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APPLICATION OF MITRA MICROSAMPLING FOR THE QUANTITATIVE BIOANALYSIS OF ANTISENSE OLIGONUCLEOTIDES

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PURPOSE

Volumetric absorptive microsampling (VAMS) has emerged as an alternative to venous sampling, offering the convenience of sampling small volumes of whole blood (<20 µL) suitable for patient self-sampling and pediatric sample collection. Coupled with liquid chromatography and mass spectrometry (LC-MS) analysis, this technique has been mainly used to support small molecule pharmacokinetic (PK) clinical studies, with limited data available on the application and utility of this approach to other therapeutic classes such as antisense oligonucleotides (ASOs).

OBJECTIVE

In this work, we describe the first application of VAMS with Neoteryx Mitra microsampling device for quantifying Fomivirsen (FME) using an automated, high throughput sample preparation workflow coupled with LC-MS/MS detection. Due to the limitations in sample volume and to discriminate closely related analogs (*i.e.,* N-1, N-2), the hybridization LC-MS platform was used to achieve high sensitivity and specificity. The analytical performance of this novel workflow was evaluated for Fomivirsen (FME), a synthetic 21-mer ASO, used in the treatment of cytomegalovirus retinitis (CMV) in immunocompromised patients.

METHOD

Human whole blood samples fortified with FME were absorbed onto 10 µL Mitra VAMS and dried at room temperature for at least 24 hours. Effective desorption of the ASO from the Mitra substrates by impact-assisted extraction (IAE) was established by screening various buffer solutions to optimize recovery while overcoming the bias from the sample hematocrit (HCT) level. For selective extraction, hybridization of the single-stranded ASO with a complimentary biotinylated capture probe, coupled to streptavidin-coated magnetic beads, was automated using the KingFisher Flex (ThermoFisher). The resultant extract was separated on an ACQUITY Oligo BEH C18 column (50 x 2.1 mm, 1.7 µm) using acetonitrile (ACN) gradient elution with ion-pairing mobile phases composed of dibutylamine (DBA), dimethylcyclohexylamine (DMCHA), and hexafluoro-2-methyl-2-propanol (HFMIP). FME and Internal Standard (IS, N+2 analog) were monitored by multiple reaction monitoring (MRM) on a SCIEX Triple Quad 6500+ (***Figure 1***).

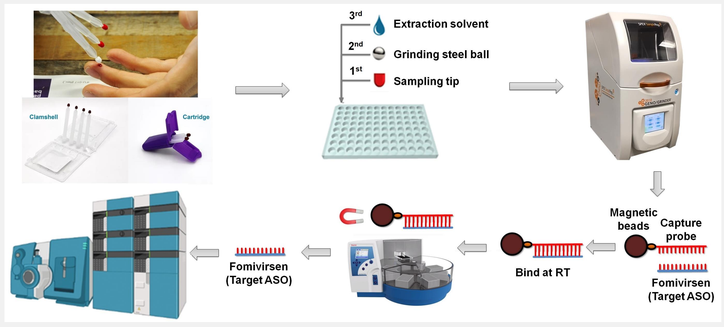

Figure 1. Microsampling, extraction and analysis schematics of Fomivirsen ASO

RESULTS

LC-MS Method Optimization

For instrument tuning, an on-column full scan was performed to obtain the Q1 mass-to-charge ratios (m/z) and charge state distribution. Exemplary Q1 spectra for FME and analog IS are shown in ***Figure 2***. The MRM parameters for FME and its analogues are outlined in ***Table 1***.

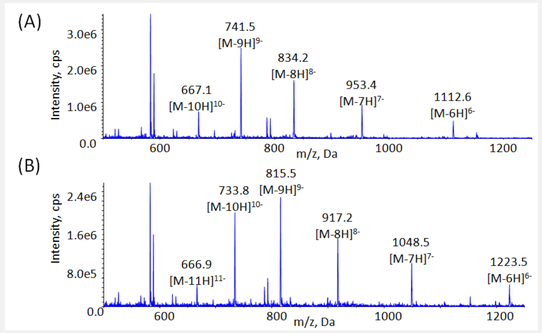

Figure 2. MS Spectra of FME (A) and IS (B)

Table 1. MRM parameters of FME and analogs

Analyte	Q1	Q3	DP	CE
FME	741.5	95.1	-30	-130
N-2 Metabolite	753.0	95.1		
N-1 Metabolite	791.1	95.1		
N+1 Analog	779.8	95.1		
N+2 Analog (IS)	815.4	95.1		

Ion-pairing (IP) is a commonly employed technique for LC separation of ASOs, traditionally utilizing a combination of triethylamine (TEA) and hexafluoroisopropanol (HFIP). In our study, the efficacy of different alkylamines, such as DBA, DMCHA, and N, N-Diisopropylethylamine (DIPEA), and their mixtures in combination with HFMIP was assessed. The results show that a combination of DBA, DMCHA, and HFMIP provides the best resolution of FME from its closely related analogs (***Figure 3***).

