Experimental Validation of the Reliability of Dilution Method for Plasma Protein Binding Assay in Human Plasma Using Commercial Compounds

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Abstract

It is a common practice to use diluted plasma for plasma protein binding assay of very highly bound drugs. By using the Correction Factor Equation (CFE) one can extrapolate the diluted fraction unbound to the undiluted unbound, which is routinely used for tissue binding. This may in theory be true for highly bound drugs but may provide misleading results if the CFE is used inappropriately. In this study, we validated the accuracy of plasma dilution method for commercial compounds using equilibrium dialysis. Results showed that dilution fold as high as 50 can result in accurate undiluted values for highly bound compounds. Both single dilution method or correction from multiple dilutions can give good prediction of undiluted unbound in plasma for highly bound compounds. For less highly bound compounds, caution is needed when interpreting data from dilution method. Multiple dilutions are recommended in order to generate the most reliable predictions for all the compounds.

Objectives

To validate the reliability of dilution method for plasma protein binding assay in human plasma using 16 commercial compounds.

Methods

- Human plasma dilution of 50-fold, 20-fold, 10-fold, and 5-fold were tested for 6 highly bound compounds. An additional 2-fold dilution as well as the undiluted neat plasma were tested for 10 moderately and low bound compounds in human plasma.
- Drug-spiked plasma (2 μM) and blank buffer were added to the donor and receiver side of the membrane of the 96-well equilibrium dialysis device, respectively.
- Samples were incubated at 37 ºC for 4 hours (moderately and low bound compounds) or 18 hours (highly bound compounds) as recommended by the previous literature [1].
- The measured diluted f_u (f_u, d) was calculated according to **Equation 1**.
- The undiluted f_u were extrapolated from the CFE using two methods: 1) a simple correction method from a single dilution factor using **Equation 2**, and 2) the linear regressions between dilution factor (D) and the measured fraction unbound values for each dilution, not including neat plasma, using **Equation 3**. Undiluted f_u can be calculated by solving the equation for the line of the best fit.

$$\text{Diluted } f_u = \frac{C_{receiver}}{C_{donor}} \qquad \text{Equation 1}$$

$$\text{Undiluted } f_u = \frac{1/D}{((1/fu,\ d)-1)+1/D} \qquad \text{Equation 2} \quad \text{Method 1}$$

$$\frac{1}{\text{Diluted fu}} = \left(\frac{1}{\text{Undiluted fu}} - 1\right)\frac{1}{D} + 1 \qquad \text{Equation 3} \quad \text{Method 2}$$

Results

Table 1. Linear regressions of 6 highly bound compounds (lines of best fit were shown in Figure 1) as well as 6 moderately and low bound compounds (figures not shown) calculated by Method 2. These compounds showed good linear relationships with R^2 ≥0.9.

Classification	Compound	Linear regression	R^2
Highly bound	Mifepristone	y = 145.02x - 1.3528	0.9962
	Rosiglitazone	y = 114.59x + 4.3766	0.9790
	Nicardipine	y = 122.2x + 1.266	0.9803
	Ritonavir	y = 66.904x + 2.3049	0.9709
	Lapatinib	y = 3019.5x - 0.0087	0.9405
	Itraconazole	y = 948.35x - 5.7976	0.9969
Moderately and low bound	Diltiazem	y = 1.245x + 1.1166	0.9713
	Imipramine	y = 3.9287x + 1.0204	0.9802
	Propranolol	y = 4.7076x + 0.8625	0.9765
	Chloroquine	y = 1.0118x + 1.207	0.9046
	Verapamil	y = 3.7425x + 1.1475	0.994
	Carbamazepine	y = 2.0751x + 1.1447	0.9822

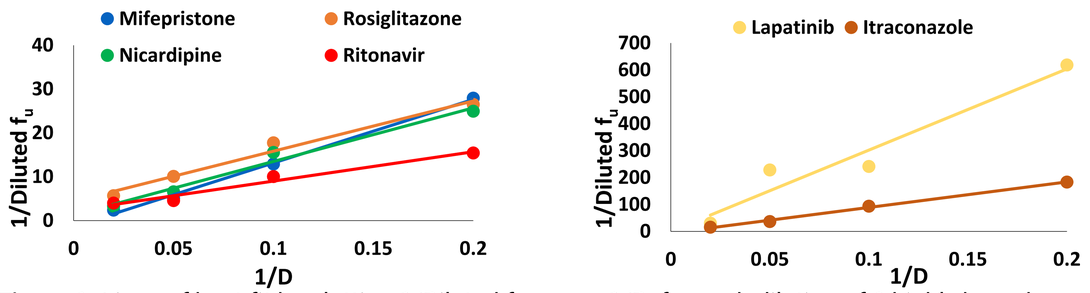

Figure 1. Lines of best fit by plotting 1/Diluted f_u versus 1/D for each dilution of 6 highly bound compounds.

Table 2. Undiluted f_u of highly bound compounds.
The undiluted f_u values calculated by both methods were close to the f_u of literature.

