Validation of 96-well Equilibrium Dialysis with Non-radiolabeled Drug for Definitive Measurement of Protein Binding and Application to Clinical Development of Highly-Bound Drugs

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ABSTRACT: Definitive plasma protein binding (PB) studies in drug development are routinely conducted with radiolabeled material, where the radiochemical purity limits quantitative PB measurement. Recent and emerging regulatory guidances increasingly expect quantitative determination of the fraction unbound (Fu) for key decision making. In the present study, PB of 11 structurally- and therapeutically-diverse drugs spanning the full range of plasma binding was determined by equilibrium dialysis of non-radiolabeled compound and was validated against the respective definitive values obtained by accepted radiolabeled protocols. The extent of plasma binding was in agreement with the radiolabeled studies; however, the methodology reported herein enables reliable quantification of Fu values for highly-bound drugs and is not limited by the radiochemical purity. In order to meet the rigor of a development study, equilibrium dialysis of unlabeled drug must be supported by an appropriately validated bioanalytical method along with studies to determine compound solubility and stability in matrix and dialysis buffer, nonspecific binding to the dialysis device, and ability to achieve equilibrium in the absence of protein. The presented methodology establishes an experimental protocol for definitive PB measurement, which enables quantitative determination of low Fu values, necessary for navigation of new regulatory guidances in clinical drug development. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:2498–2507, 2011

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INTRODUCTION

The free-drug hypothesis states that only unbound drug is available for (1) clearance and drug—drug interactions with metabolizing enzymes and transporters, (2) equilibration into tissues, and (3) pharmacological activity, that is, unbound drug concentrations drive pharmacokinetics, pharmacodynamics, and drug–drug interactions. As such, protein binding (PB) in plasma, hepatic microsomes, and relevant target tissues (e.g., brain homogenate) is routinely evaluated in drug discovery to determine the fraction unbound (Fu). The Fu parameter supports various *in vitro*-to-*in vivo* correlations and preclinical-to-clinical predictions, including microsomal and allometric clearance projections, metabolism and transport drug interactions, clinical QT prolongation liability, and understanding of *in vivo* preclinical pharmacodynamics (e.g., *in vivo* receptor occupancy, etc.).[1–6] Various approaches have been used for the measurement of PB in drug discovery including equilibrium dialysis, ultracentrifugation, ultrafiltration, and albumin-column chromatography.[7] Equilibrium dialysis is the most robust and thermodynamically-sound method for measuring PB; thus following the introduction of various 96-well equilibrium dialysis plates over the last decade, it has emerged as the method of choice in drug discovery.[8–10]

Abbreviations used: PB, protein binding; Fu, fraction unbound.

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Table 1. Human Plasma Binding of the 10 Top-Selling Small-Molecule Drugs in 2009 Reported in the Respective Prescribing Information Brochures

Drug	% Bound in Plasma
Lipitor (atorvastatin)	**≥ 98% [14]**
Plavix (clopidogrel)	**98% [15]**
Advair (fluticasone/ **salmeterol**)	fluticasone 91% [16]/**salmeterol 96% [16] (purity > 95%)[17]**
Diovan (valsartan)	95% [18]
Abilify (ariprazole)	**≥ 99% [19]**
Nexium (esomeprazole)	**97% [20]**
Zypreza (olanzapine)	93% [21]
Seroquel (quetiapine)	83% [22]
Crestor (rosuvastatin)	88% [23]
Singulair (montelukast)	**≥ 99% [24]**

Highly-bound drugs, whose binding is reported as either the radiochemical purity or equal to or greater than radiochemical purity, are listed in bold.

During clinical development of small-molecule drugs, plasma PB is reassessed in a more rigorous definitive study using radiolabeled drug material by ultracentrifugation, equilibrium dialysis, or ultrafiltration. The resulting PB parameters contribute to important development decisions (e.g., clinical drug interaction studies) and design of required biopharmaceutical studies (e.g., renal and hepatic impairment studies).[1,11–13] The definitive PB study results are reported to the clinical investigators and regulatory agencies, with the extent of binding in human plasma summarized in the Investigator's Brochure during development and ultimately in the Prescribing Information Brochure, following successful registration and launch (Table 1 and Table 3.)

The major limitation to the measurement of PB with radiolabeled drug material is radiochemical purity, which is typically 97%–99% for ^{14}C-labeled drug, and prior to the 1990s may have been as low as 95% (Tables 1 and 3).[14,17,19,24,25,26] Because unbound drug concentration (buffer receiver chamber in equilibrium dialysis, supernatant in ultracentrifugation, and filtrate in ultrafiltration) used to calculate the extent of binding is quantified by scintillation counting; for highly bound drugs, the extent of binding cannot be quantitatively measured beyond the radiochemical purity. This limitation has been a common problem because high binding is prevalent among modern drugs in clinical development and use.[2,14,19,24] Out of 50 Pfizer compounds, which progressed to clinical testing between 1998 and 2003, 40% exhibited Fu $\leq$ 3% (plasma binding $\geq$ 97%).[2] Furthermore, six of the 10 top-selling small-molecule drugs in 2009 are highly bound in plasma, with the extent of binding reported as greater than or equal to the radiochemical purity in the Prescribing Information Brochure (Table 1).[14–17,19,20,24] Definitive binding continues to be measured with radiolabeled drug material because historically scintillation counting was more robust and more sensitive than the contemporary bioanalytical methods.

