Analytical Technologies Europe: Symposium on the Practical Applications including CE, LC and MS in the Biopharmaceutical Industry (AT Europe 2017)

Symposium Co-chairs:

Christof Finkler, *F. Hoffmann - La Roche Ltd.*
Hansjörg Toll, *Sandoz GmbH*

14-17 March 2017
Radisson Blu Royal Hotel
Brussels, Belgium

*Organized by*
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The Scientific Organizing Committee gratefully acknowledges the following program partners for their generous support of this Symposium:

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- British Mass Spectrometry Society
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- German Biochemistry and Microbiology Society
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- Leiden Bio Science Park
- Sektionen för Läkemedelsanalys and Apotekarsocieteten
CASSS AT Europe Student Travel Grants

CASSS is pleased to provide a limited number of student travel grants for PhD students and post-docs who present applicable posters at the Analytical Technologies Europe: Symposium on the Practical Applications including CE, LC and MS in the Biopharmaceutical Industry (AT Europe 2017). PhD students or post-doctoral fellows conducting research at academia throughout the world are eligible.

Why you should apply:
This Symposium is an active forum for discussion of recent developments and regulatory considerations of the practical application of analytical technologies amongst industry, academia and regulatory agencies. There will be a focus on capillary electrophoresis, mass spectrometry and chromatography for product characterization, process monitoring, formulation development and release testing in the biopharmaceutical industry. As a participant, you will have an excellent opportunity to meet, network and participate in exchanging knowledge for mutual education with your peers.

Requirements are:
- Present a poster on a MS, CE, LC topics relating to biopharmaceuticals
- Proof of studentship/post-doc status
- Recommendation from your supervisor

CASSS has awarded student travel grants to the following individuals:

Separation of Synthetic Peptides and Proteins Using Specially Designed OT-CEC Column
Ashraf Ali, *Inha University, South Korea*

Strategies for the Identification and Quantitation of Virus-like Particles of Human Papillomavirus using Capillary Electrophoresis
Virginie Bettonville, *University of Liège, Belgium*

HILIC-MS: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies
Valentina D’Atri, *University of Geneva, Switzerland*

Capillary Electrophoresis and Ion Mobility Coupled to Mass Spectrometry as Complementary Tools for Cysteine Connectivity Identification in Peptides Bearing Two Intra-molecular Disulfide Bonds
Cédric Delvaux, *University of Liège, Belgium*

Three Internal Standard Based Glycan Structure Identification Method for Capillary Electrophoresis
Gábor Járvás, *University of Pannonia, Hungary*

A Novel Non-toxic Reducing Agent for Reductive Amination Based Glycan Labeling
Zsuzsanna Kovacs, *University of Debrecen, Hungary*

Characterisation of Commercial Drug Products and Comparison with In-house Produced Biosimilars
Anne Trappe, *NIBRT, Ireland*
Social Program

Wednesday, 15 March
17:45 – 19:00
Exhibitor Reception

Come network and visit exhibitors in The Capitals.

Thursday, 16 March
18:30 – 22:00
Conference Event – Chez Leon

This event is open to all full conference attendees. One day only attendees will be charged extra to attend. Please see the registration desk for pricing. Chez Leon has been serving mussels and chips to Brussels for over 100 years. Today the restaurant serves more than a thousand meals every day and has become famous for its simplicity, warm welcome and picturesque atmosphere.

Please meet CASSS staff in the lobby of the hotel at 18:30. This year’s event is a 5-minute walk from the hotel.
Acknowledgements

Symposium Co-Chairs:
Christof Finkler, F. Hoffmann – La Roche Ltd.
Hansjörg Toll, Sandoz GmbH

Scientific Program Committee:
Martin Blüggel, Protagen Protein Services GmbH
François de l’Escaille, Analis SA
Marta Germano, Janssen Infectious Diseases and Vaccines
András Guttman, University of Pannonia
Anders Lund, Sanofi
Cari Sänger - van de Griend, Kantisto BV
Birgit Schmauser, BfArM, Federal Institute for Drugs and Medical Devices
Jeremy Springall, MedImmune, A member of the AstraZeneca Group
Harold Taylor, Merz Pharmaceuticals GmbH

Audio-Visual:
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**AT Europe 2017**

**Scientific Final Program Summary**

**Tuesday, 14 March 2017**

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<th>Time</th>
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<tbody>
<tr>
<td>08:30 – 14:00</td>
<td><strong>Registration</strong> (for course attendees ONLY) in the 1st Floor Foyer</td>
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<tr>
<td>09:00 – 12:30</td>
<td><strong>Short Course</strong> in Salon 1&lt;br&gt;&lt;br&gt;&lt;strong&gt;Fundamentals of Mass Spectrometry in the Analysis of Protein Therapeutics&lt;/strong&gt;&lt;br&gt;Short Course Facilitator: Anders Lund, <em>Sanofi, Framingham, MA USA</em></td>
</tr>
<tr>
<td>12:30 – 13:30</td>
<td><strong>Lunch</strong> (for course attendees ONLY) in the Atrium Restaurant</td>
</tr>
<tr>
<td>13:30 – 17:00</td>
<td><strong>Short Course</strong> in Salon 1&lt;br&gt;&lt;br&gt;&lt;strong&gt;Applications of Mass Spectrometry to Characterize Protein Therapeutics&lt;/strong&gt;&lt;br&gt;Short Course Facilitator: Anders Lund, <em>Sanofi, Framingham, MA USA</em></td>
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### Keynote I in Royal Ballroom

**Session Chair:** Christof Finkler, *F. Hoffmann – La Roche Ltd., Basel, Switzerland*

**09:15 – 10:00**

**Higher Order Structure: Route to QC Testing?**  
Carl Jone, *UCB Pharma, Braine l’Alleud, Belgium*

**10:00 – 10:30**

**Networking Break** – Visit the Exhibits and Posters in The Capitals

### Method Development and Comparability in Royal Ballroom

**Session Chairs:** Harold Taylor, *Merz Pharmaceuticals GmbH, Frankfurt Am Main, Germany*

**10:30 – 10:55**

**Enhanced Analytical Development for Complex Antibody Formats**  
Markus Wild, *F. Hoffmann – La Roche Ltd., Basel, Switzerland*

**10:55 – 11:20**

**Analytics for Complex Biologics: FDA Examples**  
David Keire, *CDER, FDA, St. Louis, MO USA*

**11:20 – 11:45**

**Forced Degradation Comparability of an AQbD-developed Adenovirus Quantification CZE Method**  
Lars Geurink, *Janssen Infectious Diseases and Vaccines, Leiden, Netherlands*

**11:45 – 12:15**

**Discussion**

**12:15 – 13:15**

**Buffet Lunch** in the Atrium Restaurant

**13:15 – 14:15**

**Poster Session** in The Capitals

**14:15 – 14:45**

**Technical Seminar**

**Accelerating Biotherapeutics Characterization with the new Biopharma Compass 2.0 Software**  
Romano Hebeler, Detlev Suckau, *Bruker Daltonik, Bremen, Germany*

*Sponsored by Bruker Daltonik*

**Royal Ballroom**

**14:45 – 15:10**

**Flow Induced Dispersion Analysis (FIDA) for Rapid Measurement of Protein Concentration and Protein–Ligand Interactions under Native Conditions**  
Henrik Jensen, *University of Copenhagen, Copenhagen, Denmark*
Wednesday, 15 March 2017 continued

15:10 – 15:35  Sequential Injection Capillary Electrophoresis for Bioprocess Monitoring
Rosanne Guijt, University of Tasmania, Hobart, Australia

15:35 – 16:00  Development of a CGE-SDS Method for Routine Fragmentation Monitoring
in a High Complex Fusion Protein
Roberta Russo, Merck Serono SpA, Rome, Italy

16:00 – 16:30  Discussion

16:30 – 16:45  Mini Break

16:45 – 17:45  Roundtable Discussions I in Royal Ballroom
Session Chairs: Christof Finkler, F. Hoffmann – La Roche Ltd., Basel, Switzerland
and Hansjörg Toll, Sandoz GmbH, Kundl, Austria

17:45 – 19:00  Exhibitor Reception – In The Capitals
Thursday, 16 March 2017

08:30 – 17:30  Registration in the 1st Floor Foyer

Keynote II in Royal Ballroom
Session Chair: Hansjörg Toll, Sandoz GmbH, Kundl, Austria

09:00 – 09:45  Protein Glycosylation Analysis by Mass Spectrometry
Manfred Wührer, Leiden University Medical Center, Leiden, Netherlands

09:45 – 10:15  Networking Break – Visit the Exhibits and Posters in The Capitals

Glycan Analysis in Royal Ballroom
Session Chair: András Guttman, University of Pannonia, Hungary

Govert Somsen, Vrije Universiteit Amsterdam, Amsterdam, Netherlands

10:40 – 11:05  Comparison of Methods for IgG Glycosylation - A Multi Laboratory Study
Dietmar Reusch, Roche Diagnostics GmbH, Penzberg, Germany

11:05 – 11:35  Discussion

11:35 – 12:05  Technical Seminar

Fast Glycan Labelling and Analysis: High-Resolution Separation and Identification in Minutes
András Guttman, SCIEX, Brea, CA USA

Charge Heterogeneity Analysis of Intact Monoclonal Antibodies using CESI-MS
Steve Lock, SCIEX, Pudsey, United Kingdom

Sponsored by SCIEX Separations

Royal Ballroom

12:05 – 13:05  Buffet Lunch in the Atrium Restaurant

New Trends in Mass Spec in Royal Ballroom
Session Chair: Anders Lund, Sanofi, Framingham, MA USA

13:05 – 13:30  Exploratory and Targeted MS Approaches for Precise Monitoring of HCP Clearance Through DSP
Séverine Clavier, Sanofi, Vitry-sur-Seine, France

13:30 – 13:55  Modernizing the Platform Characterization Peptide Map for Accurate Assessment of Deamidation and Isomerization by LC-MS/MS
Lisa Marzilli, Pfizer, Inc., Andover, MA USA

13:55 – 14:25  Discussion
### Thursday, 16 March 2017 continued

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<tr>
<th>Time</th>
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<th>Speaker/Location</th>
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</table>
| 14:25 – 14:55 | **Is There a Need for a New Regulatory Pathway for Biologics?**  
Alan Fauconnier, Belgian Medicines Agency (FAGG-AFMPS), Brussels, Belgium | 14:25 – 14:55
| 15:55 – 16:25 | **Technical Seminar**  
**Sampling the Globe – How to Handle Unlimited Sample Diversity in Mass Spectrometry Data for Biotechnology Discovery and Development**  
Anders Michael Bernth Giessing, Novozymes A/S, Bagsvaerd, Denmark | 15:55 – 16:25
| 16:25 – 17:25 | **Roundtable Discussions II** in Royal Ballroom  
Session Chairs: Christof Finkler, F. Hoffmann – La Roche Ltd., Basel, Switzerland  
and Hansjörg Toll, Sandoz GmbH, Kundl, Austria | 16:25 – 17:25
| 18:30 – 22:00 | **Conference Event – Chez Leon**                                                      | 18:30 – 22:00

**Regulatory Insight Session in Royal Ballroom**  
Session Chair: Birgit Schmauser, BfArM, Federal Institute for Drugs and Medical Devices, Bonn, Germany

**Technical Seminar**
- **Sampling the Globe – How to Handle Unlimited Sample Diversity in Mass Spectrometry Data for Biotechnology Discovery and Development**
  - Anders Michael Bernth Giessing, Novozymes A/S, Bagsvaerd, Denmark

**Sponsored by** Genedata AG

**Royal Ballroom**
## Friday, 17 March 2017

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<tr>
<td>08:30 – 15:00</td>
<td>Registration in the 1st Floor Foyer</td>
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<td>09:00 – 09:45</td>
<td><strong>Keynote III</strong> in Royal Ballroom&lt;br&gt;Session Chair: Francois de l’Escaille, <em>Analis SA, Suralee, Belgium</em></td>
</tr>
<tr>
<td>09:00 – 09:45</td>
<td><strong>Recent Advances in Separation Sciences and Mass Spectrometry Applied to the Characterization of Monoclonal Antibodies and Antibody-drug Conjugates</strong>&lt;br&gt;Pat Sandra, <em>Research Institute for Chromatography, Kortrijk, Belgium</em></td>
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<tr>
<td>09:45 – 10:15</td>
<td>Networking Break – Visit the Exhibits and Posters in The Capitals</td>
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<tr>
<td>10:15 – 10:40</td>
<td><strong>New Trends in Chromatography</strong> in Royal Ballroom&lt;br&gt;Session Chair: Martin Blüggel, <em>Protagen Protein Services GmbH, Dortmund, Germany</em></td>
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<td>10:15 – 10:40</td>
<td><strong>Characterisation of Extractable and Leachables from Single Use Bioprocessing Solutions</strong>&lt;br&gt;Jonathan Bones, <em>NIBRT, Dublin, Ireland</em></td>
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<tr>
<td>10:40 – 11:05</td>
<td><strong>New Trends in SE-UHPLC</strong>&lt;br&gt;Jennifer Rea, <em>Genentech, a Member of the Roche Group, South San Francisco, CA USA</em></td>
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<tr>
<td>11:05 – 11:30</td>
<td><strong>HILIC-MS: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies</strong>&lt;br&gt;Valentina D’Atri, <em>University of Geneva, Geneva, Switzerland</em></td>
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<tr>
<td>11:30 – 12:00</td>
<td>Discussion</td>
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<tr>
<td>12:00 – 12:30</td>
<td>Technical Seminar&lt;br&gt;&lt;br&gt;<strong>Best of Both Worlds: Combining Quantitative Targeted Proteomics with High-content Discovery Proteomics</strong>&lt;br&gt;Florian Marty, Karel Novy, <em>Biognosys AG, Zurich, Switzerland</em></td>
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<tr>
<td>12:30 – 13:30</td>
<td><strong>Buffet Lunch</strong> in the Atrium Restaurant</td>
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Friday, 17 March 2017 continued

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<th>Time</th>
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<th>Speaker</th>
<th>Institution and Location</th>
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<tr>
<td>13:55 – 14:20</td>
<td>Analysis of Influenza Antigens: Sequence, Concentration and Glycans</td>
<td>Terry Cyr</td>
<td>Health Canada, Ottawa, ON Canada</td>
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<td>14:20 – 14:50</td>
<td>Discussion</td>
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<tr>
<td>14:50 – 15:00</td>
<td>Closing Comments in Royal Ballroom</td>
<td>Hansjörg Toll</td>
<td>Sandoz GmbH, Kundl, Austria</td>
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Higher Order Structure: Route to QC Testing?

Carl Jone

UCB Pharma SA, Braine L'Alleud, Belgium

Biopharmaceuticals are complex mixtures of closely related variants and we are fortunate to have many sensitive, accurate, quantitative and robust assays that can be used to measure their quality attributes. This is true both for powerful characterization methods and more simple assays that are routinely used for batch release and stability testing. The drive in implementing QbD is facilitating the development of better analytical tools and our understanding of critical quality attributes.

Although higher order structure has been identified as an important quality attribute for some time, in our experience, this has not yet translated into health authorities requesting to see more higher order structure data. This may change as QbD becomes more embedded into product development, and higher order structure methods are more extensively used to provide insights into quality or functional understanding that current analytical tools do not provide.

We are currently evaluating several technologies (CD, NMR and native peptide maps) to determine their sensitivity in monitoring higher order structure consistently for batch-to-batch comparison and structure-function relationships. Our aim is to determine which of these methods will be more amenable to routine testing environments.

Specific case studies will be presented to highlight the challenges of developing these techniques into QC ready higher order structure methods.

NOTES:
Enhanced Analytical Development for Complex Antibody Formats

Markus Wild

F. Hoffmann-La Roche Ltd., Basel, Switzerland

The complexity of antibody formats has increased in recent years. Analytical challenges are associated with such new formats since platform methods can usually not be applied. Thus analytical methods need to be developed using enhanced approaches in order to address complexity and challenges. Analytical quality by design (AQbD) concepts provide a science- and risk-based framework for developing enhanced understanding of analytical methods. AQbD helps to understand, reduce and control sources for variability and thus helps to develop robust and reliable methods which are applicable throughout the lifecycle of the method. This talk will give an overview about AQbD tools and aspects taking practical examples into account.

NOTES:
For surveillance purposes, facile, sensitive high-resolution assays are important to insure the quality of complex drugs with global supply chains. For example, the FDA monitors for adverse event reports that could stem poor quality drugs. In addition, the agency works to select, implement and understand modern analytical tools so that when issues with drug supply chains occur these tools can be quickly applied to investigate if the root cause of the adverse drug reactions arise from drug quality. In this presentation, examples of FDA/CDER/OPQ/OTR/DPA studies were selected to illustrate the role of analytical tools in establishing and monitoring drug quality. Examples of the application of MS, NMR and CE to assessing the quality of peptide, protein or carbohydrate therapeutics sourced from synthetic or biological manufacturing processes will be presented.

