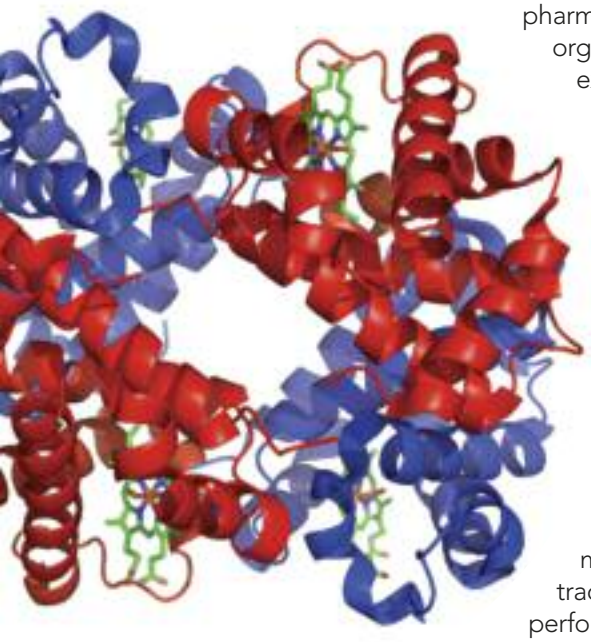
FOCUS

Alturas Advisor

FALL WINTER 2011-12

LC-MS/MS Bioanalysis of Macromolecules: Proteins and Biomarkers

As LC-MS/MS technology advances to improve instrument sensitivity and selectivity, applications of the technology continue to expand. Recently, pharmaceutical and biotech organizations have expanded their research to macromolecules in order to enhance their drug development pipeline. Additionally, the monitoring of biomarkers (both large and small molecules) is playing a larger role in drug discovery and development. Bioanalysis of these macromolecules has traditionally been performed with the use of ligand binding assays (LBA). Ligand binding assays can be sensitive with LLOQs in the low pg/mL range. However, the limitations of LBAs such as poor selectivity, limited dynamic range and extensive method development have created an opportunity for LC-MS/MS technology to be used for the analysis of macromolecules.



Structure of hemoglobin

Large molecule biomarkers (i.e., proteins) and protein based therapeutics utilize similar LC-MS/MS bioanalytical approaches. The main difference is often that the measurement of biomarkers requires a lower limit of quantitation than the delivered protein based therapeutic. Thus, biomarker assays may necessitate more selective sample preparation and LC-MS/MS analysis methods. This article discusses example approaches to improve the selectivity and sensitivity for the analysis of proteins in biological fluids.

Two approaches exist for the analysis of macromolecules by mass spectrometry. There is a "top-down" approach and "bottom-up" approach. Top-down methods rely on the analysis of the intact molecule. Although this method can have advantages, selectivity and sensitivity may suffer due to the multiple charges on the molecule and wide isotopic distribution with mass spectrometry. Thus, the majority of macromolecule analysis by mass spectrometry has been performed using "bottom-up" methods, where proteases are used to digest proteins to create smaller surrogate peptides. In order to further enhance selectivity and sensitivity, one or several of the surrogate peptides can be monitored simultaneously using MS/MS.

Sample preparation is critical for the LC-MS/MS bioanalysis of proteins. Three methods are generally accepted as viable options for sample preparation of proteins and peptides depending on the size of the protein of interest and needs for the program. The three methods include, 1) immunoaffinity (IA)

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Micro Flow Liquid Chromatography - MS/MS Research with a QTRAP 5500®

The sensitivity advantage of micro-flow liquid chromatography coupled with a mass spectrometer (MFLC-MS/MS) is well known. Recently, MFLC-MS/MS has gained renewed interest now that HPLC pumps can be manufactured to accurately deliver ≤100 µL/min of solvent and HPLC columns with an inside diameter of ≤1 mm are routinely available from many vendors.

