THERAPEUTIC OLIGONUCLEOTIDE METABOLITE IDENTIFICATION, **PROFILING AND QUANTITATION**

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EXECUTIVE SUMMARY

Therapeutic oligonucleotides are structurally diverse, chemically modified and often designed for targeted delivery. These features can improve pharmacology, but they also complicate bioanalysis. Metabolite identification, profiling, and quantitation help sponsors understand which molecular forms are present, where they distribute, how long they persist, and how bioanalytical findings may affect safety, efficacy, and regulatory strategy.

INTRODUCTION

Therapeutic oligonucleotides (ONTs) are a diverse class of sequence-directed nucleic acid-based medicines that include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs), splice-modulating oligonucleotides, and aptamers. Since the early 2010s, ONT therapeutics have advanced through new conjugation strategies that link these molecules to targeting ligands, proteins or antibodies. The ability of ONTs to modulate RNA processing, reduce or restore protein expression, and address targets that may be difficult to reach with conventional small molecules or antibodies has transformed drug discovery and development.

The field has advanced substantially through chemical stabilization and targeted delivery. Common design elements include:

- Ribose nucleotides (A, C, G, and U)
- Deoxyribose nucleotides (dA, dC, dG, and dT)
- Locked nucleic acid motifs
- Phosphorodiamidate morpholino oligomers (PMOs)
- 2'-O-methyl, 2'-O-methoxyethyl, and 2'-fluoro ribose substitutions
- Phosphodiester (PO) linkages
- Phosphorothioate (PS) linkages
- Terminal caps
- Stereospecific backbones

Conjugation to N-acetylgalactosamine (GalNAc) supports hepatocyte targeting via the asialoglycoprotein receptors (ASGPR), while hydrophobic fatty acid lipid tails (16-carbon and 22-carbon), peptides, proteins, antibodies, other ligands, and lipid nanoparticles are being explored to extend delivery beyond the liver. These modifications improve pharmacokinetics, safety, and pharmacology, but they also create structurally diverse metabolites and bioanalytical challenges that require a fit-for-purpose, multimodal strategy.

HOW THERAPEUTIC OLIGONUCLEOTIDES ARE CLEARED AND METABOLIZED

ONT disposition is dependent on a combination of protein binding, distribution, cellular uptake, endosomal escape, nuclease-mediated chain shortening, renal clearance and biliary excretion of the intact ONT and the catabolized shortmers. Depending on the modality and chemistry, metabolism may include sequential 3'- and 5'-exonuclease trimming, endonucleolytic cleavage, deconjugation, linker cleavage, biotransformation of terminal adenosine (A) to inosine (I) by adenosine deaminase (ADAR) enzymes, and metabolism of the targeting ligand. Duplex siRNAs require separate evaluation of guide (antisense) and passenger (sense) strands because each have distinct biological and functional roles, and can display distinct stability, metabolite patterns, and biodistribution.

PS-ONTs are often highly protein-binding, which leads to high tissue retention and reduces rapid renal filtration. For PO-ONTs, nuclease activity commonly generates progressively shortened n-1, n-2, and related chain-shortened metabolites. For GalNAc-conjugated ONTs, there may be rapid cleavage of the targeting ligand after hepatocyte uptake, and this should be distinguished bioanalytically from degradation of the active ONT sequence. The chain-shortened metabolites and shortmers are generally biologically active, making metabolite identification essential for the understanding of clinical pharmacology.

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WHY METABOLITE IDENTIFICATION AND QUANTITATION MATTER

Current regulatory expectations emphasize an integrated understanding of safety profile, pharmacokinetics, tissue distribution, off-target exposure, exposure-response relationships, drug-interaction potential, and immunogenicity for ONT therapeutics.

