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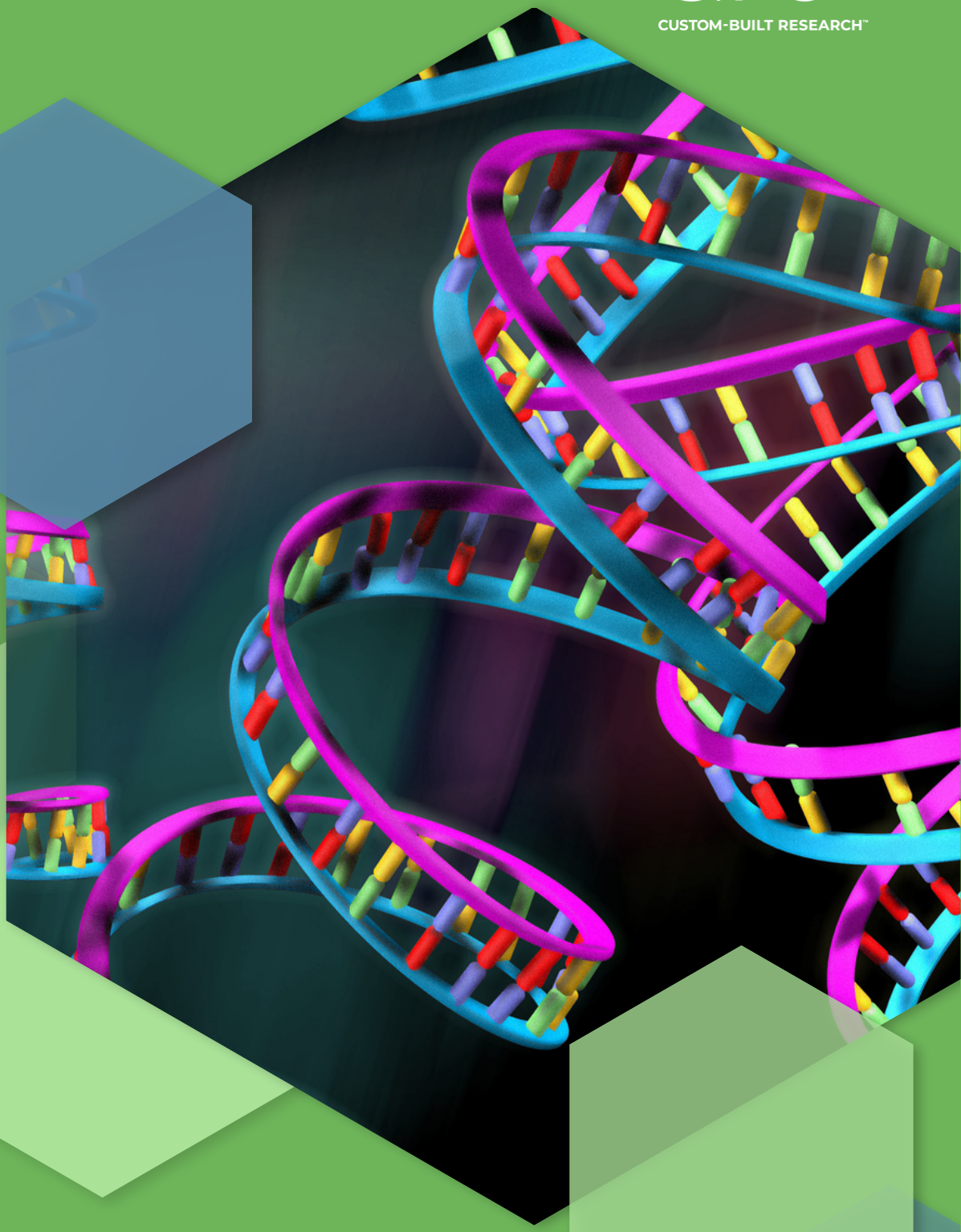

LBAs vs chromatographic platforms for oligonucleotide quantification

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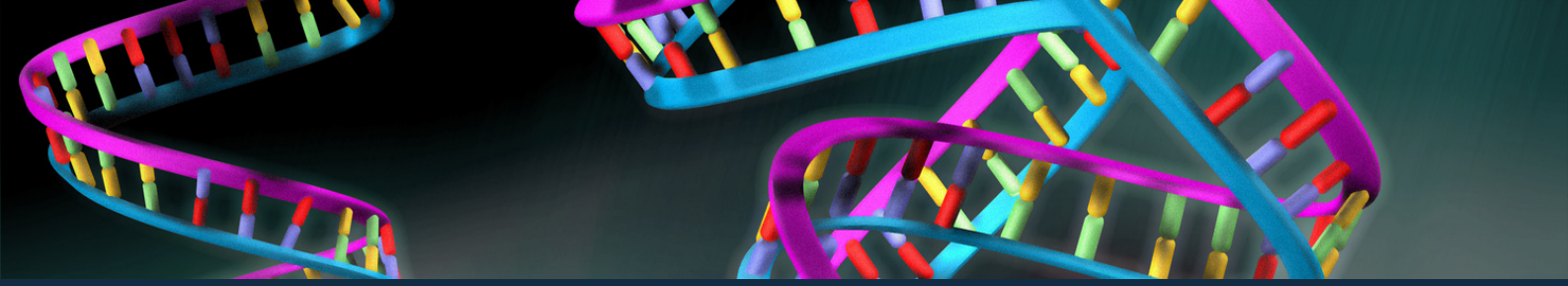


Technology Digest: LBAs vs chromatographic platforms for oligonucleotide quantification

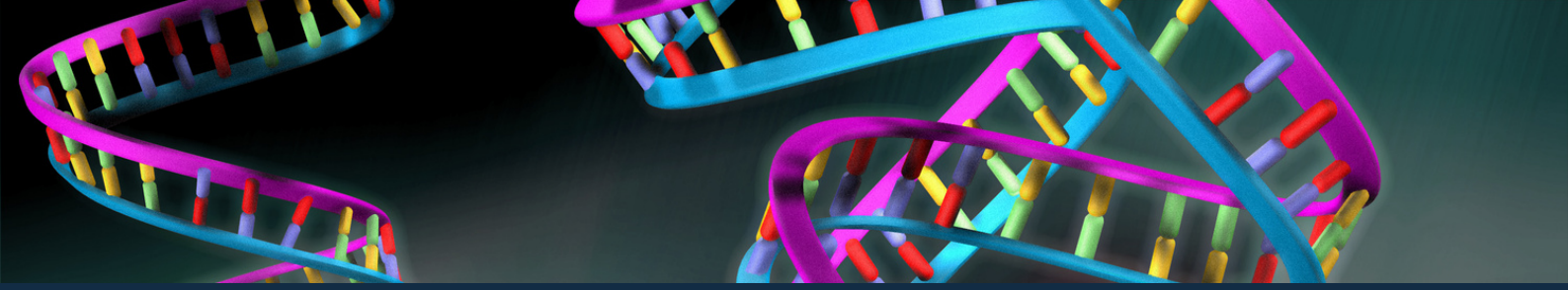
Background

Amy White
Digital Editor
Bioanalysis Zone

Oligonucleotides are, in essence, nucleic acid polymers that have the potential to treat a vast array of diseases, acting as therapeutics that predominantly focus on gene silencing [1]. Their typical mechanism of action involves binding of the oligonucleotide to a target mRNA sequence, hindering the action of ribosomes, disrupting splicing and encouraging the action of enzymes that degrade the mRNA. Oligonucleotides can be chemically modified to increase their potency. For instance, the addition a fluorine molecule to the 2' ribose position increases both the binding affinity of the oligonucleotide and its resilience to nuclease proteins [2].

The two most widely used strategies for gene silencing employ antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) [3]. There are many different oligonucleotide therapeutic modalities but all aid in targeting specific diseases that are currently untreatable by existing therapies. In general, oligonucleotide therapeutics, such as mRNA or noncoding RNA, have high specificity and can target molecules that cannot be governed by standard drugs, thus resulting in the cultivation of ground-breaking drugs against genetic diseases and cancers beneficial for the future treatment of patients [4].

Due to their many benefits, the interest in oligonucleotide therapeutics has increased over the years, thus the consideration for their analysis, regulation and validation must be a key focus moving forward. The current method of validation – oligonucleotide quantification – is an integral step in ensuring drug safety and efficacy by evaluating oligonucleotide concentration [5]. Ligand binding assays (LBAs) and LC-MS platforms are the most preferred platforms used for quantification of therapeutic modalities [6]. The essence of a LBA is that a ligand is used to capture and/or detect the target molecule (typically large molecules within biological matrices) and, due to its high specificity, can differentiate target molecules better than chromatographic methods [7]. The process of LC-MS analysis entails liquid chromatography (LC) separation combined with MS in order to detect extracted analytes, in this instance oligonucleotides, from biological matrices and, due to specificity and dynamic range, can provide more accurate quantification of oligonucleotides than LBAs [6]. Here we explore the benefits and ideal use cases for each platform when it comes to oligonucleotide quantification.



Technology Digest: LBAs vs chromatographic platforms for oligonucleotide quantification

Chromatographic platforms for oligonucleotide quantification

There are many different platforms available for the quantification and qualification of oligonucleotides, including LC and MS and combinations thereof [8]. Where other techniques offer benefits like the low detection limits offered by immunoassays, LC-MS is classed as the most accurate and thorough for the characterization of oligonucleotides – especially useful when conducting metabolite profiling [8]. Additionally, as of 2019, LC-MS was surveyed to be the second most popular assay format used to analyse nucleotides within 2 years of data collection, with 75–80% of survey members attesting to using the platform [9].

However, there are limitations to using LC-MS platforms as it can be challenging to achieve selectivity between metabolites and parent molecules in a single assay [10].

Considering the pros and cons of LC, MS and their combined uses, Daniel Schulz-Jander, Senior Director, Bioanalysis Mass Spectrometry, QPS Holdings LLC (DE, USA) said:

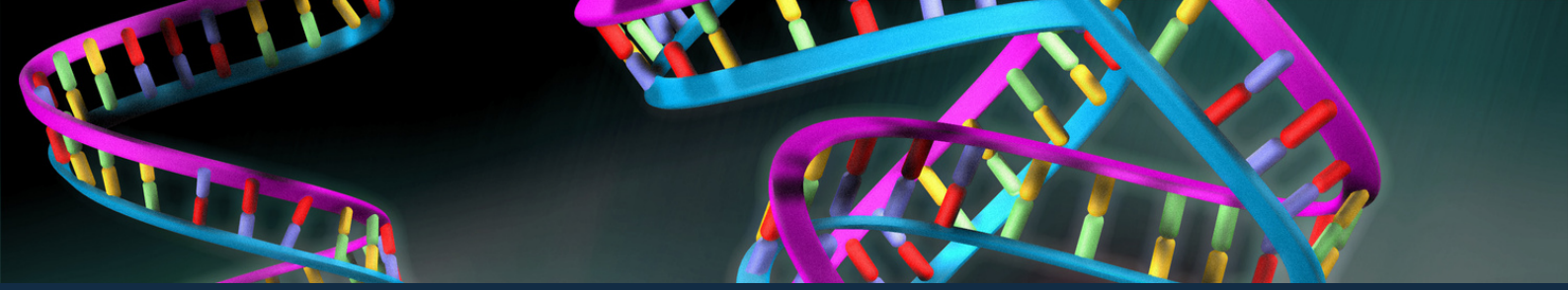
“The availability of micro and nano LC combined with mass spectrometric detection provides a great possible solution for quantitation with a high degree of selectivity, but the need for appropriate stable label internal standards add a costly and time-consuming hurdle that needs to be considered.”

High resolution MS (HRMS) is another technique employed for quantification of siRNAs and other major metabolites. In full scan mode, it provides selectivity and can vastly improve detection limit [11], and thus is an attractive technique for oligonucleotide therapeutic analysis.

Since 2003, QPS Holdings LLC has been working with chromatographic methods to examine plasma and tissue exposure to aid oligonucleotide quantification. As of 2014, ultra-performance LC–HRMS (UPLC HRMS) methodology has been its preferred choice of quantification, as it can simultaneously determine both sense and antisense strands. As a result, the company have quantitated up to 20,000 Daltons double stranded (ds) oligonucleotides with lower limit of quantification (LLOQ) at 5 ng/mL per the latest US FDA (MD, USA) Bioanalytical Method Validation guidance [12].

Ligand binding assays

Alternative to chromatographic techniques, LBAs – such as hybridization ELISA – can also be utilised for oligonucleotide quantification. Where LC-MS may be favored in the discovery phase due to its ability to use generic reagents, LBAs can provide high sensitivity, high sample throughput and ease of implementation that are beneficial once a program reaches development stage where sufficient knowledge of the drug in question and its metabolites has been gained, analytes are well defined and specific reagents



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are generated [6]. Additionally, LBA platforms can offer minimal clean up or extraction, which allows for shorter methods and an increased number of analytical runs on a weekly basis [4].

When asked what techniques or methods might become more important in the analysis of oligonucleotides, expert Zamas Lam responded:

'Hybridization followed by LBA or chromatography for better sensitivity' – Zamas Lam, Senior VP & Global Head Bioanalytical (Mass Spectrometry) & Preclinical Development, QPS [13].

QPS Holdings LLC has supported gene therapies for more than a decade, including oligonucleotide therapy quantification. Depending on the therapy's requirements, they employ probe-hybridization, ligand binding with ELISA using colorimetric, fluorescent, chemiluminescent detection as well as Gyrolab® and MSD® approach. QPS' Clinical Laboratory Improvement Amendments (CLIA) program also aids in navigating study requirements, as it provides quality laboratory testing as well as custom assay development and validation, in accordance with both GCP and CLIA, to support both diagnostic and traditional clinical trial endpoints [12].

LBAs vs chromatographic platforms

When it comes to a preferred method of quantification, LC-MS is commonly utilized because of its wide dynamic range and specificity, when compared to LBAs which have a narrow dynamic range and require the generation of specific reagents for accurate quantitation of intact oligonucleotides in the presence of truncated metabolites [6]. LBAs are limited by their narrow dynamic range and the inability to differentiate between intact and truncated metabolites, which can limit their success [6].

However, LC-MS, when compared with LBAs, is less sensitive and requires ion-paring reagents, which can limit the therapeutic modality's effectiveness when quantifying oligonucleotides [6]. Overall, LBAs and LC-MS have their differing benefits that work around different requirements within individual studies; it is a matter of finding the right method of quantification that aligns with the program's stage and requirements.

Conclusion

There are many bioanalytical techniques for quantifying oligonucleotides, and choosing the right technique depends on many different factors including modality, soluble target levels, availability of reagents and many more.

