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Automated Sample Preparation Enables LC-MS/MS as a Routine Diagnostic Analysis for Serum Testosterone.

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Guest: Dr. Judy Stone, Senior Technical Specialist in the Clinical Mass Spectrometry Laboratory at the University of California San Diego Health Center for Advanced Laboratory Medicine.

Randye Kaye:

Hello, and welcome to this edition of "JALM Talk" from *The Journal of Applied Laboratory Medicine*, a publication of the American Association for Clinical Chemistry. I'm your host, Randye Kaye.

Approximately 14 years ago, a study describing the poor performance of testosterone immunoassays, when compared to mass spectrometry, was published. Since then, a number of other studies have been carried out and standardization efforts have been implemented. Due to this, more clinical laboratories are now using liquid chromatography-tandem mass spectrometry, or LC-MS/MS, to measure testosterone, especially in pediatric and female patients. However, not all LC-MS/MS assays are created equal and can vary in terms of, for example, sample preparation, the chromatography method, and calibration, which can lead to measurement bias between methods.

An article titled, "Automated Sample Preparation Enables LC-MS/MS as a Routine Diagnostic Analysis for Serum Testosterone," published in the July 2017 issue of JALM, describes the development and validation of an LC-MS/MS method to measure testosterone that has a low measurement bias when compared to a method certified by the Centers for Disease Control Hormone Standardization Program. This method also has an automated sample preparation strategy enabling it to be used for routine testosterone analysis in place of immunoassay. The first author of this article is Dr. Judy Stone, Senior Technical Specialist in the Clinical Mass Spectrometry Laboratory at the University of California San Diego Health Center for Advanced Laboratory Medicine, and she's our guest for today's podcast.

Welcome, Dr. Stone. First question for you, LC-MS/MS has been used by clinical laboratories to quantify serum testosterone for at least 10 years, why is yet another LC-MS/MS testosterone method of interest now?

Dr. Judy Stone: Well, you mentioned in your introduction that in fact, it's been almost 15 years since that landmark paper that pointed out the problems with automated immunoassays for measuring testosterone in women and children. So you would think that by this time, there would be no question that all laboratories would be using the technique.

So the large reference laboratories like Quest, and LabCorp, Mayo, and ARUP, do use LC-MS routinely for testosterone analysis. But when you move to the next set of laboratories, let's say, that have lower test volume, fewer number of samples, then some of those laboratories might be using them. So large academic medical centers for example, core laboratories for regional health care systems, but many of them are not. And then when you move to somewhat smaller laboratories, smaller in terms of the number of samples that they test, it's much less likely that they would be using LC-MS/MS. And the reason is that although the technique clearly has some superiority, it is complex. It's challenging technically. And so, if you don't have enough workload to dedicate a certain number of people or a certain -- a laboratory and instruments -- towards doing this testing, it can become prohibitive, such that people don't actually use it.

So there's other factors, not just the sample preparation that make the technique complex, but sample preparation is a big piece of it. So, this is why we were interested. We were collaborating with Tecan who makes the AC Plate. And in reading about the way the technique works, it seemed to us like it could be potentially, like it's feasible for us to automate all of our testosterone testing, without necessarily having to add additional people or additional instruments to the laboratory setup that we already have for mass spec testing.

So we feel like that was our question. Could we use this sample preparation technique to facilitate that change, so that we could stop sending out some of our testing and doing the rest of it on automated immunoassay? And the outcome was, yes, that we were able to do that. So that's really -- it's less about a new land-breaking way that you might measure it and more about pushing out the technique so that more laboratories should be able to do it, that less specialized expertise, perhaps, is necessary in order to do this type of testing.

Randy Kaye: Okay. So let's just talk a bit about this mode of sample preparation, absorptive chemistry in a 96-well format. Now this has been described as "relatively easy to automate." So, what makes one sample preparation technique easier or more difficult than another to automate?

Dr. Judy Stone: So, if you're preparing a sample for LC-MS testing, it involves a number of steps. There is pipetting to transfer liquids, there is transferring the analyte, that is testosterone, from one phase say, to another from a liquid aqueous phase like serum to an organic liquid phase, say like hexane, there's transferring the analyte perhaps from a liquid phase to a solid or a stationary phase, or evaporating some of the solvent that you can concentrate the testosterone.

So every time you have to transfer the analyte or if you have to mix it vigorously to enable that transfer from one phase to another, it involves an additional robotic step and it involves an additional opportunity for variance. And of course, our whole goal is to reduce variance. We want to have the process be very, very reproducible for every single sample.

