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A validated capillary microsampling liquid chromatography–tandem mass spectrometry method for quantification of antisense oligonucleotides in mouse serum

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Background: Capillary microsampling (CMS) has been used for quantitative bioanalysis of small molecules; however, there is no report of applying this technique in the bioanalysis of antisense oligonucleotides (ASOs). **Results:** A CMS LC–MS method was successfully developed and validated for the quantification of ASO1 in mouse serum. The validated method was applied in a safety study in juvenile mice. Equivalent performance between CMS samples and conventional samples was demonstrated in the mouse study. **Conclusion:** This work is the first to report using CMS for liquid chromatography–tandem mass spectrometry quantitative bioanalysis of ASOs. The validated CMS method was successfully applied to support good laboratory practice safety studies in mice and the CMS strategy has subsequently been applied to other ASOs.

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Microsampling techniques, which typically collect less than 100 µl of blood, have been gaining increasing interest in drug research and development for the quantification of drugs, their metabolites or endogenous substances in liquid biological samples (e.g., blood, plasma or serum) [1,2]. One major advantage of microsampling is its significantly lower blood sample collection volume compared with conventional methods (for which typically 300 µl of blood is collected at each time point in a rat study). Thus, for studies in small animals (e.g., rats and mice), microsampling is desirable from an ethical perspective, as it can significantly reduce the number of animals and therefore be more compliant with the ‘3Rs’ (reduce, refine and replace). Juvenile animal testing is on the rise for pediatric drug development and, with the strict constraints on sample collection volumes in juvenile animals, microsampling becomes especially attractive. Commensurate with the reduced number of dosed animals, the required amount of total drug will be reduced accordingly, resulting in further cost reductions for studies.

Quantitative bioanalysis of drug candidates is an essential component of drug discovery and development. It provides critical information on the systemic exposure of drug candidates and, therefore, enables the evaluation and understanding of the toxicokinetic (TK) and pharmacokinetic (PK) properties of drug candidates. Commonly used microsampling techniques for quantitative bioanalysis include dried blood spot (DBS) and volumetric absorption microsampling (VAMS) [1,2]. These techniques involve the collection of whole blood samples and storing and using the samples in dried blood form for analysis. DBS and VAMS samples are convenient for sample collection and storage. There have been reliable and robust workflows developed and applied for the bioanalysis of these samples [1–4]. One main concern in using these types of dried blood microsampling techniques is the regulatory acceptance of blood concentration data. Traditionally, plasma or serum samples are used for the quantification of drug concentrations, and the generated plasma/serum drug concentration data are then used to determine PK/TK parameters. The blood concentration data generated from DBS or VAMS samples often need to be converted to

plasma concentrations for data interpretation and decision-making [4]. Regulatory agencies recommend conducting correlative studies between DBS microsampling and traditional sampling during drug development to establish their concordance [2,5]. In contrast, capillary microsampling (CMS) is another type of microsampling technique that uses a capillary micropipette to collect a small volume of blood via capillary force [6–11]. The collected blood in capillary tubes can be further processed into plasma or serum for analysis. The plasma/serum collected by CMS is the same matrix as conventional sampling; therefore, there is no need to convert drug concentrations or conduct a bridging study. CMS has been applied to regulated toxicity studies for small-molecule compounds under good laboratory practice (GLP) environments [8,9].

Antisense oligonucleotides (ASOs) are rapidly growing as a new class of therapeutics in drug research and development for the treatment of various diseases [12,13]. An ASO is a synthetic single-stranded oligonucleotide (typically around 20 base pairs in length) with a complementary sequence to a target mRNA. By specifically binding to the target mRNA via Watson-Crick base pairings, ASOs can modulate gene expression via various mechanisms (e.g., RNase H-mediated cleavage of mRNA or splicing modulation) and, therefore, modify the target protein expression and achieve the desired disease-modifying profile. More than ten ASO drugs had been approved by the US FDA and/or EMA by 2022 [14] and hundreds of ASO drug candidates are currently under preclinical and clinical development [15].

To support safety studies of ASO drug candidates in mice, microsampling is desired due to the reduced blood collection volume. This advantage is particularly important when conducting studies in juvenile mice, which necessitate stricter constraints on sample volumes. Therefore, to support safety studies of ASO drug candidates in juvenile mice, CMS was selected as the microsampling technique in the current work; however, two major challenges remained. First, although equivalent performance between CMS and conventional sampling has been demonstrated for small-molecule compounds [6,7], such equivalence has not been established for ASOs; there is no report of using CMS for evaluating exposure and PKs/TKs of ASOs in safety studies. Second, the significantly lower sample volume (for mouse studies, typically only 5 μ l of plasma or serum is available for analysis) requires a highly sensitive bioanalytical assay for the quantification of the ASO analyte. Traditionally, hybridization-based immunoassays, such as hybridization enzyme-linked immunosorbent assay (HELISA), are commonly used assay formats for the quantification of ASOs [16,17]. HELISA has the advantages of high sensitivity and good assay throughput. However, HELISA often lacks the specificity to distinguish the intact ASO drug and its truncated metabolites (e.g., N-1, N-2 metabolites) [16,18]. In addition, HELISA requires significant method development, especially for the quantification of tissue samples. LC–MS/MS methods have high specificity, wide assay range and relatively fast method development [19–22]. However, one major limitation of the traditional liquid–liquid extraction or solid-phase extraction-based LC–MS/MS assay is its significantly lower sensitivity compared with hybridization immunoassays. Recently, a hybridization LC–MS/MS methodology was developed for achieving highly sensitive and specific quantification of ASOs in biological samples [23–28]. This methodology utilized a DNA probe with a sequence complementary to the target ASO as the capture probe to specifically hybridize to the ASO analyte, thus, it can generate very clean sample extracts with almost complete recovery and, as a result, achieved much-improved sensitivity (below 1 ng/ml). The hybridization LC–MS/MS methodology has also been successfully applied to the quantification of double-stranded oligonucleotides, such as siRNA, by using a peptide nucleic acid capture probe [29].

