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Simultaneous measurement of plasma vitamin D_3 metabolites including 4β,25-dihydroxyvitamin D_3 using liquid chromatography-tandem mass spectrometry

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Abstract

Simultaneous and accurate measurement of circulating vitamin D metabolites is critical to studies of the metabolic regulation of vitamin D and its impact on health and disease. To that end, we developed a specific LC-MS/MS method that permits the quantification of major circulating vitamin D_3 metabolites in human plasma. Plasma samples were subjected to a protein precipitation, liquid-liquid extraction and Diels-Alder derivatization procedure prior to LC-MS/MS analysis. Importantly, in all human plasma samples tested, we identified a significant dihydroxyvitamin D_3 peak that could potentially interfere with the determination of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] concentrations. This interfering metabolite has been identified as 4β,25-dihydroxyvitamin D_3 [4β,25$(OH)_2D_3$] and was found at concentrations comparable to 1α,25$(OH)_2D_3$. Quantification of 1α,25$(OH)_2D_3$ in plasma required complete chromatographic separation of 1α,25$(OH)_2D_3$ from 4β,25$(OH)_2D_3$. An assay incorporating this feature was used to simultaneously determine the plasma concentrations of 25OHD_3, 24R,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$, and 4β,25$(OH)_2D_3$ in healthy individuals. The LC-MS/MS method developed and described here, could result in considerable improvement in the quantification of 1α,25$(OH)_2D_3$, as well as monitoring the newly identified circulating metabolite, 4β,25$(OH)_2D_3$.

Keywords

Vitamin D_3; 25-hydroxyvitamin D_3; 24R,25-dihydroxyvitamin D_3; 1α,25-dihydroxyvitamin D_3; 4β,25-dihydroxyvitamin D_3; Plasma; LC-MS/MS

Vitamin D is critical for the regulation of calcium and phosphate homeostasis and has been implicated in a number of other important biological processes including immune function [1-3]. Insufficiency or deficiency of vitamin D is a risk factor for metabolic bone diseases such as rickets, osteoporosis and osteomalacia [2, 4]. Vitamin D exists naturally in two forms, namely vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). The major

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source of vitamin D in humans is the photoconversion of 7-dehydrocholesterol to pre-vitamin D_3 in the epidermis, which isomerizes to vitamin D_3 [5, 6]. Vitamin D_3 undergoes 25-hydroxylation by 25-hydroxylase enzymes in the liver to produce the most abundant circulating form, 25-hydroxyvitamin D_3 ($25OHD_3$) [7]. $25OHD_3$ is typically used as a biomarker for vitamin D status, and a plasma level less than 20 ng/mL has been reported to be indicative of vitamin D deficiency [5]. Hydroxylation at the C-24 position is generally considered to be the main deactivating pathway for vitamin D [8]. One of the products, 24R, 25-dihydroxyvitamin D_3 [24R,$25(OH)_2D_3$], is the major circulating dihydroxyvitamin D_3 metabolite (Figure 1). Alternatively, $25OHD_3$ can be converted into the most biologically active form, 1α,25-dihydroxyvitamin D_3 [1α,$25(OH)_2D_3$], a reaction occurring predominantly in the kidney. The circulating plasma level of 1α,$25(OH)_2D_3$ is approximately 1/500 to 1/1000 lower than that of $25OHD_3$ [9, 10]. It has been proposed that the plasma concentration of 1α,$25(OH)_2D_3$ relative to that of $25OHD_3$ or 24R,$25(OH)_2D_3$ may give a better indication of vitamin D_3 status [11]. However, due to its very low circulating concentrations (picomolar), methodological difficulties with the accurate measurement of 1α,$25(OH)_2D_3$ are considerable [12]. Moreover, the inter-laboratory variability in 1α,$25(OH)_2D_3$ measurement makes direct comparisons difficult. Thus, improvements in the accuracy and reliability of vitamin D metabolite measurement, especially 1α,$25(OH)_2D_3$ in plasma/serum, would be very valuable to translational research and clinical practice.

Quantification of vitamin D metabolites in plasma/serum is a challenging task because the molecules are very lipophilic and bind tightly to vitamin D binding protein; moreover, some metabolites are found at extremely low levels [13]. A number of methodologies have been successfully developed for the quantification of vitamin D metabolites in plasma/serum [14]. These typically involve measurements by immunoassays [15-17], protein binding assays [18-21], HPLC-UV [22, 23] and LC-MS [24, 25]. Hospitals and clinical laboratories have relied on immunoassays because of their rapidity to execute. However, those methods require extensive sample preparation to remove cross-reactive interferences [15, 21, 22, 26]. HPLC-UV and LC-MS are suitable for $25OHD_3$ measurement; however, due to detection limits they can not quantitate metabolites found at very low concentrations. Accordingly, high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) methods have been developed to measure nonderivatized $25OHD_3$ [27-31] and 1α, $25(OH)_2D_3$ [32, 33], which provide much higher sensitivity and specificity [34]. Additionally, it was found that derivatization by Cookson-type triazolinediones and related reagents can enhance ionization efficiencies of vitamin D metabolites and thereby improve sensitivity [35-37]. Therefore, ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) with derivatization prior to MS analysis has been touted as more favorable analytical methodology [38-40]. These techniques allow for simultaneous quantification of concentrations for multiple endogenous vitamin D metabolites, including 1α,$25(OH)_2D_3$.

In the present study, we have also developed a sensitive LC-MS/MS method, but in the process identified a significant peak which interferes with quantifying 1α,$25(OH)_2D_3$ in human plasma extracts. Results being published elsewhere [41] show that the interfering compound is also a dihydroxyvitamin D_3 metabolite with two vicinal hydroxyl groups on the A-ring, but hydroxylated at the 4β position, rather than the 1α position (Figure 1). Initially, the presence of this metabolite proved problematic since it co-eluted with 1α, $25(OH)_2D_3$ during chromatography. Upon further effort, we successfully developed a sensitive and specific LC-MS/MS method to simultaneously quantify $25OHD_3$, 24R, $25(OH)_2D_3$, 1α,$25(OH)_2D_3$ and this interfering metabolite [4β,$25(OH)_2D_3$]. The assay described here could result in considerable improvement in the quantification of 1α, $25(OH)_2D_3$.

Materials and Methods

Chemicals

Ethylenediaminetetraacetic acid (EDTA), human serum albumin (HSA), $25OHD_3$, 24R, $25(OH)_2D_3$, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and sodium periodate were purchased from Sigma-Aldrich. $1\alpha,25(OH)_2D_3$ was purchased from Calbiochem (San Diego, CA). Deuterated standards of vitamin D_3 metabolites, d6-$25OHD_3$ and d6-$1\alpha,25(OH)_2D_3$ (containing six deuterium atoms at C-26 and C-27), were purchased from Medical Isotope Inc. (Pelham, NH). HPLC-grade solvents acetonitrile, methanol, ethyl acetate, formic acid were obtained from Fisher Chemicals. Deionized water was generated in-house for mobile phase preparation.

