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The trials and triumphs in oligonucleotide bioanalysis

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Technology Digest: The trials and triumphs in oligonucleotide bioanalysis

by Naamah Maundrell
Editor-in-Chief, Bioanalysis Zone

Emerging modalities in drug development: harnessing oligonucleotide therapeutics for challenging disease targets

Over the last decade there has been considerable evolution in the target modalities contributing to the development of pharmaceutical medicines. Joining established pipelines of traditional small molecule and monoclonal antibodies, are newer modalities including protein degraders, gene and cell therapies, RNA drugs and synthetic peptides and proteins [1,2]. Oligonucleotide therapies, which are synthetically modified nucleic acid polymers, are of particular interest because of their ability to address disease biology at the gene transcription and translation level, thus, providing an opportunity to treat previously 'undruggable' targets or disease conditions [1,3,4]. Composed of single or double-stranded RNAs or RNA/DNA hybrids, oligonucleotides exist in broad classes such as antisense oligonucleotides (ASOs), small interfering RNAs (siRNA), microRNAs and antagomirs [1,3]. Their highly specific target mechanisms, combined with tendency for low toxicity, lend to an attractive therapeutic approach [4].

The inherent challenges in oligonucleotide bioanalysis

Only the most efficacious and safe oligonucleotide drug candidates will progress through the development pipeline towards clinical trials, however, and determining pharmacokinetic (PK) and toxicokinetic (TK) profiles through bioanalytical assays is critical. There are two common bioanalytical approaches used to quantify therapeutic oligonucleotides in biological matrices: the indirect approach of hybridization immunoassays (such as ELISA) and the direct approach of chromatographic based assays (such as liquid chromatography with tandem mass spectrometry (LC–MS/MS)) [5]. Deciding on which approach to take is generally based on requirements for sensitivity, specificity, throughput and cost [3].

LC–MS/MS is routinely used to quantify a wide range of molecules in biological matrices, including oligonucleotides, due to the sensitivity, specificity and linear dynamic range it affords [4]. However, the polyanionic structure of oligonucleotides presents specific challenges for LC–MS/MS assays. Strong binding to biological proteins in the sample matrix can hinder sample extraction and free metal site interactions can result in non-specific binding to metal surfaces (for example in the chromatography systems) leading to poor and inconsistent recovery and inaccurate quantitative measurements. The impact on the quality of quantitative analysis and the ability to produce reliable data appropriate for validated bioanalytical assays means there can be a heavy requirement of time and skill needed in the method development stage [5].

> *"Oligonucleotides are notorious for their analyte loss due to their extreme protein binding in biological samples, as well as non-specific binding to metal surfaces during LC chromatographic analysis, often leading to high method and assay variability,"* commented Mary Trudeau, Consulting Scientist at Waters™ Corporation (MA, USA).

Traditional mitigating approaches to the analytical challenges

Selective extraction of the unbound oligonucleotides from the biological media is commonly achieved using either liquid-liquid extraction (LLE) or solid-phase extraction (SPE) for LC–MS/MS assays. The latter approach, often carried out within a 96 well microtiter plate format, affords throughput and recoveries required for a busy bioanalytical laboratory [4]. The strong bond that exists between oligonucleotides and endogenous proteins in the biological samples needs to be disrupted prior to extraction. This can be achieved through use of a lysis buffer containing detergents or via a protease digestion pretreatment, or a combination of both [6].

The propensity of oligonucleotides to interact and adsorb to metal surfaces within a typical LC flow path is due to their polyanionic nature, which derives from the many phosphate groups present in their structures. These interactions can result in poor peak shape (tailing), analyte recovery (peak height) and reproducibility [5]. Traditional approaches to manage these non-specific interactions include conditioning or passivating the flow path using either a high concentration of the test analyte prior to analytical testing, or adding a chelating agent, like EDTA to the mobile phases. While these measures can be effective initially, the impact can be short lived and in the case of chelating agents, can bring new challenges such as suppression of the MS signal, which may impact assay sensitivity [4].

> *"Addressing these analytical challenges and enhancing the robustness and sensitivity of quantitative oligonucleotide LC–MS workflows has been and continues to be a key focus for me and many analytical scientists here at Waters. The column chemistry and chromatography and MS instruments all factor into the robustness equation, but clearly the greatest source of variability for these assays arises from the sample preparation procedure,"* added Mary Trudeau.

The potential for low recovery and poor reproducibility can present barriers to method transferability from user-to-user and lab-to-lab. Standardized approaches to sample preparation can help and may be enabled with universal kit-based solutions and automated workflows [6].

Innovative solutions from Waters Corporation

To enhance oligonucleotide bioanalysis robustness and sensitivity, Waters provides a range of innovative tools that work together across the analytical value stream to provide reproducible extraction, LC separation and MS detection of oligonucleotide analytes [6].

Sample preparation procedures have been standardized for a wide range of oligotherapeutic modalities and addressed through the introduction of the Oligoworks™ SPE sample preparation kit, which includes optimized protease digest pretreatment reagents that disrupt oligonucleotide to protein binding and SPE devices containing OligoWorks WAX sorbent that have been batch-selected for optimal oligonucleotide recovery. If desired, automation using pre-defined protocols on the Waters Andrew+™ liquid handler can further support recovery and reproducible performance in the bioanalysis assay [4,6].

> Mary Trudeau explained: *"I am proud to be part of the team that developed the OligoWorks SPE bioanalytical sample preparation kits, as they have been shown to achieve high oligonucleotide recovery for a diversity of oligonucleotide therapeutics, while greatly reducing the need for extensive sample preparation method development. Additionally, the kits are automation friendly allowing for scalability. In our lab, we routinely utilize the Andrew+ Pipetting Robot to prepare and extract our samples."*

To maximize the benefits of optimized sample preparation a comprehensive approach to the end-to-end analytical workflow is required. Waters Xevo™ TQ Absolute Tandem Quadrupole MS delivers the sensitivity performance required for low concentration oligonucleotides of varying lengths, linkers and modifications [1,7]. This compact mass spectrometer reliably addresses the detection challenge of negative ionizing oligonucleotides. The issue of non-specific adsorption via free metal interactions is largely negated through using the ACQUITY Premier UPLC™System and ACQUITY™ Premier Oligonucleotide C18 Columns that incorporate Waters MaxPeakTM High Performance Surface (HPS) technology, which enhance sensitivity and reproducibility while eliminating the need for time consuming conditioning/passivation prior to analysis.

To protect precious samples and optimize time usage, pre-assay verification and efficient troubleshooting are crucial. Waters offers a range of oligonucleotide standards used to verify LC–MS system health and assist in all aspects of troubleshooting from instrumentation to sample extraction efficiency [7,8].

> Mary Trudeau added: *"Another key to achieving highly sensitive and repeatable LC–MS bioanalytical methods is leveraging our ACQUITY Premier Columns and LC Systems, specifically designed to mitigate adsorption of metal sensitive analytes, like oligonucleotides. Additionally, we use our Xevo TQ Absolute tandem quadrupole MS, which has improved MS analysis in negative electrospray mode. These combined technologies ensure we can routinely achieve highly sensitive and robust bioanalytical analysis of oligonucleotides."*

The augmented end-to-end workflow allows for reliable implementation of high-quality bioanalysis assays but with a much-reduced investment in method optimization than typical oligonucleotide assays [8].

Summary

As the drug development industry continues to expand to wider ranges of modalities, bioanalytical techniques and methods must also evolve to tackle the new analytical challenges associated with these new modalities [1,4].

A growing pipeline of oligonucleotide therapeutics brings with it inherent difficulties in analyte extraction recovery, non-specific adsorption and LC–MS sensitivity and reproducibility needed to achieve low detection limits. Through purposefully designed tools and technologies, these challenges across the analytical workflow can be addressed to support high quality bioanalysis of oligonucleotides now and in the future and enable their continued transition through the drug development pipeline [4,7,8].

References

1. Kotapati S,Deshpande M, Jashnani A, Thakkar D, Xu H & Dollinger G. The role of ligand-binding assay and LC–MS in the bioanalysis of complex protein and oligonucleotide therapeutics. Bioanalysis, 13(11), 931–954 (2021).
2. Waters. Quantifying Oligonucleotides Brochure. (2024): waters-brochure-QuantifyingOligonucleotidesBrochure-720008221en.pdf [Accessed 24 April 2024].
3. Xu Y, Garofolo F & Musuku A. The exciting world of oligonucleotides: a multidisciplinary complex challenge for multitasking ingenious bioanalysts. Bioanalysis, 11(21), 1905–1908 (2019).
4. Waters. An Automated, Standardized, Kit-Based Sample Preparation Workflow for Bioanalytical Quantification of Therapeutic Oligonucleotides. (2023): https://www.waters.com/content/dam/waters/en/app-notes/2023/720008068/720008068-en.pdf [Accessed 24 April 2024].
5. Nguyen JM, Gilar M, Koshel B *et al*. Assessing the impact of nonspecific binding on oligonucleotide bioanalysis. Bioanalysis, 13(16), 1233–1244 (2021).
6. Waters. Development of a Standardized, Kit-Based Approach for Selective and Reproducible Sample Preparation and Extraction for Therapeutic Oligonucleotides from Biological Matrices. (2023): https://www.waters.com/content/dam/waters/en/app-notes/2023/720008086/720008086-en.pdf [Accessed 24 April 2024].
7. Waters. Sensitive Bioanalysis of Antisense Oligonucleotides of Various Lengths and Modifications. (2023): https://www.waters.com/content/dam/waters/en/app-notes/2022/720007809/720007809-en.pdf [Accessed 24 April 2024].
8. Waters. Xevo TQ Absolute Triple Quadrupole Mass Spectrometer. (2024): https://www.waters.com/nextgen/au/en/products/mass-spectrometry/mass-spectrometry-systems/xevo-tq-absolute.html [Accessed 24 April 2024].

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Assessing the Impact of Nonspecific Binding on Oligonucleotide Bioanalysis

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Bioanalysis

Assessing the impact of nonspecific binding on oligonucleotide bioanalysis

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Aim: Accurate and reliable quantification of oligonucleotides can be difficult, which has led to an increased focus on bioanalytical methods for more robust analyses. Recent advances toward mitigating sample losses on liquid chromatography (LC) systems have produced recovery advantages for oligonucleotide separations. **Results & methodology:** LC instruments and columns constructed from MP35N metal alloy and stainless steel columns were compared against LC hardware modified with hybrid inorganic-organic silica surfaces. Designed to minimize metal-analyte adsorption, these surfaces demonstrated a 73% increase in 25-mer phosphorothioate oligonucleotide recovery using ion-pairing reversed-phase LC versus standard LC surfaces, most particularly upon initial use. **Conclusion:** Hybrid silica chromatographic surfaces improve the performance, detection limits and reproducibility of oligonucleotide bioanalytical assays.

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Keywords: bioanalysis • ion-pairing • LC-UV-MS • mass spectrometry • oligonucleotides • pH • phosphorothioate • spectrofluorometric quantitation

An emerging focus on RNA-based therapies in the biopharmaceutical industry has led to an increasing demand for improved bioanalytical technologies to support the development of these complex drugs. Antisense therapy is one form of treatment to address genetic diseases, and it employs the use of synthetic short, single stranded oligodeoxynucleotides called antisense oligonucleotides (ASOs). These ASOs can range in length but typically consist of around 20 nucleotides and bind to RNA through Watson–Crick base pairing. Through this mechanism, an ASO can alter gene expression and inhibit the translation of proteins to achieve a therapeutic effect. While the first publication describing the potential of ASOs as therapeutic agents was released in 1978 by Zamecnik and Stephenson [1], it was not until 1998 that the US FDA approved the first ASO, fomivirsen, for treating cytomegalovirus retinitis [2]. Since 2016, with the prominent release of the personalized RNA therapy, milasen [3], RNA therapies have been subject to a rapid pace of development, which has placed increasing demands on the development of bioanalytical techniques to facilitate *in vitro* cellular studies, pharmacokinetics and pharmacodynamics for not just ASOs, but also for small interfering RNA (siRNA) and other oligonucleotide modalities.

The two most common bioanalytical methods used to quantify therapeutic oligonucleotides from a complex sample matrix are hybridization immunoassays such as enzyme-linked immunosorbent assay (ELISA) and liquid chromatography coupled to mass spectrometry (LC-MS) [4,5]. Similar to developing a protein-based therapy, these methods are required in preclinical development to obtain toxicological, pharmacokinetic and metabolic properties of the oligonucleotide in animal models before beginning human trials. Hybridization-ELISA assays can be used in these preclinical trials for quantification [6]. However, while highly sensitive, hybridization-ELISA lacks specificity to distinguish intact oligonucleotides and its truncated metabolites [4,7]. In general, nucleic acid metabolism occurs through the hydrolysis of the phosphodiester bonds of nucleic acids by endo- and exonucleases [8]. Characterization of these metabolites is extremely important for assessing biotransformation events and to evaluate the pharmacokinetic impact of oligonucleotide therapeutics. Chromatographic assays, especially those paired with MS detection, have the capability of monitoring and characterizing the target oligonucleotide and its related

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metabolites. Husser and co-workers have shown that LC-MS can be sensitive enough to determine the cleavage points and metabolites of oligonucleotides conjugated with GalNAc, a ligand that can grant greater potency through high affinity liver targeting but also causes changes in the biotransformation of oligonucleotides [9,10]. More recent publications by Kim and co-authors have described the development of an LC-MS method for the 2′-*O*-methyl modified phosphorothioate ASO eluforsen and its metabolites [8], and Kilanowska and co-authors have described the *in vitro* metabolism of various modified and different length ASOs with human liver microsomes using LC-MS [11]. Nevertheless, there remain challenges in using LC-MS for quantitation. Due to the polyanionic properties of oligonucleotides, there can be a propensity toward non-specific binding, not just to biological materials such as proteins or lipids, but also to chromatographic LC hardware [7]. These surfaces are generally made from metals such as stainless steel or titanium, where pronounced sample losses with these metal surfaces can be difficult to contend with [12].

To address this challenge, many different approaches have been applied to reduce adsorption and non-specific binding. Sample passivation and conditioning of the LC system and column is a common method to mask the active sites of metals or materials that would contribute to non-specific binding [12]. Metal ions, which can be found in mobile phase reagents or shed from LC pumps, can also cause problems for an analysis [13]. In addition, metal chelators, such as EDTA or other additives, can also be added to the mobile phase or sample vial [14,15]. These chelators have been shown to improve the peak shape of oligonucleotides by chelating metal ions and preventing adduct formation with sodium and potassium. While additives like EDTA can improve peak shape, they can also cause undesirable effects, most notably ion suppression in MS detection. Therefore, the concentration of these reagents must be carefully optimized in order to ensure desired performance is achieved in terms of sensitivity and lower limits of quantitation (LLOQ) [16,17].

Ultimately, these studies of chelators reveal that there is also a need to improve LC components to minimize adsorption and sample loss. Very little has been published on refining LC system and column components to prevent oligonucleotide adsorption. Typically, systems made of alternative metals such as nickel cobalt MP35N alloy or titanium have been used for biopharmaceutical applications and to prevent corrosion that would otherwise occur with stainless steel systems [18]. These systems can still suffer from adsorptive losses and in the case of titanium, can also contribute to system contamination [19]. Polyether ether ketone (PEEK) tubing and hardware have also been employed as an alternative but lack the mechanical strength to withstand UHPLC operational pressures [20]. Though PEEK-lined steel tubing can allow higher operational pressures, higher variability exists with internal diameters of PEEK tubing versus stainless steel and titanium [20]. Moreover, the hydrophobicity of PEEK can cause issues related to hydrophobic secondary interactions [21,22].

Thus, we have investigated an alternative chromatographic surface to minimize sample losses resulting from metal-ion mediated adsorption. This new technology consists of a highly crosslinked, hybrid organic/inorganic ethylene-bridged siloxane surface that is chemically similar to bridged-ethylene hybrid (BEH) sorbent [23]. This novel surface is more hydrophilic than PEEK and more chemically resilient than fused silica. Previous studies by Tuytten and co-authors have shown that phosphorylated biomolecules adsorb strongly to stainless steel and that the number of phosphate moieties correlate to the strength of adsorption [24]. When analyzing the classes of analytes that are susceptible to metal adsorption, significant improvements in recovery and peak shape have been gained upon using these hybrid surfaces to reduce metal-sensitive analyte interactions with the electron-deficient metallic LC surfaces [23].

Here, we applied hybrid surfaces toward the reversed-phase (RP) LC-MS bioanalysis of oligonucleotides. As modifications to the oligonucleotide backbone are often incorporated to increase nuclease resistance, we examined the impact of hybrid surfaces on the quantitation of a 25-mer phosphorothioate (PS) oligonucleotide and the recoveries of various length oligodeoxythymidines [25]. Additionally, because RPLC-MS of oligonucleotides is primarily performed at neutral to high pH, we evaluated the effect of mobile phase pH on oligonucleotide recovery. Finally, to demonstrate their utility for bioanalytical assays, we compared the linearity of calibration curves generated from columns and LC systems constructed with and without hybrid silica chromatographic surfaces.

Materials & methods

Samples & reagents

Trecovirsen, also known as GEM91®, GEM132, and 39-mer oligodeoxythymidine were acquired from Integrated DNA Technologies, Inc. (IA, USA). Trecovirsen is an antisense phosphorothioate (PS) oligonucleotide (ASO) with an average mass of 7771 g/mol and a sequence of 5′d(CTC TCG CAC CCA TCT CTC TCC TTC T)3′.

GEM132 has an average mass of 6600 g/mol and a sequence of 5′d(U′G′GGGCTTACCTTGCGA'A′C′A′)3′, where the label represents 2′-*O*-methyl modifications. An equimolar mixture of lyophilized 15, 20, 25, 30 and 35-mer oligodeoxythymidines was acquired from Waters Corporation (MA, USA) in the form of the MassPREP OST Standard. LC-MS grade methanol (MeOH), acetonitrile (ACN) and acetic acid were purchased from Thermo Fisher Scientific (MA, USA), and MilliQ water was used. Triethylamine (TEA), N, N-diisopropylethylamine (DIPEA), and hexylamine (HA) were purchased from Millipore Sigma (MA, USA), and 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) was purchased from Acros Organics (NJ, USA).

Recovery of oligomers with conventional & hybrid surface columns

An equimolar mixture of 15, 20, 25, 30 and 35-mer oligodeoxythymidines was reconstituted to a 5 pmol/µl solution and analyzed by LC-UV with an ACQUITY UPLC H-Class Bio system that had been modified with hybrid silica surfaces, as described by DeLano *et al.*, including the injection needle [23]. Comparative separations were performed on unused 2.1 × 150 mm stainless steel columns packed with a 130 Å, 1.7 µm BEH C18 stationary phase and unused columns of the same dimension and stationary phase constructed with hybrid silica hardware. An ion-pairing mobile phase system comprised of 25 mM hexylammonium acetate (mobile phase A) and a 50:50 solution of mobile phase A and acetonitrile (mobile phase B) at a pH of 6 or 7 (only aqueous mobile phase A was pH adjusted using acetic acid). Samples were injected at an injection volume of 2 µl, or a mass load of 10 pmol per oligonucleotide, and run at a temperature of 60°C, flow rate of 0.4 ml/min, and gradient from 50 to 86% B in 12 min. Chromatograms were recorded with an ACQUITY UPLC PDA detector equipped with a 5 µl titanium flow cell at 260 nm using chromatography software Empower 3.0.

