MS PHARMA NETWORK 2018
SIZE DOES NOT MATTER: PHARMA AND HR-MS
"Small and Large Analytical Challenges"

Villa Aurelia, Roma
19 - 21 Febbraio 2018

New, emerging technologies for pharma & biopharma
Topics: ultra-high resolution, ion mobility, HDX, imaging and new software tools for advanced data analysis

Large molecules characterization (Antibodies & ADC)
Topics: advanced structural characterization (including PTMs), native state MS, top-down MS, challenges in formulation

DMPK and sample prep (small & large molecules)
Topics: the critical role of sample preparation, bioanalytical challenges and solutions

Small molecules and MetID
Topics: streamlining MetID with high-resolution, challenges with small molecules

Contacts: info@imass.it
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With the patronage of
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Claudio Medana (University of Torino) 

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Caterina Temporini (University of Pavia)
SCIENTIFIC PROGRAMME

MONDAY FEBRUARY 19, 2018

14.30 Conference Opening (Pietro Franceschi, IMaSS President and Organiser Representatives)

OPENING PLENARY SESSION

CHAIRMAN: PIETRO FRANCESCHI

15.00 PL1 Andrea Bizzi Corporate Marketing, Chiesi Farmaceutici, Parma, Italy
GLOBAL PHARMA SCENARIO

15.45 PL2 Carol Robinson University of Oxford, UK
MASS SPECTROMETRY AND ITS ROLE IN MEMBRANE PROTEIN DRUG DISCOVERY

SESSION 1 NEW EMERGING TECHNOLOGIES FOR PHARMA AND BIOPHARMA

CHAIRMEN: ANDREA ARMIO RTTI AND CLAUDIO MEDANA

16.30 KN1_S1 Elisabetta Boeri Erba Universite Grenoble Alpes, Grenoble, France
FROM INTACT PROTEINS TO MACROMOLECULAR COMPLEXES: THE EMERGING ROLE OF MS IN STRUCTURAL BIOLOGY

17.15 OC1_S1 Francesca Monaco CISM, University of Florence, Florence, Italy
CHARACTERIZATION OF PULMONARY COLLAGEN THROUGH IMAGING MASS SPECTROMETRY

17.35 OC2_S1 Simone Cristoni I.S.B. - Ion Source & Biotechnologies srl, Milano Italy
HIGH RESOLUTION MASS SPECTROMETRY COUPLED TO GRAPHICAL PROCESSING UNIT DATA ELABORATION APPLIED TO PHARMACEUTICAL AREA

17.55 KN2_S1 Martijn Hilhorst PRA Health Sciences, Assen and University of Groningen, the Netherlands
QUANTITATIVE BIOANALYSIS OF BIOPHARMACEUTICALS USING HIGH-RESOLUTION MASS SPECTROMETRY
Kindly sponsored by Sciex

18.35 WELCOME RECEPTION
TUESDAY FEBRUARY 20, 2018

SESSION 2  LARGE MOLECULES CHARACTERIZATION
CHAIRWOMEN: BARBARA PIOSELLI AND CATERINA TEMPORINI

8.20  KN1_S2  Michael O. Glocker  Proteome Centre Rostock, University of Rostock, Germany
ITEM-TWO: SIMULTANEOUS CHARACTERIZATION OF SPECIFICITIES AND AFFINITIES OF
EPITOPE – ANTIBODY REACTIVITIES IN THE GAS PHASE BY NANO-ELECTROSPRAY MASS
SPECTROMETRY

9.05  OC1_S2  Richard Blankley  Agilent Technologies, Cheadle, UK
A TOOLBOX OF ANALYTICAL TOOLS FOR CHARACTERIZING LARGE AND COMPLEX
BIOMOLECULES

9.35  OC2_S2  Angela Capolupo  Structural Characterization Laboratory, PDBP-Protein Chemistry Department, Merck
Serono, Guidonia (RM)
HIGH RESOLUTION MASS SPECTROMETRY FOR IN-DEPTH CHARACTERIZATION OF
BIOPHARMACEUTICALS

9.55  OC3_S2  Loredana Lupu  Steinbeis Centre for Biopolymer Analysis & Biomedical Mass Spectrometry, Rüsselsheim,
Germany
EPITOPE IDENTIFICATION AND AFFINITY QUANTIFICATION OF PROTEIN- LIGAND INTERACTIONS
USING ONLINE SPR- MS: APPLICATION TO DNA APTAMER-PROTEIN INTERACTION EPITOPES

10.15  KN2_S2  Michael Przybylski  Steinbeis Centre for Biopolymer Analysis & Biomedical Mass Spectrometry, Rüsselsheim,
Germany
SPR- BIOSENSOR- MASS SPECTROMETRY COMBINATION FOR PROTEIN EPITOPE IDENTIFICATION:
CLINICAL APPLICATION FOR NEW APPROACHES TO ENZYME REPLACEMENT THERAPY
Kindly sponsored by I&L Biosystems

10.45  Coffee Break

11.15  KN3_S2  Gianluca Degliesposti  MRC Laboratory of Molecular Biology, Cambridge UK
CROSS-LINKING AND MASS SPECTROMETRY: A POWERFUL AID TO THE STRUCTURAL
INVESTIGATION OF PROTEIN COMPLEXES

12.00  OC4_S2  Claudio Iacobucci  Martin Luther University Halle-Wittenberg, Halle/Saale, Germany
A NOVEL CROSS-LINKERS TO STUDY 3D-STRUCTURES OF PROTEINS AND PROTEIN COMPLEXES

12.20  KN4_S2  David Munoz  Thermo Fisher Scientific, Hemel Hempstead, UK
UNDERSTANDING YOUR MAB

13.05  OC5_S2  Federica Dal Bello  Molecular Biotechnology and Health Sciences Dpt, University of Turin, Italy
DEVELOPMENT OF A NANO-HPLC-HRMS METHOD TO QUANTIFY NEUROPEPTIDES AND
ANALOGUES IN BIO-PATHOLOGICAL SAMPLES

13.25  Lunch
**Tuesday February 20, 2018**

**Session 3: Small Molecules and MetID**

**Chairwomen:** Silvia Catinella and Ornela Curcuruto

14.30 **KN1_S3**  Filip Cuyckens, Janssen Pharmaceutical Companies, Antwerp Area, Belgium
**High-Resolution MS in DMPK: Current Trends and Future Perspectives**

15.15 **KN2_S3**  Ellenia Bordini, Aptuit, Exotec Company, Verona, Italy
**Metabolites in Safety Testing: Strategies to Meet the MIST Requirements**

