Oligonucleotide Biopolymers – Future Challenges for Chromatography

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Introduction

Synthetic Oligonucleotides as Therapeutic Medicines

Synthetic oligonucleotides are an exciting new class of biomolecules capable of treating many disorders, which are currently not amenable to existing drugs, including viral infections [1], respiratory disorders [2], cancers [3] and rare diseases [4]. Current interest has been largely fuelled by two key events: firstly, Fire and Mello's Nobel-prize winning discovery of gene silencing by RNA interference (which helped to improve our understanding of the genetic basis of many diseases) [5]; and secondly, the regulatory approval of two oligonucleotide-based drugs, namely Vitravene® [6] (a 21-base single stranded antisense oligonucleotide approved by the FDA in 1998 for cytomegalovirus infections) and Macugen® [7] (a pegylated aptamer approved in 2005 for treating wet macular degeneration).

Synthetic oligonucleotides currently in clinical development are comprised of single or double stranded DNA [8], RNA [9], locked nucleic acid (LNA) sequences [10], aptamers [11], speigelmers [12] and oligonucleotides conjugated to polymers [13]. Little is known about their exact mechanism of action, but they are thought to involve protein biosynthesis control *via* immunostimulation [14] or *via* interference with gene transcription/translation processes to inhibit production of potentially harmful proteins [15]. Alternatively, oligonucleotides could work *via* an exon-skipping mechanism partially restoring the functional properties of a defective protein [4].

Structural and Regulatory Considerations

The unique chemical/structural properties of oligonucleotide therapeutics has left them in somewhat of a grey area when it comes to regulatory guidance for the development and control of drug substance and product. Oligonucleotide therapeutics are macromolecules derived from DNA and RNA building blocks with a suggested optimal length in the range of about 20 bases (or 20-mer) [16]. They are manufactured in a stepwise fashion using solid-phase synthesis (i.e. one nucleotide at a time) and are specifically excluded from the current ICH guidelines Q6A [17], Q3A(R2) [18], and Q3B(R2) [19]. Whilst there is currently no specific guidance from regulatory agencies for the Chemistry, Manufacturing, and Controls (CMC) of oligonucleotide based therapeutics, the spirit of the existing guidance, particularly Q6A, around quality and safety can certainly be followed. In addition, many of the concepts discussed in ICH Q6B [20], for the analysis of biologically engineered products, are applicable to oligonucleotides as both share similar analytical challenges due to their size, polymeric and secondary structure and closely related impurities or variants.

This short review identifies the source of many impurities and focuses on the application of chromatographic methods using UV and mass spectrometric detection to characterise single and double stranded oligonucleotides and their related impurities.

Chromatographic Analysis of Oligonucleotides

Source of Impurities

Oligonucleotides are synthesised using either H-phosphonate [21] or phosphoramidite chemistry [22] on automated commercial synthesisers. The chemistry starts with an amidite starting material chemically bonded to a solid support, typically glass [23] or a polymeric resin.[24] The chain length is then extended by repeated cycles of deprotection, coupling, oxidation (or sulphurization) and capping. When the desired chain length is achieved, the crude oligonucleotide is cleaved from the solid support and purified *via* preparative ion exchange and reverse phase chromatography. Figure 1 shows a short fragment of a typical oligonucleotide chemically modified on the phosphate backbone and on the ribose sugar. The overall quality of the oligonucleotide is assured through control of the raw materials (primarily the amidite starting materials). However, the large number of synthetic steps (up to 120 steps for a typical 20-mer) coupled with the relative inefficiencies of each reaction step can give rise to a significant number of impurities. Impurities can arise from many sources including manufacturing conditions, from raw materials and from degradation. Most of these compounds fall into the following categories [25]:

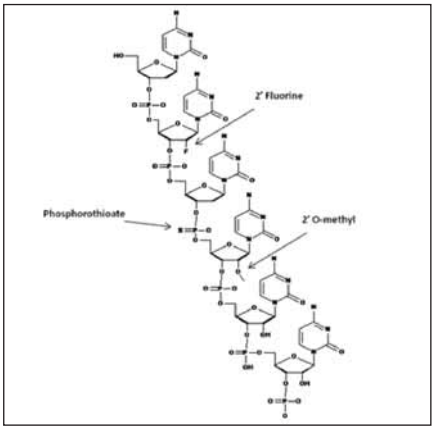


Figure 1. Short Fragment of a typical tetramer oligonucleotide showing chemical modifications.

- Shortmers, deletion or failure impurities – oligonucleotide sequences where the chain length is shorter by n-x.
- Longmers or extension impurities – oligonucleotides sequence where the chain length has been extended by n+x.

