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

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Running Title: In Vitro ASO Protein Binding Using Hybridization LC-MS/MS

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Number of text pages: 26

Number of tables: 3

Number of figures: 5

Number of references: 49

Number of words in Abstract: 192

Number of words in Introduction: 750

Number of words in Discussion: 1370

Abbreviations

ACSF, artificial cerebral spinal fluid

ASO, antisense oligonucleotide

CNS, central nervous system

CSF, cerebral spinal fluid

f_u , unbound fraction

LC-MS/MS, liquid chromatography–tandem mass spectrometry

MOE, 2'-O-(2-methoxyethyl)-oligoribonucleotides

MW, molecular weight

MWCO, molecular weight cutoff

MRM, Multiple reaction monitoring

NSB, nonspecific binding

PBS, phosphate-buffered saline

PCR, polymerase chain reaction

PD, pharmacodynamic

PK, pharmacokinetic

RED, rapid equilibrium dialysis

Abstract

The development of quantitative models for prediction of drug pharmacokinetics based on in vitro data has transformed early drug discovery. Drug unbound fraction (f_u) characterization is a key consideration in pharmacokinetic (PK) and pharmacodynamic (PD) modeling, assuming only unbound drug can interact with the target, and therefore has direct implications in the efficacy and potential toxicity of the drug. The current study describes the implementation of a hybridization LC-MS/MS platform for the direct quantitation of antisense oligonucleotide (ASO) f_u . The method provides substantial improvements including minimal matrix effects and high specificity when compared to previously used oligonucleotide f_u detection methods such as ligand binding assays or liquid scintillation. The hybridization LC-MS/MS platform was integrated with ultracentrifugation, ultrafiltration and equilibrium dialysis, and method performance for each technique was evaluated. While ASO protein binding has been previously characterized in plasma, there were no studies that quantitated ASO f_u in brain or CSF. As ASOs continue to undergo clinical trials for neurological and neuromuscular indications, f_u characterization in brain and CSF can provide invaluable information about ASO distribution and target engagement in the central nervous system, therefore providing support for in vivo PK-PD data characterization.

Significance Statement

A novel hybridization LC-MS/MS based approach was successfully developed for the determination of ASO in vitro protein binding in plasma, and for the first-time brain and CSF. Ultrafiltration, equilibrium dialysis, and ultracentrifugation were assessed for the separation of unbound ASO from biological matrices. The hybridization LC-MS/MS platform provided unique

advantages, including minimal matrix effects and high specificity, comparing to traditional ligand binding assays or liquid scintillation approaches, which enabled efficient and reliable in vitro protein binding assay.

Introduction

Antisense oligonucleotides (ASOs), defined as short synthetic oligonucleotides with single-stranded sequences complementary to certain mRNA sites, have been under drug development for approximately 30 years, with the first FDA approved drug, Fomivirsen, being approved in 1998 (Stein and Castanotto, 2017). Chemical modifications such as substitution of one non-bridging oxygen (phosphate) for a sulfur (phosphorothioate) have increased stability against nuclease degradation as well as plasma protein binding, with phosphorothioated oligonucleotides being highly protein bound (>85%) (Levin et al., 2007; Geary et al., 2015). Proper characterization of ASOs chemical modifications and its biological ramifications are important since ASO pharmacokinetic properties are largely driven by chemistry rather than sequence (Geary, 2001; Geary et al., 2015).

Drug unbound fraction (f_u) quantification in any given matrix is essential in understanding drug pharmacokinetics since it is generally accepted that only the unbound drug is available and responsible for drug efficacy and potential drug toxicity (Rowland et al., 2011; Mariappan et al., 2013; Roberts et al., 2013). f_u quantification provides valuable insight into drug absorption, distribution, metabolism, excretion, and toxicity. While ASO f_u has been previously characterized in plasma, ASO f_u in matrixes of high interest such as brain and cerebral spinal fluid (CSF) is still unknown. Due to relatively large molecular size and multiple negative charges, ASOs do not efficiently cross the blood brain barrier (Geary et al., 2015; Bennett et al., 2017). However, this limitation has been mitigated through intracerebroventricular or intrathecal delivery while less invasive delivery techniques continue to be developed (Miller et al., 2013; Wurster and Ludolph, 2018; Alarcón-Arís et al., 2020; Min et al., 2020). To better

understand ASO exposure and target occupancy in the CNS, proper f_u characterization in brain and CSF is crucial.

