

METHODS in CLINICAL CHEMISTRY

An accessory work to the 5th edition of

*Kaplan and Pesce's : Clinical Chemistry:
Theory, Analysis, Correlation**

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A work of 144 Methods of Analysis describing current methodology.

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Methods Clinical Chemistry

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Foreword

Foreword for the 2009 edition of *Methods in Clinical Chemistry*

In the mid-1980s we perceived a need for an extensive, up-to-date, compilation of methods available for use in clinical chemistry laboratories. To meet this need, we published in 1987 *Methods in Clinical Chemistry* with C.V. Mosby. This volume provided not only a review of extant methodologies, but also a critique of each method. This enabled the authors, when appropriate, to suggest one technique as a 'recommended' method. Since its initial publication, *Methods in Clinical Chemistry* has been repeatedly updated and made available in electronic form (CD-ROM or Internet) by Pesce Kaplan Publishers.

Like the previous version, this edition is published in parallel with the current edition of our textbook, *Clinical Chemistry: Theory, Analysis and Correlation* (5th edition; Elsevier, 2010). The editors of this work, Peter Hickman and Gus Koerbin, have assembled an international group of expert clinical chemists from the United State, Europe, and Australia/New Zealand. We have retained the scope of previous editions, including:

- 144 revised method reviews of available technologies for the analysis of each analyte,
- a critique of each methodology,
- analytical quality goals (when available),
- recent references,
- a suggested procedure for manual methods.

This edition is available in both electronic (CD-ROM) and printed (two volumes) formats. It is our hope that this edition will be widely used and vigorously reviewed by its users. Using new software technology, we will provide a mechanism for input from readers for future versions of this edition. We have created an Internet site (<http://www.pescekaplan.com/>) where individuals can publicly post their comments. Eventually, the Editors will redact the suggestions into changes incorporated into *Methods in Clinical Chemistry*.

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25-OH-Vitamin D

25-OH-Vitamin D

Ravinder Jit Singh

Clinical significance:

Chemical class: Steroid

Cholecalciferols

	Molecular formula	Merck Index	Molecular mass
25-Hydroxycholecalciferol (25-OH-vitamin D ₃ , calcifediol)	C ₂₇ H ₄₄ O ₂	1610	400.62
25-Hydroxycholecalciferol (25-OH-vitamin D ₂ , ergocalciferol)	C ₂₈ H ₄₆ O ₂	1610	412.62

Refer to Chapter 33, Bone Disease, in the 5th edition of *Clinical Chemistry: Theory, Analysis, Correlation*.

25-OH-Vitamin D

Principles of Analysis and Current Usage

Vitamin D deficiency is widely recognized, and various treatment options are being proposed in the literature. Vitamin D (written without the subscript) will refer to both vitamin D₂ and vitamin D₃. Vitamin D itself is biologically inert and is activated in the body through two specific hydroxylation reactions (Figures 1 and 2). Most evidence indicates that both natural vitamin D₃ and synthetic vitamin D₂ are metabolized by the same enzyme systems so that ingested vitamin D₂ is also converted first to 25-OH-vitamin D₂ and then to 1,25-(OH)₂-vitamin D₂. From the standpoint of assay development, it is critical that any vitamin D₂ metabolites present in serum be included in the total assayed fraction. It should be noted that in addition to 25-OH-vitamin D₃ and 1,25-dihydroxyvitamin D₃, other hydroxylated metabolites of vitamin D are known to circulate. These include 24,25-dihydroxyvitamin D₃, 25,26-dihydroxyvitamin D₃, and 1,24,25-trihydroxyvitamin D₃.

The use of methods for measuring these metabolites has been restricted to research, and the usefulness of the measurements is unknown; thus they will not be considered in this discussion. Analysis of 25-OH-vitamin D in circulation has been reported to be the best marker for determining vitamin D deficiency [1-6].

Various methods are available for measuring circulating concentrations of 25-OH-D. Current methods include HPLC, RIA with low throughput-to-high throughput automated chemiluminescence immunoassays, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). These methods have already aroused controversy [7-9]. Correlation and agreement studies between immunoassays and LC-MS/MS methods for 25-OH-D have been reported by several investigators [7-9]. These studies report reasonable correlations but with significant differences, the reasons for which are not transparent or well understood. Automated or manual competitive immunoassays are known to have less specificity for low-molecular-weight compounds, and immunoassays for 25-OH-D are no exception.

The first useful techniques for measurement of 25-OH-vitamin D were competitive protein-binding (CPB) assays (Table 1, Method 1) [10-13]. These procedures exploit the natural serum vitamin D-binding protein (DBP), which has a high affinity ($5 \times 10^8 \text{ M}^{-1}$) for 25-OH-vitamin D₃. The protein from many species has similar binding characteristics, widening the available sources of binding reagent. The serum does not need to be from vitamin D-deficient animals because the endogenous vitamin D metabolites occupy only a small number of the binding sites.

DBP also binds other circulating vitamin D metabolites, including 24,25-(OH)₂-vitamin D and 25,26-(OH)₂-vitamin D. Because of the lack of specificity of the binding protein and the existence of nonspecific lipid interference

in serum, measurement of 25-OH-vitamin D concentrations directly in crude serum extracts using CPB assays results in higher values than those observed when chromatography is used before assay. 25-OH-vitamin D can be readily separated from other metabolites by chromatography on a minicolumn of silica gel. Thus in the case of 25-OH-vitamin D assay, if a rapid index of overall vitamin D status is desired, the use of the CPB assay with preliminary Sep-Pak chromatography is preferable.

