Serum Concentrations of 1,25-Dihydroxyvitamin D_2 and 1,25-Dihydroxyvitamin D_3 in Response to Vitamin D_2 and Vitamin D_3 Supplementation

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Objective: The purpose of this study was to determine 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and 1,25-dihydroxyvitamin D_2 [1,25$(OH)_2D_2$] levels in healthy adults consuming 1000 IU vitamin D_2 or vitamin D_3 per day for 11 weeks.

Subjects and Design: Blood from 34 healthy male and female adults, aged 18 to 79 years, from a placebo-controlled, double-blind study who received a placebo, 1000 IU vitamin D_3, or 1000 IU vitamin D_2 daily for 11 weeks at end of winter was analyzed. Serum levels of 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, 1,25$(OH)_2D_2$, and 1,25$(OH)_2D_3$ were determined by liquid chromatography–tandem mass spectroscopy.

Results: Of the adults, 82% were vitamin D insufficient (serum 25-hydroxyvitamin D [25(OH)D $<$30 ng/mL]) at the start of the study. Administration of vitamin D_2 and vitamin D_3 induced similar increases in total 25(OH)D as well as in 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, respectively. Compared with placebo and adjusting for baseline levels, 1000 IU daily of vitamin D_2 was associated with a mean increase of 7.4 pg/mL (95% confidence interval, 4.4–10.3) in 1,25$(OH)_2D_2$, which was accompanied by a mean decrease of 9.9 pg/mL ($-$15.8 to $-$4.0) in 1,25$(OH)_2D_3$. No such differences accompanied administration of 1000 IU daily of vitamin D_3.

Conclusion: Vitamin D_2 and vitamin D_3 were effective in raising and maintaining total serum concentrations of 25(OH)D. Ingestion of vitamin D_2 also resulted in an increase in serum concentrations of 1,25$(OH)_2D_2$. This increase was accompanied by a comparable decrease in serum concentrations of 1,25$(OH)_2D_3$; therefore, the total 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] concentrations did not significantly change after 11 weeks compared with baseline levels. Ingestion of vitamin D_3 did not alter serum concentrations of 1,25$(OH)_2D_3$ or total 1,25$(OH)_2$D. Therefore, ingestion of 1000 IU vitamin D_2 or vitamin D_3 for 11 weeks was effective in raising total serum concentrations of 25(OH)D as well as sustaining serum concentrations of total 1,25$(OH)_2$D. (***J Clin Endocrinol Metab*** **98: 973–979, 2013)**

Vitamin D plays an integral role in bone health, specifically, in calcium and phosphorus homeostasis (1). Vitamin D deficiency and insufficiency, defined as serum 25-hydroxyvitamin D [25(OH)D] levels $<$20 and 21 to 29 ng/mL, respectively, is a major health concern that extends beyond skeletal ailments (2). Because there is a vitamin D receptor in most every tissue in the body, people of any age or race are at risk of becoming vitamin D deficient (3–5). It is an indiscriminate affliction that has been associated with a wide range of health issues such as autoimmune

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Abbreviations: CV, coefficient of variation; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 1,25$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D; 25$(OH)D_2$, 25-hydroxyvitamin D_2; 25$(OH)D_3$, 25-hydroxyvitamin D_3; LC-MS/MS, liquid chromatography–tandem mass spectroscopy.

diseases, diabetes, cardiovascular disease, and certain cancers (6–18). In a vitamin D–deficient state, the body only absorbs 10% to 15% of dietary calcium and approximately 60% of phosphorus. 1,25-Dihydroxyvitamin D [$1,25(OH)_2D$], the active form of vitamin D, increases the efficiency of intestinal calcium and phosphorus absorption by up to 40% and 80%, respectively (19).

