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Bioanalytical strategies for oligonucleotide assessment

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Contents

Foreword

Over the past 30 years, oligonucleotide research has become increasingly important as an avenue for new and innovative therapeutics. With this special eBook, we are excited to bring you an exploration of the current landscape of oligonucleotide research and where it stands regarding validation and regulatory challenges and future directions for the field.

Oligonucleotides consist of short, single- (sometimes double-) stranded sequences, or oligomers, of synthetic DNA or RNA. There are various types of oligonucleotide therapeutic modalities, including antisense oligonucleotides, siRNA, miRNA, splice switching oligonucleotides and aptamer RNAs. These mechanisms of therapy have been applied by researchers in various fields ranging from oncology to neurodegenerative conditions and infectious diseases. Oligonucleotides offer a promising new modality of disease targeting that were once thought to be 'undruggable' via small molecule drugs or monoclonal antibodies. While interest in oligonucleotide therapeutics has increased, their analysis, validation and regulation must also be considered. The appropriate bioanalytical strategies must be implemented to assess PK profiling, drug metabolism, assessment of compound distribution in the anatomy, immunogenicity and biomarkers. With a diverse scope of mechanisms of action and targeting approaches comes the need for an equally diverse range of bioanalytical approaches when considering oligonucleotide therapeutic development.

Through interviews, commentaries, journal articles and more, we hope to highlight this range of bioanalytical approaches and strategies in oligonucleotide analysis in this special eBook as well as shed some knowledge on the future of the field of oligonucleotide therapeutics.

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Review

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Bioanalysis

The role of ligand-binding assay and LC–MS in the bioanalysis of complex protein and oligonucleotide therapeutics

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Ligand-binding assay (LBA) and LC–MS have been the preferred bioanalytical techniques for the quantitation and biotransformation assessment of various therapeutic modalities. This review provides an overview of the applications of LBA, LC–MS/MS and LC–HRMS for the bioanalysis of complex protein therapeutics including antibody–drug conjugates, fusion proteins and PEGylated proteins as well as oligonucleotide therapeutics. The strengths and limitations of LBA and LC–MS, along with some guidelines on the choice of appropriate bioanalytical technique(s) for the bioanalysis of these therapeutic modalities are presented. With the discovery of novel and more complex therapeutic modalities, there is an increased need for the biopharmaceutical industry to develop a comprehensive bioanalytical strategy integrating both LBA and LC–MS.

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Drug discovery and development has significantly changed over the last 25 years as the field has expanded beyond small-molecule drugs to novel modalities such as protein and oligonucleotide therapeutics to address previously 'undruggable' targets or disease conditions [1]. Protein therapeutics are primarily produced from living cells using recombinant DNA technologies. Examples of protein-based therapeutics include anticoagulants, clotting factors, enzymes, fusion proteins, growth factors, hormones, monoclonal antibodies (mAbs) and related formats [2,3]. These protein-based therapeutics can be genetically engineered or chemically modified for improving their therapeutic profile or targeted delivery such as in PEGylated therapeutic proteins, Fc fusion proteins, antibody–drug conjugates (ADCs), bispecific antibodies etc. [4]. Oligonucleotide therapeutics are an emerging class of therapeutic modality with six US FDA-approved drugs in the last 5 years. The major classes of therapeutic oligonucleotides are antisense oligonucleotides (ASOs), siRNA, miRNAs and aptamers [5].

As the biopharmaceutical industry discovers and develops these novel and complex modalities, it is critical to develop bioanalytical methods for their quantification and biotransformation assessment. Traditionally, ligand-binding assay (LBA) is the gold standard for quantitation of large molecules from biological matrices, while LC–MS is the preferred technique for small-molecule quantitation [6]. However, recent advances in the MS instrumentation as well as sample preparation techniques such as affinity enrichment has propelled LC–MS as a complementary technology and in some cases the only alternative for the bioanalysis of protein and oligonucleotide therapeutics [3,6–9].

Protein therapeutics can undergo a wide range of biotransformations in systemic circulation including deamidation, isomerization, oxidation and proteolytic cleavage leading to truncated forms [7]. As more complex protein therapeutic modalities are developed, there are additional challenges in biotransformation assessment. For instance, ADCs can undergo deconjugation of payload-linker or payload, linker cleavage and metabolism of conjugated payload [10,11]. Overall, the biotransformation of protein therapeutics can lead to reduced or complete loss of

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activity and in some instances toxicity as well. Oligonucleotide therapeutics can undergo cleavage of phosphodiester or phosphorothioate bonds leading to shorter chain/truncated metabolites [12]. Also, the next generation of oligonucleotide therapeutics being developed such as oligonucleotides with chemically modified backbones or conjugated oligonucleotides [12] presents unique challenges due to the possibility of formation of novel metabolites. It is important to identify and characterize the biotransformation of protein and oligonucleotide therapeutics during drug discovery and development. This ensures best and improved forms of drugs are available to the patients now and in the future.

LBA and LC–MS are the preferred techniques for the bioanalysis of protein and oligonucleotide therapeutics. LBA is based on the principle of specific interaction of the capture and detection reagents (typically antibodies, antigens or hybridized oligonucleotide probes) with the analyte [13,14]. ELISA is the most commonly used LBA format. The analyte is typically immobilized either directly on a plate or indirectly bound to an immobilized capture reagent and detected by a detection reagent conjugated with a moiety that generates chromogenic or fluorescent readout. Other platforms such as MSD (multiarray technology with electrochemiluminescence detection), and Gyrolab (high-throughput automated microfluidic system with fluorescence detection) are routinely being used for bioanalysis because of their multiplexing and high-throughput capabilities. Additionally, platforms such as Quanterix Simoa, Singulex Erenna and Imperacer Immuno-PCR offer ultra sensitivity at picogram to femtogram per milliliter levels [15].

LC–MS analysis involves liquid chromatographic (LC) separation in combination with MS for detection of extracted analytes [16]. LC–MS quantitation of biotherapeutics typically involves digestion of the sample containing the therapeutic protein into peptides either directly, for example, pellet digestion or after extraction from biological matrix using solid-phase extraction (SPE) or affinity enrichment [6]. These peptides are then analyzed using LC–MS/MS in selective reaction mode with a unique precursor and product ion combination for each analyte, using a tandem MS instrument like a triple quadrupole [8]. More recently, quantitation of proteins at subunit or intact level using TOF or Orbitrap-based instrumentation has gained increased focus [8]. One of the advantages in LC–MS analysis is the usage of an internal standard such as stable isotope-labeled peptide or full-length biologic for accurate quantification. Another major advantage of LC–MS is its ability to identify *in vivo* biotransformation and catabolites of biotherapeutics [7].

The current review provides a comprehensive overview on the applications of LBA and LC–MS for bioanalysis of complex protein (ADC, fusion protein and PEGylated proteins) and oligonucleotide therapeutics. The goal of the review is to provide examples in this area, but not intended to be an exhaustive literature search. Furthermore, the strengths and limitations of LBA and LC–MS for the bioanalysis of each of these therapeutic modalities are discussed with specific examples from the literature. Additionally, some general guidelines and our perspective on how to choose appropriate bioanalytical technique(s) to support the discovery and development of various biotherapeutics are also provided. In summary, our hope is that this review will provide valuable insights to bioanalytical scientists on the design and development of integrated bioanalytical strategies involving LBA and LC–MS for the quantitation and biotransformation assessment of complex protein and oligonucleotide therapeutics including new therapeutic modalities.

Antibody–drug conjugates

ADCs are complex biotherapeutics designed to deliver the cytotoxic payload specifically to tumor. Currently, there are nine ADCs approved by the FDA and more than 150 ADCs in various stages of development [17]. There have been several advancements in the optimization of conjugation modes and linker chemistries over the last decade with the goal of improving the therapeutic index [17]. The payload can be conjugated to surface accessible lysine residues, hinge-cysteines or site-specifically using cysteine engineering, chemoenzymatic conjugation, unnatural amino acid incorporation etc. [18]. While bioanalysis of an mAb is relatively straightforward and typically involves the determination of its concentration over time in plasma or serum followed by calculation of pharmacokinetic (PK) parameters, the bioanalysis of ADC is complex as there are multiple species formed *in vivo*. The main species monitored include: total antibody (Ab with or without payload), conjugated antibody or total ADC (mAb with atleast one payload), conjugated payload (payload conjugated to mAb) and deconjugated payload (payload released from ADC *in vivo*) [10]. Furthermore, understanding the catabolism and *in vivo* biotransformation assessment of ADC is also critical.

Quantitation by LBA

LBA is the preferred technology for total Ab and total ADC measurements because of its good sensitivity and high sample throughput. For total Ab, the ADC is captured using an antigen, an anti-idiotypic antibody or a generic antihuman IgG reagent and detected by another antibody that binds to the antibody component of the ADC. For total ADC (conjugated antibody), the ADC is captured using anti-idiotypic antibody or a generic antibody against the mAb, while the detection is achieved with an antibody against the payload or *vice versa* [10,11]. Phillips *et al.* and Dere *et al.* developed ELISA methods for the quantification of total antibody and total ADC species of Trastuzumab-Mertansine (DM1) ADC dosed in preclinical and clinical studies [19,20]. In the case of an ADC where the payload undergoes *in vivo* biotransformation to an inactive metabolite, the PK profile of active ADC can also be determined by LBA with the availability of specific reagent that binds to the active payload but does not bind to inactive species. Myler *et al.* developed and validated a semiautomated method using microfluidic Gyrolab platform for the quantitation of multiple analytes: Total Ab, Total ADC and Active ADC using just 20 µl of sample [21]. A representative example of the common LBA assay formats for ADC quantitation is depicted in Figure 1A–B.

Quantitation by LC–MS

Although not routine, hybrid LC–MS/MS can also be used for total Ab and total ADC quantitation, especially during early discovery when appropriate LBA reagents are not available. For total Ab quantitation, the ADCs are first extracted from biological matrix by affinity capture with an anti-idiotypic antibody or generic capture reagents (such as Protein A, Protein G or antihuman IgG) against the mAb component of ADC. The affinity enriched ADCs are then digested with trypsin or other proteases into surrogate/signature peptides that are finally quantified by LC–MS/MS (Figure 1C) [22]. Stable isotope-labeled signature peptides are typically added during the trypsin digestion step for accurate quantification. For total ADC quantitation, ADC species with conjugated payload are specifically isolated by affinity capture with an anti-idiotypic antibody against the payload, followed by digestion to signature peptides that are quantified by LC–MS/MS (Figure 1C). Determination of conjugated payload is typically achieved by affinity capture of ADC, followed by cleavage of payload using proteases such as Cathepsin B and Papain (for protease cleavable linkers) or reduction with DTT or TCEP (for disulfide linkers) and LC–MS/MS multiple reaction monitoring (MRM) analysis of payload (Figure 1C) [23–25].

In case of ADCs with a noncleavable payload-linker, the conjugated payload is quantified by affinity capture of ADC from biological samples, followed by digestion of ADC by proteases such as trypsin, Lys-C etc. into peptide-linker-payload moiety, and LC–MS/MS analysis [23]. Hyung *et al.* determined the *in vivo* concentrations of conjugated payload of THIMOAB drug conjugate (TDC with Monomethyl Auristatin E, THIOMAB-vc-PAB-MMAE) using this approach. They further compared this method with the previous methodology involving papain-mediated release of MMAE from affinity captured TDC and observed a good agreement between the two methods [26]. Shi *et al.* also successfully utilized the methodology for quantification of conjugated payload of a centyrin-drug conjugate (CDC, centyrin conjugated with DM1 via a triglycine containing noncleavable peptide linker) [27]. The quantitation of deconjugated payload in systemic circulation is important to understand the toxicity, and is typically achieved by protein precipitation or SPE of analyte from biological matrix followed by LC–MS/MS analysis (Figure 1C) [23].

The various LC–MS/MS-based workflows for ADC quantitation are shown in Figure 1C. These workflows were applied to an ADC with a microtubule inhibitor conjugated via random lysine conjugation [22], MEDI4276 (aHER-2 mAb conjugated with a tubulysin analog) [28] and MEDI3726 (antiProstate-specific membrane antigen [aPSMA] mAb conjugated with a pyrrolobenzodiazepine [PBD]) [29] in preclinical and clinical studies. Jin *et al.* developed a hybrid LC–HRMS method for quantitation of an intact lysine-linked ADC, trastuzumab emtansine from rat plasma without the need to generate tryptic peptides [30]. However, very few studies have employed LC–HRMS for quantification of ADCs given the heterogeneity and complexity of this modality.

Comparison of LBA & LC–MS/MS assays for quantitation

Wang *et al.* conducted a comprehensive bioanalytical study comparing the LBA and hybrid LC–MS/MS-based quantitation of a proprietary ADC (microtubule inhibitor conjugated to an antihuman IgG mAb via lysine conjugation) dosed in rats [22]. For LBA, the assay formats were: Total Ab (capture with anti-id against the mAb, detect with antihuman IgG Fc), Conjugated Ab (payload + metabolite)/Total ADC (capture with anti-id against the mAb, detect with anti-id against both active and inactive payload) and Conjugated Ab/Active ADC (capture with anti-id against the mAb, detect with anti-id against only the active payload) [22]. For LC–MS/MS-based

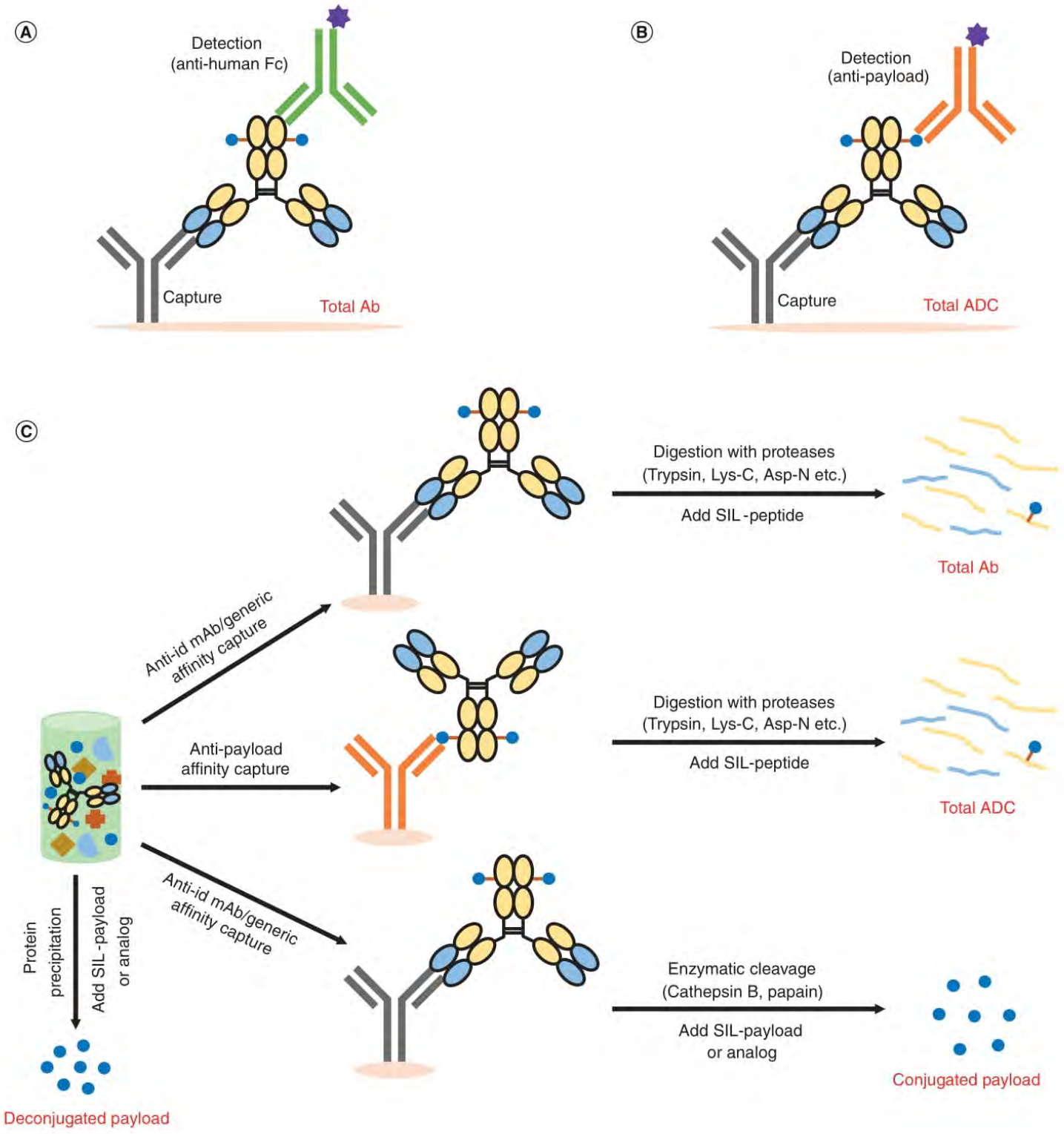

Figure 1. Summary of ligand-binding assay and LC–MS/MS workflows for antibody–drug conjugate quantitation. (A) Ligand-binding assay formats for quantitation of total Ab, **(B)** total ADC and **(C)** LC–MS/MS workflows for the quantitation of total Ab, total ADC, conjugated and deconjugated payload.
Ab: Antibody; ADC: Antibody–drug conjugate; SIL: Stable isotope labeled.

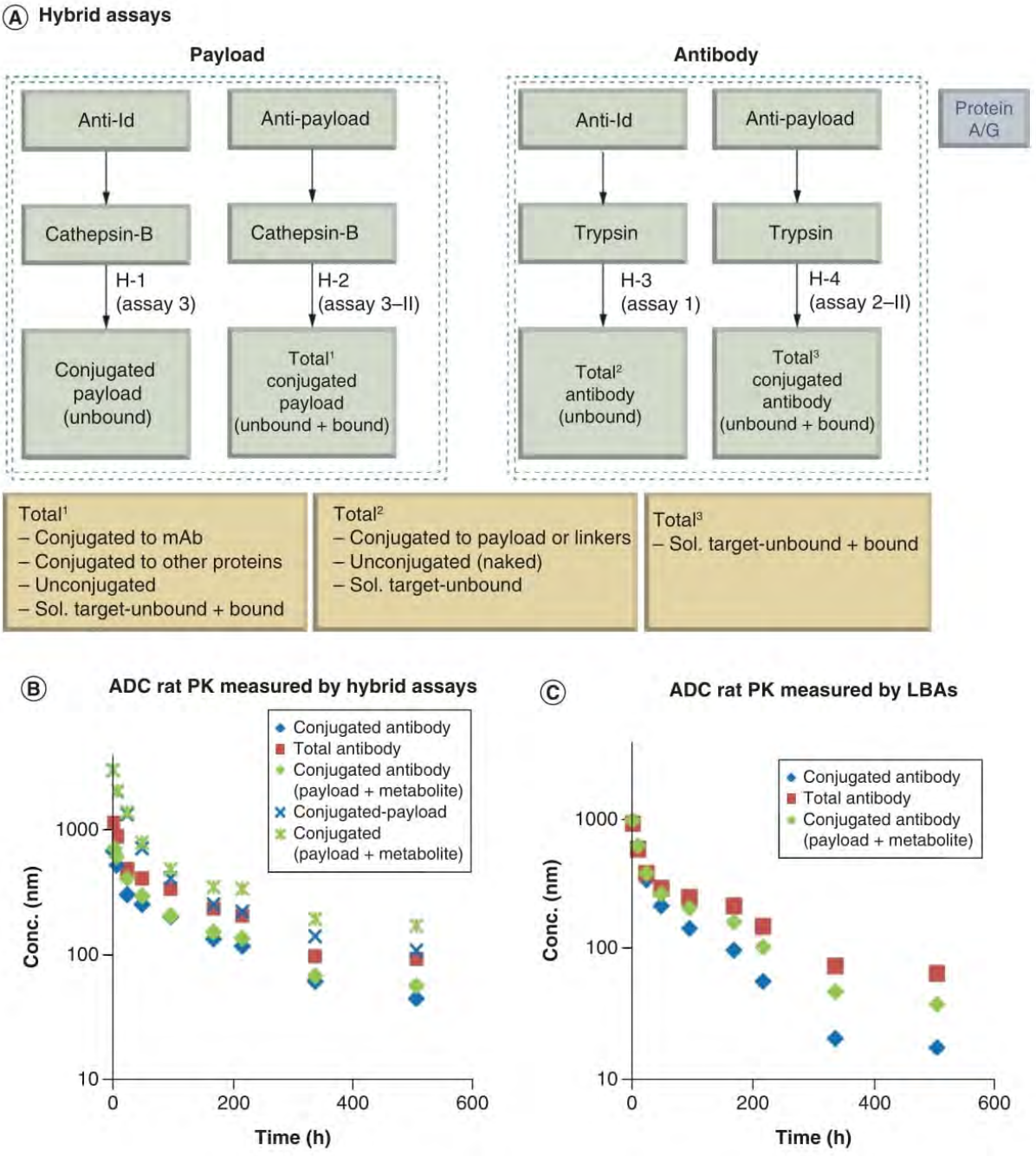

Figure 2. Comparison of ligand-binding assay and hybrid LC–MS/MS for the quantitation of a proprietary antibody–drug conjugate. (A) Hybrid LC–MS/MS workflows for quantitation of various ADC analytes, **(B)** Hybrid LC–MS/MS and **(C)** LBA-based quantitation of concentrations of various ADC analytes in rat.
ADC: Antibody–drug conjugate; LBA: Ligand-binding assay.
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quantitation, the assay workflows are shown in Figure 2A. The PK profiles obtained by both LBA and LC–MS/MS assays were well correlated for the initial time points (Figure 2B–C). However, at the later time points (>200 h), the conjugated Ab/active ADC levels determined by hybrid LC–MS/MS were two- to threefold higher than LBA (Figure 2B–C). This was attributed to the fact that the current LBA assay format underestimated the conjugated Ab levels due to decreased drug to antibody ratio (DAR) over time [22]. This example highlights the importance of understanding the capabilities and limitations of both LBA and LC–MS/MS-based formats and how they are effected by *in vivo* biotransformation. Most importantly, this also highlights the need of confirming PK data by both LBA and LC–MS, especially for complex therapeutics such as ADCs.

Biotransformation assessment

ADCs can undergo *in vivo* biotransformation such as deconjugation of payload-linker, linker cleavage resulting in payload loss, and payload metabolism which can result in changes in the DAR distribution and impact the efficacy and toxicity [10,11]. Hence, determination of these *in vivo* biotransformations is critical and is typically achieved by LC–HRMS analysis. LC–HRMS is the preferred technique for the biotransformation assessment of biotherapeutics because of its ability to resolve the various charge states of proteins with high accuracy, thereby enabling the determination of exact mass of protein after deconvolution. The ADC is captured from biological samples using affinity enrichment and analyzed as an intact ADC (with or without removal of N-Glycan) or broken down into subunit/fragments by digestion with enzymes such as IdeS and/or interchain disulfide reduction. Xu *et al.* used affinity capture followed by top-down LC–HRMS analysis of intact ADC for biotransformation assessment of a cysteine-engineered anti-MUC16 THIOMAB ADC [31]. He *et al.* further developed an affinity capture top-down LC–HRMS method using a high-resolution Orbitrap MS instrumentation for monitoring *in vivo* DAR distribution changes as well as identification of low-mass change catabolites such as deacetylation for site-specific and lysine-conjugated ADCs [32,33]. Su *et al.* developed an affinity capture 'on-bead' IdeS digestion LC–HRMS assay for Fab-conjugated site-specific ADCs. The reduced size of analyte from ~150 to ~100 kDa $F(ab')_2$ fragment resulted in enhanced sensitivity and resolution compared with the intact ADC analysis [34]. Jashnani *et al.* developed a faster and automated affinity capture top-down LC–HRMS assay for evaluating the *in vivo* DAR distribution changes of HC-Fab and HC-Fc conjugated ADCs by affinity capture with a generic capture reagent on a cartridge-based platform and 'on-cartridge' enzymatic digestion with IdeS and PNGase F, respectively [35]. Several methods involving a combination of affinity capture, enzymatic digestion and interchain disulfide reduction to generate approximately 25–50 kDa subunits were developed for the assessment of biotransformation of ADCs [36–38]. While these methods do not provide DAR distribution data, they provide catabolism and average DAR information. More recently, Kotapati *et al.* reported a universal affinity capture subunit LC–MS assay for the investigation of biotransformation of any site-specific ADC independent of antibody type, conjugation chemistry/technologies, conjugation site and payload class [39]. This universal bioanalytical methodology is shown in Figure 3A. A representative example of application of this methodology for *in vivo* biotransformation assessment of ADC conjugated with tubulysin payload on the HC-Fc is shown in Figure 3B–C. LC–HRMS analysis revealed that the payload is not completely cleaved from the ADC *in vivo* and is only partially metabolized (deacetylation, -42 Da) to form a new catabolite (HCQTag Fc/2 + DP), and the relative percentage of this catabolite increased over time compared with the parent species (HCQTag Fc/2 + AP) [39].

