Published in final edited form as:

J Pharm Biomed Anal. 2013 March 5; 75: 112–117. doi:10.1016/j.jpba.2012.11.018.

A mass balance approach for calculation of recovery and binding enables the use of ultrafiltration as a rapid method for measurement of plasma protein binding for even highly lipophilic compounds

Changguang Wang^a and Noelle S. Williams^{a,b}

Changguang Wang: Changguang.Wang@utsouthwestern.edu; Noelle S. Williams: Noelle.Williams@utsouthwestern.edu

^aDepartment of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390, U.S.A

Abstract

The aim of this study is to further validate the use of ultrafiltration (UF) as a method for determining plasma protein binding (PPB) by demonstrating that non-specific binding (NSB) is not a limitation, even for highly lipophilic compounds, because NSB sites on the apparatus are passivated in the presence of plasma. Mass balance theory was used to calculate recovery of 20 commercial and seven investigational compounds during ultrafiltration in the presence and absence of plasma. PPB was also measured using this mass balance approach for comparison to PPB determined by rapid equilibrium dialysis (RED) and as found in the literature. Compound recovery during UF was dramatically different in the presence and absence of plasma for compounds with high NSB in PBS only. A comparison of PPB calculated by ultrafiltration with literature values or calculated by RED gave concordant results. Discrepancies could be explained by changes in pH, insufficient time to equilibrium, or compound instability during RED, problems which were circumvented by ultrafiltration. Therefore, NSB, as measured by the traditional incubation of compound in PBS, need not be an issue when choosing UF as a PPB assay method. It is more appropriate to calculate compound recovery from the device in plasma as measured by mass balance to determine the suitability of the method for an individual compound. The speed with which UF can be conducted additionally avoids changes in pH or compound loss that can occur with other methods. The mass balance approach to UF is thus a preferred method for rapid determination of PPB.

Keywords

Plasma protein binding; non-specific binding; ultrafiltration; mass balance; rapid equilibrium dialysis

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^bTo whom correspondence should be addressed Department of Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390-9038, U.S.A., Tel: 214-648-5072, Fax: 214-648-8856, Noelle.Williams@UTSouthwestern.edu.

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1. INTRODUCTION

Plasma protein binding (PPB) plays an important role in the pharmacokinetics (PK) and pharmacodynamics (PD) of a drug. Free drug levels are closely related to the pharmacological effects of a compound as the principal determinant of tissue distribution, cell entry, receptor interactions, and availability for elimination. At the early stages of drug development, PPB experiments advance understanding of ADME (absorption, distribution, metabolism, excretion) properties to aid in candidate selection [1]. There are numerous methods available for measuring PPB including ultrafiltration (UF), equilibrium dialysis, ultracentrifugation, and *in vivo* microdialysis [2–4]. Each technique has its own advantages and pitfalls. Equilibrium dialysis (ED) is the most commonly used method and is frequently considered the gold standard. Recently, a rapid equilibrium dialysis (RED) device was developed in order to reduce the time to equilibrium, and many published papers have reported the use of this method [3, 5]. UF is another frequently used simple and rapid method in which centrifugal force is used to separate free drug from that bound to plasma proteins through a size exclusion membrane. However, several authors have raised questions regarding the adequacy of UF for PPB measurement because of high non-specific binding, which is traditionally calculated by recovery of the compound in the ultrafiltrate when incubated with PBS [6–9]. The filter membrane and plastic device provide NSB sites due to their polarity and charge characteristics. In short, non-specific binding is viewed as a serious impediment to the application of UF for measurement of compound binding to plasma proteins.

