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Development of a Surrogate Cerebrospinal Fluid Matrix for Quantitative Analysis of Antisense Oligonucleotides by Hybridization LC-MS/MS

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OVERVIEW

PURPOSE

To identify a suitable surrogate matrix for antisense oligonucleotide (ASO) quantitation in NHP CSF that overcomes both matrix shortage and non-specific binding issues.

METHODS

Different combinations of artificial CSF supplemented with surfactant, bovine serum albumin, and NHP serum were tested to establish a suitable proxy matrix. Samples were extracted using a novel affinity capture approach with complementary sense probes immobilized on streptavidin beads. Analytes and IS were separated using ion-paired reverse-phase chromatography, and injected on a SCIEX API 6500+ in ESI(-) mode.

RESULTS

Artificial and NHP CSF treated with 1% Tween 80 and 5% BSA w/v (f.c.) was successfully established as a surrogate approach to mitigate non-specific binding (NSB) and freeze-thaw stability issues for three candidate ASO drugs.

INTRODUCTION

ASOs are short, single-stranded synthetic oligonucleotides that can regulate gene expression by binding to mRNA (**Figure 1**). Quantitative bioanalysis in various biological matrices is critical to understanding the PK/PD parameters of ASO drug candidates for neurological and neuromuscular indications. Challenges inherent in method transfer from serum to cerebrospinal fluid (CSF) include susceptibility to NSB due to low protein background and more recently, the shortage of NHP matrices within the context of global supply chain issues caused by the COVID-19 pandemic. These important disruptions have renewed the need to advance the 3R (replacement, reduction, refinement) approach to minimize the use of animals without compromising scientific integrity. In the current study, the use of a surrogate matrix was explored to address these issues.

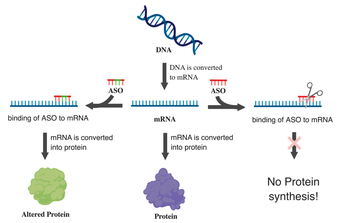

Figure 1. ASO mechanism of action (*Image by Dr. Larissa Nitschke*)

METHODS

HYBRIDIZATION LC-MS/MS

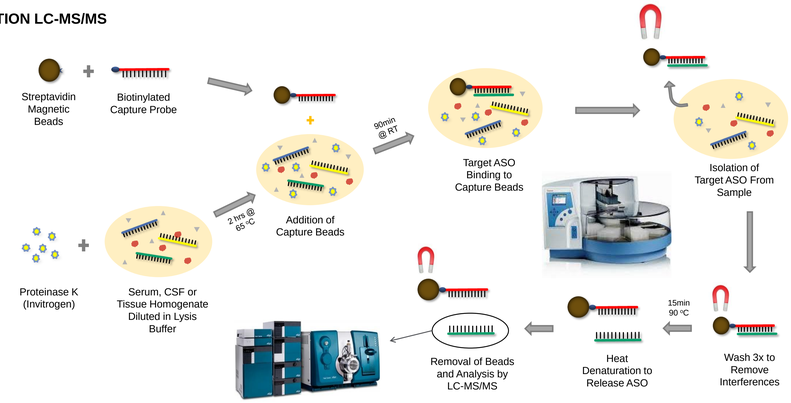

Figure 2. Hybridization LC-MS/MS workflow for the quantitation of ASOs in biological matrices

SAMPLE EXTRACTION

- Biotinylated full complementary capture probe
- Digestion with Proteinase K prior to hybridization step
- Automated magnetic bead processing (KingFisher Flex)
- Release of ASO using heat denaturation (90 °C)
- ASO concentration range from 0.5 to 500 ng/mL
- Internal standard added at end of extraction

Table 1. Summary of ASO characteristics

Oligo	MW (kDa)	Sequence length	Chemistry	MRM
ASO1	7.0	20	5-10-5 MOE gapmer	638/95
ASO2	7.1	20	5-10-5 MOE gapmer	709/95
ASO3	7.1	20	5-10-5 MOE gapmer	788/95
ASO-IS	7.9	20	MOE with PS backbone	879/95

LC-MS/MS CONDITIONS

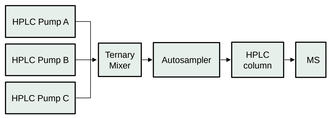

Figure 3. Schematic diagram of UHPLC setup

- Reversed-phase ion-pair chromatography (RP-IPC)
- Ternary flow for robustness
- Column: Waters ACQUITY UPLC Oligonucleotide BEH C18, 50 x 2.1 mm, 1.7 µm
- Mobile Phase:
  - MPA: H_2O
  - MPB: ACN:H_2O 90:10% v/v
  - MPC: 125mM HFMIP + 75 mM DMCHA in ACN:H_2O 50:50% v/v
- SCIEX 6500+ ESI in negative mode
- Multiple reaction monitoring (MRM)

