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Bioanalytical developments for the analysis of antisense oligonucleotides

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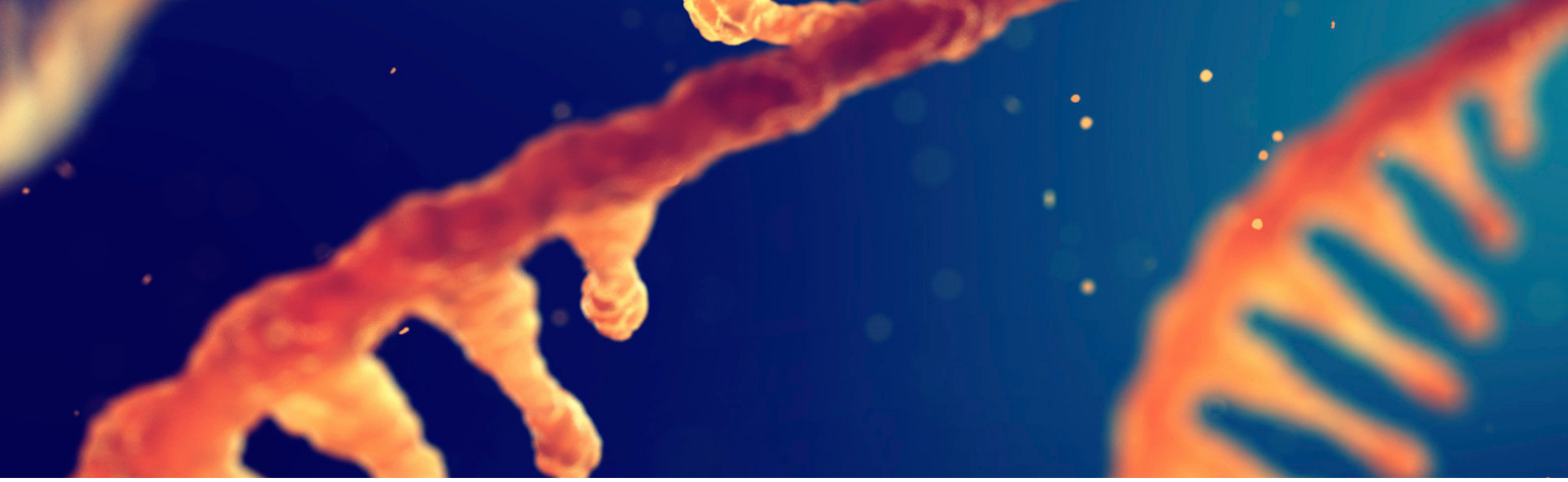

Contents

Foreword

With the ability to target the source of a diseases's pathogenesis, antisense oligonucleotides (ASOs) demonstrate a promising modality for intractable diseases and hereditary disorders. We are excited to bring you this special eBook on recent developments in the bioanalysis of ASOs.

ASOs are synthetic, single-stranded oligodeoxynucleotides modified to be complementary to an mRNA target, offering a disease-modifying therapeutic option for neurodegenerative diseases. Such therapies include the US FDA-approved Exondys 51 for Duchenne muscular dystrophy and Spinraza for spinal muscular atrophy. The development of ASO therapies has been extended to such conditions as Huntington's disease, Alzheimer's disease and Parkinson's disease. However, the bioanalytical methods utilized for studying the potential of ASO therapies are not well-standardized or regulated, and different approaches offer both positive results and related challenges. Certain assays have supported ASO analysis, such as hybridization enzyme-linked immunosorbent assay (ELISA), where cross-reactivity is an issue due to truncated metabolites generated *in vivo*. Methods such as LC–MS/MS offer quantitation of the metabolites and ASO, but with low sensitivity and labor-intensive extraction methods.

Novel hybridization approaches have gained a foothold in resolving these issues through utilizing biotinylated single-stranded DNA and capture probes with a complementary sequence to targeted ASOs. Such capture probes allow for full automation of the extraction process. Other developments include newer platforms for LBAs, which display increased assay sensitivity and specificity.

This eBook highlights the benefits and challenges of these novel approaches, with case studies demonstrating how best to align the bioanalytical technique with the goals of a particular ASO drug development program.

We hope you enjoy delving into the topic of ASO bioanalysis with us!

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Research Article

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Bioanalysis

Quantitative analysis of imetelstat in plasma with LC–MS/MS using solid-phase or hybridization extraction

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Aim: Imetelstat, a 13-mer oligonucleotide with a lipid tail is being evaluated for treating hematologic myeloid malignancies. This report describes the development of extraction and quantification methods for imetelstat. **Methodology & results:** Imetelstat was extracted using SPE (rat plasma) or by hybridization using a biotinylated capture probe (human plasma) and was quantified by LC–MS/MS. Calibration curves were established (0.1–50 μg/ml). Stability of imetelstat in plasma was demonstrated. Concentrations of imetelstat extracted using either of the methods and quantified with LC–MS/MS were comparable with a validated ELISA. **Conclusion:** Two extraction methods (solid phase and hybridization) were developed for quantifying imetelstat in plasma using LC–MS/MS. The hybridization extraction in combination with LC–MS/MS is a novel extraction approach.

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Keywords: hybridization extraction • oligonucleotide • SPE

In recent years, oligonucleotide drugs (ODNs) have gained popularity owing to their ability to target specific genes and provide specificity. ODNs comprise an oligonucleotide sequence, containing 10–50 nucleotides [1,2]. Numerous ODNs have been investigated so far [1–4]. But only six ODNs (fomivirsen, pegaptanib, mipomersen, eteplirsen, nusinersen and defibrotide sodium) have been approved by the US FDA since 1998 [5].

Pharmaceutical development of ODNs has several technical challenges such as maintaining *in vivo* stability, ensuring delivery and bioavailability, and minimizing off-target effects. These challenges can be minimized by chemical modifications of ODNs to enhance their pharmacokinetic and pharmacodynamic properties [4,6]. These chemical modifications include: sulfurization of the phosphodiester bond to avert degradation by enlindogenous exonucleases, addition of methoxy or methoxyethyl groups to sugar moieties [7,8], locked and unlocked nucleic acids, and alterations in the internucleotide linkage (e.g., amide linkage) resulting in peptide nucleic acids [1]. Sulfurization results in phosphorothioate analogs that are more hydrophobic, exhibit more complex secondary structures with higher protein binding and greater accumulation in organs than phosphodiester analogs [9–11].

Imetelstat is a novel, first-in-class telomerase inhibitor ODN [12], being investigated for the treatment of hematologic myeloid malignancies [13,14] and certain solid tumors [12,15,16]. Imetelstat is a 13-mer oligonucleotide N3′–P5′ thio-phosphoramidate (NPS) with a covalently linked C16 (palmitoyl) lipid moiety at the 5′-end (Figure 1; Supplementary Table 1). Addition of a lipid chain and the modified oligonucleotide backbone enables imetelstat to penetrate cells and tissues, with high biodistribution into normal and malignant cells [12].

Imetelstat differs from antisense oligonucleotides in its mechanism of action. Imetelstat is complementary to the template region of the RNA component of telomerase, to which it binds with high affinity and specificity, and directly competes with telomere binding, thereby inhibiting telomerase activity [17]. Thus, imetelstat acts as a classical active-site enzyme inhibitor, rather than an antisense oligonucleotide.

Preclinical and clinical pharmacokinetic and toxicokinetic studies for imetelstat are supported with a validated competitive nucleic acid hybridization ELISA [15]. Though hybridization ELISA is highly sensitive [18], it has a narrow dynamic range, is labor intensive and can lack selectivity toward truncated metabolites, potentially leading

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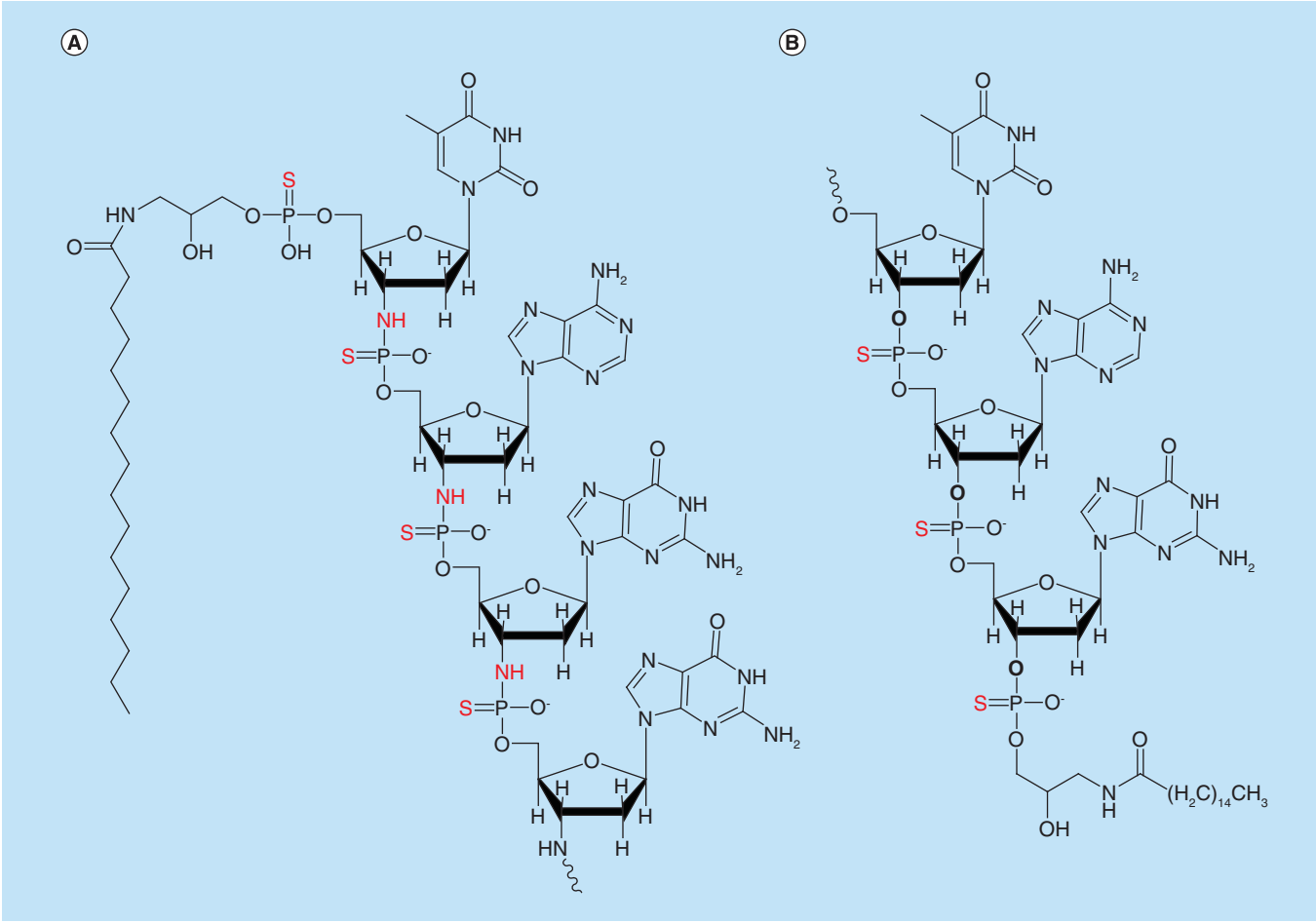


Figure 1. Partial oligonucleotide structures showing (A) imetelstat with NPS linkages and (B) internal standard with OPS linkages between subsequent nucleotides.
The palmitoyl lipid tail at the 5′ or 3′ position for both molecules is presented as well.
NPS: Thio-phosphoramidate linkage; OPS: Two bridging oxygens and one nonbridging sulfur.

to overestimation of parent ODN levels and does not provide insight into potential imetelstat metabolites. LC–MS is a potential alternative method to increase assay selectivity [19]. MS has become a method of choice to study structural properties [19–21], as well as for quantitative analyses of ODNs. Other chromatographic strategies (utilizing capillary electrophoresis or ion exchange in combination with fluorescence or UV detection [22–25]) are also reported, although these methods are often incompatible with MS.

There are several technical issues associated with LC–MS analysis of ODNs as they are large acidic molecules with limited ionization efficiency in ESI-MS. High polarity of ODNs leads to limited chromatographic retention. Ion-pair reversed-phase HPLC increases the retention and the nature and concentration of the ion-pairing reagent impacts the MS response. Applying the ion-pair reagent with acidic modifiers enables the right balance between improved MS sensitivity and LC separation [26,27]. Hydrophilic interaction LC has been proposed in combination with MS-friendly mobile phases for the analysis of synthetic and chemically modified oligonucleotides [28].

Sample cleanup is also a critical part of ODN analysis and displacement of ODNs bound to protein needs to be ensured. Various approaches for ODN extraction from the biological matrix have been used including protein precipitation, liquid–liquid extraction (LLE) and SPE, though each method has its own shortcomings and compound-dependent success rates [27,29–33].

This paper describes the development of an LC–MS/MS assay for imetelstat as an orthogonal assay to the hybridization ELISA. The assay presented is risk-based and fit-for-purpose to support limited analysis for explorative

work and a lean validation approach was taken [34]. In the event, the LC–MS assay would substitute the validated hybridization ELISA, a complete validation as per regulatory guidance would be required [35,36].

Materials & methods

Chemicals & reagents

Imetelstat, a 13-mer oligonucleotide (5′-TAGGGTTAGACAA-3′) N3′–P5′ NPS with a palmitoyl tail at the 5′-position (5′-Palm-TAGGGTTAGACAA-NH_2-3′) and the internal standard (IS), a 12-mer phosphorothioate (OPS; ACAGATTGGGAT) with a palmitoyl group linked at 3′-position (Figure 1; Supplementary Table 1) were provided by Geron Corporation (CA, USA). Two biotinylated capture probes having biotin label at the 3′- or 5′-position, in other words, TTGTCTAACCCTA-3′ TEG (tetraethylene glycol)-biotin and 5′ biotin-TEG-TTGTCTAACCCTA capture probes (Supplementary Table 1) were synthesized by Integrated DNA Technologies (Leuven, Belgium). The 16-mer reference phosphodiester (OPO) oligonucleotide (ATCTATACAAGCTGTC) was also custom made by Integrated DNA Technologies.

For SPE, Clarity® OTX™ 96-well plates (100 mg/well) were obtained from Phenomenex (Utrecht, The Netherlands). Pierce™ streptavidin magnetic beads (bead concentration: 10 mg/ml; binding capacity: 3500 pmol biotinylated fluorescein/mg of beads) were purchased from Thermo Scientific (Gent, Belgium). Human and rat blank plasma (K_2EDTA) were obtained from Bioreclamation (West Sussex, UK). LoBind DNA tubes, LoBind 96-well plates, all solvents (HPLC grade or equivalent) and all chemicals (AR or HPLC grade) were purchased from Sigma-Aldrich or Merck (Overijse, Belgium). Multivette® 600 K3E tubes were procured from Sarstedt (Nümbrecht, Germany).

The HPLC column (X-bridge C_{18} 2.1 × 50 mm, 3.5 μm) was procured from Waters (Zellik, Belgium).

Animals

Sprague Dawley rats were obtained from Charles River Laboratories (Sulzfeld, Germany).

Preparation of imetelstat standard, IS & capture probe solutions

A 2 mg/ml stock solution of imetelstat in water (HPLC grade) was prepared. Calibration standards of imetelstat were prepared by adding 5, 10 and 25 μl of the stock solution to a total volume 1 ml of plasma (rat or human K_2EDTA plasma), resulting in 10, 20 and 50 μg/ml imetelstat, respectively. Two further serial dilutions of each of these three dilutions were prepared in the appropriate plasma (rat or human; 100 + 900 μl) to obtain standard imetelstat concentrations of 0.1, 0.2, 0.5, 1, 2 and 5 μg/ml. Quality control (QC) samples were prepared by diluting an independently prepared stock solution of imetelstat to achieve concentrations of 0.8, 1.6, 8 and 40 μg/ml in human or rat plasma.

IS stock solution (2 mg/ml) and working dilutions (5 or 10 μg/ml) were prepared in water.

For both capture probes, stock solutions (1 mg/ml) were prepared in water. Working dilutions (1–10 nmol/ml) were prepared from the stock solution using buffer (15 mM sodium citrate, 150 mM sodium chloride).

LC–MS/MS system

The LC–MS/MS system consisted of a Shimadzu LC20AD HPLC with an SIL-HTC autosampler (Shimadzu Scientific Instruments, MD, USA), coupled to an API4000™ triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with Turbo Ionspray source. Additional details of LC and MS/MS parameters are provided in Supplementary Tables 2 and 3, respectively. Data acquisition and processing were performed using Analyst® software Version 1.6.2 (AB Sciex, Toronto, Canada).

For quantitation, linear regression was executed on log-transformed peak-area ratios (imetelstat-IS) against the log-transformed imetelstat concentrations. The concentrations in the incurred samples were calculated by interpolation from the calibration curve.

Imetelstat extraction from plasma

SPE from rat & human plasma

SPE of imetelstat from rat or human plasma was conducted using Clarity OTX extraction kits according to the manufacturer's protocol (Phenomenex) with certain modifications to optimize the extraction. The wash steps were modified to improve the matrix cleanup and to avoid contamination due to overfilling of wells. The final method is detailed in Supplementary Table 4.

Hybridization extraction for human plasma

The hybridization extraction method is based on the annealing of sense and antisense DNA probes (Supplementary Figure 1A). The antisense probe, also named the capture probe, includes a biotin label for affinity capture of the sense–antisense complex from the biological matrix (plasma), using streptavidin-coated magnetic beads. The method is summarized in Supplementary Table 5.

