High Content Automated Plasma Protein Binding Screening and Definitive Assays Using Rapid Equilibrium Dialysis for Drug Development

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Overview

- The purpose of this study was to develop a high content screen and a definitive automated method to determine plasma protein binding for drug discovery and development groups.
- Rapid equilibrium dialysis was used as the method of choice.
- Both screening and definitive approaches gave values similar to literature reports.
- Assays had good reproducibility.

Introduction

A pharmacokinetic/pharmacodynamic principle important in drug development is that it is the free (unbound) and not the total dosed drug that exerts a pharmacological effect or is available for distribution, metabolism and clearance from the body. Plasma proteins such as albumin have a high propensity to bind drugs and are typically responsible for the bulk of non-specific *in vivo* drug binding [1]. Therefore the extent of plasma protein binding (PPB) is a critical parameter to determine during drug development as it can influence efficacy factors such as receptor occupancy and disposition factors such as metabolic clearance. In the present study we developed automated methods for PPB screening and definitive PPB assessment on a Tecan liquid handler with endpoints measuring fraction unbound (f_u), mass balance (% recovery) and plasma stability (% remaining). Rapid equilibrium dialysis (**Figure 1**) was selected as the methodology of choice for PPB assessment as it is commonly used in the industry and amenable to automation [2]. In equilibrium dialysis, drug is spiked into plasma and placed into one cell while phosphate buffer with no drug is added to the adjacent compartment. Both cells are separated by a semipermeable membrane and the unbound drug diffuses across the membrane down its electrochemical gradient until equilibrium is established (*i.e.* unbound drug concentration is equal in both compartments).

Materials & Methods

Chemicals

Erythromycin, nifedipine, phenytoin, quinidine, rosiglitazone, sertraline, verapamil and warfarin were purchased from Sigma-Aldrich (St. Louis, MO). Pooled heparinized rat, dog and human plasma were purchased from Bioreclamation-IVT (Westbury, NY). All other reagents were of analytical grade.

Assessment of plasma protein binding by equilibrium dialysis

Plasma protein binding was assessed by 96-well rapid equilibrium dialysis (RED) purchased from Thermo Scientific (Waltham, MA).The workflow for these assays is shown in **Figure 2**.

Screening assay: A plasma protein binding screen method was developed on a Tecan Freedom EVO-200 liquid handling instrument (Tecan Group Ltd., Mannëdorf, Switzerland) utilizing a Multi-Channel Arm (MCA-96) and 8 channel Liquid Handling Arm (LiHa) on the EVOWare® v2.5 software platform (Tecan Group Ltd., Mannëdorf, Switzerland). This method can test up to 16 compounds at a single concentration with plasma from up to six different species. In the present study the binding of several compounds, namely erythromycin, nifedipine, phenytoin, quinidine, rosiglitazone, sertraline, verapamil, and warfarin (each at 2 µM in neat plasma) was evaluated in plasma from three species (human, rat and dog). Plasma samples were dialyzed against a phosphate buffer for four hours at 37°C, 95% humidity, and 5% CO_2. Compound stability was evaluated using additional plasma samples from each species at t = 0 and t = 4 hours. Incubations were quenched by the transfer of plasma into pre-aliquoted organic solvent (at an approximate 1:8 ratio) containing internal standards. Samples were then analyzed by relative LC/MS/MS quantitation as described below, followed by data processing with Galileo LIMS (Thermo Scientific, Waltham, MA) and Crystal Reports 2013 (SAP Business Objects) to determine f_u, % recovery and % remaining.

Definitive assay: A definitive plasma protein binding method was developed on a Tecan Freedom EVO-200 liquid handling instrument (Tecan Group Ltd., Mannëdorf, Switzerland) utilizing a Multi-Channel Arm (MCA-96) and 8 channel Liquid Handling Arm (LiHa) on the EVOWare® v2.5 software platform (Tecan Group Ltd., Mannëdorf, Switzerland). This method was developed to test three compounds (at a single concentration) with a positive control, or one compound (at three concentrations) with a positive control in plasma from up to six different species. The assay is then typically repeated for a total of three runs (and a total of n = 12 data points for each concentration). In the present study, warfarin was evaluated at 2, 5 and 10 µM with sertraline (2 µM) as the positive control in plasma from three species (human, rat and dog). Plasma samples (n = 4) were dialyzed against a phosphate buffer at 37°C, 95% humidity, and 5% CO_2 for four hours. Compound stability was evaluated using additional plasma samples from each species at t = 0 and t = 4 hours. Incubations were quenched by the transfer of plasma into pre-aliquoted organic solvent (at an approximate 1:8 ratio) containing internal standard. Samples were then analyzed by absolute LC/MS/MS quantitation as described below, followed by data processing with Galileo LIMS (Thermo Scientific, Waltham, MA) and Crystal Reports 2013 (SAP Business Objects) to determine fraction unbound (f_u), % recovery and stability (% remaining).