The quantitative extent of binding of the highly bound drugs in Table 1 could be determined by combining a chromatographic separation with both radiochemical and mass spectrometric detection (mass spectrometry needed to confirm absence of coeluting peaks). However, as demonstrated by the widely-used drugs in Tables 1 and 3, this approach is rarely taken in practice, and instead binding is qualified as high and reported as equal to or greater than the radiochemical purity.[14,19,24–26] The widespread use of sensitive and robust LC–MS/MS bioanalysis, which is routinely validated to support other development activities, begs the question: Why not measure definitive binding with unlabeled drug, which would allow quantitative measurement of binding for highly-bound drugs?

In drug development of the past, the goal of the definitive PB studies was characterization of the extent of binding in plasma, and not necessarily a quantitative determination of the Fu and its impact on pharmacokinetics and drug interactions. As such, reporting plasma binding as greater than the radiochemical purity (i.e., high binding) was sufficient to support drug development and registration.[14,19,24–26] For example, per the 2006 FDA drug interaction draft guidance, the decision whether a drug interaction study is necessary for a drug that inhibits a metabolic pathway is based on the total systemic concentration.[27] However, in 2010 European regulators have already reconsidered this long-standing approach in favor of simulations driven by unbound concentrations.[11] Regulatory guidances are continually evolving, and there is an increasing expectation for the quantitative determination of the Fu value, even for highly-bound drugs.[11,13] New and emerging guidances are now relying on the unbound drug concentration and Fu value to make key development recommendations.[1,11–13]

The current manuscript presents a non-radiolabeled definitive PB study protocol capable of quantitative determination of binding parameters for highly-bound drugs, and the validation of this approach against radiolabeled data. Although basic elements of the presented PB approach have been used previously for preliminary discovery studies, the definitive methodology differs in the level of bioanalytical rigor, extensive solubility and stability assessments, evaluation of device nonspecific binding, and ability to achieve equilibrium in the absence of protein. This definitive PB protocol is capable of providing Fu values between 0.01% and 100% (99.99% to 0% bound), thus enabling development of highly-bound drugs to comply with emerging regulations based on unbound drug concentrations.

EXPERIMENTAL PROCEDURES

Reagents

Atenolol, diclofenac, diltiazem, imipramine, indomethacin, loperamide, midazolam, nelfinavir, quinidine, sertraline, and warfarin were purchased from Sigma–Aldrich (St. Louis, Missouri). All other chemicals were of reagent grade and readily available from commercial sources.

Plasma was prepared by pooling three lots (A50135, A49989, and A51449) of human plasma obtained from the Blood Bank (Bangalore, India). Both plasma and phosphate buffer (100 mM) pH were adjusted to 7.4 on the day of the experiment.

Plasma Solubility and Stability

Plasma was preincubated for 15 min at 37°C prior to addition of dimethyl sulfoxide (DMSO) solution (0.4% final concentration) containing atenolol (final plasma concentration: 1 and 10 μM), diclofenac (10 and 100 μM), diltiazem (1 and 10 μM), imipramine (1 and 10 μM), indomethacin (1 and 10 μM), loperamide (1 and 10 μM), midazolam (1 and 10 μM), nelfinavir (10 and 100 μM), quinidine (1 and 10 μM), sertraline (1 and 10 μM), or warfarin (10 and 100 μM). Samples were mixed by inversion (4–5 times). Initial concentration samples were taken immediately following mixing and added directly to vials containing acetonitrile with internal standard and were mixed to minimize nonspecific binding to the vial and pipette tip. After 6-h incubation at 37°C, final concentrations were sampled. All samples were analyzed by liquid chromatography with detection by tandem mass spectrometry (LC–MS/MS).

Aqueous Solubility and Stability, Device Nonspecific Binding, and Equilibrium

The Teflon equilibrium dialysis plate (96-well, 150-μL half-cell capacity) and cellulose membranes (12–14 kDa molecular weight cutoff) were purchased from HT-Dialysis (Gales Ferry, Connecticut). On the day of the experiment, dialysis membranes were conditioned as follows: soaked for 15 min in deionized water, followed by 15 min in 25% methanol, and finally 15 min in phosphate buffer. The equilibrium dialysis plate was assembled by placing conditioned membranes between rows of wells and tightly clamping the assembled apparatus.

Aqueous solubility, device recovery, and ability to achieve equilibrium in the absence of protein were assessed in human plasma-dialyzed buffer (100 mM phosphate buffer dialyzed against human plasma for 6 h). DMSO stock solutions containing study compounds were added to dialyzed buffer (0.4% final DMSO concentration) to achieve twice the expected unbound receiver chamber concentration [the product of plasma concentration (i.e., 1, 10, or 100 μM) and the Fu value]. The donor chamber concentration was twice the expected unbound concentration, so that at equilibrium, both chambers contain the expected unbound concentration. Initial concentration samples were taken immediately following mixing (inversion 4–5 times) and added to vials containing acetonitrile with internal standard to minimize nonspecific binding. The solutions were then aliquoted into the donor chambers of the dialysis plate (100 μL per half-well), and the remaining solution was incubated at 37°C for 6 h. An equal volume of dialyzed buffer was placed in each corresponding receiver well. The dialysis plate was sealed with the kit adhesive (HT-Dialysis, Gales Ferry), and dialysis was conducted in an orbital shaker (120 rpm) maintained at 37°C with 5% carbon dioxide atmosphere for 6 h. Following 6-h incubation, aliquots from the donor and receiver chambers, as well as the stock solution incubated for 6 h at 37°C were removed and added to vials containing internal standard in acetonitrile and were mixed to prevent nonspecific binding to the vial and pipette tip. All samples were analyzed by LC–MS/MS.

