Organizing Commettee

Ornella Curcuruto (Aptuit, Verona, Italy)

Fabio Bonelli (IRMB, Roma, Italy)

Valentina Mileo (Chiesi Farmaceutici, Parma, Italy)

Angelo Palmese (MerckGroup, Roma, Italy)

Simona Scarpella (Nerviano Medical Sciences, Milano, Italy)

Caterina Temporini (University of Pavia, Italy)

Scientific Commettee

Chiara Bigogno (Aphad, Milano, Italy)

Massimo Breda (Aptuit, Verona, Italy)

Marco Mor (Università degli Studi di Parma, Italy)

Mara Rossi (MerckGroup, Roma, Italy)

IMASS PHARMA NETWORK 2015

PROGRAM

Monday 26 October 2015

14.00 - 15.00 Registration

15.00 - 15.30 ***Opening Ceremonies***

Ornella Curcuruto and Ciriaco Maraschiello (Aptuit srl, Verona, Italy)

15.30 - 16.30 ***Opening lecture***

Andrea Chiesi (Chiesi Farmaceutici S.p.A., Parma, Italy)

Holoclar®, the first stem cell—based medicinal product

16.30 - 17.10 ***Coffee break***

MS in Pharma Research

Chairman: Simona Scarpella (Nerviano Medical Sciences, Milan, Italy)

17.10 - 17.40 ***Key Note 1***

Sebastien Brier (Institute Pasteur, Paris, France)

Probing hydrogen exchange in proteins by mass spectrometry

17.40 - 18.00 ***Oral 1***

Eleonora Calandra (Istituto di Ricerca Pediatrica, Padova, Italy)

The development of a matrix-assisted laser desorption/ionization (MALDI)-based analytical method for quantification of irinotecan in human plasma

18.00 - 18.20 ***Oral 2***

Laura Tigli (Chiesi Farmaceutici S.p.A., Parma, Italy)

Investigating pulmonary drug distribution through quantitative Imaging Mass Spectrometry approach

18.20 - 18.50 ***Plenary Lecture 1***

Mario Varasi (European Institute of Oncology, Milan, Italy)

(R)evolution in drug discovery

18.50 - 20.30 ***Cocktail and welcome concert by Associazione Studenti Musicologia e Beni Culturali, Università di Pavia***

Tuesday 27- October 2015

MS from Drug Discovery to Pharma Development

Chairman: Fabio Bonelli (IRBM, Roma, Italy)

9.00 - 9.40 ***Plenary Lecture 2***

Edith Monteagudo (IRBM, Rome, Italy)

Discovery of Grazoprevir (MK-5172) a Pangenotype Inhibitor of HCV NS3/4A Protease currently under U.S. FDA marketing approval in combination with Elbasvir

9.40 - 10.00 ***Oral 3***

Marco Michi (Aptuit Verona, Italy)

Development and validation of a challenging chiral assay for (S) and (R)-Pramipexol in human plasma by LC-MS/MS

10.00 - 10.20 ***Oral 4***

Chiara Bigogno (APHAD, Milan, Italy)

Evaluation of a New Microsampling Technique for Pharmacokinetic Characterization in the Drug Discovery Phase

10.20 - 10.40 ***Oral 5***

Leonardo Mendes de Souza Mesquita (Universidade Estadual Paulista 'Júlio de Mesquita Filho', san Paulo, Brasil)

Dereplication by FIA-ESI-IT-MSn of secondary metabolites from Rhizophora mangle and antioxidant activity

10.40 – 11.10 ***Coffee break***

Chairman: Caterina Temporini, University of Pavia, Italy

11.10 - 11.40 ***Key Note 2***

Koen Sandra (Metablys and RIC. Kortrijk, Belgium)

Chromatographic tools for the analytical characterization of mAbs and ADCs

11.40 - 12.00 ***Oral 6***

Sara Tengattini (Department of Drug Sciences, University of Pavia, Italy)

Rational design and analytical characterization of potential neo-glycovaccines against tuberculosis

12.00 - 12.20 ***Oral 7***

Federica Vacondio (Department of Pharmacy, University of Parma, Italy)

Antidepressant-like and cardioprotective activity of FAAH inhibitor URB694 in a murine model of social stress: a behavioral and HPLC-ESI-MS/MS study

12.20 - 12.50 ***Key Note 3***

Cinzia Stella (Genentech, San Francisco, USA)

Characterization of Oxidative Carbonylation on Recombinant Monoclonal Antibodies

13.10 - 14.30 ***Lunch***

13.10 - 14.30 ***Lunch Seminar Agilent***

Dr. Moritz Wagner (Market Specialist EMEAI)

High-throughput mass spectrometry as tool for Screening and ADME workflows

14.30 - 16.00 ***IMaSS members assembly***

16.00 - 16.40 ***Coffee break***

Chairman: Chiara Bigogno (APHAD, Milan, Italy)

16.40 - 17.00 ***Key note 4***

David Lascoux (Waters Corporation - EHQ France)

Complete characterization of mAb and ADC using Unifi system solution

17.00 - 17.20 ***Oral 8***

Luca Regazzoni (University of Milan, Italy)

Protein analysis by means of electrospray ionization mass spectrometry: applications in drug discovery, design and development.

Wednesday 28 October 2015

MS from Pharma Development to Pharma Launch

Chairman:Andrea Raffaelli, CNR Pisa, Italy

9.30 – 10.00 ***Key note 5***

Davy Guillarme (Universities of Geneva and Lausanne,CH)

Liquid chromatography and its application to the analysis of intact protein biopharmaceutics

10.00 – 10.20 ***Oral 9***

Matteo Stocchero (S-IN, Soluzioni Informatiche, Vicenza, Italy)

QbD principles in LC method development

10.20 - 10.40 ***Oral 10***

Manuela Bartolini (Department of Pharmacy and Biotechnology, University of Bologna,Italy)

On-Line Glycan Release and Analysis by An Integrated Pngase F-IMER LC-ESI-Q-Tof Approach

10.40 - 11.00 ***Coffee break***

Chairman: Angelo Palmese (MerckGroup Rome, Italy)

11.00 - 11.30 ***Key note 6***

Alain Van Doersselaer (CNRS - Institut Pluridisciplinaire Hubert Curien, Strasbourg, France)

Proteomics and native mass spectrometry for the characterization of biopharmaceuticals

11.30 - 11.50 ***Oral 11***

Roberto Fantozzi (Hamilton Robotics,Italy)

The role of automation in Mass Spectrometry

11.50 - 12.10 ***Oral 12***

Kerstin Pohl (Sciex Germany GmbH, Darmstadt, Germany)

Increased Confidence in Host Cell Protein Workflow: Biologics Done Right

12.10 - 12.30 ***Closing Remarks***

12.30 - 14.00 ***Light Buffet***

ABSTRACT

Key note 1

Probing hydrogen exchange in proteins by mass spectrometry

Darragh P. O'Brien[1], Karine Jain[2], Véronique Hourdel[1], Maxime Le Mignon[2], François Peurois[2], Véronique Bordas[2], Julia Chamot-Rooke[1], Daniel Ladant[2], Emmanuel Nony[2], Alexandre Chenal[1], Sébastien Brier[1]

[1]Institut Pasteur, UMR CNRS 3528, Chemistry and Structural Biology Department, 75015 PARIS, France (sebastien.brier@pasteur.fr);
[2]Stallergenes, Research and Pharmaceutical Development, 92183 ANTONY, France.

Hydrogen/Deuterium eXchange measured by Mass Spectrometry (HDX-MS) is a powerful tool to probe the structure and dynamics of proteins in solution. Significant improvements in the past decade have resulted in the technology becoming an invaluable resource in the pharmaceutical sector. In particular, the implementation of robotics for sample handling and preparation, and the automation of the labor-intensive data processing step have greatly expedited current HDX-MS strategies.

In this talk, a brief introduction to the technology will be provided, along with all of the major advances which have led to the streamlined HDX-MS workflow which is commercially available today. As an example, two applications will be discussed. Specifically, the utilization of HDX-MS for both epitope mapping and to probe conformational changes associated with ligand binding in a large intrinsically-disordered protein will be described in detail.

Oral 1

The development of a matrix-assisted laser desorption/ionization (MALDI)-based analytical method for quantification of irinotecan in human plasma

E. Calandra [1], S. Crotti [1], G. Toffoli [2], M. Agostini [1,3], P. Traldi [1,4]

1. Istituto di Ricerca Pediatrica – Città della Speranza, Corso Stati Uniti 4, 35127 Padova, Italy (eleonora.calandra@irpcds.org);
2. Experimental and Clinical Pharmacology Unit, Centro di Riferimento Oncologico, IRCCS National Cancer Institute, Via Franco Gallini 2, 33081 Aviano, Italy;
3. First Surgical Clinic Section, Department of Surgical, Oncological and Gastroenterological Sciences, University of Padova, Via N.Giustiniani 2, 35128 Padova, Italy;
4. IENI CNR, Corso Stati uniti 4, 35127 Padova, Italy.