Standards and plasma samples

All standards were prepared as 50 μg/mL primary stock solutions in methanol and stored in amber vials. Quality control (QC) samples were made by serial dilutions from the stock solutions. Six different levels of calibration standards were prepared by serial 2X dilutions starting from the calibration standard mixture at the highest concentration [$1\alpha,25(OH)_2D_3$, $24R,25(OH)_2D_3$, $25OHD_3$; 0.8, 16, 80 ng/mL respectively]. A HAS solution (30 mg/mL) in phosphate-buffered saline was used as the blank matrix for generating calibration curves. A working internal standard solution was prepared containing 0.1 μg/mL d6-$1\alpha,25(OH)_2D_3$ and 0.5 μg/mL d6-$25OHD_3$. All standards were stored at -80 °C.

Outdated human plasma from the local Blood Bank was used for assay development and validation. We estimated the variability in plasma vitamin D metabolite concentrations in a group of 25 healthy, ethnically diverse adults. The University of Washington Institutional Review Board approved this study and all subjects gave written informed consent. We enrolled 12 females and 13 males between 20 and 40 years of age and their self-reported ethnicities were Blacks (n = 4), Asian (n = 5) and Caucasian (n = 16). Blood was collected in vacutainer tubes containing EDTA from subjects following an overnight fast and all plasma samples were stored at -80° C until analysis.

Sample preparation from plasma

Because vitamin D metabolites are light sensitive, the extraction procedure was conducted under low light to avoid unpredictable degradation. Different methods and conditions for sample preparation were evaluated and are further described in the “Results and Discussion” section. The optimized procedure is described below and was performed for all subsequent analyses.

After thawing at room temperature, human plasma (1 mL) was transferred to a 15 mL polypropylene tube (Sarstedt, Newton, NC), spiked with 10 μL of working internal standard solution, vortex-mixed, and allowed to equilibrate for 30 min at room temperature in the dark. Proteins were precipitated by adding 2 mL of acetonitrile, vortex-mixed, followed by centrifugation for 10 min at 2360 *g* (Jouan CR422, Rockville, MD). The supernatant was transferred to a glass tube, and the volume was reduced under a nitrogen stream. The remaining solution (~ 1 mL) was subjected to liquid-liquid extraction (LLE) by adding 5 mL ethyl acetate. After shaking vigorously for 10 min, samples were centrifuged for 20 min at 590 *g* (Jouan CR422), and the upper organic layer was transferred into a new glass tube. After complete evaporation of solvent under a nitrogen stream, the derivatization reagent, PTAD (150 μL, 1 mg/mL) in acetonitrile, was added to the residue, vortex-mixed, and left at room temperature for 1 hour to complete the reaction. The sample was then transferred to another glass tube, evaporated under a nitrogen stream and reconstituted in 40% acetonitrile (40 μL). The sample was transferred to an amber glass vial with a glass insert for analysis.

Chromatography and mass spectrometry

Chromatographic separation was performed using a Hypersil Gold (2.1 × 100 mm, 1.9 μm) column (Thermo Scientific) at 40 °C on an Agilent 1200 LC system using acetonitrile (B)/water (A, 0.1% formic acid) as a mobile phase. Starting gradient conditions were 40% B with 0.2 mL/min flow rate. The following gradient program was used: 0 to 3 min 40% B, 9 to 10.5 min 60% B, 11 to 14 min 90% B, 18 to 25 min 40% B. Under these chromatographic conditions, the interfering peak 4β,25$(OH)_2D_3$ was separated from 1α,25$(OH)_2D_3$. Samples were kept at 4 °C, and the injection volume was 5 μL. Mass spectrum (MS) analysis was carried out using a positive mode electrospray ionization method on an Agilent 6410 triple quadrupole tandem mass spectrometer. The ionization and fragmentation parameters were set as follows: capillary voltage, 5000 V; gas temperature, 300 °C; gas flow rate, 11 L/min; nebulizer: 35 psi; fragmentor: 140 V; collision energy: 14 V. Multiple Reaction Monitoring (MRM) channels of *m/z* 574 → 298, 574 → 314, 558 → 298, 580 → 314 and 564 → 298 were set to detect 24R,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$, 25OHD_3, d6-1α,25$(OH)_2D_3$ and d6-25OHD_3, respectively. The interfering compound, 4β,25$(OH)_2D_3$, appeared in the same MRM channel as 1α,25$(OH)_2D_3$ but with a different retention time (RT).

Quantification and data analysis

Data acquisition was performed using the Agilent MassHunter Workstation software. Calibration curves were constructed by plotting the peak area ratio for each vitamin D_3 metabolite and its internal standard, versus the corresponding concentration, and fitting a linear regression equation to the data. Calibration statistics are shown in Table 2. Deuterated 1α,25$(OH)_2D_3$ was used as an internal standard for the quantification of 1α,25$(OH)_2D_3$, 4β, 25$(OH)_2D_3$ and 24R,25$(OH)_2D_3$. 4β,25$(OH)_2D_3$ was quantified using 1α,25$(OH)_2D_3$ for the standard curve because 4β,25$(OH)_2D_3$ is not commercially available. Data analysis was performed using MassHunter Qualitative Analysis software (Agilent) and Microsoft Excel 2003.

Stability and recovery

Previous studies have shown that vitamin D_3 metabolites are stable unless exposed to intense light [40]. Consistent with those reports, we found metabolites were stable (residues ≥ 95%) at 4 °C (1 week) or -20 °C (2 months) and for 5 freeze-thaw cycles. The derivatized products of these metabolites were also stable in a cooled autosampler (4 °C) for 3 days. To determine recovery rates, we prepared samples in blank matrix (30 mg/mL HSA solution) at two concentrations [1α,25$(OH)_2D_3$: 50 and 200 pg/mL; 24R,25$(OH)_2D_3$: 1.0 and 4.0 ng/mL; 25OHD_3: 2.5 and 10 ng/mL] and extracted according to the LLE procedure. In control experiments, solid phase extraction (SPE) was performed using Oasis HLB 1 cc (30 mg) cartridges. Cartridges were preconditioned by sequential washing with acetonitrile and water. After loading the samples, we washed the columns with 30% acetonitrile and eluted with 100% acetonitrile. The recovery of analytes was determined by comparison of LC-MS/MS signals against those generated for the corresponding standard solutions measured without the extraction process.

