Research Article

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Bioanalysis

Volumetric absorptive microsampling coupled with hybridization LC–MS/MS for quantitation of antisense oligonucleotides

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Background: Volumetric absorptive microsampling has emerged as a less invasive alternative to venous sampling for small-molecule pharmacokinetic studies, but its application to novel therapeutics such as antisense oligonucleotides (ASOs) is not well-established. **Results:** A workflow was developed using Mitra microsampling coupled with hybridization LC–MS/MS for accurate determination of fomivirsen, a 21-mer ASO, in human blood. Quantitative recovery was achieved regardless of blood hematocrit level or microsample age by implementing impact-assisted extraction. A thorough method evaluation confirmed sensitivity, linearity, precision/accuracy, matrix effect, metabolite interference and four months of microsample stability. **Conclusion:** The combined impact-assisted extraction and hybridization LC–MS/MS workflow demonstrated the successful quantitation of fomivirsen, establishing the validity and applicability of the approach for ASO drug candidates.

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Antisense oligonucleotides (ASOs) are single-stranded, synthetic oligodeoxynucleotides typically 15–25 nucleotides long [1,2]. By sequence-specific binding to mRNA, ASOs can prevent gene expression of the target transcript and therefore mitigate the formation of disease-related proteins [3,4]. Due to their extremely high target specificity, ASOs are considered ideal drug candidates for different types of oncology, cardiomyopathies, viral infections and neurological and neuromuscular diseases [5–8]. Furthermore, advances in nucleotide chemical modifications and targeted delivery systems have accelerated the research and development of ASO therapeutics with improved efficacy, safety and pharmacokinetic (PK) profiles [9–11]. To meet increasing requirements to evaluate the drug metabolism and PKs of these novel therapeutics, including drug–drug interactions, it is critical to develop robust, sensitive and selective bioanalytical methods to determine ASO concentrations in biological samples [8].

Among the different analytical approaches to ASO quantitation, hybridization-based immunoassays have been the most implemented methodology due to their high assay sensitivity and throughput [12–15]. These assays typically use capture probes to hybridize with the target oligonucleotide, which reduces the need for extensive and tedious sample extraction while enabling the lower limits of quantitation (LLOQ) to be typically achieved in the low ng/ml concentration range [16–18]. However, these assays are plagued by narrow ranges, duplicated sample analysis and the requirement of multiple critical reagents. Moreover, the accuracy of ASO quantitation can be significantly compromised by the presence of truncated metabolites generated *in vivo* via nuclease activity. Such n-1 and n-2 metabolites often retain target affinity and activity [7]. Therefore, it is crucial to employ appropriate detection methods to overcome these limitations and improve the selectivity by which ASOs can be accurately quantified.

As an alternative to the immunoassay approach, LC–MS/MS has emerged as a robust analytical platform for quantifying ASOs, owing to its high specificity and capability to discriminate parent ASOs from truncated metabolites [19–21]. To mitigate the specificity challenges in immunoassays while retaining sensitivity through the hybridization approach, a novel workflow has recently been developed that combines hybridization-based sample extraction with LC–MS/MS-based detection [22,23]. In a 2020 study, Li *et al.* reported that the hybridization LC–

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MS/MS workflow had improved sensitivity (0.50 ng/ml LOQ achieved using 100 µl of plasma) and supported a wide linear range (0.50–500 ng/ml) for the quantitation of ASOs in both plasma and tissue samples [7]. More recently, the hybridization LC–MS/MS workflow was validated for the quantitative bioanalysis of an ASO drug candidate in various biological matrices [24].

However, the potential of the novel ASO bioanalysis approach in liquid matrices is hindered by the limited sample volume of modern microsampling devices. Additionally, collecting matrix amounts between 2–20 µl proves particularly advantageous for supporting small rodent preclinical studies or patient-centric remote sampling in clinical trials [25–27]. Regulated bioanalysis employs both plasma-based and blood-based microsampling devices [28]. Plasma capillary microsampling is one such technique that offers homogeneous samples without red blood cells, rendering the hematocrit (Ht) effect negligible [29]. However, this technique necessitates centrifugation and is generally applicable only in laboratory settings. Moreover, capillary plasma samples require reduced temperature for storage and shipment, which could potentially lead to complications in drug metabolism. This implies that the target analyte may undergo metabolism or degradation, which could adversely affect the accuracy of the measurements [30]. Furthermore, remote sampling is not possible with this technique, limiting its application in decentralized clinical trials and patient-centric drug development. Dried blood spot (DBS) is another widely used microsampling technique, but it has a significant drawback: the use of a fixed subpunch of a DBS on cellulose substrate can be problematic, as Ht levels may affect the spot size and cause a bias in sampling volume. Essentially, the collected volume of dried blood may increase with higher blood Ht due to decreased spreading as a result of increased viscosity [26,31].

To circumvent the Ht bias on sample volume in the subpunch approach, in 2014, Neoteryx introduced the Mitra volumetric absorptive microsampling (VAMS) device [25]. This small device contains a hydrophilic polymeric sorbent that utilizes capillary action to collect an accurate volume of blood (usually 10 or 20 µl), independent of Ht level [32–34]. Since its introduction, the Mitra VAMS device technology has been successfully implemented in a range of small-molecule clinical studies, supporting antiretrovirals [35], anticancer agents [36], antiepileptics [37] and immunosuppressants [38] in addition to a host of other therapeutic indications [39,40]. Recently, a study of monoclonal antibodies has also shown the feasibility of using Mitra VAMS for large-molecule bioanalysis [41]. By integrating Mitra VAMS with a hybridization LC–MS/MS workflow, the approach offers a potential solution for ASO quantitation in applications that require frequent and/or small-volume sampling. This approach can facilitate PK investigations in both preclinical and clinical settings, thereby enhancing the understanding of the therapeutic capabilities of ASO drug candidates. As a result, it provides additional support for the potential effectiveness of these treatments.

