FOCUS

Alturas Advisor

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HPLC/MS/MS Bioanalysis of Peptides

The use of peptides as therapeutic agents or as delivery agents for drugs (drug-peptide conjugates) is becoming more common; thus the quantitative measurement of peptides from biological fluids is important to obtain pharmacokinetic data. Often these peptides are measured with the use of immunoassays. However, immunoassays are often not as selective, accurate or precise as HPLC/MS/MS. Additionally, the limited dynamic range of immunoassays can make the analysis of data problematic, which makes HPLC/MS assays an attractive alternative for the measurement of peptides from biological fluids. However, traditional sample collection, preparation and analysis methods may not be optimal when combined with HPLC/MS/MS. The following discussion describes the important procedures to consider when developing HPLC/MS/MS methods for the analysis of peptides from biological fluids.

Stability of Peptides: The stability of peptides in biological fluids can be an issue. In fact, many peptides are designed to degrade to an active form in a certain biological compartment. Since hydrolysis can be pH or enzymatically-driven, the cause of the degradation must first be determined. At Alturas Analytics, we have prepared custom blood collection tubes to control the pH of the plasma to minimize degradation. Additionally, we have prepared custom blood collection tubes with Type A and/or Type B esterase inhibitors to suppress degradation. Proteases in the plasma can also cause degradation of the parent peptide, thus custom collection tubes containing protease inhibitors can also be prepared. There are peptides that have a half-life of seconds or several minutes even with the addition of esterase inhibitors or custom additives. In this case, it may be necessary to crash the entire blood sample in an accurate volume of trichloroacetic acid (TCA). All of the above methods need to be validated according to FDA guidelines for bioanalytical method validation.

Extraction of Peptides from Biological Fluids: The structure of peptides makes them water soluble and difficult to extract using conventional liquid-liquid techniques. Additionally, the poor organic solvent solubility of peptides can make precipitation methods with acetonitrile or MeOH unsuccessful. Our first choice for extraction of peptides is typically solid-phase extraction (SPE). Solid-phase extraction can be optimized for great selectivity and can be automated to process large sample batches. The ionic characteristics of peptides also allow for methods orthogonal to the typical reversed-phase HPLC method to be developed. This makes for a highly selective analysis that greatly reduces ionization effects in the source of the LC/MS. Even with SPE, the solubility and ionic characteristics of the peptides must be accounted for during the extraction. Critical steps, such as adding just the right mixtures of organic and aqueous rinse solvents, must be adjusted. Often with peptides, typical "reversed-phase" or "normal-phase" behavior may not apply.

HPLC/MS/MS Analysis of Peptides: Since peptides are generally fragile and often have large molecular

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OUTREACH 2007-08

24th Montreux Symposium on LC/MS & Related Techniques
October 10-12, 2007
Hilton Head Island, South Carolina.
Presentations pending.
(http://www.lcms2007.org/)

10th Annual Symposium on Chemical and Pharmaceutical Structure Analysis (CPSA),
Oct. 22-25, 2007
Langhorne, Pennsylvania.
Short course Monday, October 22, 2007: "Method Development for LC/MS: Traditional Approaches and Emerging Trends". (*http://www.milestonedevelopment.com/CPSA/2007/index.html*). Alturas Analytics, Inc. is a 2007 sponsor of CPSA.

AAPS 2007
November 11 – 15
San Diego, California.
Please visit Alturas Analytics at booth #1328 (*http://www.aapspharmaceutica.com/annualmeeting/*).

The 59th Annual Pittsburgh Conference
March 1 – March 7, 2008
Ernest N. Morial Convention Center
New Orleans, Louisiana.
LC/MS/MS Short Course: HPLC Method Development for LC/MS.
(*http://www.pittcon.org*)

56th ASMS Conference on Mass Spectrometry
June 1 – 5, 2008
Denver, Colorado.
Presentations pending
(*http://www.asms.org*)