In addition to CPB assays, methods based on high-performance liquid chromatography (HPLC-UV) and detection by ultraviolet absorbance have been used successfully to measure 25-OH-vitamin D in serum (Table 1, Method 2). Because of the limited sensitivity of conventional flow-through ultraviolet detectors (low-nanogram range), these methods generally require greater sample volumes than CPB assays do, but this in turn entails rigorous prepurification procedures to remove the larger amounts of contaminating lipids and proteins. In the earlier versions of HPLC procedures, serum was equilibrated with tracer quantities of ³H-25-OH-vitamin D₃ to determine the recovery of sterol during extraction and chromatography. The 25-OH-vitamin D was prefractionated either by minicolumn chromatography or by HPLC. Final resolution and quantitation of the sterol is achieved by HPLC on microparticulate columns of silica or C₁₈ bonded silica. Detection is by ultraviolet spectroscopy at 254 nm. The concentration of sterol is then determined by relating the peak area of unknown to a calibration curve generated by HPLC of known amounts of authentic 25-OH-vitamin D. The HPLC methods for 25-OH-vitamin D, though precise and accurate, are more time consuming. When the HPLC method is used, automation of sample injection and column elute collection is desirable. In recent versions of HPLC-UV methods, non-isotopic internal standards are used, and above-mentioned limitations have been addressed.

Although more laborious and expensive, the HPLC assays offer certain advantages over the CPB methods [14-20]. For example, it is possible, with the appropriate columns, to separate the natural form of 25-OH-vitamin D₃ from 25-OH-vitamin D₂, a synthetic form, and thus gain a measure of dietary versus endogenously derived vitamin D₃. Moreover, so that 25-OH-vitamin D is completely resolved from other lipids, the HPLC methods are less susceptible than the CPB methods to nonspecific interference. For these reasons, the HPLC methods are increasingly employed to measure 25-OH-vitamin D (Figures 3 and 4).

A specific radioimmunoassay for 25-OH-vitamin D has been developed, and results indicate that this approach allows direct measurement of 25-OH-vitamin D in serum extracts (Table 1, Method 3). Although it has been suggested that 25-OH-vitamin D can be reliably measured in ethanol extracts of serum

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without preliminary chromatography, comparisons of assays with and without chromatography have clearly shown that “direct” assays produce overestimation of the 25-OH-vitamin D concentration (Figure 5) [21, 22].

Implementation of LC-MS/MS (Table 1, Method 4) has revolutionized the use of mass spectrometry in clinical laboratories. In large reference labs in the United States, serum 25-OH vitamin D is now measured routinely using LC-MS/MS, the gold standard (Figures 6 and 7) [23, 24].

Reference and Preferred Methods

It is critical to have a reference method for 25-OH vitamin D against which commercial manufacturers and clinical labs can standardize their methods for better patient care. In recent years, reference procedures for cholesterol have been developed and are available as a service for commercial manufacturers and clinical laboratories, and this has made significant impact on the clinical practice. At present, there exist no reference procedures for measurement of 25-OH vitamin D.

All the methodologies described above are being used in various clinical laboratories. It is acknowledged that there are challenges in all of these methods, and high-level technical expertise is required to perform the analysis in clinical laboratories. The College of American Pathologists (CAP) and the United Kingdom-based DEQAS (Vitamin D External Quality Assessment Scheme) surveys provide independent approaches to monitor the performance of laboratories that use various methods for testing of 25-OH-D (Table 2). The survey feedback does not assess the accuracy of 25-OH-D measurements by laboratories but scores laboratories for agreement within the group using a particular method. Recent CAP data (CAP survey, 2007 Ligands Special) indicate that clinical laboratories using chemiluminescence immunoassays can report a result ranging from 41 to 96 $\mu\text{g/L}$ for a survey sample with a value of 75 $\mu\text{g/L}$ determined by LC-MS/MS (BGS-04 in Figure 8). There could be many reasons for these variations, including drifts in the reagents being manufactured, but there is a clear and urgent need for harmonization and standardization. Considerable variation is observed in results observed for the same sample, as demonstrated in DEQAS data (Table 2).

NIST is developing quality-control materials (human serum, SRM 972) that will contain 25-OH-D₂, 25-OH-D₃, and the metabolite 3-epi-25-OH-D at 4 different concentrations as characterized by LC-MS/MS. SRM is especially important for assays for which the cross-reactivity with these metabolites is not well defined [9].

LC-MS/MS is becoming the technique of choice for various reference laboratories. Laboratories which use in-house LC-MS/MS have responsibility for many steps of the assay. The LC-MS/MS technology for testing of human samples is not approved by the U.S. Food and Drug Administration (FDA), and manufacturers of LC-MS/MS instrumentation are not responsible for troubleshooting the assays. Laboratories performing 25-OH-D testing by LC-MS/MS technology have differences in their standard operating procedures, and thus inter-laboratory CVs are in

the range of 20%. The preparation of the reagents required for in-house LC-MS/MS assays is conducted by individual laboratories under their institutionally regulated standard procedures. The complexity of the LC-MS/MS technology in its present form demands a robust, fully automated platform that can meet the need for throughput, precision, and accurate testing of vitamin D and metabolites. Multiplexed immunoassays may have the potential of achieving accuracy and precision for multiple vitamin D metabolites.

Specimen

Whole or heparinized blood should be collected and the serum or plasma frozen at -80°C . 25-OH-vitamin D is stable when stored frozen at -80°C .

Serum 25-OH-Vitamin D Reference Intervals

Recent publications have proposed that a serum 25-OH-vitamin D concentration $< 30 \text{ ng/mL}$ be used as a cut-point to define vitamin D deficiency [1, 5]. Latest research has shown that deficiency of vitamin D may be associated with susceptibility to various diseases, including cancers. For prevention of these diseases, there are many individual recommendations in the literature for minimum levels of circulating 25-OH-vitamin D, but no consensus or evidence-based-medicine guidelines have been established to help patients and physicians. Since 2004, the Mayo Clinic has defined 25-OH-vitamin D deficiency in patients (using an LC-MS/MS method) based on the criteria below. We have observed that 8.5% of the U.S. patients ($n = 40,000$) have $< 10 \text{ ng/mL}$ in winter, which drops to 4% in summer. Optimum levels were present in 60% and 73% of the population in winter and summer, respectively.

