Research Article
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Bioanalysis

LC–TOF–MS methods to quantify siRNAs and major metabolite in plasma, urine and tissues

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There are a few different bioanalytical approaches that have been used for the quantification of siRNA in biological matrices, such as S1 nuclease protection 'cutting ELISA', fluorescent probe hybridization HPLC, HPLC UV, LC–MS/high-resolution accurate-mass (HRAM) and LC–MS/MS. We have developed and validated plasma assays for several oligonucleotides such as GalNAc-conjugated siRNA, using uHPLC and high-resolution mass spectrometer by TOF detection. Although the molecular weights are in the range of 7000–9000, we were able to meet the same assay acceptance criteria as for the small molecules based on regulatory bioanalytical method validation guidance. The antisense strand and the sense strand can both be monitored. The method was also used in the tissue lysate matrices without a full validation.

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To support preclinical and clinical studies, assay ruggedness and robustness, sensitivity and specificity are essential features for obtaining concentrations from biological matrices. There is a lot of interest in the application of full-scan high-resolution MS (HRMS) for the quantitative analysis of oligonucleotides [1[1]]. This approach not only provides quantitative information for the target oligonucleotides, but also provides information related to metabolites, even when standard reference materials are not available. HRMS in full-scan mode provides selectivity and can improve the detection limit considerably [2]. Therefore, HRMS is especially advantageous for the analysis of oligonucleotides. We were able achieve a 5–10 ng/ml lower limit of quantitation (LLOQ) for double stranded oligonucleotides with an 18–22-mer oligonucleotide in each strand. Compound specific tuning is not necessary for HRMS and specific high resolution (R >10000) in full-scan MS mode is sufficient.

Several attributes of the oligonucleotides make the extraction method of this class of compounds challenging. Oligonucleotides bind extensively to proteins and direct protein precipitation leads to very low recoveries (<10%). Proteinase K digestion combined with liquid–liquid extraction (LLE) did result in reasonable recoveries [3–5]; however, proteinase K digestion requires long incubation times which is not desirable for the fast paced industry. LLEs also lead to high matrix background and uHPLC column blockage, which is not cost–effective. A good extraction method is critical for processing plasma and tissue biological samples. During the method development, attention is required to minimize the effect from many other attributes. Nonspecific binding of oligonucleotides to various surfaces and containers might result in low recoveries or inconsistent recovery through the concentration range. Formation of sodium adducts can severely reduce the signal of the ion of interest and decrease the sensitivity of the assay [6]. Degradation of oligonucleotides by nucleases is another source that might affect the recovery and decrease the method precision.

In this paper, we describe a general method used for validation of 22mer oligonucleotides in plasma and the methods were used in urine and tissue lysate matrices without full validation. These are GalNAc-conjugated siRNA (see Figure 1 for the basic structure of siRNA). The duplex of the full-length oligonucleotides were used for the preparation of standards and quality control samples (QCs). Phenomenex Clarity OTX SPE plates are effective for a one-step SPE extraction of the duplex siRNA oligonucleotides from plasma, urine and tissue matrices [7–9]. The extraction procedure from the manufacturer with slight modification works well for extraction of oligonucleotides.

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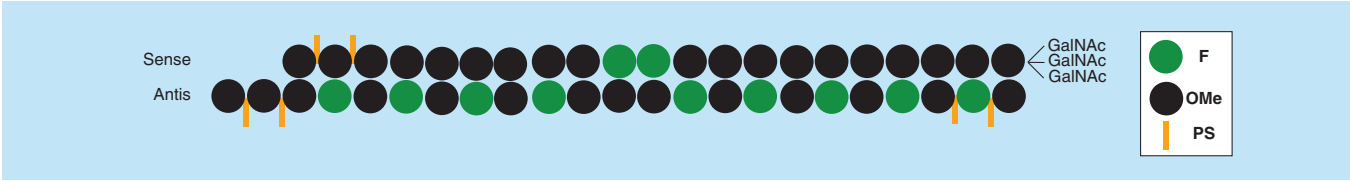

Figure 1. Basic structure of SiRNA.

To prevent the loss and degradation of siRNA, RNA recovery solution needs to be used in the final extracts to get reasonable recoveries.

Many therapeutic oligonucleotides have chemical modifications to improve their stability [10,11]. Most therapeutic oligonucleotides contain phosphorothioate in the backbone [12] and 2′-modification on the ribose ring. 2′-Modification tends to improve stability but on the other hand results in minimal fragmentation when MS/MS mode is used. In our approach, we did not use MS/MS mode but full-scan mode using TripleTOF mass spectrometer system for the quantitative analysis. The high-resolution feature provides the needed selectivity and sum of multiple ions improved sensitivity.

Experimental procedures

Materials

The oligonucleotide and internal standards (IS; purity >95%) were provided by a sponsor. Chemicals such as 1,1,1,3,3,3-hexafluoro-isopropanol (HFIP), diisopropylamine (DIPA), tetrahydrofuran, ammonium acetate and ammonium bicarbonate, were purchased from Sigma-Aldrich Inc. (MO, USA). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (PA, USA). Clarity OTX lysis buffer and SPE cartridges were obtained from Phenomenex Inc. (CA, USA).