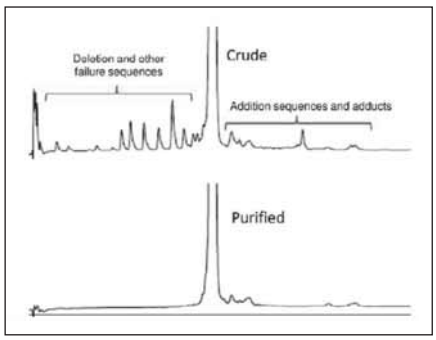


Figure 2. IP-RP-HPLC Analysis of a 21-mer Oligonucleotide in its crude and purified states. See reference [39] for more details.

- Adducts– these are modified full-length sequences which arise from incomplete deprotection of the oligonucleotides. Examples of these adducts include isobutyl, benzoyl or cyanoethyl derivatives.
- Oxidised phosphodiesters – oligonucleotide impurities found only in phosphorothioate oligonucleotides where incomplete sulphurization of the phosphate linkage leads to trace levels of phosphodieser linkages (P=O).
- Depurination – oligonucleotide impurities where the nucleoside has lost a purine base.
- Degradants – impurities formed from degradation of the oligonucleotide during synthesis or under storage conditions (heat, humidity, oxidative, etc.).
- Non-hybridized single strand– excess single strand that remain unreacted during the annealing process to form the double stranded oligonucleotide.
- Derived from structural modification – e.g. phosphothiolation of the phosphate linkages [26] and the addition of alky and alkyloxy groups at the C-2 position of ribose sugars [27], peptides conjugates [13], bridging riboses [28] and morpholino ring derivatives [29].

Chromatographic Methodology

The primary objective of any chromatographic method is to separate impurities from the desired product. This task is made more challenging for oligonucleotides because of their complex structure, multiply charged nature and the presence of myriads of structurally similar impurities. Over the years, methods for separating oligonucleotides have evolved from traditional slab gel electrophoresis [30] and P-31 NMR [31] to modern high performance liquid chromatographic (HPLC) [32] and capillary electrophoretic (CE) [33] approaches. Apart from the choice of analytical technique for oligonucleotide analysis, other considerations include the type of analysis required (i.e. impurity profiling, analysis of aggregate or assay determination), and whether the biopolymer is being analysed in its single or double stranded state, or both. Generally, all of these analytical criteria can be met with one or more of the following chromatographic techniques, ion pair reverse phase HPLC (IP-RP-HPLC) [34], anion exchange HPLC (AX-HPLC) [32] and size exclusion HPLC (SEC) [35]. Capillary gel electrophoresis (CGE) [33] has been included in this review because of its complementary and orthogonal nature to the more traditional separation techniques.

Analysis of Single-Stranded Oligonucleotides

The preferred methods for determining the purity of single stranded oligonucleotides are IP-RP-HPLC [34] and AX-HPLC [32]. Both techniques have the resolving power to separate deletion and extension sequences from the full-length oligonucleotides and for adduct impurities. Separations based on IP-RP-HPLC rely on the formation of transient ion pairs between the oligonucleotide and the ion pair reagents (as well as the more recognised interactions between the ion pair reagent and the stationary phase), resulting in increased and improved separation [36]. Commonly used ion pairing reagents include triethylammonium acetate (TEAA) [37], hexylammonium acetate (HAA) [38] and combination of 1,1,1,3,3,3- hexafluor-2-propanol (HFIP) and triethylammonia (TEA) [38]. In Figure 2, a 21-mer oligonucleotide has been analysed using IP-RP-HPLC before and after column purification [39]. In the crude sample, the increased hydrophobicity of the longmers and adduct impurities results in increased retention. Conversely, the shorter mers are more polar eluting before the main peak. Apart from the choice of ion pair reagent, other factors such as particle size and the use of non-porous stationary phases (e.g. monoliths) can enhance the oligonucleotide separation *via* improved mass transfer and enhanced peak capacity in IP-RP-HPLC [40] and UPLC [41]. In Figure 3, a capillary monolith column eluted with TEAA was used to baseline resolve a mixture of homologous oligothymidylic oligonucleotides (12 to 18-mers) with very high efficiencies within five minutes [42].

AX-HPLC offers a complementary seperation alternative to IP-RP-HPLC and resolves oligonucleotides (and related impurities) primarily based on their charge differences [32]; however, hydrophilic and hydrophobic interactions may also play a role in the separation mechanism [43]. High resolution oligonucleotide separations can be achieved by optimising key parameters such as the ion exchange sorbent (e.g. diethylaminoethyl (DEAE) bonded onto a polystyrene matrix [44]), pH, organic solvents and counterions. Figure 4 shows an AX-HPLC separation of impurities present in a phosphorothioated 19-mer antisense oligonucleotide [45]. The resolved impurities include oxidised phosphodiester (P=O) groups (impurities A1, A2 and A3) and shortmers, where n-1 (impurity A4) and n-2 (impurity A7). In CGE, the gel-filled capillary resolves oligonucleotides *via* a 'sieving' mechanism primarily according to size. To overcome the bias associated with electrokinetic injection, internal standards are