Fusion proteins

Small therapeutic proteins such as cytokines, enzymes, growth factors and hormones have short half-lives due to their metabolism (proteolytic cleavage) or faster renal clearance [40,41]. Various strategies have been developed to improve the PK profile of these small therapeutic proteins. The first strategy involves reduction of renal clearance by increasing the hydrodynamic volume. This is achieved by conjugating therapeutic proteins synthetically with PEG or carbohydrates (glycosylation, polysialylation etc.) or recombinant fusion with polypeptide repeats (XTEN, elastin like polypeptides, homo-aminoacid polymers etc.). The second strategy involves linking the therapeutic protein with another half-life extending protein such as albumin, transferrin, Fc, antibodies etc. Examples of Fc fusions currently approved include alefacept, belatacept, dulaglutide, etanercept and romiplostim [40,41]. Since the therapeutic fusion proteins have atleast two different components (protein–protein, protein–polymer, protein–peptide, protein–Fc, protein–mAb etc.), bioanalysis of these proteins is complex and a comprehensive strategy that takes into consideration the different protein components as well as the synthetic/peptide linker is desirable.

Quantitation by LBA

ELISA is routinely used for the quantification of fusion proteins. The simplistic assay format involves usage of capture and detect reagents that bind specifically to the therapeutic protein component of the fusion protein. For instance, Kim *et al.* used commercial ELISA kit for the quantitation of rhGH in plasma of rats dosed with rhGH-Fc fusion [42]. However, only the total therapeutic protein can be quantified by this approach as it does not differentiate between cleaved and intact forms. The quantification of intact fusion protein can be accomplished with the usage of capture and detect reagents binding to the two different protein components of the fusion protein. For example, Liu *et al.* employed semiautomated gyrolab platform for quantitation of TNFR2-Fc fusion protein using biotinylated anti-TNFR2 mouse mAb as capture and antihuman Fc rabbit mAb as detection reagent [43]. It has been

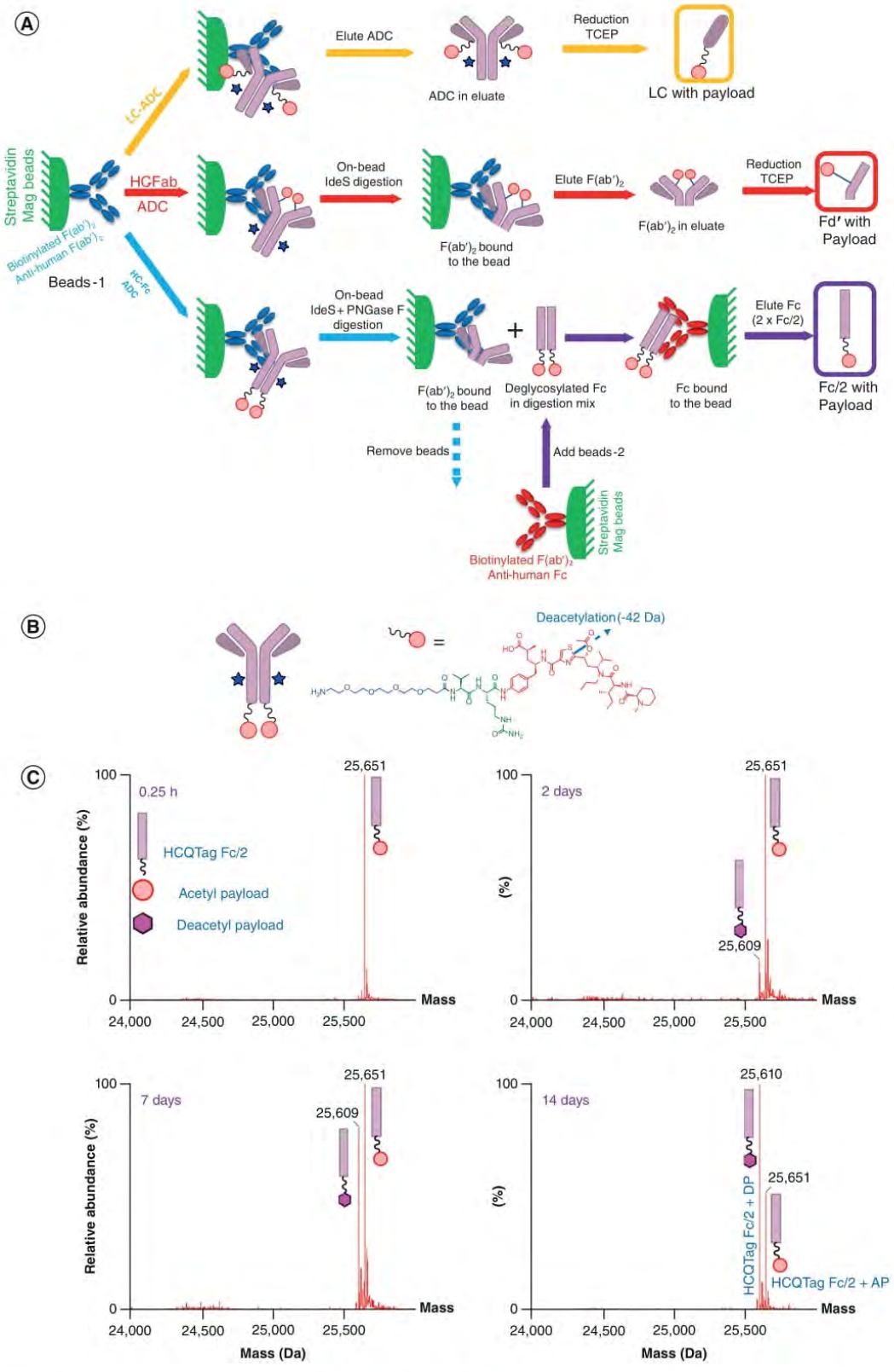

Figure 3. Universal affinity capture LC–HRMS assay for the biotransformation assessment of site-specific antibody–drug conjugates. (A) Schematic representation of universal affinity capture method for LC–, HC-Fab- and HC-Fc-conjugated ADCs, **(B)** Pictorial representation of an ADC conjugated with a tubulysin payload at HC-Fc and **(C)** Representative deconvoluted spectra showing the *in vivo* biotransformation of HC-Fc-conjugated ADC following dual affinity capture and LC–HRMS analysis.
ADC: Antibody–drug conjugate; AP: Acetylated payload; DP: Deacetylated payload; HC: Heavy chain; Q Tag: Glutamine tag; TCEP: tris(2-carboxyethyl)phosphine.
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reported that because fusion proteins are composed of two different domains, the therapeutic protein component can undergo conformational changes in systemic circulation, resulting in increased or decreased susceptibility to *in vivo* proteolytic cleavage and aggregation [44]. *In vivo* proteolysis of fusion proteins leads to formation of truncated forms (e.g., loss of N- and C-terminal residues) and the ELISA reagents in the above two methodologies may not have the specificity to differentiate between intact and truncated forms. Furthermore, aggregation may lead to immunogenicity and formation of anti-drug antibody (ADA) against the fusion protein. The presence of ADA may interfere with the accurate quantitation of fusion protein by LBA. Differential ELISA methods that employ various combinations of specific capture and detection reagents to unique epitopes on the fusion protein have been developed for the accurate quantification of fusion protein and its truncated metabolites [45]. Gan *et al.* and Kendra *et al.* developed differential ELISA assays for the quantitation of antibody-cytokine fusion protein (anti-GD2 mAb fused with IL-2) from mouse serum using various combinations of reagents against the mAb and IL-2 domains [46,47]. Giragossian *et al.* also developed differential ELISA assays for the quantification of FGF21-antibody fusion protein dosed in rats and monkeys [48]. A pictorial depiction of differential ELISA assay formats for a representative mAb-Cytokine is shown in Figure 4A–D. However, the success of this approach is dependent on identification and generation of specific reagents that can differentiate intact and multiple truncated metabolites formed *in vivo*.

Quantitation by LC–MS

If specific ELISA reagents are not available to quantify various truncated metabolites, a bioanalytical lab may prefer to develop LC–MS/MS quantitation method for fusion proteins. For LC–MS/MS-based quantitation, the fusion proteins are first extracted from the biological matrices by protein precipitation into pellet or generic/anti-id affinity capture, followed by enzymatic digestion (Trypsin, Lys-C etc.) to peptides, which can be used as surrogate analytes. A stable isotope-labeled peptide or a protein analog is added during or after sample preparation to enable accurate quantification. This methodology has been successfully employed for several fusion proteins including Alefacept (LFA3-Fc) and Fc-Adnectin [49–51]. A nanosurface and molecular-orientation limited proteolysis method to generate specific signature peptides in the therapeutic protein component of fusion proteins was developed for the LC–MS/MS-based quantification of Etanercept and Abatacept in human serum [52]. Affinity capture top-down LC–HRMS was also successfully applied for simultaneous identification and quantification of Dulaglutide, a glucagon-like peptide 1 (GLP1)−Fc fusion protein and its *in vivo* catabolites [53]. Dulaglutide and its catabolites were captured along with the spiked stable isotope-labeled mAb internal standard (SILuMab K4) from mouse plasma using biotinylated antihuman Fc capture reagent coated on streptavidin beads and analyzed by LC–HRMS. The MS spectra was deconvoluted and peak heights of the most abundant glycoform species of dulaglutide and internal standard were used for accurate quantitation [53].

Complementarity of LBA & LC–MS/MS assays for quantitation

Hager *et al.* developed differential ELISA assays for accurate quantitation of intact and truncated species of Fc-FGF21 (RG, double mutant) dosed in monkeys [45]. The four different assays are shown in Figure 5A. Assay-1 measured intact FGF21, assay-2 measured truncated FGF21, assay-3 measured total fusion (Fc-FGF21) and assay-4 measured total Fc using specific reagents [45]. As shown in Figure 5B, the levels of fusion protein as determined by various assays after day 14 were found to be: intact FGF21 <truncated FGF21 <total fusion <total Fc. This indicated that there is significant *in vivo* proteolytic cleavage at C-terminus as well as between the Fc and FGF21 domains. The exact sites of cleavage were determined by affinity capture MALDI analysis. Since the loss of more than two amino acid residues at C-terminus was observed to result in complete loss of bioactivity, it was critical to quantify and/or determine the relative percentages of various C-terminal truncated species *in vivo* [45]. This is accomplished by affinity capture of fusion protein and its catabolites using a generic antihuman Fc reagent, followed by generation of C-terminal signature peptides by Asp-N digestion and LC–MS/MS MRM analysis. The relative levels of intact and various C-terminal truncated species at various time points post *in vivo* dosing were determined by comparing the peak areas from the MRM profiles shown in Figure 5C [45].

Biotransformation assessment

As discussed in the ADC section, LC–HRMS is the preferred bioanalytical technique for the biotransformation assessment of proteins. The sample preparation typically involves affinity capture of fusion protein using generic or anti-idiotypic reagents, followed by analysis of intact fusion protein or subfragments generated after enzymatic

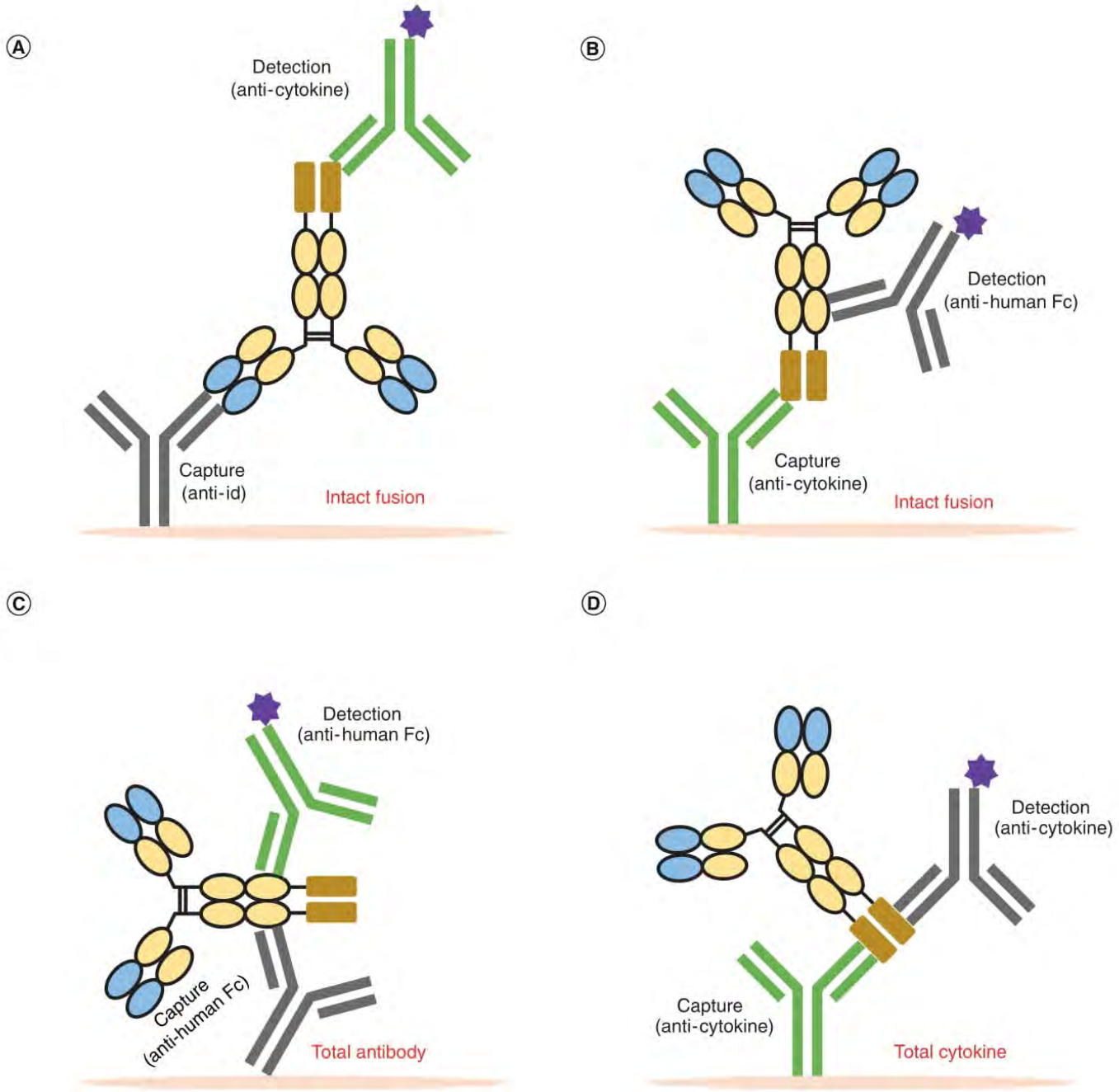

Figure 4. Differential ELISA assay for quantitation of a hypothetical monoclonal antibody-cytokine fusion protein. (A) ELISA for measurement of intact fusion (anti-id for capture, anticytokine for detection), **(B)** ELISA for measurement of intact fusion (anticytokine for capture, antihuman Fc for detection), **(C)** ELISA for total antibody measurement (antihuman Fc monoclonal antibody as capture and detection reagents) and **(D)** ELISA for total cytokine measurement (anticytokine reagents for capture and detection).

digestion and/or disulfide reduction. Affinity capture LC–HRMS was successfully applied for the biotransformation assessment of Fc-FGF21, Dulaglutide (GLP1-Fc) and TN-ApoA1 [45,53,54]. Li *et al.* evaluated the *in vivo* biotransformation of Fc-FGF21 in rats by affinity capture of the deglycosylated fusion protein with antihuman Fc reagent, followed by interchain disulfide reduction and LC–HRMS analysis on a TOF instrumentation [55]. The application of LC–HRMS in the *in vivo* biotransformation of Dulaglutide is discussed in the next subsection. Zell *et al.* also applied affinity capture LC–HRMS assay on a Q-TOF instrument for the determination of *in vivo* biotransformation of TN-ApoA1 dosed in rabbits [54]. The site of N-terminal truncations was further confirmed by Lys-N digestion of fusion protein after affinity capture and LC–MS/MS MRM analysis of the N-terminal surrogate

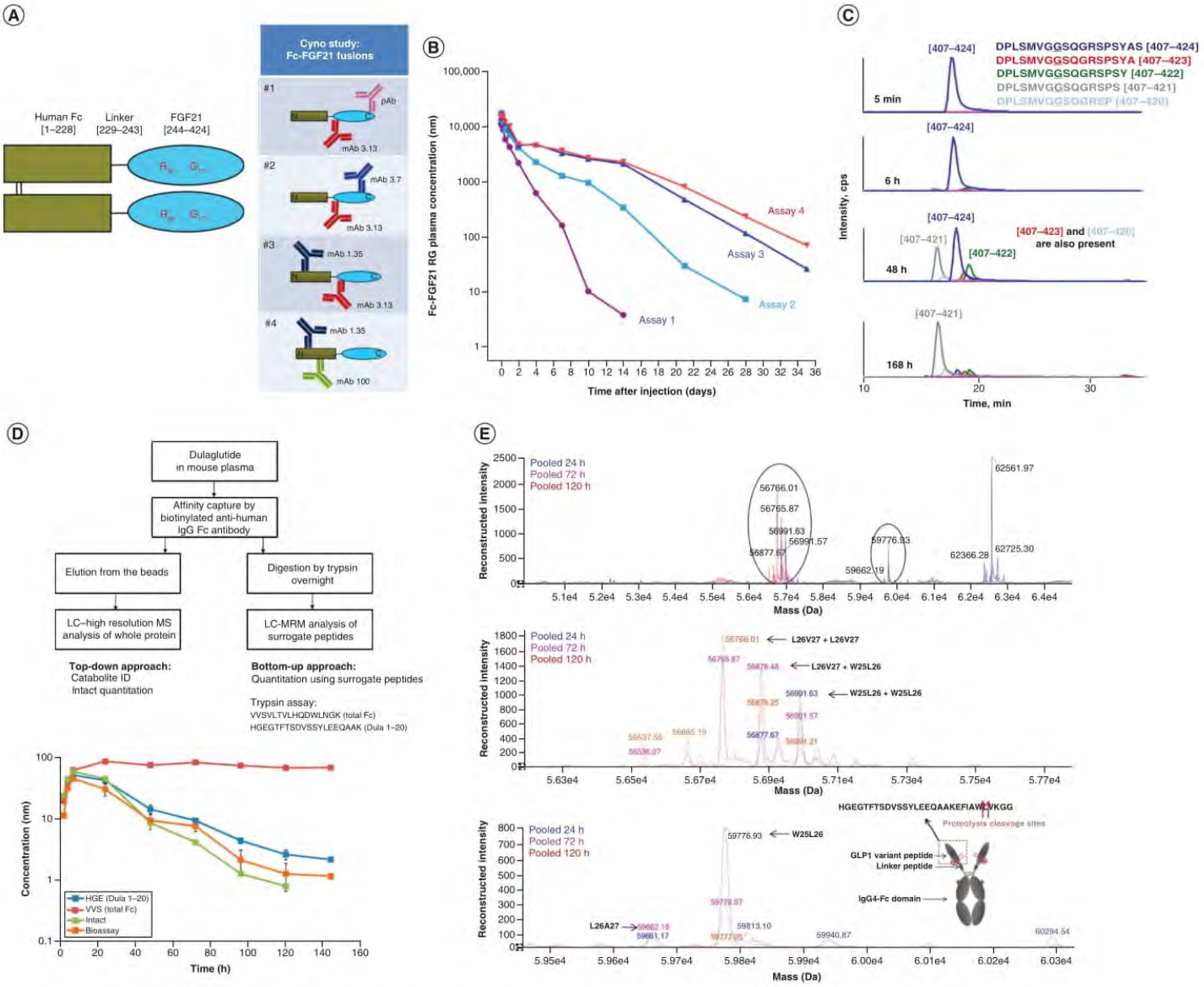

Figure 5. Complementarity of ligand-binding assay and LC–MS for the bioanalysis of Fc-FGF21 (RG) and dulaglutide. (A) Fc-FGF21 (RG) construct and schematic representation of differential ELISA assay formats used for quantitation of Fc-FGF21 (RG) in monkey serum. **(B)** PK profile of Fc-FGF21 (RG) dosed in monkeys determined by differential ELISA. **(C)** LC–MS/MS profiles of C-terminal truncated metabolites of Fc-FGF21 (RG) formed *in vivo*, **(D)** Overview of bioanalytical workflows and comparison of PK profiles of dulaglutide determined by LC–MS/MS and LC–HRMS and **(E)** LC–HRMS-based identification and characterization of *in vivo* metabolites (proteolytic sites of cleavage) of dulaglutide after dosing in mice.
LBA: Ligand-binding assay; mAb: Monoclonal antibody; MRM: Multiple reaction monitoring; pAb: Polyclonal antibody.
(A–C) Reprinted with permission from [45] © American Chemical Society (2013).
(D–E) Reprinted with permission from [53] © American Chemical Society (2017).

peptide [54]. Kullolli *et al.* developed a novel bioanalytical method for the evaluation of *in vitro* biotransformation of neurotensin human Fc (NTs-huFc) [56]. The therapeutic Fc fusion protein and its catabolites were captured from mouse serum using a generic antihuman Fc capture reagent immobilized on the beads and then tagged 'on-bead' with TMPP. The TMPP-tagged proteins were then eluted, digested, and the peptide mixture was further analyzed by data-dependent nanoflow LC–HRMS/MS on an orbitrap instrumentation to identify the proteolytic sites of cleavage [56].

Complementarity of LC–MS/MS & LC–HRMS

As described in the Fc-FGF21 example above, depending on the choice of ELISA reagents and their binding to different epitopes on the fusion protein, the *in vivo* levels of fusion protein may be under estimated or over estimated. Similarly, the choice of signature peptides used as surrogate analytes for LC–MS/MS-based quantitation is also critical for accurate quantitation. For example, in the case of Dulaglutide, two signature peptides, one corresponding to the N-terminus of the GLP1 protein and the second corresponding to the Fc domain were used as surrogate analytes for quantitation of GLP1 and total Fc, respectively (Figure 5D). The *in vivo* concentrations of GLP1 surrogate peptide decreased significantly over time, while the Fc peptide remained relatively constant (Figure 5D). The concentrations of fusion protein determined by affinity capture top-down LC–HRMS were well correlated with the GLP1 surrogate peptide levels (Figure 5D). This confirmed that the GLP1-Fc underwent proteolytic cleavage *in vivo*. The exact sites of proteolytic cleavage were determined by comparing the mass of parent and catabolites in the deconvoluted MS spectra obtained after LC–HRMS analysis of the *in vivo* samples (Figure 5E). This highlights the importance of an integrated bioanalytical strategy comprising of complementary and orthogonal methods for accurate quantitation and comprehensive *in vivo* biotransformation assessment.

PEGylated proteins

Therapeutic proteins are chemically conjugated with PEG to improve the PK properties including decreased clearance and immunogenicity, and increased *in vivo* stability. Several PEGylated therapeutic proteins are currently approved (PEGylated-Factor VIII, Pegfilgrastim, Pegaspargase, Peginterferon-α-2a etc.) or in clinical development [40,41].

