Analysis of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in Serum Using Ultra-performance Liquid Chromatography with Tandem Mass Spectrometry

Yong-Wha Lee

Department of Laboratory Medicine and Genetics, Soonchunhyang University Bucheon Hospital and Soonchunhyang University College of Medicine, Bucheon, Korea
Email: lywmd@daum.net

Abstract: Background: Measurement of serum 25-hydroxyvitamin D metabolites (25OHD) may be useful in the laboratory diagnosis and treatment of vitamin D deficiency. A standardized liquid chromatography-tandem mass spectrometry (LC-TMS) assay has been used for accurate analysis of 25-hydroxyvitamin D levels. We evaluated the performance of a recently developed ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC-TMS) for the simultaneous quantification of 25OHD3 and 25OHD2 in human serum. **Materials and Methods:** We established the measurement method of 25-hydroxyvitamin D using UPLC-TMS and evaluated the precision and made a comparison between the UPLC-TMS and the chemiluminescence immunoassay (CLIA). Proportions of vitamin D deficiency in a local population and the spectrum of vitamin D2 measured were investigated. **Results:** UPLC-TMS revealed within-run precision and between-run precision of less than 4% and showed a bias of less than 5%. The CLIA measurements of 25OHD showed a correlation of CLIA = 0.86 × UPLC-TMS + 1.16 (r^2 = 0.98). The means±SD of serum 25OHD, 25OHD2, and 25OHD3 in 1,047 individuals were 12.8±9.3 ng/mL (0.5-62.7 ng/mL), 0.6±1.6 ng/mL (0-22.4 ng/mL), and 12.2±9.2 ng/mL (0.4-62.4 ng/mL), respectively. The proportions of vitamin D insufficiency and deficiency were 34.4% (360/1047) and 48.6% (509/1047), respectively. **Discussions:** The newly developed 25OHD assay using the MSMS Vitamin D kit based on the UPLC-TMS assay showed excellent performance and is suitable for routine clinical measurements of serum 25OHD2 and 25OHD3. We expect that this method will contribute to improved accuracy and reduced interlaboratory variation versus other methods for 25OHD measurement.
[Yong-Wha Lee. **Analysis of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in Serum Using Ultra-performance Liquid Chromatography with Tandem Mass Spectrometry**. *Life Sci J* 2013; 10(3): 1648-1652]. (ISSN: 1097-8135). http://www.lifesciencesite.com 247

Keywords: 25-hydroxyvitamin D (25OHD); vitamin D; ultra-performance liquid chromatography (UPLC); tandem mass spectrometry (TMS); chemiluminescence immunoassay (CLIA)

1. Introduction

Vitamin D deficiency is a common cause of bone deformities and can induce secondary hyperparathyroidism with consequent bone loss and osteoporosis (Bikle, 2012). More recently, the vitamin D system has also been linked to overall health and well-being, including diabetes, hypertension, and cancer risk (Danik, 2012, Holick, 2007, Janssens, 2009, Vuolo, 2012, Zerwekh, 2008).

"Vitamin D" consists of two different compounds, ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Vitamin D2 is synthesized by plants while vitamin D3 is synthesized in skin exposed to sunlight. Both vitamin D3 and vitamin D2 are metabolized to 25-hydroxy forms (25OHD) in the liver. These are the predominant circulating forms of vitamin D and are considered to be the best indicator of vitamin D status (Isenor, 2010).

The candidate reference method for 25OHD assay, LC-TMS, requires technical expertise, specialized equipment, and expensive deuterated internal standards. Commercial assays such as immunoassays and chemical binding assays are used more frequently in clinical laboratories because they have automated pre-treatment steps and produce results within a shorter time. However, they vary in accuracy and precision (Carter, 2012, Ong, 2012). Methods for the quantification of vitamin D metabolites are increasingly being switched from immunoassays (IAs) and radioimmunoassays (RIAs) to liquid chromatography coupled with tandem mass spectrometry (LC-TMS) (Farrell, 2012).