In this work, to overcome the sensitivity challenge, hybridization LC–MS/MS methodology was applied to achieve highly sensitive quantification of ASO1 in mouse serum for both CMS and conventional sampling samples. The developed method was successfully validated for the quantification of ASO1 in mouse serum over the range of 1.00–500 ng/ml using 20- μ l samples. To investigate whether CMS can be used equivalently to conventional sampling for the estimation of ASO exposure, an exploratory repeat-dose range-finding study was conducted for ASO1 in juvenile mice. Two sets of samples (CMS serum samples and conventional serum samples) from the same time points were collected and analyzed by the hybridization LC–MS/MS method, and concentration results were compared. To the best of our knowledge, this is the first time CMS was evaluated for LC–MS/MS quantitative bioanalysis of an ASO and was successfully applied to PK/TK evaluation in a safety study. The validated CMS LC–MS/MS method has been used to support GLP safety studies of ASO1 in mice. The CMS methodology has also been applied to other ASOs.

Materials & methods

Chemicals, reagents, materials & instrumentation

The analyte ASO1 (a 20-mer ASO) and the analogue internal standard (IS) ASO2 were proprietary compounds obtained from Biogen (MA, USA) and its collaborator. ASO1 is a 20-mer ASO (molecular weight: 7.1 kDa) with mixed backbone. ASO2 is a 20-mer ASO (molecular weight: 7.9 kDa) with a phosphorothioate backbone. A 5' biotinylated DNA probe with full sequence reverse complementary to AS1 was obtained from Integrated DNA Technologies (IA, USA).

Methanol (MeOH), acetonitrile (ACN), N,N-dimethylcyclohexylamine (DMCHA), ethylenediaminetetraacetic acid (EDTA), 1,1,1,3,3,3-hexafluoro-2-methyl-2-propanol (HFMIP), Tween 20, tris(hydroxymethyl)aminomethane (Tris), 10 N sodium hydroxide (NaOH), sodium chloride (NaCl) and DL-dithiothreitol (DTT) were obtained from MilliporeSigma (MA, USA). Dynabeads MyOne streptavidin C1, proteinase K and blocker BSA in TBS (10×) concentrate (100 mg/ml) were purchased from Thermo Fisher Scientific (MA, USA). Clarity OTX Lysis-Loading Buffer v 2.0 was obtained from Phenomenex (CA, USA). LoBind Eppendorf 1-ml round-bottom 96-well plates and 1.5-ml LoBind tubes were purchased from Eppendorf (CT, USA). Control mouse serum and rat plasma (K₂EDTA) were obtained from BioIVT (NY, USA). Capillary tubes (5- μ l end-to-end pipettes) were purchased from Vitrex Medical (Herlev, Denmark).

The LC system used for separation was a Shimadzu Nexera X2 UHPLC system (Shimadzu, MD, USA) equipped with three LC-30AD pumps. Mass spectrometric detection was performed on a Sciex TripleQuad 6500+ mass spectrometer (Sciex, MA, USA) with Analyst software v 1.6.3. Hybridization sample preparation was conducted using a KingFisher Flex Purification System (Thermo Fisher Scientific).

Preparation of calibration standard & quality control samples

The stock solution of ASO1 (9.3 mg/ml in water) was diluted in ASO diluent (25 mM HFMIP, 15 mM DMCHA, 100 μ M EDTA and 0.05% rat plasma K₂EDTA in water:ACN 90:10% v/v) to obtain the intermediate solution (1000 μ g/ml), which was further diluted to prepare a calibration standard (STD) spiking solution at 50,000 ng/ml and quality control (QC) spiking solutions at 37,500, 100,000 and 375,000 ng/ml. All ASO solutions were stored at approximately 4°C. The IS stock solution of ASO2 was prepared at 500 μ g/ml in water, which was further diluted to prepare the IS working solution (ISWS) at 100 ng/ml in ASO diluent. The ISWS was prepared and used fresh daily.

Ten levels of STDs from 1.00 ng/ml, the lower limit of quantitation (LLOQ), to 500 ng/ml, the upper limit of quantification (ULOQ), were prepared by serial dilutions of the STD spiking solution in blank mouse serum. Similarly, QC samples, including LLOQ QC at 1.00 ng/ml, low QC at 3.00 ng/ml, geometric mean (GM) QC at 22.5 ng/ml, mid-QC at 250 ng/ml and high QC at 375 ng/ml were prepared in mouse serum using the QC spiking solution prepared at 37,500 ng/ml and dilution QC at 4250 ng/ml using the QC spiking solution at 100,000 ng/ml. A dilution QC at 42,500 ng/ml was prepared directly from the intermediate solution. CMS QCs were prepared at concentrations tenfold higher than the corresponding conventional QCs at 10.0, 30.0, 225, 2500 and 3750 ng/ml. CMS QCs were prepared by dipping the tip of a 5- μ l end-to-end pipette into the serum sample and waiting until the end-to-end pipette was full. The CMS QC sample was then transferred into a clean 1.5-ml Eppendorf LoBind tube for storage. All biological samples were stored at approximately -80°C.