Quantitation by LBA

Various ELISA formats have been developed for the quantification of PEGylated proteins. Choy *et al.* determined the concentration of PEGylated anti-TNF Fab from biological samples using direct ELISA (captured with recombinant human TNF-α and detected with generic antihuman κ light chain antibody) [57]. Song *et al.* employed competitive ELISA for quantification of PEG-hirudin by coating the assay plate with PEG-hirudin and quantifying the loss of signal due to inhibition of binding of rabbit antihirudin to the coated protein by the presence of various levels of PEG-hirudin in the biological samples [58]. Bruno *et al.* developed a sandwich ELISA for quantification of PEG-IFN-α-2a and α-2b using two different antihuman IFN antibodies (that bind to different epitopes of IFN-α-2a and α-2b) as capture and detection reagents [59]. Cheng *et al.* and Su *et al.* developed specific anti-PEG mAb reagents (that bind to the repeating PEG backbone) that were employed as capture/detection reagent pair in a sandwich ELISA for quantification of PEG-IFN-α-2a, α-2b and other PEGylated proteins [60,61]. Myler *et al.* developed a hybrid sandwich electrochemiluminescent immunosorbent assay (ECLIA) on MSD platform for the quantification of PEGylated human growth hormone (PEG-hGH) in patient samples using an anti-PEG capture reagent and anti-hGH polyclonal antibody for detection [62]. The typical ELISA formats for PEGylated protein are depicted in Figure 6A–D.

Quantitation by LC–MS

LBA has been successfully applied for quantitation of several PEGylated proteins because of their sensitivity and high throughput. Because of the wide spread exposure of humans to PEG, it has been reported that a significant percentage of the population have anti-PEG antibodies [63]. However, when LBA assays may be effected by presence of soluble target, ADA or other interferences, LC–MS has been used as a complementary technique for PEGylated protein quantitation. The PEGylated proteins are typically first extracted from biological matrices using protein precipitation with organic solvent (with or without acid dissociation of ADA complexes) or SPE. The extracted PEGylated proteins can then be digested with trypsin, followed by quantitation of signature peptide by LC–MS/MS MRM methods [49,64,65]. A stable isotope-labeled peptide is typically added during the sample preparation to ensure accurate quantitation. These methods may result in overestimation of intact PEGylated protein as they measure a surrogate peptide in the protein region and hence cannot differentiate between PEGylated and dePEGylated protein species. Affinity enrichment with anti-PEG antibodies or antiprotein reagents offers additional specificity during the sample cleanup process [65,66]. Xu *et al.* observed that the concentrations of PEG-GLP1 determined by protein precipitation workflow were higher compared to anti-PEG affinity enrichment as the former measures total protein (PEGylated and dePEGylated), while the latter only measures PEGylated protein [65]. This indicates that

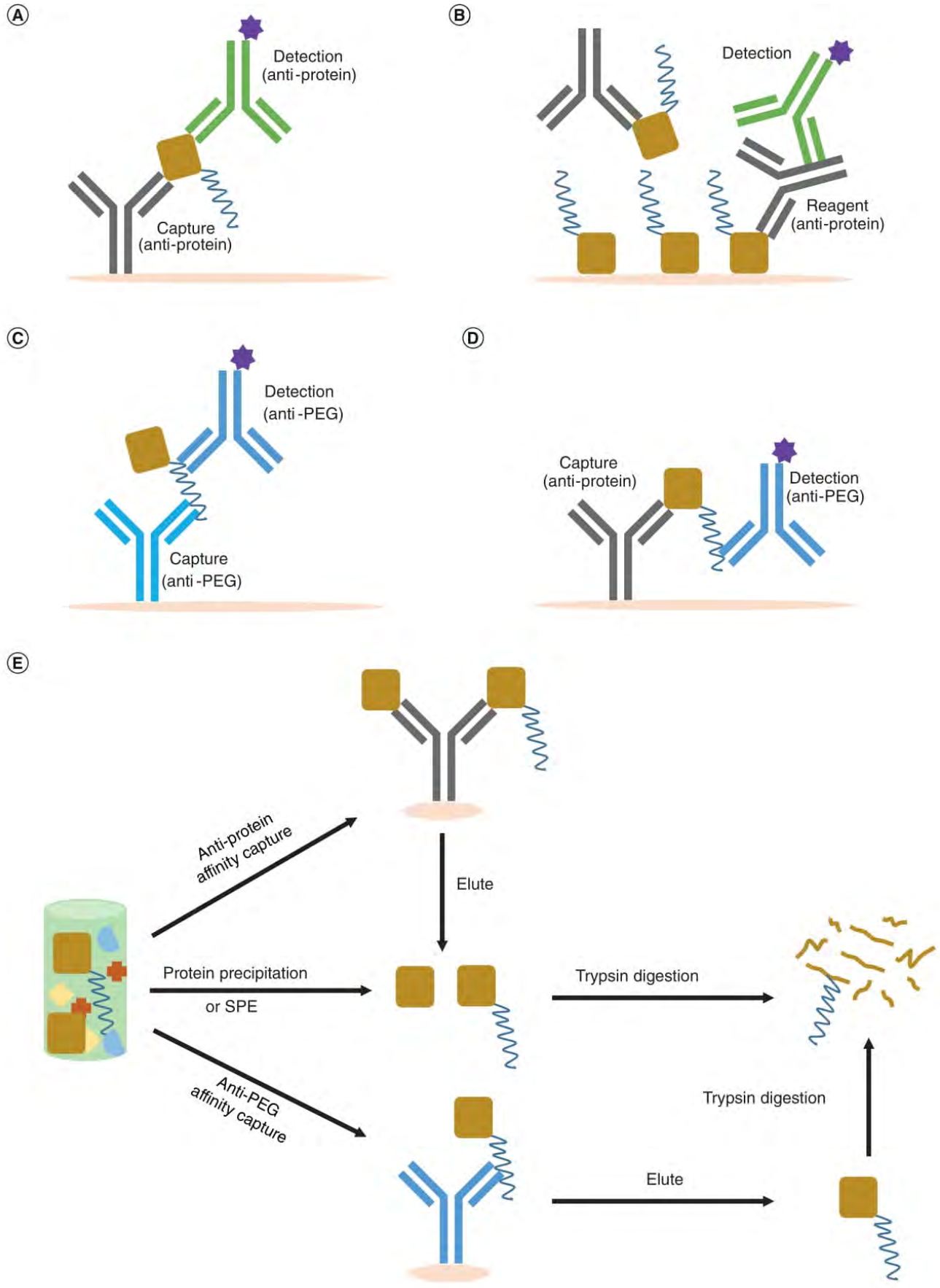

Figure 6. Ligand-binding assay and LC–MS/MS assays for the quantitation of PEGylated proteins. (A) Antiprotein sandwich ELISA, **(B)** competitive ELISA, **(C)** anti-PEG sandwich ELISA, **(D)** hybrid sandwich ELISA and **(E)** sample preparation procedures for LC–MS/MS assays. SPE: Solid phase extraction.

the choice of sample extraction procedure may influence the quantitation (depending on form of drug extracted) and hence needs to be carefully evaluated.

Liu *et al.* developed a generic affinity enrichment method using streptavidin magnetic beads coated with an antihuman capture reagent to selectively capture PEGylated-antifactor D Fab and its metabolite (dePEGylated Fab) from monkey serum [66]. The captured proteins were subjected to 'on-bead' trypsin digestion, followed by MRM of the signature peptide in the CDR region of the protein to accurately quantify the total drug (PEGylated and dePEGylated Fab) [66].

Li *et al.* developed a quantitation method involving SPE of human calcitonin peptide receptor antagonist conjugated with a 20 K PEG from monkey serum, followed by in source collision-induced dissociation (CID) to generate surrogate peptide fragments (along with gas-phase dePEGylation). This LC–MS/MS MRM method was sensitive with an LLOQ of 5 ng/ml and enables direct quantitation of intact PEG-protein without the need to generate peptides using trypsin digestion [67]. However, since the MRM is still specific to a peptide in the protein, even this method could not differentiate between PEGylated and dePEGlated species. Gong *et al.* employed a similar strategy using in source CID coupled with conventional CID to generate unique MRM transitions specific to PEG species to quantify PEGylated Adnectin as well as its metabolite, deconjugated PEG simultaneously from rat plasma [68].

Zheng *et al.* employed LC–HRMS single ion monitoring for the quantification of PEGylated disulfide-rich protein in monkey serum [69]. A large disulfide-containing peptide (essential for the activity of protein and also has a potential *in vivo* proteolytic liability) and a nondisulflide containing peptide (confirmatory peptide) were used as surrogate peptides for quantification. By comparing the concentrations of PEGylated protein obtained from these two surrogate peptides, it is possible to determine the PK profile as well as assess the *in vivo* proteolysis/stability of the protein simultaneously [69]. This approach might be beneficial for the quantification of disulfide-rich proteins (with a surrogate disulfide containing peptide) that are not amenable to MRM-based quantitation due to inefficient gaseous phase fragmentation and sensitivity issues. The summary of typical LC–MS/MS assay formats for quantitation of PEGylated proteins is depicted in Figure 6E.

Comparison of ELISA & LC–MS/MS for quantitation

Wang *et al.* determined the concentrations of a PEGylated scaffold protein dosed in monkeys using both ELISA and LC–MS/MS [70]. A sandwich ELISA using a biotinylated antigen and an anti-PEG rabbit mAb reagents was developed and employed for quantitation. For LC–MS/MS, the PEGylated protein was extracted by protein precipitation with an acidified organic solvent, followed by tryptic digestion and LC–MS/MS MRM analysis of a signature peptide in the antigen-binding region. A stable isotope-labeled signature peptide was added prior to tryptic digestion to ensure accurate quantitation. The concentration of the drug *in vivo* determined by both ELISA and LC–MS/MS were in good agreement until 96 h, while at later time points the drug concentrations determined by LC–MS/MS were significantly higher than ELISA [70]. At the terminal time point, three out of four animals did not have any quantifiable level of protein in the ELISA format. The drastic decrease in the levels of PEGylated protein at the final time points indicated the possibility of ADA. Further investigations with multiple techniques confirmed the presence of ADA and identified that the ADA binds to antigen-binding epitope of the PEGylated protein to form drug-ADA complex, which led to the under recovery of PEGylated protein in the ELISA format [70]. Hence, ELISA format only measured the free/active form of the drug. However, since LC–MS/MS used an acidified organic solvent for extraction, the drug-ADA complex was dissociated and the total drug was successfully extracted and quantified. This highlights the importance of using LC–MS/MS as a complementary technique to ELISA for the accurate quantitation of PEGylated protein.

Biotransformation assessment

LC–HRMS top-down analysis of PEGylated proteins is challenging due to the heterogeneity and polydispersity of PEG. So very few studies have investigated the application of LC–HRMS for the biotransformation assessment of PEGylated proteins. For example, Liu *et al.* employed LC–HRMS to evaluate the *in vitro* mouse serum/plasma stability of 20 K PEGylated-PPA and Glucagon [71]. By postcolumn addition of diethyl methylamine, a charge stripping reagent, it was possible to reduce the charge on PEGylated proteins and simplify the complex mass spectra, thereby enabling deconvolution and mass interpretation [71].

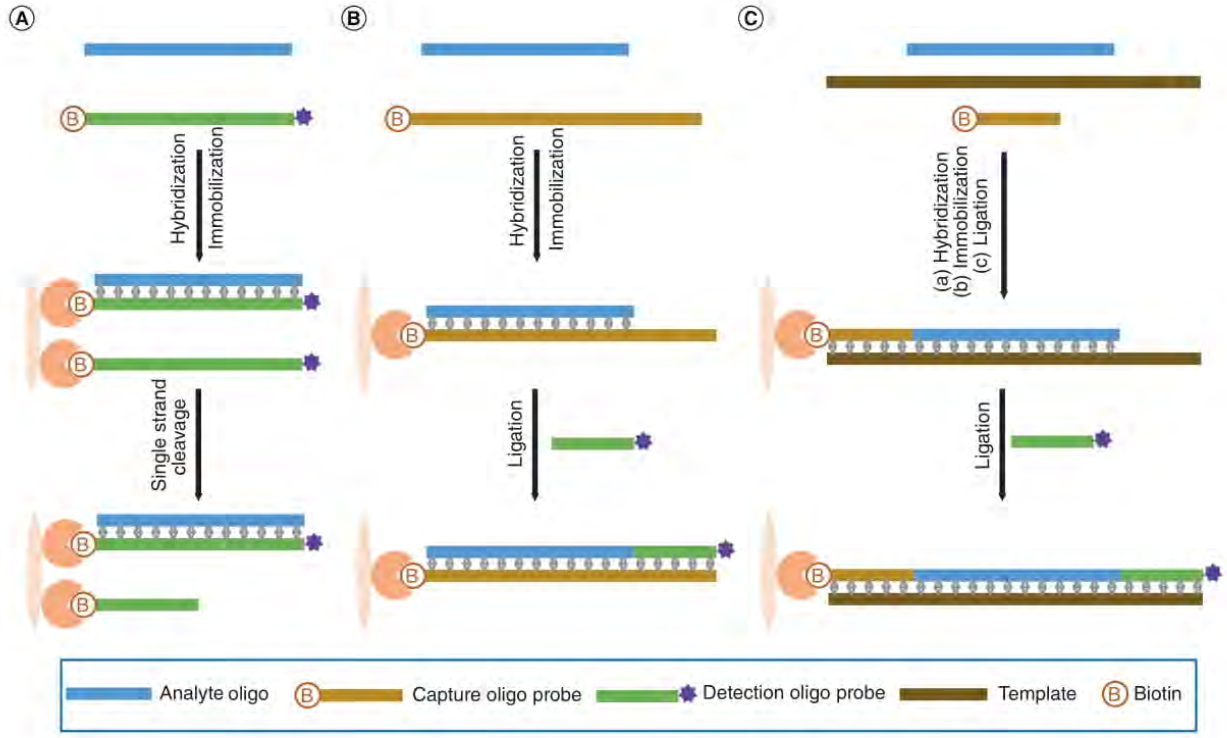

Figure 7. Hybridization ELISA formats for quantitation of therapeutic oligonucleotides. (A) One-step hybridization ELISA, **(B)** two-step hybridization ELISA and **(C)** dual ligation ELISA.

Therapeutic oligonucleotides

Oligonucleotides are nucleic acid polymers with or without modified backbones and have good therapeutic potential. The primary mechanisms of action include gene silencing, splice modulation and gene activation [5]. To date, nine therapeutic oligonucleotides were approved by FDA. The two major types of therapeutic oligonucleotides are ASOs and siRNA. ASOs are synthetic single-stranded oligonucleotides composed of 16–30 nucleotides and bind specifically to a complementary RNA target through Watson–Crick base pairing, further resulting in degradation of RNA target or RNA modulation through steric blocking [5,72]. siRNA are double-stranded oligonucleotides that have two components: a sense strand that guides the siRNA to the degrading intracellular RNA endonuclease Ago2, and an antisense strand that binds to the target RNA due to the complementarity, ultimately resulting in RNA degradation and gene silencing. Other formats that have been gaining increased attention are miRNA mimics, anti-miRNA ASOs, aptamers and cytosine-phosphate-guanosine (CpG) deoxynucleotides [72].

Quantitation by LBA

Hybridization ELISA is the preferred method for therapeutic oligonucleotide quantitation, especially because of its excellent sensitivity (pg/ml range) and high sample throughput. Various formats of hybridization ELISA including one-step hybridization, two-step hybridization, sandwich hybridization, dual ligation hybridization and competitive hybridization were developed. A one-step hybridization/nuclease-based hybridization ELISA is a format in which a complementary probe labeled with biotin at one end and a detection tag (e.g., digoxigenin) at the other end is hybridized with the analyte oligo (Figure 7A). The complex is immobilized on a streptavidin plate and incubated with S1-nuclease to remove the single-stranded complementary probe that did not form a duplex and selectively quantify the intact oligo analyte. The one-step hybridization ELISA methodology will not be able to differentiate between intact and 3′ truncated metabolites if formed *in vivo*, resulting in overestimation of intact oligonucleotide [73].

A two-step hybridization ELISA involves hybridization of a template probe (with a biotin at the 3′ end and an overhang at the 5′ end) with the analyte oligo (Figure 7B). The analyte-template oligo complex is then immobilized on streptavidin-coated plate and a ligation probe (complementary to the 5′ overhang on template probe) with a detection tag is ligated to the analyte oligo by T4 ligase. The nonligated probes are washed away and the analyte

oligo complex is finally detected [74]. Wei *et al.* modified this assay to include an additional step of incubation with S1-nuclease to cleave single-strand capture probe and nonfully formed DNA duplexes (formed by catabolism at 3′ end of analyte oligo), thereby reducing the interference and enabling accurate quantification of intact analyte oligo [73]. Although this assay does not have interference from 3′ truncated metabolites, it cannot distinguish between intact and 5′ truncated metabolites [73]. More recently, Thayer *et al.* improved this assay further with the incorporation of locked nucleic acids into the capture and detection probes. Other experimental parameters were also optimized and the assay is converted to an electrochemiluminescent format for the quantification of siRNA in serum and tissue homogenates with an increased dynamic range [75].

In a sandwich hybridization ELISA, the capture probe (complementary sequence to the 3′ end of analyte oligo) is first coated on a plate. A detection probe (e.g., biotinylated oligo probe with complementary sequence to the 5′ end of analyte oligo) is hybridized with the analyte oligo and this intermediate complex is finally hybridized with the immobilized capture probe to form a capture-analyte-detection complex, which is finally detected and quantified [76]. However, this assay quantifies N-1 and N-2 truncated metabolites from both the 5′ and 3′ ends along with the intact oligo [76].

A dual ligation hybridization assay can accurately and specifically quantify only the intact oligo (no interference from 5′ and 3′ truncated metabolites) [77]. In this assay, a complementary template probe with both 5′ and 3′ overhangs was hybridized to the analyte oligo and a biotinylated capture probe. The resulting complex is then immobilized on a plate and a detection probe was then ligated enzymatically to form the completed duplex, which is finally detected [77]. A pictorial representation of the dual ligation ELISA format is shown in Figure 7C.

A competitive hybridization ELISA assay for the quantification of analyte oligo in plasma is based on the principle of competition between the analyte oligo and a probe oligo (same sequence as the analyte oligo) with a detection tag to hybridize with an immobilized template oligo with a complementary sequence [78]. Over the years, various modifications and improvements have been made in the above five assay formats to improve the sensitivity and decrease interferences [79–81].

Since oligonucleotides have high tissue disposition, quantitation of oligonucleotides in various tissues is critical. While minimal sample preparation is needed for oligonucleotide quantitation in plasma, tissue analysis requires sample preparation steps such as liquid–liquid extraction (LLE) and Proteinase K digestion to remove the protein interference, thereby ensuring effective oligonucleotide binding to the capture reagents in the hybridization assays [82].

Quantitation by LC–MS

Although ELISA has been successfully used for quantification of therapeutic oligonucleotides and has excellent sensitivity (in pg/ml range) and requires minimal sample preparation for most sample types except tissues, the assays have a narrow dynamic range and requires the generation of specific reagents for accurate quantitation of intact oligonucleotide in the presence of truncated metabolites [83]. qPCR and hybridization LC–fluorescence-based approaches have also been used for quantification of oligonucleotides [84]. LC–MS/MS and LC–HRMS in the negative ion mode have been widely used for the quantification of oligonucleotides because of its wide dynamic range and specificity [84]. The first step in LC–MS analysis is the extraction of the analyte oligo from the biological matrix (serum, tissue homogenate etc.). One important factor to consider is that the oligo can bind to proteins in the biological samples, and hence the selection of an appropriate extraction procedure that can dissociate oligo from the proteins is critical. The major sample preparation procedures reported are protein precipitation, enzymatic digestion (e.g., proteinase K), LLE, SPE and a combination of these techniques [85]. Sips *et al.* performed a comprehensive study and determined that the percent recovery of 20 ASOs and five siRNAs from plasma using anion exchange SPE is greater than 70% [86]. However, the SPE recovery was lower from tissue homogenates. Hence, a hybridization LC–MS method was developed by hybridizing the analyte oligo from the biological matrix to a complementary biotinylated probe immobilized on streptavidin beads. By optimizing the assay parameters such as concentration of beads and capture probe, more than 90% recoveries were observed by this approach [86].

Ion-pair reversed-phase liquid chromatography (IP-LC) has been the preferred chromatographic separation method for the LC–MS-based quantification and biotransformation assessment of oligonucleotides [87,88]. Dai *et al.* employed SPE followed by IP-LC–MS/MS analysis for the identification and quantification of G3139 (18-mer ASO) and its metabolites from rat and human plasma [89]. Ewles *et al.* employed LLE coupled with SPE followed by IP-LC–MS/MS analysis for quantitation of Trabedersen (18-mer ASO) and its six metabolites [90]. Hemsley *et al.* developed and validated an online SPE-IP-LC–MS method for the quantification of 15-mer oligonucleotide

in human plasma. By replacing offline SPE with online SPE, increased sample loading and cleanup was possible, which resulted in sensitive quantitation of the oligo with an LOQ of 50 pg/ml [91]. More recently, MacNeill *et al.* employed mixed-mode SPE followed by HILIC–MS for the quantification of an 18-mer oligonucleotide from human plasma [92].

LC–HRMS is increasingly being employed for quantification of therapeutic oligonucleotides because of its ability to simultaneously identify and characterize any new metabolites formed. The applications of LC–HRMS for biotransformation assessment is discussed in the next section. The high resolution and mass accuracy offered by HRMS instruments provides the necessary selectivity and sensitivity to quantify the oligonucleotide extracted from a biological matrix. Liu *et al.* employed SPE followed by IP-LC–HRMS on an orbitrap instrument for the identification and quantification of metabolites of a short oligonucleotide REVERSIR-A dosed in rat and monkey [93]. Ramanathan *et al.* used SPE in combination with IP-LC–HRMS on a TOF instrument for the quantification of GalNac-conjugated siRNA dosed in monkeys [94]. Kim *et al.* compared hybridization affinity capture and anion-exchange SPE sample preparation methods for quantitation of Eluforsen (a 33-mer ASO) and its metabolites by LC–HRMS [95].

Biotransformation assessment

While early studies employed LC–MS/MS on quadrupole and ion trap instrumentation for identification of oligonucleotide metabolites [89,96], LC–HRMS on orbitrap and TOF instrumentation is now routinely used. As discussed previously, the major advantage of LC–HRMS is the ability to simultaneously identify and quantify oligonucleotides and its metabolites from biological matrices [87]. However, the exact identity of metabolite is typically confirmed by LC–HRMS/MS. The primary mode of biotransformation of oligonucleotides is the hydrolysis of phosphodiester or phosphorothioate backbone by endo- and/or exonucleases resulting in truncated metabolites [12]. Beverly *et al.* used LLE and/or combination with SPE followed by IP-LC–MS/MS and HRMS for the biotransformation assessment of siRNA [97–99]. Husser *et al.* developed a generic, untargeted and a sensitive LC–HRMS/MS (with capillary flow LC and column switching) assay for the biotransformation assessment of GalNac-conjugated ASO [100]. Liu *et al.* used LC–HRMS and LC–HRMS/MS on an orbitrap instrument to identify two major 3′ truncated species, Rev-N1-N9 and Rev-N1-N7 in monkey plasma [93]. A new metabolite with an increased mass of just 0.984 Da compared with the major metabolite (Rev-N1-N9) was observed in monkey liver as shown in MS spectra and extracted ion chromatograms (Figure 8B–F). The identity of the new metabolite was confirmed by tandem mass spectrometry analysis of mouse liver sample extract and comparing it with the standards of Rev-N1-N9 and the predicted metabolite (Figure 8G–I). Finally, the concentrations of REVERSIR-A and its three metabolites including the novel metabolite in monkey liver were determined by selected ion monitoring (Figure 8J) [101]. This example highlights the applicability of LC–HRMS as a versatile tool for oligonucleotide quantitation as well as biotransformation assessment.