The streptavidin-coated magnetic beads were prepared before use. For each experiment, an appropriate volume of the bead suspension (based on 500–3000 pmol beads/sample depending on the experiment, with an extra allowance) was centrifuged (2430× *g*, 22°C, 3 min). The beads were precipitated using a magnet and the supernatant fraction was discarded. The beads were washed three-times by suspending in wash buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl) using three-times the volume of the original bead suspension. After the final wash step, the beads were resuspended in wash buffer containing 2% Tween 80 to achieve the required pmol beads/200 µl. During method development, the pmol of beads in 200 µl of buffer was varied to optimize the procedure.

The annealing process was initiated by the addition of 100 µl of the capture probe (100 pmol of the 5′ probe) and 200 µl of prepared beads per well of a LoBind 96-well plate, followed by the addition of 100-µl phosphate-buffered saline with 0.01% Tween 80. The plate was subsequently incubated for 10 min at 22°C in a thermomixer. Annealing was initiated by the addition of 20-µl aliquots of samples (study samples, QC samples and calibrator human K_2EDTA plasma) to the beads-capture probe solution. The plate was incubated in the thermomixer for 90–120 min at 22°C, followed by centrifugation (2000× *g*, room temperature [RT], 2 min) and precipitation of the beads using a magnet. The plate was washed twice by discarding the supernatant fraction, adding 200 µl of ice-cold water, vortexing, cooling the plate on ice-water for 20 min, followed by centrifugation (2000× *g*, 4°C, 2 min) and precipitation of the beads using a magnet.

To denature the antisense–sense oligonucleotide complex, 200 µl of water (80°C) was then added to each well. The plate was incubated in a thermomixer (1100 rpm, 80°C, 20 min), followed by centrifugation (2000× *g*, RT, 2 min) and precipitation of the beads using a magnet. The supernatant fractions were then transferred to a new 96-well LoBind plate and 25-µl IS was added to each well and mixed before injection onto the LC–MS system.

hybridization ELISA

Quantitative analyses of preclinical (rat plasma) and clinical (human plasma) samples using the described extraction method, coupled with LC–MS/MS method were compared with the results from the validated hybridization ELISA for imetelstat [15]. The hybridization ELISA is based on the competitive binding between imetelstat and a detection probe (i.e., a DNA probe with a digoxigenin label at the 3′-position) to hybridize to the complementary DNA strand (Supplementary Figure 1B). An antidigoxigenin antibody conjugated to alkaline phosphatase allows fluorescent detection of the digoxigenin-labeled probe. Consequently, a low signal intensity in the ELISA signifies high imetelstat concentrations.

Clinical samples

Blood samples were obtained from five patients from the Baerlocher *et al.*'s study [14] in which patients with polycythemia vera or essential thrombocytopenia had received imetelstat infusion of either 7.5 or 9.4 mg/kg for 2 h. Blood samples were collected on K_2EDTA before the start of infusion (from two patients), at the end of the infusion and at 1 and 2 h after the end of the infusion. Plasma was isolated and stored in Nunc® vials at -20°C. Samples with concentrations above the ULOQ were diluted five-times in human plasma for the final analysis.

Rat pharmacokinetic study

A pharmacokinetic study of imetelstat was conducted in male Sprague-Dawley rats (N = 16; 250–280 g). An intravenous bolus of imetelstat was administered in the saphenous vein at either 1 or 5 mg/kg (n = 8 each dose). The selected doses were the same as used in the preclinical studies carried out to support clinical development of imetelstat (data on file). Blood (0.4 ml) was sampled from the tail vein at 7 and 20 min, and at 1, 2, 4, 7, 24, 48, 72 and 96 h postdose (n = 3 per time point) and collected into Multivette® 600 K3E tubes (containing K_2EDTA). Plasma was separated by centrifugation (1900× *g*, 5°C, 10 min). Each plasma sample was divided into two aliquots and stored in micronic vials at -20°C. One aliquot was used for quantification of imetelstat with the SPE–LC–MS/MS method and the other aliquot was used for quantification with the hybridization ELISA method (assay range: 0.7–3.5 µg/ml). The plasma samples of the first two time points of both groups, 5 mg/kg dose group and 1 mg/kg dose group were analyzed following five-times dilution in rat plasma.

Table 1. Intrabatch precision and accuracy of quality control data (n = 6) for imetelstat in rat plasma (the SPE–LC–MS/MS method) and in human plasma (the hybridization–LC–MS/MS method).

Method/parameter	Nominal imetelstat concentration (μg/ml) in QC samples			
	0.8	1.6	8	40
Observed imetelstat concentration (μg/ml) in rat plasma (SPE–LC–MS/MS)				
Intrabatch mean (SD)	0.746 (0.0605)	NA	8.4 (0.1)	40.0 (6.0)
Intrabatch precision (CV%)	8.1	NA	1.7	14.9
Intrabatch accuracy (%)	93.2	NA	105	100
Observed imetelstat concentration (μg/ml) in human plasma (hybridization–LC–MS/MS)				
Intrabatch mean (SD)	NA	1.68 (0.159)	8.8 (0.726)	35.9 (2.7)
Intrabatch precision (CV%)	NA	9.5	8.4	7.6
Intrabatch accuracy (%)	NA	105	108	89.8

NA: Not applicable; QC: Quality control.

Acceptance criteria for assay qualification & execution

A calibration curve covering the concentration range of interest needs to have at least five calibration points with relative errors (RE) less than 20% (<25% at the LLOQ), following application of an appropriate linear regression model. The QC samples were evaluated at three concentration levels for accuracy and precision (n = 6, Table 1) [34]. The average back-calculated value of the QCs at each concentration level should have an accuracy between 80 and 120% with a CV of less than 20%. For the analysis of study samples, calibration standards and QC samples at three concentration levels in duplicates were included in each analytical run. Results were accepted if calibration standards (at least five concentration levels) as well as four of six QC samples (with each concentration level represented) had accuracies between 80 and 120%. Selectivity was evaluated by analysis of LLOQ QCs prepared in six different lots of rat or human plasma. The average back-calculated value of the QCs should have an accuracy between 75 and 125% with a CV of less than 25%.

Ethical approval

The study was approved by appropriate institutional review board and followed the principles outlined in the Declaration of Helsinki for all clinical and preclinical experimental investigations. For clinical investigations, informed consent was obtained from the participants involved. Preclinical investigations in animals were carried out in accordance with the guidance for the care and use of laboratory animals.

Results & discussion

In this paper, we describe the development of a qualified assay to quantify imetelstat in plasma (rat and human) using LC–MS/MS assay along with two alternative extraction strategies to allow appropriate sample preparation.

LC–MS/MS method development

LC solvents

ODNs are detected in MS as multiply charged negative ions. The abundance of these different charge states is highly dependent on the choice of LC solvent. For imetelstat, the 6-charge state was the most abundant ion when hexafluoroisopropanol (HFIP; as modifier) and triethylamine (as ion-pairing reagent) were used in LC analysis while the 4-charge state was the most abundant ion with other ion-pairing reagents, such as hexylamine. The 6-charge state was selected for further analysis. A spectrum with the charge state distribution and the product ion spectrum was obtained on an API4000 instrument and presented at an European Bioanalysis Forum meeting [37]. The findings are consistent with previous results for the HPLC/MS-MS analysis of a 24-mer phosphorothioate oligonucleotide in rat plasma [38]. Using alkylamines with higher Henry's law constant (hexylamine, 1.57 vs triethylamine, 0.35) as ion-pairing agents reduces the charge distribution by forming complexes with the oligonucleotide in gaseous phase while the ion-pairing reagents with a lower Henry's law constant value are depleted in the ion spray droplets [39]. For imetelstat, increasing the concentration of the ion-pairing reagent (i.e., triethylamine) in the LC mobile phase while improving the dynamic range of the analysis resulted in deterioration of the peak shape and sensitivity (data not shown). The optimal combination for the LC mobile phase was 0.2% HFIP and 0.2% triethylamine.

The findings with LC solvents used in our analysis are consistent with previous findings [26,27] in which introducing the solvent combination HFIP/triethylamine enhanced the LC separation and increased ESI-MS efficiency. These findings have been extended by the systematic investigation of modifiers for the quantification of phosphorothioate DNA and recently other fluorinated alcohols alongside HFIP have been proposed [22,38]. Furthermore, the impact of different ion-pairing reagents has also been reported for the LC–MS/MS analysis of specific ODNs [39–41].

Attaining a robust signal

The LC–MS/MS conditions were carefully controlled and optimized. Initially, LC with new, unused silica-based C_{18} column required multiple injections (>10) of matrix-containing samples to obtain a stable signal for imetelstat. This stabilization behavior was less pronounced for the IS. During the initial phase of method development, a reference compound (16-mer natural oligonucleotide without a lipid tail; Supplementary Table 1) which was assessed in the LC–MS/MS system demonstrated a more stable signal than what was seen for both imetelstat and the IS (i.e., oligonucleotides with lipid tails).

For both imetelstat and the IS, a decline in response was observed over a typical analytical run of approximately 60–100 injections. Various modifications were evaluated to optimize the LC method. Since HFIP was considered to have a potential role in the response decline owing to its volatility, additional precautions were taken to avoid evaporation, and solvents were replenished daily. However, this resulted in only a minimal improvement. An alternative strategy of decreasing HFIP concentrations from the LC mobile phase and introducing post-LC column (to enhance ionization of the oligonucleotides) was also assessed. Decreasing the HFIP concentration from 2 to 0.1% resulted in a substantial increase in response (around 350-fold). However, this strategy proved unviable since the peak shape and column performance deteriorated rapidly. Decreasing HFIP concentration significantly increased mobile phase pH to 11–12, which reduced the life time of the LC column. Complete removal of HFIP from the mobile phase and performing LC at a higher pH with ammonium carbonate resulted in a 50–100-fold decrease in response. Hence, this method was not pursued.

Tetrahydrofuran was chosen as the ternary mobile phase to wash out the LC system at the end of the chromatographic run since it reduced the carryover for the analyte.

LC–MS/MS method performance

The LC–MS/MS method for imetelstat in plasma (Supplementary Tables 2 & 3) was optimized to balance sensitivity requirements (lowest expected concentrations in preclinical and clinical samples, 0.1 and 0.5 µg/ml, respectively) while providing a stable response across an analytical batch.

Representative chromatograms of imetelstat and of the IS are presented in Figure 2. Although there were some methodological challenges owing to the lipid tail on these oligonucleotides, the LC and MS profiles were in-line with published reports.

Imetelstat extraction from plasma

Mixed-mode anion exchange SPE method development

Initially, a similar extraction approach as described by the authors of the 18-mer Trabedersen assay was assessed [33]. This method combined an LLE method using chloroform-phenol-isoamylalcohol with a hydrophilic-lipophilic-balanced SPE. However, for imetelstat, the recovery in the LLE step was very low (<20%), despite good recovery (>70%) of the experimental reference 16-mer oligonucleotide that was tested alongside. Combinations of protein precipitation (with acetonitrile or ethanol) and SPE to extract imetelstat were also ineffective due to losses in the initial protein precipitation step. Hence, mixed-mode anion-exchange extraction on SPE was explored as a potential solution to obtain good recovery.

Initial experiments with the commercially available kit (Clarity OTX extraction kit; 100 mg/3-ml mg/3 ml cartridges) also yielded a very low (<10%) recovery for imetelstat, though recovery for the reference 16-mer oligonucleotide was more than 50% (Supplementary Table 1). Imetelstat recovery improved to ≥65%, following incubation of 20 µl plasma with 200 µl lysis buffer at 50°C for 10 min before loading on the SPE cartridge. While imetelstat recovery was acceptable under these conditions, drift in the LC–MS/MS response still occurred in larger analytical runs, most likely due to residual matrix or buffer components. The wash and elution steps were further optimized (buffer composition, solvents and pH; data not shown). Finally, the fractionated elution (Supplementary Table 4) with 500 µl fractions of 100 mM ammonium bicarbonate pH 8.8/acetonitrile (50/50 v/v) revealed no

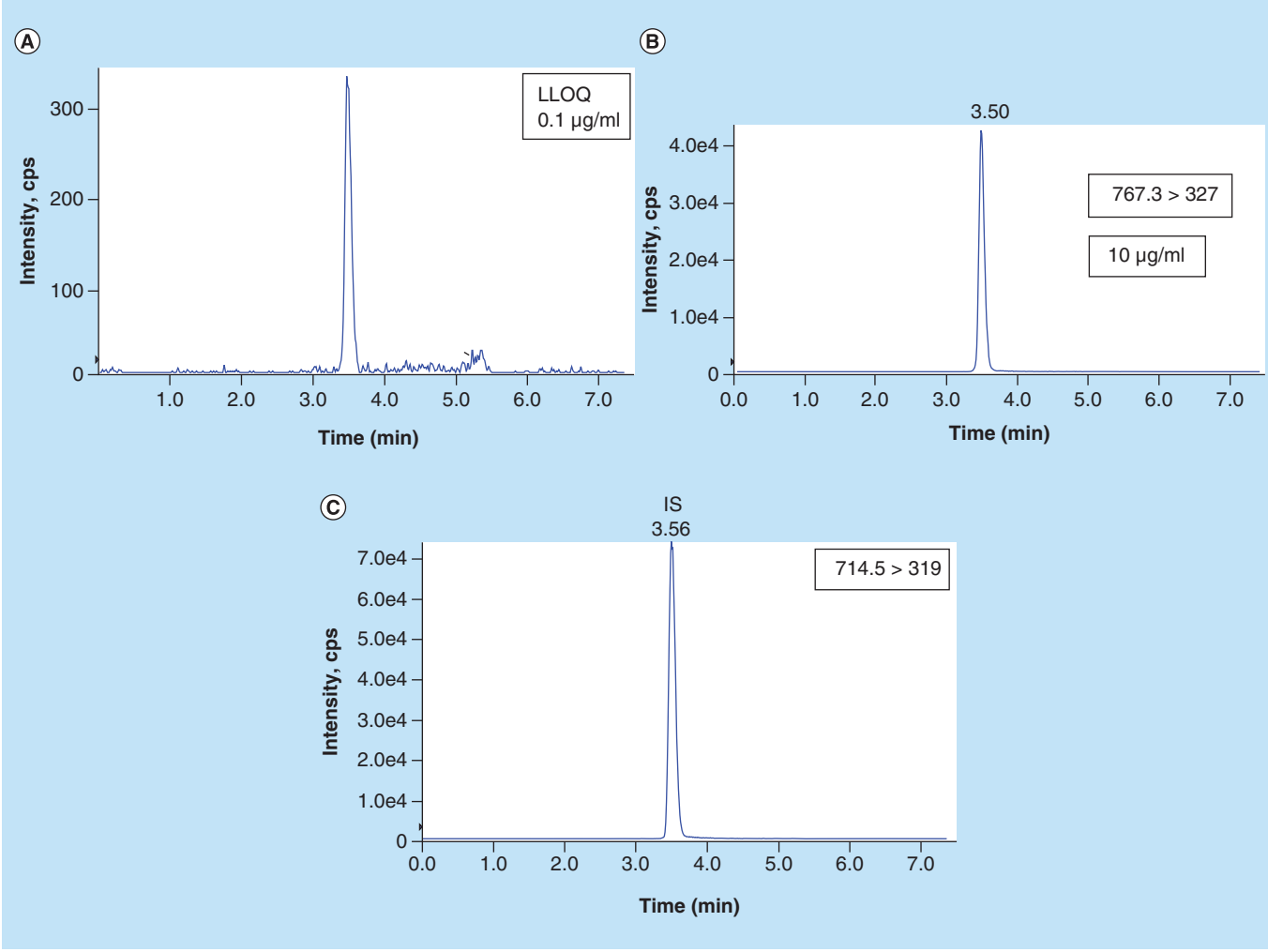
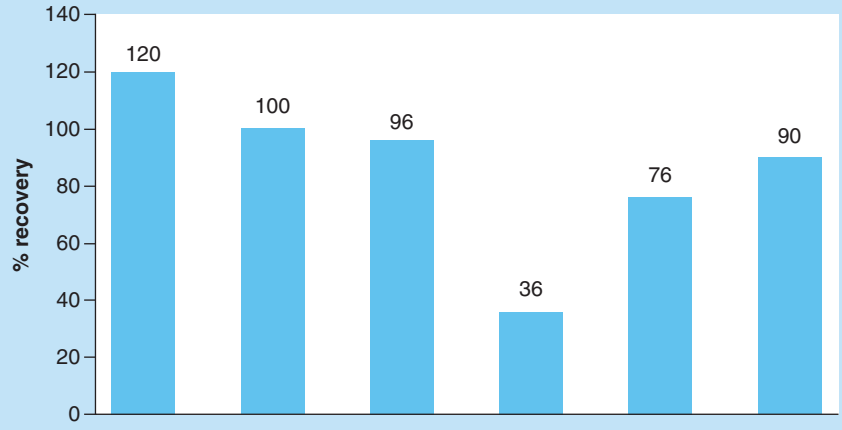


Figure 2. LC–MS/MS chromatograms of (A) imetelstat 0.1 µg/ml and (B) imetelstat 10 µg/ml, and (C) IS 20 µg/ml.
Chromatographic and MS conditions are described in Supplementary Tables 2 & 3.
cps: Counts per second; IS: Internal standard.

elution of imetelstat in the first 500 µl. Using 500 µl elution fractions (Supplementary Table 4), imetelstat recovery was 54% in the second elution and 10% in the third 500 µl elution fraction (first 500 µl removed). The IS used in our analysis contains the 12-mer 5′-nucleotide sequence of imetelstat including the lipid tail (the sequence is identical but linkage opposite from 3′ to >5′) and the NPS linkage is replaced by the OPS linkage (Figure 1; Supplementary Table 1). Other oligonucleotide structures such as the 13-mer OPO of imetelstat without a lipid tail were evaluated as well as IS but behaved more different than the 12-mer with the lipid tail. Nevertheless, this IS had a different elution pattern with approximately 40, 30 and 10% recovery in the first, second and the last 500 µl elution fractions, respectively. Combining all eluted fractions had a negative impact on signal robustness in the LC–MS/MS analysis. Since the IS showed a different elution profile compared with imetelstat, the IS was spiked after SPE elution, so that the IS only compensated for the signal drift in the LC–MS/MS but not for SPE recovery. Although addition of the IS following extraction is less optimal, all analytical runs are controlled by inclusion of QC samples prepared in the corresponding blank matrix as means of in-study validation.

