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Review

# Application of LC–MS for quantitative analysis and metabolite identification of therapeutic oligonucleotides

Zhongping John Lin [a,*], Wenkui Li [b,**], Guowei Dai [c,***]

[a] *AstraZeneca Pharmaceuticals LP, Global Development DMPK and Bioanalysis, 1800 Concord Pike, Wilmington, DE 19850, USA*
[b] *Novartis Pharmaceuticals Corporation, Drug Metabolism and Pharmacokinetics, One Health Plaza, East Hanover, NJ 07936, USA*
[c] *Bristol-Myers Squibb Pharmaceutical Research Institute, Route 206 & Province Line Road, Princeton, NJ 08543, USA*

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**Abstract**

Therapeutic oligonucleotides (OGNs) have been studied extensively in the recent years as novel agents designed to selectively and specifically inhibit target gene expression in cell culture, in animal disease models and in human. This review summarizes applications of liquid chromatography coupled with mass spectrometry or tandem mass spectrometry (LC–MS or LC–MS/MS) for quantitative analysis of therapeutic OGNs and characterization of their metabolism *in vitro* and *in vivo* described in the literature over the past 10 years. Although the applications of LC–MS or LC–MS/MS to the molecular mass measurement, sequence determination, DNA adducts identification, detection of mutations and characterization of covalent and/or noncovalent DNA/RNA complexes have been comprehensively reviewed in a few excellent review papers. The quantitative bioanalysis and metabolite identification of therapeutic OGNs using LC–MS or LC–MS/MS have not been covered. This review covers technical issues, current approaches and applications of LC–MS or LC–MS/MS for quantitative analysis of OGNs in biological matrices and characterization of their *in vitro* and *in vivo* metabolism. Finally, some conclusions are drawn and prospects of LC–MS in quantitative analysis and metabolism characterization of therapeutic OGNs are discussed.


*Keywords:* Review; Antisense; Oligonucleotides; si-RNA; LC–MS/MS; Quantification; Characterization; Metabolism; Metabolite identification

## Contents



*Abbreviations:* AS, antisense; CGE, capillary gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; IP-RP-HPLC, ion-pair reversed phase high performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; OGNs, oligonucleotides; PS, phosphorothioate; rafAON, raf antisense oligonucleotide; RNAi, RNA interference; si-RNA, short interfering RNA; TEA, triethylamine; TEAA, triethylammonium-acetate; TEAB, triethylammonium bicarbonate

* Corresponding author. Tel.: +1 302 885 0491; fax: +1 302 886 5463.
** Corresponding author. Tel.: +1 862 778 4255; fax: +1 973 781 7579.
*** Corresponding author. Tel.: +1 609 252 6342; fax: +1 609 252 7821.
*E-mail addresses:* zhongping.lin@astrazeneca.com (Z.J. Lin), wenkui.li@novartis.com (W. Li), david.dai@bms.com (G. Dai).

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## 1. Introduction

With the advancement of synthetic chemistry and biology of DNA and/or RNA, the dream of developing nucleic acid-based drugs that can be employed to target genetic abnormality in cells for the treatment of various diseases has become true in the past decades [1,2]. The widespread growth of knowledge and exponential accumulation of information on chemistry and biology of nucleic acids have enabled researchers from both academy and industry to quickly test a variety of synthetic nucleic acid-based drug candidates for target-specific knock-down of gene expression at the DNA and/or RNA levels [1–4]. One of the well-known strategies for the above goals is the activation of protein ribonucleases (RNases) using complementary nucleic acid molecules to degrade specific RNAs [1–4]. Many chemically synthesized nucleic acid-based drug candidates have been designed to hybridize to specific sequences of target RNA to form a double-stranded hybrid recognizable by specific protein RNases. Among them are various oligonucleotides (OGNs), including antisense oligonucletides (AS-OGNs) and short-interfering RNAs (si-RNAs) [1–4].

AS-OGNs are synthetic, in general, 15–20 single-stranded DNA, RNA, or their analogues, that directly inhibit gene expression by specifically binding to the complementary sequence of target mRNA or pre-mRNA via Watson-Crick base pairing [1], while short interfering RNAs (si-RNAs) are specific sequences of, generally, 19–21 double-stranded RNA that can be used to silence gene expression via endogenous RNA interference pathway (RNAi) [1–4]. Both AS-OGNs and si-RNAs hold marked therapeutic and enormous commercial potential, and are currently being extensively explored for the treatment of several diseases, including cancer, viral infections, cardiovascular disorders, macular degeneration, diabetes, and inflammatory diseases, etc. To our best knowledge, there are currently at least two AS-OGN drugs on market, which are Vitravene (Fomivirsen) of ISIS Pharmaceuticals for the treatment of cytomegalovirus (CMS) retinitis, and Macugen of Eyetech Pharmaceuticals for the treatment of age-related Macular degeneration [5,6]; more than 26 of AS-OGNs are being investigated at Phase II/Phase III clinical trial stages [1].

Quantitative analysis of therapeutic OGNs (mainly AS-OGNs and si-RNAs) and characterization of their metabolism are important aspects of drug development and evaluation. Determination of OGNs in various biological matrices (plasma, urine, tissue, etc.) and identification of their *in vitro* and *in vivo* metabolites not only yield crucial information which assists investigators in toxicokinetics (TK), pharmacokinetics (PK) and metabolism pathway studies but also enables the development of rational strategies for improving the stability, durability and bioavailability of these therapeutic agents [7–9]. Many techniques have been employed in the quantitative analysis and metabolism studies of therapeutic OGNs, which include radiolabel tracer methods, hybridization-based enzyme-linked immunosorbent assay (ELISA) methods, capillary gel electrophoresis-based methods (CGE-UV/Fluorescence) and conventional HPLC methods (HPLC-UV/fluorescence), etc. [7]. The radiolabel tracer method is based on the measurement of radioactivity of high cost $^{3}H$, $^{14}C$, $^{35}S$ or $^{125}I$-labeled OGNs in the biological matrices. The overall sensitivity of the method is poor due to limited isotope labeling. Without pre-separation via chromatography, the assay result only reflects the overall radioactivity of the parent OGNs plus their metabolites bearing the isotope labels, which leads to overestimation of the parent OGNs [10]. Information on metabolites is limited by the chemical location and metabolic fate of the isotope labels. Moreover, a radiolabled OGN has physicochemical properties that differ from the unmodified OGN, which might result in a different disposition *in vivo*. Hybridization-based ELISA method, in general, provides the best reported assay sensitivity and throughput. The method requires a minimum of sample clean-up, and has been widely used for quantitative analysis of OGNs in support of TK/PK evaluation, especially for the terminal phase PK assessment [11,12]. Unfortunately, this method could not distinguish full-length OGNs from their putative metabolites truncated by the loss of one or more single nucleotide units, which might cause cross-hybridization and, subsequently, the overestimation of the parent OGNs [7,11]. Moreover, this method is definitely not a choice for metabolite identification of OGNs in biologic matrices. Based on mass and charge selectivity of the analytes, capillary gel electrophoresis (CGE) could provide separation of OGNs from their interfering metabolites under optimized experimental conditions. Reports have been published using CGE-UV/fluorescence for the determination of OGNs in biological matrices with a reported LLOQ of 20 ng/mL when post-column derivatization followed by fluorescence detection was employed [13]. However, one of the drawbacks that limit the application of the method is the poor robustness and reproducibility due to endogenous interference, which requires extensive sample preparation [7]. Owing to its great availability and ease of operation, conventional HPLC-UV/Fluorescence methods have been widely used for quality controls of synthesized OGNs and other DNA/RNA products for purity verification. However, method sensitivity is an issue for comprehensive TK/PK assessment of OGNs with a reported LLOQ of 40 ng/mL [14]; Furthermore, the method resolution or selectivity remains to be demonstrated to separate the target OGNs from matrix materials, especially those metabolites with one or a few nucleotide units less than the parent compound

under conventionally used ion-pair reversed-phase or strong ion-exchange LC conditions. It is not unusual to see that retention time based methods give a false metabolite ID for a given peak if chromatographic resolution is questionable.