16.00 **KN3_S3**  Davide Vecchietti, Shimadzu Italia s.r.l., Milan, Italy
**Application of Nano-Surface and Molecular-Orientation Limited (nSMOL) Proteolysis to LC/MS Bioanalysis of Monoclonal Antibody Drugs**

16.45  **Coffee Break**

17.05 **KN4_S3**  Russell Mortishire-Smith, Waters Corporation, Wilmslow, Manchester, UK
**Ion Mobility Enabled Approaches to Drug Metabolism and Pharmacokinetics**

17.55 **SC1_S3**  Michele Iannone, Sapienza University of Rome, Rome, Italy
**Investigation of the Metabolic Pathways of Synthetic Isoflavones Following Oral Administration by Gas Chromatography Coupled to High Accuracy Mass Spectrometry**

18.30  **Social Event and Dinner** at Museo di Arte Farmaceutica e di Storia della Farmacia
*at the premises of Nobile Collegio Chimico Farmaceutico Universitas Aromatariorum Urbis.*
## Wednesday February 21, 2018

### Session 4 | DMPK and Sample Preparation
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**Chairmen:** Fabio Bonelli and Chiara Bigogno

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<td>Filipe Lopes ROCHE, Basel, Switzerland&lt;br&gt; DMPK of “NOVEL” BIOTHERAPEUTICS - WHAT IS CHANGING?</td>
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<td>9.15</td>
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<td>Marina Naldi Department of Pharmacy and Biotechnology, University of Bologna, and Centre for Applied Biomedical Research, S. Orsola-Malpighi Hospital, Bologna, Italy.&lt;br&gt; MASS SPECTROMETRY-BASED CHARACTERIZATION OF HUMAN SERUM ALBUMIN HETEROGENEITY IN PHARMACEUTICAL FORMULATIONS.</td>
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<td>Andrea Barbarossa University of Bologna, Italy&lt;br&gt; THE RISK OF A CRITICAL ERROR IN MS/MS DETECTION OF KETAMINE</td>
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<td>Lorenza Putignani Ospedale Pediatrico Bambin Gesu’, Rome, Italy&lt;br&gt; MONITORING HOST AND CELLULAR CHANGES IN RESPONSE TO DISEASES AND DRUGS: THE PROTEOMIC CHALLENGE</td>
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### Session 5 | Size Does Not Matter
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**Chairwoman:** Angela Capoluco

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Abstracts
PL2

Mass Spectrometry and its role in membrane protein drug discovery

Carol Robinson

Physical and Theoretical Chemistry Laboratory, University of Oxford, Oxford, UK

With over 50% of current drugs directed at membrane receptors, new technologies are required to study these demanding targets. Native mass spectrometry, in which proteins retain their folded state and interactions, is an exciting advance. Recent breakthroughs allow this technology to be applied to membrane protein assemblies. Released directly into the gas phase from micelles, or lipid-based solubilisation vehicles, we can preserve non-covalent interactions allowing small molecules to be observed directly attached to membrane receptors. In my lecture I will demonstrate how we can exploit these new mass spectrometry approaches to understand protein interactions in a number of membrane protein drug targets, from ion channels to G-protein coupled receptors. Beyond defining binding stoichiometry I will show how we can use the approach to assign affinity constants to multiple ligands simultaneously. I will also show how we are deciphering the importance of post-translational modifications and the surrounding lipid bilayer using new methodologies developed in our laboratory.
From intact proteins to macromolecular complexes: the emerging role of MS in structural biology

Elisabetta Boeri Erba

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Mass spectrometry (MS) represents a key tool to assess the mass of intact biomolecules at high resolution and sensitivity. I will illustrate examples of investigating intact proteins and biopharmaceuticals using MALDI- and ESI-instruments, underlining the importance of sample preparation.

MS performed under so-called “native conditions” (native MS) can be used to assess the mass of biomolecules that associate noncovalently. One can determine the accurate stoichiometry of intact assemblies, the direct interactions between subunits and the relative position (core vs. periphery) of subunits within a complex. By mixing subunits in a stepwise manner, a hierarchy in the assembly pathway can be determined.

I will illustrate the application of native MS to the study of the structure of macromolecular assemblies, including protein complexes involved in host-pathogen interactions. Overall, MS is useful for gaining important insights into the composition, structure and dynamics of macromolecular complexes.
Characterization of pulmonary collagen through Imaging Mass Spectrometry

Francesca Monaco¹, Riccardo Zecchi¹, Francesca Boscaro¹, Emanuele Barborini², Giuseppe Pieraccini¹, Gloriano Moneti¹

¹ Mass Spectrometry Center (CISM), University of Florence. Viale G. Pieraccini, 50139 Florence, Italy.
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² Tethis S.p.A. Via Russoli, 3 20143 Milan, Italy

Collagen is the most important member of the fibrillar and microfibrillar structures of the extracellular matrix and basal membrane, it is also an important structural member of connective tissue and interstitial tissue of parenchymal organs.
Mutations and excessive accumulation of collagen different types can be responsible of rare genetic diseases and a lot of common diseases, for example pulmonary fibrosis.
The collagen study is a difficult task due to several characteristics of this structural component: the primary sequence of individual α-chains is highly repeated, collagen molecules are large and not soluble, characterized by a lot of glycosylated amino acid residues and by abundant inter-chain and intra-chain interactions.
Despite these technical difficulties, we tried to develop a method using mass spectrometry that is one of the most important techniques in the study of collagen and proteomics applied to clinical related diseases.
In this work, we combined high resolution nanoLC-MS with MS-Imaging approaches. We identified the most abundant peptides from different types of collagen using Shotgun Proteomics approach and, then, we mapped their localization in healthy pulmonary sections by MS-Imaging analysis, using vacuum MALDI-LTQ-Orbitrap and MALDI-TOF/TOF instruments.
We have been able to develop an effective and reproducible method to evaluate the tissue distribution of collagen peptides most commonly found in the lungs (types I and III).
A substantial improvement to this approach was linked to the use of new slides ns-TiO₂ characterized by a layer of titanium dioxide nanostructures on their surface. Their use allowed us to develop an innovative method for decellularization of pulmonary sections directly on ns-TiO₂ surface, thanks to a better adhesion of the tissue sections. It was not possible to realize the decellularization method on-tissue using standard glasses.
Our actual final goal is to apply this approach to pulmonary sections of animal model of the fibrotic pathology, to detect possible pathological alterations by mapping the peptides of the collagen sequences.
OC2_S1

High resolution mass spectrometry coupled to Graphical Processing Unit Data Elaboration applied to pharmaceutical Area