Historically, f_u determination for small-molecule drug candidates has been determined by ultrafiltration, ultracentrifugation, or equilibrium dialysis, which are techniques based on the physical separation of unbound drug molecules from those bound to proteins (Pacifici and Viani, 1992). In vitro drug f_u results are one of the properties used to prioritize certain drug candidates into further stages of drug development. Due to their physical properties such as relatively high molecular weight, linear structure, and nonspecific binding, ASOs present unique challenges in f_u determination through traditional techniques. Because of the linear conformation of ASOs, the molecular weight cutoff (MWCO) for dialytic membranes needs to be much higher than the ASO molecular weight (generally ~ 7 KDa). The lack of commercially available equilibrium dialysis membranes with MWCO over 20K makes this technique incompatible with ASOs (Rocca et al., 2019). Ultrafiltration also requires a membrane medium, which becomes a major source for oligonucleotide nonspecific adsorption and therefore low recoveries. To overcome this issue, membrane pre-treatment with surfactants or sacrificial oligonucleotides that are irrelevant to the analyte have shown to provide acceptable recoveries (Watanabe et al., 2006; Humphreys et al., 2019). Ultracentrifugation bypasses the need for a membrane, however low recoveries have been reported for siRNA, potentially due to differential sedimentation of macromolecules (Hughes et al., 1938; Humphreys et al., 2019). Alternatively, electrophoretic mobility shift has been evaluated as a method for determining siRNA f_u in plasma (Rocca et al., 2019). While this technique also bypasses the need for a membrane, limited sample throughput prevents it from being widely applied in drug discovery.

Previous detection methods for oligonucleotide f_u determination included liquid scintillation and hybridization techniques such as real-time polymerase chain reaction (PCR). There are several advantages when choosing LC-MS/MS over previous methods, including the generation of more quantitative values, easier normalization, and less biased results coming from different variations of PCR techniques (Wang et al., 2013; Basiri et al., 2019). Additionally, liquid scintillation requires the synthesis of radiolabeled ASOs that are expensive to produce and challenging to dispose of.

In this work, ASO f_u in plasma, brain and CSF were characterized through an ultrafiltration hybridization LC-MS/MS platform. This is the first time LC-MS was employed for direct quantitation of ASO f_u in not only plasma, but also brain tissue and CSF, for a more complete understanding of ASO protein binding. The method allowed specific and sensitive analysis, wide linear range, and automation of the hybridization extraction (Li et al., 2020). Complete elimination of matrix effects enabled the direct comparison between unbound and bound ASO in different sample compositions, which was otherwise unachievable by previous methods. In addition to ultrafiltration, equilibrium dialysis and ultracentrifugation were also evaluated, although membrane incompatibility, limited sample throughput, and sampling bias hinders their performance. f_u was characterized for three different ASOs across different matrixes in multiple species.

Materials and Methods

Materials

Analyte ASO-1, ASO-2, ASO-3, and the internal standard (ASO-4) were proprietary assets of Biogen and provided by its collaborator, with basic information shown in Table 1.

Capture probes (biotinylated reverse complimentary DNA) were synthesized by Integrated DNA Technologies (Coralville, IA). Rapid equilibrium dialysis (RED) device [12 kDa MWCO], PierceTM BCA protein assay kit, Dynabeads MyOne streptavidin C1 and RNA grade proteinase K solution (20 mg/ml) were purchased from Thermo Fisher Scientific (Waltham, MA). Clarity OTX lysis-loading buffer was acquired from Phenomenex (Torrance, CA). Amicon Ultra 0.5-ml centrifugal filters [10, 30, 50 and 100 kDa MWCO], Trizma base, sodium chloride, Tween 20 and ethylenediaminetetraacetic acid (EDTA) were acquired from MilliporeSigma (St. Louis, MO). 1,1,1,3,3,3-Hexafluoro-2-methyl-2-propanol (HFMP) was obtained from Alfa Aesar (Tewksbury, MA). N,N-dimethylcyclohexylamine (DMCHA) was obtained from TCI America (Portland, OR). HPLC grade acetonitrile (ACN) and DL-1,4-dithiothreitol (DTT) were obtained from Fisher Scientific (Hampton, NH). Pooled rat, mouse, dog, monkey and human plasma (K2EDTA) as well as male rat brain homogenate and monkey CSF were obtained from BioIVT (Westbury, NY). Artificial cerebrospinal fluid (ACSF) was acquired from Tocris Bioscience (Minneapolis, MN). Biogen in-house Milli-Q deionized water was used.