We evaluated the performance of a recently developed ultra-performance liquid chromatography combined with tandem mass spectrometry for the simultaneous quantification of 25OHD3 and 25OHD2 in human serum. We estimated the proportion of vitamin D insufficiency or deficiency based on the results from the individuals visiting our clinics for regular health check-ups or medical care service. We also measured the spectrum of vitamin D2 compared with vitamin D3.

2. Material and Methods

2.1. Samples

In total, 1,047 patient serum samples were collected from our laboratory archives for analysis, protected from light, and stored at -80 ℃ until analysis. All samples were anonymized.

Serum 25-hydroxyvitamin D measurements were made using a routine laboratory UPLC-TMS assay and compared with results from the Roche Diagnostics Elecsys vitamin D total assay.

2.2. Measurements

2.2.1. Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-TMS)

2.2.1.1. Materials and Reagents

All solvents were of HPLC grade. Methanol, acetonitrile, and ethanol were from Burdick & Jackson (Muskegon, MI). The MSMS Vitamin D kit (PerkinElmer, Waltham, MA) includes six-level calibrators, three-level controls, and stable-isotope internal standards. These materials were prepared in charcoal-stripped human serum. $^{2}H_{6}$-25-hydroxyvitamin D2 and $^{2}H_{6}$-25-hydroxyvitamin D3 were used as standards and control materials. $^{2}H_{3}$-25-hydroxyvitamin D2 and $^{2}H_{3}$-25-hydroxyvitamin D3 were used as internal standards. The MSMS vitamin D derivatization box contains MSMS vitamin D derivatization reagent (4-phenyl-1,2,4-triazoline-3,5-dione; PTAD), MSMS vitamin D quench solution, and MSMS vitamin D HPLC solvent additive.

2.2.1.2. Sample preparation

Calibrators and controls were reconstituted by adding 2 mL of deionized water and dissolved for 1 h, and derivatization reagent was reconstituted in 30 mL acetonitrile. Daily precipitation solution was diluted with reconstituted IS 1:100, with acetonitrile containing 0.1% formic acid. Samples (200 µL) were added to the wells of a 500-µL V-bottomed microplate. The plate was shaken at 750 rpm for 10 min and centrifuged for 30 min. The plate was dried with a nitrogen gas flow and 50 µL of derivatization reagent was added, and then 50 µL of quench solution was added and sample aliquots were injected into the UPLC-TMS for analysis.

2.2.1.3. UPLC-TMS measurements

UPLC was performed on a Waters Acquity UPLC system (Waters Corporation, Milford, MA). The autosampler injected 15 µL of the extract on to an Acquity UPLC HSS C18 column (2.1×50 mm, 1.8 µm) maintained at 50 ℃ in a column oven. LC separation of the 25OHD and matrix interference was performed using a gradient profile of mobile phase A and B solutions, consisting of 0.025% solvent additive and 50% methanol and 0.025% solvent additive and 100% methanol, respectively. The flow rate was at 300-700 µL/min and the run time was 5 min.

TMS was used to detect 25OHD2 and 25OHD3 and their corresponding deuterium-labeled internal standards on a Waters Acquity TQ-Detector. At unit mass resolution, the mass analyzer had the following settings: cone voltage at 30 V, collision energy at 24 eV, source and desolvation temperatures at 150 ℃ and 400 ℃, respectively, and desolvation gas flow at 800 L/h. The analysis was performed in multiple reaction monitoring (MRM) mode and electrospray ionization positive using the following transitions: the mass to charge ratios (*m/z*) of 25OHD2, 25OHD3, 2H3-25OHD2, 2H3-25OHD3, 2H6-25OHD2, 2H6-25OHD3 are 619.4 → 298.1, 607.4 → 298.1, 622.4 → 301.1, 610.4 → 301.1, 625.4 → 298.1 and 613.4 → 298.1, respectively. Quantitation was performed using the TargetLynx Manager in the Waters MassLynx 4.1 software by linear regression of peak area ratios of 25OHD2/2H6-25OHD2 and 25OHD3/2H6-25OHD3 against the calibrator concentrations with 1/x weighting.