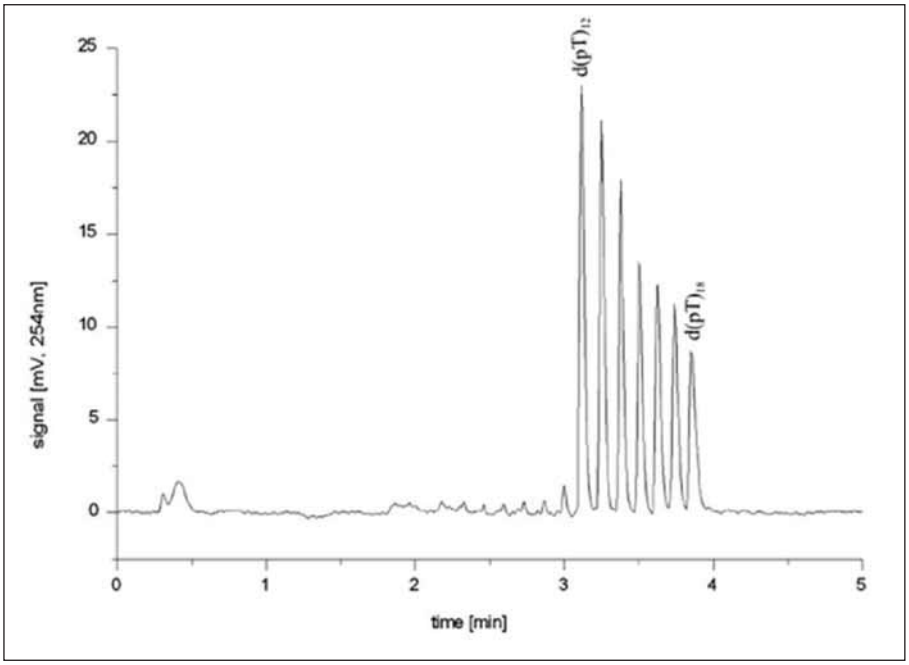
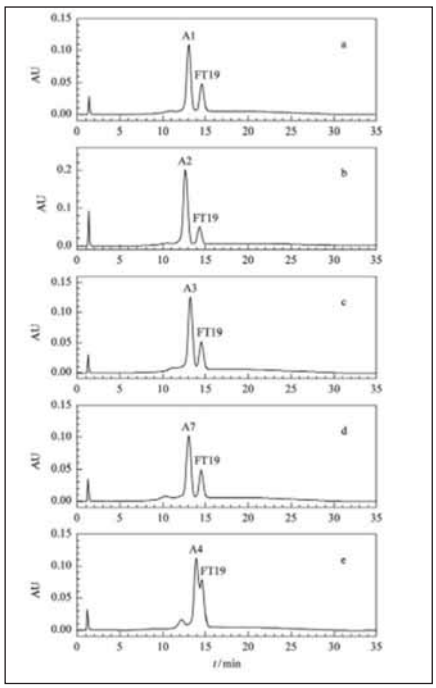


Figure 3. IP-RP HPLC Separation of oligonucleotides d(pT)12 to d(pT)18 using a monolith column. See reference [42] for more details.



Figures 4. AX-HPLC Analysis of a Phosphorothioated 19-mer oligonucleotide (FT19), showing chromatograms showing resolution of the full length sequence from P=O impurities (A1, A2 and A3 in (a) to (c)) and and n-x deletion sequence impurities (A7 and A4 in (d) to (e)). See reference [45] for more details.

often included in the analysis. This is exemplified in Figure 5 which shows a CGE electropherogram of a fully phosphorothioated 20-mer resolved into its deletion sequence impurities in the presence of a 23-mer internal standard [46].

Analysis of Double-Stranded Oligonucleotides

Of the various classes of oligonucleotide therapeutics, double stranded oligonucleotides such as siRNA (short interfering RNA) offer some of the more significant analytical challenges from a quality control perspective. The individual antisense and sense strands are non-covalently hybridized to form an alpha-helical duplex which is the active pharmaceutical ingredient (API). Non-denaturing chromatographic techniques, aimed at maintaining the native duplex structure of the oligonucleotide during analysis, are routinely used. Non-hybridized single strands may be present as impurities in the API. In addition, aggregated oligonucleotide impurities (larger than the targeted duplex conformation) can be formed during manufacture or during storage of the API [47]. The presence of these impurities is of concern from both a potency and safety perspective [48].Whilst chromatographic analysis of the intact duplex is obviously necessary, analysis of the individual sense and antisense strands is equally important. Chromatographic resolutions are improved as oligonucleotide length is reduced and on-column base pairing or secondary structure is eliminated from the analysis. Consequently, chromatographic separations of the individual single strands are more discriminating and have the ability to resolve closely related impurities formed during manufacture or on stability, resulting in a more complete measure of purity and greater understanding of the impurity profile. Analysis of the individual strands can be performed either prior to annealing the single strands or *via* the use of denaturing techniques which dissociate the duplex during the analysis, allowing for on-column separation of the individual sense and antisense strands. The latter approach can give incomplete resolution between the two strands or their related impurities and consequently, analysis of the pre-annealed single strands is preferred. However, a denaturing impurities method would still be required to ensure that no additional degradation has occurred during the subsequent manufacturing processes (i.e. annealing, desalting, lyophilisation) and during storage of the API. Figure 6 shows the separation of sense and antisense siRNA strands from their related impurities [49].