References

1. Roberts, T, Langer, R & Wood, M. Advances in oligonucleotide drug delivery. Nat Rev Drug Discov. 19, 673–694 (2020). doi: 10.1038/s41573-020-0075-7
2. Biosynthesis. 2' Fluoro RNA Modification. www.biosyn.com/oligonucleotideproduct/2-fluoro-rna-modification.aspx [Accessed 02 November 2022]
3. Watts J, Corey D. Silencing disease genes in the laboratory and the clinic. J Pathol. 226(2), 365-79 (2012) doi: 10.1002/path.2993.
4. Bioanalysis Zone. Bioanalytical strategies for oligonucleotide assessment. https://www.bioanalysis-zone.com/ebook-bioanalytical-strategies-for-oligonucleotide-assessment_ebook_oligo_ppd_qps/ [Accessed 25 October 2022]
5. Quality Assistance. Absolute Quantification of Oligonucleotides by ICP-MS/MS. (2021) https://acrobat.adobe.com/link/review?uri=urn:aaid:scds:US:7f59bfd5-a239-3f07-9194-0f800740e7f4 [Accessed 25 October 2022]
6. Kotapati S, Deshpande M, Jashnani A, Thakkar D, Xu H, Dollinger G. The role of ligand-binding assay and LC-MS in the bioanalysis of complex protein and oligonucleotide therapeutics. Bioanalysis. 13(11), 931-954. (2021) doi: 10.4155/bio-2021-0009.
7. Certara. Ligand Binding Assays. www.certara.com/knowledge-base/ligand-binding-assays/ [Accessed 25 October 2022]
8. Erb R, Leithner K, Bernkop-Schnürch A, Oberacher H. Phosphorothioate oligonucleotide quantification by μ-liquid chromatography-mass spectrometry. AAPS J. 14(4), 728-37 (2012) doi: 10.1208/s12248-012-9381-2.
9. Bioanalysis Zone. Infographic: the exciting world of oligonucleotides. https://www.bioanalysis-zone.com/infographic-exciting-world-oligonucleotides_yuduoligo/ [Accessed 02 November 2022]
10. Ewles M, Goodwin L, Schneider A & Rothhammer-Hampl T. Quantification of oligonucleotides by LC–MS/MS: the challenges of quantifying a phosphorothioate oligonucleotide and multiple metabolites. Bioanalysis. 6(4), 447-464. (2014) doi: 10.4155/bio.13.319
11. Ramanathan L & Shen H. LC–TOF–MS methods to quantify siRNAs and major metabolite in plasma, urine and tissue. Bioanalysis. 11(21). 1983-1992. (2019) doi: 10.4155/bio-2019-0134
12. QPS Holdings LLC. Gene therapy and cell therapy. https://www.qps.com/service/bioanalysis/cell-and-gene-therapy/ [Accessed 25 October 2022]
13. Bioanalysis Zone. In your opinion, what techniques or methods might become more important in the analysis of oligonucleotides? https://www.bioanalysis-zone.com/5-in-your-opinion-what-techniques-or-methods-might-become-more-important-in-the-analysis-of-oligonucleotides_ate_oligo_alturas_sciex_qps/ [Accessed 25 October 2022]

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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.

newlands press

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.

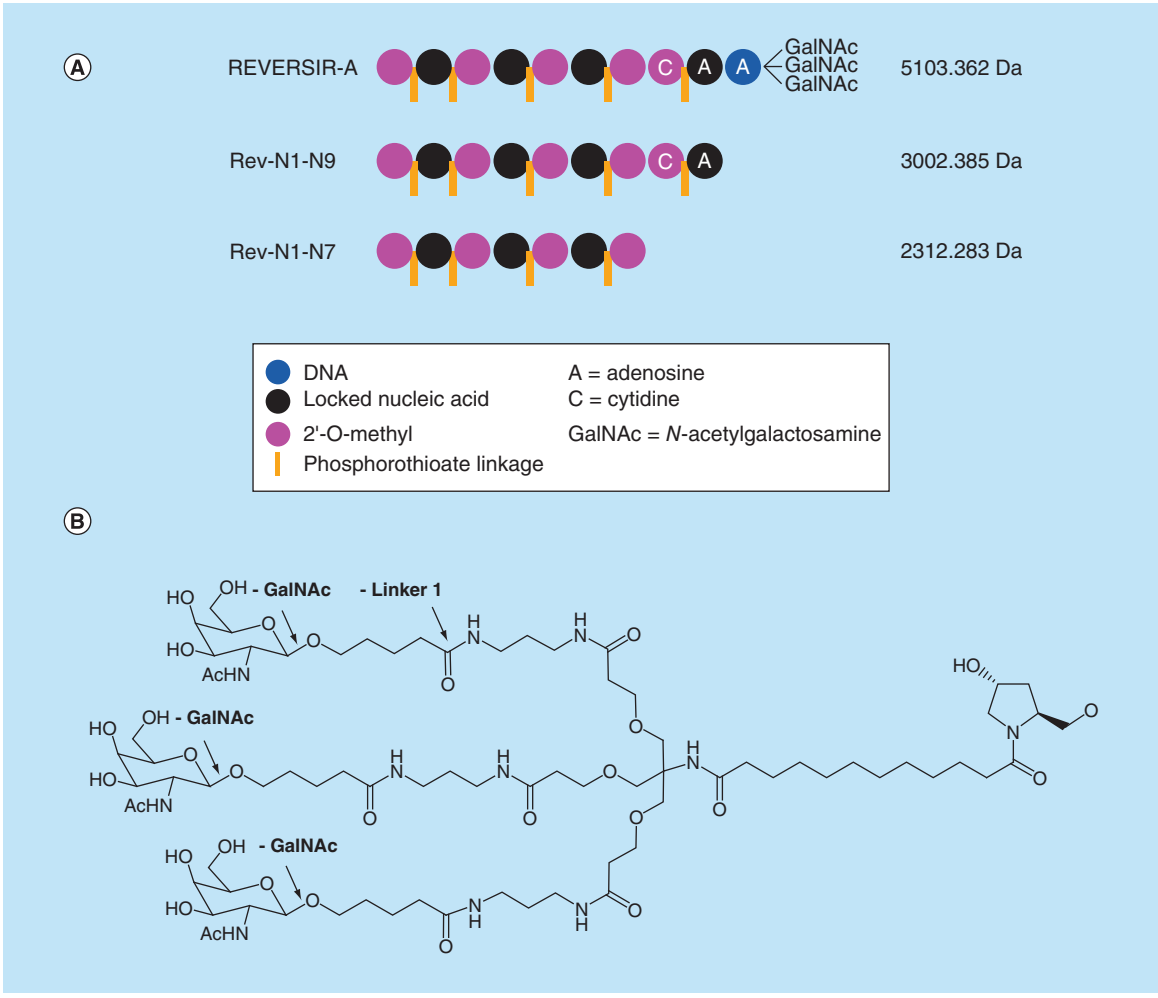

Figure 1. Sequences and structure. (A) Sequences of REVERSIR-A and metabolites. **(B)** Chemical structure and cleavage sites of metabolites of the triantennary GalNAc ligand of REVERSIR-A.

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

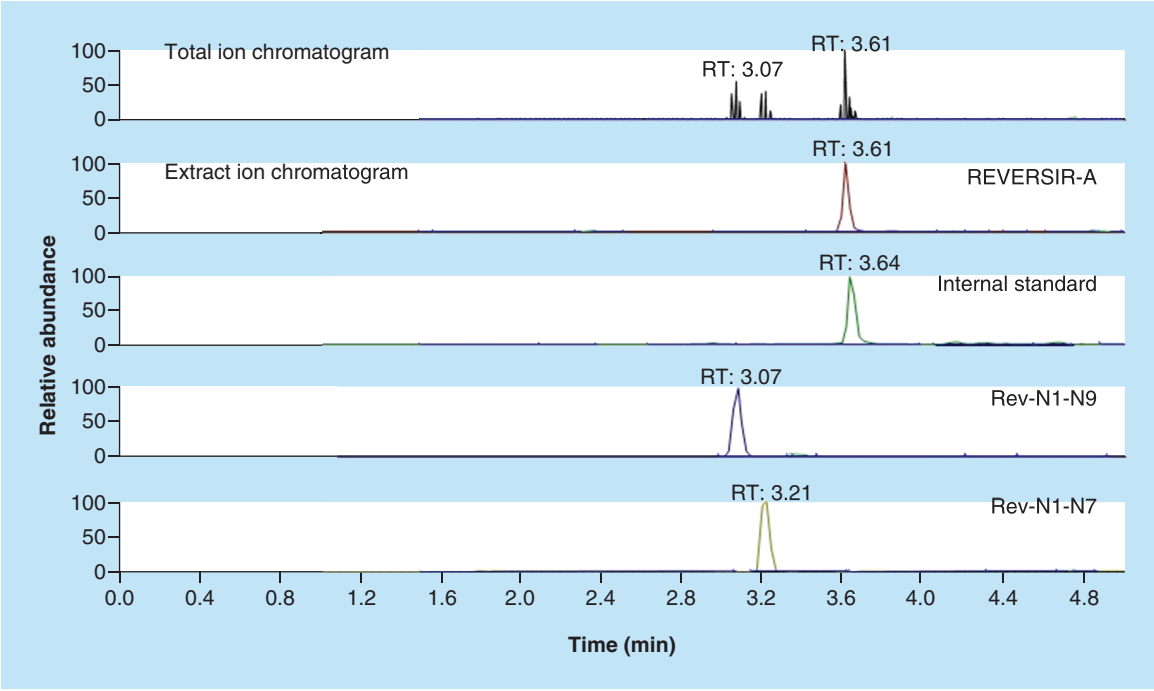

Figure 2. LC-high-resolution mass spectrometry chromatograms for spiked REVERSIR-A and metabolites in rat liver homogenate. The blank rat liver homogenate was spiked with 3500 ng/ml of REVERSIR-A and Rev-N1-N9 and Rev-N1-N7 metabolites.

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

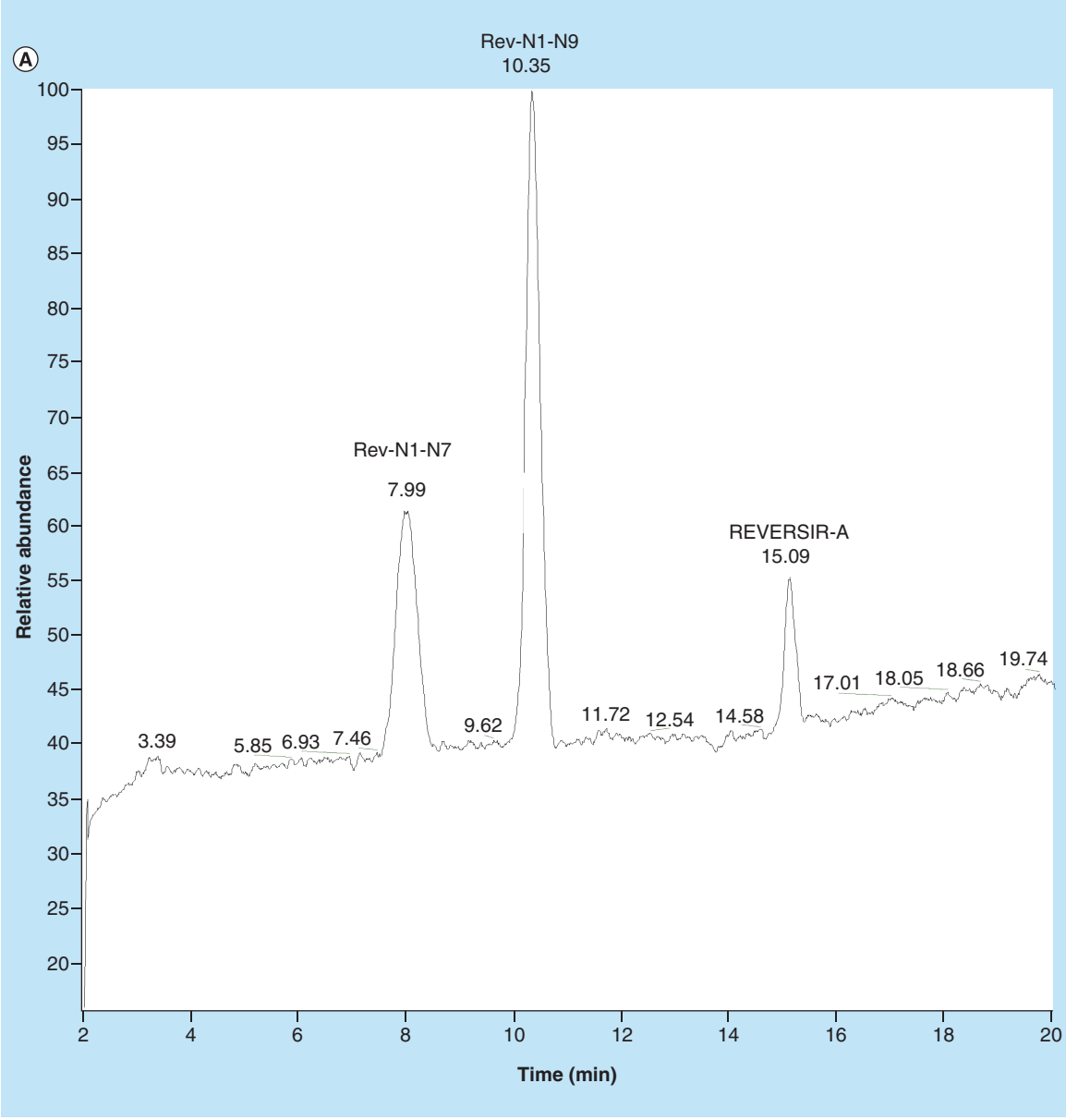

Figure 3. LC-high-resolution mass spectrometry analysis of REVERSIR-A in monkey plasma. Sample was collected 2 h after a 3.0 mg/kg sc. dose of REVERSIR-A. **(A)** Total ion chromatogram. **(B)** Electrospray ionization mass spectrum of Rev-N1-N9 metabolite. **(C)** Isotopic envelopes for the charge state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