Impact of pH on oligonucleotide recovery

Trecovirsen was analyzed by LC-UV in an MISER type experiment with an ACQUITY UPLC H-Class Bio system modified with hybrid silica hardware components. Briefly, MISER (multiple injections in single experimental run) allows numerous injections to be made while a separation is still occurring [26]. For this experiment, the column was removed and a 2.1 mm stainless steel frit was placed in a flow path between the injector and detector. The frit housing outlet was connected to a PDA detector cell using 75 µm i.d. × 40 cm PEEK tubing. Separations were performed at a flow rate of 0.2 ml/min at 30°C and employing isocratic elution using 10 mM aqueous ammonium acetate mobile phase at pH 4.5, 7.0, or 8.5. Fifty injections of a 2 pmol/µl solution of trecovirsen were made in blocks of 10 injections (0.5 minute/injection) using an injection volume of 1 µl and followed by a gap of 0.5 or 2 min after each block of injections, and the resulting peaks of all injections were recorded as a single chromatogram. The recovery of the oligonucleotide was estimated from a control experiment where the frit was replaced with a PEEK union. The signal with the PEEK union is considered to be 100%. Analyses were performed with UV detection at 260 nm using Empower 3.0 for data acquisition and analysis.

Generation of LC-UV calibration curves

A 2.6 pmol/µl solution of trecovirsen was used to run a twofold dilution series with injection volumes of 4 µl until the analyte could no longer be detected. Separations were performed on previously conditioned 2.1 × 50 mm stainless steel or hybrid surface columns packed with a 130 Å, 1.7 µm BEH C18 sorbent using an ion-pairing mobile phase system comprised of 8.6 mM TEA, 100 mM HFIP at pH 8.25 (mobile phase A) and methanol (mobile phase B). An ACQUITY UPLC H-Class PLUS Bio Binary System was used in conjunction with the stainless steel and hybrid surface columns. An equivalent system outfitted with hybrid surface components was employed for testing corresponding hybrid surface columns. Samples were run at a temperature of 60°C, flow rate of 0.2 ml/min, gradient from 14 to 24% B in 10 min, and UV detection at 260. Empower 3.0 software was used for data acquisition and analysis.

Generation of LC-MS calibration curves

Trecovirsen was analyzed by LC-MS with a UPLC and triple quadrupole mass spectrometer (ACQUITY UPLC H-Class Bio equipped with hybrid surfaces hyphenated to a Xevo TQ-XS mass spectrometer; Waters, MA, USA). A 0.75 pmol/µl (5 µg/ml) concentration of GEM132 was added to samples and used as an internal standard to compensate for variability in ionization efficiency from one run to another. Calibration curves were generated from dilutions of a 0.13 pmol/µl (1 µg/ml) concentration of trecovirsen, and the peak area ratio (analyte peak area to internal standard peak area) was used to plot linearity. Separations were performed on unused 2.1 × 50 mm

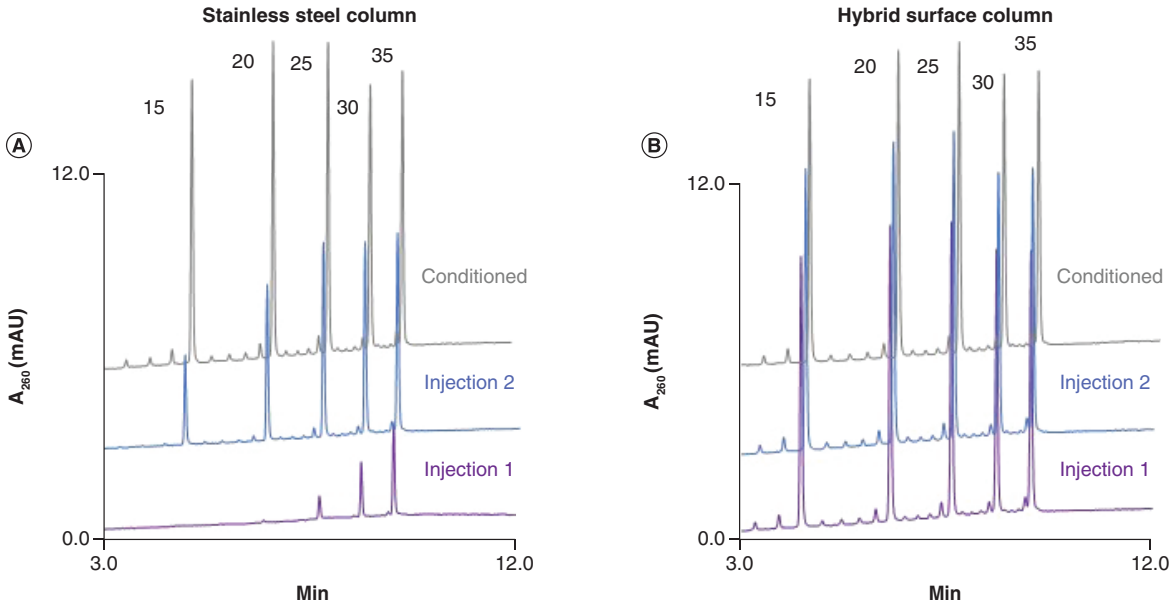

Figure 1. UV chromatograms corresponding to the first use of the columns and runs collected after high mass load conditioning. An equimolar mixture of 15, 20, 25, 30 and 35-mer oligodeoxythymidines was separated using either a 2.1 × 150 mm **(A)** stainless steel column or **(B)** a hybrid surface column and a pH 6 mobile phase.

stainless steel or hybrid surface columns packed with a 130 Å, 1.7 µm BEH C18 sorbent and using an ion-pairing mobile phase system comprised of 5.8 mM (or 0.1%) DIPEA, 98 mM (or 1%) HFIP in water (mobile phase A) and 2.2 mM (0.0375%) DIPEA, 73 mM (0.75%) HFIP in 65% acetonitrile (mobile phase B). Samples were run at a temperature of 60°C, flow rate of 0.3 ml/min, and gradient from 5 to 25% B in 20 min using injection volumes of 5 µl. MS analysis was performed in negative MRM mode using MassLynx 4.1. A capillary voltage of 2.0 kV, sampling cone at 45, source offset at 30, a source temperature of 150°C, a desolvation temperature of 600°C, desolvation gas flow set at 1000 L/h, and a collision energy of 5 eV. The transitions used, in m/z, were 732.80 → 94.90 and 824.50 → 94.96 for GEM132, and 863.10 → 94.96 and 971.03 → 94.96 for trecovirsen.

Results & discussion

Recovery of oligomers by ion pairing reversed phase chromatography

Prior research has shown that acidic analytes with anionic properties interact with electron deficient surfaces such as metals, which results in decreased analyte recovery [24]. Hybrid inorganic-organic silica surfaces based on ethylene bridged siloxane polymer can be applied to metals to mitigate these problematic ionic interactions. We sought to evaluate ethylene bridged siloxane hybrid silica surfaces for oligonucleotide LC-based quantitation by first investigating the recoveries of a sample consisting of 15, 20, 25, 30 and 35-mer oligodeoxythymidines.

Figure 1 shows a comparison of the poly dT oligomers separated with unused stainless steel versus hybrid surface columns. The injection labeled 'conditioned' was executed after an injection of 1 nmol of 39 mer oligodeoxythymidine in a separate injection. This high mass load injection served to 'condition' the columns, where the high load of the oligonucleotide sample served to saturate any potential binding sites within the columns and to therefore help minimize any subsequent analyte losses.

With the stainless steel column, poor initial recovery of each of the five components of the standard was observed. Upon seeing its first injections, this column showed recoveries for the 15, 20, 25 and 30-mer oligodeoxythymidines that were approximated to be under 20%. Meanwhile, the recovery of the 35-mer, while higher, was still less than 40%. Interestingly, problems from secondary interactions seem to lessen in severity according to elution order, which suggests that early eluting peaks encounter and then sacrificially passivate active sites during each chromatographic run. Later eluting peaks therefore stand a chance of being chromatographed with subtly better recovery. To that point, the 15-mer oligomer was not even recovered until the second injection, and it was only after conditioning the column with the 1 nmol injection of 39-mer oligodeoxythymidine that full recovery of oligonucleotide species could be achieved. Improvements in recovery were observed from injection 1 to injection 2 using the stainless steel

column, suggesting that each injection gradually 'conditions' the column, improving the sample recovery for later injection, presumably due to saturation of ionic adsorption sites present in the metal column hardware. In contrast, separations using a hybrid surface column gave nearly full recovery for all oligodeoxythymidines upon even its initial use. In another experiment, a quantitative experiment was performed to confirm that the signal observed with the hybrid surface column was indeed representative of ~90% recovery (see Supplementary Information).

Impact of pH on oligonucleotide recovery

It was hypothesized that the pH of an ion pairing reversed phase separation might affect the magnitude of adsorptive losses for oligonucleotides, as has been previously shown for the small molecule adenosine triphosphate (ATP) [23]. In addition, mass load dependence needed to be investigated further. The previously mentioned results were obtained with a pH 6 mobile phase and with sample loads that were much higher than those found in bioanalytical assays. Oligonucleotide separations are characteristically performed using high pH mobile phases, because DNA and RNA are more stable at neutral to basic pH [27]. A pH above 9 is generally not advised given that it deprotonates ion-pairing agents and thereby compromises ion-pairing based retention mechanisms [28]. Oligonucleotide separations tend to be performed at pH 7 to 9 and with organosilica or polymeric stationary phases for their increased stability over silica [29]. Since our initial investigations using conventional stainless steel and hybrid surface columns were performed at pH 6, we ran the same set of experiments but with pH increased to 7. Interestingly, at pH 7, each oligodeoxythymidine could be partially recovered upon first injection. Nevertheless, the loss of sample onto a stainless steel column could still be observed, even though it was not as pronounced as was seen with pH 6 mobile phase (Supplementary Figure 3).

To explore the impact of pH on sample adsorption and recovery, we used trecovirsen as a model analyte. Trecovirsen is a 25-mer phosphorothioate ASO that has been studied as a treatment for HIV-1 [30–32]. Phosphorothioate is a common backbone modification, where a sulfur atom replaces the non-bridging oxygen of the phosphate linkage to protect against nuclease attack [33]. This modification has been shown to increase the non-specific binding of oligonucleotides to proteins and impact the metal binding of oligonucleotides [34,35]. For this investigation, we employed the use of MISER (Multiple Injections in Single Experimental Run) for pH screening using a single 2.1 mm stainless steel frit for high throughput injections [26]. No chromatographic column was used in this experiment. The rationale of using a frit for the experiments is that highly porous frits (used in LC columns to hold the sorbent in place) represents a significant amount of the surface area that an analyte encounters. In this study, non-metallic LC components were employed to ensure that adsorption events were isolated to the frit being placed into the flow path, and the recovery of the oligonucleotide was normalized to a control experiment where the frit was replaced with a PEEK union (data not shown).

In Figure 2A are MISER chromatograms obtained at pH 4.5, 7 and 8.5. The lowest oligonucleotide recovery was observed at pH 4.5, where the first injections of trecovirsen show nearly complete sample loss. In the subsequent injections, the peaks areas and heights gradually increased, suggesting that the frit surface was progressively conditioned by the oligonucleotide sample. Presumably, the sample was adsorbed onto the metallic frit surfaces, saturating the active adsorptive sites. However, full recovery was never achieved when compared with the control experiment, even after 50 injections of sample. Similar experiments were performed at pH 7 and 8.5, respectively, which are more common pH conditions used for oligonucleotide LC analysis. Sample loss was less pronounced at these higher pH values, and the number of injections needed to condition the frit was reduced. Only at the highest pH – pH 8.5 – was the sample recovery satisfactory and reproducible across 50 injections using a stainless steel frit. In a separate experiment, we performed a study on the recovery of a 30 mer oligodeoxythymidine using a mobile phase system comprised of 25 mM hexylammonium acetate at three different pH values (pH 6, 7 and 8.5) for six previously unused stainless steel columns (Supplementary Material). We observed that elevated pH does help to mitigate sample loss, where recovery at pH 6 to pH 7 improves from 19 to 54%. However, even at pH 8.5, only 75% recovery could be achieved (Supplementary Figure 4). Alternatively, the oligonucleotide was almost fully recovered regardless of the pH when hybrid surface columns were used, with recoveries of over 95% from pH 6 to 8.5. More detail can be found in the Supplementary Material.

Figure 2A also offers an important insight regarding the mechanism of passivating a stainless steel frit as achieved by repetitive injections of oligonucleotide sample. One can notice that the buildup of oligonucleotide on the frit surface was not continuous and did not reach full frit 'saturation' even after 50 injections. In addition, when a gap of 2 min was inserted after each 10 injections, the first injection area in the next series was lower than the preceding injection in the previous injection series. This can be explained by slow bleed of adsorbed oligonucleotide from the

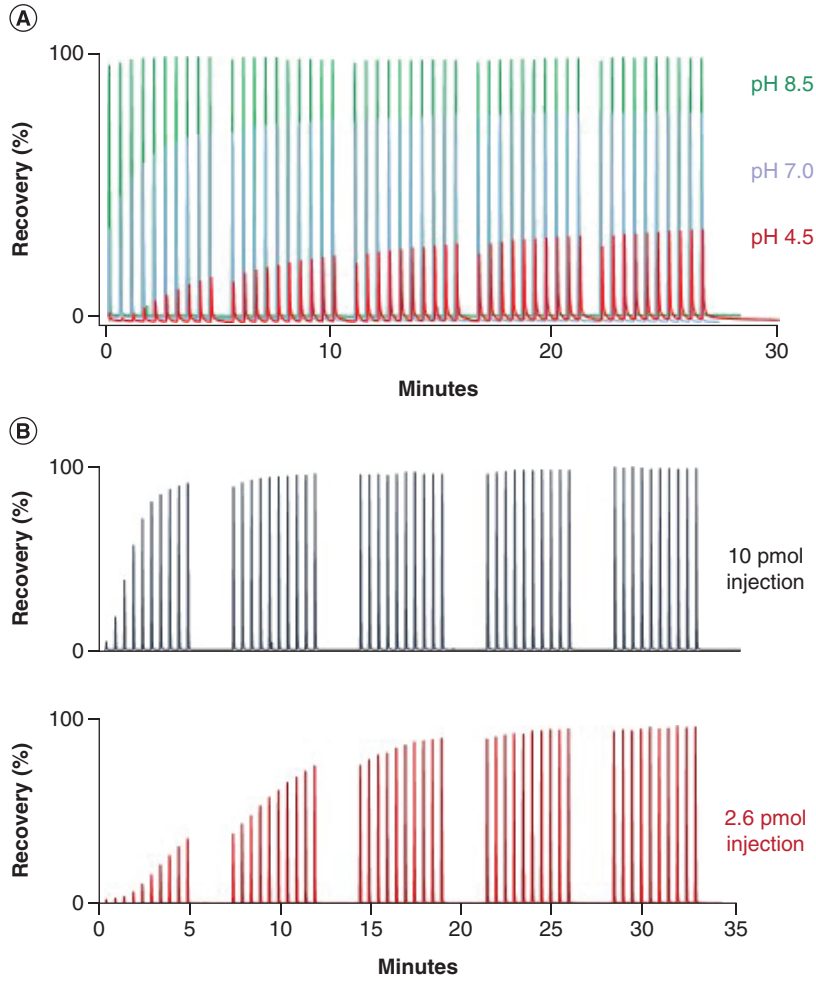

Figure 2. Effect of pH on apparent oligonucleotide adsorption on a 2.1 mm i.d. stainless steel frit. (A) 50 consecutive injections of 2 pmol of 25 mer oligonucleotide was performed at pH 4.5, pH 7.0 or at pH 8.5. **(B)** Compares 50 oligonucleotide injections using a fresh stainless steel frit as performed with 2.6 or 10 pmol sample mass per injection at pH 6.8.

stainless steel frit surface. A similar effect has been observed with other analytes, such as organic acids [36]. These observations have practical implications for the LC analysis. The conditioning of the LC system or column hardware, often practiced by analysts, can be achieved by repetitive sample injections, but the conditioning is transient in nature [37,38]. An inconsistent state of the LC system/column conditioning can have direct consequences toward the assay's accuracy.

Figure 2B further illustrates an important consideration for LC frit (column) conditioning. The available LC surfaces, in our case the 2.1 mm i.d. stainless steel frit, have finite adsorption capacity. Such capacity can be saturated more speedily with an exposure to a higher concentration (mass) of the sample. Nearly complete sample recovery was observed after 10 injections of 10 pmol oligonucleotides, while 30 to 40 injections were required to reach the recovery plateau with 2.6 pmol injections of the sample.

Generation of LC-UV calibration curves

The above experiments, in particular Figure 2B, suggest that quantitative bioanalysis of oligonucleotides performed at low concentrations would be especially challenging on conventional LC hardware. Thus, the application of hybrid surfaces to both the LC and column should prove beneficial in this respect and as demonstrated by De Lano and *et al.* [23]. To that end, we performed a systematic experiment to evaluate improvements in trecovirsen recovery when using LC hardware in three configurations: a stainless steel column with a conventional system, a hybrid surface column with a conventional system, and hybrid surfaces put to use in both the column and LC system. To

Table 1. Average peak areas and corresponding percent RSDs as performed on previously conditioned LC hardware in three configurations: stainless steel column with a conventional system, hybrid surface column with a conventional system and hybrid surfaces used for both the column and system.

Mass load (pmol)	Stainless steel column/conventional LC system		Hybrid surface column/conventional LC system		Hybrid surface column/hybrid surface LC system	
	Log(peak area) (n = 3)	%RSD	Log(peak area) (n = 3)	%RSD	Log(peak area)(n = 3)	%RSD
10.40	5.25	1.3	5.34	0.5	5.41	0.3
5.20	4.93	0.9	5.02	1.3	5.10	0.4
2.60	4.59	2.0	4.71	0.2	4.79	0.7
1.30	4.22	4.1	4.39	1.2	4.48	1.3
0.65	3.90	3.1	4.09	0.6	4.14	1.9
0.32	3.41	7.0	3.76	0.9	3.84	0.1
0.16	3.00	9.2	3.44	1.6	3.47	2.1
0.08	2.54	12.2	3.16	2.4	3.20	0.3
0.04	Not detected		2.78	8.6	2.84	2.3
0.02			Detectable but SNR <3		2.41	2.2
0.01					Detectable but SNR <3	

examine the lower limits of detection for these configurations, mass loads down to 0.01 pmol were investigated for quantitation using LC-UV, which was a 1000-times lower than the mass load used in Figure 1 and a 100-times lower than used in Figure 2.