Because of the speed and ease with which UF is conducted, many investigators have attempted to reduce NSB by the use of alternative membrane materials or chemical pretreatment of the UF device, such as the use of 0.5% Tween or benzalkonium chloride [8]. However, it was hypothesized here that such treatment is unnecessary because the NSB behavior of a compound would be very different when that compound is incubated with a UF device in PBS versus plasma. It is well known that serum proteins will adsorb to the surface of many types of materials [10, 11] potentially blocking most NSB sites. Thus, rather than calculating NSB after an incubation with PBS, it may be more appropriate to calculate total recovery of compound in plasma from the device to determine the suitability of the method. Here, mass balance analysis was used to investigate the recovery of 20 commercial drugs and seven investigational compounds in comparison with the traditionally calculated NSB value to validate the use of UF for determination of plasma protein binding.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Centrifugal filter units (Centrifree® YM-30 regenerated cellulose membrane, MWCO 30K) were obtained from Millipore (Billerica, MA). Rapid equilibrium dialysis (RED) devices were obtained from Thermo Scientific (Woburn, MA). Mouse plasma was purchased from Bioreclamation LLC (Westbury, NY). Nineteen of the commercial drugs (acetaminophen, atenolol, lovastatin, paclitaxel, propranolol, sulfamethoxazole, terbutaline, tolbutamide, warfarin, indomethacin, hydrocortisone, vinblastine, verapamil, vorinostat, sulfadiazine, sulfathiazole, sulfamethiazole, sulamerazine, and sulfadimethoxine) and the internal standard (n-benzylbenzamide) were obtained from Sigma, while vismodegib and the seven investigational compounds (P7C3 and P7C3-S10 [12], Led209 [13], IWR1 [14], compound 8.3 [15], RMT5265.HCl [16], AB-5/diazonamide [17], purity >95%) were provided by investigators at the University of Texas Southwestern Medical Center (Dallas, TX).

2.2 RED method

RED device inserts were placed in the Teflon base plate without prior preparation. Compounds were added to human plasma at 10 μ M. The plasma solutions were vortexed well and allowed to incubate at RT for 10 min before placing 200 μ l in the red chamber of the RED device. Three hundred fifty μ l of isotonic phosphate buffered saline (PBS, pH 7.4; 0.01M) was added to the corresponding white chamber, the base plate was covered with a gas-permeable membrane, and then agitated in a shaking water bath set at 100 rpm and 37°C for 4 hours. Fifty μ l aliquots of sample were removed from both chambers and an equal volume of blank plasma or PBS was added to create analytically identical sample matrices for measurement of compound concentrations (C_u – ultrafiltrate and C_p -plasma).

2.3 Ultrafiltration method

Compounds were added to 400 μ l of human or mouse plasma at 10 μ M. Two 50 μ l aliquots were removed before placing in a Centrifree® device. One 50 μ l aliquot was immediately processed for measurement of initial compound concentration (C_1) while the other was incubated along with the Centrifree® device at 37°C, 5% CO₂ for 20 min for evaluation of plasma stability (C_{37}). The top and bottom chambers of the Centrifree® device were weighed before compound addition and then again after spinning to obtain the approximate volume of the top plasma (V_2) and bottom ultrafiltrate (V_3) samples, assuming a density of one for both samples. The centrifree device was spun at $1000 \times g$ for 5 min at RT in a Thermo Scientific Sorvall T1 Centrifuge with a T41 high-capacity swing-out rotor. A 50 μ l aliquot of the top plasma (C_2) and bottom ultrafiltrate (C_3) were then removed for detection. For traditional measurement of NSB, plasma was replaced with PBS (pH 7.4), and samples were processed as above. Fifty μ l of the spiked PBS was used for initial concentration detection (C_{pbs}), and 50 μ l of the ultrafiltrate was used for detection in ultrafiltrate (C_{pbs-u}). An equal volume of blank plasma was added to all the samples to create analytically identical matrices for LC-MS/MS analysis.