FDA Disclaimer: The findings and conclusions in this article have not been formally disseminated by the Food & Drug Administration and should not be construed to represent any Agency determination or policy.

NOTES:
Forced Degradation Comparability of an AQbD-developed Adenovirus Quantification CZE Method

Lars Geurink, Cari Sänger – van de Griend, Ewoud van Tricht

Janssen Infectious Diseases and Vaccines, Leiden, Netherlands, Kantisto B.V., Baarn, Netherlands

Adenovirus vector vaccines are developed in Janssen Vaccines and Prevention to prevent the spread of infectious diseases such as Zika, Ebola, and HIV. Safe and efficacious vaccines products are required for in human use. Analytical test methods for characterization and release are needed to support development. The adenovirus particle concentration is an important quality attribute for product safety. So far a precise, accurate, fast and stability indicating method could not be developed for that purpose. An AQbD approach was used to develop a CE method for adenovirus particle quantification and was compared to (bio-)analytical techniques in a forced degradation study that was precise, accurate, and fast adenovirus quantification method for adenovirus serotypes 26 and 35. The intermediate precision was <3.7% RSD for the adenovirus concentration and 0.82% RSD for the migration time. The spiked recovery was between 97 – 103%, and analysis time per sample was 3.5 min. The LOQ was determined to be 80 pmol/l but could be enhanced at least 10-fold.

We applied temperature, oxidation, freeze/thaw and pH stress to our adenovirus products in a forced degradation study. The samples were analyzed with CZE, AEX-HPLC, RP-UPLC, FFF-MALS, QPA and QPCR. CZE resulted in an adenovirus peak decrease with temperature and oxidation stress in line with the results of AEX-HPLC, RP-HPLC, FFF-MALS and QPCR. On top of that new peaks appeared. The results of CE, AEX-PLHC, RP-UPLC, FFF-MALS and QPCR were all in line with and linked to a decrease in potency as measured by the QPA method.

In total, we developed an adenovirus quantification CZE method that is more precise, accurate, and robust with shorter analysis times than orthogonal techniques such as AEX-HPLC and QPCR. The CZE method indicated early loss of adenovirus particles and showed additional peaks, potentially degradation products, during a forced degradation study.

NOTES:
Flow Induced Dispersion Analysis (FIDA) for Rapid Measurement of Protein Concentration and Protein-Ligand Interactions under Native Conditions

Nicklas Poulsen¹, Morten Pedersen¹, Jesper Østergaard¹, Nickolaj Petersen¹, Niels Heegaard², Henrik Jensen¹

¹University of Copenhagen, Copenhagen, Denmark, ²Statens Serum Institut, Copenhagen, Denmark

Do you have in-built quality control of your protein and protein – ligand interaction assays? Are you sure what it is you are measuring – might it be an artifact related to non-specific surface adsorption or binding not resembling that in solution? Is your assay compatible with complicated sample matrixes such as plasma or serum? Most laboratories will answer “no” to these questions, which has been a key motivator for us to develop and commercialize new technologies not suffering from these shortcomings. In this work we present Flow Induced Dispersion Analysis (FIDA) as a novel capillary-based technology for assessing protein concentration in plasma samples, measuring in-solution binding under native conditions (pure plasma) and for probing immune responses such as immunogenicity towards biopharmaceuticals.

FIDA is based on the dispersion in a pressure driven flow of a ligand (indicator molecule) interacting with the protein of interest (for example an antibody-based drug). The indicator has a high apparent diffusivity when it is not bound to the antibody, but upon binding it will have a lower apparent diffusivity due to a change in the apparent hydrodynamic radius. The change in apparent diffusivity forms the basis for an accurate measure of protein concentration.¹,²

In this presentation, FIDA is illustrated for assessing in-solution protein concentration, quantification of antibody-based drug compounds and for detection of patient immune responses. Immune responses are important in relation to immunogenicity testing of biologics and for diagnosis of autoimmune diseases. For example, the rapid (minutes) measurement of autoantibodies against dsDNA (a marker for Systemic Lupus Erythematosus, SLE) is demonstrated.


NOTES:
Sequential Injection Capillary Electrophoresis for Bioprocess Monitoring

Rosanne Guijt

University of Tasmania, Hobart, Australia

Biological processes are naturally susceptible to variability because living cells consume substrates and produce metabolites and products in a dynamic way with variations over short time intervals. This presentation demonstrates the potential of capillary electrophoresis (CE) for bioprocess monitoring, with application in culture management and pharmacology. The novel injection strategy - replacing a vial by a flow-through interface - dramatically reduces sample consumption and facilitates automation. Adhesion cultures were monitored for three days using less than 2 mL of sample for 228 analyses, suspension cultures for >8 mL for 192 analyses over 4 days. Combined with electrophoretic separation, this fully automated system offers high sample throughput, good temporal resolution and low sample consumption. Combined with robustness, sensitivity and flexibility, it provides a promising new platform for pharmacological and biotechnological studies.

AlHusban, Gueven, Breadmore and Guijt, Scientific Reports, under review

AlHusban, Gueven, Breadmore and Guijt, Analytica Chimica Acta 2016, 920, 94-101


NOTES:
Development of a CGE-SDS Method for Routine Fragmentation Monitoring in a High Complex Fusion Protein

Roberta Russo, Alessia Pellegrini

Merck Serono SpA, Rome, Italy

Capillary based electrophoresis methods offer several advantages over conventional gel-based methods, such as ease of use, shortened analysis time, streamlined sample preparation and higher resolution. Due to the possibility of automation and quantitative analysis, they are the best candidate for routine QC analysis.

In the last years, several CE based methods have been developed, characterized and validated for the routine analysis of antibodies, as well as for more complex proteins (fusion protein).

The present study was aimed at developing and validating a CGE-SDS method for the analysis of fragments and clipped species of a complex fusion protein, under both reducing and non-reducing condition. Due to the complexity of the protein and to its particular fragmentation and clipping trend, the challenge was the fine-tuning of the analytical conditions in order to have a reproducible and accurate method. In particular, the sample buffer pH has been optimized in order to avoid any induced fragmentation under NR condition and to have the complete reduction of disulphide bridges under RED condition. The final profiles obtained under reducing and not reducing conditions have been characterized by comparison with artificially degraded samples and by the support LC-MS.

The final CGE-SDS method was successfully validated and transferred to QC Labs, for the routine use in release and stability study. Furthermore, the method has been proven to be applicable for process controlling since it has been found suitable for the analysis of intermediate process samples. In addition, the method under reducing condition has been found to be a useful fast screening tool for the evaluation of the level of non-glycosylated protein in the sample, in replacement of more complex LC-MS analysis.

NOTES:
Protein Glycosylation Analysis by Mass Spectrometry

Manfred Wührer¹, Kathrin Stavenhagen¹, Mehmet Kayili¹, Stephanie Holst¹, Ruchira Engel², Diana Wouter², Sacha Zeerleder², Bekir Salih³

¹Leiden University Medical Center, Leiden, Netherlands, ²Sanquin Research, Amsterdam, Netherlands, ³Hacettepe University, Ankara, Turkey

Mass spectrometry (MS) is a very useful method for the characterization of protein glycosylation, on the level of intact glycoproteins, released glycans, or glycopeptides. For this purpose, MS is often coupled with miniaturized separation techniques, such as nanoLC-MS and capillary electrophoresis employing (nano-)electrospray ionization.

Integration of various analytical levels is required for resolving the often-enormous heterogeneity of protein glycosylation as exemplified for the human complement factor C1 inhibitor. C1 inhibitor is a serine protease inhibitor and the major regulator of the contact activation pathway, as well as the classical and lectin complement pathways. In fact, it is one of the most heavily glycosylated plasma glycoproteins, but only very little is known about the glycosylation features and their biological role.

We performed the first detailed site-specific N- and O-glycosylation analysis of C1-Inh using a panel of glycomics and glycoproteomics workflows. The protein was digested with a variety of proteases and glycopeptide samples were partly treated with PNGase F and exoglycosidase treatment. Glycopeptide analysis was performed using C18-[porous graphitized carbon (PGC)]-LC-ESI-QTOF-MS/MS and C18-LC-ESI-ion trap-MS/MS under different fragmentation regimes such as stepping-energy collision induced dissociation (CID) and electron transfer dissociation (ETD).

In total, 27 O-glycosylation sites featuring core 1 O-glycans were identified on C1-Inhibitor. While ten of them could be located with their exact position, one glycosylation site was found to be either Thr27 or Ser28, next to a heavily O-glycosylated region with up to 16 occupied O-glycosylation sites within the peptide sequence Thr82-Ser121.

Additionally, all the six N-glycosylation sites were identified and five of them were characterized in a site-specific manner. N-glycoform distribution determined on the glycopeptide level was largely in agreement with the total released N-glycan profile analyzed by MALDI-TOF/TOF-MS/MS.

This data forms the basis for further studies into the function of C1-Inh glycosylation.

NOTES:
Hydrophilic Interaction Liquid Chromatography-Mass spectrometry for Glycoform Profiling of Intact Pharmaceutical Proteins

Govert Somsen¹, Jordy van Angeren¹, Sara Tengattini², Rob Haselberg¹, Elena Dominguez Vega¹

¹Vrije Universiteit Amsterdam, Amsterdam, Netherlands, ²University of Pavia, Pavia, Italy

The role of mass spectrometry (MS) in quality control and assurance of intact pharmaceutical proteins is steadily growing. Biopharmaceuticals often encompass glycoforms which may not be distinguished consistently by MS only. Separation prior to MS detection is essential to achieve reliable assignment of intact protein variants. However, traditional protein separation techniques such as size-exclusion chromatography (SEC), ion-exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC), generally show poor compatibility with MS. Reversed-phase liquid chromatography (RPLC) is MS compatible, but often provides low performance for intact proteins and lacks the selectivity to resolve glycoforms.

This lecture presents the design and application of hydrophilic interaction liquid chromatography (HILIC) with MS detection for the assessment of the microheterogeneity of intact glycoproteins. By selecting appropriate columns, eluent additives and ion-source conditions, high separation performance can be achieved, while maintaining excellent MS compatibility. It will be shown that glycoform resolution in HILIC is mainly governed by carbohydrate content, yielding efficient separations based on number and size of attached glycans. Examples will highlight the attainable glycoform assignment with focus on the analysis of proteins of pharmaceutical interest, such as interferon-beta, erythropoietin, antigen glycoconjugates and monoclonal antibodies (mAbs).

NOTES:
Comparison of Methods for IgG Glycosylation - A Multi Laboratory Study

Dietmar Reusch

Roche Diagnostics GmbH, Penzberg, Germany

Immunoglobulin G (IgG) fragment crystallisable (Fc) glycosylation is crucial for antibody effector functions, such as antibody-dependent cellular cytotoxicity, and for their pharmacokinetic and pharmacodynamics behavior. To monitor the Fc-glycosylation in bioprocess development, as well as product characterization and release analytics, reliable techniques for glycosylation analysis are needed. A wide range of analytical methods has found its way into these applications. In this study, a comprehensive comparison was performed of separation-based methods for Fc-glycosylation profiling of an IgG biopharmaceutical. A therapeutic antibody reference material was analyzed 6-fold on two different days, and the methods were compared for precision, accuracy, throughput and other features; special emphasis was placed on the detection of sialic acid-containing glycans. Seven, non-mass spectrometric methods were compared; the methods utilized liquid chromatography-based UHPLC separation of fluorescent-labeled glycans, capillary electrophoresis-based separation of fluorescent-labeled glycans, or high-performance anion exchange chromatography with pulsed amperometric detection. HILIC-UHPLC of 2-aminobenzamide (2-AB)-labeled glycans was used as a reference method. All of the methods showed excellent precision and accuracy; some differences were observed, particularly with regard to the detection and quantitation of minor glycan species, such as sialylated glycans.

NOTES:
Exploratory and Targeted MS Approaches for Precise Monitoring of HCP Clearance Through DSP

Séverine Clavier

Sanofi, Vitry-sur-Seine, France

Host cell proteins (HCPs) are bioprocess-related impurities arising from death or secretion of cells used for monoclonal antibody (mAbs) production. Clearance of thousands of HCPs through downstream purification (DSP) is required to produce safe and stable therapeutic proteins. DSP of mAbs utilizes a series of orthogonal chromatographic steps, the first of which is most often a protein A capture step, followed by one or two polishing steps. Following purification, low residual levels of HCP impurities can remain in the final drug substance. As HCPs might represent potential safety risks for patients including immunogenicity, adjuvant activity, proteolytic activity and direct biological activity, it is necessary to identify and quantify HCPs along the purification steps. This represents a major analytical challenge due to the parts-per-million (ppm) sensitivity and high dynamic range required to detect a given HCP in the presence of the mAb product.

Following identification by an exploratory approach, quantification can be achieved with a targeted approach, by LC-MRM/PRM-MS on specific HCPs of interest, and help DSP team better understand and improve HCPs clearance. In Sanofi, an HCP profiling was performed for mAb samples collected at the different steps of the DSP. Based on this HCP profiling a list of “usual suspects” was built and labelled synthetic surrogate peptides were obtained in order to quantify the corresponding HCPs along DSP. Analytical strategies used for both the discovery and quantification phases will be described with a focus on the challenge of sensitivity for the detection of these low levels impurities.

NOTES:
Modernizing the Platform Characterization Peptide Map for Accurate Assessment of Deamidation and Isomerization by LC-MS/MS

Lisa Marzilli, Elaine Stephens, Shibu Philip, Michelle English, Jason Rouse

\textsuperscript{1}Pfizer, Inc., Andover, MA, USA, \textsuperscript{2}Pfizer, Inc., Pearl River, NY USA

Accurate evaluation of chemical modifications such as asparagine deamidation and aspartic acid isomerization is an essential component of comprehensive characterization of therapeutic monoclonal antibodies (mAbs). When located in the complementarity determining regions (CDRs), these modifications can cause a loss of function, impacting product efficacy and safety, resulting in the designation of the modification as a critical quality attribute. However, artifactual modifications can be introduced by analytical procedures, and distinguishing modifications as either critical quality attributes or method-induced artifacts is an important objective for product development. Conventional peptide mapping coupled with ultrahigh-resolution mass spectrometry offers advanced capabilities for definitive characterization of protein therapeutics. However, experimental conditions such as digestion time and pH can influence the observed level of chemical modifications, usually leading to over-estimation. In this work, a new peptide mapping method was developed specifically for mAb characterization that employs optimal enzyme pH for robustness, but short digestion times and time-course elements to minimize and monitor deamidation/isomerization, respectively, allowing a more accurate assessment of potential CDR sequence liabilities.

NOTES:
Is There a Need for a New Regulatory Pathway for Biologics?

Alan Fauconnier

Belgian Medicines Agency (FAGG-AFMPS), Brussels, Belgium

Recent and more ancient history of regulation has shown on several occasions that biological health products - or biotherapeutics – paved the way to novel regulatory processes. This was observed with vaccines and plasma derived medicinal products and more recently with ATMPs and biosimilars. Today, phage therapy biotherapeutics are witnessing a renewed interest. However, this reemerging therapy does not rekindle without raising sensitive regulatory concerns. Indeed, whereas the European regulatory framework has been basically implemented to tackle ready-to-use pharmaceuticals produced on a large scale, phage therapy relies on a dynamic approach requiring a regulation on patient-customised-medicine, nonexistent at present. A new regulatory framework, referred to as “Biological Master File” or BMF could be set up for phage therapy. Interestingly, the BMF concept could prove valuable in several other contexts, including the promising field of personalized-medicine.

NOTES:
In recent years within the pharmaceutical industry and also in our research activities related to pharmaceutical analysis, a remarkable shift from small to large molecules was noticed. Being on the market since 1982, protein biopharmaceuticals have seen an enormous growth in the last decade. A dominant role is thereby played by monoclonal antibodies (mAbs) which currently account for 50% of marketed biopharmaceuticals. The successes of mAbs have furthermore triggered the development of various next generation formats including antibody-drug conjugates (ADCs). ADCs might be the magic bullets referred to by Paul Ehrlich over 100 years ago.

mAbs have a complexity far exceeding that of small molecule drugs, hence, unraveling this complexity represents an analytical challenge. This challenge becomes even bigger in case ADCs are considered since the heterogeneity of the initial antibody is superimposed with the variability associated with the cytotoxic drug conjugation strategy. It is clear that analyzing these molecules demands for the best of both separation technologies (pressure and electro-driven) and mass spectrometry.