So the fewer steps that you have to make, the fewer transfers that you have to make, it just sets you up to be able to automate in an easier way. That is assuming that the chemistry that actually isolates your testosterone and leaves the rest of your sample, we would say the matrix, the proteins, the carbohydrates, the lipids, it leaves that behind, the simpler it is, the fewer opportunities you have for something to go wrong, if I can put it that crudely.

So that makes it that much easier to automate. There are fewer steps that you have to program the robot, there's fewer opportunities for error to be introduced. And the nature of the AC Plate is everything happens in one 96-well plate and really, the only robotic steps are pipetting and shaking the plates to facilitate the transfer of the testosterone from the sample into the coding on the wells of the plates. And then the final step where you're essentially bringing it back out of the coding but now, you're transferring it into a liquid that's compatible with injecting it into the LC-MS/MS instrument.

There is one final transfer from that original plate to a second plate that we use that actually goes on to the instrument, but that's the only time that you have that transfer. So, I think that's why theoretically, it should be easier to automate. And in our experience, automation always takes much longer than you think it's going to. There's a lot of refining small, little steps, but nonetheless, compared to the other projects I've worked on, I would say that it did live up to that promise in terms of being less complex to automate.

Randy Kaye: Great! Well, that does make sense. And I think you may have already answered this next question, but I just want to see if there's anything you want to add about how this

extraction method is different from solid-phase extraction methods that have been used previously.

Dr. Judy Stone: Right. So, solid-phase extraction is a very powerful technique that's been use for a long time. One of its advantages is that it can be used for a wide range of molecules of different types of properties. So it's not as if the AC Plate is going to replace solid-phase extraction, but one of the differences is again, we are transferring the analyte from the liquid phase into a stationary or solid-phase, but with the AC Plate, the stationary phase is coded on the wells, of the 96-well plate.

With solid-phase extraction, the stationary phase is coded on tiny particles and those particles are then packed until we say it's a bit -- you know, see just a white chunk if you like, in the bottom of the 96-well plate or in a syringe, a barrel, a cartridge, if you're doing it in a non-automated fashion. And so, your liquid has to flow through that bit, and it does give some opportunity to be able to concentrate the analyte. But it also means, especially when you're talking about patient samples which are so diverse, we have diseased patients, we have samples coming from patients say, that are on anticoagulation agents, and so, it doesn't flow through the bed. You have to push it through with positive pressure or pull it through with vacuum.

And so, although sometimes it's a problem, sometimes not, there is that additional challenge of moving that liquid through the bed of the cartridge and also, subsequent liquids. It's usually just applying the sample that causes a problem. And you don't need to do that with the AC Plate. You're not pushing a fluid through a particle bed. So, I would say that's the chief difference between the two techniques. They have a similar step, you apply the sample, you wash away the material that you don't want and then, we would say you elude out, you're bringing your analyte back into a liquid that is compatible with your analysis. Those steps are the same but it's the flow through part that's different.

Randy Kaye: Got it, thank you. So, automated immunoassay works well for many diagnostic tests, but not as well for others, in this case, testosterone. Is this more about the compound or about the measurement techniques?

Dr. Judy Stone: I would love to be able to say just one or the other, but probably, it's a little of both. With immunoassay, we're relying on the antibody, that's the "immuno" part, to give us our specificity.

It recognizes some part of the molecule and says, "This is the molecule we want to measure and not that other one."

It's particularly powerful if you have a large molecule like a protein, and you can sit two different antibodies onto that molecule. For analyzing it, I think it's kind of obvious, you have double the specificity, right? Two sites have to match up with the antibody, not just one.

When you get to smaller molecules, and testosterone would fit into the category of smaller molecules, we can't fit two antibodies onto the molecule, so we have to have only one. So right there, we have somewhat less specificity. And the other thing about steroid hormones in general, this certainly applies to testosterone, is they all look a lot alike. They look very similar. Just a small change in the molecule, one bond or one chemical moiety that's different, and the antibody might not be able to detect that difference. But in your body, physiologically, it could be a big difference. It could be inactive, it could be going from active to inactive or more active.

But with mass spectrometry, we can detect the smaller differences, the combination particularly really of three dimensions. The liquid chromatography part, it separates things out, and then we have, if you want to think of it that way, two dimensions of mass spectrometry, of two stages of mass analysis. So we just have a lot more opportunities to be more specific. So for some molecules, automated immunoassay works very well. But for steroid hormones in particular like testosterone, it's one of the things for which mass spectrometry is measurably better than automated immunoassay.

Randye Kaye: So in the article, you used the word "robustness," robustness to characterize the validated method itself, and then some of the method development work that you performed. So, can you expand a bit on that concept and why you think matters?