Herein, the authors report the first application of a VAMS approach for ASO quantitation by coupling Mitra microsampling with hybridization LC–MS/MS. Fomivirsen (FME), a 21-mer ASO, was used as a representative analyte to characterize and optimize the workflow. To achieve quantitative recovery and facilitate desorption of FME from dried blood Mitra substrate (10 µl), the primary extraction solution was altered from the typical high-organic compositions used for small molecules to aqueous buffer solutions containing detergents. This extraction solution was also aligned with the subsequent hybridization process, which involved the use of a full-length complementary capture probe with an automated, high-throughput magnetic particle processor. The LC–MS/MS analysis was performed under reversed-phase (RP) conditions incorporating ion-pairing (IP) reagents to ensure the separation of FME, truncated metabolites (n-1, n-2) and closely related analogues (n + 1, n + 2).

Materials & methods

Chemicals & reagents

FME and its analogues (5′n-1, 5′n-2, 5′n + 1 and 5′n + 2), as well as the capture probe (BiotinTEG-DNA, full-length complementary to FME), were purchased from Integrated DNA Technologies (IA, USA; Supplementary Table 1). Proteinase K and Dynabeads MyOne Streptavidin C1 magnetic beads were obtained from Thermo Fisher Scientific (MA, USA); 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP) was purchased from Oakwood Products (SC, USA); 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), *N,N*-dimethyl cyclohexylamine (DMCHA), *N,N*-diisopropylethylamine (DIPEA) and DL-1,4-dithiothreitol (DTT) were purchased from MilliporeSigma (MA, USA). Dibutylamine (DBA) was purchased from Fisher Chemicals (CA, USA) and Clarity OTX Lysis-Loading Buffer v2.0 was purchased from Phenomenex (CA, USA). Nonidet P40 (NP-40), a nonionic surfactant, was purchased from Accurate Chemical & Scientific Corporation (NY, USA). LC–MS grade acetonitrile (ACN) and LC–MS-grade methanol (MeOH) were also purchased from MilliporeSigma (MA, USA). Rat plasma (K_2EDTA),

human whole blood and plasma (K_2EDTA) were sourced from BioIVT (NY, USA). Mitra VAMS microsamplers (10 µl) were obtained from Neoteryx (CA, USA). Desiccant (5-g silica gel packets) was purchased from Desiccare Inc. (NV, USA). Lysing matrix S bulk balls used for bead-beating were sourced from MP Biomedicals (OH, USA).

Solutions & standard preparation

Individual stock solutions of FME and analogues were prepared by dissolving them in H_2O at a concentration of 1.00 mg/ml. Working solutions for LC–MS/MS optimization were prepared at 1.00 µg/ml by diluting stock solutions in 0.05% rat plasma in ACN:H_2O, 10:90% v/v. Spiking solutions containing 50.0 µg/ml FME as the upper limit of quantitation (ULOQ) and 37.5 µg/ml FME for high-quality control (QC) samples were prepared in a similar manner. All stock and working solutions were stored at 4°C. The internal standard (IS) working solution (ISWS) was prepared fresh daily at a concentration of 75.0 ng/ml in 0.05% rat plasma in ACN:H_2O, 10:90% v/v. Pooled human whole blood (K_2EDTA) was used for standard/QC preparation on Mitra. Blank whole blood was fortified and gently mixed to yield the ULOQ at 1000 ng/ml and the high QC at 750 ng/ml. The remaining calibration standards and QC samples were prepared by serial dilution in whole blood (Supplementary Table 2) and sampled onto Mitra according to a previously published protocol [27]. Following Mitra sampling, the microsamples were dried for at least 2 h under ambient lab conditions and stored at room temperature in a sealed Ziploc bag in the presence of a desiccant.

Preparation of blood at different Ht levels

Blood of differing Ht levels was prepared by mixing plasma and red blood cells collected after centrifugation of human whole blood at 1500 × *g* for 10 min at 4°C, with the Ht determined using capillary centrifugation (Dynacare®, Quebec, Canada) [27].

Sample preparation

Impact-assisted extraction

Dried blood Mitra microsamples were carefully detached from the handler and placed in a 96-well plate. A 5/32" stainless steel grinding bead was then added to each well, followed by the addition of 25-µl ISWS and 200 µl of extraction solution whose compositions are detailed in Supplementary Table 3. The plate was sealed and, following 5 min of bead beating using a Geno/Grinder 2000 (SPEX CertiPrep) at 1750 strokes per min, the samples were left soaking for an additional 5 min and then placed on wet ice for 5 min to cool. The mixing step on the Geno/Grinder 2000 at 1750 strokes per min for 5 min, as well as the subsequent cooling steps, were repeated to ensure complete desorption of ASOs from the Mitra substrate. Next, the plates were centrifuged at 4612 × *g* for 10 min and the supernatant was transferred to a KingFisher 96-deep-well plate for hybridization extraction.

Streptavidin magnetic bead preparation

A total volume equaling 15 µl per sample of 10 mg/ml streptavidin-coated magnetic beads was homogeneously suspended and aliquoted into centrifuge tubes. Using a DynaMag-2 Magnet (Thermo Fisher Scientific, MA, USA), the beads were collected and washed using the binding and washing buffer (5 mM Tris, 1 M NaCl, 0.5 mM EDTA and 0.05% Tween-20 in water) three times. After resuspending the beads in the binding and washing buffer, an excess amount of capture probe was added to the bead suspension with a probe-to-bead volume ratio of 1:25, and gently mixed on a 10 HulaMixer (Thermo Fisher Scientific, MA, USA) to allow for conjugation at room temperature for 1 h. Following conjugation, the beads were carefully washed at least three times and resuspended in binding and washing buffer.

