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High-Resolution, Accurate-Mass Mass Spectrometry Urine Steroid Profiling in the Diagnosis of Adrenal Disorders

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Guest: Dr. Ravinder Singh is Director of the Mayo Clinic Endocrine Laboratory.

Bob Barrett: This is a podcast from *Clinical Chemistry,* sponsored by the Department of Laboratory Medicine at Boston Children's Hospital. I am Bob Barrett.

Adrenal steroid analysis plays an important role in the diagnosis of Cushing's syndrome, disorders of steroidogenesis, and adrenal tumors. The analytical sensitivity and specificity for steroid assays have evolved over time from individual immunoassays to multiplexed mass spectrometry-based methods. This transition has highlighted a need for simultaneous measurement of multiple steroids and their various metabolites to achieve the best diagnostic accuracy. Therefore, metabolomic profiling performed by liquid chromatography, high-resolution accurate-mass mass spectrometry might be poised to make a substantial impact on endocrine laboratory testing.

The December 2017 Issue of *Clinical Chemistry* describes such a method used to determine sex- and age-based reference intervals and to perform a limited assay evaluation in patients with different adrenal diseases.

For this podcast, we are joined by Dr. Ravinder Singh, Director of the Mayo Clinic Endocrine Laboratory. His research career is focused on discovering innovative ways to apply mass spectrometry methods to provide patients with faster and more accurate diagnosis.

So, Dr. Singh, before we get to the article, let's talk about some of your background and experience with steroid testing in the clinical lab.

Ravinder Singh: Sure, Bob. I have been practicing as a clinical chemist at Mayo Clinic for 17 years. And this time, I have been involved in developing and validating mass spec tests for various steroids which include cortisol, aldosterone, testosterone, and 17-hydroxyprogesterone. We performed these tests for various adrenal diseases like Cushing's, primary aldosteronism, or Conn's syndrome and congenital adrenal hyperplasia.

Currently, we diagnose Cushing by monitoring free cortisol in urine. We diagnose primarily aldo by measuring aldosterone in urine and plasma and we do diagnose congenital adrenal hyperplasia by measuring steroids like 17-hydroxyprogesterone, androstenedione, and testosterone.

Overall, these diseases do have high morbidity and mortality rates if they are not diagnosed and treated correctly on time. At the same time, steroid testing for this kind of disease or this disorder is not new. Historically, steroid metabolites have been measured by various methods. For example, in the 1930's chemists used to measure 17-ketosteroids by a reaction called Zimmermann, which is the reaction of 17-ketosteroids with meta-dinitrobenzene which gives a colored product at 550 nanometers. But very soon, it was realized that many endogenous compounds can interfere with this assay.

Then in 1950's chemists tried paper chromatography, thin-layer chromatography, but these kinds of approaches then were becoming laborious and very qualitative. Then, in the mid-‘80s, 15 steroid metabolites were hydrolyzed and measured by GC-FID. So, GC stands for gas chromatography, FID stands for flame-ionization detection, but flame-ionization detection is not very specific compared to the mass spectrometer. In the 1980s, good for the clinical chemistry field, Dr. Rosalyn Yalow got a Nobel Prize for developing radioimmunoassays for various proteins and peptides like insulin and serum and plasma.

As a result, the radioimmunoassays were also developed for these steroids and which were implemented in various labs. But very early on, it was realized that some of the interferences were affecting for example, when you do testosterone measurement in women or in children or do estrogens in men, the assays were not very specific.

To overcome this, the GC-MS was introduced in our clinical chemistry and was considered gold standard in the ‘90s. But at that time, the methodology of mass spec was expensive, laborious, and was not very practical for the routine clinical chemistry labs. In 2000 or so, a lot of manufacturers converted to these radioactive assays, to the chemiluminescence-based assays, and using antibody as a reagent. These platforms were very convenient to implement to the clinical chemistry lab, but they were not using any extraction to remove any interferences from our urine or serum samples.

And the clinicians were getting very frustrated because they were getting many false-positive results, and these false-

positive results were due to the cross-reactivity of the antibody with these interferences.

Now, most of the hospital labs are performing steroids testing by using mass spec to overcome the problems I just mentioned using LC-MS and low-resolution mass spec. So, the introduction of mass spec in the clinical endocrinology was not by choice but rather, the necessity to improve the specificity of the steroids method.