Humans obtain vitamin D from diet and sunlight (1, 20–22). The main source of vitamin D_3 is from exposure to sunlight, accounting for more than 90% of the body's vitamin D requirement (23). Vitamin D_3 is synthesized in the skin and naturally found in oily fish, whereas vitamin D_2 is present in sundried and UV light–exposed mushrooms (24). Vitamin D_2 is produced from the irradiation of yeast and vitamin D_3 is produced from lanolin and both are used to fortify milk, other dairy products, orange juice, and cereals (1, 25). Both vitamin D_3 and vitamin D_2 are found in dietary supplements, but vitamin D_2 is the only prescription form available in the United States (1).

Endogenous, dietary, and supplemental forms of vitamin D_2 and vitamin D_3 are metabolized in sequential hydroxylations in the liver and kidneys to 25(OH)D and $1,25(OH)_2D$, respectively (1, 26). Some studies have suggested that vitamin D_2 was 30% to 50% less effective than vitamin D_3 in maintaining serum 25(OH)D levels (27–29). However, children receiving 2000 IU vitamin D_2 daily increased their serum 25(OH)D to the same level as children receiving a daily dose of 2000 IU vitamin D_3 (30). In addition, healthy adults taking 1000 IU vitamin D_2 or 1000 IU vitamin D_3 in a capsule or in orange juice were able to raise their blood level of 25(OH)D to the same level by the end of the 11-week study (31, 32). Men and women who took 50,000 IU vitamin D_2 twice a month for up to 6 years were able to maintain their serum 25(OH)D at >30 ng/mL without toxicity (33).

The purpose of this study was to determine 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_2$] levels in adults consuming 1000 IU vitamin D_2 or vitamin D_3 daily for 11 weeks using the state-of-the-art liquid chromatography-mass spectroscopy assay.

Subjects and Methods

Subjects and serum samples

To determine the effect of ingesting 1000 IU vitamin D_2 or 1000 IU vitamin D_3 on circulating blood levels of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ and total $1,25(OH)_2D$ we evaluated blood samples from a double-blind, placebo-controlled study that was approved by our institutional review board at Boston University Medical Center and published previously (32). Of the 105 subjects in the parent study, we were able to retrieve the 1 mL of the serum required for the $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ assays from 8 subjects (7 female and 1 male) who received the placebo, 9 subjects (8 female and 1 male) who received 1000 IU vitamin D_3, and 17 subjects (10 female and 7 male) who received 1000 IU vitamin D_2 daily for 11 weeks.

Analytical methods

Serum $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ concentrations were determined by liquid chromatography–tandem mass spectroscopy (LC-MS/MS) at Quest Diagnostic Laboratory (San Juan Capistrano, California). Before analysis, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were extracted from 1 mL of the patient serum samples through the use of a cospecific antibody targeted to the A ring of $1,25(OH)_2D$. To improve ionization and fragmentation in the mass spectrometer, analytes were derivatized using a Cookson-type reagent, 4′-phenyl-1,2,4-triazoline-3,5-dione. Stable isotope-labeled internal standards for both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were used throughout to improve accuracy and precision.

A multiplexed analytical HPLC system using a reverse-phase column and reverse-phase gradient was used to effect chromatographic separation. The flow of liquid solvent from the high-performance liquid chromatograph entered a heated nebulizer interface of a LC-MS/MS analyzer (Thermo Fisher Scientific, Waltham, Massachusetts).

Analytes were measured using the selected reaction monitoring mode whereby the mass of the intact analyte was selected in the first quadrupole, collisionally induced dissociation effected in the second quadrupole, and multiple fragment ions sequentially isolated by the third quadrupole.

The areas under the chromatographic peaks were determined, and calibration curves were constructed by plotting standard concentration vs peak area ratio of analyte/internal standard. Using the calibration curves, the concentrations of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were quantitated for patient samples.