With the advent of soft electrospray ionization (ESI) mass spectrometric technique, a new avenue for overcoming the above sensitivity and selectivity obstacles was LC separation coupled with mass spectrometric detection (LC–MS) [15–18]. Unlike regular methods where the analytical result is solely based on the retention times (CGE and HPLC) or specificity/selectivity of hybridization/binding/ligation/signaling (hybridization) of the OGNs, LC–MS analytical result is based on not only the retention time, but more importantly, the molecular information (compound dependent precursor and product ions for MS detection) of the OGNs. The advantage of the method over others is in eliminating the interference for quantitative analysis of OGNs and more detailed metabolite identification in OGN metabolism studies [15–18].

The LC–MS applications to the determination of molecular mass and sequence of RNA and/or DNA, identification of DNA adducts, detection of DNA/RNA mutations and characterization of covalent/noncovalent DNA and RNA complexes have been well reviewed in a few excellent review papers [15–17]. However, the quantitative analysis and metabolite identification of therapeutic OGNs using LC–MS or LC–MS/MS has not been reviewed. The objective of the present review is to give an overview on the progress, to date, in the use of LC–ESI/MS and LC–ESI/MS/MS for the quantitative analysis and the characterization of *in vitro* and *in vivo* metabolism of therapeutic OGNs.

## 2. Quantitative analysis of oligonucletides in biological matrices using LC–MS

### 2.1. Technical issues and current approaches

Although LC–MS has become a standard technique for determination of small organic molecules, peptides, and proteins in biological fluids (plasma, serum, urine, tissues, etc.) in support of TK/PK assessment during drug discovery and development [19], the methodology has been less successful for quantitative analysis of therapeutic OGNs in biological matrices until recently [7,20,21]. This is primarily due to the low sensitivity when compared to hybridization-based ELISA method, and several technical issues associated with this type of molecules including: (1) limited ionization efficiency and signal suppression caused by extensive adduction with ubiquitous cations ($Na^+$, $K^+$, etc.) in biological samples, and (2) incompatibility of chromatographic resolution and MS detection. In order to enhance the feasibility of LC–MS or LC–MS/MS for quantitative analysis of therapeutic OGNs, a number of approaches have been proposed.

#### 2.1.1. ESI-MS detection

OGNs possess an acidic proton at each phosphodiester bond along their backbone. Generally, ESI gives a much lower detection sensitivity in the positive-ion mode than in the negative-ion

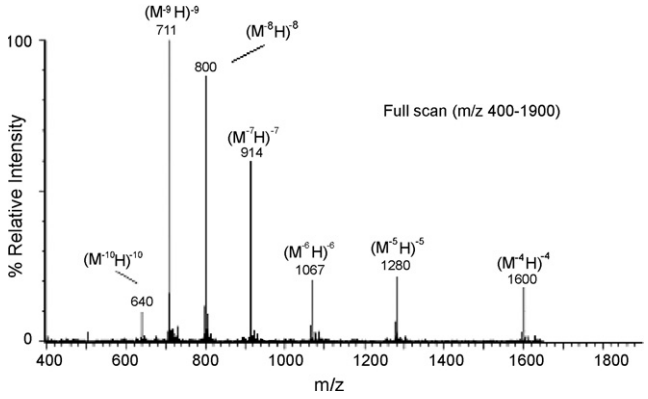


Fig. 1. Full scan of a 18-mer phosphorothiate OGN, which shows $[M–10H]^{10-}$, $[M–9H]^{9-}$, $[M–8H]^{8-}$, $[M–7H]^{7-}$, $[M–6H]^{6-}$, $[M–5H]^{5-}$, and $[M–4H]^{4-}$ at *m/z* 640, 711, 800, 914, 1067, 1280 and 1600, respectively (reproduced with permission from Ref. [37]).

mode for OGNs, where OGNs form deprotonated molecular ions with a multiplicity of different charge states and give multiple peaks corresponding to what is called an 'envelope' of charge states in full scan mass spectrum [22]. Due to the partition of the deprotonated molecular ions of OGNs into multiple peaks (Fig. 1), the signal intensity of the selected ion(s) that could be used as target ion(s) in the selected ion monitoring (SIM) or selected ion recording (SIR) with a single quadrupole mass spectrometer, or the precursor ions in selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) with a triple quadrupole mass spectrometer was significantly reduced for quantification purpose.

Various factors affect the ionization efficiency and/or signal intensity of OGNs in the negative ESI. These factors include but are not limited to organic solvent content, solution pH and cation ion content [15,22,23]. Although the ionization process for OGNs is not very clear, it is well accepted that the reduction in ionization efficiency of OGNs is mainly due to the high surface tension and high conductivity of the droplets in the gas phase when high aqueous and low organic mobile phase has to be used for chromatographic separation [23]. An increase of organic content in the infusion solution or mobile phase is normally accompanied with an increase in the signal intensity via reduction of the surface tension and acceleration of the evaporation process of the droplets in gas phase. Thus, post-column addition of organic solvents (acetonitrile, methanol or 2-propanol) is a practical approach to increase the ionization efficiency [24,25]. In addition to post-column addition of MS favorable organic solvents, signal enhancement might also be obtained by adding MS-compatible organic base, e.g., triethylamine (TEA), in the solution. Bleicher and Bayer reported a 50-fold increase in MS signal intensity by adding acetonitrile and TEA to a 20-mer OGN sample in a 0.1 M ammonium acetate [26]. According to the most common ionization model for ESI, it is critical that the analytes exist as ions in solution, consequently, for the negative ion formation of OGNs, a higher pH of the solution yields a higher MS signal intensity, which was demonstrated by Johnson et al., who noticed that the signal intensity increases significantly with the increase of pH value of the infusion solution for rafAON

(antisense oligonucleotide against Raf-1 expression), and TEAA buffer at pH 10 is the most suitable for signal intensity and analyte stability [27].