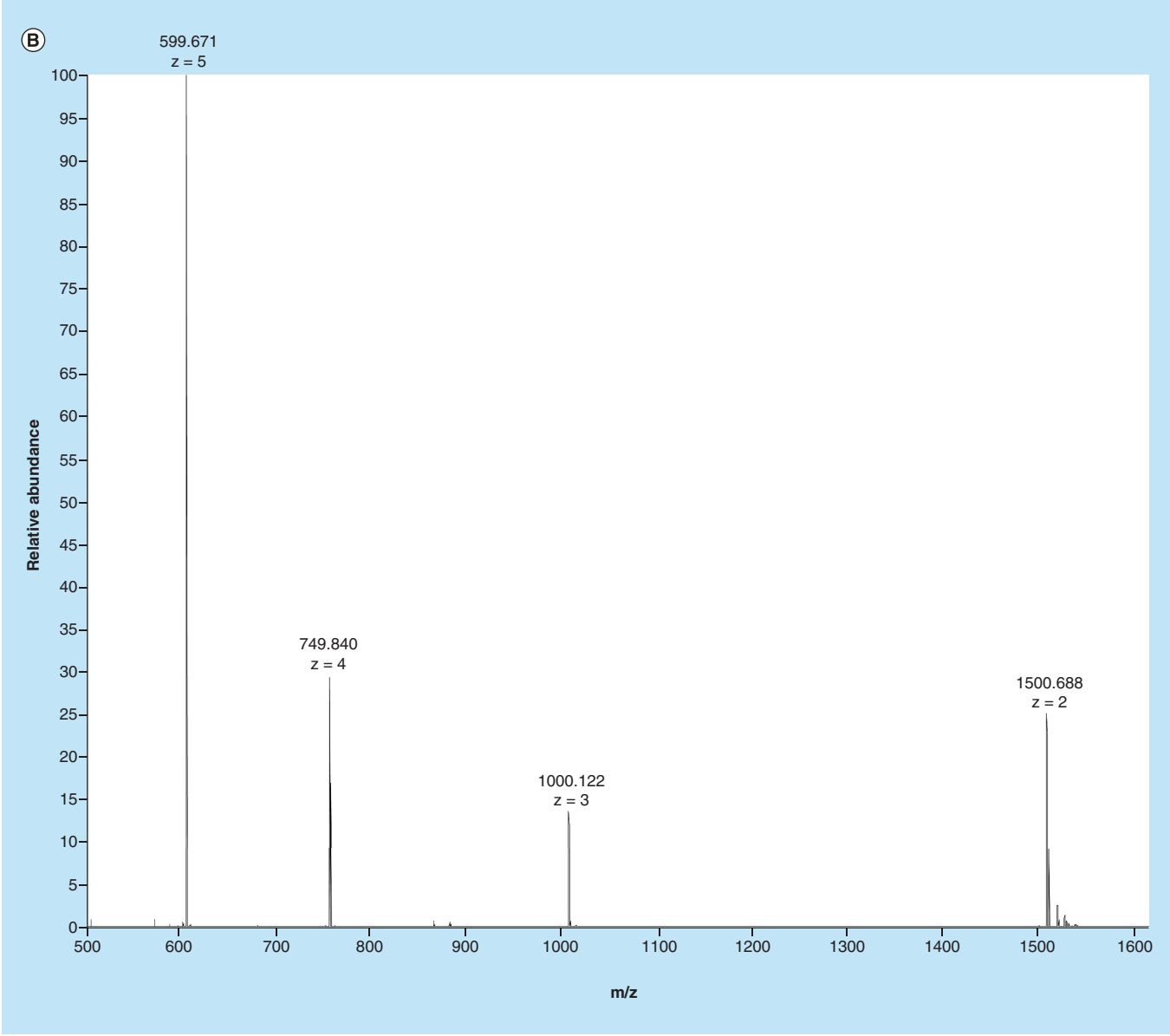

Figure 3. LC-high-resolution mass spectrometry analysis of REVERSIR-A in monkey plasma (cont.). Sample was collected 2 h after a 3.0 mg/kg sc. dose of REVERSIR-A. **(A)** Total ion chromatogram. **(B)** Electrospray ionization mass spectrum of Rev-N1-N9 metabolite. **(C)** Isotopic envelopes for the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

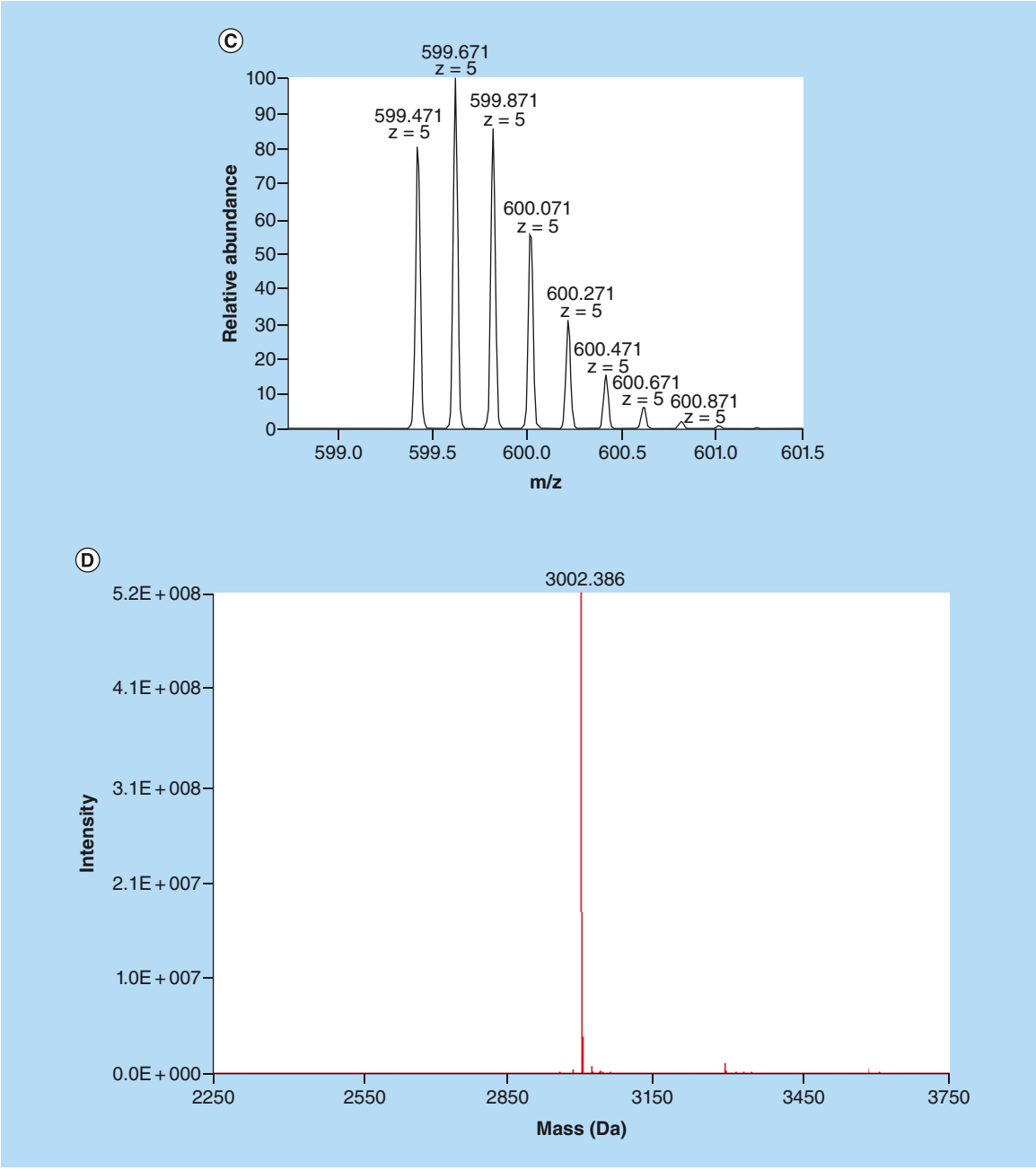

Figure 3. LC-high-resolution mass spectrometry analysis of REVERSIR-A in monkey plasma (cont.). Sample was collected 2 h after a 3.0 mg/kg sc. dose of REVERSIR-A. **(A)** Total ion chromatogram. **(B)** Electrospray ionization mass spectrum of Rev-N1-N9 metabolite. **(C)** Isotopic envelopes for the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

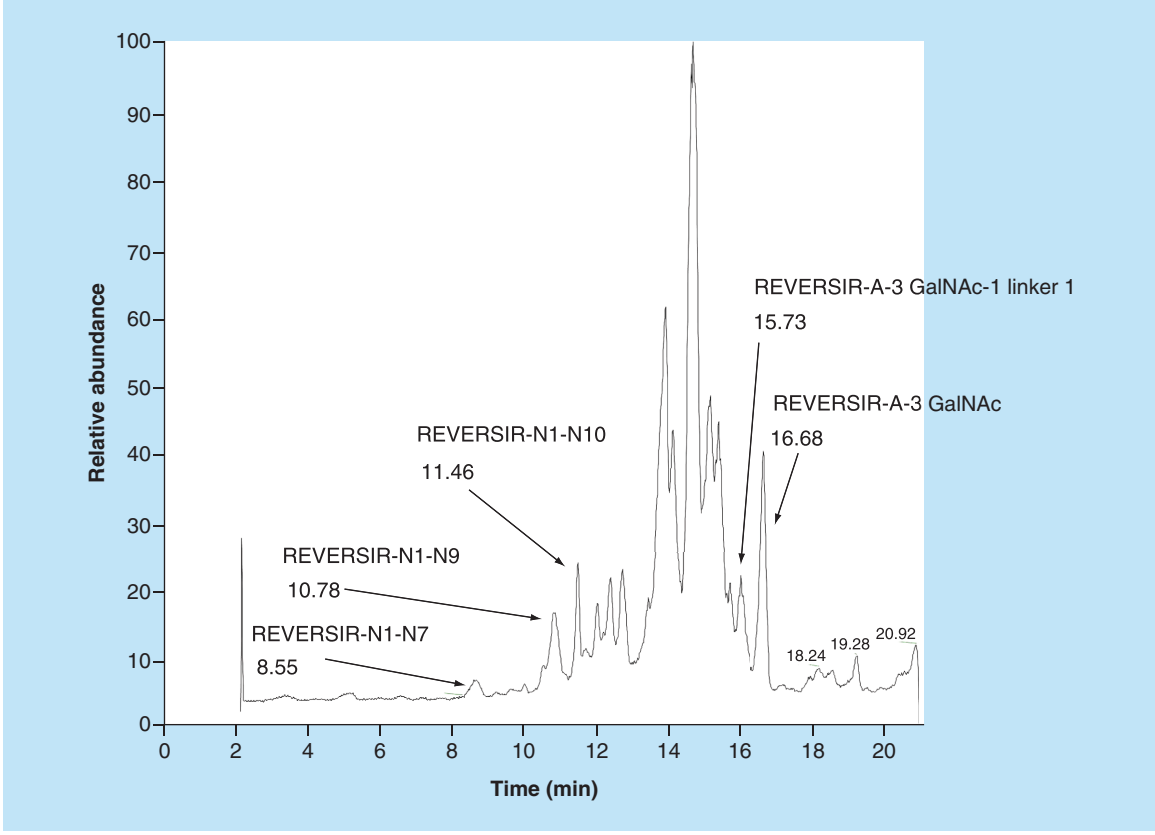

Figure 4. Total ion chromatogram of REVERSIR-A and its metabolites in a monkey liver sample by LC high-resolution accurate mass spectrometry. The sample was collected 8 h after subcutaneous administration of a 3.0 mg/kg dose of REVERSIR-A.

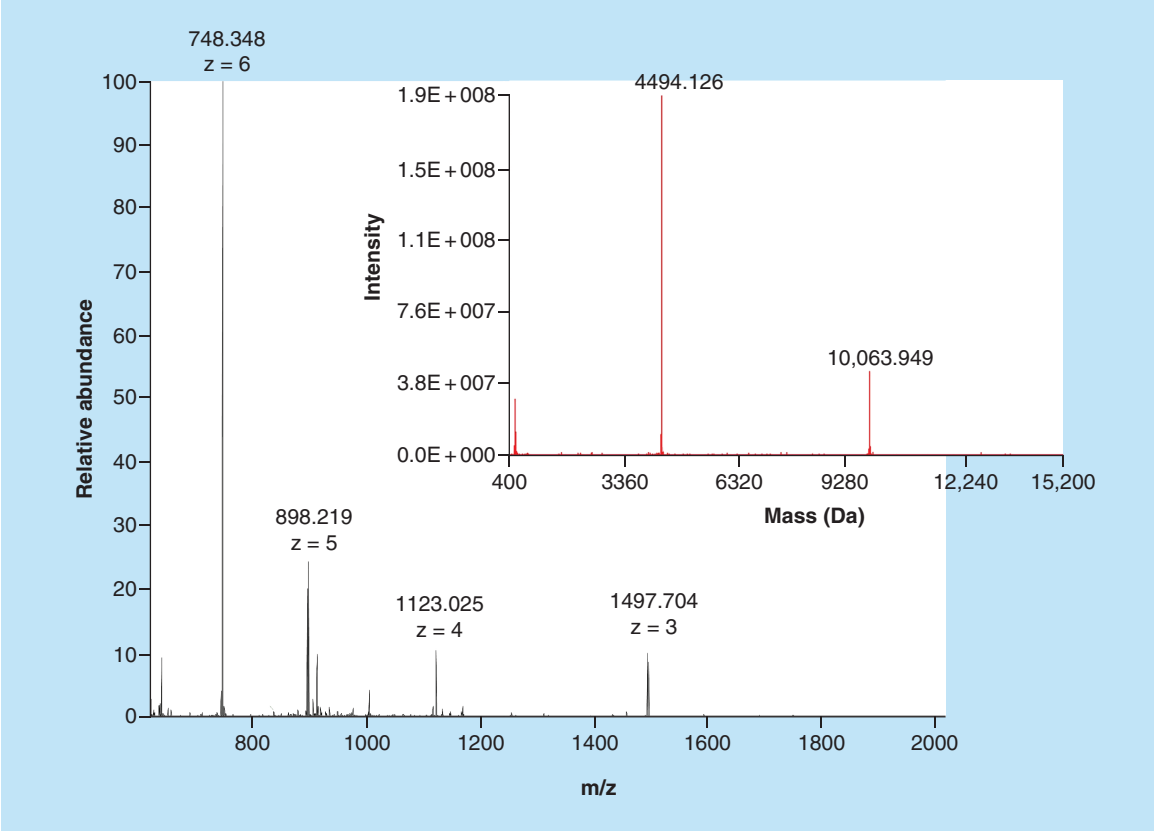

Figure 5. Electrospray ionization mass spectrum of the peak at 16.68 min in a monkey liver sample. The insert displays the deconvoluted mass spectrum of the peak. The liver sample is as described in Figure 4.

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.