Hybridization extraction method development

For imetelstat hybridization extraction, two capture probes were synthesized. The biotin labels were introduced via a TEG linker. This linker differs slightly when included at the 3′- or 5′-position as reflected in the molecular weight (Supplementary Table 1). In theory, the 3′ probe is preferred as the TEG linker is slightly longer, thereby



Streptavidin beads (pmol)	3000	3000	3000	750	750	750
5' biotin-capture probe (pmol)	1000	1000	100	100	100	100
Imetelstat (pmol)	200	100	100	500	200	100

Figure 3. Imetelstat recovery by different concentrations of beads, capture probe and imetelstat.

potentially minimizing steric hindrance. However, at this position steric hindrance could have more pronounced spatial hindrance as both the lipid tail and the biotin label are in close spatial proximity when the complementary strands hybridize (Supplementary Figure 1A), thereby limiting the recovery. Therefore, both options were explored, and recovery was assessed for 3′- and 5′-biotin probes. Following incubation of 100 pmol imetelstat with 100 pmol of capture probe and elution for 30 min at 80°C, imetelstat recovery was 97.8 and 35.2% with the 5′- and 3′-capture probes, respectively.

Regarding the optimum temperature required for adequate denaturation and elution of the double-stranded molecule, it was observed that 50°C was insufficient while elution for 30 min at 80°C resulted in lower recovery compared with the elution for 30 min at 50°C, followed by 10 min at 80°C. Therefore, elution for 20 min at 80°C was finalized as the elution condition (Supplementary Table 5) and proved to be robust as the assay acceptance criteria were met, as demonstrated by the performance of the QC samples.

The combination of the amount of beads and the concentration of the capture probe was also investigated. In theory, streptavidin has four biotin-binding sites but, in practice, steric hindrance limits binding efficiency. The streptavidin magnetic beads used in this analysis had a binding capacity of 3500 pmol/mg at a concentration of 10 mg/ml. Preliminary experiments demonstrated the loss of capture probe in the supernatant fraction at equimolar binding capacity of beads and capture probe (data not shown). Thus, a relative excess of binding capacity (i.e., amount of beads) was selected. Figure 3 shows imetelstat recovery at different concentrations of streptavidin beads and 5′ biotin-capture probe. We found that at least equal amounts of capture probe and imetelstat were a prerequisite for the sufficient recovery (>90%) of imetelstat. Excess capture probe was considered, but increased capture probe levels, in turn, required increased concentrations of streptavidin beads, thereby increasing the assay costs. The ULOQ of the assay determines the amount of capture probe needed in the assay; a slight excess (×1.5) was considered to be able to evaluate study samples with concentrations above the ULOQ.

Technologies such as polymerase chain reaction and southern blots rely on the hybridization principle, in other words, single-stranded DNA or RNA annealing to the complementary DNA or RNA strand. In a validated hybridization ELISA for imetelstat [15] (Supplementary Figure 1B), a complementary oligonucleotide sequence containing a biotin label at the 3′-position provided the scaffold for competition of imetelstat and a 3′-labeled digoxigenin analog. Application of this 'affinity' to extract oligonucleotides from a biological matrix for qualitative

Table 2. Stability of imetelstat in rat and human plasma spiked at 20 μg/ml.

Storage condition	Rat plasma		Human plasma	
	Measured concentration (μg/ml)	% vs reference (time = 0 h)	Measured concentration (μg/ml)	% vs reference (time = 0 h)
Reference control (time = 0 h)	20.0 (0.3)	100.0	21.7 (1.2)	100.0
2 h at RT (22°C)	18.6 (0.2)	92.8	21.7 (0.3)	100.0
2 h at 4°C	18.5 (0.6)	92.5	21.9 (0.7)	100.8
2 h at 37°C	17.5 (0.1)	87.4	14.9 (1.5)	68.7
24 h at RT (22°C)	15.6 (0.4)	77.9	19.8 (2.0)	91.0
5x freezing and thawing cycles	16.7 (3.3)	83.4	20.8 (1.2)	95.6

Extraction performed using SPE-Clarity approach. Results are mean (SD) of three independent samples.
RT: Room temperature.

and quantitative analysis in combination with LC–MS/MS is newly explored and has only been briefly mentioned previously [42,43].

SPE–LC–MS/MS performance

Precision & accuracy

Using the final SPE method presented in Supplementary Table 4, the method was qualified in rat plasma in a single LC–MS/MS run (Supplementary Tables 2 & 3). This qualification included nine calibration levels of imetelstat. Using log–log linear regression back-calculated values with %RE ≤12% at all concentration levels of the calibration curve were obtained. The precision and accuracy of the method were determined at three QC levels – low, medium and high imetelstat concentrations (0.8, 8 and 40 μg/ml, respectively).

In rat plasma, the calibration curve for imetelstat was established in the range of 0.1–50 μg/ml. The calibration curve for imetelstat in rat plasma exhibited good linearity. Table 1 shows the intrabatch precision and accuracy of the analysis of imetelstat in rat plasma. The intrabatch precision was acceptable, with the CV being less than 15%. The intrabatch accuracy ranged between 93.2 and 100%. Selectivity was evaluated in six different rat plasma batches at the LLOQ level and showed an average accuracy of 94.7% and a CV of 8.7%.

Since the SPE–LC–MS/MS assay in human plasma was identical apart from the plasma source, no formal qualification was carried out for human plasma, but all analytical runs included QC samples prepared in human plasma as means of in-study validation.

Imetelstat stability

The stability of imetelstat in rat and human plasma was assessed (Table 2). Imetelstat was stable in plasma stored for 2 h at either 4°C or at RT, and during repeated freezing and thawing as the measured values were more than 80% of reference control. Stability of imetelstat at RT for 24 h and after freezing and thawing in rat plasma showed more degradation compared with the human plasma, probably explained by the enhanced esterase and hydrolase activity observed in rodent plasma [44].

Hybridization extraction–LC–MS/MS performance

Precision & accuracy

Using the final hybridization method (Supplementary Table 5), the method was qualified in human plasma in a single LC–MS/MS (Supplementary Tables 2 & 3) run. This qualification included seven calibration levels of imetelstat. The back-calculated concentrations levels obtained following log–log linear regression showed RE less than 5%. The precision and accuracy of the method were determined at three QC levels – low, medium and high imetelstat concentrations (1.6, 8 and 40 μg/ml, respectively). Selectivity was evaluated in six different plasma batches at the LLOQ level and showed an average accuracy of 79.8% and a CV of 13.3%.

In human plasma, the calibration curve for imetelstat was established in the range of 0.5–50 μg/ml (Supplementary Figure 2B). The LLOQ of the method is defined as the lowest calibration point with S/N ratio more than 5 and %RE less than 25%, and can be adapted as a function of the lowest concentrations of imetelstat expected in (pre)clinical studies.

Table 3. Comparison of imetelstat concentrations in clinical plasma samples as analyzed by three independent assays on three different occasions.

Patient and sampling times	Imetelstat concentration (μg/ml) hybridization ELISA	Imetelstat concentration (μg/ml) SPE–LC–MS/MS[†]	Accuracy % of ELISA result[‡]	Imetelstat concentration (μg/ml) hybridization–LC–MS/MS	Accuracy % of ELISA result[§]
Patient #1					
Before infusion	<LLOQ	<LLOQ	–	<LLOQ	–
End of infusion	105	113	108	96.8	92.3
1 h after infusion	80.7	86.0	107	75.9	94.0
2 h after infusion	102	86.0	84.3	86.8	85.1
Patient #2					
Before infusion	<LLOQ	<LLOQ	–	<LLOQ	–
End of infusion	158	181	114	127	80.7
1 h after infusion	111	153	138	119	107
2 h after infusion	124	129	103	98.6	79.3
Patient #3					
End of infusion	98.6	108	110	98.6	100
1 h after infusion	83.6	104	124	97.3	116
2 h after infusion	81.8	85.5	105	77.3	94.5
Patient #4					
Before infusion	0.6	0.588	98.8	<LLOQ	-
End of infusion	120	119	99.2	108	89.6
1 h after infusion	99.2	93.5	94.2	88.6	89.3
2 h after infusion	85.3	78.5	92.0	78.2	91.6
Patient #5					
Before infusion	<LLOQ	<LLOQ	–	<LLOQ	–
End of infusion	133	112	83.7	100	75.1
1 h after infusion	140	133	94.9	117	83.7
2 h after infusion	116	113	96.9	99.5	85.8

[†]Samples > ULOQ were analyzed after a 1:5 dilution.
[‡]SPE–LC–MS/MS versus hybridization ELISA.
[§]Hybridization–LC–MS/MS (LLOQ LC–MS/MS assay = 0.500 μg/ml) versus hybridization ELISA.
LLOQ hELISA = 0.35 μg/ml.

Table 1 shows the intrabatch precision and accuracy of the analysis of imetelstat in human plasma. The intrabatch precision was acceptable, with the CV being less than 8%. The intrabatch accuracy ranged between 105 and 112%.

Comparison of the SPE–LC–MS/MS & hybridization extraction–LC–MS/MS methods with the validated hybridization ELISA to quantify imetelstat in study samples

Human plasma

For comparison of the SPE–LC–MS/MS and hybridization extraction–LC–MS/MS methods with the validated hybridization ELISA, 19 clinical samples from five patients who had received imetelstat infusion of either 7.5 or 9.4 mg/kg for 2 h were evaluated. Imetelstat concentrations were determined with the validated hybridization ELISA [15].

Prior to LC–MS/MS analysis, plasma samples were stored at -80°C for about 2 years. No 2-year stability data are available for this assay but in the validated ELISA 113 days stability in the freezer was proven. The study samples were analyzed at risk with the goal to encipher whether ELISA results in higher concentration levels compared with the LC–MS assay.

Table 3 presents imetelstat concentrations from the SPE–LC–MS/MS and hybridization–LC–MS/MS methods compared with those from the hybridization ELISA. With both methods, imetelstat concentrations were in agreement with the values obtained with hybridization ELISA.

Since the hybridization ELISA also measures truncated oligonucleotides, our data that show comparable imetelstat levels measured with SPE–LC–MS/MS and hybridization ELISA may suggest that imetelstat metabolite

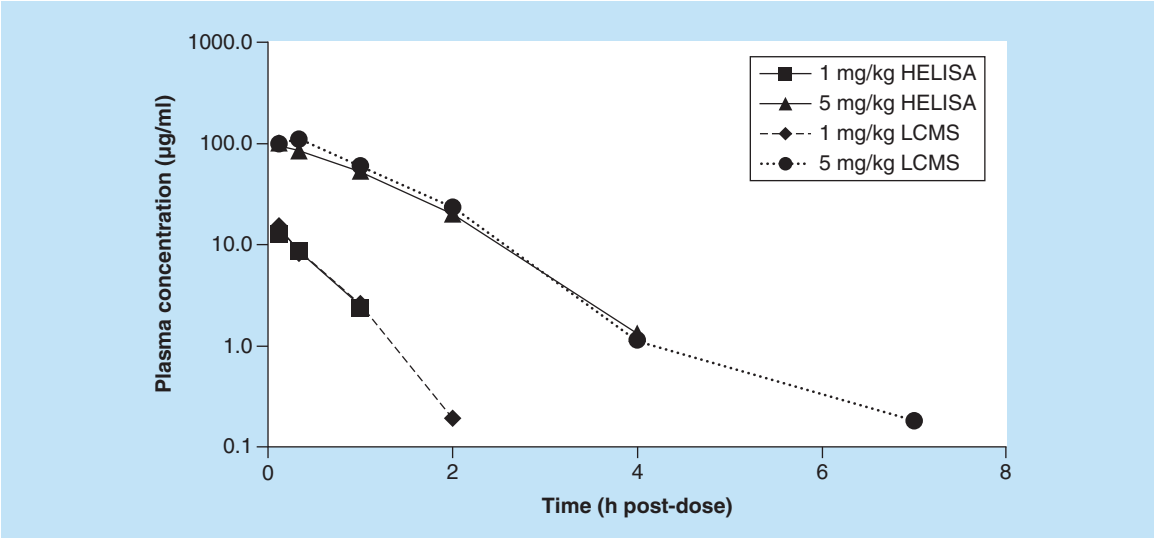


Figure 4. Concentration versus time profiles for imetelstat in rat plasma after intravenous bolus dosing at 1 and 5 mg/kg after analysis by orthogonal methods: SPE–LC–MS/MS and hybridization ELISA.

concentrations are low (though no formal data are available to prove this). Moreover, the specificity of the LC–MS/MS system for imetelstat and its metabolites has not been established. The LC–MS/MS assay separates the truncated forms (n-1) of imetelstat; however, the NPO metabolite (one S exchanged for an O) coelutes with imetelstat but very low signals were observed in the incurred samples. Nonetheless, whether other Na^+ adducts of multiple-charged ions can contribute to the imetelstat transition is unclear due to the unavailability of reference materials. It should be noted though that LC conditions with HFIP are believed to minimize adduct formation [38].

The combination of selective hybridization extraction with LC–MS/MS detection potentially offers selectivity and specificity for imetelstat evaluation. However, the selectivity of the hybridization has not yet been fully explored. It is unknown if shorter oligonucleotide fragments (n-1; n-2 metabolites) can also hybridize with the capture probe; the cutoff fragment length is unknown. If only unchanged imetelstat is present and quantified, the evaluation using hybridization extraction and LC–MS/MS is not expected to have a substantial impact. However, if metabolite identification is required, then the selectivity of this method will require further exploration.

Rat plasma

The mean plasma exposures of imetelstat after receiving an intravenous bolus dose at either 1 or 5 mg/kg as determined from both the SPE–LC–MS/MS method and the hybridization ELISA are shown in Figure 4; the data are virtually superimposable. As shown, the SPE–LC–MS/MS assay is slightly more sensitive than the hybridization ELISA and is capable of detecting plasma exposure of imetelstat above the LLOQ at one additional time point.

Conclusion

LC–MS/MS assay was developed for the quantitative analysis of imetelstat in preclinical and clinical plasma samples. The assay performance was fit for purpose and in line with typical bioanalytical acceptance criteria. Furthermore, the analysis of human and rat plasma samples using both methods, SPE–LC–MS/MS and hybridization extraction–LC–MS/MS yielded results that were comparable to those obtained using a validated hybridization ELISA assay.

The presence of the lipid tail in imetelstat alters the overall physicochemical properties of the oligonucleotide such that classical ODN extraction approaches are not applicable. Two alternative extraction strategies in combination with LC–MS/MS were explored and qualified. The SPE approach using Clarity OTX anion exchange was optimized for recovery and to minimize matrix interferences, achieved through a fractionated extraction procedure. The hybridization extraction of an oligonucleotide in combination with LC–MS/MS is a novel approach and offers a more selective extraction, with a dynamic range dependent on the amount of capture probe. The specificity of this extraction toward truncated forms of imetelstat is currently unknown, although evidence suggests that this hybridization method is comparable to the validated hybridization ELISA. However, the mass spectrometric detection offers additional selectivity.

Future perspective

Development of quantitative LC–MS/MS assays for oligonucleotides continues to encounter some methodological challenges. Nevertheless, such methods are invaluable to support results obtained with ELISA or polymerase chain reaction (PCR) technology.

Cleanup using 2D chromatography, as well as trap/elute approaches or full orthogonal 2D strategies may enhance the signal stability. MicroLC with low flow rates could enhance ionization efficiency and reduce signal variability as less matrix is introduced. Strategies to avoid using HFIP and ion-pairing reagents as constituents in the mobile phase, such as by hydrophilic interaction LC before introduction to the MS, may strengthen the MS response.

High-resolution MS provides the benefit of improved selectivity and can eliminate the fragmentation step used in the multiple reaction monitoring approach, which results in nonselective product ions in case of oligonucleotides.

Oligonucleotide extraction from a biological matrix is a critical step, especially for oligonucleotides such as imetelstat, which combine a lipid tail at the 5′-position and a hydrophilic nucleotide strand. These characteristics alter the physicochemical properties, and the more traditional liquid extractions using chloroform–phenol approaches do not result in sufficient recovery. Disrupting the strong protein binding by use of organic solvents, detergents or proteinase K treatment can also be examined. With SPE, pretreatment with lysis buffer is essential; however for the hybridization approach, currently no special treatment for dissociation of the protein binding is included. The high hybridization affinity will probably displace the oligonucleotide from the proteins, to which it is bound. For hybridization extraction, understanding the functional specificity of assay conditions in relation to the truncated variants, as well as endogenous oligonucleotides present in the sample requires further exploration to apply and adapt this extraction method to new oligonucleotide sequences.