Data produced at Alturas Analytics confirms that as the flow rate decreases, the mass spectrometer signal intensity increases. See Figure 1. of a chromatogram that compares MFLC-MS/MS with conventional HPLC-MS/MS for the analysis of propranolol. Depending on the molecule, typical signal gains are from 2-10X when comparing a conventional 2.1 mm ID column vs. a 0.3 mm ID column with the use of similar linear velocities. Our data shows that these gains come mostly from an increase in the MS signal and not improvements in the chromatographic efficiency. Although the injection volume is limited (e.g. ≈10 µL for a 0.3 mm ID column) and may not always overcome the signal gains of using a larger injection volume with conventional HPLC-MS/MS systems, the additional benefits of minimizing matrix effects and conserving solvent may outweigh this limitation. Additionally, the MFLC-MS/MS system is the optimal choice if only a small volume (≤10 µL) of sample is available for injection or for on-line extractions such as when coupling on-line dried blood spot (DBS) analysis to HPLC-MS/MS. As the investigation of MFLC-MS/MS continues, analysis of macromolecules could show even further sensitivity gains. More research with our collaborators is on-going to improve the source design to further enhance the MS signal and minimize band broadening at the lower flows. Our research suggests that these source improvements could easily lead to another 50% increase in the MS signal.

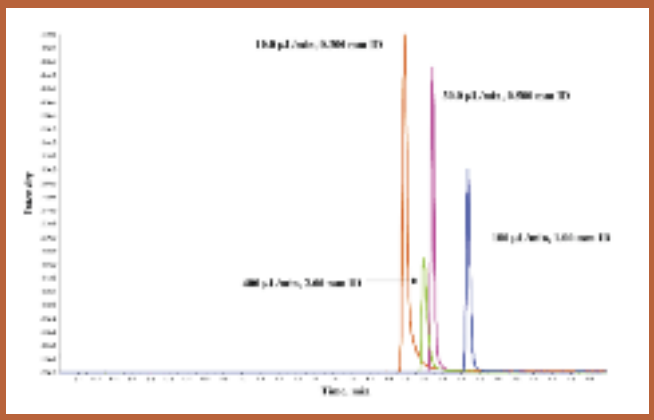


Figure 1. HPLC-MS/MS and MFLC-MS/MS chromatograms from the analysis of a 1.5 µL injection of a 5.00 ng/mL Propranolol solution

The enhancements provided by MFLC-MS/MS have been demonstrated by researchers including Alturas Analytics. In collaboration with our sponsors, research continues at Alturas Analytics including investigation of the benefits of MFLC-MS/MS for DBS, biomarker analysis and other bioanalytical assays. For more information regarding Alturas Analytics visit our website at **www.alturasanalytics.com**.

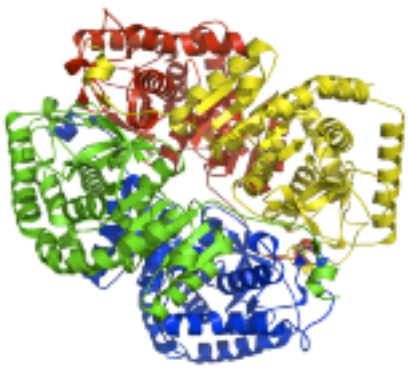
(continued from page 1)

purification, 2) solid phase extraction (SPE) and 3) protein precipitation. **Immunoaffinity purification** procedures can be bead based or column based methods. Depletion methods are often used to remove abundant endogenous proteins (e.g., albumin, IgG) from the sample to improve the selectivity of the analysis. **Solid phase extraction** offers a preparation procedure that can be fine-tuned to select the molecule of interest based on the size and charge state of the molecule. The pore sizes of typical SPE sorbents have been found to exclude proteins larger than 20 kDa. Thus, large endogenous proteins can be depleted via size exclusion before a digestion. Often mixed-mode or ion-exchange sorbents of varying pore sizes are used in combination with reversed phase HPLC to obtain the most selective 2D separation. **Protein precipitation** provides a simple, non- selective clean-up for removal of large proteins (>40 kDa). Depending on the solubility of the protein of interest, a combination of organic (MeOH, acetonitrile, etc.) and aqueous (TCA, perchloric acid, etc.) precipitation solvents can be used. For further selectivity enhancements precipitation methods can be followed by SPE clean-up procedures.