Hybridization extraction

In a KingFisher 96-deep-well plate containing 200 µl of supernatant, 100 µl of Clarity OTX Lysis buffer, 180 µl of digestion buffer (100-mM Tris, 250-mM NaCl, 10-mM DTT and 10-mM EDTA in H_2O) and 20 µl of proteinase K (20 mg/ml) were added. The plate was sealed and heated at 65°C on a Thermomixer (Eppendorf, NY, USA) running at 850 r.p.m. for 2 h. After the digestion step, 400 µl of capture buffer (10-mM Tris, 500-mM NaCl, 1-mM EDTA and 0.05% Tween-20 in H_2O) and 30 µl probe-conjugated magnetic bead suspension was added to each well and incubated at 850 r.p.m. for 90 min. The beads were then transferred and sequentially washed with 500 µl of capture buffer and 500 µl of wash buffer (10-mM Tris, 50-mM NaCl, 1-mM EDTA and 0.05% Tween-20 in water) at room temperature. Finally, the washed beads were transferred to the elution plate, containing

150 µl of the reconstitution solution (0.05% rat plasma in ACN:H_2O, 10:90% v/v) preheated to 95°C. The beads were rigorously agitated for 15 min at 95°C and collected by the magnet, leaving the elution plate ready for LC–MS/MS analysis.

LC–MS/MS conditions

A Nexera X2 UHPLC system (Shimadzu, MA, USA) with an ACQUITY Oligonucleotide BEH C_{18} column (50 × 2.1 mm 1.7 µm 130 Å, MA, USA) was used for chromatographic separation of ASOs. A ternary flow system with three UHPLC pumps were implemented with mobile phase A (5-mM DBA and 25-mM HFMIP in MeOH:H_2O, 20:80% v/v), mobile phase B (25-mM DMCHA in H_2O:ACN, 50:50% v/v) and mobile phase C (H_2O:ACN, 90:10% v/v). The column temperature was set to 60°C with gradient elution at a flow rate of 0.5 ml/min (Supplementary Table 4). Following peak elution, the analytical column was rinsed with mobile phase C to minimize on-column carryover.

MS/MS quantitation was conducted using a QTRAP 6500$^+$ mass spectrometer (SCIEX, MA, USA) equipped with an electrospray ionization source. Optimized ion-source parameters included curtain gas at 35 psi, collision gas set to high, electrospray potential at -4500 V, source temperature at 500°C and both the nebulizer and heater gas flows at 65 psi. Operating in negative-ionization mode, the multiple reaction monitoring (MRM) function was used to detect and quantify ASO; MRM transitions and optimized parameters are summarized in Supplementary Table 5. LC–MS/MS instrument control, data acquisition and processing were conducted using Analyst 1.6.3 (SCIEX, MA, USA). The peak area ratio of analyte to IS was used for standard regression and sample quantitation.

Results & discussion

Development of impact-assisted extraction

While a Mitra VAMS approach minimizes sample volume bias due to blood Ht, it does not eliminate the impact of Ht level on recovery [25,27,40]. Higher blood Ht can result in lower analyte yields due to the increased proportion of red blood cells trapped in the Mitra substrate, which can impede the analyte desorption process [42]. In addition, the extractability of analytes from the Mitra substrate may decrease with increasing sample age, a phenomenon referred to as age-related extractability [34]. Several factors can affect analyte recovery as a function of Ht level, including the choice of extraction solvent [32], the type and strength of mixing and the duration of agitation. To overcome Ht bias on recovery and mitigate age-related extractability, our group previously reported a bead-beating approach referred to as impact-assisted extraction (IAE), which demonstrated consistently high yields of recovered analyte from the Mitra substrate independent of Ht level [27]. In our previous work on IAE, 5/32" stainless steel grinding balls provided optimal results, after evaluating the performance of different bead sizes [26,27]. The current investigation of stroke speed and duration showed that using 1750 vertical strokes per min for 5 min, repeated twice, was sufficient to desorb the targeted ASOs from the Mitra substrate. Another mixing method was evaluated by gently mixing the Mitra microsamples with stainless beads at 850 r.p.m. for a longer duration (2 h). The result showed identical FME recovery to that of direct IAE with stainless beads at high QC levels (750 ng/ml). However, at low QC levels (15.0 ng/ml), recovery was lower when gently mixing compared with IAE. Further, linearity between low and high QC levels was only maintained when using IAE, demonstrating that desorption of ASOs from the Mitra substrate necessitates a more aggressive extraction approach.

To minimize the influence of Ht on FME yield, careful selection of the extraction solution was essential. Desorption solutions typically employed in small-molecule applications consist of high proportions of organic solvents (e.g., >70% MeOH or ACN) and pH modifiers. These solutions are ineffective for the desorption of ASOs from dried blood microsamples when using a downstream hybridization strategy that requires buffered aqueous conditions for full recovery. Therefore, a variety of extraction compositions were screened at low and high QC levels as outlined in Figure 1 and Supplementary Table 3. Extracting FME using water (Sol-6; Supplementary Table 3) resulted in low response and significant variability, highlighting the requirement for buffers and detergents to improve recovery and reproducibility. The impact of extraction solution pH, ranging from 5.5 to 8.0, was negligible, with similar responses obtained using buffer solutions at pH 8.0 (Sol-1) and 5.5 (Sol-2). However, an increase of the NaCl concentration in the extraction solution (Sol-3 to Sol-5) led to a decrease in FME response, indicating that a high salt concentration can inhibit the desorption of ASOs from the Mitra substrate. Sol-5 containing 50-mM NaCl showed significant variability at the high QC level, suggesting that this concentration is insufficient to desorb high levels of FME. Furthermore, it is important to highlight that detergent is required in releasing the ASO analytes from Mitra DBSs. In Sol-1, NP-40 is used for this purpose, and the transfer of