Equilibrium Dialysis

The equilibrium dialyzer was assembled as described above in *Aqueous Solubility, Device Nonspecific Binding, and Equilibrium.*

Plasma (pH 7.4) was preincubated for 15 min at 37°C prior to addition of stock DMSO solutions (0.4% final concentration) containing atenolol (final plasma concentration: 1 and 10 μM), diclofenac (10 and 100 μM), diltiazem (1 and 10 μM), imipramine (1 and 10 μM), indomethacin (1 and 10 μM), loperamide (1 and 10 μM), midazolam (1 and 10 μM), nelfinavir (10 and 100 μM), quinidine (1 and 10 μM), sertraline (1 and 10 μM), or warfarin (10 and 100 μM). Study concentration range was chosen to provide coverage for both clinically-relevant and supratherapeutic plasma concentrations.[28] Initial plasma concentration samples were taken immediately following mixing (inversion 4–5 times) and added to vials containing internal standard in acetonitrile, and were mixed to prevent nonspecific binding to the vial and pipette tip. Drug-spiked plasma was then aliquoted into the donor chambers of the dialysis plate (100 μL per half-well), and the remaining plasma was incubated at 37°C for 6 h. An equal volume of phosphate buffer (100 mM, pH 7.4) was placed in each corresponding receiver well. The dialysis plate was sealed with the kit adhesive (HT-Dialysis), and dialysis was conducted in an orbital shaker (120 rpm) maintained at 37°C with 5% carbon dioxide atmosphere for 6 h. Complete equilibrium is achieved in the HT-Dialysis apparatus within 6 h.[8] Following incubation, aliquots from the donor and receiver chambers, as well as the plasma stock solution incubated for 6 h at 37°C were removed and added to vials containing internal

standard in acetonitrile and were mixed to prevent nonspecific binding to the vial and pipette tip. All samples were analyzed by LC–MS/MS.

In order to confirm adequate clamp pressure, membrane integrity, and absence of leakage in each row of the dialysis plate, binding of a well-characterized compound should be measured in one well in each row of the 96-well plate as a positive control. On the basis of extensive experience with the binding of diclofenac in human plasma, it is used as the positive control; acceptable range is ±3-fold of the historical Fu (0.25%). The ±3-fold range was selected based on the MSR of this assay.

In order to minimize nonspecific binding associated with multiple transfers, which has the potential to result in underestimation of the extent of binding for highly-bound drugs, a separate set of experiments was used to collect data on potential volume shift over the 6-h dialysis. The donor and receiver chamber volume was measured gravimetrically (assuming 1 g/mL density) at the end of the dialysis.

Bioanalytical Method Development and Characterization

Sensitive LC–MS/MS methods were developed with a lower limit of quantification (LLOQ) to allow the measurement of 0.01% Fu of the lowest tested concentration (e.g., LLOQ = 0.10 nM for 1 μM test concentration).

Prior to solubility and binding experiments, bioanalytical methods for each analyte were characterized by performing a one-batch precision and accuracy assessment. The batch consisted of calibration curves (minimum of six concentrations) at the beginning and end of the analytical batch, quality control samples (six replicates) at LLOQ, 1% of final tested concentration in buffer, and highest tested concentration in human plasma. A minimum of five different standard curve concentrations had to meet the ±20% accuracy criteria in both the front and back calibration curve. The quality control samples had to meet the following criteria: (1) accuracy not to exceed ±15% relative error (±20% at the LLOQ) for the average at each concentration and (2) precision not to exceed 15% relative standard deviation (20% at the LLOQ) for the average at each concentration, a minimum of five acceptable results at each concentration if an individual result is rejected as an outlier by the *Q*-test. The LLOQ response had to be at least four times the response of the blank sample. Variation in internal standard area counts could not exceed ±50% in the quality control samples and back calibration curve samples relative to the front calibration curve. Carryover of the analyte and internal standard was assessed with blank samples containing internal standard injected after the highest calibration curve point; both relative carryover and absolute carryover had to be less than 25%, where

$$\%\ \text{Relative carryover} = \\ = (\text{blank peak area}/\text{average LLOQ area}) \times 100\%,$$

and

$$\%\ \text{Absolute carryover} = \\ = (\text{blank peak area}/\text{high standard peak area}) \times 100\%$$

Bioanalysis

Samples were analyzed by LC–MS/MS (Applied Biosystems API 4000 triple quadrupole with TurboIonSpray interface; MDS Sciex, Concord, Ontario, Canada). Analytes and their internal standards were eluted from a Kromasil Cyano column (4.6 × 50 mm, dp = 5 μm; Eka Chemicals, Bohus, Sweden) using isocratic elution [mobile phase = acetonitrile: 5 mM ammonium formate (70:30) + 0.05% formic acid, flow rate = 500–600 μL/min] and were detected using multiple reaction monitoring. Sertraline was used as the internal standard for atenolol, imipramine, loperamide, midazolam, nelfinavir, quinidine, and warfarin; loperamide was the internal standard for diltiazem and sertraline; flufenamic acid and diclofenac were internal standards for diclofenac and indomethacin, respectively. Internal standards were selected based on their capacity to provide acceptable response in the preferred ionization mode for a given analyte, and as necessary, match the chromatographic character of the analyte.