Sodium and potassium adducts pose a severe problem for ESI-MS anlaysis of OGNs because these cationic ions present in the liquid or gas phase are attracted by the highly negatively charged poly-anionic phosphate backbone of the OGNs and form what is called "cation adducts", which further disperse the OGN ions among multiple cation-containing species, resulting in a lower MS detection sensitivity [15,22,23]. It has been shown that the presence of a strong organic base such as TEA or piperidine effectively suppress the formation of sodium and potassium adduct via a displacement mechanism. Greig and Griffery [28] found that 1:1 co-addition of a strong base (e.g., TEA or piperidine) and a weak base (e.g., imidazole) dramatically reduced $Na^+$ and $K^+$ adducts, resulting in an improved MS sensitivity. Muddiman et al. reported similar results after investigation of OGNs in acetonitrile/water solution with or without acetic acid/formic acid/piperidine/imidozole [29]. Deguchi et al. noticed that post-column addition of acetonitrile containing a volatile and weak base, imidazole (50–100 mM), to the eluents from a regular reversed-phase column eluted with TEAA/acetonitrile gained a significant MS sensitivity enhancement [30]. The mechanism by which imidazole affords improved MS sensitivity for the LC–MS system using TEAA buffer is not clear.

### 2.1.2. LC–MS

In general, conventional reversed-phase chromatographic conditions are fully compatible with ESI. Separation of target OGNs from matrix components could be accomplished by applying an isocratic or a gradient elution with MS favorable organic solvents, such as acetonitrile and methanol, in ammonium acetate or ammonium formate buffer [19]. However, due to the extremely polar and ionic nature of OGNs, very limited retention and/or resolution could be obtained. This might partially explain why no reports have been published to describe the application of conventional reversed-phase (RP) LC–MS for the quantitative analysis of OGNs, although limited applications were reported for chromatographic separation of some single-stranded OGNs modified with trityl or dimethyoxytrityl groups [31].

Ion-pair reversed phase HPLC (IP-RP-HPLC) has been so far the chromatographic choice for quantitative analysis of OGNs due to its high-resolving power for many acidic and ionic compounds, including OGNs. The ion-pair mobile phase was comprised of a regular aqueous and organic solvent system, but was modified with ion-pair reagents which consists of an amphiphilic-ion that carries positively charged and hydrophobic groups and a small, but hydrophilic counter ion.

Early analytical LC–MS methods for OGNs were using triethylammonium-acetate (TEAA) as ion-pair reagent [32]. The positively charged TEA formed ion-pair with OGN, which was further stabilized in liquid phase. The formation of ion-pairs caused the analyte molecules to appear as "neutrals" to the stationary phase of the reversed-phase HPLC column. As a result, the ion-pairs of OGNs with TEA offered much better retention and peak shape on the reversed-phase chromatography. However, due to the presence of the ion-pair of analyte with TEA, the formed ion-pair was "pseudo-neutral" in gas phase of MS source and its charge was "masked" to the electric fields of mass spectrometer. So, the ejection of the analytes from the droplets in the gas phase of MS source was more or less inhibited, depending on the "binding affinity of TEA and the analyte". Such a phenomenon was called "signal suppression" [27,32]. As expected, the higher concentration of the ion-pair reagents in the mobile phase, the better separation of the target OGNs from matrix components, but the lower signal intensity in MS detection. TEAA at concentrations of 50–100 mM has been shown to cause significant ion suppression [32]. On the other hand, the lower concentration of ion-pair reagents, the better MS signal intensity, but the worse chromatographic separation/resolution [32,33], since it is the concentration of ion-pair reagents (e.g., TEA) rather than others that plays a crucial role in the chromatographic performance [20,21]. A few approaches have been proposed to overcome the above dilemma. An alternative ion-pairing buffer, comprised of triethylammonium bicarbonate (TEAB) or diisopropylammonium acetate with an acetonitrle gradient, was introduced by Huber and Krajete for the analysis of OGNs, but the ESI-MS signal intensity was still low [24,34]. Apffel et al. proposed that HFIP (1,1,1,3,3,3-hexafluoro-2-propanol)/TEA could substitute TEAA as an ion-pair reagent [32]. In the HFIP/TEA buffer system, TEA serves as an efficient ion-pair reagent for negatively charged OGNs. The system provides comparable chromatographic resolution to the TEAA buffer system, but MS signal intensity was significantly enhanced along with a minimum of cation adduction. The proposed mechanism is based on the dynamic adjustment of the pH in the electrospray droplet as a function of the preferential removal of anionic counterion from the droplet by evaporation. Compared to regular TEAA buffer contained acetic acid ($pK_a = 4.75$, bp = 118 °C), which is completely dissociated at pH 7.0 or above, and consequently cannot be removed by evaporation, HFIP ($pK_a \sim 9$, bp = 57 °C) is not charged at pH 7.0 and can be freely evaporated. During the electrospray and desolvation process, the volatile HFIP is depleted at the droplet surface, and as a result, the pH at the surface rises to 10, which is favorable for the dissociation of the OGN-TEA ion-pair and, subsequently, desorption of OGN into the gas phase. A buffer system with 16.3 mM TEA/400 mM HFIP (pH 7.0) in methanol was recommended for optimal LC–MS analysis of OGNs [32]. However, a recent study indicated that the above buffer system might not be optimal for some OGNs. Instead, increasing the buffer pH by reducing concentration of HFIP to 100 mM (pH 8.3) reduced the charge state of an OGN (G3139) and increased the abundance of the target precursor ion [18].

Recently, Hail et al. modified the above ion-pair mobile phase system by adding 10 μM of ethylenediaminetetraacetic acid (EDTA) to mobile phase (pH 7.3) [35]. It was assumed that the addition of EDTA to the mobile phase is beneficial in preventing formation of cation adducts ($Na^+$, $K^+$, etc.) caused by the trace amounts of these cations in the mobile phase or reconstituted samples. Although the addition of EDTA was also found to more effective than simply rising pH, which could cause degra-

dation of certain OGNs containing base-labile moieties, more applications remain to be demonstrated.

#### 2.1.3. Sample preparation

In complex biological matrices, salt, small organic and inorganic components, and other protein/non-protein macromolecules contribute to the overall matrix effect that makes it difficult for quantitative analysis of OGNs using LC–MS [18]. Thus, sample preparation/purification is a critical step to separate the target OGNs from matrix components and bulk proteins before further separation using ion-pair reversed-phase chromatography prior to MS detection. Unfortunately, general protein precipitation method for OGNs with ammonium acetate, methanol or acetonitrile results in marked signal suppression in MS detection along with low recovery [7]. Isolation of analytes via liquid–liquid extraction using phenol–chloroform–isoamyl alcohol [36,37], solid-phase extraction (SPE) [18,27,33,38] or protein precipitation coupled with SEP [39,40] with a proper preconditioning of the SPE cartridge using organic solvents (acetonitrile or methanol) followed by TEAA or TEAB buffer are useful and attractive approaches. After sample loading, the bulk interference matrices (proteins, salts, etc.) could be effectively removed with sequential elution with buffer (TEAA or TEAB) and water before the final elution of the analytes from SPE cartridge with aqueous organic solvents [18,27]. In order to achieve a good extraction efficiency, pretreatment of sample matrix with a proper amount of TEAA or TEAB buffer is necessary.