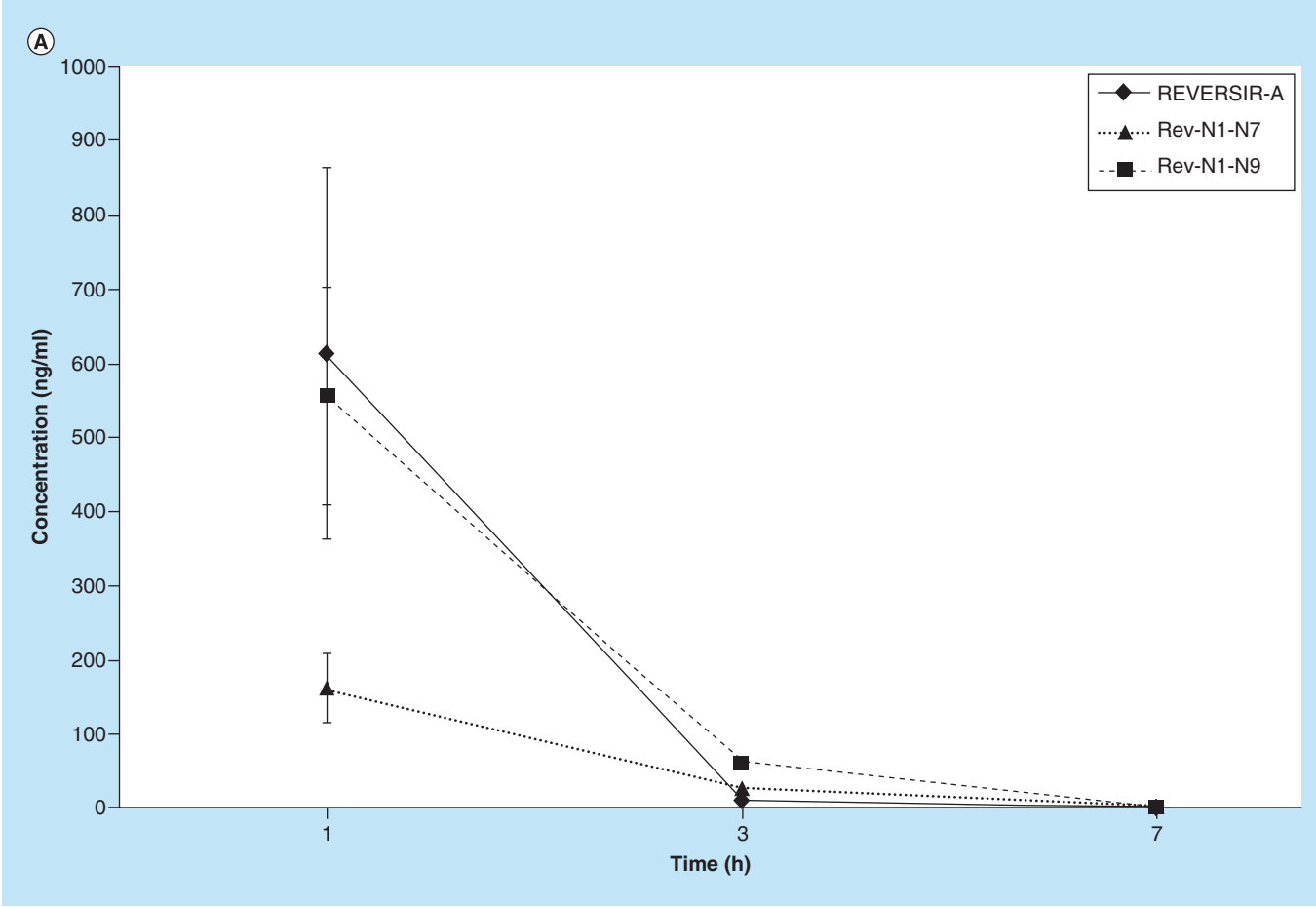

Figure 6. REVERSIR-A and metabolite concentrations in rat plasma and tissues after subcutaneous administration of a 3.0 mg/kg dose of REVERSIR-A. (A) Rat plasma (n = 3); **(B)** rat liver (n = 3); **(C)** rat kidney (n = 3).

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.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Wittrup A, Lieberman J. Knocking down disease: a progress report on siRNA therapeutics. *Nat. Rev. Genet.* 16(9), 543–552 (2015).

• **Comprehensive review of siRNA.**

2. Lares MR, Rossi JJ, Ouellet DL. RN Ai and small interfering RNAs in human disease therapeutic applications. *Trends Biotechnol.* 28(11), 570–579 (2010).

3. Angaji SA, Hedayati SS, Poor RH, Madani S, Poor SS, Panahi S. Application of RNA interference in treating human diseases. *J. Genet.* 89(4), 527–537 (2010).

• **Comprehensive review of RNA interference as therapeutics.**

4. US Food and Drug Administration Department of Health and Human Services. *Drug Approval Package: Onpattro (patisiran)* (2019). www.accessdata.fda.gov/drugsatfda_docs/nda/2018/210922Orig1s000TOC.cfm

5. European Medicines Agency. *European Public Assessment Report: Onpattro (patisiran)*(2019). http://www.ema.europa.eu/en/medicines/human/EPAR/onpattro#authorisation-details-section

6. Raymond CK, Roberts BS, Garrett-Engele P, Lim LP, Johnson JM. Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs. *RNA* 11(11), 1737–1744 (2005).

7. Chen C, Ridzon DA, Broomer AJ *et al.* Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.* 33(20), e179 (2005).

8. Holmes K, Williams CM, Chapman EA, Cross MJ. Detection of siRNA induced mRNA silencing by RT-qPCR: considerations for experimental design. *BMC Res. Notes* 3, 53 (2010).

9. Kim EJ, Park TG, Oh YK, Shim CK. Assessment of siRNA pharmacokinetics using ELISA-based quantification. *J. Control. Release* 143(1), 80–87 (2010).

10. Tian Q, Rogness J, Meng M, Li Z. Quantitative determination of a siRNA (AD00370) in rat plasma using peptide nucleic acid probe and HPLC with fluorescence detection. *Bioanalysis* 9(11), 861–872 (2017).

11. Wang L. Oligonucleotide bioanalysis: sensitivity versus specificity. *Bioanalysis* 3(12), 1299–1303 (2011).
12. Lin ZJ, Li W, Dai G. Application of LC–MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides. *J. Pharm. Biomed. Anal.* 44(2), 330–341 (2007).

• **Comprehensive review of small interfering RNA analysis by LC–MS.**

13. van Dongen WD, Niessen WMA. Bioanalytical LC–MS of therapeutic oligonucleotides. *Bioanalysis* 3(5), 541–564 (2011).

• **Comprehensive review of small interfering RNA analysis by LC–MS.**

14. Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS. Analysis of oligonucleotides by HPLC-electrospray ionization mass spectrometry. *Anal. Chem.* 69(7), 1320–1325 (1997).
15. Apffel A, Chakel JA, Fischer S, Lichtenwalter K, Hancock WS. New procedure for the use of high-performance liquid chromatography–electrospray ionization mass spectrometry for the analysis of nucleotides and oligonucleotides. *J. Chromatogr. A* 777(1), 3–21 (1997).

• **MS-based techniques first demonstrating application of the ion-pairing system triethylamine and hexafluoroisopropanol.**

16. Dai G, Wei X, Liu Z, Liu S, Marcucci G, Chan KK. Characterization and quantification of Bcl-2 antisense G3139 and metabolites in plasma and urine by ion-pair reversed phase HPLC coupled with electrospray ion-trap mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 825(2), 201–213 (2005).
17. Zlatev I, Castoreno A, Brown CR *et al.* Reversal of siRNA-mediated gene silencing *in vivo*. *Nat. Biotechnol.* 36(6), 509–511 (2018).
18. Phenomenex Chromatography Product Guide (2019). https://view.highspot.com/viewer/5d7bdf29b7b73962b4dc50db
19. Johnson JL, Guo W, Zang J *et al.* Quantification of raf antisense oligonucleotide (rafAON) in biological matrices by LC–MS/MS to support pharmacokinetics of a liposome-entrapped rafAON formulation. *Biomed. Chromatogr.* 19(4), 272–278 (2005).
20. Nuckowski L, Kaczmarkiewicz A, Studzinska S. Development of SPE method for the extraction of phosphorothioate oligonucleotides from serum samples. *Bioanalysis* 10(20), 1667–1677 (2018).
21. Meng M, Zhang J, Liu A, Reuschel S, Sazani P, Wong M. Quantitative determination of AVI-7100 (Radavirsen), a phosphorodiamidate morpholino oligomer (PMOplus®), in human plasma using LC–MS/MS. *Bioanalysis* 9(10), 827–839 (2017).
22. Zhang G, Lin J, Srinivasan K, Kavetskaia O, Duncan JN. Strategies for bioanalysis of an oligonucleotide class macromolecule from rat plasma using liquid chromatography−tandem mass spectrometry. *Anal. Chem.* 79(9), 3416–3424 (2007).
23. Fountain KJ, Gilar M, Gebler JC. Analysis of native and chemically modified oligonucleotides by tandem ion-pair reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 17(7), 646–653 (2003).
24. Birdsall RE, Gilar M, Shion H, Yu YQ, Chen W. Reduction of metal adducts in oligonucleotide mass spectra in ion-pair reversed-phase chromatography/mass spectrometry analysis. *Rapid Commun. Mass Spectrom.* 30(14), 1667–1679 (2016).
25. Hail Mark E EB, Anderson Kathleen. High-throughput analysis of oligonucleotides using automated electrospray ionization mass spectrometry. *Am. Biotechnol. Lab.* 22(1), 12–14 (2004).
26. Pesek JJ, Matyska MT, Fischer SM. Improvement of peak shape in aqueous normal phase analysis of anionic metabolites. *J. Sep. Sci.* 34(24), 3509–3516 (2011).
27. Hsiao JJ, Potter OG, Chu T-W, Yin H. Improved LC/MS methods for the analysis of metal-sensitive analytes using medronic acid as a mobile phase additive. *Anal. Chem.* 90(15), 9457–9464 (2018).
28. Lin ZJ, Li W, Dai G. Application of LC–MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides. *J. Pharm. Biomed. Anal.* 44(2), 330–341 (2007).
29. Zou Y, Tiller P, Chen I-W, Beverly M, Hochman J. Metabolite identification of small interfering RNA duplex by high-resolution accurate mass spectrometry. *Rapid Commun. Mass Spectrom.* 22(12), 1871–1881 (2008).

•• **Use of ProMass deconvolution software for automated data interpretation.**

30. Shemesh CS, Yu RZ, Gaus HJ *et al.* Elucidation of the biotransformation pathways of a Galnac3-conjugated antisense oligonucleotide in rats and monkeys. *Mol. Ther. Nucleic Acids* 5, e319 (2016).
31. Husser C, Brink A, Zell M, Muller MB, Koller E, Schadt S. Identification of GalNAc-conjugated antisense oligonucleotide metabolites using an untargeted and generic approach based on high resolution mass spectrometry. *Anal. Chem.* 89(12), 6821–6826 (2017).
32. Food and Drug Administration Center for Drug Evaluation and Research. *Guidance for Industry: Safety Testing of Drug Metabolites* (2019). www.fda.gov/media/72279/download

Research Article
SPECIAL FOCUS ISSUE: The Exciting World of Oligonucleotides

Bioanalysis

LC–MS quantification of oligonucleotides in biological matrices with SPE or hybridization extraction

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Aim: Quantitative LC–MS analysis of oligonucleotides (OGNs) in biological matrices is needed to support candidate selection of new therapeutic OGNs. **Methodology & results:** A set of 20 single stranded antisense oligonucleotides (ASO) and five siRNAs were extracted from plasma and tissue homogenates. Anion Exchange (AEX) SPE was selected as generic extraction approach, resulting in recoveries from plasma >70%. Extraction from tissue homogenates showed often more variation and lower recoveries. A proof of concept of a novel tailored hybridization extraction is demonstrated for two 16-mer reference OGNs. **Conclusion:** Two methods for extraction of OGNs were investigated and applied for quantitative analysis. The AEX-SPE is considered a more generic approach preferred when multiple compounds are evaluated. Hybridization extraction has great potential but critical reagents per analyte are needed.

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Keywords: anion-exchange extraction (AEX-SPE) • antisense oligonucleotide (ASO) • capture probe (CP) • hybridization extraction • small interfering RNA (siRNA)

DNA and RNA are natural occurring oligonucleotides (OGNs) comprising our genome and transcriptome. Enhancement or suppression of the expression of genes translating into functional proteins can be the cause of diseases. Gene mutations or single nucleotide polymorphism have also been implicated in disease states. Even noncoding RNA regions (introns) have been associated with disease pathogenesis. Not surprisingly, OGNs have generated a boost of interest, certainly the last decade, as potential therapeutic agents [1–3]. Different therapeutic mechanisms of actions are envisaged. Antisense therapy uses single or double stranded nucleotide sequences to inhibit translation of specific genes [2]. Both steric hindrance of the ribosome or recruitment of RNase H, which results in breakdown of the RNA–DNA hybrid, have been proposed as mechanism of action. RNA interference (RNAi) relies on the discovery that short double stranded RNA (small interfering RNA, siRNA) can silence targeted gene expression. Through a complex process involving loading of dsRNA on the RNA-induced silencing complex, followed by degradation of the sense strand and pairing of the antisense strand with its target RNA, the target mRNA is destroyed [4–7]. MicroRNA and larger modified mRNA constructs are currently also considered as novel therapeutic approaches, but these types of molecules are out of scope for this manuscript.

The first endeavors in OGN drug development highlighted substantial challenges but over the years significant progress has been realized. Chemical modifications are now commonly introduced to overcome nuclease instability (phosphorothioate additions in the backbone), to improve half-lives, increase affinity toward the target mRNA, reduce innate immune-stimulation (modifications at the 2′ position in the ribose) and to target delivery toward specific organs (5′ extension with N-acetyl galactosamine or lipids) [3,5]. The latest new approvals have created a new momentum [8]. The approval of Nusinersen, an 18 mer phosphorothioate antisense oligonucleotide (ASO) with 2′O-modifications, administered intrathecally, was a huge breakthrough and provided the first drug for patients with spinal muscular atrophy [9]. The approval of Eteplirsen, a 30-mer phosphomorpholidate was more controversial. Eteplirsen treats Duchenne muscular atrophy by restoring mutations in the dystrophin gene [10].

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In August 2018, Alnylam announced the first-ever FDA approval of an siRNA therapeutic, Onpattro™ (patisiran) for the treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis in adults [11]. Onpattro is administered through intravenous infusion every 3 weeks. This milestone was quickly followed by the approval in October 2018 of TEGSEDI™ (inotersen) developed by Ionis and Akcea for the same indication [12].

