UTILIZING VARIOUS PLASMA PROTEIN BINDING TOOLS FOR DETERMINATING THE FREE FRACTION OF LIPOPHILIC AND LIPOPEPTIDE DRUGS

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Introduction

Many drugs bind to circulating plasma proteins, such as human serum albumin (HAS) and alpha-acid glycoprotein (AGP). Binding of drug molecules to plasma proteins is considered an important parameter in assessing drug ADME properties. It is often debated whether it is the free fraction or instead the free concentration at the target site that is related to efficacy, therapeutic index, and half-life. However, the free drug hypothesis is widely applied in drug discovery and development to establish pharmacokinetic–pharmacodynamic relationships, to predict the therapeutically relevant dose and to monitor drug concentration in clinical studies. To this end, obtaining an accurate value for fraction unbound can often be challenging for highly bound and lipophilic compounds due to the physiochemical properties of the drug candidate.

Factors Affected by Inaccurate Determination of PPB of Drugs	
Dose	IVIVC
Free Drug Concentration	In Vitro and In Vivo Clearance
Therapeutic Index	Cross-Species Comparison
Half-Life	Modeling

Factors That Cause Inaccurate Determination of PPB of Drugs	
Membrane Diffusion Rate	Stability
Size	Solubility
Equilibration (On/Off rate)	Sensitivity (S/N)
Non-specific Binding	Extraction Efficiency
Recovery	Matrix Effects

Many methodologies have been developed to measure PPB and tissue binding. Among the most commonly applied binding methods are Rapid Equilibrium Dialysis (RED) and Ultrafiltration (UF). These methods remain a challenge for compounds with high nonspecific binding and larger molecules (New Modalities) with atypical geometry and physical chemical properties.

In this study, we evaluated Sovicell TRANSIL partitioning technology, specifically the PPB and High Sensitivity Binding (HSB) kits. The human PPB kit utilizes a serial dilution of Human serum albumin (HSA) and alpha 1-acid glycoprotein (AAG) bound to TRANSIL beads. The HSB kit incorporates lipid membranes bound to TRANSIL beads, to which plasma is added at different concentrations. The fraction unbound in plasma protein binding assays of 6 test articles were determined and a compared between with RED, UF, and TRANSIL partitioning. Compounds: Warfarin, Sulfamethoxazole, Propantheline, Dalbavancin, Liraglutide, and ent-Verticilide[1]. Results described here support that TRANSIL partitioning technology may provide an efficient option (cost and time) for accurate determination of free fraction in candidate optimization and selection of certain drug classes.

Equations for Rapid Equilibrium Dialysis

$$\text{Stability} = 100\% \times \frac{[\text{Concentration at each timepoint hour}]}{[\text{Concentration at zero hours}]}$$

$$\%\ \text{Recovery} = 100\% \times \frac{[\text{Buffer Chamber}]}{[\text{Sample Chamber}]}$$

$$\text{Fraction unbound} = \frac{[\text{Buffer Chamber}]}{[\text{Sample Chamber}]}$$

$$\%\ \text{Bound} = 100\% \times (1 - \text{Fraction unbound})$$

Equations for Ultrafiltration

$$\text{Stability} = \frac{(C_{4(1hr)})}{(C_{1(0hr)})} * 100 \qquad \%\ \text{Recovery} = \frac{(C_2 * V_2 + C_3 * V_3)}{(C_1 * V_1)} * 100$$

$$\%\ \text{Bound} = \frac{(C_2 - C_3) * V_2}{(C_2 * V_2 + C_3 * V_3)} * 100$$

$$\text{Fraction Unbound} = 1 - \text{Fraction bound} \qquad f_u = \frac{1}{f_b}$$

C_1: PLASMA-0hr: INITIAL drug concentration in plasma at time 0 hour (not in UF device)
V_1: PLASMA- volume loaded to UF device
C_2: PLASMA drug concentration on top in concentrate
V_2: PLASMA volume on top in concentrate
C_3: PLASMA drug concentration on bottom in filtrate
V_3: PLASMA volume on bottom in filtrate
C_4: PLASMA-1hr: FINAL drug concentration in plasma after 1 hour (not in UF device)

