A Simple, Rapid Atmospheric Pressure Chemical Ionization Liquid Chromatography Tandem Mass Spectrometry Method for the Determination of 25-Hydroxyvitamin D2 and D3

Uttam Garg,^{1,2*} Ada Munar,^{1,2} Clinton Frazee III,^{1,2} and David Scott^{1,2}

¹Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, Missouri

²Department of Pathology and Laboratory Medicine, University of Missouri School of Medicine, Kansas City, Missouri

Vitamin D plays a vital role not only in bone health but also in pathophysiology of many other body functions. In recent years, there has been significant increase in testing of 25-hydroxyvitamin D (25-OH vitamin D), a marker of vitamin D deficiency. The most commonly used methods for the measurement of 25-OH vitamin D are immunoassays and liquid chromatography tandem mass spectrometry (LC-MS-MS). Since immunoassays suffer from inaccuracies and interferences, LC-MS-MS is a preferred method. In LC-MS-MS methods, 25-OH vitamin D is extracted from serum or plasma by solid-phase or liquid-phase extraction. Because these extraction methods are time consuming, we de-

veloped an easy method that uses simple protein precipitation followed by injection of the supernatant to LC-MS-MS. Several mass-to-charge (m/z) ratio transitions, including commonly used transitions based on water loss, were evaluated and several tube types were tested. The optimal transitions for 25-OH vitamin D2 and D3 were $395.5 > 269.5$ and $383.4 > 257.3$, respectively. The reportable range of the method was 1–100 ng/mL, and repeatability (within-run) and within-laboratory imprecision were <4% and <6%, respectively. The method agreed well with the solid-phase extraction methods. *J. Clin. Lab. Anal.* 26:349–357, 2012. © 2012 Wiley Periodicals, Inc.

Key words: vitamin D; 25-hydroxyvitamin D2; 25-hydroxyvitamin D3; tandem mass spectrometry

INTRODUCTION

Vitamin D plays a vital role in calcium and phosphorus metabolism, and bone health. In addition to its well-known function in bone health, vitamin D plays important roles in the physiology and pathophysiology of many other body functions (1–4). The role of vitamin D has been implicated in diabetes, cardiovascular diseases, cancer, multiple sclerosis, and immune system functions (1). It has been known for years that elderly population and patients with renal and liver disease are at high risk of vitamin D deficiency (1, 5, 6). In recent years, a number of epidemiological studies have shown that vitamin D deficiency is fairly common in the general population (1, 7–9). The best markers of nutritional deficiency of vitamin D are vitamin D metabolites 25-hydroxyvitamin D2 and D3 (25-OH vitamin D2 and D3). Due to an increased

awareness of vitamin D deficiency, clinical laboratories have seen significant increase in the testing of 25-OH vitamin D2 and D3. The common methods for the measurement of vitamin D metabolites are immunoassays and chromatographic methods (10–14). Due to high sensitivity and specificity, and ability to differentiate between D2 and D3, liquid chromatography tandem mass spectrometry (LC-MS-MS) using electrospray or atmospheric pressure chemical ionization (APCI) is a preferred method.

*Correspondence to: Uttam Garg, Department of Pathology and Laboratory Medicine, Children's Mercy Hospitals and Clinics, Kansas City, MO 64108. E-mail: ugarg@cmh.edu

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Current LC-MS-MS methods for the measurement of 25-OH vitamin D2 and D3 that involve liquid–liquid or solid-phase extractions are laborious and time consuming (15,16). We describe an easy LC-MS-MS method that uses simple protein precipitation followed by injection of the supernatant to LC-MS-MS. This method of analyzing 25-OH vitamin D2 and D3 without prior extraction was compared with widely accepted solid-phase extraction methods.

MATERIALS AND METHODS

Chemicals and Reagents

25-OH vitamin D2 and D3 were purchased from Cerilliant (Round Rock, TX). In addition, certified material containing 25-OH vitamin D2 and D3 was purchased from National Institute of Standards and Technology (NIST). Internal standard, 25-OH vitamin D3 (26, 26, 26, 27, 27, 27-d6), was purchased from Cerilliant. Calibrators were prepared in 7% bovine serum albumin prepared in phosphate buffered saline and ranged from 1 to 100 ng/mL for 25-OH vitamin D2 and D3. Quality control material was purchased from UTAK Laboratories (Valencia, CA).

