Oligonucleotides: The Next Big Challenge for Analytical Science

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Introduction

With the rapid growth of interest in oligonucleotides as therapeutic medicines, the Chromatographic Society recently held at a two day symposium, from 27 – 28th October 2010, on the challenge of analysing these molecules. The venue was the very comfortable GlaxoSmithKline Amenities Centre, Ware, UK.

The presentations, stretching over two information packed days, included talks from Pharma companies, CROs, academic institutions and instrument and column vendors. Whilst the majority of presentations focussed on the chromatographic separation of oligonucleotides for various applications, there were also some very useful overview talks, putting this type of therapeutic into context, as well as a presentations on NMR characterisation and the challenge of delivering these molecules to the target cells in the body.

Glaxosmithkline Amenities Centre, Ware, Herts UK

Mini-Exhibition

As an integral part of the symposium, 9 companies had tabletop displays of their latest product offerings.

COMPANY	KEY PRODUCTS ON DISPLAY	CONTACT
Agilent Technologies	Agilent 1200 Series LC systems and Poroshell and Zorbax Columns including integration of Varian products into portfolio	www.chem.agilent.com Tel: +44 (0)845 712 592
Crawford Scientific	YMC range of LC columns and chromatographic training services	www.crawfordscientific.com +44 (0)1357 522961
Dionex	Ultimate® 3000 Rapid Separation LC (RSLC) and Acclaim® range of LC columns	www.dionex.com Tel: +44 (0)1276 691722
Girindus	cGMP compliant scale-up and manufacturing of oligonucleotides	www.girindus.com Tel: +49 (0)511 857 3535
Hichrom	Hypersil Gold, Nucleodur and Zorbax RRHT UPLC columns and chromatographic training services	www.hichrom.co.uk Tel: +44 (0)118 930 3660
ILM Publications	Scientific publications including the forthcoming title – "Analytical Characterisation and Separation of Oligonucleotides and Their Impurities"	www.ilmpublications.com Tel: +44 (0)1727 731948
Phenomenex	Chromatography and sample preparation consumables including the new Clarity™ kit for oligonucleotide extraction	www.phenomenex.com Tel: +44 (0)1625 501367
Sigma-Aldrich	Fine chemicals including DNA and RNA	www.sigmaaldrich.com Tel: (UK) 0800 26 9016
Thermo Fisher Scientific	Hypersil Gold UPLC columns and sample extraction products	www.thermo.com Tel: +44 (0)1442 233 555

The Scientific Program

Chris Bevan opened the oligonucleotide symposium with a welcome address. He also gave particular thanks to the vendors for their financial support of the meeting and to GSK for providing the excellent venue. Chris then went on to introduce the first keynote speaker, Mike Webb, who was to introduce the area of oligonucleotides as therapeutic molecules and the challenge they present to analytical chemists.

The full program is shown below with video lectures and presentations from the meeting available on the CHROMmunity website. To view the online material register at http://chrommunity.chromacademy.com and view the CHROMSOC pages using the top navigation bar. A summary of selected talks is given below as a taste of what was presented at the meeting. Anyone who has a particular interest in this area is urged to view the full set of presentations for themselves.