Dr. Judy Stone: So, robustness is a bit of generic term, isn't it? It's kind of like saying "pretty." We might all have a somewhat different definition of it. When we're talking about clinical laboratory analyses, this is where the fact that liquid chromatography tandem-mass spectrometry comes from originally a research environment. And it's fairly easy to develop a test quite quickly, maybe in a couple of weeks, maybe do a validation in five days, and run a set of specimens, and publish a paper.

However, if that method is going to be used day in, day out, not just for hundreds of samples but thousands of samples, and as I said earlier, quite diverse samples, it then becomes really a whole different matter, that we want a robust method is one that has low variance and that allows the instrument to stay clean. That sounds pretty simple. We

would say all methods need low variance. That's true, but we find one of the strengths of mass spectrometry is we have a range of different parameters that we can use with every individual result as well as for the whole batch of results to say, is this the correct answer? That's part of the power of the technique that I talked about earlier, that we have all these different levels of specificity and we have a lot of -- we would call it "metadata" which allows us to say, "Yes, this result is correct," unlike immunoassay where basically, we just get a number and we have other measures that we test during the day to say, "Yes, the instrument is working correctly," but we don't have additional information on that one sample.

So with a robust method, you put a lot of effort into reduce variance so that doing many samples per day, different analysts, when the instrument is working perfectly versus when the instrument has worn in a little bit, it's been in use for a number of years, that you want the method to perform the same way every single day. And that does take some additional effort to add that in.

The second thing I talked about, keeping the instrument clean, what do we mean by that? Well, with mass spectrometry, the sample prep process is that we want to retain our analyte, that's the testosterone, and we want to get rid of all the stuff in the sample that we don't want to measure. That's what we call the matrix -- the protein, the carbohydrates. But we never get rid of all of it. There's always a little residual left. And so, every time we inject the sample into the instrument, we're depositing some of that residue, some of that matrix residue, on the hardware of the instrument. It's under high vacuum, it's heated. You can think of it that it's like little bits of burned toast that's depositing on the inside. And you can imagine that over time, that degrades the performance of the instrument and then it needs to be cleaned. And we can determine when it needs to be cleaned by monitoring various functional parameters of the instrument. But I think you can see if we do the cleanup on the sample up front, then we have less deposit on the instrument, it stays clean longer, it has to have maintenance less frequently and that means, we're spending more of our time using the instrument for testing and less of our time to be cleaning, to be -- it's not productive while it's being cleaned and recovering from being cleaned.

So a robust method really, to me, it has to meet those two things, low variance and something that keeps the instrument clean. And it's harder than you might think to achieve those two things. But that we felt that it was worth the additional effort, as they say, to try to deliver those qualities with the method that we work on because when

you're in the business of patient testing, then it becomes very important to you. That's what allows you to meet your budget, that's what allows you to meet the quality that's necessary when you're reporting results for patient samples and patient care.

Randy Kaye: Okay, thank you. And I will say that doing all these podcast for JALM, I think that everything is harder than I might think. You've spoken about many steps and strategies that were taken to optimize this method. So my last question is, if you had to choose just one as the most significant contributor to the robustness of the method, what would that be?

Dr. Judy Stone: I think that ultimately, my experience has been both with this technique and with some other similar ones that I've worked on, the step that contributed to the best precision, that lowered the variance of the method, was how we pipetted what we call the internal standard reagent. So, this is a compound that's very similar to testosterone. It's testosterone labeled with deuterium so it has a somewhat different mass, but it performs chemically exactly like testosterone. And it's that internal standard is typically made up in methanol or in methanol or water or another organic solvent in water and it turns out that that's much more difficult to pipette accurately than it is to pipette an aqueous sample like serum. We would think of serum as an aqueous sample.

And so, as always, the devil is in the details of how much volume do you pick up in the pipette tips? Do you prime the pipette tips with the internal standards? When you pipette it, do you touch it off to the liquid that's in the well? How slowly do you dispense it? The variables seemingly are infinite. And my experience was that I directly as I could improve that pipetting step by optimizing the pipetting parameters of the robot, I can see a measurable improvement in the precision of the technique.

So that was not really what I would have expected before I started working with automated pipetting, but that really was the step that I would say, was the most useful to us for getting low variance.

Randy Kaye: That was Dr. Judy Stone from the University of California San Diego, talking about the JALM article, "Automated Sample Preparation Enables LC-MS/MS as Routine Diagnostic Analysis for Serum Testosterone" for this podcast. Thanks for tuning in for JALM Talk. See you next time, and don't forget to submit something for us to talk about.