Sample Collection and Processing

Before complete method validation, different tube types were evaluated for the method suitability. To compare different tube types, samples from three volunteers were collected in EDTA, heparin with and without gel, clot activator red top with and without gel tubes. Serum or plasma was separated by centrifugation of blood at 3,000g for 10 min. After optimizing mass-to-charge (m/z) transitions, it was determined that many tubes were suitable. However, most of the studies were performed on red-top tubes since these tubes are more versatile and suitable for other chemistry assays.

TABLE 1. HPLC Parameters

Time (min)	Events	Parameter (%)
0.00	Pump B	80
4.00	Pump B	100
5.00	Pump B	100
6.00	Pump B	80
6.10	Stop	

Column temperature was 45°C and the flow rate was 1 mL/min. Gradient was made from 100% water containing 0.1% formic acid (A) and 100% methanol containing 0.1% formic acid (B).

TABLE 2. Optimized m/z Transitions for 25-Hydroxyvitamin D2, 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D3 (26, 26, 26, 27, 27, 27-d6)

Analyte	Q1	Q3	Qualifier ion
25-Hydroxyvitamin D2	395.5	269.5	377.5
25-Hydroxyvitamin D3	383.4	257.3	365.5
25-Hydroxyvitamin D3 (26, 26, 26, 27, 27, 27-d6).	389.3	211.5	n/a

TABLE 3. MS-MS Parameters

Nebulizer, curtain, collision gas		Nitrogen
Curtain gas (psi)	40	
Nebulizer current (μA)	3	
Turbo flow pressure, Gas 1 (psi)	55	
Detector temperature	450°C	
Collision gas (GAD)	Medium	
Declustering potential (V)	D3 = 86.0, D3(d6) = 85.0 D2 = 84.0	
Entrance potential (V)	10.0	
Exit potential (V)	D3 = 9.0, D3(d6) = 11.0, D3 Qual = 12.0 D2 = 10.0, D2 Qual = 10.0	
Collision energy (eV)	D3 = 20.0, D3(d6) = 38.0, D3 Qual = 18.0 D2 = 29.0, D2 Qual = 28.0	

Sample Preparation

To 200 μL aliquots of patient sample (serum or plasma), controls or calibrators, 200 μL of refrigerated acetonitrile containing 50 ng/mL deuterated internal standard 25-OH vitamin D3 (26, 26, 26, 27, 27, 27-d6) were added. The samples were then vortexed and left at room temperature for 10 min. Next, the samples were centrifuged at 10,000g for 10 min. Finally, the supernatants were transferred to autosampler vials and 30 μL was injected on LC-MS/MS for analysis.

HPLC-MS-MS Conditions and Operation

Shimadzu Prominence HPLC (Lenexa, KS) linked to Applied Biosystems 4000 QTrap (Foster City, CA) was used for the analysis. Chromatographic separation involved reverse phase 5 μm, 5 cm × 46 mm, C18 SupelcosilTM analytical column (Bellefonte, PA), and mobile phases, water, and methanol containing 0.1% formic acid. HPLC parameters are given in Table 1. Positive ion APCI and various multiple reactions monitoring (MRM), m/z transitions, were used and optimized for MS-MS analysis. The optimized m/z transitions were 25-OH vitamin D2 (395.5 > 269.5 and 395.5 > 377.5), 25-OH vitamin D3 (383.4 > 257.3 and 383.4 > 365.5), and 25-OH vitamin D3-d6 (389.3 > 211.5; Table 2). Optimized MS-MS

Fig. 1. Representative LC-MS-MS chromatograms for 25-hydroxyvitamin D2 comparing m/z transitions 413.5 > 395.5 (left) and 395.5 > 269.5 (right). Samples were prepared by adding 1 mL of serum-based control to each tube type. Data from all the transitions were collected simultaneously from the same injection. The average concentration of 25-hydroxyvitamin D2, from different tubes, was 30.2 ng/mL. The variation in measured concentrations among different tube types was <5%. Since different transitions give different sized peaks, for better comparison, the signal-to-noise (S/N) ratios of the peaks of interest are provided.

parameters are given in Table 3. Commonly used m/z transitions involving water loss, 413.5 > 395.5 for 25-OH vitamin D2, 401.2 > 383.2 for 25-OH vitamin D3 and 407.2 > 389.2 for 25-OH vitamin D3-d6 were also opti-

mized and evaluated. The data were analyzed using Applied Biosystems Analyst Software (Foster City, CA). The data from Analyst Software were exported to Microsoft Excel for better resolution graphing.