PRESENTER	ORGANISATION	TITLE OF PRESENTATION
Mike Webb	GSK	Therapeutic oligonucleotides new medicines with new chromatographic challenges
Kathy Ackley	Girindus	Use of Orthogonal Analytical Methods for Analysis of Impurities in Oligonucleotides
William van Dongen	Proxylab [NL]	Bioanalytical LC-MS of asRNA and siRNA: A state of the art overview
Jim Thayer	Dionex	Anion exchange approaches for monitoring strand stoichiometry and stability in RNAi therapeutics (see article in this edition of CT)
Ken Cook	Dionex	High Resolution Ion Exchange Separations of a broad Spectrum of Oligonucleotides with Automated off-line desalting for MS
Mark Dickman	University of Sheffield	RNA Chromatography; From Principles to Applications (see article in this edition of CT)
Paul Newstead	GSK	Phosphorothioate oligomer ion pair chromatography – a case study
Kevin Boyce	Thermofisher	Routine analysis of oligonucleotides using the latest Ion Trap and OrbiTrap technologies
Tony Beck	GSK	Analysis of synthetic oligonucleotides by NMR Spectroscopy
Andrew Ray	AstraZeneca	Ion Mobility: mass spectrometry of oligonucleotides
James Rudge	Phenomenex	CLARITY[R]: A portfolio of advanced solutions for oligonucleotides isolation and separation (see article in this edition of CT)
Thomas Rupp	Girindus	Strategies for analytical and preparative chromatography of short RNAi oligonucleotides
Bernhard Noll	Analytics CMC, Roche Kulmbach GmbH, Germany	Analytical approaches for drug substance and impurity characterization in siRNA drug development
Nadim Akhtar	AstraZeneca	Pulmonary delivery of Oligonucleotides; Analytical, formulation and regulatory considerations in early development
Marco Smith	GSK	LCMS characterisation of a modified oligonucleotide together with its synthetic impurities using a high resolution mass accuracy instrument
Ashley Sage	Agilent Technologies	Analytical Characterisation of Therapeutic Synthetic Oligonucleotides and Associated Impurities by LC/MS
Matt Waldheim	Agilent Technologies	LCMS Analysis of Phosphoramidites
Martyn Hemsley	Covance	Approaches to the Quantitative Bioanalysis of Oligonucleotides in Plasma
Rob Wheller	GSK	Quantitative work on oligonucleotides
David Cleaver	Sigma Aldrich	Quality Control of Synthetic Oligonucleotides by Mass Spectrometry. and Chromatographic Analysis

