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Sensitive Quantitation of Antisense Oligonucleotides Using Capitainer® qDBS Microsampling Device Coupled With Hybridization LC-MS/MS

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OVERVIEW

Purpose

To sensitively determine antisense oligonucleotides (ASOs) with Capitainer® quantitative dried blood spot (qDBS) microsampling technique coupled with hybridization LC-MS/MS.

Method

Blood samples were fortified with Fomivirsen (FME) and its 5' n-1 metabolite. These samples were then absorbed onto the Capitainer qDBS device and dried with desiccant for at least 24 hours. The desorption of dried blood samples was performed by impact-assisted extraction (IAE) in the presence of detergent, followed by hybridization extraction using magnetic beads conjugated to the capture probe. The resulting extract was separated using ion-pairing reversed-phase liquid chromatography (IP-RPLC). A post-injection divert valve was used to mitigate compromised peak sharpness and reduced sensitivity at a low flow rate of 0.3 mL/min.

Results

A combination of buffers with detergents was used to introduce additional desorption solution volume, achieving an ASO recovery of over 80%. The method enabled a lower limit of quantification (LLOQ) of 0.50 ng/mL for dried blood samples from a 10 µL qDBS device. The chromatographic resolution allowed simultaneous quantitation of the parent ASO and its 5' n-1 metabolite. Precision and accuracy assessments met the acceptance criteria across the analytical range (0.50 – 500 ng/mL). Specificity and absence of matrix effect were confirmed using low and high-quality control (QC) samples from multiple donors, while blood hematocrit (HCT) level did not impact ASO quantitation.

INTRODUCTION

ASOs are short synthetic oligodeoxynucleotides (15-25 nucleotides) that specifically bind to mRNA, blocking gene expression and reducing disease-related protein synthesis. Their promising therapeutic potential has generated a strong demand for sensitive and selective analytical methods, particularly when using microsampling techniques for low-volume blood collection.

Aligned with recent initiatives for patient-centric healthcare and easing recruitment efforts for vulnerable patient populations and pediatrics in decentralized clinical trials, Capitainer AB recently introduced the qDBS microsampling device, designed to minimize discrepancies associated with conventional DBS approaches by allowing volumetrically accurate and precise capillary blood collection independent of HCT. However, the inherent limitation in low microsampling blood volume (e.g., 10 µL) can still lead to compromised LLOQ, particularly for ASOs whose total ion current is disseminated amongst multiple charge states under electrospray ionization conditions.

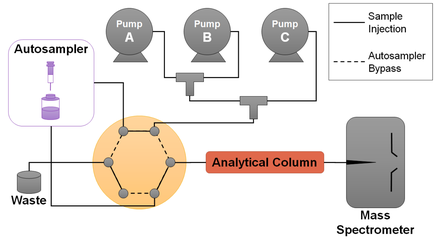
Recently, hybridization extraction coupled with LC-MS has emerged as a novel oligonucleotide quantitation strategy applied to several matrices, including plasma, serum, cerebrospinal fluid, and many tissue types. However, to date, there have been no reports of ASO quantitation from dried blood microsamples, in part due to the complexity of the extracts, which confound traditional sample preparation approaches. Therefore, by using capture probes with full-length complementary strands within a hybridization workflow to enhance bioanalytical methods' sensitivity and selectivity, the current research details the successful extraction and quantification of FME and its 5' n-1 metabolite from dried blood microsamples, which were collected using the Capitainer qDBS device.

METHODS

Sample Preparation

Human blood samples fortified with FME and 5' n-1 metabolite were absorbed onto the 10 µL Ahlstrom 222 cellulose substrate of the Capitainer qDBS device and allowed to air-dry in the presence of desiccant for a minimum of 24 hours.

Dried blood qDBS microsamples were carefully detached and placed in a 96-well plate containing 5/32" stainless steel grinding beads, followed by adding the n+2 analog of FME as internal standard (IS). The microsamples underwent desorption in the presence of detergent through IAE (1750 rpm x 5 min x 2 times). During the subsequent hybridization purification step, functionalized magnetic beads were employed to form an ASO-probe-beads complex. After multiple wash steps, the targeted ASO was released via thermal denaturation. The resulting extracts were then stored at 4ºC for LC-MS/MS analysis.