Equations for Protein Bound TRANSIL Partition Method(s)

[details *provided in SOVICELL user manuals*]

PPB Kit (and other protein fixed TRANSIL beads)

$$K_D = \frac{[A]\cdot[P]}{[AP]} \qquad [A] = f_u \cdot ([A] + [AP])$$

$$\frac{f_b}{f_u} = \frac{1}{K_D} \cdot P \qquad f_b = 1 - \frac{1}{1 + \frac{[HSA]}{K_D^{HSA}} + \frac{[AGP]}{K_D^{AGP}}}$$

A: free drug concentration
P: free concentration of plasma
AP: drug bound to plasma
HSA: concentration of HSA
K_D^{HSA}: affinity constant of drug to HSA in experiment
AAG: concentration of HSA
K_D^{AAG}: affinity constant of drug to HSA in experiment

Equations for High Sensitivity Binding TRANSIL Partition Method

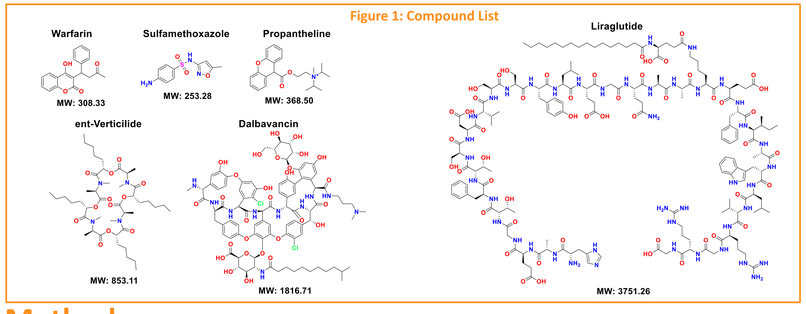
[details *provided in SOVICELL user manuals*]

High Sensitivity Binding and Membrane Affinity Kits (membrane bound beads)

$$APA = \frac{n_t \cdot (\alpha \cdot P + K_D)}{\alpha \cdot P \cdot V_b + K_D \cdot (V_b + MA \cdot V_l)} \qquad f_u = \frac{1}{1 + \frac{P}{K_D}}$$

APA: drug concentration in buffer and plasma phase
P: concentration of full plasma
α: plasma dilution
MA: membrane affinity
K_D: affinity constant of drug to plasma in experiment
n_t: total drug in experiment
V_b: buffer volume
V_l: lipid volume of membrane

Figure 1: Compound List



Methods

Protein binding by Rapid Equilibrium Dialysis was performed under standard conditions, adapted from literature methods[2,3]

Protein binding by ultrafiltration was performed under standard conditions employing Amicon® Ultra-0.5 centrifugal filter, Ultracel-30 regenerated cellulose membrane, 30 kDa MWCO (Sigma UFC5030). The assay was adapted from previously published methods.[4,5]

Protein binding by TRANSIL PPB and High Sensitivity Binding kit was performed as described in the vendor user manuals with ultra low binding plastic tubes as the plate method.[6]

Plasma extractions were performed with acetonitrile containing 1% formic acid (or 1% trifluoroacetic acid in for Liraglutide incubated in the HSB kit) and tolbutamide as an internal standard. Sample analysis was performed with a Thermo Exploris-120 Orbitrap coupled to a Vanquish HPLC equipped with a standard reverse phase C18 column. Data was collected under Full MS and Single Ion Monitoring (60k resolution) to allow for flexibility of metabolite detection (if unstable) and additional sensitivity from high background observed from extractions of test articles from plasma. Each Sovicell TRANSIL kit comes with pre-formatted worksheet for estimation of fraction unbound.

