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Hybridization LC-MS/MS for Sensitive Quantitation of Antisense Oligonucleotides in Plasma and Dried Blood Microsamples

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OVERVIEW

Purpose

To develop an ultra-sensitive and selective hybridization LC-MS/MS method for simultaneous quantitation of antisense oligonucleotides (ASOs) and primary truncated metabolites (i.e., *n*-1) in plasma and dried blood microsamples. Developed methods can support pharmacokinetic (PK) and metabolism studies in decentralized clinical trials and facilitate patient-centric sampling.

Method

Blood samples containing Fomivirsen (FME) and primary (n-1) metabolite (PM) were collected using CapitainerB microsampling devices. Following RmT drying (in presence of desiccant), ASOs were extracted with detergent using bead-beating. Targeted hybridization extraction was next implemented using a full-length complementary capture probe. Ion-pairing reversed-phase liquid chromatography (IP-RPLC) with post-injection divert valve was used to enhance sensitivity at low flow (0.30 mL/min).

Results

Optimization of buffer and detergent composition resulted in > 80% recovery for both FME and PM accompanied by high specificity. The method supported LLOQ's of 50.0 pg/mL from 100 µL of human plasma and 0.500 ng/mL from 10 µL of dried blood, the latter without hematocrit (HCT) bias. Simultaneous quantitation of FME and PM met acceptance criteria for all evaluations conducted, supporting concentration ranges of 0.500 - 500 ng/mL (blood) and 50 pg/mL - 500 pg/mL (plasma).

INTRODUCTION

Antisense oligonucleotides target specific mRNA sequences to reduce disease-related protein expression. Their clinical use requires sensitive bioanalytical methods to quantify both ASOs and their metabolites in complex matrices. While ligand-binding assays (LBAs) are sensitive, they lack specificity for metabolites, whilst LC-MS/MS, though more selective, faces challenges such as poor recovery and matrix effects when using traditional extraction methods.

We present a hybridization LC-MS/MS method combining sequence-specific capture-probe extraction alongside optimized ion-pairing chromatography, applied to both plasma and dried blood microsamples. A post-injection divert valve strategy is described that enhances chromatographic resolution and ionization efficiency, resulting in sensitivity gains (ca. 10-fold) that allow exceptionally low detection limits of 0.500 ng/mL and 50.0 pg/mL for blood and plasma, respectively.

Table 1. Multiple Reaction Monitoring Conditions

Analyte	Q1 Parent Ion *m/z*	Q3 Fragment Ion *m/z*	Dwell time (ms)
FME	741.5	95.0[1]	150
n - 1	791.1	95.0[1]	150
n - 2	753.0	95.0[1]	10[2]
n + 1	779.8	95.0[1]	10[2]
n + 2 (IS)	815.5	95.0[1]	50

[1] Fragment of Phosphorothioate (PS)
[2] Not included in the final LC-MS conditions

METHODS

Sample Preparation

Human blood was applied to the CapitainerB microsampling device (10 µL collected) and dried ≥ 24 hr with desiccant. Removed dried blood discs were placed in a 96w plate containing stainless steel beads, followed by addition of (*n*+2) FME analog (IS) and extraction buffer (20 mM Tris, 20 mM EDTA, 100 mM NaCl, 0.5% NP-40, pH 8.0). Sample preparation was performed by disc homogenization (1750 rpm, 10 min) after which hybridization purification with magnetic beads was used to isolate ASOs (Figure 1).

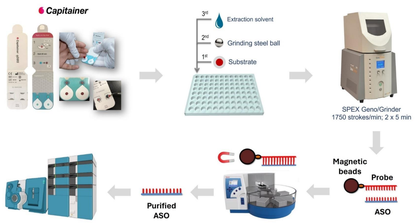

Figure 1. Microsampling, Extraction and LC-MS Analysis Workflow

LC-MS/MS

Chromatography was performed using a Shimadzu NexeraX2 configured with three pumps and a 6-port valve (**Figure 2**). Separation was achieved at 0.30 mL/min on an Acquity Premier BEH C_{18} column (50 x 2.1mm, 1.7 µm) using mobile phase A/B containing dibutylamine (DBA), dimethylcyclohexylamine (DMCHA) and hexafluoro-2-methyl-2-propanol (HFMIP) in a mixture of H_2O, ACN and MeOH. Mobile phase C, a H_2O/ACN mix, was required for column flushing. MS/MS detection using a SCIEX 6500+ was performed in negative ion electrospray mode with multiple reaction monitoring (Table 1).