Various SPE phases are available for OGNs biological sample preparation, including C18 analogues, anion exchange, mixed-mode or polymeric sorbents. Taking into consideration of complexity of biological matrix from different species, different sorbents and cleanup techniques might be optima for different biological matrices, even for the same analyte. In the case of rafAON, Johnson et al. reported that SPE with Oasis HLB gave the highest recoveries for monkey plasma samples, while Varian C18OH cartridges were optimal for mouse plasma sample analysis when the same SPE procedure was applied [27]. Unfortunately, the low recovery of the SPE methods together with the significant matrix effect (or signal suppression, ~50%) remains to be resolved [18,27].

### 2.2. Current application

Although very limited applications of LC–MS technologies have been reported for quantitative analysis of therapeutic OGNs, these pioneer work could serve as templates for future endeavors. More details of these applications are summarized in Table 1, which covers sample preparation procedures, IP-RP-HLPC conditions, mass detection, method sensitivity and dynamic ranges [18,27,33,36–41]. These methods employed LLE or SPE for sample preparation, LC–MS/MS for quantification of OGNs in plasma or tissue samples. MS (triple quadrupole or ion trap) was operated in negative ESI mode with multiple reaction monitoring. It is worthwhile to mention that Xu et al. summed the signals from five MRM transitions to enhance MS signal for quantification of an OGN with a reported LLOQ of 1.00 ng/mL when a 50 μL of sample volume was used [33].

## 3. Characterization of oligonucletide metabolism using LC–MS

One of the fundamental prerequisites for the successful development of therapeutic antisense OGN or siRNA is their metabolic stability *in vivo*. Identification of metabolic pathways for therapeutic OGNs can provide useful information about their unexpected toxicity, desired pharmacological effect, ultimately clinical utility, and improving the durability of these therapecutic OGNs. In this section, characteristics of mass spectrum, practical aspects of metabolism study and current applications of LC–MS or LC–MS/MS on *in vitro* and *in vivo* metabolism will be reviewed.

### 3.1. MS characteristics and detection of therapeutic oligonucleotides

Mass spectrometric measurements involve the determination of molecular mass or the masses of dissociation of gas-phase ions, which can then be related to various structural properties. OGNs are thermally fragile, acidic, and the most polar biopolymers. The full scan mass spectra of OGN show a series of multiply deprotonated ions $(M–n\mathrm{H})^{n-}$ as major ions forming so called 'envelope' of charge state distribution in ESI negative mode, each of which represents a multiply charged ion of the intact molecule that has a specific number of protons removed from the sugar-phosphate backbone. The signals of the multiply charged series can be readily deconvoluted to yield the molecular mass. Mass measurement of OGNs up to 120 mers was demonstrated with a mass accuracy of better than 0.01–0.1%. The pH of mobile phase [18,27,29], additives in mobile phase [28–32,42] and the MS tuning parameters [43] have a strong influence on the charge distribution and their detectability in quadrupole ion trap and triple quadrupole mass spectrometers. The protons in the sugar-phosphate backbone can also be substituted by variable numbers of other cations such as sodium and potassium resulting in highly complex spectra and a decreased sensitivity. The degree of cation adduction increases with the length of the OGNs and is more extensive with phosphorothioates than with phosphodiesters [28]. The high quality mass spectrum is fundamental requirement for the positive metabolite identification. Therefore, the effective removal of cations and careful control on instrument parameters are required for OGN metabolism studies using LC–ESI/MS or MS/MS.

### 3.2. Practical aspects of oligonucleotide metabolism using LC–MS

In addition to the above-mentioned mass spectrum quality issues, characterization of OGN metabolism by LC–ESI/MS has been rather difficult due to several key issues associated with this class of compounds: (1) metabolites are usually in low quantity; (2) detrimental matrix effect: endogenous protein, peptide and cations interfere with the extraction/purification and subsequent ionization; (3) lack of optimal ion-pairing reagents for both MS sensitivity and HPLC separation; (4) limited automatic OGN sequencing software (metabolite ID program) [17]. To overcome

Table 1
Current applications of LC–MS/MS for quantitative analysis of therapeutic oligonucleotides

| Compound | No. of Nucleotide | Sample preparation/ sample size (mL) | Reconstitution | Column | Mobile phase | MS Detection | Dynamic range (ng/mL) | References |
|---|---|---|---|---|---|---|---|---|
| PS-P | 20-mer ASO | LLE (phenol–chloroform–isoamylol)/NA | 2.5%MeOH/100 mM TEAA | | Mobile phase A: 7 mM TEA/400 mM HFIP in water; mobile phase B: TEA/HFIP/methanol (1:1:498) | Triple quadrupole MS/MS | NA | [36] |
| Compound A | WM = 7138 | SPE (Oasis HLB), TEA/MeOH (1/499) used for conditioning followed by TEA/HFIP/water (1:1:998) for equilibration/0.05 (mouse plasma) | 5% MeOH | Develsoil RPAqueous (150 mm × 2.0 mm) | Mobile phase A: TEA/HFIP/water (1:1:998); mobile phase B: TEA/HFIP/ACN (1:1:498) | Triple quadrupole MS/MS | 1–2000 | [33] |
| rafAON | 15 | SPE (Oasis HLB or Varian C18OH), 100 mM TEAA (pH 7.0) used for SPE conditioning, sample loading/0.1 (monkey, mouse plasma) | Water | PRP-1 (25 mm × 2.3 mm) column | Mobile phase A: 10 mM TEAA (pH 10.0); mobile phase B: ACN | Triple quadrupole MS/MS | 50–10,000 (monkey), 25–10,000 (mouse), 500–10,000 (tissue) | [27,38] |
| | | Protein precipitation, followed by SPE (Oasis tC812cc)/1.0 (human plasma) | 5 mM ammonium acetate (pH 7.5) | Synergi Max-RP (50 mm × 2 mm, 4 μM) | Mobile phase A: MeOH/water/$NH_4OAc$ (1 M, pH 8), 10/90/0.5 (v/v/v); mobile phase B: MeOH/water/$NH_4OAc$ (1 M, pH 8), 90/10/0.5 (v/v/v) | | 8–10,000 | [39,40] |
| G3139 | 18 | Sample (+ISTD) was mixed with 0.8 mL of 0.1 M TEAB buffer (pH 8.5) before SPE on Oasis HLB, TEAB/0.2 (rat, human plasma) | 100 mM HFIP/8.6 mM TEA (pH 8.35) | Waters Xterra MS18 2.5-μm (50 mm × 2.1 mm) | Mobile phase A: 100 mM HFIP/8.6 mM TEA in water (pH 8.35); mobile phase B: 100 mM HFIP/8.6 mM TEA in water/MeOH (1:1, v/v) (pH 8.3) | Quadrupole ion trap MS/MS | 100–10,000 | [18] |
| Compound I | 18 | LLE (phenol–chloroform–isoamyl alcohol)/50 mg tissue | 2.9 mM TEA/286 mM HFIP in water (0.2/15/500, v/v/v) | Discovery RP-Amide C16 100 mm ×2 mm, 5 μm) | Mobile phase A: 2.9 mM TEA/286 mM HFIP in water (0.2/15/500, v/v/v); mobile phase B: 2.9 mM TEA/286 mM HFIP in MeOH/ACN (0.2/15/350/150, v/v/v/v) | Triple quadrupole MS/MS | 125–10,000 ng/g | [36] |
| ISIS1082 | 21 | SPE (phenyl-bonded)/NA | NA | NA | NA | Triple quadrupole MS/MS | 5–500 | [41] |

these issues, careful sample preparation and selection of chromatographic and mass spectrometric condition are crucial for successful identification of metabolites.