Inter-individual variability in pharmacokinetics, receptor sub-types or genetic polymorphisms in transporters, enzymes and targets could lead in reduced or no clinical benefit after drug administration. Thus therapeutic drug monitoring (TDM) is becoming necessary to prevent the onset of side effects due to the use of pharmaceutical products with narrow therapeutic range.
However the use of TDM in clinical practice is still limited because currently employed methodologies to perform pharmacokinetic measurements (liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and immunobinding assay) exhibit some weak points. Indeed, LC-MS/MS is time consuming and requires high operator expertise whereas immunobinding assays are not able to discriminate between the molecule of interest and its analogues.
In this view laser desorption/ionization (LDI) mass spectrometry techniques seem suitable to overcome these limits, since they require easy sample preparation and faster analysis time.
In our work we tested different laser desorption/ionization methods, both surface-assisted and matrix-assisted, in order to select the most suitable approach for quantitative analysis. The best results in terms of intensity and reproducibility were obtained with MALDI using alpha-cyano-4-hydroxycinnamic acid as matrix[1]. After the evaluation of suitability of the technique for performing in-plasma measurements of irinotecan concentration, the method was successfully applied, in blind, to a series of patient's plasma sample[1,2]. The pharmacokinetic data obtained were in agreement with those obtained with LC-MS/MS measurement.

References

[1] E. Calandra, S. Crotti, M. Agostini, D. Nitti, M. Roverso, G. Toffoli, E. Marangon, B. Posocco, P. Traldi Eur. *J. Mass Spectrom.* 2014, **20**(6), 445-59.
[2] E. Calandra, S. Crotti, D. Nitti, M. Roverso, G. Toffoli, E. Marangon, B. Posocco, P. Traldi, M. Agostini *J. Mass Spectrom.* 2015, **50**(7), 959-62.

Oral 2

Investigating pulmonary drug distribution through quantitative Imaging Mass Spectrometry approach

Riccardo Zecchi[a], Laura Tigli[b], Marcello Trevisani[c], Maria Pittelli[c], Pamela Pedretti[c], Maria Elena Manni[c], Pietro Franceschi[e], Valentina Mileo[b], Elisa Moretti[b], Barbara Pioselli[b], Pamelard Fabien[d], Jonathan Stauber[d], Giuseppe Pieraccini[a], Gloriano Moneti[a], Silvia Catinella[b]

[a] *Mass spectrometry center CISM - University of Florence, Florence, Italy;*
[b] *Chiesi Farmaceutici SpA, Corporate Preclinical R&D - Analytics and Early Formulations Department, Parma, Italy;*
[c] *Chiesi Farmaceutici SpA, Corporate Preclinical R&D - FABER Project Group, Florence, Italy;*
[d] *ImaBiotech SAS, Loos, France;*
[e] *Edmund Mach Foundation, San Michele all'Adige (Trento), Italy.*

Objective: The development of matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) has set a new frontier for the study of distribution of pharmaceutical compounds within organs and tissues. In the present work we applied MALDI-IMS to investigate the distribution of tiotropium bromide (TIO) inside guinea pig lung tissues, comparing three different administration methods: nebulized aerosol, intratracheal administration of aqueous drug solutions and intratracheal administration of dry powder formulation. In order to obtain a better comprehension of the real quantities of delivered tiotropium inside the different pulmonary districts, we applied a quantitative approach based on internal standard method.
Method: Healthy guinea pigs were administered with TIO using dosages that bring comparable anti-bronchoconstrictive effect. Tissues sections of 20 µm thickness were coated by ImagePrep with HCCA and aniline matrix solution, containing Ipratropium bromide (IPT) as internal standard. Data were acquired in high resolution full scan mode on a MALDI-LTQ-Orbitrap XL and processed using Quantinetix software. LC-MS dosages on remaining lung tissues were conducted using IPT as internal standard; all the samples were acquired on a Nexera UHPLC coupled with a Q-Exactive.
Results: Qualitative results achieved by MALDI-IMS clearly underlined important differences in the pulmonary distribution of TIO among the three administration methods. The application of a quantitative approach allowed us to better understand which is the real drug delivery inside alveolar tissue micro-compartments, the target area of inhalatory drugs.
Conclusions: The good correlation between IMS and LC-MS quantitation assured us a robust and reproducible method to investigate the delivery efficacy of novel inhalatory devices for pulmonary drug administration. Quantitative IMS application gives the uneven advantage to evaluate the real tiotropium delivery for every tissue micro-compartment inside the lung.

Plenary Lecture 1

(R)evolution in drug discovery

Mario Varasi

Academic Drug Discovery Program, European Institute of Oncology,
via Adamello 16, Milan, Italy

The drug discovery & development is a long, expensive and high risk process. Pharmaceutical R&D evolved from being a chemistry inspired-pharmacology driven to a biology inspired-chemistry driven process, leading to the development of molecular targeted therapies. This evolution was a consequence of a less empirical approaches in the search of new drugs based on an increased knowledge of the biological targets, as well as of advances in synthetic and purification technologies, increased input from structural chemistry and modeling tools, enormous progress in the field of mass spectrometry and nuclear magnetic resonance, and computer-assisted literature and data retrieval and analysis.

Nevertheless, in the last decades , while R&D spending was growing, we faced relative low productivity and lack of innovation with a significant decrease in the rate that new drug candidates are being translated into effective therapies in the clinic.

A certainly not exhaustive list of causes of the relative decline in productivity of the pharmaceutical industry includes: the nature of diseases being investigated, the increase regulatory and testing requirements with more evidence for efficacy and safety, the restriction put in place by pricing and reimbursement authorities and manage-care organizations, CEOs and other high-level executives too often with little or no scientific/technical experience, pressures from shareholders for quick return, conformism (benchmarking mania) and merger mania. There has been a worrying rise in late-stage attrition in phase 2 and phase 3. The two single most important reasons for attrition in clinical development are lack of efficacy and clinical safety or toxicology, believed to have their “preclinical” origin in the lack of accurate and efficient methods for target identification and (continuous) validation process, and shortage of tools for patient stratification. All these aspects represent the great defiance being faced in our day.

The sequencing of the human genome was a significant landmark, although turning this data into information useful for drug discovery revealed to be much more complicated than expected, raising several genomic and post-genomic (e.g. epigenetic, non coding DNA) challenges. These challenges represent at the same time great opportunities for innovative therapies for several diseases. Relevant developments in the understanding of the structure and function of the human genome and epigenome have occurred that bring closer to becoming a reality the so called “personalized medicine”: to provide "the right patient with the right drug at the right dose at the right time”.

All these efforts are directed, at least in part, to disprove what Mr. Jean-Baptiste Poquelin, french actor & comic dramatist, said many years ago:

“Doctors pour drugs of which they know little, to cure diseases of which they know less, into human beings of whom they know nothing”.

Plenary Lecture 2

Discovery of Grazoprevir (MK-5172) a Pangenotype Inhibitor of HCV NS3/4A Protease currently under U.S. FDA marketing approval in combination with Elbasvir

Edith Monteagudo

IRBM, Rome, Italy

HCV NS3/4a protease inhibitors are proven therapeutic agents against chronic hepatitis C virus infection. Overcoming antiviral resistance, broad genotype coverage, and a convenient dosing regimen are important attributes for future agents to be used in combinations without interferon. In this communication, we report the discovery and preclinical development of MK-5172, a novel P2-P4 quinoxaline macrocyclic NS3/4a protease inhibitor under NDA approval in combination with Elbasvir. The compound demonstrates subnanomolar activity against a broad enzyme panel encompassing major hepatitis C virus (HCV) genotypes as well as variants resistant to earlier protease inhibitors. In replicon selections, MK-5172 exerted high selective pressure, which yielded few resistant colonies. In both rat and dog, MK-5172 demonstrates good plasma and liver exposures, with 24-h liver levels suggestive of once-daily dosing. When administered to HCV-infected chimpanzees harboring chronic gt1a or gt1b infections, MK-5172 suppressed viral load between 4 to 5 logs at a dose of 1 mg/kg of body weight twice daily (b.i.d.) for 7 days. Based on its preclinical profile, MK-5172 was anticipated to be broadly active against multiple HCV genotypes and clinically important resistance variants and highly suited for incorporation into newer all-oral regimens.

Oral 3

Development and validation of a challenging chiral assay for (S) and (R)-Pramipexol in human plasma by LC-MS/MS

M. Michi

Aptuit, Pharmaceutical & Preclinical Development, via Fleming 4 37135 Verona Italy

A chiral liquid chromatographic method for the enantiomeric resolution of Pramipexole (6*S*)-4,5,6,7-tetrahydro-N6-propyl-2,6-benzothiazole-diamine dihydrochloride monohydrate, a dopamine agonist for the treatment of Parkinson's disease was developed and validated in human plasma. Several methods are available in literature but none of them was able to separate and quantify the *S* enantiomer at sub nanogram levels in the presence of a high concentration of the *R* enantiomer. The challenge was not only related to the high sensitivity required for a chiral separation but also to the order of elution of the two enantiomers as the *S* form was eluted on the tail of the big peak of the *R* enantiomer on each of the several chiral columns tested. The final LLQ validated for (S)-Pramipexol was 0.1 ng/mL in the presence of 2000 ng/mL of (R)-Pramipexol.