Ultrafiltration

A volume of 0.5 ml phosphate-buffered saline (PBS; 0.137M NaCl, 0.0027M K, 0.0119M Phosphates) with 0.1% Tween 20 (v/v; PBST) was added to Amicon Ultra 0.5-ml centrifugal three times to remove residual glycerin and condition filter. Following every PBST addition, 30, 50 and 100 kDa filters were spun in a bench-top centrifuge for 10 min at $3000 \times g$ while 10 kDa filters were spun at $5000 \times g$. After the third addition of PBST, filters were incubated at room temperature for 15 min before centrifugation. Any residual treatment solution was removed, while the collection tube was retained. For the sample treatment experiment, four

conditions were evaluated: 0.1% Tween-20 in PBS, 5 μ M sacrificial ASO in PBS, 5 μ M sacrificial ASO in PBS with 1% Tween-20, and 5 μ M sacrificial ASO in PBS with 0.1% Tween-20. Spiked matrixes were added to filters immediately after treatment to ensure filters never dried out. For recovery experiments, 1 μ M ASO-1, 1 μ M warfarin and 1 μ M antipyrine were co-spiked into PBS. A volume of 0.5 ml was transferred to treated filters and incubated at room temperature for 15 min. Following incubation, 10 kDa MWCO filters were centrifuged at $3000 \times g$ for 1 min while 30, 50 and 100 kDa filters were centrifuged at $1500 \times g$. Centrifugation time varied for different matrixes and filter MWCO sizes.

Plasma (pre-equilibrated to 37 °C) was co-spiked with 1 μ M ASO, 1 μ M warfarin and 1 μ M antipyrine and incubated at 37 °C while shaking at 500 rpm. 500 μ l of spiked plasma was transferred to treated filters and 10 kDa MWCO filter centrifuged at $3000 \times g$ for 2 min, while 30, 50, and 100 kDa centrifuged at $1500 \times g$ for the same amount of time. Same procedure was repeated for brain homogenate and CSF samples, but centrifugation time varied between matrixes: brain homogenate – 10 kDa ($3000 \times g$), 30, 50, and 100 kDa ($1500 \times g$) for 3 min, CSF - 10 kDa ($3000 \times g$), 30, 50, and 100 kDa ($1500 \times g$) for 2 min. Centrifugation time and speed were optimized to ensure sufficient but no more than 20% of the sample crossed the filter. CSF was also co-spiked with 1 μ M ASO, 1 μ M warfarin and 1 μ M antipyrine, while brain homogenate was co-spiked with 1 μ M ASO and 1 μ M verapamil. Ultracentrifugation and rapid equilibrium dialysis methods, as well as the small-molecule LC-MS/MS quantification method are provided in the Supplemental Section.

ASO Quantification

For ASO quantification, 10 μ l of the filtered (representing "free") or unfiltered (representing "total") sample was combined with 90 μ l of corresponding blank matrix in a Kingfisher 96 deep-well plate (Thermo Fisher Scientific, Waltham, MA). Sample preparation and LC-MS/MS analysis were conducted following a previously published protocol from Li and co-workers with minor modifications (Li et al., 2020). Multiple reaction monitoring (MRM) transitions used were: ASO-1 (785 $\rightarrow$ 94.9 m/z), ASO-2 (637.7 $\rightarrow$ 94.9 m/z), ASO-3 (660.1 $\rightarrow$ 94.9 m/z).

Recovery and f_u were calculated as previously described by Humphreys and co-workers using the following equations:

$$\% Recovery (buffer) = \frac{[receiver]}{[donor]} \times 100 \quad \text{Eq.1}$$

$$f_u = \frac{[receiver]}{[donor]} \quad \text{Eq.2}$$

where [donor] is the original concentration of drug in the spiked matrix prior to addition to filtration apparatus, and [receiver] is the drug concentration in the ultrafiltrate (Humphreys et al., 2019).

In this study f_u is shown in terms of percentage, meaning that equation 2 is multiplied by 100. Lastly, the dilution for brain homogenate (1:8) was adjusted through the following equation described by Kalvass and co-workers (Kalvass and Maurer, 2002; Kalvass et al., 2007):

$$undiluted f_u = \frac{1/D}{((1/f_{u,measured})-1) + 1/D} \quad \text{Eq.3}$$

D represents the dilution factor of brain tissue and $f_{u,measured}$ is the ratio described in eq.2. All ultrafiltration and RED experiments had a minimum number of 3 replicates ($N \geq 3$). Due to limited throughput, ultracentrifugation experiments were not replicated ($N=1$).