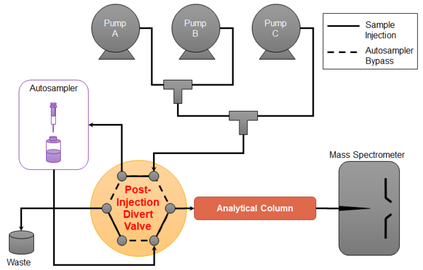

Figure 2. LC-MS Configuration Incorporating a Post-injection Divert Valve

RESULTS AND DISCUSSION

LC-MS/MS Optimization

Low LC flow rate improves ionization efficiency in ESI, particularly for ASO detection in negative polarity. However, flow rates ≤ 0.3 mL/min with 2.1 mm i.d. columns can cause peak broadening and reduced resolution. To address this challenge, a post-injection divert valve was integrated to by-pass the autosampler, minimizing void volume and improving gradient responsiveness, peak sharpness, analyte resolution, and sensitivity (**Figure 3**).

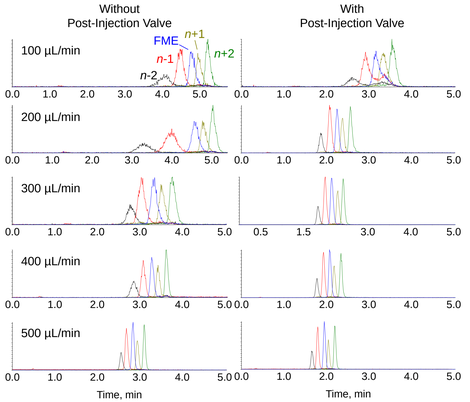

Figure 3. Chromatographic profiles of FME, metabolites and analog without post-injection divert valve (left column) and with post-injection divert valve (right column) at flow rates from 100 µL/min to 500 µL/min.

Table 2. Microsample Inter-Run Precision and Accuracy (*n* = 3)

Statistics	LLOQ QC (0.500 ng/mL)	Low QC (1.50 ng/mL)	Mid QC (250 ng/mL)	High QC (375 ng/mL)
FME				
% Nominal	99.9	103.1	103.4	96.1
% C.V.	8.5	5.0	4.3	5.7
PM				
% Nominal	108.7	104.6	103.5	95.4
% C.V.	7.5	5.6	5.4	5.3

Table 3. Evaluation of Blood HCT on ASO Quantitation

HCT (%)	Low QC (1.50 ng/mL)			High QC (375 ng/mL)		
	Average	% C.V.	%Bias	Average	% C.V.	%Bias
FME						
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
PM						
20	1.49	1.7	-0.6	378	10.2	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

Method Qualification

The LLOQ for 10 µL of extracted dried blood microsample was 0.500 ng/mL for FME and PM. Despite lower sample volume and increased matrix complexity compared with plasma, high recovery coupled with hybridization specificity resulted in an LLOQ with SNR > 10:1 (**Figure 4**).

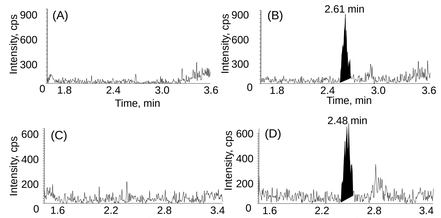

Figure 4. Representative chromatograms of: (A) extracted blank microsample at FME MRM transition, (B) FME extracted LLOQ of 0.500 ng/mL, (C) blank microsample monitored at PM MRM transition and (D) extracted LLOQ of 0.500 ng/mL for PM.