Each analytical batch contained at least three sets of quality controls, run at three concentration levels, comprising a high QC level of at least 75% of the upper limit of quantification, a low QC level that is no greater than five times the LLOQ, and an intermediate QC level. For a run to be accepted, at least 67% of the overall QCs and 50% at each concentration level had to pass the ±20% accuracy criterion. The calibration curves, quality control samples, and assay acceptance criteria were otherwise as described in *Bioanalytical Method Development and Characterization* section. Analyst software (v. 1.4) was used to acquire, integrate, regress, and quantify bioanalytical data.

Data Analysis

In *Plasma Solubility and Stability* assay, the percent (%) initial target concentration and percent remaining at 6 h were calculated using the following equations:

$$\%\ \text{Initial target concentration} = \\ = \left\{ \frac{[\text{Compound}]_{0\,\text{h}}}{[\text{Compound}]_{\text{nominal}}} \right\} \times 100\%$$

$$\%\ \text{Remaining at 6h} = \left\{\frac{[\text{Compound}]_{6h}}{[\text{Compound}]_{0h}}\right\} \times 100\%$$

where, $[\text{Compound}]_{\text{nominal}}$ is nominal compound concentration in human plasma, $[\text{Compound}]_{0h}$ is compound concentration in plasma immediately following addition of the DMSO stock, $[\text{Compound}]_{6h}$ is concentration in plasma following 6-h incubation at 37°C. The nominal concentration is only used in calculation of percent initial target concentration in plasma and dialysate to assess initial compound solubility and stability. All subsequent calculations, including all binding parameters, are based on experimentally-determined concentrations and so are not impacted by potential weighing inaccuracies.

In *Aqueous Solubility, Device Nonspecific Binding, and Equilibrium* assay, the percent initial target concentration, percent remaining at 6 h, percent equilibrium, and percent device recovery were calculated using the following equations:

$$\%\ \text{Initial target concentration} = = \left\{\frac{[\text{Compound}]_{0h}}{[\text{Compound}]_{\text{nominal}}}\right\} \times 100\%$$

$$\%\ \text{Remaining at 6 h} = \left\{\frac{[\text{Compound}]_{6h}}{[\text{Compound}]_{0h}}\right\} \times 100\%$$

$$\%\ \text{Equilibrium} = \left\{\frac{[\text{Compound}]_{\text{receiver}}}{[\text{Compound}]_{\text{donor}}}\right\} \times 100\%$$

$$\%\ \text{Device recovery} = = \left\{\frac{[\text{Compound}]_{\text{receiver}} + [\text{Compound}]_{\text{donor}}}{[\text{Compound}]_{6h}}\right\} \times 100\%$$

where, $[\text{Compound}]_{\text{nominal}}$ is nominal compound concentration in phosphate buffer, $[\text{Compound}]_{0h}$ is compound concentration in buffer immediately following addition of the DMSO stock, $[\text{Compound}]_{6h}$ is concentration in buffer following 6-h incubation at 37°C, $[\text{Compound}]_{\text{receiver}}$ is compound concentration in receiver compartment after 6-h dialysis, $[\text{Compound}]_{\text{donor}}$ is compound concentration in donor compartment after 6-h dialysis. The percent equilibrium calculation is used to experimentally confirm the assumption that compound freely diffuses through the dialysis membrane to complete equilibrium in the absence of plasma protein. The recovery equation does not contain volume terms because volume loss from the dialysis apparatus was negligible (i.e., $\text{Volume}_{\text{initial}} \approx \text{Volume}_{\text{final}}$; see *Results*), thus compound recovery was calculated solely based on concentrations because equal volume terms cancel out between the numerator and denominator.

In *Equilibrium Dialysis* assay, the percent unbound, percent bound, and percent device recovery were calculated as follows:

$$\%\text{Unbound} = \left\{\frac{[\text{Compound}]_{\text{buffer}}}{[\text{Compound}]_{\text{matrix}}}\right\} \times 100\%$$

$$\%\text{Bound} = 100\% - \%\text{Unbound}$$

$$\%\ \text{Recovery} = = \left\{\frac{[\text{Compound}]_{\text{buffer}} + [\text{Compound}]_{\text{matrix}}}{[\text{Compound}]_{6h}}\right\} \times 100\%$$

where, $[\text{Compound}]_{\text{buffer}}$ is compound concentration in buffer (receiver chamber) following 6-h dialysis, $[\text{Compound}]_{\text{matrix}}$ is compound concentration in plasma (donor chamber) following 6-h dialysis, and $[\text{Compound}]_{6h}$ is compound concentration in nondialyzed plasma (donor chamber stock) after 6-h incubation at 37°C. The recovery equation does not contain volume terms because volume loss from the dialysis apparatus was negligible (i.e., $\text{Volume}_{\text{initial}} \approx \text{Volume}_{\text{final}}$; see *Results* section), thus compound recovery was calculated solely based on concentrations because equal volume terms cancel out between the numerator and denominator.