Choice of bioanalytical technique

There is no defined set of rules on the choice of platform to be employed for bioanalysis of biotherapeutics as multiple factors need to be considered during the decision-making. Major factors that need to be considered are form of analyte measured (free or total), modality, biotransformation, ADA, soluble target levels, availability of reagents, sensitivity required based on dosing and stage of program (discovery, development etc.) [102]. Sometimes, other factors such as instrumentation/resource availabilities, costs, expertise may also be considered. For example, during the discovery phase of a program, when specific reagents are not available, LC–MS might be the preferred platform because of the ability to use generic reagents and measure a signature peptide as surrogate analyte for protein quantitation. However, as the program reaches development and sufficient knowledge on drug and its metabolites is gained, analytes to be measured are well defined and specific reagents are generated, then LBA might be the preferred method for quantitation because of its ease of implementation and high throughput [6,102]. If a therapeutic protein is dosed at low level, the main goal would be development of sensitive assay for quantitation. In this scenario, LC–MS assay would require extensive sample preparation such as antipeptide capture, usage of capillary, nanoflow or 2D LC, which might not be robust and results in low throughput. Hence, LBA would be a better fit given its ability to achieve sub-ng/ml sensitivity with identification and usage of specific capture and detect reagents [6]. However, there are multiple cases when both LBA and LC–MS are employed. For example, for an ADC program in early discovery, total Ab can be determined by generic reagents (e.g., antihuman Fc capture and antihuman kappa detection). The conjugated payload can be determined by LC–MS/MS, since specific anti-id

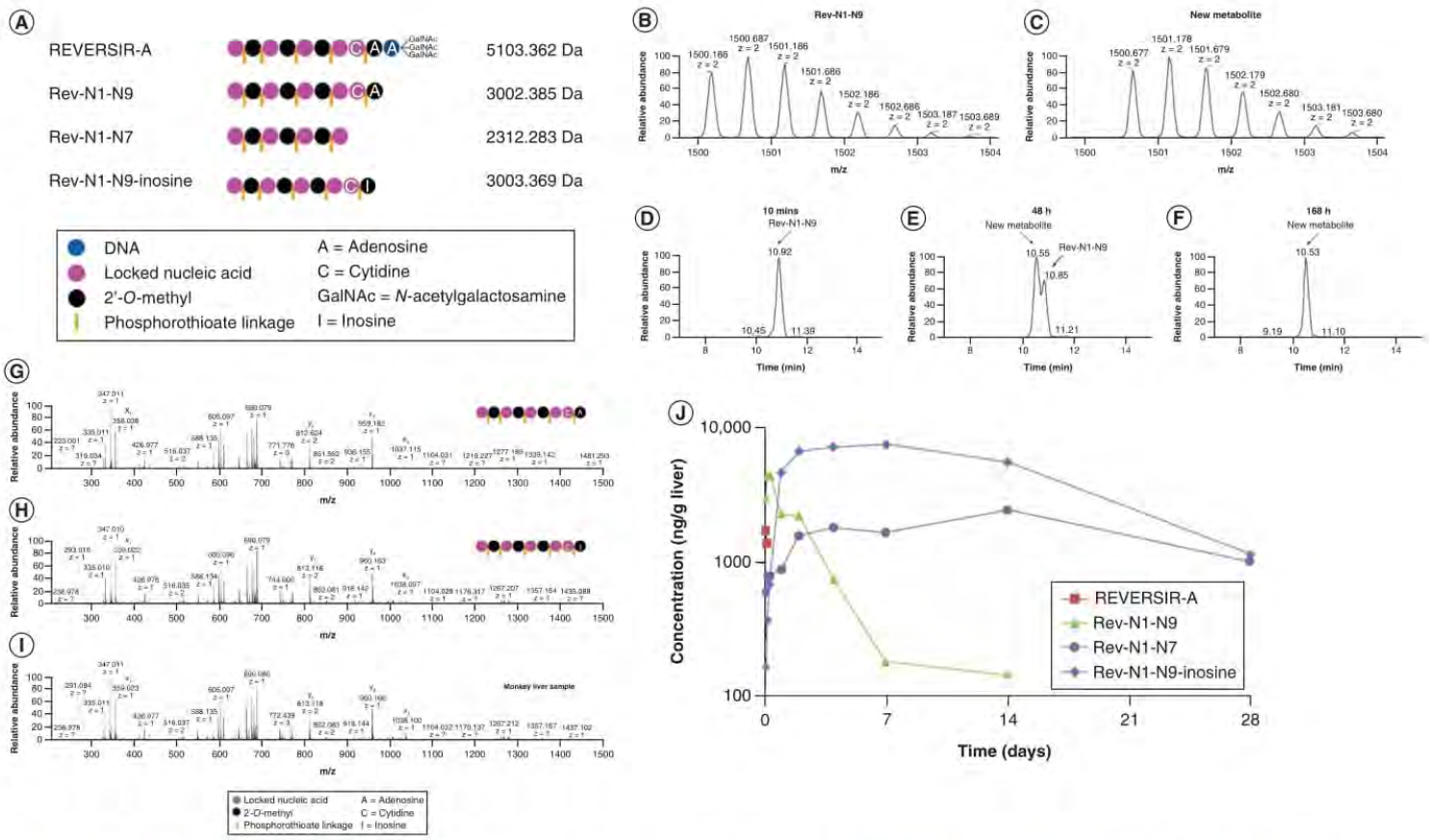

Figure 8. LC–MS/MS and LC–HRMS-based identification and quantification of REVERSIR-A and its metabolites. (A) Sequence and molecular weights of REVERSIR-A and its metabolites, **(B–C)** MS spectra and **(D–F)** Extracted ion chromatograms of Rev-N1-N9 and the new metabolite, **(G–I)** MS/MS spectra confirming the identity of the new metabolite as Rev-N1-N9-inosine and **(J)** LC–HRMS Selected ion monitoring-based quantitation of REVERSIR-A and its metabolites in monkey liver.
Reprinted with permission from [101] © Future Science Group (2019).

against the payload for LBA may not be available during this stage. Furthermore, the biotransformation of ADCs needs to be investigated and this is accomplished by LC–HRMS. In summary, the choice of platform would need to evaluated on a case by case basis depending on modality, scientific question and goals, and thorough understanding of the strengths and limitations of both of these technologies (Table 1). However, for most programs, especially during early discovery, an optimal bioanalytical strategy would involve application of both LBA and LC–MS technologies.

Conclusion

LBA has been traditionally and reliably used for the quantitation of multiple therapeutic modalities, and has several advantages such as sensitivity, no/minimal sample cleanup, easy implementation and excellent throughput. The success of LBA depends on the availability and generation of specific and selective reagents. In most cases, these reagents are available in house or through external vendors and can be readily employed to develop bioanalytical methods for quantitation. However, in some cases, especially during early discovery phase, when the reagents are not available or suitable and needs to be generated, the process can be time consuming and expensive. Furthermore, in some additional cases, either due to *in vivo* catabolism/biotransformation or ADA formation, there is a possibility of over- or underestimation of active drug.

LC–MS has been increasingly employed for the quantification of these therapeutics, especially when appropriate LBA reagents are unavailable or as a complementary technique to validate the LBA results. The initial MS-based methods for therapeutic protein quantification involved tryptic digestion of proteins followed by monitoring of surrogate peptide by LC–MS/MS. While this methodology is widely used, a major drawback is that the surrogate peptide only represents just one region of therapeutic protein and does not provide information regarding the integrity of the whole protein. Furthermore, the sample preparation is long and sometimes labor intensive, and

Table 1. Strengths and limitations of ligand-binding assay and LC–MS for the bioanalysis of various therapeutic modalities.

Modality	Assay	Strengths	Limitations
ADCs	LBA	• Good sensitivity compared with LC–MS (low ng/ml) • Requires small-sample volume • High throughput • Relatively easy to implement in lab	• Needs specific capture and detect reagents (e.g., anti-id against payload) • Does not provide DAR measurement • No biotransformation information
	LC–MS	• Can be developed with generic reagents • Provides DAR distribution changes • Provides biotransformation information • Specificity due to MRM and HRMS	• Relatively less sensitive than LBA • Affinity capture, enzymatic digestion steps can be time consuming • Advanced training and experience required for complex biotransformation assessment and interpretation
Fusion proteins	LBA	• Good sensitivity compared with LC–MS • Requires small-sample volume • Minimal sample preparation • Differential ELISA for quantification of various truncated metabolites	• Specific reagents required for differential ELISA • Reagent generation and method development can be time consuming • No biotransformation or sequence information
	LC–MS	• Affinity capture with generic reagents • Provides biotransformation information • Simultaneous identification and quantification of fusion protein and its truncated metabolites	• Relatively less sensitive than LBA • Affinity capture, enzymatic digestion steps can be time consuming • Surrogate peptide approach is not representative of the integrity of the fusion protein
PEGylated proteins	LBA	• Good sensitivity compared with LC–MS • Requires small-sample volume • Choice of multiple formats	• Specific reagents may be required • ADA interference can lead to underestimation of total drug • No biotransformation information
	LC–MS	• Can be developed with generic reagents • Sample preparation such as acid dissociation for accurate quantitation of total drug • Minimal interference from ADA and measures total drug	• Relatively low throughput • Time-consuming sample preparation steps and enzymatic digestion to peptides • Biotransformation assessment is challenging due to PEG polydispersity and heterogeneity
Oligonucleotides	LBA	• Excellent sensitivity (pg/ml LOQ) • No sample cleanup or extraction (except tissues) • High throughput	• Narrow dynamic range • Needs specific capture and detect probes • Does not differentiate intact and truncated metabolites • Assay development can be time consuming • No biotransformation information
	LC–MS	• Good dynamic range • SPE, LLE can be used for sample preparation without the need for specific hybridization probes • Accurate and simultaneous quantification of intact and truncated species • Identification and characterization of truncated metabolites	• Less sensitive compared with LBA (ng/ml LOQ) • Sample preparation can be time consuming and intensive • Requirement of ion-pairing reagents for LC • Relatively less sample throughput

Ab: Antibody; ADA: Anti-drug antibody; ADC: Antibody–drug conjugate; DAR: Drug-to-antibody ratio; LBA: Ligand-binding assay; LC: Liquid chromatographic; LLE: Liquid–liquid extraction; LOQ: Limit of quantitation; MRM: Multiple reaction monitoring; SPE: Solid-phase extraction.

throughput is less compared with LBA. These are being addressed with development and application of automated sample preparation, faster enzymatic digestion processes and multiplexing. More recently, with the improvements in MS technology as well as significant contributions from researchers around the globe, LC–HRMS is being increasingly used for the quantification of these therapeutics as it also offers an insight into the integrity and stability of the therapeutic drug.

A comprehensive bioanalytical strategy also involves the identification and/or quantification of the *in vivo* catabolites of the drug. For example, ADCs can undergo loss of cytotoxic payload resulting in changes in DAR, fusion proteins may be clipped or cleaved, while therapeutic oligonucleotides are cleaved to form truncated metabolites. LC–HRMS is the preferred method of choice to identify the *in vivo* catabolites of these therapeutic modalities. However, along with the drawbacks described above for LC–MS/MS workflows, LC–HRMS has an additional limitation of sensitivity. Some of these challenges are being addressed by using hybrid LC–MS methodology by affinity capture of therapeutic from biological matrices, low flow, 2D-LC etc. Once the identity of metabolites is determined and confirmed, and as the program reaches development, all the LBA reagents needed for accurate quantitation of the drug and its metabolites are generated and LBA methods such as differential ELISA are developed and preferred for routine quantitation because of minimal sample preparation requirements and high throughput.

Given the increased complexity and diversity of therapeutic modalities, specifically protein and oligonucleotide therapeutics, no single bioanalytical assay or technology is applicable for bioanalysis of all of these drugs. The bioanalytical researchers should carefully consider the strengths and limitations of both LBA and LC–MS technologies. A flexible and integrated strategy that incorporates both these platforms should be adopted as they provide complementary answers to important scientific questions that drive the discovery and development of these novel and complex therapeutic modalities.

Future perspective

LBA will continue to be the gold standard for quantitation of therapeutic proteins and oligonucleotides. The development of new immunoassay technologies will continue to push the limits of sensitivity and throughput. This is especially critical given the fact that some of these new therapeutic modalities are dosed at low levels, and the industry continues to adapt microsampling. LC–MS/MS will increasingly be used as a complementary technique to LBA for quantitation of biotherapeutics. With innovations in automation for sample preparation, LC and MS technologies with focus on improved sensitivity, the routine implementation of LC–HRMS for the simultaneous quantification and biotransformation assessment of these therapeutic modalities at intact level is an exciting possibility in the near future without the need to generate peptides for LC–MS/MS quantitation. Additionally, improvements in data processing software will simplify the interpretation of the complex MS data. It is our anticipation that an integrated bioanalytical strategy involving both LBA and LC–MS will be adopted as the new standard by bioanalytical laboratories around the globe. The choice of bioanalytical technique would then be solely determined by the scientific question that needs to be addressed.

Executive summary

- This review highlights the applications and examples of ligand-binding assay (LBA) and LC–MS methodologies for the quantitation and biotransformation assessment of antibody–drug conjugates (ADCs), fusion proteins, PEGylated proteins and therapeutic oligonucleotides.
- LBA is typically preferred for high-throughput quantitation of biotherapeutics when appropriate reagents are available.
- LBA assays are sensitive, easy to implement and requires minimal sample preparation and use small-sample volumes.
- LC–MS/MS quantitation using signature peptides as surrogate analyte is used for biotherapeutic quantitation when LBA assays are not available or suitable (specific reagents not available, ADA, other interferences etc.).
- LC–HRMS is the preferred bioanalytical technique for identifying the *in vitro* and *in vivo* biotransformation of biotherapeutics.
- LC–HRMS is increasingly being used in recent years for simultaneous metabolite profiling and quantitation of biotherapeutics.
- A comprehensive bioanalytical strategy that integrates both LBA and LC–MS is required for the novel and complex therapeutic modalities.

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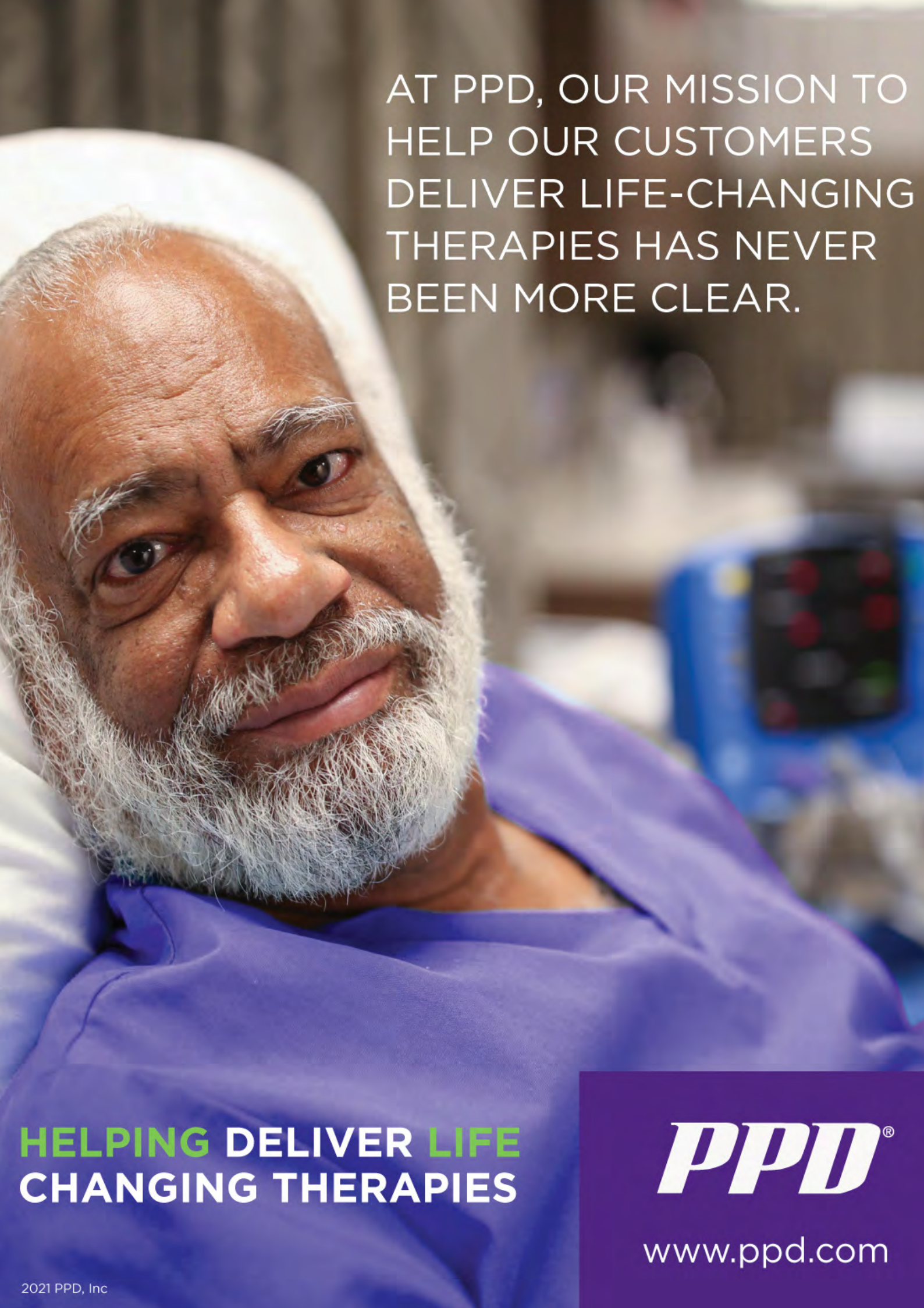
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Ligand-binding and chromatographic methodology comparison and synergies for the pharmacokinetic bioanalysis of therapeutic oligonucleotides

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Overview

Oligonucleotides, which are nucleic acid polymers that can range from approximately 10–50 base pairs, have been undergoing research and development as potential therapeutics for over 3 decades. Over that span of time, several various oligonucleotide modalities have been developed to alter gene expression and protein translation, including antisense oligonucleotides (ASO), aptamers, small interfering RNAs (siRNA), microRNAs (miRNA), splice switching oligonucleotides and, more recently, CRISPR/Cas9, among others [1,2]. In addition, considerable advances have been made to increase the half-life of oligonucleotides *in vivo* via chemical and backbone modifications as well as advances in targeted delivery of the oligonucleotides to specific tissues that are the site of disease [2–10].

Advances in the delivery of oligonucleotides to targeted tissues include adjusting the route of administration, such as intrathecal for diseases of the central nervous system (CNS), intraocular for diseases of the eye, intramuscular for muscular dystrophy [11–18]. In addition, there have been advances in targeted delivery such as the utilization of lipid nanoparticles and conjugation to N-acetylgalactosamine (GalNAc) for delivery to the liver, conjugation to monoclonal antibodies for targeted delivery to multiple tissues, such as the muscle, and conjugation to peptides [2,3,11,19–26].

Given the nature of oligonucleotides and that they are built from nucleic acids, they can be utilized to treat diseases and targets previously thought to be undruggable by small molecules and biologics such as monoclonal antibodies. As a result, oligonucleotides are being developed as therapeutics to treat a wide array of diseases including, but not limited to, cancer, infectious disease, rare diseases (such as those of the CNS and muscular dystrophy) and cardio-metabolic diseases [2].

Taken together, the advances and increased research of oligonucleotides as therapeutics has resulted in significant advancement in the field, including the regulatory approval of ten oligonucleotide therapies, including eight within the last 5 years dating back to 2016 [2]. As a result, there has been an increase in the number of biotechnology and pharmaceutical companies with a sole focus on the development of oligonucleotide-based therapeutics, as well as the number of large pharmaceutical companies that have oligonucleotides in their pipelines either through licensing agreements or independent development. Due to the advances being made in oligonucleotide development and delivery, expansion of oligonucleotide modalities and increased volume of oligonucleotides being developed as therapeutics, an appropriate bioanalytical strategy must be implemented for the quantification of oligonucleotides. The importance of an appropriate bioanalytical strategy is amplified by the generated data being utilized to assess PK and drug metabolism, time courses and anatomical distribution to drive dosing decisions. These include drug concentration and number and timing of doses to ensure efficacy while minimizing potential off-target effects, as well as toxicity and safety issues.

Historically, oligonucleotide quantification has taken place via chromatographic, or ligand-binding-based (LBA) methodologies. LC platforms such as UPLC–UV, LC–FL, LC–MS/MS and LC–HRAM can be utilized and are highly selective for the parent compound vs chain-shortened metabolites and offer a wide quantitation range. However, they require extensive sample preparation and extraction and have reduced sensitivity. An alternative to chromatographic-based methodologies are LBAs such as ELISA and electrochemiluminescent (ECL) assays. ELISA and ECL methods are highly sensitive methods that offer high sample throughput at a lower cost but have a narrower dynamic range.

They often are not selective for the full-length parent compound and may also quantitate long-chain n-1 and n-2 metabolites. The focus of the remainder of the commentary is to review chromatographic and LBA methodologies as they relate to the quantification of oligonucleotides, as well as how they can be combined to generate a potential synergistic approach for oligonucleotide PK/DM assessments.

Hybridization ELISA and ECL

The quantification of oligonucleotides via LBA typically utilizes either hybridization ELISA (hELISA) or ECL (hECL) methodologies. For both hELISA and hECL, the sample is hybridized to a complementary capture probe and, if a sandwich format is applied, then to a complementary detection probe followed by a detection reagent and substrate if needed. hELISA with a fluorescent or colorimetric readout, including a single and dual ligation hELISA [101,27–28], nuclease-based hELISA [102,29] and dual probe hELISA [30], has been historically used. hECL methodologies have also been recently developed allowing for increased dynamic range and sensitivity compared to hELISA methodologies. hECL can utilize the same method formats as hELISA with the differences being that the detection portion of the probes, or detection antibody, are ruthenium-labeled [31,32].

Benefits

Ligand-binding formats including hELISA and hECL platforms offer several benefits for the quantification of oligonucleotides. We have found that they are appropriate to measure the levels of a multitude of oligonucleotide modalities (ASO, siRNA, SSO, TLR agonist etc.) across multiple matrices (plasma, urine, cerebrospinal fluid, preclinical tissues, human biopsies etc.) via variable delivery mechanisms (LNP, GalNAc and peptide conjugated, polymers etc.). Chief amongst the benefits of using hELISA and hECL is that they offer the most sensitive platforms that are widely used to date for PK/DM assessments, with sensitivity down the picogram/picomolar levels. Additional advances in sensitivity have been made by utilization of hECL methodologies [31,32]. Increased method sensitivity will continue to be beneficial given the numerous advancements being made in oligonucleotide chemistries and delivery techniques, which can be synergistic, enabling lower and less frequent doses of oligonucleotide therapeutics [10]. While hELISA and hECL methodologies offer the most sensitive methods for quantification of oligonucleotides, the increase in sensitivity will continue to be a focus moving forward as oligonucleotide research evolves to ensure concise half-life measurements such that appropriate dosing schemes are administered.

In addition to sensitivity, hELISA and hECL methods offer a wide array of additional benefits. In contrast to chromatographic methods, there is minimal sample cleanup or extraction required. This allows for shorter methods and an increased number of analytical runs on a weekly basis. Additionally, oligonucleotide analysis with hELISA and hECL typically utilizes a 96-well format and does not require linear injections associated with chromatographic methods, meaning that analysts can run upwards of approximately 100 samples per day and a team can analyze thousands of samples over the course of a week. High-throughput analysis via hELISA and hECL, along with minimal extraction, allows for cost benefits, as samples can be analyzed quickly with minimal consumables required. Moving forward, it is anticipated that 384-well formats may be compared to 96-well formats and assessed to further increase oligonucleotide sample throughput. Additionally, hELISA and hECL methods are conducive to being automated on liquid handlers such as TECAN (Männedorf, Switzerland) and Hamilton (NV, USA). Automation is widely used to make calibrators and quality controls to reduce variability and to dilute samples with a standard MRD. Method-specific scripts can be generated to fully automate methods minimizing analyst variability and increasing the success of incurred sample re-analysis.