The assay has an intraassay coefficient of variation (CV) of 9% and interassay CV of 12%. Serum PTH was determined using immunoassay/spectrophotometry (Quest Diagnostics Nichols Institute, San Juan Capistrano, California). The assay has an intraassay CV of 8% and interassay CV of 10%. Serum 25-hydroxyvitamin D_2 [$25(OH)D_2$] and 25-hydroxyvitamin D_3 [$25(OH)D_3$] concentrations were determined by LC-MS/MS as described previously (32).

Statistical analyses

Descriptive statistics (means, medians, and SDs) were computed for baseline and total change at end of the study for each

Table 1. Baseline of Vitamin D Metabolite Concentrations, by Treatment Group[a]

	Placebo (n = 8)	D2 (n = 17)[b]	D3 (n = 9)[c]
$25(OH)D_2$, ng/mL	0.9 (2.1)	3.8 (4.9)	0.9 (2.7)
$25(OH)D_3$, ng/mL	17.6 (7.8)	15.5 (6.9)	21.3 (12.9)
$1,25(OH)_2D_2$, pg/mL	0.0 (0.0)	2.8 (6.7)	0.0 (0.0)
$1,25(OH)_2D_3$, pg/mL	30.3 (8.1)	30.5 (9.0)	35.4 (13.0)

[a] Data are means (SD).

[b] 1000 IU vitamin D_2/d.

[c] 1000 IU vitamin D_3/d.

treatment group. Comparisons of trends between groups using all time points were performed using generalized estimating equations, which acknowledge the repeated measurements on subjects in computing estimates and confidence intervals, with statistical control for baseline levels; ie, comparisons of $25(OH)D_2$ on treatment were performed with control for baseline $25(OH)D_2$ levels. Differences were considered statistically significant if null hypotheses of no difference could be rejected at the .05 level.

Results

Of the 34 subjects, 28 subjects (82%) had 25(OH)D levels <30 ng/mL at baseline. Descriptions of baseline levels of all vitamin D metabolite concentrations are given in Table 1. Baseline levels were roughly comparable, although subjects assigned to 1000 IU vitamin D_2 had slightly greater $25(OH)D_2$ levels at study start, compared with those of other subjects, and those assigned to 1000 IU vitamin D_3 had slightly greater $25(OH)D_3$ levels at study start. Most subjects began the study with $1,25(OH)_2D_2$ levels that were undetectable, whereas the overall mean (SD) $1,25(OH)_2D_3$ at study start was 32.0 (10.2) pg/mL.

The trajectory of change in 25(OH)D levels is depicted in Figure 1, with change from the first to last visit described in Table 2. Subjects assigned to 1000 IU vitamin D_2 or vitamin D_3 exhibited the expected increases in $25(OH)D_2$ and $25(OH)D_3$, respectively. In contrast, assignment to 1000 IU vitamin D_2 appeared to be associated with