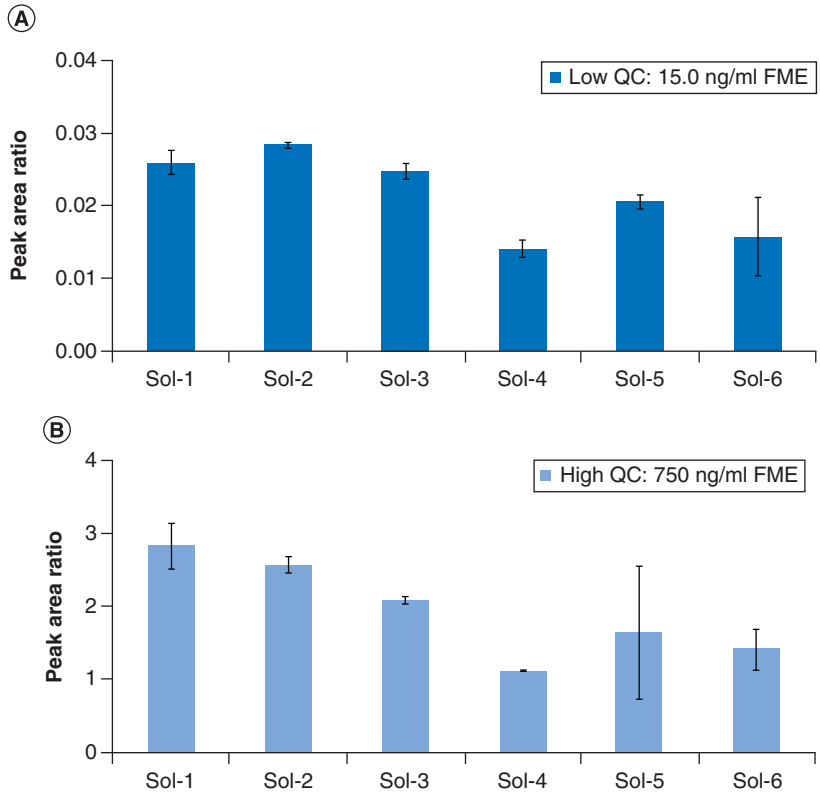

Figure 1. Optimization of impact-assisted extraction solutions described in Supplementary Table 3.
FME: Fomivirsen; QC: Quality control.

NP-40 from the sample plate to the final elution plate is negligible, leading to a minimal quantity remaining in the ultimate extracts. Thorough experiments have demonstrated that this residual NP-40 has no discernible impact on the retention times or peak profiles of ASOs, even during injections for a run comprising around 150 samples. Therefore, Sol-1 was chosen as the optimal extraction solution (adjusted to pH 8.0) for IAE containing 20-mM Tris, 20-mM EDTA, 100-mM NaCl and 0.5% NP-40, since not only was the near-quantitative recovery of FME from Mitra achieved, but the composition facilitated the downstream implementation of a hybridization workflow.

Optimization of ASO hybridization

The aqueous extraction solution used in the IAE process for Mitra has the potential to dissolve dried blood. Therefore, an efficient sample cleanup step is necessary following IAE to maintain optimal LC–MS/MS performance. In this context, a hybridization extraction method combined with IAE would be advantageous since it can isolate the targeted ASO with high specificity and limit any potential interference from the sample matrix. The hybridization extraction workflow is typically a three-stage process involving digestion to release protein-bound ASO, formation of a full-length double-stranded complex between ASO and capture probe and isolation of the ASO-probe-bead complex from the sample matrix using magnetic separation followed by subsequent release of the targeted ASO by thermal denaturation [7,24].

The first step involved the use of a nonselective protein hydrolase, proteinase K, to digest the proteins solubilized from the dried blood prior to hybridization. Proteinase K digestion offered several benefits, including the complete release of protein-bound ASO under mild buffer conditions, compatible with downstream hybridization. Additionally, protein digestion was performed at a temperature of 65°C, which is greater than the melting temperature (T_m) of most ASOs, thus allowing for the release of ASO from target mRNA [7,24]. The optimization of the buffer composition, the use of proteinase K and the duration of incubation time led to a full recovery of FME in digestion buffer (100-mM Tris, 250-mM NaCl, 10-mM DTT and 10-mM EDTA in H_2O) when using a 0.8 mg/ml concentration of proteinase K for a duration of 2 h at 65°C.

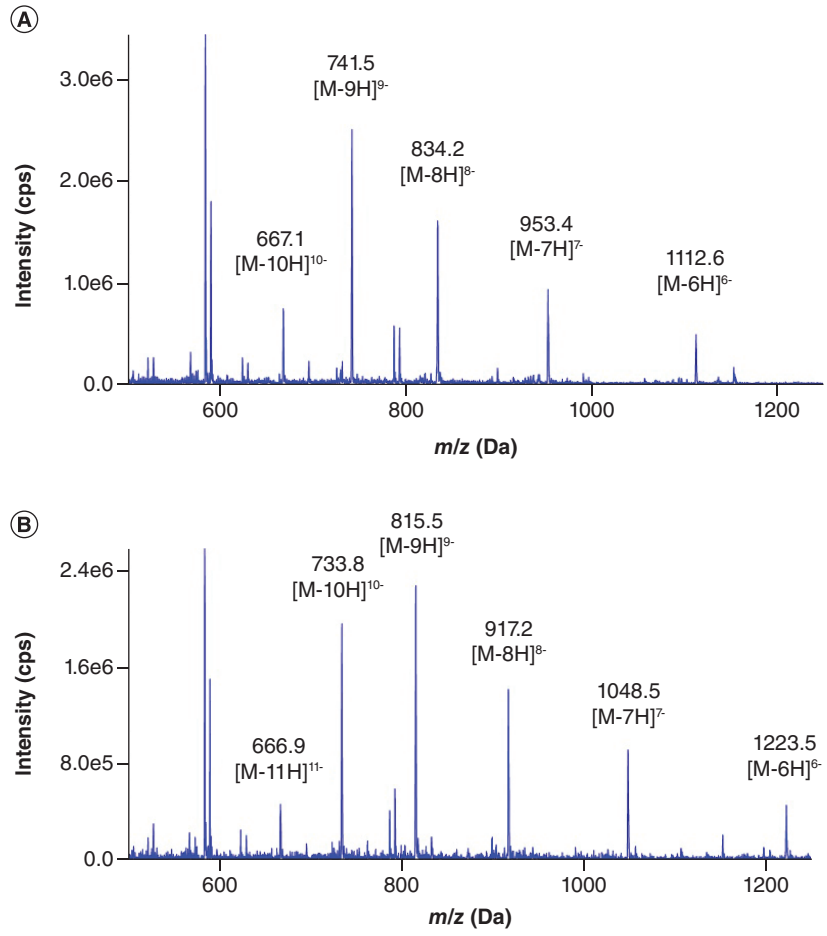

Figure 2. Full-scan single MS spectra. (A) Fomivirsen and **(B)** n + 2 analogue internal standard.