Results

Recovery Evaluation

To assess analyte nonspecific binding (NSB) to the filter membrane, the major potential limitation of ultrafiltration, recovery was evaluated for different filter pre-treatment conditions and different filter sizes by measuring the percentage of drug remaining in a protein free medium after filtration (Toma et al., 2021). Recovery determination was calculated through Eq.1. Figure 1A shows the recovery of 1 μ M ASO-1 in PBS under five different filter treatment conditions including untreated (filter rinsed with PBS), 0.1% Tween-20 in PBS, 5 μ M sacrificial ASO in PBS, 5 μ M sacrificial ASO in PBS with 1% Tween-20, and 5 μ M sacrificial ASO in PBS with 0.1% Tween-20. Recovery was assessed in 30K MWCO filters, which was the size showing significant but mitigatable loss to NSB. Consistent with previous reports, filter pre-treatment with either Tween-20 or sacrificial ASO was shown to minimize NSB (Watanabe et al., 2006; Humphreys et al., 2019). Based on recovery against a commonly accepted criterium at 80%, 0.1% and higher concentrations of Tween-20, with or without the combination of sacrificial ASO, provided sufficient anti-NSB protection, suggesting 0.1% Tween-20 to be the ideal blocking agent. Previous studies have shown Tween-20 to be the best blocking agent over several conditions including heparin, irrelevant oligonucleotides, bovine serum albumin, and others (Humphreys et al., 2019; Rocca et al., 2019).

Upon determining optimal filter treatment with 0.1% Tween-20, recovery of 1 μ M ASO-1 co-spiked with 1 μ M warfarin and antipyrine was evaluated at four different filter MWCO sizes (Figure 1B and 1C). While low recovery was observed for 10K filters, acceptable recovery was achieved for 30, 50, and 100K MWCO filters, suggesting a correlation between recovery and MWCO and further suggesting that unbound ASO cannot effectively pass 10K filter due to size constraint. MWCO of membranes is determined based upon a relationship between molecular weight and globular size as defined by Humphreys and co-workers (Humphreys et al., 2019). Given this relationship, the linear nature of oligonucleotides requires much higher MWCO, even though the MW of ASOs in this study was below 10 kDa (Humphreys et al., 2019). While filter size and treatment have direct implications on ASO recovery, these variables have no apparent impact on the recovery of small molecule controls (Figure 1C), confirming that the filter size had no impact on drug molecules that were significantly smaller than the MWCO. In summary, conditions of MWCO above 30K and treatment with 0.1% Tween-20 were considered sufficient in ensuring unbound ASO recovery. Recovery of 83% was observed at 30K MWCO in comparison to 96% recovery at 50K and 100K MWCO.

ASO f_u Determination in Plasma

Plasma f_u experiments were conducted at all filter sizes (Figure 2A), and experiments were orthogonally validated through co-spiked small molecules that are either highly protein bound (warfarin) or highly protein unbound (antipyrine). As filter MWCO increased, measured plasma f_u also increased for both ASO and small molecule controls. The upward trend suggests protein bleeding, specifically for proteins carrying bound drug molecules, in larger MWCO filter sizes as the responsible factor for artificially higher plasma f_u measurements. In the context of

this study, protein bleeding is defined as proteins with higher MW than filter cutoff or proteins carrying bound drug molecules that pass through the filter and end up in the ultrafiltrate. Previous studies have shown ASOs to be highly bound to albumin (Cossum et al., 1993; Srinivasan et al., 1995; Watanabe et al., 2006). Albumin has a relatively low MW (~ 66.5 kDa) and has been identified in the ultrafiltrate of a 50K MWCO ultrafiltration assay through electrophoretic mobility, suggesting protein bleeding even though the MW is higher than the MWCO (Humphreys et al., 2019). Similarly, warfarin is also highly bound to albumin and its concentration in the ultrafiltrate is also artificially higher when using 50K or higher MWCO filters, which results in a similar upward trend to those observed in Figure 2A (Supplemental Figure 1A). In contrast, minimal change in antipyrine concentration in the ultrafiltrate was observed in different MWCO filters, since it is highly unbound and not significantly affected by protein bleeding (Supplemental Figure 1B). To minimize the impact of protein bleeding, the smallest filter size that still achieved satisfactory recovery, in this case 30K MWCO, was chosen as the final condition. Plasma f_u measurements were repeated in two other ASOs across 5 species (Figure 2B). Interspecies differences in plasma f_u were observed following a trend in the order of rat < monkey < human $\approx$ dog < mouse, which was consistent with previously reported results (Watanabe et al., 2006). Additionally, Watanabe and co-workers described an inverse relationship between ASO sequence length and unbound concentration through plasma f_u measurements of 10 shortmer metabolites. While plasma f_u for ASO metabolites was not evaluated in this study, higher unbound concentrations were observed for ASO-3 (17mer) in comparison to the other 2 ASOs (20mers). The 30 kDa MWCO ultrafiltration method was orthogonally validated through previously characterized small-molecule controls, shown in Table 2. The results generated from the ultrafiltration method were consistent with those from standard

RED methods, as well as the literature reported values. This further demonstrated the validity of the developed ultrafiltration method.

