding work has been done using the intrinsic fluorescence technique. Recently, Epps *et al.* have demonstrated the feasibility of the intrinsic fluorescence methodology for a series of proprietary compounds (12). Here, we examine whether the intrinsic fluorescence method is capable of detecting drug binding to the warfarin and benzodiazepine binding sites of human serum albumin and extend the method to the popular multiwell format. The results show that the intrinsic fluorescence method is facile, drug nonspecific and has the potential of predicting percent bound in plasma.

MATERIALS AND METHODS

Drugs and Chemicals

Human serum albumin (HSA), α_1-acid glycoprotein (AAG), acetaminophen, chloramphenicol, chlorpromazine hydrochloride, iophenoxic acid, imipramine hydrochloride, phenylbutazone and ($\pm$) sulfinpyrazone were obtained from Sigma Chemicals (St. Louis, MO); sodium salicylate, lithium chloride and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Pittsburgh, PA); Ampicillin was obtained from Apothecon; Diazepam was obtained from Hoffmann-La Roche (Nutley, NJ); Theophylline was obtained from Nutritional Biochemicals Corporation (Cleveland, OH); The R, S and RS enantiomers of sodium warfarin were from Endo Laboratories (Garden City, NY). Dulbecco's phosphate buffered saline (D-PBS) was purchased from Life Technologies, Grand Island, NY.

Fluorescence Spectroscopy

All fluorescence studies were done at room temperature on a SLM Aminco 8000 fluorimeter with 4 mm excitation and emission slits.

Tryptophan fluorescence emission spectra over the 300–400 nm wavelength range were recorded with the excitation wavelength set at 280 nm. A 295 nm long pass filter was used during the measurements to minimize the effect of Raman bands on the emission maxima. Spectra were recorded using the 2

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mm path in I-shaped, 2 mm/10 mm dual path length cuvets to minimize the contributions of the inner filter effect.

HSA and AAG were dissolved in D-PBS at a concentration of 2.02 μM. Stock solutions of sodium warfarin (R, RS, S), lithium chloride, ampicillin, phenylbutazone, iophenoxic acid, imipramine hydrochloride and sodium salicylate were prepared in D-PBS while the diazepam, sulfinpyrazone, theophylline, chloramphenicol, acetaminophen and chlorpromazine hydrochloride were prepared in DMSO. For titrations, solutions were prepared by serially diluting the stock solution in 2.02 μM HSA (or AAG). The solutions were mixed and after allowing 30 minutes at room temperature for equilibration, the spectra were recorded.

The percent reduction in fluorescence (% Quenching) was plotted against total drug concentration, D_T. The quadratic binding equation was used for fitting instead of the Michaelis-Menten type simple binding hyperbola because under the experimental conditions used, the free drug concentration is not measured (10,13). According to the quadratic binding equation, bound drug D_b, is given by:

$$D_b = \frac{S - \sqrt{S^2 - 4D_TP_T}}{2}$$

In the equation, D_b is the concentration of the drug-protein complex, P_T is the total protein concentration, K_d is the dissociation constant and $S = D_T + P_T + K_d$. Without loss of generality, the observed fluorescence F, is related to the molar concentrations of the drug-protein complex D_b, free drug D_f and free protein P_f and their respective specific fluorescence values, f_1, f_2, and f_3.

$$F = f_1D_b + f_2D_f + f_3P_f$$

$$F = f_1D_b + f_2(D_T - D_b) + f_3(P_T - D_b)$$

$$F = (f_1 - f_2 - f_3)D_b + f_2D_T + f_3P_T$$

But $f_3P_T = F_0$, the fluorescence of protein alone in the absence of drug. Rearranging:

$$\frac{F_0 - F}{F_0} = \%\ \text{Quenching} = \frac{(f_2 + f_3 - f_1)}{f_3}\frac{D_b}{P_T} - \frac{f_2}{f_3}\frac{D_T}{P_T}$$

The $(f_2 + f_3 - f_1)/f_3$ and f_2/f_3 terms are constants and since P_T is a constant under the chosen experimental conditions, the constants can be absorbed into new constants C_1 and C_2.

$$\%\ \text{Quenching} = C_1\left(\frac{S - \sqrt{S^2 - 4D_TP_T}}{2}\right) - C_2D_T$$

This quadratic binding equation for fluorescence quenching has 3 fitted parameters, C_1, C_2 and K_d. The least squares curve fitting routine in Kaleidagraph 3.08 (Synergy Software, Reading PA) was used to determine the unknown parameters, particularly K_d from plots of % Quenching vs. D_T.