In the second step, a full-length biotinylated capture probe complementary to the sequence of FME was designed and used (Supplementary Table 1). Following the digestion step, a slight excess of probe-coated beads was added to all samples at 65°C. The temperature was gradually cooled to room temperature, allowing the formation of a highly specific double-stranded ASO-probe-bead complex that could be magnetically purified from the matrix. This approach allows the efficient isolation of not only the ASO analyte but also the analogue IS and truncated metabolites.

The hybridization extraction was performed as the third step in a 96-well plate, using a fully automated Kingfisher Flex instrument with a total run time of 30 min, which included two wash steps and a final heat denaturation elution step. To facilitate hybridization extraction, a high-salt buffer was used for the first washing step to remove unbound interferences, while a low-salt buffer was used briefly for the final wash to remove excess salts. Finally, targeted ASOs were released from the capture probe by thermal denaturation at temperatures well above T_m. This three-step approach has been demonstrated to effectively capture and purify targeted ASOs, leading to improved sensitivity and specificity when blood components are coextracted from the Mitra substrate.

Establishment of LC–MS/MS

A full-scan MS spectrum was acquired to obtain parent ion mass-to-charge ratios (m/z) and charge state distribution, with exemplary spectra for all five ASOs presented in Figure 2 & Supplementary Figures 1–3. Among the different charge states observed, the most abundant ions were selected at m/z 741.5 (-9) for FME and m/z 815.5 (-9) for the n + 2 analogue. These precursor ions were found to yield the most intense product ions with optimal signal-to-noise ratio, allowing for sensitive quantitation. The product ion scans of FME and n + 2 analogue are shown in Supplementary Figures 4 & 5, respectively. The observed ions included those corresponding to the DNA bases cytosine (110), guanine (150) and thymine (125), as well as the entire nucleotides cytidine phosphate (304),

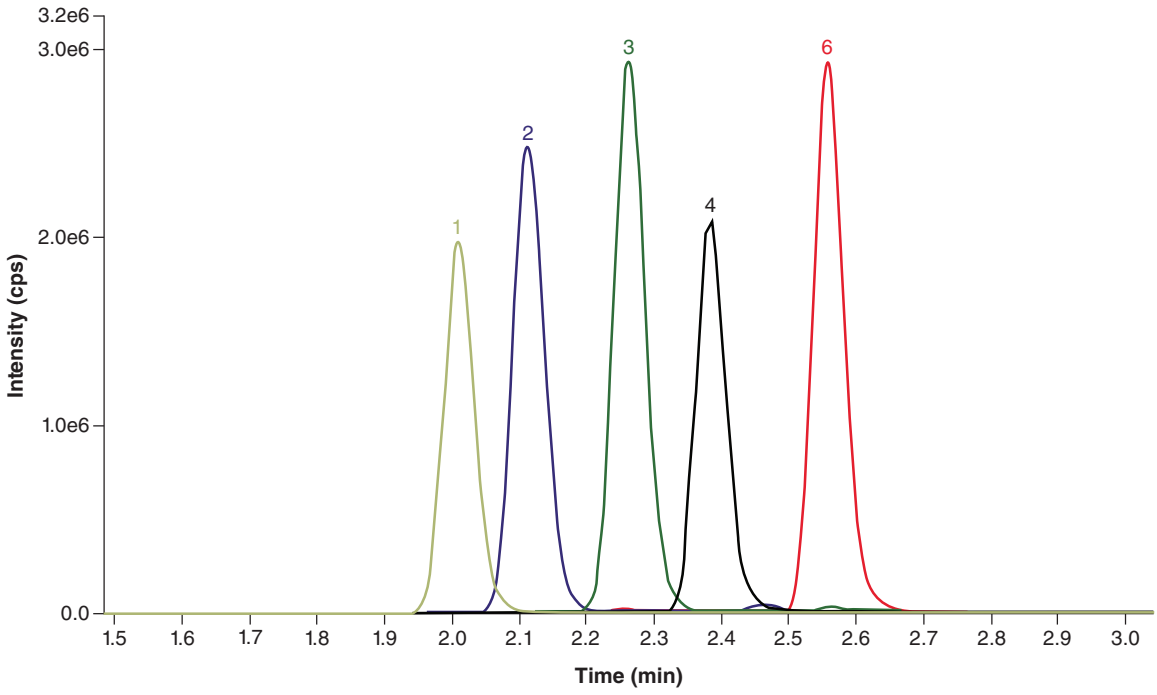

Figure 3. Chromatographic profile of fomivirsen and metabolites under optimal separation conditions. Peak order: 1 = 5′ n-2; 2 = 5′ n-1; 3 = fomivirsen; 4 = 5′ n + 1; 5. = 5′ n + 2 (IS). Mobile phase conditions are detailed in Section 3.3 of "LC–MS/MS Conditions" and Supplementary Table 4.

guanosine phosphate (344) and thymidine phosphate (319). Notably, the spectra of FME, its metabolites and analogues exhibited remarkable similarity, with no discernible product ions that were unique to any of the analytes. While several product ions with potentially higher specificity (e.g., *m*/z 304, 319 and 344) were observed, their low signal intensity limited their usefulness for achieving optimal assay sensitivity. To fine-tune the MRM parameters, the two most abundant fragments at *m*/*z* 79.0 (phosphate) and *m*/*z* 95.0 (phosphorothioate) were evaluated, with *m*/*z* 95.0 found to yield optimal signal-to-noise ratio for FME and n + 2 analogue.