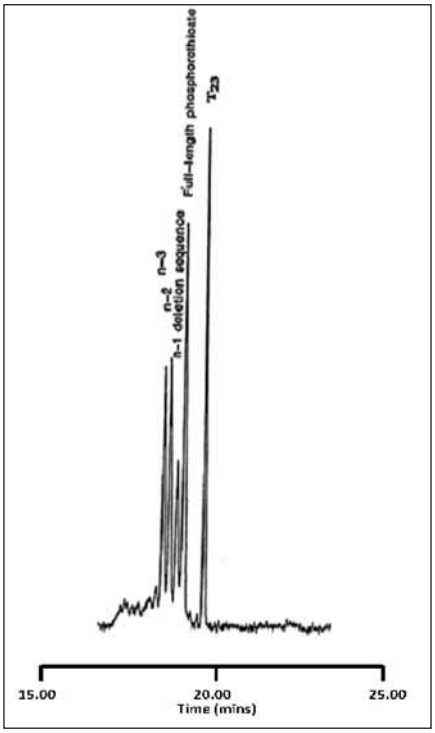


Figure 5. CGE of Phosphorothioated 20-mer oligonucleotide showing resolution of the full length sequence from n-1, n-2, n-3 deletion sequence impurities with a 23-mer internal standard (T23). See reference [46] for more details.

IP-RP-HPLC [34] and AX-HPLC [32] are capable of being run in both non-denaturing and denaturing conditions. In the latter approach, factors such as increased column temperature, increased organic modifier, extremes of pH, and low ionic strength in the mobile phase are used to dissociate or "melt" the duplex into individual strands and eliminate any secondary structures. In contrast, opposing chromatographic conditions are used to create non-denaturing methodology. In particular, the use of a high ionic strength mobile phase is critical to maintaining the duplex structure and other secondary structure conformations such as aggregated impurities. This approach can be very useful if the analytical goal is to separate a duplex oligonucleotide from aggregate impurities and excess unhybridized single stranded impurities. Figure 7 shows a SEC chromatogram of a 21-mer SiRNA duplex containing both aggregate and residual single stranded impurities [49]. Whilst non-denaturing SEC can be used to determine levels of residual single strand impurities, it suffers from poor specificity impacting on sensitivity. We have demonstrated [49] that residual single stranded impurities (≤1% w/w) co-elute under the tailing section of the duplex peak via SEC and are not detected [49]. However, non-denaturing SAX can provide excellent specificity for the detection and quantification of residual single strand impurities.

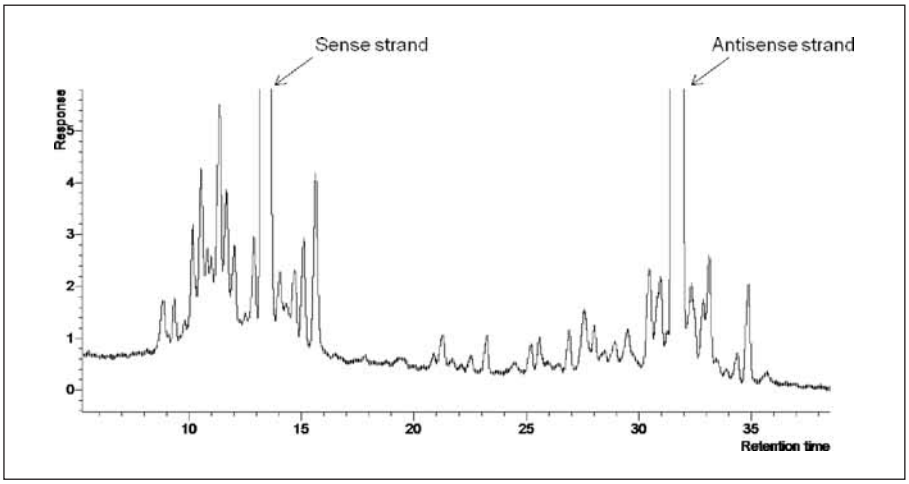


Figure 6. Denaturing RP-IP-UPLC Analysis of a 21-mer siRNA double stranded oligonucleotide showing separation of sense and antisense strands from related impurities. See reference [49] for more details.

