For reprint orders, please contact: reprints@future-science.com

The exciting world of oligonucleotides: a multidisciplinary complex challenge for multitasking ingenious bioanalysts

Yuanxin Xu¹, Fabio Garofolo^{*.2} & Adrien Musuku³

¹Alnylam Pharmaceuticals, Cambridge, MA, USA

²Angelini Pharma, Rome, Italy

³Pharmascience, Montreal, QC, Canada

*Author for correspondence: fabio garofolo@hotmail.com

“Understand drug characteristics is the first step toward development of robust bioanalytical methods.”

First draft submitted: 22 October 2019; Accepted for publication: 22 October 2019; Published online: 12 December 2019

Keywords: ASO • branched-DNA • hybridization-ELISA • hybridization-HPLC • LCMS • oligonucleotides • qPCR • WRIB

Advancement of cutting-edge therapeutics requires innovative bioanalysis, especially drugs with new modality and high complexity to treat rare diseases. Many novel drugs move to late stage development and some gained regulatory approval. The first small interference RNA (siRNA) ONPATRO[®] was approved for the treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis. An antisense oligonucleotide (ASO) SPINRAZA[®] and AAV gene therapy ZOLGENSMA[®] are now available for pediatric and adult patients with spinal muscular atrophy. Chimeric antigen receptor-T cell (CAR-T) therapies such as KYMRIAH[™] and YESCARTA[™], both genetically modified CD19 autologous T-cell immunotherapies were approved to treat diffuse large B-cell lymphoma. mRNA-based vaccines for infectious diseases/cancer and genetically modified CD34⁺ hematopoietic stem cell (via CRISPR-Cas9) therapy for β -thalassemia, sickle cell disease and other genetic diseases also entering clinical stage of development.

Bioanalysis is the foundation of novel drug development. To understand drug absorption, distribution, metabolism and excretion (ADME), mechanism of action, efficacy and safety, quality methods are critical. Although regulatory guidance documents are available for drug quantification [1–3], biomarkers [3,4] and immunogenicity [5,6], the physical, chemical and biological properties of these novel drugs would make it challenging to simply categorize them as either small molecule using mass spec methods or protein drugs using ligand-binding assays (LBAs) and/or hybrid LBA/LCMS. New methods have been developed and validated including specialty mass spec assays (not typical for small molecule drugs), novel ‘LBAs’ such as ‘cutting ELISA’ (oligonucleotide probe hybridization/S1 nuclease protection), fluorescent oligo probe hybridization HPLC, qPCR/RT-qPCR and b-DNA (branched DNA) and other innovative methods to specifically characterize the novel drugs and metabolites.

When traditional mass spec and LBA methods were adapted for use to assess these novel drugs, scientists soon realized that ‘one-size-fits-all’ approaches have limitations, thinking ‘outside of the box’ with an open mind is needed. Understand drug characteristics is the first step toward development of robust bioanalytical methods. The typical isotopically labeled internal standard (IS) for mass spec assay may not be feasible for oligonucleotide drug quantification. Primers/probes with or without fluorescent label do not function as labeled antibodies that bind to the protein drug and given the difference in binding (unlike antigen-antibody), LBA assay conditions for nucleic acid drugs need to be further optimized to enhance signal while maintain the low background. Real-time PCR (qPCR) methods have been used for nucleic acid therapies, but there are no regulatory guidelines for method development and validation although a paper from an academic initiative has provided some framework on minimal requirement of method performance [7]. Based on PCR assay principle, it is considered that ‘ligand’ (primer and

newlands
press

probe) binding plus enzymatic activity assay (DNA or RNA polymerase and/or reverse transcriptase) and criteria for assay validation remain to be better defined.

Industries and regulators worked together in 2018 and 2019 with focused discussions on bioanalytical challenges of novel drugs and published white papers to mitigate these issues [8–11].

An excerpt from the 2018 White Paper in Bioanalysis [8] highlights recent advances, future trends and impact on oligonucleotide bioanalysis:

“Analysis of oligonucleotides from biological samples is a challenging analytical task. Therapeutic oligonucleotides are either single or double stranded DNA or RNA molecules with a specific nucleotide sequence. Chemical modifications reduce endo- and/or exo-nuclease mediated degradation thereby improving *in vivo* drug metabolic stability. They differ in length from approximately 15 up to >1000 nucleotides, are sometimes conjugated with different ligands and may be formulated in LNPs or other delivery systems, such as viral vectors. Due to the heterogeneous nature of the analytes and their exposure in matrices that can vary significantly in nature, no single bioanalytical approach can be applied to cover all the different therapeutically relevant oligonucleotide classes (e.g., ASO, siRNA, miRNA mimics or inhibitors, aptamers, decoys, sgRNA, mRNA, immuno-stimulatory oligonucleotides).”