Bob Barrett: Okay. Now, in your article's introduction, you note that steroid metabolomics might be poised to make a substantial impact on endocrine laboratory testing. Let's get basic on this. What is steroid metabolomics?

Ravinder Singh: Steroids are synthesized from cholesterol with the help of many enzymes in the adrenal gland, and the steroids are big groups. One group is called glucocorticoids and cortisol is one of those examples, which plays an important role in glucose maintenance in the blood circulation. The other group is called mineralocorticoids and the aldosterone will be a good example of this category, which plays an important role in maintaining the blood pressure. And then, the third group is called sex steroids and the DHEA is an example of the sex steroids which is a precursor of testosterone and estradiol.

When we study the precursors and the metabolites of the target bioactive steroid, for example, cortisol, but we study it as a group, and that is what we call steroid metabolomics. For adrenals tumors imaging procedures like CT scan and MRI are being used very routinely these days. And our radiologist colleagues are finding adrenal nodules accidentally which are now called adrenal incidentalomas. These nodules or adrenal masses are highly prevalent to almost 5% in the general population. Although majority of these tumors may be hormonally inactive or benign adenoma, but a small percentage, up to 8% of these, depending upon the size of the tumor, do have malignancy and they are called adrenocortical carcinoma.

The survival rate for these patients with the carcinoma is very poor. CT and MRI have a very poor specificity to help these patients, and the existing lab test for steroids also don't add additional diagnostic values for confirming adrenocortical carcinoma to help these patients out.

So, the approach of steroid metabolomics has a potential in the endocrinology that by measuring multiple steroids, instead of depending upon a single steroid, we can confirm the diagnosis and differentiate adrenocortical carcinoma from adrenocortical adenoma or the benign nodules. Before our study, which is going to come out in the issue of *Clinical*

Chemistry, there were three other studies which have shown that by measuring urinary steroid metabolites, we can differentiate between adrenocortical carcinoma from adrenocortical adenoma, and this will be highly specific and accurate and will have a higher diagnostic value.

Bob Barrett: So, what benefits did high-resolution mass spectrometry provide in these illustrative cases over more traditional clinical mass spectrometry methods that have lower resolution?

Dr. Ravinder Singh: Good question again, Bob. In this method which we published in the *Clinical Chemistry* journal, we are quantifying 26 different steroid metabolites in urine. We know that urine is a very complex human matrix. Urine can contain many endogenous and exogenous chemicals including drugs and dietary components, which show up as an isobaric interference when we are doing steroid metabolomics approach to study these patients. We did start this project in a low-resolution mass spec instrument, but considering the clinical severity of the adrenal cancer, we prefer to use the high-resolution mass spec instrument. High resolution means more detail about the signal and the noise of the method, so that we can distinguish the steroid with more accuracy, and it is well known that high-resolution instruments provides twice the identification confidence in comparison to the low-resolution mass spectrometry.

For adrenal disorders, especially cancers, high accuracy and specificity is very important. In clinical diagnostics, we don't want to take any risk since the liability is very high. And now, this high-resolution technology has been shown to work in the clinical labs. And even though it is very expensive compared to the triple-quads, but the 1% chances of error on the low-resolution instrument can turn out to be very expensive for a cancer patient.

Both qualitative and quantitative analysis can be improved by using high-resolution mass spec. So, in an ideal world, we can now run the instrument, which is a high-resolution instrument, both in untargeted mode without sacrificing any quality for the target steroid. This will help us in learning more and more about diseases like adrenocortical carcinoma.

Bob Barrett: Well, this approach does sound very promising. Understanding its translation to clinical workflow, which is in the early stages, what are some of the challenges of applying high-resolution mass spectrometry for clinical applications?

Ravinder Singh: So Bob, this high-resolution instrument, or the mass spectrometer, is designed to collect a lot of information like all the molecules which are present in the urine or in the serum. But, the negative is that this generates a lot of data and we may require a big computer service. The development of the analytical method for steroids in the metabolomics approach is not difficult. But the clinical validation so that we can prove its clinical use for rare cancer will be time consuming. It will be slow because we don't have enough clinical cases to prove the value of the steroid metabolomics approach, so that is one of the challenges as well.

Once we have all this information in the computers, then we will need a dedicated bioinformatics person also, which we currently don't have in the clinical lab and who will help us to go into this data and help in finding the additional information which will have some clinical value as well.