Drawbacks

While there are many benefits to using hELISA and hECL for the quantification of oligonucleotides, there are also several drawbacks. For example, LBA have a decreased dynamic range compared to chromatographic platforms, meaning that samples often must undergo dilution to fall within the quantitation range. This requires the appropriate dilutional linearity to be established during method validation to cover the anticipated Cmax. This can also lead to an increased need for sample re-analysis if samples quantitate above the upper limit of quantitation. However, in our experience in collaboration with our sponsors, hELISA and hECL methods do not suffer from issues with dilution and we have established upwards of 100,000-fold dilutional linearity or more during validation. LBA methodologies typically are also unable to distinguish and quantitate metabolites in addition to the full-length parent compound as chromatographic platforms can do. Thus, major metabolites are typically identified initially via LC–MS methods, including which major 5’ and 3’ metabolites exist, which are most prevalent and the associated time course with which metabolites quantitate. This information can be applied to future preclinical and clinical studies and provides additional guidance on the designing of probes and the selection of hELISA/hECL methodologies during validation and sample analysis. If metabolites were to be measured via hELISA or hECL, separate methods for each metabolite would likely be required, which would require significant time and cost compared to chromatographic platforms.

The largest drawback to hELISA and hECL for the quantitation of oligonucleotides is that they lack the selectivity for the parent compound as compared to chromatographic methods. Typically, hELISA and hECL cannot distinguish between the full-length parent compound and long-chain metabolites such as n-1 and n-2. This may result in higher oligonucleotide concentrations in samples as results may be a combination of full-length parent compound and the long-chain metabolites, noting that long-chain metabolites may still be efficacious and have the desired therapeutic effect. However, it is typical that a time course of metabolites and their prevalence over time are determined prior to most preclinical or clinical studies that would utilize LBA. This information can be used to make assumptions on the actual concentration of the parent compound at specific time points. For example, if an n-1 metabolite quantitates with the parent compound, but is not seen until day 2 or 3, it may be inferred that concentrations determined on day 1 samples are attributable to the parent compound. In addition, information on the prevalence of long-chain metabolites can be applied such that if they comprise 20% of the total concentration on day 2 via chromatographic methods, then the concentration of full-length parent compound may only be 80% of the concentration measured by hELISA or hECL.

There are mechanisms with which to assess the selectivity of the parent compound during method development and validation and tools that can be utilized to increase selectivity. During method development and validation, the prevalent metabolites may be tested to assess their ability to quantitate off the calibration curve comprised of the full-length compound.

Typically, short metabolites will not quantitate. However, n-1 and n-2 metabolites often quantitate and do so accurately compared to the theoretical concentration. Therefore, long-chain metabolites will typically have an additive effect in combination with full-length parent oligonucleotides resulting in a positive bias and the overestimation of parent oligonucleotide concentrations discussed above. Amongst the tools to increase selectivity for the parent compound in hELISA and hECL are use of S1 nuclease, probe design and method design. S1 nuclease recognizes and cuts single stranded RNA and DNA, and can be added to LBA methods. If there are metabolites present in the samples, the S1 nuclease would recognize the portion of the probes that are not hybridized, inducing a cut that would remove either the capture or detection portion (digoxigenin, ruthenium, etc.) of the probe such that a signal would not be generated during readout [102]. Additionally, alteration of probe design can help with selectivity for the parent compound. For example, if it is known that there are short 3’ or 5’ metabolites, the size and lengths of capture and detection probes can be altered to minimize metabolite overhang that might cross-link capture and detection probes, resulting in a signal.

Finally, single- and dual-ligation methods have been utilized to further increase selectivity for the parent compound. A single ligation hELISA requires an intact 3′ end of the oligonucleotide for ligation to the detection probe to occur. If there is a 3′ shortened metabolite, the T4 DNA ligase is unable to anneal the oligonucleotide to the ligation probe and the S1 nuclease is added, resulting in cleavage of the hybridized capture/detection probe and a loss of signal [101,27]. While the single ligation hELISA removes interference from 3′ shortened metabolites, it does not do so for 5′ shortened metabolites. To address the 5′ shortened metabolites, Tremblay *et al.* developed a dual-ligation hELISA that utilized a template probe with both 5′ and 3′ overhangs and capture and detection probes that are complementary to the template probe overhangs. It was demonstrated that the intact full-length oligonucleotide was detected without interference from 5′ and 3′ shortened metabolites [28].

Chromatographic methodologies

Chromatography-based approaches

In addition to hybridization LBA methodologies, chromatography-based approaches are an alternative and complementary methodology for the quantitation of oligonucleotide in biological matrices. Contrary to the indirect analysis by hybridization LBA, chromatography-based approaches offer direct analysis. Liquid chromatography (LC) is the method of choice when oligonucleotide separation is required. Oligonucleotides are polyanionic ions, which exhibit challenging chromatographic behaviors. Oligonucleotides are very polar, making them unretained on widely used reversed-phase liquid chromatography (RPLC) systems.

Although polar oligonucleotides are suitable for hydrophilic interaction chromatography mode separations, the use of hydrophilic interaction chromatography for the bioanalysis of oligonucleotides is not routine [33]. To enable the use of RPLC, it is critical to mask the negative ions on the backbone of oligonucleotides using ion paring reagents [34]. Another mode of separation for oligonucleotides is ion exchange chromatography. The three major common detection methods for oligonucleotides following LC separations includes UV, fluorescence and mass spectrometry (MS).

LC–UV

UV is a universal yet low-cost detection for an array of molecules including oligonucleotides. The selectivity of LC–UV is low, depending mainly on an oligonucleotide's length and number of charges. Strong cation exchange chromatography is typically employed for the separation of oligonucleotides. Shorter oligonucleotide metabolites can be chromatographically resolved from oligonucleotides. This separation can be improved when ultra-high-performance liquid chromatography (UHPLC) is employed [35]. Hence, LC–UV can be used to profile metabolites of an oligonucleotide from dosed subject samples after *in vivo* biotransformation. Due to the similarity in structures and lack of matrix effects, LC–UV can provide semi-quantitation for an oligonucleotide's metabolites without reference materials [36]. Furthermore, the identity of metabolite(s) can be confirmed when LC–UV is combined with MS detection [37]. The m/z information confirms the presence of metabolite(s). A typical LLOQ is in the 100 ng/mL range by LC–UV with a moderate throughput, approximately 10–15 minutes per analysis.

Hybridization LC–FL

Hybridization LC–FL (LC–FL) combines the use of hybridization and chromatographic separations [38]. A sequence-specific oligonucleotide labeled with fluorescence probes hybridizes to a target oligonucleotide analyte via Watson-Crick base pairs. The resulting duplex oligonucleotides with fluorescence probes attached are then subject to separation via ion exchange chromatography and detected by a fluorescence detector. No enzymes are involved in the methodology. Assay selectivity of LC–FL is achieved via hybridization and chromatography.

The advantages from hybridization include: 1) sensitivity via fluorescence detection where a LLOQ of approximately 1 ng/mL can be reliably achieved. This is up to 10-fold and 100-fold more sensitive than that from LC–MS or LC–MS/MS and LC–UV, respectively; and 2) sample preparation can be simple. Nevertheless, proteinase K has been added to deactivate oligonucleotide protein binding and/or degrade abundance proteins in the biologic matrices (e.g., plasma or tissues) to reduce potential interference via digestion [38]. Ion exchange chromatography can provide added selectivity by resolving oligonucleotide duplexes from matrix interferences and chain-shortened metabolites. Another advantage is the good method reproducibility in terms of accuracy and precision enabled by the strong stability of FL detection. Unlike traditional LC–MS/MS assays, no internal standards are added in FL–LC assays to track assay variabilities such as hybridization and LC injection. Unlike qPCR, LC–FL tolerates chemical modifications of oligonucleotides including miRNA and siRNA therapeutics.

One distinct disadvantage, by assay design, is the need for custom-made probes for each oligonucleotide molecule, which adds additional time and cost to methods. Furthermore, FL probe design can impact its binding affinity toward a target oligonucleotide molecule, which can directly change assay attributes including linearity and extract stability [40,41]. LC–FL assay throughput is low with a cycle time of 20–30 minutes per analysis. Last but not the least, it should be noted that LC–FL technology is currently patented [103].

LC-MS

LC–MS-based methodologies have been increasingly employed for quantitative bioanalysis of therapeutic oligonucleotides since approximately 2000 [40–42] and extensively reviewed [35,43–45]. LC–MS is the established golden standard for bioanalysis of small molecules (drugs and biomarkers) due to its unparalleled accuracy, precision, specificity, dynamic range, throughput and widespread use. These attributes are equally applicable to oligonucleotide bioanalysis. There are two common types of mass spectrometers: 1) triple quadrupole (QQQ) and 2) high-resolution mass spectrometry (HRMS), such as TOF-MS and Orbitrap. QQQ is most widely utilized in support of absolute quantitation using multiple reaction mode (MRM).

Precursor ions of a specific m/z (or charge state) are filtered or selected by the first stage quadrupole and subject to collision induced dissociation (CID) in a second quadrupole, where fragment ions (i.e., fingerprint of the precursor ions) of a specific m/z are filtered by the third stage quadrupole. This is also referred to as tandem mass spectrometry mode (MS/MS) or LC–MS/MS when combined with LC. MS/MS offer unique assay selectivity intrinsic to an oligonucleotide molecule.

Oligonucleotides are ionized by electrospray ionization, where polyanionic oligonucleotides generate multiply charged negative ions. Ion-paring reserved-phase liquid chromatography is generally the choice of separation prior to mass spectrometric detection of oligonucleotides. Ion paring agents offer positively charged organic moieties to bind with the naked negatively charged polyphosphate backbones of oligonucleotides. The masking of these negative charges enhances interaction between ion-paired oligonucleotides and reversed-phase stationary phases leading to improved retention and peak shapes. Importantly, the RPLC can readily separate oligonucleotides from its metabolites (such as n-1, n-2, etc.). Hexafluoroisopropanol and triethylamine are commonly used ion-pairing agents that achieve a balance between separation and MS sensitivity [46,47]. Many ion-pairing reagents, including triethylammonium bicarbonate and triethylammonium acetate, are used for oligonucleotide separations [47,48]. Chromatographic performance of oligonucleotides such as peak width and retention time can be optimized by ion-pairing agents [49].

Sample preparation plays a key role in the development of a successful LC–MS/MS method for oligonucleotide bioanalysis. This stems from the fact that it is challenging, if not impossible, to produce stable isotope-labeled internal standards (SLISs) for oligonucleotides. In small molecule bioanalysis using LC–MS/MS, a SLIS, which is physiochemically identical to its unlabeled analyte, can seamlessly compensate an analyte's matrix effects (i.e., ionization enhancement or suppression). This attribute leads to robust assay performance. In comparison, a structurally similar oligonucleotide is often used as an analog internal standard for a target oligonucleotide analyte. Additionally, an oligonucleotide analog internal standard (IS) needs to be chromatographically separated from the oligonucleotide analyte to eliminate mass spectral interference. As such, an analog oligonucleotide IS will likely not compensate for an analyte's matrix effects to the same extent of a SLIS. Therefore, it is imperative to develop an extraction method that provides extracts that are as clean as possible to minimize downstream matrix effects in the ESI processes. To that end, a combination of liquid–liquid extraction (LLE) with solid phase extraction (SPE) can provide optimal extraction for oligonucleotides from biological matrices [32].

LLE extracts oligonucleotides from matrices while SPE further removes residual matrix components. In the authors labs, the combined LLE–SPE extraction strategy has proven to be highly effective in support of oligonucleotide bioanalysis in plasma and tissues [50]. Major drawbacks of such an elaborate extraction are that: 1) it is time consuming due to long extraction, 2) it is prone to human errors due to complexity and 3) the lack of automation. The use of Phenomenex® Clarity®-OXT SPE plates, as a single-step SPE, were reported to extract intact duplex siRNA oligonucleotides from plasma and urine [32,51]. The authors' lab has success in using Phenomenex® Clarity®-OXT SPE plates for regulated bioanalysis of siRNA.

HRMS is an alternate type of mass spectrometer that has been used for bioanalysis of oligonucleotides [44,51,52]. HRMS, e.g., TOF-MS and Orbitrap, can provide high resolving power up to over 100,000 (e.g., at m/z 200). In comparison, QQQ generally operates in the unit resolution with a full width at the half maximum (FWHM) being 0.7 Da. HRMS can separate chemical species with a very close m/z, which otherwise would be detected as one single peak in a QQQ instrument. As such, HRMS provides an additional selectivity via high mass spectral resolution. HRMS can be operated in the LC–HRMS or LC–MRM high resolution modes. The operating principle is similar between QQQ and HRMS for oligonucleotide bioanalysis. When developing LC–HRMS method, it is important to optimize required resolution to achieve sensitivity and selectivity in the time scale of a chromatographic peak [32]. One major advantage of HRMS is the ease of use, notably no need of compound specific tuning.

It is possible to process data post-acquisition, which is especially useful to investigate metabolites from study samples. The industry is still adapting LC–HRMS for regulated oligonucleotide bioanalysis. With the expanded use of the technology and the continuing technology development, the use of LC–HRMS will become more widely accepted in support of oligonucleotide drug development.

LC–MS/MS or LC–HRMS can routinely reach a LLOQ in the range of 5–25 ng/mL [47]. This level of sensitivity is adequate to support most preclinical studies in obtaining an oligonucleotide's concentrations in plasma and various tissues. Further sensitivity improvement is expected via continuing technological advancement and methodology optimization in the LC–MS field. For instance, latest mass spectrometry always provides enhanced sensitivity. The use of microflow separation [53] can increase LC–MS assay sensitivity for oligonucleotides. Optimization in workflow led to an LLOQ of 0.2 ng/mL for a GalNAc-conjugated 16-mer oligonucleotide in human plasma [54]. A novel method, combining oligonucleotide hybridization and LC–MS/MS technologies, was reported to have achieved a LLOQ of 0.5 ng/mL for an ASO using 100 µL plasma [55]. LC–MS methodology, with its versability and advantages, will be an essential tool – complimentary to LBA – to support development of oligonucleotide therapeutics.

In all, the three major chromatographic methodologies, i.e., LC–UV, LC–FL and LC–MS/MS or LC–HRMS, offer distinct advantages and disadvantages for the quantitation of therapeutic oligonucleotides in biological matrices. The selection of a suitable chromatographic approach ultimately depends on the specific project needs in the stage of an oligonucleotide development. Considerations include, but are not limited to, assay sensitivity, specificity, throughput and level of rigor (i.e., precision and accuracy). The need for profiling potential chain-shortened metabolites can also influence methodology selection [56].

Potential synergies and strategies to utilize chromatographic and LBA platforms

While chromatographic and LBAs offer diverse methodologies for the quantification of oligonucleotides, both can be utilized to establish a robust PK/DM profile by taking advantage of the benefits of both platforms. For example, the ability to differentiate the parent compound and assess the major metabolites, along with their time course, could be conducted initially via LC–MS in early exploratory preclinical models, then validated.

Once the characterization of the major metabolites is completed, a strategy can then be implemented to determine which LBA methodology is most appropriate with the goal to take advantage of the sensitivity and high-throughput analysis in preclinical and clinical studies. If it is known that the major metabolites are 3' truncated, a single ligation hELISA or hECL with S1 nuclease can be implemented. Additionally, a dual-probe method might be chosen wherein the probe design is altered as discussed above, adjusting the number of nucleotides on the capture or detection probe to minimize quantitation of the major metabolites established via LC–MS analysis.

An upfront strategy can be discussed prior to initiation of *in vitro* and preclinical exploratory studies that would consider the priorities for development of the compound and the end goals, assessing if exploration of metabolites, sensitivity, dosing etc. are most important. The strategy would allow for an initial road map of how to select platforms and methodologies and at which point an adjustment might be made moving from chromatographic to LBA methods, or if the need would be to remain with chromatographic methods, and perhaps LBA, based on the compound itself (i.e., based on the length of the oligo). As the initial studies begin, it is understood that strategies may need to be adjusted based on the results generated and, as an example, it might be found that the major metabolites are different than expected. As such, downstream methodologies may need to be reconsidered (i.e., moving from a single ligation hELISA to a dual-probe hECL). Overall, the diverse methodologies for quantification of oligonucleotides are beneficial in that they can be utilized cooperatively to put together a robust package of data characterizing the oligonucleotide therapeutic in preclinical and clinical studies.

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INFOGRAPHIC

The exciting world of oligonucleotides – how far have we come?

Over 2 years ago, Bioanalysis Zone hosted an engaging Spotlight on oligonucleotides. This brought together leading experts within the bioanalytical community to discuss their experience with oligonucleotides and the current challenges facing the field, in addition to looking at what the future could hold.

The 2017 Spotlight survey highlighted key trends regarding what types of oligonucleotides are studied, assay formats used to analyze oligonucleotides and opinions on methodology and guidance for oligonucleotide analysis. To explore how this field has changed over the last 2 years, a second survey was conducted in 2019, enabling key variations in data to be analyzed.

A full comparison of the statistical data can be seen below.

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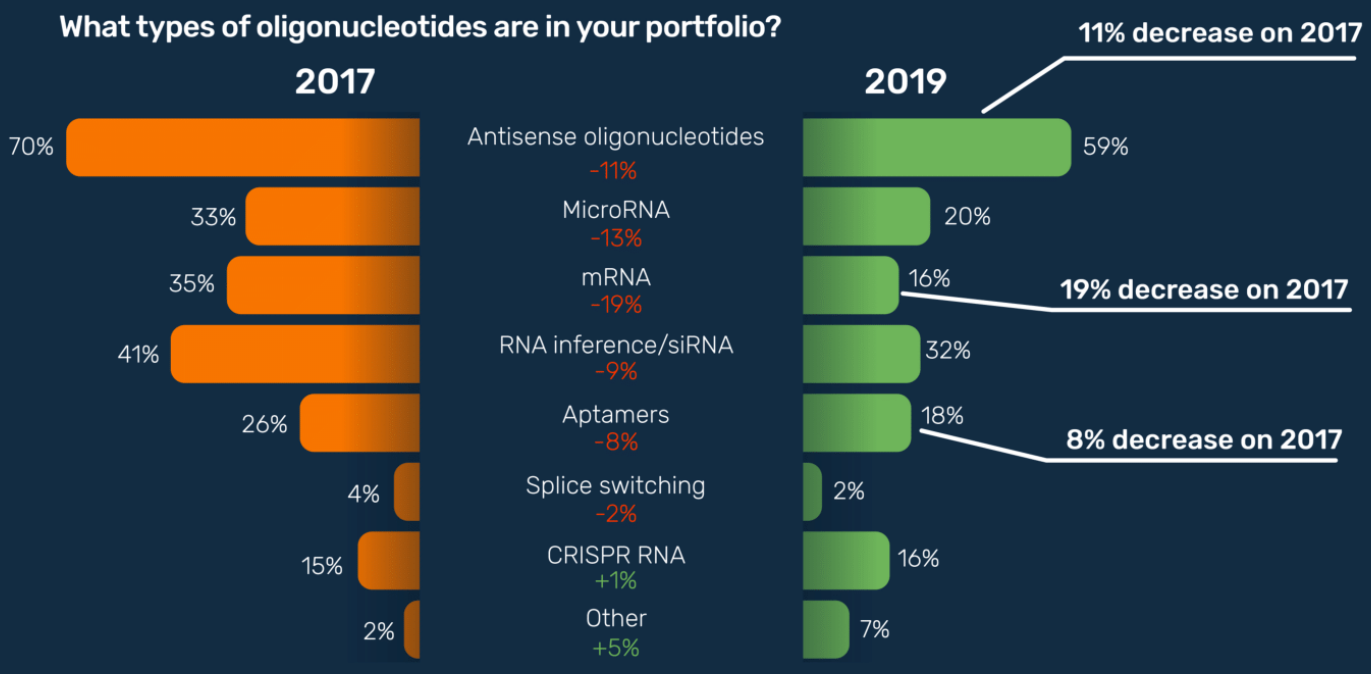
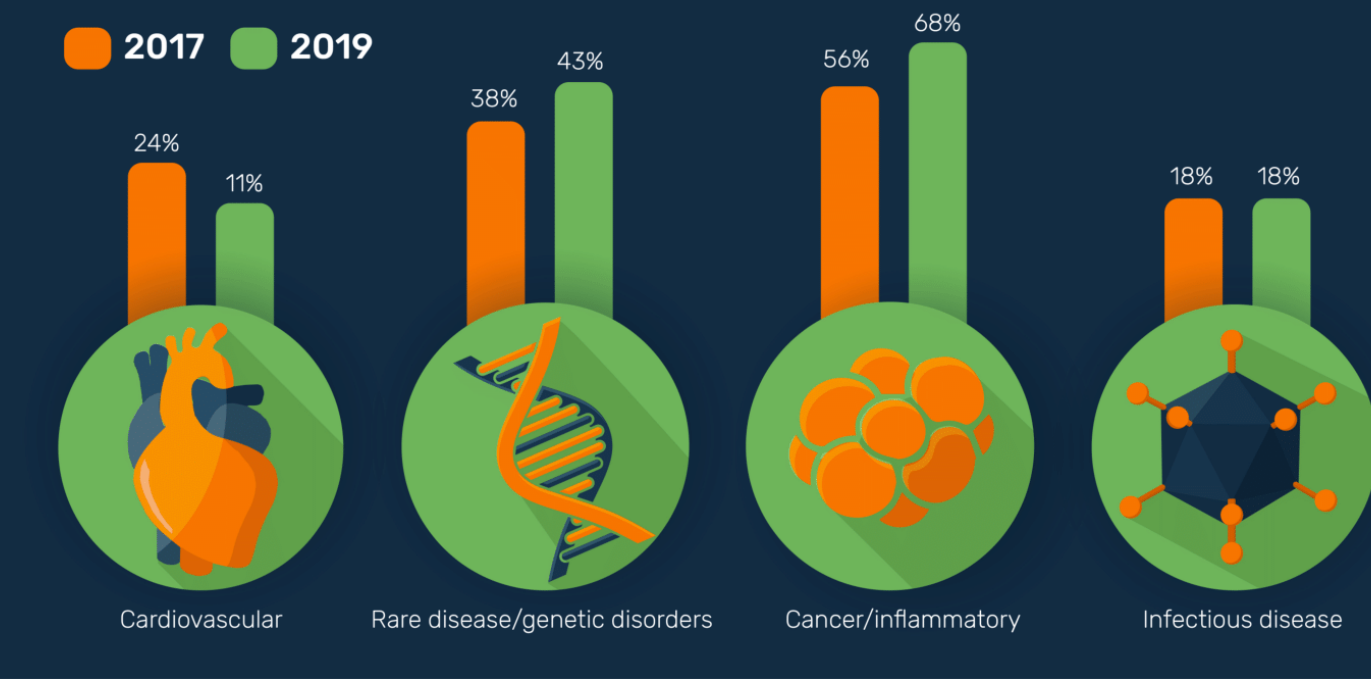
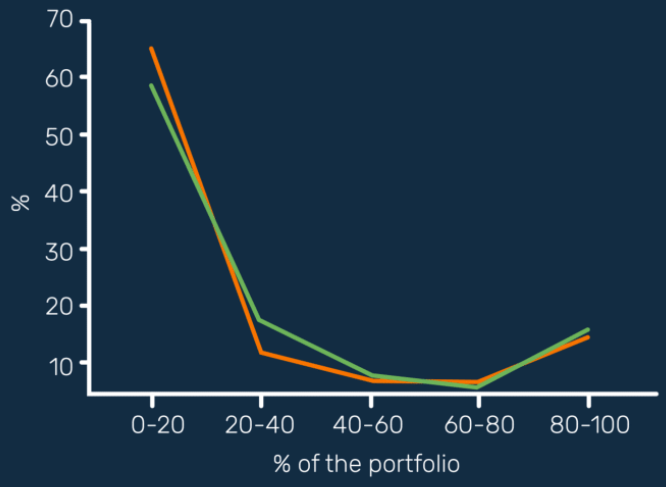
Key trends in oligonucleotide analysis

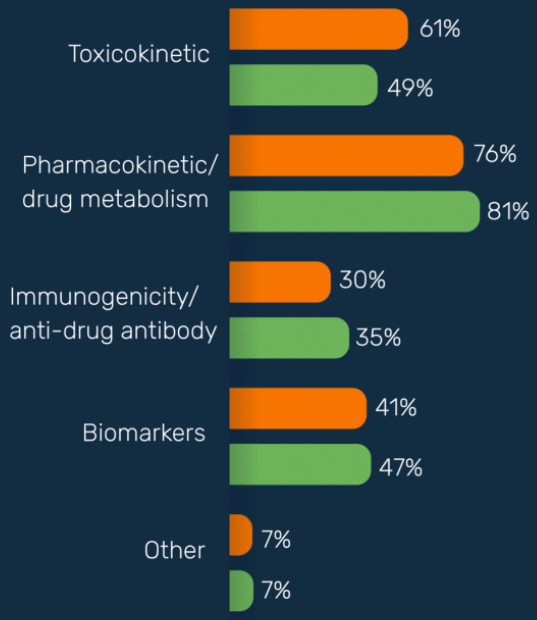




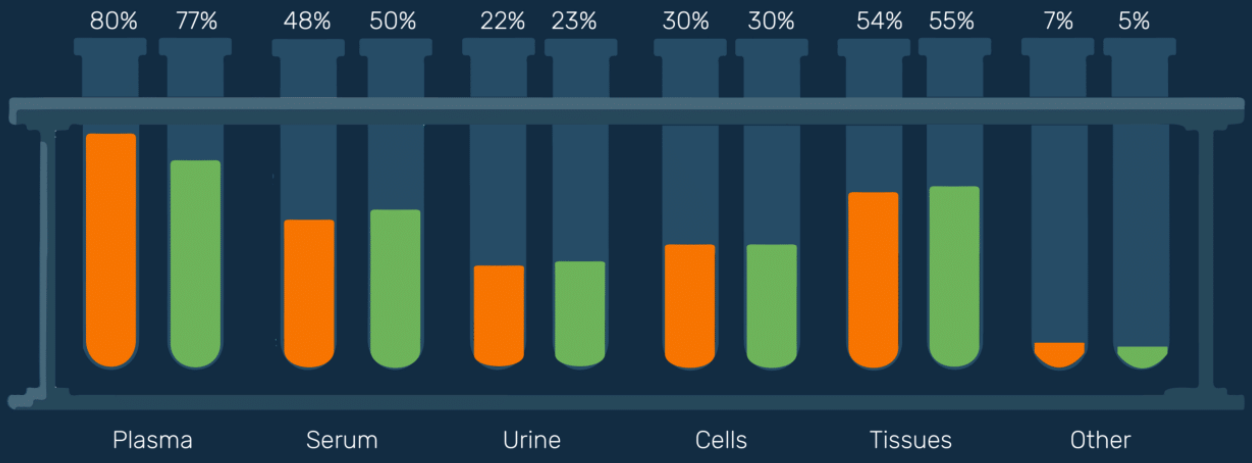
What percentage of the portfolio is oligonucleotides compared to traditional small molecules and large molecules?