Preparation of stock & working solutions

Oligonucleotide solution was prepared in deionized water to give a final concentration of 2 mg/ml. IS stock solution concentration was 300 µg/ml. Working solutions of the oligonucleotides were prepared by spiking stock solutions into plasma. The calibration samples were prepared with concentrations of 10, 20, 100, 300, 1000, 3000, 9000 and 10,000 ng/ml and QC samples with concentrations of 10, 30, 400, 4000 and 8000 ng/ml. For calibration standards 100 µl of these solutions were then spiked with 50 µl of IS working solution before extraction.

Sample preparation for plasma & urine samples

The equilibration buffer consisted of ammonium acetate, NaN_3 and K_2EDTA in water and the pH was adjusted with acetic acid to 5.5. The washing buffer 1 consisted of ammonium acetate in 50:50 water:acetonitrile and pH was adjusted with acetic acid to 5.5. The washing buffer 2 consisted of ammonium acetate in 10:90 water:acetonitrile and the pH was adjusted to 5.5 with acetic acid. The elution buffer contained ammonium bicarbonate, pH adjusted to 8.8 with ammonium hydroxide, acetonitrile and tetrahydrofuran. Extraction was performed using a semi-automated TomTec Quadra 4 SPE 96-well format liquid handler. The SPE cartridges were conditioned using methanol and equilibration buffer sequentially. Plasma samples were then mixed with IS and Clarity OTX buffer and loaded onto the column. The cartridges were washed using washing buffer 1 and 2 and the analytes were eluted using elution buffer into a plate containing RNA recovery solution. The collected solutions were evaporated to dryness and reconstituted with reconstitution solution. The reconstitution solvent contained water, methanol, DIPA, HFIP, EDTA in water, acetonitrile and ammonium hydroxide. A total of 10 µl of the reconstituted solution was injected into the uHPLC–TOF system for analysis.

Sample preparation for tissue samples

Tissue samples underwent disruption to produce a frozen powder using a SPEX Geno/Grinder (Spex Sample Prep LLC, NJ, USA). Tissue powder was homogenized by adding Clarity OTX Lysis-Loading buffer (Phenomenex, CA, USA) to produce a 100 mg/ml mixture of tissue homogenate. After mixing for 3 h at ambient temperature, the tissue homogenate was centrifuged. The tissue lysate was separated out and used for standard and QC preparations.

The calibration standards in tissue lysate ranged from 10–10,000 ng/ml and 100–100,000 ng/g in terms of tissue concentration. The extraction, LC–TOF–MS and quantitation methods for tissue were similar to plasma methods.

Instrumentation LC–TOF–MS conditions

Samples were analyzed using reversed-phase uHPLC with ESI TOF–MS detection using high resolution TripleTOF™ system (AB Sciex LLC, MA, USA). In TOF-MS quantitation, each peak in the isotopic envelope of the $[M-4]^{4-}$and $[M-3]^{3-}$ charged states was extracted using a 50–75 mDa extraction window. Accurate mass of ten most intense ions (m/z) for each strand of the analyte, antisense and sense and each strand of IS, antisense and sense were monitored in the negative ion mode. The peak area for the analyte or IS was the sum of the response from the respective ten ions. Waters Acquity BEH C18 column (2.1 × 50 mm, 1.7 µm, Waters Corporation, MA, USA) was used with a flow rate of 0.85 ml/min and the following mobile phases: Mobile phase A is H2O:DIPA:HFIP (100:0.15:0.264 v/v/v) and mobile phase B is H_2O:MeOH: DIPA:HFIP (50:50:0.15:0.264, v/v/v/v). The gradient was from 30%B increased to 50%B in 1.6 min, then to 100%B in 0.1 min and maintained for 0.5 min, followed by re-equilibration with 30%B for 0.6 min. The column temperature was maintained at 70°C.

Method validation in plasma

Plasma calibration curves were constructed using peak area ratios of the oligonucleotide to the IS and applying a weighted ($1/x^2$) quadratic regression. Precision and accuracy were determined using QC samples in five concentrations (10, 30, 400, 4000 and 8000 ng/ml) and in six replicates for each concentration. Interday accuracy and precision was determined in three separate analytical runs and two of the runs were performed on consecutive days. Evaluation of method selectivity included blank and spike-in-selectivity tests using six different lots of blank matrix. A minimum of one replicate from each of the six lots of blank matrix was spiked with analyte at lower limit of quantitation (LLOQ) to prepare the spiked-in-selectivity samples. The impact of hemolyzed plasma was also included in the selectivity assessment. The relative recoveries were determined with spiked plasma samples (n = 5) at concentrations of 30, 400 and 8000 ng/ml and the matrix effect was determined at two different concentrations (30 and 8000 ng/ml) but in six different lots of blank matrix. Relative extraction recovery was calculated by dividing the peak area for a sample spiked with oligonucleotide before extraction by the peak area for an equal concentration sample in the same matrix spiked after extraction. Matrix effects were calculated by determining the matrix factor (MF) for the analyte and IS by dividing the mean of the peak area in the presence of matrix (analyte and IS spiked into the extracts of blank matrix) by the mean of the peak area in the absence of the matrix (neat solution). Then the IS-normalized MF was calculated by dividing the analyte MF by the IS MF. Linearity of dilution was also assessed by preparing dilution QC in the same matrix as study samples, at one concentration greater than upper limit of quantitation and then diluting the samples and analyzing in five replicates. Stability of oligonucleotide during freeze–thaw (3–5 cycles), at room temperature (24 h), processed sample stability and long-term storage stability were also determined. Carryover evaluations were performed by placing an extracted blank matrix sample after each highest calibration standard in each run.