The maximal impact of various deviations from the acceptable range (e.g., incomplete aqueous solubility, incomplete equilibrium in buffer vs. buffer dialysis, etc.) on the Fu ($\%\ \text{Unbound}_{\text{max}}$) and fraction bound ($\%\ \text{Bound}_{\text{min}}$) was calculated as follows:

$$\%\text{Unbound}_{\text{max}} = = \%\text{Unbound}_{\text{experimental}} \times \frac{100\%}{\%\text{Observed}_1} \times (\cdots) \times \frac{100\%}{\%\text{Observed}_n}$$

$$\%\text{Bound}_{\text{min}} = 100\% - \%\text{Unbound}_{\text{max}}$$

where, $\%\text{Unbound}_{\text{experimental}}$ is experimentally-determined Fu, $\%\text{Observed}_{1\ldots n}$ represent observed solubility, equilibrium, etc. values outside of acceptable range (e.g., target buffer concentration < 70%, equilibrium < 70%, etc.).

Data Reporting and Acceptance Criteria

All experimental measurements are reported as mean ± standard deviation, $n = 6$, unless otherwise noted. Outliers were identified by the Q-test (95% confidence

level). Concentrations below the limit of quantification were treated as "no data" and were not used in calculations. Binding parameters are only reported where $n \geq 3$. Experimentally-determined initial concentration values in matrix and buffer must have precision and accuracy within ±30%. Equilibrium dialysis device recovery, 6-h stability in matrix and buffer, and % equilibrium must each be 100 ± 30%. Percent coefficient of variation (CV) on each Fu and fraction bound value must be less than 30% to be reportable. The ±30% acceptance criteria for these experiments were chosen based on accepted LC–MS/MS bioanalytical variability, which overall is ±15%–20% but is generally greater near the LLOQ (acceptable up to ±25%–30%), where unbound concentrations of highly-bound drugs are usually measured.[29,30] Percent unbound values were reported up to 0.01% (99.99% bound). At higher test concentrations (i.e., 10 and 100 μM), quantification of percentage unbound values below 0.01% are bioanalytically possible, but any such values were reported as <0.01% (>99.99% bound). The historical diclofenac Fu is 0.25%; the positive control in each row of the dialysis plate must be within ±3-fold of the historical value.

RESULTS

Table 2 provides a summary of plasma and dialyzed buffer solubility and stability, device nonspecific binding, and ability to achieve equilibrium in the absence of protein. All 11 drugs demonstrated acceptable (70%–130%) initial solubility in human plasma (11-compound overall % initial target concentration = 97 ± 13%) and stability following 6-h incubation at 37°C (11-compound overall % remaining at 6 h = 95 ± 12%) at the concentrations tested (1 and 10 μM or 10 and 100 μM). All drugs demonstrated acceptable initial solubility in human plasma-dialyzed buffer at the concentrations tested (plasma concentrations × Fu), except sertraline at 600 nM (69%) and loperamide at 40 nM (53%). In this validation exercise, the experiments proceeded because both sertraline and loperamide demonstrated acceptable initial solubility in human plasma-dialyzed buffer at the other concentrations tested; the potential impact of the low initial aqueous concentrations on the binding parameters is reported below. All 11 drugs demonstrated acceptable stability in human plasma-dialyzed buffer following 6-h incubation at 37°C at the concentrations tested. Except nelfinavir (35%–36% equilibrium), all the other drugs reached equilibrium between the donor and receiver chambers (10-drug overall % equilibrium = 93 ± 11%). The impact of the inability to reach equilibrium for nelfinavir on the measurement of binding parameters is reported below. Indomethacin (64 nM, 69% equilibrium) and loperamide (40 nM, 68% equilibrium) were below the 70%–130% acceptable equilibrium range, but considering that the other concentrations tested were within the acceptable range and the low deviation outside the acceptable range, the binding study proceeded with indomethacin and loperamide; the impact of these findings on the binding parameters is reported below. All drugs demonstrated acceptable dialysis apparatus recovery in the absence of plasma protein at the concentrations tested (11-compound overall recovery = 100 ± 13%).

Human plasma binding parameters for the 11 drugs are summarized in Table 3. Recovery of all 11 drugs following dialysis was within the acceptable 70%–130% range (11 drug overall recovery = 99 ± 14%). The extent of atenolol, diltiazem, and quinidine binding in human plasma was low (Fu > 10%). Plasma binding of imipramine, loperamide, and sertraline was moderate (10% > Fu ≥ 2%). Binding of diclofenac, indomethacin, midazolam, nelfinavir and warfarin in human plasma was high (Fu < 2%). For each compound, the extent of binding was comparable across the concentration range tested (1–10 μM or 10–100 μM).

The extent of plasma binding determined by equilibrium dialysis of unlabeled drugs was comparable to reported definitive values generated with radiolabeled compounds (Table 3). Atenolol was predominantly unbound in human plasma in agreement with the Prescribing Information, although numerical differences were observed in the atenolol Fu values. For the highly-bound drugs, diclofenac, indomethacin, midazolam, nelfinavir and warfarin, equilibrium dialysis of unlabeled drug yielded quantitative binding values, whereas reported radiolabeled data could only qualify the extent of binding as high and equivalent to or greater than the radiochemical purity. Concentration-independent binding is consistent with the Prescribing Information Brochures.

