Journal of Steroid Biochemistry & Molecular Biology 126 (2011) 72–77

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

The ratio of serum 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3 is predictive of 25-hydroxyvitamin D_3 response to vitamin D_3 supplementation

Dennis Wagner[a,b,*], Heather E. Hanwell[a,b], Kareena Schnabl[c,d], Mehrdad Yazdanpanah[c], Samantha Kimball[a,b], Lei Fu[d], Gloria Sidhom[e], Dérick Rousseau[f], David E.C. Cole[d], Reinhold Vieth[a,b,d]

[a] Department of Nutritional Sciences, University of Toronto, Canada
[b] Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Canada
[c] Department of Paediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Canada
[d] Department of Laboratory Medicine and Pathobiology, University of Toronto, Canada
[e] Department of Clinical and Chemical Pathology, National Research Centre, Cairo, Egypt
[f] Department of Chemistry and Biology, Ryerson University, Toronto, Canada

ARTICLE INFO

Article history:
Received 23 January 2011
Received in revised form 21 April 2011
Accepted 5 May 2011

Keywords:
24,25-Dihydroxyvitamin D
25-Hydroxyvitamin D
Vitamin D
Supplementation
Catabolism
CYP24A1

ABSTRACT

24,25-Dihydroxyvitamin D (24,25VD) is a major catabolite of 25-hydroxyvitamin D (25VD) metabolism, and may be physiologically active. Our objectives were to: (1) characterize the response of serum $24,25VD_3$ to vitamin D_3 (VD_3) supplementation; (2) test the hypothesis that a higher $24,25VD_3$ to $25VD_3$ ratio ($24,25{:}25VD_3$) predicts $25VD_3$ response.

Serum samples ($n = 160$) from wk 2 and wk 6 of a placebo-controlled, randomized clinical trial of VD_3 (28,000 IU/wk) were analyzed for serum $24,25VD_3$ and $25VD_3$ by mass spectrometry.

Serum $24,25VD_3$ was highly correlated with $25VD_3$ in placebo- and VD_3-treated subjects at each time point ($p < 0.0001$). At wk 2, the $24,25{:}25VD_3$ ratio was lower with VD_3 than with placebo ($p = 0.035$). From wk 2 to wk 6, the $24,25{:}25VD_3$ ratio increased with the VD_3 supplement ($p < 0.001$) but not with placebo, such that at wk 6 this ratio did not significantly differ between groups. After correcting for potential confounders, we found that $24,25{:}25VD_3$ at wk 2 was inversely correlated to the $25VD_3$ increment by wk 6 in the supplemented group ($r = -0.32$, $p = 0.02$) but not the controls.

There is a strong correlation between $24,25VD_3$ and $25VD_3$ that is only modestly affected by VD_3 supplementation. This indicates that the catabolism of $25VD_3$ to $24,25VD_3$ rises with increasing $25VD_3$. Furthermore, the initial ratio of serum $24,25VD_3$ to $25VD_3$ predicted the increase in $25VD_3$. The $24,25{:}25VD_3$ ratio may therefore have clinical utility as a marker for VD_3 catabolism and a predictor of serum $25VD_3$ response to VD_3 supplementation.

1. Introduction

Vitamin D (VD) has received considerable attention because of associations between low VD status and increased risk for several diseases, including osteoporosis, cancers, multiple sclerosis, diabetes, cardiovascular disease, and microbial infections [1–7]. The determinants of serum 25-hydroxyvitamin D (25VD), the classic measure of VD status, include environmental (e.g. season, latitude, sunlight, diet) [8,9], demographic [e.g. ethnicity, body mass index (BMI)] [10], and genetic factors (e.g. polymorphisms in metabolism and transport genes) [11–13]. However, the factors that modify response to VD supplementation warrant further study, especially in view of the large inter-individual variation that has been reported in serum 25VD response to supplementation with identical doses of VD [12,14,15]. An analysis of 24,25-dihydroxyvitamin D (24,25VD), the major metabolite of 25VD, could provide clinically relevant information that may shed light on these inter-individual differences.

24,25VD is produced via 24-hydroxylation of 25VD by the cytochrome P450 24-hydroxylase enzyme (CYP24A1; $V_{max} = 0.088$ mol/min/mol P450, $K_m = 160$ nM) [16]. In addition, CYP24A1 catalyzes the side-chain metabolism of 1,25-dihydroxyvitamin D (1,25VD), considered to be the primary active metabolite. CYP24A1 is expressed in many tissues [17–20] but the biological activity of 24,25VD remains controversial. The general view is that 24,25VD production is the first step to inactivate 25-hydroxylated metabolites of VD, thus regulating synthesis of 1,25VD [21,22]. However, there is considerable evidence demonstrating that 24,25VD has unique biological properties, including physiological roles in embryogenesis, cartilage development, and

* Corresponding author at: Mount Sinai Hospital, Pathology and Laboratory Medicine, 600 University Avenue, Room 6-327, Toronto, Ontario, Canada M5G 1X5. Tel.: +1 416 586 4800x2726; fax: +1 888 314 6038.
E-mail address: dennis.wagner@utoronto.ca (D. Wagner).

0960-0760/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2011.05.003

fracture repair [23–29]. Recently, Larsson et al. demonstrated that 24,25VD binds to catalase, suggesting that 24,25VD-mediated signal transduction may occur through modulating hydrogen peroxide production [30].