Linearity and detection limits

Linearity of each calibration curve was determined by spiking nine concentrations of standards in blank matrix [1α,25$(OH)_2D_3$: 0.0125 to 2 ng/mL; 24R,25$(OH)_2D_3$: 0.5 to 50 ng/mL; 25OHD_3: 1.25 to 200 ng/mL]. Calibration curves were plotted as the peak area ratio of each vitamin D_3 metabolite and its surrogate internal standard versus the corresponding concentration using linear regression. The limit of detection (LOD) was defined as a signal-to-noise ratio (S/N ratio) of 3:1, and limit of quantification (LOQ) as a S/N ratio of 10:1. Instrumental LOD were estimated as the lowest calculated injected amount on the column

that produced a S/N ratio ≥ 3 after injecting a series of dilutions (5 to 160 pg/mL in 1 mL blank matrix). LOQ was determined by three replicate injections from serial dilutions of QC samples, and estimated as the lowest concentration which produced a mean S/N ratio ≥ 10.

Precision and accuracy

The intra-assay and inter-assay precision and accuracy were evaluated using QC samples at three nominal concentrations [1α,25$(OH)_2D_3$: 50, 100 and 200 pg/mL; 24R,25$(OH)_2D_3$: 0.5, 1.0 and 2.0 ng/mL; 25OHD_3: 10, 20 and 40 ng/mL]. The QC samples were spiked into blank matrix for analysis and the endogenous concentrations of vitamin D_3 metabolites in native plasma were measured in parallel. Intra-assay precision and accuracy were determined by measuring metabolite concentrations in the QCs from six replicates on the same day. Inter-assay precision and accuracy were evaluated based on the replicate measurements of the QCs made six times over a ten day period.

Addition of standards in plasma

Human plasma (1 mL) was spiked with 10 μL of a standard mixture of 1α,25$(OH)_2D_3$, 24R, 25$(OH)_2D_3$ and 25OHD_3 in methanol (initial concentrations given in parentheses): set 1 [2.5, 50 and 125 ng/mL, respectively], set 2 [5.0, 100 and 250 ng/mL, respectively] and set 3 [10, 200 and 500 ng/mL, respectively. After equilibrating for 30 min in the dark, samples were extracted and measured as described above.

Periodate cleavage of plasma extracts

Since the interfering peak contained a characteristic *m/z* 314 fragment, we used periodate cleavage to identify the regiochemistry of the two hydroxyl groups on the A-ring. Briefly, vitamin D_3 metabolites were extracted from human plasma as described above. After PTAD derivatization, samples were evaporated and reconstituted in 300 μL methanol. Standard solutions of 1α,25$(OH)_2D_3$ (1 ng), 24R,25$(OH)_2D_3$ (1 ng) were used as controls in a parallel experiment. An aqueous solution of 5% sodium periodate ($NaIO_4$, 30 μL) was added to the plasma extracts and water was added as a negative control in parallel plasma extracts. After incubation for 1 hour, 0.5 mL water was added to mixtures and the derivatized metabolites were extracted using 2 mL ethyl acetate. The extracts were then transferred to a glass tube, evaporated and reconstituted in 40% acetonitrile (40 μL) for LC-MS/MS analysis.

Results and Discussion

Optimization of extraction

Aronov et al. proposed a general strategy for the quantification of vitamin D metabolites from plasma/serum [38]. Following their approach, we subjected samples to a three-step protein precipitation, extraction (LLE/SPE) and Diels-Alder derivatization procedure, prior to LC-MS/MS analysis. We validated and modified each step when necessary, yielding an optimized procedure that is described in “Materials and Method”. Due to its similarity to previously published procedures [38, 39], we briefly highlight our optimized extraction conditions as a complement to the published work. *1) Protein precipitation*. Because vitamin D_3 metabolites are highly lipophilic, protein precipitation prior to extraction was necessary. We tested organic solvents such as acetonitrile, acetone and acetonitrile/methanol mixtures at various volume ratios. The addition of two volumes of acetonitrile was found to be efficient for protein precipitation. In addition, partial evaporation of acetonitrile prior to LLE increased the extraction efficiency. *2) Liquid-liquid extraction*. Since both LLE and SPE have been used previously for the extraction step [38-40], we compared the efficiency of each by spiking known amounts of standards into blank matrices to evaluate the recovery rates. LLE resulted in the recovery of 84 ± 2% of 1α,25$(OH)_2D_3$, 85 ± 4% of 24R,

$25(OH)_2D_3$ and 73 ± 2% of $25OHD_3$ which was comparable to previously reported results. However, we observed lower recoveries (50% to 70%) following SPE, especially for the dihydroxyvitamin D_3 metabolites. In addition, SPE had slightly greater variability in a sample-to-sample comparison. Thus, we opted to use LLE rather than SPE for the isolation of metabolites, despite the longer processing time.

Optimization of LC-MS/MS conditions

The ionization and fragmentation parameters were optimized by the MassHunter software optimizer (Agilent), using injections of derivatized standards. Consistent with previous findings [38], the parent ions at *m/z* 592 [1α,$25(OH)_2D_3$, 24R,$25(OH)_2D_3$] and *m/z* 576 ($25OHD_3$) lost one molecule of water at the C-25 position, generating the major ions at *m/z* 574 and 558, respectively. The collision-induced dissociation (CID) spectra of derivatized 1α,$25(OH)_2D_3$, 24R,$25(OH)_2D_3$ and $25OHD_3$ were acquired from the precursor ion $[M\text{-}H_2O+H]^+$. As shown in Figure 2, the predominant product ions (m/z 314 and 298) represent moieties that include the PTAD-attached A-ring. Since this product ion was detected at high sensitivity and selectivity, we used selected reaction monitoring (SRM) for each metabolite, as listed in Table 1. Moreover, the presence of the characteristic *m/z* 314 fragment was used as an indicator of hydroxylation on the A-ring.

Derivatization with PTAD produced two epimers, 6S and 6R; as a result, two peaks may be expected for each metabolite in the ion chromatograms. As shown in Figure 3, both 24R, $25(OH)_2D_3$ and $25OHD_3$ contained two peaks, whereas 1α,$25(OH)_2D_3$ had only one peak (presumably co-eluting epimers) during chromatography. When two peaks appeared, both major and minor peaks were integrated and combined to minimize the calculation errors, although previous results showed that integration of the major peak was sufficient for quantification [38, 39].

