Published in final edited form as:
J Steroid Biochem Mol Biol. 2007 March ; 103(3-5): 473–476.

The Assessment of Circulating 25(OH)D and 1,25(OH)$_2$D: Where We Are and Where We Are Going

Bruce W. Hollis[*] and **Ronald L. Horst**[a]

***** *Departments of Pediatrics, Biochemistry and Molecular Biology Medical University of South Carolina Charleston, SC 29425*

a *National Animal Disease Center, 2300 Dayton Avenue, Ames, IA 50010, USA*

Abstract

The field of vitamin D assay technology has progressed significantly over the past four decades. Further, the clinical utility of these measurements have moved from esoteric into mainstream clinical diagnosis. This movement has been fueled by the realization that vitamin D is involved in bodily systems beyond skeletal integrity. The clinical assay techniques for circulating 25(OH)D and 1,25 (OH)$_2$D have progressed away from competitive-protein binding assay (CPBA's) that utilize tritium reporters to radioimmunoassay (RIA's) that utilize both I^{125} and chemiluminescent reporters. These advances have allowed direct serum analysis of 25(OH)D in an automated format that provides a huge sample throughput. Detection of circulating 25(OH)D can also be achieved utilizing direct high performance liquid chromatographic (hplc) or liquid chromatography coupled with mass spectrometry (LC-MS) techniques. These methods are accurate, however, they require expensive equipment and restrict sample throughput in the large clinical laboratory. Direct serum detection of 1,25(OH)$_2$D is unlikely to occur for many reasons as a sample pre-purification will always be required. However, a semi-automated chemiluminescent detection system with automated sample preparation is in final development for the determination of circulating 1,25(OH)$_2$D. These advances will allow both 25(OH)D and 1,25(OH)$_2$D to be detected in an accurate, rapid fashion to meet the clinical demands we see emerging.

Keywords

vitamin D assay; 25-hydroxyvitamin D; 1; 25-dihydroxyvitamin D

Introduction

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 (CPBA) for 25(OH)D [1] and 1,25-dihydroxyvitamin D [2]. These CPBA's were introduced more than three decades ago and were based on assessing circulating 25(OH) D or 1,25(OH)$_2$D using the vitamin D-binding protein (DBP) or chick intestinal receptor, respectively, as the primary binding agents. Both of these assays used ^{3}H-labeled compounds

*Corresponding author. Tel:843-792-6854. E-mail address:Email: hollisb@musc.edu

Presented at the 13th Workshop on Vitamin D (Victoria, British Columbia, Canada, April 8-12, 2006).

as reporters. Although these CPBA's were valid, they were also relatively cumbersome, especially the 1,25$(OH)_2$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 finally random access automated assay (RAAA) based on chemiluminescence assay technology. These various techniques will be discussed.

The Assay of 25(OH)D

The most abundant vitamin D metabolite in the circulation is 25(OH)D. 25(OH)D also serves as the indicator of nutritional vitamin D status [3]. The C_{25} hydroxylation of vitamin D occurs primarily in the liver under the control of a microsomal P450, CYP2R1 [4,5].

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 formidable 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 are 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.

Competitive Protein Binding Assays

The first valid CPBA 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 (DBP) as a primary binding agent and ^{3}H-25$(OH)D_3$ as a reporter [1]. The Haddad CPBA method gained widespread use and greatly contributed to our understanding of vitamin D metabolism. Although this CPBA 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 CPBA's for circulating 25(OH)D 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. in 1974 [6]. However, the Belsey assay could never be validated due to matrix problems originating from ethanolic sample extraction. In the late 70's these "direct" non-chromatic CPBA's for 25(OH)D are basically history at the present time with the exception of the Nichols Advantage Automated Chemiluminescent 25(OH)D CPBA [7].

Radioimmunoassay

In the mid 1980's a non-chromatographic RIA for circulating 25(OH)D was introduced [8]. The antibody was raised against an antigen that would generate an antibody that was co-specific for 25$(OH)D_2$ and 25$(OH)D_3$ [8]. 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 in to incorporate a ^{125}I-labeled reporter and calibrators in a serum matrix [9]. This assay was commercialized by DiaSorin Corporation (Stillwater, MN) and as of today it is still widely utilized. ELISA's for 25(OH)D also exist and although they are available commercially from IDS LTD [10] and ALPCO [11], they have not been well described and have not gained much commercial acceptance.

