Algorithme Pharma, 575 Armand-Frappier, Laval (Montréal), Québec, Canada
algopharm.com | contact@algopharm.com

An Ultrafiltration Assay for Assessing the Extent of Whole Plasma Protein Binding of Antisense Oligonucleotides in Human and Various Species Plasma

M. Clement [1], T. Vu [1], B. Tayefeh [2], S. Greenlee [2], R. Yu [2], T. Watanabe [2], D. Norris [2] and R. Masse [3]

OVERVIEW

This poster highlights the development of an ultrafiltration method, combined with a hybridization ELISA detection, to evaluate *in vitro* whole plasma protein binding of anti-sense oligonucleotides (ASOs). The method required:

- High levels of ASO recovery in water at 250 ng/mL (low non-specific binding)
- Low %CV between filter replicates
- Demonstration of similar whole plasma protein binding as observed with different ultrafiltration (UF) devices (i.e. no drug or matrix specific differences)

INTRODUCTION

ASOs are hydrophilic, poly-anionic, ~6-8 kDa molecules that are highly plasma protein bound (typically > 90%). ASOs bind to hydrophilic sites on plasma proteins, but do not displace small molecules, or vice versa. They display low affinity, non-specific, hydrophilic interactions with plasma proteins.

This work compares the performance between the below listed devices:

- Ultra-free-MC© ultrafiltration units (30K MWCO), low-binding regenerated cellulose membrane from Millipore
- Nanosep© centrifugal devices (30K MWCO; OMEGA membrane), low protein-binding, modified polyethersulfone on polyethylene substrate, from Pall Life Sciences

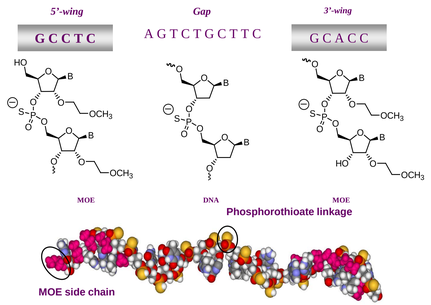

Figure 1. Structure of Representative 2nd Generation ASO Drugs

As shown above, these molecules encompass:

- Gapmers which are chimeric ASOs that contain a central block of deoxynucleotide monomers sufficiently long to induce RNase H cleavage
- MOE modification at ends

METHODS

SELECTION OF AN APPROPRIATE ULTRAFILTRATION FILTER

Several ultrafiltration filters were assessed; the Nanosep© 30K Omega filter from Pall Life Sciences was selected for the following reasons:

- Low non specific binding
- Low sample volume requirement, maximum 500 µL, compatible to our requirement

Millipore Ultra-free-MC©
0.5 mL ultrafiltration units

Nanosep© 0.5 mL ultrafiltration units
Pall Life Sciences

EXPERIMENTAL CONDITIONS TESTED

Filters were pre-conditioned with Tween 80, and then with a non-specific ISIS ASO-1

1. Filter pre-treatment 1 : 0.05%, 0.1%, 0.2%, 0.5%, 1%, 2% and 5% Tween 80
2. Filter pre-treatment 2: 2x with 200 µg/mL or 1x with 300 µg/mL of ISIS ASO-1
3. Spinning at: 3500 xg, 5000 xg, 7500 xg, 9000 xg, 12000 xg, 13000 xg and 14000 xg
4. Wash step (with or without) after pre-treatment with Tween 80
5. Wash step (with or without) after pre-treatment with ISIS ASO-1

Although filters are relatively inert, pre-treatment with Tween 80 and with ASO-1 (an oligonucleotide of a different sequence) was required to minimize non-specific binding (NSB) of the specific ASO ISIS-501861 to the filter.

Once optimized, the ultrafiltration method was combined with a nuclease-dependent hybridization ELISA detection method (range 1.0 to 100.0 ng/mL) to evaluate *in vitro* plasma protein binding of ISIS 501861, a 20-mer antisense phosphorothioate oligonucleotide with 2'methoxyethyl (MOE) modifications at the 3' and 5' wings. Protein binding was determined in human, mouse and monkey plasma.

RESULTS and DISCUSSION

OBSERVATIONS

- Filter pretreatment 1: 0.5% to 2.0% Tween 80 had the same effect on blocking non-specific binding sites
- Filter pretreatment 2: both ISIS-ASO-1 pre-treatment concentrations tested provided similar results
- Centrifugation speed: recovery was consistent at 12,000 x g. At 14,000 xg, the recovery was high (90 to 100%), however, the %bound was low (10 to 50%), indicating the protein-bound portion also passed through the filter
- Wash step after Tween 80 treatment helped to significantly improve the recovery
- Wash step after ISIS ASO-1 pre-treatment had no positive impact and was removed

KEY EXPERIMENTAL FACTORS

- Choice of filter design: a flat-bottom filter (not vertical). During centrifugation, the sample volume is gradually reduced resulting in a smaller portion of the filter membrane remaining in contact with the pre-treatment solution. As a result, the filter is not evenly blocked.
- Inclusion of a wash step after Tween 80 treatment. It is hypothesized, the wash step helped the removal of Tween 80 from the filter membrane to assure that ISIS ASO-1 passed through the membrane in the subsequent non-specific binding step.
- Selection of the optimal centrifugation speed was also an important factor. At a lower spin (i.e. 3500 xg to 7500 xg), ISIS ASO-1 did not pass through the filter. Whereas at a higher spin (i.e. 14000 xg), the protein bound portion of ASO was also collected in the filtrate. A 12000 xg spin was found to be optimal for maximizing separation of the free ASO from protein-bound ASO complex.