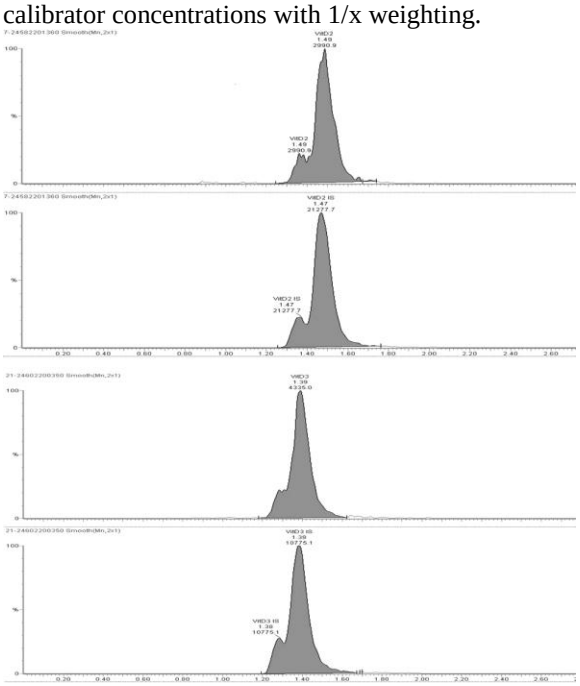


Figure 1. Chromatogram of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in serum sample measured by ultra-performance liquid chromatography with tandem mass spectrometry

2.2.2. Chemiluminescence immunoassay

The Roche Elecsys Total 25OHD assay (Roche Diagnostics GmbH, Germany) consists of pretreatment using dithiothreitol and sodium hydroxide. This is followed by incubation with ruthenium-labeled vitamin D binding protein, streptavidin-coated microparticles, and biotin-labeled 25OHD. Serum 25OHD will bind to ruthenium-labeled vitamin D binding protein. The remaining free binding sites will be occupied by labeled 25OHD to form a ruthenium-labeled vitamin D binding

protein biotinylated 25OHD complex that can bind to the solid phase via the biotin-streptavidin interaction. The mixture is then aspirated into a measuring cell where the microparticles are captured magnetically on the surface of the electrode, with measurement of the chemiluminescent emission after application of a voltage to the electrode. Three lots of reagents were provided by Roche Diagnostics for this study, before regulatory approval of the assay.

2.3. Method validation

The within-run and between-run precision of the UPLC-TMS method was assessed using duplicate low, medium and high serum control material samples for 20 days. The UPLC-TMS method was compared with the CLIA method using a convenience set of serum samples obtained from patients admitted for vitamin D assays. The samples were distributed evenly from low concentration to high concentration based on the results of the CLIA method.

In total, 1047 samples were obtained from individuals visiting our clinics for regular health check-ups or medical services, and measurements of vitamin D2 and vitamin D3 were performed on these samples; the proportion of vitamin D insufficiency or deficiency was estimated based on the results, and the spectrum of vitamin D2 measured was compared with vitamin D3.

2.4. Statistics

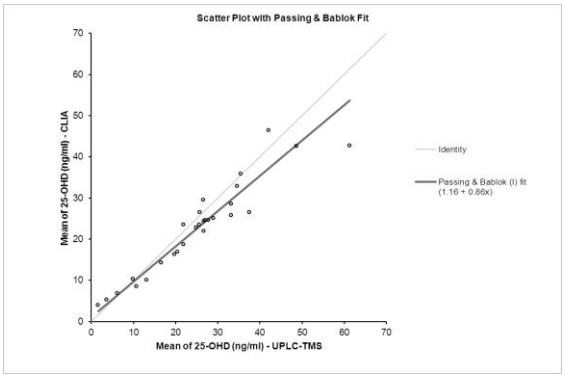
Analyse-it for Microsoft Excel (Analyse-it Software Ltd., Leeds, UK) was used to evaluate the data statistically using descriptive statistics. P-values < 0.05 were considered to indicate statistical significance. Passing and Bablok regression and a Bland-Altman plot were performed for comparisons.