Independent of the stage of a drug development project, a good bioanalytical strategy is needed to enable support of exposure evaluation after *in vivo* administration. Nowadays bioanalytical scientists can select the analytical tool in alignment with the type/size of OGN, the sensitivity, the specificity and throughput needed [13]. Over the life time of a project often multiple methods are considered. Hybridization-based ELISA, qRT-PCR, HPLC–FL/UV and LC–MS(/MS) are applied in support of OGN quantification in biological matrices [14–18]. ELISA, PCR and FL based assays rely on hybridization of the analyte of interest with a complementary strand, which introduces a new dimension toward the selectivity and the need to design and synthesize these reagents. These technologies allow less stringent conditions with respect to sample clean up. On the other hand, Elisa and PCR cannot always discriminate between full length OGN and truncated metabolites. HPLC assays based on hybridization with a complementary fluorescent probe will be able to discriminate only when chromatographic separation is realized. LC–MS based assays provide this selectivity at the level of detection [19]. OGNs are hydrophilic analytes with limited or no retention on reversed phase chromatography and poor ionization efficiency in electrospray MS. Although both ion exchange chromatography [20] and ion-pairing reversed phase have been described, the latter is the preferred option in combination with mass spectrometry. The combination of an ion-pairing reagent with an acidic modifier (hexafluoroisopropanol) still provides the best compromise between chromatographic performance and MS sensitivity and in generally these LC–MS assays can also meet requirements to allow exposure evaluation in initial *in vivo* studies [21–24].

Depending on the technique and the biological matrix of interest, extraction of the OGN can become a critical step, especially for the LC–MS assay. In a recent review, Nuckowski *et al.* [25] summarize the different strategies applied for extraction of OGNs from biological matrices.

To enable throughput and flexibility in OGN development programs, we focus our bioanalytical support on LC–MS technology in combination with a more generic anion exchange extraction to provide a solid starting point for method development for a variety of OGN molecules. As an alternative, we report on the exploration of hybridization/annealing to extract OGNs from a biological matrix with the aim to reduce matrix background and improve overall robustness of the assays. This approach is less generic and requires the availability of a complementary biotinylated probe. Future experiences will educate us whether this can be considered as a valuable alternative to extraction techniques currently applied with LC–MS detection. Hybridization extraction for the quantitation of therapeutic OGNs is novel and has only been reported recently for quantification of imetelstat and microRNA (miR) 451 [31,32].

Materials & methods

Chemicals & reagents

Most OGNs (ASO and siRNA) were synthesized and provided by internal Janssen synthesis facilities. A 16-mer reference phosphodiester (OPO), the 16-mer phosphorothioate (OPS) analog OGN (ATCTATACAAGCTGTC) as well as the 5′ biotinylated capture probe (5′ biotin-TEG-(tetraethylene glycol)-GACAGCTTGTATAGAT were custom made by Integrated DNA Technologies (Leuven, Belgium). A comprehensive list of all ASOs and siRNAs discussed in this manuscript is provided in Supplementary Table 1. The siRNAs in the table have similar modifications but the number of modifications and the nucleotide sequence (not disclosed) differ.

For SPE, Clarity® OTX™ 96-well plates (100 mg/well) and lysis buffer were obtained from Phenomenex (Utrecht, 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). Rat and mouse blank plasma (sodium ethylenediaminetetraacetic acid [Na2EDTA]) were obtained from Bioreclamation (West Sussex, UK). Proteinase K (10 ml, 20 mg/ml) was from Qiagen (Venlo, The Netherlands). Tris-EDTA (TE pH 8.0) buffer was obtained from VWR (Beerse, Belgium).

LoBind DNA tubes, LoBind 96-well plates, all solvents (HPLC grade or equivalent) and all chemicals (analytical or HPLC grade) were purchased from VWR (Beerse, Belgium).

The HPLC columns (DNAPac RP™ 2.1 × 50 mm, 4 µm) were procured from Thermo Scientific (Gent, Belgium).

Preparation of OGN standard, internal standard & capture probe solutions

10 mg/ml stock solutions of OGNs in water (HPLC grade) were prepared. Calibration standards were prepared by adding 10 µl of the stock solution to 1 ml of plasma (rat EDTA plasma) resulting in 100 µg/ml plasma. Dilutions were prepared in the appropriate plasma to obtain standard concentrations of 10,000 ng/ml (100 µl + 900 µl); 5000 ng/ml (50 µl + 950 µl) and 2000 ng/ml (20 µl + 980 µl). Further serial dilutions (100 µl + 900 µl) of each of these dilutions resulted in concentrations of 1000, 500, 200, 100, 50, 20 and 10 ng/ml. Quality control (QC) samples were prepared by diluting a solution to achieve concentrations of 40, 800 and 8000 ng/ml in rat plasma.

Internal standard (IS) stock solutions (2 mg/ml) and IS working dilutions (5 or 10 µg/ml) were prepared in water. Selected ISs were as much as possible 'equal mers' analog OGNs.

Stock solutions of the capture probe (1 mg/ml) were prepared in water. Further dilutions (0.1-10 nmol/ml) were obtained from the stock solution using buffer (15 mM sodium citrate, 150 mM sodium chloride). For each experiment, 100 µl of probe working solution was added to each sample. This contained the required amount (pmol) probe mentioned in the individual experiments.

LC–MS/MS system

A Shimadzu LC20AD HPLC with a SIL-HTC autosampler (Shimadzu Scientific Instruments, MD, USA), was coupled to an API4000™ triple quadrupole mass spectrometer (AB Sciex, Toronto, Canada) equipped with Turbo Ionspray source. Additional details of LC and MS/MS (for the 16 mer OPO and OPS OGN) parameters are given in Supplementary Tables 2 and 3, respectively. Analyst® software Version 1.6.3 (AB Sciex) was used for data acquisition and processing.

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

Extraction of OGNs from plasma, tissue homogenates and other biological matrices

Solid-phase extraction

Solid-phase extraction of OGNs from plasma and other matrices was conducted using Clarity OTX extraction kits according to the manufacturer's protocol (Phenomenex, Utrecht, The Netherlands). Modifications to optimize the extraction yield and eliminate matrix interferences were introduced. The wash steps were modified to improve the matrix clean-up and to avoid contamination due to overfilling of wells. Also, the elution step was optimized in function of the specific OGN investigated. A final generic method is detailed in Table 1 .

Further discussion on the adaptations considered at the individual steps in the protocol are included in the results and discussion section.

Hybridization extraction from plasma

Annealing of sense and antisense DNA probes forms the basis of the hybridization extraction method. The complementary probe, in other words, a capture probe, requires a biotin label for affinity capture of the sense-antisense complex from the biological matrix using streptavidin coated magnetic beads. This extraction approach was currently only explored for the phosphodiester 16 mer, two truncated phosphodiesters (3′ n-1; 3′ n-3) and the phosphorothioate modified 16 mer.

Preparation of the streptavidin coated magnetic beads before use was as follows: for each experiment, an appropriate volume of the bead suspension (based on required pmol beads/sample; e.g., 250 pmol in accuracy and precision analysis) was centrifuged (2430×*g*, 22°C, 3 min). Following centrifugation of the beads in combination with magnetic separation, the precipitated beads were washed with wash buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 2 M NaCl). This procedure was repeated twice. Finally, the beads were resuspended in wash buffer containing 2% Tween 80 to achieve 250 pmol beads in 200 µl. During method development, the amount of beads in 200 µl of buffer was adapted to evaluate and optimize the procedure.

Immunoaffinity interaction between capture probe and streptavidine beads was initiated by adding 100 µl of the capture probe (e.g., 50 pmol of the 5′ biotinylated capture probe), 200 µl (250 pmol beads) of prepared beads and 100 µl phosphate buffered saline with 0.01% Tween 80 per well in a LoBind 96-well plate. After incubation for 10 min at 22°C in a thermomixer, 20 µl sample (study, QC or calibration samples) was added to the beads capture probe solution and further incubated for 90–120 min at 22°C to start the annealing process. Thereafter

Table 1. Sample extraction procedure using solid-phase Clarity OTX 96-well plates.

Step	Procedure
1	Samples thawed and aliquoted on melting ice
2	20 (or 50) µl aliquots of study samples, QC samples and calibrator plasma, tissue homogenate or other biofluids transferred to a 1 ml LoBind 96-well plates
2′	For tissue homogenates: proteinase K treatment ([10 µl] + 50 µl TE buffer, 2 h 37°C)
3	Add 10 µl of carrier oligonucleotide (200 µg/ml)
4	100 µl lysis buffer (Phenomenex; part number ALO-8579) added to each well, plate sealed and vortexed for 10 min at RT
5	200 µl of equilibration buffer (50 mM NaH_2PO_4 pH 5.5) added to each tube
6	96-well SPE Clarity OTX plate(s) conditioned with 1 × 1 ml methanol for each well
7	Conditioned plate(s) equilibrated with 1 ml equilibration buffer/well
8	Samples transferred to the conditioned/equilibrated plate(s)
9	Wells washed with 3 × 1 ml equilibration buffer/acetonitrile (50/50; v/v)
10	Wells washed with 1 × 0.5 ml water
11	Wells washed with 1 × 0.5 ml 100 mM NH_4HCO_3 pH 10 2 mM TCEP (optional)
12	Wells eluted with 0.5 ml elution buffer (100 mM NH_4HCO_3 pH 10 [with ammonia] /acetonitrile) (50/50; v/v) - elution 1
13	Elution with 500 µl elution buffer – elution 2 (optional)
14	10 µl of IS dilution added and samples vortexed
14′	Optional: Elution fraction evaporated and redissolved in 200 µl 100 mM NH_4HCO_3
15	20 µl aliquot injected onto LC–MS system

the samples were centrifuged (2000×*g*, room temperature [RT], 2 min) to precipitate the beads. The beads were washed twice, with 200 µl ice-cold water. Beads were centrifuged (2000×*g*, 4°C, 2 min) and precipitated using a magnet.

Denaturation of the antisense-sense OGN complex was obtained by adding 200 µl of hot water (80°C) to each sample. The plate was incubated at 80°C during 20 min, followed by centrifugation (2000×*g*, RT, 2 min) and precipitation of the beads. The eluted OGNs were transferred to a new 96-well LoBind plate. A comprehensive overview of the procedure can be consulted in Table 2.

Results & discussion

A detailed list with structural information of all analytes considered in this manuscript is available as Supplementary Information. Figure 1 illustrates the different modifications apparent in the molecules evaluated. In Supplementary Table 1 more specification is given with respect to number of nucleotides and specific modifications of the analytes.

The set of OGNs consisted of four 16 mer OGNs; the two 16 mer tool compounds with a fully phosphorothioate analog and two molecules that contained some locked nucleic acids. 16 20-mer OGNs were included with a large variety of modifications in the backbone (phosphorothioate -phosphorothioamidate), at the 5′ or 3′ ends and at the ribose 2′ position. Three 24 mer OGNs were evaluated; including two molecules with lipid conjugations at the 5′ end. In addition, a set of five siRNAs, with the same number of nucleotides but different in sequence and modifications, was also included in this evaluation.

Although the focus of this manuscript is on extraction of OGNs from biological matrices, the robustness of the LC–MS/MS method is certainly critical to enable evaluation of the extraction recoveries. For MS detection, the three most abundant negatively charged peaks were considered (ranging from 7- to 12- charges) for further fragmentation. The nonselective phosphate ion (*m/z* 79) or phosphorothioate (*m/z* 95) ion was not selected as Q3 ion. Details on the LC–MS settings for the two reference OGNs (16 mers) are provided in Supplementary Tables 2 and 3. A typical chromatogram for the 16 mers is provided in Supplementary Figure 1.

Many publications address the choice and optimization of the LC–MS parameters [21–24,26–28,36]. Initially we explored the effect of different ion pairing reagents (IPR) TEA (triethylamine) versus DIEA (di isopropyl ethyl amine) and HA (hexylamine) and clear differences were observed. DIEA is a more hydrophobic ion pairing resulting in enhanced retention of the OGNs. With hexylamine as IPR the charge state envelope of the OGN shifts toward lower charge states. Finally, we decided to continue with HFIP/TEA mobile phase composition on a polymeric DNAPac™ RP column (compared with silica-based columns often used) as the gains in sensitivity with the different IPRs were minimal. In our hands this polymeric DNAPac™ column provided longer lifetime and robustness in

Table 2. Sample extraction procedure using hybridization with biotinylated capture probe.

Step	Procedure
Preparation of beads and probe – Immunoafinity reaction	
1	100 µl of capture probe (50 pmol 5′ probe or as appropriate) added per well of a 96-well LoBind plate
2	200 µl of prepared beads (10–1000 pmol) added, plate incubated in a thermomixer (1100 rpm, 22°C, 20 min)
3	100 µl phosphate buffered saline + 0.01% Tween 80 added per well
4	Plate sealed and shaken in a thermomixer (1100 rpm, 22°C, 10 min)
Annealing	
5	20 µl aliquots of study samples, QC samples and calibrator plasma added per well of the 96 LoBind plate, containing magnetic beads and capture probe
6	Plate sealed and shaken in a thermomixer (1100 rpm, 22°C, 90–120 min)
7	Plate centrifuged (2000×*g*; RT, 2 min)
8	Plate magnetized and supernatant fraction discarded
Wash	
9	200 µl of ice-cold water added to each well
10	Plate sealed, vortexed and cooled for 20 min on ice-water
11	Plate centrifuged (2000×*g*; RT, 2 min)
12	Plate magnetized and supernatant fraction discarded
13	Steps 9–12 repeated
Elution	
14	200 µl of hot water (80°C) added per well
15	Seal and place the plate in a thermomixer and shake for 20 min at 80°C (1100 rpm)
16	Plate centrifuged (2000×*g*; RT, 2 min)
17	Plate magnetized and supernatant fraction transferred to a new 96-well LoBind plate
18	10 µl of IS dilution added and plate vortexed (if applicable/available)
19	20 µl of each sample injection onto the LC–MS system

IS: Internal standard; RT: Room temperature; QC: Quality control.

daily applications. Analyte dependent carryover was observed and partially mitigated by addition of IPR in the autosampler rinsing solution (Supplementary Table 2). To avoid memory effects of TEA a dedicated LC–MS/MS system was used exclusively for OGN analyses.

Alternative IPRs such as octylamine [26] or dimethylbutyl amine (DMBA) [29,36] have also been recommended. We have recently included DMBA in an evaluation, and it performed excellent with respect to MS sensitivity. However, for the evaluations described in this manuscript DMBA has not been included.