Scheme 1. The LC-MS system setup to bypass the autosampler during sample analysis

LC-MS/MS

Chromatographic separation was performed using a Shimadzu LC-30AD NexeraX2 UPLC system, including three isocratic pumps and one 6-port valve (**Scheme 1**). Conditions were as follows:

- **Analytical column:** Acquity Premier BEH C18 column, 50 x 2.1mm, 1.7 µm
- **Mobile phase A/B for separation:** dibutylamine (DBA), dimethylcyclohexylamine (DMCHA), hexafluoro-2-methyl-2-propanol (HFMIP) in H_2O/ACN/MeOH
- **Mobile phase C for column flush:** H_2O/ACN
- **Flow rate:** 0.30 mL/min
- **Column temperature:** 60 ºC
- **MS:** SCIEX 6500+ QQQ operated in negative electrospray ionization/multiple reaction monitoring (ESI/MRM) mode (**Table 1**)

Table 1. MRM Parameters of FME, Metabolites and Analogs

Analyte	Charges	Q1	Q3
FME	9-	741.5	95.0
N-2 Metabolite	8-	753.0	
N-1 Metabolite	8-	791.1	
N+1 Analog	9-	779.8	
N+2 Analog (IS)	9-	815.4	

RESULTS AND DISCUSSION

Optimization of LC-MS/MS

Typically, the lower LC flow rates result in the generation of smaller droplets in ESI, yielding a greater surface-area-to-volume ratio. This augmented surface area delivers higher ionization efficiency, particularly for ASOs, since they are detected in negative ESI mode. Nonetheless, a low flow rate (≤ 0.3 mL/min) may exacerbate the impact of pre-column void volume, leading to consequential drawbacks, such as broadened peak width, deteriorated separation resolution, and compromised sample throughput. To address this impact, the integration of a post-injection divert valve emerges as a strategic measure (**Scheme 1**).

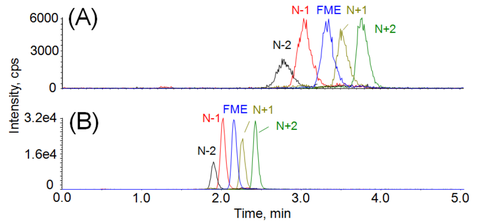


Figure 1. Representative chromatograms of FME, metabolites and analogs without (A) and with (B) post-injection divert valve at a flow rate of 0.3 mL/min

As shown in **Figure 1A**, in the absence of this post-injection divert valve, the resulting peak widths were significantly wide at the flow rate of 0.3 mL/min, leading to reduced separation resolution, compromised achievable LLOQ, and sample throughput. However, the incorporation of the post-injection divert valve effectively addresses these challenges, resulting in sharper peak profiles. This advancement directly translates to an improved separation resolution, heightened sensitivity, and an overall boost in method performance (**Figure 1B**). Under these optimal conditions, it becomes feasible to simultaneously determine FME alongside its key 5' n-1 metabolite with an achievable LLOQ at 0.50 ng/mL from 10 µL Capitainer qDBS microsamples.

Development of Impact-Assisted Extraction

Impact-assisted extraction of the targeted ASOs from the cellulose substrate of the Capitainer qDBS overcame a moderate reduction in extractability with increasing blood HCT up to a maximally evaluated level of 50%. Notably, this phenomenon has been previously reported and is not device-dependent. Furthermore, to achieve robust ASO recovery, detergent was introduced in the desorption solution. Specifically, the inclusion of components such as 20 mM Tris, 20 mM EDTA, 100 mM NaCl, and 0.5% NP-40 at pH 8.0 increased solubility and reduced surface tension, thereby facilitating the permeation of buffer into the cellulose substrate. This optimization enables the near quantitative recovery of targeted ASOs. However, this was accompanied by the complete desorption of all blood components, thereby complexifying IAE extracts. To address this, an effective sample clean-up technique before LC-MS analysis becomes essential. In this scenario, hybridization purification emerges as a suitable approach. Furthermore, to maximize volume transfer efficiency post IAE while maintaining the optimal recovery, an additional volume of Phenomenex Clarity OTX Lysis buffer was introduced following the IAE process.

Establishment of ASO Hybridization

The hybridization extraction workflow involves three main stages: (1) releasing protein-bound ASOs through digestion, (2) forming a double-stranded complex between ASO and capture probe, and (3) separating the ASO-Probe-Bead complex from the matrix using magnetic methods, followed by targeted ASO release through thermal denaturation. Initially, proteinase K was used to digest proteins from IAE extracts, ensuring complete ASO release under mild buffer conditions for downstream hybridization. In addition, a biotinylated capture probe designed to complement FME's sequence facilitated the formation of a distinct ASO-Probe-Bead complex, which was also applicable to the 5' n-1 metabolite and n+2 analog. The third stage employed hybridization extraction with the Kingfisher Flex processor, effectively capturing and purifying targeted ASOs, enhancing sensitivity and specificity, especially with co-extracted blood components from Capitainer qDBS microsamples.

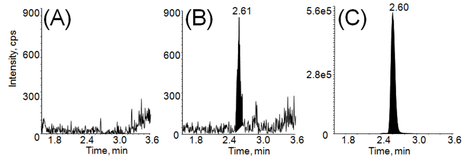


Figure 2. Representative chromatograms of extracted Blank (A), LLOQ (0.50 ng/mL FME) (B), and (C) ULOQ (500 ng/mL FME).