Figure 2: Standard Rapid Equilibrium Dialysis Device Incubation

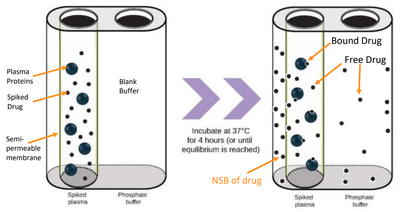


Figure 3: Standard Ultrafiltration Assay

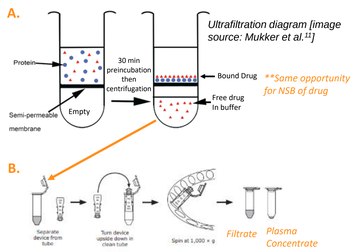
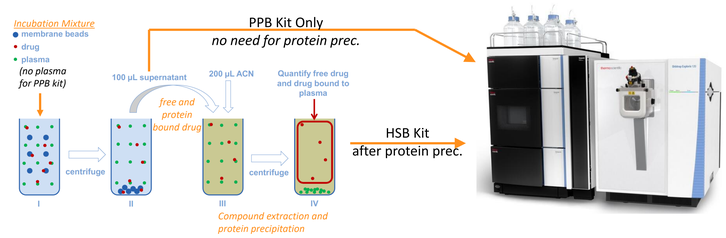
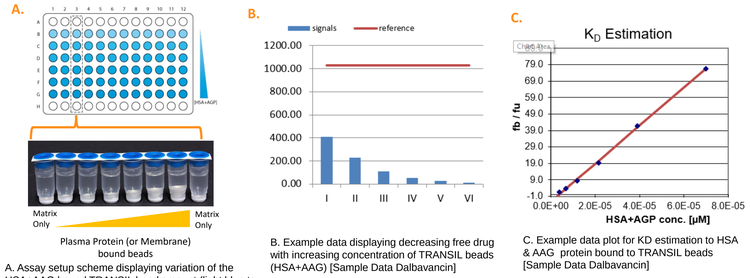


Figure 4: TRANSIL Partition Assay Workflow



Results

Figure 5: TRANSIL PPB Kit Design and Example Output Data

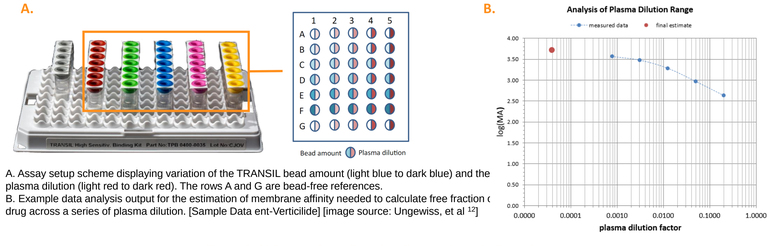


A. Assay setup scheme displaying variation of the HSA+AAG bound TRANSIL bead amount (light blue to dark blue. The rows A and G are bead-free references. [image source: Ungewiss, et al [12]]

B. Example data displaying decreasing free drug with increasing concentration of TRANSIL beads (HSA+AAG) [Sample Data Dalbavancin]

C. Example data plot for KD estimation to HSA & AAG protein bound to TRANSIL beads [Sample Data Dalbavancin]

Figure 6: TRANSIL HSB Kit Design and Example Output Data



A. Assay setup scheme displaying variation of the TRANSIL bead amount (light blue to dark blue) and the plasma dilution (light red to dark red). The rows A and G are bead-free references.
B. Example data analysis output for the estimation of membrane affinity needed to calculate free fraction of drug across a series of plasma dilution. [Sample Data ent-Verticilide] [image source: Ungewiss, et al [12]]

