

Development and validation of a liquid chromatography-tandem mass spectrometry assay for serum 25-hydroxyvitamin D₂/D₃ using a turbulent flow online extraction technology

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Abstract

Background: Vitamin D is important to health and disease. Liquid chromatography-tandem mass spectrometry (LC-MSMS) is considered the most accurate technology for quantification of serum 25-hydroxyvitamin D (25OHD) which is the best biomarker for estimating vitamin D nutritional status.

Methods: Serum was mixed with acetonitrile containing hexadeuterated 25-hydroxyvitamin D₃ (d₆-25OHD₃) and centrifuged 10 min at 15,634×g. The supernatant was injected onto a turbulent flow preparatory column then transferred to a polar endcapped C18 analytical column. The mass spectrometer was set for positive atmospheric pressure chemical ionization.

Results: The analytical cycle time was 5.5 min. Inter- and intra-assay CV for both analytes across three concentrations ranged from 3.8% to 14.2%. The method was linear from 3.0 to 283.6 nmol/L for 25-hydroxyvitamin D₃ (25OHD₃) and 4.6 to 277.9 nmol/L for 25-hydroxyvitamin D₂ (25OHD₂), with an accuracy of 88.7%–118.7% and 90.7%–100.3%, respectively. No carryover or ion suppression was observed. Comparison with a radioimmunoassay using patient specimens (n=527) showed a mean difference of 5.2%, and diagnostic agreement of 80.9% with Deming regression of slope 0.867, intercept 12.8, standard error of estimate (SEE) 17.4, and r=0.8425.

Conclusions: The LC-MSMS method coupled with turbulent flow technology for serum 25OHD quantitation is rapid, efficient, and suitable for clinical testing. Clin Chem Lab Med 2009;47:1565–72.

Keywords: 25-hydroxyvitamin D; liquid chromatography; radioimmunoassay; tandem mass spectrometry; turbulent flow.

Introduction

Vitamin D has been recognized as the sunshine vitamin and deficiency/insufficiency has been associated with poor bone metabolism, weak muscle strength, cancer risk and mortality, autoimmune disease, and cardiovascular disease (1–3). Recently published studies show links between vitamin D and various diseases including muscle strength (4), cancer risk and mortality (5–7), insulin resistance (8), and cardiovascular disease (9).

There are two common types of vitamin D: cholecalciferol (D₃) and ergocalciferol (D₂). D₃ is synthesized in the skin from 7-dehydrocholesterol in cell membranes upon exposure to UVB (290–320 nm). D₂ is derived primarily from yeast and produced exogenously by UV irradiation of ergosterol (2, 10). Both dietary supplementation and sunshine exposure are effective in preventing vitamin D deficiency (2, 10). There is clear evidence that UV light exposure, consuming vitamin D-fortified food, and/or vitamin D supplementation has a positive impact on serum 25-hydroxyvitamin D (25OHD) concentrations, and adults can tolerate vitamin D at doses above the current dietary reference intake levels (11–13). Though both D₂ and D₃ have been used as supplements, D₂ is less efficient compared with D₃ for increasing serum 25OHD (10, 14, 15). However, long-term treatment with D₂ and D₃, 1000 IU daily, showed similar effectiveness in increasing serum 25OHD (16).

Vitamin D in circulation is metabolized to 25OHD in the liver and further metabolized to the active metabolite, 1,25-dihydroxyvitamin D [1,25(OH)₂D] in the kidney (10). The concentration of 1,25(OH)₂D is highly regulated by many factors including serum parathyroid hormone and phosphorus (1, 2). The half-lives of vitamin D, 25OHD, and 1,25(OH)₂D are ~24 h, 3 weeks, and 4 h, respectively (17). Liver production of 25OHD is not regulated significantly and is primarily dependent on the availability of vitamin D (17). Therefore, measuring total serum 25OHD is considered the best estimate of vitamin D nutritional status. It is now generally accepted that serum 25OHD concentrations of 50 nmol/L (20 ng/mL) and 75 nmol/L (30 ng/mL) are the cut-off thresholds for deficiency and insufficiency, respectively (10, 18, 19). Vitamin D insufficiency is recognized as an epidemic issue in both children and adults (1, 10, 20).