3. Results

3.1. Chromatogram of 25-OHD using UPLC-TMS

Two isolated peaks were observed and the 6-S-isomer was the main product. The retention times of 25-OHD3 and 25-OHD2 were 1.40 and 1.45 min, respectively, and the same as those of calibrators and internal standards (Figure. 1). The mass of 25OHD2-PTAD and 25OHD3-PTAD was increased by 175 amu using derivatization and also increased by 31 amu by solvent addition, compared with 25OHD2 and 25OHD3 at Q1. A daughter ion fragment of 25OHD3-PTAD, 25OHD2-PTAD, was detected at 298.1 amu at Q3.

A



B

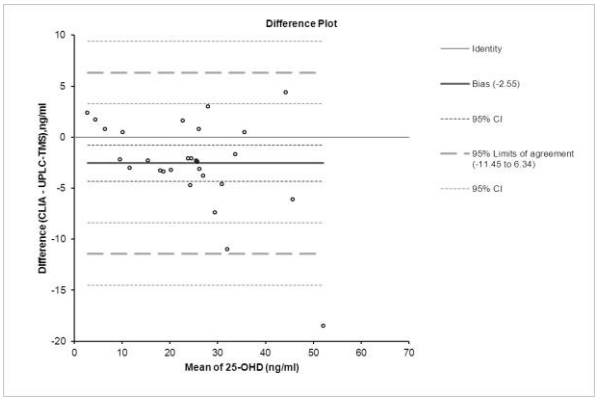


Figure 2. Comparison of 25-hydroxyvitamin D between ultra-performance liquid chromatography with tandem mass spectrometry and chemiluminescence immunoassay method.
A. Passing and Bablok regression plot.
B. Bland-Altman plot.

3.2. Performance validation of UPLC-TMS

The precision performance of the assays is summarized in Table 1, with within-run and between-run precision of quality control samples shown. Overall, the coefficients of variation were less than 4%, which is widely accepted for hormonal measurements (Table 1).

Table 1. Within-run and between-run precision for 25-hydroxyvitamin D control materials

			Within-run			Between-run		
		Target (ng/mL)	Mean (ng/mL)	SD	CV (%)	Mean (ng/mL)	SD	CV (%)
25-hydroxyvitamin D2	Level 1	9.9	10.4	0.3	3.1	10.3	0.3	2.8
	Level 2	41.7	43.5	0.7	1.6	43.0	0.9	2.0
	Level 3	85.8	85.1	1.4	1.7	85.2	1.3	1.5
25-hydroxyvitamin D3	Level 1	8.5	8.8	0.2	2.1	8.7	0.2	2.1
	Level 2	35.3	36.2	0.6	1.6	36.2	0.5	1.4
	Level 3	72.5	72.5	1.5	2.1	72.4	1.4	2.0

The results of UPLC-TMS measurement showed comparability to the CLIA method using Passing-Bablok regression analysis. The slope of the CLIA method was 0.8984 and the intercept was 2.3334 (CLIA = 0.86 × LC-MSMS + 1.16, r^2 = 0.98; Figure. 2). The mean difference between two methods was -2.55 ng/mL (95% CI -4.31 to -0.79), based on the Bland-Altman plot.

