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Editorial: The Determination of Circulating 25-Hydroxyvitamin D: No Easy Task

One of the major factors responsible for the explosion of knowledge related to vitamin D metabolism and its relation to clinical disease was the introduction of a competitive protein binding assay (CPBA) for 25-hydroxyvitamin D [25(OH)D] (1). This CPBA was introduced in *JCEM* more than three decades ago by the late Dr. John Haddad, Jr., and was based on assessing circulating 25(OH)D using the vitamin D-binding protein (DBP) as a primary binding agent and ^{3}H-25(OH)D_3 as a reporter (1). The Haddad CPBA method for assessing circulating 25(OH)D gained widespread use and has achieved citation classic status in *Current Contents*. Although this CPBA was valid, it was also relatively cumbersome. The sample had to be extracted with organic solvent, dried under N_2, and purified by column chromatography, and it required individual sample recovery estimates to account for endogenous losses of 25(OH)D during this extensive procedure. This assay was fine for the research laboratory but did not meet the requirements for a high throughput clinical laboratory. As a result, the journey began to simplify the method used to assess circulating 25(OH)D.

This issue of *JCEM* contains a report by Binkley *et al.* (2) that highlights just how difficult the journey to a simplified 25(OH)D assay has become. These authors report the unacceptable variation in circulating 25(OH)D measurements encountered from laboratory-to-laboratory as well as from method-to-method and the confounding of the two. To fully appreciate the Binkley paper, I feel it necessary to provide a brief history of 25(OH)D analysis.

The major problem in measuring 25(OH)D is attributable to the molecule itself. 25(OH)D is probably the most hydrophobic compound that is measured by protein binding assay (PBA), which constitutes CPBA or RIA. Couple this aqueous insolubility with the fact that it exists in two forms, 25(OH)D_2 and 25(OH)D_3, and you have a serious analytical problem. The lipophilic nature of 25(OH)D renders it especially vulnerable to matrix effects in any PBA. Matrix effects would be caused by something present in the sample assay tubes that is not present in the standard assay tubes. These matrix effect substances are usually lipid but in the newer direct assays could be anything contained in the serum or plasma sample. The matrix factors simply change the ability of the binding agent, antibody, or binding protein to associate with 25(OH)D in the sample or standard in an equal fashion. When this occurs, it markedly diminishes the validity of the assay. Experience has demonstrated that the DBP is more susceptible to these matrix effects than are antibodies (3). The matrix problem was overcome in the original Haddad procedure (1) by using chromatographic sample purification before CPBA. Also, matrix problems are not unique to the assay of 25(OH)D. The performance of direct estradiol assays has never been as good as the original indirect RIAs (4, 5). Nevertheless, for whatever reason, this subpar performance has become accepted by endocrinologists.

As with all analytical procedures, there was a strong desire to simplify the CPBA for circulating 25(OH)D. The goal of this second generation CPBA was to eliminate chromatographic sample purification as well as individual sample recovery using ^{3}H-25(OH)D_3. This type of assay was introduced by Belsey *et al.* (6) in 1974. However, the Belsey assay could never be validated due to matrix problems originating from ethanolic sample extraction. In 1978, after several years of trying to validate nonchromatographic CPBA measurements of circulating 25(OH)D, the methods were abandoned. The paper that sealed the fate of nonchromatographic CPBAs for 25(OH)D was published by Dorantes *et al.* (7). The Dorantes report demonstrated that unknown substances in nonchromatographed samples interacted with the DBP to cause spurious results in the CPBA. Thus, investigators and clinicians had to return to the Haddad method (1) or the newly introduced direct UV HPLC assay (8). Again, both of these assays are slow, cumbersome, and/or expensive.

My group decided in the early 1980s that a nonchromatographic RIA for circulating 25(OH)D would be the way to go. Thus, we designed an antigen that would generate an antibody that was cospecific for 25(OH)D_2 and 25(OH)D_3 (9). Furthermore, we designed a simple extraction method that allowed a simple nonchromatographic quantification of circulating 25(OH)D (9). This assay was further modified in 1992 to incorporate a ^{125}I-labeled reporter and calibrators (standards) in a serum matrix, and the mass determination of circulating 25(OH)D was under way (10).

