T1230-02-11

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

Ming-Luan Chen, Kevork Mekhssian, Jeff Plomley, and Anahita Keyhani

Altasciences, Laval, Canada

CONTACT INFORMATION: Altasciences, 575 Armand-Frappier, Laval, Québec, Canada
altasciences.com | contact@altasciences.com

aaps National Biotechnology CONFERENCE

Click here to listen to the recorded poster presentation

PURPOSE

Novel finger-stick-derived capillary blood collection, such as that supported by Capitainer's quantitative dried blood spot (qDBS) microsampling device, has recently emerged as a viable alternative to traditional venipuncture. This approach is less invasive and enables a patient-centric approach by allowing remote/at-home collection of small-volume blood samples (typically < 20 µL). Despite these advantages, there is limited data available on the applicability of dried blood as a matrix for the analysis of antisense oligonucleotides (ASOs). This is partly due to the lack of selectivity in traditional chemical extraction methods and the compromised sensitivity resulting from the combination of low sample volume and poor extractability.

OBJECTIVE

In the current research, we describe a sensitive and selective workflow that combines micro-sample homogenization with a hybridization LC-MS/MS approach for the simultaneous quantitation of a 21-mer ASO, fomivirsen (FME), and its *5′* N-1 metabolite across a concentration range from 0.50 to 500 ng/mL. Additionally, we establish a bioanalytical procedure for FME and its N-1 metabolite in human plasma, an essential step for establishing concordance with blood samples and validating the applicability of the developed workflow in clinical settings.

METHOD

Human blood samples fortified with FME and N-1 metabolite, ranging from 0.5 to 500 ng/mL, were absorbed onto the 10 µL Ahlstrom 222 pre-cut cellulose substrate of the Capitainer qDBS device and allowed to air-dry in the presence of desiccant. Dried micro-samples were carefully detached and placed in a 96-well plate, to which a 5/32" stainless steel grinding bead was added, followed by the N+2 analog of FME as an internal standard (IS). Dried blood micro samples were homogenized in the presence of surfactant, followed by affinity purification of the ASO-Probe complex using functionalized magnetic beads. After multiple wash steps, the targeted ASO was released from the capture probe via thermal denaturation. The resulting extracts were then stored at 4°C for LC-MS/MS analysis. In the human plasma method, 100 µL of matrix was spiked with FME and N-1 metabolite at concentrations ranging from 50 pg/mL to 50 ng/mL and extracted using the identical affinity purification process. Liquid chromatographic separation was performed on an Acquity Premier BEH C18 column (50 x 2.1mm, 1.7 µm) with a mobile phase containing ion-pairing reagents. FME, N-1 metabolite, and N+2 internal standard were detected using a SCIEX 6500+ QQQ operated in negative ESI/MRM mode (**Figure 1**).

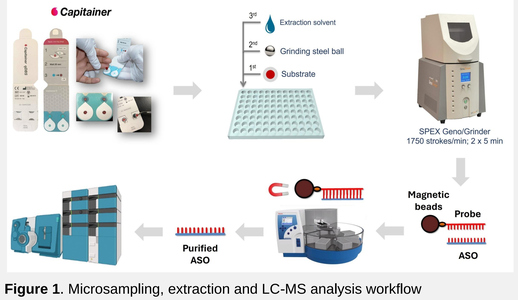

Figure 1. Microsampling, extraction and LC-MS analysis workflow

RESULTS

Optimization of LC-MS/MS

Lower LC flow rates typically produce smaller and more highly charged droplets in electrospray ionization (ESI), increasing ionization efficiency, particularly for multiply (negatively) charged ASOs. However, employing a low flow rate (≤ 0.3 mL/min) can exacerbate issues related to pre-column void volume, leading to drawbacks such as peak broadening, diminished separation resolution, and compromised sample throughput. A post-injection divert valve was integrated into the LC configuration to overcome these challenges (**Figure 2**). This valve effectively eliminates the void volume after sample injection, resulting in sharper peak profiles and thereby improving separation resolution and signal-to-noise ratio (**Figure 3**). Under optimized LC-MS conditions, the simultaneous determination of FME and N-1 metabolite was achieved, with an LLOQ of 0.5 ng/mL (10 µL blood micro-sample) and 50.0 pg/mL (100 µL plasma).

Figure 2. LC-MS sample injection configuration

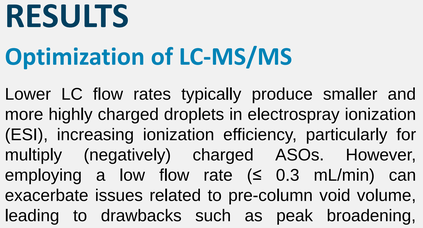

Figure 3. Representative chromatograms of FME, metabolites, and analogs without (**A**) and with post-injection divert valve (**B**) at a flow rate of 0.3 mL/min.

