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& STUDENT TRAVEL AWARD CONTEST

Washington Chromatography Discussion Group

September 21, 2016
6pm - 8pm

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Automated method for structural characterization of membrane proteins in bicelles using HDX-MS

Kyle Anderson, Jeffrey Hudgens
National Institute of Standards and Technology, Institute for Bioscience and Biotechnology Research, Rockville, MD

The majority of pharmaceutical targets are membrane proteins. However, structural characterization of membrane proteins has been a consistent challenge for LC-MS analysis. Membrane lipids are deleterious to chromatography performance and cause ion suppression of peptide signal. Typically, LC-MS hydrogen deuterium exchange (HDX) studies solubilize membrane proteins by truncation or altered amino acid sequence in the membrane domain, which can induce erroneous structural changes and prevent the measurement of allosteric effects. Alternatively, membrane proteins may be solubilized with lipids prior to HDX and the lipids can be removed after HDX using manually performed methods, which introduces errors during short exchange rates in kinetic experiments. Our method enables fully-automated deuterium labeling, proteolysis, lipid removal, desalting, analytical separation, and MS acquisition. In addition to physiological relevance of measurements in membranes, our workflow enables studies for many membrane proteins that are not even available in a soluble variant for HDX-MS analysis and have yet to be studied. Furthermore, the effect of variations in glycosylation or modifications of protein therapeutics on the binding to membrane proteins can be studied with our method. Bicelles were produced to solubilize membrane proteins using a mixture of long chain phospholipids, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and short chain phospholipids, 1,2-di-o-hexyl-sn-glycero-3-phosphocholine (DHPC). DOPC forms a bilayer around the hydrophobic transmembrane domain of the protein and the bilayer is capped by short chain DHPC. Deuterium labeling, proteolysis, lipid removal, desalting, and analytical separation were performed inline on a fully-automated HDX PAL robot coupled to a Thermo LTQ Velos Orbitrap Elite. DOPC/DHPC were removed inline using zirconium oxide beads, allowing lipid-free protein to continue to proteolysis and LC-MS analysis.

CE-nanoESI-MS for Untargeted Proteomic Characterization of Single Embryonic Cells and Small Neuron Populations

Sam Choi, Marta Zamarbide, M. Chiara Manzini, Sally Moody, Peter Nemes
George Washington University, Washington, DC

Molecular characterization of small neuronal populations presents transformative potentials for studying neurodegenerative diseases, but requires detection technologies with high sensitivity, preferably mass spectrometry (MS). Here we present a mass spectrometry platform that is sensitive and able to measure proteins in small populations of mammalian neurons. The system uses a tapered-tip metal emitter to hyphenate volume-limited capillary electrophoresis to nanoelectrospray ionization and high-resolution mass spectrometry. By measuring <500 pg of protein digest, we demonstrate that this platform is able to identify 300+ protein groups in the hippocampal neurons. Proteins were extracted from small populations of hippocampal neurons, trypsin-digested, and reconstituted in 50% acetonitrile containing 0.05% acetic acid. A 500 pg digest (1 nL) was analyzed with a custom-built CE-nanoESI-MS system. Neuronal peptide ions were sequenced using quadrupole-Orbitrap MS (QE+) to identify proteins against the mouse proteome using MaxQuant search engine with FDR < 1% against a reversed-sequence database. We custom-designed a microscale CE and a CE-nanoESI interface, and developed specialized MS methodology to enable trace-level bottom-up proteomics. The CE platform was able to analyze 1 nL, or ~500 pg protein digest. The CE-nanoESI interface featured a co-axial sheath-flow design, a tapered-tip metal emitter, and the stable cone-jet spraying regime. 2 fg angiotensin II yielded signal/noise = 17, suggesting an ~350 zmol lower limit of detection. Quantification was linear over 5 log-order concentration with a ~2% RDS in migration time and <20% RSD in peak area. Furthermore, measurement of a 500 pg of protein digest from hippocampal neurons uncovered ~300 different protein groups (<1% FDR), including known neuronal-specific gene products (e.g., synapsin 1 and 2 and the neural cell adhesion molecule). We anticipate that CE-nanoESI-MS will help also characterize the proteomic composition of smaller cells and finer brain substructures to help better understand molecular processes during normal development.

