Optimization and Qualification of Ultracentrifugation for Protein Binding Determination

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INTRODUCTION

Equilibrium dialysis (ED) has been the gold standard for protein binding determinations. However, this method has its limitations particularly for compounds with very high non-specific binding, plasma instability, or inability to diffuse through the dialysis membrane. Ultracentrifugation (UC) may be an alternative method for these types of compounds. The objective of this study is to evaluate and optimize the UC method for plasma protein binding determination and to optimize experimental conditions for UC methods.

MATERIALS AND METHODS

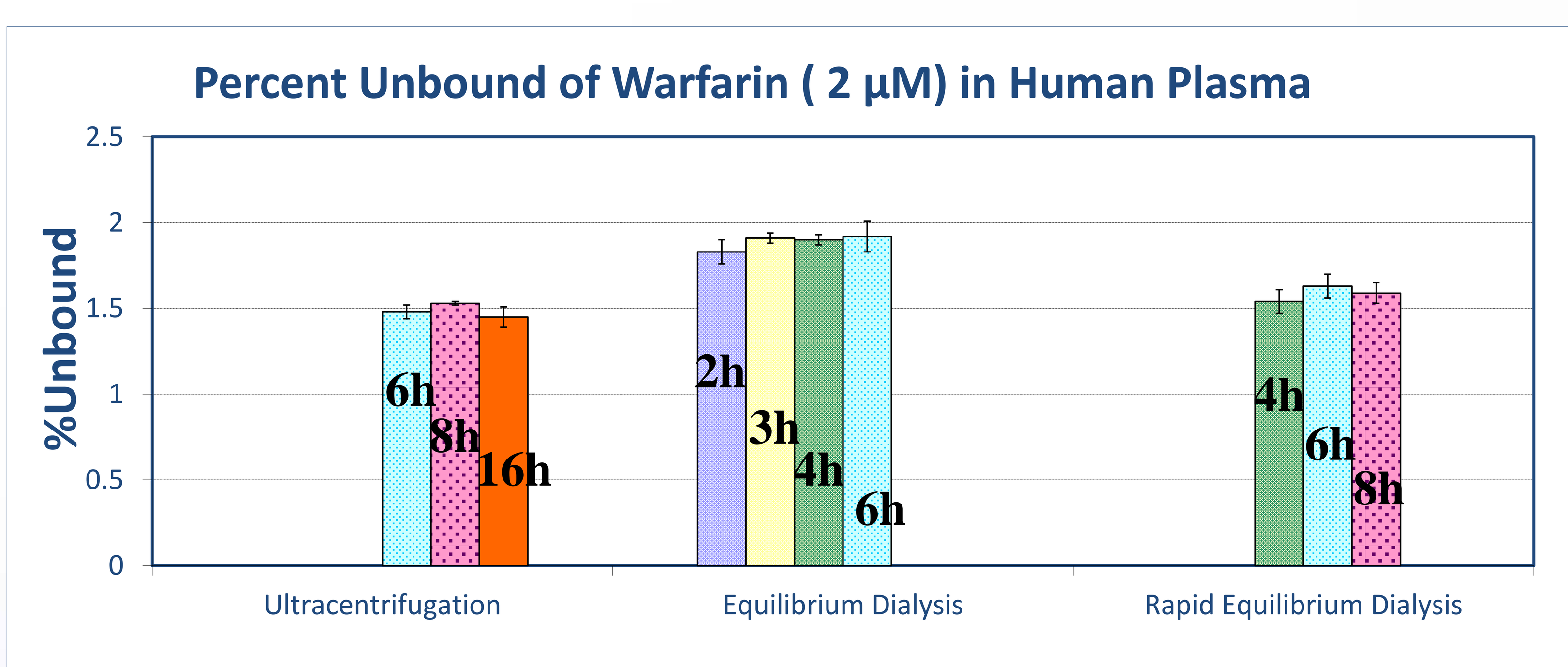
- Ultracentrifugation (UC) was conducted with spiked plasma (2 µM, ~ 1.4 mL) centrifuged (280,000 x g or 200,000 x g) at 37°C using a system consisting of a Beckman Model: L8-70M and FiberLite Ultraspeed Rotor (Model: F50L-24 × 1.5).
- Equilibrium dialysis (ED) was conducted with spiked plasma (2 µM, 1 mL) dialyzed against 0.133M potassium phosphate buffer (1 mL, pH 7.4) at 37°C using a Multi-Equilibrium Dialyzer™ system with a dialysis membrane of 5000 molecular weight cut off (5K MWCO).
- Rapid Equilibrium Dialysis (RED) was conducted with spiked plasma (2 µM, 0.3 mL) was dialyzed against 0.133M potassium phosphate buffer (0.5 mL, pH 7.4) in a 5% CO_2 humid incubator at 37°C using the Single-Use Plate with membranes (8K MWCO).
- Ultrafiltration (UF) was conducted with spiked plasma (2 µM, 1 mL) was equilibrated at 37°C for ~ 15 minutes before centrifuged for ~ 20 minutes using Centrifree® with 30K MWCO membranes.
- Time course experiments were carried out with UC, ED, and RED. Protein concentration in supernatant after UC, dialysate of ED or RED, and ultrafiltrate was measured using BCA assay. Drug concentration was determined by LSC or LC-MS/MS.
- After UC, visually clear phase separation was produced and at least 5 aliquots (100 µL each) could be removed from each UC plasma sample supernatant for analysis. Warfarin, docetaxel, and other proprietary compounds, including several peptides were used in this study.