Simone Cristoni

I.S.B. - Ion Source & Biotechnologies srl, Via Ludovico Ariosto 21, Milano Italy, simone.cristoni@gmail.com

High resolution mass spectrometry applications in industrial area are extensively grown in the recent years mainly thanks to the advent of both Time of Flight (TOF) and FT based (ORBITRAP) mass analyzer. This technology can monitor a huge amount of compounds with respect to low resolution and mass accurate one and can provide detailed qualitative information useful in the identification of unknown compounds. The high number of produced data lead to the need of data high efficient elaboration platform. Especially when complex matrices are analyzed the data extraction, inter-sample comparison and analyte quantitation become time consuming. Graphical processing unit (GPU) data elaboration approach makes possible to speed up these steps with respect to the classical elaboration system. Here we present some practical application of this technology to the pharmaceutical field.
Quantitative bioanalysis of biopharmaceuticals using high-resolution mass spectrometry

Martijn Hilhorst

Bioanalytical laboratory, PRA Health Sciences, Assen (the Netherlands)
Analytical Biochemistry, Department of Pharmacy, University of Groningen (the Netherlands)

High-resolution mass spectrometry (hrMS) is already commonly used in the field of metabolomics, peptidomics and for the qualitative investigation of drug metabolism. In recent years, it has also become more popular for quantitative bioanalysis of small molecules, peptides and larger biopharmaceuticals.

A major advantage of hrMS over triple quadrupole MS systems (QqQ-MS) is the ability to narrow the mass extraction window (MEW). The use of smaller MEW’s significantly improves the selectivity of an assay, compared with QqQ-MS where the MEW is fixed and relatively large. This can result in the removal of interfering endogenous compounds, thus resulting in cleaner chromatograms and an improved signal to noise (S/N) ratio. hrMS also has the capability of summation of different mass traces. With the help of software, multiple traces (for example multiple y-fragments, charge states or C13-isotopes) can be summed to achieve higher detection sensitivities. The ability to post-acquisition reprocess hrMS datasets with other search parameters without the need of re-analyzing samples is a great advantage. Especially in the early stages of method development one can monitor different mass channels at once and evaluate them with regard to sensitivity and selectivity.

In this presentation examples will be shown of the use of hrMS for quantitative bioanalysis. The effect of changing MEW’s and summation of multiple fragments on method selectivity and sensitivity will be presented. The quantification of the 22-kDa biopharmaceutical protein recombinant human growth hormone in rat plasma using a quadrupole-time of flight mass spectrometer will be discussed after an enzymatic digestion.
Simultaneous characterization of specificities and affinities of epitope – antibody reactivities in the gas phase by nano-electrospray mass spectrometry

Michael O. Glocker¹

¹ Proteome Center Rostock, University Rostock Medical Center and Natural Science Faculty, University of Rostock, Schillingallee 69, 18059 Rostock, Germany. e-mail: michael.glocker@med.uni-rostock.de

We have developed an electrospray mass spectrometry method, termed ITEM-TWO, which is capable to simultaneously determine both equally important antibody features, specificity and affinity, in a single gas phase experiment [1-3]. First, epitope mapping defines the antibody’s specificity. Second, the antibody’s affinity is determined by determining binding energies to which dissociation constants give quantitative information. For executing ITEM-TWO three electrospray-compatible solutions in which antibodies maintain their binding activities are analyzed. Solution 1 is a mixture of peptides from which at least one hosts the epitope. Solution 2 contains the antibody. Solution 3 is a mixture of solutions 1 and 2. Solution 3 is electro-sprayed without any further purification. Multiply charged ions are translated into the gas phase and, with the aid of a mass spectrometer’s ion filtering devices, the intact immune-complex ions are separated from unbound peptide ions. Increasing the energy in a subsequent collision cell results in collision induced dissociation of the immune-complex by which bound peptide ions are released. Measuring the masses of the released peptide ions in a time-of-flight mass analyzer identifies the epitope. A step-wise increase in the collision cell energy changes the intensity ratios of the surviving immune complex ions and of the released peptide ions. From the normalized intensity ratios we deduced the apparent activation energies (ΔH°(m,t)) and the dissociation constants (Kd) of the gas phase dissociation processes. In the investigated cases, the order of the apparent gas phase dissociation constants matched well with those obtained from in-solution measurements.

References


A toolbox of analytical tools for characterizing large and complex biomolecules

Richard Blankley Ph.D.,
Agilent Technologies, 5500 Lakeside, Cheadle, United Kingdom

A detailed quantitative and qualitative analysis of biomolecules is a pre-requisite for their use as biotherapeutics. However, the size, complexity and heterogeneity of proteins (especially protein-conjugates) presents a significant analytical challenge. No single analytical strategy or technique provides all the answers to all of the challenges, instead, a toolbox of tools covering sample preparation, separation and high resolution MS is required. I will show how Agilent scientists and our customers are using tools such as automated sample prep, 2D-LC, capillary electrophoresis (CE) and high resolution ion mobility mass spectrometry to address the challenges of characterizing complex biomolecules now and in the future.

References
High Resolution Mass Spectrometry for in-depth characterization of biopharmaceuticals

Angela Capolupo\textsuperscript{1*}, Eugenio Galano\textsuperscript{1}, Nunzio Sepe\textsuperscript{1}, Francesca Cutillo\textsuperscript{2}, Mara Rossi\textsuperscript{3}, Angelo Palmese\textsuperscript{1}

\textsuperscript{1} Structural Characterization Laboratory, PADBP-Protein Chemistry Department, Merck Serono, Via Luigi Einaudi, Guidonia (RM)
\textsuperscript{2} Scientific advisor e head of third parties’ management, PADBP-Protein Chemistry Department, Merck Serono, Via Luigi Einaudi, Guidonia (RM)
\textsuperscript{3} Head of Protein Chemistry department, PADBP-Protein Chemistry Department, Merck Serono, Via Luigi Einaudi, Guidonia (RM)

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In the past decades, proteins and peptides have emerged as very efficient drugs in the treatment of several pathophysiological states. Given the complexity of biopharmaceuticals, a deep knowledge of their structure is required, since structural features play a key role in molecule’s functionality and binding. In this scenario, mass spectrometry has acquired more and more importance, due to the huge number of information that is able to provide in one experiment. High resolution mass spectrometry has proven to be very versatile in the characterization and monitoring of these critical quality attributes. In this work, we present two case studies, which can be considered as paradigm to describe the fundamental contribute that high resolution is able to give. In particular, the first case study is focused on the unequivocal assignment of a phosphorylation site through EThcD fragmentation, while the second one describes the analysis of a pegylated protein, through post-column injection of triethylamine as stripping base [1], in order to measure its molecular weight and define the heterogeneity of PEG moiety, which can be a critical regulatory criterion for health authorities.