Routine MS Detection for USP Chromatographic Methods

Ken Blakeslee, Tom Wheat, Dan Root, Aparna Chavali, Patricia McConville
Waters Corporation, Columbia, MD

Analytical method validation includes peak identification and purity assessments where mass spectral information would be ideal. Unfortunately many LC methods use non-volatile buffers that are not compatible with MS. The ACQUITY UPLC with 2D-LC and QDa detector provide an efficient solution to this method characterization challenge.

The NISTmAb: A Comprehensively Characterized Reference Material to Support Biopharmaceutical Analytical Technology Development

Abigail Turner, John Schiel
National Institute of Standards and Technology, Institute for Bioscience and Biotechnology Research, Rockville, MD

The National Institute of Standards and Technology (NIST) has recently developed the NISTmAb Reference Material (RM 8671), an IgG1κ class-representative monoclonal antibody. The NISTmAb is intended to serve as a platform for harmonization and open innovation in the biopharmaceutical analysis community. It is a widely and longitudinally available test material that will support novel technology and method development, serve as an external system suitability control, and provide a medium for open access information sharing. Here, we provide an overview of the NISTmAb program, with special emphasis on NISTmAb analysis by capillary electrophoresis methods including CE-SDS, cIEF, CZE, and CE-ESI-MS2. We demonstrate comprehensive electrophoretic characterization of the NISTmAb reference material and discuss its utility as a reference standard in CE assay development.

Second-generation single-cell mass spectrometry finds metabolic cell heterogeneity along the left-right body axis in the developing frog (Xenopus) Embryo

Rosemary Onjiko, Erika P. Portero, Sally A. Moody, Peter Nemes
George Washington University, Washington, DC

We recently developed a single-cell mass spectrometry (MS) platform based on whole-cell microdissection integrated to our in-house capillary electrophoresis electrospray ionization (CE-ESI) high-resolution MS to enable the characterization of 40 different metabolites between single embryonic blastomeres (cells) in the 16-cell Xenopus laevis embryo. Using this system, we uncovered metabolites that are able to change the normal tissue fate of blastomeres which are precursors to nervous and epidermal tissues (RM Onjiko et al., 2015). Here, we implement technological–methodological advances to reveal metabolic heterogeneity between blastomeres derived across the left-right axis of the embryo. First, we developed a multi-step metabolite extraction protocol for the single cells to improve metabolite detection to hundreds of different signals and then proceeded to identify 56 of them with confidence. Due to higher sensitivity, we were able to assess whether there are metabolic differences between blastomeres that occupy the left versus right hemispheres of the 8-cell embryo, at which stage body asymmetry already exists but the underlying molecular players are still elusive. By reproducibly quantifying ~80 different small-molecule signals, we identified 10 different metabolites that were statistically ($p < 0.05$) and biologically significantly (fold change < 1.5) differentially enriched between left and right dorsal blastomeres fated to become nervous tissues. Furthermore, we extended these single-cell measurements to later stages of embryonic development by integrating microcapillary sampling into the workflow. Consequently, we minimized chemical interferences from sampling media, allowing us to obtain signals that have comparable–to–higher signal/noise ratios than detected from manually microdissected cells despite microsampling a miniscule (<10%) portion of the cell's cytoplasm. The ability to measure a broad diversity of small molecules from the complex cellular composition using single-cell capillary microsampling MS raises an opportunity to gain deeper insights to molecular processes underlying normal cell differentiation and development of the vertebrate embryo.

