FOCUS

Alturas Advisor

FALL WINTER 2018-19

Discovery Platform for Cystine-Dense Peptides (CDP) Therapeutics Analysis by Microflow HPLC-MS/MS

This research was conducted in collaboration with the Fred Hutchinson Cancer Research Center in Seattle, WA.

At Alturas Analytics, Inc., our research for the analysis of proteins, peptides, antibody drug conjugates, and biomarkers continues to expand [1]. Our routine use of microflow liquid chromatography-tandem mass spectrometry (MFLC-MS/MS) for the bioanalysis of biologics and new biological entities (NBE) shows promise for the improvement in the detection limits and chromatographic results for the analysis of these molecules [2]. Here we discuss the utilization of MFLC-MS/MS for the analysis of cystine-dense peptides (CDPs).

CDPs are miniproteins with a highly disulfide cross-linked arrangement and can be found in all kingdoms. Various biological actions such as toxicity, inhibition, and antimicrobial activity may be exhibited by the small proteins [3]. The cross-linked bodies of the CDP classes provide chemical stability and bioavailability making them an exceptional candidate for therapeutic research. For further classification of CDPs based on connectivity, approximately 700 structures were available and more than 680,000 accepted CDPs have been bioinformatically identified. The effective high-throughput screening (HTS) discovery platform for preclinical therapeutic candidate selection requires quantitative measurement of multiple compounds quickly with adequate sensitivity for future preclinical activities.

The preferred method of analysis for small molecules and peptides is MS paired with high-pressure liquid chromatography (HPLC). A triple quadrupole MS with operation in the multiple reaction monitoring (MRM) mode achieves outstanding results in the detection and precise quantitation of multi-target proteins in human plasma at concentrations in low ng/mL levels. This technology is quickly becoming a chosen analytical technique for proteins due to selectivity problems associated with ligand binding assays. In our study, the CDPs were analyzed both intact and without a digestion. Due to enzymatic or spontaneous modification that may take place, the strength of the intact CDP miniproteins is essential. We established a new method to concurrently extract and quantify multiple intact CDPs in order to rank the candidates. To classify the CDP candidates based on preclinical discovery data, it was necessary to develop the platform so the method has adequate detection limits and is reproducible. In order to achieve lower detection limits, microflow HPLC, instead of conventional HPLC, was utilized during analysis. The MFLC-MS/MS discovery platform provided the additional benefit of use in future pharmacokinetic studies

The data generated indicated an acceptable limit of quantification (LLOQ) can be achieved with the optimized extraction and MFLC-MS/MS methods. The LLOQ for this method is 1.00 ng/mL with a dynamic range of 1.00-500 ng/mL (8 concentrations analyzed in duplicate), accuracy within 20%, and an r value >0.990 for each CDP. Each CDP has been found to be stable in rat plasma for at least one hour with sufficient recovery. One commercially available (stable label) internal standard peptide was found to provide satisfactory precision for the quantitative analysis of all six peptides. No interfering peaks were present in the chromatograms of an extracted blank rat plasma sample.

References listed on page 2.

SCIENTIST FOCUS

Sara Underwood

Sara is the Lead Technical Writer at Alturas Analytics, Inc. She collaborates with scientific staff to deliver submission-ready reports to clients. After graduating from Washington State University with a B.S. in Genetics and Cell Biology, Sara spent 16 years in the biotechnology industry. During her tenure in biotech, Sara provided pharmacokinetic analysis in support of preclinical and clinical biologic drug development. This experience provided her with an understanding of the challenges encountered moving a drug from research into the clinic.

Sara joined us at Alturas in 2014, committed to being part of a team that is paramount in supporting the fight of diseases. Her ability to carry multiple projects while delivering quality reports ahead of schedule makes her a valuable component of Alturas' operational team and a highly respected scientist among her peers. Sara was also a significant contributor in the development of our in-house project management system, ALICE (Alturas Lab Interface Communications Equipment), which enables clear communication across departments to ensure on-time deliverables. Sara recently expanded her responsibilities, applying her expertise of pharmacokinetic and toxicokinetic analysis as a complement to Alturas' suite of bioanalytical services. Sara brings the knowledge as a scientist and a unique perspective from the biotech industry that has greatly contributed to the growth and success of Alturas Analytics.

When not at work, Sara may be found galloping her horse over the Palouse, or hiking with her husband and son exploring the beauty Northern Idaho has to offer. Sara also enjoys swimming and the camaraderie of participating on a team.