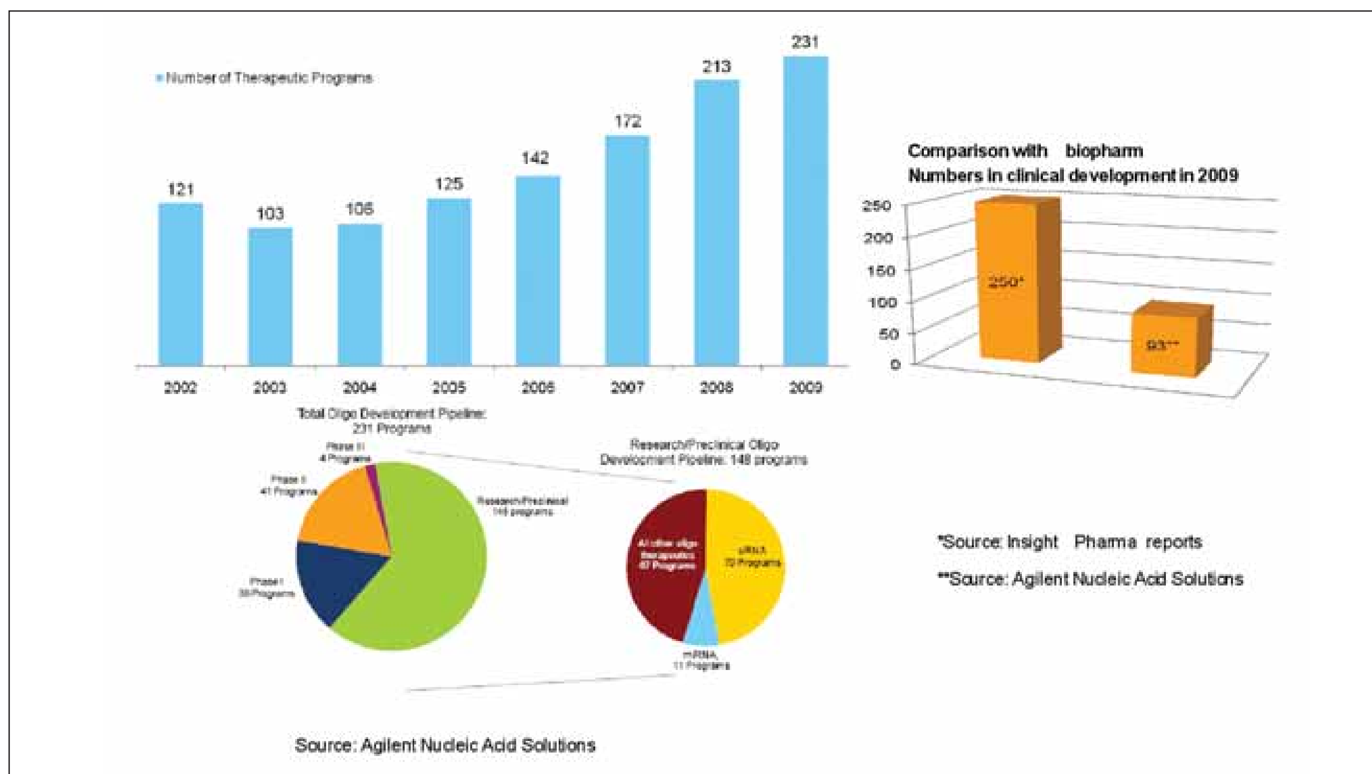


Figure 1. Rapid growth of therapeutic oligonucleotides.

A summary of selected lectures

Mike Webb (GSK) opened the scientific program with a lecture titled 'Therapeutic Oligonucleotides – New Medicine with New Analytical Challenges'. First he outlined the different types of oligonucleotides; antisense, aptamer, microRNA (miRNA) and small interfering RNA (siRNA) illustrating the importance of secondary structure in some cases on the activity of the molecules. He presented industry statistics showing the increase in the number of therapeutic development programs for oligonucleotides having more than doubled from 103 in 2003 to 231 in 2009. He also compared the number of oligonucleotides in clinical development (93) with the number of monoclonal antibody therapies (MABs) (250) demonstrating the growing importance of these molecules to Pharma companies (Figure 1).

GSK have invested heavily in recent years in building a portfolio of oligonucleotide molecules in the areas of inflammation, respiratory and rare diseases. This includes Prosensa, a therapy for the treatment of Duchenne Muscular Dystrophy (DMD) in which a genetic mutation results in a poorly transcribed protein. Dr Webb used Prosensa, an RNA modulating therapeutic, to illustrate just one example of the mechanism of action of an oligonucleotide molecule which induces exon skipping resulting in a modified aberrant protein with much greater functional use in subjects suffering the disease.

Mike moved on to emphasise the complementary nature of oligonucleotides as they have the potential to treat disease "undruggable" using small molecules or MABs. These include genetic disorders but they are also amenable to other more common diseases for example hepatitis-C, atheroscerosis, lupus, psoriasis etc. As oligonucleotides resemble endogenous molecules, they often exhibit fewer toxic side effects i.e. non genotoxic and highly target specific (fewer off-target effects). However, with the site of action in the cell cytoplasm, delivery is a major challenge and in the words of Dr Webb – "A hell of a journey for a big molecule!" Delivery options include conjugation with other molecules, liposomal formulation or formation of antibody complexes.

There is also the challenge of synthesising, characterising and purifying the active pharmaceutical ingredient. Oligonucleotides are fully synthetic molecules derived through linear elongation of the monomer units