Prior to data collection over three triplicate injections, the LC system and column were conditioned with multiple injections of trecovirsen. Table 1 lists the average of the peak area (in log) and percent deviation for the three sets of equipment configurations. Here, the stainless steel column and conventional system yielded the lowest recovery (as determined by the log of the peak area) for each injection regardless of mass load. While switching to a hybrid surface column on a conventional system improved recovery, the highest yield resulted from using hybrid surfaces within both the column and the LC hardware. The effect from the hybrid surfaces granted a lower limit of detection and lower percent RSDs throughout the dilution series.

The calibration curves of each dilution series are shown in Figure 3 to make it possible to visually assess dynamic range differences. By plotting each calibration curve in a log-log plot, one can see that different slopes (response factors) were observed for each configuration, which indicates that there are analyte recovery differences even after following sample-based conditioning procedures. Overlaying the data revealed rather significant linear differences especially at low mass data points, despite each curve appearing linear with an R-squared value close to one. Recovery, and thus linearity, was improved by using hybrid surfaces. When employed for both column and LC hardware, hybrid silica surfaces afforded the best linearity throughout the dynamic range, lowest limits of detection, and best repeatability (lowest percent RSD). Dynamic range with optical detection approached three orders of magnitude with the hybrid column and LC hardware, but was only two orders of magnitude with a conventional system and a stainless steel column.

Generation of LC-MS calibration curves

High throughput capabilities and the ability to monitor oligonucleotide drug targets and their related metabolites have made LC-MS an attractive and common alternative to immunoaffinity assays such as ELISA. Additionally, as LC instrumentation continues to be developed, LC-MS assays are becoming increasingly sensitive. Thus, experiments next turned to exploring even lower detection limits based on LC-MS detection with a triple quadrupole mass spectrometer.

In general, LC-MS bioanalysis of oligonucleotides necessitates the use of an internal standard that can be used to correct for run-to-run fluctuations in ionization efficiency [39,40]. We thus included a high concentration of GEM132 as an internal standard to neat solutions of trecovirsen and generated calibration curves from these samples on previously unused stainless steel versus hybrid surface columns (Figure 4). The data is plotted on a log/log plot to better illustrate linearity across the entire calibration range. No weighted regression is applied to this curve.

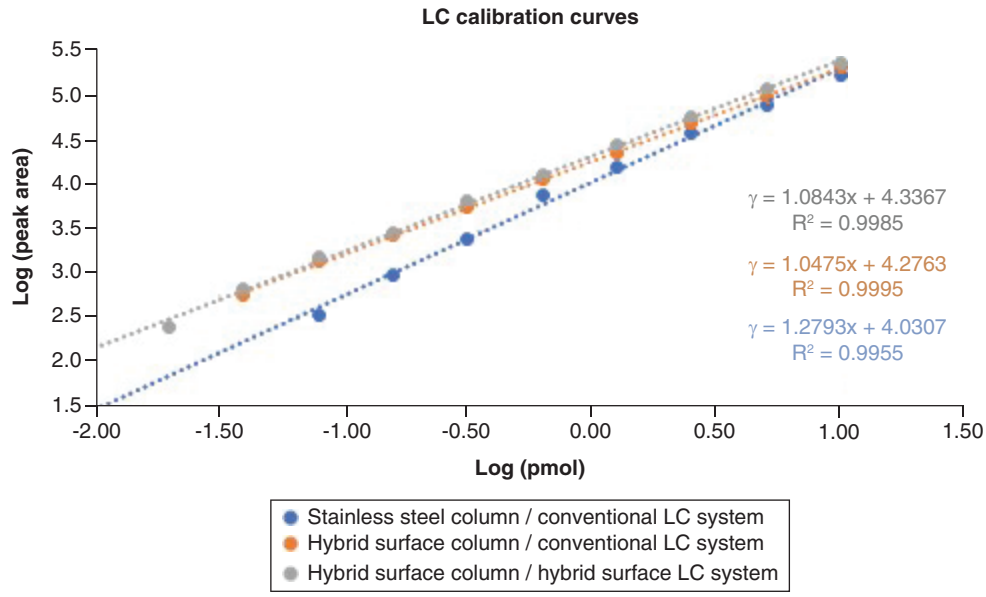

Figure 3. Comparison of the linearity of calibration curves using previously conditioned LC hardware in three configurations: stainless steel column with a conventional system (blue), hybrid surface column with a conventional system (orange), and hybrid surfaces for both column and system (gray). The graph is viewed in log/log mode to better visualize the data trend throughout the calibration range.

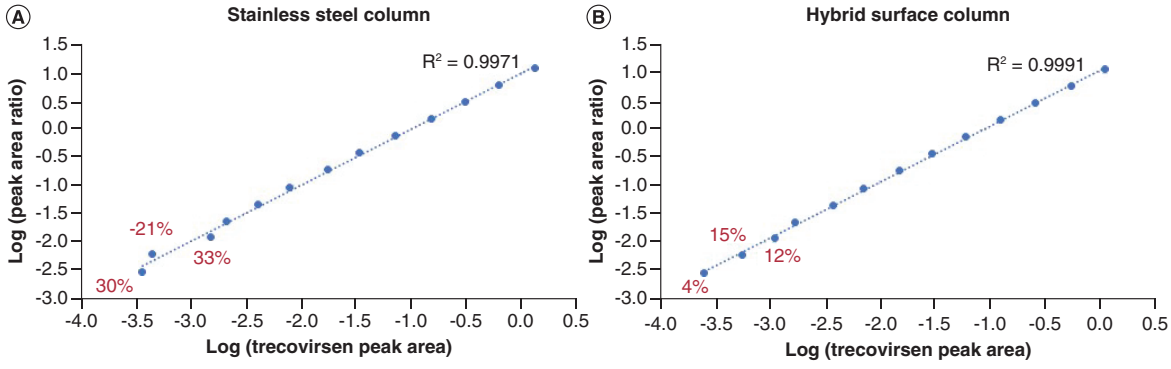

Figure 4. Comparison of the linearity at the low end of the calibration curves as generated through LC-MS separations of trecovirsen using previously unused 2.1 × 50 mm stainless steel versus hybrid surface columns. Graphs are viewed in log/log mode using the peak area ratio of the internal standard to trecovirsen to better visualize the data trend throughout the calibration range.

Much like in Figure 3, although the curves appear linear and have R-squared values close to one, when using the stainless steel column, the three lowest concentrations would fall outside of typical bioanalytical acceptance criteria [41]. Compared with the hybrid surface column, where the lowest concentration in the linear dynamic range was 0.36 fmol/µl (2.8 ng/ml, 1.8 fmol on column), the limit of quantitation for the conventional column was only down to 6.3 fmol/µl (49 ng/ml, 32 fmol on column). The elimination of non-specific binding, which is most pronounced at the low end of the calibration curve, resulted in a 17× improvement in the limit of quantitation observed. Even with the internal standard spiked into the sample, there can be challenges in performing reliable measurements with a stainless steel columns, most particularly when attempting to analyze low sample concentrations.

The non-linearity observed with the stainless steel column is likely due to there being active sites in the column that cannot be passivated with the addition of this quantity of internal standard. It might also be important to consider whether an internal standard elutes before or after the analyte peak. Nevertheless, as seen in Supplementary Figure 5, the initial recoveries from the first injections of the internal standard were poor when using a stainless

steel column, with almost a fourfold decrease upon first injection versus hybrid surface columns. However, they did progressively increase over time, from injection to injection. This can cause analytical problems because the peak area ratio (analyte peak area to internal standard peak area) is variable and changing overtime. The hybrid surface column, in turn, gave reproducible peak area and a peak area ratio from injection 1 to injection 20.

This study demonstrates that the ability to use neat standards is noteworthy for LC-MS applications. While the presence of other biological components in matrix may produce less sizable recovery differences, matrix effects are often calculated for bioanalytical applications, with the reference being the analyte in the absence of the matrix [5,42]. In addition, publications involving the treatment of neat standards of oligonucleotides with nucleases have been demonstrated to provide insight into biotransformation pathways and analysis of metabolites [43]. Appropriate MRM transitions are often chosen by infusing neat standards into the MS to monitor the target analyte, ensuring no overlap with its metabolites. Furthermore, sample matrices such as urine, consisting primarily of water and salt with little plasma proteins, may benefit from hybrid silica surfaces [7].

Conclusion

Our results demonstrate the utility of ethylene-bridged hybrid silica chromatographic surfaces for oligonucleotide bioanalysis. Hybrid surfaces improved the recovery of a metal-sensitive, phosphorothioate oligonucleotide and circumvented the need for column or system conditioning. The data showed that while standard LC and stainless steel column hardware required multiple sample injections to improve recoveries and peak widths, a configuration fully outfitted with hybrid surfaces can achieve these results with high reproducibility upon first injection. In turn, the linear dynamic range and calibration curves generated with bioanalytical assays could be enhanced with the use of alternative chromatographic surfaces, especially at lower mass loads. Based on our experiments, the performance of standard, metal-based LC systems can be improved at elevated pH, but it is only a partial solution. Recovery of oligonucleotides (and in general, acidic analytes) were improved via dynamic system passivation by repetitive injections of sample, but the effect was only transient. Improvements in recovery and reproducibility of oligonucleotides on LC hardware (column and LC system) modified with hybrid silica surfaces can help address these shortcomings and give new options for enhanced bioanalytical approaches for oligonucleotide therapeutics.

Future perspective

With the rise in RNA/DNA-based therapies, new bioanalytical approaches are required to support the development of oligonucleotide biotherapeutics and to understand their biotransformation and pharmacokinetic profiles. Here, ethylene bridged hybrid silica surfaces were employed within both an LC system and the column to improve the recovery and lower limits of detection of phosphorothioate oligonucleotides and oligodeoxythymidines, which is indicative of their potential value for other nucleic acid based analytes. As these nucleic acid therapeutics are becoming more complex, the need for better quantitation and higher reproducibility will be increasingly apparent. It appears that chromatographic surfaces based on hybrid silica will be of value in the pursuit of new approaches for LC-based quantitation.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.4155/bio-2021-0115

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

Summary points

- Aim: to investigate the use of ethylene-bridged hybrid silica as a chromatographic surface and its suitability for the quantitation of oligonucleotide biotherapeutics.
- Separations of a 25-mer PS oligonucleotide and various length oligodeoxythymidines were analyzed and quantified through spectrofluorometric detection using systems and columns constructed from standard, metal based versus hybrid silica based LC surfaces.
- Oligonucleotide recoveries at various pH conditions and sample loads were investigated.
- Calibration curves were generated to comparatively assess the dynamic range of LC-UV and LC-MS separations of a PS oligonucleotide.
- Quantitation of nanogram levels of oligonucleotide was possible using hybrid surfaces, including a 73% increase in recovery versus standard, metal based LC surfaces.
- Reproducible and robust performance was observed even upon the first injection and use of a hybrid surface column, eliminating the need for conditioning.
- Ion-pairing separations performed at higher pH can help to mitigate the need for system passivation/conditioning when standard LC surfaces are used.
- Employing hybrid surfaces extends the dynamic range of calibration curves and thus allows for lower limits of detection and improved quantitation for oligonucleotide bioanalysis.
- Hybrid chromatographic surfaces are a viable alternative to metal based LC surfaces, as seen in instances of them improving the recovery and reproducibility of LC-based quantitation of oligonucleotides.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

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

1. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl Acad. Sci. USA* 75(1), 280–284 (1978).
2. Verma A. Recent advances in antisense oligonucleotide therapy in genetic neuromuscular diseases. *Ann. Indian Acad. Neurol.* 21(1), 3–8 (2018).
3. Amariles P, Madrigal-Cadavid J. Ethical, economic, societal, clinical, and pharmacology uncertainties associated with milasen and other personalized drugs. *Ann. Pharmacother.* 54(9), 937–938 (2020).
4. Basiri B, Bartlett MG. LC-MS of oligonucleotides: applications in biomedical research. *Bioanalysis* 6(11), 1525–1542 (2014).
5. Li P, Gong Y, Kim J *et al.* Hybridization liquid chromatography-tandem mass spectrometry: an alternative bioanalytical method for antisense oligonucleotide quantitation in plasma and tissue samples. *Anal. Chem.* 92(15), 10548–10559 (2020).

• **Demonstrates LC-MS as used for the bioanalysis of antisense oligos.**

6. Tremblay G, Khalafaghian G, Legault J, Nielsen P, Bartlett A. Dual ligation hybridization assay for the specific determination of oligonucleotide therapeutics. *Bioanalysis* 3(5), 499–508 (2011).
7. Wang L. Oligonucleotide bioanalysis: sensitivity versus specificity. *Bioanalysis* 3(12), 1299–1303 (2011).

• **Review of oligo bioanalysis.**

8. Kim J, Basiri B, Hassan C *et al.* Metabolite profiling of the antisense oligonucleotide eluforsen using liquid chromatography-mass spectrometry. *Mol. Ther. Nucleic Acids* 17, 714–725 (2019).
9. Husser C, Brink A, Zell M, Muller MB, Koller E, Schadt S. Identification of GalNAc-conjugated antisense oligonucleotide metabolites using an untargeted and generic approach based on high resolution mass spectrometry. *Anal. Chem.* 89(12), 6821–6826 (2017).
10. Sewing S, Gubler M, Gerard R *et al.* GalNAc conjugation attenuates the cytotoxicity of antisense oligonucleotide drugs in renal tubular cells. *Mol. Ther. Nucleic Acids.* 14, 67–79 (2019).
11. Kilanowska A, Nuckowski L, Studzinska S. Studying *in vitro* metabolism of the first and second generation of antisense oligonucleotides with the use of ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* 412(27), 7453–7467 (2020).

12. Lauber M, Walter TH, Gilar M *et al.* Low adsorption HPLC columns based on MaxPeak high performance surfaces. *Waters White Paper 720006930EN* (2020).
13. Birdsall RE, Gilar M, Shion H, Yu YQ, Chen W. Reduction of metal adducts in oligonucleotide mass spectra in ion-pair reversed-phase chromatography/mass spectrometry analysis. *Rapid Commun. Mass Spectrom.* 30(14), 1667–1679 (2016).
14. Hsiao JJ, Potter OG, Chu TW, Yin H. Improved LC/MS methods for the analysis of metal-sensitive analytes using Medronic acid as a mobile phase additive. *Anal. Chem.* 90(15), 9457–9464 (2018).
15. Pesek JJ, Matyska MT, Fischer SM. Improvement of peak shape in aqueous normal phase analysis of anionic metabolites. *J. Sep. Sci.* 34(24), 3509–3516 (2011).
16. Liu J, Li J, Tran C *et al.* Oligonucleotide quantification and metabolite profiling by high-resolution and accurate mass spectrometry. *Bioanalysis* 11(21), 1967–1980 (2019).
17. Basiri B, Sutton JM, Hooshfar S, Byrnes CC, Murph MM, Bartlett MG. Direct identification of microribonucleic acid miR-451 from plasma using liquid chromatography mass spectrometry. *J. Chromatogr. A* 1584, 97–105 (2019).
18. In: *The HPLC Expert II.*Kromidas S (Ed.). Wiley-VCH, Weinheim, Germany (2017).
19. De Pra M, Greco G, Krajewski MP *et al.* Effects of titanium contamination caused by iron-free high-performance liquid chromatography systems on peak shape and retention of drugs with chelating properties. *J. Chromatogr. A* 1611, 460619 (2020).
20. Rao S, Rivera B, Anspach JA. Bioinert versus biocompatible: the benefits of different column materials in liquid chromatography separations. *LC GC N Am.* 36(6), 24–29 (2018).
21. Lough J, Mills MJ, Maltas J. Analyte adsorption in liquid chromatography valve injectors for samples in non-eluting solvents. *J. Chromatogr. A* 726, 67–75 (1996).
22. Hambleton P, Lough WJ, Maltas J, Mills M. Unusual analyte adsorption effects on inert LC components. *J. Liq. Chromat. Rel. Tech.* 18, 3205–3217 (1995).
23. DeLano M, Walter TH, Lauber MA *et al.* Using hybrid organic-inorganic surface technology to mitigate analyte interactions with metal surfaces in UHPLC. *Anal. Chem.* 93(14), 5773–5781 (2021).

•• **Description of the properties of hybrid surfaces.**

24. Tuytten R, Lemiere F, Witters E *et al.* Stainless steel electrospray probe: a dead end for phosphorylated organic compounds? *J. Chromatogr. A* 1104(1–2), 209–221 (2006).
25. Monia BP, Johnston JF, Sasmor H, Cummins LL. Nuclease resistance and antisense activity of modified oligonucleotides targeted to Ha-ras. *J. Biol. Chem.* 271(24), 14533–14540 (1996).
26. Welch CJ, Gong X, Schafer W *et al.* MISER chromatography (multiple injections in a single experimental run): the chromatogram is the graph. *Tetrahedron: Asymmetry* 21, 1674–1681 (2010).
27. Gilar M, Bouvier ESP. Purification of crude DNA oligonucleotides by solid-phase extraction and reversed-phase high-performance liquid chromatography. *J. Chromatogr. A* 890(1), 167–177 (2000).
28. Fountain KJ, Gilar M, Gebler JC. Analysis of native and chemically modified oligonucleotides by tandem ion-pair reversed-phase high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 17(7), 646–653 (2003).
29. Wyndham KD, O'gara JE, Walter TH *et al.* Characterization and evaluation of C18 HPLC stationary phases based on ethyl-bridged hybrid organic/inorganic particles. *Anal. Chem.* 75(24), 6781–6788 (2003).
30. Lisziewicz J, Sun D, Weichold FF *et al.* Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells. *Proc. Natl Acad. Sci. USA* 91(17), 7942–7946 (1994).
31. Sereni D, Tubiana R, Lascoux C *et al.* Pharmacokinetics and tolerability of intravenous trecovirsen (GEM 91), an antisense phosphorothioate oligonucleotide, in HIV-positive subjects. *J. Clin. Pharmacol.* 39(1), 47–54 (1999).
32. Veal GJ, Agrawal S, Byrn RA. Synergistic inhibition of HIV-1 by an antisense oligonucleotide and nucleoside analog reverse transcriptase inhibitors. *Antiviral Res.* 38(1), 63–73 (1998).
33. Gan R, Wu X, He W *et al.* DNA phosphorothioate modifications influence the global transcriptional response and protect DNA from double-stranded breaks. *Sci. Rep.* 4, 6642 (2014).
34. Zhou W, Saran R, Liu J. Metal sensing by DNA. *Chem. Rev.* 117(12), 8272–8325 (2017).
35. Saran R, Huang Z, Liu J. Phosphorothioate nucleic acids for probing metal binding, biosensing and nanotechnology. *Coord. Chem. Rev.* 428, 1–17 (2020).
36. Smith KM, Wilson ID, Rainville PD. Sensitive and reproducible mass spectrometry-compatible RP-UHPLC analysis of tricarboxylic acid cycle and related metabolites in biological fluids: application to human urine. *Anal. Chem.* 93(2), 1009–1015 (2020).
37. Stoll DR, Hsiao JJ, Staples GO. Troubleshooting LC separations of biomolecules, part I: background, and the meaning of inertness. *LC GC N Am.* 38(3), 146–150 (2020).