Few clinical studies have reported circulating 24,25VD concentrations [31–37], likely because its measurement is technically challenging and its physiological role is unclear. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has received increased attention because it is capable of measuring $25VD_2$ and $25VD_3$ separately, but the capability for measuring 24,25VD has not been widely exploited. Furthermore, the effects of VD_3 supplementation on serum $24,25VD_3$ concentrations in humans are unknown. Here, we characterize the biochemical response of serum $24,25VD_3$ to VD_3 supplementation in healthy adults using a highly sensitive and specific LC–MS/MS assay for simultaneous determination of serum $25VD_3$ and $24,25VD_3$ concentrations. We hypothesized that a higher $24,25VD_3$ to $25VD_3$ ratio ($24,25{:}25VD_3$) would predict a smaller serum $25VD_3$ response to an increased VD_3 intake because a relatively higher $24,25VD_3$ would indicate higher catabolism.

2. Materials and methods

2.1. Study samples

Human serum samples ($n = 160$) were obtained from a randomized, double-blind, placebo-controlled clinical trial carried out in Toronto, Canada (latitude 43°N). Healthy young adults, half of whom were female, received either 28,000 IU VD_3/wk as a supplement or fortified cheese of equivalent bioavailability ($n = 60$), or a placebo ($n = 20$), for 8 weeks during the winter months [38]. Serum aliquots were stored at −80 °C until analysis. Under these storage conditions, VD metabolites are stable in serum or plasma over a prolonged time and repeated freeze–thaw cycles [39,40]. Samples were available from subjects at wk 2 ($n = 80$) and wk 6 ($n = 80$) of the dosing protocol. The study protocol was approved by the Research Ethics Boards of the University of Toronto and of Mount Sinai Hospital (Toronto, Canada).

2.2. 25VD and 24,25VD assays

Aliquots of 200 μL serum were spiked with 50 μL of d_6-25-hydroxyvitamin D_3 [d_6-$25VD_3$] (Medical Isotopes Inc., Pelham, NH, USA) internal standard and extracted with 1 mL of methyl-*tert*-butyl ether. The upper ether phase was transferred to a clean borosilicate tube and the solvent evaporated under a stream of nitrogen gas at 40 °C. The residue was dissolved in 1 mL of 4:1 methanol:water and 1 mL of heptane was added. The methanol phase was transferred into clean borosilicate tubes and evaporated to dryness under a stream of nitrogen gas at 40 °C. The residue was dissolved in 100 μL of 1:1 methanol:water and transferred into an HPLC auto-sampler vial. A 20 μL aliquot was analyzed by LC–MS/MS.

The chromatographic separation of $25VD_3$, $25VD_2$, and $24,25VD_3$ was carried out using an Agilent Technologies 1200 series HPLC system in linear gradient mode at a flow rate of 0.80 ml/min on an Eclipse C8 column (50 mm × 3.0 mm, 1.8 μm) employing a mobile phase consisting of methanol–water (37:63) increasing to 100% methanol over 4 min and maintained at 100% methanol for 1 min. The column was re-equilibrated with methanol-water (37:63) for 1 min. The column temperature was maintained at 50 °C. The total chromatographic run time for each sample was 6.5 min and typical retention times for $24,25VD_3$, d6-$25VD_3$, $25VD_3$, and $25VD_2$ were 2.92, 3.65, 3.66, and 3.72 min, respectively.

An API 5000 mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada) was equipped with an atmospheric pressure chemical ionization (APCI) source and operated in the positive mode. The ion source temperature was maintained at 400 °C, the corona current adjusted to 3.0 μA, and collision gas, nebulizer gas and curtain gas pressures set to 5, 40, and 30, respectively, the collision energy set to 24 V and the declustering potential set to 100 V. The ion-transitions of m/z 417.4 → 399.4, 407.5 → 389.4, 401.4 → 383.4, and 413.4 → 395.4 were monitored to detect and quantify $24,25VD_3$, d6-$25VD_3$, $25VD_3$, and $25VD_2$, respectively. The dwell time per transition was set to 50 ms.

Analyst software (version 1.4.2) mediated data acquisition, peak-area integration and comparison against the standard curve to calculate the concentration of unknowns. The standard curve was derived from calibrators of $25VD_3$, $25VD_2$, and $24,25VD_3$ (Sigma–Aldrich) prepared in 100% ethanol that were analyzed within the same analytical run. The absolute concentrations of the calibrators were assigned using the Agilent 8453 E ultraviolet/visible spectrophotometer and calculated using the Merck Index molar absorptivity of 18,300 AU mol^{-1} L^{-1} at 265 nm.

Serum 25VD was also determined by DiaSorin "25-hydroxyvitamin D ^{125}I Radioimmunoassay (RIA)" and DiaSorin "LIAISON 25 OH Vitamin D TOTAL" chemiluminescent immunoassay (LIA), as reported previously [41], and used for confirmatory analyses. Serum $24,25VD_3$ and $25VD_3$ concentrations measured by LC–MS/MS are presented, unless otherwise indicated.

2.3. LC–MS/MS method evaluation

Between-day imprecision was assessed by measuring VD metabolites in low (L1), medium (L2), and high (L3) plasma control pools in duplicate over 20 working days. Within-run imprecision was evaluated by measuring VD metabolites in 20 different aliquots of L1, L2, and L3. Linearity of the analytical measurement range was evaluated by measuring VD metabolite calibrators in triplicate. The measurement response was classified as linear if a straight line was drawn within an allowable systemic error of 10% of each calibrator point.