Automated Instrumentation Methodology

Nichols Institute Diagnostics (San Clemente, CA) and DiaSorin Corporation (Stillwater, MN) 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 is called The Advantage® System. This instrument is similar to the Liaison System, however, the assay is very different. The Nichol's Advantage 25(OH)D assay utilizes the human DBP as a competitive binder instead of an antibody. Dispite the manufacturers claims of 100% cross reactivity with 25(OH)D_2, it appears that this assays has trouble measuring 25(OH)D_2 reliably [12].

The DiaSorin Liaison® 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 [13]. Further, it has gained wide acceptance and is utilized in most large clinical laboratories in the U.S. The sample throughput is 180 samples per hour.

Direct Detection Methodology

Direct detection methodology for the determination of circulating 25(OH)D include both hplc [14–16]and LC-MS procedures [17]. The HPLC methods offer the advantage of separating and detecting 25(OH)D_2 and 25(OH)D_3. The HPLC followed by UV detection methods are highly repeatable and in general is considered the gold standard method. However, these methods can be cumbersome and sample throughput is slow as well as possessing its own set of unique problems. It is not suited for a high capacity clinical lab.

LC-MS has recently been revitalized as a viable method to assess circulating 25(OH)D [17]. When properly performed it is a very accurate testing method. However, the equipment is very expensive and throughput cannot match that of the automated instrumentation format. Recently LC-MS and RIA comparisons have proven to be excellent [17]. One problem that LC-MS has is its relative inability to discriminate between 25(OH)D_3 and its inactive isomer 3-epi-25(OH) D_3, which has been shown to be especially troublesome in the circulation of newborn infants (Singh, 2006, personal communications).

The Assay of 1,25(OH)$_2$D

Of all the steroid hormones, 1,25(OH)$_2$D represented the most difficult challenge to the analytical biochemist with respect to quantitation. 1,25(OH)$_2$D circulates at pmol concentrations (too low for direct UV or MS quantitation), is highly lipophilic and its precursor, 25(OH)D, circulates at nmol levels. The development of a simple, rapid assay for this compound has proven to be a daunting task.

Radioreceptor Assays

The first radioreceptor assay (RRA) for 1,25(OH)$_2$D was introduced in 1974 [2]. Although this initial assay was extremely cumbersome, it did provide invaluable information with respect to

vitamin D homeostasis. This initial RRA required a 20 ml serum sample, which was extracted using Bligh-Dyer organics. The 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 RRA had been reduced to a 5 ml sample and sample pre-purification had been modified to include hplc [18,19]. 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 RRA determination of circulating $1,25(OH)_2D$ [20]. This new RRA utilized solid-phase extraction of $1,25(OH)_2D$ from serum along with silica cartridge purification of $1,25(OH)_2D$. 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 $1,25(OH)_2D$. This procedure also resulted in the production of the first commercial kit for $1,25(OH)_2D$ measurement. This RRA was further simplified in 1986 by decreasing the required chromatographic purification steps [21].

As good as the calf thymus RRA for $1,25(OH)_2D$ 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 legend, only ^3H-$1,25(OH)_2D_3$ could be used as a reporter, eliminating the use of a ^{125}I or chemiluminscent reporter. This was a major handicap, especially for the commercial laboratory.

Radioimmunoassay

In 1978, the first RIA for $1,25(OH)_2D$ was introduced [22]. 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 RIA's developed for $1,25(OH)_2D$ suffered from the same shortcomings. In 1996, we developed the first significant advance in $1,25(OH)_2D$ quantification in a decade [23]. This RIA incorporated and ^{125}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 RRA procedure [21]. This assay has 100% cross-reactivity between $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ and is FDA-approved for clinical diagnosis in humans.

Another ^{125}I-based RIA for $1,25(OH)_2D$ is also commercially available from IDS Ltd. The basis of this kit is a selective immunoextraction of $1,25(OH)_2D$ from serum or plasma with a specific monoclonal antibody bound to a solid support. This antibody is directed toward the H-hydroxylated A ring of $1,25(OH)_2D$ [24]. 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 $1,25(OH)_2D$ levels. Evidence of this overestimation is evident in a recent publication which shows a correlation of circulating 25(OH)D and $1,25(OH)_2D$ at physiologic levels [25] indicating that 25(OH)D maybe interfering with the assay.