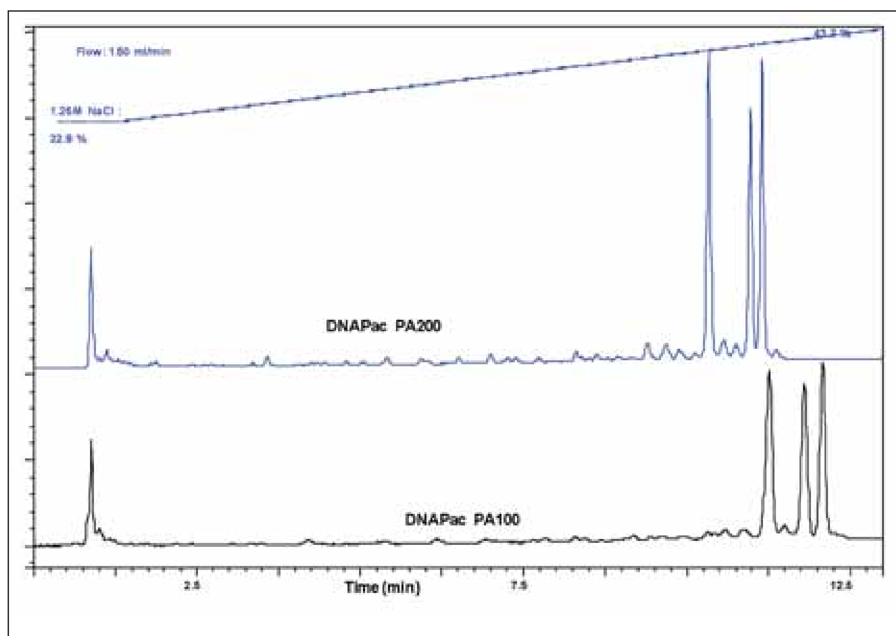


Figure 2. An AEX Method showing the potential for separating "shortmers"

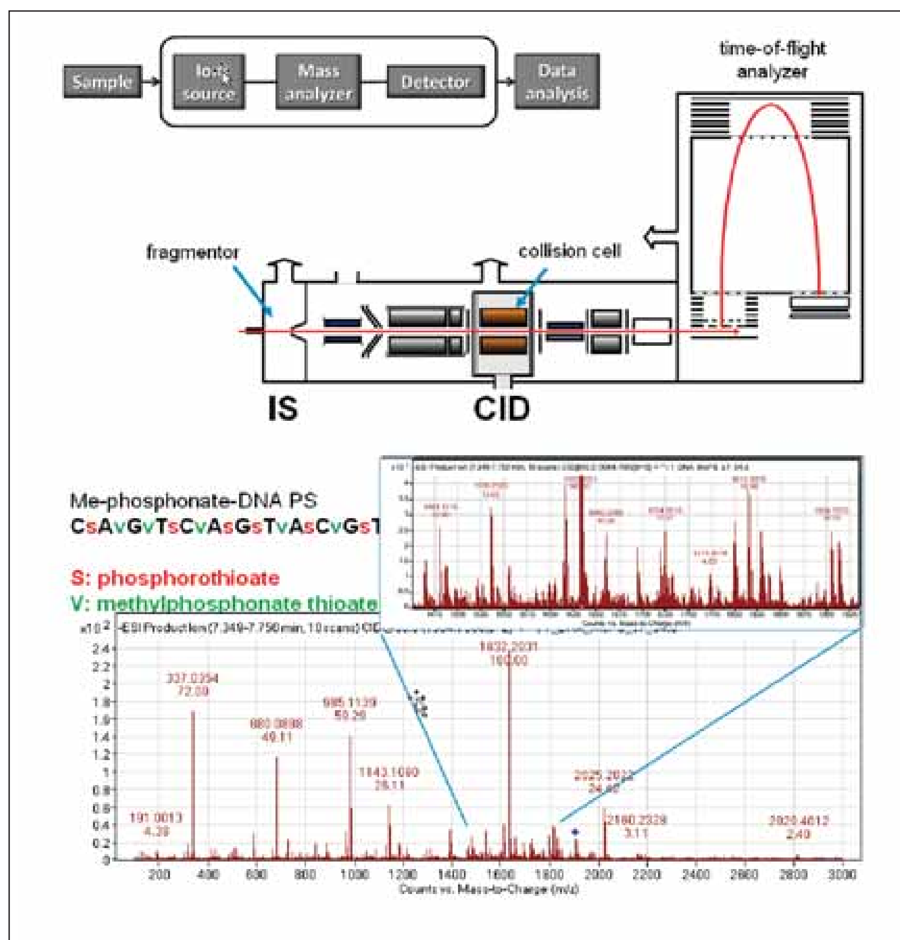


Figure 3. High resolution QTOF MS/MS data used for sequence determination of oligonucleotides