Immunoassays and chromatography-based methods have been used to measure 25OHD in serum or plasma. The International Vitamin D Quality Assess-

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ment Scheme demonstrated that most commercial 25OHD methods were capable of producing reliable results for samples containing only 25-hydroxyvitamin D₃ (25OHD3), but most methods had significant bias compared to HPLC for samples with a substantial proportion of 25-hydroxyvitamin D₂ (25OHD2) (21). Evaluation of current radioimmunoassay (RIA) and chemiluminescent methods for serum 25OHD using patient samples showed substantial variability among the six methods, and the same methods used in different laboratories (22). Compared to a HPLC method for 25OHD, significant positive proportional bias was observed for immunoassays in the range of 20–50 nmol/L in serum samples before the patients had D2 treatment. Also, all the immunoassays that were evaluated under-estimated the 25OHD2 in samples following D2 treatment (23). A comprehensive evaluation of seven methods using 291 EDTA plasma samples (277 had no detectable 25OHD2 and 14 had 25OHD2 between 5 and 8 nmol/L) showed that all methods, except HPLC, demonstrated considerable negative bias compared to liquid chromatography-tandem mass spectrometry (LC-MSMS) (24). For most immunoassays, the deviation was more significant at 25OHD >75 nmol/L compared with those >75 nmol/L (24).

Most HPLC methods require lengthy sample preparation, including solid-phase extraction (25) and liquid-liquid extraction (26), with a long HPLC chromatography time ranging from 10 to 30 min. Attention should be given to the late elution peaks which might interfere with the analysis of succeeding samples (25). Although LC-MSMS is considered the most accurate technology for 25OHD quantification (17, 27), 1 α -hydroxyvitamin D₃ (1 α OHD3) (28) and the C3 epimer of 25OHD (3-epi 25OHD) (29) could be significant interferences due to the same molecular weight if not separated by LC. Most LC-MSMS methods employ deuterated 25OHD3 as internal standard. To improve ionization efficiency, some methods employ a derivatization strategy with a Cookson-type reagent (30) or Diels-Alder derivatization (31). For direct measurement, sample preparation could be cumbersome. In general, sample preparation includes protein precipitation followed by solid-phase extraction (32–34) or liquid-liquid extraction (28, 35, 36). Turbulent flow technology is a robust and rapid online purification tool for high efficiency extraction (37, 38), and has been used for online sample cleaning for serum 25OHD quantification (29). Here, we report a simple and fast LC-MSMS method with online turbulent flow extraction. We compared this method with a RIA method using a large number of patient samples.

Materials and methods

Reagents and solutions

Methanol, acetone, acetonitrile, and isopropanol (Burdick and Jackson High Purity Solvent) were from VWR (West Chester, PA, USA). Type 1 water was from Millipore Synergy System (Billerica, MA, USA). 25OHD2 and 25OHD3 were

$\geq 98\%$ and 1 α OHD3 was $\geq 97\%$ pure by HPLC (Sigma-Aldrich, St. Louis, MO, USA). Hexadeuterated-25-hydroxyvitamin D₃ (d₆-25OHD3) (99%⁻²H) and hexadeuterated-25-hydroxyvitamin D₂ (d₆-25OHD2) (99%⁻²H) were procured from Medical Isotopes (Pelham, NH, USA). A turbo preparatory column, Cyclone-P 1.0 \times 50 mm, and a polar endcapped C18 column, Hypersil GOLD aQ 2.1 \times 50 mm, 5 μ m, were purchased from Thermo Fisher (Waltham, MA, USA). The solvent mixer (P/N G1312-87330) was from Agilent (Santa Clara, CA, USA). The sampling vials (Waters, Milford, MA, USA) were LC-MS certified and Axygen 96-well plates were from VWR. ClinChek Serum 25OHD2 and 25OHD3 controls were purchased from IRIS Technologies Inc (Olathe, KS, USA). A stock solution for each 25OHD3 (nmol/L=2.496 \times ng/mL), d₆-25OHD3 (nmol/L=2.459 \times ng/mL), and 25OHD2 (nmol/L=2.423 \times ng/mL) was made at a nominal concentration of 250 μ mol/L. The final stock concentration was calculated using a Beckman Coulter DU 800 UV/visible spectrophotometer (Fullerton, CA, USA) with molar extinction coefficients of 17,950 (39) for 25OHD2 and 18,000 (40) for 25OHD3 at 265 nm. A set of calibration standards with combined 25OHD3 and 25OHD2 at 200.0, 100.0, 50.0, 25.0, and 12.5 nmol/L for 25OHD3 and 194.0, 97.0, 48.5, 24.3, 12.1 nmol/L for 25OHD2 was prepared by serial dilution in absolute ethanol and stored at -70°C . Ethanolic calibrators were used because human serum, bovine serum albumin, and commercial steroid free serum all contained significant amounts of 25OHD, based on our evaluation. To ensure the matrix difference did not have an adverse impact on quantification of patient samples, serum-based commercial controls were included in each run. In addition, serum-based samples with known added values were used in the precision and linearity studies to show the validity of ethanol-based calibrators. A precipitation/internal standard solution containing d₆-25OHD3 at 103.3 nmol/L in acetonitrile was stored at -20°C .