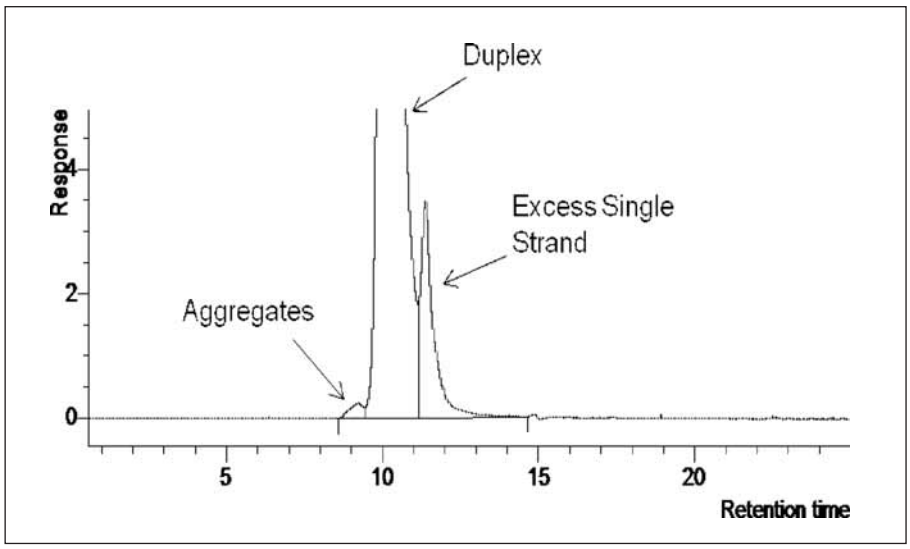


Figure 7. Non-denaturing SEC Analysis of a 21-mer siRNA double stranded oligonucleotide showing separation of the duplex from aggregates and excess single strand. See reference [49] for more details.

agents have been explored based of their differences in structure and overall hydrophobicity [56-58], [37].

Conclusions and Future Directions

Despite the complexities associated with synthetic oligonucleotides, chromatography is still the analytical technique of choice for characterising these biopolymers. However, no one separation technique can fully characterise either the API, or the related impurities. Consequently, orthogonal and complementary separation techniques are routinely utilised, e.g. IP-RP-HPLC, AX-HPLC and SEC. The data from these separation techniques then need to be consolidated and integrated to provide an overall assessment of both purity and related impurities. Industry is working with the regulators to provide guidance in this evolving field. One of the biggest challenges to the regulatory mind-set

Oligonucleotide Analysis using Mass Spectrometric Detection

Mass spectrometry (MS) is routinely interfaced with HPLC separations to provide improved specificity, sensitivity, and structural characterization capabilities. HPLC-MS or HPLC-MS/MS is routinely used for the confirmation of base sequences or to structurally characterise impurities. However, some authors are also reporting its use in release testing of API for the control of impurities co-eluting with the main peak [50]. This latter approach is important as it is directly related to the specificity achievable *via* the chromatographic separation, which in turn determines the necessity for high sensitivity impurity quantification via MS detection. Figure 8 demonstrates the collection of the MS and MS/MS spectra for the sense strand of a 21-mer siRNA from a IP-RP-UPLC separation [51]. Many chromatographic separations used for the analysis of oligonucleotides are not MS compatible, particularly non-denaturing techniques, due to the use of non-volatile buffers in the mobile phase. However, IP-RP HPLC is MS compatible and has become a standard separation platform for the analysis of oligonucleotides [52-58], [37]. The retention of oligonucleotide on the column is critically impacted by the concentration of the ion pair reagent. The more ion pairing agent used the greater the retention. However, high ion pair concentrations can significantly suppress the electrospray ionization efficiency adversely impacting on mass spectrometric detection/analysis [53]. As a result, optimization of the ion pairing agent concentration is commonly performed to enable suitable mass spectrometric detection [52-54]. In addition to TEA, other ion pairing