Sample preparation

Streptavidin magnetic beads (15 μ l per sample) were washed three times and resuspended in binding and washing buffer (5 mM Tris, 1 M NaCl, 0.5 mM EDTA and 0.05% Tween 20 in water). After incubation with capture probes (~0.5 nmol probe per 100 μ l of beads) at room temperature for 1 h, the coated beads were washed three times and resuspended to the original volume using binding and washing buffer.

For hybridization sample extraction, in each well of a 96-well plate, the following were added: 20 μ l of serum sample, 100 μ l of Clarity OTX Lysis-Loading Buffer, 180 μ l of digestion buffer (100 mM Tris, 250 mM NaCl, 10 mM EDTA and 10 mM DTT in water, prepared fresh daily) and 20 μ l of proteinase K (20 mg/ml). The plate was briefly centrifuged, mixed and incubated for at least 2 h at 65°C; 400 μ l of capture buffer (10 mM Tris, 500 mM NaCl, 1 mM EDTA and 0.05% Tween 20 in water) and 15 μ l of coated streptavidin beads were then added to the samples. The sample plate was mixed for at least 90 min at room temperature and then washed twice with 500 μ l of capture buffer, and once with 500 μ l of 10 mM Tris, 50 mM NaCl, 1 mM EDTA and 0.05% Tween 20 in water at room temperature. The elution was conducted by adding 100 μ l of ASO diluent and incubating at

95°C for 20 min; 25 µl of ISWS was then added and mixed well. The sample extract was transferred into a LoBind Eppendorf plate, centrifuged at $4612 \times g$ for 5 min and then submitted for LC–MS/MS analysis.

For CMS samples, 45 µl of blank mouse serum was added to the tubes containing the end-to-end pipette filled with 5 µl serum samples. The samples were centrifuged at $2000 \times g$ for 5 min, vortexed for 1 min and then centrifuged again at $2000 \times g$ for 5 min. The resulting diluted samples (tenfold dilution) were then used for further sample preparation. All CMS samples were prediluted tenfold with blank mouse serum using this approach prior to further dilution and/or hybridization sample extraction.

UHPLC–MS/MS conditions

An Acquity UPLC Oligonucleotide BEH C18 (2.1 × 50 mm, 1.7 µm particle size; Waters, MA, USA) coupled with an Acquity BEH C18 guard column (VanGuard 2.1 × 5 mm, 1.7 µm particle size) was used for chromatographic separation. The column temperature was set at 60°C and the autosampler temperature was set at 4°C. The mobile phases used were water (mobile phase A), ACN/water 90/10 (mobile phase B) and 125 mM HFMIP, 75 mM DMCHA in ACN/water 50/50 (mobile phase C). The LC method was adopted from a previous publication with slight modifications [30]. Briefly, the total flow rate was 0.50 ml/min with 0.40 ml/min from mobile phase A/B and 0.10 ml/min from mobile phase C. The gradient started from 0% B and increased to 25% B in 3 min. The column then was washed with multiple gradient cycles of 25–100% B using 0.60 ml/min of mobile phase A/B. After the wash, the column was equilibrated at the initial condition for 2 min. The total run time was 7 min. The injection volume was validated between 10 and 15 µl.

The mass spectrometer was operated in electrospray negative ionization mode using the following optimized operating parameters: ion spray voltage: -4500 V; temperature: 550°C; curtain gas: 40 units; ion source gas 1: 80 units; ion source gas 2: 60 units; and dwell time: 250 and 50 ms; declustering potential: -75 and -65 V; collision energy: -130 and -150 eV for ASO1 and the IS, respectively. Multiple reaction monitoring (MRM) transitions monitored were m/z 709.7 → 95 for ASO1 and m/z 879.5 → 95 for the IS, ASO2.

TK study in mice

A repeat-dose range-finding study was conducted for ASO1 in juvenile CD-1 mice. Mice were treated with 25, 75 and 150 mg/kg ASO1 once every other week on postnatal days (PNDs) 10, 24, 38 and 52 by subcutaneous injection. For TK evaluation, following administration on PNDs 10 and 52, blood samples ($n = 3$, sex was 1 male and 2 females or 2 males and 1 female at each time point) were collected at 0, 1, 2, 4, 8 and 24-h post-dose, and a single time point on PNDs 24 and 38 (1-h post-dose). Approximately 32 µl (PND 10) or 64 µl (PNDs 24, 38 and 52) of CMS blood samples were collected by jugular venipuncture using hematocrit tubes without anticoagulant. For terminal samples (8- and 24-h timepoints) at PND 52, two sets of samples were collected: a CMS sample (~64 µl by jugular venipuncture) prior to necropsy procedures and a conventional terminal sample (~0.25 ml collected in a serum separator tube) via the abdominal aorta following isoflurane anesthesia. CMS blood samples were processed to serum as follows: blood samples were allowed to clot for a minimum of 30 min following collection and then centrifuged at $1500 \times g$ at 4°C for 15 min. The resultant serum was separated, and duplicate aliquots of 5 µl each were transferred to a 5-µl Vitrex end-to-end pipette. The end-to-end pipette was put into an appropriately labeled polypropylene tube and stored in a freezer at approximately -80°C.