References
Epitope identification and affinity quantification of protein-ligand interactions using online SPR-MS: Application to DNA Aptamer-Protein Interaction Epitopes

Loredana Lupu¹, Hendrik Rusche¹, Zdenek Kukacka¹, Yannick Baschung¹, Nico Hüttmann¹,², Maxim Berezovski³, and Michael Przybylski¹,²

¹ Steinbeis Center for Biopolymer Analysis & Biomedical Mass Spectrometry, Bahnhofsplatz 1, 65428 Rüsselsheim, Germany; e-mail
² University of Konstanz, Department of Chemistry, 78457 Konstanz, Germany
³ University of Ottawa, Department of Chemistry and Biomolecular Sciences, Ottawa, Canada

Bioaffinity analysis using biosensors has become an established technique for detection and quantification of biomolecular interactions. However, a principal limitation of biosensors is their lack of providing chemical structure information of affinity-bound ligands. Proteolytic excision/extraction (Protex-MS), hydrogen-deuterium exchange (HDX-MS) of peptide backbone hydrogens, and Fast-Photochemical Oxidation (FPOP) are major techniques for mass spectrometry based elucidation of protein-ligand interactions, but none of these tools alone provide quantitative affinity data. Using a surface plasmon resonance (SPR) biosensor, we have developed a continuous online biosensor-MS combination with electrospray ionization mass spectrometry that enables the simultaneous affinity isolation, structure identification and affinity quantification of biopolymer ligands from a protein-ligand complex immobilized on a gold chip. Key tool of the online biosensor-MS epitope analyzer is a new integrated, automated interface that provides sample concentration and in-situ elution for direct MS analysis of the ligand epitope(s) [1]. ESI-MS systems from all major manufacturers can be coupled, using a newly developed software.

Here we report a study of the C-Met interaction with two DNA aptamers that bind the target protein (C-Met) with high affinity and specificity. Nucleic acid-based aptamers have very similar properties with monoclonal antibodies, however they are substantially smaller and are chemically synthesized. To determine the affinity of the two aptamers, SPR biosensor analysis was employed. Furthermore, by using epitope excision-MS, we could determine specific epitope regions on the C-Met protein. The interactions of both aptamers showed very strong affinities and two binding events, and revealed that the aptamers undergo conformational changes after the first binding event. The second binding event was found to be approx. 100-times stronger. Further analysis revealed the binding epitopes for both aptamers and confirmed that the protein has 2 different binding sites for each aptamer.

References
**KN2_S2**

**SPR- Biosensor- Mass Spectrometry Combination for Protein Epitope Identification: Clinical Application for New Approaches to Enzyme Replacement Therapy**

**Michael Przybylski**, Stefan Maeser, Zdenek Kukacka, Fabio Borri, Loredana Lupu, Lorenzo Altamore, Julia Hennermann and Anna Maria Papini

Biosensor analysis using surface plasmon resonance (SPR) has become an established technique for detection and quantification of biomolecular interactions; however, a principal limitation is their lack of providing chemical structure information of affinity-bound epitope ligands. Proteolytic excision/extraction (PROTEX-MS), hydrogen-deuterium exchange (HDX-MS) of peptide backbone hydrogens, and Fast Photochemical Oxidation (FPOP) are major techniques for mass spectrometry based elucidation of protein-ligand interactions, but none of these tools alone provide quantitative affinity data. We have developed a most efficient PROTEX-MS epitope analyzer by online SPR-ESIMS combination that enables the simultaneous affinity isolation, structure identification and affinity quantification of biopolymer epitopes from a protein-ligand (e.g., antibody complex). Key tools of the PROTEX-SPR-MS Epitope Analyzer are (i), a new SPR biosensor, that - unlike any other SPR - enables the application of microfluidics for ESI-MS without any buffer change; (ii), an integrated interface that provides direct MS analysis of the eluted epitope peptides [1]; and (iii), a new proteolytic system with substantially improved efficiency of protease digestion. First clinical applications of the SPR-MS combination will be presented in Enzyme replacement therapy (ERT) which has been successfully developed for the treatment of lysosomal storage diseases (LSDs). While effective for a number of LSDs, severe therapeutic limitations for immunogenic LSDs are caused by the formation of neutralizing antibodies, which may be associated with allergic reactions, from mild symptoms to life threatening complications. The formation of antibody-enzyme complexes can prevent the uptake of enzymes into lysosomes and diminish the efficacy of ERT. At present there is poor understanding of the mechanisms of immune reactions and antibodies upon ERT, and there is currently no approach to modulate or prevent immune reactivity and impairment of therapy. Antibodies formed upon ERT bind to a defined epitope of the lysosomal enzyme. SPR-biosensor-MS analysis was successfully applied to the epitope elucidation and affinity characterization of antibodies from antisera of patients with Fabry’s Disease (alpha-galactosidase deficiency). A single sequence epitope peptide (αGal-309-322) was identified and prepared by solid phase peptide synthesis (SPPS). The peptide epitope showed high affinity binding to the antibody (KD ca. 40 nM), comparable to the affinity of the full length enzyme (16 nM) [2]. The identification of epitope(s) of lysosomal enzymes to neutralizing antibodies is opening new therapeutic approaches (i), by hyposensitizing patients from allergic reactions prior to ERT using stabilized synthetic epitope peptides, and (ii), by molecular apheresis in order to reconstitute therapeutic efficiency of ERT.

**References**

Cross-linking and Mass Spectrometry (XL-MS) have proved to be a powerful combination for the identification of protein-protein interactions in both functional and structural investigations. XL-MS provides data useful for interpreting the three-dimensional arrangement of protein complexes and when used as spatial restraints can help the fitting of subunits into structural models. Interest in XL-MS has increased with the growing popularity of cryo-electron microscopy (cryo-EM). In recent years, the structures of a number of complexes differing in subunit complexity and solubility (e.g. cytosolic or membrane bound proteins) have been solved by cryo-EM aided by XL-MS. Here I present how XL-MS provided key information for the structural characterisation of two different complexes: the membrane bound mitochondrial NADH:ubiquinone oxidoreductase (Complex I) and the soluble eukaryotic mRNA 3’-end processing machinery CPF (Cleavage and Polyadenylation Factor).