The validation workflow includes acquisition of full scan TOF MS spectra and use of high resolution extracted ion (XIC) for quantitation, summing multiple ions (m/z) to achieve sensitivity. The post-acquisition data mining is a significant advantage of this workflow and it allowed us to easily identify and mine metabolites. Data were acquired using Analyst TF™1.7 and quantitation was performed using MultiQuant™ software.

Pharmacokinetic & toxicology studies

To evaluate the pharmacokinetic profile, a single or multiple dose of the test oligonucleotide was administered subcutaneously to cynomolgus monkeys (Covance Laboratories, WI, USA and Charles River Laboratories, OH, USA). As per the *in vivo* study protocol, all in-life procedures were in compliance with the Animal Welfare Act. The study animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals and the Office of Laboratory Animal Welfare. The age of the animals at initiation of dosing was 24–60 months and the animals weighed around 2–4 kg. The animals were assigned to groups and test article was administered subcutaneously at 30, 100 and 300 mg/kg. The control group received vehicle control (0.9% sodium chloride). Approximately 1 ml of blood was collected per timepoint using K_2EDTA as the anticoagulant. For most of the studies, blood samples were collected at predose and 0.25, 0.5, 1, 2, 4, 8, 24, 48, 96 and 168 h post dose. Plasma was obtained by centrifuging the collected blood samples within 30 min of collection. Liver (terminal or biopsy)

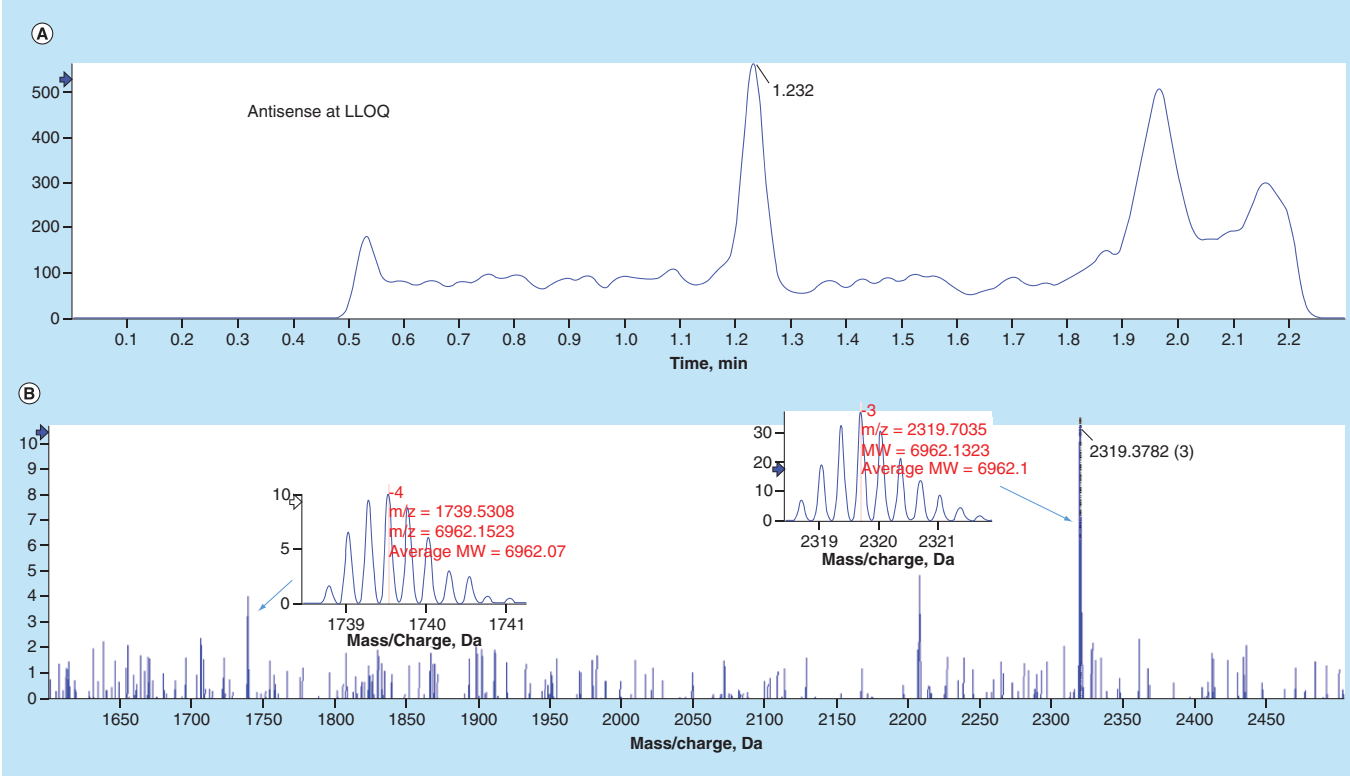

Figure 2. Typical chromatogram and full scan mass spectrum of siRNA. (A) Full-length oligonucleotide extracted from plasma **(B)** showing different charge state and isotopic peaks for each charge state. Mass spectrometer response of five isotopic peaks from charge state 3^- and charge state 4^- were combined and used to construct calibration curve and regression analysis.

and kidney tissue was collected in some of the studies. Samples were shipped on dry ice to our facility and stored at -70°C until extraction and sample analysis. The samples were analyzed for the quantitation of the oligonucleotides.