Results & discussion

Method development & qualification

Based on the Q1 MS full scan of ASO1, the ions at m/z 709.7 (-10 charged) and 788.7 (-9 charged) were the two most abundant ions and were chosen as the precursor ions for further optimization of MRM transitions. The product ion at m/z 95 (phosphorothioate ion) is the most commonly used product ion for oligonucleotides with phosphorothioate backbones for its good sensitivity [26]. For the analysis of ASO1, m/z 95 was also chosen as the product ion because of its best sensitivity. After further comparison of the sensitivity and selectivity of m/z 709.7 and 788.7 as the precursor ions, the MRM transition of m/z 709.7 → 95 was selected as the final method for the detection of ASO1. LC separation was achieved using ion-pair chromatography with the commonly used alkylamine (15 mM DMCHA) and fluoroalcohol (25 mM HFMIP) combination, which was adapted from previously published methods with slight modification [26,30].

Due to the limited sample volume, 20 µl of mouse serum sample (or diluted serum sample for CMS samples) was used for the assay. To maximize the sensitivity of the assay, the hybridization method was chosen for extraction

Table 1. Comparison of assays for analysis of ASO1 regular mouse serum quality controls and capillary microsampling mouse serum quality controls.

	LLOQ QC 1.00 ng/ml	Low QC 3.00 ng/ml	Mid QC 250 ng/ml	High QC 375 ng/ml	Low QC (CMS) [†] 3.00 ng/ml	High QC (CMS) [†] 375 ng/ml
	1.05	2.97	248.59	407.89	2.87	376.41
	1.15	3.19	229.04	346.05	3.22	380.08
	1.02	2.97	273.16	394.13	2.87	426.84
	1.07	3.04	238.68	381.09	3.04	344.35
Mean	1.07	3.04	247.37	382.29	3.00	381.92
SD	0.05	0.11	18.96	26.52	0.17	33.98
n	4	4	4	4	4	4
%CV	5.0	3.5	7.7	6.9	5.6	8.9
%Nominal	107.0	101.4	98.9	101.9	100.0	101.8

Calibration curve was prepared using conventional mouse serum samples.
[†] CMS QCs were prepared at 10× higher of the nominal concentrations at 30.0 and 3750 ng/ml, respectively, and were prediluted tenfold for analysis.
 CMS: Capillary microsampling; CV: Coefficient of variation; LLOQ: Lower limit of quantification; QC: Quality control; SD: Standard deviation.

to achieve highly effective and specific purification of the samples. A DNA probe with a 100% complementary sequence to ASO1 was used as the capture probe. The hybridization extraction generated a clean sample extract (minimal matrix effect) with almost complete (~90%) recovery of the ASO analyte from the serum sample. As a result, an LLOQ of 1.00 ng/ml was achieved using only 20 µl of mouse serum. For CMS samples, considering the tenfold dilution used for the predilution of the samples, the final assay sensitivity was 10.0 ng/ml, which was acceptable to support the safety study in mice.

The developed method was first tested and qualified using regular serum STD and QC samples for curve linearity, accuracy, precision, recovery, matrix effect, dilution integrity and stability. The results of all tests passed the acceptance criteria, demonstrating the good performance of the assay for regular serum samples. The method was then evaluated for the analysis of CMS samples using CMS QCs. The accuracy, precision and stability of CMS QCs were evaluated against a calibration curve prepared using regular serum STDs, and the results were compared with the results generated from regular QCs. As shown in Table 1, all CMS QCs (low and high) in the run passed the acceptance criteria of within ±15% of nominal concentrations and the results were comparable to the regular QCs. The CMS stability QCs (6 days at -80°C) also passed the acceptance criteria. These results showed that the assay had good performance in the analysis of CMS samples. The optimized method was then moved forward for validation.

Method validation

The method was validated for the quantification of ASO1 in both regular and CMS serum samples following the US FDA Guidance for Industry-Bioanalytical Method Validation [5] and the EMA Guideline on Bioanalytical Method Validation [31]. The validation was fully compliant with GLP. Standard curve linearity, sensitivity (LLOQ), selectivity, accuracy and precision (A&P), matrix effect, recovery, dilution integrity and stability were evaluated using regular serum samples. For CMS samples, A&P and long-term storage stability were evaluated.

Curve linearity & A&P

Ten levels of STDs ranging from 1.00 ng/ml to 500 ng/ml of ASO1 in mouse serum were analyzed in duplicate to establish the linearity of the calibration curve. A quadratic $1/x^2$ weighted regression model ($y = ax^2 + bx + c$) was found to provide the best statistical fit with the coefficient of determination (R^2) ≥ 0.9931 in all seven validation runs extracted with a calibration curve. The within-run and between-run A&P of the method were assessed for QCs in mouse serum at four concentration levels in three A&P runs. Table 2 summarizes the A&P data. Based on the four levels of analytical QCs (low, GM, mid and high), the within-run accuracy (%bias) was -7.5–11.0%, the precision (coefficient of variation [CV]) was 2.9–6.8%, and the between-run A&P were -0.8–4.0% and 6.2–8.4% CV, respectively, all well within the 15% acceptance criteria. The A&P of the method were also assessed for CMS QCs in mouse serum in one A&P run. CMS QCs were quantified against a calibration curve prepared using conventional serum STDs. The within-run A&P were -5.3–3.5% and 2.7–7.1% CV, respectively (Table 3). These

Table 2. Within-run and between-run accuracy and precision of ASO1 quality controls in mouse serum.