The limit of detection (LOD) and limit of quantification (LOQ) are defined as the peaks that give signal to noise ratios of 3:1 and 10:1, respectively, and were determined by running the calibration curve in triplicate with the following calculations: $LOD = (3 \times SD_{0\ calibrator})/slope_{curve}$, $LOQ = (10 \times SD_{0\ calibrator})/slope_{curve}$. Functional sensitivity was evaluated by diluting L1 and measuring it 5 times to determine the concentration that gives a coefficient of variation (CV) near 20%.

The specificity of the LC–MS/MS assay to measure $24,25VD_3$ and $25VD_3$ separately was evaluated by spiking pooled serum with either $25VD_3$ (~500 nmol/L), $24,25VD_3$ (~50 nmol/L), or both, and assaying as described above. Samples were run in triplicate on 2 separate days. The method was evaluated for potential interference of high bilirubin, hemoglobin, and lipemic conditions by spiking separate control plasma pools with bilirubin (800 μmol/L), hemoglobin (3 g/L), and lipids (100 mmol/L), and assaying as described above.

LC–MS/MS assay 25VD measurements were compared to Diasorin RIA ($n = 160$) and Diasorin LIA ($n = 160$) values. Method comparisons were not performed for $24,25VD_3$ measurements because there is no published reference method for this metabolite.

2.4. Other biochemical measurements

Calcium, phosphate, and creatinine in serum and urine, as well as serum parathyroid hormone (PTH), were measured on the Modular Analytics Serum Work Area (Roche) as previously described (36). Glomerular filtration rate (GFR) was estimated from serum

creatinine using the Modification of Diet in Renal Disease (MDRD) Study equation [42].

2.5. Statistical analyses

The study was powered for a probability of 80% to detect a difference of 1 SD in 25VD; this required a sample size of at least 34. Results are presented as means ± SD. All data were analyzed with SPSS software (version 18.0). Associations between biochemical measures were assessed using Pearson correlation coefficients (r). For regression lines plotted non-parametrically, we used the locally estimated scatterplot smoothing (LOESS) approach. Within-group changes in biochemical variables over time were analyzed with paired 2-tailed t tests. Between-group differences in biochemical measures at each time point were analyzed with independent sample 2-tailed t tests. The cut-off for statistical significance was set at $p < 0.05$.

3. Results

3.1. LC–MS/MS method evaluation

All data were normally distributed, as indicated by the Kolmogorov–Smirnov test. LC–MS/MS assay performance characteristics are shown in Table 1. Total imprecision for all VD metabolites (CV = 7.3–14%) was comparable to immunoassays (5–15%) [40]. Linearity was confirmed across the analytical measurement range for all VD metabolites. The functional sensitivity for all VD metabolites (≤1 nmol/L) was lower (i.e. higher sensitivity) than immunoassays (≤10 nmol/L). $25VD_2$ was not detected in any sample. Specificity experiments indicated no cross-reactivity (i.e. complete resolution) between $24,25VD_3$ and $25VD_3$. Bilirubin, hemolysis, and triglycerides did not interfere with measurement of VD metabolites. Lastly, serum $25VD_3$ concentrations determined by LC–MS/MS correlated well with those measured by RIA ($r = 0.915$, $p < 0.0001$) and LIA ($r = 0.907$, $p < 0.0001$). However, both the RIA and LIA 25VD methods demonstrated significant positive bias compared to LC–MS/MS (15.0 and 13.6 nmol/L, respectively, $p < 0.0001$), likely because these immunoassays have 100% cross-reactivity with 24,25VD [34].

3.2. Biochemical responses

Linear regression analysis indicated that serum $24,25VD_3$ and $25VD_3$ were highly correlated in the total sample (Fig. 1), and separately in the placebo- and VD_3-treated sub-groups at wk 2 ($r = 0.81$, $r = 0.86$, respectively; $p < 0.0001$) and wk 6 ($r = 0.92$, $r = 0.81$, respectively; $p < 0.0001$). LOESS fitting supported these findings but suggested slight deviation from linearity at $25VD_3$ concentrations >100 nmol/L. All correlations persisted when serum 25VD values previously measured by RIA and LIA were used ($p < 0.0001$). On average, serum $24,25VD_3$ values were 14% of $25VD_3$ concentrations.

In the VD_3 group, serum $24,25VD_3$ and $24,25{:}25VD_3$ ratio also correlated with serum creatinine at wk 2 ($r = -0.46$, $r = -0.39$, respectively; $p < 0.005$) and wk 6 ($r = -0.39$, $r = -0.39$, respectively; $p < 0.005$). However, neither serum $24,25VD_3$ nor $24,25{:}25VD_3$ ratio correlated significantly with estimated GFR, nor with PTH, calcium, or phosphate in serum or urine.

Table 2 shows the absolute $25VD_3$ and $24,25VD_3$ concentrations at wk 2 and wk 6. After 2 wk of treatment, both serum $25VD_3$ and $24,25VD_3$ concentrations were significantly greater in the VD_3 group (69.6 ± 17.5 and 8.9 ± 3.1 nmol/L, respectively) compared to placebo (40.2 ± 17.2 and 5.9 ± 2.5 nmol/L, respectively) ($p < 0.0001$). By wk 6, serum $25VD_3$ and $24,25VD_3$ had increased to 90.5 ± 19.7 and 12.8 ± 3.6 nmol/L with VD_3 supplementation,

Table 1
LC–MS/MS assay performance characteristics.