assembled on a solid support to form the final oligomer. With each monomer cycle consisting of 4 chemical synthetic steps, a 20mer (20 monomer units) requires 76 synthetic steps with each potentially resulting in impurity products. The ICH-Q3 guidance on impurities in New Drug Substance and Products are not really applicable to

oligonucleotides and are in fact specifically excluded. GSK have therefore developed their own specifications based on the application of good science where identification must include sequence confirmation by mass spectrometry and melting point (Tm). Purity analysis (% area) is then carried out by employing ion-pair reversed phase HPLC with acceptance criteria of "N-1" & "N+1" groups individually <3.0%, any unqualified impurity <1.0% and total impurities <10.0%.

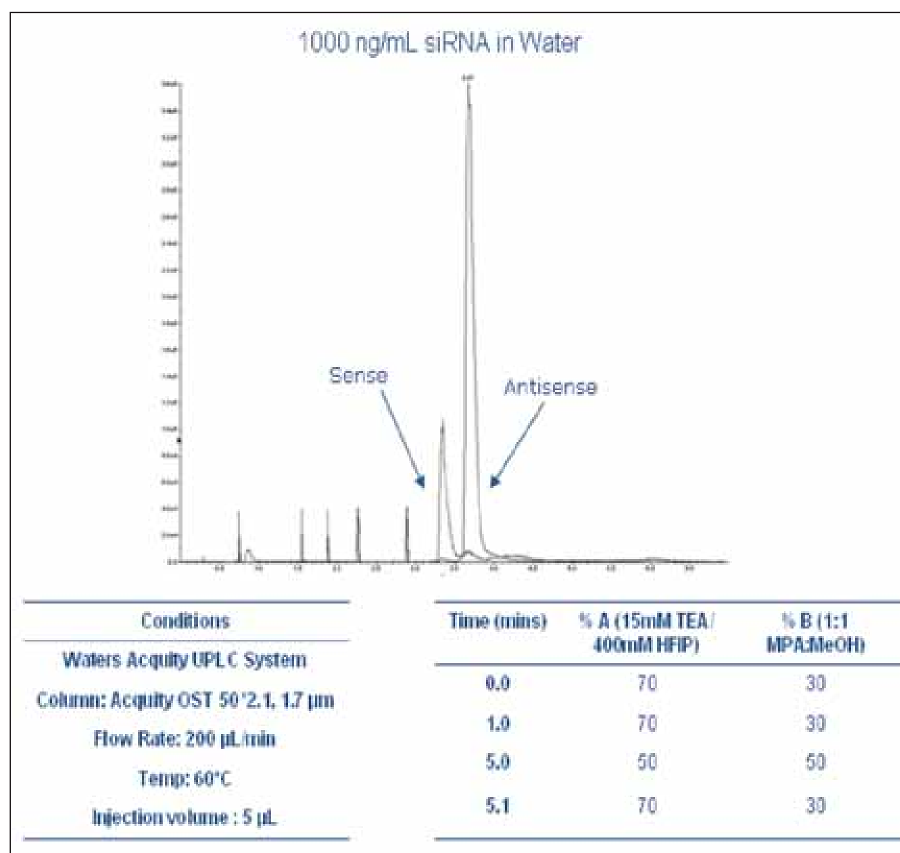
An excellent introductory talk was concluded by reiterating that oligonucleotide therapy is a rapidly growing area for the pharmaceutical industry. It is a major change from small molecules but is also different from biopharmaceuticals. Although there are many challenges to be overcome, with it being such a new area, there is plenty of opportunity to be a pioneer.