In this presentation, analytical platforms for full characterization and analysis of mAbs (originator and biosimilars) and ADCs will be presented and illustrated. Emphasis will be on the different HPLC formats (AC, IEC, SEC, HIC, HILIC, RPLC) applied in unidimensional and multidimensional (multiple heart-cutting (mLC-LC) and comprehensive (LC×LC)) mode combined with high resolution mass spectrometry.
Characterisation of Extractable and Leachables from Single Use Bioprocessing Solutions

Jonathan Bones, Noemi Dorival-Garcia, Sara Carillo, Christine Ta

NIBRT, Dublin, Ireland

Single use bags (SUBs) are increasingly being used for mammalian cell bioprocessing and represent considerable advantages over traditional stainless steel vessels, namely elimination of cleaning validation requirements and faster facility start up and batch-to-batch turnaround. Despite these attractive features, a concern exists that certain compounds may be extracted from or may leach out of the plastic container into the cell culture media, so called extractable and leachables (E&Ls), and that these compounds may adversely affect the cell culture. One particular leachable, bis(2,4-di-tert-butylphenyl) phosphate (bDtBPP), was shown to be cytotoxic to Chinese hamster ovary (CHO) cells, even at parts per billion concentrations. A pressing need therefore exists for in depth methods for the sample preparation and analytical characterisation of E&Ls.

Here, we optimised the extraction conditions for E&Ls using a design of experiments approach. Mixtures of isopropanol water for found to be optimum for broad scale extraction of E&Ls. Sample suppression effects were noted during the analysis of leachables. However, the inclusion of a dispersive liquid-liquid microextraction step within the workflow resulted in sample clean up and removal of the matrix effect. Using our optimised workflow, we applied the platform to the characterisation of E&Ls present in 34 SUBs spanning different manufacturers and different brands from specific manufacturers. To ensure deep characterisation of potential E&Ls present headspace GC analysis was performed for residual solvents, high resolution accurate mass GC-MS for semi-volatiles on the Q-Exactive GC Orbitrap platform, high resolution accurate mass LC-MS for non-volatiles and trace element analysis by inductively coupled plasma mass spectrometry were performed. Resulting data was annotated based on accurate mass and high resolution MS/MS based data combined with library searching. Analysis of the resulting data using multivariate statistics revealed strong correlations with bag age and the type of polymeric material of the innermost fluid contact layer.

NOTES:
New Trends in SE-UHPLC

Jennifer Rea

Genentech, a Member of the Roche Group, South San Francisco, CA USA

Size exclusion chromatography (SEC) is widely used in the biopharmaceutical industry for routine characterization and quality control of aggregates in protein therapeutics. Aggregation is a carefully monitored quality attribute from the earliest stages of clinical development owing to the possibility of eliciting an immunogenic response in the patient. Size Exclusion High Performance Liquid Chromatography (SE-HPLC) has been used for molecular size distribution analysis of proteins for decades, although more recently, Size Exclusion Ultra High Performance Liquid Chromatography (SE-UHPLC) columns, containing sub-2-µm porous particles, have been developed for biopharmaceutical analysis. This presentation will review recent applications of SE-UHPLC from routine characterization to novel applications in the biopharmaceutical industry.

NOTES:
HILIC-MS: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies

Valentina D'Atri¹, Szabolcs Fekete¹, Alain Beck², Jean-Luc Veuthey¹, Davy Guillarme¹

¹University of Geneva, Geneva, Switzerland, ²Centre d'Immunologie Pierre Fabre, St Julien-en-Genevois, France

Monoclonal Antibodies (mAbs) represent the largest and fastest growing category of biopharmaceutical proteins. They are manufactured through recombinant DNA technology, custom-designed to target chosen antigens, and employed for a wide range of applications, particularly in cancer, immune disorder, and infectious disease. In addition, because several commercial mAbs are approaching patent expiry, a market for so-called biosimilars is becoming increasingly attractive and is accordingly undergoing rapid growth.

The approval processes of biosimilar mAbs depend on their comparability to originators in terms of product quality and safety. Therefore, sophisticated analytical comparisons are required to assess structural features and post-translational modifications (PTM) and thereby minimize the possible risks of clinically meaningful differences between biosimilars and originators mAbs.

The glycosylation pattern of mAbs is considered to be an important critical quality attribute (CQA), and several analytical approaches have been proposed for monitoring the glycosylation profile, albeit mainly at a glycan and glycopeptide level of analysis. In this study, we demonstrate the utility of hydrophilic interaction chromatography (HILIC) hyphenated with mass spectrometry (MS) for the qualitative profiling of glycosylation patterns at the protein level, by comparing originator and biosimilars mAbs (Remicade®-Remsina®-Inflectra®, Herceptin®-Trastuzumab B, and Erbitux®-Cetuximab B). For this purpose, a middle-up approach, consisting of digestion and reduction of the mAbs to obtain sub-units ranging from 25 to 50 kDa, was applied.

The ability of HILIC to resolve hydrophilic variants of protein biopharmaceuticals will be demonstrated. Moreover, the complementarity of HILIC to reversed phase liquid chromatography (RPLC), and its hyphenation to MS will be illustrated, to prove that HILIC-MS can be considered as an innovative and powerful analytical tool for the comparison of originator and biosimilar therapeutic mAbs.

NOTES:
Understanding the Structure-function Relationship Between Disulfide Bridging and Potency in Etanercept

Robert Mayer

Sandoz GmbH, Kundl, Austria

GP2015 – an approved biosimilar product to the reference product Enbrel® – consists of 934 amino acids and comprises a homodimer of the extracellular ligand-binding portion of human tumor necrosis factor receptor (p75) linked to the Fc portion of an human IgG1 as the reference molecule does.

The GP2015 manufacturing process was designed to deliver a product with a bioactivity well within the overall distribution of Enbrel® batches. As all Enbrel® batches were sourced from the markets and represent released product, the measured overall distribution in bioactivity defined the range of acceptable bioactivity for the biosimilar product GP2015. Notably, recent batches of Enbrel with expiry dates from 2012 onwards showed a bioactivity in the lower half of the overall distribution, which triggered further investigations. The lower activity as measured by the TNF-α reporter gene assay was found to correlate with higher amounts of wrongly bridged variants (WBV). Within this talk, the structure-function relationship impacting the bioactivity of etanercept will be elucidated in more detail, as an extensive understanding thereof was crucial for the approval of GP2015 as first biosimilar to Enbrel® in the US.

NOTES:
Analysis of Influenza Antigens: Sequence, Concentration and Glycans

Terry Cyr, Lisa Walrond, Yi-Min She, Marybeth Creskey, Aaron Farnsworth, Sean Li

Health Canada, Ottawa, ON Canada

Influenza vaccines are very widely used to prevent or mitigate symptoms from the annual circulating influenza virus strains. There are three or four virus strains that are targeted: two HA stains and either one or two HB stains. The current means to control the amount of the key antigen is by single radial immune-diffusion (SRID). This method relies on the comparison of a Reference antigen to each vaccine lot using the relative radius of interaction in an antiserum for each Reference HA or HB versus sample HA or HB. This method normally works very well, but it requires the Reference antigen and the antiserum to be prepared and calibrated. We, and others, have been developing alternative methods by a variety of means. In this presentation, I will focus on our recent work using mass spectrometry with and without labels. The Hi-3 method is compared to the various means of generating labelled peptides. One key limitation is that the peptide sequence must be unique after trypsin digestion of the antigen. Practical advantages and limitations of the various approaches will be discussed.

NOTES:
Technical Seminar

Wednesdays, 15 March 2017
14:15 – 14:45
Royal Ballroom
Sponsored by Bruker Daltonik

Accelerating Biotherapeutics Characterization with the new Biopharma Compass 2.0 Software

Peter Hufnagel¹, Guillaume Tremintin², Romano Hebeler¹, Detlev Suckau¹, Jason S. Wood³

¹Bruker Daltonik, Bremen, Germany, ²Bruker Daltonics, Fremont, CA USA, ³Bruker Daltonics, Billerica, MA USA

Attendees of this technology seminar will hear about the new Biopharma Compass 2.0 Software. Biopharma Compass 2.0 combines the data acquisition, data analysis and results reporting of the most important biopharma workflows including the following:

- Intact mass screening of proteins, antibodies or antibody fragments for sequence errors and post-translational modification (PTM) profiles
- Fast and specific Top-down and middle-down sequence analysis of large proteins with both ESI-UHR-QTOF and MALDI-TOF/TOF for sequence confirmation, and to confirm protein N- or C-terminal modifications
- Peptide screening with routine PTM and artifact quantification with highest dynamic range, fully integrating LC-UV, MS, and MS/MS data in predefined report formats.

Biopharma Compass 2.0 utilizes Bruker's industry-leading SNAP II algorithm, yielding reliable monoisotopic mass determination on Bruker’s ultrahigh-resolution time-of-flight hardware with sub-ppm mass accuracy, up to the heavy chain of antibodies. It comes with a single, integrated user interface and has the tools onboard to help you bring 21 CFR part 11 compliance to your laboratory.

NOTES:
Thursday, 16 March 2017  
11:35 – 12:05  
Royal Ballroom  
Sponsored by SCIEX Separations

Fast Glycan Labelling and Analysis: High-Resolution Separation and Identification in Minutes

András Guttman

*SCIEX, Brea, CA USA*

There is a growing demand in the biopharmaceutical industry for rapid N-glycosylation profiling of therapeutic antibodies. The need is greatest in bioprocessing where cell culture development and clone selection require rapid analysis of hundreds of samples to accelerate products through the pipeline.

We recently developed a fast glycan labeling and analysis approach using a novel magnetic bead-mediated process and a CE gel-buffer separation system to quickly profile glycoprotein N-linked carbohydrates. Glycan release, fluorophore labeling and clean-up parameters were optimized, resulting in a 60-min sample preparation. The rapid CE-LIF separation gave excellent yield, high reproducibility, supported large scale 96-well plate sample processing and was easy to automate.

A triple-internal standard glycoinformatics method* removed the need to run the maltooligosaccharide ladder. This significantly speeded up the glycoinformatics tasks and enabled precise glucose unit (GU) assignment of human IgG glycans (relative standard deviation ≤1.07%).

* Patent pending

Charge Heterogeneity Analysis of Intact Monoclonal Antibodies Using CESI-MS

Steve Lock

*SCIEX, Pudsey, United Kingdom*

Therapeutic mAbs can exhibit significant micro-heterogeneity due to the numerous post-translational modifications (PTMs), sequence variants, and degradation products that occur during production and storage.

Integrating CE and electrospray ionization (ESI) into one dynamic process (CESI-MS) simplifies coupling CE with MS. In this work, we demonstrate how CESI-MS can be used for the comprehensive, rapid and robust characterization of intact mAb charge variants.

By coupling CESI with a QTOF-MS we were able to achieve high quality, reproducible mass spectra for the intact mAbs. Mass spectral deconvolution of each peak revealed that charge variants were attributed to mass shifts of as little as +1 Da, possibly from known asparagine deamination. This work has shown the ability of CESI-MS to resolve and characterize acidic charge variants.

NOTES:
At Novozymes we produce a wide range of industrial enzymes and microorganisms. Enzymes are proteins, and in nature they initiate biochemical reactions in all living organisms. It is enzymes that convert the food in our stomachs to energy and turn the falling leaves in the forest to compost. Novozymes finds enzymes in nature and optimizes them for use in industry. In industry, enzymes replace chemicals and accelerate production processes. They help our customers make more from less, while saving energy and generating less waste. Enzymes are widely used in laundry and dishwashing detergents and to improve the quality of bread, beer and wine, or increase the nutritional value of animal feed. Enzymes are also used in the production of biofuels where they turn starch or cellulose from biomass into sugars which can be fermented to ethanol.

Mass spectrometry (MS) is a key technology for characterizing the sequence, structure and function of enzymes. Before mass spectrometry data can be transformed into innovation, the raw instrument code needs to be translated from machine language into protein sequence. This is typically done using proprietary software developed by the instrument vendor, a peptide search engine such as Mascot if doing bottom-up proteomics, or one of an ever-growing suite of open-source dedicated software developed by academia. At the Novozymes Mass Spectrometry core facility in Bagsværd, Denmark we have for the past two years used Genedata Expressionist® as a platform for all MS data analysis. In this presentation I will demonstrate the versatility of the Genedata Expressionist platform for MS data analysis across some of our industries, from simple protein identification using classical SDS-Page in-gel analysis, through intact protein top-down sequencing, to more complex bottom-up proteomics experiments of host cell proteins and the influence of dietary interventions on endogenous proteins in chickens.
Best of Both Worlds: Combining Quantitative Targeted Proteomics with High-content Discovery Proteomics

Florian Marty, Karel Novy

Biognosys AG, Zurich, Switzerland

Biognosys is the leading proteomics company offering services and tools for highly multiplexed protein quantification from any species in any biological matrix to facilitate translational research. Equipped with the newest LC-MS/MS systems for discovery and quantitative proteomics the R&D department of Biognosys strives for improvements in all aspects of proteomics. Our landmark discovery proteomics platform allows unbiased quantification of up to 9’000 proteins per sample, while up to 150 pre-defined proteins can be analyzed in a high-throughput mode in thousands of samples using our targeted proteomics approach.

In the upcoming technical seminar we will present an overview of both platforms and its implementation into our service and product solutions. Furthermore, we will show a new approach that combines the quantitative power of stable isotope-labelled (SIS) reference peptides with the discovery potential of Biognosys’ data independent acquisition (DIA) based workflow called Hyper Reaction Monitoring (HRM). Using the reference peptides from our PlasmaDive and PlasmaDeepDive multiplexed panels we have obtained absolute quantitative information of 173 proteins in un-depleted human plasma. Additionally, over 400 plasma proteins were identified in a single shot HRM experiment. These proteins can be quantified over a large cohort with high reproducibility to obtain relative quantitative information. The quantification values obtained for the SIS peptides can be used to extrapolate the absolute quantitative values of all proteins identified in the HRM experiment. This principle can be applied to any predefined panel of identified biomarkers for any sample type.

NOTES:
Round Robin Discussions

Wednesday, 15 March, 2017
16:45 – 17:45

Round Robin Discussions should be active discussions, not presentations or lectures. There are eleven topics. Participation will be on a first come, first serve basis. Each topic will include a facilitator, whose role is to help assist the discussion and ensure a lively exchange, and a scribe, whose role is to make general, anonymous notes about the discussion. Notes will be posted on the CASSS website two weeks after the conference.

Listed below is a quick view of the roundtable topics, facilitators and scribes.

Table 1  Emerging Technologies in Protein Characterization, including High Throughput Analysis
Pierre Allemand, Protien Metrics, Inc.
Lars Geurink, Janssen Infectious Diseases and Vaccines

Table 2  New Trends in Analytical Technologies for Process Control (USP, DSP, fill and finish), Including Analysis of Process-related Impurities
Helen Nyhlen, Genovis AB
Harold Taylor, Merz Pharmaceuticals GmbH

Table 3  Method Lifecycle Management - From Early Development to Method Transfer and Maintenance
Richard Shannon, MedImmune Limited
Markus Wild, F. Hoffmann - La Roche Ltd.

Table 4  Role of Analytics in Understanding Critical Quality Attributes
Anders Lund, Sanofi
Cari Sänger-van de Griend, Kantisto BV

Table 5  New Technologies for Analysis of HCPs
Séverine Clavier, Sanofi
Thomas Flad, Protagen Protein Services GmbH

Table 6  Analytical Technologies for Formulation Development of Biopharmaceuticals
Christof Finkler, F. Hoffmann - La Roche Ltd.
Marta Germano, Janssen Infectious Diseases and Vaccines
Table 7  HOS Methods - Capabilities and Limitations
András Guttman, University of Pannonia
David Keire, CDER, FDA

Table 8  New Trends in Mass Spec
Lisa Marzilli, Pfizer, Inc.
Stephen Lock, SCIEX

Table 9  Comparability
Terry Cyr, Health Canada
Anne Trappe, NIBRT

Table 10  Developments in Analytical Strategies for Bispecifics, ADCs, or New Formats
Virginie Bettonville, University of Liege
Jennifer Rea, Genentech, a Member of the Roche Group

Table 11  The Need for Regulatory Guidance on Product Characterization
Martin Blüggel, Protagen Protein Services GmbH
Birgit Schmauser, BfArM, Federal Institute for Drugs and Medical Devices
Round Robin Discussions should be active discussions, not presentations or lectures. There are eleven topics. Participation **will be on a first come, first serve basis**. Each topic will include a facilitator, whose role is to help assist the discussion and ensure a lively exchange, and a scribe, whose role is to make general, anonymous notes about the discussion. Notes will be posted on the CASSS website two weeks after the conference.

Listed below is a quick view of the roundtable topics. See addendum for facilitators and scribes.