Oral 4

Evaluation of a New Microsampling Technique for Pharmacokinetic Characterization in the Drug Discovery Phase

C. Bigogno[1], U. Bastrup[1], G. Dondio[1] Aphad Srl, Via della Resistenza 65, 20090 Buccinasco (MI), Italy. c.bigogno@aphad.eu, A. Chiorazzi[2], V.A. Carozzi[2], C. Meregalli[2], A. Canta[2] N. Oggioni[2], G. Cavaletti[2] and M. Pinza[1], Department of Surgery and Translational Medicine, University of Milano-Bicocca, via Cadore 48, 20900 Monza (MB), Italy

The application of multiparametric approaches that simultaneously optimize activity, selectivity, physicochemical properties and *in vitro* ADME properties, resulted in an increased number of potential drug candidates for early pharmacokinetic characterization.
On the other hand, there is a strong demand from several institutions and in particular from the UK national organization NC3Rs [1] for the application of new technologies and approaches to replace, reduce and refine (3R) the use of animals for scientific purposes.
Microsampling may be regarded as a modern and ethical technique able of serving the needs of pharmacokinetic characterization of novel chemical entities (NCEs) and in line with the 3R principles regarding the animal experimentation in all the phases of the drug discovery process.
A novel microsampling technology Mitra™ was designed to allow a precise volumetric absorptive microsampling that substantially reduces the volumetric bias caused by sampling blood samples with different hematocrit as in dried blood spots (DBS) [2].
Comparison of the set-up of a Multiple Reaction Monitoring (MRM) method for a PK study of a NCE using classical serial sampling *vs.* a serial sampling using Mitra™ devices will be presented highlighting the pros and cons of the new technique in terms of LOQ, matrix effect and recovery. The use of Mitra™ for analysis of compounds unstable in plasma will also be discussed.

[1] hiip://www.nc3rs.org.uk/
[2] Neil Spooner, Philip Denniff, Luc Michielsen, Ronald De Vries, Qin C Ji, Mark E Arnold, Karen Woods, Eric J Woolf, Yang Xu, Valérie Boutet, Patricia Zane, Stuart Kushon, James B Rudge. A device for dried blood microsampling in quantitative bioanalysis: overcoming the issues associated with blood hematocrit. *Bioanalysis*. 2015 7(6), **653–659**.

Oral 5

Dereplication by FIA-ESI-IT-MS^n of secondary metabolites from *Rhizophora mangle* and antioxidant activity

Leonardo Mendes de Souza Mesquita[1], Caroline Fabri Bittencourt Rodrigues[1], Cláudia Quintino da Rocha[1], Henrique Hessel Gaeta[1], Mariana Novo Belchor[1], Marcos Hikari Toyama[1], Wagner Vilegas[1]

1. Biosciences Institute, São Paulo State University - UNESP, Coastal Campus. – Bioprospection and natural products laboratory – mesquitalms@gmail.com - Pça Infante Dom Henrique S/N – zip code: 11330-900 – São Vicente, SP (Brazil).

Rhizophora mangle L. is a coastal species, natural from mangrove ecosystems, is commonly used by traditional populations, especially for the treatment of diabetes, analgesia, ulcers and colitis [1]. The aim of this study was to evaluate the acetonic extracts of the stem bark in relation to their chemical characterization by mass spectrometry and antioxidant activity. The collections of the stem barks from the specimens were conducted between the months of January and March of 2015, in mangroves of Santos (São Paulo - Brazil). The material was dried and grinded in a Wiley mill. Extraction was carried out by maceration, soaking the particulate material in acetone (70%). The extract was solubilized in water fractionated by liquid-liquid partition using hexane, ethyl acetate and butanol, in this sequence. Full scan ESI-MS and collision induced dissociation were performed on a mass spectrometer equipped with an ion trap analyser (Parameters of Direct injection: flow rate: 5µL/min, capillary temperature: 270°C, sheath gas (nitrogen) flow rate 80 (arb) and auxiliary gas flow rate was 5 (arb)), data were acquired in the negative ion MS and MS^n mode. In order to evaluate the variation of *R. mangle* extracts, PCA was carried out using Primer 6. The antioxidant test of the extract and fractions were compared to the standards quercetin and gallic acid, following the protocol of DPPH assay (scavaning activity percentage) [2]. The principal component analysis revealed that the hexane fraction does not show similarity with the other fractions. The total extract shows 40% similarity with the ethyl acetate fraction. The butanol and aqueous fraction gathered in 20% similarity. The full characterization of the extract showed the presence of phenolic compounds derived from catechin, quinic acid and anthocyanin. The derivatives of catechins (*m/z* 289) occurs on its polymeric form (condensed tannins), dimer (*m/z* 577) (Fig. 1A), trimer (*m/z* 865) and tetramers (*m/z* 1153). Glycosylated dimers were also detected (*m/z* 739) (Fig. 1C). All catechins derivatives were identified in the ethyl acetate fraction and butanol. Catechins show gastrointestinal protection, antiulcer, anti-inflammatory and antioxidant effects [3]. Caffeoylqunic acids (*m/z 515* and *m/z* 353) (Fig. 1B) were detected in the total extract and in the ethyl acetate fraction. There are several advantageous health properties associated to this class of compounds, such as anti-inflammatory, and against diabetes type 2 and Alzheimer's disease [4]. The acetone extract showed higher antioxidant activity (IC_{50}: 1.58), than the quercetin (IC_{50}: 3.15) and gallic acid (IC_{50}: 2.53) standards. The hexane fraction does not show antioxidant capacity. Ethyl acetate (IC_{50}: 2.53) and butanol (IC_{50}: 3.79) fractions have high

antioxidant capacity. The aqueous fraction, despite having an IC_{50}: 6.76, does not show a prononced activity when compared to the extract and fractions ethyl acetate and butanol. It is reported that tannins have a high antioxidant capacity. Condensed tannins from two mangrove species (*Kandelia candel* and *Rhizophora mangle*) showed significantly higher percentage inhibition of DPPH radical compared to ascorbic acid [2]. Thus, *R. mangle* extract has medicinal interest molecules. Caffeoylqunic acids derivates were not previously reported in the genus *Rhizophora* and have interesting medicinal properties. So, it is a plant with proven medicinal properties, but there is still no detailed studies on their chemical characterization, and probably there are medicinal properties still unknown.

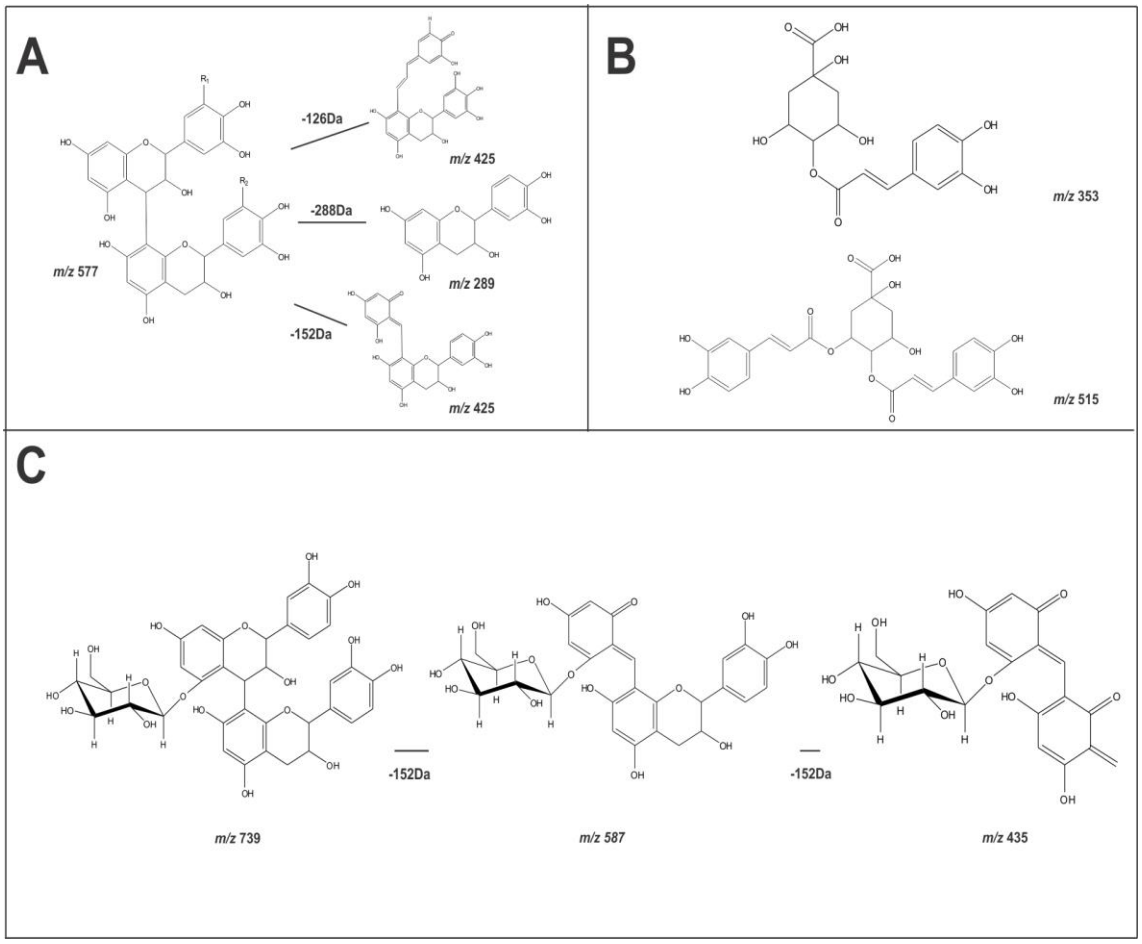


Fig. 1 **(A)** dimers of catechins and possible fragmentations; **(B)** caffeoylquinic acids derivates; **(C)** domers of catechins glycosides and possible fragmentation.