38. Hsiao JJ, Chu TW, Potter OG, Staples GO, Stoll D. Troubleshooting LC separations of biomolecules, part 2: passivation and mobile-phase additives. *LC GC Eur.* 33(8), 388–392 (2020).
39. Zhang G, Lin J, Srinivasan K, Kavetskaia O, Duncan JN. Strategies for bioanalysis of an oligonucleotide class macromolecule from rat plasma using liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 79(9), 3416–3424 (2007).
40. Chen B, Bartlett M. A one-step solid phase extraction method for bioanalysis of a phosphorothioate oligonucleotide and its 3′ n-1 metabolite from rat plasma by uHPLC-MS/MS. *AAPS J.* 14(4), 772–780 (2012).
41. Kadian N, Raju KS, Rashid M, Malik MY, Taneja I, Wahajuddin M. Comparative assessment of bioanalytical method validation guidelines for pharmaceutical industry. *J. Pharm. Biomed. Anal.* 126, 83–97 (2016).
42. Ramanathan L, Shen H. LC-TOF-MS methods to quantify siRNAs and major metabolite in plasma, urine and tissues. *Bioanalysis* 11(21), 1983–1992 (2019).
43. Shemesh CS, Yu RZ, Gaus HJ *et al.* Elucidation of the biotransformation pathways of a galnac3-conjugated antisense oligonucleotide in rats and monkeys. *Mol. Ther. Nucleic Acids* 5, e319 (2016).

Waters™

Application Note

Sensitive Bioanalysis of Antisense Oligonucleotides of Various Lengths and Modifications

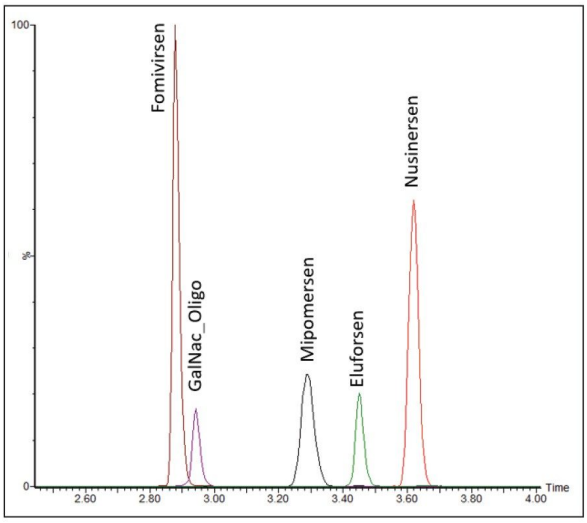
Suma Veeramachineni, Mark D. Wrona

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

Abstract

This application brief demonstrates the sensitivity and suitability of the Xevo™ TQ Absolute Triple Quadrupole MS for bioanalysis of oligonucleotides in human plasma matrix with varying lengths (18 to 33 nucleotides), linkers, and modifications.



Left: Waters Xevo TQ Absolute System with Waters Acquity Premier UPLC;

Right: Representative MRM traces for 5 ASO compounds using 4 min bioanalytical LC-MS/MS IP method.

Benefits

Coupled with the ACQUITY™ Premier UPLC System and ACQUITY Premier Oligonucleotide C_{18} Column, the Xevo TQ Absolute MS demonstrates enhanced chromatographic recovery of oligonucleotides along with excellent (sub ng/mL) system sensitivity enabling support of challenging LC-MS/MS assays and PK studies.

Introduction

High sensitivity and five orders of dynamic range performance were described previously using GEM91/Trecovirsen.[1] This work extends this methodology to antisense oligonucleotides (ASOs) of varying length and modifications.

Experimental

Plasma samples were spiked at concentrations from 0.1 ng/mL to 1000 ng/mL with multiple ASOs containing 2′-MOE modified bases, GalNAc conjugate, or phosphorothioate linkers (Eluforsen, Fomivirsen, Mipomersen, Nusinersen, and a GalNAc conjugated oligonucleotide). 100 µL were extracted using liquid-liquid extraction of plasma standards. GEM91 (100 ng/mL) was used as internal standard to quantitate all oligonucleotides. 100 mM hexafluoroisopropanol (HFIP) + 15mM N, N-diisopropylethylamine (DIPEA) in water and in 90% acetonitrile were used as mobile phase A and B, respectively.

For complete method details, please refer to Waters™ application note 720007574.

Name	Mol wt	Size (mers)	Linkers	Modifications	Parent (*m/z*)	Daughter (*m/z*)
GEM91	7776	25 nts	PO_2S^{3}-	N/A	597.2	319.1
Fomivirsen	6682	21 nts	PO_2S^{3}-	N/A	741.4	319.1
Nusinersen	7127	18 nts	PO_2S^{3}-	2′-MOE	889.8	393.1
Eluforsen	11,469	33 nts	PO_2S^{3}-	2′OMe	673.7	335.2
Mipomersen	7177	20 nts	PO_2S^{3}-	2′-MOE ; 5-Me rC	716.6	319.0
GalNAc_Oligo	~8000	21 nts	PO_3^{3}-	3'-triantennary GalNAc	A	B

Table 1. Details of the oligonucleotides used in study.

Results and Discussion

To demonstrate reproducibility, duplicates of calibration standards and six replicates of each QC level of ASO panel (table 1) in three runs on three separate days. The calibration curves were linear with r^2 values >0.99 ($1/x^2$ weighting) with >75% non-zero calibrator levels and QCs meeting acceptance criteria in each run *i.e.*, non-zero calibrators and QCs should be ±15%, except at LLOQ where the calibrator or QCs should be ±20% of nominal concentrations in each run as shown in Table 2 and 3.

		Eluforsen		Fomivirsen		Mipomersen		Nusinersen		GalNAc_Oligo	
Name	Std conc (ng/mL)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Std-1	0.10	102.0	10.8	100.8	3.6	98.4	10.4	100.1	13.3	–	–
Std-2	0.20	94.7	12.9	97.3	4.8	100.1	8.7	98.0	6.8	98.3	9.8
Std-3	0.50	96.0	5.6	100.2	5.4	103.5	7.2	99.6	5.4	102.4	2.4
Std-4	1.00	101.9	3.7	103.4	3.5	106.0	4.6	105.7	3.7	103.1	7.1
Std-5	2.00	106.6	6.0	105.9	5.0	105.9	5.2	108.7	3.6	104.1	4.8
Std-6	10.0	98.8	3.7	102.6	3.3	101.3	4.0	102.2	2.3	103.9	3.6
Std-7	100	102.9	4.0	102.6	5.3	99.2	6.0	100.1	4.2	100.6	3.5
Std-8	1000	94.9	2.8	89.3	2.9	89.0	4.5	86.6	1.2	90.1	2.9

Table 2. Statistics for calibration standards.

		Eluforsen		Fomivirsen		Mipomersen		Nusinersen		GalNAc_Oligo	
Name	Std conc (ng/mL)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
QC1	0.10	94.4	14.6	94.5	12.2	99.9	11.4	92.8	6.9	–	–
QC2	0.20	94.8	7.9	99.3	7.0	100.5	9.7	98.4	7.8	110.2	6.9
QC3	0.50	104.4	5.8	104.1	6.5	102.8	6.3	104.3	4.4	103.0	7.5
QC4	50.0	101.2	2.9	102.1	3.7	98.9	4.3	99.7	2.5	100.1	2.5
QC5	800	97.9	2.1	91.4	3.7	89.0	3.5	87.3	2.1	93.8	2.2

Table 3. Statistics for QC samples.

Method was developed with optimized transitions, which were evaluated across a broad range of parent charge states and resulting fragment masses. Enhanced negative ion mode detection capabilities enabled improved counts, S/N and detection limits of the assay. The lower limit of quantification (LLOQ) of 0.1 ng/mL (0.2 ng/mL for GalNAc oligo) was achieved over a calibration range of 0.1 to 1000 ng/mL in human plasma and is shown in Figure 1 with representative chromatograms of lowest calibration standards of all oligonucleotides.

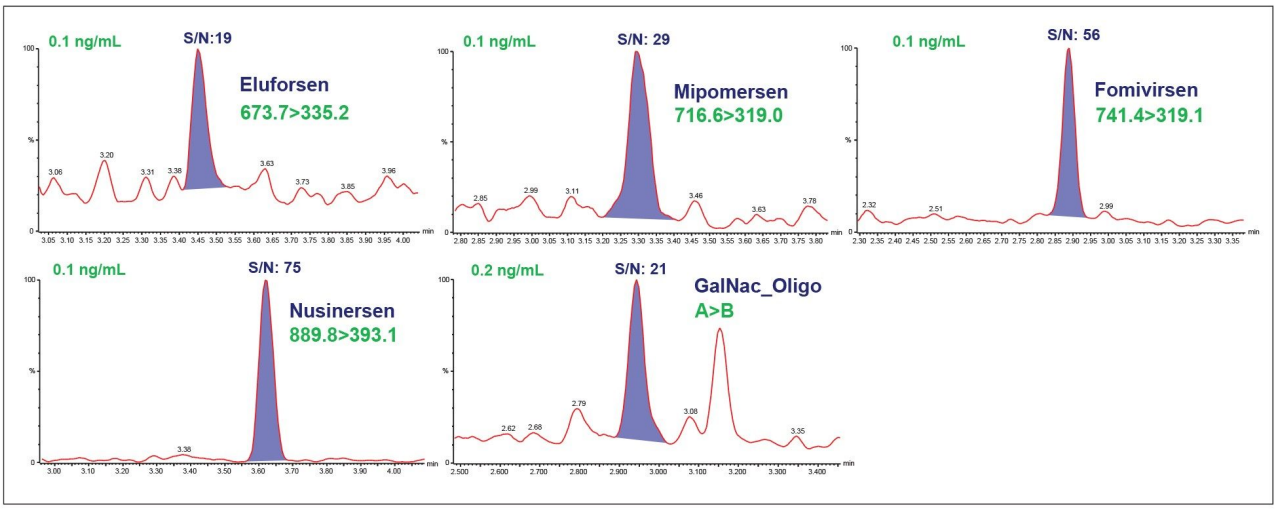


Figure 1. Representative chromatograms showing peaks at LLOQ levels.

Conclusion

- Sub ng/ml levels of sensitivity, with good dynamic range performance was observed in human plasma for antisense oligonucleotides of different lengths (18 to 33 nts) and with a variety of linkers and modifications.
- MaxPeak™ HPS technology reduces nonspecific binding, metal absorption, and enabled excellent sensitivity and low-level detection.
- With enhanced sensitivity for challenging negative ionization compounds, the Xevo TQ Absolute tandem MS can generate high quality data for routine LC-MS/MS based quantitation of antisense oligonucleotides in biological matrices.

Acknowledgement

The authors thank Greg Jones and Alnylam Pharmaceuticals for donation of 21 mer GalNac oligonucleotide for our experiments.

This is the same as application note 720007418 by Mary Trudeau.

References

1. Suma Veeramachineni, Mark D Wrona, 'Sensitive LC-MS/MS Bioanalytical Quantitation of Antisense Oligonucleotides', Waters, Application Notes, 720007574, March 2022.

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Bioanalysis

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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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Keywords: ADC • antibody–drug conjugate • bioanalysis • biotransformation • ELISA • fusion protein • LBA • LC–MS • PEGylated protein • quantitation • therapeutic oligonucleotide

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

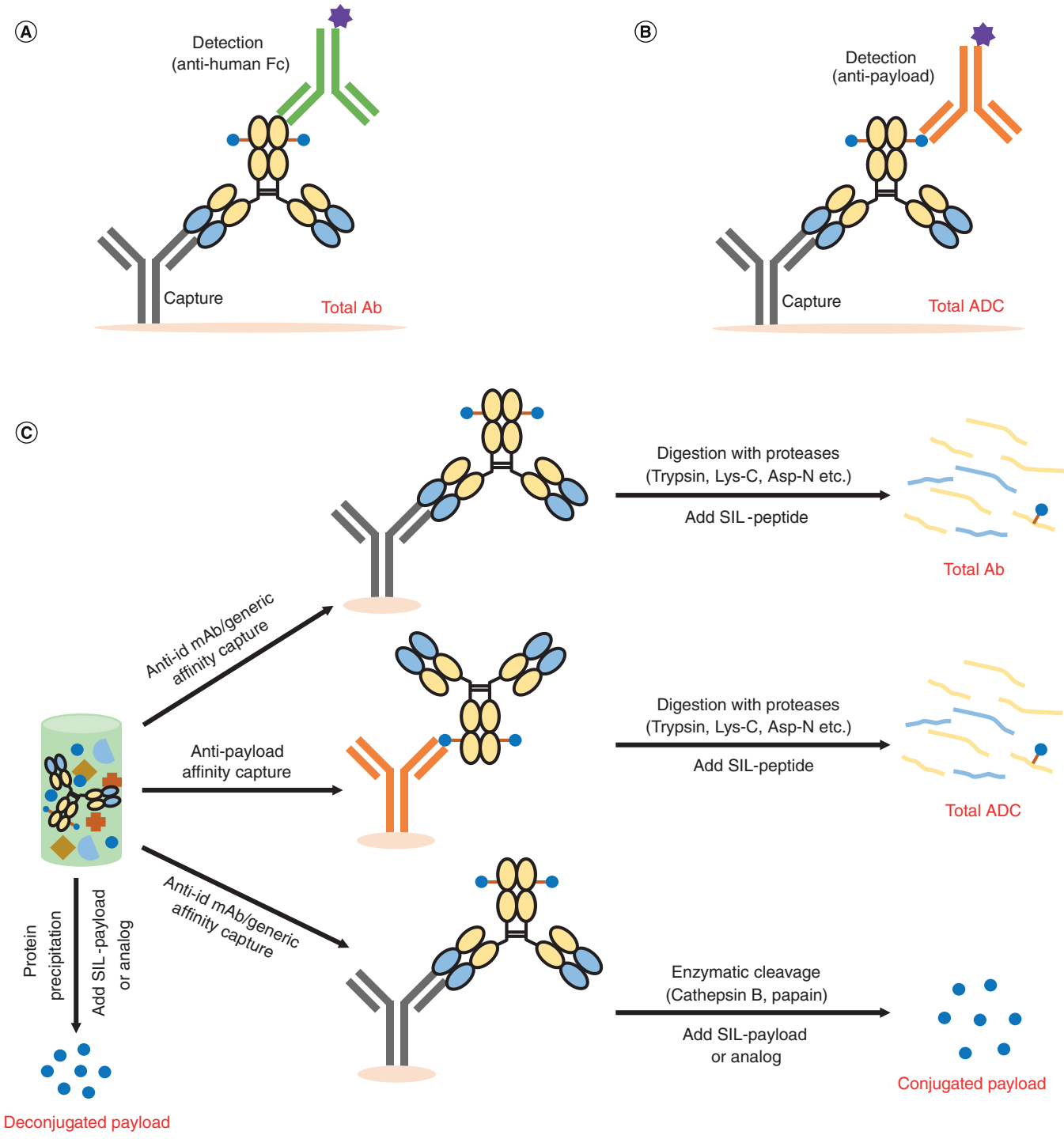

Figure 1. Summary of ligand-binding assay and LC–MS/MS workflows for antibody–drug conjugate quantitation. (A) Ligand-binding assay formats for quantitation of total Ab, **(B)** total ADC and **(C)** LC–MS/MS workflows for the quantitation of total Ab, total ADC, conjugated and deconjugated payload.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

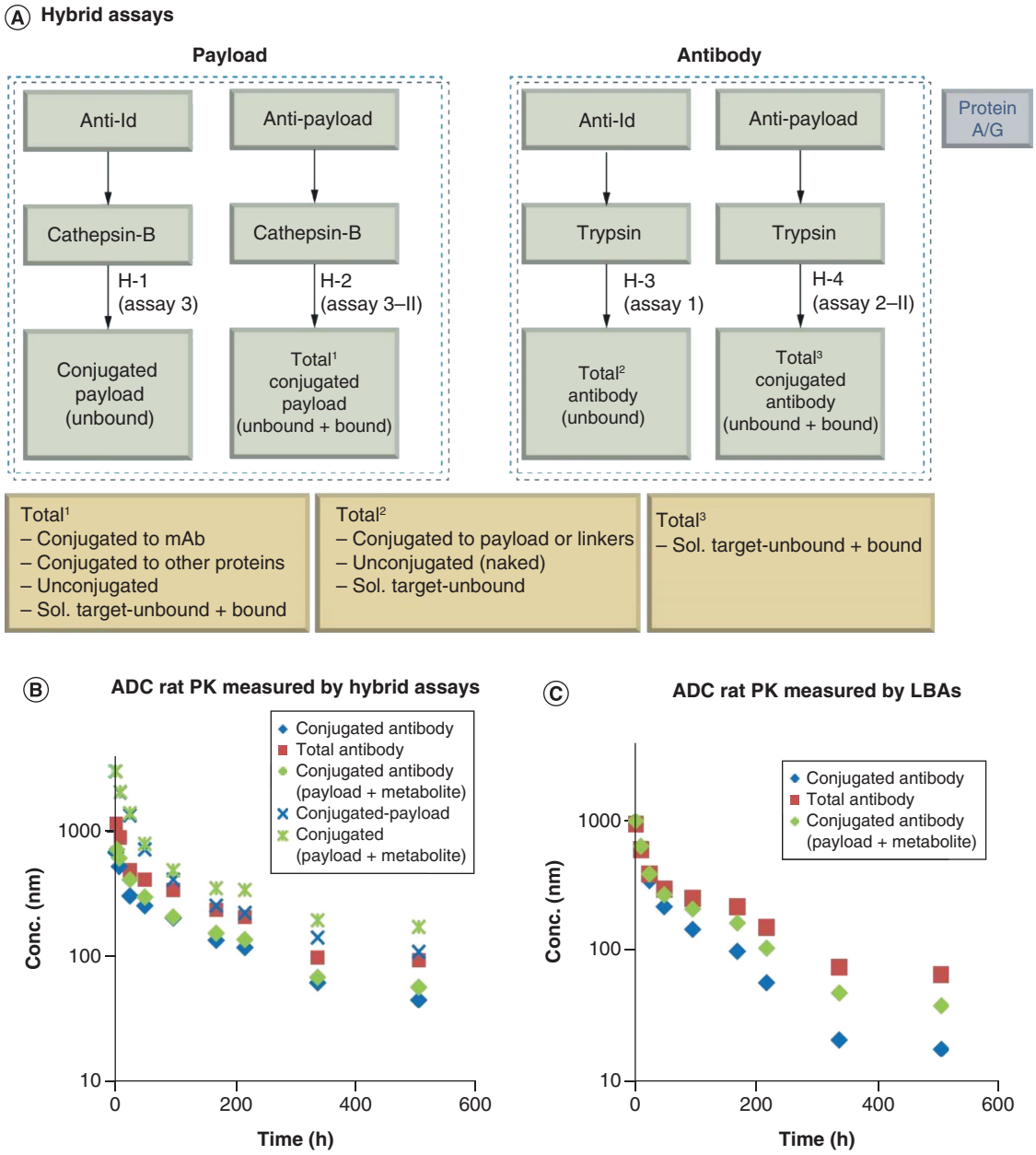

Figure 2. Comparison of ligand-binding assay and hybrid LC–MS/MS for the quantitation of a proprietary antibody–drug conjugate. (A) Hybrid LC–MS/MS workflows for quantitation of various ADC analytes, **(B)** Hybrid LC–MS/MS and **(C)** LBA-based quantitation of concentrations of various ADC analytes 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