ASO f_u Determination in Brain Tissue Homogenate

Ultrafiltration experiments were similarly conducted in rat brain tissue homogenate. ASO f_u determination at 1 μ M was orthogonally validated through co-spiked 1 μ M verapamil, a better characterized compound for brain f_u . Brain f_u determination was carried out at four different MWCO sizes, across 3 different ASOs. As seen in Figure 3, similar upward trend in measured brain f_u was observed as filter sizes increased, suggesting protein bleeding to influence measured brain f_u . Just like in plasma samples, the most suitable filter size was 30 kDa MWCO. Overall, rat brain f_u was 7-fold lower in comparison to rat plasma f_u . Differences in f_u in brain homogenate in comparison to plasma f_u are likely due to differences in protein concentration in the two matrices. The correlation between higher protein content and higher protein binding has been discussed by Liu and co-workers (Liu et al., 2014). Based on this correlation, lower brain f_u is likely due to brain's higher protein content in comparison to protein content in plasma.

ASO f_u in Cerebral Spinal Fluid

The final biological matrix in which ASO f_u was characterized was monkey CSF. As expected, CSF f_u was significantly higher in comparison to other biological matrices due to its relatively low protein composition. Initially, protein binding experiments were carried out in CSF and protein-free artificial CSF (ACSF). Figure 4A showed ASO-1 f_u in CSF and ACSF in different filter sizes. ACSF protein binding results were similar to recovery experiments, since both ACSF and PBS are protein-free. Differences between 30K and 50K filters in CSF were proportional to

differences between these sizes in ACSF. Furthermore, 30K CSF f_u values can be corrected in accordance to differences in recovery between 30K and 50K filters in ACSF. The f_u corrected is similar to f_u in 50K filters (Supplemental Figure 2), suggesting that protein bleeding is less concerning in CSF, likely due to its relative low protein composition. Overall, these results suggested that 50K filters were the appropriate size for CSF f_u quantification without the need for recovery normalization. CSF f_u for different ASOs was quantified in 50K MWCO filters (Figure 4B). Co-spiked warfarin and antipyrine CSF and ACSF f_u is available in supplemental figure 3. Similar to warfarin f_u in plasma and brain homogenate, there is an upward trend in CSF f_u as the filter MWCO increases, which can be attributed to protein bleeding. However, warfarin CSF f_u measurements were not significantly different between 30K and 50K MWCO filters, due to the low concentrations of CSF proteins within this narrow MW range, and therefore limited impact from protein bleeding. For antipyrine, due to its highly unbound nature, filter size had no impact on CSF f_u measurements.

Equilibrium Dialysis

Rapid equilibrium dialysis (RED) is a popular technique for high throughput protein binding assays and often referred to as the "gold standard". (Trainor, 2007) As previously shown, filter MWCO plays a key role in ASO recovery. The first step in RED was to confirm if ASO concentrations both in the sample compartment and solvent compartment reached equilibrium in a protein free medium. Despite different membrane pre-treatments and prolonged incubations, system equilibrium was never reached with the biggest available MWCO at 12K, suggesting ASOs were not physically able to cross the membrane limited by the pore size. Results were also comparable to poor ASO permeability of similar MWCO at 10K demonstrated in the

ultrafiltration section. Both small molecule controls (warfarin and antipyrine) reached equilibrium under the assessed experimental conditions (Figure 5), which not only orthogonally confirmed the validity of the experiment, but also pointed the loss of ASO to its higher MW and unique NSB.

Ultracentrifugation

In addition to ultrafiltration and RED, an ultracentrifugation method was explored and its compatibility with ASOs was assessed. Polycarbonate tubes were filled with either 1 ml of rat plasma or PBS, co-spiked with 1 μ M ASO-1 and 1 μ M warfarin. Spiked samples were centrifuged at $400,000 \times g$ for 1, 2, 3 and 4 hours. Upon collection of each time point, 1 ml sample was divided into 4 vertical quadrants of equal volumes (250 μ l each). With the top layer (first 250 μ l) being "Q1", second 250 μ l being "Q2" and so on. ASO-1 and warfarin concentrations were quantified for every quadrant, and their recoveries were calculated against pre-centrifuge samples and shown in Table 3. Protein concentration for different quadrants is shown in Supplemental Table 1.