Using the sample preparation methods described above, endogenous proteins may be removed before or after the digestion process. A typical protocol would be to add urea to the sample to denature proteins. This is followed by reduction of the denatured proteins with dithiolthreitol. Alkylation is then performed with iodoacetamide. Trypsin is added to the mixture to digest the protein. After a thorough digestion, the sample is ready for additional clean-up or LC-MS/MS analysis.

Along with the sample preparation challenges, good MS/MS selection for MRM analysis, good chromatography, attention to potential adsorption and stability issues and an optimal selection of a suitable internal standard are imperative for successful analysis of proteins.

Whether it is for monoclonal antibody (mAb), antibody drug conjugate (ADC), or biomarker analysis, LC-MS/MS is increasingly becoming a tool for the quantitative analysis of macromolecules. Continued gains in the sensitivity and selectivity of LC-MS/MS will accelerate the use of LC-MS/MS in support of drug discovery and development programs of macromolecules. Research in the analysis of macromolecules at Alturas Analytics continues and we look forward to expanding our suite of these LC-MS/MS assays. For more information regarding LC-MS/MS of macromolecules and other bioanalytical assays at Alturas Analytics visit our website at **www.alturasanalytics.com**.



Structure of lactate dehydrogenase (tetramer).

OUTREACH 2011-12

Chemical and Pharmaceutical Structure Analysis (CPSA)
Short Course: Method Development for LC/MS: Traditional Approaches and Emerging Trends
Oral Presentation
October 3-6, 2011
Sheraton Bucks County Hotel
Langhorne, PA

American Association of Pharmaceutical Scientists (AAPS)
2011 Annual Meeting and Exposition
Exhibit and Poster Presentation
October 23 - 27, 2011
Washington Convention Center
Washington, D.C.

Pittcon
Short Course: HPLC Methods Development for LC/MS
March 11-15, 2012
Orange County Convention Center
Orlando, FL

Society of Toxicology Annual Meeting
March 11-15, 2012
Mascone Convention Center
San Francisco, CA

CVG Group
The 6th Workshop on Recent Issues in Regulated Bioanalysis
March 26-29, 2012
San Antonio River Walk Marriott
San Antonio, TX

AAPS National Biotechnology Conference
Exhibit
May 21-23, 2012
Sheraton San Diego and Marina
San Diego, CA

60th ASMS Conference on Mass Spectrometry
Presentation (pending), Exhibit
May 20 - 24, 2012
Vancouver, Canada

13th Annual Land O'Lakes Bioanalytical Conference
Presentation
July 2012
Devil's Head Resort
Merrimac, WI

STAFF PROFILE: Derek Laine

Derek Laine has been a scientist with Alturas Analytics since 2008. His responsibilities as a scientist include bioanalytical method development, validation, and sample analysis using HPLC/MS/MS instrumentation in support of GLP and non-GLP studies. Derek has also played a role in fundamental research projects at Alturas Analytics, including investigations of Dried Blood Spot (DBS) and Dried Matrix Spot (DMS) analysis and derivitization procedures for small molecule HPLC/MS/MS analysis.

Derek earned a Bachelor of Science degree in Chemistry from the University of Montana where he gained an interest in analytical chemistry. As an undergraduate Derek did research measuring alkalinity, pH and dissolved oxygen content in local rivers.

Derek went on to earn a Ph.D. in analytical chemistry from the University of Idaho working in Dr. Frank Cheng's lab. His doctoral research included studying the mechanistic pathways to a chemical system that degrades organic pollutants and chemical warfare agents. The system makes use of EDTA (which is also used as an anticoagulant in bioanalysis). His research later showed that EDTA, when complexed to iron, could be employed for the electrochemical detection of peroxide based explosives.

Derek lives in Moscow, Idaho, with his wife Rachel. They enjoy all the outdoor activities north Idaho has to offer, including fishing, hiking and picking huckleberries.