Compound	Undiluted fu calculated by Method 1				Undiluted fu calculated by Method 2	fu of literature	Ref No.
	50-fold	20-fold	10-fold	5-fold			
Mifepristone	0.0143	0.0122	0.0083	0.0074	0.0070	0.0043	[2]
Rosiglitazone	0.0043	0.0055	0.0059	0.0078	0.0084	0.0039	[3]
Nicardipine	0.0087	0.0089	0.0068	0.0083	0.0081	0.0072	[2]
Ritonavir	0.0067	0.0140	0.0109	0.0137	0.0144	0.035 ± 0.001	[4]
Lapatinib	0.0005	0.0002	0.0004	0.0003	0.0003	0.00037	[1]
Itraconazole	0.0014	0.0014	0.0011	0.0011	0.0011	0.0014	[1]

Table 3. Undiluted f_u of moderately and low bound compounds.
When the dilution was equal to or less than 10-fold, single correction method can result in consistent data with in house f_u of neat plasma, except for chloroquine. Otherwise, higher variation between replicates were observed for these compounds. Besides, the corrected undiluted f_u may result in >2 fold differences compared to the data from neat plasma.

Compound	Undiluted f_u calculated by Method 1					Undiluted f_u calculated by Method 2	In house f_u (neat plasma)	f_u of literature	Ref No.
	50-fold	20-fold	10-fold	5-fold	2-fold				
Diltiazem	0.1175	0.1910	0.3170	0.3329	0.4106	0.4234	0.3667	0.45 ± 0.04	[4]
Imipramine	0.0924	0.1310	0.2301	0.2350	0.1965	0.2021	0.1702	0.24 ± 0.03	[4]
Propranolol	0.1397	0.2441	0.2145	0.2569	0.1786	0.1795	0.1730	0.231 ± 0.046	[5]
Chloroquine	0.1270	0.2512	0.2101	0.4243	0.4171	0.4507	0.5038	0.43	[6]
Verapamil	0.0610	0.1486	0.1675	0.1921	0.1966	0.2045	0.1908	0.30 ± 0.02	[4]
Carbamazepine	0.0701	0.2118	0.2924	0.2542	0.2971	0.3106	0.2931	0.291 ± 0.029	[5]

Table 4. Undiluted f_u of additional 4 moderately and low bound compounds.
Accurate f_u values can be obtained by method 2 with 5 dilutions, even though the linearity is not good (R^2 <0.9).

Compound	Linear regression of method 2	R^2	Undiluted f_u calculated by Method 2	In house f_u (neat plasma)	Literature f_u	Ref No.
Metoprolol	y = -0.2784x + 1.1431	0.7195	1.0	0.9591	0.89 ± 0.05	[4]
Rivastigmine	y = 0.4739x + 1.0761	0.7075	0.6452	0.6349	0.6	[6]
Zonisamide	y = 0.3046x + 1.0346	0.8179	0.7467	0.5982	0.6	[7]
Tizanidine	y = -0.0428x + 1.2767	0.0114	0.8104	0.5710	0.7	[6]

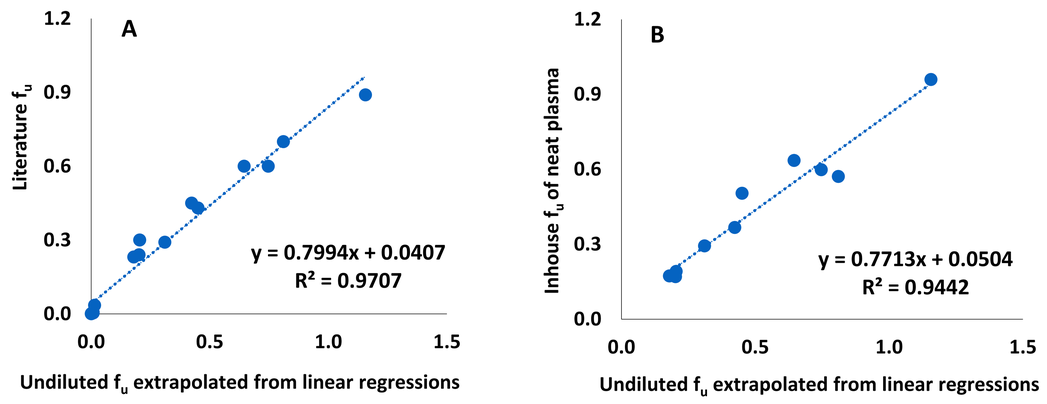

Figure 2. (A) Correlation between the in house undiluted f_u values extrapolated from linear regressions (Method 2) and data of literature for all of the 16 compounds. (B) Correlation between the undiluted f_u values extrapolated from linear regressions (Method 2) and the in house f_u of neat plasma for 10 moderately and low bound compounds.

Conclusion

1. Dilution method using one dilution fold could be used for PPB evaluations during early screening phases.
   - For highly bound compounds, dilution fold as high as 50 can provide reliable data.
   - For moderately and low bound compounds, in cases when dilution steps are required due to limited sample volumes or technical requirements, dilution fold should not be higher than 10 fold.
2. Multiple dilutions are recommended in the late development phases in order to generate the most reliable predictions.

Contact

References

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