

## Assessment of Circulating 25(OH)D and 1,25(OH)<sub>2</sub>D: Emergence as Clinically Important Diagnostic Tools

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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 competitive protein-binding assays for 25(OH)D<sup>1</sup> and 1,25(OH)<sub>2</sub>D.<sup>2</sup> These assays were introduced more than three decades ago, and were based on assessing circulating 25(OH)D or 1,25(OH)<sub>2</sub>D using the vitamin D-binding protein or chick intestinal receptor, respectively, as the primary binding agents. Both of these assays used <sup>3</sup>H-labeled compounds as reporters. Although these assays were valid, they were also relatively cumbersome, especially the 1,25(OH)<sub>2</sub>D procedure.

Through the years, assays for both of these metabolites have advanced. Some advances have been for the better, some have not. These methodologies include radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC-MS), and random access automated assay (RAAA) based on chemiluminescence assay technology.

### THE ASSAY OF 25(OH)D

The most abundant vitamin D metabolite in the circulation is 25(OH)D, which serves as the indicator of nutritional vitamin D status.<sup>3</sup> 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 (i.e., competitive protein-binding assays or RIA). This aqueous insolubility coupled with the fact that it exists in two forms, 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>, poses a formidable analytical

problem. The lipophilic nature of 25(OH)D renders it especially vulnerable to matrix effects in any protein-binding assay caused by something present in 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 it 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. These solubility issues are not a big factor if one chooses to use a physical detection method for 25(OH)D such as HPLC or LC-MS. However, these techniques have their own specific problems that will be discussed later.

The first valid competitive protein binding assay for measuring circulating 25(OH)D was introduced more than three decades ago by the late Dr. John Haddad, Jr., and was based on using the vitamin D-binding protein as a primary binding agent and <sup>3</sup>H-25(OH)D<sub>3</sub> as a reporter.<sup>1</sup> The Haddad competitive protein-binding assay method gained widespread use and greatly contributed to our understanding of vitamin D metabolism. Although this assay was valid, it was relatively cumbersome due to organic extraction, nitrogen drying, and preparative chromatography of the sample prior to assay. This assay was fine for the research laboratory but did not meet the requirements for a high-throughput clinical laboratory. As a result, the quest for assay simplification began.

The goal of the second generation of competitive protein-binding assays for circulating 25(OH)D was to eliminate chromatographic sample purification as well as individual sample recovery using <sup>3</sup>H-25(OH)D<sub>3</sub>. This type of assay was introduced by Belsey et al. in 1974.<sup>4</sup> However, the Belsey assay could never be validated due to matrix problems originating from ethanolic sample extraction. In the late 1970s, these “direct” non-chromatographic competitive protein-binding assays for 25(OH)D, including the Nichols Advantage Automated Chemiluminescent 25(OH)D competitive protein-binding assay,<sup>5</sup> were abandoned and are basically history at the present time.

In the mid-1980s, a non-chromatographic RIA for circulating 25(OH)D was introduced.<sup>6</sup> The antibody was

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doi: 10.1301/nr.2007.aug.S87–S90

raised against an antigen that would generate an antibody that was co-specific for 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>.<sup>6</sup> The extraction method involved the use of acetonitrile, which allowed for the simple, non-chromatographic quantification of total circulating 25(OH)D. This assay was further modified to incorporate a <sup>125</sup>I-labeled reporter and calibrators in a serum matrix.<sup>7</sup> The assay was commercialized by DiaSorin Corporation (Stillwater, MN) and is still widely utilized today. ELISAs for 25(OH)D also exist and are available commercially. They have not been well described.

Nichols Institute Diagnostics (San Clemente, CA) and DiaSorin Corporation have both introduced methods for the direct (no extraction) quantitative determination of 25(OH)D in serum or plasma utilizing competitive chemiluminescence technology. These assays on the surface appear quite similar but they are not.

The Nichols Institute platform was called the Advantage<sup>®</sup> System. This instrument was similar to the Liaison System, but the assay was very different. The Nichol's Advantage 25(OH)D assay utilized the human vitamin D-binding protein as a competitive binder instead of an antibody. Despite the manufacturer's claims of 100% cross-reactivity with 25(OH)D<sub>2</sub>, it appeared that this assay had trouble measuring 25(OH)D<sub>2</sub> reliably. As a result of this problem, the assay was withdrawn at governmental insistence.

The DiaSorin Liaison<sup>®</sup> platform utilizes a specific antibody to 25(OH)D that is coated onto magnetic particles (solid phase). The tracer D is linked to an isoluminol derivative. During the incubation of sample, 25(OH)D is dissociated from its binding protein and competes with the labeled vitamin D for binding sites on the antibody. This procedure has been published elsewhere in detail.<sup>8</sup> Further, it has gained wide acceptance and is utilized in most large clinical laboratories in the United States.

Direct detection methodology for the determination of circulating 25(OH)D include both HPLC<sup>9,10</sup> and LC-MS procedures.<sup>11,12</sup> The HPLC methods offer the advantage of separating and detecting 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>. The method of using HPLC followed by UV detection is highly repeatable and in general is considered the gold standard. However, this method can be cumbersome and sample throughput is slow and has its own set of unique problems. It is not suited for a high-capacity clinical laboratory.

