Antisense Oligonucleotide In Vitro Protein Binding Determination in Plasma, Brain and Cerebral Spinal Fluid Using Hybridization LC-MS/MS

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Supplemental Data

Methods

Ultracentrifugation

Rat plasma and PBS were co-spiked with 1 µM ASO and 1 µM warfarin. Upon equilibration at 37 ºC, 1 ml of spiked plasma or PBS was transferred to 2ml polycarbonate tubes. With 10 spots in the rotor, four spots were filled with spiked PBS and four spots were filled with spiked plasma. Samples were spun at 400,000g for four hours at 37 ºC. After every hour, the centrifuge was stopped, and a spiked PBS and spiked plasma sample were removed totaling 1 spiked plasma and 1 spiked PBS sample for every hour. For data analysis, every tube was divided into four 4 vertical quadrants of equal volumes (250 µl each). With the top layer (first 250 µl) being "Q1", second 250 µl being "Q2" and so on. 10ul from each quadrant was used for ASO quantification, 20 µl was used for warfarin quantification and 25 µl was used for protein quantification (spiked plasma samples only) using a Pierce™ BCA Protein Assay Kit. Original plasma and quadrant 4 samples were diluted 80-fold in PBS prior to protein quantification.

Rapid Equilibrium Dialysis (RED)

Single use RED plate with 12kDa MWCO inserts were pre-treated under four different conditions: 0.1% Tween 20 in PBS, 5 µM sacrificial ASO in 1% Tween-20, and 5 µM sacrificial ASO in 0.1% Tween-20. 500 µl and 750 µl of pre-treatment solution were added to the sample and buffer chamber respectively. The plate was covered and incubated at room temperature for 5 h while shaking at 250 rpm. Pre-treatment solution was removed, and the plate was spun upside down at 3000 rpm for 3 min to ensure any pre-treatment solution excess was removed. Upon pre-treatment, 300 µl of spiked PBS (1 µM ASO, 1 µM antipyrine and 1 µM warfarin) was added to

the sample chamber while 550 µl of PBS was added to the buffer chamber. The plate was sealed with sealing tape and incubated at 37 °C overnight while shaking at 250 rpm. Following incubation, 10 µl from each chamber was removed for ASO quantification, while 20 µl was removed from each chamber for small molecule quantification. Per the manufacturer instruction, the first step in RED analysis should be to evaluate if at a given time, both sides reach equilibrium in a protein free medium. Equilibrium was determined through the following equation:

$$\%\ Equilibrium = \frac{[sample\ chamber]}{[buffer\ chamber]} X100$$

Equilibrium values of 100% would indicate the system has reached equilibrium.