The desire for simplicity never ends; thus, the drive to develop assays for circulating 25(OH)D, which eliminates radioactive reporters, and function in a direct random access format became the next goal. This was achieved in 2001 when Nichols Diagnostics introduced the fully automated chemiluminescence ADVANTAGE 25(OH)D assay system (11). In this assay system, nonextracted serum or plasma is introduced directly into a mixture containing human DBP, acridinium-ester labeled anti-DBP, and 25(OH)D_3-coated magnetic particles. Note that the primary binding agent is human DBP. Thus, this assay is a CPBA much like the manual procedure introduced in 1974 by Belsey *et al.* (6). The major difference between these procedures is that Belsey deproteinized the sample with ethanol before assay. The calibrators for the Belsey assay were in ethanol. In the ADVANTAGE assay, the calibrators are in a serum-based matrix, and

Abbreviations: CPBA, Competitive PBA; DBP, vitamin D-binding protein; DEQAS, Vitamin D External Quality Assessment Scheme; 25(OH)D, 25-hydroxyvitamin D; PBA, protein binding assay.

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it is assumed that this matrix will replicate the serum or plasma sample introduced directly into the assay system.

Another direct random access chemiluminescence assay has recently been introduced by the DiaSorin Corporation (12). The assay, LIAISON 25(OH)D, is very similar to the ADVANTAGE assay with one major difference. The LIAISON assay uses an antibody as a primary binding agent as opposed to the ADVANTAGE using the human DBP. Thus, the LIAISON is an RIA method, whereas the ADVANTAGE is a CPBA procedure. Both of the assays have received FDA approval for clinical diagnostic use.

Finally, I would like to address the assessment of circulating 25(OH)D using direct UV detection after HPLC. This type of assay was introduced in 1977 from the laboratory of Dr. Hector DeLuca (8). The HPLC method for the detection of circulating 25(OH)D is often referred to as the standard by which other methods are compared. Furthermore, HPLC detection will allow the individual quantitation of $25(OH)D_2$ and $25(OH)D_3$. This type of assay is very accurate if validated and performed by experienced personnel. However, be warned that inaccurate HPLC methods exist and can provide misleading results just as the PBA methods can. This is especially true for the quantitation of $25(OH)D_2$ by HPLC. Spurious UV-absorbing peaks unrelated to $25(OH)D_2$ may be interpreted by inexperienced laboratory personnel to be $25(OH)D_2$, and thus overestimation will occur. I myself have more than two decades of experience with this assay and thus know how difficult it is to perform properly. Other disadvantages of the HPLC procedure include large sample volume requirements, slow sample throughput, the requirement of highly trained laboratory personnel and expensive dedicated equipment.

Using the information that I have just presented, what can be derived from the Binkley report (2)? The first assumption I will make is that Dr. DeLuca's laboratory knows how to perform a valid HPLC-based circulating 25(OH)D assay. Dr. DeLuca's laboratory was laboratory H in the Binkley paper, and it performed the HPLC analysis for this publication. Also, I assume then that the values for the other laboratories/methods in the report should coincide with the HPLC values generated in Dr. DeLuca's laboratory. How then did the other laboratories/methods perform in this regard? From the inspection of the data, the obvious answer is, for the most part, poorly. Of the laboratories that were used in the comparative aspect of this report, three used the Nichols ADVANTAGE chemiluminescent assay (laboratories D, E, and G), two used the DiaSorin RIA (laboratories C and E), and one used HPLC (laboratory H). I would like to state that, as a reviewer of this report, I believed it was important that the laboratories performing the testing be identified. Identification of the testing method in the Binkley report does not go far enough in identifying the best choice of laboratory to which the specimen should be sent. Hospital laboratory managers simply do not have the time or expertise to search out this information. According to the author, except for laboratories C and H, the request was denied. Laboratory C is the Mayo Medical Laboratory and laboratory H, as previously mentioned, is Dr. DeLuca's laboratory.

