

Evidence that vitamin D₃ increases serum 25-hydroxyvitamin D more efficiently than does vitamin D₂¹⁻³

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ABSTRACT In all species tested, except humans, biological differences between vitamins D₂ and D₃ are accepted as fact. To test the presumption of equivalence in humans, we compared the ability of equal molar quantities of vitamin D₂ or D₃ to increase serum 25-hydroxyvitamin D [25(OH)D], the measure of vitamin D nutrition. Subjects took 260 nmol (≈4000 IU) vitamin D₂ ($n = 17$) or vitamin D₃ ($n = 55$) daily for 14 d. 25(OH)D was assayed with a method that detects both the vitamin D₂ and D₃ forms. With vitamin D₃, mean (±SD) serum 25(OH)D increased from 41.3 ± 17.7 nmol/L before to 64.6 ± 17.2 nmol/L after treatment. With vitamin D₂, the 25(OH)D concentration went from 43.7 ± 17.7 nmol/L before to 57.4 ± 13.0 nmol/L after. The increase in 25(OH)D with vitamin D₃ was 23.3 ± 15.7 nmol/L, or 1.7 times the increase obtained with vitamin D₂ (13.7 ± 11.4 nmol/L; $P = 0.03$). There was an inverse relation between the increase in 25(OH)D and the initial 25(OH)D concentration. The lowest 2 tertiles for basal 25(OH)D showed larger increases in 25(OH)D: 30.6 and 25.5 nmol/L, respectively, for the first and second tertiles. In the highest tertile [25(OH)D >49 nmol/L] the mean increase in 25(OH)D was 13.3 nmol/L ($P \leq 0.03$ for comparison with each lower tertile). Although the 1.7-times greater efficacy for vitamin D₃ shown here may seem small, it is more than what others have shown for 25(OH)D increases when comparing 2-fold differences in vitamin D₃ dose. The assumption that vitamins D₂ and D₃ have equal nutritional value is probably wrong and should be reconsidered. *Am J Clin Nutr* 1998;68:854-8.

KEY WORDS Cholecalciferol, ergocalciferol, 25-hydroxyvitamin D, vitamin D₂, vitamin D₃, 25-hydroxycholecalciferol, 25-hydroxyergocalciferol, 25-hydroxycalciferol, adults, humans

INTRODUCTION

Although 1,25-dihydroxyvitamin D [1,25(OH)₂D] is the most potent vitamin D metabolite, there is now clear evidence that serum concentrations of its precursor, 25-hydroxyvitamin D [25(OH)D], correlate better with observed calcium absorption efficiency (1, 2). It was shown in humans that 25(OH)D affects calcium absorption efficiency without any changes in circulating total 1,25(OH)₂D (3). These findings substantiate the relatively recent concept that the most objective measure of vitamin D nutritional status in humans is the circulating concentration of 25(OH)D (4).

On the basis of studies done in the 1930s, it has been assumed that vitamin D₂ and vitamin D₃ are equally effective in humans. No conclusive difference, in terms of preventing infantile rickets, was shown for the different forms of vitamin D. Therefore, although recognizing the difficulties with earlier studies that compared vitamins D₂ and D₃, Park concluded in 1940 that, "For practical purposes, the vitamin D in vitosterol (vitamin D₂) may be regarded as being equal to the vitamin D of cod liver oil (vitamin D₃)" (5). On the basis of such evidence, both the British and American pharmacopoeias continue to define the units of vitamin D with the simple conversion of gram quantity, where 1 international unit (IU) equals 25 ng of either form of the vitamin (6-8). This is despite the obvious difference in molecular weight (399 compared with 384 for vitamin D₂ and vitamin D₃, respectively). There is no objective contemporary evidence that in humans vitamins D₂ and D₃ are of equivalent value in terms of increasing circulating 25(OH)D.

All nonhuman species tested showed differences in response to vitamins D₂ and D₃. In birds, vitamin D₂ is only one-tenth as effective as vitamin D₃ at increasing 25(OH)D (9). In monkeys, vitamin D₃ is far more effective than vitamin D₂ (10). Surprisingly, in rats vitamin D₂ has been reported to be more effective than vitamin D₃ (11).