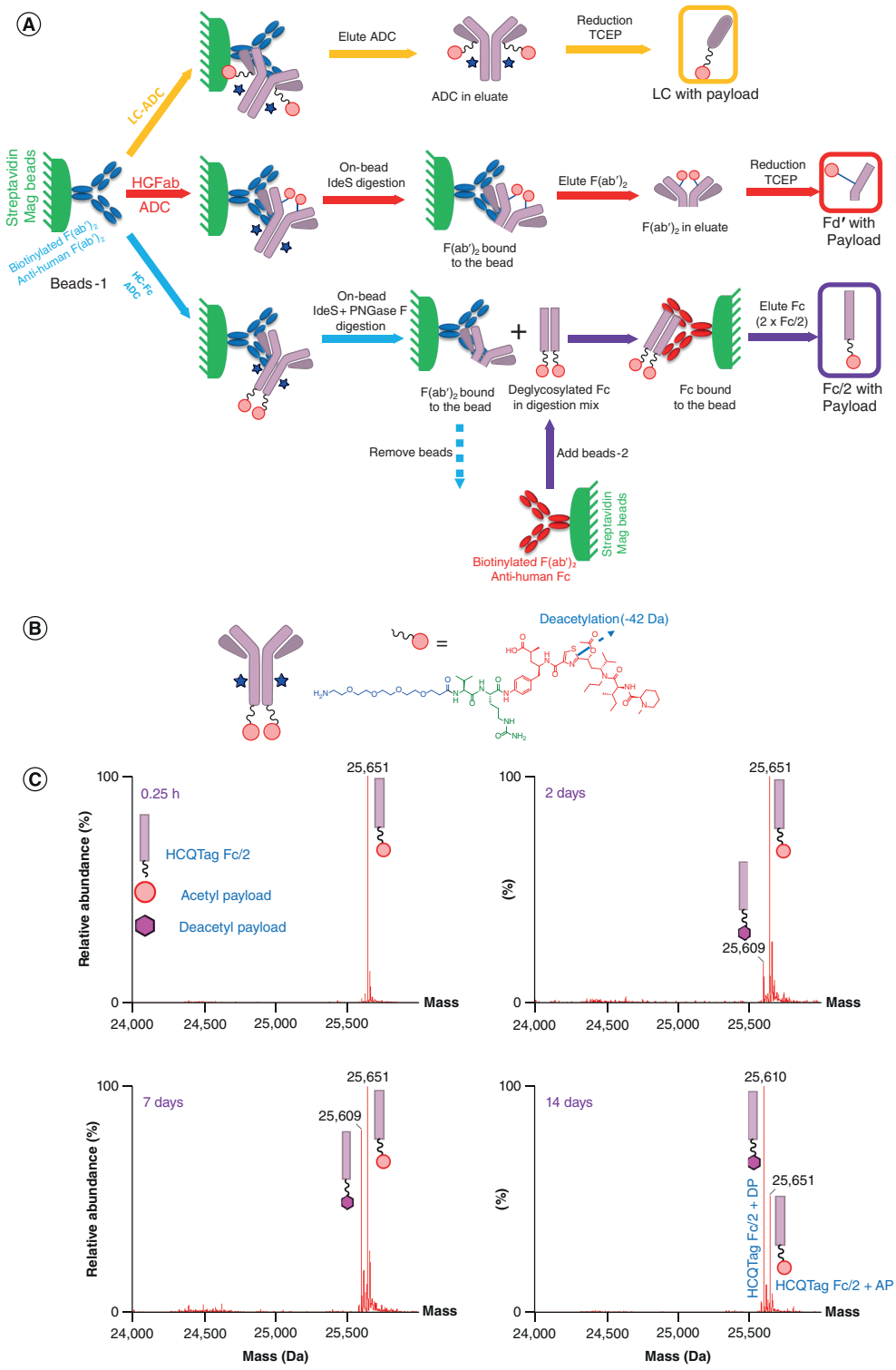

Figure 3. Universal affinity capture LC–HRMS assay for the biotransformation assessment of site-specific antibody–drug conjugates. (A) Schematic representation of universal affinity capture method for LC–, HC-Fab- and HC-Fc-conjugated ADCs, **(B)** Pictorial representation of an ADC conjugated with a 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

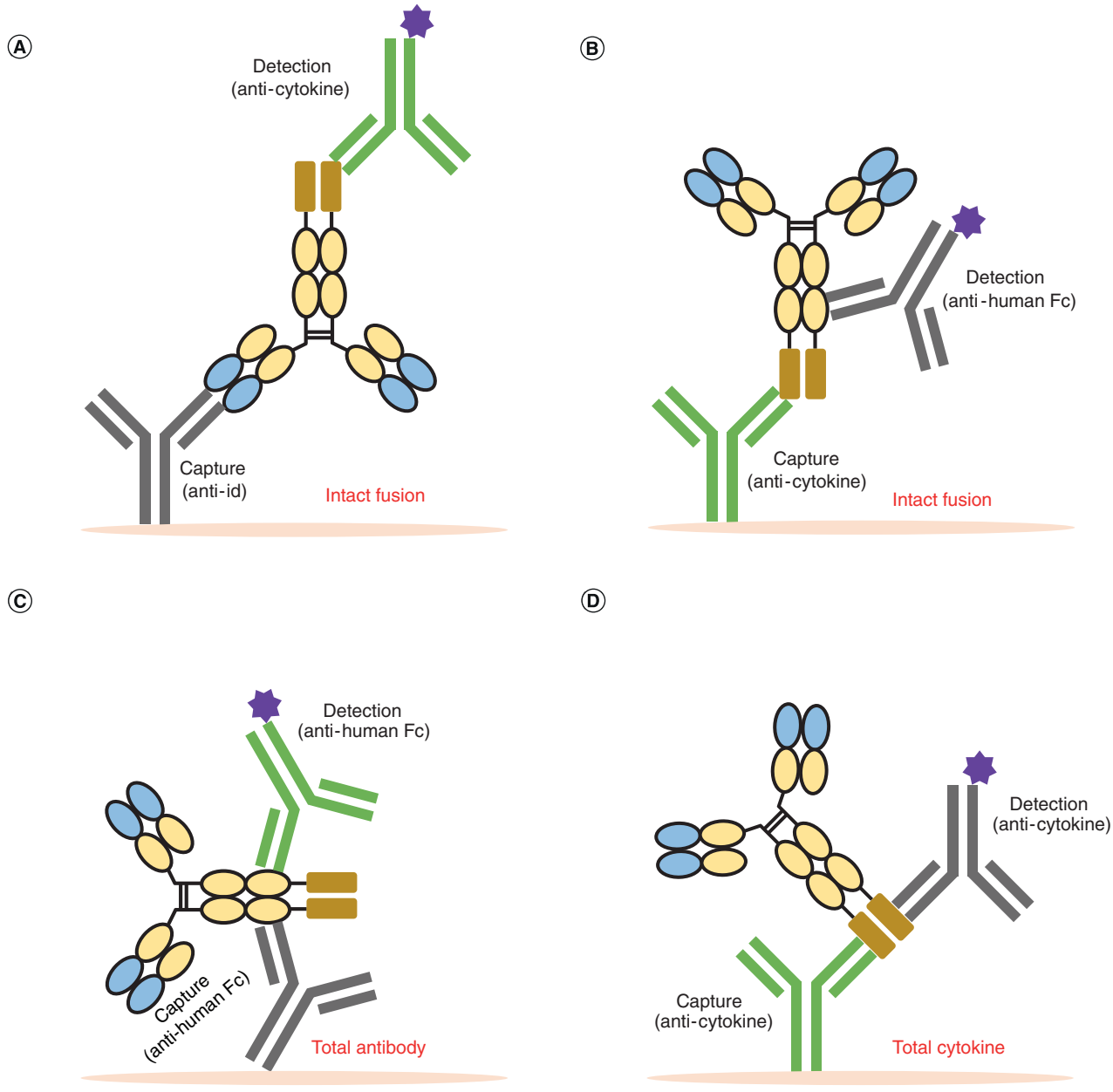

Figure 4. Differential ELISA assay for quantitation of a hypothetical monoclonal antibody-cytokine fusion protein. (A) ELISA for measurement of intact fusion (anti-id for capture, anticytokine for detection), **(B)** ELISA for measurement of intact fusion (anticytokine for capture, antihuman Fc for detection),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

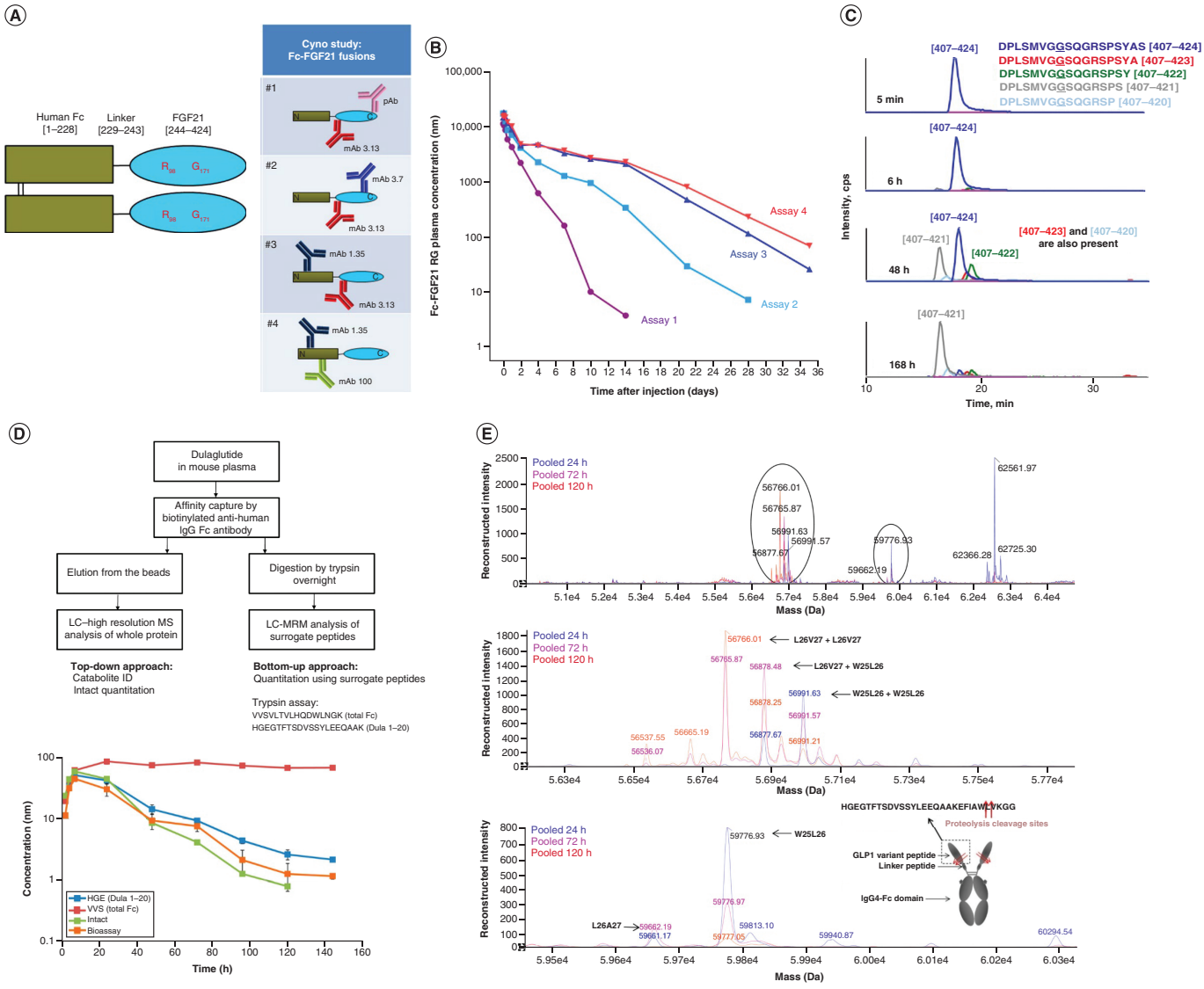

Figure 5. Complementarity of ligand-binding assay and LC–MS for the bioanalysis of Fc-FGF21 (RG) and dulaglutide. (A) Fc-FGF21 (RG) construct and schematic representation of differential ELISA assay formats used for quantitation of Fc-FGF21 (RG) in monkey serum. **(B)** PK profile of Fc-FGF21 (RG) dosed 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

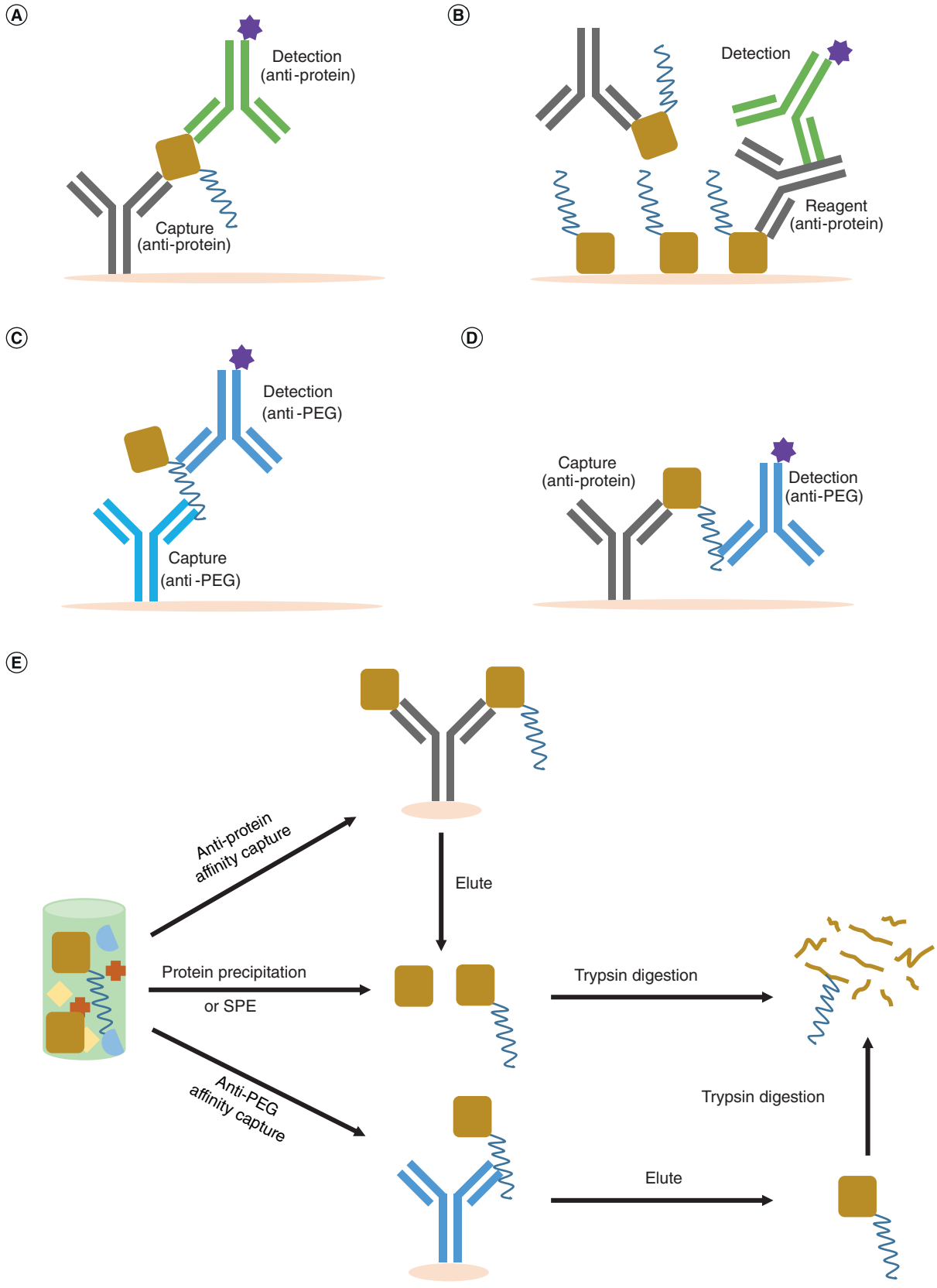

Figure 6. Ligand-binding assay and LC–MS/MS assays for the quantitation of PEGylated proteins. (A) Antiprotein sandwich ELISA, **(B)** competitive ELISA, **(C)** anti-PEG sandwich ELISA, **(D)** hybrid sandwich ELISA and **(E)** sample preparation procedures for LC–MS/MS assays. SPE: Solid phase extraction.

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].

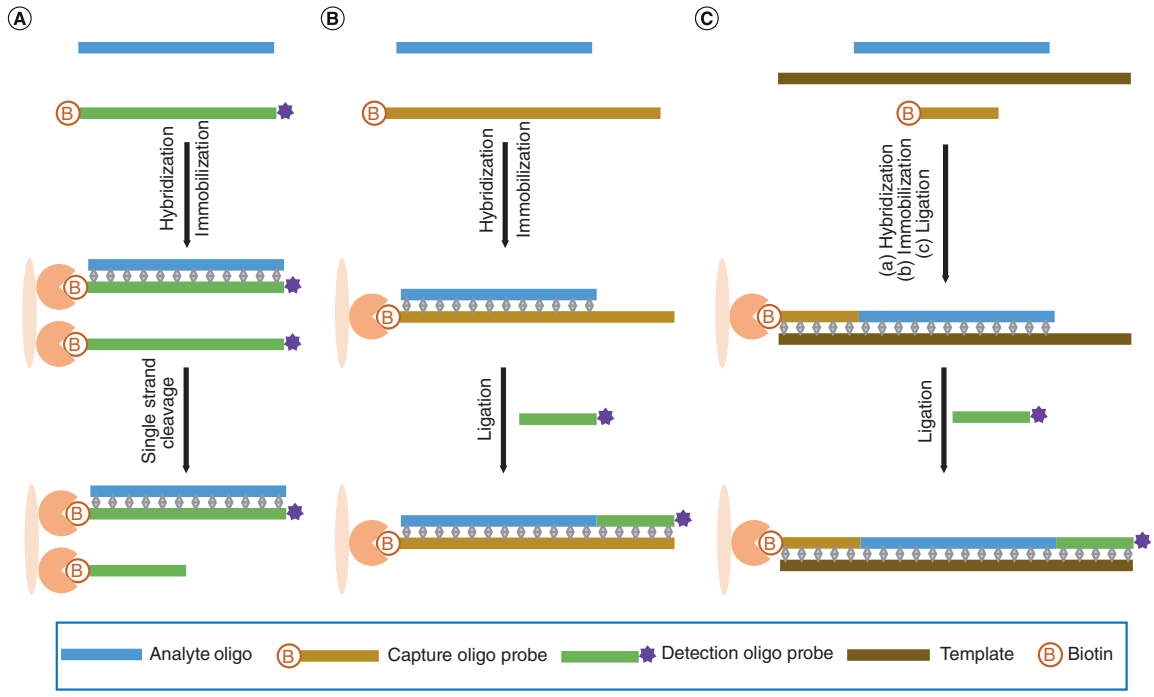

Figure 7. Hybridization ELISA formats for quantitation of therapeutic oligonucleotides. (A) One-step hybridization ELISA, **(B)** two-step hybridization ELISA and **(C)** dual ligation ELISA.