HPLC Method Development and Validation for the Assay and Organic Impurities of Sulfisoxasole Acetyl

Eduardo Lim, Shane Tan, John Simpson
U.S. Pharmacopeia, Rockville, MD

The current USP Sulfisoxazole Acetyl monograph uses titration for Assay and Thin Layer Chromatography (TLC) for Organic Impurities. In an effort to strengthen and modernize the monograph, a stability indicating HPLC method was developed for both the assay and organic impurities tests. Methods: Chromatography was carried out on a C18 column, 15-cm x 4.6-mm, 3.5-µm, at 25º. The mobile phase consisted of acetonitrile and 0.1 % phosphoric acid (55:45) at a flow rate of 1.0 mL/min. Detection was at 272 nm. The HPLC method separated sulfisoxazole acetyl from the specified impurity, 4,5-dimethylisoxazol-3-amine with a resolution of more than 5. Forced degradation studies were carried out to demonstrate the stability-indicating ability of the method. The major hydrolysis product, sulfisoxazole was also completely separated. The sulfisoxazole acetyl peak was spectrally pure in all the stressed and the unstressed samples using photo diode array detection. No co-elutions were detected on the LC/MS analysis. The method was robust under all the variations allowed in USP General Chapter <621>. The validation results for both the Organic impurities and Assay procedures met all the preset acceptance criteria. A single HPLC method was developed and validated for the assay and organic impurities procedures for USP Sulfisoxazole Acetyl monograph. The method was specific, linear, accurate and precise

In vivo Analysis of Metabolites in Single Embryonic Cells in the Developing Frog (Xenopus) Embryo using Microcapillary-Sampling CE-ESI-MS

Erika Portero, Rosemary M. Onjiko, Sally A. Moody, Peter Nemes
George Washington University, Washington, DC

In vivo investigation of the single-cell metabolome provides in depth molecular information on cell differentiation. Single-cell mass spectrometry enables the detection of diverse types of metabolites in single cells, typically under ex vivo conditions. Here, we develop microcapillary sampling for single embryonic cells , utilizing a custom-built single-cell capillary electrophoresis mass spectrometry (CE-ESI-MS) to detect and quantify metabolites in cells fated to give rise to different tissue types in the South African clawed frog (Xenopus laevis). Additional to identifying a number of metabolites, this work provides exciting new data to further our molecular understanding of cell differentiation during development of the vertebrate embryo. Each microcapillary was mounted on a micromanipulator to fine-position its tip into identified cells in the Xenopus embryo, where n ~ 10nL of the cell were withdrawn. Subsequently, the cell extract was transferred into a microvial containing 4 µL of a metabolite extraction mixture. Metabolites in the resulting extract were separated using a custom-built CE-ESI system and detected using a high resolution mass spectrometer (Impact HD Qq-TOF, Bruker). This technique allowed us to detect ~130 different molecular features (unique m/z vs. migration time) and identify fifty-five of these molecular features with high confidence as small molecules (metabolites) based on accurate mass measurements, tandem MS, and migration time comparison to standards. Using microcapillary sampling, we enabled the investigation of metabolites in single cells during normal embryonic development. Next, we microcapillary-sampled the four cell types (V11, V12, D11, and D12) in the same 16-cell Xenopus embryo in n = 4-5 biological replicates. The resulting metabolic data enabled us to find metabolite gradients across cell types in the same embryo that could uncover metabolic networks during cell differentiation, while minimizing biological variability between embryos. Microcapillary sampling followed by CE-ESI-MS raises a potential to be adapted to other animal models.