In general, intensive sample clean-up procedure is required for metabolism studies comparing to quantitative analysis for OGN using LC–MS or MS/MS. Commonly used methods for sample preparation are based on precipitation of OGN using ammonium acetate, methanol or acetonitrile but low recovery remains a matter of concern. Ultrafiltration and size-exclusion spin columns do not provide a sufficient degree of desalting. Dialysis allows for a high degree of desalting, but it is time-consuming and costly for large number of samples. A two-step solid-phase extraction procedure has been developed by Leeds et al. [44]. Plasma samples containing OGNs were first loaded onto a strong-anion cartridge to remove proteins and lipids, followed by a reverse phase C18 SPE to remove salts generated from the first step and desalted by dialysis. The final desalting step by membrane dialysis is not necessary if LC–ESI/MS/MS method is employed since reversed-phase chromatography can efficiently remove cations associated with OGNs. The recovery of strong-anion SPE was greater than 90% but dropped to 40% after the C18 SPE. This procedure was modified to extract metabolites from tissues. Homogenized tissues were first extracted using phenol/chloroform liquid–liquid extraction method, followed by the two-step SPE method. The modified method has been utilized in OGN metabolism studies using LC–ESI/MS method [8,45–47] or MALDI-TOF [48]. A single step SPE method was developed to extract antisense OGN and metabolites from plasma and urine samples. Bulk interference matrix was removed from Oasis HLB cartridge by sequential elution with TEAB buffer, water, and TEAB-acetonitrile gradient [18]. Another simple method for extraction is liquid–liquid extraction using either acetonitrile containing 0.1% TEA [27] or phenol–chloroform–isoamyl alcohol containing ammonium hydroxide [37]. Acetonitrile with 0.1% TEA provided recovery of 22.8% and matrix effect was measured to be 48% [27]. The recovery for the latter method was not reported. For si-RNA sample preparation, more attention is needed. Beverly et al. described a modified procedure for purification of si-RNA from a commercially available RNA extraction kit [49]. Tissues lyses were mixed with phenol/chloroform/isoamyl alcohol adjusted to pH 4.7. si-RNA duplex and its metabolites were precipitated by 80% ethanol on a glass fiber then eluted by water.

### 3.3. Current application

#### 3.3.1. In vitro metabolism

An *ex vivo* liver homogenate model derived from rat liver was used to study the mechanism of phosphorothioate OGN (PS-OGN) [50]. Nuclease activity of liver homogenate was durable with respect to degradation of a 21-mer PS-OGN. It was found that 3′-exonuclease activity was predominant and 5′-exonuclease and endonucleases activity were also observed. The metabolism of PS-OGN depends on sequence, length, and chemical modification. LC–ESI/MS results revealed that oxidation of phosphorothioate linkage did not occur in rat liver under the tested experiment conditions.

Under optimal chromatographic conditions using 400 mM HFIP/16.3 mM TEA (pH 7.9), ion-pair reversed-phase HPLC coupled with a high-resolution ESI-TOF mass spectrometry permit a highly efficient separation of chemically modified antisense OGNs from their metabolites [20,51]. A 21-mer PS-OGN with four 2′-OMe modified nucleotides at each end was incubated with bovine intestinal mucosa phophodiesterase (3′-exonuclease) to generate 3′ chain-shortened metabolites. Baseline separation of metabolites was achieved on a 50 mm × 1 mm capillary column.

#### 3.3.2. In vivo metabolism

The *in vivo* metabolism of the first generation antisense OGNs has been studied extensively using ion-pair reversed phase LC–ESI/MS/MS. The metabolism of two antisense PS-OGN ISIS11637 and ISIS11061 in rats were examined with both CGE and LC–ESI/MS methods [52]. The optimized LC–ESI/MS condition in this report was ion-pair reversed-phase HPLC using ODS PVA-C18 column with gradients of 60% isopropanol in 20 mM aqueous triprophylamine. LC-ESI/MS method unequivocally proved that the removal of bases from the 3′-end of the OGN is the major metabolic pathway in plasma. Metabolites isolated from liver and kidney were degraded by 3′-exonuclease, 5′-exonculeases, or a combination of both enzymes. There were extensive degraded metabolites from the 3′-end in kidneys. 5′-terminal metabolites were predominant species in liver with much less extent as compared to kidney samples. Although CGE analysis was able to provide efficient separation based on the length of metabolites, the complexity of metabolites in kidney and liver requires further understanding of base composition and base modification. LC–ESI/MS has been demonstrated in this report to be a valuable tool to characterize metabolism of the antisense OGN. However, sequence verification by tandem MS was not provided.

Using LC–ESI/MS/MS with the quadrupole ion trap mass spectrometry, Griffey and co-workers, for the first time, identified first-generation antisense OGN metabolites generated *in vivo* [53]. Strong anion-exchange chromatography in combination with desalting by TEAA gradient was used to extract and purify the metabolites. Shorter metabolites were separated from longer metabolites and then subject to ion-pair reversed-phase HPLC using 400 mM HFIP adjusted to pH 7.0 with TEA. The sequence of metabolites was confirmed via MS/MS using an user-written computer program. The mass spectrum of a 12-mer metabolite was shown in Fig. 2(a). The deconvolution gave a molecular mass of 3814.5 Da which was used to identify the base composition. Then MS/MS was used to confirm the sequence of this metabolite by isolating the lowest charge state ion $[M–3H]^{3-}$ at $m/z$ 1270.8. Tandem mass spectrum with ion assignments was presented in Fig. 2(b) and ion assignment of signature fragment ions from precursor ion 1270.8 was listed in Table 2. The widely accepted nomenclature for OGN fragment ions proposed by McLuckey et al. was utilized to assign w (3′-terminal) and a-Base (5′-terminal) series ions [17]. In theory, the procedures such as one proposed by Ni et al. can be used for sequencing of completely unknown OGNs [54]. However, *de novo* sequence of unknown