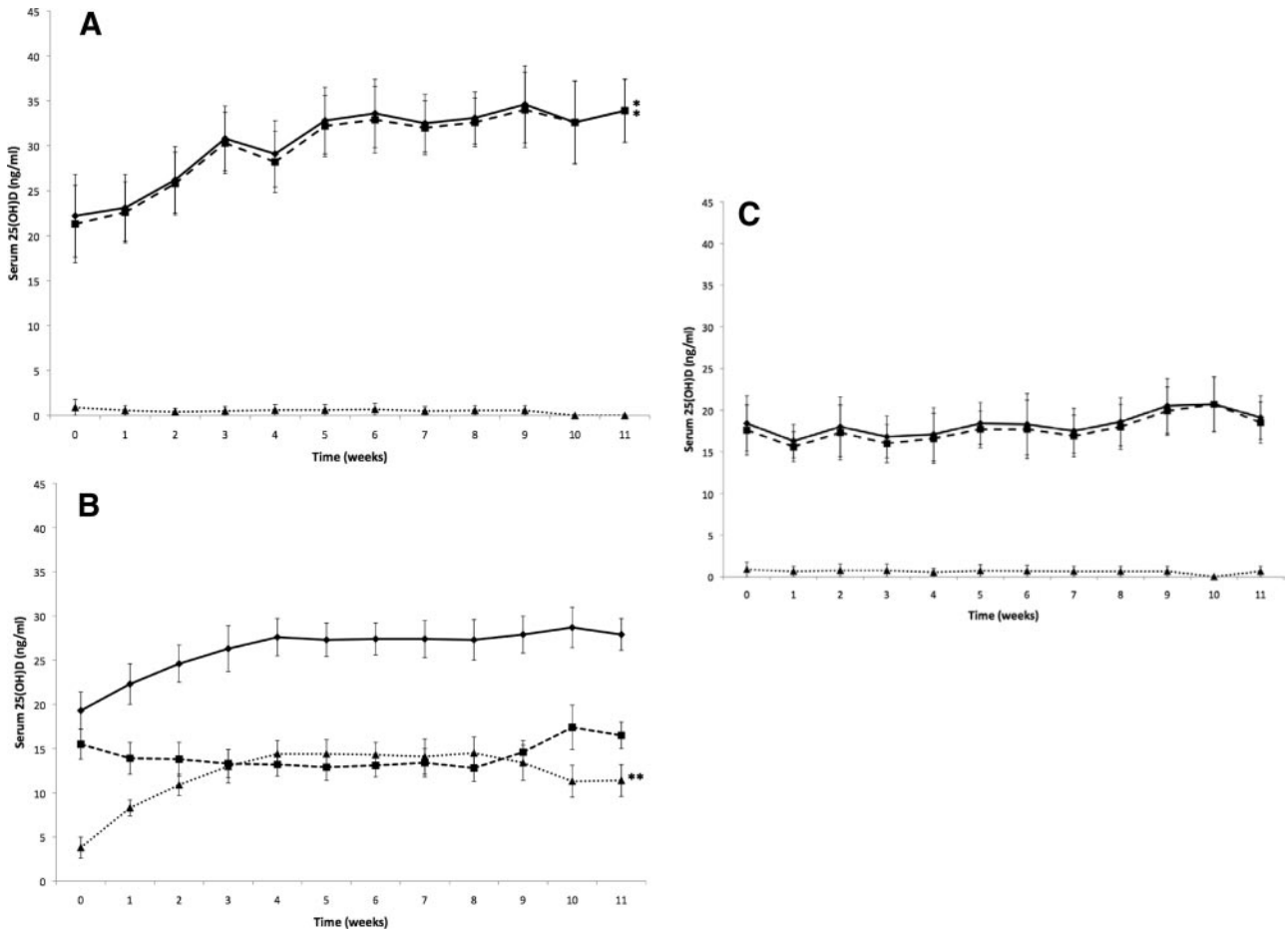

Figure 1. A, Total 25(OH)D levels are demonstrated over time. Shown are mean (± SEM) serum total 25(OH)D (♦; n = 9), serum $25(OH)D_3$ (■; n = 9), and serum $25(OH)D_2$ (▲; n = 9) after oral administration of either 1000 IU vitamin D_3 in orange juice or vitamin D_3 in capsules. There were no statistically significant differences in serum $25(OH)D_2$ over time in the groups receiving either 1000 IU vitamin D_3 in orange juice or vitamin D_3 in capsules (2-tailed paired *t* test, $P > .05$). *$P < .05$ comparing serum total 25(OH)D and $25(OH)D_3$ over time in the groups receiving either 1000 IU vitamin D_3 in orange juice or vitamin D_3 in capsules. B, Total 25(OH)D levels are demonstrated over time. Shown are mean (± SEM) serum total 25(OH)D (♦; n = 17), serum $25(OH)D_3$ (■; n = 17), and serum $25(OH)D_2$ (▲; n = 17) after oral administration of either 1000 IU vitamin D_2 in orange juice or vitamin D_2 in capsules. There were no statistically significant differences in serum total 25(OH)D and $25(OH)D_3$ over time in the groups receiving either 1000 IU vitamin D_2 in orange juice or vitamin D_2 in capsule (2-tailed paired *t* test, $P > .05$). **$P = .0005$ comparing serum $25(OH)D_2$ over time in the groups receiving either 1000 IU vitamin D_2 in orange juice or vitamin D_2 in capsules. C, Total 25(OH)D levels are demonstrated over time. Shown are mean (± SEM) serum total 25(OH)D (♦; n = 8), serum $25(OH)D_3$ (■; n = 8), and serum $25(OH)D_2$ (▲; n = 8) after oral administration of either unfortified orange juice or placebo capsule. There were no statistically significant differences in serum total 25(OH)D, $25(OH)D_3$, and $25(OH)D_2$ over time in the groups receiving either unfortified orange juice or placebo capsule (2-tailed paired *t* test, $P > .05$).