Parameter	$24,25VD_3$	$25VD_3$	$25VD_2$
Imprecision (%CV)			
Between-day	7.3–9.6	5.3–6.5	7.5–13
Within-run	5.2–7.4	4.7–6.6	5.3–7.0
Total linearity	9.1–12	7.3–8.5	10.0–14.0
Analytical measurement			
Range (nmol/L)	0–60	0–437	0–403
Linear calibration curve	$y = 0.024x - 0.002$[a] $r = 0.9992$	$y = 0.034x + 0.003$ $r = 1.0000$	$y = 0.025x - 0.079$ $r = 0.9997$
Sensitivity (nmol/L)			
Limit of detection[b]	0.24	0.25	0.17
Limit of quantification[c]	0.8	0.83	0.57
Functional sensitivity[d]	1.13	0.59	1.14
Specificity (nmol/L)			
−Cross-reactant	5 ± 0.1	31 ± 0.2	ND[e]
+$25VD_3$ (~500 nmol/L)	4 ± 1.0	534 ± 10	ND
+$24,25VD_3$ (~50 nmol/L)	66 ± 9.0	30 ± 1.0	ND
+Both	69 ± 2.0	491 ± 23.0	ND
Interference (nmol/L, %change)			
−Interferant	10.6	75.3	10.6
+Bilirubin (800 μmol/L)	12.1, +14%	77.5, +2.9%	12.1, +14%
+Hemoglobin (3 g/L)	11.3, +6.6%	79.6, +5.7%	11.3, +6.6%
+Triglyceride (100 mmol/L)	10.9, +2.8%	71.7, −4.8%	10.9, +2.8%
Method comparison			
Regression/correlation	Not reported No reference method for $24,25VD_3$	LC–MS/MS = 0.82 (RIA) + 0.32 $r = 0.92$, $n = 160$ LC–MS/MS = 0.82 (LIA) + 1.24 $r = 0.91$, $n = 160$	ND
Agreement	Not reported No reference method for $24,25VD_3$	RIA bias = 15.0 ± 13.4 nmol/L LIA bias = 13.6 ± 13.8 nmol/L	ND

[a] y = peak area VD/peak area IS; x = concentration (nmol/L).
[b] Calculated as (3 × SD)/slope.
[c] Calculated as (10 × SD)/slope.
[d] CV = 20%.
[e] Not detected.

Table 2
Serum $25VD_3$ and $24,25VD_3$ concentrations over time in subjects consuming placebo ($n = 20$) or VD_3 ($n = 60$)[a].

Measurement	Placebo-treated	Vitamin D-treated
$25VD_3$ (nmol/L)		
wk 2	40.2 ± 17.2	69.6 ± 17.5**
wk 6	39.2 ± 17.1	90.5 ± 19.7**,†
Change	−1.1 ± 4.1	21.2 ± 9.1**
$24,25VD_3$ (nmol/L)		
wk 2	5.9 ± 2.5	8.9 ± 3.1**
wk 6	5.6 ± 2.6	12.8 ± 3.6**,†
Change	−0.3 ± 1.3	4.1 ± 2.0**
$24,25{:}25VD_3$ ratio		
wk 2	0.155 ± 0.05	0.127 ± 0.02*
wk 6	0.146 ± 0.04	0.142 ± 0.02†
Change	−0.009 ± 0.04	0.016 ± 0.02*

[a] Values are means ± SD.
* $p < 0.05$ (compared to placebo).
** $p < 0.0001$ (compared to placebo).
† $p < 0.001$ (compared to wk 2).

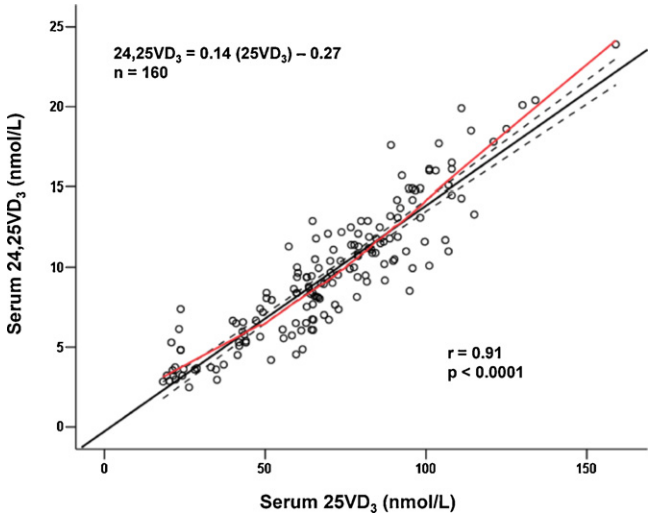

Fig. 1. Strong correlation between serum 24,25VD_3 and 25VD_3 concentrations in the total sample ($n = 160$), with slight deviation from linearity at serum 25VD concentrations >100 nmol/L. Linear regression line, with 95% confidence intervals (curved lines), shown in black. LOESS fit line depicted in red. The linear regression equation and Pearson correlation coefficient (r) are also indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

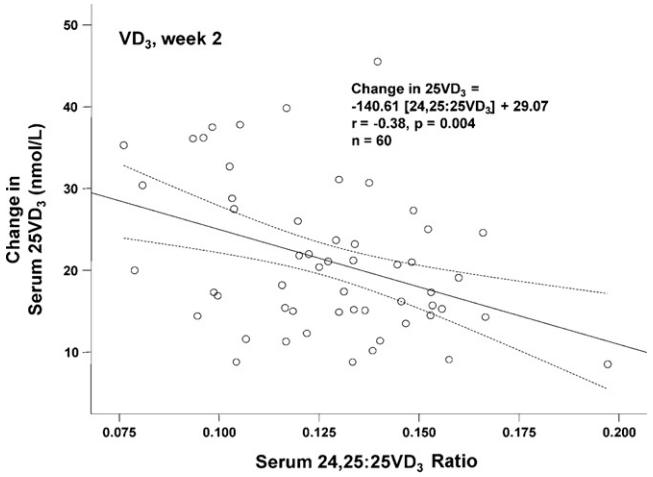

Fig. 2. Inverse correlation between the serum 24,25VD_3 to 25VD_3 ratio at wk 2 and change (wk 6–wk 2) in 25VD_3 concentrations in subjects consuming VD_3 ($n = 60$). Linear regression line, with 95% confidence intervals (curved lines), is shown. The linear regression equation and Pearson correlation coefficient (r) are also indicated.