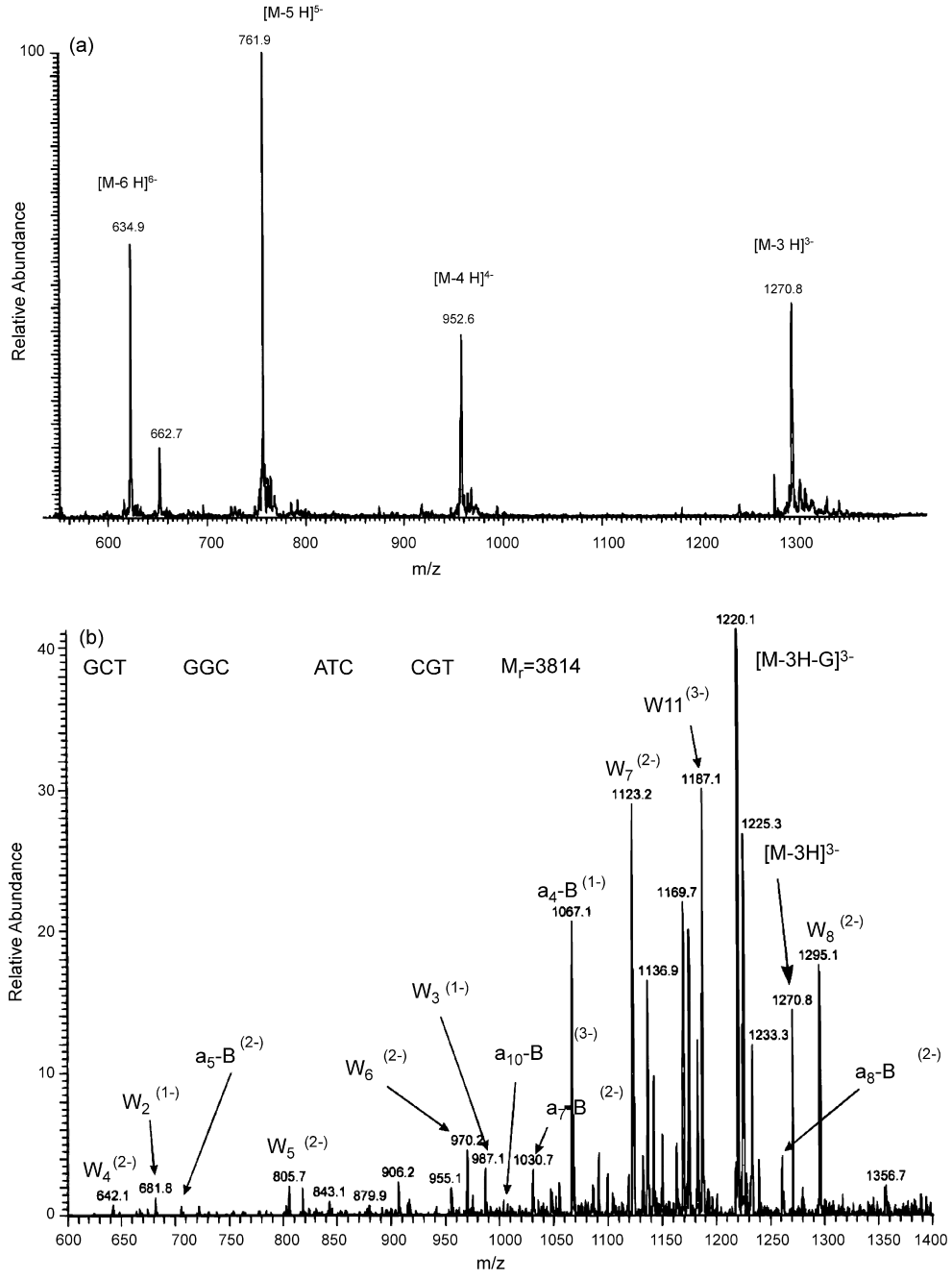


Fig. 2. LC/ESI mass and tandem mass spectra obtained from a 12 mer OGN 5′-GCTGGCATCCGT-3′. (a) Raw mass spectrum obtained from 2.1 to 3.4 min in the liquid chromatogram; (b) tandem mass spectrum produced following isolation of the ion at *m/z* 1270.8. A window of 1.5 Da was employed for ion isolation. A mass range of 450–1750 was scanned in the second dimension Ion masses and assignments are listed in Table 2 (reproduced with permission from Ref. [53]. Copyright 1997 John Wiley and Sons Ltd.).

metabolites was not necessary in this study. Subsequently, eleven product ions including eight w ions and five a-B ions were accurately assigned and the sequence of this 12-mer metabolite was unambiguously verified in light of accurate mass information. Interestingly, the authors confirmed the involvement of 5′-endonuclease in PS-OGN metabolism although endonuclease activity is relative low compared to exonuclease activity. Additional so-called "*N* + 1" metabolites were also observed at longer retention time than the parent drug. The mass difference of these "*N* + 1" metabolites were consistent with addition of either phosphodiester cytosine, guanosine or adenosine to the parent drug. Another possibility is the addition of phosphorothioate deoxyguanosine.

Cummins et al. utilized a LC–ESI/MS/MS method and confirmed that the major metabolic pathways for PS-OGNs are either 3′-exonuclease or 5′-exonuclease [55]. Additional metabolites with slower electrophoretic mobility than the parent drug were also found in the kidney. Attempt to *de novo* sequence these "*N*+" metabolites was not successful. Based on mass differences, it was hypothesized that these metabolites were generated

Table 2
Assignment of fragment ions from MS/MS collision-induced dissociation of *m/z* 1270.8

| *m/z* | Ion assignment | Sequence |
|---|---|---|
| 681.8 | $w_2^{(1-)}$ | GT-3′ |
| 987.1 | $w_3^{(2-)}$ | CGT |
| 642.1 | $w_4^{(2-)}$ | CCGT |
| 805.7 | $w_5^{(2-)}$ | TCCGT |
| 970.2 | $w_6^{(2-)}$ | ATC CGT |
| 1123.2 | $w_7^{(2-)}$ | CATC CGT |
| 1295.1 | $w_8^{(2-)}$ | GC ATCCGT |
| 1187.1 | $w_{11}^{(3-)}$ | CTGGCATCCGT |
| 1067.1 | $a_4$-$B^{(1-)}$ | 5′-GCTG |
| 705.1 | $a_5$-$B^{(2-)}$ | GCTGG |
| 1030.7 | $a_7$-$B^{(2-)}$ | GCTGGCA |
| 1261.4 | $a_8$-$B^{(2-)}$ | GCTGGCAT |
| 1004.7 | $a_{10}$-$B^{(2-)}$ | GCTGGCATCC |
| 1220.1 | -GH | GCTGGCATCCGT |

through the addition of one or more nucleotide bases to the parent compound.

There has been some speculation that PS-OGNs undergo metabolic oxidation, i.e., sulfur for oxygen oxidation at the phosphorothioate linkage *in vivo* via the CYP450s [55]. Phillips et al. [45] have detected minor products consistent with sulfur for oxygen exchange by LC–ESI/MS/MS. However, another study demonstrated that oxidation did not contribute to metabolism of PS-OGNs in whole liver homogenates over an 8 h period [50]. The discrepancy could be explained by different processing and handling of tissue samples.

Dai et al. investigated *in vivo* metabolism of G3139, a 18 mer PS-OGN targeted to human Bcl-2, using LC–ESI/MS/MS [18]. Major metabolites were identified and quantified in plasma obtained from patients treated with G3139. A modified TEA/HFIP mobile phase (8.6 mM TEA and 100 mM HFIP, pH 8.3) was employed to achieve optimal ion-pair chromatographic separation and mass spectrometric performance. Subsequently, Xterra MS C18 column was chosen as it provided reasonable column lifetime with high pH mobile phase. Mass measurement accuracy is critical in metabolism study. Using an ion trap instrument, mass accuracy ranging from ±0.009% to ±0.038% was observed for five metabolites. The TIC and mass spectra of three metabolites are presented in Fig. 3. Multiple charged ions are evident in Fig. 3B–D and in all cases the $3^-$ charge state was dominant. Deconvolution as well as CID sequencing were used to elucidate the base composition of those metabolites. The detailed assignment of fragment ions for both the parent and $3'N-1$ metabolites are listed in this report. The results indicated that the major metabolic pathway for G3139 is 3′-exonuclease mediated and there is similarity across various animal species.