Interestingly, a peak interfering with 1α,$25(OH)_2D_3$ quantification was present in plasma extracts prepared by both LLE or SPE (Figure 4) and could not be suppressed by adjusting instrumental parameters. Because the interfering peak had the same MRM characteristics to 1α,$25(OH)_2D_3$, we speculated that it could also be a dihydroxyvitamin D_3 metabolite, with mono-hydroxylation of the A-ring of vitamin D_3. The interfering peak co-eluted with 1α, $25(OH)_2D_3$ if the mobile phase gradient contained a high percentage of organic solvent, i.e., greater than 70% acetonitrile or 90% methanol. We tested different columns and mobile phase conditions to separate 1α,$25(OH)_2D_3$ from the interfering peak. Initially, methanol was used as an organic solvent to give higher signal intensity; however, we substituted it with acetonitrile due to the higher backpressure. Using optimized LC conditions, the interfering peak eluted ~0.3 min earlier than 1α,$25(OH)_2D_3$ (Figure 4), which was acceptable for quantification of 1α,$25(OH)_2D_3$.

Method validation

For each metabolite, we plotted the calibration curve as the peak area ratios of each metabolite and its surrogate internal standard versus the corresponding concentrations. Since a deuterated standard was not available for 24R,$25(OH)_2D_3$, we used d6-1α,$25(OH)_2D_3$ as its internal standard. The interfering metabolite was semi-quantified using the 1α, $25(OH)_2D_3$ calibration curve. We obtained the linear range and correlation coefficients using linear regression analysis from the set of six data points in the calibration curves (Table 2). We obtained excellent linearity over the calibration range, and the R^2 values for 1α,$25(OH)_2D_3$, 24R,$25(OH)_2D_3$ and $25OHD_3$ were 0.999, 0.999 and 0.996, respectively. As shown in Table 2, these metabolites had relatively similar instrumental LOD and LOQ, possibly because of the similarities in structures and ion fragmentation.

We estimated the accuracy and precision of the assay using QC samples at three concentration levels and a plasma sample for which the endogenous levels of the analytes were measured (Table 3). The accuracies were calculated by the equation: mean of determined concentration/nominal concentration. As shown in Table 3, good accuracies (bias < 10%) were observed for all analytes at all three concentrations. The coefficient of variance (% CV), which is used for evaluating precision, ranged from 1.1% to 12.4% (intra-assay) and 1.0% to 13.8% (inter-assay). We also determined the accuracy and precision by adding known amounts of vitamin D_3 metabolites to a human plasma sample (Table 4). Because vitamin D_3 metabolites are endogenous compounds, the addition of known amounts of metabolites would increase their total plasma levels. As summarized in Table 4, increases were observed in proportion to the amounts added to plasma. The precisions (% CV) of all analytes were < 10% and the accuracies were in the range of 96% to 106%. These data further confirmed that the method was reliable and reproducible for measurement of all targeted analytes in plasma.

Periodate cleavage of plasma extracts

Since the interfering metabolite underwent Diels-Alder derivatization with PTAD and had the same characteristic *m/z* 314 fragment as 1α,25$(OH)_2D_3$, we hypothesized that it was a vitamin D_3 metabolite with two hydroxyl groups on the A-ring. A similar observation was reported previously [38], where the authors suggested an endogenous metabolite from epimerization of 1α,25$(OH)_2D_3$, 1α,25-dihydroxy-3-epi-vitamin D_3 [1α,25$(OH)_2$-3-epi-D_3] as a possible candidate. However, 1α,25$(OH)_2$-3-epi-D_3 (a gift from Dr. T. Fujishima, Tokushima Bunri University, Japan) did not co-elute with the interfering peak with RT at 12.35 min (Figure 5A). In another experiment, we treated derivatized plasma extracts with sodium periodate, which selectively cleaves C-C bonds with vicinal hydroxyl (or vicinal hydroxyl-ketone) groups. As shown in Figure 5B, upon periodate treatment, the interfering peak disappeared completely, indicating that the putative metabolite contains two vicinal hydroxyl groups. As expected in the control experiments, the 24R,25$(OH)_2D_3$ peak was diminished ~95%, but there was no significant change for 1α,25$(OH)_2D_3$,. Thus, we speculated this putative vitamin D_3 metabolite might be 2,25$(OH)_2D_3$ or 4,25$(OH)_2D_3$ [42-44]. We performed further experiments for structure elucidation, and the accumulated evidence suggest that it is 4β,25$(OH)_2D_3$, and is a metabolite of 25OHD_3 produced by cytochrome P450 3A4 (CYP3A4). These findings have been submitted for publication elsewhere [41].

Population levels of vitamin D_3 metabolites

Using the newly developed method, we measured plasma concentrations of vitamin D_3 metabolites in 25 healthy individuals uniformly recruited from the Seattle, Washington area throughout year (Figure 6). In these healthy subjects, the average (range) plasma concentrations of 25OHD_3, 24R,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$ and 4β,25$(OH)_2D_3$ were 25.6 ng/mL (6.9 to 52.6 ng/mL), 2.28 ng/mL (0.13 to 6.19 ng/mL), 61 pg/mL (17 to 101 pg/mL) and 40 pg/mL (2 to 128 pg/mL), respectively. These values are consistent with quantitative data reported previously [38, 40]. Interestingly, both the level of 4β,25$(OH)_2D_3$ (R^2 = 0.727) and 24R,25$(OH)_2D_3$ (R^2 = 0. 789) correlated well with the level of 25OHD_3. In contrast, the level of 1α,25$(OH)_2D_3$ (R^2 = 0.455) was only weekly correlated with the level of 25OHD_3.

Conclusions

The accurate measurement of circulating vitamin D metabolites is critical to studies of the metabolic regulation of vitamin D and its impact on health and disease. In this report, we describe a sensitive and more specific method that permits the quantification of major circulating vitamin D_3 metabolites in human plasma. Importantly, we found that another

dihydroxylvitamin D_3 metabolite, 4β,25$(OH)_2D_3$, circulates in human plasma at concentrations that could interfere with 1α,25$(OH)_2D_3$ measurements. Consistent with our findings, an immunoaffinity extraction method has been developed recently to remove interfering substances in the 1α,25$(OH)_2D_3$ quantification prior to LC-MS/MS analysis [45]. In the present study, a complete chromatographic separation of 1α,25$(OH)_2D_3$ from 4β, 25$(OH)_2D_3$ was critical for the quantification of the individual metabolites. The LOQ for 1α, 25$(OH)_2D_3$ was 25 pg/mL and in some "healthy" subjects, the estimated concentrations of both 1α,25$(OH)_2D_3$ and 4β,25$(OH)_2D_3$ metabolites were below 25 pg/mL. Thus, for clinical practice, sample enrichment might be required prior to LC-MS/MS analysis to capture accurate concentrations in individuals with apparent vitamin D deficiency, when reporting that the analyte was found at less than the lower detection limit is insufficient.