Well-designed metabolite profiling and characterization studies support interpretation of exposure, pharmacological activity, tissue persistence, and safety. They help determine whether circulating or tissue-resident shortened sequences retain sequence-specific activity, whether conjugate or linker-derived products require separate assessment, and whether animal studies adequately cover human exposures. Most of the initial in vitro studies are performed using plasma, serum, tritosomes, liver S9 fractions, and long-term hepatocytes co-culture from mice, rats, monkeys, and humans. Following-on studies are performed on plasma, urine, and critical organs (liver, kidney, ...) samples from toxicology studies, then single ascending dose (SAD) and multiple ascending dose (MAD) samples from first-in-human (FIH) studies.

No single platform provides a comprehensive metabolic picture. High-resolution mass spectrometry (HRMS) offers molecular specificity and structural information, targeted liquid chromatography (LC)-MS/MS (LC tandem mass spectrometry) supports robust quantitation of parent and metabolites, sequence-specific hybridization assays can provide exceptional sensitivity, while radiolabeled studies can deliver mass balance and recovery information independent of assay types. The most robust picture are derived from a combination of orthogonal methods and clear definitions of what molecular information each assay measures: the full-length parent, total ONT, active strand, duplex, conjugated form, deconjugated form or selected metabolites.

AN INTEGRATED BIOANALYTICAL WORKFLOW

1. Study Design and Analyte Definition

A strong bioanalytical workflow starts by defining the biological question the assay is intended to answer. For pharmacokinetic analysis and biodistribution, the priority is the full-length active ONT and major metabolites in plasma, urine, and tissues. For biotransformation, the objective may be broad detection of chain-shortened products and conjugate-related metabolites. For mass balance and excretion studies, the workflow must account for both the total administered material and the elimination profile. A sampling scheme should reflect rapid distribution, potentially prolonged tissue retention, and the possibility that plasma concentrations do not represent the pharmacologically relevant tissue compartment.

These planning decisions help ensure the assay measures the right molecular forms in the right matrices and produces data that can be interpreted in the context of the study objective:

- Define full-length, total, and analytes separately
- Assess guide (antisense) and passenger (sense) strands independently and as necessary for duplex siRNA
- Design matrix-specific methods for plasma, urine, liver, kidney, cerebrospinal fluid, if CNS target, and other relevant target tissues, as necessary
- Use time- or exposure-weighted pooling for profiling, while retaining individual samples for quantitative interpretation
- Include untreated matrix, pre-dose samples, extraction blanks, and process control samples

2. Sample Preparation

Modern ONT extraction typically combines matrix disruption, protein digestion or denaturation, selective capture and concentration. Tissue samples will require homogenization under conditions that minimize ex vivo nuclease activity and must be representative of the tissues or organs. Proteinase K digestion, lysis buffer, surfactants, and strong chaotropic agents, such as urea, if necessary, can improve the release of highly protein-bound ONTs.

Sample cleanup may use strong anion-exchange, mixed-mode or reversed-phase solid-phase extraction. Sequence-specific hybridization capture can provide high selectivity for targeted assays of parent, n-1, n-2, and liquid-liquid extraction is better for identifying potential shortmers and the released targeting ligand. Automated liquid handling improves throughput and reduces variability for large studies.

Extraction efficiency and recovery are driven by the length of the ONTs, backbone chemistry, and targeting ligand, and should be evaluated across parent and representative

metabolites. Stability experiments should address bench-top, freeze-thaw, processed-sample and long-term storage conditions, as well as potential ex vivo conversion during collection and preparation. Where possible, stable-isotope-labeled or structurally close analogue internal standards should be added early enough to correct for extraction and bioanalytical variability.

3. Chromatographic Separation

Ion-pair reversed-phase LC, coupled with negative-mode electrospray for PO- and PS-ONTs and positive-mode electrospray for PMOs, remains widely used because it separates ONTs by length and hydrophobicity while providing MS-compatible elution. The choice and concentration of ion-pair reagents, organic modifiers, column chemistry, particle size, pore size, temperature, and mobile-phase additives must be optimized to balance retention time, peak shape, sensitivity, adduct formation, and instrument cleanliness. Dedicated LC flow paths and careful control of metal interactions can improve robustness.