Four drugs, nelfinavir, loperamide, indomethacin, and sertraline, exhibited deviations from the 70%–130% acceptable range in one or more of the following categories: plasma or buffer solubility, stability, device nonspecific binding, and ability to attain equilibrium (Table 2). The maximal impact of these deviations on the binding parameters reported in Table 3 was calculated using the $\%Unbound_{max}$ and $\%Bound_{min}$ equations described in *Experimental Procedures* section. The most serious deviation was observed with nelfinavir, which did not achieve complete equilibrium between the donor and acceptor chambers in the absence of plasma protein (35%–36% equilibrium at the two concentrations tested). The incomplete equilibrium may have caused the plasma Fu to have been underestimated experimentally; however, the magnitude of the inaccuracy is expected

Table 2. Summary of Mean Solubility, Stability, Device Nonspecific Binding, and Equilibrium Data

	Human Plasma			Human Plasma-Dialyzed Buffer				
Compound	Conc (μM)	% Initial Target Conc	% Remaining at 6 h	Conc (nM)	% Initial Target Conc	% Remaining at 6 h	% Equilibrium	% Device Recovery
Atenolol	1	118	127	1440	125	101	108	100
	10	90	92	13120	127	127	96	82
Diclofenac	10	91	96	40	119	94	92	109
	100	97	91	480	99	105	86	100
Diltiazem	1	99	87	480	96	92	99	90
	10	101	85	4440	83	93	101	96
Imipramine	1	94	89	120	79	96	98	108
	10	87	91	1480	83	92	98	88
Indomethacin	1	97	89	6	74	102	80	107
	10	98	92	64	109	96	69	124
Loperamide	1	85	92	40	53	101	68	124
	10	81	76	1000	80	95	93	75
Midazolam	1	97	100	13	101	102	92	115
	10	100	107	164	98	100	96	109
Nelfinavir	10	76	82	2	117	107	35	84
	100	83	87	60	82	88	36	91
Quinidine	1	120	107	320	98	93	110	105
	10	125	123	3760	85	101	96	105
Sertraline	1	98	96	40	77	79	89	94
	10	117	110	600	69	81	92	94
Warfarin	10	97	89	160	117	91	96	96
	100	89	90	2000	101	89	100	113

to be no greater than 2.9-fold (100%/35%). Therefore, nelfinavir plasma $\%Unbound_{max}$ is 0.09 ± 0.03 and $\%Bound_{min}$ 99.91 ± 0.03, which is an order of magnitude (23-fold) lower than the Fu estimated with radiolabeled compound (< 2%). Loperamide at the 40 nM concentration exhibited initial buffer concentration 53% of nominal value and did not attain complete equilibrium (68%). The maximal impact of the solubility (1.9-fold = 100%/53%) and incomplete equilibrium (1.5-fold = 100%/68%) on the binding values is 2.8-fold (1.9 × 1.5); therefore, the $\%Unbound_{max}$ is 5.91 ± 1.30 and $\%Bound_{min}$ is 94.09 ± 1.30, which is in

Table 3. Binding Parameters for the 11 Drug Validation Set in Human Plasma

	Prescribing Information		Experimental			
Compound	% Bound (Reported)	% Unbound (Calculated)	Concentration (μM)	% Bound	% Unbound	% Recovery
Atenolol	6–16[31]	94–84	1	$28.53 \pm 3.00(n = 4)$	$71.47 \pm 3.00(n = 4)$	$81 \pm 1.4(n = 4)$
			10	$34.30 \pm 7.24(n = 6)$	$65.70 \pm 7.24(n = 6)$	$105 \pm 4.8(n = 6)$
Diclofenac	>99[25]	< 1	10	$99.85 \pm 0.01(n = 6)$	$0.15 \pm 0.01(n = 6)$	$129 \pm 5.5(n = 6)$
			100	$99.97 \pm 0.04(n = 6)$	$0.23 \pm 0.04(n = 6)$	$106 \pm 15.9(n = 6)$
Diltiazem	70–80[32]	30–20	1	$75.99 \pm 1.05(n = 6)$	$24.01 \pm 1.05(n = 6)$	$91 \pm 2.8(n = 6)$
			10	$77.80 \pm 1.04(n = 6)$	$22.20 \pm 1.04(n = 6)$	$97 \pm 2.1(n = 6)$
Imipramine	90.1 ± 1.4[28]	9.9 ± 1.4	1	$93.89 \pm 0.57(n = 5)$	$6.11 \pm 0.57(n = 5)$	$107 \pm 2.3(n = 5)$
			10	$92.55 \pm 0.24(n = 5)$	$7.45 \pm 0.24(n = 5)$	$110 \pm 2.2(n = 5)$
Indomethacin	ap. 99[33]	ap. 1	1	$99.69 \pm 0.03(n = 6)$	$0.31 \pm 0.03(n = 6)$	$107 \pm 5.4(n = 6)$
			10	$99.68 \pm 0.02(n = 6)$	$0.32 \pm 0.02(n = 6)$	$108 \pm 2.8(n = 6)$
Loperamide	97[34]	3	1	$97.87 \pm 0.47(n = 6)$	$2.13 \pm 0.47(n = 6)$	$103 \pm 6.1(n = 6)$
			10	$94.96 \pm 1.04(n = 6)$	$5.04 \pm 1.04(n = 6)$	$99 \pm 1.6(n = 6)$
Midazolam	98[28]	2	1	$99.33 \pm 0.05(n = 6)$	$0.67 \pm 0.05(n = 6)$	$112 \pm 6.4(n = 6)$
			10	$99.17 \pm 0.12(n = 6)$	$0.83 \pm 0.12(n = 6)$	$108 \pm 15.2(n = 6)$
Nelfinavir	>98[26]	< 2	10	$99.99 \pm 0.002(n = 6)$	$0.01 \pm 0.002(n = 6)$	$110 \pm 3.7(n = 6)$
			100	$99.97 \pm 0.01(n = 4)$	$0.03 \pm 0.01(n = 4)$	$102 \pm 1.5(n = 4)$
Quinidine	80–88[35]	20–12	1	$83.16 \pm 1.22(n = 5)$	$16.84 \pm 1.22(n = 5)$	$74 \pm 0.6(n = 5)$
			10	$81.30 \pm 0.81(n = 5)$	$18.70 \pm 0.81(n = 5)$	$70 \pm 2.9(n = 5)$
Sertraline	98[36]	2	1	$97.82 \pm 0.62(n = 6)$	$2.18 \pm 0.62(n = 6)$	$96 \pm 1.3(n = 6)$
			10	$97.09 \pm 0.56(n = 5)$	$2.91 \pm 0.56(n = 5)$	$85 \pm 2.2(n = 5)$
Warfarin	ap. 99[37]	ap. 1	10	$99.29 \pm 0.07(n = 5)$	$0.71 \pm 0.07(n = 5)$	$91 \pm 0.9(n = 5)$
			100	$99.00 \pm 0.13(n = 3)$	$1.00 \pm 0.13(n = 3)$	$91 \pm 9.4(n = 3)$