Analytical Methods

Samples were analyzed by liquid chromatography (LC) with tandem mass spectrometric detection (MS/MS) on an AB Sciex 4000 or 5500QTrap mass spectrometer (Foster City, CA) with a Shimadzu Prominence or Nexera LC system (Columbia, MD) or a Waters Acquity UHPLC system (Milford, MA) interfaced by electrospray ionization (ESI). Relative or absolute quantitation of each individual analyte was performed using Analyst v.1.6.2 or more recent (AB Sciex, Foster City, CA).

Screening assay: Generic LC gradients comprising formic acid modified water and acetonitrile mobile phases ramping up to 98% organic over approximately 2 min were employed with a Waters Atlantis dC18 column (Milford, MA). For MS detection, a custom partial-tuning approach was developed in-house. Briefly, solutions of the analytes were evaluated to establish the de-/protonated molecule *m/z* and appropriate fragment ion to build MRM transitions for the analytes. Rudimentary optimization of fragmentation parameters was performed. Several ionization parameters were kept at generic values appropriate for the specific MS employed. Generic internal standards (*e.g.,* terfenadine) were employed for relative quantitation.

Definitive assay: Warfarin and sertraline samples were analyzed with an LC gradient method comprising formic acid modified water and acetonitrile mobile phases ramping from 30% to 98% organic over 2.3 min applied to a Waters Atlantis dC18 column (Milford, MA). Warfarin-d_5 and sertraline-d_3 were used as internal standards. Mass spectral detection employed selected reaction monitoring (SRM) with the following ionization parameters: 4500V electrospray voltage, 20 psi curtain gas pressure, 50V declustering potential and 550°C source temperature. The SRM transitions used for warfarin detection were 309/251 and 314/256 for warfarin and warfarin-d_5, respectively, with 29 eV collision energy (CE). The transitions employed for sertraline detection were 306/159 and 309/159 for sertraline and sertraline-d_3, respectively, with 33 eV CE. Analytes were quantified against calibration curves (1- 3000 nM for 2 µM PPB assays; 2.5-7500 nM for 5 µM; 5-15000 nM for 10 µM) with linear least-squares regression and 1/x weighting.

Results

As shown in **Table 1**, a panel of eight compounds was tested (at 2 µM each) for plasma protein binding with the screening method. The results were consistent with literature reported ranges, with nifedipine, rosiglitazone, sertraline and warfarin binding highly to plasma proteins regardless of species (96-99% bound). Phenytoin was found to be moderately bound to plasma proteins (72-85% bound). Additionally, species dependent plasma protein binding was observed for erythromycin (26-47% bound in rat; 85-93% in dog; 54-67% in human), quinidine (78% bound in rat; 98% in dog; 71% in human), and verapamil (92-96% bound in rat; 90-94% in dog; 77-86% in human). All compounds were fully recoverable and had good stability.

For the definitive assay, as shown in **Table 2**, warfarin was tested at 2, 5 and 10 µM with sertraline (2 µM) used as the positive control. The results were consistent with literature warfarin and sertraline binding values with assay %CVs <15% and 90%+ recovery and stability.

Figure 1. The basis of plasma protein binding assessment by equilibrium dialysis

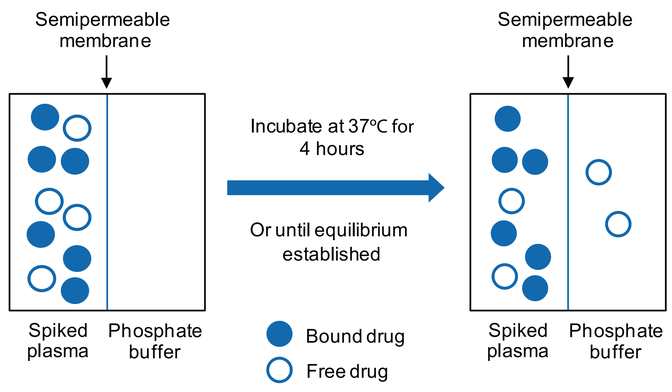

Figure 2. Plasma protein binding assay workflow for both screening and definitive studies