Results & discussion

Chromatogram & mass spectrum

Figure 2 shows the typical full scan chromatogram of full-length oligonucleotide extracted from plasma, different charged states and the isotopic envelopes of each charge state. Figure 3 shows the chromatogram for the full length siRNA, the antisense, sense strand of test oligonucleotide and IS. For the HRMS analysis, as long as there are no mass overlaps between analytes, retention time separations are not mandated.

Bioanalytical method validation in plasma

Selectivity

Blank and spike-in-selectivity (spiked at 10 ng/ml, LLOQ-QC) using different lots of plasma (n >6) were extracted and analyzed. No significant peaks were observed in either the analyte or the IS channels for blank matrix samples, indicating that the method was highly selective. The precision and accuracy data for LLOQ-QC spiked in six different lots of plasma were within the acceptable range (±20%RE). Hemolyzed plasma (containing 2% whole blood) did not have any impact on selectivity assessment.

Calibration curve

Good linearity was demonstrated for both calibration curves by the sense and antisense strands where the peak area ratio of sense to IS sense and the peak area ratio of antisense to IS antisense were each independently used for two separate calibration curves. Quadratic regression analysis were performed with $1/x^2$ weighting. A quadratic fit gave a consistently better % relative error for the calibration standard. The coefficients of correlation were greater than 0.99 for both calibration curves for the antisense and sense strands. Figure 4 shows an example calibration curve generated for the antisense strand using quadratic regression.

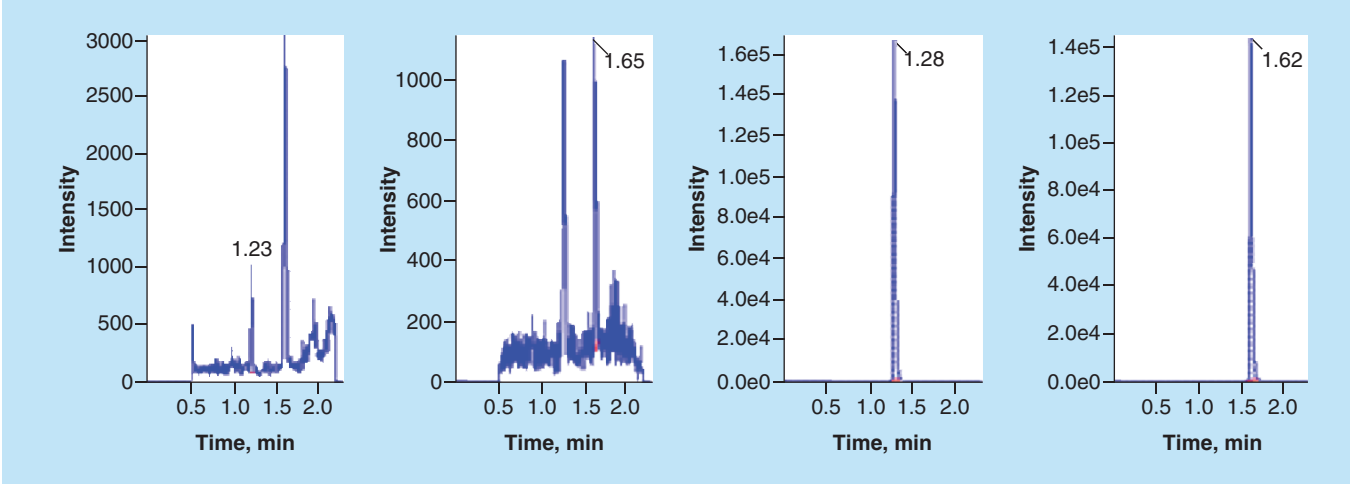

Figure 3. Typical extracted ion chromatogram of siRNA and IS. There are four columns for each chromatogram, the first two columns are for the full length siRNA monitored for the antisense (at retention time [RT] of 1.23) and sense strand (at RT of 1.65); third and fourth columns are for the internal standard (by antisense at RT of 1.28 and by sense at RT of 1.62).