Sample preparation for the experiments employing multiwell plates was analogous. The samples (200 μl) were transferred to black 96 well plates and read on a SpectraMax Gemini fluorescence microplate reader (Molecular Dynamics, Sunnyvale, CA) with excitation and emission wavelengths of 280 nm and 340 nm, respectively. Data were analyzed using the % Quenching equation with the linear term set to zero because the additional linear term C_2, was not significant, i.e., its confidence intervals spanned the origin. The changes in the K_d estimates, with and without the linear term, were not statistically significant.

All the K_d values are reported in the form, average $K_d \pm$ average standard error of the K_d estimate, and these terms respectively, refer to the arithmetic means of the K_d and standard error estimates obtained from separately fitting data from multiple experiments.

RESULTS

Intrinsic Fluorescence Quenching of HSA Measures Binding to the Warfarin Site

The warfarin binding site is a distinct drug binding site that has been identified on HSA and is used by drugs such as phenylbutazone, sulfonamides, phenytoin and valproic acid. Because fluorescence quenching measures the response of only the amino acids, tryptophan and to a lesser extent tyrosine, we decided to challenge the feasibility and sensitivity of the method by testing the hypothesis that warfarin binding to the warfarin binding site would result in concentration-dependent fluorescence quenching. We selected sodium warfarin as a probe for the site and Fig. 1A shows fluorescence spectra of 2.02 μM HSA in PBS in the presence of varying concentrations of sodium warfarin. The spectra show concentration-dependent decreases in HSA fluorescence. The fluorescence peak of HSA alone is at 342 nm which suggests that the average environment of the two tryptophans is shielded from the surrounding water molecules. At higher drug concentrations, an additional peak corresponding to the fluorescence of the drug appears at longer wavelengths.

Figure 1B shows the percent quenching at 340 nm as a function of drug concentration. The data in Fig. 1B show linearity at low drug concentrations and saturation at high drug concentrations—characteristics usually associated with specific binding to proteins. The data were fitted to a quadratic binding equation and the. average K_d ($\pm$average error) value was found to be 5.3 $\pm$ 1.5 μM using the cuvet method and 6.8 $\pm$ 1.5 μM using the multiwell format. These results show that the warfarin

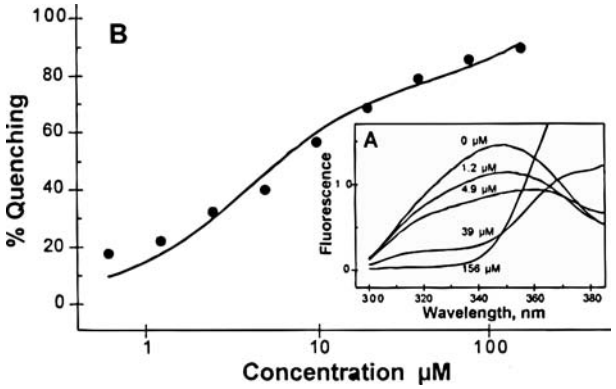

Fig. 1. Figure 1A shows the emission spectra of HSA in the absence and presence of the indicated concentrations of RS sodium warfarin. Figure 1B plots the percent quenching as a function of the RS sodium warfarin concentration. The solid line is the best fit quadratic binding curve.

binding site responds to drug binding with changes in fluorescence quenching.

Intrinsic Fluorescence Quenching of HSA Measures Binding to the Benzodiazepine Site

The benzodiazepine binding site on HSA binds drugs such as diazepam, probenicid and the penicillins and is the other distinct drug binding site on HSA. We tested the hypothesis that HSA fluorescence is modulated by drug binding to the benzodiazepine site using diazepam as a probe for this site.