[1] de Faria, F.M. et al *Evid Based Complement Alternat Med.* 2012, **11**, pages.
[2] Sochor, J. et al *Molecules*. 2010, **23**, pages.
[3] Zhang, L-L. et al *Molecules*. 2010, **12**, pages.
[4] Gouveia, S.C. et al *Food res int*. 2012, **7**, pages.

Key note 2

Strategies for the characterization of protein biopharmaceuticals

Koen Sandra

R&D Director Life Sciences, Metablys, President Kennedypark 26, 8500, Kortrijk, Belgium, T +32(0)56204031, F +32(0)56204859, info@richrom.com, www.richrom.com, koen.sandra@richrom.com

Monoclonal antibodies and recombinant proteins have emerged as important therapeutics for the treatment of various life-threatening diseases including cancer and autoimmune diseases. Protein biopharmaceuticals have a complexity far exceeding that of small molecule drugs, hence, unraveling this complexity represents an analytical challenge. The present contribution reports on the power of liquid chromatography and mass spectrometry in the characterization of protein biopharmaceuticals and biosimilars.

Oral 6

Rational design and analytical characterization of potential *neo*-glycovaccines against tuberculosis

S. Tengattini [1], C. Temporini [1], T. Bavaro [2], L. Piubelli [3], F. Rinaldi [1], F. Mangione [4], A. Pedrali [1], G. Marrubini [1], I. Serra [2], G. Speranza [5], L. Pollegioni [3], M. Terreni [2], G. Massolini [1]

1. Department of Drug Sciences, University of Pavia, viale Taramelli 12, Pavia, Italy (presenting author: sara.tengattini01@ateneopv.it); 2. Italian biocatalysis center, University of Pavia, viale Taramelli 12, Pavia, Italy; 3. The Protein Factory, Interuniversity Centre Politecnico di Milano, ICRM CNR Milano and University of Insubria, Milan, Italy; 4. Department of Infection Diseases, Fondazione IRCCS-Policlinico San Matteo, viale Golgi 19, Pavia, Italy; 5. Department of Organic and Industrial Chemistry, via Golgi 19, University of Milan, Milan, Italy.

Designing a second-generation vaccine against *Mycobacterium tuberculosis* (MTB) is an international research priority as tuberculosis remains one of the leading cause of morbidity and mortality in the world.

The focus of this work is the rational design and the characterization of glycoconjugate vaccines obtained by conjugation of highly immunogenic proteins over-expressed by MTB with glycocomponents based on the glycoportion exposed on bacterial cells, named lipoarabinomannan (LAM). The glycosylation of immunogenic proteins can in fact result either in a co-adjuvant effect, or in an additional stimulation of humoral response over the T-cell mediated one.

Two proteins, namely TB10.4 (11 kDa) and Ag85B (31 kDa), were selected and produced by recombinant DNA technology. An optimization of protein preparation protocol, followed by direct infusion (DI) in ESI-MS, allowed us to obtain TB10.4 and Ag85B preparation higly pure and enough stable to be glycosylated [1].

A convergent synthetic approach, in which the unmodified protein is conjugated with saccharides previously activated, was selected as coupling strategy and two chemical activations, namely homobifunctional (4-nitrophenyl ester) glycoside (pNO_2) and iminomethoxyethyl thioglycoside (IME), were considered. Both the conjugation methods may involve different surface amino acids and then lead to a mixture of glycoproteins with variable saccharide loading number and positioning.

To compare the two activations, a MS-based approach was developed. The combination between DI-MS and *on-line* SPE-LC-MS^n peptide mapping allowed the systematic characterization of the reactivity of the surface amino acids in TB10.4 glycosylation. Significantly different chemoselectivity and glycosylation efficiency were demonstrated for the two chemical functionalization and, based on the results, IME activation was selected for the glycosylation of the antigenic proteins [2].

A rational design of experiments (DoE) was then carried out to optimize the coupling reaction and identify the suitable experimental conditions for the preparation of highly pure and homogeneous glycoconjugates [2].

Under optimized conditions TB10.4 and Ag85B were coupled with mono- and disaccharides [mannose, mannose(1→6)mannose, arabinose(1→6)mannose] and the resulting *neo*-glycoproteins characterized using the previously developed analytical approach.

In order to achieve a comprehensive knowledge of product composition, a stability evaluation of antigenic proteins under glycosylation conditions was performed by capillary electrophoresis (CE)-high resolution (HR)-MS, resulting in the identification, based on the accurate mass shown in the deconvoluted spectra, of synthetic impurities and degradation products.

In parallel, to obtain a fast and easy determination of glycosylation yield and glycoform composition, an HILIC-UV-MS method was developed for the analysis of intact *neo*-glycoproteins. The method was optimized using a model glycoprotein, Ribonuclease B (RNase B) [3], and applied to the characterization of the glycoderivatives of TB10.4 and Ag85B.

Finally, after their complete characterization, the antigenic proteins and their glycoconjugates were evaluated for their immunogenicity by *ex-vivo* assays. The observed biological activity was correlated with the preservation of amino acids *in-silico* identified as involved in the epitope formation and the results led to the rational design and the production of mutated species of Ag85B with a potentially better activity profile.

References

[1] L. Piubelli, M. Campa, C. Temporini, E. Binda, F. Mangione, M. Amicosante, M. Terreni, F. Marinelli, L. Pollegioni *Microbial Cell Factories* 2013, **12**, 115.

[2] C. Temporini, T. Bavaro, S. Tengattini, I. Serra, G. Marrubini, E. Calleri, F. Fasanella, L. Piubelli, F. Marinelli, L. Pollegioni, G. Speranza, G. Massolini, M. Terreni *J. Chromatogr. A* 2014, **1367**, 57-67.

[3] A. Pedrali, S. Tengattini, G. Marrubini, T. Bavaro, P. Hemström, G. Massolini, M. Terreni, C. Temporini *Molecules*, 2014, **19**, 9070-9088.

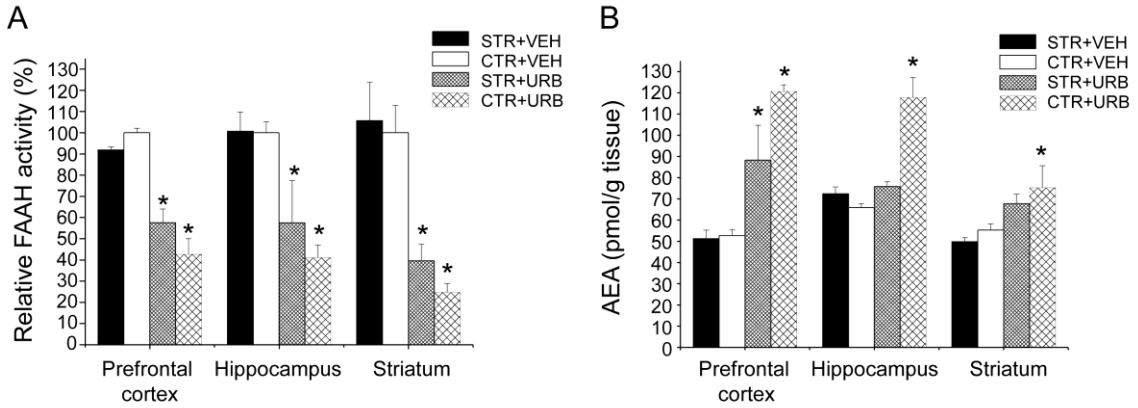
Oral 7

Antidepressant-like and cardioprotective activity of FAAH inhibitor URB694 in a murine model of social stress: a behavioral and HPLC-ESI-MS/MS study

F. Vacondio [1], L. Carnevali [2], A. Sgoifo [2], G. Spadoni [3], A. Bedini [3], S. Rossi [4], S. Callegari [5], E. Macchi [4], S. Rivara [1] and M. Mor [1]

1. Department of Pharmacy, University of Parma, Parco Area delle Scienze 27/a, 43124 Parma, Italy (federica.vacondio@unipr.it);
2. Department of Neuroscience, University of Parma, Parma, Italy;
3. Department of Biomolecular Sciences, University of Urbino "Carlo Bo", Urbino, Italy;
4. Department of Life Sciences, University of Parma, Italy; 5. Division of Cardiology, Vaio Hospital, Fidenza, Parma, Italy.