IP-RPLC is a well-established technique that is particularly effective for hydrophilic ASOs, which are otherwise difficult to retain on traditional C_{18} columns due to their multiple negative charges. This separation technique involves the addition of organic alkylamines to the mobile phase to neutralize the negative charges on the ASO backbone, thereby promoting hydrophobic retention resulting in enhanced chromatographic separation. Moreover, the addition of fluorinated alcohols, traditionally HFIP, in the mobile phase can improve chromatographic separation and enhance ionization efficiency, leading to improved sensitivity and specificity [19]. In our study, we evaluated the performance of different alkylamines, including DBA, DMCHA and DIPEA, and their mixtures with HFMIP. Results demonstrated that DBA provided ideal retention and optimal separation resolution for all five ASOs (Supplementary Figure 6A). Conversely, DIPEA resulted in less retention and wider peak profiles (Supplementary Figure 6B), while retention and separation with DMCHA were similar to DIPEA, but with narrower peaks of less tailing (Supplementary Figure 6C). We further investigated the DBA/DMCHA combination for its separation performance using different organic solvents (ACN and MeOH) and fluorinated alcohols (HFIP and HFMIP). The results indicated that ACN provided a lower column back-pressure, allowing a more effective column rinsing by use of a higher flow rate. Although it was reported that MeOH can provide enhanced ionization efficiency relative to ACN, no significant advantage was observed in the tested ASOs with the DBA/DMCHA combination. Therefore, ACN was used in mobile phase B, while a small proportion of MeOH was added in mobile phase A to prevent column performance deterioration resulting from 100% aqueous as the initial mobile phase. Compared with HFIP, HFMIP is ACN-soluble and provided slightly better peak responses for the tested ASOs. Previous studies also support the use of HFMIP over HFIP for analyzing phosphorothioate oligonucleotides [7,43]. Taken together, optimized peak sharpness and separation resolution of FME and its analogues were achieved with a mobile phase consisting of binary alkylamines along with HFMIP and ACN gradient conditions (Figure 3).

We also conducted experiments with different temperature conditions and higher temperatures not only reduce ASO retentions but also adversely affect the column's durability. After a thorough evaluation, we determined that maintaining a column temperature of 60°C offers the optimal balance between ASO retentions and column longevity. This temperature selection allows us to achieve the desired separation and analytical performance while ensuring the column's durability.

Another method challenge encountered was on-column carryover resulting from incomplete elution of ASOs from high-concentration samples. This issue could lead to contamination of subsequent samples, thereby impacting the accuracy and reproducibility of analysis. To mitigate ASO on-column lingering, a ternary pump configuration was implemented using mobile phase C containing 90% ACN. Multiple mobile phase C gradient cycles were used for each injection, with a gradual increase in flow rate up to 1.0 ml/min. With these precautionary measures, carryover was controlled with detection in two blank samples at levels of ~15% and < 5% of the LLOQ following two ULOQ injections.

Method qualification

Sensitivity & linearity

A typical hybridization LC–MS/MS workflow can achieve ASO method detection limits of *ca*. 0.5 ng/ml from a plasma volume of 100 µl. In the current study, since the Mitra substrate only wicks 10 µl of blood, the targeted LLOQ for FME was 5.00 ng/ml with an overall 15-fold extract dilution factor (1.6 pg on-column). The chromatograms in Figure 4 demonstrated a robust response for the LLOQ achieved with a signal-to-noise ratio >10:1.

The primary factor contributing to the high sensitivity was the employment of a capture probe in the hybridization extraction process, resulting in highly selective and reproducible quantitative recovery without matrix effect. This hybridization approach provided an interference-free background that was not achievable with conventional solid-phase extraction methods [7]. Another crucial element was the use of IP-RPLC, which consisted of binary alkylamines and HFMIP with ACN gradient conditions, as this separation effectively resolved any potential isobaric cross-talk interference due to truncated metabolites.

To establish linearity, ten calibrants ranging from 5.00 to 1000 ng/ml were quantitated for each analytical run. The best fit for the standard peak area ratio response versus concentration data was achieved using a weighted ($1/x^2$) linear ($y = mx + b$) least squares regression. Six calibration curves were generated, with the coefficient of determination (R^2) between 0.9921 and 0.9959, indicating consistency in the regression model (Supplementary Table 6).

Precision & accuracy

When analyzing ASOs from Mitra dried blood microsamples, a key challenge is the selection of IS and its point of introduction in the extraction process. In a typical hybridization LC–MS/MS workflow, IS fortification occurs at the end of the extraction and is often an analogue that is immune to probe capture [7]. However, extraction of ASOs from dried blood microsamples without the addition of IS earlier in the process was observed to lead to variability and compromised assay performance. Truncated metabolites cannot serve as ISs due to their presence in the study samples. Instead, the n + 1 or n + 2 analogues of FME are ideal choices since they are not formed *in vivo*. These analogues share similar sequences with FME, ensuring comparable desorption efficiency during the IAE process, identical hybridization with a full-length capture probe, equivalent ionization efficiency and closely matched retention times. They effectively monitor method variability, with the n + 2 analogue exhibiting superior separation resolution from the target analyte compared with the n + 1 analogue, thus reducing isobaric interference in LC–MS. As a result, the n + 2 analogue was selected as the preferred IS and was introduced into the extraction solvent prior to IAE.

Precision and accuracy (P&A) were evaluated with the previously described IS approach using replicate analysis of QC samples at LLOQ (5.00 ng/ml), low (15.0 ng/ml), medium (500 ng/ml) and high (750 ng/ml) concentrations. Precision was determined by calculating the coefficient of variation (%CV) from four replicate QC measurements, while accuracy was expressed as the percent nominal (%nominal), which represented the deviation of the observed sample concentration from its theoretical value. All extracted batches of QC samples met acceptance criteria for precision (%CV ≤ 20.0%, except for LLOQ at ≤ 25.0%) and accuracy (%nominal 80–120%, except for LLOQ at 75–125%). Inter-run precision and accuracy were also within acceptance criteria, as demonstrated in Table 1. These results demonstrate that, by employing an analogue IS with a well-controlled Mitra VAMS sampling technique,