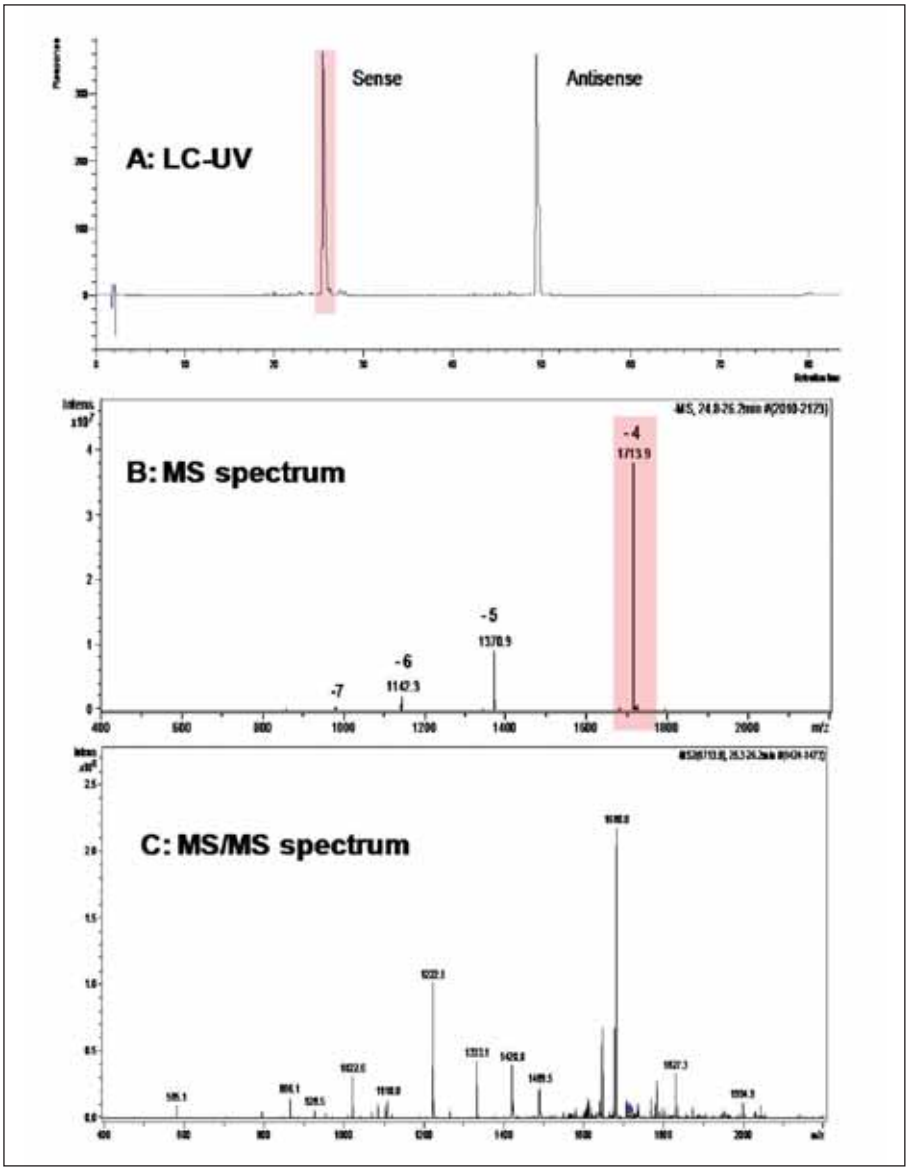


Figure 8. RP-IP-UPLC-MS data showing A) siRNA UV chromatogram under denaturing conditions (sense strand has been highlighted); b) An MS spectrum of the sense strand peak (6860 MW) showing the multiple charge states of this oligonucleotide (-4 through -7) formed as a result of the electrospray ionization; and C) The MS/MS spectrum of the 1713.9 m/z ion (-4 charge state) showing the various fragment ions associated with the specific nucleotide sequence. See reference [51] for more details.

is the need to re-define impurities. Classically, impurities have been regarded as related compounds that provide no beneficial attributes and thus need to be tightly controlled based on safety considerations, e.g. ICH Q3A and Q3B. In contrast, oligonucleotide derived related impurities (especially longmers /shortmers) may have similarly efficacy/safety to the parent API. Therefore, there are less safety concerns and thus greater levels can be tolerated in the final drug product.

Future progress is likely to be focussed on improving the selectivity and sensitivity of these existing chromatographic approaches. However, CE and related electrochromatographic techniques may play a greater role in the future. Recent advances with interfacing CE with ESI-MS (sheathless ESI) [59], have dramatically improved sensitivity to levels comparable with HPLC-ESI-MS, and hold much promise. This coupled with on-line pre-concentration mechanisms like ITP [60], electrokinetic supercharging [61] or immunoaffinity microreactors [62] has ensured that CE-ESI-MS is now one of the most selective and sensitive analytical separation techniques available.

Biography

Dr George Okafo is currently a Chemistry Science Director in Scinovo, a group in GSK dedicated to providing integrated pre-clinical drug development consultancy and technical diligence for GSK's internal and external collaborators. Dr Okafo has been at GSK for over 20 years and has worked in all aspects of chemical development to support numerous projects. Dr Okafo has interests in all aspect of analytical chemistry and, more recently in oligonucleotides analysis.

Dr Daren Levin is currently an Investigator within Exploratory Development Sciences, a group at GSK focusing on pre-clinical drug product development. Dr Levin has spent the past four years at GSK developing internal capabilities/knowledge around the analysis and control of short interfering RNA (siRNA) therapeutic compounds. In addition, Dr Levin has been focused on the development of formulations used to enhance the cellular uptake of oligonucleotide therapeutics. Prior to joining GSK, Dr Levin spent seven years at Alkermes, Inc. developing high efficiency inhalation products as well as sustained release microsphere injectable products.