**Table 1**  Emerging Technologies in Protein Characterization, including High Throughput Analysis  
Henrik Jensen, *University of Copenhagen*  
Cari Sänger-van de Griend, *Kantisto BV*

**Table 2**  New Trends in Analytical Technologies for Process Control (USP, DSP, fill and finish), Including Analysis of Process-related Impurities  
Arnaud Delobel, *Quality Assistance s.a.*  
Lars Geurink, *Janssen Infectious Diseases and Vaccines*

**Table 3**  Method Lifecycle Management - From Early Development to Method Transfer and Maintenance  
Robert Mayer, *Sandoz GmbH*  
Dietmar Strehlow, *Wacker Biotech GmbH*

**Table 4**  Role of Analytics in Understanding Critical Quality Attributes  
András Guttman, *University of Pannonia*  
Jim Thorn, *SCIEX Separations*

**Table 5**  New Technologies for Analysis of HCPs  
Séverine Clavier, *Sanofi*  
Thomas Flad, *Protagen Protien Services GmbH*

**Table 6**  Analytical Technologies for Formulation Development of Biopharmaceuticals  
Harold Taylor, *Merz Pharmaceuticals GmbH*

**Table 7**  HOS Methods - Capabilities and Limitations  
Gábor Járvás, *University of Pannonia*  
Birgi Schmauser, *BfArM, Federal Institute for Drugs and Medical Devices*
Table 8  New Trends in Mass Spec
Valentina D’Atri, *University of Geneva*
Hansjörg Toll, *Sandoz GmbH*

Table 9  Comparability
Martin Blüggel, *Protagen Protein Services GmbH*
Robert Mayer, *Sandoz GmbH*

Table 10  Developments in Analytical Strategies for Bispecifics, ADCs, or New Formats
Julie Bonvin, *F. Hoffmann - La Roche Ltd.*
Cédric Delvaux, *University of Liege*

Table 11  The Need for Regulatory Guidance on Product Characterization
Ashraf Ali, *Inha University*
Marta Germano, *Janssen Infectious Diseases and Vaccines*
Analysis of Sialylated N-Glycans using an Optimized HILIC-FLR-MS Method

Guillaume Bechade¹, Qi Wang², Matthew Lauber²

¹Waters Corporation, Baden Dättwil, Switzerland, ²Waters Corporation, Milford, MA USA

In protein therapeutics, sialic acid residues are of interest because they can impact serum half-lives and they can serve as indicators of cell culture conditions. N-glycolylneuraminic acid, a non-human mammalian sialic acid with a structure similar to the human sialic acid N-acetylneuraminic acid, can contaminate biotherapeutic glycoproteins via production in non-human cell lines or from animal-derived media, which will result in different levels of immune responses in humans. Accordingly, sialylated glycan profiling is often needed in order to monitor a critical quality attribute related either to drug stability, activity, or immunogenicity. Due to the lability of sialic acid residues, accurate mass spectrometric analysis of sialylated glycans has sometimes been elusive or required neutralization of the sialic acids. However, with rapid release and labeling of N-glycans with a labeling reagent known as RapFluor-MS, it is now possible to obtain enhanced sensitivity for fluorescence (FLR) and mass spectrometry (MS) based detection of all types of N-glycan species, including those that are sialylated.

In this work, we have applied hydrophilic interaction chromatography (HILIC)-FLR-MS analyses to the study of RapFluor-MS labeled sialylated N-glycans. A wide-pore (300 Å) amide-bonded, sub-2 μm HILIC stationary phase was used with mobile phases having optimized ionic strength to improve the chromatographic resolution of sialylated N-glycans. Combined with optimized electrospray ionization (ESI) source parameters and acquisition settings, a high-resolution separation was achieved along with improved MS sensitivity and quality. Moreover, within this work, it has been shown that these methods can be effectively combined with neuraminidase digestion to perform a cursory level sialic acid linkage analysis.

NOTES:
Production of recombinant proteins requires the use of a host organism to produce proteins using recombinant DNA; in modern biopharmaceutical facilities, the most commonly used host organisms are bacteria or mammalian cells. Mammalian cells are preferred over bacterial cells for the production of many therapeutic proteins as they produce human-like post-translational modifications. Chinese hamster ovary (CHO) cell lines are widely used due to an established regulatory track record, their ability to grow in suspension culture under chemically defined media conditions and their safety record. CHO cells produce human like glycosylation, however, also some non-human epitopes. One of the disadvantages related to CHO cell lines usage is that protein glycosylation may vary from lot to lot based upon to slight changes in environment conditions encountered by cells during the bioprocess.

The characterization of cellular glycome is technically demanding as differences in the composition can change the analytical approach needed to identify high and low abundant structures. Deciphering all these glycan structures is important to understand the glycosylation pattern of produced biopharmaceuticals.

The glycome of CHO cells has been recently investigated. The ability of CHO cells to produce large and complex glycans was previously shown using MALDI-ToF mass spectrometry. However, no in-depth studies to characterise the glycosylation present on CHO cells have been undertaken.

In this study, glycans from IgG1 expressing DP-12 CHO cells have been obtained following cell culture in chemically defined media. The cells were collected by centrifugation and the glycans from the total cell lysate were fluorescently labelled and analysed. The complex N-glycome has been analysed with two-dimensional WAX × HILIC liquid chromatographic strategy hyphenated to ESI-MS. Using this deep glycomic approach an excess of 200 glycans have been identified, each displaying unique structures with a potentially important role in cellular function and ultimately biopharmaceutical quality and stability.

NOTES:
Evaluating Structure-Function Relationships of Biosimilars with an Orthogonal Approach to Analytics

Martin De Cecco, Stuart Wright, Laura Munro, Catriona Thomson

Sartorius Stedim BioOutsource, Glasgow, United Kingdom

The biggest challenge in developing a biosimilar monoclonal antibody is to comprehensively characterise the molecule’s structure and function in order to demonstrate comparability to the innovator. State-of-the-art, orthogonal methods are necessary to obtain a ‘totality of evidence’ data package required for regulatory acceptance. Given the complexity and diversity of glycosylation, a key part of this involves understanding the functional significance of different glycan structures.

In this case study, we have explored the impact of glycan variation on antibody function by comparison of a candidate biosimilar to the innovator molecule. Orthogonal methods, including intact mass and released N-glycan analysis, were used to assess antibody glycosylation. By combining these data with those from binding and functional assays, we were able to measure the effect of structural differences on ADCC and CDC activity.

The data presented highlight the benefits of carrying out relevant structural and functional analysis at each stage of biosimilar development and the positive impact this can have on the progress of a biosimilar development program. This analytical approach provides biosimilar developers with a comprehensive testing package that aims to provide a totality of evidence fit for regulatory submissions.

NOTES:
P-104-T

State-of-the art LC/MS Methods Applied to the Characterization of a Highly-glycosylated Fusion Protein: Etanercept

Arnaud Delobel, Eric Largy, Fabrice Cantais

Quality Assistance s.a., Donstiennes, Belgium

A large number of pharmaceuticals are glycosylated proteins, including monoclonal antibodies (mAbs) and other recombinant proteins (fusion proteins, cytokines, growth factors). An adequate glycosylation is critical for therapeutic glycoproteins in terms of safety, bioactivity, solubility, stability, and pharmacokinetics and dynamics. Consequently, the glycosylation profile of therapeutic glycoproteins must be thoroughly analysed. However, these proteins are typically produced in different expression systems, whose glycosylation machineries function through sequential and competitive steps, hence creating micro- (glycans nature for a given site) and macro heterogeneities (number and location of sites) of glycosylation. This creates a challenging analytical puzzle that requires a number of orthogonal analytical techniques, at different levels of analysis (released glycans, peptides, intact and subunits), to be solved.

We will present the use of mass spectrometry to characterise both the N- and O-glycosylation of Etanercept, a tumor necrosis factor-a (TNFa) antagonist, commercialised as Enbrel®. It is produced by recombinant DNA technology as a fully human, dimeric fusion protein, each monomer consisting of a TNFa-receptor linked to an Fc/2 of IgG1 (minus its CH1 domain). Etanercept glycosylation accounts for roughly a third of the protein’s apparent molecular weight (150 kDa).

The methods used for this complete characterisation were:

- N-glycans profiling by UPLC/FLR/MS using RapiFluor-MS labelling
- O-glycans profiling by LC/MS using a porous graphitic carbon column
- Sialylation profiling by mixed-mode chromatography with fluorescence detection
- Sialylation quantification by RP-UPLC/FLR
- Site-specific N-glycans profiling by HILIC-ESI-QTOF/MS
- Determination of O-glycosylation sites by RP-UPLC-ESI/MS with ETD fragmentation
- Characterisation at the subunit level using Widepore HILIC ESI-QTOF/MS

The combination of all these methods allowed a full characterisation of Etanercept N- and O-glycosylation.

NOTES:
Analysis of Highly Sialylated and Low Input Glycoprotein Samples on the GlycanAssure™ System

Parita Ghia

Thermo Fisher Scientific, South San Francisco, CA USA

In this study, we evaluated APTS-labeling conditions on highly sialylated glycans, specifically Fetuin. Furthermore, we also tested 3500xl CE detection capability for analysis of glycans at low sample inputs and labeling with APTS dye. Results reinforce the robustness of the GlycanAssure workflow, even with shorter labeling time and low protein inputs. The 3500xl CE platform achieves quantitative, high resolution glycan analysis, with high-sensitivity and high-reproducibility.

NOTES:
Fast Glycan Labeling and Analysis: High-Resolution Separation and Identification in Minutes

András Guttman¹, Marton Szigeti², Anna Lou¹, Mervin Gutierrez¹

¹SCIEX, Brea, CA, USA, ²University of Debrecen, Debrecen, Hungary

There is a growing demand in the biopharmaceutical industry for rapid N-glycosylation profiling of carbohydrates associated with therapeutic antibodies in all phases of biologics development. Glycan identification historically has been a time-consuming method. Liquid phase and gel based glycanalytical methods (e.g., HPLC, UHPLC, CE, and PAGE) generally require labor intensive, time consuming sample preparation including glycan release, purification, fluorophore labeling and pre-concentration prior to analysis. In addition, numerous centrifugation and vacuum-centrifugation steps were necessary - making full automation of such protocols complex and expensive. This poster demonstrates the optimization of all steps using a rapid magnetic bead based protocol. Modifications include rapid endoglycosidase digestion and fluorophore labeling reaction time, optimized labeling temperature and rapid Capillary Electrophoresis (CE) separation. The procedure can be performed for as few as a single sample to as many as 96 samples at a time using automated liquid handling platforms without the need for centrifugation or vacuum centrifugation, commonly used in other glycan sample preparation methods. Capillary electrophoresis separation with laser-induced fluorescence detection (CE-LIF) of the fluorophore labeled glycans was also optimized to provide fast and high resolution separations of the labeled sugars in 5 minutes, enabling rapid analysis after both the manual and automated sample preparation processes.

NOTES:
High Resolution Glycan Analysis by Temperature Gradient Capillary Electrophoresis

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Temperature gradient capillary electrophoresis was introduced to enhance separation selectivities for branched glycans of biotherapeutic interest. A mixture of afucosylated, fucosylated and high mannose oligosaccharides was separated in the range of 15°C to 45°C at 5°C temperature intervals. It was found that within this temperature range the separation selectivity was carbohydrate structure dependent. The resolution between some glycan structures was greater at elevated temperatures, while others separated better at lower temperatures. More interestingly, the temperature of resolution maximum was different for most structures. The components of a nine-glycan mixture showed resolution maximums at different temperatures, implying the applicability of a temperature gradient to enhance their capillary electrophoresis separation. We suggest that the same activation energy requirement concept plays a role here that was reportedly associated with the earlier observed electromigration differences between linear and branched oligosaccharide structures. Thus, temperature gradient capillary electrophoresis can open up a new chapter in high resolution glycan analysis. Our results also emphasized the high importance of tight temperature control to provide the option of setting up an appropriate temperature gradient during capillary electrophoresis separation of complex carbohydrates. Based on these encouraging results, in the future we plan to investigate the effect of temperature on the kinetic and transport properties in the capillary to further enhance separation selectivities.

NOTES:
Automated Online Monitoring and Quality Control of Glycan Profiles from Various Antibodies Using FabRICATOR Digestion and Subunit Separation in Combination with Ultrahigh-Resolution QTOF Mass Spectrometric Analysis and the Latest Biopharma Software Tools

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The industrial manufacturing of a monoclonal antibody is generally preceded by a process developing phase that can take several years to complete. During this work, it is crucial to achieve sufficient understanding of the design space and its impact on the drug substance specifications. Software and analytical tools that can facilitate the fast and accurate acquisition of this knowledge are required to save valuable downstream resources.

High-resolution mass spectrometry is a powerful tool for the monitoring of quality attributes related to a typical bioproduction process. This, due to its high sensitivity, specificity and exceedingly short assay time. However, for mass spectrometry to reach full compatibility with the production environment, a robust, integrated analytical platform (including easy to use, open access software) is required, which provides automated online sampling, sample handling and enrichment of the target molecule as well as instant data evaluation.

In this work, we present a standalone analytical platform developed for the direct connection to a bioreactor with subsequent high-resolution mass spectrometric detection (including the latest in Biopharma software), for real time, at-line monitoring, of monoclonal antibody quality.

NOTES:
P-109-W

The Application of a Novel Mass Detector in Biopharmaceutical Glycan Analysis

Mark Hilliard¹, Sinead Hallinan Hallinan¹, William Alley², Ying Qing Yu², Pauline Rudd¹

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Oligosaccharides are attached to many therapeutic proteins and influence both protein structure and function. In order to elucidate their specific roles, it is necessary to characterize glycans with as much detail as possible, in terms of monosaccharide composition and linkage. Using traditional methods, complete glycan analysis is analytically very challenging. In this presentation, we discuss improved methods for analysing N- and O-linked glycans, which require different approaches due their unique chemistries.

In collaboration with Waters Corporation, we describe a method for N-linked glycan analysis based on a novel fluorescent label, RapiFluor-MS (RFMS), which enables UPLC mass detection using a QDa. In this experimental design, the 1:1 stereospecific fluorescent labelling of the glycans with RFMS allows for relative quantitation. Additionally, the ability to incorporate mass detection into UPLC-HILIC-FLR platforms for glycan analyses has proven to be very advantageous. Here, mass data was used to confirm of glucose unit (GU) based assignments from the FLR data. To demonstrate that the QDa is compatible with our standard N-linked glycan methods, we have analysed the N-linked glycans derived from Herceptin. We demonstrate the ability to collect GU values and analyse the products of exoglycosidase digestion arrays with the QDa system.

The QDa also enabled’s studies of O-linked glycans, which are typically more challenging to analyse than N-linked glycans, in part because the diversity of the core structures and the lack of a universal O-glycanase. Researchers typically employ chemical techniques for O-linked glycan release. Here, we present an O-linked glycan workflow in which the release was microwave-assisted and reduced the number of degradation products routinely associated with chemical releases. The released glycans were then fluorescently labelled and analysed by UPLC coupled with QDa mass detection. This workflow has facilitated the generation of GU data collected from the fluorescent channel as well as orthogonal mass confirmation.

NOTES:
Implementation of the Triple-Internal Standard Based Glycan Structure Assignment Method in High-Throughput Environment

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In spite of the continuously increasing use of capillary electrophoresis methods for glycosylation analysis in the biopharmaceutical industry, data interpretation methods are lagging behind. In this poster, we show the design and implementation of a co-injected triple-internal standard method to alleviate the need of an accompanying run of the maltooligosaccharide ladder for glucose unit (GU) calculation and concomitant structural interpretation. Based on the migration times of the three coinjected internal standards of maltose, maltotriose, and maltopentadecaose (bracketing the peaks of interest), a data processing approach was designed and developed to establish a virtual ladder that was used for GU calculation. Data processing was tested in terms of the calculated GU values of human IgG glycans, and the resulting relative standard deviation was ≤ 1.07%. As an additional benefit, the maltotriose also acted as an internal labeling standard and in this way provided valuable information about the efficiency of the derivatization reaction. The triple-internal standard method was integrated in a commercial software package (32 Karat) and proved to be system independent as it overcame the bottleneck of the traditional GU calculation approach, which was sensitive to various experimental conditions. Thanks to the fact that besides the temperature, most separation conditions did not influence the calculated GU values, the implemented method fulfilled high-throughput requirements, thus should be of broad industrial and academic interest.