The endocannabinoid (eCB) system plays an important role in the physiology and behavioral expression of stress responses. Stress evokes bidirectional changes in the two eCB transmitters, arachidonoylethanolamide (anandamide, AEA)[1] and 2-arachidonoylglycerol (2-AG)[2], with stress exposure reducing AEA levels and increasing 2-AG levels. Additionally, exposure to chronic stress causes a down regulation or loss of cannabinoid type 1 (CB_1) receptors. Decline in AEA appears to contribute to the manifestation of the stress response, including activation of the hypothalamic-pituitary-adrenal (HPA) axis[3]. The enzyme Fatty Acid Amide Hydrolase (FAAH)[4], a mammalian member of the 'amidase signature' family, terminates the action of AEA catalyzing its hydrolytic cleavage. FAAH inhibition can therefore represent a useful strategy to upregulate anandamide signaling at cannabinoid receptors in those districts in which it is needed.



In the last decade, our group focused on the class of cyclohexylcarbamic acid biphenyl-3-yl esters, as potent and selective FAAH inhibitors[5,6]. Structure-property studies showed that the introduction of a small polar electron-donating group (i.e. OH) at the para position of the proximal phenyl ring, as in URB694, allowed to retain FAAH inhibitory potency, while increasing in vitro hydrolytic stability; this turned into an

improved in vivo distribution and selectivity towards known off-targets of carbamate-based inhibitors such as liver carboxylesterases[7,8].

In the present study, the antidepressant-like and cardioprotective effects of URB694 were evaluated in socially stressed Wistar Kyoto (WK) rats[9]. Male rats were exposed to five weeks of repeated social stress or control procedure. Starting from the third week, they received daily administration of URB694 (0.1 mg/kg, i.p.) or vehicle. A fast and sensitive HPLC-ESI-MS/MS method was set up and validated for the quantification of the endocannabinoid AEA, the related non-cannabinoid fatty acid amides oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) and the stress biomarker corticosterone (CST) in WK rat plasma. AEA, OEA and PEA levels were also dosed and the activity of FAAH enzyme was monitored in those rat brain regions (hippocampus, striatum, prefrontal cortex) involved in the regulation of behavioral responses to stress and in the heart (atria and ventricles). The social stress protocol induced in rats, if compared to controls, changes that recalled the symptoms of human depression such as reduction in body weight gain and in sucrose preference, increased immobility, hyperactivity of the HPA axis, as revealed by a marked increase of CST plasma levels. The group of stressed rats showed also marked alterations in the heart function, including a larger incidence of spontaneous arrhythmias. Daily treatment with URB694 normalized plasma CST levels, increased brain, plasma, atrial and ventricular levels of AEA, and, more importantly, protected the heart against the adverse effects of social stress.

Taken together, these biochemical and behavioral data suggest that FAAH inhibition could represent a promising new strategy for the treatment of depression/cardiovascular comorbidity under chronic stress conditions.

[1] W. A. Devane, L. Hanuš, A. Breuer, et al. *Science* 1992, **258**, 1946-1949.
[2] R. Mechoulam, S. Ben-Shabat, et al. *Biochem. Pharmacol.,* 1995, **50**, 83–90.
[3] C. J. Riebe, C. T. Wotjak *Stress*, 2011, **14**, 384-397.
[4] F. Desarnaud, H. Cadas, D. Piomelli *J. Biol. Chem.* 1995, **270**, 6030-6035.
[5] S. Kathuria, S. Gaetani, D. Fegley, et al. *Nat. Med.* 2003, **9**, 76–81.
[6] M. Mor, S. Rivara, A. Lodola, et al. *J. Med. Chem.* 2004, **47**, 4998–5008.
[7] F. Vacondio, C. Silva, A. Lodola, A. Fioni, et al. *ChemMedChem* 2009, **4**, 1495-1504.
[8] J. R. Clapper, F. Vacondio, A. R. King, A. Duranti, et al. *ChemMedChem* 2009, **4**, 1505-1513.
[9] L. Carnevali, F. Vacondio, S. Rossi, S. Callegari, et al. *Eur. Neuropsychopharmacol.* 2015, doi: 10.1016/j.euroneuro.2015.07.015.

Key note 3

Characterization of Oxidative Carbonylation on Recombinant Monoclonal Antibodies

C. Stella [1], Y. Yang[1], W. Wang[1], C. Schoneich [2], L. Gennaro[1]

1. Genentech Inc., South San Francisco, California 94080, USA (stellac@gene.com);
2. University of Kansas, Lawrence, Kansas 66047, USA

In the biotechnology industry, oxidative carbonylation as a post-translational modification of protein pharmaceuticals has not been studied in detail. Using Quality by Design (QbD) principles, understanding the impact of oxidative carbonylation on product quality of protein pharmaceuticals, particularly from a site-specific perspective, is critical. However, comprehensive identification of carbonylation sites has so far remained a very difficult analytical challenge for the industry. In this paper, we report for the first time the identification of specific carbonylation sites on recombinant monoclonal antibodies with a new analytical approach via derivatization with Girard's Reagent T (GRT) and subsequent peptide mapping with high-resolution mass spectrometry. Enhanced ionization efficiency and high quality MS2 data resulted from GRT derivatization were observed as key benefits of this approach, which enabled direct identification of carbonylation sites without any fractionation or affinity enrichment steps. A simple data filtering process was also incorporated to significantly reduce false positive assignments. Sensitivity and efficiency of this approach were demonstrated by identification of carbonylation sites on both unstressed and oxidized antibody bulk drug substances. The applicability of this approach was further demonstrated by identification of 14 common carbonylation sites on three highly similar IgG1s. Our approach represents a significant improvement to the existing analytical methodologies and facilitates extended characterization of oxidative carbonylation on recombinant monoclonal antibodies and potentially other protein pharmaceuticals in the biotechnology industry.

Lunch Seminar

High-throughput mass spectrometry as tool for Screening and ADME workflows

M. Wagner

Agilent Technologies, Hewlett-Packard-Str. 8, 76337 Waldbronn, Germany
moritz_wagner@agilent.com

The RapidFire High-throughput Mass Spectrometry System provides drug discovery researchers with mass spectrometry-based, high-throughput screening solutions for targets that have proven challenging to screen using conventional approaches. These intractable targets have substrates and products that are either too small to label or undergo modifications that are difficult to detect.
Furthermore, the same technology can be applied to a variety of in vitro ADME assays such as cytochrome P450, permeability and protein interaction assays. The desire to eliminate weak candidates at earlier phases of the drug discovery process has caused *in vitro* ADME analysis to shift earlier as well, resulting in the need to evaluate a larger number of samples. Therefore, an efficient means of analyzing these assays, in a fast and cost-effective manner is required.
Agilent RapidFire/MS systems combine high-throughput sample processing with triple quadrupole (QQQ) or time-of flight (TOF) mass spectrometry (MS) to streamline drug discovery analysis processes, herewith presenting the latest findings and application examples.

Key note 4

Complete characterization of mAb and ADC using Unifi system solution

Lascoux David

Biopharmaceutical Business Development Manager, Europe and India, WATERS

These last 20 years the pharmaceutical market did change extensively. News drugs based on protein are now coming and become widely produced and delivered. These news bio-therapeutics required different approaches considering process, development and production. As a consequence pharmaceutical companies have to deal with news constraints to insure product quality.
Characterization of these new therapeutic proteins such as mAb and or ADC is highly complex.
In this lecture you will see how Waters based on its knowledge on sample preparation, separation and mass detection has develop complete solution to allow companies to fully and deeply characterized their bio-product regarding regulation guidelines.
We will explain how we can confirm the sequence, identify and localized all potentials modifications, determine and localize disulfide bridges and monitor glycosylation. For ADC's samples you will discover complete workflow able to determine easily and rapidly the DAR, the distribution and fixation's site of drugs.

Oral 8

Protein analysis by means of electrospray ionization mass spectrometry: applications in drug discovery, design and development

Luca Regazzoni

Department of Pharmaceutical Sciences, Università degli Studi di Milano, via Mangiagalli 25, 20133, Milan, Italy

The main reason for mass spectrometry popularity lies in its high sensitivity and flexibility along with its unsurpassed resolution in the determination of the molecular weight of the analytes.
In the last decades, the routinely use of electrospray led to the design of several methods for protein analysis by means of mass spectrometry, which besides the determination of molecular weight allowed a more comprehensive characterization of proteins structure including information on the folding state and supramolecular chemistry. Notably, many of these techniques were specifically designed to support pharmaceutical and biomedical research.
Herein, they will be described some of these approaches through some illustrative examples in the field of drug discovery, design and development. In details, it will be firstly discussed the role of mass spectrometry in quality control by showing the case of recombinant ribonuclease UK114 and in particular the characterization of its purity and structural integrity. Secondly, it will be shown the use of mass spectrometry for the identification of protein covalent interactions applied to toxicity studies either on a marketed drug (i.e. amoxicillin) or on developing molecules (i.e. cysteine proteases inhibitors). Finally, the cases of the characterization of conformation and non-covalent interactions of beta2-microglobulin and Hsp90 will give clues about the modern use of mass spectrometry for fragment-based drug discovery.