2.4 LC-MS/MS analysis

Samples pre-cleared of protein by extraction with a 2:1 ratio of an equal mixture of methanol and acetonitrile containing 300 ng/ml n-benzylbenzamide as an internal standard and 0.15% formic acid were analyzed by LC-MS/MS for compound levels using an AB Sciex (Framingham, MA) 4000 Qtrap LC-MS/MS coupled to a Shimadzu Prominence HPLC (Torrance, CA). An Agilent (Santa Clara, CA) XDB-C₁₈ (5 μ m, 4.6 $\times$ 50mm) column was used for sample analysis. The mobile phases consisted of 0.1% formic acid in water or acetonitrile. The flow rate was 1.5ml/min with a gradient elution. Multiple reaction monitoring was performed using nitrogen as collision gas with a dwell time of 100 ms and analysis time of 5 min per sample. Data collection and analysis were performed using Analyst 1.5 (AB Sciex). The analyte response area for each sample was normalized for IS peak areas. Experiments were performed with a minimum of three replicates. %PPB and recovery are reported as mean $\pm$ SEM.

2.5 Calculation of NSB, PPB, recovery, and stability

%Protein binding using the RED device was calculated as described in Waters, *et al* [3]:

$$\%PPB = (1 - (C_u/C_p)) \times 100\% \quad (1)$$

where, C_u is the ultrafiltrate drug concentration and C_p is the plasma drug concentration after incubation. % Protein Binding, % Recovery and % Stability using the Centrifree device with plasma were calculated by mass balance as follows:

$$\%PPB = ((C_2 - C_3) * V_2 / (C_2 * V_2 + C_3 * V_3)) \times 100\% \quad (2)$$

$$\%Recovery = ((C_2 * V_2 + C_3 * V_3) / (C_1 * V_1)) \times 100\% \quad (3)$$

$$\%Stability = (C_{37} / C_1) \times 100\% \quad (4)$$

where C_1 is initial drug concentration in plasma, V_1 is plasma volume loaded to Centrifree® device, C_2 and V_2 are the plasma drug concentration and volume on the top, C_3 and V_3 are the ultrafiltrate drug concentration and volume on the bottom, and C_{37} is the drug concentration in the plasma aliquot set at 37°C for 20 min. All tested compounds showed stability >90% for the 20 minute incubation period. Nonspecific binding to the Centrifree device was calculated as follows.

$$\%NSB = (1 - C_{pbs-u} / C_{pbs}) \times 100\% \quad (5)$$

Where C_{pbs-u} is the concentration of plasma in the PBS ultrafiltrate and C_{pbs} is the concentration of compound in the initial PBS solution.

3. RESULTS and DISCUSSION

3.1 NSB measurement

Most protocols describing the use of ultrafiltration for the determination of plasma protein binding recommend that non-specific binding (NSB) to the device in PBS be calculated to determine the suitability of the method for the compound to be evaluated [6–9, 18]. The NSB values for 20 commercial and seven investigational compounds (Fig. 1) tested in PBS in Centrifree® ultrafiltration (UF) devices are listed in Tables 1 and 2. Forty percent of the compounds showed NSB in excess of 25% in the PBS solution. Many investigational compounds had NSB values significantly higher than most commercial drugs, possibly due to their highly lipophilic nature, making progress in determining %PPB difficult. However, it was hypothesized here that the NSB behavior of a compound would be very different when that compound was incubated with a UF device in PBS versus plasma. Thus, mass balance analysis was used to calculate the NSB value of these same compounds in the presence of plasma, according to equation (3) shown in the Experimental section. Table 2 shows that 24 of the compounds could be recovered in excess of 90% and the remaining 3 compounds in excess of 80% in mouse plasma. Similar improvements were noted in human plasma (Table 1). These data suggest that it may not be necessary to determine NSB in PBS for determination of plasma protein binding by ultrafiltration because the binding behavior of compounds to the device is significantly altered in the presence of plasma. Instead it may be more important to use mass balance to determine total recovery of the compound from the device after incubation in plasma to ascertain whether ultrafiltration will be a valid approach for measuring plasma protein binding. However, validation of PPB values determined by this approach is needed.