Li *et al.* [26] claim that the sequence of the OGN strongly influences the choice of IPR and provided a predictive algorithm that guides selection of the IPR to enhance chromatographic performance. However, only a limited number of non-modified phosphodiester and phosphorothioate OGNs were evaluated in this study. Also, alternative modifiers have been proposed [27]; however, most alternatives are close HFIP analogs (but often even more expensive).

AEX-SPE Extraction of ASOs & siRNAs from plasma

In this manuscript results are obtained with SPE extraction for a set of OGNs. Although many studies describe elaborated extraction protocols, often combining liquid–liquid extraction with SPE [16,17,29], also one-step SPE has been proposed [30]. Table 1 shows the details of the extraction procedure applied for weak anion exchange on the Clarity OTX. As mentioned earlier a generic protocol is proposed, which should be evaluated per analyte (or group of analytes). Often recovery from plasma is acceptable (>70%) and reproducible, but in case insufficient recovery is experienced the individual steps in the extraction process, discussed below, can be adapted for further optimization. A key aspect in all extraction protocols is disruption of the protein binding of the OGNs in combination with the instability and adsorption challenges. The protocol includes the addition of a lysis buffer (composition not disclosed by the vendor) in a ratio 1:2 sample versus lysis buffer to disrupt protein binding. Several other ratios have been investigated with variable success. Also increasing the incubation time of the sample with the lysis buffer as well as elevation of the temperature can improve the protein release for some analytes but more often it did

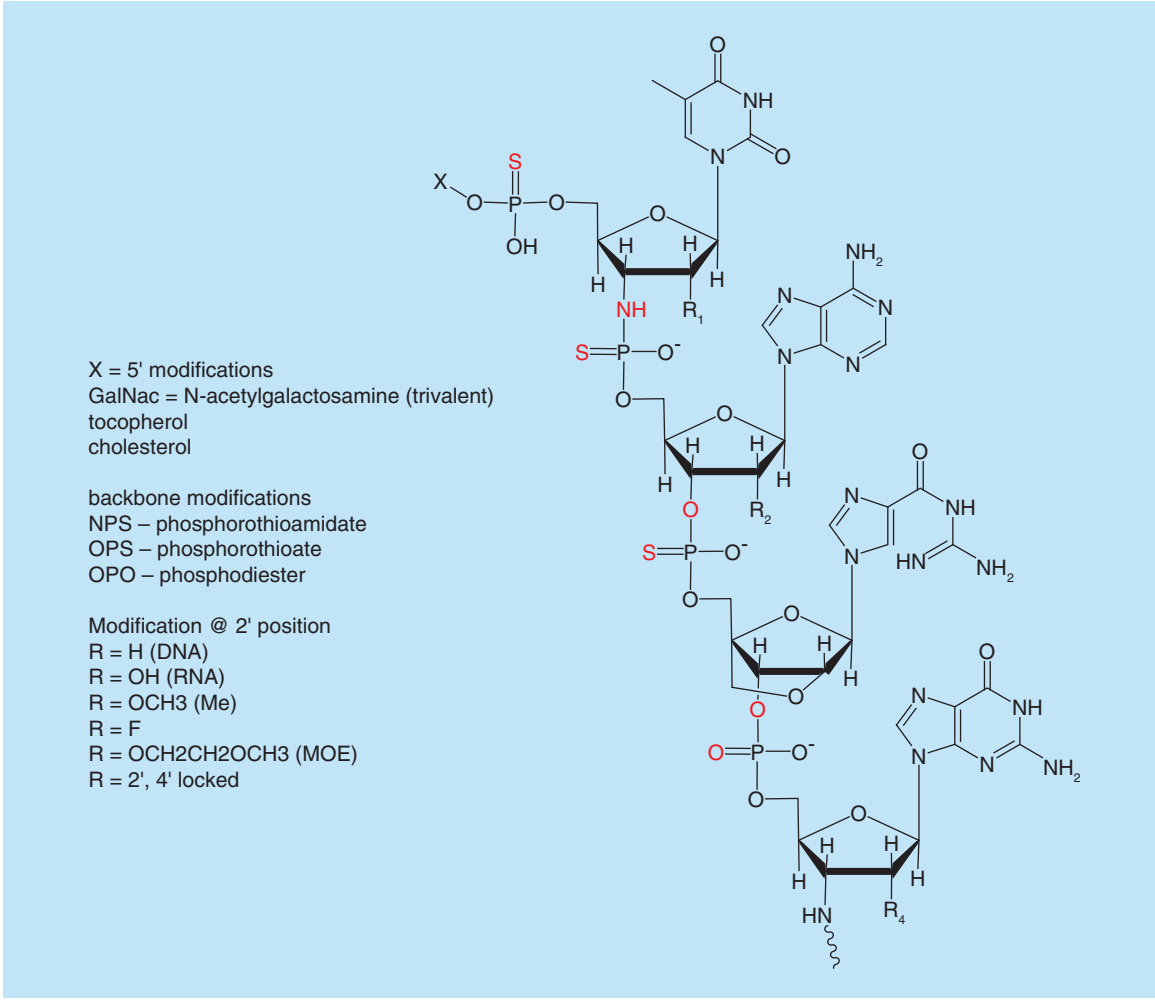

Figure 1. Structural information on the modifications encountered in the antisense oligonucleotide and siRNA molecules evaluated.

not improve recovery. The introduction of lysis buffer is a compromise between good recovery of the OGN and reduced clean-up of the matrix. For some OGN structures, the presence of lysis buffer is not a prerequisite for good recovery from plasma but for other OGNs it was key. However, no correlation of the effect of the lysis buffer could be found in relation to the OGN structures or modifications. For example, for the five siRNAs evaluated, adding lysis buffer to plasma samples did not affect recovery, but lysis buffer was needed for tissue homogenates. If recovery is rather low, often the OGN is lost in the flow-through during sample clean-up, related to insufficient protein release. Additionally, it is also critical that the equilibration buffer (pH 5.5) brings the extraction columns at the correct charge state. The equilibration buffer should provide conditions for application of the samples of pH 5.5 or lower to avoid neutralization of the + charge of the OTX cartridge during application of the sample.

Following the application of the samples, no major losses in the washing steps were experienced. Nevertheless, the wash steps provide additional clean-up and therefore are critically important with respect to LC–column life time. For several OGNs many different wash step conditions were tested. The changes evaluated for the initial 20 mer OGNs consisted of increasing or decreasing the organic content in the solvent (30, 50 and 70% acetonitrile/methanol) and including an extra water wash to remove phosphate ions from the equilibrium buffer. Recently, an additional step with 100 mM NH_4HCO_3 pH 10 was successfully included for the latest OGNs (three 20 mer OGNs with the 2′ MOE modification). This improved the robustness of the LC–MS method (increased lifetime of the column with reduced backpressure) but increased the risk of recovery loss. Therefore, systematic evaluation of each OGN of interest is advised.

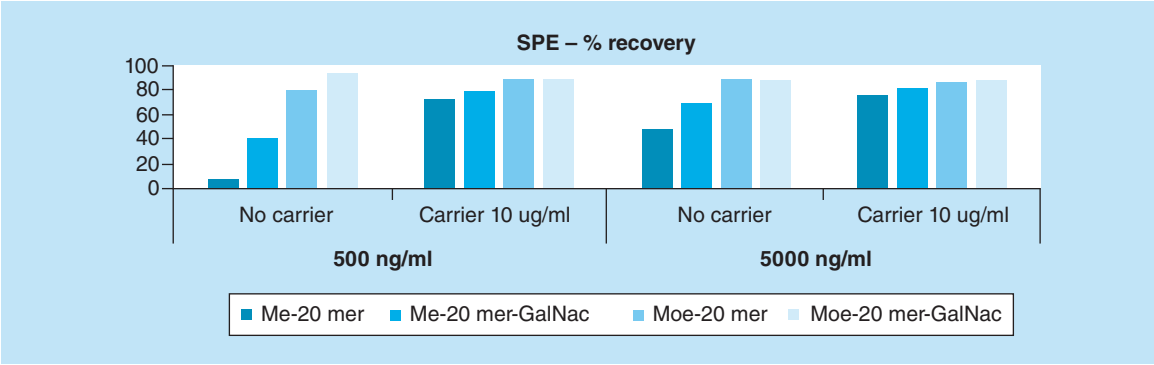

Figure 2. Improvement of extraction recovery for antisense oligonucleotides from plasma through addition of a carrier oligonucleotide. See Table 1 for details on procedure.

Efficient elution from the OTX cartridges is primordial, and this step has been substantially investigated. The Phenomenex protocol advises 100 mM NH_4HCO_3 pH 8.8. We were unsuccessful for the first set of compounds, especially those compounds including a lipid tail. Recoveries substantially improved by increasing pH to 10 during elution (2 mM TCEP added as antioxidant). With recent shipments of the Phenomenex SPE columns, we have noticed that the recommendations have been adapted and elution at pH 9.5 is proposed.

Addition or inclusion of higher elution strength solvents such as tetrahydrofuran (THF) and acetone were evaluated and some improvements for the lipid containing OGNs were noticed but at the expense of recovery for the polar non-modified OGNs. Often the best choice comprised an extra elution fraction (two or more fractions of 0.5 ml). For the lipid containing OGNs, THF increased recovery on the one hand, but on the other hand the presence of THF in the evaporation step (if included) was – for all OGNs evaluated (also for the lipid containing OGNs) – detrimental for recovery.

Figure 2 demonstrates for a limited set of four ASO compounds the effect of adding a carrier OGN (at a concentration of 10 µg/ml) to the sample before initiation of the extraction. Especially at lower concentrations, substantial improvement in recovery could be realized for non-GalNAc modified OGNs. It is hypothesised that especially the lower concentrations benefit from reduced aspecific binding in presence of carrier OGN.

For the GalNAc modified OGNs in general elution at pH 8.8 resulted in improved recovery (not shown) compared to the recovery with elution at pH 10. However, in the case of GalNAc modified OGNs, the primary analyte of interest in the target liver tissue often is the non-conjugated OGN. As a generic approach elution at pH 10 was retained for improved recovery of the nonconjugated OGN.

An overview of recoveries in plasma, obtained with elution at pH 10, for several classes of OGNs is provided in Table 3. Lowest recovery is obtained for the tocopherol modified OGN. Starting from the generic protocol, further optimization can be realized within 2–3 days, delivering extraction conditions that are sufficiently robust to allow exposure evaluation in the initial *in vivo* studies.

Tables 4 and 5 include the accuracy results for the calibration curves and the accuracy and precision of the QCs at three concentration levels for the 16 mer OGN and its phosphorothioate analog in plasma after SPE extraction.

SPE extraction of ASOs & siRNAs from tissue homogenates & other matrices

As OGNs interact with their intracellular target in a specific organ or tissue, determination of the intracellular concentrations in tissues is primordial to understand PD and safety aspects. When analysing with LC–MS, extraction of OGNs from tissue homogenates is more challenging since more clean-up is required. Hybridization based analytical methods (hybridization ELISA or hybridization-fluorescence LC) typically only need proteinase K treatment to digest all proteins as sample treatment [15]. As for the plasma extractions, we have not combined LLE and solid phase extraction but preferred to optimize the extraction of OGNs from tissue homogenates with mixed mode anion exchange solid phase extraction. We have experienced that more diluted tissue homogenates, in other words, a 1:10 or even 1:20 (w:w) tissue homogenate instead of a 1:4 or 1:5, could improve analyte recovery. For liver and kidney tissues, the homogenate is also subjected to proteinase K treatment before application to the SPE cartridges. Recovery from brain homogenates is less influenced by the proteinase treatment. Despite these manipulations, recoveries from tissue homogenates were often lower (Table 6) compared with recoveries of the

Table 3. Extraction recoveries (average of n = 2) from plasma with SPE – Clarity OTX extraction. Extraction recovery was evaluated at concentration of 10 μg/ml or 50 μg/ml. Reference sample consisted of a blank extracted matrix sample spiked post extraction.

# mers	Linkage	2′ ribose/5′/3′ modification	% Recovery
16	OPO	none	92
16	OPS	none	96
16	OPS	Locked 2′-4′	85
16	OPS	Locked 2′-4′	85
20	NPS-OPS	none	73
20	NPS-OPS	5′ GalNac	79
20	NPS-OPS	2′ F	77
20	NPS-OPS	2′ F/ 3′ GalNac	71
20	NPS-OPS	2′ MOE	94
20	NPS-OPS	2′ MOE / 5′ GalNac	69
20	OPS	2′ Methyl	64
20	OPS	2′ Methyl / 3′ GalNac	73
20	OPS	2′ MOE	92
20	OPS	2′ MOE / 3′ GalNac	69
20	NPS	2′ MOE	87
20	OPS	2′ MOE	85
20	OPS	2′ MOE	101
20	NPS-OPS	2′ Methyl	98
20	NPS-OPS	2′ Methyl / 2′ MOE	96
20	OPS	2′ Methyl / 2′ MOE	102
24	OPO-OPS	5′ tocopherol	60
24	OPO-OPS	5′ cholesterol	85
24	OPO-OPS	none	107
21	OPO-OPS	2′ Methyl / 2′ F /5′vinyl	92
21	OPO-OPS	2′ Methyl / 2′ F	99
21	OPO-OPS	2′ Methyl / 2′ F	95
21	OPO-OPS	2′ Methyl / 2′ F	98
21	OPO-OPS	2′ Methyl / 2′ F	94

NPS: Phosphorothioamidate; OPS: Phosphorothioate.

Table 4. % Accuracy for 16 mer oligonucleotides (phosphodiester OPO vs phosphorothioate OPS) calibration curves analyzed following SPE or hybridization extraction from plasma. Hybridization experiment with 250 pmol beads and 50 pmol capture probe.

Concentration (ng/ml)	% Acc following SPE		% Acc following hybridization	
	16 mer OPO	16 mer OPS	16 mer OPO	16 mer OPS
10	96.6	94.0	98.4	BQL
20	101.5	106.7	102.5	92.5
50	102.1	99.6	91.7	83.7
100	94.8	97.7	100.5	100.5
200	100.3	96.5	109.5	118.2
500	107.2	110.5	103.5	118.9
1000	98.9	99.3	97.9	110.1
2000	106.9	101.8	100.0	102.6
5000	96.9	97.5	98.1	95.0
10,000	95.7	97.6	98.9	85.1

BQL: Below quantitation limit; OPO: Phosphodiester; OPS: Phosphorothioate.

Table 5. Accuracy and precision of quality controls analysed in sixfold in plasma with the two extraction techniques. Hybridization experiment with 250 pmol beads and 50 pmol capture probe.