Key note 5

Liquid chromatography and its application to the analysis of intact protein biopharmaceutics

Davy Guillarme

School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland

The characterization of therapeutic monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs), which combines the specificity of a mAb with a potent cytotoxic agent covalently bound via a linker to the antibody, is a tremendous challenge to state-of-the-art separation technologies. Indeed, subtle changes in these large (> 145 kDa) molecules at the amino acid level can have profound effects on efficacy and pharmacokinetic properties, thus it is important to have the ability to rapidly and accurately assess changes in the distribution of different isoforms (e.g., glycosylation, oxidation, deamidation, lysine truncation…) of such biomolecules.

The aim of this work is to provide an overview of the recent trends in reversed phase liquid chromatography (RPLC), ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC) for the analysis, at the protein level, of biopharmaceuticals including mAbs and ADCs.

Then, the possibility to combine orthogonal chromatographic techniques in a 2D setup will be also shown as it offers a powerful compromise between analytical throughput, and very high resolution separation. As a first example, online selective two-dimensional liquid chromatography coupled to time-of-flight mass spectrometry (sLCxLC-TOF-MS) will be described, using IEX in the first dimension, and RPLC in the second one. This approach provides, in a single analysis, information about the charge variants of each fragment and its masses of a model therapeutic mAb, namely rituximab. As a second example, the combination of HIC and RPLC withQqTOF/MS detection was evaluated for the determination of DAR distribution and the positional isomers of the same DARs of a commercially available ADC (Brentuximab-Vedotin). The possibility to make HIC and RPLC compatible is also demonstrated and the main advantage of this setup is to have HIC analysis, compatible with mass spectrometric (MS) detection.

Oral 9

QbD principles in LC method development

Matteo Stocchero [1], Albert van Wyk[2]

1. M. Stocchero, S-IN Soluzioni Informatiche, via G. Ferrari 14, Vicenza, Italy (matteo.stocchero@s-in.it);
2. Albert van Wyk, Advanced Chemistry Development, Inc., 8 King Street East, Toronto, ON, Canada.

Quality by Design (QbD) has become popular within the pharmaceutical industry and the FDA has cited a risk-based approach to drug development as desirable for the near future [1]. More complete information and transparency relating to risk assessment will be made available for new drug applications, speeding the approval process and preventing late-stage failures. QbD can be applied at every stage in the development and manufacturing process. Recently, analytical chemists have begun to apply QbD approach to the development of chromatographic methods for impurities and degradant studies [2,3]. Indeed, ensuring both robustness and optimization from an efficiency standpoint is time-consuming and difficult when method development is carried out applying the commonly used trial-and-error approach. As a result, a large amount of manual data interpretation is required and poor conditions are often obtained. The QbD philosophy advocates a more systematic, fact-based approach and focuses decision-making systems on key measurable attributes of the method to ensure it is fit for purpose. The result is a method that gives faster and more consistent chromatographic measurements. In this respect, Advanced Chemistry Development, Inc. (ACD/Labs) developed the software solution ACD/AutoChrom where the principles of QbD and advanced computational approaches to predict separations and fast data collection and processing are combined to support decision-making during chromatographic method development. A case study where ACD/AutoChrom is applied for developing a LC method for detecting impurities in a stability study is presented highlighting the advantages in the optimization stage, the reduction in time and the improvement in quality.

References

[1] Guidance for Industry. Q8(R2) Pharmaceutical Development (2009) U.S Food and Drug Administration.
[2] P.F. Gavin, B.A. Olsen, *J. Pharm. Biomed. Anal.* 2008, **46**, 431.
[3] I. Molnár, H.J. Rieger, K.E. Monks, *J. Chromatogr. A* 2010, **1217**, 3193.

Oral 10

On-Line Glycan Release and Analysis by An Integrated Pngase F-IMER LC-ESI-Q-Tof Approach

M. Bartolini,[1] M. Naldi,[1,2] V. Andrisano[3]

1. M. Bartolini, M. Naldi. Department of Pharmacy and Biotechnology, University of Bologna, via Belmeloro 6, Bologna, Italy; manuela.bartolini3@unibo.it;
2. M. Naldi, Center for Applied Biomedical Research (C.R.B.A.), S. Orsola-Malpighi Hospital, Via Massarenti 9, Bologna, Italy;
3. V. Andrisano, Department for Life Quality Studies, University of Bologna, Rimini Campus, Corso di Augusto 237, Rimini, Italy.

Protein glycosylation is acknowledged to be one of the major post-translational modifications, which significantly affects protein folding, conformation, stability and activity. Since more than one-third of approved biotherapeutics are glycoproteins [1], glycosylation state is a key issue in the production of biotherapeutics and biosimilars. Indeed, the presence and type of glycans dramatically affect efficacy and safety of this class of drugs. Thus, the increasing use of biotherapeutics translates into an urgent need to characterize glycosylation state and detect its alteration, in view of quality control reliable analytical methods. In this context, mass spectrometry (MS) coupled with liquid chromatography (LC) has already been shown to be an effective tool for analyzing glycoproteins and glycosylation sites. However, glycosylation analysis still remains highly challenging. The basic pipeline for glycosylation analysis is time consuming and includes protein deglycosylation and/or glycoprotein digestion (or combination of) followed by the analysis of intact proteins, glycopeptides and free glycans (either not labeled or labeled). To overcome low automation rate and long analysis time, glycan release by an immobilized enzyme reactors, i.e., enzymes immobilized on a suitable solid support (IMERs), can be used as a valuable alternative to the in solution assay.
This presentation focuses on the development of a novel Peptide-N-glycosidase F (PNGase F) immobilized enzyme reactor, its characterization and its integration into a LC-ESI-Q-ToF platform, which enables the sequential analysis of deglycosilated proteins and released glycans. PNGase F is one of the most frequently used endoglycosidases employed to release N-linked glycans, the most common form of glycans.
The PNGase F-IMER was obtained by oriented covalent immobilization of the target enzyme onto the surface of a short bed, high performance monolithic column (epoxy CIMac™ Analytical column). The monolith material, which has excellent permeability and fast mass transfer, has previously been shown to be a suitable support for IMER preparation when integration into a separative system is required.
The LC-MS integrated platform consisted of a series of two analytical columns able to selectively trap and separate in parallel both the proteins and the free glycans released by the IMER. In the proposed set up, glycoproteins were first on-line deglycosylated by the PNGaseF-IMER, the deglycolysalted proteins were subsequently collected by a small C4 CIMac™ analytical column (0.1 mL bed volume), and analysed by ESI-Q-ToF. In

parallel, released glycans were trapped onto a Hypercarb porous graphitic carbon (PGC) column and sent to ESI-Q-ToF for their analysis and identification. Each step was optimized to be perfectly interfaced with the subsequent MS analysis as well as with the enzyme requirements for stability and activity.

[1] G. Walsh, R. Jefferis R. *Nat. Biotechnol.* 2006, **24**, 1241-52.

Key note 6

Proteomics and native mass spectrometry for the characterization of biopharmaceuticals.

Alain VAN DORSSELAER

Laboratoire de Spectrométrie de Masse Bio-Organique, Département des Sciences Analytiques, Institut Pluridisciplinaire Hubert Curien, UMR 7178 (CNRS-UdS), ECPM, 25 rue Becquerel F67087-Strasbourg-Cedex 2

The use of recombinant proteins as pharmaceutical has been tightly dependant of the progress of the full characterization of these large and complex molecules. Nano-chromatography coupled with tandem mass spectrometry (LC-MS/MS) has played a key role from this point of view with the development of proteomic analysis which becomes continuously more sensitive and more automatized.

Indeed, the possibility offered by a series of new proteomic methodologies are today widely used for the full characterization of drug proteins including their expected heterogeneity, for example in the case of N-glycosylation where the optimal biological activity is dependent on the presence of different glycoforms in a defined ration. Proteomic analysis is also particularly useful for the detection and identification of protein impurities derived from the drug protein itself, but also from host cell protein.

The interpretation of MS/MS data in an automated way relies on bioinformatics, which is still one of the major bottlenecks of proteomic analysis. A wide diversity of bioinformatics tools is developed by the proteomic community to fill this gap. The identification of proteins by de novo interpretation of MS/MS spectra when the protein sequence data bases contain error or are not complete, is an example of development in this field which will be presented with a series of specific tools. (hiip://msda.unistra.fr).

The measurement of protein molecular masses is today routinely done up to several hundred thousand Daltons with accuracy, in particular when they are performed native conditions. When native conditions are used (strictly aqueous buffer), the native conformation of proteins can be maintained. In this case, the mass of protein/protein or protein/ligand complexes can be measured. A series of examples will be given, including for monoclonal antibodies. Also, when using native mass spectrometry, for example in the case of mixtures of several monoclonal antibodies, it is observed that a better spectral separation between the different molecular species can be obtained at m/z values. This allows a better detection of impurities and a better mass measurement.

Recently, a new technique (Ion Mobility Spectrometry: IMS) that can separate gaseous ions based on their size and shape (collision cross-section: CCS), has been introduced and allows improving the characterization of large proteins when coupled with a mass spectrometer (IMMS).

A series of examples will be given illustrating the new possibilities of IMMS for the characterization of biopharmaceuticals.