The means ±SD of serum 25OHD, 25OHD2, and 25OHD3 status for the 1047 individuals were 12.8 ±9.3ng/mL (0.5-62.7 ng/mL), 0.6 ±1.6 ng/mL (0-22.4 ng/mL), and 12.2 ±9.2 ng/mL (0.4-62.4 ng/mL), respectively. The proportions of vitamin D insufficiency (less than 20 ng/mL) and deficiency (less than 10 ng/mL) were 34.4 % (360/1047) and 48.6% (509/1047), respectively (Figure. 3). The ratio of $25OHD_2$ to 25OHD was 5%, on average (0-77%). There was no correlation between $25OHD_2$ and $25OHD_3$.

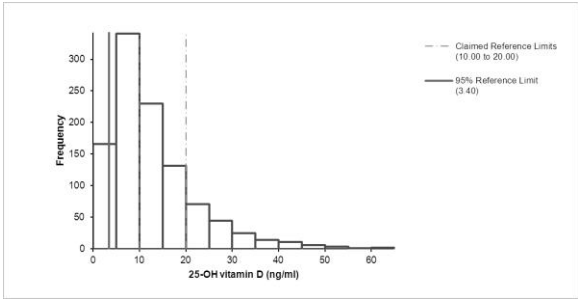


Figure 3. Distribution of serum concentrations of 25-OH vitamin D from 1,047 Korean subjects

4. Discussions

It is clear that clinical laboratories are moving more towards mass spectrometry-based technology for routine quantitative analyses. The increasing demand for vitamin D metabolite analysis in recent years has resulted in the development of LC/MS-based methods for analysis. In this study, we evaluated a newly developed UPLC-TMS system for 25OHD measurements in clinical serum samples.

PTAD, which improves the ionization efficiency and TMS signal intensity of the analytes, was used as a derivatizing material in this study. PTAD is selectively added to the cis-diene double bonds of the 25OHD molecules, resulting in the generation of a new chiral center and the formation of a new pair of 25OHD diastereomers. Both 6S and 6R isomers are combined for the quantitative determination of 25OHD2 and 25OHD3 in the samples.

The UPLC-TMS method correlated with the CLIA method based on the Passing-Bablok regression equation, as has been shown previously (Costelloe, 2009). However, some data from the CLIA method were lower than those from UPLC-TMS, by -2.55 ng/mL, which could be due to matrix interference or poorer sensitivity (Vieth, 2000). Another study found that some CLIA and RIA methods do not show good correlations with LC-TMS (Emmen, 2012). Our data are generally in agreement with other laboratories performing LC-TMS quantitation of 25OHD3 and 25OHD2.

The within-run precision and between-run precision of UPLC-TMS were consistent with those reported by the manufacturer. Using biological variation as a guide, this assay was considered valuable.

In this study, the proportions of vitamin D insufficiency and deficiency were 34.4 % (360/1047) and 48.6% (509/1047), respectively. 25OHD concentrations determined for vitamin D deficiency and insufficiency cutoffs were derived from studies that used, mostly, RIA methods (Bischoff-Ferrari, 2004, Bischoff-Ferrari, 2006). This may also confuse the use of cutoff points for the diagnosis of vitamin D deficiency in the clinical setting.

The US Institute of Medicine committee concluded that a serum 25OHD level of 20 ng/mL (50 nmol/L) is desirable for bone and overall health. The Institute found that serum 25OHD concentrations above 30 ng/mL are not consistently associated with increased benefit. There was a lower risk of cardiovascular disease when vitamin D ranged from 8 to 24 ng/mL (https://en.wikipedia.org/wiki/Vitamin_D).

In our study, the mean ±SD of serum 25OHD2 was 0.6 ±1.6 ng/mL (from 0 to 22.4 ng/mL) and the proportion of 25OHD2 to 25OHD was 5%, on average (0-77%). Our study demonstrates that it is necessary to separate and quantify vitamin D compounds individually for accurate measurement of specific metabolites. It is difficult to anticipate the amount of 25OHD2 because some clinical samples have 25OHD2 at very high concentrations (up to 22.4 ng/mL) even though, generally, vitamin D2 is not common in supplements, and even less common in food fortification, if at all. Regular nutrition and exposure to sunlight provide the body with the D3 form only (Tripkovic, 2012).