Sample collection and preparation

Blood was collected in Vacutainer tubes with no additives (BD, Franklin Lake, NJ, USA). The specimens were centrifuged immediately upon arrival at $\sim 1500\times g$ at room temperature and assayed for 25OHD by RIA. Leftover samples of serum were aliquoted, de-identified, and frozen at -70°C for the comparison study. Sample preparation consisted of adding 250 μ L internal standard to 100 μ L of serum in either polypropylene microcentrifuge tubes or 96 well plates. After vortexing for 1 min, the mixture was centrifuged for 10 min at 15,634 $\times g$ for the microcentrifuge tubes and 3300 $\times g$ for the 96 well plates. In the microcentrifuge tubes, the supernatant was transferred to sample vials and placed on the autosampler. The plates were directly loaded into the autosampler with 100 μ L of the supernatant injected into the system.

Turbulent flow LC-MSMS method

This method was developed on the Thermo TSQ Quantum Access with a Cohesive front end using turbulent flow technology. Instrument software for this study consisted of Tune Master 1.5, Aria 1.6.1, and Xcalibur 2.0.7. The duplex Cohesive system consisted of a robotic sampling arm and a refrigerated sampling compartment for six 96-well plates followed by two parallel and independent inline degassers, binary HPLC pumps, and quaternary HPLC pumps. A solvent mixer was placed between the sample injection port and the turbo column to mix the different solvents in the samples (acetonitrile) and the mobile phase (methanol), which could cause significant peak broadening. Samples were loaded on the

Cyclone-P turbo column which was washed afterwards. At the transfer step, 100 μ L of methanol from the transfer loop eluted the analytes from the Cyclone-P turbo column to the Hypersil GOLD aQ analytical column where separation occurred with an isocratic elution of 95% methanol. The total run time between two injections was 5.5 min if only one channel was used. The mass spectrometer was set to positive ion mode with the atmospheric pressure chemical ionization probe. The discharge current was set to 2.0 A with the capillary temperature at 200°C and the vaporizer temperature at 290°C. The sheath gas was set to 55 U and aux gas to 25 U. Multiple reaction monitoring was set at the transitions of 401.3 \rightarrow 383.2 m/z for 25OHD3, 407.3 \rightarrow 389.2 m/z for d_6 -25OHD3, and 413.3 \rightarrow 395.0 m/z 25OHD2. The collision energy was 8 eV for 25OHD3 and 25OHD2 and 7 eV for d_6 -25OHD3 and the tube lens for 25OHD3 was 110 V and for d_6 -25OHD3 and 25OHD2 was 130 V. Data collection started at 2.5 min into the HPLC run and continued for 1.25 min. A representative patient chromatogram is displayed in Figure 1A. The turbulent flow and HPLC methodology is outlined in Table 1.

RIA assay

The primary method for comparison was the DiaSorin RIA (Stillwater, MN, USA) which was linear from 17.5 to

249.6 nmol/L. The intra-day variation ($n=10$) was 6.0% at 34.4 nmol/L and 5.5% at 120.6 nmol/L. Inter-day variation ($n=15$) was 11.0% at 36.9 nmol/L and 10.5% at 136.8 nmol/L. The lower limit of quantification (LLOQ) was 17.5 nmol/L.

Validation methods

Ion suppression was evaluated by post-column infusion of a pure deuterated 25OHD2 and 25OHD3 solution while patient samples were injected in the system. Two phospholipids (lyso-phosphatidylcholine C16:0 and C18:0) were monitored for potential coelution. Since 1α OHD3 has the same molecular weight as 25OHD3 and was noted in a previous publication (28) as a possible interferent, we performed testing by injecting an ethanolic solution containing both 1α OHD3 and 25OHD3.