Orthogonal separations can be valuable. Anion-exchange LC offers strong resolution based on charge and can help resolve chain-length variants. Hydrophilic interaction LC (HILIC) or mixed-mode approaches may support selected chemistries. Conventional standard analytical flow provides greater ruggedness for higher-throughput regulated bioanalysis, while microflow LC increases sensitivity and reduces solvent consumption. Longer, multiple-step gradients may be needed for complex metabolite profiling, whereas shorter, shallower gradients are sufficient for targeted quantitation.

4. High-Resolution Mass Spectrometry for Profiling

High-resolution accurate-mass platforms, including quadrupole time-of-flight (QTOF) and Orbitrap instruments, can detect multi-charged ONT ions and distinguish metabolites using accurate intact mass, isotopic patterns, retention time, and tandem-MS fragments. Modern workflows use charge-state deconvolution and sequence-aware searching to propose 3'-truncations, 5'-truncations, internal cleavages, deconjugation products, and a combination of biotransformation. Data-dependent or targeted MS/MS using collision-induced dissociation (CID) or electron-activated dissociation (EAD) can then confirm sequence and cleavage location.

Using accurate intact mass data exclusively only supports tentative assignment. Matching isotopic pattern, diagnostic fragments, and retention behavior increases confidence of the metabolites assignment. Nevertheless, comparison with an authentic standard provides the strongest confirmation. Low-abundance metabolites should not be excluded solely because they have weak full-scan intensity if their MS/MS data help explain a coherent pathway or are detected consistently across matrices, animals or time points.

5. Targeted Quantitation and Hybrid Approaches

If the ONT does not undergo metabolism, sequence-specific hybridization assays in a ligand-binding format remain valuable when sensitivity is the primary requirement.

LC-MS/MS data from triple-quadrupole or QTOF can provide sensitive, selective, and high-throughput quantitation for parents and metabolites when the appropriate precursor-to-product transitions are available. Historically, for very low concentrations or challenging matrices, the most viable methodology has been hybridization LC-fluorescence detection, in which a complementary capture probe is used to enrich the target before fluorescence detection. Further adaptations have led to hybridization LC-MS/MS detection. This approach combines sequence selectivity with the structural specificity of mass spectrometry. However, shortmers cannot be characterized using the parent or n-1, n-2, n-3 metabolites in a single run with this hybridization methodology.

An integrated assay strategy often includes one highly sensitive method for pharmacokinetics and one structurally informative method for metabolite characterization. Agreement and differences between platforms can reveal whether an assay measures the full-length parent, a family of cross-reactive metabolites or total sequence-related material.

New-generation QTOF mass spectrometers can achieve sensitivity comparable to that of hybridization assays while simultaneously quantifying the intact parent, shortened sequences, sequences with the targeting ligand and shortmers, and providing detailed metabolite information in a single run under ICH M10 criteria.

6. Radiolabeled and Orthogonal Detection

Radiolabeled studies remain important when the objective is mass balance and excretion. Radio-chromatography can reveal components that ionize poorly by MS, provided the radiolabel remains associated with the relevant molecular region. Label position should therefore be selected with likely cleavage pathways in mind. Liquid scintillation counting (LSC) in parallel to the mass spectrometer or fraction collection followed by offline radio-detection or, as necessary, accelerator mass spectrometry, may extend sensitivity, while HRMS provides structural assignments for radioactive peaks.