“Multiple methods are required for adequate characterization of the PK of ASOs and siRNAs. LCMS, LBA (hybridization ELISA), RT-qPCR (or equivalent), FL-HPLC are all options; drivers of analytical platform selection are associated to the objective of the study and typically depends on the specificity, sensitivity and matrix.”

A full day advanced training session with numerous case studies and panel discussions was organized during the 12th WRIB (9–12 April 2018) in Philadelphia, USA to harmonize industry practice and regulatory expectations in oligonucleotide bioanalysis. This session entitled, “The Exciting World of Oligonucleotides: a multidisciplinary complex challenge for multitasking ingenious bioanalysts” has been extremely successful, and it has been expanded in this special issue aimed to heighten an appropriate attention to all you need to know to enter in this fascinating field: small and large molecule approaches, LCMS, HRMS and LBA-advanced techniques, hybridization HPLC-FL, expressed protein analysis, hybridization ELISA, branched DNA (bDNA), RT-qPCR and new technologies.

Articles in this issue have been inspired by the interactive discussion during the 12th WRIB, they will discuss specific case studies, in-depth evaluations of the findings to illustrate the complexity of oligonucleotide bioanalysis.

Daniel Norris (Ionis Pharmaceuticals Inc; CA, USA), begins this special issue with his editorial considering pharmacokinetic evaluation of antisense oligonucleotides as therapeutics [12]. It goes on to discuss the advantages and limitations of the main assays used for quantifying antisense oligonucleotides.

Following this, Stebbins *et al.* (Biogen Inc; MA, USA) considered the immunogenicity risk assessment process of antisense oligonucleotides [13].

Next, Bennett *et al.* (PPD; VA, USA) presented their method for quantification of oligodeoxynucleotides in human plasma [14]. They also reported on the impact of PNA probe design on the performance of the hybridization-based LC–fluorescence assay.

In another methodology article, Rocca *et al.* (Alnylam Pharmaceuticals Inc; MA, USA) evaluate an electrophoretic mobility shift assay, as an alternative to ultrafiltration, to determine plasma protein binding of small interfering RNAs and antisense oligonucleotides [15]. They report their method characterized concentration-dependent plasma protein binding of small interfering RNAs successfully, unlike ultrafiltration.

In their research article, Dillen *et al.* (Janssen Research and Development; Beerse, Belgium) describe two extraction strategies that were investigated for quantification of oligonucleotides from biological matrices [16]. The anion-exchange solid phase extract method is reported to be a more generic approach compared with the hybridization extraction method which, they report, has greater potential overall.

Li *et al.* (Alnylam Pharmaceuticals, Inc.; MA, USA) report the first observation of a long-chain deaminated metabolite of a single-stranded REVERSIRTM oligonucleotide *in vivo* in monkey liver [17].

Liu *et al.* (Alnylam Pharmaceuticals, Inc.) goes on to describe their HPLC coupled with high-resolution mass spectrometry method that was developed to quantify REVERSIR oligonucleotide molecules and their metabolites in plasma and tissue [18].

In the final research article of this issue, Helen Shen and Lakshmi Ramanathan (QPS; DE, USA) describe their LC–TOF–MS method for the simultaneous quantitation of a test oligonucleotide and its metabolites in plasma and tissue matrices also [19].

In a bioanalytical challenge, Henderson *et al.* (AstraZeneca R&D Gothenburg; Goteborg, Sweden) discuss how to overcome challenges in generating data related to lipid nanoparticle encapsulated modified mRNA therapeutic distribution [20].

Henderson and Wilson (AstraZeneca; Cambridge, UK) also discuss challenges and strategies for performing regulated bioanalysis of modified mRNA therapeutics in their special report [21]. In this, they compare two main analytical approaches: quantitative reverse transcription polymerase chain reaction and branched DNA.

Finally, Gorovits *et al.* (Pfizer Inc; MA, USA) complete the issue with their perspective article discussing the current regulatory recommendations related to adeno-associated virus gene therapy development [22].

Acknowledgments

The guest editors would like to thank Rhiannon Finnie (Commissioning Editor, Bioanalysis) for her great enthusiasm, dedication and continuous support during the various development stages of this Special Issue on Oligonucleotides.