closer agreement with the binding parameters determined at the higher loperamide concentration (Fu = 5.04 ± 1.04%), where no experimental issues were observed. Deviations observed with 600 nM sertraline (% initial target concentration = 69%) and 109 nM indomethacin (% equilibrium = 69%) were relatively minor and not observed at the other concentrations tested; the maximal impact on the Fu values is 1.4-fold (100%/69%).

Volume shift over the 6-h dialysis was assessed in a separate set of experiments (n = 492). A marginal volume shift was observed from the buffer receiver chamber (92 ± 5% of initial volume) to the plasma donor chamber (97 ± 5% of initial volume). The approximately 5% volume shift from buffer to plasma (i.e., dilution of plasma) was considered negligible because it has minimal impact on the binding data (5% error in Fu value). Volume loss from the receiver and donor wells over the 6-h dialysis was negligible under these experimental conditions, that is, $Volume_{initial} \approx Volume_{final}$.

DISCUSSION

Human plasma binding was determined using equilibrium dialysis of non-radiolabeled compound for 11 structurally-diverse drugs from a variety of therapeutic areas, spanning the full range of low, moderate, and high plasma binding. The study was supported with an appropriately validated LC–MS/MS bioanalytical assay, as well as thorough evaluation of matrix and buffer solubility and stability, device nonspecific binding, and ability to achieve equilibrium in the absence of protein. The extent of binding of the 11 drugs was in agreement with Prescribing Information values obtained by accepted definitive methods using radiolabeled compound.

The quantitative extent of binding was in agreement between this study and the reported definitive values for drugs with low plasma binding (atenolol, diltiazem, and quinidine) and moderate plasma binding (imipramine, loperamide, and sertraline).[28,31,32,34–36] Binding of diclofenac, indomethacin, midazolam, nelfinavir, and warfarin in human plasma was high. The present study was qualitatively in agreement with the radiolabeled data for these highly-bound drugs. However, equilibrium dialysis of unlabeled drug was capable of quantitative determination of low Fu values; in contrast, reported radiolabeled data could only qualify the extent of such binding as high and equivalent to or greater than the radiochemical purity.[25,26,28,33,37]

Definitive PB studies with radiolabeled drug have been used almost exclusively in drug development because scintillation counting is robust and circumvents the ±15%–20% variability of LC–MS/MS bioanalysis inherent even to asssays with the highest levels of validation.[29,30] However, conduct of radiolabeled PB studies has several major disadvantages. Radiolabeled PB studies are unable to quantify binding beyond the radiochemical purity, essentially making radiolabeled PB studies qualitative for highly-bound drugs.[14,19,24–26] In radiolabeled equilibrium dialysis, radiolabel recovery may appear high and provide a false sense of accuracy, when in fact the receiver chamber concentration, and thus the Fu were underestimated. For example, consider a drug with Fu equal to 5% and radiochemical purity equal to 99%: following dialysis, plasma concentration should be 95% of the original concentration, and buffer concentration should be 5%. However, the receiver concentration may be 1% with recovery of at least 96%, resulting in a 5-fold underestimation of the Fu, if any of the following occurred: (1) compound precipitated or was bound to the device in the receiver chamber in the absence of protein, (2) unbound compound formed micelles in the donor chamber and only a part of the donor chamber concentration was available for dialysis, (3) for other reasons (e.g., membrane binding), full equilibrium was not achieved. Furthermore, it is unknowable whether the apparent Fu value of 1% is due to actual PB of the drug, or far higher drug PB but low binding of the radiochemical impurity. Equilibrium dialysis is the most robust PB methodology; other methods used in definitive PB studies, such as ultracentrifugation or ultrafiltration, present additional experimental pitfalls.[7]