respectively ($p < 0.0001$), but remained unchanged in the placebo group ($p = 0.26$). The increases in serum 25VD_3 and 24,25VD_3 during this time period was significantly greater in the supplemented group (21.2 ± 9.1 and 4.1 ± 2.0 nmol/L, respectively) than in controls (-1.1 ± 4.1 and -0.3 ± 1.3 nmol/L, respectively) ($p < 0.0001$).

At 2 wk of treatment, the ratio of 24,25VD_3 to 25VD_3 was lower in the VD_3 group (0.127 ± 0.02) than in the placebo group (0.155 ± 0.05) ($p = 0.03$). From wk 2 to wk 6, the 24,25:25VD_3 ratio increased with the VD_3 supplement to 0.142 ± 0.02 ($p < 0.001$) but remained unchanged in controls (wk 6: 0.146 ± 0.04, $p = 0.34$). At wk 6, the 24,25VD_3 to 25VD_3 ratio did not differ significantly between VD_3- and placebo-treated subjects ($p = 0.61$). These results were confirmed when using RIA and LIA serum 25VD values. When stratifying by gender in the VD_3 group, the 24,25:25VD_3 ratio was not significantly different between genders at wk 2 (females: 0.131 ± 0.02, males: 0.122 ± 0.02; $p = 0.20$) but at wk 6 it was significantly higher in females (0.149 ± 0.02) compared to males (0.134 ± 0.02) ($p = 0.01$).

Linear regression indicated that the 24,25:25VD_3 ratio at wk 2 and wk 6 was significantly inversely correlated with the change in serum 25VD_3 (i.e. wk 6–wk 2) in the VD_3 group ($r = -0.38$, $p = 0.004$; $r = -0.30$, $p = 0.03$; respectively) but not in the placebo group ($p > 0.40$) (Fig. 2). These correlations were essentially the same, irrespective of 25VD measurement method ($p < 0.05$, data not shown). Significant correlation between 24,25:25VD_3 and 25VD_3 response to VD_3 persisted at wk 2 and wk 6 after controlling for serum 25VD_3 (wk 2 and 6), 24,25VD_3 (wk 6), BMI, age, gender, PTH, calcium, phosphate, and creatinine ($p < 0.05$). After controlling for baseline serum 25VD (as measured by RIA and LIA) and 24,25VD_3 at wk 2, the association between 24,25:25VD_3 and 25VD_3 response was attenuated at wk 6 ($r = -0.21$, $p = 0.14$) but not at wk 2 ($r = -0.35$, $p = 0.01$). The 24,25:25VD_3 ratio at wk 2 also correlated significantly with the overall change in serum 25VD_3 (i.e. wk 8–wk 0) ($r = -0.40$, $p = 0.003$) in the VD_3 group but not in the placebo group ($p = 0.22$).

4. Discussion

Our data suggest a new clinical indication utility for measuring serum 24,25VD_3, the major metabolite of 25VD_3, by a novel LC–MS/MS assay for simultaneous determination of 25VD_3 and 24,25VD_3. The developed LC–MS/MS method was highly sensitive, specific, and the first to quantify 24,25VD_3 in serum. Investigators should therefore exploit the capability of LC–MS/MS methods to measure both serum 24,25VD and 25VD simultaneously. Indeed, 24,25VD is the most abundant 25VD metabolite and its roles in fracture healing and cartilage growth [25–29] support its physiological relevance beyond VD catabolism.

We found that serum 24,25VD_3 concentrations were highly correlated with serum 25VD_3, indicating that the catabolism of 25VD_3 into 24,25VD_3 rises with increasing 25VD_3 concentrations. This is consistent with the findings of other investigators [34,36,37]. In our study, the correlation between these variables was remarkably strong; indeed, 82% of the variation in serum 24,25VD_3 could be explained by 25VD_3 concentrations. Furthermore, serum 24,25VD_3 increased in parallel with 25VD_3 levels during the 4 weeks of 28,000 IU/wk VD_3 supplementation. In fact, the two variables are so closely related that one might argue that serum 24,25VD_3 could serve as an alternative marker of VD status. Taken together, the strong correlation and similar response of serum 24,25VD_3 with 25VD_3 indicate that 24,25VD_3 measurement provides clinically useful information pertaining to VD status and supplementation.

Since 24,25VD_3 concentration changed in proportion to that of 25VD_3, we normalized serum 24,25VD_3 response by calculating the ratio of 24,25VD_3 to 25VD_3. This ratio served as an index of VD_3 clearance since 24,25VD_3 is the major initial catabolite of 25VD_3 metabolism. Interestingly, the 24,25:25VD_3 ratio at wk 2 was significantly lower in the VD_3 group than placebo, indicating a possible lag in 24-hydroxylation during the early phase of supplementation. We speculate that this lag effect is the result of: (1) the large incremental increase in 25VD_3 observed during the first 2 wk of dosing, which was greater than that observed at any other time interval, and (2) the slower reaction kinetics of CYP24A1 [turnover number (TN) = 2–20 min^{-1}] compared to CYP27A1 (25-hydroxylase; TN = 40–50 min^{-1}) [16,19]. By wk 6, however, the 24,25:25VD_3 ratio had increased significantly with supplementation, as a response to the VD_3 loading. Overall, these results suggest that catabolism is induced with VD_3 supplementation but these adaptations may occur over weeks not days. Indeed, in vitro studies indicate that a variety of molecular mechanisms may be involved, including gene expression up-regulation and enzyme trafficking [43]. Future studies should investigate the genetic influences of CYP24A1 genotypes on VD catalytic activity and biochemical response.