Table 2. Change From Baseline in Vitamin D Parameters, by Treatment Group[a]

	Placebo (n = 8)	D2 (n = 17)[b]	D3 (n = 9)[c]
$25(OH)D_2$, ng/mL	−1.0 (2.4)	6.5 (5.5)	−0.9 (2.7)
P	.31	<.001	.35
$25(OH)D_3$, ng/mL	1.8 (4.0)	1.3 (11.2)	12.3 (7.5)
P	.28	.65	.001
$1,25(OH)_2D_2$, pg/mL	0.0 (0.0)	5.2 (11.4)	0.0 (0.0)
P	>.99	.09	>.99
$1,25(OH)_2D_3$, pg/mL	0.7 (7.2)	−6.8 (14.1)	−1.1 (13.8)
P	.81	.07	.82

[a] Data are means (SD). Baseline represents the last visit minus baseline, other visits ignored. *P* values from 2-sided Student's *t* test of hypothesis of zero change.

[b] 1000 IU vitamin D_2/d.

[c] 1000 IU vitamin D_3/d.

increases in $1,25(OH)_2D_2$ and similar decreases in $1,25(OH)_2D_3$ at study end, whereas those subjects assigned to 1000 IU vitamin D_3 exhibited no appreciable change in either $1,25(OH)_2D_2$ or $1,25(OH)_2D_3$; this observation was consistent with trends over the course of the treatment period (Figure 2).

Results of longitudinal regression analysis were consistent with these exploratory findings (Table 3). Compared with placebo, assignment to 1000 IU vitamin D_2 was associated with mean (95% confidence interval) on-treatment increases of 7.4 (4.4–10.3) pg/mL in $1,25(OH)_2D_2$ accompanied by decreases of similar magnitude in $1,25(OH)_2D_3$, whereas those assigned to 1000 IU vitamin D_3 exhibited little appreciable change in either parameter. The mean total 25(OH)D increase was greater in those assigned to 1000 IU vitamin D_3 than in those assigned to 1000 IU vitamin D_2, but evidence in favor of a greater increase associated with 1000 IU vitamin D_3 did not achieve statistical significance when all data were taken into consideration in the longitudinal model ($P = .07$).

Discussion

Although there continues to be debate as to whether vitamin D_2 is as effective as vitamin D_3 in maintaining blood levels of 25(OH)D, less is known about how $25(OH)D_2$ is metabolized to $1,25(OH)_2D_2$ and what happens to $1,25(OH)_2D_3$ and total $1,25(OH)_2D$ concentrations. It had been reported previously that the concentrations of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were proportional to distribution of $25(OH)D_2$ and $25(OH)D_3$ (34). Hartwell et al (35) observed that premenopausal women who received 4000 IU vitamin D_2 for 8 weeks demonstrated significant increases in serum $1,25(OH)_2D_2$ at 4 weeks that continued to increase at the end of the 8-week study with comparable declines in the serum concentrations of $1,25(OH)_2D_3$. They further observed over this 8-week period that the total serum concentrations of $1,25(OH)_2D$ did not significantly change. It remained to be determined whether and when the $1,25(OH)_2D_2$ would reach a plateau and what the consequences would be on the serum concentrations of $1,25(OH)_2D_3$ because Hartwell et al (35) only measured blood levels at 4 and 8 weeks. Furthermore, because they only performed their study in premenopausal women, it is not known whether men and postmenopausal women would also metabolize vitamin D_2 in a manner similar to that of premenopausal women.