The conduct of definitive PB studies with equilibrium dialysis and non-radiolabeled compound does not automatically circumvent the technical challenges associated with poor solubility, instability, nonspecific binding, and inability to achieve equilibrium. These basic underlying assumptions of equilibrium dialysis must be evaluated independently. In the presented definitive PB protocol, a series of experiments was conducted to confirm that the compound does not violate any of these assumptions. The *Experimental Procedures* section provides a detailed description of how these experiments should be conducted in support of definitive PB. If a compound deviates outside the acceptable range in any of the evaluated parameters, the maximal impact of such a deviation on the Fu value can be quantified and reported. Furthermore, if a compound is determined to have such poor solubility, stability, high nonspecific binding, or cannot attain equilibrium, such that equilibrium dialysis is not appropriate, these results will assist in the choice of an alternative PB methodology, for example ultracentrifugation (poor aqueous solubility, inability to achieve equilibrium) or ultrafiltration (instability).[7]

The goal of the definitive PB determination in drug development has been primarily characterization of the extent of binding in plasma; therefore, reporting

plasma binding as greater than the radiochemical purity (i.e., qualitatively high binding) was sufficient to support drug development and registration.[14,19,24–26] However, recent and emerging regulatory framework is increasingly aligned with the free-drug hypothesis and makes key recommendations with respect to drug clearance characterization and drug interactions based on unbound drug concentrations.[1,11–13] The 2010 EMA drug interaction draft guidance supports simulations with unbound drug concentrations for evaluation of whether *in vitro* inhibition of a metabolic pathway poses a clinical drug interaction risk.[11] The new EMA guidance also presented the expectation for comparison of renal clearance to the unbound glomerular filtration rate (Fu × glomerular filtration rate) to determine whether a drug is cleared passively by filtration or is actively secreted.[11] Likewise, the 2010 FDA draft guidance on pharmacokinetics in patients with impaired renal function clearly expects quantitative determination of potential binding differences between groups of patients with varying degrees of renal function.[13] Drugs with low binding (< 80% bound, Fu > 20%) are exempted from the PB determination, but quantitative assessment of potential binding differences is expected for drugs with moderate and high binding.[13] The seven transporters recommended for evaluation during drug development by the International Transporter Consortium have been voted for inclusion into the revision of the FDA drug interaction draft guidance by the FDA advisory committee in March 2010.[1] The International Transporter Consortium decision trees are uniformly driven by unbound, and not total, systemic drug concentrations.[1] The regulatory guidances continue to evolve, and there is an increasing expectation for the quantitative determination of the Fu value, including for highly-bound drugs.[1,11,13] As such, it is becoming increasingly insufficient to report definitive drug binding as qualitatively high and equal to or greater than the radiochemical purity.

Another important PB consideration in drug development is the evaluation of potential binding differences in special populations. Although the selection of special populations is ultimately driven by the indication, the regulatory expectation is that pharmacokinetics (including potential changes in binding) be characterized in patients with hepatic or renal impairment, if hepatic or renal clearance accounts for more than 20% and more than 30% of systemic clearance, respectively.[12,13] Because nearly all small-molecule drugs are hepatically and/or renally cleared, these studies are conducted for most drugs during clinical development.[38] The guidance specifically emphasizes evaluation of quantitative PB differences for moderately- and highly-bound drugs (hepatic impairment Fu < 10%, renal impairment Fu < 20%).[12,13] For example, quinidine plasma binding is 2.5-fold lower in infants and neonates (Fu = 30%–50% vs. adult Fu = 12%–20%), but is increased in renal-failure patients.[35] Because PB of quinidine is low, radiolabeled PB studies were able to quantitatively assess these changes. However, for a drug with Fu equal to 0.3% in normal adults, if the Fu changes by three fold to 0.1% or 1% in special populations, a radiolabeled method limited by 97%–99% radiochemical purity will be incapable of quantifying any differences in binding. As such, the need exists for definitive PB methodology capable of quantifying high binding.

Although not specifically discussed in this manuscript, the presented definitive PB methodology can be applied to matrices other than plasma. For example, the experiment can be conducted at the physiological albumin concentration or the lower and upper limit of the alpha-1-acid glycoprotein physiological range to determine the predominant plasma-binding protein. In addition, for development decisions regarding drug interaction studies based on *in vitro*-to-*in vivo* extrapolation of metabolism inhibition data, definitive microsomal PB study may be conducted.

In summary, a non-radiolabeled definitive PB study protocol capable of quantitative determination of binding parameters for highly-bound drugs was validated and verified against definitive radiolabeled data. This validation study demonstrated that with adequate bioanalytical rigor, solubility, stability, nonspecific binding, and equilibrium characterization, equilibrium dialysis of non-radiolabeled drug is a suitable method for definitive determination of plasma PB. Application of this PB approach to support clinical drug development is novel and could serve as an invaluable tool for rational navigation of new regulatory framework with highly-bound drugs.

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