Mitochondrial complex I is the largest protein assembly of the respiratory chain with a total mass of 970 kDa. It transfers electrons from NADH to ubiquinone concomitant with the translocation of protons across the membrane. The combined approach of cryo-EM and XL-MS solved a nearly complete atomic structure to 3.9 Å resolution. All 14 conserved core subunits and 31 mitochondria-specific supernumerary subunits were unambiguously assigned using the data from XL-MS investigation and resolved within the characteristic L-shaped assembly. The hydrophilic matrix arm comprises one molecule of flavin mononucleotide and 8 iron–sulfur clusters involved in electron transfer, while the membrane arm contains 78 transmembrane helices, mostly associated with the antiporter-like subunits involved in proton translocation. Supernumerary subunits form an interlinked, stabilizing shell around the conserved core. The structure provides insight into the mechanism, assembly, maturation and dysfunction of mitochondrial complex I, and allows detailed molecular analysis of disease-causing mutations.

The cleavage and polyadenylation factor (CPF) is a ~1 MDa multiprotein complex responsible of the 3’ ends processing of newly transcribed eukaryotic precursor messenger RNAs (pre-mRNAs). CPF cleaves pre-mRNAs, adds a polyadenylate tail and triggers transcription termination. However, it is unclear how its various enzymes are coordinated and assembled. The combination of nanoESI-MS, cryo-EM, XL-MS described that the nuclease, polymerase, and phosphatase activities of yeast CPF are organized into three modules. Using electron cryomicroscopy aided by XL-MS, a 3.5 Å resolution structure of the ~200 kDa polymerase module was determined and validated. This revealed four β propellers, in an assembly markedly similar to those of other protein complexes that bind nucleic acid. Combined with in vitro reconstitution experiments, these data show that the polymerase module brings together factors required for specific and efficient polyadenylation, to help coordinate mRNA 3’-end processing.

References
Novel Cross-linkers to Study 3D-Structures of Proteins and Protein Complexes.

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Cross-linking/mass spectrometry (MS) is a powerful tool to derive 3D protein structural information and to investigate protein interaction networks. A chemical cross-linker acts as a kind of “molecular ruler” by connecting functional groups of amino acid side chains. These covalent connections then serve as basis for deriving distance constraints within the protein(s) under investigation and can be used for a computational modeling of protein structures.

While non-cleavable cross-linkers, such as bissulfosuccinimidyl suberate (BS3) or disuccinimidylsuserate (DSS), have been employed for more than 15 years for conducting structural studies of proteins, MS-cleavable cross-linkers are now moving more and more into the focus of the cross-linking/MS approach. Briefly, an MS-cleavable cross-linker exhibits a labile moiety, guaranteeing a specific fragmentation under collisional activation conditions. When MS-cleavable cross-linkers are employed, a characteristic product ion pattern is created and serves as basis for the unambiguous assignment of cross-linked amino acids. They have an enormous potential for a reliable, automated identification of cross-links and for conducting proteome-wide cross-linking studies.

Four novel MS-cleavable (photo)-cross-linking paradigms, developed by our group during the last months, will be presented. They include 2,2'-azobis(2-methylpropanimidate) (ABI)1,2 1,1-carbonyldiimidazole (CDI)3,4, diallylurea (DAU)5, and diazirine-based reagents6. With these reagents, amine, hydroxy, thiol, and carboxylic groups can be targeted in proteins.

References
Understanding your mAb

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Monoclonal antibodies (mAbs) are an increasing point of interest for biopharmaceutical companies. Last year the U.S. Food & Drug Administration approved 68 such therapeutics1 and more than 50 are being evaluated in late-stage clinical studies2. Most mAbs are produced from mammalian cultured cells using recombinant DNA technology. Due to the production process including cell culture, purification, formulation and storage, mAbs are subject to possible degradation and chemical modifications. This lack of purity and the inherent molecular heterogeneity makes mAbs very complex molecules. In order to characterize and monitor the modifications present in therapeutic antibodies, several analytical techniques are used, mass spectrometry being one of the most versatile. It allows users to obtain information about the mAbs purity, molecular weight, aminoacid sequence, post translational modifications (PTMs) among several other attributes. The possibility of analyzing many of these attributes simultaneously makes mass spectrometry an invaluable tool for the biopharma industry. For this reason mass spectrometry is rapidly being implemented in all stages of drug development.

References
Development of a nanoHPLC-HRMS method to quantify neuropeptides and analogues in bio-pathological samples

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GnRH, the gonadotropin-releasing hormones, are a group of endogenous neuropeptides involved in the sexual hormones equilibrium. There are 24 isoforms isolated from vertebrate and invertebrate species, and the N- and C-terminus regions, responsible for the receptor binding, are always conserved [1, 2].

The mammalian GnRH (mGnRH) is a 96 amino acids pro-hormone and through protease enzymes it is converted in a decapeptide. The neurohormone has a ultradian rhythm (30-120 minutes) with a serum concentration of 8-10 and 2-8 pg/ml for women and men respectively. During pregnancy GnRH passes the blood–brain barrier (BBB) and acts as sexual hormone in fetus development [3]. When an abnormal secretion of GnRH occurs, the whole organism is affected by reproductive system pathologies, such as hypothalamic amenorrhea and polycystic ovary syndrome (PCOS). Because of its very short half-life, the GnRH as is could not be used as drug in therapy. Instead, the synthetic molecule of GnRH, the gonadorelin, and analogues structures of the hormone, such as cetrorelix, are employed.

In our laboratory we developed a new nanoHPLC-HRMS analytical method to quantify the concentration both of GnRH and cetrorelix in different bio-pathological samples. The sample preparation work flow was the first crucial step. Protein removal and analytes isolation with SPE were optimized. For chromatography separation many stationary and mobile phases were tested to obtain the best performances. The HRMS parameters were changed to attain an appropriate lower limit of detection and quantitation of analytes.

With the developed method, we were able to measure the concentration of GnRH in PCOS women serum and cetrorelix in murine brain in the pg/ml range.

References
High-resolution MS in drug metabolism & pharmacokinetics: past, present and future

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High-resolution mass spectrometry (HRMS) is already for many years the analytical technique of choice for metabolite profiling and identification. The new generation HRMS systems now also offer the right performance for quantitative analyses, i.e., sensitivity, dynamic range, resolution, accuracy and scan-to-scan reproducibility, making them a worthwhile alternative for the ‘golden standard’ triple quadrupole MS systems. This provides a huge potential since quantitative and qualitative (quan-qual) information can be obtained from a single analysis but also requires a different mindset and expertise to make the right choices and compromises to obtain the best results.\(^1\)

While more and more smaller, cheaper and user friendly HRMS systems are available for quantitative and quan/qual analyses, the high end HRMS instruments are further advancing in resolving power or providing additional capabilities thanks to combinations with techniques such as ion mobility separation\(^2\) and infrared ion spectroscopy\(^3\).