3.2 Comparison of PPB values obtained by UF with literature reported values

As discussed below in more detail, PPB values reported in the literature vary widely because of the use of different methods and variations in those methods. Nonetheless, an attempt was made to compare human PPB values obtained by ultrafiltration using a mass balance approach to commonly reported values for 15 commercial compounds (Table 1). Where appropriate, the range of values observed in the literature was reported. Compounds with a

wide range of physiochemical features, including those with very different observed NSB values, were chosen for analysis. There was generally good agreement between PPB determined by UF here and values reported in the literature. Importantly, %PPB as measured by ultrafiltration and calculated using mass balance was never overestimated which might have been expected if NSB was contributing to the values obtained.

3.3 Comparison of PPB values obtained by UF and RED

Because of the difficulty in ascertaining detailed methods for literature-reported values and a desire to assess the PPB characteristics of novel compounds, a comparison was made between %PPB determined by ultrafiltration and that obtained using a modification of equilibrium dialysis known as rapid equilibrium dialysis (RED). Values were obtained for both methods in human (Table 1) and mouse plasma (Table 2). In general, similar values were reported using both methods (Fig. 2). However, a number of significant exceptions were noted that are worth exploring. IWR1 is an investigational compound targeting the Wnt pathway [14]. %PPB determined by ultrafiltration was 97.2% but was only 35.0% when measured by RED. Although, IWR1 is stable in plasma during the 20 min incubation used for ultrafiltration, over longer periods of time, it suffers from known instability [14], leading to the altered %PPB value noted using RED, which requires four hours of incubation. Although, this instability could be easily discerned in a test conducted prior to performing RED, it points to a limitation in this technique for evaluating slightly unstable compounds, despite the reduced time of this assay relative to traditional equilibrium dialysis. For another set of compounds, most notably those of a higher mw, including RMT5265.2HCl, vinblastine, and verapamil, %PPB determined by RED was higher than that observed by ultrafiltration. Evaluation of compound distribution in PBS in the RED device for these three compounds indicated that the four hour time chosen for analysis of PPB by RED was insufficient to reach equilibrium. Only 22% of RMT5265, 29% of vinblastine, and 42% of verapamil had reached the opposite side of the membrane after four hours of incubation (data not shown). Thus, for larger mw compounds, care must be taken to optimize the equilibrium time used for RED so that %PPB is not overestimated. In addition, pH dependence has been noted by Kochansky *et al* for protein binding observed for both vinblastine and verapamil [19] with higher binding reported at higher pH. The starting pH of the plasma used for the experiments reported here was pH 7.8. A minimal shift to pH 7.9 was noted after incubation of the Centrifree device at 37°C, 5% CO₂; however, a more significant change was noted in the pH of the plasma in the RED device after four hour incubation at 37°C atmospheric CO₂ to pH 8.2 (data not shown). Thus, pH shifts during incubation may also account for some of the variability between %PPB noted by UF versus RED. Unfortunately, as reported in [19] there is no discernible feature of a compound, including pKa, that can predict whether pH changes will affect protein binding. As the incubation time for UF is much shorter than that for RED and traditional equilibrium dialysis, it may be easier to control pH in ultrafiltration than these other techniques. Finally, for several of the compounds, such as atenolol and terbutaline, %PPB determined by ultrafiltration more closely approximated values reported in the literature than did RED, at least as measured here.

4. CONCLUSION

The data presented herein reinforce the benefits of ultrafiltration for determining plasma protein binding during early drug discovery, namely speed, accuracy, and ease of conduct, while at the same time demonstrating that nonspecific binding need not be an impediment to obtaining valid data. This study showed that NSB is dramatically decreased in the presence of plasma; thus, evaluation of NSB in PBS is not relevant for determining the suitability of ultrafiltration for PPB. Instead it is more appropriate to use mass balance to determine total recovery of the compound from the device in the presence of plasma. This validation greatly

extends the value of the ultrafiltration technique in protein binding determinations, especially for lipophilic compounds often explored in early drug development.