Oral 12

Increased Confidence in Host Cell Protein Workflow: Biologics Done Right

Kerstin Pohl[1], Milla Neffling[2]

1. K. Pohl, Sciex Germany GmbH, Landwehrstraße 54, Darmstadt, Germany (kerstin.poh@sciex.com);
2. M. Neffling, Sciex UK Ltd, Phoenix House Lakeside Drive Centre Park, Warrington, UK.

Biologics characterization reguires high confidence analytical tools and automated processing for the identity and integrity of the product. Crucial part of a Biologics' safety and efficacy assessment is the control over Host Cell Host protein Complement. Analysis of the Host Cell Proteins of Biotherapeutics by MS provides a logical way to inform, supplement, and improve assays developed with other techniques. Earlier LC-MS studies relied on proteomics techniques, were sometimes selective, and mostly required some pre-knowledge of the HCP complement and considerable expertise. Routine, time-effective HCP analysis provides the industry with cost and time benefits for early process understanding in biotherapeutics development. We present routine methodology for unbiased and comprehensive HCP analysis. A standard instrument platform is used to detect and quantify a number of contaminant proteins in the presence of highly abundant product protein with unambiguous evidence for identity and concentration. Routine chromatography provides a robust methodology that is complete within a few hours.

The proven SCIEX technology provides information on the level of 1) profile of the HCP complement up to 1000s of proteins to sub 1ppm level, 2) the detection allows you to identify the HCPs without bias [without inclusion/ exclusion lists]. In this way you can provide a catalogue of HCPs for a process for further development and process optimization.

Due to the technology, the information is easily transferred to another platform, as it provides precursor and fragment information to allow for easy monitoring.

Poster 1

Characterization of the major phase I metabolic pathways of non-steroidal anabolic compounds by liquid chromatography-mass spectrometry-based techniques following in vitro metabolism assays.

Anna Pia Dima [1,3], *Xavier de La Torre*[1], *Monica Mazzarino* [1], *Francesco Botrè* [1,2]

1. *Laboratorio Antidoping, Federazione Medico Sportiva Italiana, Largo Giulio Onesti 1, 00197 Roma Italy*
2. *Dipartimento di Medicina Sperimentale, "Sapienza" Università di Roma, Viale Regina Elena 324, 00161 Rome, Italy*
3. *Dipartimento di Chimica e Tecnologia del Farmaco, "Sapienza" Università di Roma, Piazzale Aldo Moro 5, 00185 Rome, Italy.*

Selective androgen receptor modulators (SARMs) are a class of non-steroidal anabolic compounds that since 2008 are included in the section S1 "Anabolic Agents" of the World Anti-Doping Agency list of prohibited substances and methods. SARMs represent enhanced tissue selectivity, binding to the androgen receptor with affinity similar to testosterone, but exhibiting only partial agonist properties in androgenic tissue. For these characteristics, they represent a prominent group of compounds for being misused in sports and, therefore, although not currently being marked, knowledge of their metabolic and analytical behavior is of the utmost importance in preventive doping control. Here we present an *in vitro* investigations aimed to characterize the metabolic reactions and the enzymatic isoforms involved in the biotransformation pathways of several SARMs.

The *in vitro* studies were performed on human liver microsomes and isolated CYP isoforms using protocols optimized evaluating different incubation times and concentrations of the selected substrates and of the enzymatic proteins.

The samples obtained from the *in vitro* incubations were then pre-treated using a liquid/liquid extraction in basic field with tert-butylmethyl ether (following enzymatic hydrolysis in case of phase II metabolism assays). The chromatographic separation was performed with an octadecyl reverse-phase column (2.1 ×150 mm, 5 µm) using acetonitrile and water with 0.1% of formic acid as mobile phase. The detection was carried out using a triple quadrupole mass spectrometers under positive electrospray ionization conditions and different acquisition modes.

The phase I metabolic reactions detected include hydroxylation in different positions, de-halogenation, dephenylation, reduction, deacetylation and combinations of them. The CYP450 isoforms mainly involved in the phase I metabolism of the compounds selected were the CYP3A4, CYP3A5, CYP1A2, CYP2D6, CYP2C19 and CYP2B6. In turn, most of the phase-I metabolites underwent conjugation reaction to form the corresponding glucuro-conjugated.

Poster 2

Optimization and application of a medium-high throughput method for lipophilicity profiling of pharmaceutical entities using HPLC-MS

F. Ferlenghi[1], F. Vacondio[1], M. Mor[1], V. Mileo[2], S. Catinella[2]

1. Dipartimento di farmacia, Università degli studi di Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy;
2. Chiesi Farmaceutici S.p.A, Largo F. Belloli 11/A, 43122 Parma, Italy.

Among drug-like properties, lipophilicity plays a major role because it affects both in vitro and in vivo properties (i.e. solubility, permeability, protein binding and GI absorption). This awareness has been leading scientists to introduce rapid methods in order to estimate hydrophobicity. Early screening allows to select and promote to development only the most suitable candidates. It also results in saving money and time in pharmaceutical research[1, 2].

This project aimed at setting up a medium/high-throughput method to estimate drug lipophilicity and to rank structural homologous. The method employed High Performance Liquid Chromatography (HPLC) coupled to both UV (λ=254 nm) and Mass Spectrometry detection (MS). MS was interfaced with an electrospray ion source (ESI), working alternatively in positive and negative ion mode and setting mass to charge (m/z) scan between 100 and 1000 amu. Selected Ion Recording mode (SIR) was used when sensitivity needed to be enhanced.

UV detection allowed to record retention time (t_R) and MS analysis confirmed molecular identity, integrity and purity, and also improved selectivity and specificity.

The study could be divided into two phases: first we reproduced a general method (-2<Log $D_{7.4}$ <5.5) described by Kerns et al.[3].

Stock solutions of nineteen diverse drugs were prepared in dimetilsulfoxide (DMSO) and, among them, six were chosen to calibrate the method. t_R were plotted versus Log $D_{7.4}$ values measured by the reference shake flask technique.

Then t_R was obtained for thirteen test compounds and their Log $D_{7.4}$ values were calculated applying linear regression model coefficients.

To test the repeatability of the method, the coumpounds were run two times under the same conditions; to prove the feasibility of combinatorial analysis a mixture of the test compounds was analyzed.

The second phase focused on adapting the general method to structurally-related compounds: it was applied to steroids, melatonin derivatives[4], H_3 antagonists[5] and a class of Chiesi compounds.

Since homologous were extremely similar within each class, the original gradient was optimized to suit a narrower lipophilicity range (0<Log $D_{7.4}$<3); standards were selected by structural and hydrophobicity characteristics and then the method was applied to a suitable test set.

The correlation of HPLC Log $D_{7.4}$ values to shake flask ones was considered and predictivity, accuracy and fitting to experimental values were examined employing appropriate statistical parameters[6].

In the end lipophilicity scale derived from high-throughput method and reference system were compared, to determine the reliability of the former.

HPLC-MS technique proved to suit discovery requirements being rapid and reliable: expected lipophilicity ranking was confirmed, except for compounds that showed a reduced variance in molecular weight and hydrophobicity ($\Delta MW < 10$ and $\Delta \text{Log } D_{7.4} < 0.09$) at once, or that strongly interacted with stationary phase (i.e. H_3 antagonists).

Technical problems, such as column and detector contamination, could be easily solved diluiting stock solutions in acetonitrile and programming daily cycles of washing for HPLC column.

The method could be implemented testing more chemical classes and comparing results to data obtained by different lipophilicity profiling methods (i.e. scaled-down shake flask, pH-metric technique, other HPLC-based systems).

For the ease of preparation, low cost and wide feasibility, the technique here described turned out to be a useful tool to evaluate hydrophobicity of NCEs in the early drug discovery screening, even if it is not supposed to replace conventional in-depht methods.

[1] E. H. Kerns, L. Di Drug-like properties: concepts, structure, design and methods from ADME to toxicity optimization.

[2] E. H. Kerns *Int. J. of Pharm. Sci.* 2001, **90 (11)**, 1838-1858.

[3] E. H.Kerns, L. Di, S. Petuskya, T. Kleintopa, D. Huryn, O. McConnell, G. Carter, *J. Chrom. B* 2003, **791**, 381-388.

[4] M. Mor, C. Silva, F. Vacondio, P. V. Plazzi, S. Bertoni, G. Spadoni, G. Diamantini, A. Bedini, G. Tarzia, M. Zusso, D. Franceschini, P. Giusti *J. Pineal Res.* 2004, **36(2)**, 95-102; 2006, **40(3)**, 259-69.

[5] F. Vacondio, M. Mor, C. Silva, V. Zuliani, M. Rivara, S. Rivara, F. Bordi, P. V. Plazzi, F. Magnanini, S. Bertoni, V. Ballabeni, E. Barocelli, P. A. Carrupt, B. Testa *Eur. J. Pharm Sci.* 2004, **23(1)**, 89-98.

[6] A. Golbraikh, M. Shen, Z. Xiao, K. H. Lee, A. Tropsha *J. of Computer-Aided Mol. Des.* 2003, **17**, 241-253.