Finally, extraction of oligonucleotides from other biological matrices, such as tissue homogenates, is often a requirement in development programs. Whether the proposed approaches can successfully be applied to these matrices requires further elaboration.

Summary points

- Imetelstat is a novel, first-in-class telomerase inhibitor oligonucleotide, being developed for the treatment of hematologic myeloid malignancies. This report describes a quantitative LC–MS/MS method using solid phase or hybridization extractions for the quantitation of imetelstat as an orthogonal method for the validated hybridization ELISA.
- Two independent extraction approaches were developed to capture imetelstat from plasma: an anion exchange SPE method with fractionated elution, and a hybridization extraction approach using a complementary biotinylated DNA probe.
- The developed method was qualified and was sufficiently sensitive to detect the range of imetelstat concentrations expected in preclinical (rat plasma) and clinical (human plasma) samples.
- Imetelstat concentrations in incurred human and rat plasma samples with the qualified LC–MS/MS assay with solid phase or hybridization extraction were in agreement with the concentration values obtained with the validated ELISA.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.future-science.com/doi/full/10.4155/bio-2017-0145

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved. Animal experiments were carried out in accordance with the guide for the care and use of laboratory animals.

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60 seconds with Richard Geary: an interview on developments for the bioanalysis of ASOs

Richard Geary is Executive Vice President of Drug Development at Ionis Pharmaceuticals (CA, USA). He is responsible for preclinical development, global clinical development, and regulatory and manufacturing for Ionis' antisense drugs. Since joining Ionis in 1995, Geary has been involved in discovery and development, including the regulatory submission of more than 40 investigational new drug applications and five successful antisense medicine late-stage development programs through approvals in multiple jurisdictions.

1 How have ASOs evolved over the last 25 years?

Twenty-five years ago ASOs were a hope and dream for a new discovery technology that had the potential for truly disruptive medicine. Today, that hope and dream has had its initial realization with more than ten approved medicines, predominantly in rare and severe diseases, and its first blockbuster medicine Spinraza (nusinersen) that has given a treatment option for over 10 thousand patients who previously had no drug therapy available for their often fatal spinal muscular atrophy disease. The future is bright for transformative medicines previously unimagined!

2 What were some of the challenges experienced in bringing ASOs to market?

The challenges were extensive, from requiring new medicinal chemistry breakthroughs to analytical method inventions, health authority experience, and understanding of a new modality and delivery innovations.

3 What key benefits might be realized by standardizing bioanalytical methods for ASO analysis?

Of course, front and center is the ease of replication, from medicine to medicine, speed, and assured validations. Another benefit would be the ease of outsourcing a known approach, ensuring resource and expansion of high-quality expertise and laboratories to handle a rapidly growing and expanding technology.

4 Where do you anticipate this field will be in 5–10 years' time?

The goal and expected reality is to expand access to both rare and broad diseases, treating millions of patients, and meeting the unmet needs not currently possible for patients.

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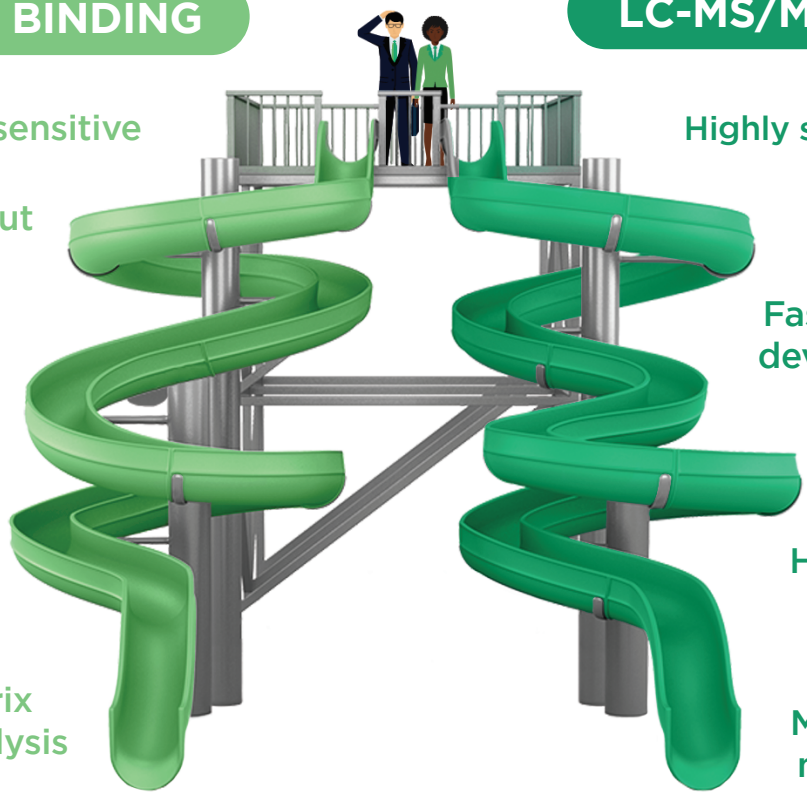

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Trends and challenges in ASO bioanalysis: a written interview with Eric David

Eric David is Principal Scientist at Biogen, Inc. (MA, USA). He has over 25 years of experience in regulated bioanalysis, having spent his career in biotech and contract research in Canada and in the United States. At Biogen, Eric created and led a GLP-compliant bioanalytical laboratory with expertise in both LBA and LC–MS assays. Over the past five years, he has focused on preclinical and clinical ASO bioanalysis. He holds a BSc in chemistry from McGill University (Montréal, Canada).

1 What are some fundamental differences between LC–MS and LBA for the quantification of ASOs?

LC–MS assays are intrinsically more selective than LBAs. Hybridization ELISA assays with full-length capture probes are the most common LBA assays utilized. However, they often cross-react to a certain extent with n-1 metabolites. Metabolite cross-reactivity can be more prevalent with dual ligation hybridization ELISAs or electrochemiluminescence (ECLIA) assays. Although LC–MS assays can also leverage hybridization for ASO capture, additional selectivity for the measurement of the intact full-length ASO can be achieved through several modalities of detection, including high-resolution accurate mass measurement and collision-activated dissociation (MS/MS). In addition, LC–MS assays often have much wider quantitation dynamic ranges than hybridization ELISA assays (approximately 10-fold), making it easier to evaluate study samples either without predilution, or by reducing the guesswork in determining a suitable predilution factor. Considering the current state of technology, LBA assays such as hybridization ECLIA still represent the most sensitive approach when pg/mL detection limits are required, with LC–MS assays slowly closing the gap.

2 What bioanalytical techniques do you see thriving in the analysis of ASOs in the future?

LC–MS utilizing hybridization capture probes will quickly become the norm for ASO bioanalysis, especially when there is no need for very low limits of quantitation. The assay reagents are quick and easy to obtain. The technology allows multiplexing, wherein both the parent ASO and major metabolites can be monitored simultaneously. As the sensitivity of the technique improves, it will ultimately surpass that of the most sensitive current LBA assays while maintaining selectivity.

However, for now when the required sensitivity cannot be achieved, we can quickly pivot towards basic LBA assays and move to more complex ones when challenged by high background noise or lack of sensitivity.

3 What are some of the challenges of standardizing bioanalytical techniques for ASO drug development programs?

Basic LC–MS and LBA assays can sometimes fail when new ASO chemistries are brought into the pipeline. Capture probes that work with common 2′-O-MOE ASOs may not bind as well for new generation ASOs such as 2′-O-NMA. New generation ASO chemistries may prove a tougher challenge for LC–MS, affecting their potential limit of quantitation. The advent of ASO–antibody conjugates will also mean that both LC–MS and LBA techniques will remain relevant, with the need to quantitate both the ASO and the antibody adding complexity and cost to the bioanalytical portion of studies.

4 At what stages of a drug development program do you consider LBA versus LC–MS?

We develop an LC–MS assay as soon as the ASO enters investigational new drug (IND)-enabling studies. The LC–MS assays generally possess adequate sensitivity at that stage and can also be leveraged to evaluate the presence of metabolites. Once the ASO reaches the clinical stage, depending on its mode of administration, a more sensitive LBA assay can sometimes be necessary in order to fully characterize the PK profile, including trough concentrations. In that case, a hybridization ECLIA assay has often been utilized.

Research Article
SPECIAL FOCUS ISSUE: The Exciting World of Oligonucleotides

Bioanalysis

Oligonucleotide quantification and metabolite profiling by high-resolution and accurate mass spectrometry

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Aim: Advancements in RNA interference therapeutics have triggered development of improved bioanalytical methods for oligonucleotide metabolite profiling and high-throughput quantification in biological matrices. **Results & methodology:** HPLC coupled with high-resolution mass spectrometry (LC-HRMS) methods were developed to investigate the metabolism of a REVERSIR™ molecule *in vivo*. Plasma and tissue samples were extracted using solid-phase extraction followed by LC-HRMS analysis for metabolite profiling and quantification. The method was qualified from 10 to 5000 ng/ml (plasma) and 100 to 50000 ng/g (liver and kidney). In rat liver, intra and interday accuracy ranged from 80.9 to 118.5% and 88.4 to 111.9%, respectively, with acceptable precision (<20% CV). **Conclusion:** The LC-HRMS method can be applied for metabolite profiling and quantification of oligonucleotides in biological matrices.

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Keywords: mass spectrometry • metabolite profiling • oligonucleotide • pharmacokinetic • RNAi • siRNA • small interfering RNA

Small interfering RNA (siRNA) provides a promising approach to silence disease-causing genes through the mechanism of RNA interference (RNAi) and represents an opportunity to transform the treatment of many diseases [1–3]. The first RNAi-based drug, ONPATTRO® (patisiran), has been approved by the US FDA [4] and the European Medicines Agency [5]. As more RNAi compounds move into late-stage clinical development, it becomes increasingly important to establish a fundamental understanding of the metabolism and pharmacokinetics (PK) of these compounds to facilitate RNAi therapeutic drug design during drug discovery and development.

Measurement of siRNA concentration and identification and quantification of metabolites in a variety of biological matrices are critical initial steps, providing essential information for PK and toxicokinetic (TK) evaluation, and elucidation of metabolic pathways. A robust, selective, sensitive method is highly desirable for the analysis of therapeutic oligonucleotides and their metabolites.

Although bioanalysis of RNAi therapeutics is challenging due to the similarity to the vast amount of endogenous DNA and RNA present and the difficulty in differential cleanup and isolation of the siRNA and metabolites, a number of methods have been reported for quantitative analysis of siRNA *in vitro* and *in vivo*, including several PCR-based methods, such as primer-extension PCR [6], stem-loop reverse transcription (RT)-PCR [7] and real-time quantitative PCR (RT-qPCR) [8], as well as ELISA [9] and HPLC with fluorescence detection [10]. These methods can provide robust and sensitive quantification of siRNA. The success of the PCR methods was critically dependent on the design of the primers. Hybridization assays including hybridization-ELISA and hybridization-HPLC with fluorescence detection usually involve the hybridization of the target oligonucleotide to a capture probe and a detection probe [11]. Thus, the hybridization assay robustness relies on the design and availability of the reagents. Both PCR- and hybridization-based assays can be time-consuming and costly particularly with respect to the design and optimization of the primers or reagent probes. Moreover, it is very challenging to differentiate metabolites from parent siRNAs by PCR or hybridization assays.

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Until recently, application of LC coupled to MS for oligonucleotide analysis has lagged behind its use for small molecules, peptides and proteins due to its low sensitivity compared with qPCR and ELISA format assays [12]. Negative ion ESI–MS has been used for oligonucleotide detection and analysis [12,13] to meet the challenge of the charged phosphodiester backbone. Ion-pairing (IP) reversed-phase LC has also been used for the analysis of oligonucleotides due to its compatibility with MS-based techniques as first demonstrated by Apffel and colleagues using the IP system triethylamine (TEA) and hexafluoroisopropanol (HFIP) [14,15].

Complex matrices such as plasma and tissue homogenates present additional challenges in sample preparation. Biological matrices contain organic and inorganic components as well as micro- and macromolecules which result in interference with analyte detection and quantification [16]. Various sample preparation techniques such as protein precipitation, proteinase K digestion, phenol/chloroform liquid–liquid extraction and solid phase extraction (SPE), have been used in oligonucleotide analysis. Various SPE methods have been developed which yield high recovery with effective depletion of interfering compounds.

Unlike small molecule drugs, which are mainly metabolized by cytochrome P450-mediated mechanisms, oligonucleotides are metabolized mainly by cleavage of the phosphodiester bonds by nucleases. Cleavage at the 3′-terminus by exonucleases is the major metabolic fate for oligonucleotides followed by 5′-exonuclease and endonuclease cleavage events. Oligonucleotide metabolites produced by exo- and endo-nuclease activity have been identified using MS/MS with accurate mass measurement; however, data interpretation is difficult and time-intensive mainly due to the large number of fragment ions generated from siRNAs. This issue has been mitigated through improvements and advances in software such as automated spectrum deconvolution and data analysis.

A HPLC with high-resolution accurate mass spectrometry (LC-HRMS) method is capable of resolving the sense and antisense strands of siRNA using the denaturing conditions of high temperature and IP reagents. It can qualify and quantify double-stranded oligonucleotides, such as siRNA, by measuring either sense- or antisense-strand RNA. In this paper, we applied the LC-HRMS method to investigate the metabolite profiles and quantification of REVERSIR-A, a single-stranded *N*-acetylgalactosamine (GalNAc)-conjugated oligonucleotide, targeting the antisense strand of siRNA. REVERSIR™ molecules have been developed to provide control of RNAi pharmacology by rapid reversal of target-gene silencing effects of RNAi therapeutics [17]. A selective, sensitive, robust and high throughput LC-HRMS MS method was developed for the quantification of REVERSIRs as well as their metabolites in plasma and tissues to support preclinical/clinical PK and TK studies.

Materials & methods

Chemicals & oligonucleotide samples

Acetonitrile (ACN), methanol (MeOH) and water (H_2O) were purchased from Fisher Scientific (PA, USA). *N, N*-diisopropylethylamine (DIEA) and HFIP with LC–MS grade purity were purchased from Sigma (MO, USA). REVERSIR-A and metabolite standards (Figure 1) as well as the internal standard (an analog of REVERSIR-A having a different molecular weight) were synthesized at Alnylam Pharmaceuticals (MA, USA) as described previously [17] and characterized by ESI–MS and anion exchange HPLC.

In vivo rat & monkey studies

All animal studies were conducted in accordance with local, state and federal regulations as applicable and all study protocols were approved by the Institutional Animal Care and Use Committee at Alnylam Pharmaceuticals. Sprague–Dawley rats and cynomolgus monkeys received a single subcutaneous (SC) dose of 3 mg/kg REVERSIR-A. Rat blood, the right lateral lobe of the liver and both whole kidneys were collected, processed and analyzed for quantification. Monkey blood, liver biopsy tissue and urine were collected, processed and analyzed for metabolite profiling as described below.

Instrument conditions

A Dionex HPLC system (Thermo Fisher Scientific, MA, USA) equipped with an autosampler and a Q Exactive™ mass spectrometer (Thermo Fisher Scientific) was used for the LC-HRMS analysis. All devices were controlled through Xcalibur software version 1.2 (Thermo Fisher Scientific). Samples were injected on the autosampler with a PolymerX™ RP-1 column (5 µm, 100 Å, 2.0 × 50 mm; Phenomenex, CA, USA) [18].

The Q Exactive™ mass spectrometer was set at full scan monitoring mode and negative ionization mode. Typically, a scan range of 500–2000 m/z; automatic gain control target of 1×10^6. Maximum injection time of 200 ms and resolution of 35,000 full-width half maximum were set for the metabolite profiling of REVERSIR-A.

