EVALUATION OF TRANSIL HIGH SENSITIVITY BINDING ASSAY FOR HIGHLY PLASMA PROTEIN BOUND COMPOUNDS

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ABSTRACT

Plasma protein binding (PPB) is an important property of drug candidates. PPB values are widely applied in drug discovery and development for scaling of in vitro intrinsic clearance measured from liver microsomes or hepatocytes to in vivo plasma clearance for development of IVIVC and for prediction of human clearance. Species difference in PPB can affect free drug exposure between toxicity species and human and impact therapeutic index and safety window calculations. The inaccuracy of PPB measurement can lead to mis-prediction of human PK and clinical drug-drug interaction. Determination of fraction unbound (f_u) for highly plasma protein bound compounds poses a great challenge in drug discovery, due to high non-specific binding, poor recovery, or long time-to-equilibrium. Measuring PPB for these compounds using the existing methods, such as equilibrium dialysis (EqD), ultrafiltration, or ultracentrifugation proved to be problematic. A new approach, the TRANSIL High Sensitivity Binding (HSB) assay, was developed to measure PPB of highly lipophilic and bound compounds. This TRANSIL HSB assay assesses PPB via partition between silica beads coated with immobilized phosphate lipid membranes and plasma (any species). By varying the ratio between the beads and plasma, the binding constant to plasma and membrane affinity can be derived. The TRANSIL HSB assay design overcomes the issues of non-specific binding and the need for analytical sensitivity due to the large concentration difference between bound and unbound for high PPB compounds. In this study, twelve very highly bound structurally diverse compounds with reported f_u ranging from 0.0002 to 0.012 were tested in the TRANSIL HSB assay using pooled human plasma to evaluate the accuracy and precision of the assay. The f_u values were comparable with those obtained from the traditional methods reported in the literature or measured in-house. The results were highly reproducible from run to run and from day to day. The data demonstrated that TRANSIL HSB assay has a great potential for PPB measurement of very sticky and highly bound compounds. It can be applied to compounds that are not amenable to traditional PPB methods due to high non-specific binding, instability, analytical challenges, or extremely long time-to-equilibrium.

INTRODUCTION

Measuring plasma protein binding of highly bound compounds is technically challenging. Inaccuracy of f_u for highly bound compounds can translate to poor prediction of in vivo free drug exposure and therapeutic index (TI). This is particularly sensitive for drug candidates with narrow TI (e.g., oncology drugs) as it can change from acceptable TI to no TI. The successes vary with compounds using currently available technologies when measuring f_u of highly plasma protein bound compounds. Here, we discuss a new method for PPB, TRANSIL HSB, as an alternative approach for highly bound compounds and its potential application in drug discovery.

MATERIALS AND METHODS

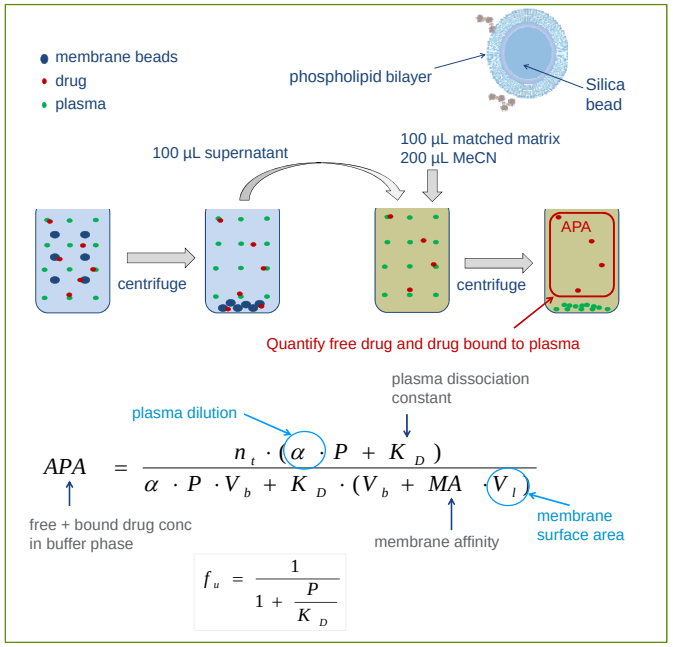
TRANSIL membrane affinity (MA) and HSB kits were provided by Sovicell (Leipzig, Germany). A batch of pooled male and female human plasma in K_3EDTA was purchased from Bioreclamation in 5 ml frozen aliquots.

Incubations were conducted at a substrate concentration of 2 µM, typically with a final DMSO content of 1% (amiodarone and itraconazole 5% DMSO). See Table 1 for list of test compounds. HSB incubations were conducted at 37 °C for 30 minutes. Supernatants were matrix matched to normalize the fraction of plasma in each sample, followed by protein precipitation using acetonitrile containing internal standard. Samples were analyzed by LC-MS/MS (LEAP autosampler, Shimadzu LC-20AD pumps, Sciex API 4000 mass spectrometer). Mobile phase A: 0.1% formic acid in water; mobile phase B: 0.1% formic acid in acetonitrile. Raw data were analyzed using Analyst 1.5 software. MA and f_u were calculated using the data analysis templates provided by Sovicell with the kits.