Fig. 1. Continued.

RESULTS

The method was evaluated for accuracy, linearity (reportable range), limits of quantitation, interferences, repeatability, and within-laboratory imprecision. The method was also evaluated for ion suppression and compared with widely used solid-phase extraction methods. Before validation of these parameters, the m/z transitions were optimized. For the assay of 25-OH vitamin D2 and D3, commonly used m/z transitions are water loss from the molecular ions; m/z 413.5 > 395.5 for 25-OH vitamin D2, and m/z 401.2 > 383.2 for 25-OH vitamin D3, respectively. These m/z transitions yielded peaks with poor resolution and significant baseline noise and showed numerous erroneous peaks (Figs. 1 and 2). These artifacts were seen in all the tubes tested (EDTA, heparin green top with and without gel, and clot activator red-top tubes with and without gel). This led to investigation and optimization of other m/z transitions that did not involve water loss from the molecular ions. Table 2 shows the optimized m/z transitions for 25-OH vitamin D2, 25-OH vitamin

D3, and 25-OH vitamin D3-d6. Figures 1 and 2 show the chromatograms for 25-OH vitamin D2 and 25-OH vitamin D3 comparing m/z transitions based on water loss and m/z transitions listed in Table 2. The data for different m/z transitions were collected simultaneously from the same sample injections. The transitions listed in Table 2 yielded better resolution, improved baseline, and no significant erroneous peaks. This was true with all the tube types tested.

Linearity (reportable range) was determined by analyzing seven levels of calibrators. Linearity (reportable range) of the method was established from 1 to 100 ng/mL for 25-OH vitamin D2 and D3 (Fig. 3). The lower limit of quantitation was 1 ng/mL. This was based on the fact that the signal-to-noise (S/N) ratios for 1 ng/mL 25-OH vitamin D2 and D3 calibrators were 18.1 and 11.2 (>10), respectively. Concentration that gives S/N ratio of >10 is generally acceptable as limit of quantitation. Accuracy/recovery was also calculated from these data. The measured values deviated <10% (<20% for the

Fig. 2. Representative LC-MS-MS chromatograms for 25-hydroxyvitamin D₃ comparing m/z transitions 401.2 > 383.2 (left) and 383.4 > 257.3 (right). The samples were collected in different tube types from a volunteer, and prepared as described in Materials and Methods. Data from all the transitions were collected simultaneously from the same injection. The average concentration of 25-hydroxyvitamin D₃, from different tubes, was 41.5 ng/mL. The variation in measured concentrations among different tube types was <5%. Since different transitions give different sized peaks, for better comparison, the signal-to-noise (S/N) ratios of the peaks of interest are provided.

Fig. 2. Continued.

Fig. 3. Linearity (reportable range) of 25-hydroxyvitamin D2 (left) and 25-hydroxyvitamin D3 (right). The concentrations of calibrators were 1, 2.5, 5, 10, 25, 50, and 100 ng/mL.

TABLE 4. Repeatability and Within-Laboratory Imprecision for Three Levels of Quality Controls of 25-OH Vitamin D2 and D3

	25-OH Vitamin D2			25-OH Vitamin D3		
Mean (ng/mL)	13.8	40.8	99.9	11.0	24.8	61.5
Repeatability CV	4.4	3.2	2.8	4.4	4.1	3.4
Within-laboratory CV	5.7	4.6	3.7	5.7	5.7	5.1

Repeatability (formerly called within-run imprecision) was calculated by taking the average of variances of replicates from different days, and within-laboratory imprecision was calculated from repeatability and variances of daily means as described in Clinical Laboratory Standards Institute (CLSI) EP15 A2 guidelines (31).