Collectively, the principal metabolic pathway for the first generation antisense OGNs is the cleavage via exonucleases. There is no evidence that cytochrome P450 family of enzymes is involved in the metabolism of this class of compounds. The patterns of metabolites suggest that the exonucleolytic cleavage by 3′-exonucleases is the major route of metabolism followed by 5′-exonuclease pathway. Metabolism mediated by endonucleases pathway is minor one. In most cases, there is a pattern of OGN metabolism, which consists of progressively chain-shortened OGN, typically forming a right-triangle ladder.

The second generation antisense OGNs, i.e., mixed backbone OGNs, possess improved physicochemical properties and considerably different pharmacokinetic and metabolic attributes from the first generation drugs. Among various 2′-modifications, one chimeric design of antisense OGNs has been proven to overcome many of the limitations of the first-generation antisense drugs. 2′-*O*-(2-methoxyethyl) or 2′-MOE modification at 3′- and 5′-terminus with phosphorothioate nucleotides in the middle provides enhanced nuclease stability *in vivo,* higher binding affinity to mRNA and longer tissue half-life [8,48]. The metabolism of ISIS104838 has been characterized in plasma and urine samples obtained from mouse, monkey and human using LC–ESI/MS [48]. There was only low abundance of metabolites in plasma. In contrast, 6-mer to 13-mer metabolites were identified in urine with mass accuracy <0.04%. Because five 2′-MOE modified nucleotides were placed on each end, progressively chain-shortened metabolites were not detected in plasma or urine, indicating that the 3′- or 5′-exonuclease metabolism from each terminus, if present, is minimal. The earliest cleavage occurred in the 10-base gap followed by exonuclease cleavage. Impact of different 2′-MOE modifications on metabolism was evaluated using CGE/UV and LC–ESI/MS [48]. Three distinctly different 2′-MOE modifications were studied: the first one had phosphorothioate backbone with 3′- and 5′-terminal 2′-MOE modifications (ISIS13650); another had phosphorothioate backbone but only containing 2′-MOE at 3′-end (ISIS15839); the third one had phosphodiester backbone with 2′-MOE modification at each nucleotide (ISIS16952). The first OGN was the most stable one followed by the second one. 2′-MOE with phosphodiester backbone did not protect it from nuclease degradation. Due to very low levels of metabolites generated from ISIS13650, LC–ESI/MS failed to detect shortened OGN in plasma and only trace amount of $3'N-1$ was detected in urine. In contrast, $3'N-1$ and $N-2$ metabolites of ISIS16952 were detected in plasma, urine and kidney, indicating phosphorothioate backbone is superior to unmodified backbone.

### 3.3.3. Metabolism of duplex oligonucleotides

The power of LC–ESI/MS/MS in delineating metabolism of therapeutic OGN was also demonstrated in the area of synthetic double-stranded complex si-RNA. The ocular metabolism of a model si-RNA duplex and SIRNA-027 designed against vascular endothelial growth factor receptor 1 (VEGFR1) were examined by LC–ESI/MS/MS using HFIP/TEA ion-pairing agents [49,56]. Either the model si-RNA or SIRNA-027 has similar RNA chemistry in that the sense strand contains one inverted deoxy abasic residue and two deoxy-thymines at 3′-end (BTT) and one inverted deoxy abasic residue at 5′-end whereas antisense strand contains a phosphorothioate linkage between the two deoxy-thymines at 3′-end. A dose of 15 μg si-RNA was injected intraocularly into rabbits and vitreous samples were collected at various time points. The chromatograms revealed