Our data provide insight on the in vivo effects of this altered expression and kinetic behaviour of the CYP24A1 enzyme. Firstly, the correlation in the VD_3-treated group of serum 24,25VD_3 and 24,25:25VD_3 ratio with serum creatinine, a measure of renal function, is supportive of the idea of variable renal CYP24A1 action in 25VD_3 metabolism. Accordingly, the 24,25:25VD_3 ratio may be useful in monitoring kidney function during VD_3 supplementation but this needs to be studied directly. The increase in 24,25:25VD_3 over time is consistent with the induction of renal CYP24A1 catabolic capacity with increasing VD_3 loading. The concept of induction proportional to load is also supported by the LOESS fit line (Fig. 1), which appears to become more curvilinear at serum 25VD_3 concentrations exceeding 100 nmol/L. Lastly, we found that the 24,25:25VD_3 ratio was significantly higher in supplemented women compared to men at wk 6. This suggests that females were catabolising 25VD_3 at a slightly faster rate than males during the later parts of VD_3 supplementation, an effect that may be related to estrogen. Further research is needed to elucidate the regulation of CYP24A1 activity by gender and varying 25VD_3 concentrations.

A major finding of this study was that the 24,25:25VD_3 ratio alone predicted the magnitude of the serum 25VD_3 change resulting from VD_3 supplementation. This inverse correlation remained significant at wk 2 after controlling for other variables that may affect serum 25VD_3 response, including baseline 25VD, BMI, gender, serum PTH, and serum calcium. Although moderate ($r = -0.38$), this correlation was similar to those commonly reported with more conventional correlates of vitamin D response and status, including BMI ($r = -0.41$) [10] and PTH ($r = -0.34$) [38]. Taken together, these results suggest that relative 24,25VD_3 concentration, as assessed by a ratio of circulating 24,25VD_3 to 25VD_3 early after dosing commences, is a potentially important determinant of serum 25VD_3 response to supplementation. Consequently, this ratio may assist in identifying individuals who are more likely to experience a lower serum 25VD_3 response and thereby require more VD_3 due to a higher 24,25:25VD_3 ratio (i.e. higher 25VD_3 catabolism) during the early loading stage (i.e. wk 2) of the supplementation protocol.

Data on 24,25VD can also be evaluated from the perspective of the biological activities of the VD metabolites. Differential 24,25VD production and 25VD response may impact bioactive 1,25VD levels, particularly in extra-renal 1,25VD synthesis, which may well depend on 25VD substrate supply, and in the renal failure population, which exhibit abnormalities in renal VD metabolism. Also, 25VD itself has been reported to be a functional ligand of VDR and to exert genomic actions independent of 1,25VD [44]. Therefore, differences in serum 25VD responses due to increased VD catabolism or other factors might directly affect 25VD-mediated responses such as cell growth regulation. Lastly, there is substantial evidence supporting unique biological properties for 24,25VD, particularly with respect to bone and cartilage [23,25–27,29]. In fact, preliminary evidence for the presence of a unique, non-nuclear membrane receptor for 24,25VD has been reported [45].

The availability of robust LC–MS/MS methods for simultaneous determination of 25VD_3 and 24,25VD_3, like the one presented here, will also help elucidate the functional role of 24,25VD in human physiology. Furthermore, the 24,25:25VD_3 ratio may indicate not only metabolic differences in serum 25VD response but also differential functioning of 24,25VD between individuals and/or target tissues. For instance, the 24,25:25VD_3 ratio (i.e. local or systemic), may be important in investigating the rate of putative 24,25VD-dependent processes, such as fracture healing, whereby a higher ratio could hypothetically indicate faster healing.

Several limitations bear mention. Serum 24,25VD_3 concentrations at baseline and end-of-study were not available. However, baseline 24,25VD_3 levels in the VD_3 group would, in all probability, be similar to those at wk 2 in the placebo group, particularly since baseline 25VD concentrations did not differ significantly between groups. However, end-of-study (wk 8) 24,25VD_3 determination may have provided additional meaningful data. The relatively small increment in 25VD in the VD_3 group from 6 to 8 wk is certainly compatible with the notion of proportional catabolism, but a direct test of this supposition is warranted. Nonetheless, our evidence indicates that metabolic clearance rate at wk 2 appears to be the key determinant of 25VD_3 response, such that the inclusion of baseline or end-of-study 24,25VD_3 measurements would not have substantially changed our findings.

In conclusion, the measurement of serum 24,25VD_3 in conjunction with 25VD_3 shows promise as a novel marker of VD_3 catabolism and predictor of serum 25VD_3 response to VD_3 supplementation. It should be emphasized that LC–MS/MS assay methods can be modified to measure both serum 25VD_3 and 24,25VD_3 simultaneously, thus providing more comprehensive data regarding VD status and repletion. Moreover, further in vivo evidence may confirm the biological activity of 24,25VD in physiological processes such as fracture repair, making its measurement ever more important. Future research should continue to explore the clinical utility of 24,25VD measurement in VD testing. Ultimately, this information may aid clinicians in adjusting VD_3 dose for optimum individual benefit, thus contributing to the goal of personalized medicine and nutrition.