The limit of detection was assessed by running saline ($n=20$) and was determined to be 3 standard deviations (SD) over the mean. A pool of patient samples with <12.5 nmol/L of 25OHD2 and 25OHD3 was spiked with both 25OHD2 and 25OHD3. Then, linearity was examined in triplicate by serially diluting the spiked pool with saline to achieve the appropriate concentrations. The LLOQ was determined by the lowest concentration levels in the linearity study with accuracy within $100\% \pm 20\%$ and total CV within 20%. Both intra-assay and inter-assay CVs were determined using three

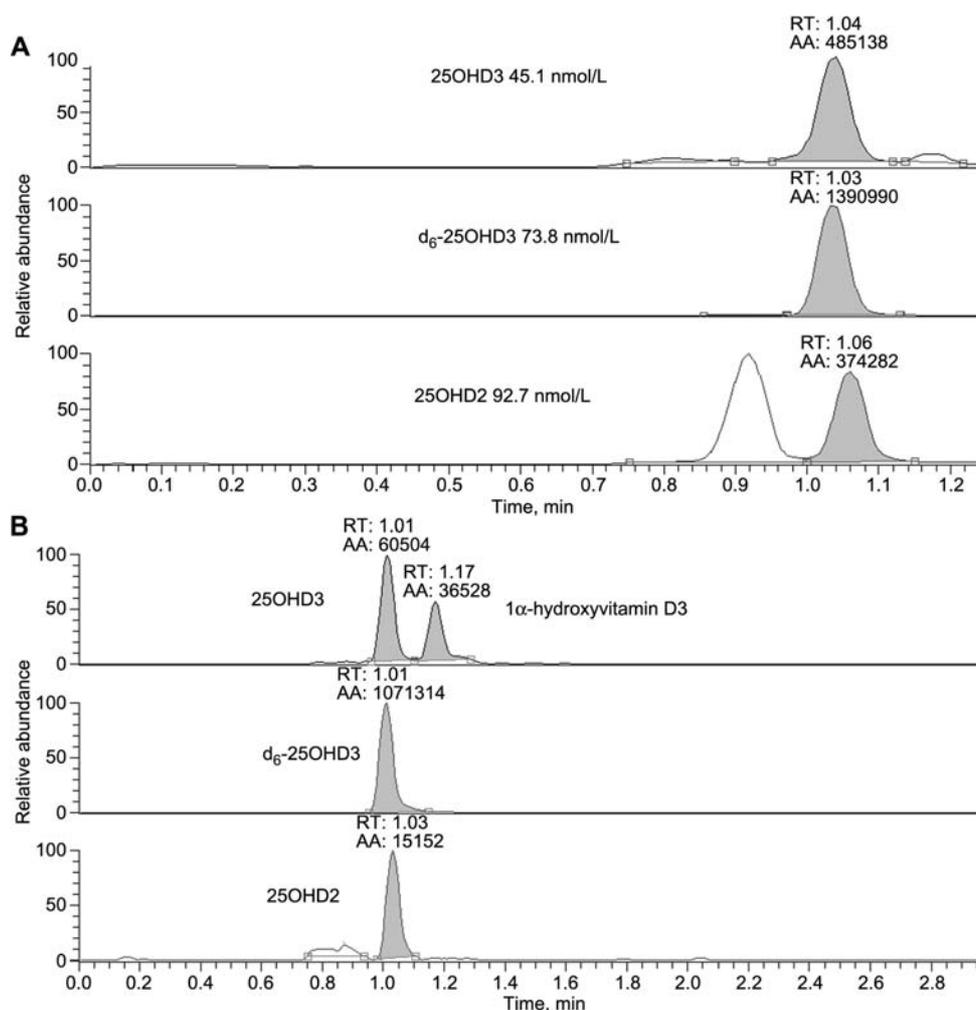


Figure 1 Chromatograms.

(A) A typical patient sample with mid levels of 25OHD2 and 25OHD3. Acceptable retention times (RT) for standards and patients are 25OHD3 and d_6 -25OHD3 at 1.01 ± 0.03 min and 25OHD2 at 1.04 ± 0.03 min. (B) Chromatographic resolution between 25OHD3 and 1α OHD3 with 25OHD3 eluting at 1.01 min and 1α OHD3 eluting at 1.17 min.

Table 1 LC conditions for turbo and analytical columns.

