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Immunoextraction - tandem mass spectrometry method for measuring intact human chorionic gonadotropin, free beta, and beta core fragment in urine

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Guest:
Dr. Anthony Butch is a Professor in the Department of Pathology and Laboratory Medicine, and Director of the Olympic Analytical Laboratory at the Geffen School of Medicine at UCLA.

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

Analysis of Human Chorionic Gonadotropin or hCG is usually associated with monitoring or detecting pregnancy. It is also a protein tumor marker for some cancers. In males, hCG stimulates testosterone production and has the potential to be abused by athletes in an attempt to enhance performance in sports.

The use of hCG by male athletes is banned in most sports, however, detecting potential hCG use is complicated by the low threshold concentrations and lack of specificity in some immunoassays.

In the August 2014 issue of *Clinical Chemistry,* a paper was published describing a new assay using immunoextraction and LC Tandem Mass Spectrometry to measure hCG in its many isoforms.

The senior author of that paper is Dr. Anthony Butch. He is professor in the Department of Pathology and Laboratory Medicine, and Director of the Olympic Analytical Laboratory at the Geffen School of Medicine at the University of California in Los Angeles, and Dr. Butch joins us in this podcast.

Doctor, why did you develop a Liquid Chromatography Tandem Mass Spectrometry method to measure isoforms of Human Chorionic Gonadotropin, or hCG, in urine?

Dr. Anthony Butch: The major reason we developed this testing method is because there are currently no tests available to measure urinary isoforms, or hCG, which is Human Chorionic Gonadotropin, separately in a single assay. The major isoforms present in urine are intact hCG, free beta-subunit and beta subunit core fragment.

Although intact hCG and total hCG beta-subunit immunoassays are routinely used by clinical laboratories,

they are only used to measure hCG in serum or plasma blood samples. In fact, none of the quantitative hCG tests in the US are FDA-approved for testing of urine samples. There are, of course, qualitative urine hCG tests that are marketed as pregnancy tests, but these tests only provide a positive or negative result for hCG based on a cut-off value that is usually around 20 international units per liter.

Now as people know, hCG use is banned in males according to the World Anti-Doping Agency Prohibited List, so accredited anti-doping laboratories must develop a method to measure hCG in the urine of the male athletes. I believe that all anti-doping laboratories validate and are currently using blood hCG immunoassays for this purpose.

The major problem with this approach is that commercially available hCG immunoassays differ in their cross-reactivity against beta-subunit core fragment. This is problematic since beta-subunit core fragment is the predominant isoform in urine, so results among laboratories can very widely depending upon the cross-reactivity of the antibody in the immunoassay for beta-subunit core fragment. This of course is not an issue when these assays are used to test serum plasma samples because beta-subunit core fragment is not found in blood.

The Liquid Chromatography Tandem Mass Spectrometry method we developed recognizes all three isoforms of hCG equally and can be used to help harmonize hCG results across various doping control laboratories. This is another reason why we developed the method.

And finally there is a need for a method to measure hCG isoforms that is not affected by the composition of the urine for doping control purposes. We all know that the composition of urine or the urine matrix, as it's often called, varies considerably from sample-to-sample, depending on many variables including exercise and food and fluid intake.

Over the past few years we have identified several urine samples that screened positive for hCG using a total hCG beta-subunit immunoassay based on a value above the cut-off, that is five international units per liter. When confirmation testing is performed on these samples using an intact hCG immunoassay as recommended by the World Anti-Doping Agency Guideline document, the concentration of intact hCG is well below the cut-off and it's often below the detection limit. These samples are considered negative. This combination of results is consistent with the presence of free beta-subunit and no intact hCG.

However, when the samples are analyzed by our Liquid Chromatography Tandem Mass Spectrometry method they

contain extremely low concentrations of all hCG isoforms that in total are well below the cut-off value. This indicates that the results from the total hCG beta-subunit test are false positive and are due to a matrix effect.

Urine matrix effect has been noted from many of the total hCG beta-subunit immunoassays, but the frequency differs among assays. Because the Liquid Chromatography Tandem Mass Spectrometry method is not subject to matrix effects, it can be used to conform false-positive screening results.

Bob Barrett: So doctor, why would a male athlete use hCG? What advantage might that give him?

Dr. Anthony Butch: That is a great question. I can imagine why anyone would inject hCG in their body unless they had a legitimate medical condition for using hCG, such as a male with hypogonadotropic hypogonadism. Since hCG stimulates interstitial cells of the testes to produce androgens including testosterone, an athlete might be tempted to inject hCG to boost blood testosterone concentrations in order to increase strength and muscle mass.

However, the most common reason for using hCG is probably to normalize endogenous testosterone production after discontinuing a cycle of high-dose anabolic androgenic steroid use. High-dose steroid use suppresses a hypothalamic pituitary axis and results in low blood testosterone concentrations.