The Mayo Medical Laboratory, which uses the DiaSorin RIA, demonstrated excellent results when compared with the HPLC standard method. This was true for both basal level comparison and exogenously added $25(OH)D_3$ recovery experiments. These results are in contrast to laboratory E which also used the DiaSorin RIA. Results of laboratory E compared poorly from both basal level comparison and exogenous recovery. The results between these two laboratories basically come down to a single factor, experience with the assay. The Mayo Clinic has been using the DiaSorin RIA for many years and has validated it in their clinical laboratory. Laboratory E had not previously used this technique, and thus, had not validated it in their laboratory.

Laboratories D, F, and G all used the Nichols ADVANTAGE 25(OH)D assay system, and the comparison to the HPLC-based method was poor. All of these laboratories significantly overestimated the basal concentration of circulating 25(OH)D and greatly underestimated exogenously added $25(OH)D_3$. The overestimation of basal 25(OH)D concentrations by this method has been previously reported by the Vitamin D External Quality Assessment Scheme (DEQAS) (13). DEQAS is an international program for monitoring the accuracy and precision of 25(OH)D and 1,25-dihydroxyvitamin D assays run out of the United Kingdom. Currently, there are over 100 worldwide laboratories participating in this quarterly quality control survey. I would strongly suggest that anyone concerned with the performance of their 25(OH)D analysis participate in this program (www.deqas.org). I believe that the inconsistencies observed with this direct detection system are based on assay limitations discussed earlier in this editorial. The other automated direct detection system, DiaSorin's LIAISION 25(OH)D assay, has just received U.S. Food and Drug Administration (FDA) approval and has not yet been proven in the clinical laboratory. Whether it is plagued by the same problems as the ADVANTAGE 25(OH)D assay remains to be determined.

I would like to address one final extremely important point not covered in the Binkley report, the monitoring of vitamin D therapy by these various assay methods. The sole therapeutic form of vitamin D in the United States is Drisdol (ergocalciferol, vitamin D_2) (Sanofi-Synthelabo Inc., New York, NY) and is supplied in 50,000 IU dosages. It is absolutely critical that any assay used in the clinical laboratory detect total circulating 25(OH)D [$25(OH)D_2$ and $25(OH)D_3$]. I have personally received calls from clinicians who have placed patients on Drisdol to correct vitamin D deficiency and who, upon monitoring the serum measurement of 25(OH)D, ask me why the subjects are not responding by increasing circulating 25(OH)D. The reason has now become apparent. The three most used testing methods in the world for assessing circulating 25(OH)D are the DiaSorin RIA, Nichols ADVANTAGE CPBA and Immunodiagnostic Systems (IDS) RIA. The product inserts from these tests state that they cross-react with $25(OH)D_2$ 100%, 100%, and 75%, respectively. In essence, they all claim to measure total circulating 25(OH)D status. However, according to the latest DEQAS survey two of them do not (14). In this latest DEQAS report, a patient with vitamin D deficiency was treated with vitamin D_2, and thus, the majority of circulating 25(OH)D was of the $25(OH)D_2$ form. This specimen was provided to all DEQAS participants. These data clearly demonstrated that the DiaSorin RIA is very effective at detecting endogenous $25(OH)D_2$

and 25(OH)D_3 in human serum. The IDS RIA has a greatly diminished capacity to detect 25(OH)D_2, whereas the Nichols ADVANTAGE CPBA was unable to detect the 25(OH)D_2 contribution to overall 25(OH)D status (14). These last results are in direct conflict with the manufacturer label claims. The importance of this cannot be ignored. Apparently, vitamin D_2 therapy cannot effectively be monitored by the IDS RIA or ADVANTAGE CPBA system. These misleading results could lead to misdiagnosis and subsequent dangerous consequences for the patient such as hypervitaminosis D. This must be addressed by the manufacturers and/or the FDA.

In conclusion, any circulating 25(OH)D assay system must be validated in the user's laboratory regardless of manufacturer claims. Performance and comparison of that system must be maintained at all times, and I cannot think of a better way to achieve this than participating in the UK assay service, DEQAS.

Bruce W. Hollis
Department of Pediatrics
Medical University of South Carolina
Charleston, South Carolina 29425

Acknowledgments

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Address all correspondence and requests for reprints to: Bruce W. Hollis, Ph.D., 114 Doughty Street, Department of Pediatrics, Medical University of South Carolina, Charleston, South Carolina 29425. E-mail: hollisb@musc.edu.

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