Acknowledgments

This work was supported by NIH/NIAID grant U01 AI077853 and NIH/NCI grant P01 CA095471. The authors would like to thank Drs. Lawrence Lum, Andrew Pieper, Vanessa Sperandio, Beatriz Fontoura, and Patrick Harran for providing the investigational compounds used in this study.

ABBREVIATIONS

ADME	Absorption, distribution, metabolism, excretion
ED	Equilibrium dialysis
EP	Eppendorf
NSB	Non-specific binding
PD	Pharmacodynamics
PK	Pharmacokinetics
PPB	Plasma protein binding
RED	Rapid equilibrium dialysis
UF	Ultrafiltration

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Highlights

- The calculation of non-specific binding in PBS during ultrafiltration is challenged.
- Mass balance analysis shows recovery is significantly different in plasma versus PBS.
- Total compound recovery in plasma is a more valid measure of method utility.
- This approach shows good concordance with other plasma protein binding methods.
- The speed and ease of ultrafiltration avoids pitfalls seen with other methods.

Figure 1.
Structures of investigational compounds

Figure 2.

Correlation of mean %PPB in mouse plasma of 27 compounds determined by ultrafiltration versus rapid equilibrium dialysis. %PPB was determined by mass balance as described in equation (2) using ultrafiltration or as described in equation (1) using RED for twenty seven known and investigational compounds in mouse plasma. The values were plotted and a linear regression line plotted with the y intercept set to 0. Compounds deviating most significantly from the regression line are indicated by a lighter degree of shading and are discussed in the text.

Table 1

Compound properties and %PPB in human plasma determined by UF and RED in comparison to literature values (n=3)^a

Compound	MW	CLog P ^b	NSB	%Recovery from plasma	%Stability	%PPB (UF)	%PPB (RED)	%PPB (Literature)
Acetaminophen	151.2	0.5	0.0	102.6±0.9	98.6±2.6	26.7±3.6	21.0±2.3	24±7 [20]
Atenolol	266.3	-0.1	0.0	102.2±0.8	104.2±1.0	9.3±1.0	33.2±1.8	<5 [21]
Hydrocortisone	362.4	-0.9	3.1	103.8±3.3	98.5±1.1	63.2±1.0	72.1±0.4	65 [8], 80 [23]
Indomethacin	357.8	3.4	21.0	89.7±7.2	111.0±8.8	99.6±0.0	99.8±0.0	99 [23]
Lovastatin	404.5	3.2	90.3	87.6±3.6	96.2±5.1	97.4±0.3	99.6±0.0	>95 [21]
Paclitaxel	853.9	3.5	86.8	87.6±2.2	104.8±2.0	86.8±0.5	97.0±0.1	88 [23]
Propranolol HCl	295.8	2.8	0.0	100.0±1.6	98.7±1.2	67.2±1.2	83.4±0.2	65–70 [8]
Sulfamethiazole	270.3	0.4	0.0	99.4±1.7	101.9±0.4	81.6±0.4	89.2±0.2	85–90 [22]
Sulfamethoxazole	252.3	-0.2	0.2	106.0±2.7	98.1±1.8	67.4±1.2	71.2±0.7	66 [21], 77 [23]
Terbutaline	225.3	0.5	0.0	104.2±2.1	106.5±3.3	18.1±1.8	37.3±2.6	20 [21], 25 [23]
Tolbutamide	270.4	2.5	2.0	97.4±0.2	102.2±0.8	95.8±0.0	97.6±0.0	96 [21]
Verapamil	454.6	4.5	9.3	91.3±0.5	97.4±0.7	74.6±0.9	93.0±0.1	92±3 (pH8.7), 73±1 (pH7.4) [19]
Vinblastine	811.0	4.2	67.7	79.1±2.1	97.5±3.1	62.6±1.6	88.7±0.2	82±3 (pH8.7), 74±3 (pH7.4) [19]
Vorinostat	264.3	1.0	3.4	100.1±2.1	101.1±1.9	60.4±0.8	75.9±0.2	71 [23]
Warfarin	308.3	2.9	2.2	94.2±3.8	100.4±4.5	98.9±0.1	99.6±0.0	99 [21]