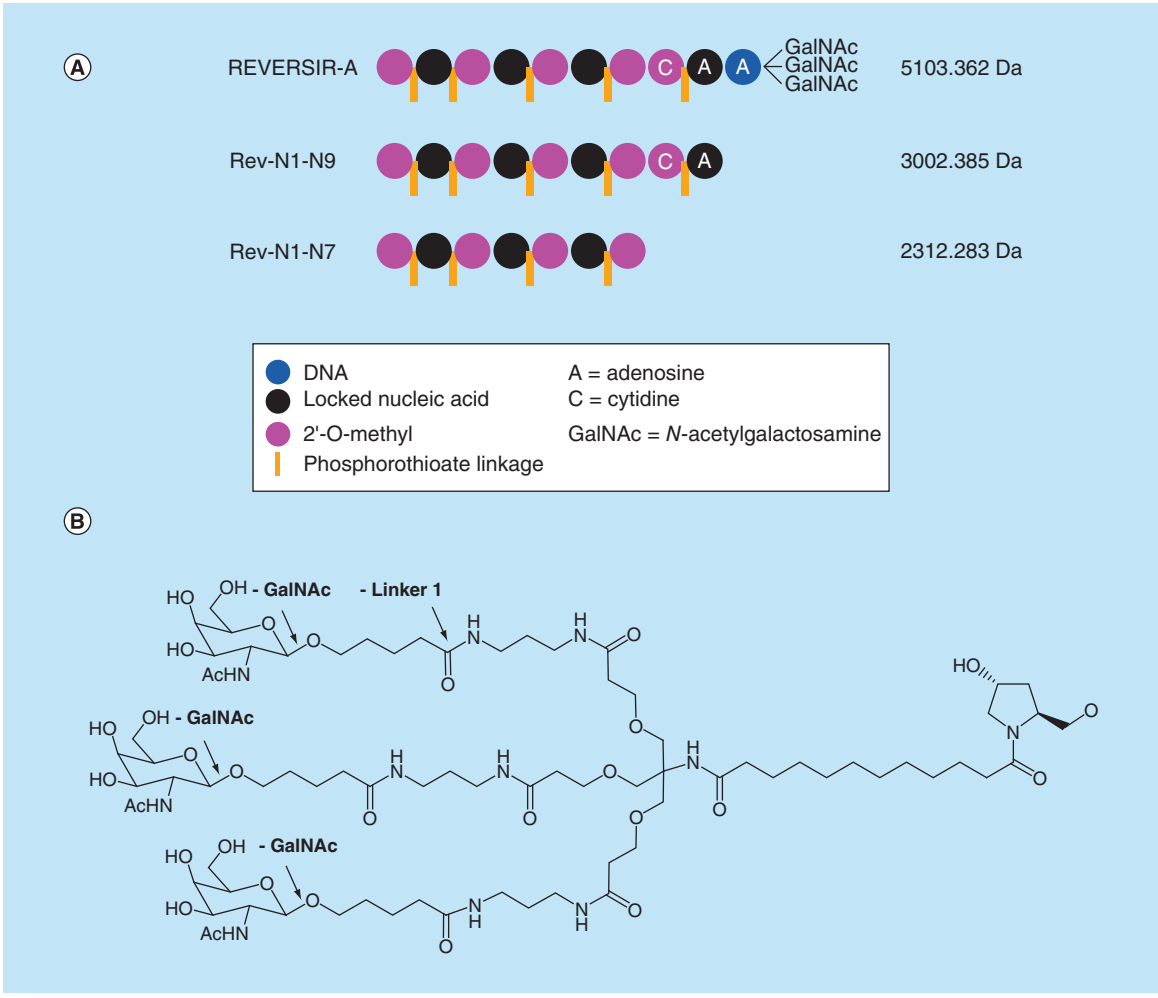

Figure 1. Sequences and structure. (A) Sequences of REVERSIR-A and metabolites. **(B)** Chemical structure and cleavage sites of metabolites of the triantennary GalNAc ligand of REVERSIR-A.

The Q Exactive™ mass spectrometer was set at targeted selective ion monitoring mode and negative ionization mode. Typically, a scan window of 5 m/z; automatic gain control target of 2×10^5, maximum injection time of 300 ms and resolution of 70,000 full-width half maximum were set for quantification of REVERSIR-A and metabolites.

Chromatographic conditions

The LC–MS mobile phases used were as follows: Mobile Phase A: 1.0% HFIP, 0.1% DIEA in water, 10 µM EDTA; Mobile Phase B: 0.75% HFIP, 0.0375% DIEA in 65% ACN and 35% water, 10 µM EDTA; Mobile Phase C: 10% water, 45% MeOH and 45% ACN.

For metabolite profiling of REVERSIR-A, the typical gradient started with 5% mobile phase B and progressed to 25% B over 20 min, then increased to 70% B in 0.1 min. The column was washed with mobile phase B for 1.9 min, and then with mobile phase C for 4.8 min. The column was re-equilibrated with 5% mobile phase B for 3 min. The flow rate was 0.5 ml/min for 1.9 min, decreased to 0.3 ml/min at 2 min, and then increased to 0.5 ml/min at 22.1 min; column temperature was 80°C.

For the quantitation of REVERSIR-A and metabolites, the typical gradient started with 0% mobile phase B, progressed to 40% B over 4 min and increased to 60% B in 0.1 min. The column was washed with mobile phase B for 0.9 min, and then washed with mobile phase C for another minute. The column was re-equilibrated with 0% B for 3.9 min. The flow rate was 0.25 ml/min; column temperature was 80°C.

Table 1. Recovery of REVERSIR-A and metabolites from rat liver homogenate using Clarity OTX solid phase extraction.

Nominal concentration (ng/ml)	REVERSIR-A			Rev-N1-N9			Rev-N1-N7		
	Pre-SPE area ratio†	Post-SPE area ratio‡	Recovery (%)	Pre-extraction area ratio†	Postextraction area ratio‡	Recovery (%)	Pre-extraction area rRatio†	Postextraction area ratio‡	Recovery (%)
25	0.03	0.03	97	0.02	0.02	91	0.04	0.04	106
75	0.10	0.09	91	0.06	0.05	87	0.11	0.11	101
400	0.50	0.44	89	0.31	0.27	87	0.54	0.53	97
1500	1.79	1.82	102	1.10	1.12	102	1.94	2.15	111
3500	3.80	3.76	99	2.24	2.27	101	3.99	4.16	104

Area ratio = analyte peak area/IS peak area.
†n = 2 replicates.
‡n = 5 replicates.
IS: Internal standard; SPE: Solid phase extraction.

Sample processing

Approximately 50 mg frozen liver and kidney samples from treated and untreated animals were ground at cryogenic temperatures in a 2010 Geno/Grinder (SPEX SamplePrep, NJ, USA) without the addition of water or buffer. Ground tissue powder samples were stored at -80°C until analysis at which time samples were resuspended in lysis buffer (Phenomenex) at 100 mg/ml tissue concentration and incubated for 3 h at ambient temperature with shaking. The different siRNA compounds were shown to be stable during processing by spiking into tissues at the time of homogenization and varying lysis times and temperature prior to analysis. All were stable under the conditions used (data not included). Pooled liver and kidney lysates from untreated animals were spiked with REVERSIR-A and metabolite standards to generate calibration standards and quality control (QC) samples. The standard curve range for both liver and kidney tissues was 100–50,000 ng/g for REVERSIR-A and metabolites. For quantification, 50 µl of tissue lysate was extracted, while 300 µl of tissue lysate was extracted for metabolite profiling.

A 50 µl aliquot of each plasma sample was diluted tenfold in lysis buffer, held at room temperature for 10 min, and then extracted for quantification of REVERSIR-A and metabolites. Calibrators and QCs were prepared by spiking REVERSIR-A and metabolite standards into pooled plasma from untreated animals. The standard curve ranged from 10 to 5000 ng/ml for REVERSIR-A and metabolites. A 100 µl aliquot of each plasma sample was diluted fivefold with lysis buffer and extracted for metabolite profiling. A 200-µl urine sample diluted tenfold with lysis buffer was extracted for metabolite profiling.

Sample extraction

All the lysed samples, standards and QCs from plasma, urine, liver and kidney were mixed with lysis-loading buffer and loaded onto equilibrated Clarity® OTX 96-well plate cartridges (Phenomenex).

The SPE cartridges were washed three-times with 1 ml of 50 mM ammonium acetate in 50:50 (v/v) water/ACN (pH 5.5) and three-times with 1 ml of 5 mM ammonium acetate in 10:90 (v/v) water/ACN (pH 5.5). Samples were eluted with 1 ml of 9:36:46:9 (v/v/v/v) 0.1 M ammonium bicarbonate (AmBicarb)/water/ACN/tetrahydrofuran solution (pH = 8.8). The eluents were dried in a TurboVap® (Caliper Life Sciences, MA, USA) under nitrogen for 1–2 h at 40°C. The dried samples were resuspended in 200 µl of Mobile Phase A. A volume of 75 µl was subjected to LC-HRMS for metabolite profiling analysis and a volume of 10 µl was subjected to LC-HRMS for qualification analysis. For assessment of recovery, matrices from untreated animals were loaded onto SPE plates, washed, eluted and then spiked with REVERSIR-A, Rev-N1-N9 and Rev-N1-N7.

Results & discussion

Efficiency of sample extraction procedure

Rat liver samples were extracted using SPE and analyzed by LC-HRMS. The SPE plates utilize a mixed-mode SPE sorbent with a wash and elution procedure that can efficiently extract oligonucleotides from biological matrices and remove interfering compounds. This methodology provides greater than 85% recovery (Table 1) from rat liver homogenate samples. The extraction efficiency for other matrices has been tested and demonstrated to be acceptable as part of method validation procedures at Contract Research Organizations (CROs). Other solid-phase extraction technologies used for oligonucleotide sample preparation, including Oasis HLB, Varian C18OH and

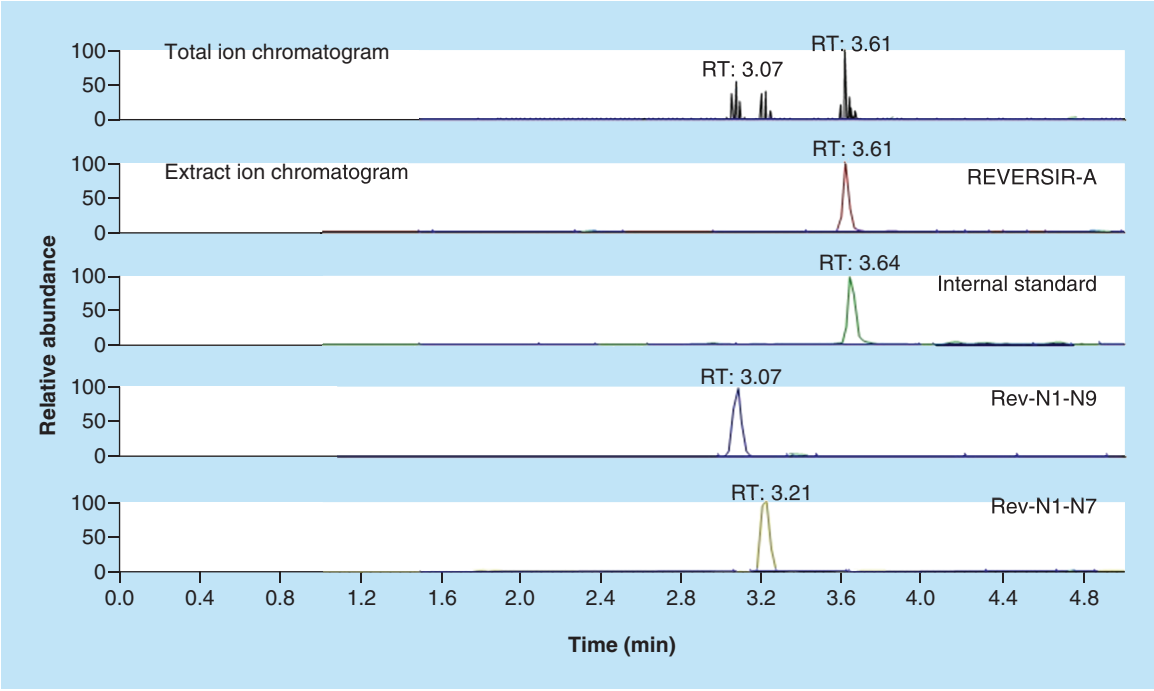

Figure 2. LC-high-resolution mass spectrometry chromatograms for spiked REVERSIR-A and metabolites in rat liver homogenate. The blank rat liver homogenate was spiked with 3500 ng/ml of REVERSIR-A and Rev-N1-N9 and Rev-N1-N7 metabolites.

Oasis® μHLB [19–21], exhibit poor recovery [16,22]. In addition to the excellent recovery of spiked REVERSIR-A and metabolites with SPE extraction, minimal interference from the biological matrices was observed for the LC-HRMS analysis. The optimized SPE extraction procedure provides a robust, high-throughput method of sample preparation for oligonucleotide analysis and has been used by CRO partners for oligonucleotide quantification in nonclinical and clinical studies in different matrices.

HRMS method for quantification of REVERSIR-A & metabolites & metabolite profiling of REVERSIR-A

Due to the high polarity of the oligonucleotides, IP chromatography is used to increase retention to a sufficient level on a reversed phase column. Ion-paring reagents such as TEA and HFIP have been commonly used and the composition of TEA/HFIP plays an important role in the separation and MS signal of oligonucleotides. The concentrations of the TEA/HFIP, pH and metal ions have been extensively evaluated for the separation and ionization of oligonucleotides by LC/MS [23,24]. Mobile phase A and B were optimized with ion pairing reagents HFIP and DIEA to provide a sensitive method with a lower limit of quantitation (LLOQ) of 10 ng/ml in plasma and 100 ng/g in tissues.

The addition of EDTA to the mobile phase greatly improves the peak shape of oligonucleotides since metal ions can be responsible for distorted analyte peak shapes via chelation [25–27]. EDTA can also prevent formation of sodium, potassium and iron adducts caused by the presence of trace amounts of these cations in the samples or HPLC system. However, EDTA causes the ion suppression of target analytes [25,26]. In this study, EDTA concentration was optimized at 10 μM and an acceptable LLOQ was reached. The sensitivity of this method is comparable with values reported in the literatures [28].

HPLC was performed at a high column temperature (80°C) to denature the REVERSIR-A and release protein-bound REVERSIR-A. The high column temperature and IP reagents in ACN buffer facilitate the separation and retention of REVERSIR-A and metabolites (Figure 2).

High-resolution accurate mass is needed for quantitative analysis of oligonucleotides to resolve the charge state distribution. Second, high mass resolution is necessary to separate or resolve the ions of interest from all possible

Table 2. Intra and interday accuracy and precision of quality control samples in rat liver homogenate.

	REVERSIR-A	Rev-N1-N9	Rev-N1-N7
Accuracy (% of nominal)			
Intraday	86.7–106.0	80.9–111.7	84.1–118.5
Interday	93.0–100.2	90.0–102.4	88.4–111.9
Precision (%CV)			
Intraday	<12.3	<19.9	<15.9
Interday	<7.6	<9.4	<6.6

Intraday: n = 4 replicates.
Interday: n = 4 replicates; n = 4 runs.
Quality controls were run at 25, 75, 400, 1500 and 3500 ng/ml spiked in rat liver lysate as in Table 1.

interferences originating from both matrix ions as well as other impurities. In addition to quantitative information for the target oligonucleotides, HRMS can simultaneously provide accurate mass for the metabolites.

The method precision and accuracy were evaluated in rat liver homogenates (Table 2). Intra and interday precision coefficient of variation (CV) was less than 20% for all three analytes in rat liver homogenate (Table 2).

The methods have been applied for quantification of other siRNAs and their most abundant metabolites in plasma and urine from multiple species. These methods have been successfully transferred to and validated by CRO partners to meet the regulatory requirements for Good Laboratory Practice (GLP) and clinical studies.

LC-HRMS method for metabolite profiling

LC-HRMS was applied for REVERSIR-A metabolite profiling because detection of less than 10 p.p.m. difference in exact mass is achievable and unambiguous metabolite identification is possible. For these studies a Q Exactive™ mass spectrometer, which provides a mass accuracy of less than 10 p.p.m. using external calibration, was used.

Figure 3 presents the results of a plasma sample collected 2 h postdose from a monkey administered 3.0 mg/kg REVERSIR-A SC and analyzed by LC-HRMS. Figure 3A shows the total ion chromatogram. The parent molecule, REVERSIR-A, eluted at 15.09 min with metabolites Rev-N1-N9 (metabolite formed by loss of the triantennary GalNAc linker with the 3′-terminal deoxyadenosine [dA-$GalNAc_3$]) and Rev-N1-N7 (metabolite formed by loss of the dA-$GalNAc_3$ plus the following cytidine and adenosine nucleotides) at 10.35 and 7.99 min, respectively. The ESI accurate mass spectrum of metabolite Rev-N1-N9 at 10.35 min is presented in Figure 3B. A mass-to-charge ratio envelope has charge states ranging from -2 to -5. The isotopic envelopes from each charge state are clearly resolved (Figure 3C) with resolution of 35,000. The deconvoluted mass spectrum of this peak reveals the deconvoluted monoisotopic mass of 3002.386 Da (Figure 3D). Given the theoretical monoisotopic mass of 3002.385 Da, the mass accuracy of this metabolite is 0.3 p.p.m., which confirms that the metabolite is Rev-N1-N9 which is formed by cleavage of dA-$GalNAc_3$ from the 3′ end of REVERSIR-A.

ProMass data processing

After LC-HRMS analysis, data were processed using ProMass HR Deconvolution™ software, version 3.0 (Novatia, LLC, PA, USA) to identify metabolites from REVERSIR-A. The software obtains the exact (monoisotopic) mass of each component found in the samples and matches the experimentally observed mass with calculated (or theoretical) masses for possible metabolites predicted by *in silico* cleavage of the sample oligonucleotide at any point along the oligonucleotide phosphodiester/phosphorothioate backbone from either direction (i.e., all possible cleavage products). Typically, a match tolerance of 5–10 p.p.m. was applied to the candidate metabolites to reject as matrix background with coincidentally nearly identical masses. An intensity threshold, typically 0.1% of the most intense target-related substance found in a particular sample, was also applied to reject as background-related peaks. Positive metabolite 'hits' were also manually assessed by inspection of both the raw and deconvoluted mass spectra to confirm the assignments. The typical metabolites of REVERSIR-A observed in monkey plasma, liver and urine are shown in Table 3.

