Understanding the Technical Challenges of Measuring Vitamin D

An Interview with James Freeman
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Vitamin D, specifically 25(OH) vitamin D, has been challenging to accurately measure. James Freeman, Director, Assay Development for the ADVIA Centaur® Vitamin D Total assay, provides insight into why vitamin D is such a complex test and considerations to keep in mind when comparing different assays and methodologies.

Why is vitamin D measurement so complex?

Vitamin D is 100% bound to binding proteins in human serum, and unbound vitamin D does not exist. There is sufficient protein in human serum to bind hundreds of nanograms of vitamin D. In order to measure vitamin D, it has to be released from the binding proteins. Since the binding constant or affinity constant to the binding protein is fairly high (107 - 109), it has to be broken down. With liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods the protein is first precipitated, followed by an organic extraction of the vitamin D from the sample and then analyzed on the LC-MS/MS.

For immunoassays, removing vitamin D from the vitamin D binding protein is handled differently among manufacturers. For the ADVIA Centaur assay, there is no pretreatment step prior to loading the sample on the instrument. On the ADVIA Centaur, the releasing agent is added to the sample through an ancillary pack by the system before any of the immunoreagents of the ReadyPack are added. For approximately 6 minutes, 20 µL of sample and 200 µL releasing agent are incubated in the cuvette. Two events occur during this time. Firstly, the proteins are denatured to release vitamin D, and secondly a compound displaces vitamin D from the binding protein to provide free vitamin D in solution. Following these procedures, the antibody is added to the cuvette so the assay can commence to produce a result. Siemens has filed a patent application for the releasing agent technology.

Why is antibody selection so important for vitamin D measurement?

First of all, there are several types of vitamin D, but the most important forms to measure are 25(OH) vitamin D_2 and 25(OH) vitamin D_3, the circulating forms of vitamin D. Supplementation can occur in the form of 25(OH) vitamin D_2 (plants) or 25(OH) vitamin D_3 (animals), and it is consequently important to ensure a vitamin D test has full recognition of both forms.

Immunoassays using polyclonal antibodies from goat and sheep are more likely to have heterophilic interferences due to goat and sheep food products like milk, cheese, and meat consumption. Polyclonal antibodies are limited by the animals being able to produce them, and specificity can change between lots. An assay utilizing monoclonal antibodies will most likely have better lot-to-lot consistency as the monoclonal antibodies are not subject to the variations seen with polyclonal antibodies.

The proprietary ADVIA Centaur Vitamin D total assay monoclonal antibodies were selected specifically for equimolar 25(OH) vitamin D_2 and D_3 recognition per the ADVIA Centaur Vitamin D Total assay design requirements and should not have interferences due to heterophilic antibodies. We see the same performance with the ADVIA Centaur Vitamin D Total assay whether it is lot-to-lot or system-to-system. Slope is typically 0.95-1.05, intercept less than 3 ng/mL, and correlation coefficient 0.95-0.97 (system-to-system)/ 0.97-0.98 (lot-to-lot).

Why are there differences between methods?

Since there are no internationally recognized reference materials, biases in slopes across methods and manufacturers can be observed. Scatter in assay comparisons between manufacturers are apparent and may be due to differences in the way the different manufacturers systems measure vitamin D, such as varying vitamin D releasing processes, and heterophilic interferences. If assays are not designed with specific blockers for a given sample and that sample contains heterophilic antibodies, then an erroneous result may be expected. This is typically evident at high or low values. This also may contribute to lower correlation coefficients when comparing methods.

Between manufacturers correlation coefficients of >0.90 and slopes of 0.8-1.2 are expected if samples are measured across the range of the assay.[1] The best way to resolve discordance between immunoassays is LC-MS/MS, since this method does not have heterophilic interference.[2] It is important to note that LC-MS/MS (unless modified to allow detection)[3] cannot distinguish 3-epi-25(OH) vitamin D (3-epi) from 25(OH) vitamin D_3, which is primarily found in infants from 25(OH) vitamin D_2 or D_3, contributing in over recovery in measurement.[4]

Seasonal variance can also influence individual results. For example, when the U.S. enters the winter months the majority of the population north of Atlanta will have lower values due to the seasonal

angle of the Earth. During the winter months the atmosphere diffuses the UVB rays, the UVB rays are needed for vitamin D synthesis.[5]

How do you standardize vitamin D?

In the absence of an international standard, this has been an area of concern. The National Institute of Standards and Technology (NIST) material became available a few years ago, but it is not a true standard like a World Health Organization (WHO) standard. The NIST material is unsuitable for immunoassays since some of the levels are of non-human origin, and it is not possible to standardize based on a single point. (The NIST SRM 972 Level 1 (23.9 ng/mL) is the only level that could be used for immunoassays.) A WHO standard-like material at a high concentration can be diluted down across the range of the assay. This is not the case with the NIST. Additionally, variances in results between laboratories can be observed, depending on how a laboratory standardizes to the NIST material. Sometimes they agree and sometimes they disagree, and that's why it is important to know both how the laboratories standardize and how the LC-MS/MS results perform within the lab. The current Siemens Vitamin D Total assay standards are gravimetrically prepared and traceable to LC-MS/MS.

In 2011, the NIH Officeof Dietary Supplements initiated the Vitamin D Standardization Program (VDSP), which will span across the range of the assay. It is not expected that any commutability challenges will be found with the ADVIA Centaur systems because these are native samples. At the end of 2011, Siemens R&D blindly measured 50 unadulterated human serum samples and submitted our results. Based on the project's timeline, it is expected that standardization will happen in 2012.

How do you interpret vitamin D DEQAS and CAP results in light of the lack a worldwide vitamin D standard?

Siemens enrolled in the January 2012 DEQAS proficiency scheme and that of the College of American Pathologists (CAP). Samples from CAP have been assayed internally and a very similar performance was seen to the VDSP values given in the CAP survey. Proficiency schemes such as the Vitamin D External Quality Assurance Scheme (DEQAS) and CAP have been good indicators of vitamin D performance, but it's not an absolute and it is important to look at the trending of DEQAS and CAP performance over time. The lack of a single worldwide standardization is reflected in these proficiency schemes. If one point in time is considered with these surveys and high CVs are seen, it may be indicative of a standardization adjustment, not an issue with the assay. There are also other factors to consider like DEQAS sample 405 that included 3-epi-D_3, which demonstrated discordant results between LC-MS/MS and some immunoassays.

References

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