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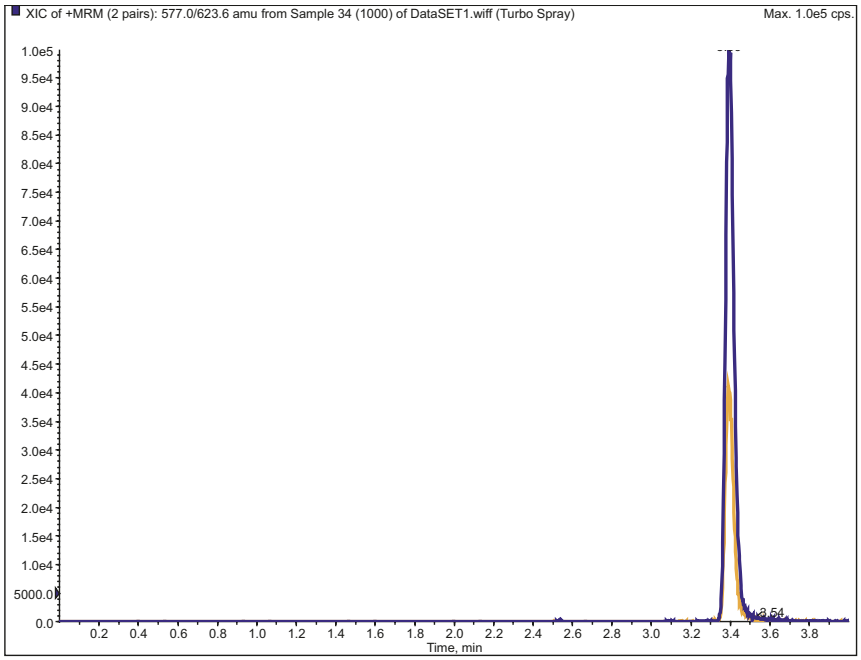
weights, the only ionization mode of choice is ESI/MS. Adequate sample preparation as shown above is critical to reduce the possibility of ionization effects in the source.

Peptides are often polar in nature, so developing a reversed-phase method with adequate retention and good peak shape can be a challenge. Traditional ion-pair or ion-suppressing agents used in conventional HPLC analyses to improve the retention and peak shape of peptides should be avoided with HPLC/MS/MS assays as these reagents have been shown to reduce the MS signal. If silica-based reversed-phase columns are used, the columns should be highly base deactivated. Traditional C18 phases may also not be the optimum choice for the reversed-phase stationary phase. A phenyl type column or other phase may give better peak shape and retention. Non reversed-phase methods such as hydrophilic interaction liquid chromatography (HILIC) may give better peak shape and retention for select peptides. Other critical issues to be aware of for the HPLC/MS/MS analysis of peptides are injection effects, such as carry-over, and mismatched solvent effects. For peptides that are sensitive to slight ionic and polar characteristics in the sample, injection effects can cause poor peak shape and diminished signal.

At Alturas Analytics, we have developed several HPLC/MS/MS assays for the quantitative determination of peptides from plasma. We have developed a general extraction and analysis method that works for many different types of peptides, including cyclic peptides, glycopeptides and linear peptides ranging from 10 to over 100 residues. The methods take advantage of the ionic, polar and non-polar characteristics of peptides for extraction and HPLC/MS/MS analysis. Our LC/MS/MS methods are accurate and precise for the measurement of the peptides to the low ng/mL level.



HPLC/MS/MS Chromatogram from the Analysis of a Peptide and It's Stable Labeled Internal Standard from Plasma

STAFF PROFILE: CHAD CHRISTIANSON

Chad Christianson serves as a Senior Scientist for Alturas Analytics, Inc. Chad's primary focus at Alturas Analytics is LC/MS/MS method development, validation and sample analysis for small molecule drugs and peptides in accordance with GLP guidelines. He has nearly seven years experience as an analytical chemist and a diverse background in the analysis of organic compounds and instrumentation. In addition to his LC/MS/MS method development and sample analysis duties, Chad is also responsible for other duties such as impurity identification using HPLC/UV/MS/MS, protein binding determination, and instrument calibration and maintenance.