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

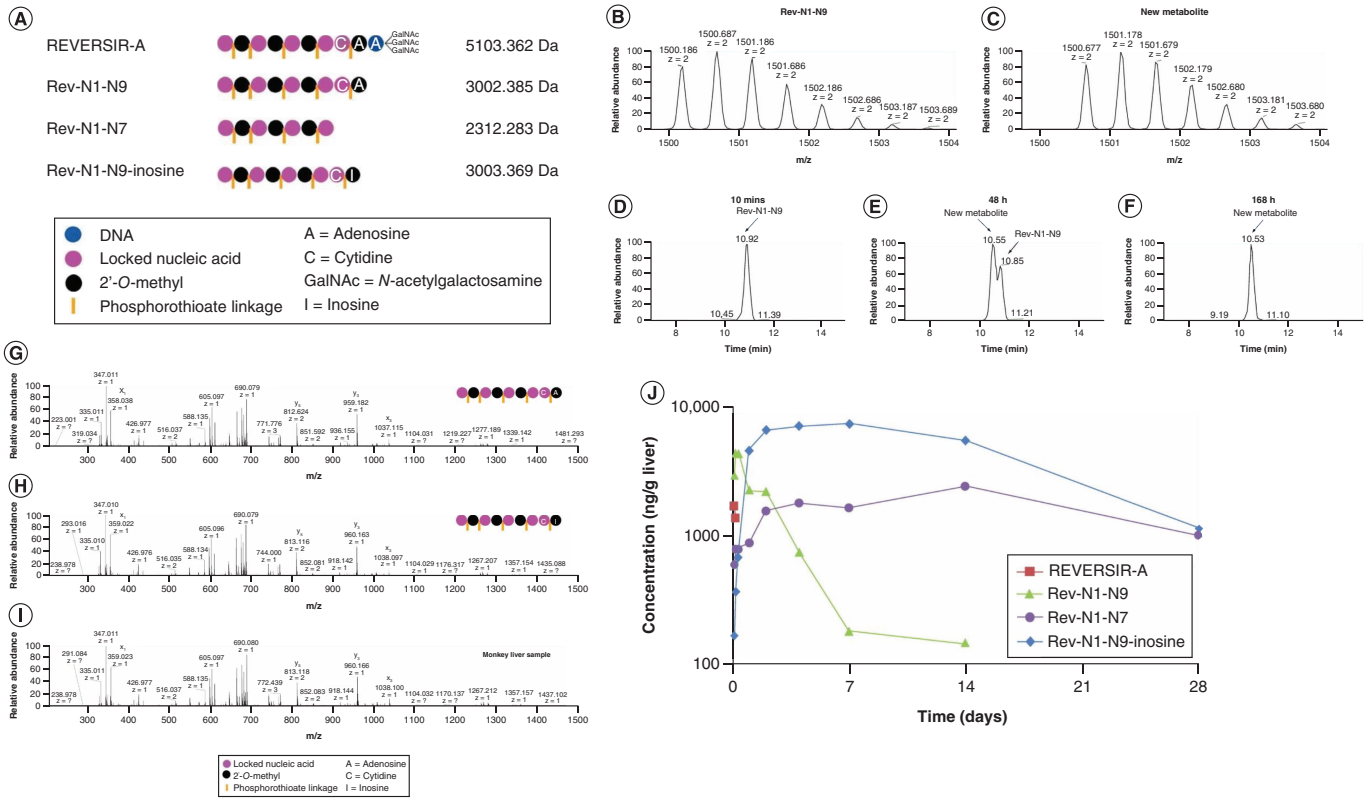

Figure 8. LC–MS/MS and LC–HRMS-based identification and quantification of REVERSIR-A and its metabolites. (A) Sequence and molecular weights of REVERSIR-A and its metabolites, **(B–C)** MS spectra and **(D–F)** Extracted ion chromatograms of Rev-N1-N9 and the new metabolite, **(G–I)** MS/MS spectra confirming 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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References

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

1. Blanco MJ, Gardinier KM. New chemical modalities and strategic thinking in early drug discovery. *ACS Med. Chem. Lett.* 11(3), 228–231 (2020).
2. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discov.* 7(1), 21–39 (2008).

3. Hall MP. Biotransformation and *in vivo* stability of protein biotherapeutics: impact on candidate selection and pharmacokinetic profiling. *Drug Metab. Dispos.* 42(11), 1873–1880 (2014).
4. Kintzing JR, Filsinger Interrante MV, Cochran JR. Emerging strategies for developing next-generation protein therapeutics for cancer treatment. *Trends Pharmacol. Sci.* 37(12), 993–1008 (2016).
5. Roberts TC, Langer R, Wood MJA. Advances in oligonucleotide drug delivery. *Nat. Rev. Drug Discov.* 19(10), 673–694 (2020).
6. Kaur S, Bateman KP, Glick J *et al.* IQ consortium perspective: complementary LBA and LC–MS in protein therapeutics bioanalysis and biotransformation assessment. *Bioanalysis* 12(4), 257–270 (2020).
7. Kellie JF, Karlinsey MZ. Review of approaches and examples for monitoring biotransformation in protein and peptide therapeutics by MS. *Bioanalysis* 10(22), 1877–1890 (2018).
8. van de Merbel NC. Protein quantification by LC–MS: a decade of progress through the pages of *Bioanalysis*. *Bioanalysis* 11(7), 629–644 (2019).

•• **Comprehensive review on LC–MS-based quantification of proteins.**

9. Kang L, Weng N, Jian W. LC–MS bioanalysis of intact proteins and peptides. *Biomed. Chromatogr.* 34(1), e4633 (2020).
10. Kaur S, Xu K, Saad OM, Dere RC, Carrasco-Triguero M. Bioanalytical assay strategies for the development of antibody–drug conjugate biotherapeutics. *Bioanalysis* 5(2), 201–226 (2013).

•• **Comprehensive overview of various bioanalytical methods for antibody–drug conjugate (ADC) quantitation and biotransformation assessment.**

11. Rangan VS, Myler H, Kozhich A, Wang J, Randazzo R, Deshpande S. Biotransformation and stability of antibody–drug conjugates: payload metabolism and linker cleavage delineation. *Bioanalysis* 7(11), 1319–1323 (2015).
12. Andersson S, Antonsson M, Elebring M, Jansson-Lofmark R, Weidolf L. Drug metabolism and pharmacokinetic strategies for oligonucleotide- and mRNA-based drug development. *Drug Discov. Today* 23(10), 1733–1745 (2018).
13. Ezan E, Bitsch F. Critical comparison of MS and immunoassays for the bioanalysis of therapeutic antibodies. *Bioanalysis* 1(8), 1375–1388 (2009).
14. Thway TM. Fundamentals of large-molecule protein therapeutic bioanalysis using ligand-binding assays. *Bioanalysis* 8(1), 11–17 (2016).
15. Eangoor P. A guided approach to preclinical bioanalysis of proteins using immunoassays for pharmacokinetic and pharmacodynamic assessments. *Bioanalysis* 12(16), 1105–1110 (2020).
16. Sugimoto H, Wei D, Dong L, Ghosh D, Chen S, Qian MG. Perspectives on potentiating immunocapture-LC–MS for the bioanalysis of biotherapeutics and biomarkers. *Bioanalysis* 10(20), 1679–1690 (2018).
17. Joubert N, Beck A, Dumontet C, Denevault-Sabourin C. Antibody–drug conjugates: the last decade. *Pharmaceuticals (Basel)* 13(9), 245 (2020).
18. Tsuchikama K, An Z. Antibody–drug conjugates: recent advances in conjugation and linker chemistries. *Protein Cell* 9(1), 33–46 (2018).
19. Lewis Phillips GD, Li G, Dugger DL *et al.* Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer Res.* 68(22), 9280–9290 (2008).
20. Dere R, Yi JH, Lei C *et al.* PK assays for antibody–drug conjugates: case study with ado-trastuzumab emtansine. *Bioanalysis* 5(9), 1025–1040 (2013).
21. Myler H, Rangan VS, Kozhich A *et al.* Validation of an integrated series of ligand-binding assays for the quantitative determination of antibody–drug conjugates in biological matrices. *Bioanalysis* 8(6), 519–531 (2016).
22. Wang J, Gu H, Liu A *et al.* Antibody–drug conjugate bioanalysis using LB-LC–MS/MS hybrid assays: strategies, methodology and correlation to ligand-binding assays. *Bioanalysis* 8(13), 1383–1401 (2016).

• **A comprehensive study comparing various ligand-binding assay and hybrid LC–MS/MS assays for quantitation of different ADC components.**

23. Zhu X, Huo S, Xue C, An B, Qu J. Current LC–MS-based strategies for characterization and quantification of antibody–drug conjugates. *J. Pharm. Anal.* 10(3), 209–220 (2020).
24. Liu A, Kozhich A, Passmore D *et al.* Quantitative bioanalysis of antibody-conjugated payload in monkey plasma using a hybrid immuno-capture LC–MS/MS approach: assay development, validation, and a case study. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 1002, 54–62 (2015).
25. Sanderson RJ, Nicholas ND, Baker Lee C *et al.* Antibody-conjugated drug assay for protease-cleavable antibody–drug conjugates. *Bioanalysis* 8(1), 55–63 (2016).
26. Hyung SJ, Li D, Koppada N, Kaur S, Saad OM. Method development of a novel PK assay for antibody-conjugated drug measurement of ADCs using peptide-linker drug analyte. *Anal. Bioanal. Chem.* 411(12), 2587–2596 (2019).
27. Shi C, Goldberg S, Lin T *et al.* Bioanalytical workflow for novel scaffold protein-drug conjugates: quantitation of total Centyrin protein, conjugated Centyrin and free payload for Centyrin-drug conjugate in plasma and tissue samples using liquid chromatography-tandem mass spectrometry. *Bioanalysis* 10(20), 1651–1665 (2018).

28. Faria M, Peay M, Lam B *et al.* Multiplex LC–MS/MS assays for clinical bioanalysis of MEDI4276, an antibody–drug conjugate of tubulysin analogue attached via cleavable linker to a biparatopic humanized antibody against HER-2. *Antibodies (Basel)* 8(1), 11 (2019).
29. Huang Y, Del Nagro CJ, Balic K *et al.* Multifaceted bioanalytical methods for the comprehensive pharmacokinetic and catabolic assessment of MEDI3726, an anti-prostate-specific membrane antigen pyrrolobenzodiazepine antibody–drug conjugate. *Anal. Chem.* 92(16), 11135–11144 (2020).
30. Jin W, Burton L, Moore I. LC-HRMS quantitation of intact antibody drug conjugate trastuzumab emtansine from rat plasma. *Bioanalysis* 10(11), 851–862 (2018).
31. Xu K, Liu L, Saad OM *et al.* Characterization of intact antibody–drug conjugates from plasma/serum *in vivo* by affinity capture capillary liquid chromatography-mass spectrometry. *Anal. Biochem.* 412(1), 56–66 (2011).

• **First report of development of a novel affinity capture LC–MS assay for ADC biotranformation assessment.**

32. He J, Su D, Ng C *et al.* High-resolution accurate-mass mass spectrometry enabling in-depth characterization of *in vivo* biotransformations for intact antibody–drug conjugates. *Anal. Chem.* 89(10), 5476–5483 (2017).
33. He J, Yu SF, Yee S, Kaur S, Xu K. Characterization of *in vivo* biotransformations for trastuzumab emtansine by high-resolution accurate-mass mass spectrometry. *MAbs* 10(7), 960–967 (2018).
34. Su D, Ng C, Khosraviani M *et al.* Custom-designed affinity capture LC–MS F(ab')2 assay for biotransformation assessment of site-specific antibody drug conjugates. *Anal. Chem.* 88(23), 11340–11346 (2016).
35. Jashnani A, Kotapati S, Deshpande M *et al.* Automated and faster affinity capture method for biotransformation assessment of site-specific antibody drug conjugates. *Anal. Chem.* 93(13), 5371−5376 (2021).
36. Grafmuller L, Wei C, Ramanathan R, Barletta F, Steenwyk R, Tweed J. Unconjugated payload quantification and DAR characterization of antibody–drug conjugates using high-resolution MS. *Bioanalysis* 8(16), 1663–1678 (2016).
37. Rago B, Clark T, King L *et al.* Calculated conjugated payload from immunoassay and LC–MS intact protein analysis measurements of antibody–drug conjugate. *Bioanalysis* 8(21), 2205–2217 (2016).
38. Davis JA, Kagan M, Read J, Walles M, Hatsis P. Immunoprecipitation middle-up LC–MS for *in vivo* drug-to-antibody ratio determination for antibody–drug conjugates. *Bioanalysis* 9(20), 1535–1549 (2017).
39. Kotapati S, Passmore D, Yamazoe S *et al.* Universal affinity capture liquid chromatography-mass spectrometry assay for evaluation of biotransformation of site-specific antibody drug conjugates in preclinical studies. *Anal. Chem.* 92(2), 2065–2073 (2020).

• **Development of a universal and generic affinity capture LC–MS assay for ADC biotransformation assessment independent of antibody type, conjugation site, chemistry and payload.**

40. Zaman R, Islam RA, Ibnat N *et al.* Current strategies in extending half-lives of therapeutic proteins. *J. Control. Rel.* 301, 176–189 (2019).
41. Iyengar ARS, Gupta S, Jawalekar S, Pande AH. Protein chimerization: a new frontier for engineering protein therapeutics with improved pharmacokinetics. *J. Pharmacol. Exp. Ther.* 370(3), 703–714 (2019).
42. Kim SJ, Kwak HH, Cho SY *et al.* Pharmacokinetics, pharmacodynamics, and efficacy of a novel long-acting human growth hormone: Fc fusion protein. *Mol. Pharm.* 12(10), 3759–3765 (2015).
43. Liu L, Gomathinayagam S, Hamuro L *et al.* The impact of glycosylation on the pharmacokinetics of a TNFR2: fc fusion protein expressed in Glycoengineered Pichia Pastoris. *Pharm. Res.* 30(3), 803–812 (2013).
44. Pearson JT, Rock DA. Bioanalytical approaches to assess the proteolytic stability of therapeutic fusion proteins. *Bioanalysis* 7(23), 3035–3051 (2015).
45. Hager T, Spahr C, Xu J, Salimi-Moosavi H, Hall M. Differential enzyme-linked immunosorbent assay and ligand-binding mass spectrometry for analysis of biotransformation of protein therapeutics: application to various FGF21 modalities. *Anal. Chem.* 85(5), 2731–2738 (2013).

• **Describes an integreated bioanalytical strategy involving both differential ELISA and hybrid LC–MS for quantitation and biotransformation assessment of a fusion protein.**

46. Gan J, Kendra K, Ricci M, Hank JA, Gillies SD, Sondel PM. Specific enzyme-linked immunosorbent assays for quantitation of antibody-cytokine fusion proteins. *Clin. Diagn. Lab. Immunol.* 6(2), 236–242 (1999).
47. Kendra K, Gan J, Ricci M *et al.* Pharmacokinetics and stability of the ch14.18-interleukin-2 fusion protein in mice. *Cancer Immunol. Immunother.* 48(5), 219–229 (1999).
48. Giragossian C, Vage C, Li J *et al.* Mechanistic investigation of the preclinical pharmacokinetics and interspecies scaling of PF-05231023, a fibroblast growth factor 21-antibody protein conjugate. *Drug Metab. Dispos.* 43(6), 803–811 (2015).
49. Sleczka BG, D'Arienzo CJ, Tymiak AA, Olah TV. Quantitation of therapeutic proteins following direct trypsin digestion of dried blood spot samples and detection by LC–MS-based bioanalytical methods in drug discovery. *Bioanalysis* 4(1), 29–40 (2012).
50. Zhu Y, D'Arienzo C, Lou Z *et al.* LC–MS/MS multiplexed assay for the quantitation of a therapeutic protein BMS-986089 and the target protein Myostatin. *Bioanalysis* 8(3), 193–204 (2016).

51. Halquist MS, Karnes HT. Quantification of Alefacept, an immunosuppressive fusion protein in human plasma using a protein analogue internal standard, trypsin cleaved signature peptides and liquid chromatography tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 879(11–12), 789–798 (2011).
52. Iwamoto N, Yokoyama K, Takanashi M, Yonezawa A, Matsubara K, Shimada T. Application of nSMOL coupled with LC–MS bioanalysis for monitoring the Fc-fusion biopharmaceuticals Etanercept and Abatacept in human serum. *Pharmacol. Res. Perspect.* 6(4), e00422 (2018).
53. Kang L, Camacho RC, Li W *et al.* Simultaneous catabolite identification and quantitation of large therapeutic protein at the intact level by immunoaffinity capture liquid chromatography-high-resolution mass spectrometry. *Anal. Chem.* 89(11), 6065–6075 (2017).
54. Zell M, Husser C, Staack RF *et al. In vivo* biotransformation of the fusion protein tetranectin-apolipoprotein A1 analyzed by ligand-binding mass spectrometry combined with quantitation by ELISA. *Anal. Chem.* 88(23), 11670–11677 (2016).
55. Li F, Weng Y, Zhang G, Han X, Li D, Neubert H. Characterization and quantification of an Fc-FGF21 fusion protein in rat serum using immunoaffinity LC–MS. *AAPS J.* 21(5), 84 (2019).
56. Kullolli M, Rock DA, Ma J. Immuno-affinity capture followed by TMPP N-terminus tagging to study catabolism of therapeutic proteins. *J. Proteome Res.* 16(2), 911–919 (2017).
57. Choy EH, Hazleman B, Smith M *et al.* Efficacy of a novel PEGylated humanized anti-TNF fragment (CDP870) in patients with rheumatoid arthritis: a Phase II double-blinded, randomized, dose-escalating trial. *Rheumatology (Oxford)* 41(10), 1133–1137 (2002).
58. Song XH, Huhle G, Wang LC, Harenberg J. Quantitative determination of PEG-hirudin in human plasma using a competitive enzyme-linked immunosorbent assay. *Thromb. Res.* 99(2), 195–202 (2000).
59. Bruno R, Sacchi P, Ciappina V *et al.* Viral dynamics and pharmacokinetics of peginterferon alpha-2a and peginterferon alpha-2b in naive patients with chronic hepatitis c: a randomized, controlled study. *Antivir. Ther.* 9(4), 491–497 (2004).
60. Cheng TL, Cheng CM, Chen BM *et al.* Monoclonal antibody-based quantitation of poly(ethylene glycol)-derivatized proteins, liposomes, and nanoparticles. *Bioconjug. Chem.* 16(5), 1225–1231 (2005).

