Alturas **Advisor**

The Bioanalyst: Challenges and Solutions

Bioanalysis Zone "In The Zone" Podcast Summary

Three of the most common challenges in developing bioanalytical assays are sensitivity, selectivity, and sample preparation time. These challenges are augmented when it comes to working with large molecules, prodrug assays, and multi-analyte assays. Alturas scientists have developed reliable ways to address these issues. With extensive experience and collaboration, senior scientists Chad Christianson, Katherine Yahvah, Ph.D., and Michael Williams, Ph.D., have come up with innovative ways to incorporate challenging assays into routine workflows in support of regulated studies.

Large Molecules by LC-MS/MS – Chad Christianson, Senior Principal Scientist

When analyzing large molecules using liquid chromatography tandem mass spectrometry (LC-MS/MS), key challenges are sensitivity, selectivity, internal standard selection, and sample preparation time. Digestion of large molecules in biological matrices results in the formation of thousands of peptides due to the high concentration of proteins present. Many of these endogenous peptides may interfere with your target molecule. Selection of a target peptide with at least eight amino acids is beneficial, but further cleanup of the sample using immunocapture may be required in order to meet the desired lower limit of quantitation (LLOQ) interference free. Many different platforms for immunocapture exist to remove the interferences present in the samples while preserving the large molecule. Magnetic bead capture is the most widely used, but other tip based methods such as MSIA™ are now on the market for commercial use. These alternative methods permit the use of robotics to perform the immunocapture allowing virtually hands-free sample preparation for most of the labor intensive steps required with magnetic bead preparation. Other sensitivity gains can be made at the instrument level by using microflow LC. Ionization efficiency increases as the flow rate going into the source is decreased. A several fold increase in signal may be obtained by reducing the flow from 0.7 mL/min to 10.0 µL/min.

When selecting the internal standard, a labeled large molecule is the best solution. This internal standard will be subjected to the same intense preparation procedure (denature, reduce, alkylate and digest) as the large molecule, so this is the best solution to compensate for any volume or procedural discrepancies. The cost of synthesizing stable label large molecules to be used as internal standards can be reduced by purchasing less expensive stable label peptides that match your target sequence. These stable label peptides are co-eluted with the target peptides so they will compensate for potential suppression and enhancement that may be present during the analysis. However, these internal standards will not compensate for any inefficiency that may have occurred during the denaturing, reduction, alkylation and digestion steps.

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OUTREACH

American Association of Pharmaceutical Scientists (AAPS) PharmSci 360
November 3-6, 2019
Henry B. Gonzalez Convention Center, San Antonio, TX
Presenting

European Bioanalysis Forum
November 20-22, 2019
Hesperia Hotel,
Barcelona, ESP
Presenting

Society of Toxicology (SOT) 59th Annual Meeting and ToxExpo
March 15-19, 2020
Anaheim Convention Center,
Anaheim, CA

14th Workshop on Recent Issues in Bioanalysis (WRIB)
March 30-April 3, 2020
Phoenix Desert Resort,
Phoenix, AZ
Presenting

Analyticon 2020
April 27-29, 2020
DoubleTree by Hilton Hotel San Francisco Airport,
Burlingame, CA
Presenting

TIDES: Oligonucleotide and Peptide Therapeutics
May 11-14, 2020
Hynes Convention Center,
Boston, MA
Presenting

68th Annual American Society for Mass Spectrometry (ASMS) Conference
May 31-June 4, 2020
George R. Brown Convention Center, Houston, TX
Presenting

23rd International Society for the Study of Xenobiotics (ISSX) North American Meeting
October 4-8, 2020
Hilton Waikoloa Village,
Waikoloa, HI
Presenting

When it comes to minimizing sample processing time, optimizing each preparation step is key. Using surfactants such as RapiGest™ (Waters Corporation) decreases the denaturing time compared to urea, and it doesn't require further dilution prior to the digestion step, which may increase the sensitivity. The biggest time savings occurs at the digestion step. Overnight digestion isn't always necessary, so a time course experiment should be performed to find the optimum digestion time. New trypsin 96-well plate technology is also becoming available. These membrane-immobilized trypsin flow through plates theoretically reduce the digestion time from hours to minutes.