Figure 2A shows the fluorescence spectra for 2.02 μM HSA in the absence and presence of varying diazepam concentrations. The spectra showed concentration-dependent changes in fluorescence intensity. The peak position did not shift suggesting that the hydrophobicity changes that occur on drug binding are either negligible or are offset. The percent quenching curve at 340 nm is shown in Fig. 2B. The quenching curve shows saturation as a function of drug concentration and K_d obtained by fitting a quadratic binding curve was 12 ± 4.7 μM. The K_d value using the multiwell format was 28 ± 4 μM. The exact reasons for the relatively small, 2.3-fold discrepancy between the multiwell and cuvet methods for this drug are currently unclear.

Intrinsic Fluorescence Quenching of HSA Measures Binding of Acidic Drugs

Because HSA commonly binds acidic drugs, we challenged the generality of the method by testing the hypothesis that the binding of sodium salicylate, a salt of salicylic acid, would quench HSA fluorescence in a concentration-dependent manner.

The percent quenching of HSA in the presence of sodium salicylate is shown in Fig. 3. The quenching curve shows saturation and demonstrates that the intrinsic fluorescence of HSA is modulated by the binding of salicylate. From the fitting, a K_d of 76 ± 8.5 μM was estimated for the cuvet method. The experiments in the multiwell format yielded K_d of 71 ± 12 μM.

Intrinsic Fluorescence Quenching of HSA Is Not Altered by DMSO

Frequently, drugs in development have poor aqueous solubility and stock solutions are prepared in DMSO. To extend the usefulness of the method for drug development applications, we tested the effects of DMSO on HSA fluorescence quenching. Over a concentration range of 0–30% v/v, DMSO did not quench HSA fluorescence (data not shown).

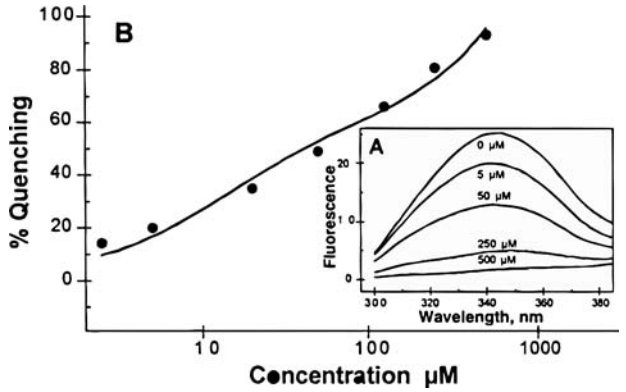

Fig. 2. Figure 2A shows the emission spectra of HSA in the absence and presence of the indicated concentrations of diazepam. Figure 2B is the corresponding percent quenching curve. The solid line is the best fit quadratic binding curve.

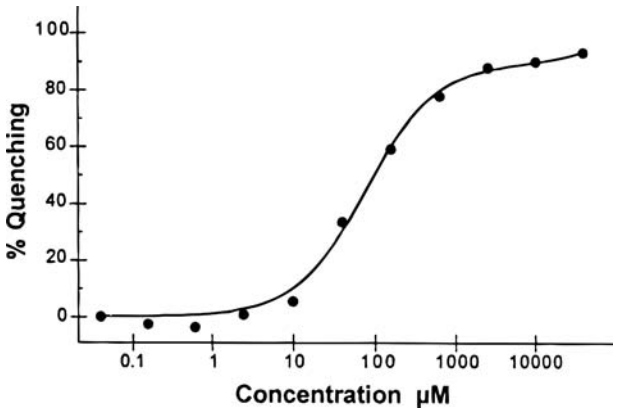

Fig. 3. The percent quenching curve for salicylic acid. The solid line is the best fit quadratic binding curve.