In our previous studies, we observed that healthy male and female adults with a wide age range (18–79 years) who ingested 1000 IU vitamin D_2 or vitamin D_3 in a capsule or in orange juice were able to raise their blood levels of $25(OH)D_2$ and $25(OH)D_3$ by ~10 ng/mL (31, 32). Furthermore, the groups who received vitamin D_2 demonstrated no significant change in the circulating concentrations of $25(OH)D_3$, and the total blood levels of 25(OH)D were the same whether the adults ingested 1000 IU vitamin D_2 or vitamin D_3. These blood samples were archived and stored at −80°C. Because the $1,25(OH)_2D$ assay required 1 mL of serum, we recovered 34 samples meeting this requirement; 28.1% were from male participants. The concentrations of $25(OH)D_2$ and $25(OH)D_3$ gradually increased in a similar fashion over a period of 6 weeks to a plateau level that was sustained for the duration of the trial period. In this analysis, we confirmed that there was no significant difference using our longitudinal model in the increase in serum $25(OH)D_2$ for the group who received vitamin D_2 compared with the increase in $25(OH)D_3$ in the group who received vitamin D_3. This was reflected by the observation that the total serum 25(OH)D concentrations were no different for the groups ingesting 1000 IU vitamin D_2 or 1000 IU vitamin D_3 for 11 weeks in this study and the parent studies (31, 32). However, when only the change from baseline in serum $25(OH)D_2$ and $25(OH)D_3$ concentrations in this smaller subset of samples from the parent study were compared with the levels at 11 weeks, there was a small, significant ($P < .04$) difference with a 2-sample *t* test. No significant change was observed in the group who received placebo (Figure 1).

Serum concentrations of $1,25(OH)_2D_3$ and total $1,25(OH)_2D$ did not change throughout the 11 weeks in the adults who ingested 1000 IU vitamin D_3 daily nor in the group who had no additional vitamin D, ie, the placebo group. However, for the group who received 1000 IU vitamin D_2, there was a gradual increase in the serum concentrations of $1,25(OH)_2D_2$, which reached a peak concentration at 6 weeks that was sustained for the ensuing 5 weeks and mirrored the increase in the serum $25(OH)D_2$