What type of oligonucleotide analysis are you conducting?

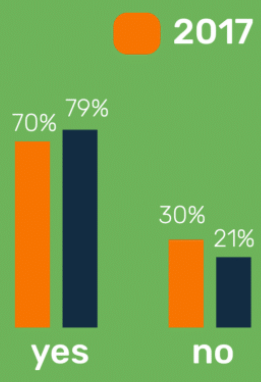


Types of matrices that are used for analyzing oligonucleotides:

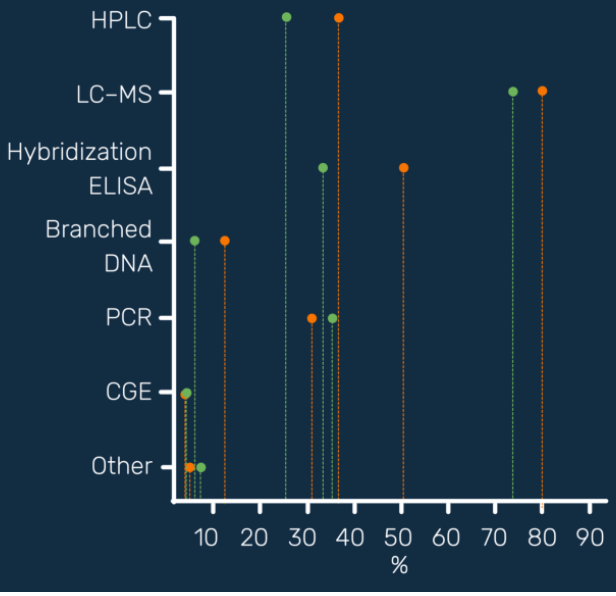


Methodology and guidance for oligonucleotide analysis

For the majority of the time, does existing methodology meet the project needs?



Assay formats used to analyze oligonucleotides:



Where are the gaps in oligonucleotide research?

- Specificity/ selectivity — 2017 71% 2019 40%
- Sensitivity — 2017 79% 2019 60%
- Robustness — 2017 64% 2019 30%
- Regulation of tissue analysis — 2017 29% 2019 30%
- Other — 2017 7% 2019 10%

Are the current regulatory guidance's satisfactory for the analysis of oligonucleotides?

YES	NO
2017 52%	2017 48%
2019 48%	2019 52%

What do you think are the main challenges of bringing oligonucleotides to market?

	2017	2019
Cost	28%	44%
Production time	7%	21%
Facility capacity	11%	28%
Issues with technology	35%	36%
Requiring highly skilled experts	23%	26%
Issues with off target toxicity	19%	26%
Competition from other companies	2%	3%
Regulatory acceptance	32%	23%

Future trends in oligonucleotide analysis

In the next 5 years, do you think the percentage of oligonucleotides in your company portfolio will...

	2017	2019
Increase	52%	71%
Stay the same	44%	24%
Decrease	4%	5%

What new methodologies/instrumentation do you think will improve current analysis?

2017

- Better cleaning of instruments
- New PCR
- Microflow LC-HRMS, qPCR
- One that offers more sensitive assays

2019

- HPLC
- HRMS
- Hybrid LC–MS assays
- More sensitive detection

About the respondents

Location:

	2017	2019
North America	52%	58%
Europe	22%	25%
Asia	21%	13%
Rest of World	5%	4%

Job title:

	2017	2019
Technician	7%	8%
Chemist/Scientist	34%	34%
Manager/Group Leader	31%	29%
Director/CEO/VP	19%	26%
Other	9%	3%

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60 seconds with Eric Tewalt: oligonucleotide quantification and validation

Dr Eric Tewalt serves as an associate director in the immunochemistry department at PPD Laboratories Bioanalytical Lab (VA, USA). He oversees an oligonucleotide and nucleic acid modality-dedicated team that conducts method development, validation and bioanalysis of immunogenicity, pharmacokinetic and regulated biomarker samples in support of sponsor preclinical and clinical trials in a regulated environment. Dr Tewalt has over 9 years of experience in the bioanalytical field and an additional 9 years of experience in immunology, infectious disease and cancer research with extensive knowledge in assay and experimental design.

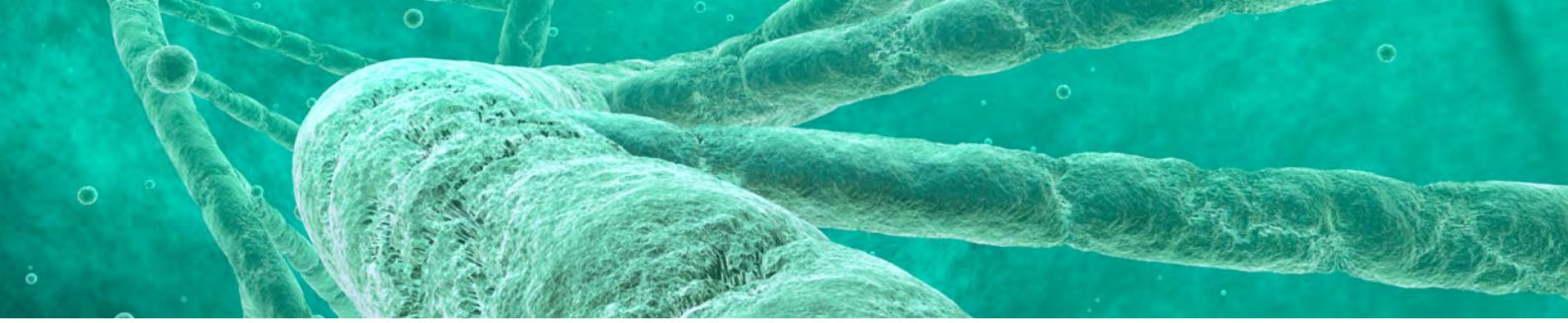
Q In what medical fields are oligonuclotides currently paving the way for drug and therapeutic development?

Oligonucleotides are currently being utilized to focus on the treatment of a wide array of diseases. In general terms, there are many being developed to target cancer, viral infections and rare diseases. Given the unique nature of oligonucleotides and their ability to target and potentially alter the production of any protein, the possibilities are endless. Oligonucleotides offer the potential to now target diseases that were previously thought to be undruggable with more traditional large and small molecule therapeutics. With that in mind, there is currently an increased focus in the rare disease space, specifically including muscular dystrophy, diseases of the central nervous system and cardio-metabolic diseases, among others.

Q What advantages do oligonucleotides provide in enabling targeted therapeutics and what challenges do they pose?

As indicated, the main advantage of oligonucleotides is that they can target a multitude of diseases, including those thought ot be undruggable via small molecule or biologics. The ability to target multiple disease states is possible due to the ability to design oligonucleotides to target and alter protein expression. Examples include, but are not limited to, protein degradation (antisense oligonucleotides and siRNA), increased or altered protein expression (splice-switching oligonucleotides), protein inhibition (aptamers) etc. Additionally, since oligonucleotides are comprised of naturally occurring nucleotides, there is a reduced possibility of immunogenicity specifically against the oligonucleotide itself (noting that the delivery mechanism could induce immunogenicity or have pre-existing antibodies). Alternatively, the challenges posed by oligonucleotides as therapeutics include concerns regarding half-life and delivery to the site of disease, though recent advances have helped to overcome these obstacles.

Historically, oligonucleotides administered in the periphery were rapidly degraded within minutes. However, chemical and backbone modifications enabling nuclease resistance increased half-life significantly. Despite this, there were limited options for targeted delivery and as such, highly concentrated levels of oligonucleotides as therapeutics were administered and on a more frequent basis. While high concentrations ensured that oligonucleotides reached the site of action, oligonucleotides would also deposit throughout the body. However, advances have been able to overcome such concerns, either via an altered route of administration (intrathecal for the central nervous system, intravitreal for diseases of the eye, intranasal for disease of the lung etc.), or via drug delivery mechanisms (GalNAc conjugation and lipid nanoparticles for liver, monoclonal antibody conjugation to target muscle etc).



Q What are the current methods of validation for oligonucleotides and how do you anticipate these will develop in the future?

The majority of oligonucleotide quantification takes place via chromatographic platforms, such as LC–MS and LC–FL (patented technology) and ligand binding assays such as ELISA and electrochemiluminescent platforms. The two platforms really complement one another and when combined, offer appropriate sensitivity and selectivity for the parent compounds. Depending on the length of the oligonucleotides, molecular techniques such as qPCR can be utilized, noting that it combines the sensitivity of ligand binding assays with the large dynamic range typically seen with chromatographic methods. While not currently widely used, platforms such as the Quanterix and Gyros, amongst others, may be utilized as they offer additional advances over standard platforms (further increased sensitivity, fully automated, minimized sample volumes), but can be limited due to kit availability and consumables that are produced via a single sourced vendor.

Q What is the current landscape of oligonucleotide quantification with regards to instrumentation, techniques etc.?

As discussed, the majority of oligonucleotide quantification takes place via chromatographic or ligand binding methodologies. Currently, we utilize the 2018 US FDA Bioanalytical Method Validation Guidance for Industry as well as the 2012 EMA Guideline on bioanalytical method validation. The 2018 US FDA guidance clearly outlines experimental design and criteria expectations for both chromatographic and ligand binding assays and includes information on biomarkers, diagnostic kits and more. Since regulatory guidances are not released on a yearly basis, White Papers produced from various conferences, such as WRIB and the Global CRO Consortium, provide additional up-to-date information and perspectives on any potential alterations or adjustments that could be utilized to produce more robust methodologies. Our current paradigm with respect to oligonucleotide method validation typically includes following the regulatory guidances, as well as to include additional experimentation that might be relevant to oligonucleotides specifically such as metabolite quantitation and potential interference testing. During method development, if there are any adjustments that are to be made to criteria specified in the regulatory guidances, it must be made and discussed with sound scientific justification. We have typically found that oligonucleotide PK methods fall within the criteria limits within the regulatory guidances, while methods to validate regulated biomarkers may be more variable and require alteration of accuracy and precision criteria with scientific justification. Moving forward, we believe that oligonucleotide methods will continue to utilize chromatographic and ligand binding methodologies.

However, there will be an increased focus on developing more sensitive methods and mechanisms to increase selectivity for the parent compound. In addition, we are seeing an expansion in the number of matrices that are being utilized for PK assessment as well as bioanalytical distribution in preclinical models so that correlations can be inferred in human studies based on drug levels in plasma or serum. With that in mind, discussions on how to assess oligonucleotides in tissue and rarer matrices, such as cerebrospinal fluid and matrices of the eye, are gaining importance. Examples of issues that would require further discussion include stability assessments, potential use of surrogate or artificial matrices, method qualification vs validation and, at the current time, supply chain issues and matrix availability. Finally, while not considered oligonucleotide quantification, we have seen an increase in the validation of immunogenicity methods for oligonucleotides. We currently utilize the 2019 FDA Immunogenicity Method Validation Guidance (Immunogenicity Testing of Therapeutic Protein Products – Developing and Validation Assays for Anti-Drug Antibody Detection) to develop and validate oligonucleotide immunogenicity methods. Historically, it was thought that oligonucleotides would not be immunogenic given that they are made from nucleotides that are endogenous, but the chemical and backbone modifications, as well as the various drug delivery mechanisms, have driven the need for a more thorough assessment of the potential to elicit anti-drug antibody responses *in vivo.*

Research Article
SPECIAL FOCUS ISSUE: The Exciting World of Oligonucleotides

Bioanalysis

Discovery of a novel deaminated metabolite of a single-stranded oligonucleotide *in vivo* by mass spectrometry

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Aim: A novel single-stranded deaminated oligonucleotide metabolite resulting from a REVERSIR™ oligonucleotide was discovered and identified in monkey liver after subcutaneous administration. **Results & methodology:** REVERSIR-A and its metabolites were extracted from biological matrices by solid phase extraction and analyzed using LC coupled with high-resolution MS under negative ionization mode. A novel 9-mer metabolite of REVERSIR-A, resulting from deamination of the 3′ terminal 2′-*O*-methyl-adenosine nucleotide to 2′-*O*-methyl-inosine, was discovered at significant levels in monkey liver. The metabolite's identity was confirmed by LC–MS/MS. **Conclusion:** This report describes the first observation of a long-chain deaminated metabolite of a single-stranded REVERSIR oligonucleotide *in vivo* in monkey liver.

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Keywords: A-to-I editing • adenosine • deaminated metabolite • deamination • inosine • MS • metabolism • oligonucleotide • RNAi • small interfering RNA

Small interfering RNAs (siRNAs), a class of double-stranded RNA molecules, operate by silencing the expression of specific genes through incorporation into the RNA-induced silencing complex, which then degrades the targeted mRNA that has a nucleotide sequence complementary to the guide strand of the siRNA [1,2]. Thus, RNAi therapeutics are useful for treating diseases that stem from problematic proteins by reducing synthesis of the targeted proteins through gene expression silencing at the mRNA level before translation begins. The first RNAi therapeutic, ONPATTRO® (patisiran), was approved in the USA and in Europe in 2018 [3,4], and innovative RNAi medicines are under development for the treatment of other serious life-threatening diseases [5–7]. With advances in delivery, design and chemistry of siRNA compounds, potent mRNA knockdown in the liver can last for several months [8,9]. The prolonged pharmacological effect, an attribute unique to RNAi therapeutics, provides the opportunity of dosing at less frequent intervals. In cases where fine control of siRNA pharmacological activity and rapid reversal of target gene silencing effects would be beneficial, REVERSIR™ molecules can be administered. REVERSIRs are short single-stranded oligonucleotides designed to bind to the guide strands of siRNA with high affinity [10]. Upon binding to a RNA-induced silencing complex-loaded siRNA guide strand, REVERSIR molecules block the recognition and cleavage of target mRNAs, reversing the effect of gene silencing.

Understanding the metabolism of therapeutic oligonucleotides *in vivo* is essential for successful drug development. Metabolite identification provides important information to guide the design of better oligonucleotide therapeutics, to understand the fate of oligonucleotide drugs *in vivo*, and to confirm adequate exposure of clinically relevant metabolites in nonclinical safety studies. In the past decade, the metabolism of oligonucleotide drugs has been studied extensively using LC–MS methods because of the high selectivity and sensitivity such methods can provide.

Oligonucleotides are primarily metabolized *in vivo* by exo- and endonucleases that cleave phosphodiester or phosphorothioate linkages at the ends of the strand or at internal positions, respectively. Beverly *et al.* studied the ocular metabolism of siRNA-027 *in vivo* using ion-pair reversed-phase LC-ESI–MS and identified that the siRNA-027 duplex was cleaved primarily from the 5′-end of the guide strand and the 3′-end of the passenger strand [11].

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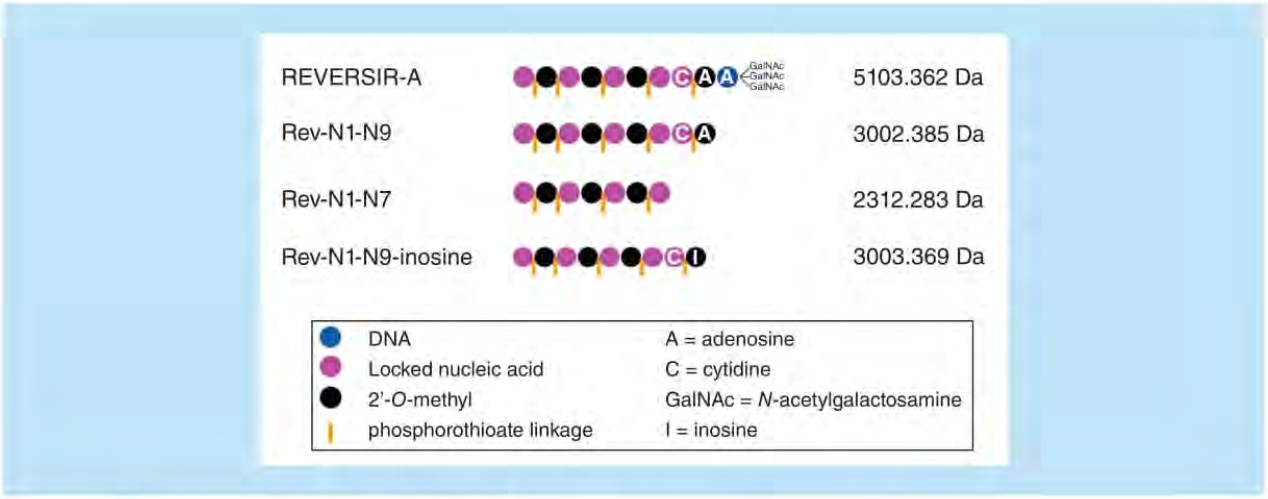

Figure 1. Sequence and monoisotopic mass of REVERSIR-A and its major metabolites.

Zou *et al.* investigated the *in vitro* metabolism of a siRNA, HBV263, in serum and liver microsomes using high-resolution accurate MS [12]. They found that the passenger strand was more vulnerable to exonuclease activity in serum, while the guide strand was preferentially degraded by endonucleases and possibly also by exonucleases in liver microsomes. The *in vivo* and *in vitro* metabolism of antisense oligonucleotides (ASOs) has also been studied extensively using ion-pair reversed-phase LC-ESI–MS [13–15]. The primary metabolic pathway observed for ASOs was cleavage by 3′-exonucleases followed by cleavage by 5′-exonucleases. Metabolism mediated by endonucleases was minor.

N-acetylgalactosamine (GalNAc)-conjugated oligonucleotides have been developed to improve uptake by hepatocytes and thereby increase potency of liver-targeted therapeutics [16]. The metabolism of the trivalent GalNAc ligand has been investigated in rat and monkey by radiolabeling the linker part of the ligand [17]. GalNAc ligand-associated metabolites identified by LC–MS included loss of GalNAc sugars from the ligand, and mono-, di-, tri- and tetraoxidations on the different branching arms, and cyclic metabolites formed by internal esterification.

Collectively, to date, metabolites identified for therapeutic oligonucleotides have been limited to chain-shortened metabolites generated by nuclease activity, and metabolites produced from the biotransformation of the GalNAc ligand [11–15,17–23]. In the present paper, we describe the discovery and characterization of a novel-deaminated metabolite of REVERSIR-A *in vivo* in monkey liver using LC coupled with high-resolution mass spectrometry (LC-HRMS). The identity of this deaminated metabolite, the most abundant metabolite in monkey liver, was confirmed by LC–MS/MS analysis and the concentration was determined by LC-HRMS analysis.

Experimental

Chemicals & oligonucleotide compounds

Optima™ LC–MS grade acetonitrile (ACN), methanol (MeOH) and water (H_2O) were purchased from Fisher Scientific (PA, USA). N,N-diisopropylethylamine (DIEA, ≥99%), EDTA, ≥99%, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, ≥99%), ammonium acetate (≥98%), ammonium bicarbonate (≥99.5%) and tetrahydrofuran (THF, ≥99.9%) were purchased from Sigma-Aldrich (MO, USA). REVERSIR-A (Figure 1), metabolite standards and the internal standard (an analog of REVERSIR-A having a different molecular weight) were synthesized at Alnylam Pharmaceuticals (MA, USA) to ≥85% purity as described previously [10]. The identities and purities of all oligonucleotides were confirmed by ESI–MS and ion exchange HPLC, respectively.

In vivo monkey study

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. Cynomolgus monkeys were dosed subcutaneously in the mid-scapular region. In one study, a single subcutaneous dose of 3 mg/kg REVERSIR-A was administered. Plasma (predose, 0.25, 0.5, 1, 2, 4, 8, 24 and 48 h postdose, ~0.25 ml each time point), liver biopsy (0.083, 0.167, 0.33, 1, 2, 4, 7, 14 and 28 days postdose, two specimens ~100 mg each time point) and urine (0–8, 8–24 and 24–48 h postdose, ~20 ml

per animal) samples were collected. In another study, cynomolgus monkeys received five weekly doses of 50 mg/kg REVERSIR-A subcutaneously on day 0, 7, 14, 21 and 28. Animals were anesthetized with sodium pentobarbital, exsanguinated and necropsied on day 29. Following necropsy, liver samples (two specimens ~1 g each) were collected. All samples were stored frozen at approximately -70°C until analysis.

Sample preparation

Frozen liver samples were ground at cryogenic temperatures in a 2010 Geno/Grinder (SPEX SamplePrep, NJ, USA) without addition of water or buffer. Ground liver powder samples were resuspended in Clarity® OTX™ Lysis-Loading Buffer (Phenomenex, CA, USA) at 100 mg/ml and incubated at ambient temperature for 3 h with shaking. siRNA compounds were confirmed to be stable during this incubation process by spiking known concentrations of compounds into ground liver powders and measuring the concentrations after incubation. The measured concentrations were within ±15% of spiked concentrations (data not shown). Pooled liver lysate, plasma and urine from untreated animals were spiked with REVERSIR-A and metabolite standards to generate calibration standards and quality control (QC) samples. Internal standard was spiked into calibration standards, QCs and samples at 400 ng/ml concentration. The calibration curve ranged from 100 to 50,000 ng/g for liver samples, and from 10 to 5000 ng/ml for plasma and urine samples. A weighted $1/x^2$ linear regression was applied for the calibration curves.