In this presentation the added value of HRMS will be exemplified by real life examples in the different stages of the metID work flow where advanced selectivity and the ability to assign elemental compositions made a difference. Current trends such as the use of ultra high resolution MS and ion mobility separation in metabolism studies will be discussed as well as the potential of generating infrared spectra with the selectivity and sensitivity of mass spectrometry.

References
The Drug(s) withdrawn from the market due to unforeseen adverse events have triggered changes in the way New Chemical Entities (NCE) are discovered and developed. This has resulted in an emphasis on truly understanding the efficacy and toxicity profile of NCEs including contributions from their metabolites. Members of the pharmaceutical industry, scientific community and regulatory agencies have held dialogues, since 1999, with respect to metabolites in safety testing (MIST). In particular, recently issued regulatory guidelines on MIST, have attempted to provide a framework for conducting studies to ensure that the safety of metabolites found in humans is evaluated adequately in preclinical toxicological studies. In these documents regulatory agencies encourage an early understanding of the human metabolism, suggesting that human circulating metabolites above a defined threshold should be observed at equal or greater levels in preclinical species in order for them to be considered ‘covered’. Overall the regulatory guidelines have led to much discussion and debate on how to apply these recommendations in today’s resource-constrained pharmaceutical environment, considering also the potential contribution of relevant metabolites when conducting DDI risk assessment. The main challenge is how to balance the recommendation of front-loading of metabolism studies with the need to invest resources appropriately according to the stage of drug development. A suggested option is to adopt “alternative approaches”, early in the drug development process, able to generate reliable data for enabling prompt and informed decisions, without always resorting to resource-intensive validated bio-analytical assays or ADME studies in human and preclinical species, that traditionally were used to provide the initial information on metabolite exposure. In this presentation various “alternative approaches” for an early estimate (during Phase I studies) of the major human circulating metabolites and for the assessment of their toxicological cover in preclinical species, in the absence of synthetic metabolite reference standards, will be described.
KN3_S3

Application of Nano-Surface and Molecular-Orientation Limited (nSMOL) Proteolysis to LC/MS bioanalysis of Monoclonal Antibody drugs

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Therapeutic monoclonal antibodies (mAbs) are developed for treatment of diverse cancers and autoimmune diseases. For expansion of mAbs approval against unapproved diseases and pharmaceutical development, pharmacokinetics study is very important. Bioanalysis provides one of the most essential index against pharmacokinetics information. Current methods used for measuring drug concentration in blood is enzyme-linked immunosorbent assay (ELISA). However, there are critical issues with ELISA, including influences from cross-reaction and inhibitory materials. In contrast, by MS, analysis is performed based on the structural information. The LCMS analysis of high-molecular-weight proteins, such as antibodies, is normally performed after fragmentation of the protein into smaller peptides using a protease, such as trypsin or lysyl endopeptidase. However, this process also generates a large number of peptides including the signature peptides. These peptides increase the background noise and ionization suppression, and become a major cause of instability in the LCMS system. So far, we developed useful method for bioanalysis of mAbs in plasma or serum, nSMOL: nano-surface and molecular-orientation limited proteolysis. nSMOL can decrease these issues by selective proteolysis on the analytical target region of the antibody Fab. Therefore, the use of this approach can improve the reproducibility and robustness of the analytical system with maintaining antibody specificity. This method can provide accurate and reproducible value of mAbs content in plasma. We consider that nSMOL method will contribute to understanding of mAb PK data and therapeutic reference.
Ion Mobility Enabled Approaches to Drug Metabolism and Pharmacokinetics

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Of the three analytical S’s – speed, sensitivity and selectivity, the latter has had the greatest impact in drug metabolism studies, with the revolution of high resolution mass spectrometry, which now underpins much of the qualitative analysis undertaken in DMPK laboratories – and increasingly, quantitation as well. Ion mobility-enhanced high resolution mass spectrometry takes selectivity to an even higher level, with the concomitant measurement of collisional cross section (CCS), which can be used either as an additional, orthogonal, dimension of separation, or as a target-specific property allowing isobaric entities to be discriminated or tracked across differing analytical methodologies. Current generation platforms also leverage CCS separations to achieve improved quantitative outcomes, both in targeted and untargeted modes of operation. I will describe a variety of applications of IMS-HR-MS in the drug metabolism field, and share thoughts on where this field may take us in the future.
Investigation of the metabolic pathways of synthetic isoflavones following oral administration by gas chromatography coupled to high accuracy mass spectrometry

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Flavonoids are a broad class of low molecular weight compounds, characterized by a flavan nucleus. They are commonly found in plants and fruits and are commercially available and used as anti-oxidants, antiestrogenic agents and “anabolic promoters”, being promoted as such by fitness and supplements for bodybuilding websites [1-2]. Isoflavones are a group of flavonoids whose basic chemical structure consists of two benzyl rings joined by a three-carbon bridge, which may or may not closed in a pyran ring. These compounds may be of interest in sport doping because they can be used by athletes in recovering periods after the administration of anabolic steroids to increase the natural production of luteinizing hormone (LH) and consequently the synthesis of natural androgens.

In this work we have evaluated the \textit{in vivo} metabolism of two synthetic isoflavones, methoxyisoflavone (5-methyl-7-methoxy-isoflavone) and ipriflavone (7-isopropoxy-isoflavone) respectively present in a dietary supplement and in a pharmaceutical preparation. The \textit{in vivo} study was carried out by the analysis of urinary samples collected from male caucasican volunteers before, during and after single/multiple administration of methoxyisoflavone and ipriflavone. After enzymatic hydrolysis and liquid-liquid extraction, all urinary samples were analyzed by GC-q-TOF MS with electronic impact (EI) ionization. Eight metabolites of methoxyisoflavone and six metabolites of ipriflavone were isolated. The accurate mass spectra obtained are specific of isoflavones structure and revealed also a retro Diels-Alder fragmentation. When excreted in large amounts, the metabolites of methoxyisoflavone and ipriflavone can act as confounding factors in doping analysis. For indeed, being their retention times similar to those of some among the endogenous steroids normally screened for by the WADA-accredited laboratories, their presence in urine may make more problematic their correct quantification. For this reason their monitoring might be helpful in routine doping control analysis.