Characteristic seasonal fluctuations are seen for serum 25-OH-vitamin D concentration. These changes reflect the amount of sunlight to which a person is exposed. Concentrations of 25-OH-vitamin D are highest in late summer and lowest in spring.

Interpretation

Upon entering the bloodstream, vitamin D is bound by a specific transport protein, vitamin D-binding protein (DBP). In the liver, vitamin D is hydroxylated at the carbon-25 position, giving rise to 25-OH-vitamin D₃, the most abundant circulating form of the vitamin. The final hydroxylation step is catalyzed in the kidney by a 1- α -hydroxylase enzyme, resulting in the production of the biologically active form, 1,25-(OH)₂-vitamin D.

The activity of the renal α -hydroxylase is under tight control, so the production of 1,25-(OH)₂-vitamin D remains constant over a wide range of substrate (25-OH-vitamin D) concentrations. The main regulators of 1- α -hydroxylase activity are calcium, parathyroid hormone, and phosphate. Low serum calcium stimulates the secretion of parathyroid hormone, which acts to increase the conversion of 25-OH-vitamin D to 1,25-(OH)₂-vitamin D.

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Hypophosphatemia also stimulates conversion of 25-OH-vitamin D to 1,25-(OH)₂-vitamin D, but this process does not require parathyroid hormone.

Vitamin D, through its active form 1,25-(OH)₂-vitamin D, has its most important effects on the intestine, where it stimulates intestinal calcium and phosphate transport. It is believed that 1,25-(OH)₂-vitamin D acts at the intestinal

Reference Intervals for 25-OH vitamin D

25-OH Vit D concentration	Clinical state
<10 ng/mL	Severe deficiency*
10 ng/mL-25 ng/mL	Mild to moderate 25OHD deficiency**
25 ng/mL-80 ng/mL	Optimum 25OHD levels†
80 ng/mL	Toxicity possible‡

* Could be associated with osteomalacia or rickets

**May be associated with increased risk of osteoporosis or secondary hyperparathyroidism

† Optimum levels in the normal population

‡ 80 ng/mL is the lowest reported level associated with toxicity in patients without primary hyperparathyroidism and with normal renal function

brush-border membrane, altering the properties of the cells to allow greater permeability to calcium and phosphate ions. The enhanced absorption of these ions raises their concentration in blood to the levels necessary to permit normal skeletal mineralization.

Vitamin D also acts directly on bone and kidney. In bone, 1,25-(OH)₂-vitamin D causes bone-mineral resorption by increasing osteoclastic resorption but probably does not play a direct role in bone mineralization. In the kidney, 1,25-(OH)₂-vitamin D decreases the excretion of both calcium and phosphate by affecting their renal tubular reabsorption.

Of the circulating vitamin D metabolites, 25-OH-vitamin D is the most abundant form and has the longest half-life (approximately 1 to 2 weeks). Its concentration serves as the best index of skin synthesis and dietary intake of vitamin D. Nutritional vitamin D deficiency, rickets or osteomalacia, is associated with chronic low 25-OH-vitamin D levels, and subjects who are intoxicated with vitamin D have concentrations above 200 ng/mL. Chronic therapy with some anticonvulsants decreases 25-OH-vitamin D concentrations by induction of hepatic clearance. Abnormally low concentrations of the metabolite (resulting from malabsorption) are also observed in patients with inflammatory bowel disease, bowel resection, or biliary cirrhosis (Table 3) [3].

Performance Goals

25-OH-vitamin D is ordered and interpreted clinically in light of calcium balance and homeostasis. The quality and performance goals for calcium analysis in the clinical labs have been revolutionized. Calcium measurement is performed on highly automated instruments and has a precision of less than 1% coefficient of variation (CV) for day-to-day performance. There is no reason to accept inferior performance of the 25-OH-vitamin D assays in the 21st century. For better patient care, the goal should be not only to have an accurate 25-OH-D value but also precision

for 25-OH-D testing, with a CV < 1%. We would propose the following other goals for the 25-OH-vitamin D assay.

Desirable interassay precision would be a 1% CV, but the current technologies used for 25-OH-vitamin D analysis cannot achieve this. A CV < 10% is acceptable, but most current assays do not perform to even this level (see Table 2). Current external quality-assessment programs indicate that accuracy is also a significant problem. Currently, commercial assays for 25-OH-vitamin D do not perform to a satisfactory standard.

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Table 1: Methods for 25-OH-Vitamin D Assay

Analyte: 25-OH-vitamin D

Method 1: Competitive protein-binding assay (CPB); employs serum or tissue-binding protein, quantitative

Principle of analysis: Competition between 25-OH-vitamin D in serum sample extract and ³H-labeled analog for binding to tissue or serum D-binding protein; separation of bound and free labeled ligand by treatment with dextran-coated charcoal

Comments: Preliminary chromatography essential for accurate measurement at high concentrations of 25-OH-vitamin D

Method 2: High-performance liquid chromatography (HPLC); chromatographic, quantitative

Principle of analysis: Serum extracts are resolved on silica columns, and the concentration of 25-OH-vitamin D is calculated by peak area (A₂₅₄) integration.

Comments: Accuracy depends on efficiency of separation of 25-OH-vitamin D from interfering peaks.