The testosterone concentrations slowly return to normal over time after discontinuing the steroid use. Although the hCG that they would use will quickly normalize testosterone production, unfortunately it does not reverse suppression of the hypothalamic pituitary axis, so the problem will persist after the athlete stops using the hCG.

Bob Barrett: What is the significance of being able to distinguish between free beta-subunits and free beta that's just part of the intact hCG molecule?

Dr. Anthony Butch: There are two reasons why we want to know if beta subunits are free or are associated with the intact hCG molecule. The first reason is to better understand why a small percentage of urine samples screen positive using a total hCG beta immunoassay and our negative when tested using an intact hCG immunoassay. I have already discussed this.

The second reason for distinguishing between the two hCG isoforms was so we could determine if free beta-subunits predominated after the administration of pregnancy purified or recombinant formulations of hCG in order to determine the best test to use to detect open with hCG, when we did

these studies we found that free beta-subunit was a low concentration at all time points after administration of either hCG formulations and that an intact hCG test is the best marker to detect doping with hCG.

Bob Barrett: Just what is hCG beta core fragment; why measure this?

Dr. Anthony Butch: Beta-subunit core fragment is a degradation product of free beta-subunits that is produced by the kidneys during renal excretion. It contains approximately half the number of amino acids present in free beta-subunit and consists of two fragments of free beta-subunit that are linked by disulfide bonds. Under normal conditions beta-subunit core fragment is the predominant isoform found in urine.

Now why would we want it measured? Well we want to measure beta-subunit core fragment separately from the other isoforms, so we could determine if it is present in urine samples in significant concentrations. This is important because total hCG beta immunoassays have variable cross-reactivity of the core fragment and this variable cross-reactivity might contribute to the widely varying results that are often observed using different immunoassays.

In addition beta-subunit core fragment could produce a positive result in the total hCG beta immunoassay screen, if the antibody used in immunoassay has a significant cross-reactivity with beta-subunit core fragment and it is present in high enough concentrations. This would help explain why intact hCG immunoassays produce negative confirmation test results when the screening test is positive. Since most intact hCG immunoassays do not cross-react with beta-subunit core fragment.

In the majority of these cases we identified, we detected very low concentrations of beta-subunit core fragment by the Liquid Chromatography Tandem Mass Spectrometry method we developed that indicates that beta-subunit core fragment was not responsible for the positive immunoassay screen results.

The other reason for measuring beta-subunit core fragment is to determine the most sensitive test for identifying doping athletes. Although we found a rapid increase in beta-subunit core fragment following administration of pregnancy purified hCG, intact hCG turned out to be the most sensitive marker for detecting hCG use.

Interestingly, we found that the rapid increase in beta-subunit core fragment following administration of the pregnancy purified hCG formulation was due to contamination of the hCG with 27% beta-subunit core fragment.

Bob Barrett: Dr. Butch, talk about the advantage this method has over immunoassays that are currently in use to measure Human Chorionic Gonadotropin?

Dr. Anthony Butch: Well, I've already touched on some of the advantages of Liquid Chromatography Tandem Mass Spectrometry method for measuring hCG. The method can distinguish between the three major isoforms in urine, and can also provide quantitative results. This is not true for any of the commercially available immunoassays that are out there.

Our method also recognizes all three isoforms equally, and does not suffer from matrix effects which are major limitations of immunoassays.

The Liquid Chromatography Tandem Mass Spectrometry method also has excellent analytical sensitivity. Since the procedure removes hCG isoforms from urine and then monitors several transitions from unique tryptic peptides. And lastly, the lower limit of quantitation of our method is at least 10-fold lower than for commercially available immunoassays. The superior lower limit of quantitation will be really essential for revising hCG cutoff values for doping control purposes.

Bob Barrett: Well finally, doctor, we know that this Liquid Chromatography Tandem Mass Spectrometry method was developed for doping control purposes, but does it have any clinical application?

Dr. Anthony Butch: Yes, I believe that the method has clinical applications. Since many qualitative hCG point-of-care devices that are used for pregnancy are susceptible to the false negative results from beta-subunit core fragment our method can be used to prove or disprove that a false-negative urinary hCG result is really due to the presence of high concentrations of the beta-subunit core fragment.

Another clinical application for our testing method would be in potential cases of familial hCG syndrome. Familial hCG syndrome has been described in the literature as a rare benign condition that is associated with free beta-subunit and free beta-subunit missing the C-terminal peptide, in urine at concentrations that can produce a positive hCG screen result.

Since the antibodies used in our immunoextraction procedure already capture free beta-subunit missing C-terminal peptide, we could easily incorporate the precursor product ions for this isotope into our method. This would allow us to monitor concentrations of free beta-subunit

missing C-terminal peptide in potential cases like this to help us better understand this unusual clinical entity.

Bob Barrett: Dr. Anthony Butch is a Professor in the Department of Pathology and Laboratory Medicine, and Director of the Olympic Analytical Laboratory at the Geffen School of Medicine at UCLA. He has been our guest in this podcast from *Clinical Chemistry*.

I am Bob Barrett. Thank for listening!