Intrinsic Fluorescence Quenching of AAG is Altered on Drug Binding

Although AAG is present at much lower concentrations in serum than HSA, it often binds certain drugs with high affinity (14). We hypothesized that binding of drugs to AAG would also affect the environment of tryptophan residues and cause concentration dependent fluorescence quenching of AAG spectra. We selected chlorpromazine hydrochloride to test this hypothesis and Fig. 4A shows fluorescence spectra of 2.02 μM AAG in PBS in the presence of varying concentrations of chlorpromazine hydrochloride. The spectra show concentration-dependent decreases in AAG fluorescence. Figure 4B shows the percent quenching at 340 nm as a function of drug concentration. The data were fitted to a quadratic binding equation and the K_d value was found to be 0.47 ± 0.18 μM. These results show that the AAG drug binding site responds to chlorpromazine hydrochloride.

To confirm that the quenching of AAG fluorescence was not unique to chlorpromazine hydrochloride, we carried out experiments with another drug, imipramine hydrochloride. As shown in Fig. 4B, concentration-dependent quenching was again observed with a K_d value of 1.2 ± 0.44 μM.

Comparison of Intrinsic Fluorescence Measurements to Extrinsic Fluorescence Methodology

Epps *et al.* proposed extrinsic fluorescence based assay for measuring drug binding to HSA (10). To validate the intrinsic fluorescence based method proposed to the extrinsic fluorescence based technique, we carried out experiments with three drugs, phenylbutazone, diazepam and warfarin sodium, whose dissociation constants were reported by Epps *et al.* The results in Table I compare the dissociation constants obtained using the two techniques. With the cuvet method, the dissociation constants for warfarin sodium and diazepam were fairly close to those estimated by Epps *et al.* but the intrinsic fluorescence estimate for the dissociation constant for phenylbutazone was

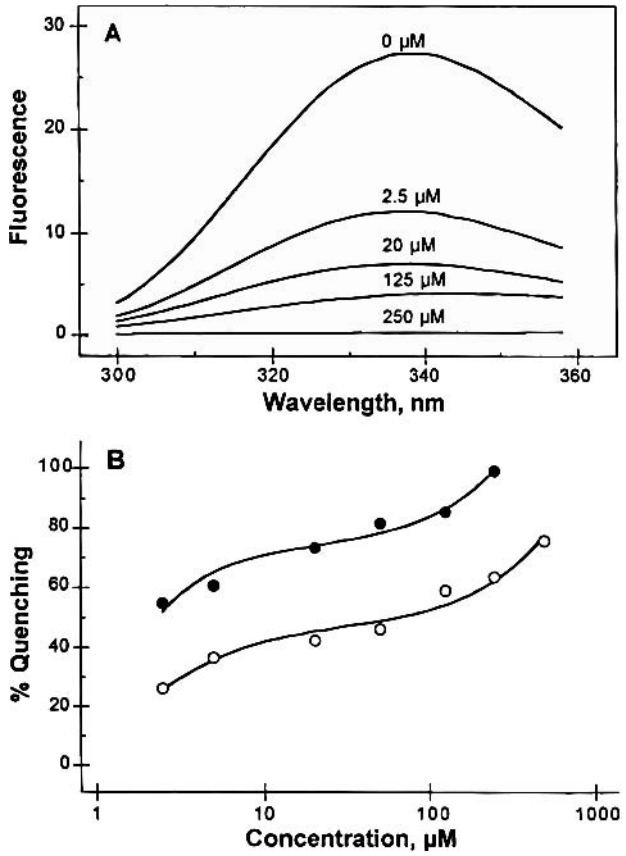

Fig. 4. Figure 4A shows the emission spectra of AAG in the absence and presence of the indicated concentrations of chlorpromazine hydrochloride. Figure 4B is the corresponding percent quenching curve for imipramine hydrochloride (open circles) and chlorpromazine hydrochloride (filled circles). The solid line is the best fit quadratic binding curve.

approximately six-fold greater than that estimated from the extrinsic fluorescence method.

Comparison of Intrinsic Fluorescence Measurements to Equilibrium Dialysis

Equilibrium dialysis is the usual reference method against which other methods are evaluated (14). We compared the dissociation constants obtained using the intrinsic fluorescence method to the equilibrium dialysis measurements of the displacement of 5-dimethylaminonapthalene-1-sulfonamide (DNSA) reported by Sudlow *et al.* (9,15).