Method 3: RIA and CLIA

Principle of analysis: Competition between 25-OH-vitamin D in serum sample extract and ¹²⁵I-labeled or acridinium ester-labeled analog for binding to antibody. In competitive RIA, the signal is monitored by counting radioactivity, and assay is mostly manual. In comparison, in automated CLIA, the signal is chemiluminescence, with moderate throughput.

Comments: Expensive; accuracy depends on standardization against gold-standard reference assay, precision depends on consistent manufacturing and least lot-to-lot variations

Method 4: Liquid chromatography-mass spectrometry (LC-MS/MS)

Principle of analysis: Non-isotopically labeled internal standard is used to account for variable recovery. Tandem mass spec is not only sensitive but also provides specificity and does not require extensive chromatography, as required in HPLC. Concentration of 25-OH-vitamin D is calculated by ratio of peak area/height of analyte/internal standard.

Comments: Initially requires expensive capital investment

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Table 2: Performance of 25-OH-Vitamin D Methods in Various Laboratories as Reported to DEQAS

Sample Method	n	Method Mean	SD	CV
Automated IDS EIA	13	44.7	10.7	23.9
Chromatographic ligand-binding assay	1	48	0	0
DiaSorin liaison	37	40.7	6.4	15.8
DiaSorin liaison, total	8	41.2	5.7	13.9
DiaSorin RIA	50	44.1	6.4	14.5
HPLC	10	48.5	16.6	34.3
IDS EIA	56	49.3	9.4	19
IDS RIA	31	45.2	4.5	10
LC-MS	20	50.8	9.3	18.2
Unknown	2	57	12.5	22
All Methods	228	45.4	7.5	16.6

n, Number of laboratories using a particular method.

Table 3: Vitamin D Metabolite Concentrations in Disorders of Calcium Homeostasis

Condition: “Nutritional” rickets or osteomalacia

25-OH-D: Low

1,25-(OH)₂-D: Low/low-normal

Condition: Vitamin D-dependent rickets

a. Type I

b. Type II

25-OH-D:

a. Normal

b. Normal

1,25-(OH)₂-D:

a. Low

b. High

Condition: Vitamin D intoxication

25-OH-D: High

1,25-(OH)₂-D: Low/normal

Condition: Hypophosphatemic rickets

25-OH-D: Normal

1,25-(OH)₂-D: Low/normal

Condition: Primary hyperparathyroidism

25-OH-D: Normal

1,25-(OH)₂-D: High/normal

Condition: Pseudohyperparathyroidism

25-OH-D: Normal

1,25-(OH)₂-D: Low/normal

Condition: Sarcoidosis with hypercalcemia

25-OH-D: Normal

1,25-(OH)₂-D: High

25-OH-Vitamin D

Figures

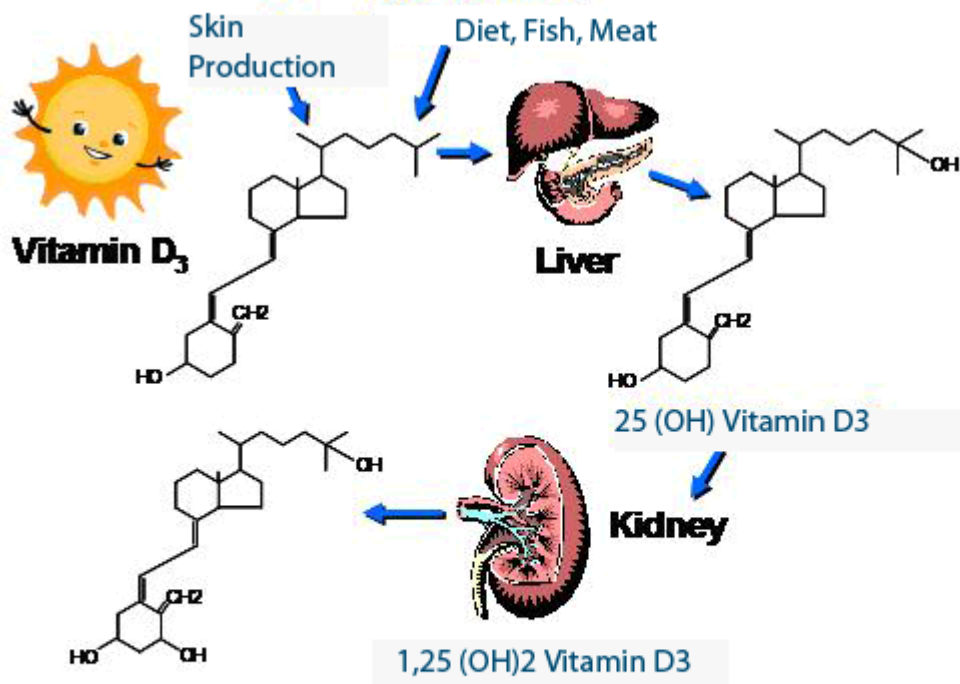


Figure 1: Pathways in the metabolism of vitamin D₃. Vitamin D₃ ingested in diet or made in skin accumulates in liver, where it is converted to 25-OH-vitamin D₃, the major circulating form. Subsequent metabolism of 25-OH-vitamin D₃ in kidney gives rise to 1,25-(OH)₂-vitamin D₃, the hormonal form.

