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Optimized Dried Blood Microsampling Extraction Coupled with Hybridization LC-MS/MS for the Sensitive Quantitation of Antisense Oligonucleotides

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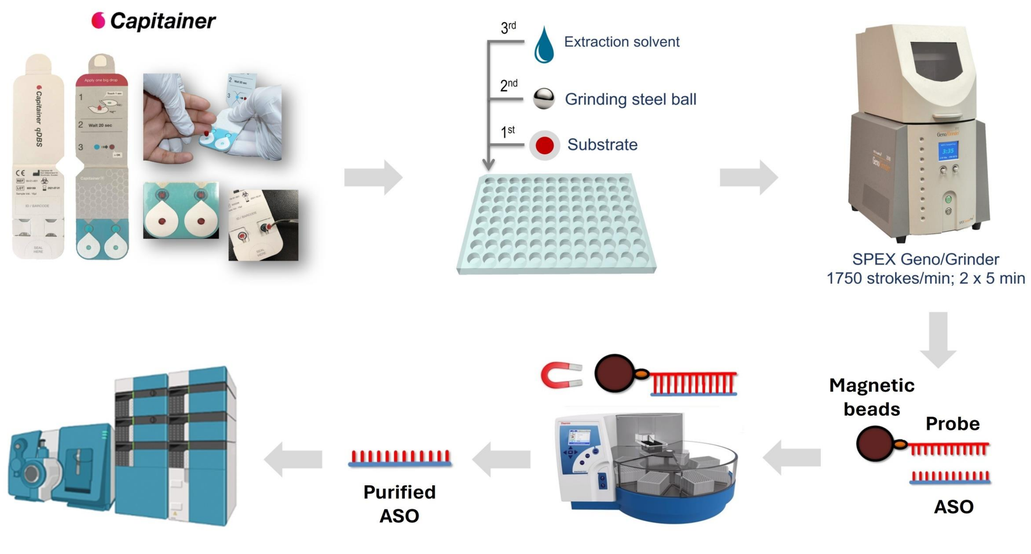
INTRODUCTION

Capillary blood collection using the Capitainer®B microsampling device offers a minimally invasive alternative to venipuncture. It is increasingly used in decentralized and patient-centric clinical sampling, providing accurate volumetric sampling independent of hematocrit (HCT). However, due to poor recovery from dried blood, its application for the bioanalytical determination of antisense oligonucleotides (ASOs) remains limited. Desorption solutions commonly used for small molecules often contain organic solvents, which are generally incompatible with ASOs. However, surfactant-containing aqueous solutions do aid ASO extractability; however, the co-extraction of endogenous matrix components can increase the likelihood for interference and ionization suppression.

In the current research, we systematically optimized the extraction of Fomivirsen (FME), a 21-mer ASO, and its primary (n-1) metabolite (PM) from Capitainer®B dried blood microsamples. By refining both extraction solution composition and agitation conditions, a workflow was established that achieved high yields without HCT-induced recovery bias. The sample preparation workflow is fully compatible with hybridization-based LC-MS/MS and supports sensitive and reproducible quantitation of ASOs from only 10 µL of dried blood sample.

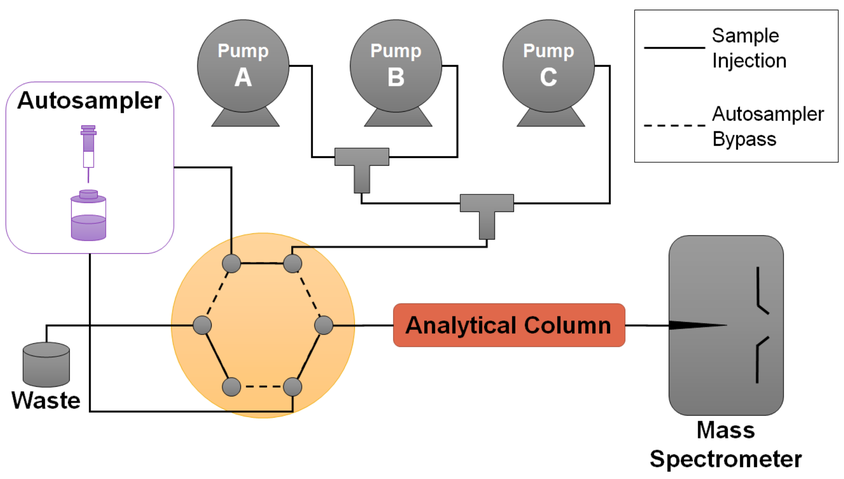


Scheme 1. Microsampling, Extraction and LC-MS Analysis Workflow

METHODS

Extraction of FME and PM from 10 µL dried blood microsamples was initially performed by pre-soaking followed by three cycles of vertical agitation (1750 rpm, 5 min/cycle) using a SPEX Geno/Grinder, carefully avoiding homogenization of the cellulose disc as resulting fibril formation notably hindered volumetric transfer and increased recovery variability. FME and PM were then selectively isolated from the primary extract using biotinylated capture probes for sequence-specific hybridization, with FME N+2 analogue as internal standard (**Scheme 1**).

