Bioanalytical LC–MS of therapeutic oligonucleotides

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ABSTRACT
Therapeutic oligonucleotides (OGNTs) are important biopharmaceutical drugs for the future, because of their ability to selectively reduce or knock-out the expression of target genes. For the development of OGNTs, reliable and relatively high-throughput bioanalytical methods are required to perform the quantitative bioanalysis of OGNTs and their metabolites in biological fluids (plasma, urine, tissue, etc.). Although immunoaffinity methods, especially ELISA, are currently widely applied for this purpose, the potential of liquid chromatography coupled to mass spectrometry (LC–MS) in OGNT analysis is under investigation. Because of its inherent ability to monitor the individual target OGNTs as well as their metabolites, LC–MS is now evolving into the method-of-choice for the bioanalysis of OGNTs. In this paper, the state-of-the-art of bioanalytical LC–MS of OGNTs and their metabolites in biological fluids (plasma, urine, tissue, etc.) is critically reviewed and its advantages and limitations are highlighted. Finally, the future perspective of bioanalytical LC–MS, i.e., lower detection levels and potential generic LC–MS methodology, is discussed.

INTRODUCTION
At present, short-chain oligomers are applied and under investigation as oligonucleotide therapeutics (OGNTs), typically between 15 and 50 nucleotide units long. These OGNTs are built to interfere with the processing of genetic information by acting on the DNA or RNA and thereby interfere with the production of (disease-related) proteins in the cell [1]. Typical examples of OGNTs are Vitravene to treat cytomegalovirus retinitis in AIDS patients and Macugen to treat age-related macular degeneration. Other OGNTs are currently under development for the treatment of cancers, diabetes, ALS, Duchenne muscular dystrophy and diseases such as asthma and arthritis with an inflammatory component. Antisense oligonucleotides (asRNAs) are currently the most successful representatives of the first generation OGNTs. The asRNA hybridizes to a specific sequence of the mRNA, thereby interfering with normal translation and inhibiting or decreasing expression of the target protein (2). Another type of OGNTs is based on a naturally occurring gene silencing mechanism called RNA interference (RNAi), where short double-stranded RNAs knock down gene expression in cells [3]. The synthetic 19- to 25-base pair (bp) double-stranded RNAs used as OGNTs of this type are called siRNAs. Challenges of the use of OGNTs are issues related to stability, drug delivery, off-target effects, and interactions by immune response. In order to enhance the stability of OGNTs upon administration to the body, the phosphate groups in the backbone are often replaced by phospho-ribothioates (PS) groups. Nevertheless, the ability to reduce or knock-out the expression of target genes with relatively straightforward designed drugs make OGNTs “the next big thing” and it is expected that more synthetic OGNTs will be developed and marketed in the near future [1]. Successful drug development can only take place if reliable bioanalytical methods are available to perform quantitative bioanalysis of OGNTs and their metabolites in biological fluids (plasma, urine, tissue, etc.). Because of their unmatched sensitivities, ELISA and qPCR are currently the methods-of-choice in most cases [4]. However, the major limitation of both techniques is that often full-length oligonucleotides cannot be distinguished from their truncated shorter metabolites, resulting in overestimation of the parent oligonucleotides. Because of its selectivity and ability to individually monitor the target OGNTs and their metabolites, the state-of-the-art of LC–MS in the quantitative bioanalysis of OGNTs and their metabolites in biological fluids is critically reviewed in this paper.

The difficulty in obtaining high sensitivity for bioanalytical LC–MS of OGNTs is mainly caused by the poly-anionic phosphate backbone (pK_a<2) which strongly dominates their extraction, chromatographic elution and electrospray ionisation behaviour.