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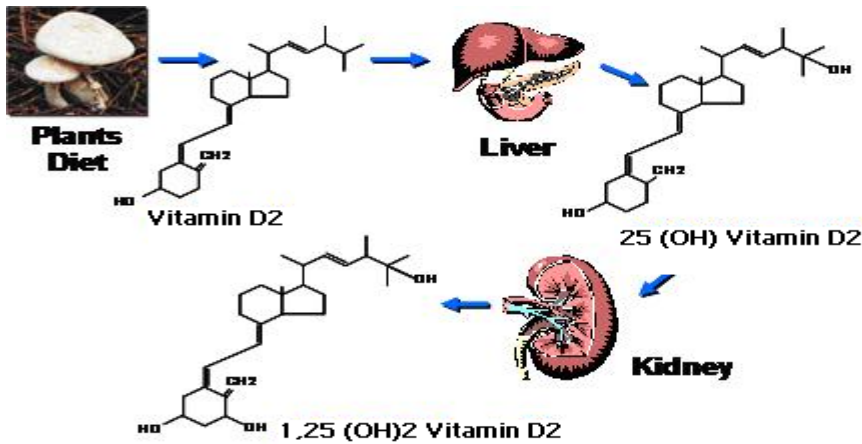


Figure 2: Pathways in metabolism of vitamin D₂. Vitamin D₂ ingested in diet or made in skin is accumulated in liver where it is converted to 25-OH-vitamin D₃, the major circulating form. Subsequent metabolism of 25-OH-vitamin D₂ in kidney gives rise to 1,25-(OH)₂-vitamin D₂, the hormonal form.

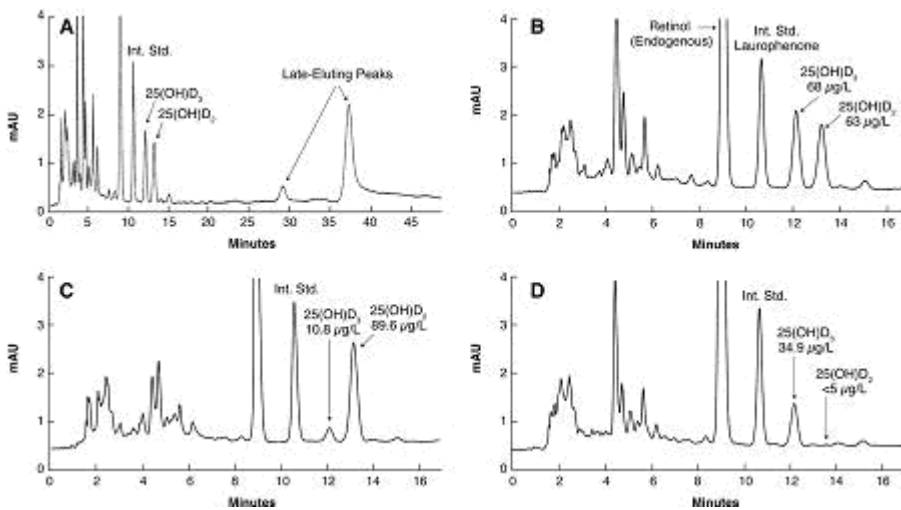


Figure 3: Representative HPLC-UV chromatograms.

(A), late-eluting peaks; (B), calibrator in extracted serum; (C), sample from patient with low 25(OH)D₃ treated with vitamin D₂; (D), sample from patient with high concentrations of 25(OH)D₃. *Int. Std.*, internal standard; *mAU*, milliabsorbance units.

25-OH-Vitamin D

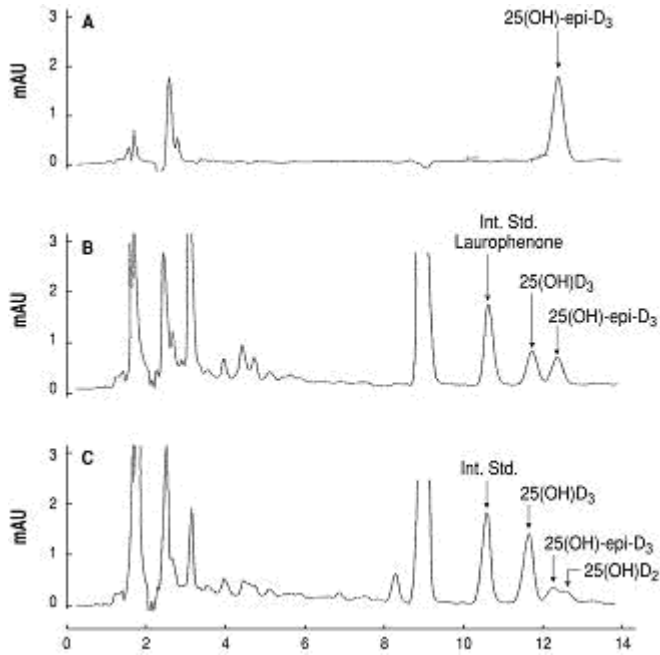


Figure 4: HPLC-UV Chromatograms showing separation of 25(OH)-epi-D₃ a recently discovered metabolite of Vit-D. (A), elution of 25(OH)-epi-D₃ (not extracted); (B), a serum extract containing 25(OH)D₃ (40 µg/L) and 25(OH)-epi-D₃ (30 µg/L); and (C), a serum extract containing 25(OH)D₃ (69 µg/L), 25(OH)-epi-D₃ (15 µg/L), and 25(OH)D₂ (12 µg/L). (26)