Concentration (ng/ml)	SPE				Hybridization			
	16 mer OPO		16 mer OPS		16 mer OPO		16 mer OPS	
	% Acc	%CV	% Acc	%CV	% Acc	%CV	% Acc	%CV
40	102.5	5.4	103.5	6.1	98.2	6.9	111.7	8.4
800	110.6	2.0	106.8	4.7	99.2	4.5	113.1	5.2
8000	103.3	3.2	106.1	1.9	104.4	2.0	88.5	3.1

CV: Coefficient of variation; OPO: Phosphodiester; OPS: Phosphorothioate; QC: Quality Control.

Table 6. Extraction recoveries from tissue homogenates (average of n = 2) with SPE – Clarity OTX extraction. Extraction recovery was evaluated at concentration of 10 μg/ml or 50 μg/ml. Reference sample consisted of a blank extracted matrix sample spiked post extraction. .

# mers	Linkage	2′ Ribose/ 5′/3′ modification	% Recovery Liver	% Recovery Kidney	% recovery Brain
20	NPS-OPS	2′ F	31	na	na
20	NPS-OPS	2′ F / 3′ GalNac	37	na	na
20	NPS-OPS	2′ MOE	57	na	na
20	NPS-OPS	2′ MOE / 5′ GalNac	35	na	na
20	OPS	2′ Methyl	35	na	na
20	OPS	2′ Methyl / 3′ GalNac	40	na	na
20	OPS	2′ MOE	73	na	na
20	OPS	2′ MOE / 3′ GalNac	36	na	na
20	NPS	2′ MOE	53	95	83
20	OPS	2′ MOE	78	99	90
20	OPS	2′ MOE	88	92	97
20	NPS-OPS	2′ Methyl	97	58	na
20	NPS-OPS	2′ Methyl / 2′ MOE	92	62	na
20	OPS	2′ Methyl / 2′ MOE	90	61	na
24	OPO-OPS	5′ tocopherol	<20	na	na
24	OPO-OPS	5′ cholesterol	<20	na	na
24	OPO-OPS	none	67	na	na
21	OPO-OPS	2′ Methyl / 2′ F/ 5′vinyl	89	na	na
21	OPO-OPS	2′ Methyl / 2′ F	89	na	na
21	OPO-OPS	2′ Methyl / 2′ F	86	na	na
21	OPO-OPS	2′ Methyl / 2′ F	94	na	na
21	OPO-OPS	2′ Methyl / 2′ F	97	na	na

na: Not analyzed; NPS: Phosphorothioamidate; OPS: Phosphorothioate.

same analyte from plasma (Table 3). However, the recovery in the table for the first set of 20 mers did not include a proteinase K treatment at that time. Especially with lipid tail modifications, improving recoveries remains a future objective since no satisfying recoveries were obtained. Of interest is that lipid tail containing OGNs do not extract at all in the phenol/chloroform extraction due to their amphiphilic nature.

For quantitative analysis of OGNs in cerebrospinal fluid or urine, samples were diluted with plasma or BSA solutions and further extracted as plasma samples. Although a few analytes have been successfully quantified in these matrices, our experience is still limited.

Proof of concept of hybridization-based extraction of OGNs from plasma

Hybridization based extraction has not yet been fully explored as an alternative, although many analytical methods (hybridization Elisa, qPCR, LC–fluorescence) rely on this principle for detection of OGNs.

The hybridization extraction strategy is based on annealing of a biotinylated complementary probe (capture probe, CP) with the analyte (OGN) of interest. The results discussed below are obtained for two 16 mer reference

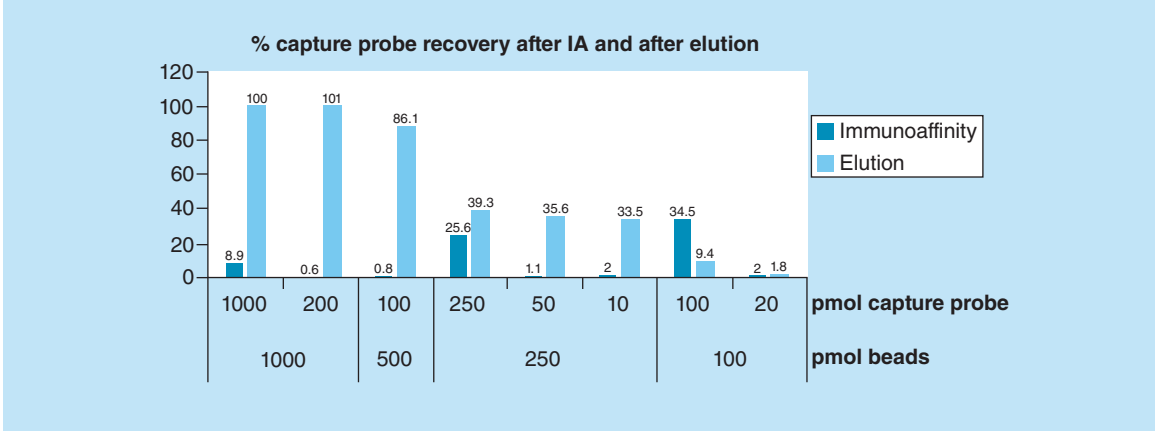

Figure 3. Recovery of the biotinylated capture probe after immunoaffinity step and following the elution step as a function of the capture probe concentration and the bead concentration.

OGNs, one with a full phosphorothioate backbone (see Supplementary Table 1). The procedure starts with binding the biotinylated probe to streptavidine (SA) magnetic beads. Temperature (22 and 37°C) and incubation time (30, 60, 120 min) were varied for both the immunoaffinity and annealing step next to the concentration ratios of streptavidine beads versus capture probe. Also, elution time and temperature were optimized (data not shown). The detailed procedure applied is provided in Table 2.

With the LC–MS method the efficiency of the immunoaffinity reaction (the affinity of the biotinylated capture probe toward streptavidine) could be monitored, and the results are presented in Figure 3. The concentration of the capture probe is measured as well in the supernatant (dark blue bars) after immunoaffinity of streptavidin beads and biotinylated probe (sampled following step 4 in Table 2) as in the elution fraction (blue bars) obtained after denaturation at 80°C (step 17 in Table 2). At equimolar concentration of beads and capture probe, variable amount of capture probe is recovered in the supernatant, ranging from 8.9% (1000 pmol beads and 1000 pmol CP) to 34.5% (100 pmol beads and 100 pmol CP). A fivefold excess of beads results for all concentrations of beads tested in almost complete binding of the CP to the SA beads (<2% remained in the SN with 20 pmol CP and 100 pmol beads). However, upon elution, the CP elutes from the SA beads in a bead concentration dependency (blue bars). As the biotine-streptavidine interaction is described as a very high affinity binding which can withstand harsh conditions, this observation was unexpected. Apparently, the denaturation at 80°C also denatures the biotine-streptavidine complex in a concentration dependent manner. At the highest concentration beads (1000 pmol) almost all CP is recovered in the elution fraction independent of the CP concentration. With 250 pmol beads, around 35% of CP is eluted while with 100 pmol beads <10% is retrieved. The recovery of the CP in the elution shows a relation to the amount of beads applied in the experiment but is independent of the CP and/or UD concentration.

When including the analyte of interest (unchanged drug, UD), similar results were obtained for the CP. In Figure 4, CP recovery in the elution fraction is >95% with 1000 pmol of beads, identical as shown in Figure 3 (blue color bars). With respect to the recovery of the OGN of interest, depending on the probe to analyte ratio, expected recoveries are obtained. In the situation, where twofold excess of UD is presented to the probe, only 50% UD is recovered as expected both with 1000 and 250 pmol of probe (first dark blue bar per section of pmol beads). Equimolar amounts of probe and UD result in >90 or >80% recovery with 1000 and 250 pmol beads. Lower amounts of beads (100 pmol) resulted in lower recoveries and were not further pursued.

To evaluate the hybridization extraction, individual calibration curves for a 16 mer OGN and its OPS analog were spiked to plasma. Concentration range was from 10 to 10000 ng/ml (20 µl plasma, ULOQ was about 40 pmol), 250 pmol SA beads and 50 pmol CP were used in these experiments.

Tables 4 and 5 show results for the calibration curve and quality control samples (% accuracy and % CV) obtained for both analytes. All results were within acceptance criteria (relative error < 20%). In comparison to the SPE method, the phosphorothioate analog showed slightly reduced accuracies with the hybridization extraction, while for the phosphodiester analog similar performance was noticed.

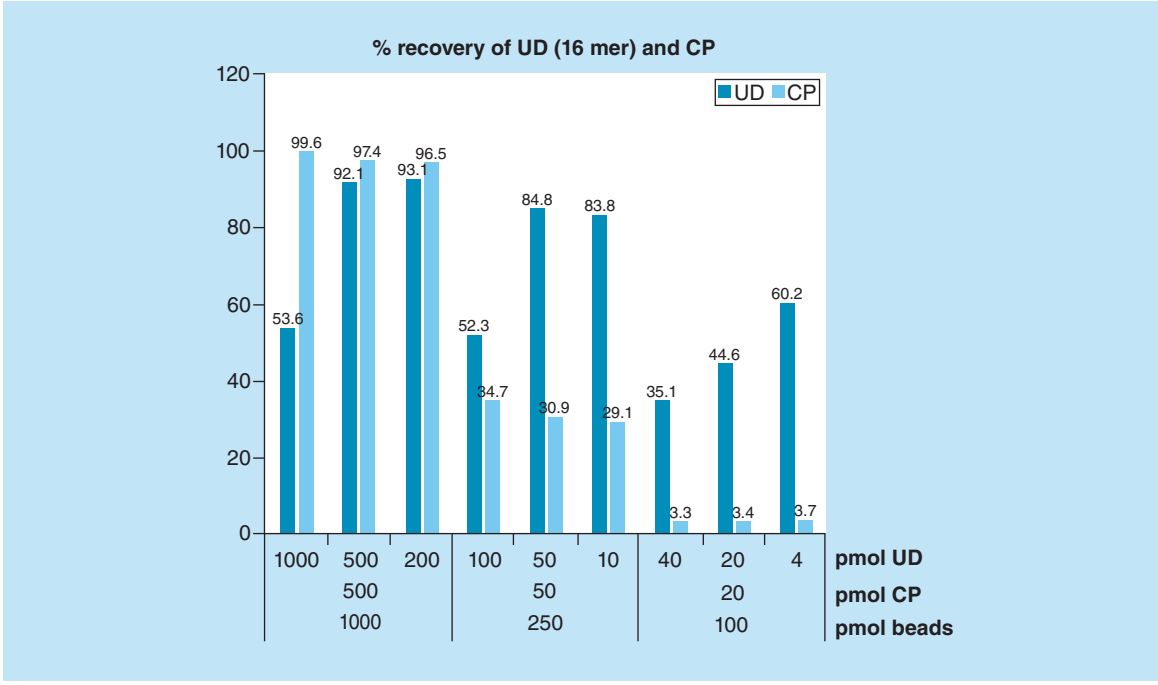

Figure 4. Recovery of both oligonucleotide UD and CP as a function of the concentration of the 3 variables: concentration of UD, CP and beads.
CP: Capture probe; UD: Unchanged drug.

With the two tool compounds no incurred samples were generated. But, for the 13-mer palmitoyl conjugated OGN imetelstat, both preclinical and clinical samples were analyzed with LC–MS/MS following AEX-SPE and hybridization extraction protocols as proposed in this manuscript and superimposable data were obtained [31].

During the preparation of this manuscript, Basiri *et al.* [32] published a method for the quantification of miR-451 in plasma exploiting a similar approach. A denaturing and annealing step is included in their protocol for ds RNA while we have only worked with ASO (single stranded) molecules. As miRs are nonmodified RNA molecules they had to overcome several challenges, but even with optimization using ssDNA, recovery was initially low which was attributed to protein binding. A proteinase K digestion step was included to degrade proteins and to inhibit RNAses. We have not experienced these challenges to the same extent for the two reference ASO compounds. Nevertheless, including a proteinase K treatment might be a preferred option assuming no influence on the biotine streptavidine binding. An interesting strategy proposed in this manuscript [32] is the use of the probe as IS. Our observations of the elution of the probe are confirmed by their results and our data substantiate that recovery of the probe is constant but depending on the amount of beads applied and therefore can be applied as IS, which would bring a major benefit for the method. In our current workflow, the ISs (if used) can only be added post extraction since a non-related sequence cannot hybridize with the capture probe, while on the other hand a similar sequence would compete during the hybridization.

We have investigated the affinity of the capture probe for truncated OGNs (shortmers) as well as for the phosphorothioate analog. In Table 7, truncated OGNs of the 16-mer with one and 3-nucleotide truncations at the 3′ end, were evaluated in different concentration ratios versus the unchanged drug. Results show that the shortmer with one nucleotide missing at the 3′ end has similar affinity toward the CP. However, a three-nucleotides deletion at the 3′ end reduces affinity, especially in competition with the full-length OGN. A ninefold excess of UD versus the n-3 shortmer resulted in reduced recovery for the shortmer (64%). These observations are in line with results obtained in a hybridization-ligation ELISA where n-3 mers showed a substantial decrease in the response [33]. For the phosphorothioate OGN, without any competition of the phosphodiester analog, only 78% recovery was obtained in the experimental setup studied. With a ninefold excess of phosphodiester only 16% of the phosphorothioate was retained. Although hybridization occurs at the level of the nucleobases, the impact of different modifications on the affinity for the probe is not fully studied and/or understood.

Table 7. Selectivity and affinity of the capture probe for truncated oligonucleotide and for phosphorothioate analogs. The capture probe was complementary to the 16 mer DNA oligonucleotide. The hybridization extraction was performed with 250 pmol beads and 50 pmol capture probe.

truncated OPO or 16 mer OPS	OPO 16 mer (pmol)	OPO/S X-mer (pmol)	% recovery OPO	% recovery truncated OPO or OPS
n-1 3′	40	0	113	1
n-1 3′	0	40	1	102
n-1 3′	20	20	120	98
n-1 3′	10	10	116	104
n-1 3′	4	36	113	97
n-1 3′	36	4	111	95
n-3 3′	40	0	116	0
n-3 3′	0	40	0	90
n-3 3′	20	20	127	85
n-3 3′	4	36	105	85
n-3 3′	36	4	114	64
OPS	40	0	108	0
OPS	0	40	0	78
OPS	20	20	149	45
OPS	4	36	153	80
OPS	36	4	113	16

OPO: Phosphodiester; OPS: Phosphorothioate.