Chromatographic retention and ionisation efficiency
Negative-ion electrospray ionisation (ESI–MS) is the obvious choice for OGNTs, given their poly-anionic phosphate backbone. Solvent properties influence ESI–MS efficiency in a variety of ways. Both ESI–MS and LC retention characteristics of OGNTs are strongly determined by eluent composition. As a consequence, the solvent systems for LC and ESI–MS cannot be independently optimized, thus requiring a compromise to be struck. The ESI mass spectra of OGNTs show a series of multiple deprotonated molecules [M–nH]^” resulting in a multiple charge state distribution (Figures 1A and 2A). In selected-reaction monitoring (SRM), the preferred acquisition mode in quantitative analysis, one or eventually only a few multiple charged ions are used in detection. Since the charge state distributions can be affected by several experimental parameters and thus can influence OGNT quantitation, it is usually required to apply suitable internal standards. For ESI–MS, it is critical that the analytes exist as [preformed] ions in solution. Consequently, for negative-ion ESI–MS, the signal intensity increases with the increase of pH value, with an optimal response at pH 10.
However, at pH 10, the deprotonated poly-anionic phosphates are only poorly retained by conventional reversed-phase LC (RPLC) [5]. In OGN analysis, this incompatibility of ESI-MS and RPLC almost always leads to unacceptable LC–MS results [6]. As a consequence ion-pair RPLC (IPLC) using ESI-MS compatible organic bases has become the LC method of choice for OGNs.

**IPLC–MS of OGNs**

Apffel et al. significantly contributed to OGN analysis by the introduction of the HFIP [1,1,1,3,3,3-hexafluoro-2-propanol]/triethylammonium (TEA), as an ion-pair system [7]. The HFIP/TEA ion-pair system can bypass the above dilemma of LC separation and efficiency vs. ESI–MS response. HFIP is thought to contribute to both the hydrophobicity of the ion-pairing reagent used, and to enhance the MS signal intensity along with a reduced cation adduction [8]. Depending on the number nucleotides, concentration range and the required resolution of the OGN analysed, TEA concentrations in the range of 1.7-8.6 mM and HFIP concentrations in the range of 100-400 mM have been used for bioanalytical applications [9-16].

**H⁺–Alkali⁺-exchange ions**

For poly-anions like OGNs, the exchange of H⁺ of the phosphate or phosphorothioates groups of the OGN backbone for Na⁺ and K⁺ pose a severe problem for ESI-MS analysis of oligonucleotides. As a result of the H⁺–Alkali⁺-exchange, the multiple-charge OGN ions will be further dispersed among multiple cation-containing species with a m/z 22/n difference for sodium exchange (with n being the charge state of the ion). This results in lower MS detection sensitivity [5, 6, 17]. Therefore, the effective removal of alkali cations from the sample and careful control on instrument parameters are required for both sensitive and reproducible OGN quantitation to support TK, PK and bioavailability studies and for identification OGN metabolism studies using LC–ESI-MS or MS/MS [18].

**Fragmentation efficiency**

The extent of fragmentation is another essential parameter for both the bioanalytical quantitation of the target OGNs and its known metabolites and the identification of unknown metabolites. Fundamental studies of oligonucleotide fragmentation by CID MS/MS with triple quadrupoles revealed that precursor ions with higher charge states give only a few low-m/z, non-specific fragment ions (Figure 1) [9, 14]. In contrast, lower charge states, are due to their greater stability harder to fragment and give numerous higher sequence specific m/z fragment ions (Figure 2) [19, 20]. In SRM, one or eventually only a few fragment ions are used in detection. With OGNs, SRM of the higher charge states potentially results in higher sensitivity and of the lower charge states in higher specificity.

For identification purposes, on the other hand, one would need fragments covering the complete OGN sequence. In that case, CID on precursor ions with the lower charge states generates the characteristic fingerprint patterns, which for instance allow the sequencing of unknown metabolites [20].

**SAMPLE CLEAN-UP FOR OGN BIOANALYSIS**

Important factors is that OGNs appear to be highly associated to matrix proteins in the biological samples. Also, OGNs are also sensitive to nonspecific binding to storage containers, all glass volumetric bottles, auto sampler vial inserts, glass tubes and centrifuge tubes, etc.

Protein precipitation results in significant ionisation suppression in ESI-MS detection along with low recovery [13, 21]. The strong plasma protein binding of OGNs makes it likely that protein precipitation results in significant losses of OGNs during sample preparation.