Kathy Ackley (Girindus) spoke on the subject of 'Use of orthogonal Analytical Methods for Analysis of impurities in Oligonucleotides'. The opportunity for the formation of impurities during the synthesis of an oligonucleotide is almost bewildering and increases with the size of the molecule. Potential impurities include shortmers (n-1, n-2 etc.) caused by failure to couple, longmers (n+1, n+2 etc.) caused by double coupling and branching, phosphodiester impurities (in oligonucleotides with phosphorothioate backbones), depurinated species and impurities originating from incomplete deprotection (i.e. n+CNET, n+iBu etc). Analytical methods available for impurity characterisation include ion-pair reversed phase HPLC or UPLC, anion exchange chromatography (AEX), size exclusion chromatography (SEC), capillary electrophoresis (CGE) and ^{31}P NMR. The impurity profile observed is highly dependant upon the analytical method used. Kathy illustrated this point by showing chromatograms of the same "positive-sense" DNA analysed by AEX (97.0% purity with 5 impurities), ion-pair chromatography (96.6% with 7 impurities) and CGE (95.6% purity with 15 impurities).

Kathy commented that methods for oligonucleotide analysis had improved dramatically in the last few years with ion-pair UPLC capable of resolving PO impurities from PS DNA oligonucleotides and AEX methods capable of separating shortmers (Figure 2) and RNA sequences with 2′-5′ linkages. Two case studies were presented which illustrated the importance of applying orthogonal chromatography separations in order to measure product purity. She concluded by saying that oligonucleotide impurities are structurally very similar to the active pharmaceutical ingredient (API), analytical methods capable of resolving impurities are needed to establish product specifications and a combination of orthogonal chromatographic methods can supply more information in early stages of the drug development and manufacturing process.

Ashley Sage's (Agilent Technologies) presentation was titled "Analytical Characterisation of Therapeutic Synthetic Oligonucleotides and Associated Impurities by TOF LC-MS" and focused on identification and sequencing using electrospray (ESI) LC-MS. Negative ion ESI allows the determination of the molecular weight of the main species and use of MS/MS enables the sequence to be elucidated. Time-of-flight (TOF) instruments are particularly useful for characterization because high resolution data is ideal for deconvolution of complex spectra and high sensitivity provides the ability to detect impurities in the presence of the main component. Dr Sage illustrated this with an example of how the multiply charged envelope mass spectrum of a single stranded oligonucleotide could be deconvoluted using software to reveal the mass of the full length product and similarly for an n-1 impurity peak. He then moved on to show how a QTOF MS instrument can be used to generate high resolution MS/MS spectra which can be used for sequence verification (Figure 3). By entering the sequence of an oligonucleotide into sequencer software, the calculated mass spectrum can be generated and compared to the actual mass spectrum to allow confirmation of identity.

Rob Wheller (GSK) focused his talk on "Bioanalysis of oligonucleotides". Current bioanalytical methods include stem-loop PCR, which is a very sensitive technique but of questionable robustness and time consuming, ELISA techniques, providing good sensitivity and throughput of samples but non-selective for some metabolites and LC-MS/MS, providing high selectivity and throughput but currently lacking the required sensitivity of



Conditions
Waters Acquity UPLC System
Column: Acquity OST 50*2.1, 1.7 µm
Flow Rate: 200 µL/min
Temp: 60°C
Injection volume : 5 µL

Time (mins)	% A (15mM TEA/ 400mM HFIP)	% B (1:1 MPA:MeOH)
0.0	70	30
1.0	70	30
5.0	50	50
5.1	70	30

Figure 4. LC-MS/MS conditions for the analysis of siRNA

detection. The core of the presentation was around overcoming the challenge associated with analysis of oligonucleotides in plasma by LC-MS/MS and the results of an evaluation of the Waters TQ-S instrument in combination with the Trizaic microfluidic chromatography system.