High Resolution CESI-MS Analysis of APTS-labeled N-glycans of Biopharmaceutical Interest

Robert Swart,[1] Bryan Fonslow,[1] Andras Guttman,[1] Boglarka Donczo,[2] Marton Szigeti,[2] Horvath Csaba[2]
[1]SCIEX; [2]Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

Glycosylation plays an important role in cellular interactions, protein folding, and monoclonal antibody (mAb) stability. For characterization, N-linked glycosylation on mAbs is routinely analyzed by CE-LIF after endoglycosidase based glycan release and fluorophore labeling. CE separation provides structural resolution and migration time-based identification of glycans, but coupling with MS detection offers additional structural information. The integration of CE and ESI into a single dynamic process (CESI) provides the capability of performing CE separation and MS ionization with ultra-low flow rates, resulting in reduced ion suppression and improved sensitivity. CESI-MS has been optimized and evaluated for APTS-labeled mAb and formalin-fixed parafin-embedded glycan analysis.

Evaluation of Polycyclic Aromatic Hydrocarbon Standard Reference Materials 869b and 1647f on Different Stationary Phases for Liquid Chromatography

Walter Wilson, Lane C. Sander, Jorge O. Ona-Ruales, Stephen A. Wise
National Institute of Standards and Technology, Gaithersburg, MD

Polycyclic aromatic hydrocarbons (PAHs) are important environmental pollutants originating from a wide variety of natural and anthropogenic sources. PAHs are generally formed during incomplete combustion of organic matter containing carbon and hydrogen. Due to the carcinogenic nature of some PAHs, their chemical analysis is of great environmental and toxicological importance. Among the hundreds of PAHs present in the environment, the U.S. Environmental Protection Agency (EPA) have included sixteen in their priority pollutants list. Liquid chromatography (LC) is a standard analysis technique used for determining the 16 EPA-PAHs. Currently at the National Institute of Standard Technology (NIST), standard reference materials (SRM) 869b and 1647f are available for evaluating current and new LC columns. SRM 869b is a mixture of three PAHs for characterizing a LC column selectivity for separation of PAHs. Depending on the elution order of the three PAHs, column selectivity can be predicted for complex PAH mixtures. SRM 1647f is a calibration solution for use in LC methods for the determination of the 16 EPA-PAHs. In this study, multiple LC columns consisting of C18, C30, phenyl-hexyl and penta-fluoro-phenyl stationary phases are evaluated with SRM 869b and SRM 1647f.

LC-MSn-ELSD analysis of plant derived oligosaccharides and human milk glycans

Connie A. Remoroza,[1] Ma. Lorna M. De Leoz,[1] Henk A. Schols,[2] Stephen E. Stein[1]
[1]National Institute of Standards and Technology, Gaithersburg, MD; [2]Wageningen University and Research Center, Netherlands

Analysis of complex mixtures of plant cell wall derived oligosaccharides and human milk glycans is often required multiple techniques for separation and characterization of these mixtures. In this study it was demonstrated that hydrophilic interaction chromatography with online mass spectrometry and/or evaporative light scattering detection (HILIC–MSn/ELSD) is a valuable tool for identification of a wide range of neutral and acidic oligosaccharides. Enzymatic degradation of plant cell wall material was done by the combined action of pectinases [1]. The released oligosaccharides were analysed. Recently, the LC-HILIC-MSn approach was extended for the analysis of glycan composition using the NIST standard reference material (SRM) 1954, a fortified human milk. Lactosyl and sialylated with and without fucose glycans from SRM1954 were isolated as described [3]. Results showed the separation of many different oligosaccharides present in plant cell wall digest. Identification and quantification of methylesterified and acetylated acidic oligosaccharides with the same charge yet with different degrees of polymerization can be obtained [1,2]. Moreover, the HILIC column enabled the separation and identification of various fucosyl and lacto-N derivatives as well as lacto-sialylated oligosaccharides present in human milk. The elution profile showed that the isomeric structures were distinguished using MSn detection. The fast analytical profiling of oligosaccharides using LC-HILIC and online MSn approach will allow the development of mass spectrometry library for plant materials and milk samples.