		LLOQ QC 1.00 ng/ml	Low QC 3.00 ng/ml	GM QC 22.5 ng/ml	Mid QC 250 ng/ml	High QC 375 ng/ml
Within-run	Mean ± SD (ng/ml)	1.05 ± 0.0709	3.05 ± 0.0952	22.7 ± 0.745	241 ± 7.92	347 ± 23.7
	%CV	6.8	3.1	3.3	3.3	6.8
	%bias	5.0	1.7	0.9	-3.6	-7.5
	n	6	6	6	6	6
	Mean ± SD (ng/ml)	1.01 ± 0.0655	2.99 ± 0.106	21.5 ± 0.615	240 ± 7.24	375 ± 15.3
	%CV	6.5	3.5	2.9	3.0	4.1
	%bias	1.0	-0.3	-4.4	-4.0	0.0
	n	5	6	6	6	6
	Mean ± SD (ng/ml)	1.05 ± 0.0589	3.33 ± 0.0178	24.8 ± 1.63	263 ± 16.7	405 ± 23.5
	%CV	5.6	5.3	6.6	6.3	5.8
Between-run	Mean ± SD (ng/ml)	1.04 ± 0.0639	3.12 ± 0.196	23.0 ± 1.76	248 ± 15.4	376 ± 31.5
	%CV	6.1	6.3	7.7	6.2	8.4
	%bias	4.0	4.0	2.2	-0.8	0.3
	n	17	18	18	18	18

CV: Coefficient of variation; GM: Geometric mean; LLOQ: Lower limit of quantification; QC: Quality control; SD: Standard deviation.

Table 3. Within-run accuracy and precision of ASO1 quality controls in capillary microsampling mouse serum.

	LLOQ QC 1.00 ng/ml	Low QC 3.00 ng/ml	GM QC 22.5 ng/ml	Mid QC 250 ng/ml	High QC 375 ng/ml
	1.11	2.99	21.3	240	405
	0.943	2.99	20.9	262	401
	1.10	3.11	21.8	248	373
	1.06	3.07	20.4	259	412
	1.16	2.79	21.9	239	355
	0.987	2.56	21.6	245	380
Mean	1.06	2.92	21.3	249	388
SD	0.0814	0.207	0.578	9.66	22.0
%CV	7.7	7.1	2.7	3.9	5.7
%bias	6.0	-2.7	-5.3	-0.4	3.5
n	6	6	6	6	6

Calibration curve was prepared using conventional mouse serum samples.

CMS QCs were prepared at 10× higher of the nominal concentrations at 10.0, 30.0, 225, 2500 and 3750 ng/ml, respectively, and were prediluted tenfold for analysis.

CMS: Capillary microsampling; CV: Coefficient of variation; GM: Geometric mean; LLOQ: Lower limit of quantification; QC: Quality control; SD: Standard deviation.

results demonstrated that the method was accurate and precise for the analysis of ASO1 in mouse serum over the dynamic range of 1.00–500 ng/ml for regular serum samples, as well as CMS serum samples.

Specificity/selectivity

The specificity of the assay was evaluated using six different normal lots and one hemolyzed lot of mouse serum. For the different lots of blank mouse serum samples, no significant interfering peaks were detected at the retention time of ASO1 or the IS, indicating good specificity of the assay. Representative LC–MS/MS chromatograms of ASO1 and the IS in blank mouse serum, blank mouse serum spiked with IS only, LLOQ and ULOQ samples are presented in Figure 1. In addition, assay selectivity was assessed by quantifying LLOQ samples (1.00 ng/ml, n = 3 for each lot) prepared in the six different normal lots and one hemolyzed lot of mouse serum. All three hemolyzed LLOQ samples and 17 of 18 normal LLOQ samples passed the ±20% acceptance criteria. Only one normal LLOQ sample was -20.7% bias of the nominal concentration, which was just slightly outside the ±20% acceptance criteria. The average bias was -4.3% and the CV was 8.4%, which further demonstrated the good selectivity of the method.