Zou *et al.* [29] reported using ProMass deconvolution software for automated data interpretation for siRNA metabolite identification. In this study, we report using HR Deconvolution™ software to facilitate automated data interpretation for GalNAc-conjugated oligonucleotide metabolite profiling analysis. This software was used to establish the metabolite profile of the backbone of oligonucleotide as well as the metabolites of GalNAc and linkers. ProMass HR Deconvolution™ software can provide a report summary of mass error in parts per million

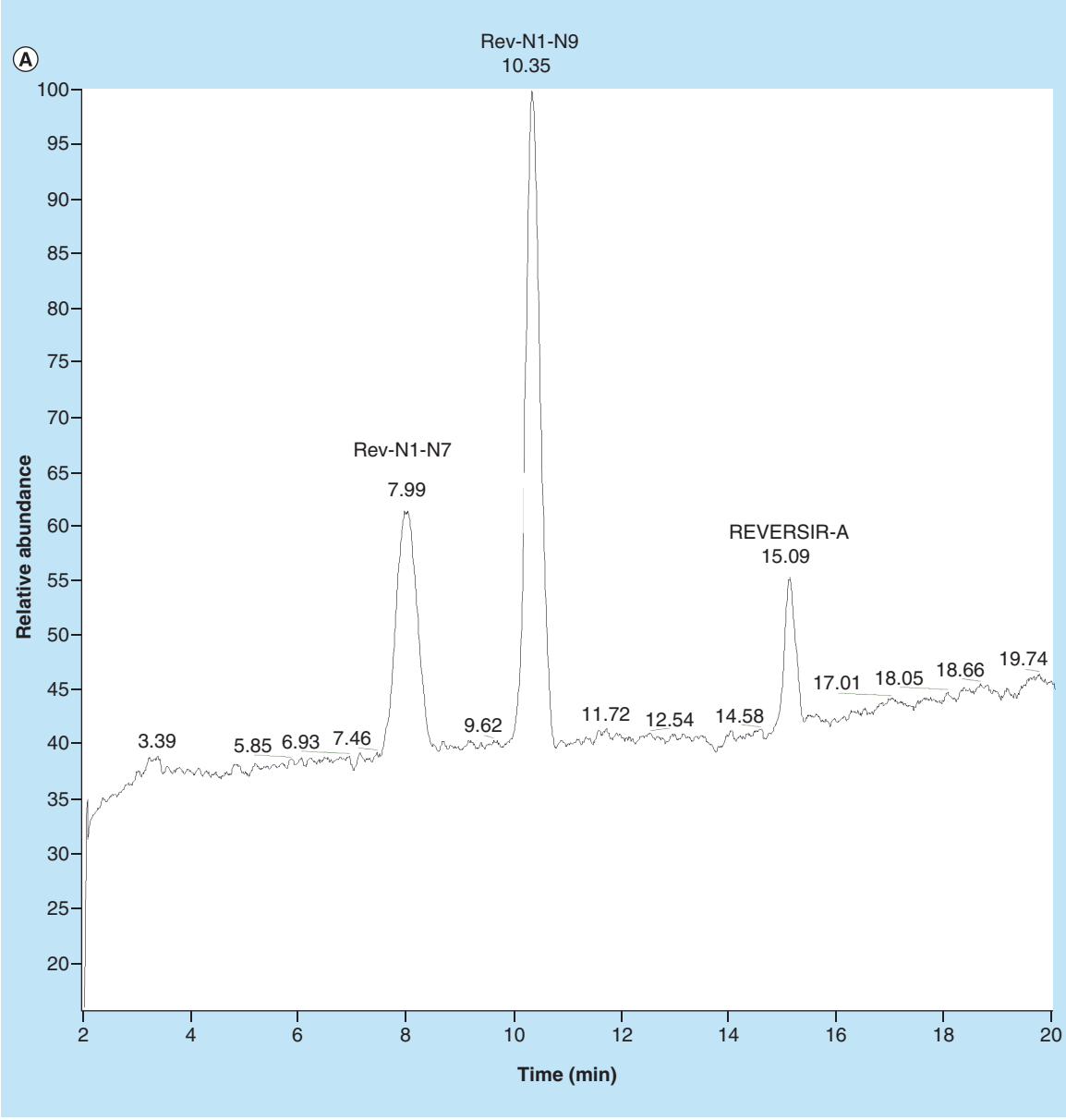

Figure 3. LC-high-resolution mass spectrometry analysis of REVERSIR-A in monkey plasma. Sample was collected 2 h after a 3.0 mg/kg sc. dose of REVERSIR-A. **(A)** Total ion chromatogram. **(B)** Electrospray ionization mass spectrum of Rev-N1-N9 metabolite. **(C)** Isotopic envelopes for the charge state of five for the Rev-N1-N9 metabolite. **(D)** Deconvoluted mass spectrum of the Rev-N1-N9 metabolite.

and possible matched sequences with peak intensities. Based on the peak intensity, the relative percentages of the metabolites in the sample were calculated.

N-acetylgalactosamine ligand metabolism

The total ion chromatogram of REVERSIR-A in a liver sample collected 8-h postdose from a monkey administered 3.0 mg/kg REVERSIR-A SC is shown in Figure 4. The full-length parent compound REVERSIR-A was not observed in this sample although the REVERSIR-A standard in monkey liver homogenate was shown to have a deconvoluted mass of 5103.367 Da. The deconvoluted peak at 16.68 min has a mass of 4494.126 Da (Figure 5) which corresponds to the most abundant metabolite of REVERSIR-A. This metabolite, designated REVERSIR-A -3 GalNAc, results from deletion of all 3 *N*-acetylgalactosamine (GalNAc) sugars (-m/z 609 Da), shown in Table 3. Another GalNAc metabolite (REVERSIR-A -3 GalNAc-1 Linker1) with deletion of all 3 GalNAc sugars and one

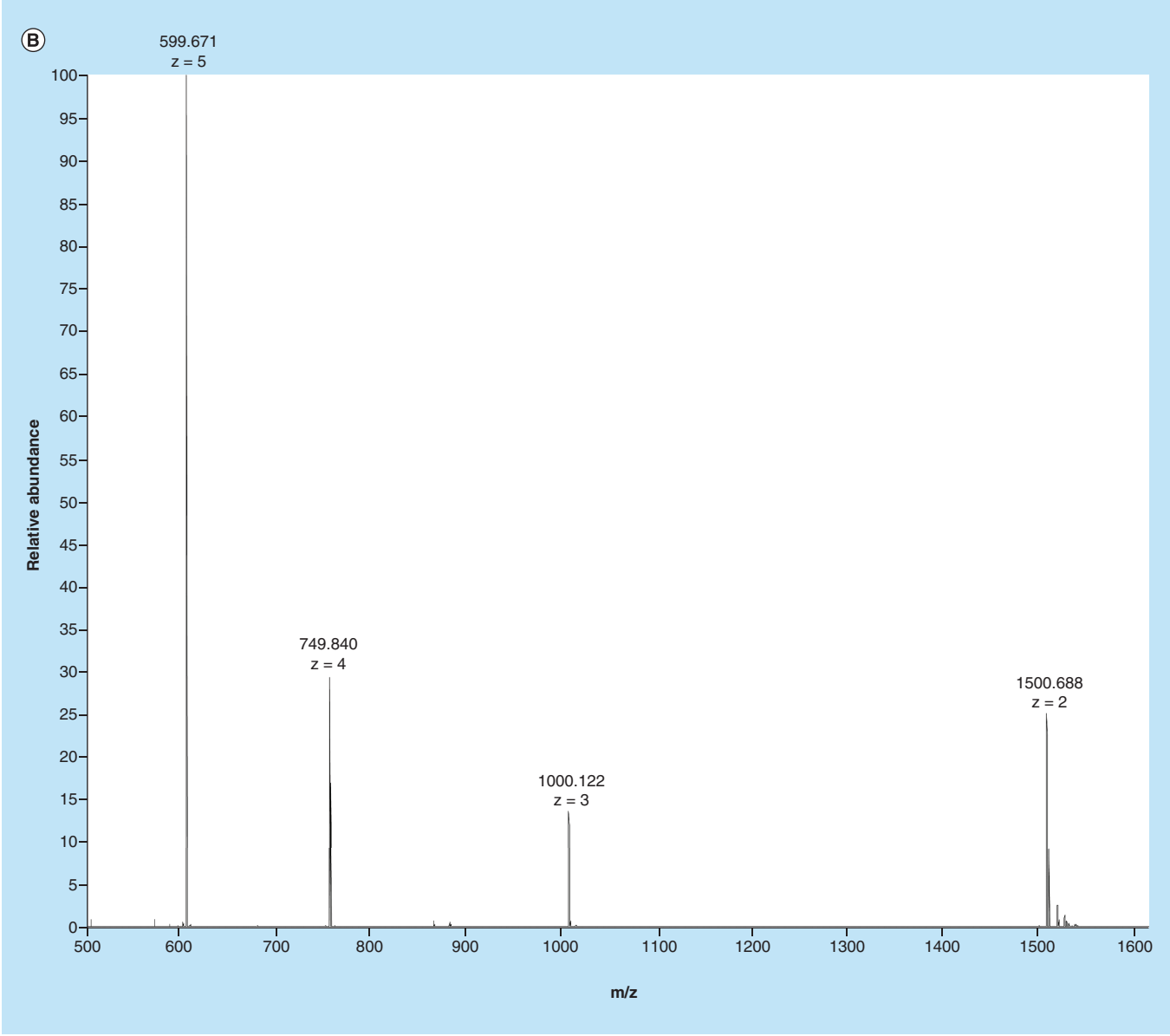

Figure 3. LC-high-resolution mass spectrometry analysis of REVERSIR-A in monkey plasma (cont.). Sample was collected 2 h after a 3.0 mg/kg sc. dose of REVERSIR-A. **(A)** Total ion chromatogram. **(B)** Electrospray ionization mass spectrum of Rev-N1-N9 metabolite. **(C)** Isotopic envelopes for the charge state of five for the Rev-N1-N9 metabolite. **(D)** Deconvoluted mass spectrum of the Rev-N1-N9 metabolite.

Linker1 (-*m*/*z* 709 Da) was also observed (Figure 1 & Table 3). The data suggest that the triantennary GalNAc ligand of REVERSIR-A was metabolized by loss of up to three GalNAc sugars and one Linker1 in monkey liver. Cleavage sites are shown in Figure 1. These results are consistent with published reports of the metabolism of triantennary GalNAc conjugates of antisense oligonucleotides [30,31].

The data are consistent with the targeted delivery of GalNAc-siRNAs to the liver via binding to the asialoglycoprotein receptor.

Metabolite profiling of REVERSIR-A in monkey plasma, liver & urine

The *in vivo* metabolite profiling of REVERSIR-A in monkey plasma, liver and urine samples following REVERSIR-A SC administration shows the formation of similar metabolites in all matrices (Table 3). REVERSIR-A was mainly metabolized in plasma to metabolites Rev-N1-N9 and Rev-N1-N7 most likely by exonuclease cleavages at the 3′

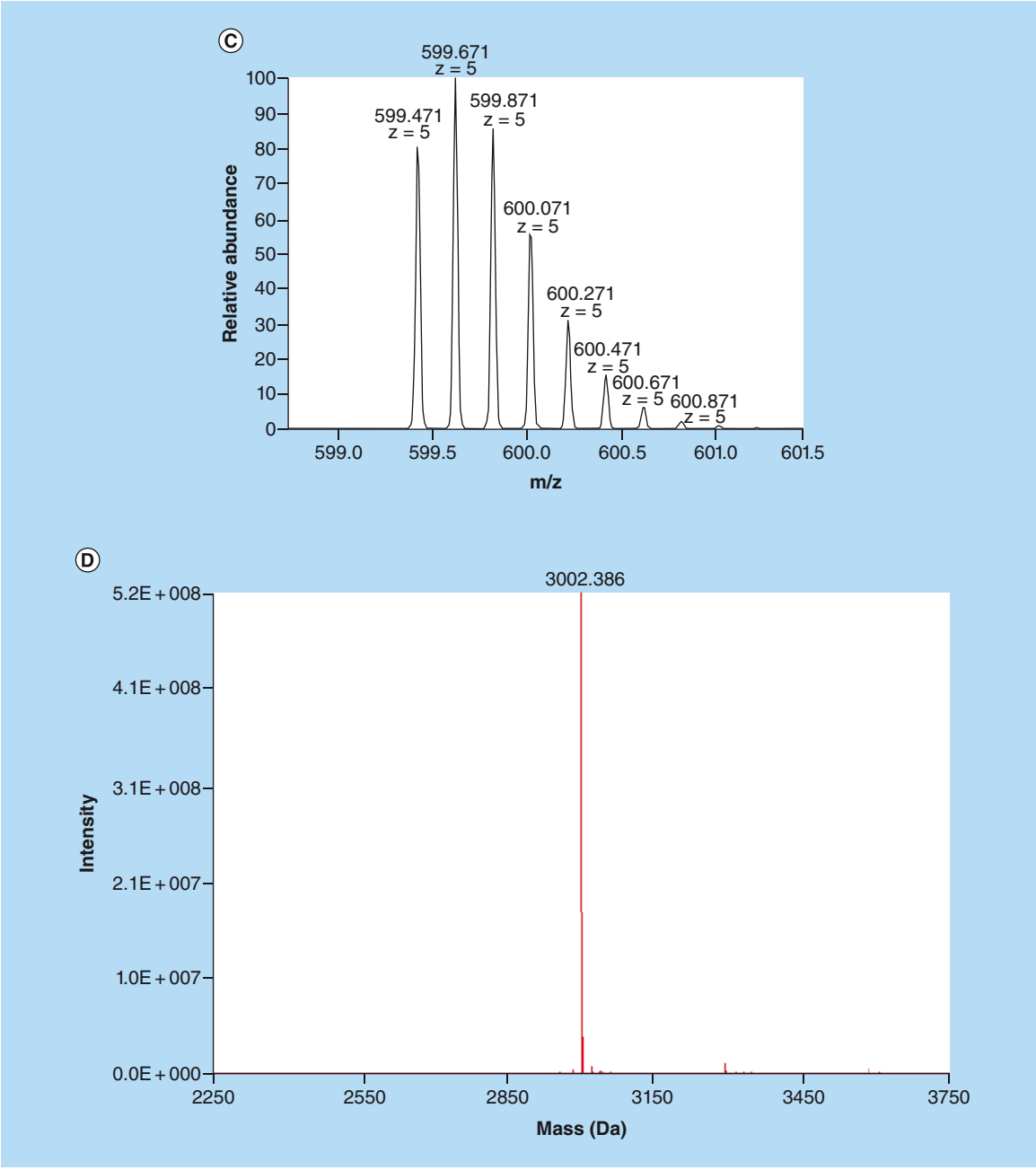

Figure 3. LC-high-resolution mass spectrometry analysis of REVERSIR-A in monkey plasma (cont.). Sample was collected 2 h after a 3.0 mg/kg sc. dose of REVERSIR-A. **(A)** Total ion chromatogram. **(B)** Electrospray ionization mass spectrum of Rev-N1-N9 metabolite. **(C)** Isotopic envelopes for the charge state of five for the Rev-N1-N9 metabolite. **(D)** Deconvoluted mass spectrum of the Rev-N1-N9 metabolite.

ends. After uptake to the liver by endocytosis via the asialoglycoprotein receptor, REVERSIR-A was mainly metabolized at the GalNAc ligand as described above. Additionally, the next most abundant metabolite observed was Rev-N1-N9 most likely produced by exonuclease activity from the 3′ end at the phosphodiester bonds. REVERSIR-A and metabolites were observed in urine indicating that they were excreted renally. Rat metabolite profiling was not included in the study protocol and was therefore not analyzed.

The metabolite profiling data can provide information on the metabolic pathway of oligonucleotides. In addition, the data also provide a good estimation of the relative percentage of the metabolites based on the individual peak intensity to the total peak intensities (Table 3). Each peak may have a different ionization efficiency in the MS;

Table 3. Representative *in vivo* metabolite profile of REVERSIR-A in monkey plasma, liver and urine samples after subcutaneous administration of 3.0 mg/kg REVERSIR-A.

Sample time point (h postdose)	RT (min)	Theoretical mass (Da)	Observed mass (Da)	Mass error (Da/p.p.m.)	Oligonucleotide	Intensity	Percent of total† (%)
Monkey plasma							
	15.09	5103.362	5103.366	0.0038/0.7	REVERSIR-A	9.57E+07	11.8
2	10.35	3002.385	3002.386	0.0010/0.3	Rev-N1-N9	5.17E+08	63.7
	7.99	2312.283	2312.283	0.0003/0.1	Rev-N1-N7	1.96E+08	24.1
Monkey liver							
	16.68	4494.124	4494.124	-0.0001 /-0.0	REVERSIR-A −3 GalNAc	5.06E+08	65.2
	15.73	4394.072	1394.078	0.0063/1.4	REVERSIR-A −3 GalNAc −1 Linker1	2.81E+07	3.6
8	11.46	3315.443	3315.443	0.0004/-0.1	Rev-N1-N10	1.29E+07	1.7
	10.78	3002.385	3002.384	-0.0010/-0.3	Rev-N1-N9	1.73E+08	22.3
	8.55	2312.283	2312.283	0.0003 /0.1	Rev-N1-N7	5.00E+07	6.4
Monkey urine							
	12.94	5103.362	5103.329	-0.0332/-6.5	REVERSIR-A	1.44E+07	1.0
8–24	8.81	3002.385	3002.364	-0.0210/-7.0	Rev-N1-N9	8.55E+08	58.3
	7.06	2312.283	2312.262	-0.0207/-9.0	Rev-N1-N7	5.90E+08	40.2

†% of total = peak intensity of each peak/sum of all peak intensities in mass spectra at the same sample. Each peak may have different ionization efficiency in mass spectrometer, therefore, the percentage reported is an estimation of relative abundance.
GalNAc: N-acetylgalactosamine; RT: Retention time.