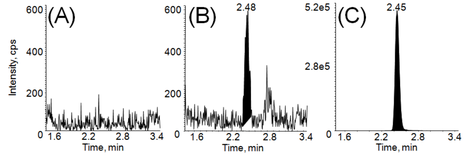


Figure 3. Representative chromatograms of extracted Blank (A), LLOQ (0.50 ng/mL 5' n-1 metabolite) (B), and (C) ULOQ (500 ng/mL 5' n-1 metabolite).

Method Qualification

The calibration curve was constructed with a range of 0.50 – 500 ng/mL established for both FME and 5' n-1 metabolite using peak area ratios to the IS and applying a weighted ($1/x^2$) linear regression. Representative chromatograms (blank [BL], LLOQ, and upper limit of quantification [ULOQ]) are shown in **Figure 2** and **Figure 3**, respectively.

Table 2. Inter-Run Precision and Accuracy (2 Runs, 8 QC Replicates)

Parameters	QCLOQ 0.50 ng/mL	Low QC 1.50 ng/mL	Mid QC 250 ng/mL	High QC 375 ng/mL
Fomivirsen				
% Nominal	97.8	105.5	104.5	94.0
% C.V.	9.4	3.4	4.7	5.2
5' n-1 Metabolite				
% Nominal	106.0	106.4	105.6	93.1
% C.V.	7.3	5.2	5.3	4.0

Precision and accuracy assessments were performed at four QC concentration levels (LLOQ, 3x LLOQ, geometric mean, and 75% of ULOQ), all within acceptance criteria (**Table 2**). Specificity and lack of matrix effect bias were confirmed from individual blood donors, maintaining consistent quantitation for each FME and 5' n-1 metabolite (**Table 3**). The high level of overall assay specificity may be attributed to the selectivity with which targeted ASOs could be isolated from complex dried blood extracts, facilitated by using the capture probe comprised of a full-length complementary sequence.

As shown in **Table 4**, the back-calculated concentrations of FME and 5' n-1 metabolite from the QC samples prepared at different HCT levels (0, 20, 40, and 50%) were within 20% of the nominal concentrations, and the coefficient of variation (%C.V.) below 20%. These results demonstrate that the assay is independent of blood HCT in a range from 0% to 50% levels.

Table 3. Matrix Effect Evaluation on the Quantitation of FME and 5' n-1 Metabolite

Donors	Low QC: 1.50 ng/mL			High QC: 375 ng/mL		
	Mean	%C.V.	%Bias	Mean	%C.V.	%Bias
Fomivirsen						
Donor #1	1.47	3.3	-1.9	341	1.6	-9.2
Donor #2	1.50	11.9	0.1	361	0.6	-3.7
Donor #3	1.42	1.0	-5.7	326	0.8	-13.1
Donor #4	1.42	9.4	-5.5	352	4.3	-6.1
Donor #5	1.34	2.0	-10.4	335	3.5	-10.6
5' n-1 Metabolite						
Donor #1	1.50	4.9	-0.1	344	3.5	-8.3
Donor #2	1.44	5.1	-3.7	367	1.6	-2.3
Donor #3	1.35	0.6	-9.9	328	1.1	-12.7
Donor #4	1.38	3.6	-7.7	349	4.9	-7.0
Donor #5	1.41	4.0	-5.8	335	1.4	-10.6

Table 4. Impact of Blood HCT on the Quantitation of FME and 5' n-1 Metabolite

HCT Levels	Low QC: 1.50 ng/mL			High QC: 375 ng/mL		
	Mean	%C.V.	%Bias	Mean	%C.V.	%Bias
Fomivirsen						
0%	1.53	13.1	1.8	388	6.3	3.4
20%	1.44	7.6	-3.8	388	9.7	3.5
40%	1.37	1.6	-8.7	407	6.6	8.6
50%	1.42	8.0	-5.3	366	0.6	-2.5
5' n-1 Metabolite						
0%	1.44	9.8	-4.0	377	7.9	0.5
20%	1.49	1.7	-0.6	378	15.2	0.7
40%	1.55	3.9	3.3	396	9.8	5.7
50%	1.50	4.1	0.1	344	2.1	-8.3

CONCLUSIONS

The research reported herein comprises the first report for ASO quantitation derived from dried blood samples, using FME and its 5' n-1 metabolite as representative analytes for assay development, establishing performance attributes for extraction from the Ahlstrom 222 cellulose substrate incorporated into the Capitainer qDBS microsampling device. The method evaluation met acceptance criteria for specificity, matrix effect, HCT bias, and precision and accuracy for a quantifiable range of 0.50 – 500 ng/mL, requiring only 10 µL of the collected blood sample. Our novel workflow consisting of IAE coupled with highly specific and sensitive hybridization LC-MS/MS carries significant implications for the advancement of ASO therapeutic development in that critical patient populations may be more readily accessed for decentralized clinical trials.