Liver lysate, plasma and urine samples as well as QC samples and calibrators were subjected to solid phase extraction (SPE) on Clarity® OTX™, 100 mg/well, 96-well plates (Phenomenex). The SPE plate was conditioned according to the manufacturer's recommended protocol for biological fluids. Liver lysate, plasma and urine samples, controls and QCs were mixed with lysis-loading buffer (1:9, v/v) and loaded onto the SPE plate. The plate was washed once with 1 ml of 50 mM ammonium acetate in H_2O (pH 5.5), followed by 1 ml of 50 mM ammonium acetate in H_2O/ACN (50:50, v/v, pH 5.5) and 1 ml of 5 mM ammonium acetate in H_2O/ACN (10:90, v/v, pH 5.5). Oligonucleotides were eluted with 0.6 ml of 100 mM ammonium bicarbonate in H_2O/ACN/THF (45:46:9, v/v/v, pH 8.8). Eluates were dried in a TurboVap® (Caliper Life Sciences, MA, USA) under nitrogen for 1–2 h. The dried samples were reconstituted in 150–200 µl of H_2O/HFIP/DIEA (100:1:0.1, v/v/v) with 10 µM EDTA.

Chromatographic conditions

The LC–MS mobile phases used were as follows: mobile phase A: H_2O/HFIP/DIEA (100:1:0.1, v/v/v) with 10 µM EDTA, mobile phase B: H_2O/ACN/HFIP/DIEA (35:65:0.75:0.0375, v/v/v/v) with 10 µM EDTA, mobile phase C: H_2O/MeOH/ACN (10:45:45, v/v/v).

For metabolite profiling of REVERSIR-A, a 75-µl aliquot of reconstituted sample was injected onto a PolymerX RP-1 column (5 µm, 100 Å, 50 × 2 mm; Phenomenex). Column temperature was 80°C and flow rate was 0.3 ml/min. The gradient started with 5% B, progressed to 25% B over 20 min, then increased to 70% B in 0.1 min and maintained for 1.9 min, and then washed with mobile phase C for 4.8 min. The column was re-equilibrated with 5% B for 3 min.

For the quantitation of REVERSIR-A and metabolites, a 10-µl aliquot of the reconstituted sample was injected onto a PolymerX RP-1 column (5 µm, 100 Å, 50 × 2 mm; Phenomenex). Column temperature was 80°C and flow rate was 0.25 ml/min. The gradient started with 0% B, progressed to 40% B over 4 min, and then increased to 60% B in 0.1 min and maintained for 0.9 min; the column was then washed with mobile phase C for 1 min and re-equilibrated with 0% B for 3.9 min.

For the baseline separation of the metabolites Rev-N1-N9 (REVERSIR-A metabolite formed by loss of the 3′ terminal 2′-deoxyadenosine nucleotide and the triantennary GalNAc ligand) and Rev-N1-N9-inosine (Rev-N1-N9 deaminated at the 3′ terminal 2′-*O*-methyl-adenosine nucleotide; Figure 1), a 20-µl aliquot of the synthetic standard (at 5 µg/ml) or reconstituted sample was injected onto an ACQUITY UPLC Oligonucleotide BEH C18 column (1.7 µm, 130 Å, 50 × 2.1 mm; Waters, MA, USA). Column temperature was 80°C and flow rate was 0.2 ml/min. At the start of the gradient, mobile phase B was kept at 5% B for 2 min, progressed to 20% B over 18 min, then increased to 100% in 0.1 min and maintained for 1.9 min. The column was re-equilibrated with 5% B for 8 min.

LC–MS instrument & mass spectrometric conditions

A Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, MA, USA) in combination with an Accela Open Autosampler (Thermo Fisher Scientific) and a Q Exactive mass spectrometer (Thermo Fisher Scientific)

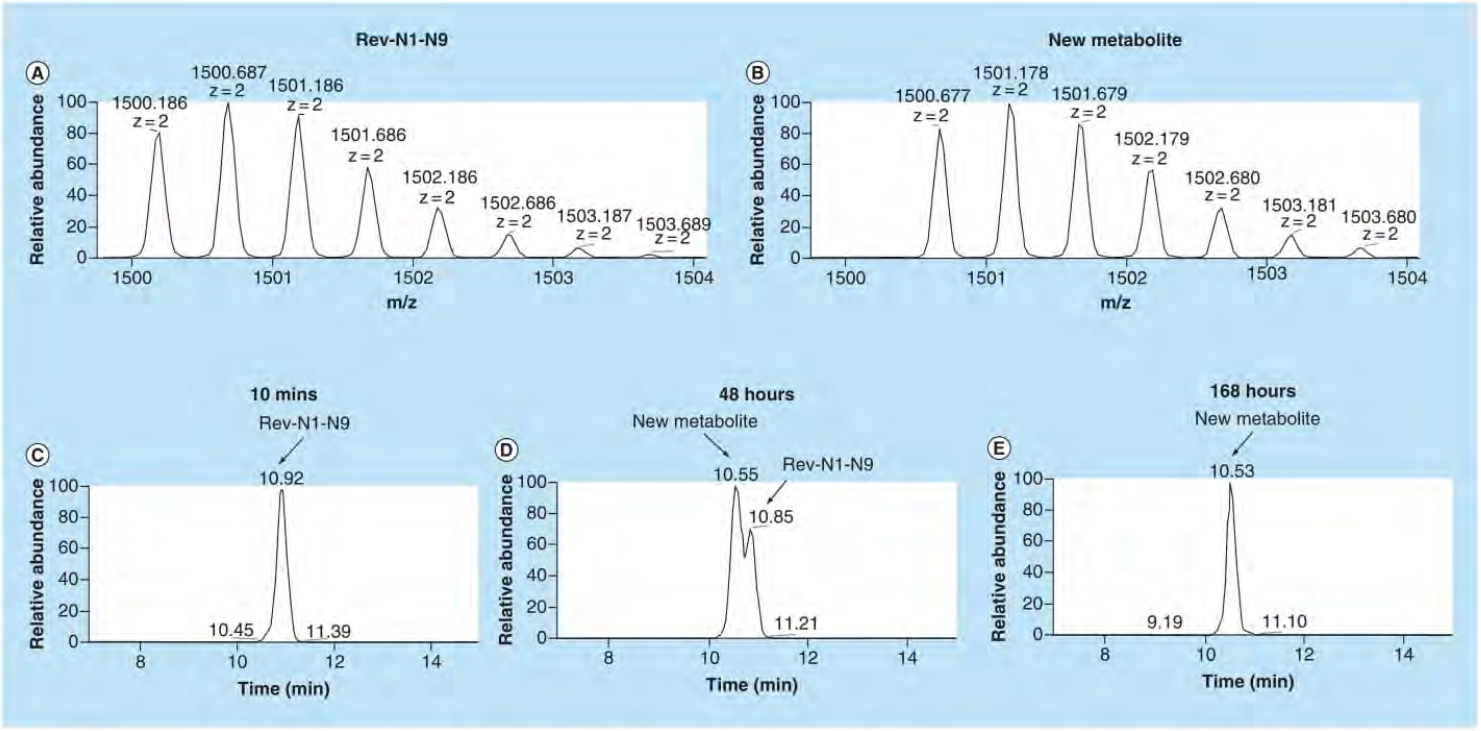

Figure 2. Mass spectra and extracted ion chromatograms of Rev-N1-N9 and the new metabolite. Mass spectra of the doubly charged ion of Rev-N1-N9 **(A)** and the new metabolite **(B)**, and extracted ion chromatograms of *m/z* 1500.68 from monkey liver samples at **(C)** 10 min, **(D)** 48 h and **(E)** 168 h following a 3 mg/kg REVERSIR-A dose.

equipped with an electrospray ionization source was used for the LC–MS analysis. All devices were controlled through Xcalibur software version 1.2 (Thermo Fisher Scientific).

The oligonucleotides were analyzed in negative ionization mode. The following ion source parameters were used: sheath gas, 40; auxiliary gas, 10; sweep gas, 1; spray voltage, 3500 V; capillary temperature, 300°C; S-lens, 60 V; auxiliary gas heater temperature, 350°C. For the metabolite profiling experiments, the Q Exactive mass spectrometer was set at full scan mode, with a scan range of 500–2000 *m/z* and resolution of 35,000 full width at half-maximum (FWHM). For the quantitation experiments, the Q Exactive mass spectrometer was set at targeted selected ion monitoring mode with an isolation window of 5 *m/z* and resolution of 70,000 FWHM. For the MS/MS experiment, the Q Exactive mass spectrometer was set at parallel reaction monitoring mode with an isolation window of 4 *m/z*, resolution of 35,000 FWHM and normalized collision energy of 15–25%.

Results & discussion

Discovery of a novel deaminated metabolite of REVERSIR-A *in vivo* in monkey liver

To understand the *in vivo* metabolism of REVERSIR-A, cynomolgus monkeys were dosed sc. with 3 mg/kg REVERSIR-A. Plasma, liver and urine samples were collected, and metabolite profiling of REVERISIR-A in each matrix was conducted [24]. Metabolites resulting from cleavage of the internucleotide phosphodiester bonds by nuclease activity were observed in monkey plasma, liver and urine, with Rev-N1-N9 and Rev-N1-N7 (REVERSIR-A metabolite with loss of three nucleotides from the 3′ end and the triantennary GalNAc ligand), being the major metabolites (Figure 1). Surprisingly, another metabolite with an observed monoisotopic mass of 3003.369 Da was found in significant abundance in monkey liver. The mass of this new metabolite was 0.984 Da greater than that of Rev-N1-N9, but did not match any phosphodiester or phosphorothioate cleavage product of REVERSIR-A. This new metabolite was observed in measurable amounts only in monkey liver, but not in monkey plasma or urine. The new metabolite eluted slightly earlier than Rev-N1-N9 under the HPLC conditions employed for metabolite profiling analysis. Representative mass spectra of the doubly-charged ions $(M\text{-}2H)^{2-}$ of Rev-N1-N9 and this new metabolite are shown in Figure 2 A & B, respectively. Because the mass difference between Rev-N1-N9 and the new metabolite was only 0.984 Da, the spectra overlap for the most part, except that the first isotopic peak at *m/z* 1500.186 was unique to Rev-N1-N9. Extracted ion chromatograms of one of the overlapping isotopes at *m/z* 1500.68 from monkey liver samples at 10 min, 48- and 168-h postdose are shown in Figure 2 C–E, respectively. At

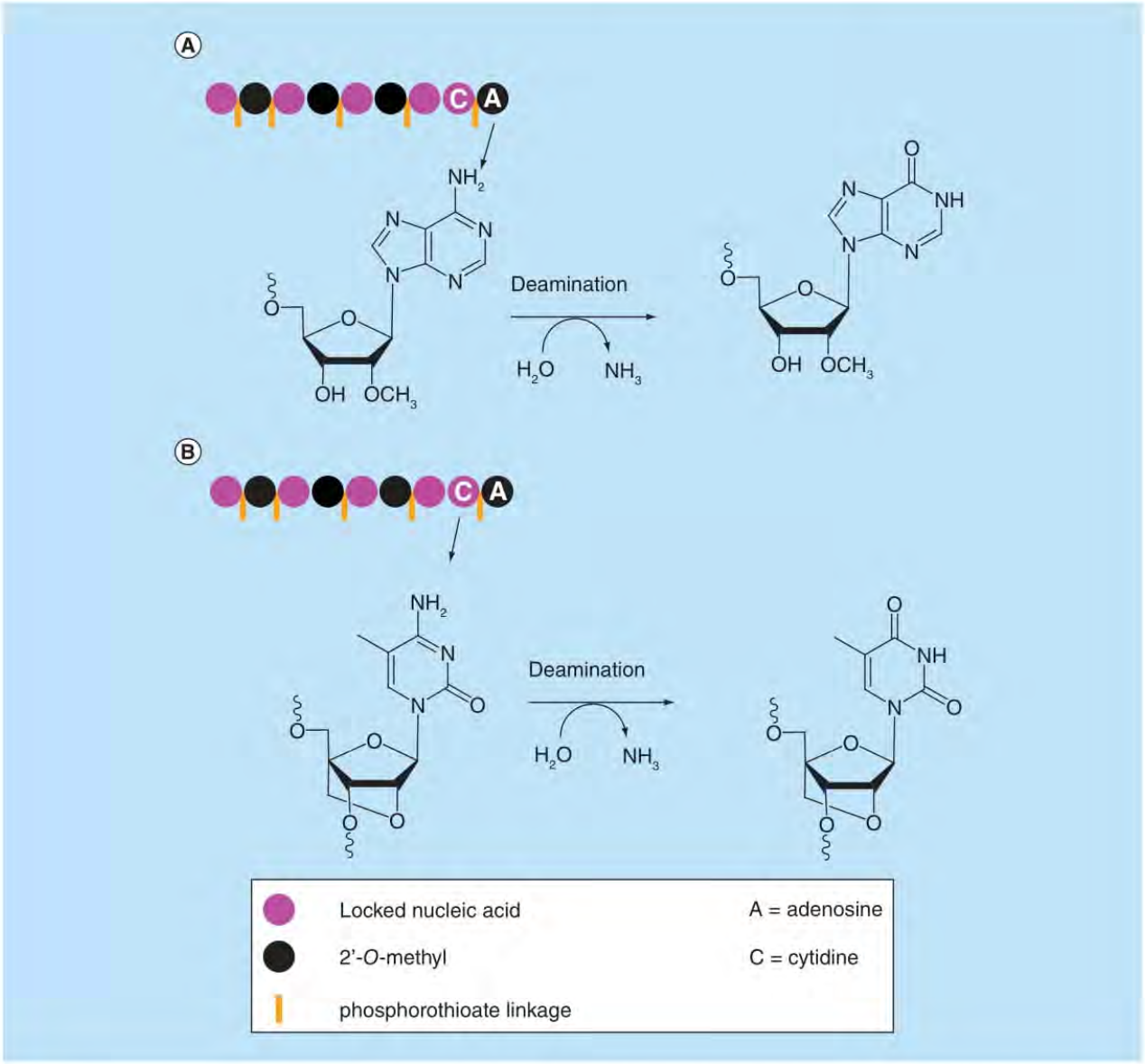

Figure 3. Deamination hypothesis. Possible deamination sites and deamination transformations of Rev-N1-N9 resulting a mass increase of 0.984 Da: **(A)** 2′-*O*-methyl-adenosine to 2′-*O*-methyl-inosine transformation or **(B)** 2′-*O*,4′-*C*-methylene 5-methyl-cytidine to 2′-*O*,4′-*C*-methylene 5-methyl-uridine transformation.

10-min postdose, only one chromatographic peak at 10.92 min was observed, the mass spectrum of which matched Rev-N1-N9. At 48-h postdose, two closely eluted chromatographic peaks were observed. Mass spectra showed that the peak eluted at 10.55 min was the new metabolite, and the peak eluted at 10.85 min was Rev-N1-N9. The relative abundance of the new metabolite continued to increase with time, while that of Rev-N1-N9 continued to decrease. At 168-h postdose, only one chromatographic peak at 10.53 min was observed, the mass spectrum of which corresponded to the new metabolite. The mass increase of 0.984 Da, which is a signature of deamination transformation, could have been the result of either an adenosine-to-inosine conversion or a cytidine-to-uridine conversion.

Confirmation of the identity of the deaminated metabolite of REVERSIR-A

The mass increase of 0.984 Da suggested deamination by addition of a water molecule and elimination of an ammonia molecule. Since an approximately 1 Da mass increase was observed with Rev-N1-N9 but not with the other major metabolite Rev-N1-N7, deamination likely occurred on either the 2′-*O*-methyl-adenosine or 2′-*O*,4′-*C*-methylene 5-methyl-cytidine at the 3′ terminus of Rev-N1-N9 (Figure 3). To determine which one of these two nucleotides was deaminated, tandem mass spectra were obtained from a monkey liver sample where the new

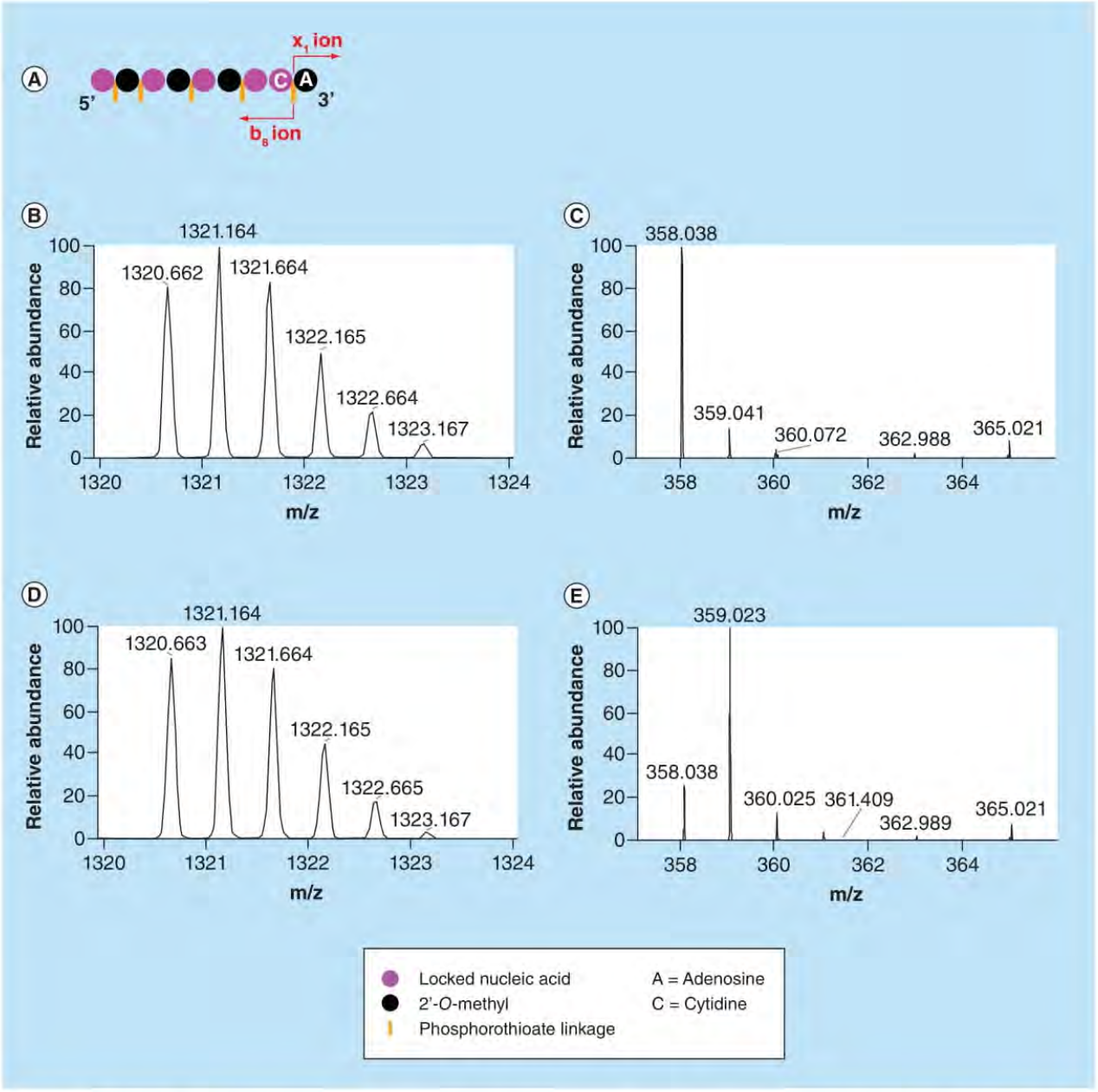

Figure 4. Tandem mass spectra showing b8 and x1 fragments of Rev-N1-N9 synthetic standard and monkey liver sample. (A) Schematic representation of b_8 ion and x_1 ion fragments on Rev-N1-N9 sequence, and tandem mass spectra showing **(B)** b_8 ion and **(C)** x_1 ion from Rev-N1-N9 synthetic standard, and **(D)** b_8 ion and **(E)** x_1 ion from monkey liver sample at day 29 after five weekly doses of 50 mg/kg REVERSIR-A.

metabolite was observed and were compared with that of the synthetic standard of Rev-N1-N9 (Figure 4). Two ions, x_1 and b_8, resulting from cleavage of the phosphorothioate bond between 2′-*O*,4′-*C*-methylene 5-methyl-cytidine and 2′-*O*-methyl-adenosine were identified in the tandem mass spectra (Figure 4). For Rev-N1-N9, the theoretical m/z of a singly-charged x_1 ion $(M\text{-}H)^-$ is 358.038, and the theoretical m/z of a doubly-charged b_8 ion $(M\text{-}2H)^{2-}$ is 1320.662 in negative ionization mode. The observed m/z values of the x_1 ion at 358.038 and the b_8 ion at 1320.662 in the synthetic standard of Rev-N1-N9 matched well with the theoretical values. In the tandem mass spectrum obtained from the monkey liver sample, the doubly-charged b_8 ion was observed at m/z 1320.663, which matched closely with the theoretical value, suggesting that nucleotides 1–8 from the 5′ terminus of Rev-N1-N9 did not change in the new metabolite. The x_1 ion of the new metabolite was observed at m/z 359.023, which is 0.985 greater than the observed m/z of x_1 ion in Rev-N1-N9 standard, confirming that the mass increase occurred on the 3′ terminal 2′-*O*-methyl-adenosine nucleotide. Therefore, the new metabolite was likely a result of deamination of the 2′-*O*-methyl-adenosine at the 3′ terminus of Rev-N1-N9 to form 2′-*O*-methyl-inosine. A less abundant

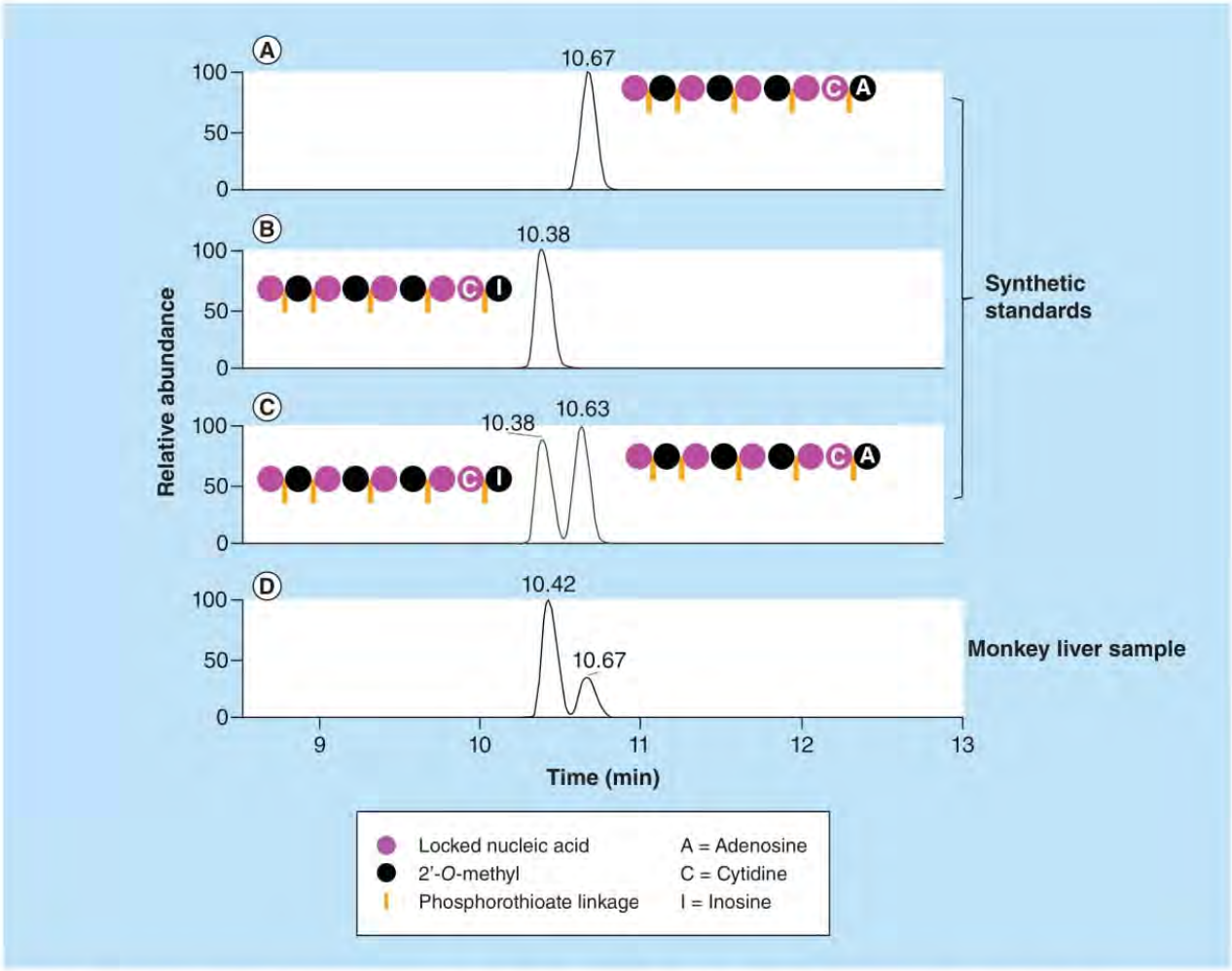

Figure 5. Extracted ion chromatograms of synthetic standards and monkey liver sample. Extracted ion chromatograms of m/z 1500.68 from **(A)** Rev-N1-N9 synthetic standard, **(B)** Rev-N1-N9-inosine synthetic standard, **(C)** mixture of Rev-N1-N9 and Rev-N1-N9-inosine synthetic standards and **(D)** monkey liver sample at day 29 after five weekly doses of 50 mg/kg REVERSIR-A.

ion at m/z 358.038 was also observed in the tandem mass spectrum acquired from the monkey liver sample and was attributed to the x_1 ion of Rev-N1-N9 which co-eluted with the new metabolite under the chromatographic conditions used in this experiment.