Process	Time, s	Turbo column solvent A:B:C	Flow rate, mL/min	Analytical column solvent A:B	Flow rate, mL/min
Turbulent flow extraction on turbo column	55	60:40:0	4.0	60:40	0.80
Slow down pumps	5	60:40:0	0.2	60:40	0.55
Begin transfer from turbo to analytical column	90	60:40:0	0.2	60:40	0.55
Clean turbo column, perform separation on analytical column	45	0:0:100	2.0	5:95	0.85
Clean turbo column, perform separation on analytical column	30	100:0:0	2.0	5:95	0.85
Load transfer loop and clean analytical columns	60	0:100:0	4.0	0:100	0.85
Equilibrate turbo and analytical columns	45	60:40:0	4.0	60:40	0.80

Mobile phase A, water; mobile phase B, methanol; mobile phase C, organic cocktail containing 70% acetonitrile, 20% isopropanol, and 10% acetone.

concentrations of patient samples in the sequence mid-high-low-mid-mid-low-low-high-high-mid run twice a day for 5 days using CLSI EP10-A3 guidelines (Wayne, PA, USA). Carryover was determined by analysis of three extractions of the sequence (low₁-high-low₂) where low₂ was a re-injection of the low₁. A passing test meant low₁ and low₂ differed by <20% and the low₂ was within 3 SDs of the low₁ value. The SD was determined using low₁ values. High values above the linearity of the assay were diluted to within the linear range and the value was back calculated. Comparisons using Deming regression, Passing-Bablok regression, and analysis for diagnostic agreement were performed between the new LC-MSMS assay and the RIA method. Statistics were calculated using Excel (Microsoft, Redmond, WA, USA), EP Evaluator Release 8 (David G. Rhoads Associates, Kennett Square, PA, USA), and Analyze-it® (Analyze-it Software, Ltd, Leeds, UK).

Ethical approval

Utilizing leftover de-identified clinical samples did not require institutional Review Board review.

Results and discussion

Chromatography

During the chromatography optimization, we found that there was a narrow range of mobile phase compositions for efficiently retaining the 25OHD analytes on the turbo column for purification. We found that 60% water with 40% methanol retained the analytes on the turbo column best, without causing peak broadening. Acid is often added to mobile phases for LC-MSMS because it improves ionization (41). Thus, formic acid was tested for ionization improvement by infusing the analytes and d₆-25OHD3 in the presence of mobile phase with and without 0.1% formic acid. There was no significant difference between the two conditions. The chromatographic run time was 5.5 min/injection in a single channel. When two LC channels were used at the same time, the average run time in a batch was reduced to 3.0 min/injection.

The retention time (mean ± SD) for 25OHD3 and d₆-25OHD3 was 1.01 ± 0.1 min (n=571) and for 25OHD2 was 1.04 ± 0.1 min (n=432) in serum samples (Figure 1). There was a significant peak in the 25OHD2 channel at 0.9 min (Figure 1A). A full spectra scan was performed (data not shown) and it was found to have a significantly different fragmentation spectrum compared with 25OHD2.

Interferences

Interferences are less common with LC-MSMS methods due to the inherent specificity of MSMS. However, a compound with the same molecular weight and ionization characteristics can possibly interfere. Interference studies were conducted by obtaining pure compound and spiking these into ethanolic standards with known 25OHD concentrations. This was injected into the system to see if it showed up on the chromatogram with a close retention time to the analytes. Due to previously published data, 1αOHD3 was considered a possible interferent for 25OHD3 and therefore was tested. The retention time of this compound was 1.17 min compared to 1.01 min for 25OHD3 with baseline separation (Figure 1B). In addition, a 250 nmol/L solution of 1αOHD3 had a peak height similar to a 12.5 nmol/L peak of 25OHD3 meaning a much lower ionization efficiency of 1αOHD3 under the current conditions compared with 25OHD3. The 3-epi 25OHD is a known interferent for patients under the age of 1 year (29). At the time of method validation, a commercial 3-epi 25OHD was not available for interference testing. Therefore, caution should be used for interpreting results in this age group. Other possible interferents are phospholipids found naturally in human blood. These compounds have similar characteristics as 25OHD2 and 25OHD3. Lyso-phosphatidylcholine C16:0 and C18:0 were monitored during development and showed no interference with either 25OHD3 or 25OHD2 (data not shown). Also, due to their relatively high molecular weight (500–1000) compared to 25OHD, they were not

Table 2 Linearity and recovery.