One of the inherent problems associated with the analysis of oligonucleotides is that the phosphate groups, which make up part of the backbone of the molecule, can give rise to multiple ionisation states and also form adducts with Na^+ & K^+ ions in solution. This envelope of differing charge states and adduct formation results in a spread of MS response reducing the overall sensitivity of detection. Whilst it is not possible to prevent the multiple ionization states, adduct formation can be minimized by the use of mobile phase additives; a combination of triethylamine and hexafluoroisopropanol (HFIP) being the favoured system by most doing reversed phase chromatography (Figure 4).

Rob then went on to discuss the optimization of SPE extraction of oligonucleotides from plasma. The best results obtained thus far in their laboratories are using the Phenomenex Clarity[R] OTX weak anion exchange SPE sample extraction kit including the use of lysis buffer containing chaotropic agents for disruption of the protein-nucleotide binding. Using this extraction system, 85% recovery was obtained from the plasma matrix. Preliminary evaluation of the Water Xevo TQ-S with the Trizaic system would appear to offer a significant increase in sensitivity over other quantitative LC-MS/MS instrumentation currently available.

Marco Smith (GSK) presented on "LC-MS characterization of modified oligonucleotides" focusing on the use of accurate mass measurement for the determination of molecular weight, elemental composition and sequence verification. Reinforcing the presentation given by Ashley Sage, he illustrated that by obtaining negative ion mass spectra of the oligonucleotide, it is possible to use deconvolution techniques to determine an accurate molecular weight. The empirical formula derived from the accurate mass data can then be used to infer nucleobase subunit composition. Marco illustrated the characterization process with a number of spectra obtained from analysis of a modified oligonucleotide on an Orbitrap instrument. However, Clinical Trials Applications also require sequence confirmation. Due to the complexity of signals from both NMR and IR techniques for this class of compounds, there is a greater reliance on mass spectrometry data. Chemically induced dissociation MS/MS ofparent molecule into characteristic fragment ions (X & Y ions) allows sequence determination of nucleobases in an analogous way to the determination of amino acid sequencing in protein and peptide analysis. He concluded in saying that accurate mass measurements have successfully allowed full sequence determination of modified oligonucleotides and is an invaluable analytical tool in supporting the progression of this class of therapeutic into Clinical Development.

William van Dogen (Proxylab) delivered a review of oligonucleotide bioanalysis titled "Bioanalytical LC-MS of asRNA and siRNA – A State of the Art Overview". After describing the analytical challenge of these molecules and the dilemma between obtaining adequate LC retention and good ionization, he described the current preferred LC-MS conditions of ion-pair chromatography in conjunction with an electrospray source. He summarized a number of published literature methods used to extract different RNAs, varying in chain length from 15-25 nucleotides units, and illustrated this with supporting validation data. He concluded that the best recovery of analyte from these published methods was obtained by liquid:liquid extraction using phenol:chloroform:isoamyl alcohol rather than Oasis HLB SPE. However, these comments were at odds with other speakers at the symposium who advocated the used of weak-anion exchange SPE. Finally, he threw down the challenge to the audience stating that the current low ng/mL limits of quantification were not adequate enough to determine the pharmacokinetics of these molecules but that ever more sensitive mass spectrometers, like the Waters Xevo TQ-S, and the use of nano-UPLC and chip technology offer the promise of greater capability.

Closing comments on the symposium

There were 147 delegates registered for this conference, some having travelled from as far as Europe or the US, which reflects the growing interest in this area. Over the two days, there was a tangible buzz around the symposium as both the knowledgeable and the novice learnt something new and made plenty of contacts to help them pursue their research further. Much thanks for the success of the conference must go to Chris Bevan (ChromSoc) and George Okafo (GSK) for their organisation of a very successful 2-day symposium. David Elder (GSK) was co-chair for the day 2 sessions with Dave Rudd (GSK) as the pub quiz master as part of the evening social event. Thanks also go to Ajit Shah (ChromSoc), Malcolm Printer (GSK) and Marco Smith (GSK) who ably assisted with security compliance and crowd control during the event.