The two methods are directly compared using iophenoxic acid, phenylbutazone and sulfinpyrazone binding to HSA in Table I. The rank order for DNSA displacement is inversely related to the rank order for the dissociation constants, i.e., drugs with lower dissociation constants displace DNSA to a greater extent.

Because a rank order correlation does not always imply a quantitative correlation, we further challenged the intrinsic fluorescence method against the DNSA fluorophore displacement method of equilibrium dialysis using the enantiomers of sodium warfarin. These measurements were carried out in the multiwell format and compared to the results of Sudlow *et al.* (15). The results are shown in Table I and again, the results demonstrate that even for compounds that are structurally related, the equilibrium dialysis and intrinsic fluorescence methods correlate well with each other: the enantiomers with higher K_d values cause more DNSA to remain bound.

The work of Sudlow and coworkers have also established that the analysis of drugs using fluorophore displacement in equilibrium dialysis method correlates very well with equilibrium dialysis using radioisotopically labeled drugs. Thus, our findings suggest that the intrinsic fluorescence method will correlate well with equilibrium dialysis (9,15).

Comparison of Intrinsic Fluorescence Measurements to Drug Binding in Plasma

The principal goal of drug binding assays is to determine the fraction of drug bound in plasma at therapeutic concentrations. However, for high throughput screens in discovery, many of the therapeutic parameters are usually unknown and it may be sufficient if a method could simply classify drugs as high, intermediate or poorly bound.

Table II compares the dissociation constants for various HSA binding drugs against the percent bound in plasma. The drugs known to be highly bound, warfarin, sulfinpyrazone, phenylbutazone, diazepam and sodium salicylate, all had K_d values of less than 100 μM. The intermediate binding compounds, chloramphenicol and theophylline had K_d values of 150 μM and 260 μM, while the poorly bound drugs, acetaminophen, lithium ion, ampicillin either had K_d values greater than 1 mM or did not quench. Thus, the intrinsic fluorescence method is capable of satisfactorily classifying drugs for protein binding characteristics.

DISCUSSION

We have systematically analyzed the fluorescence spectra of HSA and AAG, two important drug binding proteins in the presence of a wide range of drugs. We found that the intrinsic fluorescence of these proteins is quenched by drug binding. The fitting equations proposed fitted the observations well and yielded K_d values that were consistent with the values reported using extrinsic fluorescence and equilibrium dialysis methodology. The findings can be easily extended for use in high throughput, drug nonspecific assays for protein binding in multiwell format.

The use of fluorescence based assays offers many advantages over conventional techniques such as dialysis and ultrafiltration. The advantages arise primarily because fluorescence data are obtained without separating the bound and unbound species which reduces the time required for the experiment and eliminates the need for a size selective membrane. The dialysis and ultrafiltration require analysis of free and total drug concentration which can be resource and time consuming. Additionally, these methods cannot be used when the drugs bind extensively to the membrane (14); this is often a serious problem with highly hydrophobic drugs.

The displacement of extrinsic fluorophores such as warfarin and dansylglycine have been proposed as the basis for a rapid protein binding assay (10). Such assays too, are drug nonspecific and rapid but are indirect because they utilize the interaction between two drugs to produce a signal. As shown

Table I. Comparisons of the Intrinsic Fluorescence Method with Other Methods

	Intrinsic vs. extrinsic fluorescence K_d values, μM		
	Intrinsic fluorescence		
Drug	Cuvet	Multiwell	Extrinsic fluorescence[a]
RS sodium warfarin	5.3 ± 1.5	6.8 ± 1.5	3.4 ± 0.69
Diazepam	12. ± 4.7	28 ± 4.0	7.7 ± 1.0
Phenylbutazone	11. ± 5.1	8.4 ± 1.7	1.9 ± 0.3

	Intrinsic fluorescence vs. equilibrium dialysis		
	K_d values, μM by intrinsic fluorescence		
Drug	Cuvet	Multiwell	% DNSA displacement by equilibrium dialysis[b]
Iophenoxic acid	0.081 ± 0.012	Not done	69.6
Phenylbutazone	11. ± 5.1	8.4 ± 1.7	54.6
Sulfinpyrazone	28. ± 5.6	15. ± 3.1	29.5