NOTES:
N-Glycan Analysis by Orthogonal Methods: UHPLC and Multi-Capillary CE

Baburaj Kunnummal

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Sample preparation for glycoprofiling of glycoproteins has been a cumbersome, multi-day process including overnight labeling, and multiple lengthy centrifugation and vacuum drying steps. Most sample preparation protocols also use toxic reagents during the dye labeling step of glycans. Traditionally single capillary electrophoresis (CE) and liquid chromatography (LC) have been two of the most common methods used to profile N-glycans, with an average analytical time of 15-35 min for CE, and 60 min for high resolution LC. We have improved the glycoprofiling process at several levels, enabling short analysis time by highly sensitive multi-capillary CE. In addition, we have reduced overall workflow duration, hands on time, and eliminated centrifugation and vacuum drying steps. Experiments presented utilize 8-aminopyrene-1,3,6-trisulfonic acid (APTS); a well-recognized dye in regulated environments. Here we present the comparability of N-Glycan profiles of human IgG prepared with GlycanAssure™ assay kit reagents on two high performance systems—the Applied Biosystems™ CE3500xL protein quality analyzer, capable of analyzing 24 samples simultaneously, and ultra-high performance liquid chromatography (UHPLC). Utilizing this workflow, we demonstrate strong similarity of N-glycan profiles between the two platforms with low %RSD of ~5% for the entire sample preparation and analysis process.
Structural and Physicochemical Characterization of Idursulfase Beta

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Green Cross, Youngin-si, Gyeonggi-do, South Korea

Mucopolysaccharidosis type II (MPS II, Hunter syndrome; OMIM 309900) is an X-linked lysosomal storage disease caused by a deficiency of the enzyme iduronate-2-sulfatase (IDS), leading to accumulation of glycosaminoglycans (GAGs). For enzyme replacement therapy (ERT) of Hunter syndrome, two recombinant enzymes, idursulfase (Elaprase, Shire Human Genetic Therapies) and idursulfase beta (Hunterase, Green Cross Corporation), are currently available.

This study investigated the structural and physicochemical properties of idursulfase beta as part of its characterization. The results showed that idursulfase beta consisted of 525 amino acids and two disulfide bridges (Cys146-Cys159, Cys397-Cys407) and that the cysteine residue at position 59 was converted considerably to formylglycine, the key catalytic residue in the active site. Moreover, idursulfase beta contained various glycan structures such as fucosylated or afucosylated complex type (bi-, tri- and tetra-antennary structures), high-mannose type, and hybrid type. Particularly, considerable amounts of sialylated and phosphorylated glycans were detected, which contributed to the acidic pI characteristic of idursulfase beta.

The structural and physicochemical characterization of idursulfase beta may help to understand and reduce the risk associated with product quality.

NOTES:
Development of a 5-Minute Deglycosylation Method and Instant Labeling Dye for High-throughput N-Glycan Analysis by Mass Spectrometry

Michael Kimzey, Aled Jones, John Yan, Vaishali Sharma, Andres Guerrero, Alexander Gyenes, Justin Hyche, Emily Dale, Ted Haxo, Sergey Vlasenko

ProZyme, Inc, Hayward, CA USA

The structure of N-linked glycans can play a critical role in the pharmacology of therapeutic proteins, potentially affecting immunogenicity, pharmacokinetics and pharmacodynamics. This makes the characterization of N-glycans an essential part of the biotherapeutic development process. Analysis of N-glycans typically involve the labeling of enzymatically-released glycans with a tag to allow for fluorescence detection; a process that often requires numerous hours or days to complete. In addition to fluorescence (FLR) detection, mass spectrometry (MS) is also often utilized. Unfortunately, many of the commonly-used fluorescent tags are limited with regard to MS sensitivity.

We present a rapid N-glycan sample preparation workflow which uses a 5-minute in-solution digestion, instant labeling, and cleanup of excess label and denaturant prior to analysis. The workflow can be completed in as little as 45 minutes and includes InstantPC, a new instant glycan labeling reagent that provides markedly increased MS and FLR sensitivity. InstantPC-labeled N-glycans are suitable for hydrophilic interaction liquid chromatography (HILIC) utilizing both FLR and MS detection allowing flexibility for screening applications as well as in-depth characterization of N-glycans.

NOTES:
An Integrated System for High-throughput, User-friendly N-Glycan Analysis Using Rapid Separation by Capillary Electrophoresis

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Glycan characterization is becoming necessary in the earliest stages of biotherapeutic cell line development, to the point where cell culture screening often requires glycan profiling. This entails significantly increased throughput for sample preparation, analytical instrumentation, data processing and expertise in glycan characterization. Unfortunately, these factors can cause a bottleneck to results.

We present a glycan analysis solution that provides rapid sample preparation and analysis combined with a simplified data processing approach. The sample preparation includes a 5-minute deglycosylation step to release N-glycans, followed by glycan labeling and cleanup, and may be completed in under 1 hour. Labeled N-glycans are separated using a small and user-friendly capillary electrophoresis (CE) instrument, with a run time of 2 minutes per sample. Using custom software, glycan migration times are processed into glucose unit (GU values), and compared against a library of known N-glycan GU values. The relative percent area for each peak is automatically calculated. This process enables relative N-glycan quantification for up to 96 cell culture samples within a single workday.

NOTES:
Monoclonal antibodies (mAbs) and their related products require extensive characterization. Accurate mass determination is a challenging step in the analytical characterization of antibodies because of their large size and the presence of post-translational modifications such as glycosylation. These characteristics also make determining the location of modifications difficult. To overcome the challenges associated with antibody mass determination, a number of complementary approaches are typically used. Antibodies can be treated with PGNase F to remove the N-Glycans, digested with proteases such as IdeS to generate antibody fragments, or reduced to generate light and heavy chains prior to measuring the mass. Various combinations of the above approaches can also be used. Here, we demonstrate how these approaches can be streamlined by automation on the AssayMAP Bravo to increase reproducibility, decrease labor, and reduce the probability of human error.

Trastuzumab was affinity purified from cell culture supernatant using biotinylated Her2 extracellular domain (ECD) or biotinylated protein L immobilized on streptavidin cartridges (SA-W); Her2 ECD is the antigen for Trastuzumab and protein L is an affinity reagent for antibody kappa light chains. Immobilized Trastuzumab was either left intact, deglycosylated with PNGase F, or digested with IdeS by flowing the respective enzymes through the cartridges. The glycans and Fc/2 cleaved off the antibody were collected in the flow through. The intact, deglycosylated and F(ab’)_2 fragments were eluted from the cartridge into reducing and non-reducing buffers. The Fc/2 fragment was also treated with and without reducing agents. Proteins in both the flow through and the elution were analyzed with a UHPLC coupled to a Q-TOF mass spectrometer to acquire accurate protein mass data.

NOTES:
Comparison of Protein Concentration Determination of a mAb Drug Substance using Variable Path Length Spectroscopy and UV/VIS Spectroscopy – The Regulatory Dilemma: Choosing the Scientifically Sound Analytical Technique or Consistency with Clinical Trials?

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In recent years a new technology known as “Variable Path Length Spectroscopy” (e.g. SoloVPE from C Technologies Inc.) has been developed for the determination of protein concentration in biologics such as monoclonal antibody drug substances (mAb DS). The advantage of this new technique is its increased dynamic range and resulting minimized sample handling, which leads to increased throughput and also mitigates the risk of dilution errors compared to conventional UV. In this case study for a mAb in late-stage clinical development, it was desirable to change from a traditional UV method to SoloVPE for DS release, and it was shown that the protein concentration was significantly impacted by the choice of method (SoloVPE or SoloVPE LS (Corrected for Light Scattering) compared to the historical conventional UV method; p=0.0135 (% Difference = 1.5%) and p<0.0001 (% Difference 4.4%) respectively). Means of results of the SoloVPE and the SoloVPE LS were found to be statistically different (p< 0.0001 (% Difference = 2.9%), demonstrating that the impact of light scattering must be considered when SoloVPE is evaluated for determination of protein concentration. The variation within replicate results was found to be considerably lower when using the SoloVPE instead of the UV method ((SoloVPE %RSD = 1.2%; SoloVPE LS = 1.1%; UV = 3.3%) (n=36)). The difference between the SoloVPE methods and the historical UV method were considered using a risk-based approach along with other factors such as method performance, impact to the specifications, alignment of techniques through the manufacturing process and into QC, clinical experience, and impact to the labs to drive a decision for implementation of the new technology for DS release.

NOTES:
Influenza Virus Proteins CGE Method Development; the Effects of the Injection Mode and Gel Buffer Dilution

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New seasonal Influenza vaccines are developed each year in Janssen Vaccines and Prevention to prevent global flu outbreaks. Influenza viruses are produced and their immunogenic proteins hemagglutinin (HA) and neuraminidase are reformulated into non-infectious virosomes.

Rapid, precise and accurate methods are needed to quantify the HA concentration for vaccine development. For this purpose a CGE method was developed based on the CE-SDS MW application. Default kit conditions were not optimal and needed adjustment.

Analysis of different influenza strains of NIBSC reference material, virosomes, and inactivated virus bulk showed that CGE was able to identify and quantify influenza proteins. The CGE method was optimized for separation time and resolution of influenza proteins by diluting the commercial proprietary gel buffer, which resulted in a resolution increase from 0.6 to 1.3 and a migration time reduction from >25 to 16 minutes. Dilution of the gel buffer had an effect on the viscosity and ionic strength of the BGE. Both the viscosity and ionic strength had an impact on the electric field strength difference between the BGE and the sample. This electric field strength difference between the BGE and the sample caused opposing effects during electrokinetic injection compared to hydrodynamic injection with respect to injected amount, migration time, peak areas, and separation. After optimization, the method showed to be precise (<10%), accurate (90-110%), and linear in the range of 7 to 76 µg HA/ml. The LOQ was determined as 5 µg HA/ml.

In total, we developed a fast, precise, and accurate CGE method for HA quantification and influenza virus identification by understanding and applying the fundamental principles of influencing the injection and separation efficiency by adapting the local electric field strength difference between sample and BGE.

NOTES:
Streamlining of Primary Sequence Verification and PTM Quantification Workflows with Integrated Solution for Peptide Maps Acquisition and Processing

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The development of recombinant proteins requires an in-depth understanding of the candidate molecule’s quality attributes. It is important to introduce the characterization steps as early in the development as possible in order to assess various expected and unexpected liabilities linked to a given candidate. In the case of biosimilar molecules, building a complete analytical package is the base for further development.

Mass spectrometry is an important tool for those quality assessments. Intact and subunit analysis can be used to rapidly measure primary sequence deviation or detect unexpected PTMs. Once this initial characterization step is performed, peptide maps are often needed to shed light on detected discrepancies or to more thoroughly quantify PTMs. This raises a data processing challenge as an LC-MS peptide map can identify heterogeneities with extreme sensitivity, which yields a very complex dataset. Tools to reduce this complexity are required to satisfy throughput requirements.

In this work, we utilized Bruker BioPharma Compass 2.0 which facilitates the acquisition and processing of peptide maps experiments in a single interface. The NISTmab reference material was degraded under various stress conditions (oxidizing agent, alkaline conditions) and analyzed by LC-MS peptide mapping on the Bruker maxis II UHR-QTOF platform. The degradation products were identified by MS/MS and subsequently used to build a screening method that can measure these heterogeneities with minimal user intervention and simplify the tracking of important quality attributes.

NOTES:
Changes in production method of a biological product may necessitate an assessment of comparability to ensure that these manufacturing changes have not affected the safety, purity, or efficacy of the product. Depending on the nature of the protein or change, this assessment consists of a hierarchy of sequential tests in analytical testing, preclinical animal studies and clinical studies. Differences in analytical test results between pre- and post-change products may require functional testing to establish the biological or clinical significance of the observed difference. Although manufacturing is not the most common case of product failure, product quality issues can delay antibody development. Applying product knowledge proactively to manufacturing may allow greater flexibility and maintain or improve product quality. However, the ability to compare biological materials is solely dependent on the tests used, since no single analytical method is able to compare every aspects of protein structure or function. With the recent introduction of regulatory pathways for follow-on versions of complex biologics, the role of analytical technologies in comparing biosimilars with the corresponding reference product is attracting substantial interest in establishing the development requirements for biosimilars. Here, we discuss the current state of the art in analytical technologies to assess three characteristics of protein biopharmaceuticals that regulatory authorities have identified as being important in development strategies for biosimilars: post-translational modifications, three-dimensional structures and protein aggregation.
Complementary Liquid Phase Separation Techniques for N-glycosylation Analysis of a Therapeutic Glycoprotein

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The recent increase in the number of approved protein therapeutics calls for complementary characterization methods for the analysis of these new industry and regulatory agencies. Biotherapeutics possess various post-glycosylation being one of the most important ones that may affect the efficacy and immunogenicity of the products. In this presentation, the complementarity of various liquid phase bioseparation methods are discussed, including capillary electrophoresis and liquid chromatography. N-glycosylated glycoprotein having multiple glycosylation sites using these orthogonal methods demonstrated their importance in solving the glycosylation puzzle. Most structures were simply identified by public database search based on their glucose unit (GU) values. Structures not included in the databases were deciphered by exoglycosidase nonreductive end of the glycan structures followed by analysis of the resulting products.

NOTES:
Evaluation of Optimal Conditions for Antibody Subunit Analysis on a Hybrid Quadrupole-Orbitrap Mass Spectrometer

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Mass spectrometric analysis of antibodies at the protein and peptide levels is critical during development and production of biopharmaceuticals. The compositions of current generation therapeutic proteins are often complex due to various modifications which may affect efficacy. Intact mass and peptide mapping analyses have become essential techniques in mAb analysis. The analysis of antibody subunits often provides additional and complementary information with the advantage of requiring only very little sample preparation. Reduction is an optional step in the sample preparation and is resulting in ~23-25 kDa subunits when combined with digestion using the Immunoglobulin-degrading enzyme from Streptococcus pyogenes, often referred to as IdeS. Subunits in that molecular weight range are most amenable to top-down analysis and can provide highest sequence coverage.

Here we have reviewed three commercially available monoclonal antibodies under different reduction conditions aiming at complete reduction of inter- and intra-chain disulfide bridges and most efficient fragmentation and sequence coverage in top-down experiments. The mass spectrometer used for all experiments was a commercially available hybrid quadrupole-Orbitrap mass spectrometer equipped with the new BioPharma option. For top-down analysis the presence of disulfide bridges is a generally limiting factor preventing efficient fragmentation in particular in the regions between two cysteine residues involved in a disulfide bridge. Whereas the reduction of inter-chain disulfide bridges is often very efficient, complete reduction of the intra-chain disulfide bridges often requires more stringent conditions and shows higher variability amongst different antibodies.

Here we discuss results obtained for different reduction conditions as well as optimized chromatography and mass spectrometry conditions and parameter settings as well as method options for optimized antibody subunit characterization.

NOTES:
Antibody Drug Conjugates (ADCs) are monoclonal antibodies in which cytotoxic drugs are covalently conjugated. The antibody brings specificity to deliver drug to the target by binding to epitopes at the site of action. The drug is then slowly released at the target site. These ADCs are gaining a lot of attention as better cancer treatments. ADCs being protein molecules can undergo number of chemical alterations during drug conjugation, formulation and storage. Thus they demand reliable and sensitive methods for characterization. Liquid chromatography/mass spectrometry (LC/MS) technology provides a good platform to investigate ADCs at intact, subunit level and also to calculate drug antibody ratio (DAR). This study describes an optimized liquid chromatography/mass spectrometry method for analysis of ADC molecules.

Initial experiments were performed to optimize the ADC separation and to achieve best mass spectrum signals. Initial results showed that the ADC mass spectrum under study is heterogeneous with different populations of ADC due to different types of glycans and the attached drugs. The deconvoluted spectrum of intact ADC was difficult to interpret due to complex nature of the sample. In order to simplify the interpretation, the ADC was deglycosylated. The deglycosylated mass spectrum showed well resolved charge states which suggested the removal of the glycans from ADC leaving only the covalently attached drug molecules. The deconvoluted spectrum showed 7 major drug attachments and from this the drug antibody ratio was calculated. Further studies are in progress to analyze the light and heavy chains after reduction of ADC molecule.

NOTES:
Drug-to-Antibody Ratio Determination for Antibody-Drug Conjugates in Serum Enabled by a Sample Preparation Platform that Automates Affinity Purification and Deglycosylation

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Antibody-drug conjugates (ADCs) are designed to provide targeted drug delivery by linking drugs to monoclonal antibodies. Unlike small molecule drugs, ADCs are not single molecular entities but are instead a heterogeneous population of antibodies that vary by the number of drugs attached to each antibody and by variability in post-translational modifications.\textsuperscript{1,2} The drug-to-antibody ratio (DAR) is the average number of drugs coupled to an ADC. DAR is a critical quality attribute of an ADC that is optimized and closely monitored during ADC development.

Here we present a solution for determining the DAR of ADCs in serum samples that uses the AssayMAP Bravo Platform to automate ADC affinity purification from serum and on-cartridge deglycosylation. The purified ADC was analyzed by UHPLC coupled to a Q-TOF mass spectrometer to acquire accurate intact protein mass data. MS data was analyzed by MassHunter BioConfirm and DAR Calculator software to determine the ADC mass and DAR. This workflow decreases labor required for the ADC DAR determination, variability, probability of human error, and allows the number of samples to be scaled with minimal additional effort.