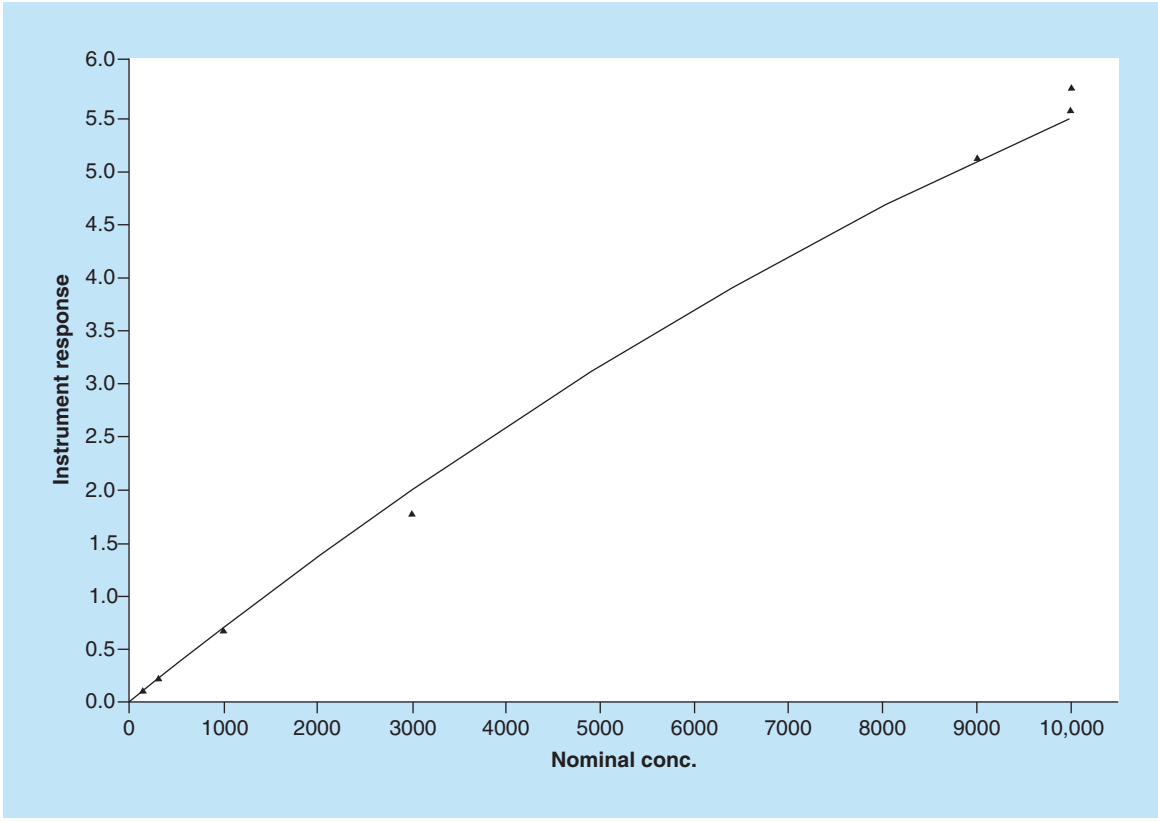

Figure 4. Standard curve for the antisense oligonucleotide.

Calibration standards met acceptance criteria that a minimum of 75% of the total number of calibration standards in the calibration range (between and including the LLOQ and the ULOQ [upper limit of quantification]) did not deviate by greater than ±15.0% (±20.0% at the method LLOQ concentration) from their nominal values.

Table 1. Intraday and interday accuracy and precision of QC samples in rat plasma by measuring antisense strand.

QC Concentration of the duplex (ng/ml)	Intrarun (n = 6)			Interrun (n = 18)		
	Mean concentration	%RE	%CV	Mean concentration	%RE	%CV
10	10.1 ± 0.9	0.7	9.4	10.0 ± 1.7	0.3	17.4
30	31.7 ± 2.4	5.8	7.6	31.9 ± 2.3	6.4	7.2
400	431 ± 10	7.8	2.3	430 ± 15	7.5	3.4
4000	3623 ± 94	-9.4	2.6	3774 ± 256	-5.7	6.8
8000	8049 ± 360	0.6	4.5	8091 ± 387	1.1	4.8

QC: Quality Control; %CV: Percent coefficient of variation, for precision assessment; %RE: Percent relative error = (measured-nominal)/nominal x 100%, for accuracy assessment.

Table 2. Extraction recovery from spiked plasma.

QC concentration (ng/ml)	Recovery (%)	
	Antisense oligonucleotide	Internal standard
30	100.4	96.9
400	105.1	94.0
8000	78.9	83.3
Mean recovery	94.8	91.4
Precision (%)	14.8	7.9

Precision & accuracy

Precision and accuracy of the method evaluated at five different concentrations (10, 30, 400, 4000 and 8000 ng/ml) met the acceptance criteria where more than 2/3 did not deviate by more than ±15.0% (±20.0% at LLOQ QC). The LLOQ QC was the same concentration as the lowest calibration standard, the next lowest QC concentration was ≤three-times the concentration of the lowest calibration standard and the high QC was 75–90% of the highest calibration standard. The intraday and interday accuracy and precision of QC samples in an oligonucleotide in rat plasma are summarized in Table 1.

Recovery & matrix effect

The oligonucleotide extraction recovery is summarized in Table 2. The average extraction recovery was between 90 and 100% at the concentration range that we tested. The matrix effect was determined for the oligonucleotide (analyte) in six individual lots of plasma at two concentrations (30 and 8000 ng/ml, n = 3) and for the IS at one concentration. Matrix effect was evaluated by calculating the matrix factor for the analyte and IS. The %CV of the IS-normalized matrix factor did not exceed 15.0%. The IS-normalized matrix factor was close to 1, indicating that there was no suppression or enhancement of the analyte and IS responses by the matrix.