RESULTS

- The measured protein concentration in the UC supernatant (6 hours at 280,000 x g) was approximately 0.8% of the initial plasma concentration, whereas approximately 0.4% was found in the dialysate from ED.
- Warfarin plasma protein binding results were consistent when determined using UC, ED, RED (rapid equilibrium dialysis), and UF (ultrafiltration).

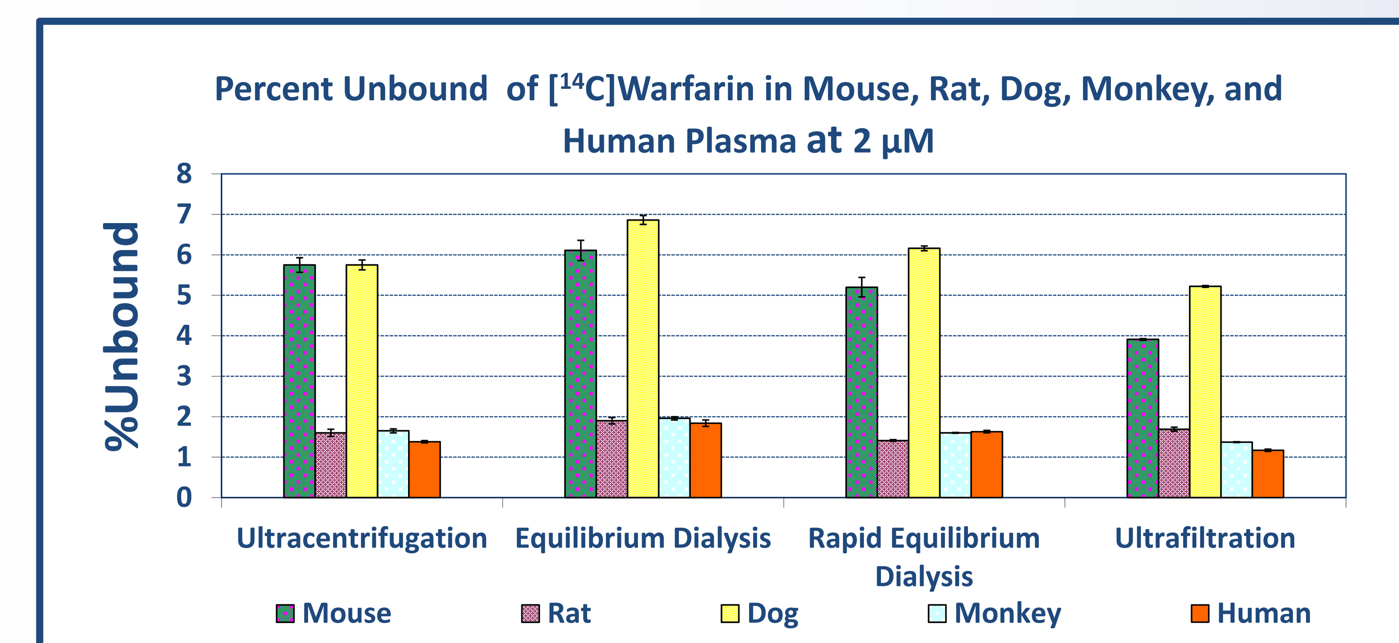
Time Course of Protein Binding Methods – Protein Conc.