NOTES:
Characterization of a Novel Protease (IgdE) for Above Hinge Antibody Subunit Generation

Magdalena Widgren Sandberg

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Subunit MS analysis utilizing IdeS enzyme has evolved as a standard analytical method for rapid characterization of MAbs. IdeS enzyme digests IgG just below the hinge generating F(ab’)2 and Fc fragments. Digestion above hinge, resulting in Fab fragments, is preferred in applications such as characterization of bispecific antibodies, crystallization and NMR studies of higher order structure (HOS) of antibodies.

A novel cysteine protease, IgdE from Streptococcus agalactiae, is characterized. IgdE digests human IgG1 at one specific site just above the hinge cysteines, thus generating intact Fab and Fc fragments. The recombinant enzyme is active in a broad range of pH and salt concentrations without the need of any cofactors or reducing reagents. This makes the enzyme suitable not only for subunit MS analysis but potentially also for crystallization and higher order structure studies of antibodies.

NOTES:
Automated Workflow for the Host Cell Protein Monitoring by Mass Spectrometry: From Raw Data to Final Report

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Mass spectrometry (MS) is emerging as the most promising technique to supplement immunoassays for the analysis of host cell proteins (HCPs). We present an automated approach for the identification, quantification, and routine monitoring of HCPs by MS based on Genedata Expressionist®, the instrument- and vendor-independent software platform for MS data processing, analysis, reporting and management. HCP contaminations can span over a wide range of concentrations, with low abundant species present at the ppm level. In our approach, highly efficient algorithms for data pretreatment are applied to obtain optimal peak detection even for low abundant species.

Two specific workflows for the analysis of HCP are described in details. In a first example monitoring of low abundance HCPs was accomplished using a two-stage identification procedure. The whole collection of signals belonging to the protein biotherapeutics (e.g. peptides, glycopeptides, modifications, etc.) was identified before submitting the data to conventional peptide spectrum match (PSM) searches. This not only allowed to monitor and quantify the expected peptides, but also to reduce the chance of low abundance features from the biotherapeutics to be falsely identified as HCP signals (false positives). However, submitting mass spectra from low-abundant signals to PSM algorithms also poses the risk of missing the identification of HCPs (false negatives). In a second example, we present a strategy to mitigate this risk. A potential HCPs is identified by PSM search on samples with enriched HCP content, and the information pertaining to the respective peptides can then be used by the software as identification criteria for any other samples. In particular, retention time and m/z coordinates of known impurities are stored in a corporate knowledge base and used for matching of the respective signals in single stage MS data even in cases where no fragmentation data is available.

NOTES:
New Trends in Capillary Electrophoresis

MCE-SDS Evaluation of the LabChip® GXII Touch for Protein Analysis

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Biopharmaceuticals such as monoclonal antibodies became indispensable in the therapy of various diseases. These drugs are highly complex molecules and therefore very challenging for quality control. Capillary electrophoresis (CE) is an important supplement to HPLC (High Pressure Liquid Chromatography) especially for protein analysis. Microchip Capillary Electrophoresis (MCE) combines the strengths of CE and microtechnology. It has been established providing new possibilities in high sample throughput (≤ 45 s per sample). In this work the LabChip® GXII instrument from PerkinElmer is evaluated including its Protein Express and Protein QC Assays. Both perform MCE-SDS applications for protein analysis. The QC Assay recently became available and is developed for quality control of monoclonal antibodies. The four proteins myoglobin, carbonic anhydrase, ovalbumin and BSA were measured in eight series of 40 runs each. Considering the extreme short time of analysis, the results were excellent. For carbonic anhydrase, the relative standard deviation (RSD) of the migration times for all 320 runs is 4.6 % for the Express Assay and 2.4 % for the QC Assay. The RSD of the %Areas is 5.4 % for the Express Assay and 4.0 % for the QC Assay. The new QC Assay provides more precise results. Further quality attributes such as linearity and resolution also show positive results. Moreover, the system of complete solutions for specific applications is very user-friendly.

NOTES:
The Application of Capillary Electrospray Ionization to the Analysis of Intact Proteins and the Study of their Degradation Products

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In this study, we describe use of CESI-MS [which is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device] for the analysis of mixture of proteins of various sizes and different isoelectric points (pI). The proteins ranged in size from <5KDa to 150KDa (monoclonal antibodies) and from acidic to basic proteins.

The aim of the study was to investigate the conditions affecting protein separation with a view to highlighting some of the factors that affect CESI-MS of intact proteins including biopharmaceuticals. In this work, we have used neutrally and positively coated capillaries to reduce wall effects on protein separations. The study investigates the effect of mass and charge of proteins on CE separations with CESI-MS and the effects of salt concentration in the sample, organic, pressure and voltage as well as background electrolyte buffer on the separations achieved. Finally, we have used test proteins and investigated how CESI-MS can be used to detect degradation products from forced degradation of test samples.

In conclusion results will show that CESI-MS is a complementary technique to LC for the separation of intact proteins separating proteins based on their charge rather than their size or interactions with a stationary phase. By varying the CE conditions and using the high-resolution separations achieved we were capable of separating charge variants, which differ in size < 5 amu, as well as proteins that have undergone post translational modifications or are of similar size but have a different primary sequence.

NOTES:
The Application of Capillary Electrospray Ionization to the Detection of Neuropeptides

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Vasoactive intestinal peptide (VIP) and Pituitary adenylate cyclase-activating polypeptide (PACAP) are both important neuropeptides. These peptides are very basic and are difficult to analyze by LC-MS methods as they bind to columns and have very poor chromatographic properties. CESI is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into a single process in a single device. In this work we describe the development of a CESI-MS method for the analysis of intact peptides, such as VIP and PACAP, as an alternative to other approaches.

The CESI-MS/MS method was developed using commercially available peptide standards. To assess the effects of matrix, serum was spiked with neuropeptides. The peptides were extracted by solid phase extraction and the reconstituted extracts injected by pressure onto either a Polyethylenimine (PEI) coated bare-fused-silica capillary or a neutrally coated capillary and detected on a SCIEX QTRAP® 5500 mass spectrometer fitted with the NanoSpray® III source and used in MRM mode. This initial study confirmed that these peptides could be detected at physiological levels in plasma, which are typically at low ng/mL. The signal to noise observed for both peptides exceeded 25:1 for a 1 ng/mL serum spike. CESI-MS has been shown to be a viable approach to detect important neuropeptides in biological samples. As the SPE extraction can also be further optimized there is the potential to achieve lower detection levels for both peptides in biological samples. This should enable this approach to be applied to other sample volume limited matrices, such as mouse models, and opens up this methodology for other peptide assays which are challenging for standard LC-MS approaches.

NOTES:
Novel CZE Method for Quantification of Intact Virus Particles in Complex Matrices – Quality by Design Method Development Implementation in a GMP Environment

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Analytical and biological methods are required to monitor the quantities (in virus particles per ml) of viruses throughout the production process, to ensure the safety, efficacy and quality of the vaccines. The current methods are either not sufficiently accurate due to carry-over and recovery issues, or require very long analysis times in order to reach adequate analytical precision. Therefore, capillary electrophoresis (CE) was evaluated for fast, accurate and precise quantification of adenovirus particles.

An analytical quality by design (AQbD) method development approach was embraced. With CE, the intact adenovirus particles were separated from sample matrix components such as cell debris, residual cell DNA, proteins, and/or salts. The background electrolyte (BGE) composition, analysis time, and sample pretreatment were optimized using a full factorial design of experiments. BGE additives, capillary temperature, the use of a coated capillary and capillary conditioning were investigated and proved vital to reduce virus adsorption, particulate matter and carry-over, and to allow long series of measurements.

The main challenge during development was to prevent adsorption of the virus and matrix components to the capillary wall. A capillary electrophoresis method was developed suitable for the accurate and precise analysis of all samples from the full process containing either cell lysate and cell debris, or high salt concentrations. The method was validated and will be implemented in the quality control department to monitor the quantity of virus particles.

NOTES:
P-130-T

Capillary Electrophoresis – Mass Spectrometry for Intact Mass Analysis of Antibodies and Antibody-Drug-Conjugates

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Monoclonal antibodies (mAbs) and antibody-drug-conjugates (ADCs) are now the most widely used class of therapeutical compounds in biopharma research and development for a wide variety of diseases. mAbs and ADCs are complex compounds with a molecular weight of ~150 kD. Since regulatory requirements demand a complete characterization, effective and meaningful analytical methods need to be developed.

We used the ZipChip system from 908 Devices to separate the charge variants of the NIST mAb standard and an ADC derived from it. The separated peaks were injected via electro spray to an extended mass range Thermo Fisher Scientific QE HF Biopharma.

The NIST mAb has three published charge variants with 0, 1 or 2 lysine residues, which can be separated within 3 minutes. Despite the acid separation conditions used, the MS of the mAb has a charge envelope ranging from +27 to +32, similar to what to be expected under truly native conditions. We also applied ammonium acetate buffers around pH 6 to successfully push the charge envelop even lower. Using the Biopharma 2.0 software package, accurate intact mass for at least 15 glyco forms were obtained. It was crucial to have the extended mass range in the 3000 – 8000 D range of the QE HF Biopharma to record these low charge states of the sample.

The NIST mAb has only one glycan site per heavy chain, which was modified using click chemistry. The resulting model ADC also had charge variants, which were successfully separated and annotated with the correct MS1 by CEMS.

In conclusion, the 908 Devices ZipChip system coupled to a high resolution extended mass range Orbitrap mass spectrometer allows fast separation of mAb and ADC charge variants and their accurate MS1 mass determination. This will allow novel QC method development for these complex drugs in the biopharma industry.

NOTES:
Sub-2 μm C18 Bound Silica Monolith Particles as HPLC Stationary Phase of Excellent Separation Efficiency

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Sub-2μm silica monolith particles have been prepared successfully on a large scale by sol-gel process. Very small particles, less than 1.5μm, were removed by sedimentation using Imhoff sedimentation cone. Sub-2μm silica monolith particles were derivatized with C18 ligand followed by end-capping with a mixture of hexamethyldisilazane (HMDS), and trimethylchlorosilane (TMCS). C18 bound sub-2μm silica monolith particles stationary phase was washed with acetone and was packed in glass lined stainless steel micro-columns (1mm ID, 300 mm length) using axial compression pump. The packing was carried out very carefully, using a specially designed tapering packing reservoir columns and various pressure sequences, i.e. 1800 psi for 10 minutes, 15000 psi for 10 minutes and 12000 psi for 30 minutes. The C18 bound sub-2μm silica monolith particles packed column was attached to HPLC system and number of theoretical plates, 170,000 plates/m were achieved for the separation of benzene and its four derivatives using 60/40 acetonitrile/water with 0.1 % TFA, as mobile phase. The separation efficiency of this new phase is much better than that of our previous C18 phases and commercial C-18 phase and the scale of production is also high. This study offers a promising vision towards commercialization of chromatographic phases based on silica monolith particles.

NOTES:
Centrifugal Field-Flow Fractionation Coupled with Online Dynamic Light Scattering for the Characterization of PLGA Particles in Cell Medium

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Centrifugal Field-Flow Fractionation (CF3) is a powerful subtechnique of Field-Flow Fractionation. While in Asymmetrical Flow Field-Flow Fractionation (AF4) separation is only based on the hydrodynamic size, CF3 additionally exploits density differences of the analytes resulting in an enhanced separation force and thus improved resolution. Moreover, Online Dynamic Light Scattering (DLS) enables recording of hydrodynamic sizes in real-time rendering this setup a powerful hyphenation (CF3-DLS) for the separation and characterization of nano- and microparticles over a wide size range.

In this presentation, CF3-DLS is used to characterize PLGA (Poly(lactic-co-glycolic acid)) sub-microparticles. In order to investigate their behavior under physiological conditions, the PLGA-particles were dissolved in cell medium and incubated for defined time durations at 37 °C. CF3-DLS analysis was subsequently performed using cell medium as carrier solution thereby closely mimicking the conditions during incubation. By these means, CF3-DLS provides valuable insights in the behavior of a biocompatible particle system, which shows promising properties towards the application in pharmaceutical applications, such as e.g. drug delivery.

Acknowledgement:

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NOTES:
Asymmetrical Flow Field-Flow Fractionation (AF4) is a powerful separation and characterization technique for biological macromolecules, lipids, viruses and cell particulates. In AF4 the separation takes place in an open channel and is based on the hydrodynamic diameter or the diffusion coefficient of the respective sample components [1]. The AF4 channel consists of two parallel walls with a thickness of 0.0190 - 0.0500 cm. Sample components are pushed toward the semi-permeable lower channel wall by the separation field (cross flow) and transported along the channel at different flow velocities via a secondary laminar flow (channel flow). The AF4 channel can also be made of a semi-permeable ultrafiltration hollow fiber, where the cross flow exits radially through the fiber wall and the channel flow moves axially along the fiber length [2]. Resolution, reproducibility and sample recovery of the hollow fiber cartridge was studied using a Bovine Serum Albumin (BSA) standard for more than 100 consecutive injections. The average retention time RSD of the monomer and dimer peaks was found to be below 1%. The hollow fiber flow FFF system was hyphenated with a dynamic light scattering (DLS) detector in order to characterize protein aggregates. Aggregation of BSA and gamma globulin standards was triggered by heat-induced stress at low pH buffer condition. The aggregated samples were subsequently analyzed by the hollow fiber flow FFF-DLS system. The results showed the separation of the large protein aggregates from a few tens to hundreds of nanometers.


NOTES:
Development of a Rapid SE-UHPLC Method for the Determination of Product-related Impurities in Bispecific Antibody Formats

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The development of protein-based therapies, using a wide variety of biotherapeutics such as monoclonal antibody (mAb) products, has expanded in recent years. Production of complex bispecific antibody formats, which are produced widely in mammalian cell lines, still struggle with numerous, unwanted process- and product-related side products, having a potential effect on drug efficacy or even immunogenicity. This underlines the absolute importance of the removal and monitoring of product-related impurities.

Size exclusion-high performance liquid chromatography (SE-HPLC) is the predominant method for the separation of antibody monomer, high molecular weight (HMW) forms, e.g. dimers and other aggregates as well as low molecular weight (LMW) forms, e.g. fragments, used in mAb characterization and quality control. Advantages of this method are its sensitivity, relatively high sample throughput and reproducibility, which are mainly dependent on the mobile phase, temperature, flow rate and column parameters (e.g. column length, resin type or pore size). Development of size-exclusion ultra-high performance liquid chromatography (SE-UHPLC) using new columns with packings of particles ≤ 2µm, coupled with appropriate UHPLC instrumentations, able to generate high pressure, provide significant gains in chromatographic efficiency by a higher resolution, allowing separations between closely related compounds, higher peak capacity and shorter run times.

Here we developed a SE-UHPLC method using the TSKgel UP-SW3000 column (2µm, 4.6 x 30mm, Tosoh) for the characterization of bi-specific CrossMAb formats and compared the results with our routinely used SE-HPLC method (using the TSK-Gel G3000SWXL column, Tosoh). By optimizing the mobile phase, flow rate, temperature and loading conditions, we were able to establish a robust SE-UHPLC method with a higher resolution and shorter run time compared to the standard SE-HPLC method used for process optimization, stability testing and release analytics.

NOTES:
Monoclonal antibodies (mAbs) are prone to modifications such as sialylation, deamidation or C-terminal lysine truncation. Traditionally, salt gradient cation exchange chromatography has successfully been used for the assessment of the mAb charge variant profile. However, significant efforts are often required to tailor salt gradient methods to individual mAbs and generally long run times are needed to achieve the desired resolution. In the fast-paced drug development environment, standardized, rapid and robust platform methods are desirable, accommodating the majority of mAbs analyzed.

Here, we present the charge variant profile of top-selling mAbs, analyzed by strong cation exchange with a linear pH gradient method utilizing next generation UHPLC technology. The pH gradient method serves as a platform method for the mAb charge variant analysis, covering a pH range from 5.6 – 10.2, allowing to determine the pI value of the charge variants when combined with an on-line pH monitor. Bevacizumab, Cetuximab, Infliximab, and Trastuzumab were analyzed on a small particle MAbPac SCX-10 column using a full pH gradient of 10 min. Separations of multiple charge variants was achieved for all mAbs analyzed. Additional resolution improvements and a significant shortening of the analysis time was achieved by optimizing the utilized pH range for each mAb in conjunction with the application of an elevated flow rate to further decrease the applied gradient slope. This easy and fast method optimization approach allowed the registration of the charge variant profile for each mAb within 5 min while retaining the high-resolution separation normally only associated with longer gradient runs.