Liquid-Liquid Extraction (LLE) of OGNs from plasma resulted in much higher recoveries of 80-90 percent [15]. The reported analyte recoveries for most of the SPE columns are generally relatively low [10-50 percent] [9, 11-13, 22-25], except for the mixed-mode Phenomenex Clarus Biosolutions SPE column with a promising 80 percent recovery [16].

Figure 1. (A) Full scan of the phosphorothioate oligonucleotide PF-ODN and (B) precursor ion scan of the m/z 591 ([M–13H]13⁻) charge state precursor ion. m/z 95 can be assigned to the phosphoric acid group (PO2S⁻) and m/z 124.9 to PO4C2H6⁻ (reprinted from Ref. [24] with permission from Elsevier).

Figure 2. ESI LC–MS mass spectrum (A) and precursor ion spectrum of [M−3H]3⁻ (B) of G3139 (phosphorothioate-TCTCCCAGCCTGGCGCCAT). The charge states (−3 to −8) of the multiply charged ions are indicated above each ion in (A). Assignment of fragment ions obtained from collision-induced dissociation of [M–3H]3⁻ of G3139 at m/z 1893.7 in (B) (reprinted from Ref. [22] with permission from Elsevier).
At present, the combination of LLE and SPE appears highly effective for both the extraction of OGNTs and removal of matrix compounds; recoveries of 70-80 percent and less than 6 percent ionization suppression have been reported (13).

**BIOANALYTICAL LC–MS OF OGNTs**

The current state-of-the-art of bioanalytical LC-MS of OGNTs is laid down in several publications (9-16, 22-30). The majority of the bioanalytical LC-MS applications concern quantitative determination of as-RNA drugs and their known metabolites in plasma, whereas few applications concerned analysis in tissue (23, 26-28, 30) (see also Figure 3).

Most of the bioanalytical applications use conventional LC columns with C18 material from several manufacturers in combination with the TEA/HFIP ion-pair mobile phase system. Surprisingly, the application of UPLC technology is only once reported (26).

The quantitative LC–MS methods for OGNTs in tissue and plasma typically have a linear range from the low nanogram to low microgram per millilitre (8, 12, 13, 21, 26). Two applications (9, 14) combine the fragmentation transitions of several charge state signals to enhance sensitivity within reach. Technological developments such as increasingly more sensitive triple quadrupole mass spectrometers, (nano-) UPLC, and chip technology, make LOQs in the range of low pg/ml feasible. Therefore, and because of its selectivity and ability to individually monitor the target and their metabolites, LC–MS is anticipated to become the method-of-choice for bioanalysis of OGNTs.

**FUTURE PERSPECTIVE**

Because of their unmatched sensitivity and ease-of-use, ELISA and qPCR are currently preferred over LC-MS. qPCR can also be applied for determination of OGNTs for very small sample volumes, such as tissue biopsies. Currently, several validated bioanalytical LC–MS methods have been established that are capable of quantifying single stranded OGNTs and their metabolites in plasma and tissue at the low ng/ml level. Although PK and PK/PD studies of potent OGNTs require LOQs of at sub ng/ml level, the application of recently developed separation and MS technology make significant improvements in sensitivity within reach. Technological developments such as increasingly more sensitive triple quadrupole mass spectrometers, (nano-) UPLC, and chip technology, make LOQs in the range of low pg/ml feasible. Therefore, and because of its selectivity and ability to individually monitor the target and their metabolites, LC–MS is anticipated to become the method-of-choice for bioanalysis of OGNTs.

**REFERENCES AND NOTES**


**ANALYTICAL TECHNOLOGIES**

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**REFERENCES AND NOTES**


**Figure 3. A representative total-ion current of 1 µg/mL of G3139 and its three major metabolites and 20 µg/mL internal standard spiked in rat plasma (A), and its corresponding SRM chromatograms (B).** (reprinted from Ref. (22) with permission from Elsevier).