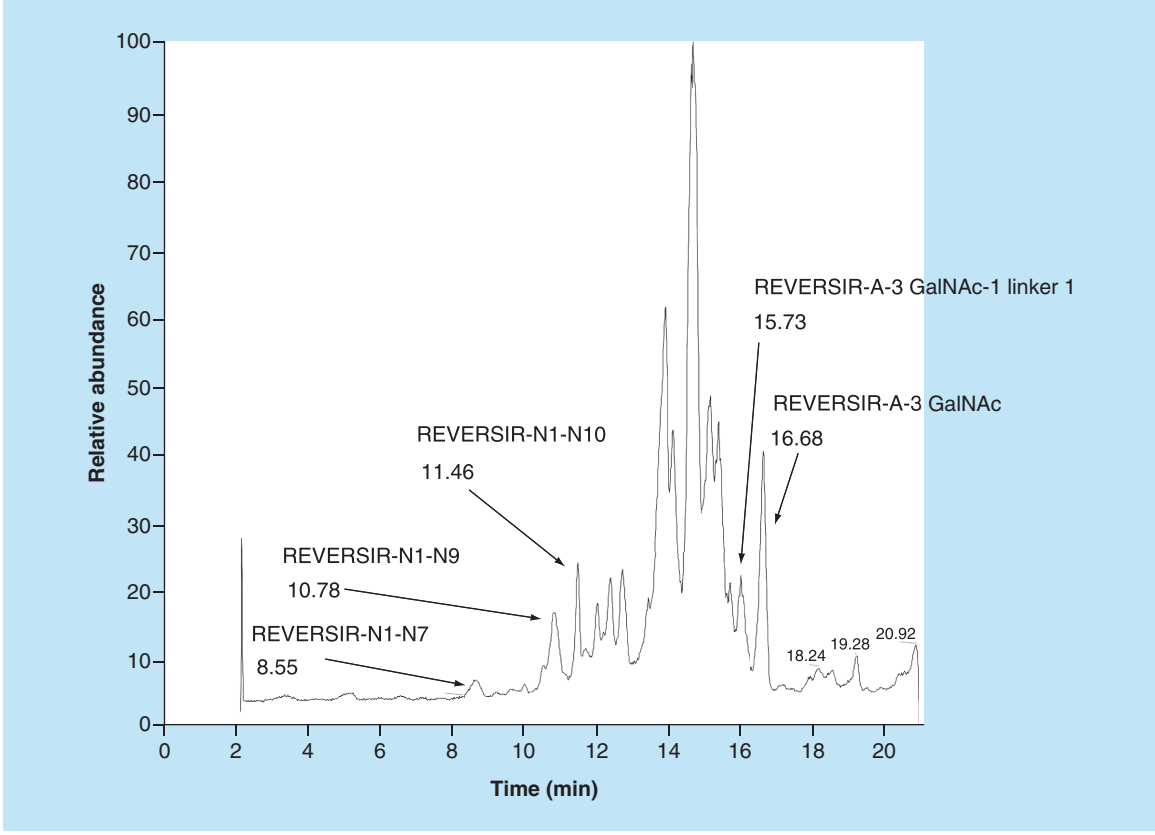

Figure 4. Total ion chromatogram of REVERSIR-A and its metabolites in a monkey liver sample by LC high-resolution accurate mass spectrometry. The sample was collected 8 h after subcutaneous administration of a 3.0 mg/kg dose of REVERSIR-A.

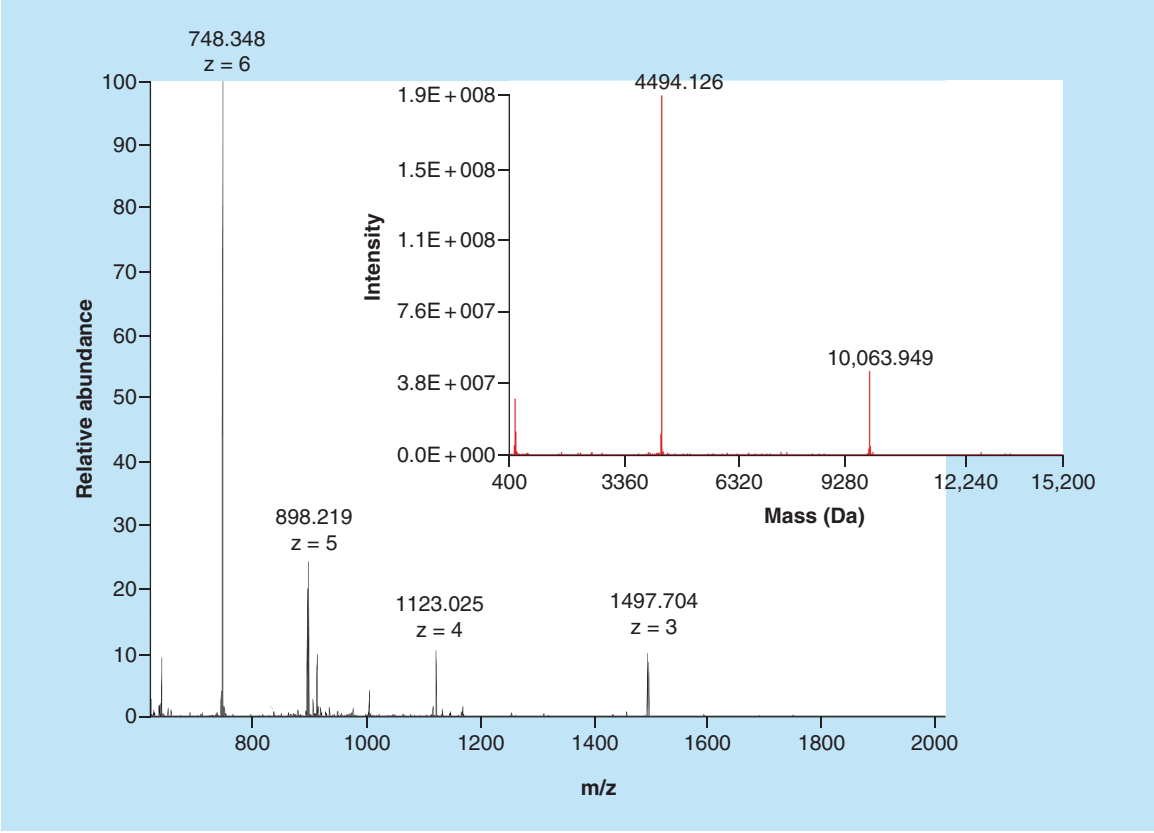

Figure 5. Electrospray ionization mass spectrum of the peak at 16.68 min in a monkey liver sample. The insert displays the deconvoluted mass spectrum of the peak. The liver sample is as described in Figure 4.

therefore, the percentage reported is an estimation of relative abundance. The estimation of metabolite abundance during drug development provides critical information that informs decisions regarding the need for quantitative assays. When the concentration of the parent compound is known, metabolite abundance can be estimated based on the metabolite peak intensity relative to the parent compound peak intensity. This approach is especially valuable during the drug discovery stage when reference compounds for metabolites are not available.

Quantification of REVERSIR-A & metabolites in rat plasma, liver & kidney

Based on FDA guidance, metabolites that are present at greater than 10% of total drug in human plasma must be quantified using a fully validated assay [32]. The qualified method was applied to quantification of REVERSIR-A and its most abundant metabolites in rat plasma, liver and kidney. The standard curve range is 10–5,000 ng/ml in plasma and 100–50,000 ng/g in both liver and kidney. The analyte concentrations were calculated using a $1/x^2$ weighted linear regression model.

Plasma PK profiles of REVERSIR-A after SC administration at 3 mg/kg showed that the plasma C_{max} value was 614 ng/ml, while Rev-N1-N9 and Rev-N1-N7 have plasma C_{max} values of 557 and 163 ng/ml, respectively (Figure 6A).

Liver PK profiles show that Rev-N1-N9 and Rev-N1-N7 have C_{max} values of 10,700 and 3,590 ng/g, respectively, compared with REVERSIR-A with a C_{max} value of 3060 ng/g (Figure 6B). Rev-N1-N9 showed a longer half-life ($t_{1/2}$) of 91.3 h, compared with REVERSIR-A which was quickly metabolized to levels below the LLOQ. The data suggest the long pharmacodynamic effect of REVERSIR-A is because of the prolonged half-life of the metabolites Rev-N1-N9 and Rev-N1-N7.

Kidney PK profiles show that Rev-N1-N9 and Rev-N1-N7 have C_{max} values equal to 2,960 and 407 ng/g compared with Reversir-A with a C_{max} of 1750 ng/g (Figure 6C). Rev-N1-N9 and Rev-N1-N7 have $t_{1/2}$ values of 112 and 149 ng/g, respectively, while REVERSIR-A was quickly metabolized to levels below the LLOQ.

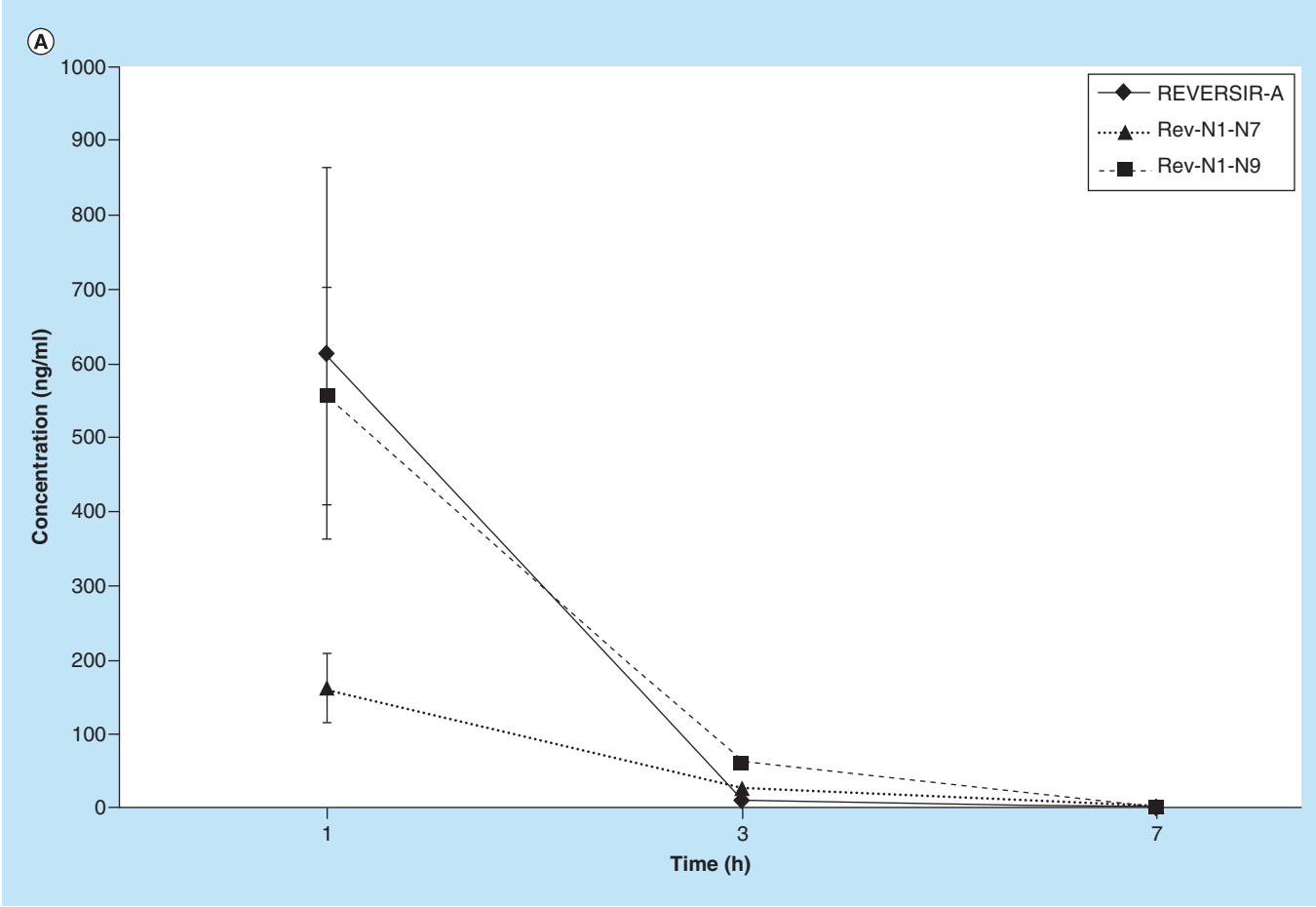

Figure 6. REVERSIR-A and metabolite concentrations in rat plasma and tissues after subcutaneous administration of a 3.0 mg/kg dose of REVERSIR-A. (A) Rat plasma (n = 3); **(B)** rat liver (n = 3); **(C)** rat kidney (n = 3).

Conclusion

LC-HRMS with Q Exactive™ mass spectrometer detection was used for metabolite profiling of oligonucleotides in biological matrices. The LC-HRMS method described here is able to distinguish mass differences of 10 p.p.m., which enables unambiguous metabolite assignments. ProMass software facilitates metabolite identification of oligonucleotides in an automated fashion with significantly reduced analysis time. Based on information available from metabolite profiling, appropriate metabolites can be identified for quantitative analysis.

A sensitive, robust and high-throughput method for quantitation of oligonucleotides and metabolites in biological matrices has been developed and qualified for application in nonclinical and clinical studies.

Future perspective

To support the development of oligonucleotides for RNAi therapeutics, new methods are needed to expand our understanding of the metabolism and PK of these compounds. The HPLC with LC-HRMS method described here is selective, sensitive, robust and high-throughput. It can be employed for the quantification of oligonucleotides as well as their metabolites in plasma and tissues to support preclinical/clinical PK and TK studies.

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Financial & competing interests disclosure

The authors are employees and stockholders of Alnylam Pharmaceuticals. The authors have no other 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 apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

All animal procedures were conducted by certified laboratory personnel using protocols consistent with local, state, and federal regulations, as applicable, and approved by the Institutional Animal Care and Use Committee at Alnylam Pharmaceuticals.

Summary points

Background

- To develop a LC–MS method to qualification and quantification of a single-stranded oligonucleotide REVERSIR-A as well as metabolites *in vivo*.

Results & discussion

- A selective, sensitive, robust and high throughput LC-HRMS method was developed for the metabolite profiling of REVERSIRs in plasma and tissues.
- The method precision and accuracy were evaluated using a REVERSIR molecule in rat liver. The method has been used by Contract Research Organization partners for oligonucleotide quantification in nonclinical and clinical studies in different matrices.
- The qualified method was applied to quantification of REVERSIR-A and metabolites in rat plasma, liver and kidney.
- *In vivo* samples were extracted by Clarity OTX solid phase extraction with >85% recovery.
- HR Deconvolution™ software greatly facilitated automated data interpretation for GalNAc-conjugated oligonucleotide metabolite profiling analysis.
- The triantennary GalNAc ligand of REVERSIR-A was taken up by liver and mainly metabolized by loss of up to three GalNAc sugars and one Linker1 and formation of two other major metabolites, Rev-N1-N9 and Rev-N1-N7.

Conclusion

- A HPLC with high-resolution accurate mass spectrometry method was employed to investigate the metabolite profiles and quantification of oligonucleotides.

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Overview of LBA and LC–MS/MS analysis of ASOs: an interview with Danielle Salha and Kevork Mekhssian

Danielle Salha

Senior Director, Immunochemistry & Immunology Altasciences (Montréal, Canada)

Danielle Salha joined Altasciences in 2017 and is Senior Director, Immunochemistry & Immunology, Ligand Binding Assays. She leads a team of 45 scientists, QCs and analysts dedicated to method development, validation and sample analysis to support preclinical and clinical PK, PD and immunogenicity studies. Dr. Salha has over 20 years pharmaceutical and CRO experience in bioanalysis supporting drug development from preclinical to Phase I and II clinical studies, including vaccines, monoclonal antibodies, ADCs and oligonucleotides. Dr. Salha has authored and co-authored several peer-reviewed publications and is an inventor, with four patent applications to her credit. She received her BSc at the University of Montréal and her PhD at McGill University from the Department of Immunology and Microbiology (both Montréal, Canada).

Kevork Mekhssian

Scientific Director Altasciences (Montréal, Canada)

Kevork Mekhssian joined Altasciences in 2013. He has over 15 years of pharmaceutical and CRO experience in mass spectrometry-based characterization and quantitation of biotherapeutic proteins using LC–MS and hybrid LBA/LC–MS workflows. He has actively participated in setting up high-throughput biotherapeutic quantitation methods and has greatly contributed to establishing Altasciences as an industry leader in this field. Kevork has authored and co-authored several peer-reviewed publications and has presented at numerous bioanalytical and mass spectrometry international meetings. Kevork completed his MSc in biochemistry at Concordia University (Montréal, Canada).