References from Page One

1. Shane R Needham and Gary A Valoskovic, "Peptide and Protein Bioanalysis using Integrated Column-to-Source Technology for High-Flow Nanospray" Chapter 5, Protein Analysis Using Mass Spectrometry: Accelerating Protein Biotherapeutics from Lab to Patient, Wiley, (2017), Mike S. Lee Editor.

2. Shane R Needham, "Microspray and microflow liquid chromatography: the way forward for LC–MS bioanalysis" Bioanalysis, Vol. 9, No. 24: 1935-1937. (2017). Editorial.

3. Gracy et al. Structure and modeling of knottins, a promising molecular scaffold for drug discovery. Curr Pharm Des. 17, 4337-4350 (2011).

DISCUSSION CORNER

Quantitative Microflow HPLC-MS/MS Analysis of the Antibody Drug Conjugate SigmaMAb Extracted from Rat Plasma

In this article we examine an accurate, precise, and cost-effective extraction procedure using microflow liquid chromatography coupled with mass spectrometry (MFLC-MS/MS) for the analysis of peptides derived from the antibody drug conjugate (ADC) SigmaMAb that can be applied to the analysis of other ADCs. ADCs are potent and specific biopharmaceuticals that continue to show significant therapeutic promise. ADCs combine the specific targeting abilities of an antibody with a drug that attacks cells that contain a unique marker. This approach significantly improves efficacy while decreasing side effects by minimizing exposure to healthy cells.

The complex nature of ADCs present unique bioanalytical challenges. Biomolecules are typically analyzed using ligand binding assays, which do not have the selectivity to accurately quantify an ADC alone and can often take six months to one year to develop and validate. Traditionally, bioanalysis of ADCs necessitates a combination of ligand binding and LC-MS/MS techniques in order to quantify all components of the molecule. HPLC-MS/MS analysis of the digested antibody is an alternative approach that provides better selectivity and dramatically reduces method development time and associated costs. The drug, linker, metabolites, as well as signature peptides for quantitative analysis of the antibody, can be performed on a single

Outreach 2018-2019

14th Annual Applied Pharmaceutical Analysis (APA)
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platform. Additionally, coupling the mass spectrometer with microflow HPLC (MFLC) results in a significant increase in sensitivity while greatly reducing run times. A rapid universal method for digestion, extraction, and MFLC-MS/MS was developed for potential applications to other biomolecules for quantitative analysis.

The method was developed in rat plasma using SigmaMAb (MilliporeSigma), a commercially available recombinant monoclonal IgG1 human antibody linked to dansyl-fluorophores (Figure 1), with SILu™MAb, an isotope-labeled antibody, as the internal standard. The internal standard was added prior to denaturing the ADC to compensate for any preparation variability. After adding SILu™MAb to the sample plates, the pellet was precipitated with acetonitrile, vortexed/centrifuged, and supernatant removed. Next, the pellet was re-suspended and denatured, extracted using a strong anion exchange solid phase extraction (SPE) method and analyzed using MFLC-MS/MS.

The data indicated that a pellet digested using acetonitrile 0.1% formic acid removed a substantial amount of interfering proteins. Urea, RapiGest™ SF, and Octyl-ß-d-glucopyranoside were evaluated to provide optimal antibody denaturing. It was found that RapiGest™ SF provided superior denaturing to Octyl-ß-d-glucopyranoside. It is also faster to denature than urea and doesn't require further dilution of the sample to reduce the concentration prior to the trypsin digestion.

Further clean-up of the sample was evaluated using various SPE phases, and it was determined that a simple TCA crash provided the highest recovery of the target peptide.

An 8 point calibration curve was extracted and analyzed in duplicate using the optimized methods. The concentration range was 100-10,000 ng/mL. The results of the analysis indicate the method is accurate and precise. Using a 1/X*X linear regression the r value was found to be 0.9979 with the percent difference of the standard points less than 15% from the nominal concentrations. Using the MFLC system instead of the conventional HPLC resulted in a >40% increase in peptide signal.

The extraction and MFLC-MS/MS method developed is accurate and precise and can be used as a starting point for other ADC or biomolecule analysis. This extraction method is cost effective and can be completed in only a few hours.