25-OH-Vitamin D

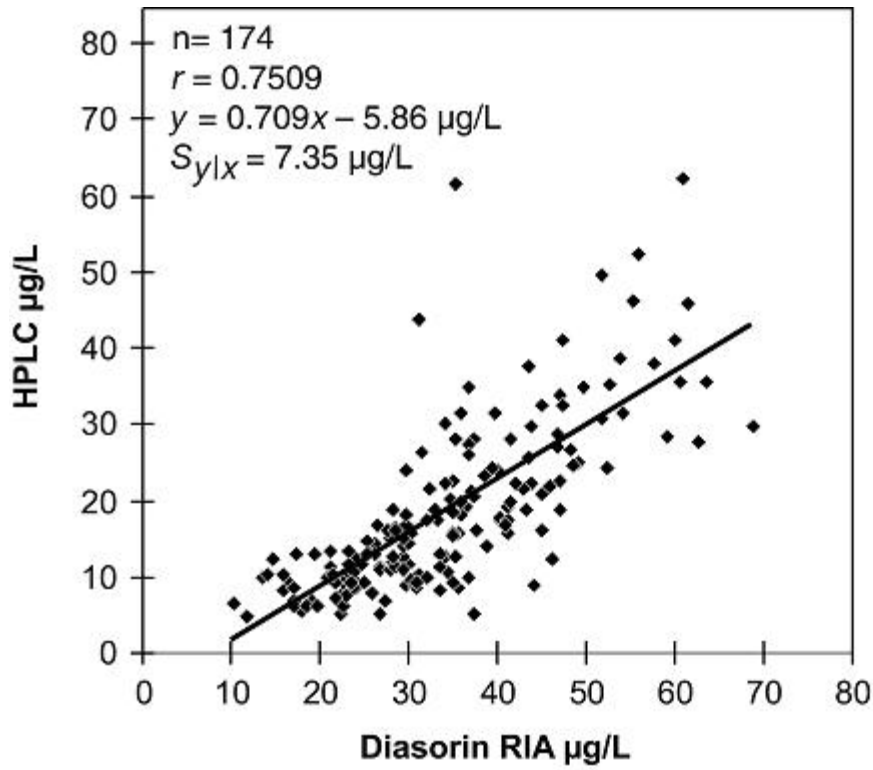


Figure 5: Comparison of the proposed HPLC method with the Diasorin RIA [21]

25-OH-Vitamin D

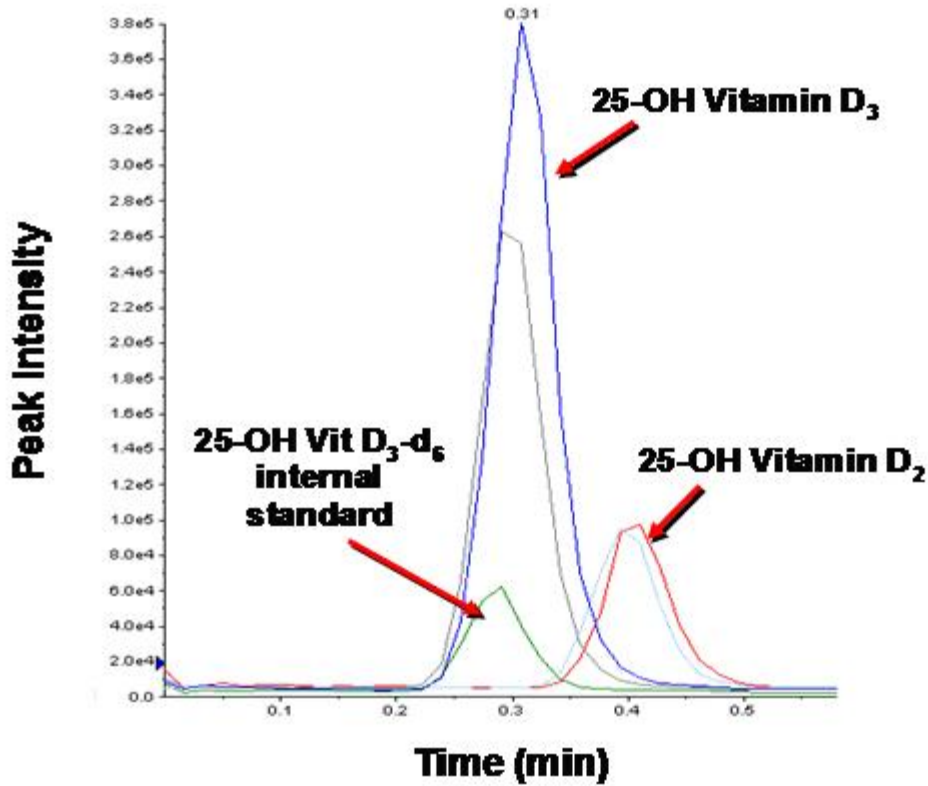


Figure 6: 25-OH-Vitamin D Metabolites. LC-MS/MS Chromatograms-MRM ion peaks for 25-(OH)-D₃ and 25-(OH)-D₂.

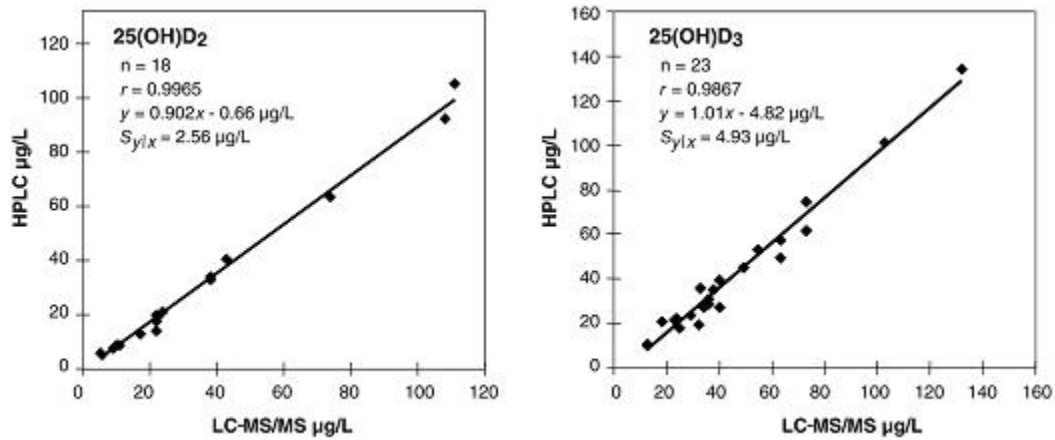


Figure 7: Comparison of the proposed HPLC method with LC-MS/MS. [19]