Analyte	Add-in, nmol/L	Expected, nmol/L	Mean, nmol/L	Analytical recovery, %	%CV
25OHD2	2.36	2.39	2.98	125.0	29.2
	4.73	4.78	4.60	96.2	4.3
	7.10	7.18	7.20	100.3	7.5
	9.46	9.57	9.45	98.8	6.9
	18.93	19.13	18.78	98.1	5.3
	37.86	38.26	36.66	95.8	5.0
	75.72	76.53	72.57	94.8	4.8
	151.44	153.06	147.15	96.1	3.0
	302.88	306.12	277.92	90.7	1.8
25OHD3	2.44	2.49	2.95	118.7	14.5
	4.87	4.98	5.42	109.0	10.2
	7.31	7.47	7.81	104.7	3.1
	9.75	9.96	10.43	104.9	2.3
	19.50	19.92	18.89	94.7	3.9
	39.00	39.84	37.94	95.1	1.4
	78.00	79.68	72.63	91.0	1.1
	156.00	159.37	144.82	90.7	2.3
	312.00	318.74	283.55	88.7	2.3

Table 3 Precision data according to CLSI EP10-A3 protocol.

	25OHD3			25OHD2		
	Low	Mid	High	Low	Mid	High
Mean, nmol/L	33.4	61.0	120.5	18.5	70.6	139.9
Total SD	3.0	4.6	11.4	3.1	7.8	15.6
Intra-assay SD	1.6	2.3	7.4	1.7	3.2	9.0
Inter-assay %CV	7.7	6.6	7.1	14.2	10.0	9.2
Intra-assay %CV	4.9	3.8	6.1	9.3	4.6	6.4

Table 4 Method comparison results by Deming regression.

Comparison method	Analyte	n	Slope (95% CI)	Intercept, nmol/L (95% CI)	Standard error of estimate, nmol/L	r	% difference of mean
RIA	25OHD	527	0.867 (0.825–0.909)	12.8 (9.5–16.1)	17.4	0.8425	5.2
RIA	25OHD3 ^a	216	0.865 (0.802–0.927)	9.0 (4.9–13.2)	14.0	0.8595	1.7

^a25OHD2 is below LLOQ.

viewed as a major interferent (42). Ion suppression was examined by monitoring total ion current with 20 patient samples individually injected through a T-connection with a post-column line prior to the mass spectrometer with a d₆-25OHD3 and d₆-25OHD2 ethanol solution infused at 5 μL/min. No ion suppression was observed.

Assay validation

Assay calibration for 25OHD3 and 25OHD2 was performed with 25OHD2 and 25OHD3 at five concentrations prepared by serial dilution in absolute ethanol and stored at –70°C until use. Quantitation was achieved based on the peak area ratios of analytes to the internal standard. The d₆-25OHD3 was used as the internal standard for both 25OHD3 and 25OHD2 due to the fact that d₆-25OHD2, while recently available, has a molecular weight of 418.2. With the loss of a water molecule from d₆-25OHD2 parent ion, a fragment with 401.2 m/z was formed interfering with 25OHD3 quantification. The limit of detection was

1.7 nmol/L and 1.2 nmol/L for 25OHD3 and 25OHD2, respectively, determined using 20 replicates of saline. From serial dilutions, linearity was determined to be 3.0–283.6 nmol/L for 25OHD3 and 4.6–277.9 nmol/L for 25OHD2, with an accuracy of 88.7%–118.7% and 90.7%–100.3%, respectively. The LLOQ was 3.0 nmol/L for 25OHD3 and 4.6 nmol/L for 25OHD2, respectively based on the precision and accuracy data in the linearity study (Table 2). The intra-assay and inter-assay CVs (3.8–14.2, Table 3) were determined using patient samples at three concentrations based on CLSI EP10-A3 guidelines (43). No significant carryover was observed by testing the spiked patient samples with low₁ (mean: 10.2 nmol/L)–high (713.3 nmol/L)–low₂ (10.2 nmol/L) for 25OHD2 and low₁ (38.9 nmol/L)–high (577.1 nmol/L)–low₂ (41.2 nmol/L) for 25OHD3.