Financial & competing interests disclosure

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

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

References

1. European Medicines Agency. Committee for Medicinal Products for Human Use (CHMP). Guideline on Bioanalytical Method Validation. London, UK (2011). EMEA/CHMP/EWP/192217/2009. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf
2. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Center for Veterinary Medicine. Guidance for Industry, Bioanalytical Method Validation. Rockville, MD, USA (2018).
3. ICH M10, Draft Bioanalytical Method Validation (February 2019). https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M10/M10EWG_Step2_DraftGuideline_2019_0226.pdf
4. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research. Draft Guidance for industry. Biomarker Qualification: Evidentiary Framework. Rockville, MD, USA (2018). <https://www.fda.gov/media/122319/download>
5. Guideline on Immunogenicity assessment of therapeutic proteins, EMA 2016. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-immunogenicity-assessment-therapeutic-proteins-revision-1_en.pdf
6. US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research. Immunogenicity Testing of Therapeutic Protein Products – Developing and Validating Assays for Anti-Drug Antibody Detection. Rockville, MD, USA (2019). <https://www.fda.gov/media/119788/download>
7. Bustin SA, Benes V, Garson JA *et al.* The MIQE Guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55(4), 611–622 (2019).
8. Welink J, Xu Y, Yang E *et al.* 2018 White Paper on Recent Issues in Bioanalysis: 'A global bioanalytical community perspective on last decade of incurred samples reanalysis (ISR)' (Part 1 – small molecule regulated bioanalysis, small molecule biomarkers, peptides & oligonucleotide bioanalysis). *Bioanalysis* 10(22), 1781–1801 (2018).
9. Stevenson L, Richards S, Pillutla R *et al.* 2018 White Paper on Recent Issues in Bioanalysis: focus on flow cytometry, gene therapy, cut points and key clarifications on BAV (Part 3 – LBA/cell-based assays: immunogenicity, biomarkers and PK assays). *Bioanalysis* 10(24), 1973–2001 (2018).
10. Fandozzi C, Evans C, Wilson A *et al.* 2019 White Paper on Recent Issues in Bioanalysis: Chromatographic Assays (Part 1 – Innovation in Small Molecules and Oligonucleotides & Mass Spectrometric Method Development Strategies for Large Molecules Bioanalysis). *Bioanalysis* 11(22), (2019) (in press).
11. Piccoli S, Mehta D, Vitaliti A *et al.* 2019 White Paper on Recent Issues in Bioanalysis: FDA Immunogenicity Guidance, Gene Therapy, Critical Reagents, Biomarkers and Flow Cytometry Validation (Part 3 – Recommendations on 2019 FDA Immunogenicity Guidance, Gene Therapy Bioanalytical Challenges, Strategies for Critical Reagent Management, Biomarkers Assays Validation, Flow Cytometry Validation & CLSI H62). *Bioanalysis* 11(24), (2019) (in press).
12. Norris DA. Bioanalysis considerations on the pharmacokinetic evaluation of antisense therapeutics. *Bioanalysis* 11(21), 1909–1912 (2019).
13. Stebbins CC, Petrillo M, Stevenson LF. Immunogenicity for ASOs: A risk-based assessment. *Bioanalysis* 11(21), 1913–1916 (2019).
14. Bennett P, Ji Y, Liu Y *et al.* Importance of probe design for bioanalysis of oligonucleotides using hybridization based LC-fluorescence assays. *Bioanalysis* 11(21), 1917–1925 (2019).

15. Rocca C, Dennin S, Gu Y *et al.* Evaluation of electrophoretic mobility shift assay as a method to determine plasma protein binding of siRNA. *Bioanalysis* 11(21), 1927–1939 (2019).
16. Sips L, Njumbe Ediage E, Ingelse B, Verhaeghe T, Dillen L. LC–MS quantification of oligonucleotides with SPE or hybridization extraction in biological matrices. *Bioanalysis* 11(21), 1941–1954 (2019).
17. 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), 1955–1966 (2019).
18. Liu J, Li J, Tran C *et al.* Oligonucleotide quantification and metabolite profiling by high-resolution and accurate mass spectrometry. *Bioanalysis* 11(21), 1967–1981 (2019).
19. Shen H, Ramanathan L. LC–TOF–MS methods to quantify siRNAs and major metabolite in plasma, urine, and tissues. *Bioanalysis* 11(21), 1983–1992 (2019).
20. Hawthorne G, Henderson N, Hölttä M, Khan S, Lindqvist J, Wilson A. Overcoming analytical challenges to generate data critical to understanding LNP delivered modified mRNA biodistribution. *Bioanalysis* 11(21), 1993–2001 (2019).
21. Henderson N, Wilson A. Measurement of mRNA therapeutics: method development & validation challenges. *Bioanalysis* 11(21), 2003–2010 (2019).
22. Gorovits B, Marshall J-C, Smith J, Whiteley LO, Neubert H. Bioanalysis of adeno-associated virus gene therapy therapeutics: regulatory expectations. *Bioanalysis* 11(21), 2011–2024 (2019).