The newly developed 25OHD assay using the MSMS Vitamin D kit, based on the UPLC-TMS assay, showed excellent performance and was suitable for routine clinical measurements of serum 25OHD2 and 25OHD3. We expect that this method will contribute to improved accuracy and reduced interlaboratory variation versus other methods for 25OHD measurement.

Acknowledgements:

This work was supported by the

Soonchunhyang University Research Fund.

Corresponding Author:
Yong-Wha Lee, M.D., Ph.D.
Department of Laboratory Medicine and Genetics, Soonchunhyang University Bucheon Hospital and Soonchunhyang University College of Medicine, 1174 Jung-dong, Wonmi-gu, Bucheon, 420-767, Korea.
E-mail: lywmd@daum.net

References

[1] Bikle DD. Vitamin D and bone. Curr Osteoporos Rep 2012;10:151-9.
[2] Bischoff-Ferrari HA, Dietrich T, Orav EJ, Hu FB, Zhang Y, Karlson EW, Dawson-Hughes B. Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged > or =60 y. Am J Clin Nutr 2004;80:752-8.
[3] Bischoff-Ferrari HA, Giovannucci E, Willett WC, Dietrich T, Dawson-Hughes B. Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. Am J Clin Nutr 2006;84:18-28.
[4] Carter GD. 25-hydroxyvitamin D: a difficult analyte. Clin Chem 2012;58:486-8.
[5] Costelloe SJ, Woolman E, Rainbow S, Stratiotis L, O'Garro G, Whiting S, Thomas M. Is high-throughput measurement of 25-hydroxyvitamin D3 without 25-hydroxyvitamin D2 appropriate for routine clinical use? Ann Clin Biochem 2009;46:86-7; author reply 7-8.
[6] Danik JS, Manson JE. Vitamin d and cardiovascular disease. Curr Treat Options Cardiovasc Med 2012;14:414-24.
[7] Emmen JM, Wielders JP, Boer AK, van den Ouweland JM, Vader HL. The new Roche Vitamin D Total assay: fit for its purpose? Clin Chem Lab Med 2012;0:1-4.
[8] Farrell CJ, Martin S, McWhinney B, Straub I, Williams P, Herrmann M. State-of-the-art vitamin D assays: a comparison of automated immunoassays with liquid chromatography-tandem mass spectrometry methods. Clin Chem 2012; 58:531-42.
[9] Holick MF. Vitamin D deficiency. N Engl J Med 2007;357:266-81.
[10] Isenor JE, Ensom MH. Is there a role for therapeutic drug monitoring of vitamin D level as a surrogate marker for fracture risk? Pharmacotherapy 2010; 30: 254-64.
[11] Janssens W, Lehouck A, Carremans C, Bouillon R, Mathieu C, Decramer M. Vitamin D beyond bones in chronic obstructive pulmonary disease: time to act. Am J Respir Crit Care Med 2009;179:630-6.
[12] Ong L, Saw S, Sahabdeen NB, Tey KT, Ho CS, Sethi SK. Current 25-hydroxyvitamin D assays: do they pass the test? Clin Chim Acta 2012; 413: 1127-34.
[13] Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, Penson S, Chope G, Hypponen E, Berry J, Vieth R, Lanham-New S. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. Am J Clin Nutr 2012;95:1357-64.
[14] Vieth R. Problems with direct 25-hydroxyvitamin D assays, and the target amount of vitamin D nutrition desirable for patients with osteoporosis. Osteoporos Int 2000;11:635-6.
[15] Vuolo L, Di Somma C, Faggiano A, Colao A. Vitamin D and cancer. Front Endocrinol (Lausanne) 2012;3:58.
[16] Zerwekh JE. Blood biomarkers of vitamin D status. Am J Clin Nutr 2008;87:1087S-91S.

8/19/2013