Bioanalytical LC-MS/MS of therapeutic oligonucleotides

W.D. van Dongen
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Oligonucleotide therapeutics

• RNA chains 15-25 nucleotide units
• cytosine (C) guanine (G), adenine (A) uracil (U)
• interferes with processing of genetic material
  – inhibits/decreases expression of therapeutically relevant protein
  – blocks expression of virus mRNA
• can treat diseases “undruggable” using small molecules or MABs
  – diseases with a genetic background e.g. DMD, cystic fibrosis, specific cancers and rare diseases
  – common diseases e.g. Hep-C, atherosclerosis, lupus, psoriasis
• Two types
  – single stranded: *e.g.* antisense RNA
  – double stranded: *e.g.* short interfering RNA
Oligonucleotide therapeutics

• Currently two drugs on the market
  – Vitravene® for cytomegalovirus infection (herpes)
  – Macugen® for wet macular degeneration (loss of vision in the center of the visual field)

• Many in development
  – >250 therapeutic programs
  – >100 in the clinic (2011)
  – >5 in phase III

*Source: Insight Pharma reports
**Source: Agilent Nucleic Acid Solutions
Bioanalysis of oligonucleotides

• Method-of-choice: ELISA
  – based on hybridization of a probe or a capture and/or detection strand complementary to asRNA or siRNA
  – unsurpassed sensitivity (25 pg/ml)

• Drawbacks
  – cannot distinguish full-length oligonucleotides from truncated shortmer metabolites
  – overestimation of parent oligonucleotide
  – Cannot determine intact siRNA
Bioanalysis of oligonucleotides

- LC-MS
  - unsurpassed selectivity: accurate levels
  - identify and quantify metabolites

- Drawbacks
Bioanalytical LC-MS/MS of therapeutic oligonucleotides

Highly challenging from an analytical perspective:

- Acidic proton at each phosphodiester bond
- Highly charged poly-anionic backbone
- Extremely polar
- Phosphorothioate linkage
Bioanalytical LC-MS/MS of oligonucleotides

Critical issues:

• LC-MS efficiency
  — retention vs. ionisation
• ESI-MS
  — multiple negative charge states
  — H⁺-alkali⁺ exchange at phosphate groups
• MS/MS
  — fragmentation of multiple charged OGNs
• Quantitation
  — internal standard selection
• Sample preparation
  — SPE
  — LLE

William D van Dongen - bioanalytical LC-MS of therapeutic oligonucleotides -
LC-MS efficiency:
chromatographic retention vs. ionisation efficiency dilemma

LC
low
low
ionpair

ESI
high
high
low

organic
pH
ions
IPLC-MS of oligonucleotides

- C18 column
- 10 mM triethylamine (TEA)
- 100 mM hexafluoro-2-propanol (HFIP)
- MeOH/MeCN gradient
IPLC-MS of oligonucleotides

• LC: HFIP increases hydrophobicity ion-pair
• ESI: HFIP dynamic liquid/gas phase pH adjuster
  – pK$_a$ 9, 99% not charged at pH 7, bp 57°C
  – evaporates during ESI
  – volatile HFIP depletes at droplet surface
  – pH at the surface rises to 10
  – OGN-TEA ion pair dissociation
  – desorption OGN into the gas phase.
• And: HFIP reduces cation exchange
Single-quadrupole S/N ratios (n=3) of 20 pg (dT)25 IPLC-MS

**TEA/HFIP:**
15mM triethylammonium /400mM hexafluoro-2-propanol

**TEAA:**
100mM triethylammonium acetate

**DMBAA:**
100mM butyldimethylammonium acetate

**TPAA:**
100mM tripropylammonium acetate

**TBAA:**
100mM tributylammonium acetate

**HAA:**
100mM hexyl ammonium acetate

*McCarthy et al. 5th Symposium on the Practical Applications of Mass Spectrometry in the Biotechnology and Pharmaceutical Industries. The Meritage Resort, Napa, CA, USA, 9–11 September 2008*
ESI-MS:
formation of multiple negative charge states

5’-GGC CAA ACC UCG GCU UAC CU-3’: C\textsubscript{208}H\textsubscript{277}N\textsubscript{72}O\textsubscript{119}P\textsubscript{19}S\textsubscript{19}
Monoisotopic mass: 6882.75Da
Average mass: 6887.66Da
ESI-MS:
formation of multiple negative charge states

5’-GGC CAA ACC UCG GCU UAC CU-3’: C_{208}H_{277}N_{72}O_{119}P_{19}S_{19}
Monoisotopic mass: 6882.75Da
Average mass: 6887.66Da

[\text{M} - 9\text{H}]^{9-}

[\text{M} - 10\text{H}]^{10-}

[\text{M} - 8\text{H}]^{8-}
5′-GAGACTGCAAGCG-3′

100 mM NH₄OAc

Z = 7

2.0 mM NH₄OAc

Z = 5

100 mM NH₄OAc

Z = 3

WD van Dongen - bioanalytical LC-MS of therapeutic oligonucleotides

Griffey et al., JAmSoc 8, 155-160 (1997)
## Complex MS/MS spectra

**5’-GGC CAA ACC UCG GCU UCU**

### Monoisotopic mass:

\[ \text{6882.75Da} \]

### Average mass:

\[ \text{6887.66Da} \]

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5’-GGCCAAACCACUCGUUCUACCUCU-3’