An oligonucleotide standard, Rev-N1-N9-inosine, with 2′-*O*-methyl-inosine replacing the 3′ terminal 2′-*O*-methyl-adenosine of Rev-N1-N9 was synthesized. A LC method was developed to baseline separate Rev-N1-N9 and Rev-N1-N9-inosine standards, and MS was used to detect the oligonucleotides. Figure 5 shows the extracted ion chromatograms of Rev-N1-N9 and Rev-N1-N9-inosine from the synthetic standards and from a monkey liver sample. The synthetic standards of Rev-N1-N9-inosine and Rev-N1-N9 eluted at 10.38 and 10.67 min, respectively. In the monkey liver sample, two chromatographic peaks at 10.42 and 10.67 min were observed, the retention time and mass spectra of which matched well with Rev-N1-N9-inosine and Rev-N1-N9 standard, respectively. The tandem mass spectra of the deaminated metabolite in monkey liver at various collision conditions also matched very well with the major fragments of Rev-N1-N9-inosine standard (Figure 6). Both results further confirmed that Rev-N1-N9-inosine was the new deaminated metabolite observed *in vivo* in monkey liver.

Determination of Rev-N1-N9-inosine concentrations in monkey liver

Concentrations of drug and primary metabolites Rev-N1-N9 and Rev-N1-N7 in monkey plasma, liver and urine, were measured by an LC–MS method using selected ion monitoring in negative ionization mode [24]. To quantitate Rev-N1-N9, the doubly charged ion $(M\text{-}2H)^{2-}$ at m/z 1500.186 and its isotopic peaks were integrated. As shown in Figure 2 A & B, the doubly charged ions of Rev-N1-N9 and Rev-N1-N9-inosine overlap for the most part. Rev-N1-N9 and Rev-N1-N9-inosine were not chromatographically separated under the LC conditions employed

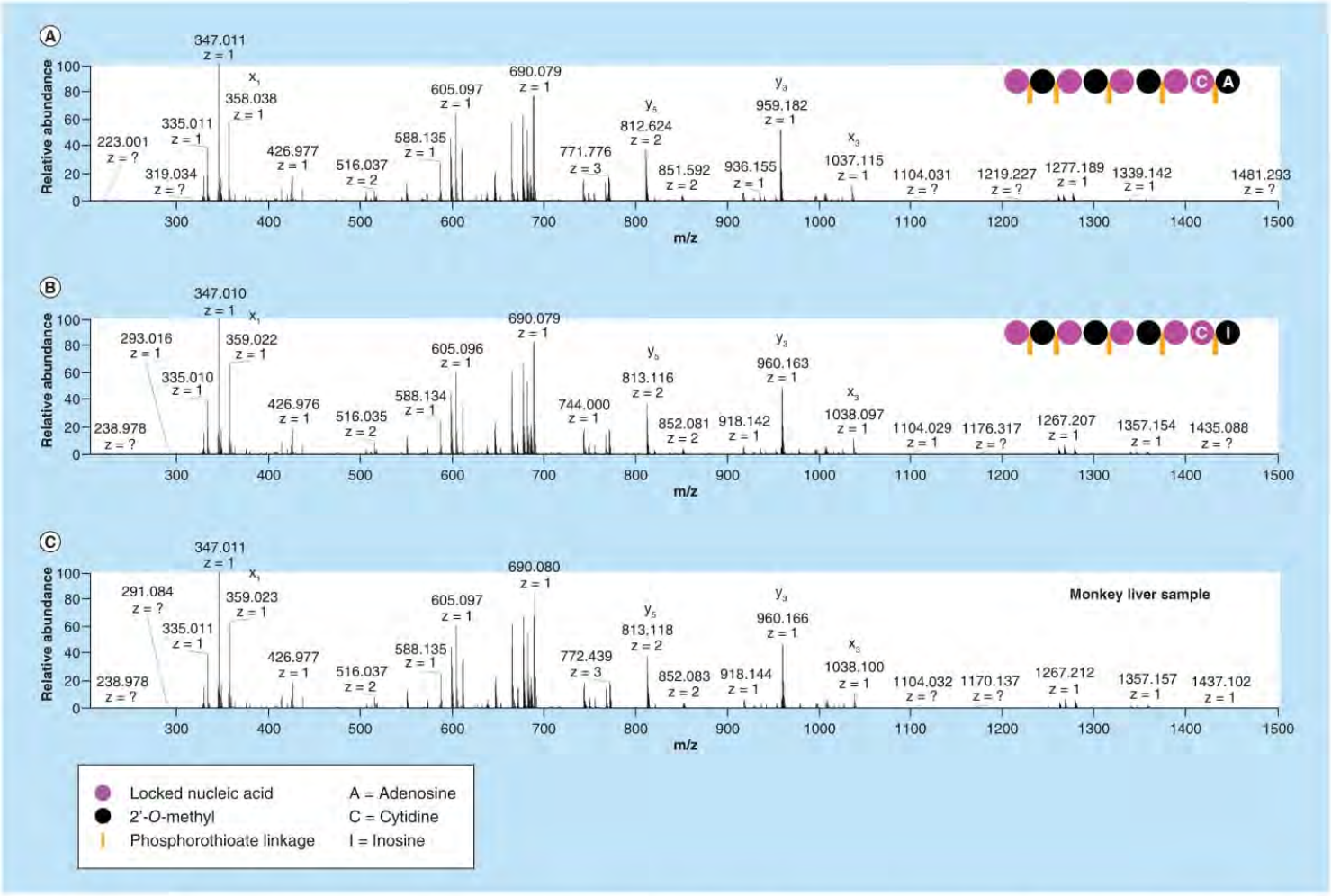

Figure 6. Tandem mass spectra of synthetic standards and monkey liver sample. Representative tandem mass spectra of **(A)** Rev-N1-N9 standard, **(B)** Rev-N1-N9-inosine standard and **(C)** monkey liver sample at day 29 after five weekly doses of 50 mg/kg REVERSIR-A. Selected fragment ions (x_1, x_3, y_3 and y_5) that can differentiate Rev-N1-N9-inosine from Rev-N1-N9 are annotated.

for the quantitation assay. Therefore, the results for Rev-N1-N9 also included Rev-N1-N9-inosine, if present in the sample. To investigate if concentrations of Rev-N1-N9 and Rev-N1-N9-inosine could be determined separately and accurately from the LC–MS data, QC samples spiked with mixtures of Rev-N1-N9 and Rev-N1-N9-inosine standards at varying percentages were prepared and quantitated against a Rev-N1-N9 calibration curve. Rev-N1-N9 concentrations were determined by integrating only the first isotopic peak at m/z 1500.186, which is unique to Rev-N1-N9, for calibration standards and QC samples. Combined Rev-N1-N9 and Rev-N1-N9-inosine total concentrations were determined by integrating all isotopic peaks from m/z 1500.186 to m/z 1503.187 for calibration standards and QC samples. Rev-N1-N9-inosine concentrations were determined by subtracting Rev-N1-N9 concentrations from the total concentrations. Using this data analysis method, the calculated Rev-N1-N9 and Rev-N1-N9-inosine concentrations were all within ±15% of the spiked concentrations (Table 1), which confirmed that Rev- N1-N9 and Rev-N1-N9-inosine concentrations could be determined separately and accurately from the overlapping mass spectrum.

This data analysis method was applied to determine Rev-N1-N9 and Rev-N1-N9-inosine concentrations in monkey liver samples from existing LC–MS data since the original liver samples had been depleted and were not available for reanalysis. The mean concentration-time profiles for REVERSIR-A and its major metabolites, Rev-N1-N9, Rev-N1-N9-inosine and Rev-N1-N7, in monkey liver following a single sc. dose of 3 mg/kg are shown in Figure 7. The profiles revealed that REVERSIR-A was quickly metabolized in monkey liver by nuclease activity to form Rev-N1-N9 and Rev-N1-N7. Once formed, Rev-N1-N9 was then converted to Rev-N1-N9-inosine via deamination. Rev-N1-N9-inosine was the most abundant metabolite in monkey liver from 24-h postdose to 28

Table 1. Spiked and calculated concentrations of Rev-N1-N9 and Rev-N1-N9-inosine when quantitating against a Rev-N1-N9 calibration curve.

Spiked concentration (ng/ml)		Observed concentration (ng/ml)		Calculated concentration (ng/ml)	% Difference = 100 x (calculated-spiked)/spiked	
Rev-N1-N9	Rev-N1-N9-inosine	Rev-N1-N9 + Rev-N1-N9-inosine total	Rev-N1-N9	Rev-N1-N9-inosine	Rev-N1-N9	Rev-N1-N9-inosine
2000	0	2003	2002	1	0	NC
1600	400	2072	1671	401	4	0
1200	800	1922	1171	751	-2	-6
800	1200	2177	869	1308	9	9
400	1600	2169	449	1720	12	7
0	2000	2239	<LLOQ	2239	NC	12

Note: LLOQ = 10 ng/ml. For calculations, <LLOQ was assigned a value of 0.
LLOQ: Lower limit of quantitation; NC: Not calculable.

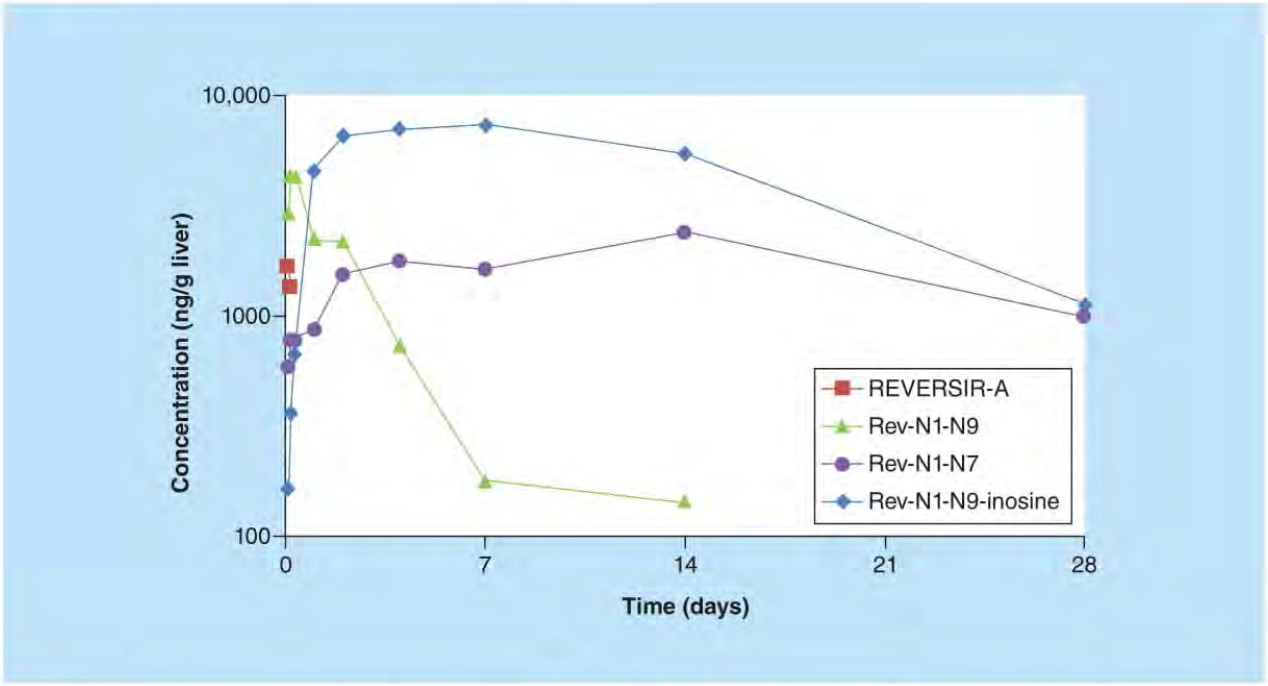

Figure 7. Mean concentration-time profiles of REVERSIR-A and its major metabolites in monkey liver following a 3 mg/kg REVERSIR-A dose. Samples were collected at 0.083, 0.167, 0.33, 1, 2, 4, 7, 14 and 28 days postdose.
Note: n = 2 animals/time point, lower limit of quantitation (LLOQ) = 100 ng/g, <LLOQ time points were not plotted.

days postdose. Rev-N1-N9 and Rev-N1-N7 were also observed as major metabolites of REVERSIR-A in monkey plasma and urine samples, but Rev-N1-N9-inosine metabolite was not detected in monkey plasma or urine.

In vivo metabolism of REVERSIR-A in monkey liver

To date, metabolites identified for therapeutic oligonucleotides have been limited to chain-shortened metabolites generated by nuclease activity and from the biotransformation of the GalNAc ligand [11–15,17–23]. In this study, REVERSIR-A, a single-stranded, GalNAc-conjugated oligonucleotide, underwent nuclease cleavage to form the chain-shortened metabolites, Rev-N1-N9 and Rev-N1-N7, consistent with the nuclease-mediated metabolism reported in the literature. In addition, a metabolite of REVERSIR-A, resulting from conversion of the 3′ terminal 2′-*O*-methyl-adenosine to 2′-*O*-methyl-inosine, was observed as the most abundant metabolite in monkey liver by 24-h postdose. The deamination of adenosine to inosine (A-to-I editing) has been well documented in literature on internal adenosines for endogenous double-stranded RNAs, precursors of certain microRNAs and tRNAs [25–31]. However, to the best of our knowledge, this report describes the first observation of A-to-I editing on the terminal adenosine of an exogenously dosed oligonucleotide. This discovery was facilitated by the high resolution and high

accuracy of the MS method used for oligonucleotide metabolite profiling and quantitation, which made detection of even a small mass shift (<1 Da) in a much larger oligonucleotide (MW> 3000 Da) feasible and straightforward. The mechanism and impact of deamination on pharmacological activity of REVERSIR-A will be investigated and are beyond the scope of this paper.

Conclusion

This paper describes the discovery and identification of a novel deaminated metabolite of a single-stranded REVERSIR oligonucleotide *in vivo* in monkey liver using LC-HRMS. This metabolite was confirmed to result from deamination of the 3′ terminal 2′-*O*-methyl-adenosine to form 2′-*O*-methyl-inosine. Quantitation by LC-HRMS revealed that the deaminated metabolite was the most abundant metabolite in monkey liver although it was not observed in measurable amounts in monkey plasma or urine. To the best of our knowledge, this report describes the first observation of A-to-I RNA editing on the terminal adenosine of an exogenously dosed oligonucleotide.

Future perspective

To support development of oligonucleotide therapeutics such as RNAi and ASO, it is critical to understand the metabolism of these new drug modalities. The analytical approach used here to identify a novel deaminated metabolite of REVERSIR-A can be applied to metabolite profiling of other oligonucleotide therapeutics since this type of deamination could possibly occur on other oligonucleotide drugs. If deamination occurs at a substantial level, the impact on pharmacological activity of the oligonucleotide drug should be evaluated.

Summary points

Background

- To identify major metabolites of a single-stranded oligonucleotide REVERSIR-A *in vivo*.

Results & discussion

- REVERSIR-A and its metabolites were extracted from *in vivo* biological samples by solid phase extraction and analyzed using LC coupled with high-resolution mass spectrometry in negative ionization mode.
- A novel deaminated metabolite, resulting from the conversion of 3′ terminal 2′-*O*-methyl-adenosine to 2′-*O*-methyl-inosine was discovered in monkey liver.
- The deaminated metabolite was confirmed by LC–MS/MS analysis.
- The deaminated metabolite was observed in monkey liver in significant amount but was not observed in monkey plasma or urine.
- A-to-I editing has been reported on internal adenosines for endogenous double-stranded RNAs, precursors of certain microRNAs and tRNAs, but not on exogenously dosed therapeutic oligonucleotide.

Conclusion

- This report describes the first observation of A-to-I RNA editing on the terminal adenosine of an exogenously dosed oligonucleotide.

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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.

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60 seconds with Zamas Lam: oligonucleotide analysis and therapeutics

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Zamas Lam, PhD, holds the position of Senior Vice President and Global Head of Preclinical Development for QPS, a global contract research organization providing discovery, preclinical and clinical drug development services since 1995. Lam is one of the world's few high-resolution mass spectrometrists by training and by trade, with a passion for biologics mass spectrometry and gene therapy.

Q What are some recent bioanalytical methods or technologies being utilized for oligonucleotide analysis?

Traditional methodologies that are still being used are hybridization ligand binding assays, hybridization LC–fluorescence assay and LC–MS/MS assays using MRM. The newer methodologies are LC–HRMS using full scan or high-resolution MRM and hybridization LC–MS/MS.

Q What is more important when analyzing oligonucleotides, mass characterization or structural characterization?

Traditionally, mass characterization has been more important as there are various options for structural characterization. Furthermore, there have been no real methods to understand the structure of minor metabolites or impurities. Moreover, the 3D structure of oligonucleotides are not easily assessable other than with x-ray crystallography. However, with the advances in software solution, new databases and newer mass spectrometry techniques such as ion mobility, charge distribution mass spectrometry and native mass spectrometry, the metabolism, 3D structure and stacking can now be evaluated.

Q In your opinion, what techniques or methods might become more important in the analysis of oligonucleotides?

Hybridization followed by LBA or chromatography for better sensitivity and LC–HRMS for its ability to quantitate and identify metabolites in the same run.

Q What are the key benefits to standardizing methods for oligonucleotide analysis?

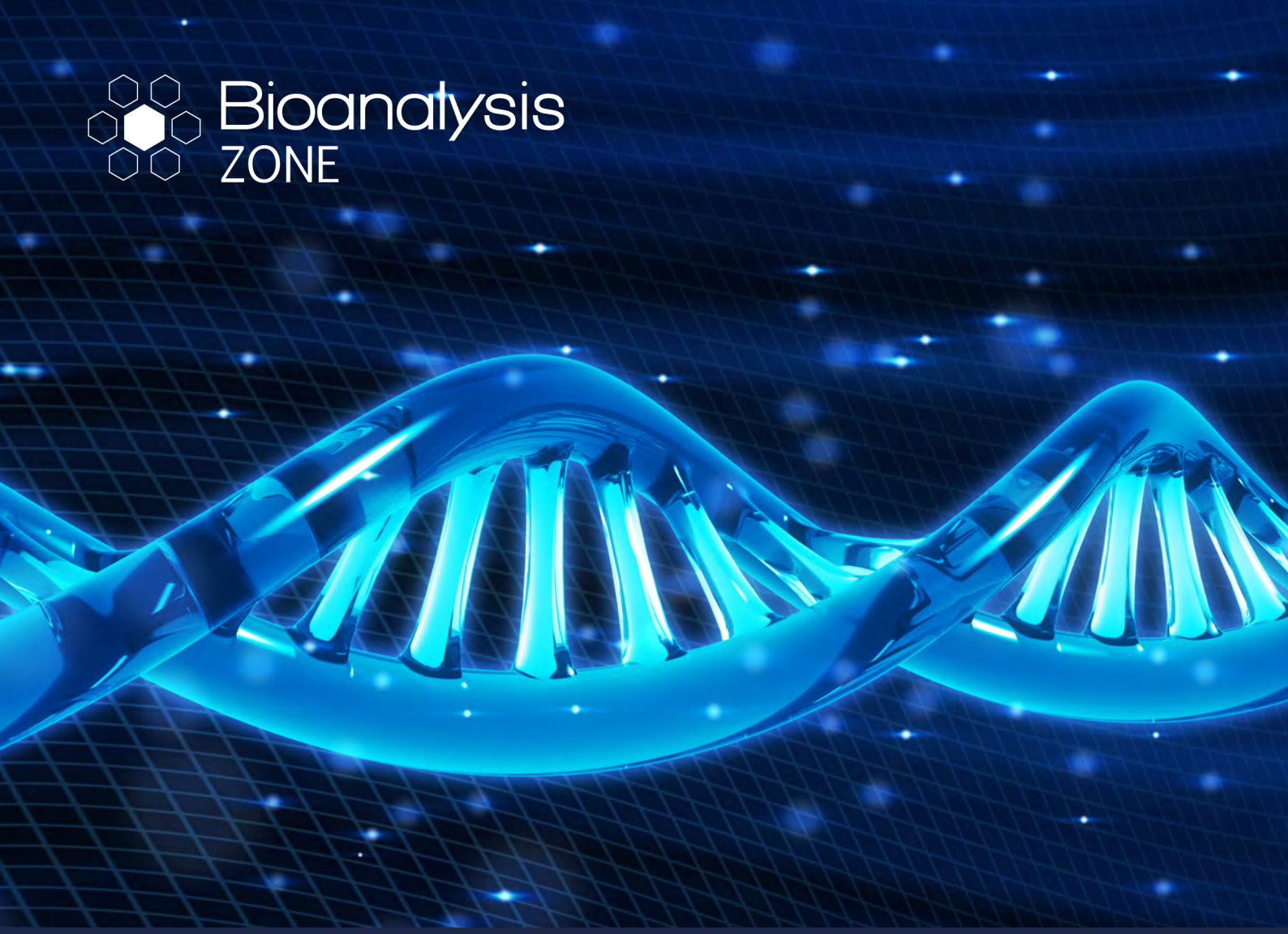
The current trend in oligonucleotides analysis and characterization is similar to what happened to protein analysis and characterizations in the 2000s. Having some standardization will provide a quicker collection and harmonization of data. However, for bioanalysis, it will continue to be the best techniques that fit the modalities, modification of the backbone and the secondary/tertiary structure.

Q In what medical fields do you see oligonucleotide therapeutics gaining interest?

Oligonucleotides will probably continue with 'interference and silencing'. Traditionally, these have been in rare and ultra-rare diseases. However, with the positive approval of Inclisiran against hypercholesterolemia, oligo therapeutics are now being developed for more common indications, such as HBV infection, dry eye in Sjögren's Syndrome, acute kidney injury and others.

Q How have current regulations affected the development of oligonucleotide therapeutics?

Oligonucleotides bioanalysis fits into the FDA 2018 BMV guidance and the current EMA ICH M10 guidance. Potential confusion may arise where some oligonucleotides can be analyzed by chromatographic or LBA assays, so the acceptance criteria can shift from 15/20 to 20/25.



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