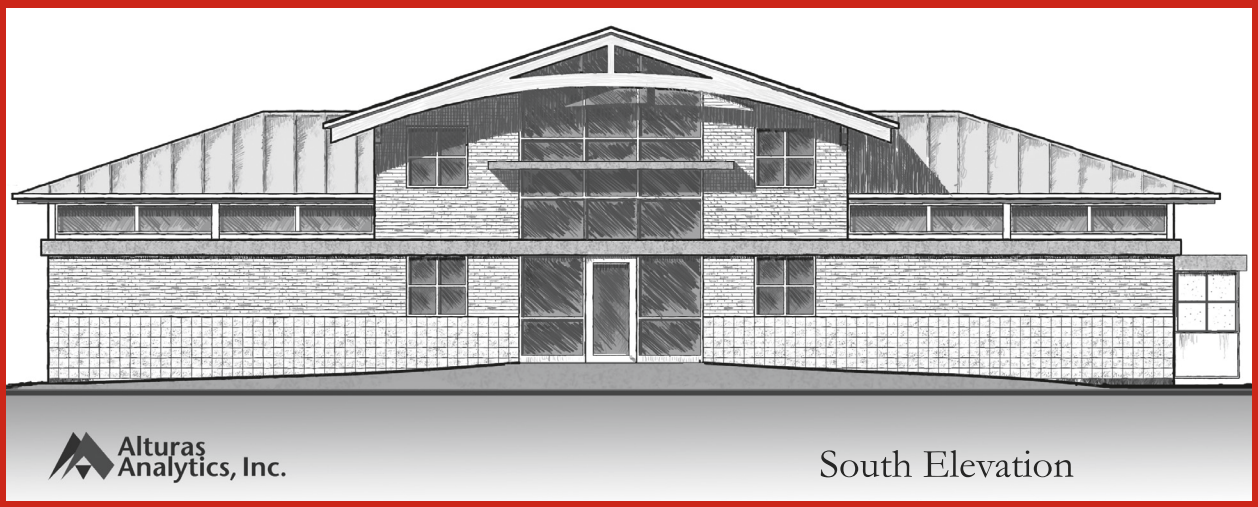
After serving three years in the army, Chad began his education at Washington State University where he received his B.S. degree in Chemical Engineering. Shortly thereafter, he began his analytical chemistry career in the environmental testing field. He was responsible for analyzing water and soil samples for semivolatile fuels, pesticides, PCBs, herbicides, and other organic compounds by GC/FID, GC/ECD, HPLC, and GC/MS.

Chad lives in Pullman, WA, with his wife, Heather, and son, Rudy. They enjoy camping, hiking, and watching WSU football and basketball.

LC/MS DISCUSSION CORNER

Minimization of Carryover with LC/MS/MS Methods on the API-5000

As more sensitive instruments are developed, such as the API5000 LC/MS/MS, sample carryover from injection to injection becomes more critical. Complete elimination of carryover may be impossible. However, if the dynamic range of the assay is three orders of magnitude, carryover should be reduced to <0.01% for optimal results. Carryover is dependent on many variables, including analyte, matrix, HPLC column, frits, tubing and the autosampler. Although carryover can be difficult to isolate, the source of carryover is often from the autosampler. Thus for this discussion we will focus on the autosampler design options available to minimize carryover. Our experience with the various autosamplers with the API5000 shows that generally two options of autosampler design are available. One design is what we will call a "continuous rinse" autosampler, where the injector components are continually rinsed with solvents of choice. The other design we will call a "sequential rinse" autosampler. In a "sequential rinse" design the injector components are usually rinsed sequentially with the use of a separate syringe and various solvents of choice. In our experience, the "continuous rinse" autosampler typically provides acceptable carryover for most compounds with the use of only acetonitrile and water mixtures. The disadvantage of a continuous rinse autosampler is that typically only mobile phase or two different rinse solvent combinations are available for use. On the other hand, for problematic compounds, a "sequential rinse" autosampler can use up to five different rinse solvents to provide more options in eliminating carryover. For example, a strong organic solvent, basified solvent, acidic solvent and various combinations may be used to reduce carryover. A recent example of using a "sequential rinse" autosampler to reduce carryover to an acceptable level was presented at ASMS 2007 by S. Wintermute, et. al. In order to provide the most versatile problem solving tools at Alturas Analytics, we employ both types of autosampler designs.



February 2008: Double Capacity

Since its inception in 2000, Alturas Analytics has developed into and become recognized as a leader in LC/MS/MS bioanalysis. Over the last 7 years, our client base has increased steadily. As a result, we at Alturas Analytics have outgrown our present space and are constructing a new facility to better meet the needs of our growing clientele.

On September 6, Alturas Analytics held a groundbreaking ceremony for a new building. The new, expanded facility will double our current laboratory and administrative space, and allow us to take full advantage of the latest improvements in laboratory technology. We expect that the new facility will allow us to double our capacity in 2008.

Alturas' new building will feature expanded laboratory space, providing support for additional equipment, personnel, and sample storage and preparation. The building will also feature enhanced security systems, laboratory noise abatement technology, and enclosed office spaces for analysts and support personnel. Security alarmed and temperature controlled sample storage areas (including additional -20 and -80°C freezers) will provide room for additional sample storage and archiving. Our communication, archiving, and information systems will be housed in a temperature-controlled, fire-resistant area, and our high-speed fiber optic technology will have redundant back-up to ensure sustained quick and efficient communications with our clients. The entire facility will be connected to an uninterruptible power supply (UPS) with generator backup to ensure constant and controlled electrical power, preventing work stoppages and keeping our clients' samples and data secure.

Alturas Analytics' new facility is scheduled for February 2008 completion. We are excited about the opportunity to continue providing quality analytical service from our new home.

The LC/MS Experts™

www.alturasanalytics.com

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