1 Can you provide an overview of ASO analysis for the most commonly encountered oligo therapeutics?

K

Antisense therapies have been established as an important class of oligonucleotide-based therapeutics and are considered ideal drug candidates for neuromuscular and neurological diseases. These short 15–22 nucleotide single-stranded ASOs act by binding to specific sequences of mRNA and inhibit or alter the expression of the targeted protein. Recently, novel strategies for ASO therapeutic development, including chemical modification for added stability and affinity as well as conjugation to different ligands for specific targeting, have been investigated. These new modality drugs have generated a growing demand for more sensitive, specific and robust assays for bioanalytical support. Several quantitative analytical platforms, including hybridization ELISA, LC-fluorescence and LC–MS/MS, are currently used to determine ASO concentrations in a variety of biological matrices. These assay types are generally classified into two main categories: hybridization-based and chromatographic. Consequently, selection of the appropriate analytical platform is driven by the study objective, the biological matrix of interest and the requirement for sensitivity, specificity and throughput. The hybridization ELISA assay offers high sensitivity and throughput compared to other methods. The approach involves the hybridization of the target ASO to an immobilized capture probe and/or a detection probe for signaling. However, the assay has a narrow dynamic range and lacks specificity towards larger truncated metabolites (n-1, n-2). In comparison, LC–MS/MS assays are highly specific due to three levels of discrimination: chromatographic separation, molecular weight (*m/z* ratio) and the generation of a unique signature product ion. The increased specificity allows for multiplexing with simultaneous determination of parent ASO and truncated metabolites in the same assay. Notably, LC–MS/MS assays using LLE or SPE for sample extraction are

labor-intensive and less sensitive than hybridization methods, especially with increasing oligonucleotide length. Another assay platform is hybridization LC-fluorescence. The approach is more sensitive than LC–MS due to the ability to hybridize a fluorescent probe to the target and is more specific than ELISA due to the upfront chromatographic separation of the parent from critical metabolites. The challenge of hybridization LC-fluorescence is the requirement for appropriate probe design, leading to longer development times and increased costs. Since no single technology is currently considered the gold standard for oligonucleotide analysis, the selection of the proper assay platform becomes critical and demands a thorough understanding of the study objectives.

D

Hybridization ELISA is an effective and accurate approach to quantify parental ASOs, both circulating and in targeted tissue, and is capable of supporting TK/PK GLP and non-GLP studies. Hybridization-based methods typically provide the highest assay sensitivity and throughput when compared with other bioanalytical methods for ASOs and typically require little or no sample cleanup. There are three main hybridization ELISA formats currently used in the industry: dual-hybridization, nuclease dependent hybridization and ligation-hybridization. Although each assay format inherently demonstrates unique advantages, there can be limitations. For example, the dual-hybridization assay has an enhanced sensitivity over the ligation assay, however cross-hybridization with 3′ or 5′ metabolites may be also detected. On the other hand, the ligation-hybridization assay has minimal 3′ metabolite cross-reactivity. The nuclease dependent hybridization assay relies on a single-strand specific enzyme that cleaves all free capture probe and imperfectly formed duplexes, which provides the advantage of quantifying only full-length ASOs. With these assay formats, we are able to support single- and double-stranded DNA and RNA up to 24 nucleic acids in length, siRNA, chemically modifed ASOs, oligodeoxynucleotides and peptide-PMOs (phosphorodiamidate morpholino oligonucleotides).

2 What predominant challenges do you face in supporting ASO analysis?

K

In recent years, the development of next-generation antisense therapies with high target specificity and stability has greatly increased the structural complexity of ASOs. The diversity of these new modality drugs has made their analysis challenging, necessitating the successful development of new bioanalytical workflows and methods for quantitation. Multiple analytical platforms are available for ASO analysis. The challenge for the bioanalytical scientist is to choose the optimal platform by carefully considering the advantages and limitations for each approach, as well as understanding the objectives of the study in terms of sensitivity, specificity and throughput.

The limitations of LC–MS/MS methods in terms of sensitivity and throughput compared to hybridization-based assays are well known. However, with the novel hybridization LC–MS/MS assay workflow established in our lab, many of these shortcomings are addressed; sensitivity levels are on-par with hybridization ELISA assays and throughput is increased using automated magnetic bead-based sample processing. For the chromatographic separation of ASOs, the combination of ion-pairing reagent with acid modifier still provides the best compromise between LC separation performance and MS sensitivity. However, ion-pairing reagents tend to stick to LC components, the analytical column and MS source. This can lead to signal suppression in subsequent analyses. To avoid long and costly decontamination between runs, LC–MS systems are specifically dedicated towards ASO analysis.

D

While instrumentation and sample preparation is less of an issue for LBA because any ELISA or ECLIA analyzer plate reader can be used, methods are characterized by a reduced dynamic range when compared against MS detection. Consequently, samples may require dilution so that analyte response falls within the supported concentration range of the assay, which is particularly challenging when conducting a nonclinical study with a high C_{max}. In these situations, our approach involves an examination of a few judiciously selected samples to best establish the appropriate dilution scheme, which is then applied to each group.

Cross-reactivity between parent ASO and metabolites can also be an issue depending upon the chosen ELISA format. Consequently, we recommend an evaluation of cross-reactivity with (n-1) and (n-2) metabolites, with results formally reported as part of method validation.

Our experience has taught that hybridization ELISAs are the more robust assay type when compared to large molecule ELISAs, most likely attributable to the fact that the bond strength between probe and ASO is much greater than the affinity of capture antibody with target protein. Therefore, a hybridization ELISA approach allows flexibility and ease when transferring assays between matrices. However, challenges persist when working with certain matrices, such as cerebrospinal fluid (CSF), which necessitates the screening of several control lots for QC preparation, crucial when surrogate CSF is used for calibrant preparation. Selected control donors would then need to be used throughout the entirety of the study program for consistency, as the qualification of new CSF lots can prove time-consuming.

3 What are some of the more recent advances in LBA and LC–MS/MS analysis for ASOs?

D

New generation ASOs deliver improved potency and biodistribution due to advancements in drug delivery and chemical modifications. Thus, higher method sensitivity is required for concentration determination in order to achieve an accurate PK profile at the preclinical stage, critical for ultimately driving clinical dosing. Therefore, we see the industry shifting from the ELISA to ECLIA platform to achieve the necessary sensitivity gains whilst concomitantly increasing assay dynamic range. Migrating from ELISA to ECLIA does require careful optimization in order to reduce background interference, thereby ensuring optimal response separation from blank matrix. Typically, this includes optimization of assay format, probe design, wash buffers, incubation temperature and time.

K

For LC–MS analysis of ASOs, recent improvements in sample preparation workflows, LC separation and MS technologies have all contributed to the increase in assay sensitivity, specificity and throughput. Sample preparation and extraction of oligos from various biological matrices can be quite complex and labor intensive. New sample extraction solutions based on mixed-mode SPE are now available and provide improved sample recovery and throughput for a wide range of therapeutic oligonucleotides. Additionally, advances in column chemistry and packing techniques have led to more durable and higher resolution columns. In terms of MS instrumentation, the new generations of triple quadrupoles and high-resolution mass spectrometers have demonstrated considerable improvements in analyte detection sensitivity, dynamic range and general performance.

However, in our lab, the most significant advance in ASO analysis has been the implementation and validation of a novel hybridization LC–MS/MS assay platform for the quantification of ASOs in serum, CSF and various nonhuman primate tissues. The approach, combining both hybridization ELISA and LC–MS/MS technologies, was first reported by Dillen *et al.* in 2017. Hybridization is achieved by using a biotinylated capture probe complementary to the ASO strand. The assay parameters and throughput were further optimized in a recent publication by Li *et al.*, demonstrating solid quantitative performance and universal compatibility in most common matrices. Compared to traditional SPE-based LC–MS/MS methods, sensitivity is largely improved to levels comparable to those reported for hybridization ELISA assays (for example, a 0.5 ng/mL LOQ). Throughput is significantly increased by automating sample extraction in a 96-well plate format using Thermo Fisher's (MA, USA) KingFisher Flex™ magnetic sample processor. Another major advantage is multiplexing, where the method can specifically distinguish and simultaneously quantitate the n-1 metabolite together with parent ASO using the identical capture probe. In our experience, the hybridization LC–MS/MS approach delivers superior performance over traditional assay platforms.

4 What are your predictions for the future of ASO analysis?

D

Some of the big assets of LBA are throughput and multiplexing capabilities through ECLIA. Further, since hybridization assays are standardized methods that can be applied to various matrices with very small adjustments to the method, introducing automation can increase sample throughput to better support large clinical studies. The multiplexing capabilities of ECLIA are expected to predominate, translating to capabilities for evaluating multiple targets in one sample when different ASOs are formulated together in one drug product.

K

With the growing number of oligonucleotides in development and the increasing complexity of these products, we expect innovative analytical approaches to be in high demand for the foreseeable future. MS-based technologies like LC–MS/MS and LC–HRMS are central for ASO analysis and will continue to demonstrate improvements in selectivity and sensitivity. Finally, emerging assay technologies such as hybridization LC–MS/MS can be considered an improved alternative to traditional LC–MS/MS and hybridization ELISA methods for quantitation of ASOs and metabolites across different biological matrices.

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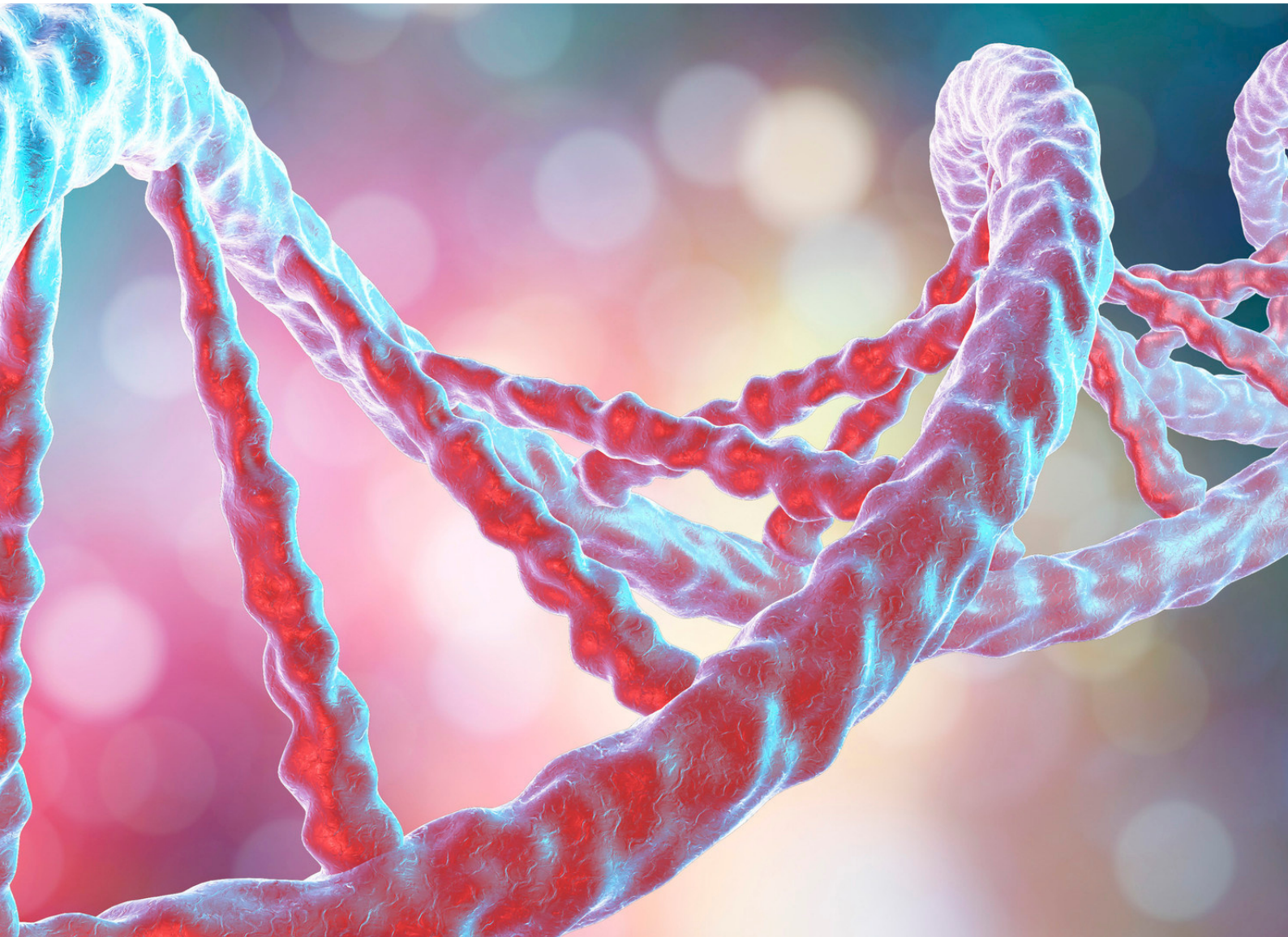


Bioanalytical developments for oligonucleotides: a walk down memory lane

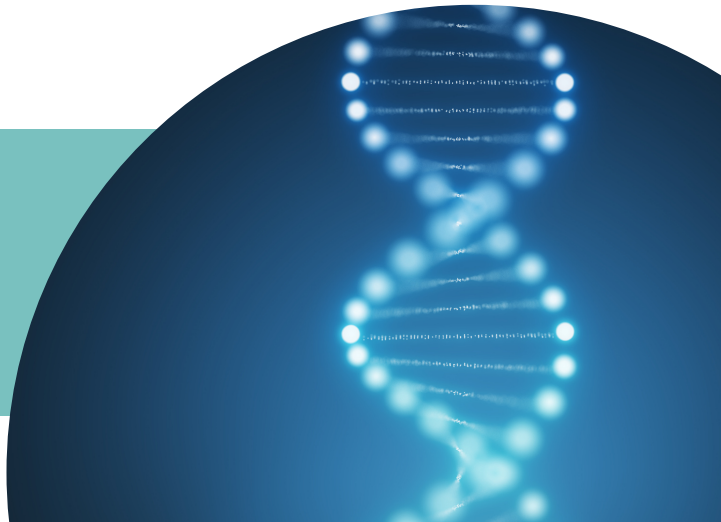
by Richard Geary

Richard Geary is Executive Vice President of Drug Development at Ionis Pharmaceuticals (CA, USA). He is responsible for preclinical development, global clinical development and regulatory and manufacturing for Ionis' antisense drugs. Since joining Ionis in 1995, Geary has been involved in discovery and development including the regulatory submission of more than 40 investigational new drug applications and five successful antisense medicine late-stage development programs through approvals in multiple jurisdictions.

Development of sensitive, precise, reproducible and specific bioassays to support pharmacokinetic assessment of oligonucleotides has evolved, expanded and improved remarkably over time. Some of the earliest methods included capillary gel electrophoresis (CGE) [1], which brought extreme validation challenges at a time when there was keen attention to validation detailing for bioanalytical methods [2]. The desire to outsource bioanalytical programs produced additional complexities as CGE was rarely to be found in larger CRO support labs, bringing another level of difficulty. Under the leadership of Rosie Yu and colleagues (Isis Pharmaceuticals, CA, USA) [3], more robust LBAs (hybridization ELISA) were invented to not only expand utility but, more importantly, to drive the sensitivity of the methods to stunning new levels that provided a greater understanding of the underlying mechanisms of clearance and half-life.

During these early days, additional progress was made by identifying stable radiolabel methods for monitoring mass balance and metabolism in animal models. Radiolabel compounds allow further assessment on what's missing from specific chemical analytical methods. One early study evaluating the connection between chemical and radiolabel compounds provided good confidence that the chemical analysis was capturing the full story for newer stabilized chemistries for ASOs [4]. Ultimately, the choice was made to incorporate a nonexchangeable tritium at the 5-position on a 2'-MOE modified ribose sugar to allow mass balance assessments for the newer stabilized chemistries. This was an important milestone at a time of substantial change from first to second generation chemistry [5].

As innovation in ASO chemistry and delivery accrued, improved potency supported lower doses (LICA), and with more ASOs delivered by multiple routes of administration such as intrathecal (SPINRAZA®), pulmonary (ENAC) and oral [6], there was a growing need for improvements to the bioanalytical armamentarium. Most of these new assays were focused on remarkably low limits of detection (pg/mL) while maintaining specificity and reliability. Furthermore, assays involving more rigorous extraction and sample manipulations were required to perform these analyses in multiple biological matrices beyond plasma. This involved complex tissue matrices, as well as additional biological fluids such as urine and cerebrospinal fluid. Some of the published methods still relied on the initial advancement in hybridization ELISA but with ever more sensitive detection modalities such as ECLIA [7].



The earliest metabolite profiling was facilitated by CGE for unmodified or phosphorothioate first generation ASOs [8]. The ability to separate single nucleotide deletions from either the 3′ or 5′ end (known as exonuclease digestion) with CGE helped elaborate the character and kinetics of metabolism. As new modified backbones began to be developed in the early 2000s and ever-growing conjugation chemistries were identified, more attention was needed to characterize metabolic pathways and metabolite identification. Advances in MS methods, including time-of-flight (TOF) applied to siRNA molecules and ion-pairing LC–MS/MS applied to ASOs [9] brought forward additional learnings.

Today bioanalytical methods used for quantification of ASOs are either ultrasensitive LBAs or less sensitive though more specific chromatographic-based assays with either UV or mass spectrophotometric detection. The choice of which method is optimal requires understanding of the question being asked and the potential limitations of each method, often depending on biological matrix or required sensitivity.

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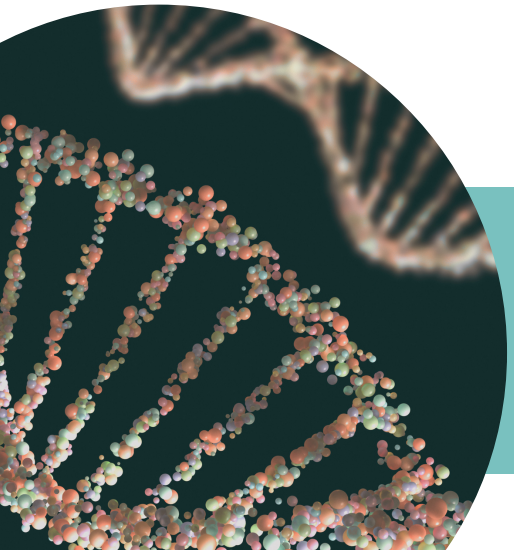

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