LC-MS has recently been revitalized as a viable method to assess circulating 25(OH)D.<sup>11,12</sup> When properly performed, it is an accurate testing method. However, the equipment is very expensive and the throughput cannot match that of the automated instrumentation format. Recently, LC-MS and RIA comparisons have proven to be excellent.<sup>11</sup> One problem that LC-MS has is

its relative inability to discriminate between 25(OH)D<sub>3</sub> and its inactive isomer 3-epi-25(OH)D<sub>3</sub>, which has been shown to be especially troublesome in the circulation of newborn infants.<sup>12</sup> Proponents of this technology have made claims that LC-MS is the method of choice for the determination of circulating 25(OH)D. In reality, this claim is not supported by data generated from the vitamin D External Quality Assessment Scheme (DEQAS) from London ([www.deqas.org](http://www.deqas.org)). Data from this large, ongoing survey show values generated by the DiaSorin RIA and Liaison platform to be at least equal to those generated by laboratories performing LC-MS determinations. Further, data suggest that individual reporting of circulating levels of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> does nothing more than confuse the diagnosing physician.<sup>13</sup> It is only important to report a total circulating 25(OH)D for diagnostic purposes.

## THE ASSAY OF 1,25(OH)<sub>2</sub>D

Of all of the steroid hormones, 1,25(OH)<sub>2</sub>D represented the most difficult challenge to the analytical biochemist with respect to quantitation. 1,25(OH)<sub>2</sub>D circulates at picomolar concentrations (too low for direct UV or MS quantitation), is highly lipophilic, and its precursor, 25(OH)D, circulates at nanomolar levels. The development of a simple, rapid assay for this compound has proven to be a daunting task.

The first radio receptor assay for 1,25(OH)<sub>2</sub>D was introduced in 1974.<sup>2</sup> Although this initial assay was extremely cumbersome, it did provide invaluable information with respect to vitamin D homeostasis. This initial radio receptor assay required a 20-mL serum sample, which was extracted using Bligh-Dyer organics. This extract had to be purified by three successive chromatographic systems, and chickens had to be sacrificed and the vitamin D receptor (VDR) harvested from their intestines. By 1976, the volume requirement for this radio receptor assay had been reduced to a 5-mL sample and sample pre-purification had been modified to include HPLC.<sup>14</sup> However, the sample still had to be extracted using a modified Bligh-Dyer procedure and then pre-purified on Sephadex LH-20. Chicken intestinal VDR was still utilized as a binding agent.

A major advancement occurred in 1984 with the introduction of a radically new concept for the radio receptor assay determination of circulating 1,25(OH)<sub>2</sub>D.<sup>15</sup> This new assay utilized solid-phase extraction of 1,25(OH)<sub>2</sub>D from serum along with silica cartridge purification of 1,25(OH)<sub>2</sub>D. As a result, the need for HPLC sample pre-purification was eliminated. Also, this assay utilized VDR isolated from calf thymus, which proved to be quite stable and thus had to be prepared only periodically. Further, the volume requirement was reduced to

1 mL of serum or plasma. This assay opened the way for any laboratory to measure circulating 1,25(OH)<sub>2</sub>D, and also resulted in the production of the first commercial kit for 1,25(OH)<sub>2</sub>D measurement. This radio receptor assay was further simplified in 1986 by decreasing the required chromatographic purification steps.<sup>16</sup>

As good as the calf thymus radio receptor assay for 1,25(OH)<sub>2</sub>D was, it still possessed two serious shortcomings. First, VDR had to be isolated from thymus glands. Second, because the VDR is so specific for its ligand, only <sup>3</sup>H-1,25(OH)<sub>2</sub>D<sub>3</sub> could be used as a reporter, eliminating the use of a <sup>125</sup>I or chemiluminescent reporter. This was a major handicap, especially for the commercial laboratory.

In 1978, the first RIA for 1,25(OH)<sub>2</sub>D was introduced.<sup>17</sup> Although it was an advantage not to have to isolate the VDR as a binding agent, this RIA was relatively nonspecific, so the cumbersome sample preparative steps were still required. Over the next 18 years, all RIAs developed for 1,25(OH)<sub>2</sub>D suffered from the same shortcomings. In 1996, the first significant advance in 1,25(OH)<sub>2</sub>D quantification in a decade was achieved.<sup>18</sup> This RIA incorporated a <sup>125</sup>I-reporter, as well as standards in an equivalent serum matrix, so individual sample recoveries were no longer required. The sample purification procedure is the same one previously used for the rapid radio receptor assay procedure.<sup>16</sup> This assay has 100% cross-reactivity between 1,25(OH)<sub>2</sub>D<sub>2</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> and is FDA-approved for clinical diagnosis in humans.

Another <sup>125</sup>I-based RIA for 1,25(OH)<sub>2</sub>D is also commercially available from Immunodiagnosics Systems (IDS), Ltd. (Bolden, Tyne and Wear, UK). The basis of this kit is a selective immunoextraction of 1,25(OH)<sub>2</sub>D from serum or plasma with a specific monoclonal antibody bound to a solid support. This antibody is directed toward the hydroxylated A-ring of 1,25(OH)<sub>2</sub>D.<sup>19</sup> This assay procedure has never been published in detail, so critical evaluation is difficult. We concluded that this immunoextraction procedure was highly specific for the 1-hydroxylated forms of vitamin D. However, we also believe that this procedure overestimates circulating 1,25(OH)<sub>2</sub>D levels. Evidence of this overestimation is evident in a recent publication showing a correlation between circulating 25(OH)D and 1,25(OH)<sub>2</sub>D at physiologic levels,<sup>20</sup> indicating that 25(OH)D may be interfering with the assay.

ELISAs for circulating 1,25(OH)<sub>2</sub>D determination do exist commercially but their performance has never been published in detail. The diagnostic field awaits a random access automated assay test for circulating 1,25(OH)<sub>2</sub>D determination. However, this advance is proving to be a daunting task for diagnostic companies at the present time.

## ACKNOWLEDGEMENT

BWH is a consultant for Dia Sorin. Dia Sorin markets *in vitro* diagnostic products.

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