NOTES:
Enabling a Multi-Attribute Method Comparison of Infliximab

Pierre Allemand

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The pace of biosimilar development and pressures for rapid commercialization have led to the widespread adoption of mass spectrometry to support or disprove similarity to an innovator product. Analysts routinely use a wide array of biophysical and biochemical methods to characterize protein samples. As mass spectrometry development has progressed, the ability to have “multi-attribute methods” to efficiently report the quality attributes of a well-characterized biotherapeutic product has become more realistic. It is only possible, however, with deep data mining of peptide maps and other data. In order to maximize the amount of information available and report it automatically, human intervention is at a premium. Although the development of mass spectrometer based methods are rapid, the tools needed to process the explosion of data are in short supply. Total and efficient analysis with the Protein Metrics suite of software reduces the human burden.

In this study, commercial samples of originator and biosimilar forms of infliximab were analyzed by LC-MS/MS in replicate and were compared for relative amounts of oxidation, deamidation, glycation, glycosylation, terminal Lys truncation and disulfide bond shuffling using Protein Metrics biopharma suite of software. Samples were quantitatively compared, and reports prepared and exported automatically. The comprehensive comparisons within reports provide time savings of weeks compared to expert, manual processing, and reduces significantly the scope for human error.

NOTES:
Characterization of protein therapeutics is a constantly evolving challenge where biochemical complexity is growing and where regulatory requirements are steadily increasing. Data processing software is the critical link between acquisition of high quality raw data and accurate automatic interpretation of results. The Sliding Window algorithm is a powerful method for deconvoluting intact protein ESI mass spectra. This algorithm specifically accommodates separations-based mass spectrometry analysis and proves to be useful and necessary for all types of biotherapeutic proteins. Sliding window acts an overlay for intact protein analysis by utilizing a deconvolution algorithm multiple times in succession, ‘sliding’ along a chromatogram or electropherogram to produce a time-integrated result. This approach allows a user to analyze mass spectra of protein masses which exhibit unique elution profiles, such as separation of mAb subunits or partial separation of intact antibody-drug conjugate (ADC) drug loads. In this study, we focus on the native size exclusion chromatography (SEC) Orbitrap MS analysis of an intact, non-deglycosylated, commercial ADC. This sample allows us to study a highly complex mixture, focusing on the top 90 expected masses. We performed Sliding Window analysis using a range of window widths and window offsets. Our experiment shows that an optimized Sliding Window analysis can be achieved using a Window Width which is equivalent to the average full width half max (FWHM) of the individual eluting components, and using the smallest possible Window Offset time (equivalent to 1 scan shift). Additionally, we compare an optimized Sliding Window deconvolution to conventional “single spectrum” deconvolution. Optimal Sliding Window improved our intact ADC analysis both in terms of qualitative purposes (absolute number of correct mass assignments) as well as in terms of relative quantitation (correct distribution of drug load across all glycoforms).

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Therapeutic proteins, such as antibody-drug conjugates (ADCs), face various challenges. Complementary MS approaches such as peptide mapping and intact mass analysis are needed for complete characterization of therapeutic proteins. The use of 100% aqueous buffers at neutral pH in native MS analysis will reduce the charge state value and, as a result, the distribution of ions will be presented at higher m/z. Using native MS to improve mass separation of heterogeneous mixtures is a powerful strategy when considering analysis of complex biotherapeutics such as random lysine-linked ADCs. We have utilized a benchtop quadrupole-Orbitrap mapping and high resolution native MS intact mass analysis. In this study, we demonstrate integrated characterization of a lysine-linked ADC standard mode for peptide mapping.

Trastuzumab Emtansine was prepared without pretreatment for trypsin peptide mapping or native intact analysis. For intact analysis protein samples were desalted online using size exclusion chromatography (SEC) or reversed phase chromatography coupled directly to the mass spectrometer. Peptide mapping analysis was performed using RP-MS, and for mass spectrometry, we utilized a commercially available Thermo Scientific™ Q Exactive™ Plus mass spectrometer with High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to m/z 8000 for native intact analysis.

We measured the intact native average drug to antibody ratio (DAR) using online native SEC-MS. Intact analysis of the untreated ADC showed a distribution of 0 to 8 linker value showing an expected distribution of N-glycans. Additionally, we observed an average DAR of 3.7, with abundant species detected at < 10 ppm mass accuracy. Peptide mapping analysis resulted in 100% coverage of the amino acid sequence and we identified conjugated peptides as well as linker-only conjugated peptides.

NOTES:
Automated Data Processing for Quality Monitoring of Biotherapeutics by Multi-attribute Methods (MAMs)

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Biopharmaceutical firms adopt complex and costly process monitoring strategies and quality systems to ensure final product quality. Critical quality attributes (CQAs) are currently monitored using an array of analytical techniques. Although routinely used as release tests, these techniques generally do not measure attributes at the molecular level. In this context, many industrial players are exploring the adoption of innovative analytical approaches employing mass spectrometry (MS) to enable direct measurement of CQAs at the molecular level. In addition, MS-based methodologies offer the benefit of measuring many different quality attribute on a given biotherapeutic with a single test. These multi-attribute methods (MAMs) can potentially reduce development and manufacturing costs and at the same time increase product quality.

We present an implementation of MAMs using a single software platform for the data processing, analysis, and management of MS data. In this approach, dedicated workflows were tailored to measure the CQAs for a given biomolecule, while testing for impurities (new peak detection), as well as checking the instrument qualification (system suitability). Optimized data processing was applied to large data sets and execution times scaled linearly with the number of samples. Browsing and downstream data analyses, including statistical tests, visual verification of the results, and generation of customized reports, were performed. This approach can be fully automated and employed as part of a bioprocess control strategy. In this case, we show as an example the real-time monitoring of quality attributes of the materials produced in a bioreactor. A compliance module including GxP functionalities such as audit trails, electronic signatures and data security allows the deployment of this MAM implementation in regulated environments.

NOTES:
Comprehensive PTM Characterization of the NIST mAb Reference Standard using an IMS QTOF Mass Spectrometer

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The NIST RM 8671 reference standard material can function as a common standard for the biopharmaceutical industry, analytical instrument/software companies, and regulators. RM 8671 can be used to evaluate and improve current analytical technologies and capabilities for determining the physicochemical and biophysical attributes of monoclonal antibodies. An exhaustive amount of characterization data has been collected from multiple mass spectrometers and published (ACS Book series: "State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization"). In this study, we demonstrate the use of a new bench top IMS QTOF MS controlled by a workflow driven software for common PTM characterization of the new NIST mAb reference standard (RM 8671).

The IMS QTOF MS system performance was bench marked using NIST RM 8671. PTM characterizations were performed using the following streamlined analytical workflows: 1) intact protein/sub unit analysis for MW determination and C-terminal lysine variant analysis, 2) RapiFluor-MS labeled N-linked glycan profiling, and 3) peptide mapping using a data independent acquisition method. The potential benefits of incorporating ion mobility separation into routine analytical workflows are also discussed.

NOTES:
Absolute Quantification of Proteins and Peptides by ICP/MS

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Despite all recent advances in analytical technologies dedicated to biotherapeutics, accurate protein quantification remains a challenge for the biopharmaceutical industry. UV spectrophotometry is commonly used for batch testing, but it requires the knowledge of the extinction coefficient of the protein, whose experimental determination requires the accurate concentration of a reference standard obtained by an absolute quantification method.

Most protein quantification techniques (separation techniques such as LC or CE, colorimetric assays, immunoassays…) cannot be considered as absolute as they also require a reference standard.

Amino acids analysis after complete hydrolysis of a protein is probably the most commonly used method for the absolute quantification of a single protein or peptide. However, hydrolysis and derivatization are time-consuming procedures and, very often, result in low precision and accuracy.

In order to address the need for a fast-analytical method capable to accurately quantify a protein without any specific reference substance, an isotope dilution ICP-MS method was developed and validated, based on sulfur determination, allowing very accurate determination of a single protein in solution after microwave digestion.

The method was validated (ref. ICH Q2A) using a NIST certified BSA solution: precision is < 1 % RSD and accuracy shows less than 2 % bias over the range of concentrations tested.

NOTES:
Multi-level Mass Spectrometric Characterisation of Antibody-Drug Conjugates in a Regulated Environment

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Antibody-drug conjugates (ADCs) are antibodies engineered to deliver a cytotoxic agent specifically to tumor cells expressing a defined antigen. Their physico-chemical characterisation requires a large number of assays aimed at verifying their sequence identity, post-translational modifications, and range/average number of conjugated drugs (Drug-to-Antibody Ratio: DAR). Cysteine and lysine ADCs are two classes of ADCs that differ by the nature of the amino-acids conjugated, and hence call for well-adapted assays.

In this context, we have developed LC/MS and 2D-LC/MS workflows for the reliable analysis of ADCs, where data processing and reporting is integrated in UNIFI 1.8 (Waters) for increased speed and robustness. An efficient sample preparation methodology generates deglycosylated ADCs, as well as their sub-structures (LC and HC) and proteolytic fragments (e.g. Fab, Fc) in 30 minutes. Moreover, the use of a state-of-the art HRMS setup yields accurate results with low amounts of ADC required, a salient point when analysing valuable therapeutic agents.

Intact ADCs, as well as their substructures/fragments are analysed by RP-LC/MS to confirm their sequence identity and glycosylation pattern, and calculate their DAR based on the MS or UV responses. For cysteine conjugates, we perform native-SEC/MS to calculate the DAR on deglycosylated ADCs. HIC-RP/MS is carried out on a 2D-LC/MS setup in order to couple a high-salt HIC, which separates the ADC molecules according to their DAR, to MS via a RP desalting. A peptide mapping methodology is used to localise the conjugation sites. For highly heterogeneous lysine conjugates, a fingerprint of the conjugated peptides can be easily obtained by studying specific drug fragments, and the fine characterisation of conjugation sites can be done using ETD fragmentation of the peptides.

Overall, we developed fast and efficient workflows that offer a comprehensive, unambiguous characterisation of ADCs.

NOTES:
Mass Spectrometry as a Powerful Tool Box for Host Cell Protein Analysis

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Developing and producing recombinant biopharmaceuticals in mammalian cells requires unambiguous monitoring of HCPs impurities. Immunoassays are still the method of choice for release testing, for which it is recommended to demonstrate the suitability of antisera in a QM-regulated environment to meet regulatory demands. However, peptide analysis by MS has been proven to be a powerful tool box by providing complementary data for HCP characterization.

We show that MS supports the data interpretation of methods for antisera characterization, like high resolution 2D gel electrophoresis combined with immunoblotting to demonstrate antisera coverage, and anti-CHO affinity chromatography to specify the antigen coverage.

Furthermore, peptide analysis by MS offers orthogonal solutions for detection and monitoring of residual HCPs. The advantage compared to immunological methods is the unbiased discovery of HCP impurities to reveal the HCP identities.

By using label-free MS quantification the removal of HCPs during downstream processing was monitored and demonstrated the suitability of the technique for quantitative analysis of trace impurities and thus elucidating effectiveness of individual downstream processing steps.

Additionally, isotopic labeled peptides were applied to quantify single host cell proteins within the processed drug substance. The use of internal standard combined with mass spectrometric LC-SRM (selected reaction monitoring) is a reliable method for absolute quantification. The robust nature of this approach offers the technical potential for validation and usage as GMP release testing for biopharmaceuticals to address the purity. However, multiple challenges need to be solved, to use MS within a release testing.

NOTES:
Sampling the Globe – How to Handle Unlimited Sample Diversity in Mass Spectrometry Data for Biotechnology Discovery and Development

Anders Michael Bernth Giessing

Novozymes A/S, Bagsvær, Denmark

At Novozymes® we produce a wide range of industrial enzymes and microorganisms. Enzymes are proteins, and in nature they initiate biochemical reactions in all living organisms. It is enzymes that convert the food in our stomachs to energy and turn the falling leaves in the forest to compost. Novozymes finds enzymes in nature and optimizes them for use in industry. In industry, enzymes replace chemicals and accelerate production processes. They help our customers make more from less, while saving energy and generating less waste. Enzymes are widely used in laundry and dishwashing detergents and to improve the quality of bread, beer and wine, or increase the nutritional value of animal feed. Enzymes are also used in the production of biofuels where they turn starch or cellulose from biomass into sugars which can be fermented to ethanol.

Mass spectrometry (MS) is a key technology for characterizing the sequence, structure and function of enzymes. Before mass spectrometry data can be transformed into innovation, the raw instrument code needs to be translated from machine language into protein sequence. This is typically done using proprietary software developed by the instrument vendor, a peptide search engine such as Mascot if doing bottom-up proteomics, or one of an ever-growing suite of open-source dedicated software developed by academia. At the Novozymes Mass Spectrometry core facility in Bagsvær, Denmark we have for the past two years used Genedata Expressionist® as a platform for all MS data analysis. In this presentation I will demonstrate the versatility of the Expressionist platform for MS data analysis across some of our industries, from simple protein identification using classical SDS-Page in-gel analysis, through intact protein top-down sequencing, to more complex bottom-up proteomics experiments of host cell proteins and the influence of dietary interventions on endogenous proteins in chickens.

NOTES:
Fast Identification and Quantification Assay for Host Cell Proteins

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Residual host-cell-proteins (HCPs) are a major component of biopharmaceutical process related impurities. They are sometimes present in minute quantities in the final biopharmaceutical product (typically expressed as ng/mg of recombinant protein or ppm concentrations), but they can potentially have a negative impact. HCPs could be responsible for unwanted immune responses in patients, they might interfere with drug activity or with drug formulation. Thus, characterization of low-abundance HCP impurities (1-100 ppm) is very important for biopharmaceutical industry.

We developed a generic LC/MS-based assay for fast HCP identification and quantification across multiple batches of mAb samples.

A therapeutic mAb (Inflectra) was denatured, reduced, alkylated and digested with trypsin and five protein digest standards were spiked in the concentration range of 10 – 500 ppm to simulate the presence of HCPs in the drug substance. The complex peptide mixture was separated by low pH RP chromatography using a 30-min gradient and identification and monitoring of spiked proteins was performed using data independent acquisitions on a QTOF mass spectrometer. The assay was able to detect the spiked proteins down to the 10 ppm level.

These results indicate that a single dimension chromatographic assay can be used for identification and quantification of HCP impurities across multiple samples in a timely fashion. The assay achieved comparable sensitivity to traditional HCP assays (LBAs), while offering the unique advantage in providing unambiguous HCP identification.

NOTES:
QuiC - A Fast, Easy to use QC Monitor that Allows you to Generate QC Readouts in Real Time from Raw Files of Various Vendors and Workflows (MRM, PRM, DIA, DDA)

Florian Marty

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LC-MS based proteomics has become the method of choice for the identification and quantification of a high number of proteins in large sample sets, for example in large cohort studies. Robust and accurate quality controls (QC) during data acquisition are essential to ensure that high quality data with low systematic errors is collected. Biognosys has developed a QC tool that is simple, easy to use and interpretable by non-mass spec experts. The tool handles all proteomics workflows across different vendors: it generates QC readouts from raw files of Thermo Fisher and Sciex mass spectrometers across multiple workflows (MRM, PRM, DIA, DDA). The tool provides an active queue of per-run QC analyses that supports real-time folder monitoring. Moreover, iRT peptide-based readouts are also provided for all workflows, while for DIA and DDA a background library can be specified and additionally targeted to better QC the samples and sample processing. This tool enables users to judge data quality and intervene as needed to prevent sample loss, LC-MS troubles and low data quality.

NOTES:
Complete Characterization of Biotherapeutic Proteins by Automated Data Processing on High Resolution Accurate Mass Spectrometry with SWATH™ Acquisition

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Crucial part of ADC development is to have all the components of the ADC – the mAb, the linker, as well as the drug to be conjugated - well defined. Confidence in data quality and reproducibility is vital for the successful development and characterisation of ADCs.

A single platform able to handle multiple levels of characterisation is desired. High resolution LC-QTOF(MS) allows for mAb full characterization on intact and subunit analysis, as well as peptide mapping with data dependent (DDA) and – independent (SWATH) acquisitions. Automated and reproducible data processing achieves the desired levels of information.

The instrumentation used was an ExionLC analytical flow instrument coupled to an X500B (SCIEX). The data analysis (data reconstruction and peptide mapping) was performed with BioPharmaView™ software.

1. The intact protein analysis provides a comprehensive fingerprint of the product, including the glycosylation pattern.
2. At the subunit level, we drill down to the smaller PTMs, where we show analysis on the level of oxidation and pyroglutamate formation. On Both 1) and 2) we demonstrate the resolving power, mass accuracy and reproducibility.
3. On peptide mapping level, we demonstrate mass accuracy, greater than 95% sequence coverage and MS/MS performance to elucidate the positional information of PTMs.