Fig. 4. Ion suppression studies based on internal standard counts in the blank, controls, calibrators, and patient samples. No significant ion suppression was observed.

lowest calibrator) from the target values. Furthermore, the measured values on NIST-certified materials were within 10% of the target values.

Repeatability and within-laboratory precision were evaluated by assaying three levels of controls in duplicate from 40 independent runs. Clinical Laboratory Standards Institute's (CLSI) EP15-A2 guidelines were used to calculate repeatability and within-laboratory precision. Coefficients of variations for repeatability and within-laboratory precisions for vitamin D2 and D3 were less than 5% and 6%, respectively (Table 4).

Ion suppression was determined by comparing the peak areas of internal standard in the blank samples with that of areas of internal standard in the controls, calibrators, and patient samples. Blank samples refer to the samples in which deionized water instead of other matrices such as 7% BSA, serum, or plasma was used. Internal standard counts in the controls, calibrators, and patient samples were within 10% of each other. As shown in Figure 4, no significant ion suppression was noted.

The results of the current method were compared with the well-established solid-phase extraction methods as reported by our reference laboratories (Mayo Laboratories or Quest Diagnostics). For comparison, reference samples with sufficient volume were split into two aliquots, one for the reference laboratory and the other for our own laboratory. Samples were stored at -20°C and tested in batches of 10–20 samples. As shown in Figure 5, the current method is in agreement with the solid-phase extraction methods. Since vitamin D2 comes only from exogenous sources and is not widely prescribed, the number of samples with 25-OH vitamin D2 was only 21.

DISCUSSION

It has been well established that vitamin D plays important role not only in bone health, but in the physiology and pathophysiology of many other body functions (1–4). In recent years, it has become evident that vitamin D deficiency is a common health problem (1, 5, 6). Measurement of vitamin D metabolites, 25-OH vitamin D2 and D3 are considered the best markers for the evaluation of nutritional deficiency of vitamin D (4). This is due to the fact that 25-OH vitamin D2 and D3 have higher concentrations in the blood and have longer half-lives of three weeks as compared to the active metabolite 1,25-dihydroxyvitamin D. Measurement of 1,25-dihydroxyvitamin D is not considered adequate for checking nutritional status of vitamin D because of its shorter half-life of only 4 h (15, 17). However, the analysis of 1,25-dihydroxyvitamin D is useful in those patient who suffer from certain diseases such as lymphoma, sarcoidosis, hypocalcemia, chronic renal disease, hypophosphatemia, hypomagnesemia, vitamin D dependent rickets, hyperparathyroidism, or hypoparathyroidism (15, 17, 18).

Commonly used methods for the measurement of 25-OH vitamin D2 and D3 include competitive immunoassays such as competitive protein binding assay, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and chemiluminescent immunoassay (13–15), and chromatographic methods such as gas chromatography mass spectrometry or liquid chromatography with ultraviolet or mass spectrometer detectors (10, 19–23).

Immunoassays are fast and convenient, but have a number of limitations and disadvantages. Immunoassays suffer from cross-reactivity with other compounds (13, 14, 24), do not provide separate concentrations of 25-OH vitamin D2 and D3, and often are less accurate as compared to chromatographic methods. Also, since both 25-OH vitamin D2 and D3 are metabolized to active forms, the ideal immunoassays should measure these compounds equimolarly. However, this is not the case because certain immunoassays detect 25-OH vitamin D3

Fig. 5. Comparison of 25-hydroxyvitamin D2 (left) and 25-hydroxyvitamin D3 (right) concentrations using solid-phase extraction method and current simple protein precipitation method. Samples represented by closed diamonds were compared with Mayo Laboratories ($N = 21$ for 25-hydroxyvitamin D2 and $N = 221$ for 25-hydroxyvitamin D3), and the samples represented by open squares were compared with Quest Diagnostics ($N = 0$ for 25-hydroxyvitamin D2 and $N = 20$ for 25-hydroxyvitamin D3).

more preferentially than 25-OH vitamin D2 (17). It may be important to measure both 25-OH vitamin D2 and D3 as it has been shown that 1,25-dihydroxyvitamin D3 may be more active than 1,25-dihydroxyvitamin D2 (25, 26).