Poster 3

Bottom-up mass spectrometry proteomics strategies for the identification of metallodrug binding sites on proteins: the search for a general protocol to assess adduct stability

Elena Michelucci[1], Giuseppe Pieraccini[1], Gloriano Moneti[1], Luigi Messori[2]

1. Mass Spectrometry Center (CISM), University of Florence, via U. Schiff 6, 50019 Sesto Fiorentino (FI), Italy;
2. Department of Chemistry "Ugo Schiff", University of Florence, via della Lastruccia 3, 50019 Sesto Fiorentino (FI), Italy.

The bottom-up mass spectrometry approach (reduction, alkylation and enzymatic digestion followed by MS/MS analysis) is one of the main methods used in proteomics to characterize the binding site of metal-based anticancer drugs on proteins. Nevertheless, in our opinion, the study of the stability of the metal fragment-protein coordination bond along the whole process has not received so far adequate attention.[1]
Previous studies[2] on myoglobin, that were confirmed in our mass spectrometry facility, showed the instability of Fe-histidine coordination bond under certain preparative (pH, presence of an organic co-solvent) and instrumental (capillary temperature, tube lens voltage) conditions.
These findings convinced us to draw up a general protocol to test metal fragment–protein adduct stability under the typical condition of the bottom-up approach (from the solution containing the metal-protein adduct to the MS/MS experiments), especially when the metal complex involved is not the well known and studied cisplatin (cisPt) but a new metal complex containing other metals as Ru and Au.
We identified eight critical conditions as potential sources of metal-protein coordination bond impairment during the bottom-up process, when using a LTQ-Orbitrap mass spectrometer:
1) sample permanence in ammonium bicarbonate;
2) dithiothreitol reduction;
3) iodoacetamide alkylation;
4) permanence in loading mobile phases (presence of organic co-solvent and pH);
5) permanence in mobile phases (presence of organic co-solvent and pH);
6) ESI process;
7) transfer through ion transfer tube and tube lens; 8) collision induced dissociation in ion trap.
An experimental protocol was thus developed to assess the relevance of the above conditions on the stability of the metal-protein coordinative bond. First of all, the protocol was applied to the well known, model system cisPt-cytochrome C: cisPt-CytC adducts proved their stability toward all conditions listed above and so they showed to be ideal candidates for a binding site investigation using a bottom-up approach.

In our opinion it is not necessary to apply again the full protocol for cisPt to test the complex with proteins different from CytC since, during the protocol application, the protein is denatured. Instead, it is strongly suggested to use it studying Pt-complexes different from cisPt or with other metals (Ru, Au).

References

[1] E. Moreno-Gordaliza, B. Cañas, M. A. Palacios, M. M. Gómez-Gómez *Analyst*, 2010, **135**, 1288-1298.
[2] M. Karas, U. Bahr, T. Dülcks *Fresenius. J. Anal. Chem*., 2000, **366**, 669-676.

Poster 4

An Automated Workflow for the Analysis, Validation and Visualization of Large HDX-MS Datasets: Application to the Study of the Adenylate Cyclase Toxin

Darragh P. O'Brien[1], Véronique Hourdel[1], Stevenn Volant[2], Marie-Agnès Dillies[2], Julia Chamot-Rooke[1], Daniel Ladant[1], Alexandre Chenal[1], and Sébastien Brier[1]

[1]*Chemistry and Structural Biology Department, UMR CNRS 3528, Institut Pasteur, 75015 Paris, France;*
[2]*Hub Bioinformatics and Biostatistics C3BI, Institut Pasteur, 75015, Paris, France.*

Hydrogen Deuterium Exchange detected by Mass Spectrometry (HDX-MS) is a powerful technique to probe the conformation and dynamics of proteins. Over the past ten years, the HDX-MS workflow has evolved substantially in both academic and pharmaceutical environments, enabling the investigation of more complex biological systems, including large protein complexes and membrane proteins. Accordingly, new pipelines for data processing and statistical analysis of large HDX-MS datasets are urgently required.

Herein, we present a fully automated HDX-MS workflow for sample preparation, injection, MS data acquisition and validation. Several statistical tools have been developed for HDX data interpretation to-date [1], with most considering data at one time point *only* and not accounting for the time dependency of the HDX reaction. To address this issue, we propose a linear mixed-effects model which is a generalization of the ANCOVA model developed by Liu and co-workers [2]. By integrating replicates as a random effect, our statistical approach accounts for both time dependency and the variability between replicates. We implemented our approach into an R package named "MEMHDX" (Mixed-Effects Model for HDX-MS experiments), which also provides an in-house data visualization of the output (plot of the data, the fitted model for each peptide, a plot of the calculated *p*-values, and a global visualization of the experiment). MEMHDX complements existing HDX-MS pipelines as it directly utilizes the output of the commercially available HDX software DynamX 3.0 (Waters).

As an application, we firstly sought to investigate the behaviour of the adenylate cyclase (CyaA) catalytic domain (AC) upon interaction with calmodulin (CaM) and secondly, the structural changes taking place in the receptor-binding Repeat-in-Toxin (RTX) domain of CyaA upon calcium binding [3]. Using our approach, we were able to locate and validate those regions of CaM which are modified upon interaction with AC and to pinpoint the RTX regions undergoing structural and conformational changes upon cofactor binding.

[1] Houde D., *et al*., (2010) *J Pharm Sci.,* 100 (6):2071-86.
[2] Liu S., *et al*., (2011) *BMC Bioinformatics*, 15;12 Suppl 1:S43.
[3] O'Brien D. P., *et al*., (2015) *Sci. Rep*. 5, 14223.

Poster 5

Rapid assay of letrozole in dried blood spots by liquid chromatography-tandem mass spectrometry

M. Pacenti[1], C. Di Serio[2], G. Pieraccini[1], G. Moneti[1], S. Materazzi[3], C. Lupi[3], S. Benemei[3], T. Susini[3]

1. Mass Spectrometry Center, Department of Health Sciences, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy (marco.pacenti@unifi.it);
2. Department of Experimental and Clinical Medicine, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy;
3. Department of Health Sciences, University of Florence, Viale Pieraccini 6, 50139 Florence, Italy.

Letrozole (LTZ), an aromatase inhibitor, is an efficient endocrine treatment of postmenopausal breast cancer. However, its efficacy is subjected to inter-individual variability and not all patients derive benefit from this treatment (1): therefore, the measurement of blood LTZ concentration in steady-state condition is crucial. LC-MS/MS is widely applied in measuring LTZ using venous blood samples (2). Our aim was the development of a selective and sensitive method for the determination of LTZ using a capillary sampling on filter paper (dried blood spot,-DBS) and LC-MS/MS with d4-LTZ as internal standard (3, 4). DBS sampling is easier to perform, less painful and requires microliters of blood.
DBSs were collected from 9 LTZ-treated postmenopausal breast cancer patients; a calibration curve was prepared using whole blood samples from not LTZ-treated women, enriched with scalar amounts of LTZ and a fixed amount of d4-LTZ, and then applied to filter paper. The procedure required a simple extraction of analytes from a 6 mm circle punched out from DBS using methanol. LC separation was performed on a Phenomenex Gemini C6-Phenyl column (100 x 2.0 mm, 3 μm) using a gradient of acetonitrile in 0.1% acetic acid in water. The eluate was directly transferred to the ESI source of a triple quadrupole mass spectrometer and MS/MS detection, performed in positive ion and MRM mode. LC-MS/MS runtime was 9 minutes, followed by a 8 minutes re-equilibration time. No interfering peaks were observed analysing blank whole blood samples at the retention time of analyte and internal standard. The calibration curve was linear in the range of interest (12.3–200 ng/ml R^2 = 0.997). The lower limit of quantitation was 12.3 ng/ml and the lower limit of detection was 4 ng/ml. The method was used to quantify LTZ in DBS obtained from postmenopausal patients with breast cancer under treatment: LTZ concentrations varied 3.5-fold in a range between 46 and 166 ng/ml and the mean levels (± SD) were 112 ± 40 ng/ml. These data are in agreement with those of literature demonstrating that: i) this is a valid and good method to determine LTZ concentrations in postmenopausal breast cancer patients (even if not corrected by haematocrit); ii) there is a considerable inter-individual variability of LTZ. In conclusion, the advantages of this method are an easy sampling, easy transport, reduced sample preparation, a potential reduction of costs.

DBS can potentially allow at-home sampling, making easier the therapeutic drug for LTZ treated patients.

References

1. B. Thurlimann, A. Keshaviah, A.S. Coates, H. Mouridsen, L. Mauriac, J.F. Forbes, R. Paridaens, M. Castiglione-Gertsch, R.D. Gelber, M. Rabaglio, I Smith, A. Wardley, KN Price, A. Goldhirsch. *N. Engl. J.Med.* 2005, **353**, 2747-2757.

2. JC. Precht, B. Ganchev, G. Heinkele, H. Brauch, M. Schwab, T.E. Murdter. *Anal. Bioanal. Chem.* 2012, **403**, 301-308.

3. J.V. Mei, J.R. Alexander, B.W. Adam, W.H. Hannon. *J. Nutr.* 2001, **131**, 1631S-1636S.

4. G. la Marca, S. Malvagia, L. Filippi, F. Luceri, G. Moneti, R. Guerrini. *Epilepsia*. 2009, **50**, 2658-2662.