In addressing the challenges of sensitivity, selectivity, and sample preparation time, selective transitions, good LC separations, adjustment of flow rate, and of course efficient sample preparation through immunocapture make a crucial difference.

Development and Validation of Assays for Unstable Prodrugs and Their Metabolites in Support of Regulated Bioanalysis
– Katherine Yahvah, Ph.D., Senior Scientist

When it comes to working with prodrug assays, the leading challenge to confront is stability of the analyte in matrix. The best way to address this issue is to modify the collection process at the in-life or clinical facility. Use of alternative anti-coagulants or additives in blood collection tubes is an excellent place to start. For example, Disodium EDTA/Sodium Fluoride is an additive that is commonly used for collection of patient blood samples for the measurement of glucose. It is effective in this case because it is an enzyme inhibitor that aids in prevention of glycolysis. We have found that use of this additive in collection tubes greatly enhances stability of prodrugs that are prone to degradation by esterase activity. This is just one example of many enzyme inhibitors that can be added to samples to preserve the integrity of prodrugs susceptible to enzymatic degradation.

Further enhancement of stability can be achieved by optimizing the pH of the matrix. Often, the addition of a small amount of acid or base can greatly enhance prodrug and metabolite stability. For the purpose of reproducibility, we will commonly provide a detailed collection protocol for the in-life facilities or clinical collection sites to follow, specifying the volumes of matrix and acid or base to combine and mix before freezing the samples. We then follow these same sample handling procedures in the lab when preparing our standard curves and QCs to assure that we are accurately mimicking the conditions to which the incurred samples are exposed.

We have also found that minimizing sample handling and exposure to warm temperatures improves assay performance when working with unstable prodrugs. Specifically, minimizing the number of freeze-thaw events and keeping samples on wet ice while processing them can help with maintaining stability and generating reproducible results.

Another arm of prodrug analysis is the use of gas chromatography to measure volatile metabolites that are incompatible with LC systems. For this work, derivatization of the molecule provides an acute advantage. The ideal derivatization agent will increase the sensitivity and selectivity of the assay. Additionally, selecting the optimum column will improve the selectivity and decrease the run time. Where it is applicable, selectivity can also be enhanced by using head space analysis. Injecting the sample in gas form at a temperature specific to the analyte keeps the instrumentation clean and lends to an improved signal to noise ratio.

Using these strategies, we have succeeded in fully validating assays for prodrugs with limited stability, in addition to performing bioanalytical analysis for subsequent studies requiring analysis of several thousand samples.

9-analyte Assay with Multiple Glucuronide, Sulfate Metabolites, from Method Development, Validation and Routine Rapid Turnaround of Phase 1 Studies
– Michael Williams, Ph.D., Senior Scientist

Initial work with the bioanalysis of a drug showed that it metabolized so quickly after administration that all but extremely high doses measured at <LLOQ. This drug is quickly metabolized into a number of phase II metabolites through sulfation or glucuronidation on either or both ends of the molecule. Because there are multiple conjugation sites on the molecule, this metabolism results in several isomeric metabolites. After preliminary evaluation of the data with the sponsor, the project goal was set to develop a method for the analysis of the primary drug plus eight metabolites for a total of nine analytes.

In order to surmount challenges encountered in method development, we systematically tuned for and analyzed analytes, adjusting chromatographic methods supplied by the client to improve throughput and optimize separation of isomeric species. Several different gradients, mobile phases, and column combinations were tried in order to optimize analyte and isomer separation. To improve selectivity of the analytes, we also explored different derivatization methods.

Eventually, two chromatographic and extraction methods emerged that worked best for two groups of analytes, with four in each method. One method involved derivatization, the other a simple precipitation. The two extraction methods were similar up to the derivatization step, so we combined them into a single written procedure that has instructions for each sub-set of analytes. The ability to use one extraction method that effectively splits the eight analytes into two sub-groups of four, which are extracted concurrently, but analyzed via two separate LC-MS/MS methods, minimizes freeze-thaw cycles for the samples.