Drug	K_d values, μM by intrinsic fluorescence (Multiwell)	% DNSA bound by equilibrium dialysis[c]
R(+)-sodium warfarin	12. ± 2.1	68.0
RS-sodium warfarin	6.8 ± 1.5	62.4
S(−)-sodium warfarin	4.6 ± 0.81	60.5

[a] Data from (10).
[b] Data from (9).
[c] Data from (15).

in Table I, our results compare favorably with those of Epps *et al.* (10). The exact reasons for the relatively small, four to six-fold discrepancy in dissociation constant of phenylbutazone and diazepam are currently unclear but it is possible that some differences between the experimental protocols is a contributing factor. However, intrinsic fluorescence offers advantages over extrinsic fluorescence and has the potential to yield better estimates of K_d when, for example, the drug of interest is physically incompatible with the fluorophores or if there are interactions between the binding sites. Physical incompatibility could occur, for example, if the drug of interest caused the fluorophore to precipitate.

Table II. Comparison of Intrinsic Fluorescence K_d Values Against Percent Bound in Human Plasma

Drug	K_d μM by intrinsic fluorescence (multiwell)	% Bound in plasma[a]
RS-sodium warfarin	6.8 ± 1.5	99 ± 1
Phenylbutazone	8.4 ± 1.7	96 ± 1
Sulfinpyrazone	15. ± 3.2	98 ± 0.3
Diazepam	28 ± 4	98.7 ± 0.2
Sodium salicylate	71 ± 12	80 to 95
Theophylline	260 ± 92	56 ± 4
Chloramphenicol	150 ± 48	53 ± 5
Acetaminophen	1279 ± 690	0
Lithium chloride	No quenching	0
Ampicillin	No quenching	18

[a] Data from Goodman and Gilman's (17).

Fluorescence methods differ from ultrafiltration and equilibrium dialysis in that low, non-physiological concentrations of serum protein are employed to avoid the nonlinearities caused by the inner filter effect and the extent of serum binding is inferred from the K_d estimates. An empirical alternative to this "K_d estimation and inference of free fraction" procedure would be to include several drugs with known extents of plasma binding as positive controls within the experiment and to directly compare the quenching curve for the drug of interest to those of the controls. Also, because the fluorescence methods do not directly measure drug concentration using analytical methods, the results may be compromised by protein binding contaminants in the drug preparation. A possible additional limitation is that the linear term in the quadratic binding equation may prove inadequate for drugs that fluoresce strongly under the same excitation and emission conditions as protein.

In fluorescence measurements, the inner filter effect (caused by the absorbance contributions of protein and drug) can be a potentially serious confounding factor and is usually compensated for by making concurrent absorbance measurements (16). Because additional absorbance measurements would add another step to the screen and potentially reduce its throughput, we used other approaches to reducing the impact of the inner filter effect. First, we examined the albumin fluorescence as a function of concentration over the range of 0–100 μM in the absence of drug. Because of the inner filter effect, the albumin intrinsic fluorescence showed apparent saturation and functionally, these observations could be modeled using a simple E_{max} or Michaelis-Menten equation ($r^2 > 0.99$). The range of EC_{50} values from the fitting was 27 μM to 36 μM and based on these values, the 2 μM HSA concentration used

in our experiments was 6–7% of EC_{50}-within in the linear range. Secondly, to ensure uniform illumination, we used a 2 mm path length cuvet. These decisions were supported *post facto* by the excellent correspondence between the fluorescence method and the other approaches. Ultimately, we plan to rigorously correct for the inner filter effect by serially processing samples through a multiwell fluorimeter and a multiwell absorbance spectrophotometer. Two-site and multi-site models for fitting have been explored in detail by Epps (12) but were not used in the analysis of our results because our regression coefficients were usually greater than 95% and the addition of parameters frequently caused some parameters to lose statistical significance.

In conclusion, we hope that rapid high throughput screens for pharmaceutical properties such as protein binding will ultimately result in a combinatorial pharmaceutics revolution to match the combinatorial chemistry revolution in drug discovery.

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