RESULTS

For the identification of a suitable proxy CSF matrix, the use of commercial artificial CSF (buffer + salts) alone showed a negative bias compared to samples extracted in serum (**Figure 4**). To mitigate NSB, aCSF composition was modified with the addition of surfactant (Tween 80) and/or bovine serum albumin (BSA) at concentrations equivalent to normal protein levels in CSF.

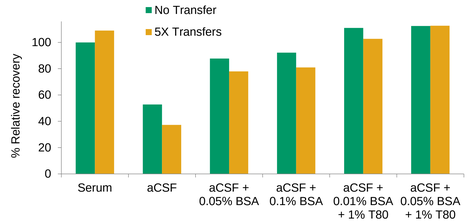

Figure 4. Relative recovery of ASO1 in different aCSF proxy solutions following 5x transfer test in LoBind tubes at low QC level (1.50 ng/mL)

ASO1 prepared in NHP CSF supplemented with 1% Tween 80 and 0.05% BSA was subjected to one freeze-thaw (F/T) cycle, and compared to freshly prepared samples in treated aCSF (**Figure 5**). A negative bias was observed in NHP CSF with an additional decrease in recovery following one freeze-thaw cycle.

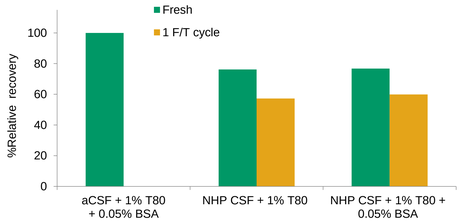

Figure 5. Freeze-thaw stability of ASO1 in treated NHP CSF at low QC level (1.50 ng/mL)

To mitigate sample loss following freeze-thaw cycles, NHP CSF samples were treated with a higher protein content (1-4% BSA or 10-50% NHP serum) prior to freezing (**Figure 6**). Recovery of ASO1 in 4% BSA or 10% NHP serum (f.c.) after two F/T cycles was identical to fresh samples prepared in proxy CSF solution.

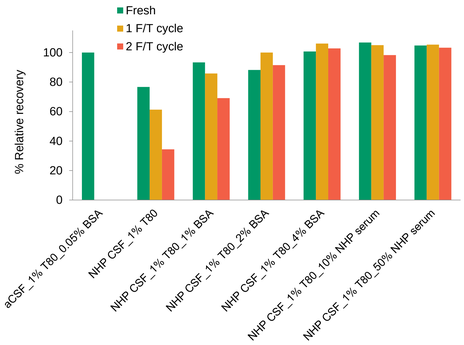

Figure 6. Freeze-thaw stability of ASO1 in treated NHP CSF at low QC level

A mix of 10% BSA + 2% Tween 80 was used as anti-adsorptive solution to treat (1:1) aCSF and NHP CSF samples. Calibration curve and QC samples were prepared in treated aCSF, and matrix QCs were prepared in treated NHP CSF. This surrogate approach was evaluated successfully with three different candidate ASOs (**Table 2**).

Sample ID	5X Transfer Test		Stability (2 F/T cycles)	
	Treated aCSF (Proxy)	Treated NHP CSF	Treated aCSF (Proxy)	Treated NHP CSF
ASO1	98.2	97.8	99.8	86.1
ASO2	95.1	101.6	102.7	95.1
ASO3	105.3	99.8	106.5	107.2

Table 2. Freeze-thaw stability and 5x transfer test of ASO1, ASO2, and ASO3 in treated aCSF (proxy) and treated NHP CSF at low QC level (1.50 ng/mL)

CONCLUSION

A surrogate CSF matrix for the quantitation of ASOs in NHP was established that overcomes NHP matrix procurement challenges and non-specific binding issues inherent in the analysis of oligonucleotides. The proxy CSF assay from 0.5 to 500 ng/mL was validated using hybridization LC-MS/MS, and successfully applied to a monkey toxicology study.

REFERENCES

Li, P. Validation and application of hybridization liquid chromatography-tandem mass spectrometry methods for quantitative bioanalysis of antisense oligonucleotides *Bioanalysis* 2022

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