^aNSB, %Recovery from plasma, %Stability, %PPB (UF) and %PPB (RED) were determined as described in Section 2.^bCLog P values were determined using ChemBioDraw Ultra 12.0 (Perkin Elmer Informatics, Cambridge, MA)

Table 2
Compound properties and %PPB values determined by UF versus RED using mouse plasma (n=3)^a

Compound	MW	CLog ^b	NSB	% Recovery from plasma	%PPB (UF)	%PPB (RED)
AB-5	696.8	4.1	100.0	103.3±4.3	100.0±0.0	99.9±0.0
P7C3	474.2	6.4	99.9	100.0±9.4	100.0±0.0	100.0±0.0
P7C3-S10	506.2	7.8	99.9	95.0±2.6	100.0±0.0	100.0±0.0
Lovastatin	404.5	3.2	90.3	82.1±8.8	97.1±0.6	91.6±2.7
Paclitaxel	853.9	3.5	86.8	90.8±3.2	95.4±0.1	97.6±0.2
LED209E	383.5	3.0	83.7	101.0±1.5	99.3±0.1	99.2±0.1
Vinblastine	811.0	4.2	67.7	87.5±2.5	82.6±2.1	92.7±0.4
IWR1	409.4	2.5	59.2	81.1±2.1	95.5±0.9	35.0±11.6
Vismodegib	421.3	2.7	47.3	94.3±3.7	97.5±0.5	99.1±0.1
RMTS265.2HCl	969.2	NA	43.1	92.4±0.6	64.0±0.3	92.3±0.6
8.3	394.5	4.4	24.1	111.0±4.2	97.9±0.2	98.8±0.1
Indomethacin	357.8	3.4	20.9	99.0±9.7	98.9±0.3	98.7±0.2
Verapamil	454.6	4.5	9.3	90.8±1.4	77.2±1.8	91.8±1.6
Vorinostat	264.3	1.0	3.4	98.7±0.6	37.8±0.5	46.4±1.9
Hydrocortisone	362.4	-0.9	3.1	102.1±4.0	48.1±1.0	58.8±3.6
Sulfadiazine	250.3	0.1	2.9	107.6±2.0	59.7±2.5	53.0±2.5
Sulfathiazole	255.3	0.7	2.6	107.5±2.1	59.8±2.1	58.2±1.6
Warfarin	308.3	2.9	2.2	99.8±3.9	87.8±2.4	91.6±0.6
Tolbutamide	270.4	2.5	2.0	106.1±3.1	94.1±1.1	95.7±0.3
Sulfamethoxazole	252.3	-0.2	0.2	112.5±3.7	52.9±3.5	45.0±9.0
Acetaminophen	151.2	0.5	0.0	97.2±2.5	29.9±2.3	38.9±2.2
Atenolol	266.3	-0.1	0.0	98.0±5.1	6.6±0.1	36.0±4.9
Propranolol HCl	295.8	2.8	0.0	103.6±7.6	67.4±3.6	73.6±1.8
Sulfamethiazole	270.3	0.4	0.0	104.1±1.2	65.2±1.9	64.7±2.2
Sulfamerazine	264.3	0.6	0.0	107.4±1.8	81.6±1.2	80.5±1.3
Sulfadimethoxine	310.3	2.0	0.0	108.6±1.1	96.0±0.2	95.6±0.4
Terbutaline	225.3	0.5	0.0	99.62±8.4	19.0±2.1	36.2±2.8

^aNSB, %Recovery from plasma, %PPB (UF) and %PPB (RED) were determined as described in Section 2.

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^bCLog P values were determined using ChemBioDraw Ultra 12.0 (Perkin Elmer Informatics, Cambridge, MA)