MS/MS of [M-3H]3-

C_{208}H_{277}N_{72}O_{119}P_{19}S_{19}

Monoisotopic mass: 6882.75Da

Average mass: 6887.66Da
5’-TCTCCCAGCGTGCGCCAT-3’
MS/MS of [M-13H]^{13-}

Full scan m/z 400-800

Product ion scan of m/z 591 [M–13H]^{13-}


WD van Dongen - bioanalytical LC-MS of therapeutic oligonucleotides -
ESI-MS

H⁺-alkali⁺ exchange at phosphate groups

200 ng/ml IS

1000 ng/ml analyte
Quantitation: internal standard selection

**IS:** [M-9H]$^9$→ m/z 95

**Analyte:** [M-9H]$^9$→ m/z 95
Internal standard affairs: case 1

Drug substance:
rafAON $G_S$TGCTCCATTGATG$_S$C mol mass: 4590
IS $G_S$UGCUCCAUUGAUG$_S$C mol mass: 4521

Chemical modifications:
lower case $S$ = PS linkage

LC:
no separation of rafAON and IS

SRM:
rafAON, [M-3H]$^{3-}$: $1529 \rightarrow 322 + 1529 \rightarrow 746$
IS , [M-3H]$^{3-}$ : $1506 \rightarrow 289$

LOQ: 50 ng/ml
Internal standard affairs: case 2

Drug substance:                                   mol mass:
PF-ODN    TCGTCGTTTTTTGTTCGTTTTTGTTCGTTT       7697
IS:       TTTTTTTTTTTTTTTTTTTTTTTTTTTTT        6327

LC:
no separation of PF-ODN and IS

SRM:
PF-ODN, [M-xH]^x-        698.8, 640.1, 591.1 (n=11-13) → 95
IS, [M-10H]^{10-}:       631.7 (n=10) → 125

LOQ: 4 ng/ml

## Sample preparation of plasma samples

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<td>G3139</td>
<td>SPE OASIS HLB</td>
<td>40-50%</td>
<td>na</td>
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<tr>
<td>rafAON</td>
<td>SPE OASIS HLB</td>
<td>22.8 ± 6.5%</td>
<td>48.2 ± 3.5%</td>
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<tr>
<td>rafAON</td>
<td>LLE Varian C18</td>
<td>80%</td>
<td>50%</td>
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<td>Compound X</td>
<td>LLE OASIS WAX</td>
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<td>Oligo1</td>
<td>LLE Clarity OTX</td>
<td>&gt;80%</td>
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<td>Compounds</td>
<td>LLE phenol/phenol</td>
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<tr>
<td>PF-ODN</td>
<td>LLE &amp; SPE chlorof/phenol &amp; Oasis HLB</td>
<td>70-80%</td>
<td>0-6%</td>
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WD van Dongen and WMA Niessen, *Bioanalysis* (2011) 3(5), 541-564
# Bioanalytical LC-MS methods of asRNA

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<td>ISIS 1083</td>
<td>SPE Phenyl</td>
<td>5-500 ng/ml</td>
<td>89-107% RSD 2-15%</td>
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<td>21-mer</td>
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<td>rafAON</td>
<td>monkey (OASIS HLB)</td>
<td>50-10,000 ng/ml</td>
<td>94–102% RSD 6–14%</td>
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<td>15-mer</td>
<td>mouse (OASIS C18)</td>
<td>25-5,000 ng/ml</td>
<td>95–101% RSD 3–11%</td>
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<td>PF-ODN</td>
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<td><strong>PF-ODN:</strong></td>
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<td>97-101% RSD 2-12%</td>
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<td>(n-1)5’/(n-1)3’:</td>
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<td>102-106% RSD 1-12%</td>
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<td>99 - 94% RSD 1-12 %</td>
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<td>90– 100 % RSD 6-7%</td>
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WD van Dongen and WMA Niessen, *Bioanalysis* (2011) 3(5), 541-564

5’-TCGTCGTTTTTGTGTTTTTGTGTTT-3’
Metabolites of antisense G3139 in human plasma obtained from *in vivo* study

Human metabolites of G3139

G3139 5’TCTCCCAGCGTGCGCCAT’3

(n−1)3’

(n−2)3’

Dai et al.
*J. Chromatogr. B*
825 (2005) 201
Future perspective: asRNA

• Current 4 ng/ml, must and will be improved
  – UPLC
  – sensitive triple quadrupole MS (Xevo TQS, API 5500, Agilent 6490)
  – (nano-)UPLC and chip technology
  – make LLQ’s in the range of low pg/ml potentially possible
Bioanalytical LC–MS of therapeutic oligonucleotides

Therapeutic oligonucleotides (OGNTs) are important biopharmaceutical drugs for the future, due to their ability to selectively reduce or knockout 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 (e.g., plasma, urine and tissue). Although immunoaffinity methods, especially ELISA, are currently widely applied for this purpose, the potential of LC–MS in OGNT analysis is under investigation. Owing to 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 is critically reviewed and its advantages and limitations highlighted. Finally, the future perspective of bioanalytical LC–MS, that is, lower detection levels and potential generic LC–MS methodology, is discussed.

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 DNA or RNA [1]. Unlike many other drugs, which target the functioning of proteins by interfering with their receptor site, OGNTs target the gene directly or at the mRNA-expression stage and thereby interfere with the production of the gene product.

Another type of OGNTs is based on a naturally occurring gene silencing mechanism called RNA interference (RNAi), where short dsRNAs knock down gene expression in cells [2-5]. The synthetic 19- to 25-base pair (bp) dsRNAs used as OGNTs of this type are called short interfering RNA (siRNAs). The RNAi process is a multistep process: the double-stranded siRNAs are incorporated into the RNA-induced silencing complex (RISC). The central defining component of the RISC is formed by an Argonaute

WD van Dongen and WMA Niessen, Bioanalysis (2011) 3(5), 541-564
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Wilfried Niessen

Mibiton

Proensa therapeutics