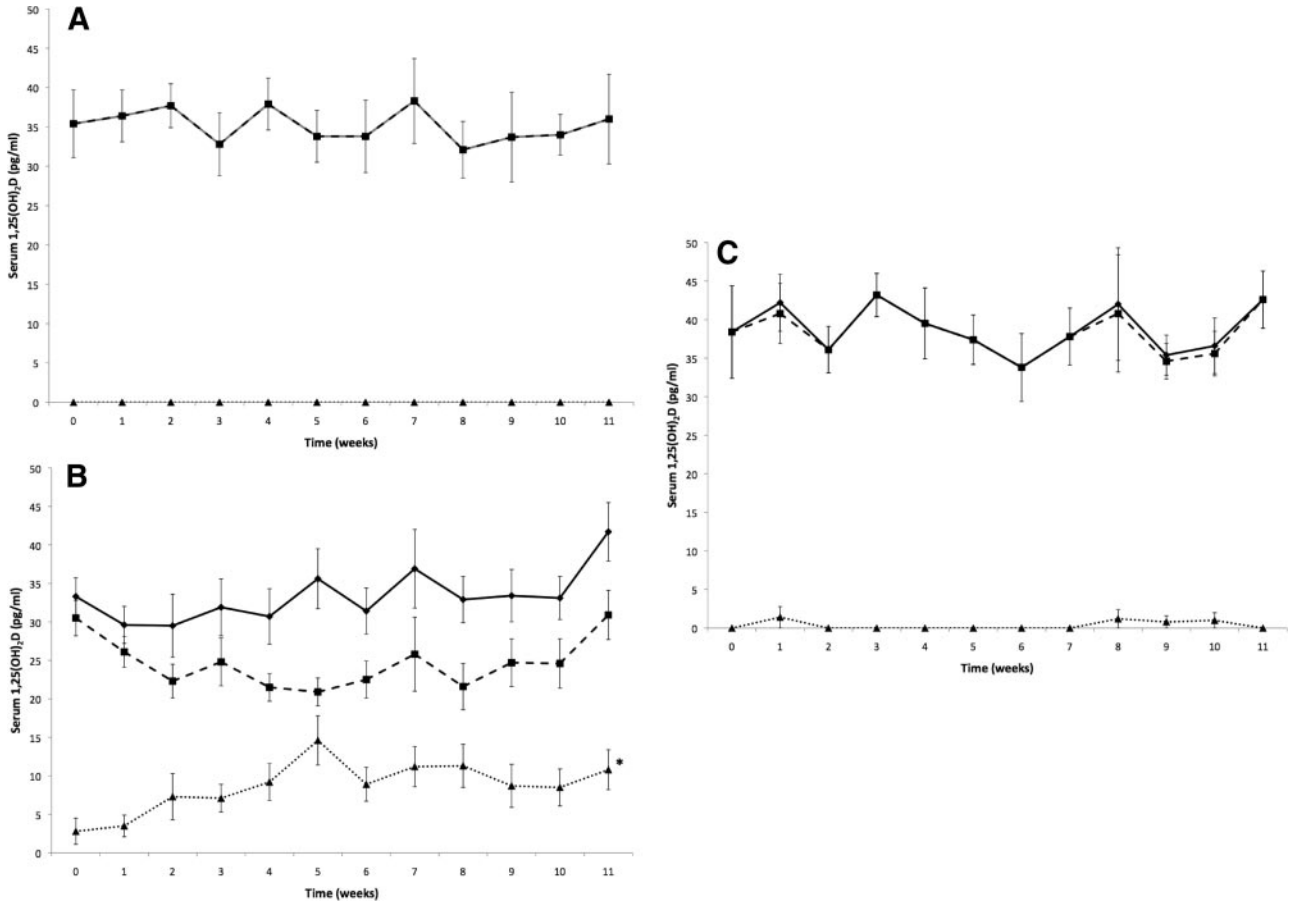

Figure 2. A, Total $1,25(OH)_2D$ levels are demonstrated over time. Shown are mean (± SEM) serum total $1,25(OH)_2D$ (♦; n = 9), serum $1,25(OH)_2D_3$ (■; n = 9), and serum $1,25(OH)_2D_2$ (▲; n = 9) after oral administration of either 1000 IU vitamin D_3 in orange juice or vitamin D_3 in capsules. There were no statistically significant differences in serum $1,25(OH)_2D$, $1,25(OH)_2D_3$, or $1,25(OH)_2D_2$ over time in the groups receiving either 1000 IU vitamin D_3 in orange juice or vitamin D_3 in capsules (2-tailed paired *t* test, $P > .05$). B, Total $1,25(OH)_2D$ levels are demonstrated over time. Shown are mean (± SEM) serum total $1,25(OH)_2D$ (♦; n = 17), serum $1,25(OH)_2D_3$ (■; n = 17), and serum $1,25(OH)_2D_2$ (▲; n = 17) after oral administration of either 1000 IU vitamin D_2 in orange juice or vitamin D_2 in capsules. There were no statistically significant differences in serum total $1,25(OH)_2D$ and $1,25(OH)_2D_3$ over time in the groups receiving either 1000 IU vitamin D_2 in orange juice or vitamin D_2 in capsules (2-tailed paired *t* test, $P > .05$). $^*P < .05$ comparing serum $1,25(OH)_2D_2$ over time in the groups receiving 1000 IU vitamin D_2 in orange juice or in capsules. C, Total $1,25(OH)_2D$ levels demonstrated over time. Shown are mean (± SEM) serum total $1,25(OH)_2D$ (♦; n = 8), serum $1,25(OH)_2D_3$ (■; n = 8), and serum $1,25(OH)_2D_2$ (▲; n = 8) after oral administration of either unfortified orange juice or placebo capsule. There were no statistically significant differences in serum total $1,25(OH)_2D$, $1,25(OH)_2D_3$, and $1,25(OH)_2D_2$ over time in the groups receiving either unfortified orange juice or placebo capsule (2-tailed paired *t* test, $P > .05$).