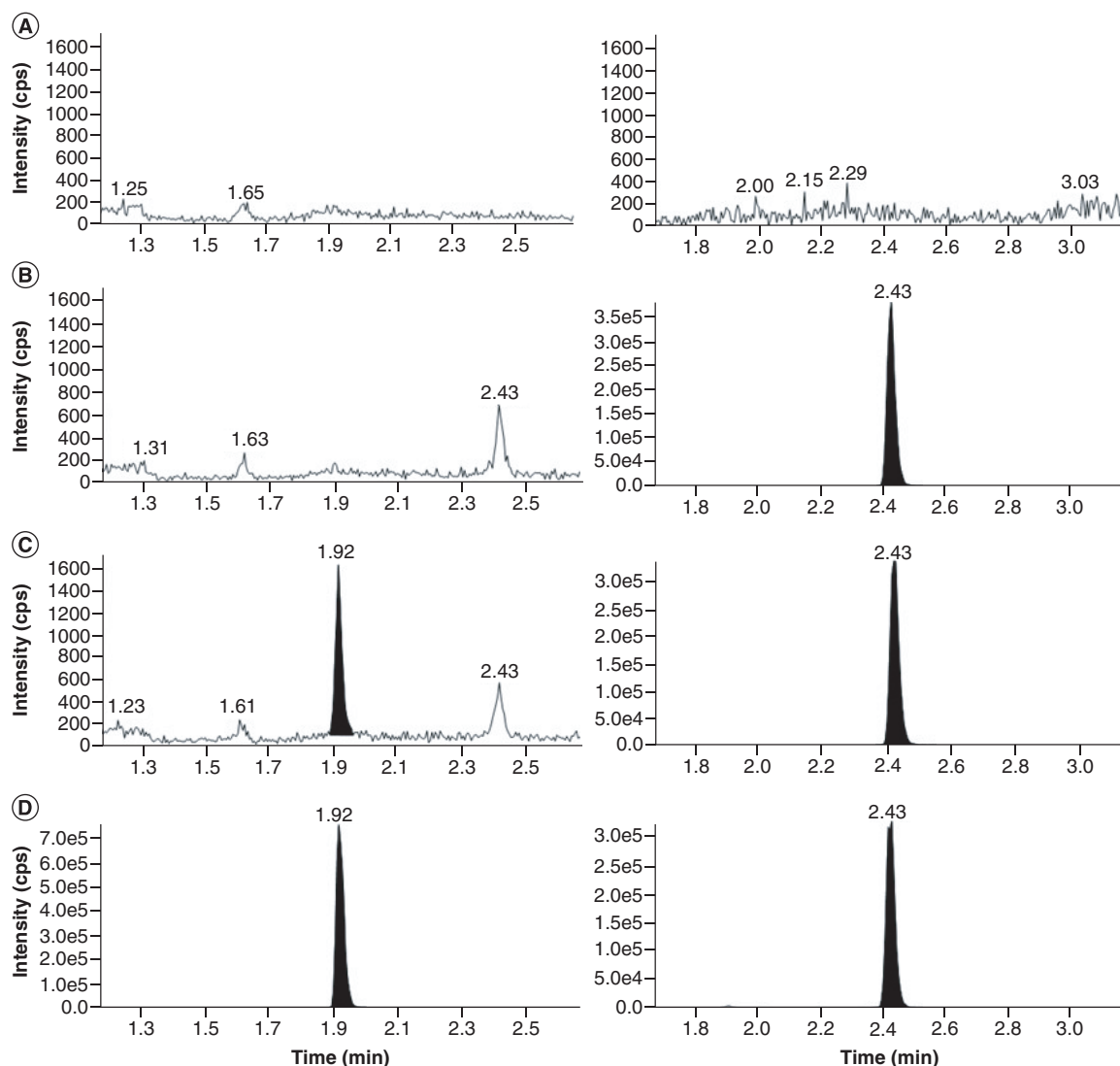


Figure 1. Representative LC–MS/MS chromatograms of (left) ASO1 and internal standard (right) ASO2 in (A) blank mouse serum, (B) blank mouse serum spiked with internal standard only, (C) LLOQ (1.00 ng/ml) and (D) ULOQ (500 ng/ml) in mouse serum.

LLOQ: Lower limit of quantification; ULOQ: Upper limit of quantification.

Lower limit of quantification

The sensitivity (LLOQ) of the assay in mouse serum was assessed using LLOQ QCs (1.00 ng/ml) in the A&P runs. The signal-to-noise ratios of the LLOQ samples were all >5 (as demonstrated by the representative chromatogram in Figure 1). The intrarun and interrune A&P at 1.00 ng/ml were all within the acceptance criteria of 20% (Table 2), demonstrating the establishment of LLOQ at 1.00 ng/ml in mouse serum. For CMS samples, the intrarun accuracy (%bias) was 6.0% and the precision (%CV) was 7.7%, all well within the 20% acceptance criteria (Table 3), which also demonstrated the establishment of LLOQ. The CMS serum samples were diluted tenfold before hybridization extraction, so the actual LLOQ for the measurement of CMS samples was 10.0 ng/ml.

Matrix effect

The matrix effect, expressed as matrix factor (MF), was evaluated using six different lots of normal mouse serum and one lot of hemolyzed serum. The MF of the analyte was determined by calculating the ratio of the analyte response area in mouse serum extract spiked postextraction to the analyte response in the ASO diluent solution without matrix. The MF of the IS was similarly calculated. The seven different lots of mouse serum were evaluated

in triplicate for the MF at 3.00 and 375 ng/ml. The MF of the analyte at 3.00 and 375 ng/ml were 0.96–1.09 and 0.95–1.09, respectively, and the MF of the IS was 0.94–1.05. The IS-normalized MF at 3.00 and 375 ng/ml were 0.95–1.05 and 0.97–1.07, with %CV at 2.9% and 2.3%, respectively, indicating minimal matrix effect of the assay on the analysis of the analyte.

Recovery

The extraction recovery of ASO1 was evaluated in six replicates at four levels of QC (low QC: 3.00 ng/ml, GM QC: 22.5 ng/ml, mid-QC: 250 ng/ml and high QC: 375 ng/ml). The recovery was calculated by comparing the response ratios in mouse serum QC samples with those blank serum samples spiked with the analyte postextraction. The extraction recovery of ASO1 at low, GM, mid and high QCs was 92.1%, 85.5%, 90.1% and 89.8%, respectively, indicating good and consistent extraction of ASO1 from mouse serum across the assay range.