Similar to ultrafiltration, analyte recovery was first assessed in PBS and confirmed that there was no significant loss of ASO-1 or warfarin to the experimental system without the impact of any protein binding. It was noticed that ASO-1 quickly formed a concentration gradient 74.3 - 153.5% from Q1 to Q4 at 1 hour and maintained the similar gradient to the end, which was not observed in warfarin. Warfarin initially formed a concentration gradient in PBS (potentially due to poor solubility and insufficient mixing), but equilibrium between the quadrants was observed in hours 3 and 4 as expected for a small molecule. The concentration gradient observed for ASO suggested that the relatively higher MW of ASO compared to small molecules

led to slight precipitation of unbound ASO at such strong ultracentrifugation conditions, though approximately 80% recovery might still be considered acceptable.

In plasma samples, significant vertical gradients were observed for both ASO-1 and warfarin, due to the precipitation of protein bound analytes as expected. After hours, the solution system started to reach equilibrium, in which Q1 was considered protein free as confirmed by total protein quantitated at approximately 0.4% of original. Therefore, ASO-1 rat plasma f_u was determined to be 2.0% based on the Q1 concentration at 4 hours, which was 2-fold higher than the f_u determined through ultrafiltration (0.89%). Warfarin plasma f_u was measured at 0.6%, lower in comparison to ultrafiltration experiments (1%) and literature (1.1%). Such different directions of bias between ASO and warfarin made it difficult to interpret the results from a systematic perspective. Considering a few facts that 1) the experiment was not strictly validated by co-spiked warfarin, 2) unbound ASO also forms a concentration gradient under high centrifugal forces, 3) measured ASO f_u was higher than references, and 4) the procedure suffered from low throughput and therefore limited replicates ($n = 1$), the approach of ultracentrifugation was deemed impractical for ASO f_u measurement.

Discussion

The current study assessed ultrafiltration, ultracentrifugation and equilibrium dialysis to measure ASO f_u in plasma, brain homogenate, and CSF, while successfully implementing LC-MS/MS as a sensitive and direct quantitation method. This is the first time LC-MS/MS has been employed for quantification of ASO in vitro protein binding and the first time ultracentrifugation and equilibrium dialysis are evaluated for such application.

Hybridization LC-MS/MS integration. Both the hybridization extraction and LC-MS/MS quantification offer major advantages over previously published methods (Watanabe et al., 2006). On the detection side, MS provides high specificity and simple signal standardization through the implementation of an internal standard. However, one of the typical drawbacks when coupling MS to the analysis of biological samples is matrix effect, which is defined as the variations in the analyte signal caused by matrix. To minimize the impact of matrix effects, calibration curves are usually prepared in the same matrix as the study samples, which becomes an inefficient process as the number of matrix components grow. In the case of protein binding experiments, matrix interference becomes problematic when measuring separated unbound ASO and total ASO existing in matrices of different compositions (one protein free and the other protein rich), as none of previously available analytical methods was able to completely avoid matrix effects. The adopted hybridization protocol overcomes matrix effect limitations by capturing the desired ASO from the biological matrix, washing residual matrix through several washes, and finally eluting the analyte in neat solution containing internal standard. Matrix removal allows for cross-matrix quantification, therefore increasing sample preparation efficiency (Li et al., 2020). Due to the mechanism of hybridization and the need of a specific capture probe, the ultrafiltration-based hybridization LC-MS/MS method is suitable for accurate protein binding assessments of identified lead ASOs, instead of screening large numbers of ASOs as a high-throughput method. Multiple ASOs can be analyzed in a batched manner as long as separate capture probes are used. The hybridization LC-MS/MS platform is robust, and compatible with ultrafiltration, RED and ultracentrifugation experiments.

Ultracentrifugation limitations. In theory, ultracentrifugation was a promising technique in ASO f_u quantification because it does not require a filter membrane, therefore avoiding ASO NSB to filter apparatus, ASO poor membrane permeability, and protein bleeding. Recently, low recovery, likely due to sedimentation, was reported in ultracentrifugation of siRNA (Humphreys et al., 2019). However, throughout the ultracentrifugation experiments we did not observe the same recovery loss, perhaps due to the lower molecular weight of ASOs (approximately 7 kDa) in comparison to siRNA (approximately 14 kDa). Even though appropriate recovery was observed in the final (4 hour) time point (82.5%), variance from the recoveries in previous time points suggests sampling challenges. The special division of four quadrants was arbitrary and practically difficult by manual pipetting. Another major limitation with ultracentrifugation is sample throughput, as high-speed rotors have limited capacity. In this study, only 10 samples were able to be analyzed per run, largely limiting the number of replicates to reliably make measurements. Additionally, ultracentrifuges able to handle speeds over $400,000 \times g$ for multiple hours are not widely available. Because of lower sample throughput, and lower reproducibility, ultracentrifugation was a less ideal choice compared to ultrafiltration.