In general, higher chromatographic resolution and sensitivity with reduction in analysis run time can be achieved with UPLC-MS/MS allowing greater throughput of samples [46]. Recently, two groups reported UPLC-MS/MS methods for the quantification of 1α, 25$(OH)_2D_3$ in plasma, both of which had a run time one-half that of our newly developed method [38, 39]. However, it appears that 1α,25$(OH)_2D_3$ was not fully separated chromatographically from 4β,25$(OH)_2D_3$ under those LC conditions, possibly due to the higher percentage of organic solvent in their mobile phases. Indeed, using lower organic composition in the mobile phase and a longer UPLC column, an interfering peak of 1α, 25$(OH)_2D_3$ was observed [38]. In addition to these two UPLC-based methods, a microflow LC-MS/MS method was used for the quantification of 1α,25$(OH)_2D_3$ [39]. Again, a single peak was observed using the same MRM channel (*m/z* 574 → 314), indicating that 4β, 25$(OH)_2D_3$ might be co-eluting with 1α,25$(OH)_2D_3$. Thus, 1α,25$(OH)_2D_3$ concentrations might be over-estimated by incorporation of 4β,25$(OH)_2D_3$ into the 1α,25$(OH)_2D_3$ quantitative signal under certain LC-MS-based conditions.

We noted marked inter-individual variability in the concentrations of all of the vitamin D_3 metabolites measured, including the newly identified 4β,25$(OH)_2D_3$ molecule. As indicated by the strong correlation coefficients, much of the variability in 4β,25$(OH)_2D_3$ and 24R, 25$(OH)_2D_3$ concentration could be attributed to differences in the plasma 25OHD_3 precursor. However, as the metabolite/parent ratios also varied substantially (not shown), significant differences in either the formation or elimination of the 4β,25$(OH)_2D_3$ and 24R, 25$(OH)_2D_3$ metabolites may be present in vivo. We also observed ethnic and gender differences in the metabolite concentrations or metabolite/parent ratios. Future work is warranted to confirm and explore the biological basis for these differences. Finally, there was more limited variability in plasma 1α,25$(OH)_2D_3$ concentrations in these healthy volunteers, perhaps indicative of tighter regulation of 1α,25$(OH)_2D_3$.

In summary, we have established a sensitive LC-MS/MS method for quantification of vitamin D_3 metabolites. For 1α,25$(OH)_2D_3$ quantification, chromatographic separation of 1α,25$(OH)_2D_3$ from another dihydroxyvitamin D_3 metabolite, 4β,25$(OH)_2D_3$ is necessary.

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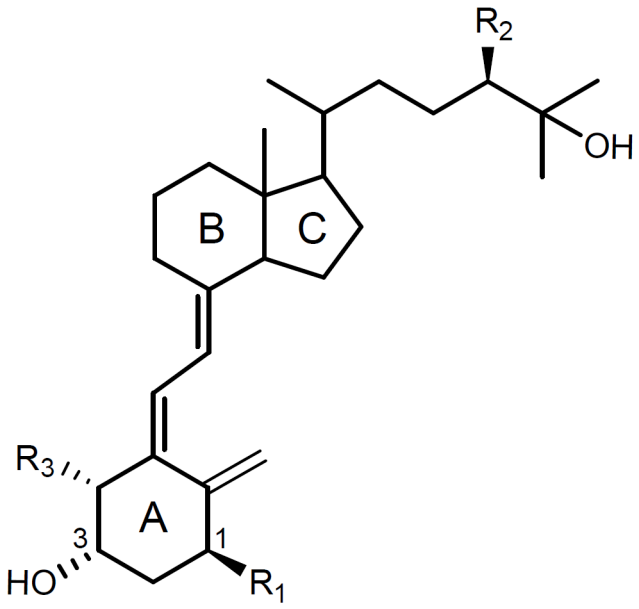
Abbreviation

CID	collision-induced dissociation
LC-MS/MS	liquid chromatography tandem mass spectrometry
HAS	human serum albumin
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
MRM	multiple reaction monitoring
1α,25(OH)$_2$D$_3$	1α,25-dihydroxyvitamin D_3
1α,25(OH)$_2$-3-epi-D$_3$	1α,25-dihydroxy-3-epi-vitamin D_3
4β,25(OH)$_2$D$_3$	4β,25-dihydroxyvitamin D_3
24R,25(OH)$_2$D$_3$	24R,25-dihydroxyvitamin D_3
25OHD$_3$	25-hydroxyvitamin D_3
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
QC	quality control
SPE	solid-phase extraction



Metabolites	R1	R2	R3
1α,25(OH)$_2$D$_3$	OH	H	H
24R,25(OH)$_2$D$_3$	H	OH	H
25OHD$_3$	H	H	H
4β,25(OH)$_2$D$_3$	H	H	OH

Figure 1.
Structures of 1α,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$, 25OHD$_3$ and 4β,25(OH)$_2$D$_3$.