Final and optimal experimental conditions are summarized below:

1. Pre-treatment 1 with Tween 80

- Add 300µL 0.05% Tween 80
- Incubate at room temperature (rt) for 10-15 min
- Centrifuge at 12,000 rpm, 10 min, rt
- Wash membrane with water (300µL x 2)

2. Pre-treatment 2 with ISIS ASO-1

- Add 300 µL of 300 µg ISIS ASO-1
- Incubate at rt for 10-15 min
- Centrifuge at 12,000 rpm, 10 min, rt

3. Plasma sample ultrafiltration

- Add 300 µL of recovery sample (250 ng/mL of specific ASO in water) or plasma protein binding samples (pre-incubated at 37º C for at least 30 minutes to allow the ASO to bind to plasma protein) to the pre-treated filter, in three replicates. Incubate at rt for 10-15 min
- Centrifuge at 12,000 rpm, 7 min, rt
- Collect about 300 µL of the recovery filtrate
- Collect about 40 – 60 µL of the protein binding sample filtrate.

4. Nuclease-dependent Hybridization ELISA

Typical hybridization ELISA data from back-calculated calibrations standards prepared in human plasma is presented in Table 1.

	Concentration (ng/mL)								
STD	**0.50**	**1.00**	**2.00**	**5.00**	**10.0**	**25.0**	**50.0**	**80.0**	**100.0**
Acceptance range	**NA**	**(0.75 -1.25)**	**(1.60 - 2.40)**	**(4.00 - 6.00)**	**(8.00 - 12.0)**	**(20.0 - 30.0)**	**(40.0 - 60.0)**	**(64.0 - 96.0)**	**(80.0 - 120)**
Measured	**0.49**	**1.05**	**2.00**	**4.83**	**10.0**	**25.4**	**50.8**	**77.6**	**101.3**
(%) Nominal	**97.2**	**104.9**	**100**	**96.6**	**100.4**	**101.5**	**101.7**	**97.1**	**101.3**

Table 1: Back-calculated calibrations standards in human plasma

The extent of whole plasma protein binding of ISIS 501861 at two different concentrations (5 and 150 µg/mL) in plasma from three different species (human, cynomolgus monkey, and mouse) was evaluated. Data is summarized in Table 2.

The lowest evaluated plasma concentration tested is expected to be near the anticipated clinical therapeutic dose peak plasma exposure levels. In addition, the highest evaluated plasma concentration tested exceeded peak plasma exposure levels observed in monkey toxicology studies.

Species	Nominal Total Plasma Conc. of ISIS 501861 (µg/mL)	Measured Initial Total Plasma Conc. (ng/mL)	Measured Ultrafiltrate Conc. (ng/mL)	% Free	% Free, Adjusted for Recovery[a]	% Free, Mean	% Bound, Mean (SD)
Human	5	5789.8	40.294	0.70	0.75	0.84	99.16 (0.09)
			46.126	0.80	0.86		
			49.771	0.86	0.92		
	150	183351.9	2758.150	1.50	1.62	1.75	98.25 (0.16)
			2914.889	1.59	1.71		
			3295.372	1.80	1.93		
Monkey	5	5945.6	17.672	0.30	0.32	0.35	99.65 (0.05)
			22.576	0.38	0.41		
			17.723	0.30	0.32		
	150	182418.6	886.522	0.49	0.52	0.51	99.49 (0.04)
			923.763	0.51	0.54		
			791.671	0.43	0.47		
Mouse	5	7497.7	41.869	0.56	0.60	0.65	99.35 (0.04)
			45.511	0.61	0.65		
			47.549	0.63	0.68		
	150	205407.5	2367.786	1.15	1.24	1.19	98.81 (0.11)
			2033.108	0.99	1.06		
			2428.062	1.18	1.27		

Comparison of the performance of Millipore and Pall Life Sciences Nanosep© UF devices is summarized in Figure 2. Data shows that both UF devices provided very similar % protein-binding results and differences in % protein binding are within the intrinsic variability of the ELISA assay.

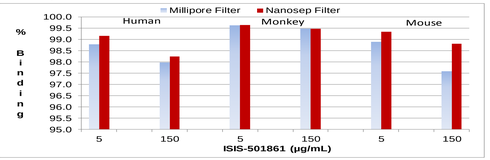

Figure 2. Comparative % protein binding of ISIS 501861 on Millipore and Nanosep © UF devices

CONCLUSION

A robust and reliable ultrafiltration method was developed to determine the extent of the protein binding of antisense oligonucleotides in human, cynomolgus monkey and mouse plasma. Details of the experimental approach used were presented along with key indicators that were found to be critical to the successful development of this ultrafiltration methodology.

Protein binding data reported herein are consistent with data previously reported for other ASO products. The reliability of this ultrafiltration method makes it a generic approach for the assessment of a wide variety of antisense oligonucleotides in biological matrix, such as plasma.

[1]Algorithme Pharma Inc., Laval (Montreal), QC, Canada
[2] Ionis Pharmaceuticals, Carlsbad, CA, USA
[3] Independent consultant