Figure 1: SigmaMAb Antibody Drug Conjugate Mimic (Recombinant Monoclonal IgG1 Human Antibody Linked to dansyl-fluorophores)

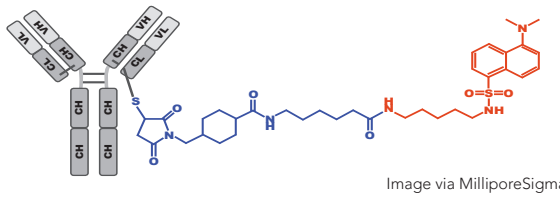
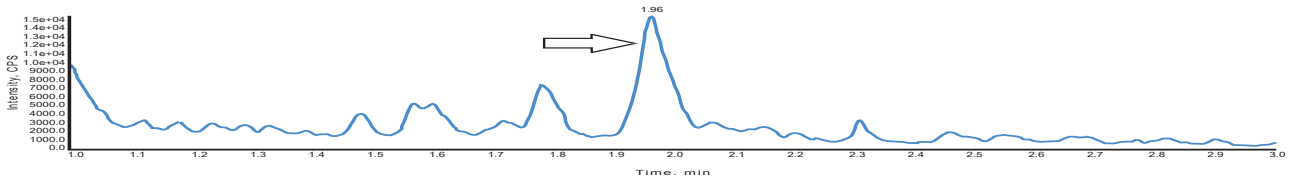


Image via MilliporeSigma

Figure 2: SigmaMAb ADC Mimic (Digested to peptide) 100 ng/mL Extracted from Rat Plasma Chromatogram



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Portland Convention Center, Portland, OR

A MESSAGE FROM THE FOUNDERS

Alturas Analytics, Inc.
The LC/MS Experts™

The Future is Bright for Alturas Analytics, Inc.: Expansion and Growth Continue

At Alturas Analytics, our focus in 2017 was building infrastructure and preparing for company growth to meet new market-driven challenges. Today, we have investments in capital equipment, increased scientific staff, and full implementation of company-wide software integration. Our company is moving forward with greater capacity and streamlined processes operated by an enhanced team of talented professionals.

In 2015, we set ambitious goals for our team called "Vision 2020," titled for our vision of what Alturas will accomplish by the year 2020. We are delighted to say that our team will meet those goals ahead of schedule! We continue to exceed our goals for the same reasons since inception: we maintain our vision of "Build an Enduring Company," and mission of "MS/MS Bioanalytical Experts, Leading the Future of Bioanalysis." We lead the industry with our personalized service and sound scientific approaches. Moreover, we continue to be entirely privately invested and funded, giving us better focus on long-term relationships with our sponsors.

As a continuation of our company's development, we have expanded the offerings of our LC-MS/MS portfolio to include bioanalysis of antibody drug conjugates (ADCs) and monoclonal antibodies (MAbs), peptides, biomarkers, and new biological entities (NBE) in support of regulated studies. We are also pleased to announce that Alturas recently expanded in-house capabilities to include pharmacokinetics (PK) and toxicokinetics (TK) data analysis and reporting.

Microflow LC (MFLC)-MS/MS and the use of specialized immunocapture are routine techniques practiced at Alturas. Since our expert staff of scientists incorporated these non-conventional methods, sample processing and run times of sponsor-transferred methods have been successfully reduced by 30%. We have completed thousands of study samples using fully validated methods with on-time data delivery. MFLC-MS/MS continues to expand in our laboratory with the purchase of two additional dedicated MFLC instruments.

Additionally, the Alturas staff engages in continuous performance improvement by evaluating new program tools to improve efficiencies in all areas of lab operations. These tools consist of a customized bioanalytical laboratory information system, named ALICE (Alturas Lab Interface Communications Equipment), electronic master schedule management systems, software to expedite report writing, paperless document management system to control SOPs and controlled documents, and tools to make the documentation review process of Quality Assurance audits fully paperless. These implementations have further reduced timelines by up to 50% for report writing and data delivery to our sponsors.

From those initial days of two AB Sciex API 3000™ instruments and a few team members to now nearly 20 MS/MS instruments, including Sciex QTRAP® 6500+ and a staff of nearly 50, we continue to thrive and look to the future. Most importantly, we continue to add to our incredible team culture. Our success requires further expansion to such a degree that Alturas recently purchased land adjacent to our current bioanalytical facility. The plans in development will more than double our capacity and size.

Alturas has much to look forward to in 2019 and beyond with enhanced, streamlined operations, a deepened portfolio, and a continued commitment to our clients. The future is bright and one thing is certain, we will maintain our vision and mission and lead the industry in MS/MS bioanalysis, with the ultimate goal of enhancing the health of our communities.

Regards,

Shane Needham, Ph.D, Co-Founder

Robin Woods, President, Co-Founder