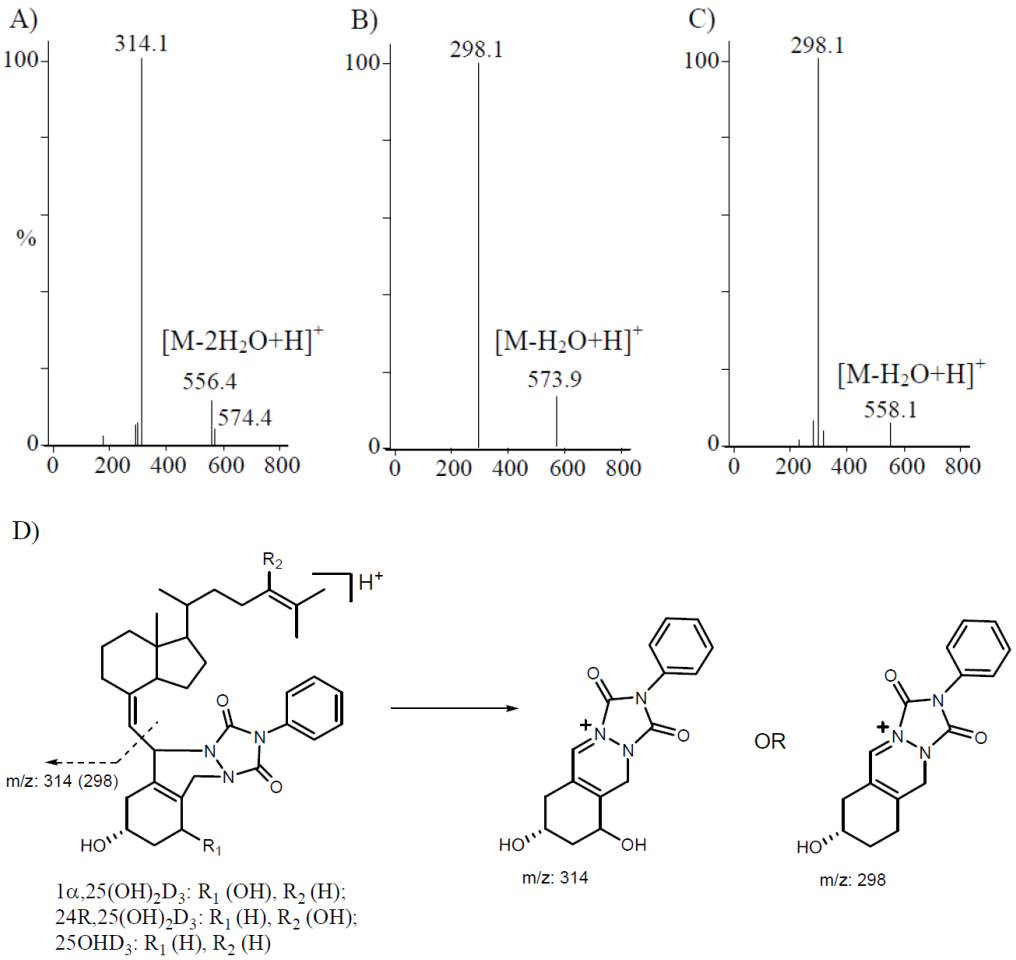


Figure 2.

Product ion mass spectra of the derivatized vitamin D_3 metabolites. A) $1\alpha,25(OH)_2D_3$; B) $24R,25(OH)_2D_3$; and C) $25OHD_3$. The product ion spectra were acquired from the dominant $[M\text{-}H_2O+H]^+$ precursor ion. Proposed fragmentation reaction is shown in figure D. Multiple reaction monitoring (MRM) channel *m/z* 574 → 314 indicates two hydroxyl groups on the A-ring.

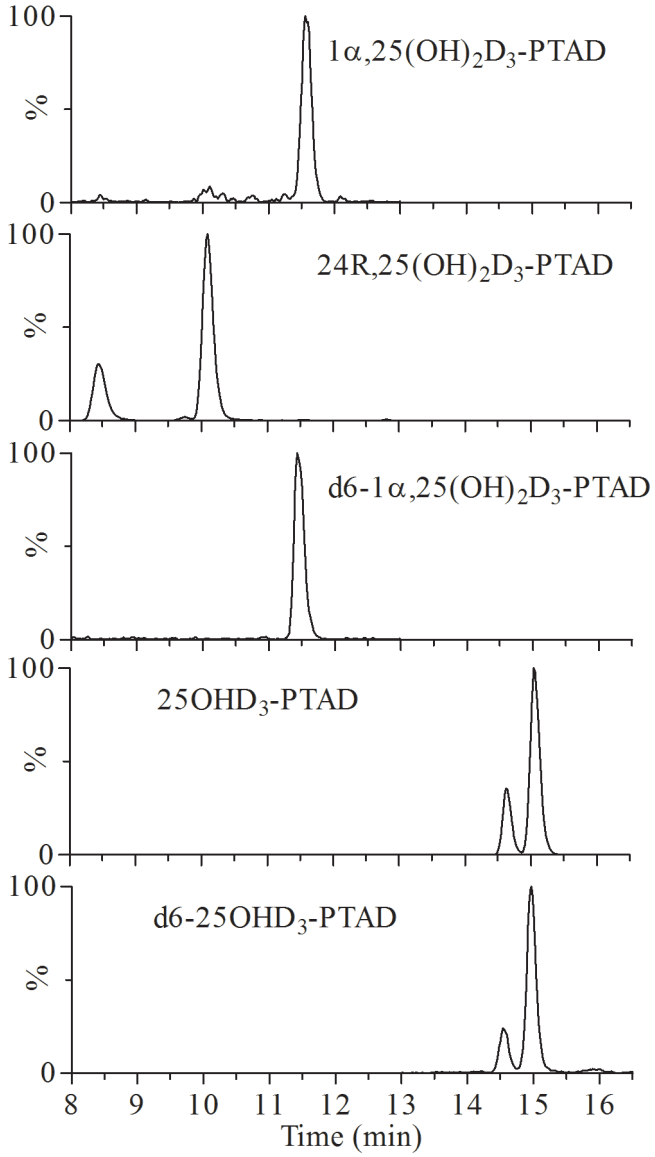


Figure 3.

Representative ion chromatograms of the derivatized vitamin D_3 metabolites by LC-MS/MS. The MRM chromatograms were obtained from a standard solution containing d_6-1α, $25(OH)_2D_3$ (1 ng/mL), 1α,$25(OH)_2D_3$ (0.4 ng/mL), 24R,$25(OH)_2D_3$ (8 ng/mL), d_6-$25OHD_3$ (1 ng/mL) and $25OHD_3$ (20 ng/mL). The deuterated internal standards of vitamin D_3 metabolites were found to elute ~0.1 min earlier than their natural analogs.

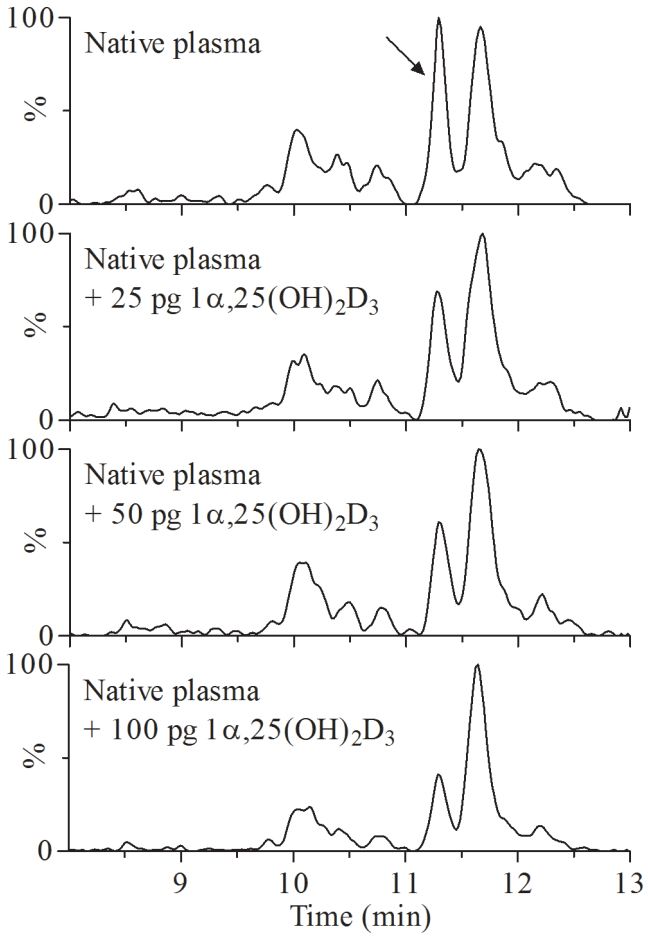


Figure 4.