Small molecule quantification

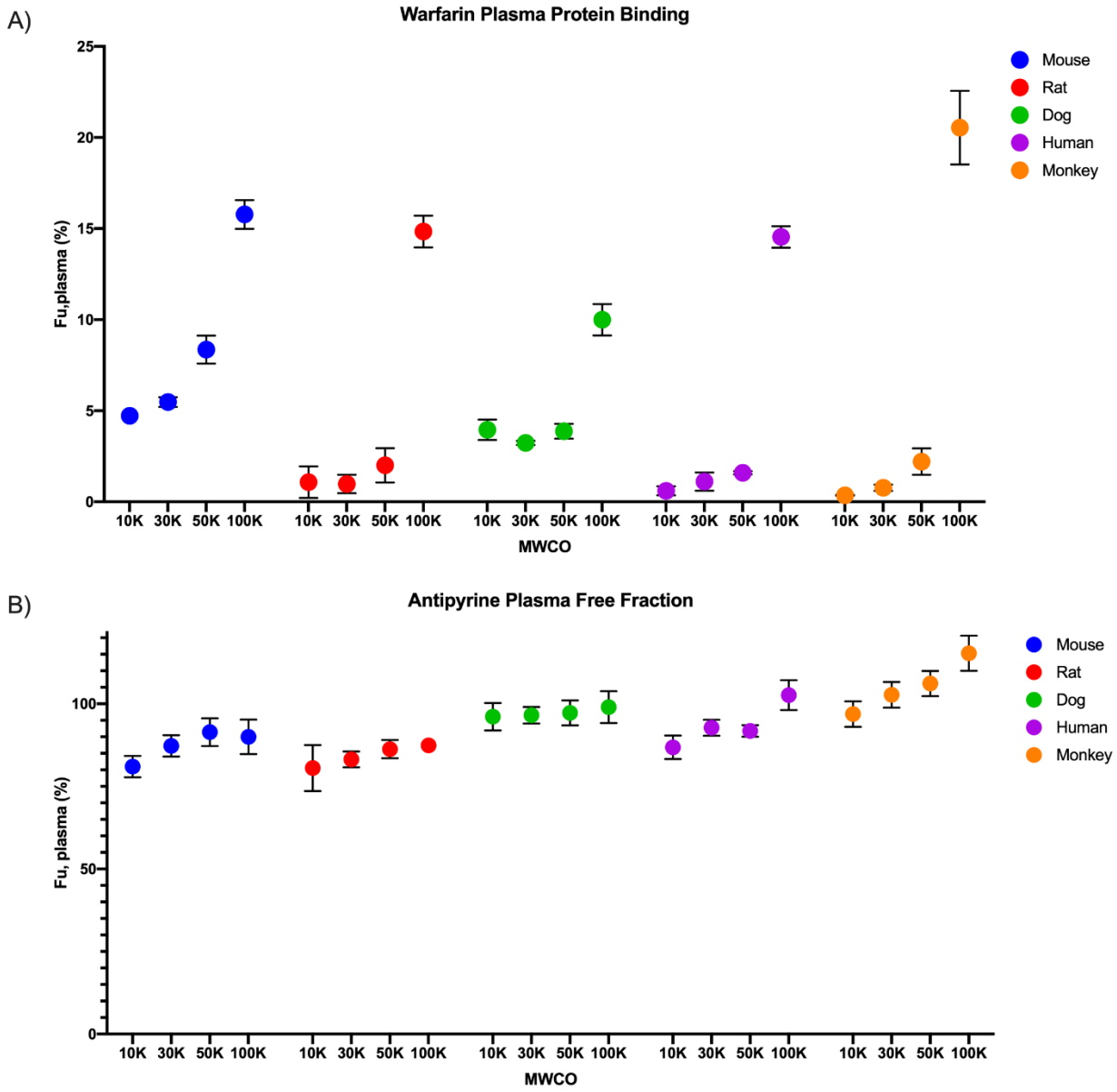
Sample preparation: 20 µl of the ultrafiltrate was transferred to a 96-well plate (plasma, brain homogenate or CSF). To account for matrix effects, 20 µl of corresponding blank matrix was added. Following matrix matching, 200 µl of acetonitrile (ACN) was added to each well. The plate was vortexed at 1000 rpm for 10 min then spun down at 3500 rpm for 10 min. 20 µl of the supernatant was transferred to 180 µl of water. Upon mixing, samples were injected into LC-MS.

LC-MS/MS conditions: Small molecule LC-MS/MS experiments were performed using a Shimadzu ExionLC™ AD HPLC system (Kyoto, Japan) coupled to an AB Sciex Qtrap 5500 (Sciex, Framingham, MA) with an electrospray ionization (ESI) source. The LC method consisted of Mobile Phase A (0.1% formic acid) and Mobile Phase B (0.1% formic acid in 50:50 ACN:MeOH). Separation was achieved using an Ascentis® Express 90° C18 (2 × 2.1mm, 2.7µm) column (Supelco, Bellefonte, PA). Injection volume was set at 10 µl. Column

