Clinical Chemistry

Quantification of 1&α,25 Dihydroxy Vitamin D by Immunoextraction and Liquid Chromatography-Tandem Mass Spectrometry

Bob Barrett: This is the podcast from *'Clinical Chemistry'*. I am Bob Barrett. Liquid chromatography-tandem mass spectrometry or LC-MSMS has changed the face of laboratory testing by providing multiplexed, high-throughput analysis, with better analytical specificity than currently possible using antibody-only detection method.

Various LC-MSMS based methods for measuring the initial metabolized form of vitamin D are available. However, the metabolism of vitamin D includes two separate hydroxylation steps. The first occurs in the liver and produces the initial 25-hydroxylated form of vitamin D. This 25-hydroxylated form serves as an indicator of vitamin D stores, but has low affinity for the vitamin D receptor and consequently, low biological activity.

Transit to the kidneys bound by vitamin D binding protein is followed by a second hydroxylation reaction to the dihydroxy form of vitamin D. It is this dihydroxylated form that is responsible for the biological activities ascribed to vitamin D, including increased intestinal absorption of calcium and phosphate, bone mineralization, suppression of parathyroid hormone synthesis and secretion and parathyroid cell proliferation.

The measurement of the initial 25-hydroxylated form of vitamin D is useful in determining vitamin D stores, but analysis of this vitamin D metabolite provides no information about the active dihydroxylated metabolite of vitamin D.

Measuring the plasma concentrations of the dihydroxylated form of vitamin D is important for the diagnosis and management of patients with chronic kidney disease, oncogenic osteomalacia syndrome, and acquired or inborn errors of phosphate homeostasis.

The concentration of this form of vitamin D and plasma is much smaller when compared with the more widely measured precursor, and inferior sample preparation techniques can negatively impact the performance of clinical LC-MSMS-based methods.

In the September issue of Clinical Chemistry, Dr. Andrew Hoofnagle, one of the Medical Directors of the Clinical Laboratories at the University of Washington in Seattle, presented a new method of detecting the dihydroxylated form of vitamin D with improved results.

Dr. Hoofnagle is our guest in this podcast. Dr., mass spectrometry continues to become more and more popular in clinical chemistry labs, why is that?

Dr. Andrew Hoofnagle: I think that there are two reasons that mass spec is making more and more inroads into the clinical laboratory. One is the potential for improvement in patient care, and the other is cost savings. In terms of improving patient care, mass spectrometry, because of the way it analyzes molecules and how it detects molecules, offers improved specificity, such that interference of the molecule that we are not looking for in the assay is not as much of a problem.

In addition, many of the lab tests that we use to measure things like proteins and different small molecules or immunoassays, the antibody is not specific, in that other molecules can interact with that antibody. And the mass spectrometer, because it can tell the difference between many of these molecules that the antibody cannot, adds an improvement and the certainty with which our measurements are performed.

In terms of cost, while the instruments themselves are expensive, one doesn't have to pay for as many reagents or as many expensive reagents in general than one has to pay for using an immunoassay platform, which generally every test can be, and the dollars for mass spectrometry, that's generally not the case.

In addition, mass spectrometers can be thought of as open platforms where the laboratory can add whatever tests to that platform they can, whereas other instruments are more limited. For that reason the testing on the immunoassay platforms can be more expensive.

Bob Barrett: Can mass spectrometers be used to measure all types of analytes in the clinical lab?

Dr. Andrew Hoofnagle: We have seen mass spectrometers used to measure things in toxicology, so exogenous substances that are present in human samples at levels that are easy to detect.

We have now used mass spectrometry in endocrinology to measure substances that are lower abundance, but are acting as hormones in the human body.

We have been using it to measure heavy metals, so exposure to different heavy metals we can use a different type of mass spectrometer than what we normally use to measure analytes and toxicology and endocrinology.

There are even some very exciting reports about using mass spectrometers and database searching algorithms to identify bacteria from smeared colonies on what we call MALDI targets, which are basically just pieces of metal that we can use to help ionize the bacteria and have proteins and other small molecules and larger small molecules fight on the plate too.

And using a database searching algorithm we can identify what bacteria we smeared on the metal plate. I think that's a huge advance in terms of improved patient care, because it's just so much faster than more of the standard biochemical approaches to identifying bacteria. So that's pretty exciting.

And then finally proteins, we have been experimenting in my laboratory, and many other laboratories are trying to prove that we can robustly measure the concentrations of proteins using mass spectrometers, and those experiments are still ongoing. I don't think we have a perfect platform yet for measuring proteins by mass spec, but we are certainly working on it.

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So can we measure all types of analytes in the clinical laboratory? I don't know if we can measure all of them, but we have certainly taken a big bite out of the bread and butter tests in the clinical laboratory.

Bob Barrett: We are getting into some tests. Why did your laboratory decide to study the dihydroxy form of vitamin D?

Dr. Andrew Hoofnagle: Well, vitamin D is a, is, was, and still is, I guess, a hot topic. It was a very hot topic a couple of years ago. We have certainly documented that about 50% of Americans are vitamin D insufficient, just given the current diet and how much sunscreen we apply and the different ethnicities that we have and skin tones; many, many, many Americans do not have enough vitamin D.

That has been proposed to lead, not only to problems with bone metabolism, which we certainly know it does, but it has also now been proposed to be related to immunology, cancer, rheumatology, etcetera. So it's hard to know exactly whether vitamin D is playing an important role or not given the lab tests that we have. The dihydroxyvitamin D immunoassays for whatever reason have never really panned out in terms of good correlation with outcomes.