Dr David Elder is currently a pharmaceutical development Science Director in Scinovo. Dr Elder has spent 33-years in the Pharmaceutical industry, with over half this period with GSK. He has a specific interest in drug impurities and is a member of the EfPIA, PhRMA and PQRI sub-groups on genotoxic impurities. ..He is a committee member of Joint Pharmaceutical Analysis Group and a member of the BP-Committee PCY: Pharmacy.

References

[1] S. Agrawal: Trends Biotechnol. Sci. 10 (1992) 3499-3507.

[2] K. Sobczak, N. Bangel-Ruland, J. Semmler, H. Lindemann, R. Heermann und W.-M. Weber , HNO (2009), 1106 – 1112.

[3] G. Degols, J.P. Leonitti, N. Mechti, B. Lebleu. Nucl. Acids. Res. 19 (1991) 945-948.

[4] A. Aartmus-rus, W.E.Kaman, J.T. den Dunnen, G.J. van Ommen, J.C.van Deutekim, Mol. Ther.14(3) (2006);401-407.

[5] E.J. Sontheimer, RW. Carthew, Cell 122(1) (2005) 9–12.

[6] US FDA: Drug approval package:vitravene (fomivirisen sodium intraveal injectable) injection; www.fda.gov/cder/foi/nda/98/20961_vitravene.html.

[7] US FDA:FDA approves new drug treatment for age-rlated macular degeneration; www.fda.gov/bbs/topics/news/2004/new01146.html.

[8] R. Bhindi, R.G. Fahmy, H.C. Lowe . Am J. Pathol. 171(4), (2007), 1079-1088.

[9] S. Sel, W.Henke, A.Dietrich, U.Herz, H.Renz, Curr. Pharm. Des. 12(25) (2006);3293-3304.

[10] J.Kurreck, E.Wyszko, C.Gillen, V.A.Erdmann, Nucleic Acid Research, 30(9), (2002), 1911-1918.

[11] L.Gold, B.Polisky, O.C.Uhlenbeck, M.Yarus, Ann. Rev. Biochem 64 (1995);763-797.

[12] G.Purschke, F.Radtke, F.Kleinjung, S.Klussmann, Nucleic Acids Res.31 (2003); 3027-3032.

[13] M.E. Zubin, E.A. Romanova, E.M. Volkov, V.N. Tashlitsky, G.A. Korshunova, Z.A. Shabarova, T.S. Oretskaya, Febs Letters, 456 (1) (1999) 59-62.

[14] A. Dorn, S. Kippenberger. Curr. Opin. Mol. Ther. 10(1) (2008) 10-20.

[15] N.K. Sahu,G. Shilakari, A. Nayak, D.V. Kohli, Curr. Pharm. Biotechnol. 8(5) (2007) 291-304.

[16] J.S. Cohen. Oligodeoxynucleotides:. Antisense inhibitors of gene expression, 1989; CRC Press, Boca Raton FL, p.255.

[17] ICH Q6A, Specifications:Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances, 1999.

[18] ICH Q3A (R2), Impurities in Drug Substance, 26th October 2006.

[19] ICH Q3B (R2); Impurities in New Drug Products (Revised Guideline), 2006.

[20] ICH Q6B, Specifications:Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, 1999.

[21] B.L. Gaffney, R.A. Jones, Tetrahedron Letters, 29 (22) (1988) 2619-2622.[22] S.L. Beaucage, M.H. Caruthers. Tetrahedron Letters 22 (1982) 90461-7.[23] H.Koster, J.Biernat, J.McManus, A.Wolter, A. Stumpe, Ch.K.Narang, N.D.Sinha,Tetrahedron, 40(1), (1984), 103-112.

[24] J.F.Labadie, Current Opinion in Chemical Biology, 2(3), (1998), 346-352.

[25] G.M.Blackburn (Editor), Nucleic Acids in Chemistry and Biology, Royal Society of Chemistry (Great Britain), 2006, 143-165.

[26] J.F. Milligan, M.D. Matteucci, J. Martin. J. Med.Chem. 36 (1993) 1923-1935.

[27] X. Chen, N. Dudgeon, L. Shen, J.H. Wang, Drug Discovery Today, 10(8) (2005) 587-593

[28] N.Dias, C.A.Stein, Molecular Cancer Therapeutics, 1, (2002), 347-355.

[29] D.R Corey, J.M. Abrams Genome Biology, 2(5) (2001) 1015.1 - 1015.3.

[30] R.E.Lockard, B.Alzner-Deweerd, J.E.Heckman, J.MacGee, M.W.Tabor,U.RajBhandary, Nucleic Acid Research, 5(1), 1978, 37-56.