Now that the LC-MS/MS method is established, the most difficult hurdle is stability. Using a buffered whole blood approach to stabilize ALL analytes as much as possible has proven to be the best solution. The difficulty of maintaining stability is always compounded when more than one analyte is present in an assay. Not all analytes are always stable under the same conditions but they must be analyzed from the same samples. Other options

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Scientist Focus

Chad Christianson
Senior Principal Scientist

Chad has 20 years of related experience as an analytical scientist with over 15 years focused on bioanalysis at Alturas Analytics. This depth of experience in applied LC-MS/MS and GC-MS/MS, along with an education in chemical engineering, provides the backbone for productive and innovative science. Chad leads the protein quantitation group at Alturas, applying novel techniques to a regulated, high-throughput production environment.

Chad was inspired to become a scientist in grade school after learning the scientific method. He has always wanted to know why something worked and the knowledge he gained laid the groundwork for his career. Science fiction movies, books, and television shows nurtured that curiosity and impacted his realization of the possibilities of science. Now, Chad is inspired by playing a small part in the approval of drugs and therapeutic biologicals that can transform the lives of people suffering from disorders that were previously untreatable.

While working in a bioanalytical laboratory he is prepared to embrace failure and use it as a learning tool. The knowledge of what does not work makes it easier for him to discover what does work and why. "Science allows an individual to ask a question, research and experiment and come up with an answer. Sometimes that answer isn't what is expected but the knowledge obtained getting to the answer can be a tool used for the rest of your life," he says.

When asked what accomplishment he is most proud of he said, "Watching Alturas blossom into a real force in the bioanalytical community. When I started in 2004 we only had a handful of employees and a few instruments. Now we have ten times the number of employees and instruments and have the reputation of taking on projects that have failed elsewhere or are too difficult for most labs to handle. We have a fantastic team assembled and I am honored to be part of it."

"The knowledge obtained getting to the answer can be a tool used for the rest of your life."

to explore may include alternate plasma types, such as plasma containing the anticoagulant Potassium Oxalate, rather than K2EDTA. These alternate anticoagulants can have a mitigating effect on certain types of metabolism, potentially increasing stability of certain drug metabolites.

Another challenge is the widely varying dynamic ranges amongst the analytes. This makes consistent creation of standard and QC working solutions, and obtaining appropriate amounts of drug from the supplier, difficult. Our strategy with this is to use more concentrated stock solutions than usual for each analyte. This requires more material for each stock solution preparation but minimizes the volume change with the addition for each analyte to the mix. When materials are precious and time-consuming to produce, coordinating with the supplier well ahead of time helps to ensure that we have enough of each analyte on hand to cover project needs.

In conclusion, after method development, we were able to separate (via chromatography) all metabolites with the exception of two isomeric compounds. These isomeric metabolites will be analyzed together as a sum peak, leaving us with eight analytes in the end. In bioanalysis, anything more than one analyte complicates matters, and with eight plus analytes those complications can create a real bottleneck in the assay development portion of the project. With careful attention to detail, a little ingenuity, careful planning, and active communication, developing and validating an assay for the bioanalysis of larger numbers of analytes simultaneously can be accomplished.

These three scenarios illustrate Alturas' ability to adapt to challenges and rise to meet them, achieving good results with a quick turnaround time and without compromising assays. What it all comes down to is that we don't look at bioanalysis as a one size fits all technique. We aim to tailor our methods to meet the specific challenges of the analyte.

Listen to the podcast for more detailed discussion of all these topics and to hear how Alturas scientists are working together to creatively meet challenges in bioanalysis. The full interview can be found at https://www.bioanalysis-zone.com/2019/09/26/bioanalyst-analytics-discussion_itz_alturas/.

COMMUNITY COMMITMENT

At Alturas Analytics, our vision is to build an enduring company of integrity in an environment that provides rewarding career paths, and empowers individuals to engage in the well-being of the communities in which they reside. Alturas supports research on a national scale as well as local community programs through participation in charitable events, matching gifts and volunteerism.

We seek to inspire the next generation of scientists through involvement of educational programs at every level. From making science fun for elementary-aged students, active participation in science fairs and guest lecturing at the undergraduate university level, we are stepping towards fostering a generation where science is exciting and an integral component of everyday life.

Recent activities include "Science on Ice", combining science lessons, physical education and ice skating for 5th and 6th graders, "Bike MS" raising funds and awareness in support of the National Multiple Sclerosis Society, "The Pink Tea", for the Gritman Foundation, "The Hope Glow Run" for the National Cancer Foundation, and Christmas for Kids providing Christmas gifts and warm clothing for the children of low-income families.