References
Protein biotherapeutics are rapidly becoming an important modality used to treat a diverse array of important diseases as well as a significant source of revenue to major pharmaceutical companies. They are evolving from previously called standard protein molecules, to more complex fusion proteins and complicated constructs. Up until recently the DMPK/ADME properties of standard biotherapeutics was thought to be understood as these were composed solely of natural amino acids, where end-stage peptide and amino acid catabolites do not generally pose toxicity concerns. As the complexity of the structure and mode of action of biotherapeutics is evolving the latter is no longer necessarily true and in addition peripherally generated intermediate circulating catabolites prior to end-stage degradation or elimination, may impact in-vivo stability, and potency/clearance. As of yet, contrary to small molecules, there are no harmonised methodologies for understanding in-vivo/vitro biotherapeutics ADME processes and its impact on drug discovery and development programs. Nor is there clear guidance from regulatory agencies on how and when these studies should be conducted.

In this keynote, a reflection is presented on the on-going journey to develop tools, technologies and approaches to investigate key factors that influence the ADME of recombinant biotherapeutics drugs, and demonstrate how ADME studies will facilitate their future development. Examples will be provided where a better understanding of ADME properties of novel biotherapeutics allowed for a better 1) evaluation on the impact on potency and clearance, 2) selection of most stable candidates or direct protein re-engineering efforts and 3) improved bioanalytical approaches for more robust drug quantification and subsequent reliable pharmacokinetic profiling and exposure assessment.
OC1_S4

Mass spectrometry-based characterization of human serum albumin heterogeneity in pharmaceutical formulations.

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Human serum albumin (HSA) is one of the most widely characterized proteins in the pharmaceutical field. HSA purified from plasma is used for therapeutic applications in case of shock, hypoproteinemia, burns and hyperbilirubinemia. Notwithstanding the therapeutic indication is mostly based on its oncotic capacity, recently the non-oncotic properties of HSA have raised scientists’ attention and administration of HSA is nowadays suggested in the case of life-threatening conditions in patients with decompensated cirrhosis¹. Since the intrinsic microheterogeneity of circulating HSA, as identified by liquid chromatography-mass spectrometry (LC-MS)-based studies,² can influence the final therapeutic effect, the structural integrity of HSA from different commercially available pharmaceutical formulations was investigated by a LC-MS method exploiting the high resolution capacity of the quadrupole-time of flight (Q-TOF) mass analyzer. Results showed that in commercial formulations HSA structure was significantly altered when compared to HSA from healthy volunteers. Furthermore in vitro studies highlighted that the antioxidant capacity worsened with increased structural modifications, potentially limiting its therapeutic effects. Since one of the most abundant alteration involves the cysteinylation of the Cys34, a high quality (hq)-HSA formulation, with fully reduced Cys34 residue, was obtained and characterized in terms of antioxidant activity and binding capacity.

Bibliography
KN2_S4

A nano-LC-MS approach for high sensitivity metabolite identification after IV microdose administration

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The development of a high sensitivity method using nano-liquid chromatography coupled with high resolution mass spectrometry is presented as a new strategy for the detection of metabolites after microdose administration. When a standard dose of 2-4 mg/kg is administered, a conventional liquid chromatography system equipped with a reverse phase column of a length between 50 mm and 100 mm and an internal diameter between 2.1 mm and 4.6 mm, is the technology of choice for metabolite identification and structural characterization. However, the standard analytical set-up might not always provide the required sensitivity when a low dose is administered (< 0.5 mg/kg). Therefore, high-sensitivity analytical methods are required to obtain definitive data. This work focused on the development of a high sensitivity method for metabolite identification and characterization using nano-liquid chromatography – high resolution mass spectrometry for the analysis of mouse plasma and brain samples collected after intravenous administration of an investigational compound. Samples were analyzed using both the standard UPLC-MS analytical conditions and the nano-LC-MS system, the latter providing a sensitivity gain of 10 fold or higher, allowing the detection of four additional low-abundant metabolites in brain samples. The advantages and disadvantages of the nano-LC approach for metabolite profiling were evaluated with particular attention to method robustness and suitability of the generated data to software-assisted data analysis.
OC2_S4

Quantitative LC-MRM/MS analysis of maternal serum proteins during pregnancy is capable to predict the risk of IUGR in preterm babies

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A multiplexing LC-MRM/MS assay was applied for distinguishing maternal peripheral blood serum samples of pregnant women that either belonged to the IUGR group (n=15) or to the control group (n=15) [1]. The first serum sample work-up (MS1) was done at the Proteome Center Rostock, Germany. Peptide mixtures (NAT peptides) were prepared according to previously published protocols [2]. In brief, after tryptic digestion, 15 µL of the NAT peptide solutions were added to 10 µL of re-solubilized SIS peptide mixtures (SIS peptide mixtures were previously prepared at the UVic Genome BC Proteomics Centre, Canada, and sent by post to the Proteome Center Rostock, Germany). The eluted peptide mixtures (containing NAT and SIS peptides) were lyophilized and transported at room temperature to the UVic Genome BC Proteomics Centre, Canada, for re-solubilization. For the second serum sample work-up (MS2), 10 µL of each serum sample were lyophilized at the Proteome Center Rostock, Germany. The dried proteins were then transported at room temperature to the UVic Genome BC Proteomics Centre, Canada, where they were re-solubilized and worked-up, again applying published protocols [2] on site. Both peptide sample sets were then subjected to LC-MRM/MS analysis following identical measurement protocols. The protein concentration data of the two different work-up procedures (MS1 and MS2) showed that both gave comparable results, indicating that either serum proteins or peptides therefore can be shipped unharmed at room temperature as lyophilized powders prior to LC-MRM/MS analysis. Of all 15 investigated proteins, the serum concentrations of apolipoprotein B100 showed the greatest power for discriminating IUGR from CTRL samples.

References
The risk of a critical error in MS/MS detection of ketamine metabolites

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Ketamine is a potent dissociative agent of common use in both human and, more recently, veterinary clinical practice. Ketamine is a chiral compound, and the two enantiomers show significantly different pharmacokinetic profile and pharmacodynamic activity [1]. The main metabolite of ketamine is norketamine (NK), which has similar activity to the parent drug, but a number of other compounds, including the enantiomeric dehydronorketamine (DHNK), results from ketamine and norketamine hydroxylation [2]. Due to its high selectivity, tandem mass spectrometry is often employed for the quantitative determination of ketamine and its metabolites in biological matrices. However, the structural similarity between norketamine and dehydronorketamine can represent a critical aspect. These molecules differ exclusively in two hydrogen atoms, but the resulting two-units-difference in their mass/charge ratio ([NK+H]+ = 224 m/z; [DHNK+H]+ = 222 m/z) is partially nullified by the isotopic abundance of the chlorine atom present in their structure. This, along with their similar fragmentation pattern (loss of -NH3 and -CO groups) and chromatographic retention, can result in the incorrect identification of these metabolites when the most abundant but shared transitions are monitored in LC-MS/MS. In order to avoid this problem, the key is using analyte-specific transitions (NK: 224>125 m/z; DHNK: 222>142 m/z) for quantification.