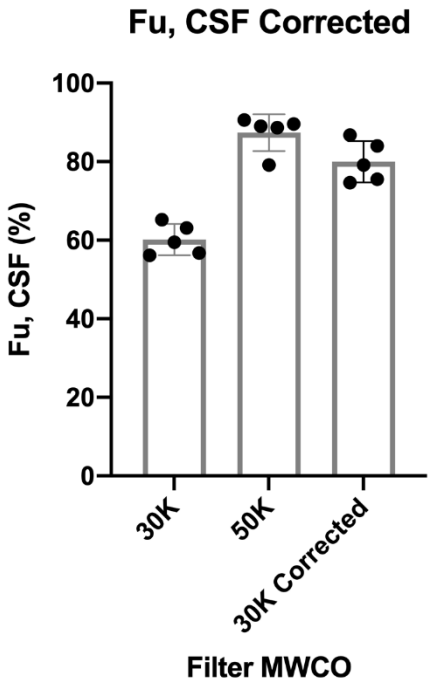
temperature was set to 40 °C and flow rate was 0.5 ml/min with the following gradient: 10% B from 0 to 0.2 min, 10-100% B from 0.2 to 1.6 min, 100% B hold from 1.6 to 2.1 min, 100-10% B from 2.10 to 2.11 and 10% B hold from 2.11 to 2.6 min. Optimized ion source parameters were the following: curtain gas (CUR) at 20, collision gas (CAD) at 8, IonSpray voltage (IS) at 5000 V, temperature (TEM) at 550 °C, ion source Gas 1 (GS1) at 60 and Gas 2 (GS2) at 60, respectively. Operating under the positive ion mode, the multiple reaction monitoring (MRM) function was used to quantitate warfarin, antipyrine and verapamil, as well as internal standard propanolol. MRM transitions were (Q1→Q3; collision energy, eV): warfarin (309.1→163.1 m/z; 25 eV), antipyrine (189.0→56.0 m/z; 25 eV), verapamil (454.6→165.1 m/z; 35 eV), propranolol (259.8→116.0 m/z; 25 eV). Analyte peak areas were integrated using Analyst 1.6.2 software (Sciex) and normalized to the internal standard. For verapamil unbound fraction quantification in brain homogenate, Eq. 3 was used to account for the matrix dilution factor.

Figures

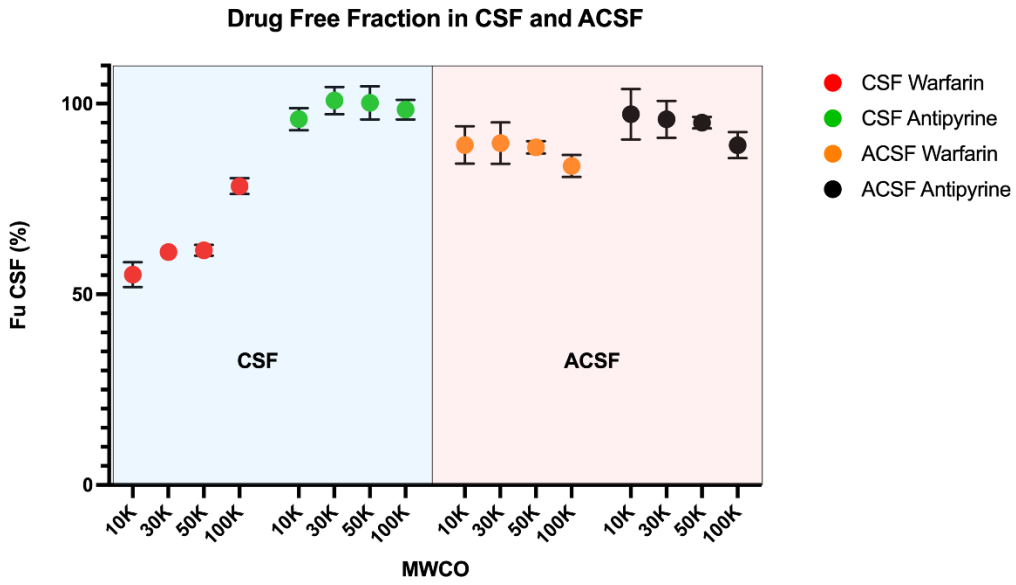
Supplemental Figure 1: Warfarin and antipyrine f_u in different filter sizes and matrixes.



Supplemental Figure 2: Cerebral spinal fluid (CSF) f_u in 30K and 50K MWCO filters, as well as 30K corrected values. 30K corrected was calculated by multiplying 30K values by the correction factor derived from recovery experiments in ACSF.



Supplemental Figure 3: Cerebral spinal fluid (CSF) and artificial CSF (ACSF) f_u for co-spiked warfarin, and antipyrine



Table

Supplemental Table 1: Protein concentration in different quadrants from ultracentrifugation experiments

	Protein Concentration (%)			
	1 hour	2 hours	3 hours	4 hours
Q1	4.0	0.4	0.4	0.3
Q2	3.9	2.3	0.4	0.4
Q3	2.8	2.6	3.2	2.7
Q4	247.7	230.1	NA	NA