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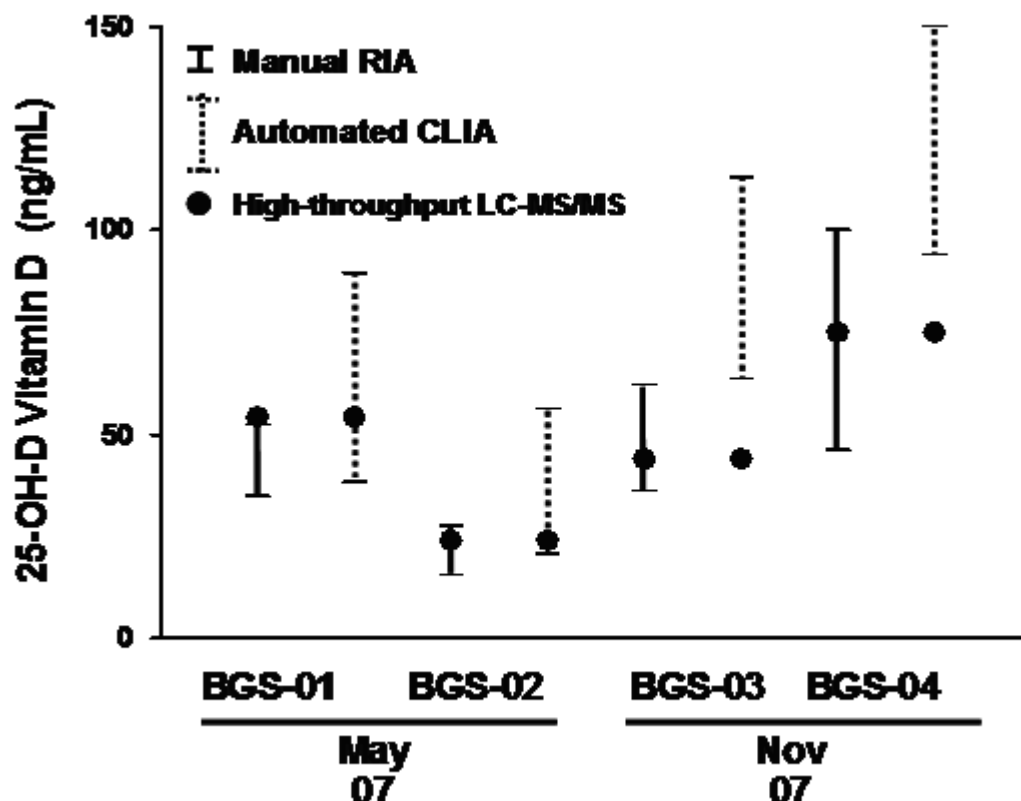


Figure 8: CAP survey data for 2007 on commonly used immunoassays for 25-OH-D.

The ranges of results for survey materials BGS-01 through BGS-04 are shown for laboratories using either RIA (*solid lines*, n = 16) or automated chemiluminescent immunoassays (*broken lines*, n = 18). Single lab LC-MS/MS 25-OH-D test results are shown (*closed circles*) for comparison. CLIA, chemiluminescence immunoassay.

Procedure: 25-OH-Vitamin D-Commercial Assays.
Follow the latest kit inserts supplied with the reagents and the instrumentation.

25-OH-Vitamin D -HPLC-UV Method [19]

Reagents

The vitamin D metabolites 25(OH)D₃ and 25(OH)D₂ can be obtained from Fluka Chemicals. ACS reagent-grade acetonitrile (CH₃CN), and ethyl acetate were obtained from Fisher. Methanol (HPLC grade) was obtained from Mallinckrodt Chemicals. Ultrapure water (18.2 MΩ/cm) was obtained from a MilliQ water purification system (Millipore). The precipitation reagent contained the internal standard laurophenone (400 µg/L) in CH₃CN and was stored in an amber bottle. Strata-X (surface-modified styrene-divinylbenzene resin) 60-mg (1 mL) extraction cartridges were from Phenomenex. An automated extraction instrument, the Gilson ASPEC XL4 (Gilson

Instruments), consisted of a 4-syringe pump module and a 4-needle sampler module with four 2-way solvent ports. Areas in the sampler racks were defined as the sample zone, reagent zone, result zone, and a disposable extraction column (DEC) zone. Acetonitrile was delivered via solvent ports. Acetonitrile-water (35:65 by volume) was stored and delivered from tubes within the reagent zone. The main reservoir contained water. The solvent evaporator was a Turbo Vap™ LV (Caliper Life Sciences). Temperature was set at 35°C, nitrogen flow was adjusted to 10 psi on the instrument gauge, and the typical drying time setting was 25 min. The HPLC unit was an integrated system with a UV3000 detector set at 275 nm, a P4000 pump set at 1.2 mL/min, an AS2000 autosampler, and an SCM1000 solvent system, all from Thermo Separation Products. A silica-saturator column (250 × 4.6 mm [i.d.] stainless steel column; Alltech) packed with ICN silica gel (particle size, 63 to 100 µm; MP Biochemicals) was installed in the oven between the pump and injector and is

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necessary here to prevent deterioration of the analytical column. The guard column (12.5 × 4.6 mm [i.d.]) and analytical column (250 × 4.6 mm [i.d.]), both containing 5- μ m Stable Bond™ Cyanopropyl (SB-CN), were from Agilent Technologies. All columns were operated at 50°C. The methanol–water (67:33 by volume) used as mobile phase was filtered and degassed.