ELISA's for circulating $1,25(OH)_2D$ determinations do exist commercially from Immunodiagnostik and IDS.. However, their performance has never been published in detail.

Deciding on an assay platform

Several factors need to be considered when deciding on which platform to utilize. If assay are performed on an infrequent basis, establishing an in house assay utilizing an hplc procedure would be preferable to purchasing commercial kits. A recent study suggested that the commercial kits give more variable results when performed by inexperienced users [26]. Utilizing the hplc methods one can monitor the performance of standards in real time and recovery estimates are essential, making troubleshooting more straightforward. For laboratories needing higher throughput, one of the commercially available RIA, ELISA or instrumentation methods would be more appropriate. When considering commercially available procedures, one needs to be sure that there is equal recognition of the Vitamin D_2 and Vitamin D_3 forms. Several of the commercial assay discriminate in some manner against the vitamin D_2 forms, which can lead to underestimation of the vitamin D status of patients being treated with Vitamin D_2. Also, if an RIA or ELISA better suits the needs of the laboratory, the results of these assays should correlate closely with result from the physical chemical "gold standard" methods of hplc or mass spectrometry. There have been a few reports with head-to-head comparison of the different methodologies. A recent report suggested that the current assays for 25(OH)D, including the DiaSorin and IDS RIAs and the Nichols Chemoluminescense assays, may overestimate the 25(OH)D_3 and underestimate the 25(OH) D_2 concentrations when compared to their in house hplc method [27]. However. other reports suggest that the the DiaSorin RIA and DiaSorin Liason methods correlated very closely with LC-MS/MS results and does not distinguish between 25(OH)D_2 and 25(OH)D_3 [17]. A recent study reported by DEQAS at the 13th Workshop on vitamin D monitored the ability of several assays platforms to recovered added 25(OH)D_2 and 25(OH)D_3. The recovery of the 25(OH) D_2 and 25(OH)D_3 for the different platforms were as follows: DiaSorin RIA; 82,1% and 83.3%; DiaSorin Liason, 81.4% and 88.6%; IDS RIA, 54.2% and 29.1%; IDS OCTEIA, 78.8% and 56.4%; Nichols Advantage, 58.9% and 46.4%; HPLC 112% and 97.1%l LC/MS, 111.5% and 118.1%. The study organizers concluded that the IDS RIA and Nichols assay gave unexpectedly low recoveries. The final choice of which method to use will depend in large part on the equipment and expertise available in the laboratory. A with many assays the accuracy and repeatability depends in large part on the experience and capabilities of the individual performing the assay.

References

1. Haddad JG, Chyu KJ. Competitive protein-binding radioassay for 25-hydroxycholecalciferol. Journal of Clinical Endocrinology and Metabolism 1971;33:992–995. [PubMed: 4332615]
2. Brumbaugh PF, Haussler DH, Bressler R, Haussler MR. Radioreceptor assay for 1α,25-dihydroxyvitamin D_3. Science 1974;183:1089–1091. [PubMed: 4812038]
3. Hollis BW. Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. J Nutr 2005;135(2): 317–322. [PubMed: 15671234]
4. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW. Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci U S A 2004;101(20):7711–7715. [PubMed: 15128933]
5. Shinkyo R, Sakaki T, Kamakura M, Ohta M, Inouye K. Metabolism of vitamin D by human microsomal CYP2R1. Biochem Biophys Res Commun 2004;324(1):451–457. [PubMed: 15465040]
6. Belsey RE, DeLuca HF, Potts JT Jr. A rapid assay for 25-OH-vitamin D3 without preparative chromatography. J Clin Endocrinol Metab 1974;38(6):1046–1051. [PubMed: 4364805]
7. Roth HJ, Zahn I, Alkier R, Schmidt H. Validation of the first automated chemiluminescence protein-binding assay for the detection of 25-hydroxycalciferol. Clin Lab 2001;47(7–8):357–365. [PubMed: 11499797]