Chromatography was performed on a Shimadzu LC-30AD Nexera X2 UPLC system equipped with three isocratic pumps and a 6-port valve (**Scheme 2**). Separation was achieved using a Waters Acquity Oligonucleotide BEH C_{18} column (50 × 2.1 mm, 1.7 µm) at a flow rate of 0.30 mL/min at 60 ºC. Mobile phases consisted of dibutylamine (DBA), dimethylcyclohexylamine (DMCHA), and hexafluoro-2-methyl-2-propanol (HFMIP) for separation, and water/acetonitrile for column flushing. Detection was carried out on a SCIEX 6500+ triple-stage quadrupole mass spectrometer operated in negative ESI/MRM mode.



Scheme 2. LC Configuration for Autosampler By-Pass Functionality.

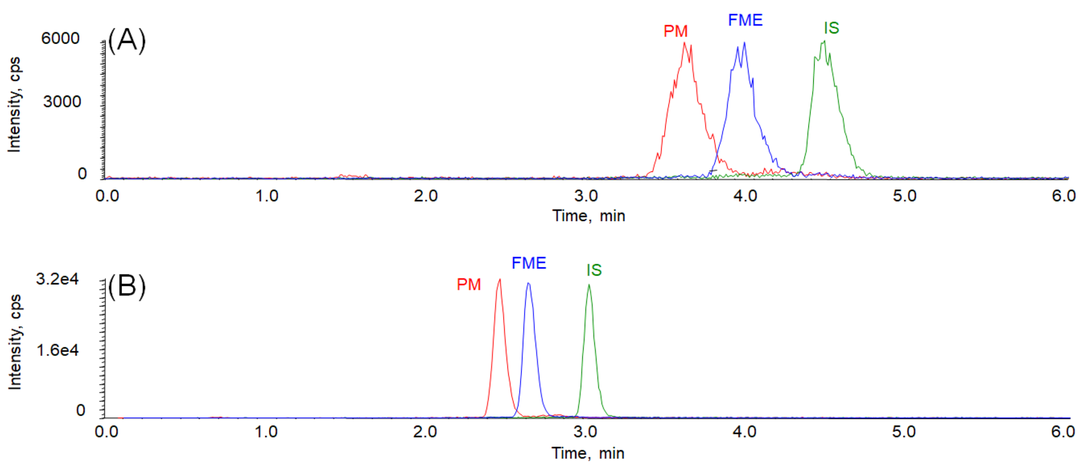


Figure 1. Representative Chromatograms of FME, Metabolites and Analogs Without (**A**) and With Post-Injection Divert Valve (**B**).

RESULTS AND DISCUSSION

Lower LC flow rates enhance ionization efficiency in ESI by generating smaller more highly charged droplets, advantageous for multiply charged ASOs detected in negative ion mode. However, flow rates ≤ 0.3 mL/min can amplify the effects of pre-column void volume, resulting in broader peaks, reduced resolution, and lower throughput. To address this, a post-injection divert valve was integrated into the LC system, effectively eliminating void volume after injection (**Scheme 2**). This adjustment yielded sharper peaks, improved separation resolution, and enhanced signal-to-noise ratio (**Figure 1**). Under optimized conditions, FME and PM were simultaneously quantified with a lower limit of quantitation (LLOQ) of 0.500 ng/mL.

Various desorption solutions, including surfactant ones, were screened to maximize ASO recovery. The optimal aqueous extraction solution contained 60 mM Tris, 15 mM EDTA, 175 mM NaCl, and 0.25% NP-40 (pH 8.0). Further, different agitation methods were evaluated, including gentle mixing (12 hr) and vigorous vertical agitation with and without bead beating. Bead beating caused complete disc homogenization and fibril formation, impairing volumetric transfer and recovery variability. The optimal workflow, which preserved disc integrity, involved pre-soaking followed by three cycles of vertical agitation without beads (1750 rpm, 5 min/cycle). Recoveries were reproducible under these conditions and ranged between 85% and 90%, independent of blood hematocrit evaluated at 25% and 50% (**Table 1**).