Method comparison

We compared the newly developed method with the DiaSorin RIA using left-over patient samples. The distribution of vitamin D concentrations was near a nor-

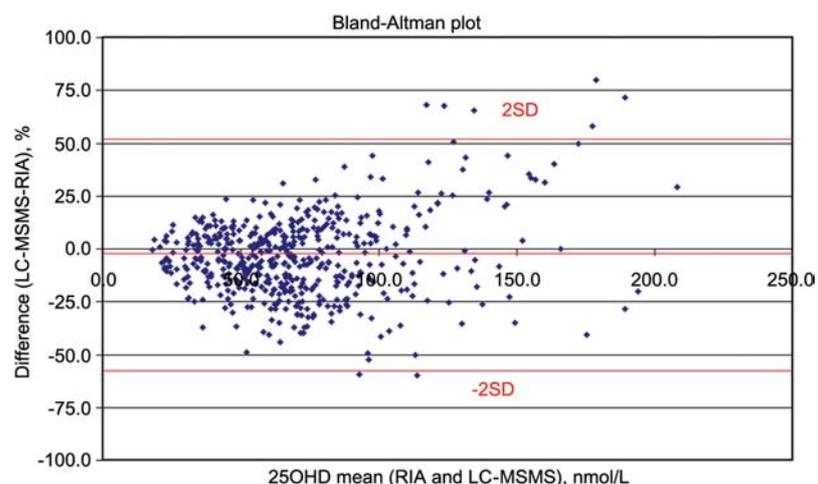


Figure 2 Bland-Altman plot. The mean of percent differences was -7.6% with a standard deviation of 26.2% .

mal distribution based upon visual inspection of the histogram of patient data (data not shown). Table 4 contains the comparison data using Deming regression between our LC-MSMS and the DiaSorin RIA method ($n=527$). The regressions had a slope 0.87 , $r>0.84$, and mean difference within 5.2% using all samples ($n=527$) or samples ($n=216$) with 25OHD_2 concentrations below the LLOQ, indicating equivalent recoveries of both 25OHD_2 and 25OHD_3 for the RIA method. To compare our results with a previously published comparison conducted between a LC-MSMS method and several immunoassays, non-parametric Passing-Bablok regression ($n=527$) was performed. The regression showed a slope of 0.96 (95% CI: $0.90-1.02$) and an intercept of 6.64 nmol/L ($3.74-10.14$) which compared favorably to the slope of 0.64 (reverse relationship) between IDS-RIA (IDS Ltd., Tyne and Wear, UK) and LC-MSMS (24). A Bland-Altman plot did not reveal concentration-dependent bias between LC-MSMS and the RIA (Figure 2), as opposed to a previous observation between IDS-RIA and LC-MSMS (24). We calculated diagnostic agreement between LC-MSMS and RIA using ≥ 75 nmol/L as sufficient vitamin D status and found this to be 80.9% . For $0-50$, $51-74$ and ≥ 75.0 nmol/L cut-offs, agreement was 68.5% based on the deficient, insufficient, and sufficient levels commonly described in the literature (10). Under the 2nd diagnostic agreement breakdowns, LC-MSMS overestimated values 22.9% of the time and underestimated 8.6% , compared to RIA. Multivariate regression was performed using all patient data ($n=527$) to determine the contribution of 25OHD_3 and 25OHD_2 by our LC-MSMS to the total by RIA. The multivariate equation with 95% CI in parentheses was $\text{RIA} = 0.93(0.89-0.97) \times 25\text{OHD}_3 + 1.04(0.98-1.10) \times 25\text{OHD}_2$, $R^2 = 0.90$. This equation along with the correlation data demonstrated that the RIA method and LC-MSMS were comparable in determining 25OHD concentrations in patient samples. Also, the difference between the two methods was much smaller than the difference observed between another LC-MSMS and the IDS RIA in a previous report (24). Therefore, our LC-MSMS method

may be used in the clinical evaluation of serum 25OHD without significant overall deviation (mean difference = 5.2%) from the RIA method, which has been used in many clinical trials. It is important to have comparable results obtained using different methodologies in clinical laboratories to avoid complication of clinical interpretation (44).

In conclusion, we developed and validated a novel LC-MSMS method with a turbulent flow online extraction technology to quantitate serum 25OHD_2 and 25OHD_3 . The method is rapid and requires little manual sample preparation. Our comparison study showed that the LC-MSMS provided comparable results with the DiaSorin RIA with a mean difference of only 5.2% .

Authors' disclosures of potential conflicts of interest

No authors declared any potential conflicts of interest. The funding organizations played no role in the design of the study, review and interpretation of data, or preparation or approval of the manuscript.

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