Acknowledgments

This work was supported by grants from the Dairy Farmers of Canada and the Natural Sciences and Engineering Research Council of Canada (NSERC). We thank the laboratory of Dr. Eleftherios P. Diamandis (University Health Network, Toronto, Canada) for performing the LC–MS/MS analyses. We also thank DiaSorin for the in kind donation of the LIAISON 25 OH Vitamin D TOTAL kits.

References

[1] H.A. Bischoff-Ferrari, W.C. Willett, J.B. Wong, E. Giovannucci, T. Dietrich, B. Dawson-Hughes, Fracture prevention with vitamin D supplementation: a meta-analysis of randomized controlled trials, JAMA 293 (2005) 2257–2264.
[2] E. Giovannucci, Y. Liu, B.W. Hollis, E.B. Rimm, 25-Hydroxyvitamin D and risk of myocardial infarction in men: a prospective study, Arch. Intern. Med. 168 (2008) 1174–1180.
[3] J.M. Lappe, D. Travers-Gustafson, K.M. Davies, R.R. Recker, R.P. Heaney, Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial, Am. J. Clin. Nutr. 85 (2007) 1586–1591.
[4] P.T. Liu, S. Stenger, H. Li, et al., Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response, Science 311 (2006) 1770–1773.
[5] K.L. Munger, L.I. Levin, B.W. Hollis, N.S. Howard, A. Ascherio, Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis, JAMA 296 (2006) 2832–2838.
[6] M. Urashima, T. Segawa, M. Okazaki, M. Kurihara, Y. Wada, H. Ida, Randomized trial of vitamin D supplementation to prevent seasonal influenza A in school children, Am. J. Clin. Nutr. 91 (2010) 1255–1260.
[7] P.R. von Hurst, W. Stonehouse, J. Coad, Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient—a randomised, placebo-controlled trial, Br. J. Nutr. 103 (2010) 549–555.
[8] K. Brock, W.Y. Huang, D.R. Fraser, et al., Low vitamin D status is associated with physical inactivity, obesity and low vitamin D intake in a large US sample of healthy middle-aged men and women, J. Steroid Biochem. Mol. Biol. 121 (2010) 462–466.
[9] L.M. Hall, M.G. Kimlin, P.A. Aronov, et al., Vitamin D intake needed to maintain target serum 25-hydroxyvitamin D concentrations in participants with low sun exposure and dark skin pigmentation is substantially higher than current recommendations, J. Nutr. 140 (2010) 542–550.
[10] R. Jorde, M. Sneve, N. Emaus, Y. Figenschau, G. Grimnes, Cross-sectional and longitudinal relation between serum 25-hydroxyvitamin D and body mass index: the Tromso study, Eur. J. Nutr. 49 (2010) 401–407.
[11] J. Ahn, K. Yu, R. Stolzenberg-Solomon, et al., Genome-wide association study of circulating vitamin D levels, Hum. Mol. Genet. 19 (2010) 2739–2745.
[12] L. Fu, F. Yun, M. Oczak, B.Y. Wong, R. Vieth, D.E. Cole, Common genetic variants of the vitamin D binding protein (DBP) predict differences in response of serum 25-hydroxyvitamin D [25(OH)D] to vitamin D supplementation, Clin. Biochem. 42 (2009) 1174–1177.