- **Report describing the development and application of a specific anti-PEG monoclonal antibody for oligonucleotide quantitation.**

61. Su YC, Chen BM, Chuang KH, Cheng TL, Roffler SR. Sensitive quantification of PEGylated compounds by second-generation anti-poly(ethylene glycol) monoclonal antibodies. *Bioconjug. Chem.* 21(7), 1264–1270 (2010).
62. Myler HA, McVay S, Kratzsch J. Troubleshooting PEG-hGH detection supporting pharmacokinetic evaluation in growth hormone deficient patients. *J. Pharmacol. Toxicol. Methods* 61(2), 92–97 (2010).
63. Gorovits B, Clements-Egan A, Birchler M *et al.* Pre-existing antibody: biotherapeutic modality-based review. *AAPS J.* 18(2), 311–320 (2016).
64. Yang Z, Ke J, Hayes M, Bryant M, Tse FL. A sensitive and high-throughput LC–MS/MS method for the quantification of PEGylated-interferon-alpha2a in human serum using monolithic C18 solid phase extraction for enrichment. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 877(18–19), 1737–1742 (2009).
65. Xu Y, Mehl JT, Bakhtiar R, Woolf EJ. Immunoaffinity purification using anti-PEG antibody followed by two-dimensional liquid chromatography/tandem mass spectrometry for the quantification of a PEGylated therapeutic peptide in human plasma. *Anal. Chem.* 82(16), 6877–6886 (2010).
66. Liu L, Liu Y, Dai L *et al.* Immunoaffinity LC–MS/MS to quantify a PEGylated anti-Factor D Fab biotherapeutic in cynomolgus monkey serum. *Bioanalysis* 11(23), 2161–2173 (2019).
67. Li H, Rose MJ, Holder JR, Wright M, Miranda LP, James CA. Direct quantitative analysis of a 20 kDa PEGylated human calcitonin gene peptide antagonist in cynomolgus monkey serum using in-source CID and UPLC–MS/MS. *J. Am. Soc. Mass Spectrom.* 22(9), 1660–1667 (2011).
68. Gong J, Gu X, Achanzar WE *et al.* Quantitative analysis of polyethylene glycol (PEG) and PEGylated proteins in animal tissues by LC–MS/MS coupled with in-source CID. *Anal. Chem.* 86(15), 7642–7649 (2014).
69. Zheng N, Zeng J, Manney A *et al.* Quantitation of a PEGylated protein in monkey serum by UHPLC-HRMS using a surrogate disulfide-containing peptide: a new approach to bioanalysis and *in vivo* stability evaluation of disulfide-rich protein therapeutics. *Anal Chim. Acta* 916, 42–51 (2016).
70. Wang SJ, Wu ST, Gokemeijer J *et al.* Attribution of the discrepancy between ELISA and LC–MS/MS assay results of a PEGylated scaffold protein in post-dose monkey plasma samples due to the presence of anti-drug antibodies. *Anal. Bioanal. Chem.* 402(3), 1229–1239 (2012).
71. Liu Q, De Felippis MR, Huang L. Method for characterization of PEGylated bioproducts in biological matrixes. *Anal. Chem.* 85(20), 9630–9637 (2013).
72. Goyon A, Yehl P, Zhang K. Characterization of therapeutic oligonucleotides by liquid chromatography. *J. Pharm. Biomed. Anal.* 182, 113105 (2020).
73. Wei X, Dai G, Marcucci G *et al.* A specific picomolar hybridization-based ELISA assay for the determination of phosphorothioate oligonucleotides in plasma and cellular matrices. *Pharm. Res.* 23(6), 1251–1264 (2006).

74. Yu RZ, Baker B, Chappell A, Geary RS, Cheung E, Levin AA. Development of an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligodeoxynucleotide in plasma. *Anal. Biochem.* 304(1), 19–25 (2002).

75. Thayer MB, Lade JM, Doherty D *et al.* Application of locked nucleic acid oligonucleotides for siRNA preclinical bioanalytics. *Sci. Rep.* 9(1), 3566 (2019).

- **A novel and sensitive hybridization ELISA incorporating locked nucleic acid into capture and detect probes.**

76. Efler SM, Zhang L, Noll BO, Uhlmann E, Davis HL. Quantification of oligodeoxynucleotides in human plasma with a novel hybridization assay offers greatly enhanced sensitivity over capillary gel electrophoresis. *Oligonucleotides* 15(2), 119–131 (2005).

77. Tremblay G, Khalafaghian G, Legault J, Nielsen P, Bartlett A. Dual ligation hybridization assay for the specific determination of oligonucleotide therapeutics. *Bioanalysis* 3(5), 499–508 (2011).

78. Deverre JR, Boutet V, Boquet D, Ezan E, Grassi J, Grognet JM. A competitive enzyme hybridization assay for plasma determination of phosphodiester and phosphorothioate antisense oligonucleotides. *Nucleic Acids Res.* 25(18), 3584–3589 (1997).

79. Chan KK, Liu Z, Xie Z *et al.* A novel ultrasensitive hybridization-based ELISA method for 2-methoxyphosphorothiolate microRNAs and its *in vitro* and *in vivo* application. *AAPS J.* 12(4), 556–568 (2010).

80. Lorenson MY, Chen KE, Walker AM. Enzyme-linked oligonucleotide hybridization assay for direct oligo measurement in blood. *Biol. Methods Protoc.* 4(1), bpy014 (2019).

81. Humphreys SC, Thayer MB, Campuzano IDG, Netirojjanakul C, Rock BM. Quantification of siRNA-antibody conjugates in biological matrices by triplex-forming oligonucleotide ELISA. *Nucleic Acid Ther.* 29(3), 161–166 (2019).

82. Tremblay GA, Oldfield PR. Bioanalysis of siRNA and oligonucleotide therapeutics in biological fluids and tissues. *Bioanalysis* 1(3), 595–609 (2009).

83. Wang L. Oligonucleotide bioanalysis: sensitivity versus specificity. *Bioanalysis* 3(12), 1299–1303 (2011).

84. Wang L, Ji C. Advances in quantitative bioanalysis of oligonucleotide biomarkers and therapeutics. *Bioanalysis* 8(2), 143–155 (2016).

85. Nuckowski L, Kaczmarkiewicz A, Studzinska S. Review on sample preparation methods for oligonucleotides analysis by liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 1090, 90–100 (2018).

- **Comprehensive review on various sample preparation methods for LC–MS quantitation of oligonucleotides.**

86. Sips L, Ediage EN, Ingelse B, Verhaeghe T, Dillen L. LC–MS quantification of oligonucleotides in biological matrices with SPE or hybridization extraction. *Bioanalysis* 11(21), 1941–1954 (2019).

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

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

89. Dai G, Wei X, Liu Z, Liu S, Marcucci G, Chan KK. Characterization and quantification of Bcl-2 antisense G3139 and metabolites in plasma and urine by ion-pair reversed phase HPLC coupled with electrospray ion-trap mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 825(2), 201–213 (2005).

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

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

92. MacNeill R, Hutchinson T, Acharya V, Stromeyer R, Ohorodnik S. An oligonucleotide bioanalytical LC-SRM methodology entirely liberated from ion-pairing. *Bioanalysis* 11(12), 1157–1169 (2019).

93. Liu J, Li J, Tran C *et al.* Oligonucleotide quantification and metabolite profiling by high-resolution and accurate mass spectrometry. *Bioanalysis* 11(21), 1967–1980 (2019).

94. Ramanathan L, Shen H. LC-TOF-MS methods to quantify siRNAs and major metabolite in plasma, urine and tissues. *Bioanalysis* 11(21), 1983–1992 (2019).

95. Kim J, Basiri B, Hassan C *et al.* Metabolite profiling of the antisense oligonucleotide eluforsen using liquid chromatography-mass spectrometry. *Mol. Ther. Nucleic Acids* 17, 714–725 (2019).

96. Gaus HJ, Owens SR, Winniman M, Cooper S, Cummins LL. On-line HPLC electrospray mass spectrometry of phosphorothioate oligonucleotide metabolites. *Anal. Chem.* 69(3), 313–319 (1997).

97. Beverly M, Hartsough K, Machemer L. Liquid chromatography/electrospray mass spectrometric analysis of metabolites from an inhibitory RNA duplex. *Rapid Commun. Mass Spectrom.* 19(12), 1675–1682 (2005).

98. Beverly M, Hartsough K, Machemer L, Pavco P, Lockridge J. Liquid chromatography electrospray ionization mass spectrometry analysis of the ocular metabolites from a short interfering RNA duplex. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 835(1–2), 62–70 (2006).

99. Zou Y, Tiller P, Chen IW, Beverly M, Hochman J. Metabolite identification of small interfering RNA duplex by high-resolution accurate mass spectrometry. *Rapid Commun. Mass Spectrom.* 22(12), 1871–1881 (2008).

100. Husser C, Brink A, Zell M, Muller MB, Koller E, Schadt S. Identification of GalNAc-conjugated antisense oligonucleotide metabolites using an untargeted and generic approach based on high resolution mass spectrometry. *Anal. Chem.* 89(12), 6821–6826 (2017).

101. Li J, Liu J, Enders J *et al.* Discovery of a novel deaminated metabolite of a single-stranded oligonucleotide *in vivo* by mass spectrometry. *Bioanalysis* 11(21), 1955–1965 (2019).

- **Application of LC–HRMS for simultaneous quantification and metabolite identification of a therapeutic oligonucleotide.**

102. Zhang YJ, Olah TV, Zeng J. The integration of ligand binding and LC–MS-based assays into bioanalytical strategies for protein analysis. *Bioanalysis* 6(13), 1827–1841 (2014).

Waters™

Application Note

An Automated, Standardized, Kit-Based Sample Preparation Workflow for Bioanalytical Quantification of Therapeutic Oligonucleotides

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Abstract

Oligonucleotide Therapeutics (ONTs) are a key focus area for many drug developers today given their powerful ability to address disease biology at the level of gene transcription and translation, and for their high target specificity and low toxicity. As the pipeline for this therapeutic class of drugs continues to expand, so does the need for sensitive, accurate, and robust bioanalytical assays to support this drug discovery and development pipeline. LC-MS detection and quantification is a widely accepted technology for bioanalytical studies, for the many benefits it affords (*i.e.*, broad drug applicability, sensitivity, selectivity, and broad linear dynamic range). However, achieving reproducible performance with LC-MS based bioanalytical assays can be challenging. In general, the greatest source of variability for these assays arises from the sample preparation needed to extract the drug and its metabolites from biofluids, and this is especially true for oligonucleotide extractions. Liquid-Liquid Extraction (LLE) and Solid Phase Extraction (SPE) are the two most widely used techniques for the extraction of ONTs from biofluids for LC-MS based quantification. LLE is a low throughput, difficult to automate technique which skilled and experienced scientists to develop, optimize, and implement these methods within a lab or across an organization. SPE is a more automation friendly, higher throughput assay, but may require

systematic optimization of every step to achieve desired recovery, reproducibility, and sensitivity. To this end, a simple, broadly applicable sample preparation workflow for ONTs that reduces the need for method development and brings greater consistency and reproducibility to LC-MS bioanalytical results is therefore highly desired. The OligoWorks™ SPE Microplate Kit (OligoWorks Kit) from Waters has been designed with this in mind. It utilizes standardized, detergent free reagents, and a robust optimized protocol that works across a diverse range of ONTs with little to no method development needed. The automation friendly reagents and SPE devices provided in each kit make it easy to automate the sample preparation procedure on an automated liquid handler, like the Andrew+™ Pipetting Robot, which can further enhance analytical performance and productivity and reduce human error/variability.

This work uses the OligoWorks Kit components and standard protocol (Figure 1) automated on the Andrew+ Pipetting Robot to successfully extract a diverse range of ONTs from plasma and achieve accurate, robust, and reproducible bioanalytical performance, with little to no need for method development.

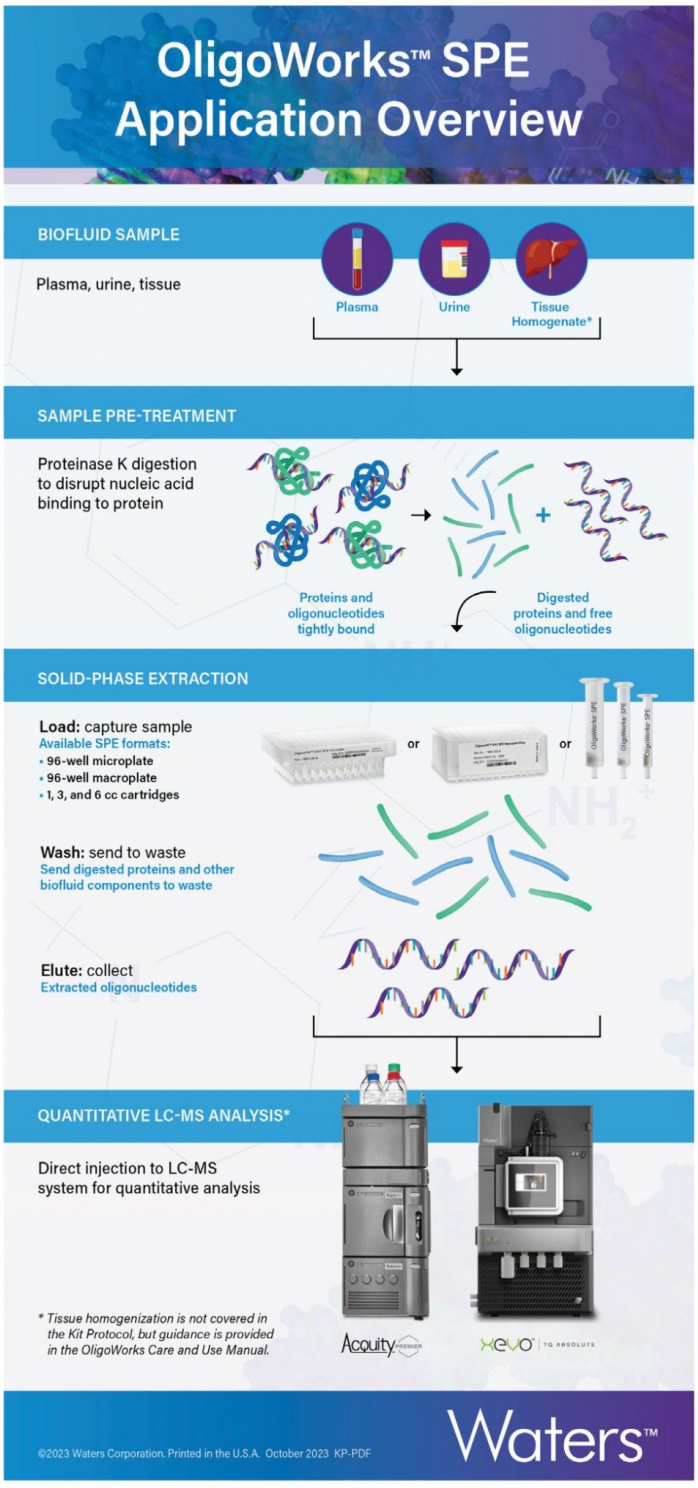

Figure 1. Graphical illustration of oligonucleotide bioanalytical quantification sample

preparation, extraction and LC-MS workflow.

Benefits

- A standardized, detergent free, kit-based solution for the extraction and LC-MS quantification of therapeutic oligonucleotides from biomatrices that requires little to no method development
- Achieve excellent recoveries (>80%) with low %CV (<15%) across a diverse range of ONTs
- Automation friendly workflow as demonstrated with the Andrew+ Pipetting Robot, with Click & Execute OneLab™ Software Library Methods that make implementation easy and improve assay performance
- Accurate, sensitive, and reproducible quantification across a diverse set of therapeutic oligonucleotides from extracted plasma samples

Introduction

The Solution

OligoWorks Kits are simple, standardized, flexible, and automation friendly sample preparation kits designed to enable accurate and robust LC-MS based bioanalytical quantitation across a diversity of oligonucleotides. The kits use an effective enzyme-based digestion sample pretreatment step with RapiZyme™ Proteinase K Digestion module to effectively disrupt oligonucleotide-biomatrix protein binding followed by selective purification using the OligoWorks SPE device, which contains a mixed-mode anion exchange SPE sorbent, designed, and QC verified for oligonucleotide performance. Each kit contains pre-measured, lot traceable, detergent free reagents, and a universal protocol to streamline the oligonucleotide sample preparation workflow and facilitate implementation by users at all experience levels.

The goals of this work were to demonstrate efficient extraction and accurate quantification of oligonucleotides from plasma using the OligoWorks Microplate Kit, automated on the Andrew+ Pipetting Robot. For this evaluation, gene-expression modulator 91 (GEM91), a 25-mer phosphorothioated antisense oligonucleotide (MWT 7771), GEM 132, a 20-mer phosphorothioated antisense oligonucleotide with 2′ methoxy caps (MWT 6600), a N-Acetylalactoseamine (GalNAc) conjugated siRNA (MWT 8590), and a 20-mer single-stranded DNA (ssDNA)

oligonucleotide (MWT 6122) were used.

Experimental

LC-MS Chromatographic Separation and Experimental Conditions

LC system:	ACQUITY™ Premier UPLC System with FTN
Column:	ACQUITY Premier Oligonucleotide C_{18} Column, 130 Å, 1.7 µm, 2.1 x 50 mm, 1/pk (p/n: 186009484)
Column temperature (°C):	55 °C
Sample temperature (°C):	10 °C
Mobile phase A:	1% HFIP (Hexafluoro-2-propanol) 0.1% DIPEA (N, N-Diisopropylethylamine) in H_2O
Mobile phase B:	0.75% HFIP (Hexafluoro-2-propanol), 0.0375% DIPEA (N, N-Diisopropylethylamine, 65% ACN 35% H_2O
Purge solvent:	25:25:25:25 Methanol:Acetonitrile:Isopropanol:Water
Injection volume (µL):	10 µL

LC Gradient table

Time (min)	Flow (mL/min)	%A	%B	Curve
Initial	0.600	95	5	6
3.25	0.600	77	23	6
3.75	0.600	10	90	6
4.10	0.600	10	90	6
4.25	0.600	95	5	6

MS System Conditions

MS system:	Xevo™ TQ Absolute MS
Ionisation mode:	ESI Negative
Acquisition mode:	MRM
Capillary voltage (kV):	3
Desolvation temperature (°C):	600
Desolvation gas flow (L/Hr):	1000
Cone gas flow (L/Hr):	150
Collision gas flow (L/Hr):	0.2
Nebulizer (Bar):	7

MRM Transitions

MRM transitions				
Oligonucleotide	**Precursor (*m/z*)**	**Product (*m/z*)**	**Cone voltage (V)**	**Collison energy (eV)**
GEM91	646.6	95.0	40	30
GEM132	824.5	94.9	40	40
GalNAc	714.6	227.4	40	20
ss DNA (20-mer)	764.3	125.1	40	30

Data Management

Instrument control software:	MassLynx™ (v4.2)
Quantification software:	TargetLynx™ (v4.2)
Automation software:	OneLab (1.19.2)

Chemicals, reagents, materials and solvents

GEM91 and GEM132 were sourced from Avecia Nitto Denko (MA, USA), GalNAc conjugated siRNA was kindly donated by Alnylam Pharmaceuticals (Cambridge, MA). The ssDNA 20-mer oligonucleotide was sourced from Waters Corporation (Milford, MA).