Calibration curves for both FME and the PM were established from 0.500 ng/mL to 500 ng/mL characterized by a linear regression weighted 1/x². Precision and accuracy at four QC concentrations met all acceptance criteria (**Table 2**). Representative chromatograms are shown in **Figure 2**.

Table 1. Impact of Blood HCT

HCT Levels	Low QC: 1.50 ng/mL			High QC: 375 ng/mL		
	Mean	%C.V.	%Bias	Mean	%C.V.	%Bias
			FME			
0%	1.56	7.8	3.8	382	5.3	1.9
20%	1.52	7.3	1.4	376	5.9	0.4
40%	1.58	3.2	5.4	364	2.7	-3.0
50%	1.47	4.1	-2.3	380	7.0	1.4
			PM			
0%	1.44	1.2	-3.8	415	5.6	10.6
20%	1.49	2.8	-0.7	402	4.6	7.2
40%	1.46	3.3	-2.5	385	2.0	2.6
50%	1.40	1.8	-7.0	402	7.7	7.3

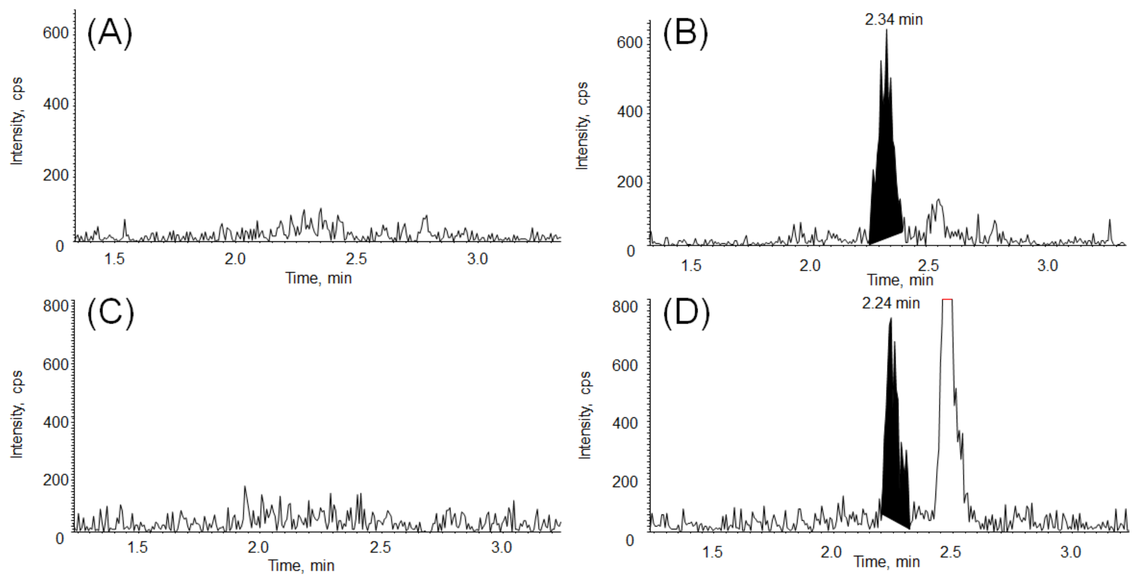


Figure 2. Representative Chromatograms of Extracted Microsamples: (**A**) Blank FME, (**B**) FME LLOQ (**C**) Blank PM and (**D**) PM LLOQ.

Table 2. Intra-Run Precision and Accuracy

Parameters	QC LOQ 0.500 ng/mL	Low QC 1.50 ng/mL	Mid QC 250 ng/mL	High QC 375 ng/mL
		FME		
%Nominal	94.9	100.4	109.9	109.0
%C.V.	10.0	9.7	5.5	3.4
		PM		
%Nominal	105.5	98.2	113.0	110.5
%C.V.	8.8	7.8	4.3	4.9

CONCLUSIONS

Through systematic optimization of extraction from Capitainer®B dried blood microsamples, a robust method was established for recovering FME and PM in high yield without HCT bias by combining pre-soaking with controlled vertical agitation. Coupled with hybridization-based purification and LC-MRM detection, the method enables sensitive and reproducible quantitation supporting an LLOQ of 0.500 ng/mL. The assay demonstrates that, with an appropriate extraction strategy, the Capitainer®B device can be fully leveraged for minimally invasive collection of capillary blood for ASO bioanalysis.