[13] T.J. Wang, F. Zhang, J.B. Richards, et al., Common genetic determinants of vitamin D insufficiency: a genome-wide association study, Lancet 376 (2010) 180–188.
[14] J.M. Burton, S. Kimball, R. Vieth, et al., A phase I/II dose-escalation trial of vitamin D3 and calcium in multiple sclerosis, Neurology 74 (2010) 1852–1859.
[15] R. Vieth, Vitamin D supplementation, 25-hydroxyvitamin D concentrations, and safety, Am. J. Clin. Nutr. 69 (1999) 842–56.
[16] T. Sakaki, N. Kagawa, K. Yamamoto, K. Inouye, Metabolism of vitamin D3 by cytochromes P450, Front. Biosci. 10 (2005) 119–134.
[17] H.J. Armbrecht, M.A. Boltz, Expression of 25-hydroxyvitamin D 24-hydroxylase cytochrome P450 in kidney and intestine. Effect of 1,25-dihydroxyvitamin D and age, FEBS Lett. 292 (1991) 17–20.
[18] D. Fischer, S. Becker, T. Cordes, et al., Vitamin D-24-hydroxylase in benign and malignant breast tissue and cell lines, Anticancer Res. 29 (2009) 3641–3645.
[19] J.L. Omdahl, B. May, The 25-hydroxyvitamin D 24-hydroxylase, in: D. Feldman, J.W. Pike, F.H. Glorieux (Eds.), Vitamin D, second ed., Elsevier Academic Press, London, 2005, pp. 85–104.
[20] T. Oyama, K. Sugio, T. Isse, et al., Expression of cytochrome P450 in non-small cell lung cancer, Front. Biosci. 13 (2008) 5787–5793.
[21] A.M. Parfitt, C.H. Mathews, R. Brommage, K. Jarnagin, H.F. DeLuca, Calcitriol but no other metabolite of vitamin D is essential for normal bone growth and development in the rat, J. Clin. Invest. 73 (1984) 576–586.
[22] J.L. Sebert, A. Fournier, G. Lambrey, et al., Does 24,25 dihydroxycholecalciferol have a physiological and pathophysiological role? Nephrologie 3 (1982) 133–141.
[23] A. Gal-Moscovici, M. Gal, M.M. Popovtzer, Treatment of osteoporotic ovariectomized rats with 24,25(OH)2D3, Eur. J. Clin. Invest. 35 (2005) 375–379.
[24] H.L. Henry, A.W. Norman, Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability, Science 201 (1978) 835–837.
[25] T. Ono, H. Tanaka, T. Yamate, Y. Nagai, T. Nakamura, Y. Seino, 24R,25-dihydroxyvitamin D3 promotes bone formation without causing excessive resorption in hypophosphatemic mice, Endocrinology 137 (1996) 2633–2637.
[26] A. Ornoy, D. Goodwin, D. Noff, S. Edelstein, 24, 25-dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation, Nature 276 (1978) 517–519.
[27] E.G. Seo, A.W. Norman, Three-fold induction of renal 25-hydroxyvitamin D3-24-hydroxylase activity and increased serum 24,25-dihydroxyvitamin D3 levels are correlated with the healing process after chick tibial fracture, J. Bone Miner. Res. 12 (1997) 598–606.
[28] M.L. Sunde, C.M. Turk, H.F. DeLuca, The essentiality of vitamin D metabolites for embryonic chick development, Science 200 (1978) 1067–1069.
[29] A.W. Norman, W.H. Okamura, J.E. Bishop, H.L. Henry, Update on biological actions of 1alpha, 25(OH)2-vitamin D3 (rapid effects) and 24R,25(OH)2-vitamin D3, Mol. Cell Endocrinol. 197 (2002) 1–13.
[30] D. Larsson, D. Anderson, N.M. Smith, I. Nemere, 24,25-dihydroxyvitamin D3 binds to catalase, J. Cell Biochem. 97 (2006) 1259–1266.
[31] T. Higashi, K. Mitamura, H. Ohmi, et al., Levels of 24,25-dihydroxyvitamin D3, 25-hydroxyvitamin D3 and 25-hydroxyvitamin D3 3-sulphate in human plasma, Ann. Clin. Biochem. 36 (Pt 1) (1999) 43–47.
[32] S. Masuda, T. Okano, M. Kamao, Y. Kanedai, T. Kobayashi, A novel high-performance liquid chromatographic assay for vitamin D metabolites using a coulometric electrochemical detector, J. Pharm. Biomed. Anal. 15 (1997) 1497–1502.
[33] J.M. Mata-Granados, d.C. Luque, J.M. Quesada Gomez, Inappropriate serum levels of retinol, alpha-tocopherol, 25 hydroxyvitamin D3 and 24,25 dihydroxyvitamin D3 levels in healthy Spanish adults: simultaneous assessment by HPLC, Clin. Biochem. 41 (2008) 676–680.
[34] I. Schoenmakers, F. Ginty, L.M. Jarjou, et al., Interrelation of parathyroid hormone and vitamin D metabolites in adolescents from the UK and The Gambia, J. Steroid Biochem. Mol. Biol. 121 (2010) 217–220.
[35] G.S. Reddy, A.W. Norman, D.M. Willis, et al., Regulation of vitamin D metabolism in normal human pregnancy, J. Clin. Endocrinol. Metab. 56 (1983) 363–370.
[36] L. Aksnes, D. Aarskog, Plasma concentrations of vitamin D metabolites in puberty: effect of sexual maturation and implications for growth, J. Clin. Endocrinol. Metab. 55 (1982) 94–101.
[37] A.F. Taylor, M.E. Norman, Vitamin D metabolite levels in normal children, Pediatr. Res. 18 (1984) 886–890.
[38] D. Wagner, G. Sidhom, S.J. Whiting, D. Rousseau, R. Vieth, The bioavailability of vitamin D from fortified cheeses and supplements is equivalent in adults, J. Nutr. 138 (2008) 1365–1371.
[39] D.M. Antoniucci, D.M. Black, D.E. Sellmeyer, Serum 25-hydroxyvitamin D is unaffected by multiple freeze–thaw cycles, Clin. Chem. 51 (2005) 258–261.
[40] D. Lissner, R.S. Mason, S. Posen, Stability of vitamin D metabolites in human blood serum and plasma, Clin. Chem. 27 (1981) 773–774.
[41] D. Wagner, H.E. Hanwell, R. Vieth, An evaluation of automated methods for measurement of serum 25-hydroxyvitamin D, Clin. Biochem. 42 (2009) 1549–1556.
[42] A.S. Levey, J.P. Bosch, J.B. Lewis, T. Greene, N. Rogers, D. Roth, A more accurate method to estimate glomerular filtration rate from serum creatinine: a new prediction equation. Modification of Diet in renal Disease Study Group, Ann. Intern. Med. 130 (1999) 461–470.
[43] J.S. Adams, H. Chen, R. Chun, et al., Substrate and enzyme trafficking as a means of regulating 1,25-dihydroxyvitamin D synthesis and action: the human innate immune response, J. Bone Miner. Res. 22 (Suppl. 2) (2007) V20–V24.
[44] Y.R. Lou, F. Molnar, M. Perakyla, et al., 25-Hydroxyvitamin D(3) is an agonistic vitamin D receptor ligand, J. Steroid Biochem. Mol. Biol. 118 (2010) 162–170.
[45] R. St-Arnaud, CYP24A1-deficient mice as a tool to uncover a biological activity for vitamin D metabolites hydroxylated at position 24, J. Steroid Biochem. Mol. Biol. 121 (2010) 254–256.