8. Hollis BW, Napoli JL. Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. Clin Chem 1985;31(11):1815–1819. [PubMed: 4053351]
9. Hollis BW, Kamerud JQ, Selvaag SR, Lorenz JD, Napoli JL. Determination of vitamin D status by radioimmunoassay with an ^{125}I-labeled tracer. Clinical Chemistry 1993;39:529–533. [PubMed: 8448871]
10. IDSLTD. Gamma-B 25-Hydroxy Vitamin D RIA. Product Insert. 2005.
11. ALPCODiagnostic. 25-OH Vitamin D EPBA. Product Insert. 2005.
12. Carter GD, Carter R, Jones J, Berry J. How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. Clin Chem 2004;50(11):2195–2197. [PubMed: 15375018]
13. Ersfeld DL, Rao DS, Body JJ, Sackrison JL Jr, Miller AB, Parikh N, Eskridge TL, Polinske A, Olson GT, MacFarlane GD. Analytical and clinical validation of the 25 OH vitamin D assay for the LIAISON automated analyzer. Clin Biochem 2004;37(10):867–74. [PubMed: 15369717]
14. Dabek JT, Harkonen M, Wahlroos O, Aldercreutz H. Assay for plasma 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 by “high-performance” liquid chromatography. Clinical Chemistry 1981;27:1346–1351. [PubMed: 6974062]
15. Eisman JA, Shepard RM, DeLuca HF. Determination of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in human plasma using high pressure liquid chromatography. Analytical Biochemistry 1977;80:298–305. [PubMed: 883638]
16. Jones G. Assay of vitamins D_2 and D_3, and 25-hydroxyvitamins D_2 and D_3 in human plasma by high-performance liquid chromatography. Clinical Chemistry 1978;24:287–298. [PubMed: 203413]
17. Maunsell Z, Wright DJ, Rainbow SJ. Routine isotope-dilution liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D2 and D3. Clin Chem 2005;51(9):1683–1690. [PubMed: 16020493]
18. Eisman JA, Hamstra AJ, Kream BE, DeLuca HF. 1,25-Dihydroxyvitamin D in biological fluids: a simplified and sensitive assay. Science 1976;193(4257):1021–1023. [PubMed: 1085035]
19. Eisman JA, Hamstra AJ, Kream BE, DeLuca HF. A sensitive, precise, and convenient method for determination of 1,25-dihydroxyvitamin D in human plasma. Arch Biochem Biophys 1976;176(1): 235–243. [PubMed: 9906]
20. Reinhardt TA, Horst RL, Orf JW, Hollis BW. A microassay for 1,25-dihydroxyvitamin D not requiring high performance liquid chromatography: Application to clinical studies. Journal of Clinical Endocrinology and Metabolism 1984;58:91–98. [PubMed: 6605973]
21. Hollis BW. Assay of circulating 1,25-dihydroxyvitamin D involving a novel single-cartridge extraction and purification procedure. Clin Chem 1986;32(11):2060–2063. [PubMed: 3022966]
22. Clemens TL, Hendy GN, Graham RF, Baggiolini EG, Uskokovic MR, O’Riordan JLH. A radioimmunoassay for 1,25-dihydroxycholecalciferol. Clinical Science and Molecular Medicine 1978;54:329–332. [PubMed: 630809]
23. Hollis BW, Kamerud JQ, Kurkowski A, Beaulieu J, Napoli JL. Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with ^{125}I-labeled tracer. Clin Chem 1996;42(4):586–592. [PubMed: 8605676]
24. Fraser WD, Durham BH, Berry JL, Mawer EB. Measurement of plasma 1,25 dihydroxyvitamin D using a novel immunoextraction technique and immunoassay with iodine labelled vitamin D tracer. Ann Clin Biochem 1997;34(Pt 6):632–637. [PubMed: 9367000]
25. El-Hajj Fuleihan G, Nabulsi M, Tamim H, Maalouf J, Salamoun M, Khalife H, Choucair M, Arabi A, Vieth R. Effect of vitamin D replacement on musculoskeletal parameters in school children: a randomized controlled trial. J Clin Endocrinol Metab 2006;91(2):405–412. [PubMed: 16278262]
26. Binkley N, Krueger D, Cowgill CS, Plum L, Lake E, Hansen KE, DeLuca HF, Drezner MK. Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. J Clin Endocrinol Metab 2004;89(7):3152–3157. [PubMed: 15240586]
27. Glendenning P, Taranto M, Noble JM, Musk AA, Hammond C, Goldswain PR, Fraser WD, Vasikaran SD. Current assays overestimate 25-hydroxyvitamin D3 and underestimate 25-hydroxyvitamin D2 compared with HPLC: need for assay-specific decision limits and metabolite-specific assays. Ann Clin Biochem 2006;43(Pt 1):23–30. [PubMed: 16390606]