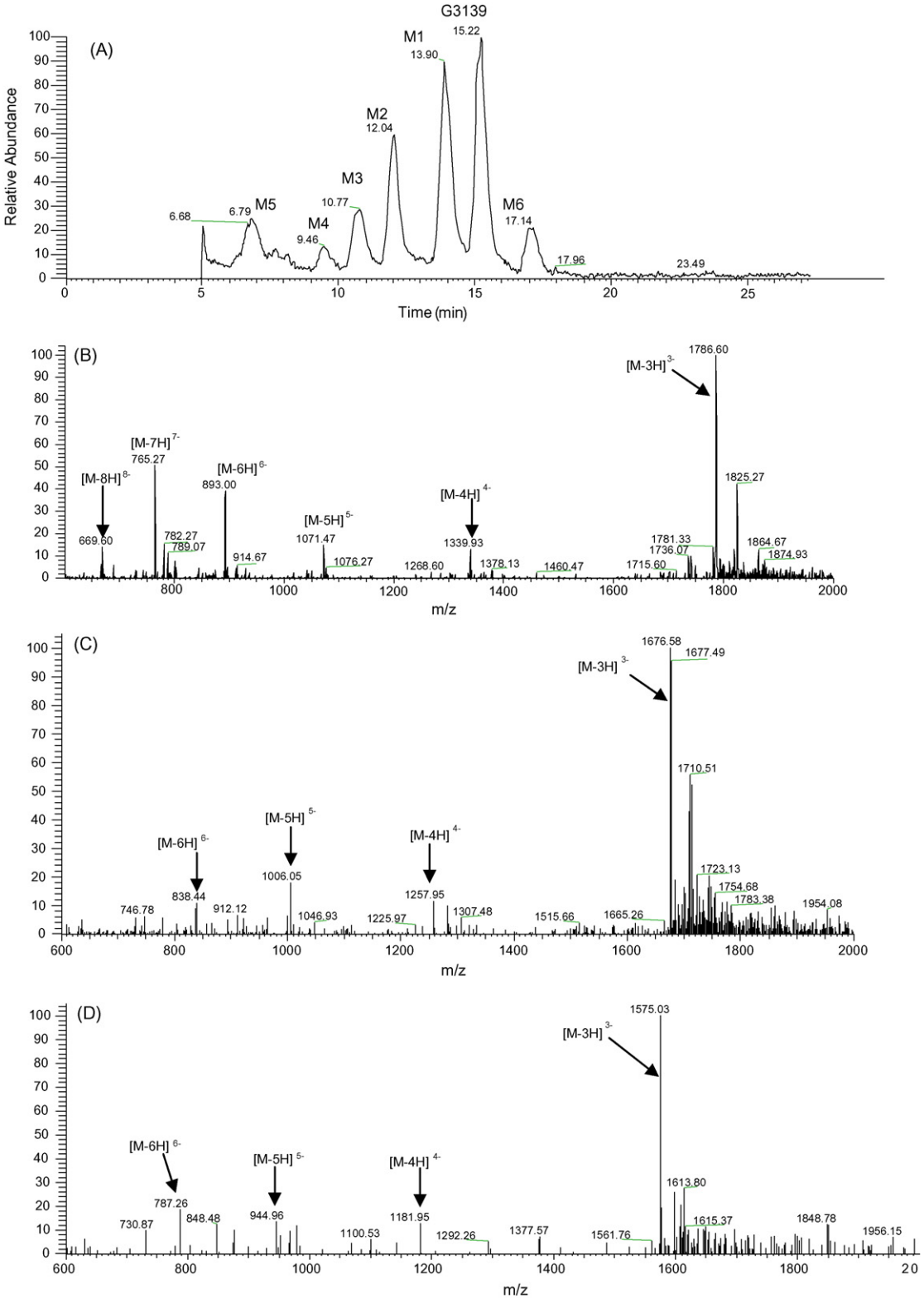


Fig. 3. Identification of the metabolites of G3139 in human plasma extract obtained from a patient treated with G3139. (A) The TIC of metabolites; (B–D) are mass spectra of $3'N-1$, $N-2$, $N-3$ metabolites. Ion-pair reversed phase HPLC column: Xterra MS C18, 2.5 μm, 50 mm × 2.1 mm; mobile phase A: 100 mM HFIP/8.6 mM TEA, pH 8.35; mobile phase B: 50% methanol in A; gradient: 75% A to 50% A in 30 min. Mass spectrometry: LCQ ion trap operated in the negative ion model (reprinted from Ref. [18]. Copyright 2005 with permission from Elsevier).

a large peak containing both parent duplex and metabolites. Deconvoluted ESI mass spectra were obtained for the main peaks. Three duplex and one single-strand metabolites were observed in mass spectrum of vitreous extract at day 7. The duplex structure plays a significant role in the metabolism of si-RNA. Because 20 nucleotides from 3′-end of the sense strand are base paired with 20 nucleotides from 5′-end of antisense strand, there was little degradation of the sense strand of the duplex while antisense strand began to degrade from $3'N-2$ and $N-3$ residues.

A subsequent study investigated the ocular metabolism of SIRNA-027, a synthetic si-RNA duplex currently in phase I study, in the retina/choroid samples. A modified RNA extract procedure involving tissue lysis, phenol/chloroform/isoamyl alcohol extraction was used to extract si-RNA duplex. The recovery was reported to be 14% for 10 μg/mL and 40% for 100 ug/mL samples. Analysis of SIRNA-027 *in vivo* using both denaturing and non-denaturing chromatography revealed that the si-RNA duplex was cleaved primarily from one end, i.e., the 5′-end of the sense strand and 3′-end antisense strand of the duplex. Under the denaturing reversed-phase conditions (65 °C, mobile phase B ramped to 80% in 20 min), the $5'N-3$ metabolite of the sense strand was predominant while other 5′ chain-shorted metabolites of the sense strand were observed. On the other hand, $3'N-1$ through $N-4$ metabolites were observed for antisense degradation products. Using a non-denaturing chromatographic condition (room temperature and shallow gradient), intact duplex and its metabolites in duplex form were identified (Fig. 4). Although broad peak prevent the separation of metabolites from parent compound, several metabolites were identified in the deconvoluted mass spectrum obtained from 14 to 16 min (Fig. 4(B)). However, only four of single-strand species identified in denaturing conditions were detected as duplexes, underscoring the detection limit for duplex need to be improved in the future.

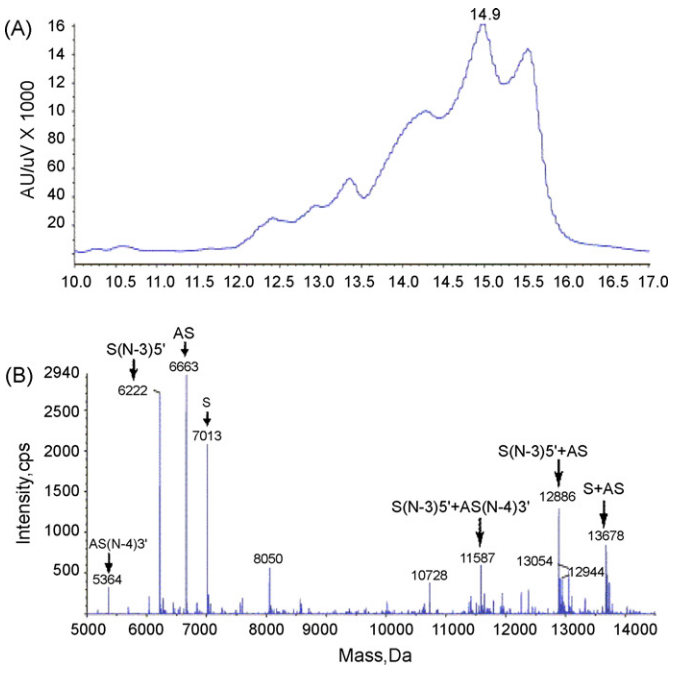


Fig. 4. (A) HPLC-UV chromatogram of day 4 vitreous extracts analyzed under non-denaturing conditions. (B) Deconvoluted mass spectrum obtained from mass spectra from 14 to 16 min in the UV chromatogram. The parent duplex is observed at mass 13,678 and two metabolites: $S(N-3)5'/AS$ and $S(N-3)5'/AS(N-4)3'$. The single-strand components of the parent duplex are also visible at mass 6663 (antisense-strand) and 7013 (sense-strand) (reprinted from Ref. [49]. Copyright 2006 with permission from Elsevier).

## 4. Summary and future perspective

Up to date, at least two antisense-OGN drugs (Vitranene and Macugen) are on market, from which many patients have reportedly benefited. Additionlly, many OGNs (AS-OGNs or si-RNAs) are in preclinical or clinical stages, for which a significant amount of toxicokinetic (TK), pharmacokinetic (PK) and other study data need to be generated in support of drug discovery and development.

Although the application of LC–MS to routine analysis of OGNs is relatively new, there is increasing evidence that LC–MS appears to be an attractive choice for quantitative analysis and metabolite identification of OGNs due to the improvements on the technical issues. Future efforts will include, but are not limited to, the following areas: (1) development of new chromatographic buffer system which offers a better chromatographic separation and less signal suppression; (2) new stationary phase for better chromatography; (3) novel SPE method for a higher extraction recovery; (4) instrument software allowing several major ion transitions to be summed up for an enhanced MRM detection sensitivity and a decreased assay variability caused by the dynamic change of charge states of OGN during LC–ESI/MS analysis; (5) better sample clean-up (desalting) procedure, including on-line sample preparation techniques or 2D LC system; (6) better chromatography system to overcome incompatibility issue of chromatographic resolution and MS detection [i.e., hydrophilic interaction chromatography (HILIC) mass spectrometry system]. LC–ESI/MS or MS/MS analysis of OGNs has low tolerance to salt contamination, and it is, therefore, a good practice to use deionized water and high purity solvents for mobile phase and sample preparation reagents.

It has been reported that the emerging technology—capillary electrochromatography coupled with electrospray mass spectrometry (CE–MS) was applied to the detection and characterization of OGNs [57], which might offer better separation and MS detection of OGNs for the metabolism studies.

To date, there is no single analytical method with high sensitivity, selectivity, ability of identifying and quantifying metabolites, good robustness and reproducibility and amenability for high throughput available for routine use to support PK and PK/PD studies. However, LC–MS based methods may ultimately provide the full characterization of absorption, distribution, metabolism and excretion (ADME) properties of therapeutic OGNs.

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