Linearity of dilution

Dilution QC was prepared at 100,000 ng/ml and diluted with pooled blank plasma at a dilution factor of 200 in five replicates. The results showed that after the dilution factor was applied, the mean concentrations of the diluted samples were within 100 ± 15% of the nominal value before dilution and the %CV did not exceed 15%.

Stability & carryover

Stability in plasma was demonstrated for five freeze/thaw cycles at -20 and -70°C, 24 h at ambient temperature and for an entire year of long-term storage at -20 and -70°C. The stability data are summarized in Table 3. Stability was also demonstrated in whole blood in an ice bath for 2 h, in stock solution at -20°C for 76 days, in stock solution at ambient temperature for 16 h and in processed samples for 112 h at ambient temperature. Carryover was <20% of the LLOQ peak area when a new column was used. Column carryover was observed over time and column could not be used for more than 250–300 injections. During sample analysis carryover was monitored closely and additional blank samples were included to minimize the effect of carryover on samples. All tests that are typically performed for small molecules have been applied to the oligonucleotides that we studied and the acceptance criteria are based on the bioanalytical method validation guidance [13].

Table 3. Stability of antisense oligonucleotide in rat plasma under four different conditions (n = 5).

Storage condition	Low QC (30 ng/ml, n = 5)			High QC (8000 ng/ml, n = 5)		
	Mean concentration	%RE	%CV	Mean concentration	%RE	%CV
Room temperature (24 h)	35.6	8.6	6.0	7702	-3.7	2.7
Freeze–thaw (-20°C, 5 cycles)	30.6	2.1	5.0	8821	10.3	4.8
Freeze–thaw (-70°C, 5 cycles)	29.8	0.6	2.9	8407	5.1	2.3
Processed samples (112 h at RT)	32.9	9.7	3.7	8622	7.8	4.9
Long-term stability (370 days at -20°C)	26.4	-12.1	12.0	7617	-4.8	3.9
Long-term stability (370 days at -70°C)	27.0	-9.9	12.3	7720	-3.5	2.3

QC: Quality Control; %CV: Percent coefficient of variation; %RE: Percent relative error = (measured-nominal)/nominal x 100%.

Table 4. Intraday accuracy and precision of QC samples in rabbit liver and kidney by measuring antisense strand.

QC concentration of the duplex (ng/ml)	Intrarun (n = 6) – rabbit liver			Intrarun (n = 6) – rabbit kidney		
	Mean concentration	%RE	%CV	Mean concentration	%RE	%CV
20	18.0 ± 1.2	-9.8	6.7	21.9 ± 2.2	9.6	9.8
60	51.7 ± 3.7	-13.9	7.1	61.1 ± 4.4	1.9	7.2
400	436 ± 10	9.0	2.2	465 ± 20	16.2	4.4
4000	4064 ± 160	1.6	3.9	4413 ± 244	10.3	5.5
8000	8552 ± 611	6.9	7.1	8749 ± 268	9.4	3.1

QC: Quality Control; %CV: Percent coefficient of variation, for precision assessment; %RE: Percent relative error = (measured-nominal)/nominal x 100%, for accuracy assessment.

Bioanalytical method qualification for urine & tissues

Given the difficulty of extracting tissue samples, the tissue sample acceptability criteria was different from plasma, a QC sample was accepted if its determined concentration did not deviate from its nominal concentration by more than ±25.0% (±30.0% at LLOQ). The tissue extractions also showed good linearity as well as precision/accuracy. Table 4 summarizes the intraday accuracy and precision of QC samples in rabbit liver and kidney by measuring antisense strand. The tissue concentrations were reported in ng/ml of tissue lysates and ng/g of tissue. Urine method qualification showed good linearity as well as precision and accuracy. Stability was also established at bench top for 24 h, three cycles of freeze thaw and up to 3 months of long-term frozen storage stability was established at -70°C. For both urine and tissue samples, carryover was observed over time and there was a need to replace column after 250 injections.

Pharmacokinetic data in monkeys

Following subcutaneous injection, increases in doses resulted in proportional increases in exposure. Figure 5 shows plasma concentration of full-length oligonucleotide in monkeys following SC administration of 30, 100 and 300 mg/kg dose. Oligonucleotide distribution was similar across dose groups. Plasma concentrations declined over time in a similar manner across different dose groups and measurable plasma concentrations ranged from 8 to 24 h post dose. There were no gender differences in pharmacokinetic profile following subcutaneous dose. Figure 6 shows the liver concentration of full-length oligonucleotide in monkeys following SC administration of 10 mg/kg dose. In general for plasma and tissues, the duplex concentrations were based on monitoring of the antisense strand. In plasma, the sense strand was monitored and was quantifiable but for most tissues, the sense strand concentration was very low to below quantitation limit (BQL).

Figure 7 depicts the mean plasma concentration of full-length oligonucleotide in three different studies following SC administration of 30 mg/kg dose, showing reproducibility over a period of 3 years. In addition, incurred sample reproducibility was also determined once per method. The incurred sample reproducibility was good, >97% of the samples reanalyzed met the acceptance criteria of ±20% difference between the original and the reassay results.