HSB assay overview and basic protocol (see also Figure 1):

- The HSB kit includes vials pre-filled with five TRANSIL bead dilutions.
- Five plasma dilutions are added by the user to generate an array of TRANSIL bead and plasma dilutions.
- Substrate is spiked in at the same concentration in each vial.
- Supernatants are collected and extracted using protein precipitation.
- Substrate is measured using LC-MS/MS (APA).
- Data analyzed in worksheet supplied with kit, which calculates f_u.

Figure 1. TRANSIL HSB assay overview. Plasma and TRANSIL bead concentrations are varied and APA is measured to calculate f_u.



RESULTS AND DISCUSSION

Table 1. log(MA) and HSB results for twelve highly bound compounds, compared to reported literature values

compound	TRANSIL log(MA)	HSB kit lipid content	f_u, TRANSIL HSB kit [a]	f_u, literature (Ref. 1)
amiodarone	3.6	1 µl	0.0037	0.0002
diclofenac	3.1	3 µl	0.0034	0.005
diflunisal	3.1	3 µl	0.0056	0.0016
fluvastatin	3.6	1 µl	0.0036	0.0079
furosemide	2.1	3 µl	0.015	0.012
ibuprofen	2.0	30 µl	0.0025	0.006
itraconazole	3.2	3 µl	0.051	0.002
meloxicam	2.3	3 µl	0.0067	0.003
mibefradil	4.6	1 µl	0.0067	0.005
rosiglitazone	3.2	3 µl	0.0064	0.002
telmisartan	2.9	3 µl	0.0031	0.004
tolcapone	3.5	3 µl	0.0045	0.0012

[a] TRANSIL quality index > 8

Figure 2. HSB versus literature f_u for twelve highly bound compounds

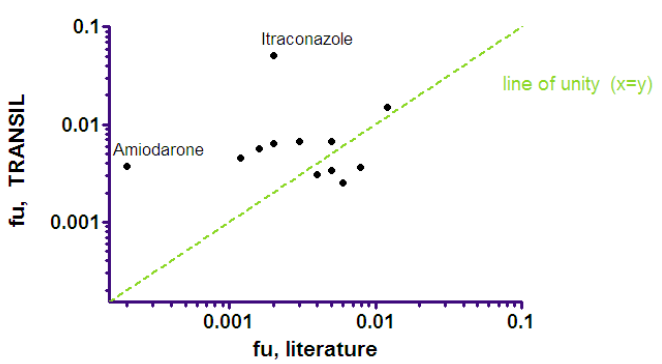


Table 2. Diclofenac HSB inter-day variability

Measurement	1	2	3	4	5
f_u	0.0039	0.0029	0.0031	0.0035	0.0035
Average	0.0034				
St Dev	0.0004				
CV	12%				

Figure 3. Plasma protein binding screening strategy

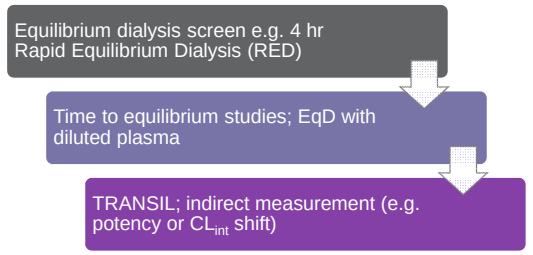
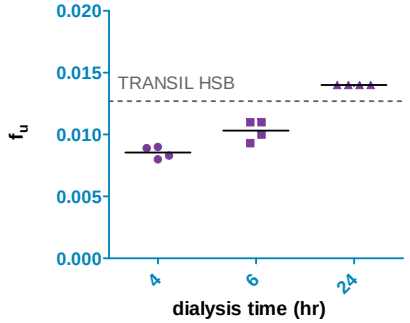


Figure 4. An example applying HSB technology to discovery project Compound A, a lipophilic acid (cLogP 6.1, MW 503) with long time to equilibrium using traditional EqD; HSB results are similar to EqD values at longer incubation times.



Summary of assay advantages

Equilibrium Dialysis	TRANSIL HSB
Low cost and high throughput Gold standard approach; widely accepted Straight forward protocol	Less demanding on analytical sensitivity Minimal non-specific binding issues Good for poorly soluble compounds Short incubation time; can assay compounds with moderate plasma instability

CONCLUSIONS

The TRANSIL HSB assay shows promise for the facile measurement of PPB of compounds with low f_u, high non-specific binding, poor analytical sensitivity, impaired solubility, or plasma instability. HSB f_u values are comparable to literature reports. Additional work is ongoing to evaluate compounds where disagreement between HSB and literature values is observed (e.g. itraconazole, amiodarone). Additional f_u data will be generated for these twelve compounds in-house using a 24 hr EqD protocol. A statistical analysis will be applied to measure agreement between HSB and EqD or literature values. Detailed results will be described in an upcoming publication.

REFERENCE

1. Obach RS, Lombardo F, Waters NJ. Trend analysis of a database of intravenous pharmacokinetic parameters in humans for 670 drug compounds. Drug Metab Dispos. 2008 Jul;36(7):1385-405.

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