MS grade Methanol, water, acetonitrile, isopropanol, Hexafluoro-2-propanol (HFIP), N,N-Diisopropylethylamine (DIPEA) and ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). K_2 EDTA rat plasma was procured from BioIVT (Westbury, NY, USA). DNase/RNase-free distilled water was purchased from ThermoFisher Scientific (p/n: 10977015) and was used for oligonucleotide standard preparation and SPE sample eluate dilution. OligoWorks Kit (p/n: 186010614 <https://prod1-author.waters.com/nextgen/global/shop/application-kits/186010614-oligoworks-spe-microplate-kit.html>) was procured from Waters Corporation (Milford, MA, USA).

OligoWorks Kit Wash Reagent Preparation

OligoWorks Kit SPE Wash 1: 50 mM Ammonium Acetate buffer, pH 5.5 was prepared by weighing out 3.84 g ammonium acetate and bringing to 1 Liter volume and adjusting pH to 5.5.

OligoWorks Kit SPE Wash 2: 30% Methanol/70% Water solution was prepared by adding 300 mL of methanol to 700 mLs of water.

Stock solutions, Calibration Curve, and QC Sample Preparation

GEM91, GEM132, GalNAc conjugated siRNA, and ssDNA were reconstituted in RNase/DNase-free distilled Water to provide a 1 mg/mL stock solution using Eppendorf DNA LoBind™ Tubes (p/n: 022431021 and 022431005). A combined working stock solution for all four oligonucleotides at 10 µg/mL each was created by adding 10 µL of each of the 1 mg/mL stock solution to 960 µL of water in DNA LoBind tubes. Calibration curve (0.25–1000 ng/mL) and quality control (QC) samples (LQC-0.75 ng/mL, MQC-50 ng/mL and HQC-750 ng/mL) in plasma were prepared using the Andrew+ Pipetting Robot.

Sample Pretreatment and SPE Extraction using the OligoWorks Microplate Kit

Prepared calibration curve and QC samples (100 µL) were added to an Eppendorf 1mL deep well plate and digested using the reagents and protocol supplied in the RapiZyme Proteinase K Digestion Module and subsequently extracted using the OligoWorks Kit SPE Microplate and eluent, following the protocol provided in the OligoWorks Kit and OligoWorks care and use manual (720008066 < https://www.waters.com/waters/support.htm?lid=135127508>). This protocol is illustrated in Figure 2. (Note: reagent volume of Proteinase K in the OligoWorks kit is sufficient to automate a full plate of 96 samples with 10% overage. If higher overage is desired, additional RapiZyme Proteinase K Digestion Module (p/n: 186010601 < https://www.waters.com/nextgen/global/shop/standards--reagents/186010601-rapizyme-proteinase-k-digestion-module.html>) can be procured separately.)

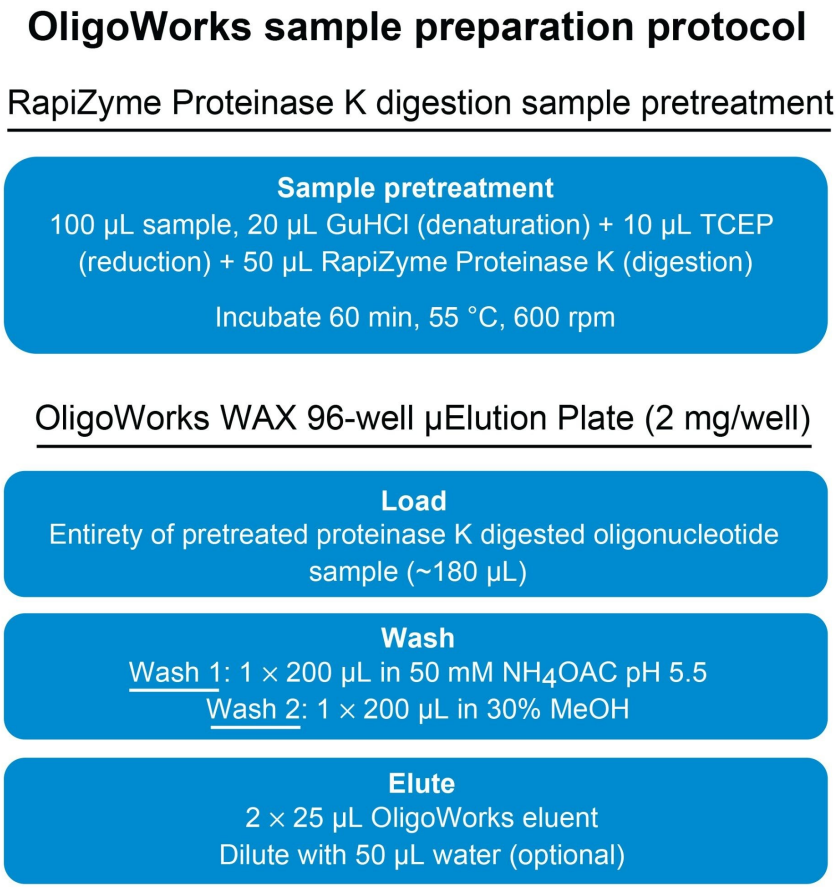

Figure 2. Graphical representation of the OligoWorks Kit Protocol (p/n: 186010614), optimized for 100 µL starting plasma/sera sample.

Automation platform

Andrew+ Pipetting Robot was used to generate calibration curves and QC's of plasma samples in a Waters QuanRecovery 700 µL plate by downloading and modifying the Simple Serial Dilution Preparation < https://onelab.andrewalliance.com/app/lab/GK6ovDkA/library/simple-serial-dilution-preparation-9jn2GGwa> method from the OneLab Software Library. All calibration curves and QC's were then extracted in triplicate by downloading the Click & Execute OligoWorks RapiZyme Proteinase K Digestion method (Figure 3A) and OligoWorks WAX SPE Microplate method (Figure 3B) from the OneLab Software Methods Library. The complete workflow, from creating plasma calibration curves and QC samples to digesting and extracting the oligonucleotides with the OligoWorks Microplate Kit was fully automated on Andrew+ Pipetting Robot

configured with the Heater-Shaker+ and Extraction+ Connected Devices.

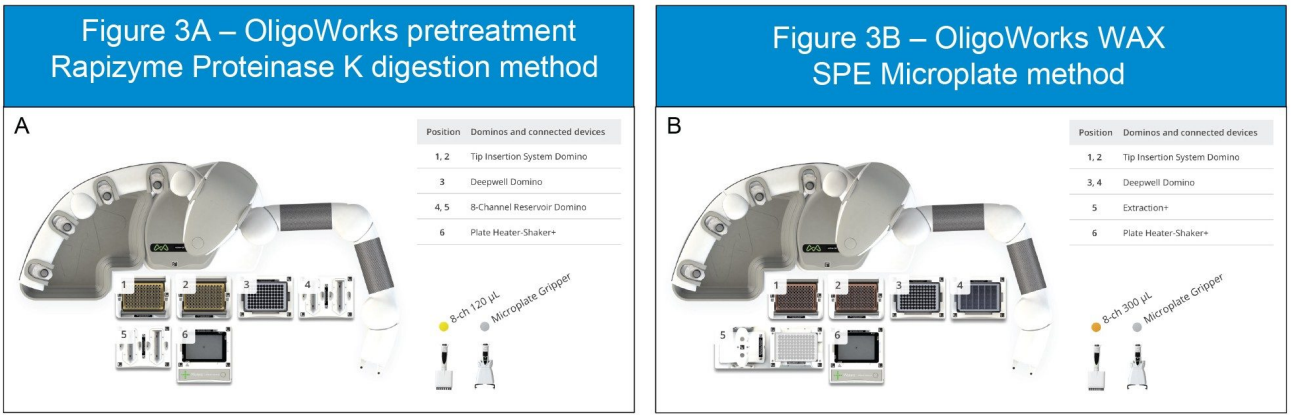

Figure 3. Representative Andrew+ Pipetting Robot Deck Layouts for oligonucleotide sample pretreatment using a Proteinase K Digestion (A) and OligoWorks WAX SPE 96-well Microplate (B). Both layouts illustrate the placement of all dominos, connected devices, and appropriate pipettes required for execution of these methods.

Results and Discussion

Therapeutic Oligonucleotides have proven to be very effective therapies for certain types of genetic or translational dysregulation diseases. With the increase in interest in exploring this therapeutic class for a variety of clinical conditions comes the need for simple, accurate, and robust analytical techniques to analyze and quantify these molecules. Achieving efficient and reproducible extraction of these analytes from complex biological matrices with high recoveries, using relatively simple sample preparation protocols is critically important for LC-MS quantification. Many ADME/DMPK workflows are highly automated for increased efficiency and reproducibility. Liquid-liquid extraction (LLE)-SPE is commonly used for extraction of oligonucleotides from biological matrices. Although effective, LLE is a slow, low-throughput manual process that is not easily automatable or scalable. Other commercially available SPE kits for this workflow use detergent-based reagents, which require extensive washing during SPE to remove these detergents and often require evaporation and reconstitution before injection into LC-MS systems to ensure SPE eluent compatibility. These steps add time, and often increase assay variability with the potential of oligonucleotide loss due to adsorption, solubility, and

potential degradation.

In contrast the OligoWorks Kit-based solution utilizes a simple, detergent-free workflow for LC-MS quantification of ONTs from biofluids that works well across a diverse range of ONTs with little to no method development, and as demonstrated in this study, is easily automated. Sample pretreatment with the RapiZyme Proteinase K Digestion Module effectively disrupts the strong oligonucleotide protein binding that occurs in biological fluids without the use of detergents, thereby removing the need for extensive washing and dry down steps prior to LC-MS analysis. The OligoWorks WAX SPE sorbent is designed to selectively bind oligonucleotides and wash away unwanted matrix components contained within the sample, resulting in a clean SPE eluate that can be directly injected onto an LC-MS system. As described in application note 720008086, the OligoWorks solution demonstrates excellent performance, with high recovery and repeatability across a diverse range of oligonucleotides and with various starting biological sample volumes. The Click and Execute OneLab Software Library Methods for OligoWorks sample pretreatment & SPE enable rapid method deployment, execution, and scalability while lowering risk of human error thus enhancing reproducibility and enabling robust analytical performance. Use of the OligoWorks Kit starting protocol, fully automated on the Andrew+ resulted in excellent oligonucleotide recovery from plasma (>96%) with less than 5% difference seen between manual and automated sample processing as illustrated in Figure 4.

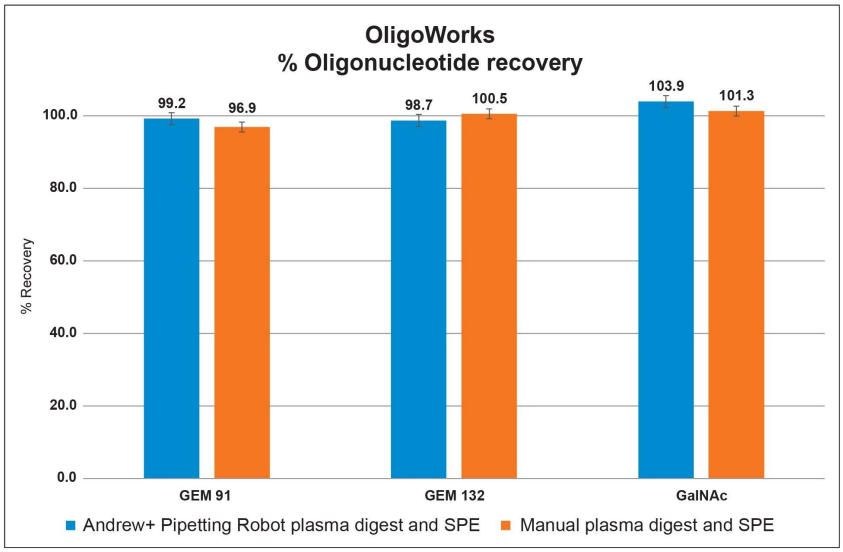

Figure 4. Comparable automated (Andrew+ Pipetting Robot) vs manual sample preparation and extraction performance using the OligoWorks Kits for GEM91, GEM132, and GalNAc oligonucleotides with >96% recovery and <5% difference from manual to automated sample processing ensuring a fit-for-purpose automated OligoWorks sample preparation and extraction solution.

The OligoWorks kit utilized in this study required no method development and facilitated the accurate quantification of four oligonucleotide therapies yielding excellent quantitative performance (no internal standard correction) from 100 µL plasma samples. Automating the workflow on the Andrew+ Pipetting Robot, lower limits of quantification (LLOQ's) of 250 pg/mL for GEM91, GEM132, and GalNAc conjugated siRNA, and 0.50 ng/mL for ssDNA 20-mer oligonucleotides were observed. Calibration curves were linear (r^2>0.99) from 0.25–1000 ng/mL (GEM91, GEM132, and GalNAc-siRNA) and 0.5–1000 ng/mL (ss DNA), with % bias and coefficient of variations (CV's) <15% for all triplicate points at each level achieving the recommended small molecule bioanalytical method validation criteria (as shown in Table 1). Specifically, accuracies and CVs across the calibration curves for GEM91, GEM 132, GalNAc, and ss DNA ranged from 85.2–119.2% and 1.97–13.87%, respectively.

Accuracy and precision for all QC levels across triplicate extractions was also within the bioanalytical method validation guidelines of ±15%. Mean accuracies for QC points for GEM91, GEM132, GalNAc conjugated siRNA, and ssDNA 20-mer oligonucleotides were between 92.30–104.07% with mean CVs between 2.82–6.77%,

respectively (as shown in Table 1). Area response for QC points increased linearly across the concentration range, as illustrated in Figure 5.

Sensitive, linear, accurate and precise

Calibration curve statistics

Analyte	Range	Weighting	Linear regression	% Accuracy range	% CV range
GEM91	0.25–1000 ng/mL	1/x	>0.99	85.4–114.7	2.01–11.43
GalNAc				85.2–114.4	2.01–13.44
GEM132				85.9–119.2*	1.97–9.67
ss DNA (20-mer)	0.50–1000 ng/mL			85.7–112.4	0.99–13.87

*% Accuracy of 119.2 for LLOQ - Acceptable per Bioanalytical method validation guidelines

QC statistics

Analyte	QC level	Expected concentration (ng/mL)	Mean observed concentration (ng/mL) (N=3)	Mean % accuracy (N=3)	Mean % CV (N=3)
GEM91	LQC	0.75	0.74	98.17	6.42
GalNAc			0.69	92.30	2.90
GEM132			0.78	104.07	5.13
ss DNA (20-mer)			0.69	92.63	8.69
GEM91	MQC	50	52.97	105.95	2.82
GalNAc			49.79	99.56	6.42
GEM132			51.72	103.43	7.41
ss DNA (20-mer)			55.15	110.29	0.99
GEM91	HQC	750	756.55	100.87	4.61
GalNAc			733.97	97.87	13.44
GEM132			748.28	99.82	6.77
ss DNA (20-mer)			763.63	101.84	4.66

Table 1. Linear accurate and precise quantitation calibration curve sample (A) and QC sample (B) performance statistics for GEM91, GEM132, GalNAc, and ss-DNA 20-mer oligonucleotides from plasma, using the OligoWorks kit, automated on the Andrew+ Pipetting Robot followed by and subsequent LC-MS/MS analysis.

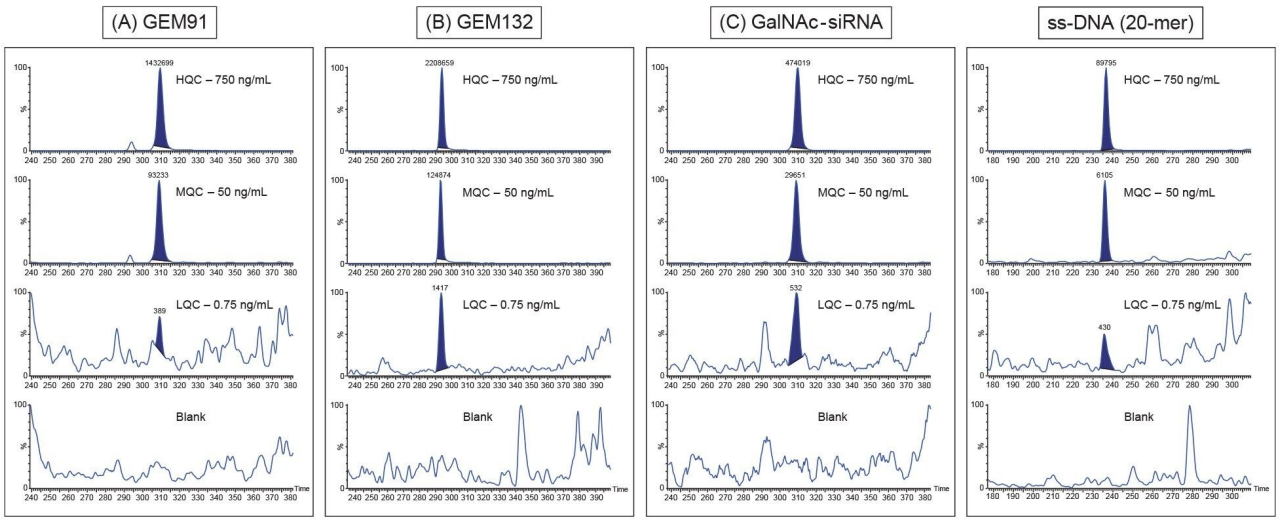

Figure 5. Representative QC chromatograms for GEM91 (A), GEM132(B), GalNAc (C), and ss DNA (D).

Conclusion

Accurate and robust oligonucleotide quantification from plasma was achieved using the OligoWorks SPE Microplate kit (with simple stepwise protocols and standardized, pre-measured, detergent free reagents). This workflow was fully automated on the Andrew+ Pipetting Robot with downloadable OneLab Click & Execute Library Methods to facilitate easy and reproducible execution of the OligoWorks Kit sample preparation and extraction workflow from day-to-day, user-to-user, and lab-to-lab. This fully automated and standardized approach (achieving high oligonucleotide recovery) greatly simplified and streamlined sample extraction, maximized lab productivity, reduced errors, and ensured overall analytical method performance.

References

1. Margot Lee, Nikunj Tanna, Mary Trudeau. Development of a Standardized, Kit-Based Approach for Selective and Reproducible Sample Preparation and Extraction for Therapeutic Oligonucleotides from Biological

Matrices, Waters Application Note 720008086, September 2023.

2. OligoWorks SPE Kits and Components, Waters User Manual, 720008066 <https://www.waters.com/waters/support.htm?lid=135127508> .

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