Individual calibrator stock solutions (40 mg/L) of each metabolite are prepared in ethanol, and the concentration was verified on a Beckman DU 7500 spectrophotometer, using molar absorptivities at 265 nm (1-cm pathlength) of 19,400 and 18,300 for 25(OH)D₂ and 25(OH)D₃, respectively. From these primary stocks, dilute, combined-stock solutions of the compounds at 10,000 μ g/L each in ethanol can be prepared, which are stable for at least 1 year at –20°C. Multiple working calibrators in the range of 5 to 200 μ g/L for each of the 2 vitamin D metabolites were combined in a drug-free serum pool. The concentrations of endogenous 25(OH)D₃ and 25(OH)D₂ present in the pool were taken into account when assigning the final concentration to the calibrator. Serum calibrators were stored frozen at –20°C in 10-mL glass vials sealed with Teflon-lined caps (Quorpak™, Fisher Scientific) and were stable for at least 6 months. Controls were prepared and used in the same manner. Commercial lyophilized serum controls were custom-prepared for us by UtaK Laboratories, Inc. Reconstituted UtaK controls and thawed calibrators/controls were stable for at least 1 month stored at 4°C.

Procedure

To prepare samples, we dispensed 2 mL of precipitation reagent with internal standard into a 13 × 100 mm disposable glass test tube; we then added 1.0 mL of serum (calibrator, control, or patient sample) to the tube without mixing of contents to avoid “balling” of the protein. The tube was allowed to sit for 5 min at room temperature, after which it was vortex-mixed for 10 sec to obtain a flocculent precipitate. After another 5-min wait, the tube was vortex-mixed and centrifuged at 2000 g for 10 min. The clear supernatant was decanted into a 10 × 75 mm disposable glass test tube, which was then transferred to the sample zone of the ASPEC XL4 and protected from exposure to natural sunlight to prevent degradation of analytes. The extraction conditions are defined in detail in ref 21. The XL4 processed 4 samples simultaneously and unattended in ~ 15 min. The unit sequentially conditioned the Strata-X cartridge in the DEC zone with 2.0 mL of CH₃CN followed by 2.0 mL of 35:65 CH₃CN–water; added 1.0 mL of water to each extract; transferred 3.5 mL of extract mixture to the DEC; rinsed the DEC with 2.0 mL of 35:65 CH₃CN–water; and eluted the Strata-X cartridge in the DEC zone with 2.0 mL of CH₃CN. The eluate was dried at 35°C under a stream of nitrogen; the

dry extract was then reconstituted with 150 μ L of ethyl acetate–CH₃CN (5:95 by volume) and vortex-mixed for 5 sec. Water (110 μ L) was then added to the tube, and the contents were vortex-mixed for 5 sec. The sample was centrifuged at 2000 g for 10 min to settle the precipitate. The clear liquid was transferred to a glass microvial insert positioned in an amber-colored vial. The sample was capped and placed in the autosampler unit of the HPLC. The extract was stable for at least 3 days at room temperature. The processor software calculated relative retention time for peak identification and peak-height ratio for quantification.

25-OH-Vitamin D LC-MS/MS Method [24]

For the standard 25-OH-D method, online extraction and HPLC chromatography of the supernatants were performed using a TX4 Turbo Flow system (Cohesive Technologies, Franklin, MA) with 1.0 × 50 mm Cyclone extraction columns and 3.3 cm × 4.6 mm, 3- μ m LC-18 (Supelco, St. Louis, MO) analytical columns. After online extraction, the analytes were eluted onto the analytical column for 90 sec with a mobile phase of 39.5% vol/vol methanol, 0.005% vol/vol formic acid. There was a step gradient to 87% vol/vol methanol, 0.005% vol/vol formic acid for the analytical column. The analytes then entered an API 4000 triple-quadrupole mass spectrometer (ABI-Sciex, Toronto, Canada) and were ionized in an atmospheric-pressure chemical-ionization source and detected by multiple reaction monitoring of the following ion pairs: *m/z* 413.0/395.3 for 25-OH-D₂, *m/z* 401.4/383.3 for 25-OH-D₃, and *m/z* 407.4/389.5 for 25-OH-D₃-d₆. The raw signals of 25-OH-D₂ and 25-OH-D₃ in the calibrators, controls, and samples were normalized to their respective internal standard 25-OH-D₃-d₆ signals, and concentrations in the samples and controls were calculated off the normalized six-point calibration curves (0 to 200 ng/mL (0 to 500 nmol/L)). Samples with concentrations that exceeded the highest calibrator were diluted and run again. The total 25-OH-D concentrations of each control and sample were calculated by summing the measured values of 25-OH-D₂ and 25-OH-D₃.

For separation of epimers, the standard LC-18 column was replaced with a longer 5-dinitrobenzoyl-(R)-phenylglycine column (Chirex-PGLY and DNB 250 × 4.6 mm; Phenomenex, Torrance, CA), and 100 μ L of the supernatant was injected. The step gradient extends only up to 67% vol/vol methanol, 0.005% vol/vol formic acid at an analytical column flow rate of 0.9 mL/min. The mass spectrometer settings remained unchanged. The concentrations of 25-OH-D₂, 25-OH-D₃, and 25-OH-D were calculated as above. The concentrations of any detected C-3 epimers of 25-OH-D₂ or 25-OH-D₃ were also calculated off the normalized 25-OH-D₂ and 25-OH-D₃

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calibration curves, and the total 3-epi-25-OH-D concentration is the sum of 3-epi-25-OH-D₂ and 3-epi-25-OH-D₃ concentrations. Intraassay CVs were 3.8%, 2.4%, and 4.7% at 25, 54, and 140 ng/mL, respectively. Interassay CVs were 6.4%, 6.8%, and 5.0% at 24, 52, and 140 ng/mL respectively.