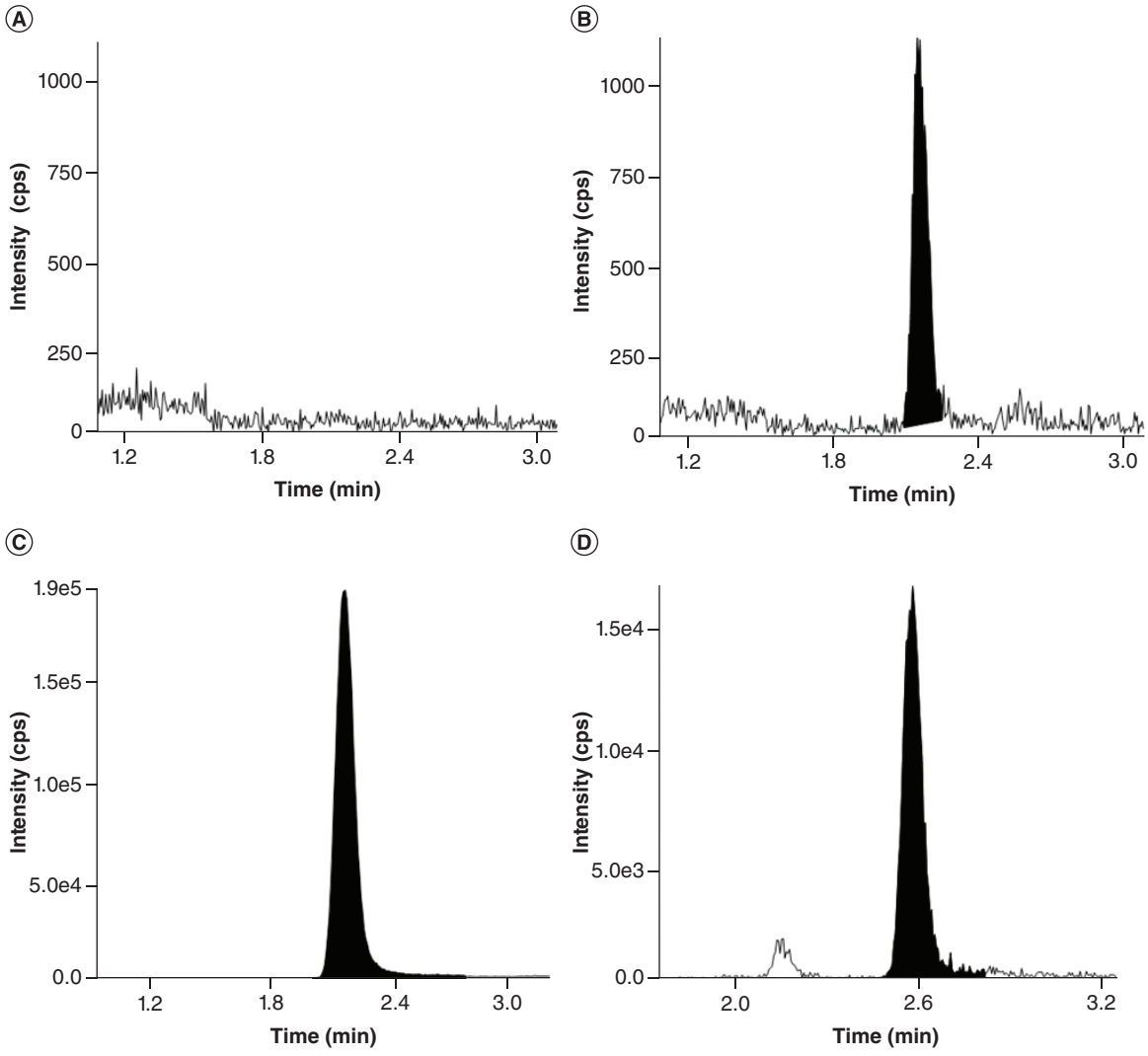

Figure 4. Chromatograms. (A) Extracted blank dried blood Mitra microsamples, **(B)** extracted lower limit of quantification microsample at 5.00 ng/ml, **(C)** extracted upper limit of quantification microsample at 1000 ng/ml and **(D)** n + 2 internal standard response (75.0 ng/ml).

optimized hybridization extraction and fine-tuned LC–MS/MS parameters, excellent P&A can be achieved despite the increase in matrix complexity relative to plasma. In addition to within-range quantitation, P&A was also determined for dilution QC samples using an approach different from that typically applied for wet matrices. In dried blood assays, an aliquot of the primary sample extract is diluted with the primary extract (containing IS) derived from Mitra dried blood microsamples prepared from blank control donors. The results confirmed successful dilution integrity for QCs at twice the ULOQ diluted fivefold (Supplementary Table 7).

Specificity, matrix effect & metabolite interference

Assay specificity was verified by examining the presence of background interference at the retention times and mass transitions of FME in four independent sources of human blood, compared against the LLOQ. Additionally, interference at the retention times and mass transitions of the IS was compared with the mean IS response of evaluable QC samples and nonzero standards used to define the curve. While regulatory guidelines may recommend testing six separate lots, we conducted tests on four lots due to resource constraints and research objectives. Although testing more lots would have been preferable, this study was designed within these limitations, and it showed excellent specificity as no significant interference was detected in any of these four control donors (Supplementary Table 8). Although isotopic cross-talk was observed between FME and the IS due to their similarity in m/z

Table 1. Inter-run precision and accuracy for fomivirsen with Mitra VAMS and hybridization LC–MS/MS analysis.

Run no.	QC LLOQ (5.00 ng/ml)	Low QC (15.0 ng/ml)	Mid QC (500 ng/ml)	High QC (750 ng/ml)
1	4.57	15.7	553	812
	5.01	15.5	570	856
	4.83	14.7	521	844
	5.37	16.7	494	828
2	4.66	15.4	565	813
	5.79	15.5	504	760
	5.16	13.9	518	839
	5.40	14.9	546	860
3	4.93	15.9	553	799
	4.45	16.6	550	807
	5.42	16.2	496	790
	5.01	16.4	481	778
4	5.50	15.7	566	817
	5.83	16.8	556	804
	5.56	15.9	569	760
	5.27	16.9	541	834
5	4.13	15.6	522	737
	4.50	13.4	540	750
	5.82	14.4	515	775
	5.09	15.5	579	842
6	4.83	14.8	524	779
	4.47	16.4	511	795
	4.82	15.7	487	698
	4.13	16.7	489	741
Mean	5.02	15.6	531	797
SD	0.51	0.9	30	42
n	24	24	24	24
%CV	10.1	5.9	5.6	5.2
%Nominal	100.4	104.2	106.2	106.2

CV: Coefficient of variation; LLOQ: Lower limit of quantification; QC: Quality control; SD: Standard deviation; ULOQ: Upper limit of quantification.

ratios and fragmentation patterns [44,45], the impact was negligible as FME and (n + 2) IS were well-resolved chromatographically.