Well, now I think rather than saying for whatever reason, I think we know the reason, the problem with immunoassay is that it lacks specificity. And so we weren't sure what these immunoassays were telling us way back when, now we might have some tricks to try to enrich that dihydroxyvitamin D and actually have a specific test that we could roll out in a clinical research environment to see if in fact there are good correlations of dihydroxyvitamin D with outcomes or some other phenotype with special attention being paid to patients with kidney disease.

So not only from the academic interest of what is dihydroxyvitamin D and how does it correlate with human disease, it's also one of the higher testing volumes that we have is dihydroxyvitamin D. Probably not for any good reason, it was probably because care providers were checking the wrong box on the Requisition Form.

Nonetheless, we were getting a large number of requests for dihydroxyvitamin D testing, and we didn't feel good about sending it out for immunoassay. So we decided to try to bring it in-house, again, as I mentioned before, to try to save money and improve patient care.

So it was an interesting academic question as well as a practical laboratory management question.

Bob Barrett: And what were the results of the studies?

Dr. Andrew Hoofnagle: Well, we first tried to quantify dihydroxyvitamin D just by extracting it straight from serum and plasma, and what we learned was that the mass spectrometers we have were not sensitive enough to detect the dihydroxyvitamin D when we extracted it.

We then had to try different ways of derivatizing the dihydroxyvitamin D, some of which have been published, others that have not.

That still wasn't enough to have the sensitivity that we needed to detect the analyte that we were interested in. So we focused on something called immuno-enrichment, which basically uses an antibody that's been raised either in mice or rabbits or something else, that can recognize small molecules. And these antibodies are the same antibodies actually that were used in radioimmunoassays. Other assays used competitive basis for measuring the analyte.

So any cross-reactivity of our analyte of interest with something else that's present endogenously in blood would lead to erroneous results, but we were going to use the mass spectrometer to tease out those interferences.

So the hypothesis that we had was that we could improve the sensitivity, but also because we were using a mass spectrometer, improve the specificity of the radioimmunoassay, and that's exactly what we found.

What we were surprised by was that we actually had to prepare the samples. We actually had to do an extraction in order to get rid of a lot of the proteins and plasma and serum before we used the antibodies, which were attached to beads, to purify those small molecules. That was surprising to us.

However, there is another paper now published on literature that uses a different product, where you don't need to prepare the samples, you can actually just add the antibody-coated beads to the serum and plasma and immuno-enrich the dihydroxyvitamin D.

So we were a little bit surprised by the need to prepare the samples, but it seems to be antibody-specific, and we thought that was very interesting.

What we were able to do though was reach a sensitivity with the assay that we could measure dihydroxyvitamin D in serum and plasma. It was sensitive enough, because we used those antibodies to enrich. It was sensitive enough to measure dihydroxyvitamin D concentration.

So even in patients who have kidney disease have very low concentrations of 1, 25 dihydroxyvitamin D. And so the antibody gave us the sensitivity and the mass spectrometer gave us the specificity that we needed for a good assay.

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I don't want to say that we invented, because people have been purifying small molecules with antibodies for a really long time. Jack Henion, who used to or I think he still teaches at Cornell University, I think is one of the first people to try purifying small molecules using antibodies and then using mass spec afterwards for the improved specificity. So it wasn't our idea, but what we found was a very sensitive assay.

Then in terms of specificity, we were actually able to monitor many different molecules, many different channels using the mass spectrometer. What we were able to show was that the antibody does bind lots of things, including 25-hydroxyvitamin D, which is at a thousand-fold higher concentration than the analyte that we were interested in, 1, 25 dihydroxy D, but we also found other molecules like 24, 25, and actually another dihydroxyvitamin D metabolite, and we are actually still working on figuring out exactly what that molecules is.

But these analytes were obviously at either comparable concentrations or even higher concentrations than the 1, 25, which really raises a serious concern for a lack of specificity in the radioimmunoassay, which has been the foundation of clinical research for the last two decades, and it makes us realize that the results from those clinical studies, which have frequently been very boring may be due to the quality of the assay, rather than the quality of the question.

Bob Barrett: Well, since mass spectrometry measures molecules and parts of molecules directly, those who have said that a mass spectrometer needs an antibody, like a fish needs a bicycle, would you say that's about right?

Dr. Andrew Hoofnagle: Well, a couple of years ago I would have agreed, but the problem is sensitivity. As you said, we are measuring molecules and pieces of molecules, and we are measuring them directly, or directly detecting those. We are not using an amplification system, which immunoassays have the benefit of. And so the antibody can be useful.

It's embarrassing to have to use an antibody, because as a mass spectrometrist, I love the idea that mass spectrometry can do everything. But when you see an antibody and its ability to enrich, like we

did with this assay, I am not so sure anymore. I think that maybe the fish could get to the other side of the island by bicycling instead of swimming, and it might be a better route.

Bob Barrett: Well, what tests are you working on now, and when might we read about these in an upcoming issue?

Dr. Andrew Hoofnagle: Good question! There are a number of different protein analytes, especially very low abundance protein analytes that we are trying to measure in blood that would probably be considered novel biomarkers. We are focusing mostly on the area of nephrology and biomarkers of renal disease and potential renal outcomes.

We are also looking at proteins associated with cardiovascular disease, specifically the apolipoproteins and some of their related molecules and whether or not they will be good markers of cardiovascular disease.

So a couple of different things and hopefully they will be interesting enough to be published in *'Clinical Chemistry'*.

Bob Barrett: Dr. Andrew Hoofnagle is one of the Medical Directors of the Clinical Laboratories at the University of Washington in Seattle, and has been our guest in this podcast from *'Clinical Chemistry'*.

I am Bob Barrett. Thanks for listening.

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