The probes used in this proof of concept experiment were DNA strands complementary to the 16mer OGNs. Peptide nucleic acid (PNA) strands have been described with improved hybridization capabilities [34]. In a diagnostic study on fungal infections, differentiation of *Candida albicans* from other Candida species was evaluated with fluorescence *in situ* hybridization [35]. PNA probes outperformed their DNA counterparts even with shorter designed PNA probes. Future endeavors with biotinylated PNA probes can explore whether also improved extraction recoveries and sample clean up can be obtained with biotinylated PNA probes. On the other hand, elution might be more difficult if Tm of the PNA–DNA or PNA–RNA complex is high.

Conclusion

In this study, two extraction strategies have been proposed for quantification of OGNs from biological matrices. The AEX-SPE method is presented as the more generic and preferred approach in early development when a variety of OGNs with different sequences and modifications are evaluated. Although proposed as a generic protocol, structure and matrix dependent protocol adaptations may be warranted. The relatively novel hybridization extraction requires the design and synthesis of a complementary biotinylated capture probe, which can delay the start of method development. The capture probe is critical and impacts whether truncated metabolites are included in the extract. Variations in the modifications but with a fixed nucleotide sequence allows the use of an identical capture probe. Therefore, this strategy is proposed at later stage when only one or a few OGNs are evaluated. Overall, both extraction methods have proven to result in accuracy and precision data that comply with current bioanalytical standards.

Future perspective

Many OGN therapeutics are currently in development for treatment of a variety of diseases. Exposure evaluation after *in vivo* administration in animals and man is required for decision making. Although different assays are implemented for OGN quantitation, LC–MS based methods gain in popularity. One critical aspect is related to extraction of OGNs from biological matrices. In this manuscript an AEX-SPE extraction was presented that can be applied with analyte dependent optimization for different classes of OGNs (ASO, siRNAs, different modifications/conjugations). As an alternative extraction, a proof-of-concept for hybridization-based pull-down was presented. This approach provides many opportunities: it is more specific and results in improved clean-up but specific reagents (a complementary capture probe) are required. Currently limited experience is described

in literature and further scientific understanding of the processes and behaviors of analyte and capture probe during extraction is needed. Affinity of the capture probes toward shortmers and/or backbone/ribose modified OGNs, mismatch toleration and the use of PNA probes need further investigation. There is the expectation that hybridization extraction can provide an alternative in case limited extraction recovery is obtained with any of the current extraction methods (e.g., lipid-modified OGNs in tissues).

Summary points

AEX-SPE extraction

- AEX-SPE extraction of oligonucleotides (OGN; antisense oligonucleotides and siRNA) can be implemented as generic extraction approach for sample preparation for LC–MS quantification in different matrices.

Hybridization extraction

- Hybridization extraction is a promising strategy with the potential of improved clean-up but specific capture probe reagents need to be synthesized.
- In the elution phase of the hybridization extraction the capture probe elutes as well and is dependent on the bead concentrations applied.
- Affinity of the capture probe for a phosphorothioate analog and for shortmers with three nucleotide truncations was reduced compared with the phosphodiester OGN.

Conclusion

- Both extraction approaches provided accuracy and precision results in line with current bioanalytical requirements (<20% RE and CV) for large molecules.

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References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1 Crooke ST, Vickers T, Lima W, Wu H. Mechanisms of anti-sense drug action, an introduction. In: *Antisense Drug Technology: Principles, Strategies and Applications.* Crooke ST (Ed.). CRC Press, FL, USA, 3–46 (2008).

2 Sharma VK, Sharma RK, Singh SK. Antisense oligonucleotides: modifications and clinical trials. *Med. Chem. Commun.* 5(10), 1454–1471 (2014).

3 Sharma VK, Watts JK. Oligonucleotide therapeutics: chemistry, delivery and clinical progress. *Future Med. Chem.* 7(16), 2221–2242 (2015).

4 Mello CC, Conte D. Revealing the world of RNA interference. *Nature* 431, 338–342 (2004).

5 Andersson S, Antonsson M, Elebring M, Jansson-Löfmark R, Weidolf L. Drug metabolism and pharmacokinetic strategies for oligonucleotide- and mRNA-based drug development. *Drug Discov. Today* 2252, 1–13 (2018).

•• **Provides an overview of the current DMPK aspects for oligonucleotide therapeutics.**

6 Bennett F, Swayze E. RNA targeting therapeutics: molecular mechanism of antisnese oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* 50, 259–293 (2010).

7 Crooke S, Witztum J, Bennett F, Baker B. RNA-targeted therapeutics. *Cell Metabol.* 27, 714–739 (2018).

•• **perspective on RNA therapeutics.**

8 Stein AC, Castabotto D. FDA-approved oligonucleotide therapeutics in 2017. *Mol. Ther.* 25(5), 1069–1075 (2017).

9 Hua Y, Sahashi K, Hung G *et al.* Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev.* 24, 1634–1644 (2010).

10 Lim KR, Maruyama R, Yokota T. Eteplirsen in the treatment of Duchenne muscular dystrophy. *Drug Des. Devel. Ther.* 11, 533–545 (2017).

11 http://investors.alnylam.com/news-releases/news-release-details/alnylam-announces-first-ever-fda-approval-rnai-therapeutic.

12 https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=reportsSearch.process.

13 Welink J, Xu Y, Yang E *et al.* 2018 White Paper on Recent Issues in Bioanalysis: 'A global bioanalytical community perspective on last decade of incurred samples reanalysis (ISR)' (Part 1 – small molecule regulated bioanalysis, small molecule biomarkers, peptides & oligonucleotide bioanalysis). *Bioanalysis* 10(22), 1781–1801 (2018).

14 Yu RZ, Baker B, Chappell A, Greary RS, Cheung E, Levin AA. Development of an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligonucleotide in plasma. *Anal. Biochem.* 304, 19–25 (2002).

15 Tian Q, Rogness J, Meng M, Li Z. Quantitative determination of a siRNA (AD00370) in rat plasma using peptide nucleic acid probe and HPLC with fluorescence detection. *Bioanalysis* 9(11), 861–872 (2017).

16 Hemsley M, Ewles M, Goodwin L. Development of a bioanalytical method for quantification of a 15-mer oligonucleotide at sub-ng/ml concentrations using LC–MS/MS. *Bioanalysis* 4(12), 1457–1469 (2012).

17 Ewles M, Goodwin L, Schneider A, Rothhammer-Hampl T. Quantification of oligonucleotides by LC–MS/MS: the challenges of quantifying a phosphorothioate oligonucleotide and multiple metabolites. *Bioanalysis* 6(4), 447–464 (2014).

18 Studzinska S, Buszewski B. Analysis of microRNA and modified oligonucleotides with the use of ultra high performance liquid chromatography couples with mass spectrometry. *J. Chromatogr. A* 1554, 71–80 (2018).

19 Studzinska S. Review on investigations of antisense oligonucleotides with the use of mass spectrometry. *Talanta* 176, 329–343 (2018).

20 Cook K, Thayer J. Advantages of ion-exchange chromatography for oligonucleotide analysis. *Bioanalysis* 3(10), 1109–1120 (2011).

21 Chen B, Mason S, Bartlett M. The effect of organic modifiers on electrospray ionization charge-state distribution and desorption efficiency for oligonucleotides. *J. Am. Soc. Mass Spectrom.* 24(2), 257–264 (2013).

22 Chen B, Bartlett M. Evaluation of mobile phase composition for enhancing sensitivity of targeted quantification of oligonucleotides using ultra-high-performance liquid chromatography and mass spectrometry: application to phosphorothioate deoxyribonucleic acid. *J. Chromatogr. A* 1288, 73–81 (2013).

23 McGinnis A, Grubb E, Bartlett M. Systematic optimization of ion-pairing agents and hexafluoroisopropanol for enhanced electrospray ionization mass spectrometry of oligonucleotides. *Rapid Commun. Mass Spectrom.* 27(23), 2655–2664 (2013).

24 Weng G, Liu Z, Chen J, Wang F, Pan Y, Zhang Y. Enhancing the mass spectrometry sensitivity for oligonucleotide detection by organic vapor assisted electrospray. *Anal. Chem.* 89, 10256–10263 (2017).

25 Nuckowski L, Kaczmarkiewicz A, Studzinska S. Review on sample preparation for oligonucleotides analysis by liquid chromatography. *J. Chromatogr. B* 1090, 90–100 (2018).

26 Li N, El Zahar NM, Saad JG, van der Hage ER, Bartlett MG. Alkylamine ion-pairing reagents and the chromatographic separation of oligonucleotides. *J. Chromatogr. A* 1580, 110–119 (2018).

• **Explains role and impact of ion pairing in chromatography.**

27 Basiri B, van Hattum H, van Dongen WD, Murph MM, Bartlett MG. The role of fluorinated alcohols as mobile phase modifiers for LC–MS analysis of oligonucleotides. *J. Am. Soc. Mass Spectrom.* 28(1), 190–199 (2017).

28 Basiri B, Murph MM, Bartlett MG. Assessing the interplay between the physicochemical parameters of ion-pairing reagents and the analyte sequence on the electrospray desorption process for oligonucleotides. *J. Am. Soc. Mass Spectrom.* 28, 1647–1656 (2017).

•• **Explains role and impact of ion pairing on the MS response.**

29 Nuckowski L, Kaczmarkiewicz A, Studzinska S. Development of a SPE method for the extraction of phosphorothioate oligonucleotides from serum samples. *Bioanalysis* 10(20), 1667–1677 (2018).

30 Chen B, Bartlett M. A one-step solid phase extraction method for bioanalysis of a phosphorothioate oligonucleotide and its 3′ n-1 metabolite from rat plasma by uHPLC–MS/MS. *AAPS J.* 14(4), 772–780 (2012).

31 Dillen L, Sips L, Greway T, Verhaeghe T. Quantitative analysis of imetelstat in plasma with LC–MS/MS using solid-phase extraction or hybridization extraction. *Bioanalysis* 9(23), 1859–1872 (2017).

32 Basiri B, Sutton MJ, Hooshfar S, Byrnes CC, Murph M, Bartlett MG. Direct identification of microribonucleic acid miR-451 from plasma using liquid chromatography mass spectrometry. *J. Chromatogr. A* 1584, 97–105 (2019).

33 Thayer MB, Lade JM, Doherthy D *et al.* Application of locked nucleid acid oligonucleotides for siRNA preclinical bioanalytics. *Nature Sci. Rep.* 9, 3566 (2019).

34 Jensen K, Orum H, Nielsen PE, Norden B. Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studies with the BIAcore Technique. *Biochemistry* 36, 5072–5077 (1997).

35 Kim HJ, Brehm-Stecher BF. Design and evaluation of peptide nucleic acid probes for specific identification of *Candida albicans*. *J. Clin.l Microbiol.* 53(2), 511–521 (2015).

36 Kaczmarkiewicz A, Nuckowski L, Studzinska S. Analysis of the first and second generation of antisense oligonucleotides in serum samples with the use of ultra-high-performance liquid chromatography with tandem mass spectrometry. *Talanta* 196, 54–63 (2019).

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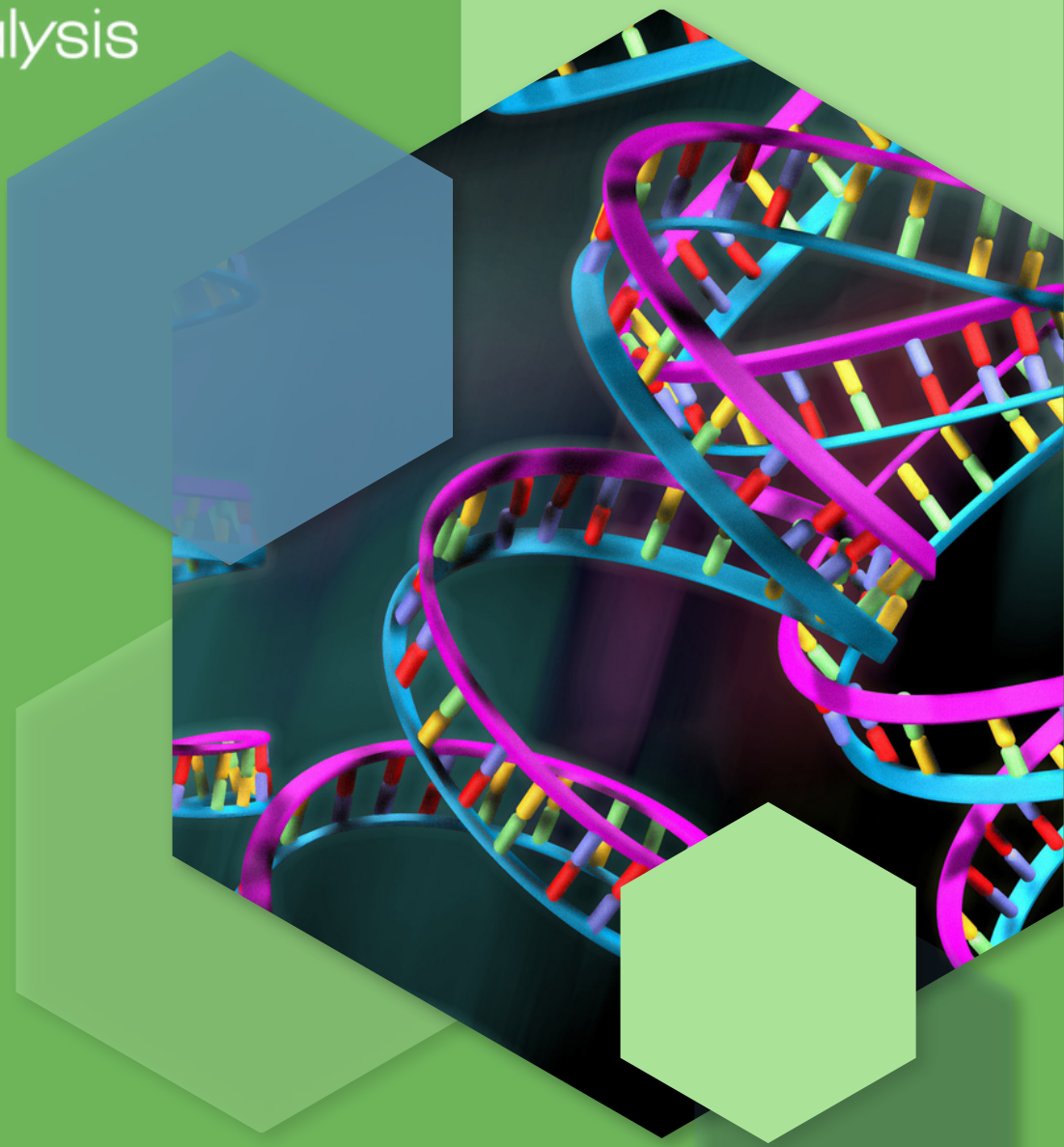

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