7. Quantitation of Known and Unknown Metabolites

Authentic standards with internal standard(s) remain the preferred basis for absolute quantitation. When standards are unavailable, relative or semi-quantitative estimates should be qualified rather than assuming equal response across all ONTs. The detector response can vary with chain length, sequence, chemical modification, charge-state distribution, matrix, and ion-pairing conditions. In most cases, the intact parent and its n-1, n-2 metabolite produce similar mass spectrometer responses when present at the same molar concentration. However, the detector response becomes less consistent with further nuclease trimming, which produces shorter fragments. As a result, the parent ONT and shortmers may generate very different responses, which can lead to misleading quantitation. A tiered defensible strategy should include:

- Authentic standards for major or safety-relevant metabolites
- Closely related surrogate standards with experimentally evaluated response factors
- Parent-equivalent estimates with clearly stated uncertainty
- Radioactivity-based proportions when radiolabeled material is available

Method validation of plasma, urine, and critical tissues should follow ICH M10 criteria. Method qualification of other significant tissues should follow fit-for-purpose principles. Selectivity, accuracy, precision, recovery, matrix effect, calibration model, dilution integrity, carryover, stability, and incurred-sample reproducibility should be considered and evaluated in the context of the bioanalytical platform and intended decision.

DATA PROCESSING AND PROCESS UNDERSTANDING

A comprehensive data workflow should connect accurate mass, chromatographic behavior, isotope patterns, and MS/MS evidence to a time- and tissue-resolved biotransformation map. Current software can support metabolite prediction and data review, but expert interpretation and thorough review of LC and MS data is essential to eliminate adducts, in-source fragments, background ions, and false sequence assignments. Results should integrate chromatographic behavior, charge envelopes, isotope patterns, MS/MS evidence, matrix distribution, and time-course trends.

Extensive experience, in-depth knowledge of the workflow, catabolic and metabolic processing, and ONT molecular

design provide an understanding of the profiling data. For example, recurring cleavage at a specific linkage may identify a nuclease-sensitive region; persistence of a shortened product may suggest retained protein binding or cellular trapping; and rapid deconjugation may confirm expected intracellular activation. These insights can guide sequence optimization, backbone modification, linker design, formulation, dose selection, and selection of toxicology species.

CURRENT EQUIPMENT AND TECHNOLOGY PLATFORM

A contemporary ONT bioanalysis laboratory should combine the following capabilities:

- Biocompatible UHPLC systems configured for ion-pair reversed-phase, anion-exchange, standard flow and microflow separations
- High-resolution accurate-mass MS using QTOF or Orbitrap instruments for targeted, high-throughput quantitation plus intact-mass profiling and MS/MS sequence confirmation
- Triple-quadrupole LC-MS/MS for targeted, high-throughput quantitation
- Automated extraction and hybridization-capture workflows using programmable liquid-handling workstations
- Radio-HPLC or fraction collection coupled with scintillation counting, or other radio-detection for mass-balance and recovery studies
- Sequence-aware software for charge deconvolution, isotope matching, fragment assignment, metabolite searching, and pathway visualization
- Orthogonal hybridization or ligand-binding platforms for ultra-low-concentration pharmacokinetic measurements at trough level (Cmin)

CONCLUSION

Therapeutic oligonucleotide bioanalysis has moved beyond simple measurement of a full-length sequence. Today's ONT must distinguish active and inactive molecular forms, characterize tissue-specific metabolism, understand conjugate and linker transformations, and integrate highly sensitive quantitation with structurally informative profiling.

For sponsors, the value of metabolite identification and quantification lies in connecting bioanalytical results to development decisions. The right strategy can help clarify exposure, tissue persistence, pharmacological activity, safety findings, and regulatory readiness.

QPS IS COMMITTED TO WORKING WITH YOU

QPS supports ONT drug development with customized bioanalytical methods, advanced LC-MS and HRMS capabilities, hybridization-based approaches, and experience designing compliant data packages for complex therapeutic modalities. By integrating method development, profiling, quantitation and interpretation, QPS helps sponsors generate data that are not only technically sound but meaningful for the next stage of development.

BROAD ACCESS

QPS provides clients with broad strategic access to its nonclinical and clinical development capabilities and experience to conduct nonclinical and clinical development of a diverse portfolio of new drug candidates across various therapeutic areas. Our preferred vendor agreements also provide for the establishment of a client dedicated unit within our organization.

TIMELY DELIVERY

Partnering with QPS will position you for success, allowing timely delivery of your mRNA therapeutics to the marketplace.

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