[1] Remoroza, C. et al. (2012). Carbohydrate Polymers.
[2] Leijdekkers, M. et al. (2011). Journal of Chromatography A.
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Differentiation for closely related botanical species by pharmacopeial monographs

Cuiying Ma, Nandakumara Sarma, Gabriel I. Giancaspro
U.S. Pharmacopeia, Rockville, MD

To distinguish the closely related botanical species, selective analytical procedures in the pharmacopeial monographs are used to reduce the possibility of adulteration by substitutes with potential confounders. In this work, we present the differentiations between Rhodiola crenulata Root and Rhizome and R. rosea Root and Rhizome, as well as the differentiations between Citrus reticulata Pericarp and C. maxima Pericarp based on the monographs developed for Rhodiola crenulata Root and Rhizome [1], R. rosea Root and Rhizome [2] and Citrus reticulata Pericarp [3]. Both R. crenulata Root and Rhizome and R. rosea Root and Rhizome contain salidroside and tyrosol but R. rosea Root and Rhizome also contains rosavin and rosarian. With the HPTLC method [1], no rosavin band was observed in R. crenulata Root and Rhizome while rosavin band was observed in R. rosea Root and Rhizome sample solution. With the HPLC method [2], R. rosea Root and Rhizome displayed peaks of salidroside, tyrosol, rosavin and rosarian; R. crenulata Root and Rhizome only displayed salidroside and tyrosol, no rosavin and rosarian peaks were observed. Both C. reticulata Pericarp and C. maxima Pericarp contain dihydroflavone glycosides, however, C. reticulata Pericarp mainly contain hesperidin and C. maxima Pericarp mainly contain naringin. With the HPTLC and HPLC methods[3], these two species could be distinguished due to hesperidin and naringin with different retention times and RF values. Orthogonality between HPLC and HPTLC technics can provide greater assurances for botanical identification than the application of only one of these procedures. The combination of traditional morphological, microscopic and modern chromatographic procedures in the identification sections of the monographs can efficiently distinguish the closely related botanical species materials. Acknowledgements: 1. State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, China; 2. Department of Pharmaceutical Analysis, Shenyang Pharmaceutical University, Shenyang, China; 3. CAMAG Laboratory References: [1] Rhodiola crenulata Root and Rhizome. [2] Rhodiola rosea Root and Rhizome. [3] Citrus reticulata Pericarp. Technical tips online. Available at http://hmc.usp.org

New Protein Separation Technologies: Spiral Countercurrent Chromatography and Centrifugal Precipitation Chromatography

Rodrigo Lazo-Portugal, Martha Knight
CC Biotech LLC, Rockville, MD

New centrifuge-based technologies are developed for large molecule fractionation by two different physical techniques. The first is a modern development of countercurrent chromatography carried out in the Ito planetary centrifuge; spiral countercurrent chromatography (SpCCC) which uses continuous tubing arrayed in layers of loops, instead of spools of open tubing for the solvent flow. The separation rotors are spun on a parallel axis displaced from a central axis, hence the name planetary centrifuge. The advantage in the new rotor design is that the stationary phase of a two phase solvent system is very high and good resolution in the aqueous two-phase solvent systems (ATPS) is possible. From the studies of extractions of ATPS for proteins, it is possible to chromatograph proteins, maximally separating impurities. Recent rotors, the Spiral Tubing Support and the Mixer-Settler Spiral Disk will be described and capabilities with large molecules will be presented. The second technology is centrifugal precipitation chromatography as embodied in the new Rotify®, a bench-top centrifuge with a membrane bearing rotor that has flow through a spiral channel on the top and flow in the opposite direction below the MW cut-off membrane. A protein sample passes into the rotor and all low MW entities pass through the membrane and the individual proteins continue passing along and are precipitated at individual rates according to a salt or solvent gradient applied. The elution of the separated proteins or polymers come out the end collected in fractions.

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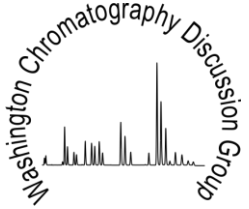

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