NOTES:
P-148-T

Sensitive and Precise Measurement of Protein Therapeutics Using LC/Q-TOF

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LC/MS has been widely used in the biopharmaceutical industry for the therapeutic protein molecular weight confirmation. It is fast, accurate and relatively quantitative. The accurate mass measurement helps to determine whether the correct protein has been expressed with the expected post translational modifications. It also provides relative abundance of different proteins or modifications present in the sample. A highly sensitive and reliable Q-TOF system has been developed to streamline this analysis. In this study, examples of characterization of monoclonal antibodies, antibody drug conjugate will be shown. Low level variants can be clearly detected using the newly developed highly sensitive system.

NOTES:
Towards Robust Analysis of Human Growth Hormone by Capillary Electrophoresis-Mass Spectrometry – Essential Considerations

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Current analysis of human growth hormone (hGH) is based on two differential immunoassays. The ratio of the two main isoforms allows to distinguish between the administrated recombinant form and endogenous hGH. However, these immunoassays lack specificity. To increase the specificity, an integrated approach was developed that combines selective sample clean-up with the separation power of CE and the sensitivity of MS.

A co-immunoprecipitation kit containing protein A/G immobilized magnetic beads was used to purify hGH isoforms. Sheathless CE-MS was performed on a CESI8000 coupled to a MaXis MS. The analyses were performed on a fused silica capillary of 90 cm with an ID of 30 µm. The BGE contained 75 mM ammonium formate (pH 8.5) and 10 % acetonitrile.

The two main isoforms of hGH were baseline separated. The addition of 10 % acetonitrile was required to prevent protein adsorption. To the best of our knowledge this is the first time that these two isoforms have been separated in their intact form. Due to the volatile nature of the BGE, evaporation was a significant problem for long-term stability of the method, resulting in a pH drop of almost 1 unit, a considerable shift in migration times (MT) and loss of the analyte peak. A mineral oil overlay applied on top of the BGE prevented evaporation of acetonitrile and ammonium, leading to prolonged stable MTs and peak areas. Additionally, the nature and order of conditioning showed to have a big impact on MT stability. After optimization, MT RSDs remained constant and were below 0.5% for 48 consecutive injections. To increase the sensitivity of the method, the possibility of on-line large-volume sample stacking was investigated. After optimization of plug-length and sample solvent, a concentration factor of ten could be reliably achieved without severely impacting the CE performance.

NOTES:
Challenges of Charge Variant Analysis for Non-platform Antibody Formats

Richard Shannon

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Full characterisation of an antibody therapeutic is a regulatory requirement prior to initiating clinical trials. One of the more challenging characterisation tests for early development is the qualitative and quantitative analysis of heterogeneity related to charged variants.

Charged variants of a molecule are due to post-translational modifications and/or chemical modifications that take place during manufacture, purification and storage. These modifications may affect binding and biological activity, physicochemical properties and pharmacokinetic properties, and thus need to be monitored and controlled.

During pre-clinical product development, the manufacturing and purification processes are being optimised concurrently with the development and evaluation of analytical lot release tests. Some attributes of the molecule that will be controlled by the process in later stages of development remain to be fully understood or controlled in the early stages, leading to some batch-to-batch variability.

For successful development, lot release and stability assays are required that are not only specific to monitor particular critical quality attributes of the molecule, but also robust enough to withstand changes to the manufacturing process. This poster describes how we approached this challenge for the analysis of charged variants. Case studies for non-platform antibody formats such as antibody-drug conjugates and Fc-fusion molecules will be discussed.

NOTES:
P-151-W

Analytical Technologies Enabling Advanced Process Control for Drug Substance Manufacture and Release

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Traditional approaches to drug substance (DS) manufacture and release have been designed as fixed manufacturing processes followed by end product release in the Quality Control laboratory. Advanced process control (APC) utilizes analytical technologies and process models to adjust process parameters during routine batch manufacturing in order to control, predict, and/or measure product quality attributes. APC thus enables a flexible and adaptive process as well as real time release (RTR) of certain key product quality attributes. This presentation highlights analytical tools such as at-line UPLC for feed-forward aggregation control, at-line and rapid microbial technologies, and multi-attribute methods that can be leveraged for APC and RTR over the entire DS process from start to finish, beginning with raw materials to final drug substance.

NOTES:
P-152-T

Separation of Synthetic Peptides and Proteins Using Specially Designed O T-CEC Column

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Pretreated silica capillary column (50 µm internal diameter and 110 cm length) was chemically modified with 4-(Chloromethyl)phenyl isocyanate in the presence of dibutyl tin dichloride as catalyst. The terminal halogen (Cl) of the bound ligand (4-(Chloromethyl)phenyl isocyanate) was reacted with sodium diethyl dithiocarbamate to incorporate the initiator moieties. A thin polymer layer was made on the inner surface of capillary by reversible addition-fragmentation transfer polymerization upon the initiator moieties using a mixture of styrene, N-phenylacrylamide, and methacrylic acid. The copolymer immobilized open tubular capillary column was used for the separation of synthetic mixture of five peptides and tryptic digest of cytochrome C sample in capillary electrochromatography. Very high separation efficiency (over one million per meter) was obtained for synthetic peptides while (Ca. 220,000 plates/column) for some of the peptide in tryptic digest of cytochrome C under optimized elution conditions.

**NOTES:**
Capillary electrophoresis (CE) is a very interesting alternative technique compared to those currently used in viral analysis, such as SDS-PAGE, Western blot or protein assay that are destructive and semi-quantitative or non-specific. Considering that a main issue in virus analysis is the absence of reference material, strategies for the identification and quantitation of HPV-VLP must be developed. In this study, HPV-VLP peak assignment was done using two indirect approaches: by comparison with a production made using a wild-type baculovirus and with VLP-based vaccine, Gardasil®, after adjuvant dissolution. The peak identity was then confirmed by the study of complex formation between HPV16-VLP and conformational H16.V5 antibody using affinity CE. Regarding the quantitation purpose, HPV16-VLP concentration was determined using ELISA with Gardasil® after adjuvant dissolution as reference material and H16.V5 antibody. HPV16-VLP concentration was found to influence particles electrophoretic mobility until a plateau was reached for concentrations ≤ 50 µg ml⁻¹. As zeta potential is directly proportional to the electrophoretic mobility, it was measured at different HPV-VLP concentrations and the results were in complete accordance with the measured electrophoretic mobilities. The concentration dependence of the electrophoretic mobility could be explained by an overlap of the electrical double layers of adjacent particles. Finally, the CE method was successfully validated following the ICH Q2R1 guidelines.

NOTES:
HILIC-MS: A Powerful Analytical Tool for the Comparison of Originator and Biosimilar Therapeutic Monoclonal Antibodies

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Monoclonal Antibodies (mAbs) represent the largest and fastest growing category of biopharmaceutical proteins. They are manufactured through recombinant DNA technology, custom-designed to target chosen antigens, and employed for a wide range of applications, particularly in cancer, immune disorder, and infectious disease. In addition, because several commercial mAbs are approaching patent expiry, a market for so-called biosimilars is becoming increasingly attractive and is accordingly undergoing rapid growth.

The approval processes of biosimilar mAbs depend on their comparability to originators in terms of product quality and safety. Therefore, sophisticated analytical comparisons are required to assess structural features and post-translational modifications (PTM) and thereby minimize the possible risks of clinically meaningful differences between biosimilars and originators mAbs.

The glycosylation pattern of mAbs is considered to be an important critical quality attribute (CQA), and several analytical approaches have been proposed for monitoring the glycosylation profile, albeit mainly at a glycan and glycopeptide level of analysis. In this study, we demonstrate the utility of hydrophilic interaction chromatography (HILIC) hyphenated with mass spectrometry (MS) for the qualitative profiling of glycosylation patterns at the protein level, by comparing originator and biosimilars mAbs (Remicade®-Remsina®-Inflectra®, Herceptin®-Trastuzumab B, and Erbitux®-Cetuximab B). For this purpose, a middle-up approach, consisting of digestion and reduction of the mAbs to obtain sub-units ranging from 25 to 50 kDa, was applied.

The ability of HILIC to resolve hydrophilic variants of protein biopharmaceuticals will be demonstrated. Moreover, the complementarity of HILIC to reversed phase liquid chromatography (RPLC), and its hyphenation to MS will be illustrated, to prove that HILIC-MS can be considered as an innovative and powerful analytical tool for the comparison of originator and biosimilar therapeutic mAbs.

NOTES:
Capillary Electrophoresis and Ion Mobility Coupled to Mass Spectrometry as Complementary Tools for Cysteine Connectivity Identification in Peptides Bearing Two Intra-molecular Disulfide Bonds

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Disulfide bonds between cysteine residues are post translational modification (PTM) playing essential roles in the biological activity and the stability of numerous peptides or proteins. Intra-molecular disulfide bonds are found in various natural-occurring peptides such as animal venoms. In such peptides, the appropriate cysteine connectivity is mandatory to ensure their bioactivity, by providing the perfect peptide conformation to allow an efficient binding to their molecular targets. Disulfide bonds connectivity characterization is still challenging and is a key issue in the analysis of structured peptides/proteins, targeting pharmaceutical or pharmacological utilizations. Thus, the use of sensitive, robust and efficient characterization techniques to access the cysteine pairing is crucial. This study describes the development of new and fast gas-phase and in-solution methods coupled to mass spectrometry to characterize disulfide bond connectivities.

Disulfide isomers of three peptides bearing two intra-molecular disulfide bonds but different cysteine connectivities have been investigated. Capillary Zone Electrophoresis (CZE) and Ion Mobility (IM) coupled to Mass Spectrometry (MS) were used to perform the separation in both aqueous and gas phases, respectively. The ability of each technique to separate the isomers with different connectivities has been investigated and compared.

Results show that CZE-MS and IM-MS can act as complementary techniques to unambiguously determine (or to confirm) the cysteine connectivity of a given peptide. Indeed, the combination of the relative migration time to a reference peptide in CZE-MS, the drift time in IM-MS and the generation of fragments by Collision Induced Dissociation (CID) lead to the attribution of the disulfide connectivities in all studied cases. Finally, theoretical modelling and calculations were performed to support and explain the experimental data on the basis of their predicted physicochemical properties.

NOTES:
Despite the ever-growing use of capillary electrophoresis in biomedical research and the biopharmaceutical industry, the development of simplified data interpretation methods is lagging behind. In this poster, we report the design and implementation of a co-injected triple-internal standard method, to alleviate the need of an accompanying run of the maltooligosaccharide ladder for glucose unit (GU) calculation. Based on the migration times of the co-injected standards of maltose, maltotriose and maltopentadecaose (bracketing the peaks of interest), a data processing approach was designed and developed to set up a virtual ladder that was used for GU calculation. In turn the resulted GU values can be used to assign the structures from a suitable database. The data processing was tested in terms of the calculated GU values of human IgG glycans and the resulting relative standard deviation was ≤1.07%. This approach readily supports high-throughput capillary electrophoresis systems by significantly speeding-up the processing time for glycan structural assignment.
A Novel Non-toxic Reducing Agent for Reductive Amination Based Glycan Labeling

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One of the most frequently used high resolution glycan analysis methods in the biopharmaceutical field is capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection. Glycans are usually labeled by reductive amination with a charged fluorophore containing a primary amine that reacts the aldehyde group at the reducing end of the glycan structures. In this reaction, first a Schiff base is formed that is reduced to form a stable conjugate by a hydrogenation reagent, such as sodium cyanoborohydride. In large scale applications, such as clone section for glycoprotein therapeutics, hundreds of reactions are accomplished simultaneously so the resulting HCN represents a safety issue. Here, we propose the use of a water-soluble iridium(III) dihydride complex for catalytic dehydrogenation of formic acid as a novel hydrogenation agent and non-toxic alternative.

The easily synthesized water-soluble iridium(III) dihydride showed high catalytic activity and could be reused several times with no loss of performance. The application of this non-toxic gas forming compound is environmentally friendly and reduces the health risks for the industry. Using carbohydrate standards, oligosaccharides released from glycoproteins with highly sialylated, high mannose and neutral structures (fetuin, ribonuclease B and IgG, respectively) as well as plasma N-glycans, we demonstrated similar labeling efficiencies for iridium(III) dihydride that of with the conventionally used sodium cyanoborohydride based reaction. The derivatization reaction time was less than 20 min with no bias towards the above mentioned specific glycan structures.

NOTES:
Development, manufacture and commercialisation of new therapeutic monoclonal antibodies (mAbs) remains a long and expensive process. MAbs offer a higher degree of success due to their inherent specificity though many mAbs fail during development due to problems associated with expression, purification, formulation and stable storage of the mAb candidate. Therefore, the availability of highly informative analytical platforms to assess the ‘developability’ of a mAb candidate to derisk the scale up, manufacturing and formulation processes, at the earliest stage possible, is highly desirable. The expression of the mAbs using a common IgG1 framework under identical culture conditions facilitates an investigation into the role of the variable gene sequences on the modulation of product critical quality attributes.

Infliximab, Cetuximab, Trastuzumab, Bevacizumab and Rituximab were the panel selected and represent some of the highest selling mAbs therapies on the market. The variable region sequences of these mAbs were cloned into a common human IgG1 framework and expressed using transient transfection in HEK 293 and CHO-S cells. Using state of the art chromatography techniques our analysis showed significant differences between the charge variant profiles between the commercial product and our in-house mAbs. Our initial analysis showed that a number of our in-house HEK produced biosimilars display a more acidic and complex charge variant profile than the commercial drug product while CHO produced mAbs had a more similar but still different profile. Other in-house mAbs were observed to be less complicated than their commercial equivalents.

To investigate these differences further, in depth characterisation of the expressed mAbs was carried out using advanced LC-MS methods. The resulting data provides an interesting insight into the challenges associated with lead candidate selection and potential biosimilar development due to differences in expression systems and process parameters when compared to those used for bioproduction by innovator companies.

NOTES:
Late Breaking

LB-01

Analytical Ultracentrifugation Assays for the Characterization of Purity in AAV Gene Delivery Vectors

Chris Sucato

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The amount of gene therapy-related research and development occurring in the US and worldwide continues to grow at a fast rate. In recent years, recombinant adeno-associated viral (AAV) vectors have been exploited in a number of gene delivery approaches. The use of these vectors in clinical applications has increased the demand for the manufacture, characterization, and quality control of AAV-based drug products. A notable feature of AAV vector generation in cell culture is the formation of an excess of “empty” capsids, which lack the vector genome and are therefore unable to provide a therapeutic benefit. The effect of the empty capsids on clinical outcome is unclear, as there is some indication of increased immunogenicity, but also possibly enhanced gene transfer as a function of the amount of empty capsids in the drug product. While the ultimate effect of empty capsids---and other impurities associated with AAV production---on safety and efficacy is yet to be fully established, an accurate and precise means of characterization of the relative abundance of variants in AAV drug products will be a necessary feature in the development of this class of biologics. Here we explore the use of analytical ultracentrifugation (AUC) in the characterization of the size-distribution profile of species in recombinant AAV samples. In particular, we discuss the applicability of band-forming sample loading, which may greatly reduce the sample amount requirements, and facilitate the use of AUC as a sensitive, precise method for the characterization of AAV purity.

NOTES:
Comprehensive Profiling, Characterization and Detailed MS-based Structural Understanding of N-glycans

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We describe a strategy uniting quantitative N-glycan profiling with comprehensive characterization where detailed structural understanding of biopharmaceutical products is a fundamental requirement. Fluorescence profiling by high performance liquid chromatography is carried out in combination with accurate mass analysis and online MS/MS and integrated with spectral-library database matching. These protocols enable the rapid and confident assignment of known structures and promotes de novo interpretation of unknowns to provide exacting characterization of any unusual structures encountered in glycoengineered products, non-traditional protein expression systems and glycosidase treated samples. Standard exoglycosidase treatments are augmented with permethylated MSn characterization, allowing detailed resolution of linkage and branching complexities to provide orthogonal evaluation of enzymatic activities. In-depth characterization of bioactive motifs by mass spectrometry augments or supplants the need for biological inference and structural designations using bracketed cartoon compositions, where terminal glycan epitopes transparent to accurate mass analysis are approached with explicit analytical data. Key aspects of optimal sample preparation, including fluorescent labeling and sample purification prior to LC-MS to remove background components, HPLC column selection, and preferred mobile phases for MS/MS spectral quality are discussed based on needed sensitivity and a minimization of signal-diluting adducts. An important consideration is the reproducibility of high vs. low energy collisions on Q-TOF vs. Ion Trap (IT) instruments. The use of MS/MS and IT-MSn spectra for spectrum-matching is treated systematically with novel consideration given to ion m/z vs. intensity plots necessary for library development.

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