Conclusion

We have developed a sensitive LC–TOF–MS method for the simultaneous quantitation of a test oligonucleotide and its metabolite in plasma and tissue matrices. The method was successfully validated in plasma with respect to linearity, sensitivity, accuracy, precision, dilution linearity, selectivity, hemolysis effect, recovery, matrix effect and

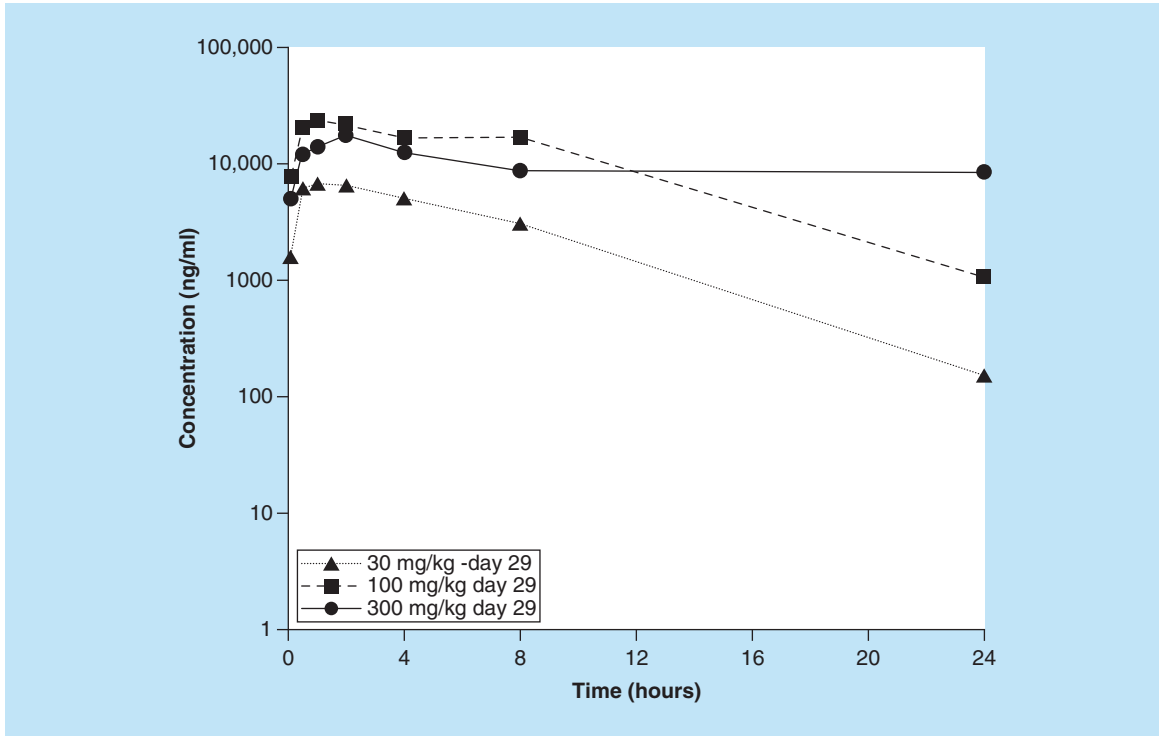

Figure 5. Plasma concentration of full-length oligonucleotide in monkeys following SC administration of 30, 100 and 300 mg/kg dose.