Stability

The short-term and long-term solution stability of ASO1 were evaluated. ASO1 was stable in ASO diluent for at least 23.7 h at room temperature ($\sim 22^{\circ}\text{C}$) and 196 days at approximately 4°C . The short-term, freeze–thaw and long-term stability of ASO1 in mouse serum were evaluated ($n = 6$) at low QC (3.00 ng/ml) and high QC (375 ng/ml). ASO1 was stable in mouse serum for at least 24.2 h at room temperature (22°C), after 4 freeze–thaw cycles and 91 days at -80°C . The long-term stability of ASO1 in CMS serum samples was also tested and the stability was established for at least 91 days at -80°C . Autosampler stability was established for at least 137.4 h at 4°C .

Dilution integrity

The dilution integrity of the assay was evaluated at dilution factors 10 and 100. Dilution QC samples were extracted with tenfold dilution (dilution QC1: 4250 ng/ml in mouse serum, $n = 6$) or 100-fold dilution (dilution QC2: 42,500 ng/ml in mouse serum, $n = 6$). The accuracy (%bias) was 0.5% and 3.8%, and the precision (%CV) was 3.3% and 2.5%, for dilution QC1 and dilution QC2, respectively. These were all well within the 15% acceptance criteria, demonstrating satisfactory dilution integrity of the assay at tenfold and 100-fold dilutions.

Application to safety study in mice

The validated assay was applied to a repeat-dose range-finding study of ASO1 in juvenile CD-1 mice. A total of four runs were conducted for the analysis of CMS samples and the runs met the acceptance criteria for STD and QC performance (%CV < 15.0%; %bias within $\pm 15.0\%$, 20% for LLOQ). For the four levels of QCs (low, GM, mid and high) analyzed in the four runs ($n = 4$ for each run), the mean %bias was -1.0 – 1.9% , and the mean %CV was 3.4–6.3%, indicating the good performance of the assay for analyzing real study samples.

Figure 2 shows the PK profile of ASO1 in female juvenile CD-1 mice following subcutaneous administration of 25, 75 and 150 mg/kg ASO1 on PND 52. The serum exposure of ASO1 increased in a dose-proportional manner over the dose range evaluated on PND 52. The concentration data and PK profile were as expected and consistent with previous studies on similar ASOs using conventional sampling, demonstrating the successful application of the CMS strategy to the mouse study, as well as the application of hybridization LC–MS/MS method for the quantification of ASO1 in CMS samples.

CMS versus conventional sampling in mouse study

To compare the performance of CMS and conventional sampling, for terminal samples at PND 52, two sets of samples (CMS serum samples and conventional serum samples) from the same time points were collected. A total of 16 samples were collected with both sampling methods and their concentrations were analyzed by the validated method. As shown in Table 4, for the 16 samples, the %Difference for 15 of 16 (93.8%) samples were within 30%, the acceptance criteria of incurred sample reanalysis (ISR) for biologics. The %Difference of the only sample (sample 7) outside of the 30% range was 32.7%, which was just slightly higher than the acceptable limit. Even when applying the stricter 20% acceptance criteria used for small molecules, 11 of 16 (68.8%) samples were within 20%, which is still more than two-thirds of the samples that pass the acceptance criteria of the ISR test. This demonstrated the equivalence of CMS samples and conventional samples for ASO concentration estimation and therefore the PK/TK evaluation. In addition, for the ISR test, the same incurred samples are analyzed twice using the same bioanalytical method. In this comparison, the CMS samples were collected from the jugular vein while

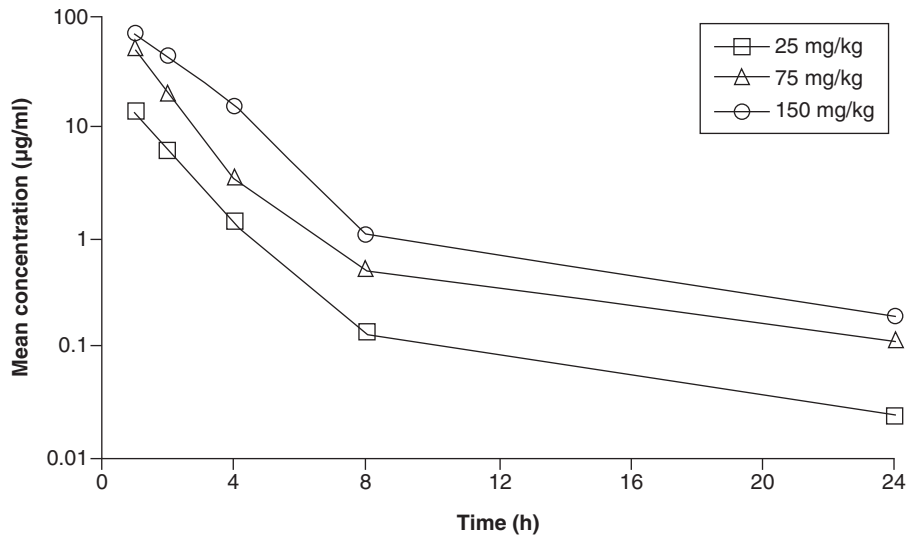


Figure 2. Mean ASO1 concentrations in female juvenile CD-1 mouse capillary microsampling serum samples following subcutaneous administration of 25, 75 and 150 mg/kg ASO1 on postnatal day 52.