The matrix effect was also evaluated at low and high QC concentrations prepared from four control donors. Each QC level was extracted in triplicate and its concentration was back-calculated against the FME calibration curve. No matrix effect was observed as the %CV of back-calculated concentrations were ≤ 7.5% with a %bias of -9.4–12.8% (Supplementary Table 9).

Nuclease cleavage *in vivo* can generate truncated metabolites (e.g., n-1, n-2 etc.) and, therefore, putative metabolites (including 5′ n-1 and 5′ n-2) were evaluated for potential interference with FME. The metabolites were spiked into blood samples at 20% of the FME concentration, with the parent ASO still expected to be predominant. The results showed a %bias of -1.7–10.7% with a %CV of 4.9% and 5.4% for low and high QC levels, respectively (Supplementary Table 10 & Supplementary Figure 7). The lack of interference can be attributed to the complete chromatographic resolution of FME from closely related analogues, demonstrating the ability to simultaneously quantify both parent ASO and truncated metabolites in a single analytical run (Figure 3).

Recovery & Ht effect

Recovery, which measures the ability to reproducibly and linearly extract an analyte from dried blood Mitra microsamples in consistent yields, was evaluated by comparing extracted QC samples with blank samples spiked postextraction. Consistent recovery is critical as the IS cannot be fortified directly in the dried blood microsample to compensate for variability in extraction yield. For both low and high QC concentration levels, recoveries of 101.4% and 91.2% were achieved, respectively (Supplementary Table 11); IS recovery was 107.1% (Supplementary

Table 2. Impact of blood hematocrit on fomivirsen quantitation.

Hematocrit	Concentration					
	Low QC: 15.0 ng/ml			High QC: 750 ng/ml		
	Mean	%CV	%Bias	Mean	%CV	%Bias
0%	16.5	1.2	10.0	823	2.9	9.7
20%	17.0	0.6	13.3	815	4.7	8.7
40%	16.8	1.2	12.0	804	5.2	7.2
66%	16.7	2.4	11.3	803	4.5	7.1

QC samples were measured against a calibration curve with 40% hematocrit.
CV: Coefficient of variation; QC: Quality control.

Table 12). These assessments support the effectiveness of the selected n + 2 analogue IS for compensating potential matrix effects, suggesting general applicability for other ASOs and biological matrices. During the evaluation of different blood Ht levels, the recovery of FME using IAE was consistent from 0% (plasma) to 66% Ht. The back-calculated concentrations of FME from both low and high QC samples prepared at different Ht values (0%, 20%, 40% and 66%) were measured against a calibration curve with 40% Ht, the latter representative of the healthy population. Results demonstrate a low bias range of 7.1–13.3% and a %CV < 5.8% (Table 2).

Stability

The stability of FME dried blood Mitra microsamples was evaluated to determine the allowed time frame within which samples must be analyzed. To this end, FME microsamples at low and high QC levels were stored at room temperature for a period of 115 days, after which FME concentrations were measured against freshly prepared microsamples dried 24 h in the presence of desiccant. As no apparent change in FME response was noted in comparison to 24-h microsamples, it was concluded that neither age-related extractability nor degradation during this 115-day storage period occurred. The %nominal was within 94.8–99.1%, the %CV < 5.8% and %deviation against freshly prepared QC microsamples were -6.3% (low-QC) and -5.6% (high-QC) as summarized in Supplementary Table 13. The stability results provided strong evidence supporting the reliability and stability of Mitra as a suitable microsampling substrate for ASO determination, particularly in situations where immediate sample processing is not feasible, such as remote collection of clinical samples. However, it is crucial to recognize and address the potential challenges that may arise from sample storage under extreme conditions, including high humidity and temperature. To mitigate these challenges and ensure optimal sample integrity, it is imperative to emphasize the significance of adhering to proper handling and storage protocols in future studies. By doing so, the potential impact of extreme storage conditions can be mitigated, thereby maintaining the integrity of collected samples.

Conclusion

In this novel investigation, a dried blood Mitra VAMS workflow was successfully combined with hybridization LC–MS/MS to accurately quantify the ASO analyte, FME, despite the complexity of the primary extract resulting from IAE using buffers and detergents (i.e., all blood components were desorbed from the substrate and solubilized). A high desorption efficiency of FME from Mitra microsamples was achieved, providing consistent recovery across the calibration range independent of blood Ht level. This novel workflow for dried blood microsampling applied to ASOs has important implications for both preclinical and clinical drug development. To our knowledge, there have been no confirmed reports detailing a bioanalytical workflow that supports the accurate and selective determination of ASO concentrations from dried blood microsamples. Given the convenience and simplicity of blood microsampling for decentralized clinical trials involving patient self-sampling, it is expected that other closely related ASOs (e.g., truncated metabolites) will be investigated using the workflows described herein. Furthermore, dried blood microsampling allows for improved alignment between preclinical and clinical sample collection methods since the former typically utilizes capillary microsampling, which is not amenable to remote sample collection.

Summary points

- The first successful application of volumetric absorptive microsampling coupled with hybridization LC–MS/MS for the quantitative bioanalysis of antisense oligonucleotides from dried blood microsamples is described.
- The work demonstrated the feasibility of using an n + 2 analogue as an internal standard, allowing the successful tracking of assay variability and ensuring accuracy and precision meet acceptance criteria.
- The use of binary alkylamine and 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol mobile phase components combined with an acetonitrile gradient to separate fomivirsen and related metabolites, allowing multicomponent analysis in a single assay is also described.
- The developed microsampling method coupled with hybridization LC–MS/MS was fully qualified and provides a valuable workflow for supporting future antisense oligonucleotide drug research leveraging the collection of dried blood microsamples.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2023-0092

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