Ultrafiltration filter considerations. Due to their linear structure, ASOs require MWCO filters that are substantially higher than the ASO's MW. Humphreys et al. suggested through a siRNA crystal structure that hydrodynamic radius must be taken into consideration when choosing MWCO filter sizes for molecules of linear conformations (Humphreys et al., 2019). In their study, a 50K MWCO filter had to be used over 30K MWCO filters in order to achieve appropriate recovery for a GalNAc-conjugated siRNA. Watanabe et. al. reports over 85% recovery when using 30K MWCO filters treated with 5 μ M irrelevant ASO (not radiolabeled) for

the analysis of a 20mer phosphonothioate ASO (Watanabe et al., 2006). These results suggest that double stranded conformations are more rigid in comparison to single stranded conformations, and therefore smaller MWCO filters (30K) are compatible with ASOs. When choosing between different MWCO sizes, the main consideration becomes ASO bound to small proteins crossing the filter membrane.

In this study, f_u was determined by Eq2 but in reality, protein bound ASOs that cross high MWCO membranes must be taken into consideration. A more realistically representation would be:

$$f_u = \frac{[receiver]}{[donor]} = \frac{[ASO\ unbound\ in\ ultrafiltrate] + [ASO\ protein\ bound\ in\ ultrafiltrate]}{[donor]} \quad Eq. 4$$

The choice of filter size should be the size at which [ASO protein bound in ultrafiltrate] is the least, while [ASO unbound in ultrafiltrate] is recovered as much as possible. For these reasons, 30K filters were chosen in the analysis of protein rich matrices (brain homogenate and plasma).

Interestingly, our results show that filter size is also matrix dependent. For protein rich matrixes such as brain homogenate and plasma, significant differences between 30K and 50K filters were observed, but in CSF (low protein concentration), differences between 30K and 50K filters were proportional to their differences in recovery. With this in mind, the filter size that was used for f_u determination in CSF was 50K MWCO, while 30K might also be considered when corrected by recovery.

Throughout the ultrafiltration experiments we noticed occasional outliers likely resulting from defective filters or tube-to-tube variations from the manufacture. All the observed outliers were substantially higher than the average for both ASOs and small molecules. Outliers were

removed using Pierce's criterion, which was selected as a pre-determined elimination criterion (Ross, 2003). A potential limitation from ultrafiltration experiments is that even when using 30K MWCO filters, it is possible that ASO bound to smaller proteins or biomolecules may pass through the filter and end up in the ultrafiltrate, which is, however, an inevitable scenario for any protein binding assay based on the molecular weight difference between bound and unbound ASO.

Brain homogenate and CSF f_u . Development of quantitative models for prediction of drug absorption, distribution, and excretion has transformed early drug discovery. In vivo measurements in many instances are possible, but it requires expensive and complex techniques with low sample throughput, which limits its application in early drug discovery and highlights the importance of in vitro data based prediction models (Trainor, 2007). Total drug and unbound concentrations in various matrixes, including plasma and brain provide valuable information about drug pharmacokinetics.

The ratio of total drug concentration in the tissue to total concentration of drug in the plasma at steady state (K_p), may be used to predict the extent of tissue distribution (Jones and Rowland-Yeo, 2013). K_p has been previously measured in vivo, however tissue distribution predictive models based on physicochemical and *in vitro* binding characteristics can effectively replace in vivo models in early-stage drug discovery (Poulin and Theil, 2002; Rodgers and Rowland, 2007; Poulin, 2015). In the CNS, K_p (brain, plasma) does not always correlate to predicted efficacy. Discrepancies are likely due to drug nonspecific binding to proteins in the brain. Brain f_u quantification is paramount and must be used as a correction factor for total brain concentration, therefore improving mechanistic PK/PD evaluations (Read and Braggio, 2010). In vivo validation of brain f_u can be difficult to measure, although drug concentration in the CSF

has been used as a surrogate method for quantifying brain f_u (Liu et al., 2006; Lin, 2008). While differences between small molecules and ASOs (high molecular weight and polyanionic character) must be taken into consideration, ASO in-vitro protein binding may provide important information on drug potency, distribution, and clearance.

Overall, the ability to modulate protein expression makes ASOs a promising therapeutic modality for the treatment of neurodegenerative diseases as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) have been linked to toxic protein accumulation (Bossy-Wetzel et al., 2004; Smith et al., 2006; DeVos and Miller, 2013). Due to relatively large molecular size and negative charge, ASOs do not efficiently cross the blood brain barrier, but regardless of this limitation, and through novel delivery methods, ASOs continue to undergo clinical trials for CNS related diseases (Hammarlund-Udenaes et al., 2008; Bennett et al., 2019; Tabrizi et al., 2019; Leavitt Blair and Tabrizi Sarah, 2020). With the exciting progress in ASO treatment of neurological diseases, this study provides valuable insight into ASO pharmacokinetics in the brain and CSF and can be an invaluable tool in the support and characterization of in vivo PK-PD data.