In addition, we will have to make sure that we follow HIPAA rules, that we get consent from our patients to explore this data which could also be one of the challenges in the clinical labs as well. Dr. Alan Wu from the University of California, San Francisco, has been using high-resolution accurate mass spec instruments in the field of toxicology for the patients who were coming to the emergency department. As newer and newer drugs of abuse were coming to the market, it was necessary to detect those drugs in an untargeted high-resolution instrument so that physicians could help these patients even though we don't have the validated methods for these new drugs on the market. But the challenge for him for legal reasons would be that we cannot synthesize this compound very quickly, and because we don't have good calibrators and good controls, and that may not stand in the court. So currently, the cost of the high-resolution instrument is also very high compared to the triple-quad, but these prices may come lower as we follow the field.

Bob Barrett: Doctor, when describing your method, you specifically discussed how you optimize the hydrolysis conditions. Why is this an important consideration in the analysis of urinary steroids?

Ravinder Singh: Steroids like cortisol and testosterone are very potent hormones for human body to have their effect on a timely basis. They do have short half lives. They are inactivated by various enzymes, and they are cleared from the human circulation and excreted in the urine. Before excretion, hydrophobic steroids are converted into hydrophilic water-soluble glucolytes and sulfates. Direct measurement of this steroid conjugate is very complex than the unconjugated

steroids, so labs have to perform the hydrolysis of these conjugates.

It is essential for the assays that the hydrolysis of this urine sample is complete before we do this analysis on the mass spec. Hydrolysis converts these conjugate steroids back to the unconjugated steroids simplifying mass spectrometry analysis. In our study, we compared the hydrolysis between acid and the enzymatic hydrolysis, and the enzyme we used is called Glusulase which has both glucuronidase and sulfatase activities.

What we found out was that enzyme hydrolysis gave us better yield of unconjugated steroids compared to the acid hydrolysis. In which case, we found out that some steroids even degraded and we couldn't see any signal. So in short, we take 150 microliters of thawed urine. We add 50 microliters of internal standard. Sample is vortex-mixed and we let it sit for 10 minutes, then we add 50 microliters of 3 monosodium acetate buffer of pH 5.2, then we add 10 microliters of Glusulase enzyme to the tubes. We incubated it at 2 hours at 50 degrees centigrade, then we stop the reaction with 50 microliters of potassium carbonate.

As you can see, this is a very complicated and laborious recipe. In clinical labs, the precision of the test or our procedure is very important to make a positive impact for the patient care. In addition to that, our test needs to be sensitive and specific, but the precision of our recipe is really dependent upon the reagents we use. What we found out was this particular enzyme by the supplier when it was given to us, it had a lot of variability between lot to lot. So to answer your question, each lab would have to worry about the quality of the enzyme to optimize the hydrolysis, and it can be tricky, time consuming, very involved, and could be steroid dependent and the hydrolysis is also very highly temperature, and time, and pH dependent, and there is a possibility that one lot of Glusulase may hydrolyze one steroid better than the other steroids. So these are the some of the challenges, Bob.

Bob Barrett: Okay. Well finally, doctor, do you think the future of steroid metabolomics involves a targeted or untargeted approach, or is there a role for both? And are there implications for research beyond the clinical utility?

Ravinder Singh This is a very complex question, Bob, and it reminds me that it's almost like the example we use of chicken and egg, which came first? As you are well aware, that the next-gen sequencing has brought revolution to the medicine, instead of performing a single genetic mutation test, we now perform next-gen sequencing where we get the data for all the mutations in the patient's DNA sample. And I strongly

believe now that a similar effort has to be required in steroid metabolomics or rather, metabolomics of various compounds we test in the clinical chemistry area. Unfortunately, the amount of the effort and the investment made in metabolomics and proteomics has been a fraction of that which has been invested into next-gen sequencing.

The future medicine will require that we integrate all biochemical genetic testing information and use bioinformatics to get the most value of all the various lab tests we do on our patients, especially in the cancer population. In the past, we had a big separation between research and the clinical factors. Going forward, one will not survive without the other. We will have to work together to get the most benefit for our patient. So in summary, the steroid metabolomics is emerging as a powerful tool with significant potential for improving the management for the patients with adrenal tumors.

Bob Barrett: Dr. Ravinder Singh is director of the Mayo Clinic Endocrine Laboratory. He has been our guest in this podcast from *Clinical Chemistry*. I'm Bob Barrett. Thanks for listening.