Chromatographic separation of $1\alpha,25(OH)_2D_3$ from an unknown dihydroxyvitamin D_3 metabolite in human plasma. Varying amounts of $1\alpha,25(OH)_2D_3$ were separately spiked into plasma for validation. An arrow indicates the interfering peak with two hydroxyl groups on the A-ring, which could co-elute with $1\alpha,25(OH)_2D_3$ under certain conditions, e.g., 70% acetonitrile.

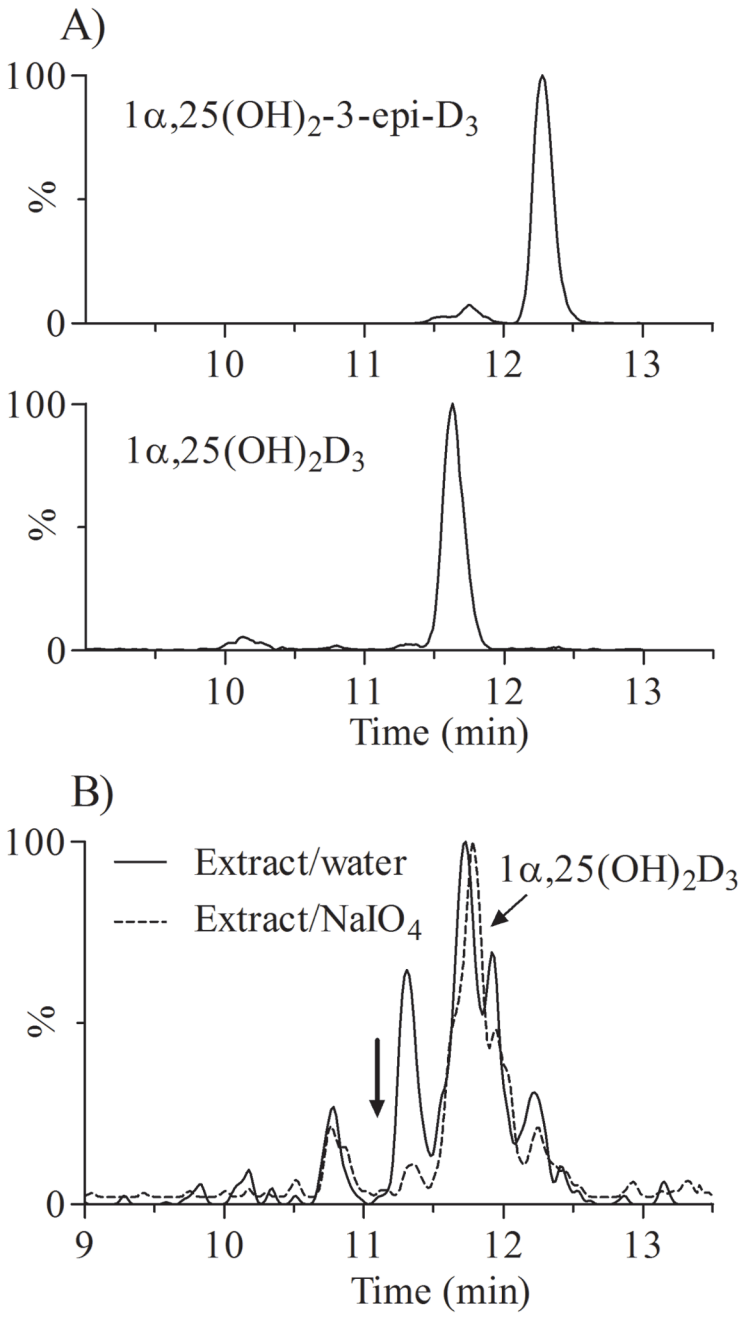


Figure 5.

The MRM (*m/z* 547 → 314) chromatograms of 1α,25(OH)$_2$-3-epi-D$_3$ and periodate cleavage of the unknown dihydroxyvitamin D$_3$ in human plasma extracts. A) Chromatograms of 1α, 25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-3-epi-D$_3$; B) Chromatogram of plasma extracts after periodate cleavage. The disappearance of the unknown metabolite peak indicated vicinal hydroxyl groups on the A-ring, subsequently identified as 4β,25(OH)$_2$D$_3$ [41]. As expected, no significant changes was observed with the peak for 1α,25(OH)$_2$D$_3$.