Table 1: % Bound Drug Fraction Determined in Each Assay

Compound	MW	LogD (7.4)[a]	Fraction Bound								
			RED		UF		TRANSIL PPB	TRANSIL HSB	Reported %-Bound	Ref
Warfarin	308.33	0.3-0.78	97.5%	+/-0.2%	103.5%	1.4%	98.0%	+/-0.2%	NA	NA	97-99.9%	*13*
Sulfamethoxazole	253.28	0.14	59.8%	+/-3.8%	50.0%	3.2%	72.1%	+/-5.5%	NA	NA	70.0%	*14*
Propantheline	368.50	0.36	unstable	-	Unstable	-	90.3%	+/-2.0%	NA	NA	NA	-
Verticilide	853.11	8 (cLogP)	*Low Recovery*	-	*Low Recovery*	-	98.2%	+/-0.6%	98.92%	+/-0.04%	NA	-
Dalbavancin	1816.71	-1.68	97.7%	+/-11.0%	*Low Recovery*	-	99.9%	+/-0.0%	99.92%	+/-0.01%	90.4-94.6, 99%	*15*
Liraglutide	3751.26	8.6	>99% (*Moderate Recovery*)	-	*Low Recovery*	-	99.8%	+/-0.1%	98.84%	+/-0.05%	99.49%	*12*

[a] calculated and experimentally determined values (see references 7-10)

Figure 7: Plasma Stability of Each Compound in Human Plasma (37C)

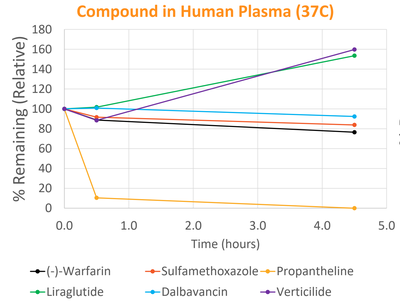


Figure 8: Estimation of Compound Recovery in Each Assay

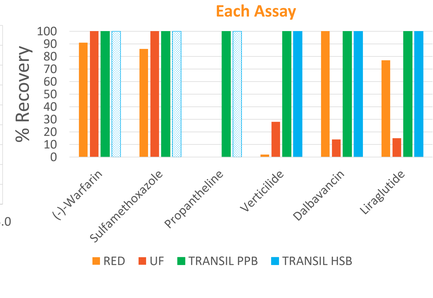


Table 2: Various Utility of Each PPB Assay Design for Compound of Different Physical Chemical Properties

Protein Binding Technique	Throughput	Small Molecules	New Modalities (bRo5)	Labile	Sticky	Low Sensitivity	High Binding
Rapid Equilibrium Dialysis (RED) Device	+++	+++	+	-	-	+	+
Ultrafiltration (size exclusion ultra centrifugal filters)	++	+++	+++	+	-	+	+
TRANSIL PPB Binding Kit(s) (plasma free)	++*	+++*	+++*	+++*	++*	+*	++*
TRANSIL High Sensitivity Binding (HSB) Kit	+	+++	+++	++	+++	+++	+++

\+ = rank of utility based on common issues associated with each category of drug/physical chemical property
\- = standard use of assay not advised
* =Assumes HSA and/or AAG are the primary plasma proteins responsible for binding

Conclusions

- The results from these experiments provide support for the utility of TRANSIL technology for the estimation of fraction unbound of drug candidates, particularly for compounds strongly bind to plastics, are highly protein bound, and unstable in plasma.
- The TRANSIL technology is limited in throughput 1 to 12 compounds per plate (depending on kit used), but the removal of membrane barriers to separate free drug from bound drug addresses the reoccurring challenge of accurate determination of free fraction for these challenging drug candidates.
- The Sovicell PPB kits that incorporates immobilized proteins on TRANSIL beads assume HSA and/or AAG are the primary plasma proteins responsible for binding in vivo (different species/organ kits available).
- The high sensitivity binding kit utilizes plasma dilutions and serve as an alternative for highly bound compounds. The HSB kit addresses the obstacle of measuring low concentrations of free drug associated with membrane filter-based PPB assays. The assay incorporates competitive binding of drug to plasma proteins against immobilized lipid membranes
- Utilization of the Exploris-120 Orbitrap (this poster) produced high quality analytical data similarly obtained with a Sciex 7500 (data not presented).

References

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