concentrations (Figure 2). Although there appeared to be a slight decline in the serum concentrations of $1,25(OH)_2D_3$, it was not statistically significant, and, furthermore, at the end of 11 weeks, the blood levels were the same as they were at baseline. There was a trend for an increase in total $1,25(OH)_2D$, but the total at baseline (33 ± 2.4 pg/mL) was not statistically significantly different from that at 11 weeks (41.7 ± 3.8 pg/mL).

These results add to the initial observation (35) that vitamin D_2 was as effective as vitamin D_3, not only in

Table 3. Comparison of Change in Vitamin D Metabolite Concentrations[a]

	25(OH)D_2, ng/mL	25(OH)D_3, ng/mL	$1,25(OH)_2D_2$, pg/mL	$1,25(OH)_2D_3$, pg/mL
Placebo (n = 8)	Referent	Referent	Referent	Referent
D2 (n = 17)[b]	8.7 (7.1 to 10.3)	−1.9 (−3.8 to 0.01)	7.4 (4.4 to 10.3)	−9.9 (−15.8 to −4.0)
D3 (n = 9)[c]	−0.2 (−1.1 to 0.7)	11.1 (7.4 to 14.8)	−0.3 (−0.7 to 0.1)	−0.6 (−6.9 to 5.7)

Data are means (95% confidence interval) for on-treatment minus baseline. Estimates were obtained via generalized estimating equations, controlling for baseline vitamin D metabolite levels.

[a] 25(OH)D and $1,25(OH)_2D$.

[b] 1000 IU vitamin D_2/d.

[c] 1000 IU vitamin D_3/d.

raising blood levels of total 25(OH)D (31, 32) but also in sustaining blood levels of total $1,25(OH)_2D$ because the total $1,25(OH)_2D$ was the same after 11 weeks in both of the groups who received 1000 IU vitamin D_2 or 1000 IU vitamin D_3. Results from this study suggest that in both young and older men and women $25(OH)D_2$ was recognized by the kidneys and efficiently converted to $1,25(OH)_2D_2$. The increase in the blood level was approximately 10 pg/mL for 1000 IU vitamin D_2, which was 1000 times less than the 10 ng/mL increase observed for $25(OH)D_2$. What remains unknown is whether $25(OH)D_2$ is recognized differently by the kidneys and, instead of substituting for $25(OH)D_3$, it acts as an additional substrate possibly increasing the total blood levels of $1,25(OH)_2D$ as suggested in Figure 2. Although this trial was not powered to detect such an increase, it is intriguing to speculate that ingesting vitamin D_2 could potentially increase total circulating concentrations of $1,25(OH)_2D$.

In conclusion, our results demonstrate that vitamin D_2 is metabolized in men and women in a fashion similar to that of vitamin D_3 to both its 25-hydroxy and 1,25-dihydroxy metabolites.

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