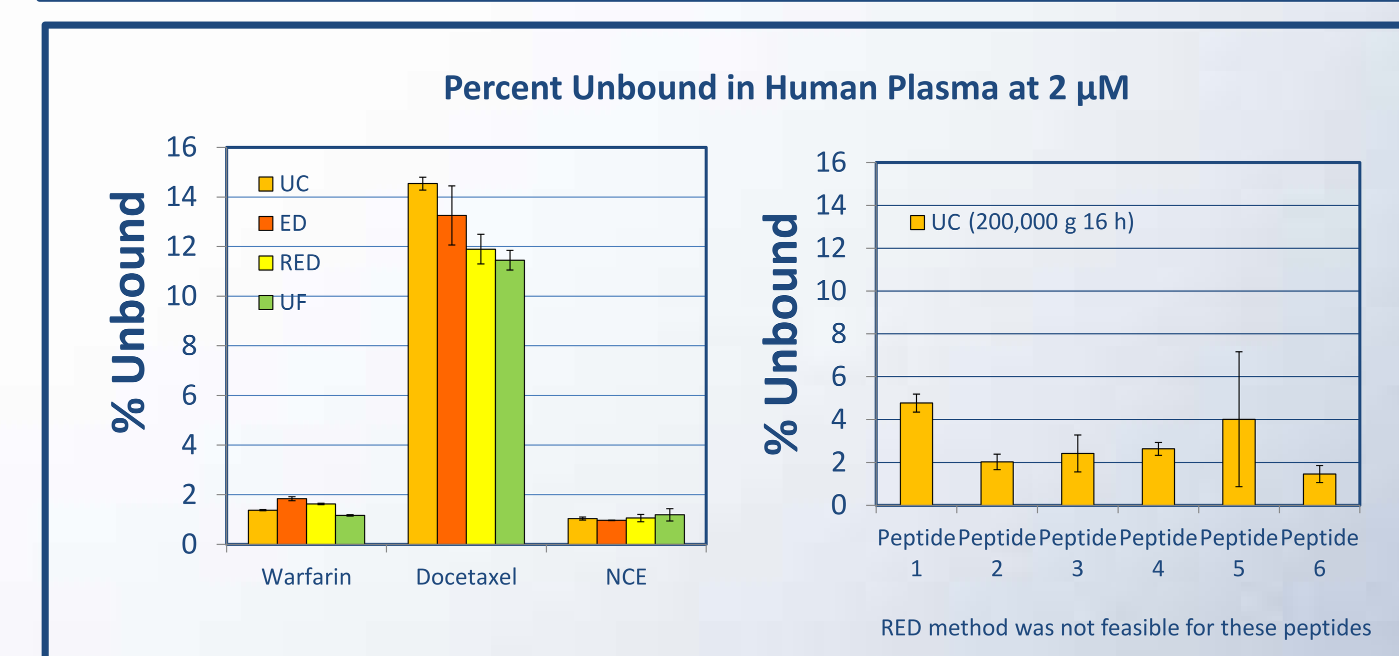
Method	Time (h)	Protein Conc. (µg/mL)			% of initial plasma conc.	
		T_0	Post-dialysis Buffer	Post-dialysis Plasma	Buffer	Plasma
ED	2		233	44978	0.40%	76%
	3		220	45329	0.37%	77%
	4		219	52149	0.37%	89%
	6		208	46901	0.35%	80%
RED	4		161	57984	0.27%	99%
	6		171	63165	0.29%	107%
	8		171	60817	0.29%	103%
UC	6		506	NA	0.86%	NA
	8		507	NA	0.86%	NA
	16		464	NA	0.79%	NA
Initial Plasma	T_0	58876				100%



[14C]Warfarin was used in this experiment

- The mean percent bound was ~ 98% in rat, monkey, and human plasma, and ~ 94% in mouse and dog plasma.
- Docetaxel was ~ 88% protein bound in human plasma using UC, ED, or UF.
- Another small molecule compound was ~ 99% bound in human plasma using UC, ED, or UF methods.
- The extent of plasma protein binding could not be determined for the peptides (MW 4000-5000) investigated using RED method with 25K membrane. The peptides were unable to move across the dialysis membrane even in buffer solutions. However, using UC the extent of plasma protein binding could be determined for all compounds, with the percent free from 1.5% to 4.8%.





CONCLUSIONS

These results demonstrate the potential advantage of the UC method. Ultracentrifugation can be useful for assessing plasma protein binding of compounds with high molecular weight or high non-specific binding. Further investigation is needed for UC method at higher speed and shorter time, which may be an alternative for compounds with limited plasma stability