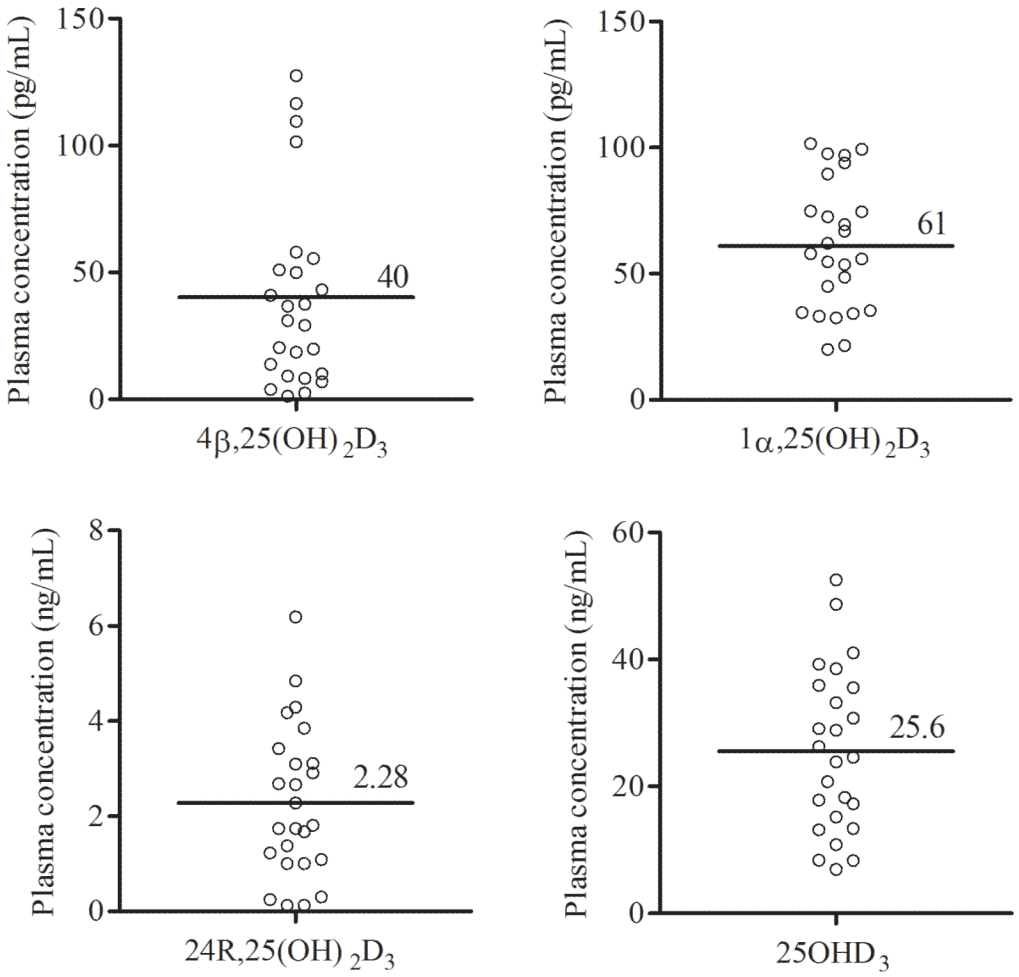


Figure 6.

Plasma concentrations of vitamin D_3 metabolites in healthy volunteers (n = 25). Four vitamin D_3 metabolites [1α,25(OH)$_2$D$_3$, 4β,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$, and 25OHD$_3$] were measured by this newly developed LC-MS/MS method. Each data point represents the mean of two determinations.

Table 1

Selected transitions from precursor ions to product ions for quantification.

PTAD derivatives	Precursor ion (*m/z*)[a]	Product ion (*m/z*)
1α,25(OH)$_2$D$_3$	574.4	314.1
4β,25(OH)$_2$D$_3$	574.4	314.1
24R,25(OH)$_2$D$_3$	574.4	298.1
25OHD$_3$	558.1	298.1
d$_6$-1α,25(OH)$_2$D$_3$	580.4	314.1
d$_6$-25OHD$_3$	564.1	298.1

[a] $[M\text{-}H_2O+H]^+$

Table 2

Sensitivity, quantification range and linearity.

Instrumental LOD and LOQ were estimated from a series of dilutions of standards in blank matrix. The linear range was determined by spiking six concentrations of standards into blank matrix. Experimental details are described in Materials and Methods.

	1α,25(OH)$_2$D$_3$	**24R,25(OH)$_2$D$_3$**	**25OHD$_3$**
LOD (pg)	1.0	2.5	4.0
LOQ (pg/mL)	25	50	50
R^2	0.999	0.999	0.996
Linear Range (pg/mL)	25 – 800	500 – 16000	2500 – 80000

Table 3

Precision and accuracy of vitamin D_3 metabolite measurement.

Compound and concentrations		[a]Intra-assay (n = 6)		Inter-assay (n = 6)	
		[b]Measured	[c]%CV	Measured	%CV
1α,25(OH)$_2$D$_3$					
	50 pg/mL	48.0 ± 3.3 (96.0%)	7.0%	46.4 ± 2.6 (92.8%)	5.6%
	100 pg/mL	100.3 ± 12.0 (100%)	11.9%	102.5 ± 6.8 (103%)	6.6%
	200 pg/mL	213.1 ± 6.3 (107%)	2.9%	206.7 ± 10.6 (103%)	5.1%
	native plasma	85.9 ± 4.6	5.3%	93.4 ± 6.7	7.1%
24R,25(OH)$_2$D$_3$					
	0.5 ng/mL	0.50 ± 0.04 (100%)	8.5%	0.50 ± 0.07 (100%)	13.8%
	1.0 ng/mL	1.04 ± 0.07 (104%)	7.0%	1.03 ± 0.05 (103%)	4.6%
	2.0 ng/mL	2.08 ± 0.26 (104%)	12.4%	2.07 ± 0.12 (104%)	5.9%
	native plasma	1.19 ± 0.06	5.3%	1.33 ± 0.11	8.3%
25OHD$_3$					
	10 ng/mL	9.5 ± 0.2 (95.0%)	2.1%	10.0 ± 0.7 (100%)	7.0%
	20.0 ng/mL	18.8 ± 0.2 (94.0%)	1.1%	18.8 ± 0.2 (94.0%)	1.0%
	40.0 ng/mL	39.8 ± 1.1 (99.5%)	2.8%	40.3 ± 0.8 (101%)	2.1%
	native plasma	21.6 ± 0.7	3.4%	23.6 ± 1.1	4.7%

[a]Intra-assay precision was obtained from 6 replicates measured in a single assay.
Inter-assay precision was obtained from 6 assays run over a 10 day period.

[b]Reported as mean ± standard deviation (accuracy).

[c]CV: coefficient of variance is defined as the ratio of the standard deviation to the mean.

Table 4

Standard addition of vitamin D_3 metabolites into human plasma. We extracted spiked human plasma (1 mL) and analyzed the vitamin D_3 metabolites by LC-MS/MS. Standard mixtures (10 μL) were spiked into native plasma at various concentrations and methanol (10 μL) was used as vehicle.

	Native plasma	Set 1[a]	Set 2	Set 3
1α,25(OH)$_2$D$_3$ (pg/mL)	97 ± 5.7[b]	125 ± 3.0 (102%)	156 ± 6.7 (106%)	199 ± 9.0 (101%)
24R,25(OH)$_2$D$_3$ (ng/mL)	1.4 ± 0.04	1.9 ± 0.10 (100%)	2.5 ± 0.14 (104%)	3.6 ± 0.14 (106%)
25OHD$_3$ (ng/mL)	24.3 ± 0.43	24.4 ± 0.39 (96%)	27.2 ± 0.33 (101%)	30.6 ± 0.60 (104%)

[a] Amounts of 1α,25(OH)$_2$D$_3$, 24R,25(OH)$_2$D$_3$ and 25OHD$_3$:
Set 1: 25 pg, 0.5 ng and 1.25 ng
Set 2: 50 pg, 1.0 ng and 2.50 ng
Set 3: 100 pg, 2.0 ng and 5.00 ng

[b] Concentration was calculated from the standard curve as described in Materials and Methods. Data reported as mean ± standard deviation (accuracy).