Table 4. Comparison of ASO1 concentration measured in capillary microsampling and conventional serum samples from a mouse safety study.

Sample ID	CMS sample (ng/ml)	Conventional sample (ng/ml)	% Difference [†]
1	77.8	76.2	2.1
2	238	240	-0.8
3	26	31.2	-18.2
4	23.8	26.1	-9.2
5	138	102	30.0
6	146	130	11.6
7	2490	1790	32.7
8	130	118	9.7
9	520	466	11.0
10	106	112	-5.5
11	373	288	25.7
12	2230	1740	24.7
13	223	210	6.0
14	798	816	-2.2
15	170	159	6.7
16	1370	1080	23.7

[†] % Difference = (CMS sample concentration – conventional sample concentration)/mean value × 100.
 CMS: Capillary microsampling.

the conventional samples were collected via the abdominal aorta (i.e., different locations). The exact collection time also varied slightly between the two samples. These considerations may lead to variability adding to the difficulty of achieving equivalent results between the two sampling methods.

A Bland–Altman analysis was also conducted to assess the agreement between CMS and conventional sampling for the measurement of ASO1 concentration in mouse serum samples using the concentration results of the 16 samples tested. As shown in Figure 3 in the Bland–Altman plot, the %Difference between the two different sampling methods for all tested samples were within the upper and lower 95% limits of agreement (38.40% and -19.92% difference, respectively), demonstrating good agreement between the two methods. This result further confirmed that the CMS samples behaved the same as the conventional samples, and the validated hybridization LC–MS/MS method accurately measured both types of serum samples. The results from this mouse study demonstrated the

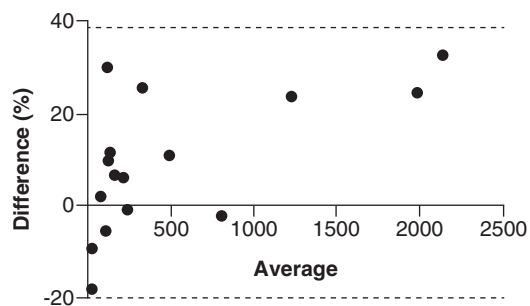


Figure 3. Bland–Altman plot assessing agreement between capillary microsampling and conventional sampling for measurement of ASO1 concentration in serum samples from a mouse safety study. Dashed lines represent upper and lower 95% limits of agreement.

validity of the CMS strategy for ASO bioanalysis. CMS was later successfully implemented in GLP mouse studies of ASO1 and other ASOs.

Conclusion

A hybridization LC–MS/MS method was successfully developed and validated for the quantification of ASO1 in mouse serum over the range of 1.00–500 ng/ml using 20- μ l samples for both regular and CMS samples. A microsampling strategy using the CMS technique was successfully applied to a safety study of ASO1 in juvenile mice. The equivalent performance of CMS serum samples and conventional serum samples was demonstrated in the mouse study, which confirmed the accuracy and reliability of the CMS strategy for ASO bioanalysis and PK/TK evaluation. This is the first report on applying CMS for LC–MS/MS quantitative bioanalysis of ASO and PK/TK evaluation in a safety study. The validated CMS LC–MS/MS method has also been used to support GLP studies of ASO1 in mice. The CMS methodology has also been applied to other ASOs.

CMS is a promising microsampling technique that has the advantage of much-reduced blood collection volume while maintaining the same plasma or serum matrix as the conventional sampling method. There will be increased applications of CMS in nonclinical studies, including GLP studies, particularly in small animals (rats, mice, etc.). In this work, we have demonstrated that CMS can be successfully applied to mouse safety studies for the quantitative bioanalysis of ASO. With the rapid growth of oligonucleotide therapeutics in drug discovery and development, the application of CMS for ASO bioanalysis will continuously increase in the future. CMS is also expected to be applied to other oligonucleotide therapeutics, such as siRNA. Sensitivity is one of the main challenges for the application of CMS due to its significantly reduced sample volume compared with conventional sampling. The recently developed hybridization LC–MS/MS methodology combines the advantages of LC–MS/MS assay and hybridization immunoassay and can achieve specific and sensitive quantification of oligonucleotides in various biological matrices. Hybridization LC–MS/MS, as demonstrated in this study, is a suitable platform to be used in combination with microsampling techniques with its superior sensitivity. With the continuous advancement in mass spectrometry and chromatography technologies and instrumentation, the sensitivity of hybridization LC–MS/MS methods can be further improved and will be more widely applied to CMS and other microsampling techniques for oligonucleotides.

Summary points

- Capillary microsampling (CMS) has been used for quantitative bioanalysis of small molecules. However, it has not been reported for the bioanalysis of antisense oligonucleotides (ASOs).
- A hybridization LC–MS method was successfully developed and validated for the quantification of ASO1 in mouse serum for both CMS samples and conventional samples.
- The validated method was successfully applied to a safety study in mice for the analysis of ASO1 in CMS samples.
- Equivalent performance between CMS samples and conventional samples was demonstrated in a safety study in mice.
- This is the first report on applying CMS for ASO bioanalysis and pharmacokinetic/toxicokinetic evaluation.

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Ethical conduct of research

The mice study in this work was reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee and was conducted in compliance with all applicable animal welfare regulations.

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