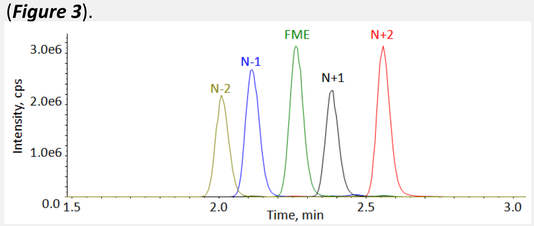

Figure 3. Representative chromatogram of FME and analogs

Impact-Assisted Extraction

Various buffers and surfactants were screened for the effective desorption of FME and IS from Mitra substrates using IAE. Addition of detergents in the desorption buffer significantly improved the extraction of ASOs from the Mitra sorbent, probably due to enhanced protein solubility and decreased surface tension, which helps buffer permeation into the porous Mitra substrates. Extraction solution containing 20 mM Tris, 20 mM EDTA, 100 mM NaCl, 0.5% NP-40, pH 8.0 was selected to characterize the assay performance.

Method Qualification

The calibration curve was constructed with a range of 5.00-1000.00 ng/mL using peak area ratios of FME to the IS and applying a weighted ($1/x^2$) linear regression. Representative chromatograms (blank [BL], lower limit of quantitation [LLOQ], IS) are shown in ***Figure 4***.

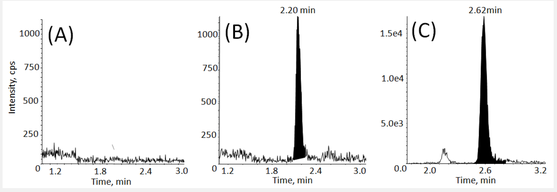

Figure 4. Representative chromatograms of extracted Blank (A), LLOQ (5 ng/mL) (B), and IS (C) samples

Precision and accuracy of the method were evaluated after replicate analysis of four quality control (QC) levels (***Table 3***). QC samples in all extracted batches met the acceptance criteria for precision and accuracy.

Table 2. Inter-run precision and accuracy (5 runs, 20 QC replicates)

Parameters	QC LOQ 5.00 ng/mL	Low QC 15.0 ng/mL	Mid QC 500 ng/mL	High QC 750 ng/mL
%Nominal	9.5	6.1	5.3	4.5
%C.V.	102.3	103.8	107.4	107.4

As shown in ***Table 3***, the back-calculated concentrations of FME from the QC samples prepared at different HCT values (0, 20, 40, and 66%) were within 20% of the nominal concentrations, and the coefficient of variation (%C.V.) below 20%. These results demonstrate that the assay is independent from blood HCT in a range from 0% to 66% HCT levels.

Table 3. Impact of HCT levels on the quantitation of FME

Sample	Concentrations at Different HCT Levels			
	0%	20%	40%	66%
Low QC (15.00 ng/mL)	16.5 ± 0.2	17.0 ± 0.1	16.8 ± 0.2	16.8 ± 0.8
High QC 750.00 ng/mL	823 ± 24	815 ± 38	804 ± 42	803 ± 36

The long-term stability of the ASO on Mitra substrates was demonstrated for 114 days at ambient temperature, and no impact from metabolites (5′ N-1, N-2) was observed in the assay (***Table 4***).

Table 4. Impact of metabolites on the quantitation of FME

Parameters	Low QC[1] 15.00 ng/mL	High QC[2] 750.00 ng/mL
Mean (*N=4*)	15.80	778.00
% Nominal	105.6	103.7
% C.V.	4.9	5.4

[1] Low QC fortified with 3.00 ng/mL each of 5′ N-1 and 5′ N-2 metabolites
[2] High QC fortified with 150 ng/mL each of 5′ N-1 and 5′ N-2 metabolites

CONCLUSIONS

A bioanalytical method for the determination of FME ASO in human blood was successfully developed with an analytical range from 5.00 to 1000 ng/ml, using Mitra VAMS (10 µL) coupled with hybridization LC-MS/MS. The method is linear, precise, and accurate, with all evaluable bioanalytical assessments meeting the acceptance criteria for chromatographic assays. The application of Mitra VAMS to the quantification of ASOs from micro samples would greatly facilitate convenient and accurate remote sampling in support of decentralized clinical trials and patient-centric drug development.

ALTASCIENCES