An additional limitation of immunoassays is that a number of these methods suffer from inaccuracies. Recently Farrell et al. (13) studied the performance of five automated immunoassays and a radioimmunoassay with two LC-MS-MS methods. Although most immunoassays had good precision, many suffered from inaccuracies. Radioimmunoassay performed better than the automated immunoassays. This was due to the fact that it involves clean-up using an acetonitrile extraction step. In addition, the use of organic solvents such as acetonitrile eliminates interference from heterophile antibodies. The use of organic solvents as clean-up step is not possible with automated immunoassays and makes these methods more prone to interferences and inaccuracies. This is evident from the fact that radioimmunoassay and LIASION-automated immunoassay use the same antibody, but the former assay shows better concordance with LC-MS-MS method (13). Furthermore, many automated immunoassays suffer from significant positive bias, particularly at low levels. For example, Roche and LIASION assays showed bias of +35% for 25-OH vitamin D at levels <8 ng/mL (13). It should be noted that although radioimmunoassay is better than automated immunoassays, it is not preferred because it uses radioactivity and is very time consuming. Furthermore, many immunoassays suffer from inaccuracies that are vitamin D binding protein concentration dependent. A recent study looked at the affect of vitamin D binding protein concentrations on the accuracy of five immunoassays as compared to tandem mass spectrometry (14). Most immunoassays suffered from vitamin D binding protein concentrations de-

pendent inaccuracies. For example, when samples from pregnant women were tested, one method categorized 67% of the patients as vitamin D sufficient, where as another method categorized only 24% as vitamin D sufficient (14).

Given the above limitations of immunoassays, chromatographic methods for the measurement of 25-OH vitamin D2 and D3 are preferred. Both gas and liquid chromatographic methods that use different detectors are available for the measurement of 25-OH vitamin D2 and D3. Liquid chromatography methods with ultraviolet or mass spectrometry detectors are preferred over gas chromatography. In recent years, LC-MS-MS has become the most widely used chromatographic method for the assay of 25-OH vitamin D2 and D3, and it is considered a "gold standard" method (21, 24, 27).

In chromatographic methods, a common step involved in the measurement of 25-OH vitamin D2 and D3 is the release of these compounds from the proteins followed by liquid-liquid or solid-phase extraction. In liquid-liquid extraction method, internal standard is added to the sample followed by extraction of 25-OH vitamin D2 and D3 with an organic solvent such as hexane or n-heptane (16). Organic solvent is then evaporated and reconstituted in an organic solvent for analysis. In a typical solid-phase extraction method, the sample is diluted with water after addition of internal standard, and then extracted with reverse solid-phase cartridges (21). Methods involving on-line solid-phase extractions are also available (28). Once 25-OH vitamin D2 and D3 are extracted, they are analyzed by tandem mass spectrometry using electrospray or APCI. Both liquid-liquid and solid-phase extraction methods are time consuming and produce a lot of organic solvent and other waste. In the present study, we explored the possibility of extracting 25-OH vitamin D2 and D3

directly into acetonitrile and then analyzing the extract without further liquid–liquid or solid-phase extraction.

Upon beginning of our study, the commonly used m/z transitions based on water loss from the molecular ions (16,21,29) were optimized using positive ion APCI. These m/z transitions yielded poor resolution and erroneous peaks. These problems were seen in all the tube types tested, that is, EDTA, heparin with and without gel, and red top with and without gel tubes. Similar issues of erroneous peaks and interferences with certain brands of polypropylene containers have been reported (30). Also, it is important to note that the interferences and erroneous peaks were different in different tube types. For example, heparin-gel tubes showed the largest interfering peaks. Therefore, it is important to validate different tube types before using the method for patient testing. In our study, this led to optimization of other m/z transitions that were not based on water loss. These m/z transitions, as shown in the results, yielded better resolution, higher sensitivity, and no erroneous peaks that interfered with the peaks of interest. The method presented here showed high sensitivity, low imprecision, and wide analytical measurement range, and yielded results that were in agreement with the results obtained by solid-phase extraction methods.

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