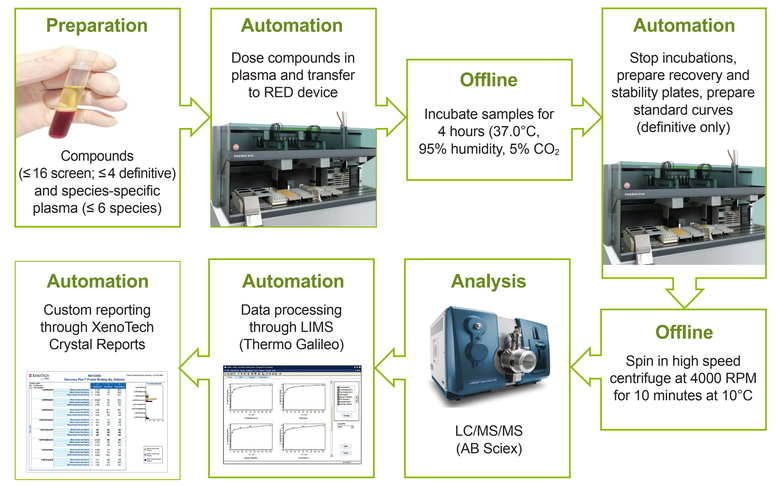

Table 1. Plasma protein binding values for 8 compounds determined with the screening approach in rat, dog and human plasma

Compound	Percent Bound						Runs (n)
	Rat		Dog		Human		
	Experimental	Literature	Experimental	Literature	Experimental	Literature	
Erythromycin	33.96 ± 11.59	22	88.53 ± 3.30	73-81	58.10 ± 3.01	75-95	4
Nifedipine	99.86 ± 0.01	99.4	95.60 ± 0.11	99	97.39 ± 0.30	92-98	4
Phenytoin	83.98 ± 1.01	>90	78.94 ± 1.19	77	83.17 ± 1.01	90	4
Quinidine	77.70 ± NA	86.5	98.10 ± NA	70-80	70.80 ± NA	80-88	1
Rosiglitazone	99.71 ± 0.21	98.8-99.5	99.07 ± 0.09	99	99.39 ± 0.38	99.8	4
Sertraline	98.76 ± 0.13	97.2	99.44 ± 0.23	98.9	98.10 ± 0.12	98	4
Verapamil	92.85 ± 1.04	93.7	90.25 ± 0.54	90.1-91.1	76.67 ± 0.32	89.6	4
Warfarin	99.44 ± 0.14	99.5	97.77 ± 0.16	96.2	98.97 ± 0.07	99	4

NA: Not applicable

Table 2. Plasma protein binding values for warfarin and sertraline determined with the definitive assay in plasma from rat, dog and human

		Rat			Dog			Human		
		2 µM	5 µM	10 µM	2 µM	5 µM	10 µM	2 µM	5 µM	10 µM
Warfarin	% Bound	99.35	99.29	99.33	97.83	97.60	97.05	99.09	99.10	98.92
	Std Dev	0.03	0.04	0.46	0.10	0.09	0.19	0.10	0.05	0.06
	% CV	5.07	5.70	15.04	4.62	3.73	6.40	11.14	5.29	5.86
Sertraline	% Bound	99.12	ND		99.70	ND		98.65	ND	
	Std Dev	0.04			0.04			0.10		
	% CV	4.52			13.02			7.34		

ND: No data

Conclusions

Overall the results from these automated methods demonstrated their robustness and repeatability to determine plasma protein binding. Both screening and definitive assays are suitable for use in drug discovery and development.

References

1 Ramanathan V and Vachharajani N (2010) Protein Binding in Drug Discovery and Development, in: *Evaluation of Drug Candidates for Preclinical Development: Pharmacokinetics, Metabolism, Pharmaceutics, and Toxicology* (Han C, Davis CB and Wang B eds). John Wiley & Sons, Inc, Hoboken, NJ: pp 135-167.

2 Waters NJ, et al. (2008) Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. *J Pharm Sci* **97**(10): 4586-4595.