The (*n*+2) FME analog was implemented as internal standard, with selective annealing achieved. Linearity (1/x²) spanned three orders of magnitude with r² between 0.9949 – 0.9984 (FME) and 0.9936 – 0.9965 (PM). Further, assay precision and accuracy met acceptance criteria with comparable statistics between plasma and blood microsamples (**Tables 2 and 4**). Microsample recoveries were ca. 80% (FME, PM) and 88% (IS), whilst quantitation remained accurate up to 50% HCT (**Table 3**). No significant matrix effect was observed (**Table 5**).

Table 4. Plasma Inter-Run Precision and Accuracy (*n* = 3)

Statistics	LLOQ QC (50.0 pg/mL)	Low QC (150 pg/mL)	Mid QC (25.0 ng/mL)	High QC (37.5 ng/mL)
FME				
% Nominal	104.5	103.6	106.1	103.8
% C.V.	8.1	5.4	5.5	6.2
PM				
% Nominal	97.3	101.5	107.2	104.8
% C.V.	6.7	6.6	6.5	6.6

Table 5. Matrix Effect Evaluation of Microsample Extracts

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

The achievable LLOQ for FME and PM extracted from plasma was 50.0 pg/mL (**Figure 5**). Linear regressions (1/x²) yielded r² of 0.9946 – 0.9972 (FME) and 0.9934 – 0.9976 (PM). Analyte recovery was consistent (87% – 92%, C.V. ≤ 6.9%) and specificity was confirmed across 10 donors, including lipemic and hemolyzed plasma. Matrix effect demonstrated C.V. ≤ 8.1% and % bias of -7.6% – 10.1% (**Table 6**).

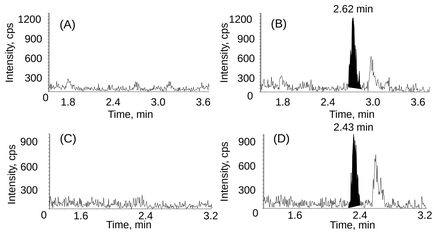

Figure 5. Representative chromatograms of: (A) extracted blank plasma monitored at FME MRM transition, (B) extracted plasma LLOQ of 50.0 pg/mL for FME, (C) blank plasma monitored at the MRM transition for PM and (D) extracted plasma LLOQ of 50.0 pg/mL for PM.

Table 6. Matrix Effect Evaluation of Plasma Extracts

Donor	Low QC (150 pg/mL)			High QC (37.5 ng/mL)		
	Average	% C.V.	%Bias	Average	% C.V.	%Bias
FME						
1	150	8.1	0.0	40.1	6.2	7.0
2	144	3.0	-4.2	39.7	0.9	6.0
3	145	5.9	-3.1	40.6	4.1	8.2
4	152	2.9	1.6	39.2	3.4	4.5
5	150	2.1	-0.2	39.0	1.2	4.1
6	161	4.7	7.6	40.7	0.5	8.5
7	147	6.9	-1.9	40.3	4.9	7.4
8	151	2.8	0.9	40.4	1.6	7.8
Lipemic	146	2.4	-2.6	40.8	1.8	8.9
Hemolyzed	160	1.3	6.9	39.4	5.9	5.0
PM						
1	152	7.9	1.0	41.3	6.7	10.0
2	149	9.6	-0.6	39.8	0.7	6.3
3	144	1.6	-4.0	40.6	3.0	8.2
4	149	2.6	-0.5	40.3	4.5	7.5
5	146	5.4	-2.9	39.6	5.2	5.7
6	151	6.4	0.4	41.3	0.1	10.1
7	139	2.2	-7.6	40.6	0.2	8.4
8	154	0.9	2.7	41.0	2.2	9.4
Lipemic	144	2.4	-4.0	41.3	2.3	10.1
Hemolyzed	150	2.5	-0.2	39.9	4.6	6.5

CONCLUSIONS

This research study presents a sensitive hybridization LC-MS/MS method for quantifying FME and PM in plasma and dried blood microsamples. It addresses potential HCT bias and resolves inherent matrix complexity through highly selective complementary strand annealing. Further, the work highlights the potential to support patient-centric microsampling for this molecular class, leveraging the CapitainerB device specifically.