References
KN2_S4

Monitoring host and cellular changes in response to diseases and drugs: the proteomic challenge

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Mass spectrometry (MS) has become the predominant technology to analyze proteomes due to its ability to identify and characterize proteins and their modifications with high sensitivity and selectivity [1]. In particular, proteomics’ studies need to deal with several issues about complexity of matrices, sample preparation, and, hence variety of MS applications. Particularly, for bottom-up shotgun proteomics, optimal proteolytic peptide’ solutions are necessary to rule out drawbacks in both chromatography and MS. This task requires many pre-analytical steps such as protein extraction from cells or membranes, gel-based, solid phase extraction fractionation and depletion of most abundant proteins.

Therefore, sample preparation is a key step to obtain a good proteomic profile. Particularly, herein we present three different cases of analytical strategies: i) characterization of extracellular vesicles in the bone loss of osteoporotic patients; ii) serum biomarkers’ discovery in Pulmonary Arterial Hypertension (PAH)-affected patients; and iii) identification of bacterial surface virulence factors of carbapenem-resistant Acinetobacter baumannii (CRAb), agent of nosocomial infections. The use of MS in clinical studies can represent a powerful tool in the laboratory medicine procedures, starting from advanced translational medicine studies.

References
Contribution of LC-HRMS to structural characterization of two heterogeneous biological drugs: Orgaran and Copaxone

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The focus of this presentation is on the relevant contribution of high resolution mass spectrometry and accurate mass measurement in the elucidation and identification of numerous species contained in very complex biological drugs as it gives information about their molecular mass and structural features. Among the analytical approaches used for structure investigation, NMR spectroscopy and mass spectrometry (MS) represent the most effective techniques [1,2]. Especially, a growing interest has been registered in the last two decades in the application of MS to the analysis of this type of samples by increasingly mass spectrometers performances in conjunction to their direct connection to liquid chromatography systems (LC-MS) [3,4]. Two very interesting examples are reported regarding to the MS analysis of a mixture of low-molecular-weight glycosaminoglycans (Danaparoid, the active component of ORGARAN® marketed by Aspen) and a pool of synthetic polypeptides (Glatiramer acetate, the active component of COPAXONE® developed by Teva Pharmaceutical Industries). Danaparoid sodium salt is an anticoagulant and antithrombotic drug constituted of three GAGs obtained from porcine intestinal mucosa extracts: heparin sulfate, representing the major component, dermanan sulfate and chondroitin sulfate being the minor ones, and an average molecular weight of 50 kDa [5,6,7].

Glatiramer acetate (GA) is indicated for the treatment of patients with relapsing forms of multiple sclerosis [8]. The optimized methods resulted very efficient to compare competitor drugs to the commercial ones, verify the presence of impurities and ensure the drug safety.

References
Preclinical characterization of GABA<sub>B</sub> PAM COR659: In vitro metabolism and in vivo PK in rats

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GABA<sub>B</sub> positive allosteric modulators (PAMs) are a promising class of ligands with therapeutic potential for psychiatric disorders, such as anxiety, substance use and alcohol use disorder (AUD).

The compound COR659 [1] suppresses self-administration of alcohol in a rodent model of AUD (Sardinian alcohol-preferring sP rats) and of highly palatable food in Wistar rats. [2] In order to investigate its composite in vivo pharmacology, which could be related to an in vivo bioconversion, the metabolic fate of COR659, first in rat liver microsomes (RLM) and then in vivo was studied. Analysis, by HPLC/UV, of RLM incubates allowed the identification of several Phase I metabolites, one of them (M1) accounting for more than 50% of all other metabolite areas. The metabolite M1 was identified as the cleavage product of the methyl ester group at C-3 of the thiophene ring, leading to the acid COR740. Its formation was not observed in rat plasma or in –NADPH RLM incubates, suggesting an oxidative rather than hydrolytic biotransformation. The second most abundant metabolite in RLM (COR1110) was the oxidation product of the –CH<sub>3</sub> group at C-5 to –COOH. Other minor metabolites having M = M<sub>COR659</sub>+O (n=1); M+O-2H (n=2); M+2O-2H (n=2) were discovered by HR-MS (Δm<2 ppm). In vivo, 30 min after i.p. administration of a 10 mg/kg dose to rats, COR659 plasma levels reached a C<sub>max</sub> of 550 nM, and showed a biphasic PK profile. COR740 reached a plasma concentration of 1.3 μM, 15 min after administration, maintaining stable μM levels up to 24h from administration. The high levels of M1/COR740, even if inactive in vitro as GABA<sub>B</sub> PAM, could complicate the assessment of the in vivo pharmacology of parent compound; further chemical optimization of COR659 is thus needed to improve its PK profile.

References
Epitope identification and affinity determination of *Mycobacterium tuberculosis* Ag85B antigen towards anti-Ag85 antibodies from different sources

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Tuberculosis (TB) is the first cause of death from an infectious disease worldwide. Nowadays, only one anti-TB vaccine is available for clinical use, but various studies highlighted that its efficacy is not always achieved.

The aim of this work is to provide a basis for the rational design of a neo-glycoconjugate vaccine against TB.

After initial structural characterization of recombinant antigenic proteins from *Mycobacterium tuberculosis* Ag85B (wild-type, variants and semi-synthetic glycoconjugates), identification of antibody epitopes by proteolytic affinity-mass spectrometry and surface plasmon resonance (SPR) biosensor analysis were used to qualitatively and quantitatively characterize interactions of the antigens with antibodies from different sources. A commercial monoclonal antibody and polyclonal antibodies from patients with active TB, vaccinated individuals and a healthy control were employed to analyze antigen-antibody interactions. The combination approach provided the identification of different protein epitope regions involved in the interaction with specific antibodies, and a quantitative comparison between the affinities of the selected antigens.
SC3_S5

Serial micro sampling for mice PK studies of preclinical candidates

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 Advances in bioanalytical techniques allow the use of smaller sample volumes to assess the plasma concentration of drug candidates from preclinical standard and/or micro dose pharmacokinetic studies. In this presentation, we describe a methodology for serial blood collection in mice together with an efficient plasma preparation and analysis that allows the use of just 10 µL of plasma. This approach takes advantage of the Tecan D300e Digital Dispenser performance and of sensitive LC-MS/MS methods to deliver a reliable methodology with the following benefits: 3Rs improvement, time and money saving and higher PK data quality due to blood collection from the same animal.