[31] P.M.Macdonald, M.J.Damha, K.Ganeshan, R.Braich, S.V.Zabarylo, Nucleic Acids Research , 24(15), (1996), 2868-2876.

[32] J.R. Thayer, K.J. Flook, A. Woodruff, S. Rao, C.A. Pohl, J. Chromatography B, 878 (2010) 933 - 941.

[33] L. Szekely, S. Kiessig, M.A. Schwarz, F. Kalman, Electrophoresis, 30 (2009) 1579-1586.

[34] L. Shu, D-D Lu, Y-L Zhang, S-Q Wang, Chromatographia, published on line June 2010.

[35] K. Makino, H. Wada, H. Ozaki, T. Takeuchi, H. Hatano, T. Fukui, K. Noguchi, Y.Yanagikara, Chromatographia 20 (1985) 713-716.

[36] M. Gilar, , K.J. Fountain, Y Budman, U.D. Neue, K.R. Yardley, P.D. Rainville, R.J.Russell II, J.C. Gebler, Journal of Chromatography A, 958 (2002) 167-182

[37] S.M. McCarthy, M. Gilar J. Gebler, Analytical Biochemistry 390(2) (2009) 181-188

[38] D.T. Gjerde, L. Hoang, D. Hornby (editors) RNA Purification and Analysis: Sample Preparation, Extraction, Chromatography, Wiley-VCH, 2009, 81-90.

[39] G.S.Srivatsa, M.F.Chan, Chimica Oggi/Chemistry Today, 28(6), (2010), 22-24.

[40] M.Gilar, K.J.Fountain, Y.Budman,U.D.Neue, K.R.Yardley, P.D.Rainville, R.J.Russell, J.C,Gebler, J. Chromatography A, 958(1-2), (2002), 167-182.

[41] M.Gilar, U.D, Neue, J. Chromatography A, 1169, (2007), 139-150.

[42] A.Greider, S.C.Ligon, C.W.Huck, G.K.Bonn, J. Sep. Sci, 32, (2009), 2510-2520.

[43] H.Engelhardt, E.Schweinheim,, Chromatographia 22(7-12) (1986) 425-429

[44] J.D. Pearson,F.E. Regnier, Journal of Chromatography A, 255 (1983) 137-149

[45] Z.Hongyan,L.U.Dandan, W.U.Lixia,Z. Zhe, W.Shengqi, Chin. J. Chromatogr, 26(5), (2008), 540-543.

[46] G.S.Srivatsa, P.Klopchin, M.Batt, Feldman X, R.H.Carlson, D.L.Cole, J. Pharmaceutical and Biomedical Analysis, 16, (1997), 619-630.

[47] Levin, D.S. Podium presentation, TIDES, Boston, MA, USA, April 2010

[48] K.Suzuki, T.Doi, T.Imanishi, T.Kodama, T.Tanaka, Eur. J. Biochem., 260, (1999), 855 - 866.

[49] Levin, D.S. Podium presentation, DIA 3rd Oligonucleotide Based Therapeutics Conference, Bethesda, MD, USA, March 2010.

[50] D.C.Capaldi; A.N.Scozzari, Antisense Drug Technology, 2nd Edition, Crooke, S.T., ed., CRC Press, Boca Raton, FL, 2006, Chapter 14.

[51] Personal communication, Daren Levin, GSK, Jan 2011.

[52] K.J.Fountain, M.Gilar, J.C.Gebler, Rapid Comm. In Mass Spectrom. 17, (2003), 646-653.

[53] A.Apfell, J.A.Chakel, S.Fischer, K.Lichtenwalter, W.S.Hancock, J. Chromatography A. 777, (1997), 3-21

[54] Q.Liao, N.H.L.Chiu, C.Shen,Y. Chen, P.Vouros, Anal. Chem. 79, (2007), 1907-1917

[55] W.Xiong, J.Glick, Y.Lin, P.Vouros, Anal. Chem. 79, (2007), 5312-5321

[56] H.J.Gaus,S.R.Owens, M.Winniman, S.Cooper,L.L.Cummins, Anal. Chem., 69, (1997), 313-319

[57] M.Gilar, K.J.Fountain, Oligonucleotides, 13, (2003), 229-243

[58] A.P.Mckeown, P.N.Shaw; D.A.Barrett, Chromatographia, 55(5/6) (2002), XXX

[59] L.H.Shi, Y.X.Jin, D.C.Moon, S.K.Kim, S.R.Park, Electrophoresis, 30 (2009), 1661-1669.

[60] E.Olvecka, D.Kaniansky, B.Pollak, B.Stanislawski, Electroophoresis, 25 (2004), 3865-3874.

[61] M.Dawod, M.C.Breadmore, R.M.Guijt, P.R.Haddad, J. Chromatogr. A, 1189(1-2), (2008), 278-284.

[62] N.A.Guzman, Electrophoresis, 24(21), (2003), 3718-3727.