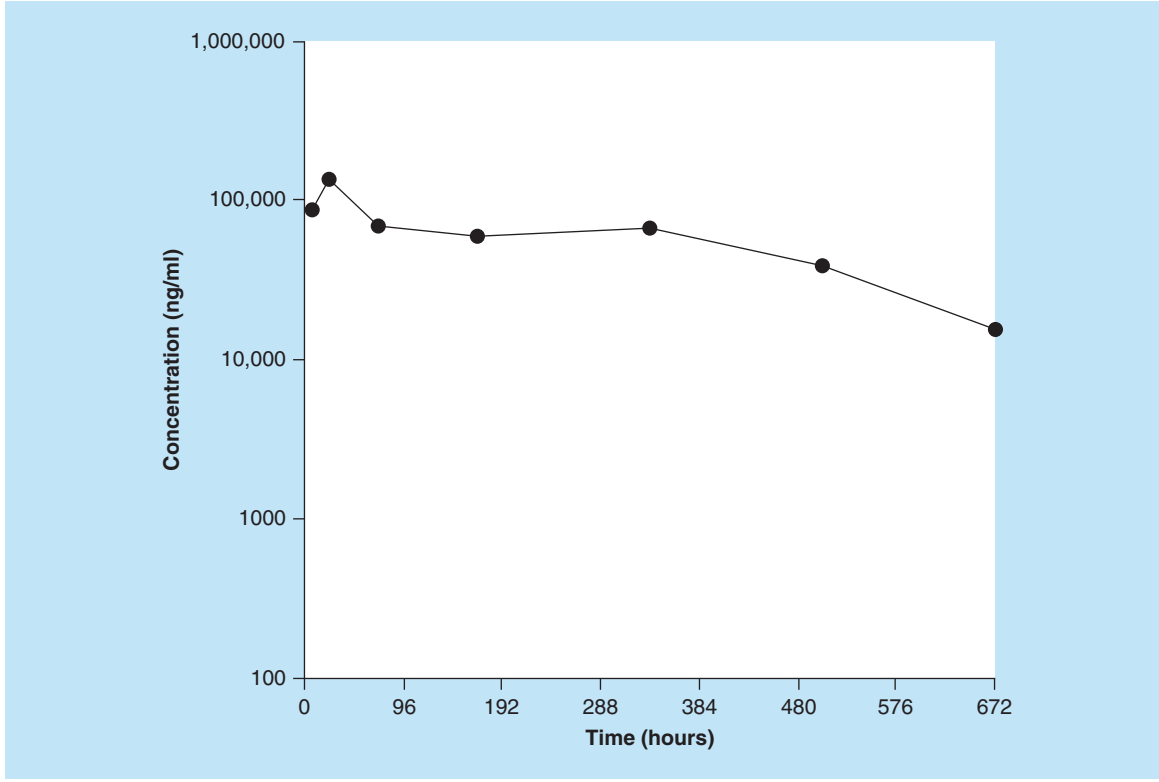

Figure 6. Mean liver concentration of full-length oligonucleotide in monkeys following SC administration of 10 mg/kg dose.

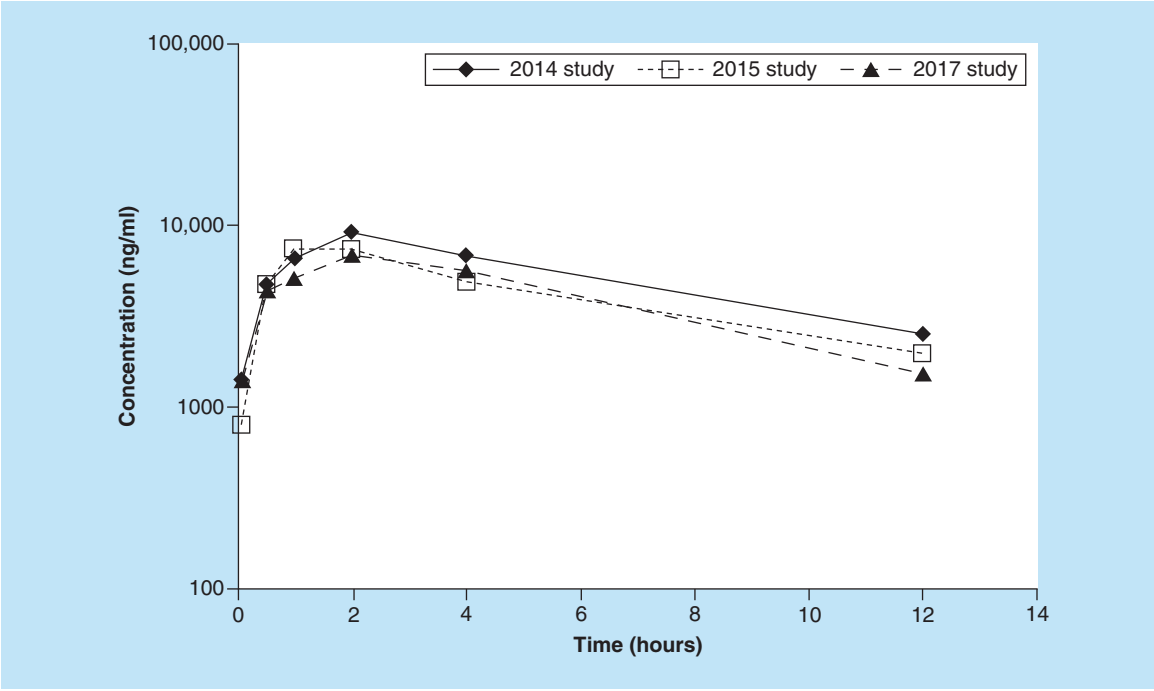

Figure 7. Mean plasma concentration of full-length oligonucleotide in monkeys following SC administration of 30 mg/kg dose, showing reproducibility over a period of 3 years.

carryover. Validated method was used for regulated preclinical studies, using a 100 µl sample volume, the dynamic range was 10–10,000 ng/ml.

Future perspective

The TripleTOF system offers sensitive, high resolution analysis of oligonucleotides and gives an opportunity to mine the data to perform qualitative and quantitative analysis in a single run. The ability to quantify oligonucleotides from complex matrices with minimal assay optimization results in consistent, reproducible analysis of oligonucleotides. For some of the oligonucleotides, a lower limit of quantitation has been requested to capture complete terminal elimination phase at clinically relevant doses. This leads to some lingering questions such as how can sensitivity of current LC–TOF–MS set up be further increased? Is this really needed and what will be the expectation in future?

Summary points

Background

- An LC–HRMS method was validated for GalNac conjugated oligonucleotides.

Experiments

- LC–TOF–MS full scan chromatography method was developed for plasma, urine and tissues. Simple SPE method could be used to extract most matrices. Chromatographic conditions were similar for most matrices and this gives the advantage of running multiple matrix using one system.
- TOF–MS full scan has the advantage of mining data for metabolites without re-extracting and re-running the samples.

Results & discussions

- GalNac conjugated oligonucleotides were successfully validated in multiple matrices.

Conclusion

- Ion pairing reversed phase chromatography allowed higher specificity and sensitivity. Reproducibility required for regulated study was achievable.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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