Authorship Contributions

Participated in research design: Guimaraes, Yuan, Li.

Conducted experiments: Guimaraes, Li

Performed data analysis: Guimaraes, Li

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Footnotes:

This work received no external funding. No author has an actual or perceived conflict of interest with the contents of this article.

Figure Legends

Figure 1. A) Recovery of 1 μ M ASO-1 in PBS through different filter pre-treatments. B) Recovery of 1 μ M ASO-1 in different MWCO filter sizes pre-treated with 0.1% Tween-20 (PBST). C) Recovery of 1 μ M warfarin and antipyrine in different MWCO filter sizes pre-treated with 0.1% Tween-20 (PBST).

Figure 2. A) ASO-1 plasma *fu* in different MWCO filter sizes. B) ASO-1, ASO-2 and ASO-3 plasma *fu* in 30K MWCO filters.

Figure 3. ASO brain *fu* across different filter sizes.

Figure 4. A) ASO-1 drug unbound fraction in CSF and artificial CSF. B) ASO CSF unbound fraction in 50K MWCO filters.

Figure 5. Rapid equilibrium dialysis (RED) system equilibrium through different membrane pre-treatments.

Tables

Table 1. ASO Analytes and Internal Standard

Name	MW (kDa)	Sequence length	Chemistry*	Use
ASO-1	7.1	20	5–10–5 MOE-gapmer	analyte
ASO-2	7.1	20	5–10–5 MOE-gapmer	analyte
ASO-3	5.9	17	MOE/cEt-gapmer	analyte
ASO-4	7.9	20	uniform MOE with PS backbone	internal standard
* MOE, 2'-O-(2-methoxyethyl)-oligoribonucleotides; cEt, constrained ethyl motif; PS, phosphorothioate linkage.				

Table 2. Orthogonal validation of 30 kDa MWCO ultrafiltration method through previously characterized small molecules.

	Experimental Values (%)	Experimental Generated Using a Standard RED Protocol (%)	Values Literature Values (%)
Plasma			
<i>Warfarin</i>			
Mouse	5.5 ± 0.3	4.3 ± 0.9	4.6 ± 0.4 ^a
Rat	1.0 ± 0.5	0.8 ± 0.4	1.1 ^b
Dog	3.2 ± 0.1	3.9 ± 0.7	4.0 ± 0.1 ^a
Human	1.1 ± 0.5	1.5 ± 0.3	1.0 ^c
Monkey	0.8 ± 0.2	1.0 ± 0.7	0.7 ± 0.1 ^a
<i>Antipyrine</i>			
Human	92.8 ± 2.2		90 ^d
Brain			
<i>Verapamil</i>			
Rat	2.8 ± 0.3	3.8 ± 1.4	3.3 ± 2.0 ^e

a (Isbell et al., 2019) b (Hirate et al., 1990) c (Zhang et al., 2012) d (Humphreys et al., 2019) e (Hirate et al., 1990)

Table 3: Ultracentrifugation plasma f_u and recovery in PBS values for ASO-1 and warfarin (n = 1)

Plasma f_u (%)								
	ASO-1				Warfarin			
	1 hour	2 hours	3 hours	4 hours	1 hour	2 hours	3 hours	4 hours
Q1	14.3	3.4	2.6	2.0	15.7	0.5	0.6	0.6
Q2	63.0	10.7	3.8	2.9	90.8	6.2	0.7	0.5
Q3	122.7	160.7	55.4	58.6	150.0	195.7	71.1	82.2
Q4	204.8	244.5	NA	NA	169.2	184.4	NA	NA
Total Recovery	101.2	104.8	NA	NA	106.4	96.7	NA	NA

Recovery in PBS (%)								
	ASO-1				Warfarin			
	1 hour	2 hours	3 hours	4 hours	1 hour	2 hours	3 hours	4 hours
Q1	74.3	75.2	78.5	82.5	62.5	73.0	109.1	106.1
Q2	97.9	77.7	79.6	80.1	77.8	72.4	107.5	104.0
Q3	87.7	85.9	84.1	101.2	79.0	76.1	111.9	107.8
Q4	153.5	152.0	110.9	129.5	210.2	216.8	107.5	119.5
Total Recovery	103.4	97.7	88.3	98.3	107.4	109.6	109.0	109.4

NA: recovery was unmeasurable due to solidified Q4 matrix after ultracentrifugation

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5