Optimization of Sample Extraction

A complete micro-sample homogenization approach was used to address the challenge of reduced extractability of ASOs posed by increasing blood hematocrit levels. In addition, detergent was introduced into the desorption solution to facilitate buffer permeation into the cellulose substrate, enhancing ASO solubility and enabling near-complete recovery. This treatment leads to the complete desorption of all blood components from the substrate. As a result, a highly selective hybridization-based workflow was implemented to purify the ASO before analysis. Specifically, a biotinylated complementary capture probe was used for the hybridization and purification steps of the target ASO, as well as its N-1 metabolite and N+2 internal standard.

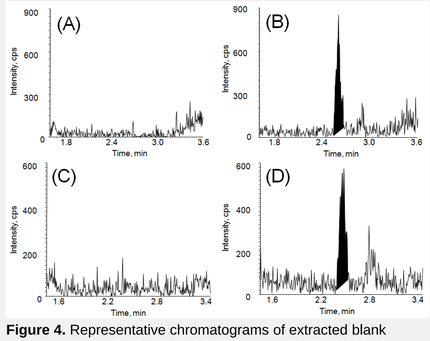

Figure 4. Representative chromatograms of extracted blank microsample for FME (**A**), LLOQ (0.5 ng/mL FME) (**B**), blank microsample for N-1 metabolite (**C**) and LLOQ (0.5 ng/mL N-1 metabolite) (**D**).

Method Qualification

The calibration curves were constructed with a range of 0.5 – 500 ng/mL established for both FME and N-1 metabolite using peak area ratios to the IS and applying a weighted ($1/x^2$) linear regression, with precision and accuracy assessments performed at four QC concentration levels meeting all acceptance criteria (**Table 1**). Representative chromatograms are shown in **Figure 4**.

Table 1. Inter-run precision and accuracy (2 runs, 8 QC replicates) in Capitainer qDBS microsamples

Parameters	QCLOQ 0.5 ng/mL	Low QC 1.5 ng/mL	Mid QC 250 ng/mL	High QC 375 ng/mL
FME ASO				
% Nominal	97.8	105.5	104.5	94.0
% C.V.	9.4	3.4	4.7	5.2
N-1 ASO				
% Nominal	106.0	106.4	105.6	93.1
% C.V.	7.3	5.2	5.3	4.0

Calibration curves were established for both FME and N-1 metabolite in plasma (0.05 – 50 ng/mL) with precision and accuracy assessments performed at four QC concentration levels. The results meet all acceptance criteria (**Table 2**). Representative chromatograms are shown in **Figure 5**.

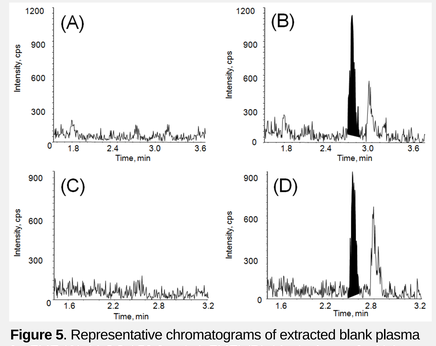

Figure 5. Representative chromatograms of extracted blank plasma for FME (**A**), LLOQ (50.0 pg/mL FME) (**B**), blank plasma for N-1 metabolite (**C**) and LLOQ (50.0 pg/mL N-1 metabolite) (**D**).

Specificity and lack of matrix effect bias were confirmed from individual blood and plasma donors, maintaining consistent quantitation for each FME and N-1 metabolite. 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 (**Table 3**).

Table 2. Inter-run precision and accuracy (3 runs, 12 QC replicates) in plasma samples

Parameters	QCLOQ 50.0 pg/mL	Low QC 150 pg/mL	Mid QC 25.0 ng/mL	High QC 37.5 ng/mL
FME ASO				
% Nominal	104.5	103.6	106.1	103.8
% C.V.	8.1	5.4	5.5	6.2
N-1 ASO				
% Nominal	97.3	101.5	107.2	104.8
% C.V.	6.7	6.6	6.5	6.6

As shown in **Table 4**, the back-calculated concentrations of FME and 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 for the quantitation of FME and N-1 metabolite in Capitainer qDBS microsamples

Donors	Low QC: 1.50 ng/mL			High QC: 375 ng/mL		
	Mean	%C.V.	%Bias	Mean	%C.V.	%Bias
FME ASO						
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
N-1 ASO						
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 N-1 metabolite in Capitainer qDBS microsamples

HCT Levels	Low QC: 1.50 ng/mL			High QC: 375 ng/mL		
	Mean	%C.V.	%Bias	Mean	%C.V.	%Bias
FME ASO						
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
N-1 ASO						
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

A sensitive and selective bioanalytical method for determining FME and N-1 metabolite has been successfully developed for an analytical range of 0.50 – 500 ng/ml. Dried blood micro-samples collected using the Capitainer qDBS (10 µL) were analyzed by hybridization LC-MS/MS. The developed method demonstrates linearity, precision, and accuracy, with all evaluated bioanalytical assessments meeting acceptance criteria. Furthermore, the same workflow has been established in plasma, providing a valuable tool for bridging pharmacokinetic data between matrices. This encourages the future use of blood microsampling in decentralized clinical trials to support the development of antisense therapeutic drugs.

ALTASCIENCES