Human studies comparing the increase in 25(OH)D with intake of vitamins D₂ and D₃ have yielded inconsistent results. All studies show greater efficacy with vitamin D₃, but usually sample sizes have been too small to be statistically conclusive (12, 13). One study found vitamin D₃ to be more effective than vitamin D₂ (14), but the sample size was small (<11 subjects per group). Furthermore, previous studies did not consider the confounding effects of vitamin D stability or seasonal solar exposure on background concentrations of vitamin D. To help resolve the issue of equivalence, we compared the ability of an equal molar quantity of either vitamin D₂ or vitamin D₃ to elevate serum 25(OH)D over a short period, between February and

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early May when vitamin D concentrations and human solar exposure are minimal.

SUBJECTS AND METHODS

Both vitamins were purchased in crystalline form from Sigma (St Louis) and dissolved in US Pharmacopoeia (USP)-grade ethanol. Appropriately controlled ultraviolet absorption spectra remained identical before and after the study for each. The molar concentration of vitamins D₂ and D₃ was adjusted to 260 µmol/0.6 L ethanol, based on absorbance at 265 nm [7.90 absorbance units (AU), using the extinction coefficient 18300 AU·mol⁻¹·L⁻¹] on a Hewlett-Packard 8452A diode array spectrophotometer (Palo Alto, CA). In addition, chromatographic analysis consistently indicated only the one peak appropriate for each vitamin D preparation.

The protocol was carried out between February and early May, when serum 25(OH)D is at its annual low concentration in Toronto. There were 72 volunteer subjects taking vitamin D whose mean (±SD) age was 38 ± 9 y. Of these, 34 were randomly assigned in a double-blind manner to take either vitamin D₃ or vitamin D₂. The rest of the subjects were given vitamin D₃ because another objective was to understand the effects of vitamin D supplementation on the change in serum 25(OH)D. The subjects took 260 nmol (≈100 µg, or 4000 IU) vitamin D/d for 14 consecutive days. The vitamin D₂-treated group consisted of 5 men and 12 women; the vitamin D₃-treated group had 19 men and 36 women. A third group consisted of 17 untreated subjects who did not wish to take the vitamin D supplement but who agreed to have blood drawn at the appropriate times. None of the subjects had been or were taking vitamin D supplements in excess of the recommended nutrient intake (200 IU/d, or 5 µg/d). Individuals who had taken or were about to take a southern vacation during the winter were excluded from the study. This protocol was approved by the University of Toronto Ethics Committee and each subject signed a consent form.

25(OH)D concentrations were determined by using the Incstar radioimmunoassay kit (Stillwater, MN). Serum samples from each patient (before and after dosing) were analyzed together in the same run. In our laboratory, the results of the 25(OH)D assay method were consistently within 1 SD of the method group mean in the international External Quality Assessment Scheme proficiency survey for this metabolite. Serum 1,25(OH)₂D was extracted and purified on C₁₈-OH cartridges and then assayed by using the classic radioreceptor assay involving competitive binding to 1,25(OH)₂D receptor prepared from calf thymus.

Statistical calculations were performed by using SPSS version 7.5 (SPSS Inc, Chicago). All data are expressed as means ± SDs. Relations between variables were analyzed by linear regression and bivariate correlation. Means comparisons were performed by paired and unpaired *t* test and by analysis of covariance (ANCOVA) to correct for slight differences in baseline 25(OH)D concentrations between groups. The increase in 25(OH)D in the vitamin D₃-supplemented group, divided into tertiles, was analyzed by one-way analysis of variance (ANOVA), and Tukey's honestly significant differences (HSD) test was used to detect significant differences. Reported *P* values are two sided.

RESULTS

The ratio of men to women in the 2 vitamin D treatment groups was 5:12 for vitamin D₂ and 18:36 for vitamin D₃— essentially

TABLE 1
25-Hydroxyvitamin D concentrations before and after vitamin D supplementation¹

	Study group		
	Vitamin D ₂ (n = 17)	Vitamin D ₃ (n = 55)	Untreated (n = 17)
	nmol/L		
Baseline	43.7 ± 17.7	41.3 ± 17.7	39.8 ± 18.7
Final	57.4 ± 13.0 ²	64.6 ± 17.2 ²	42.8 ± 20.7
Change	13.7 ± 11.4	23.3 ± 15.7 ³	3.0 ± 8.1

¹ $\bar{x} \pm SD$. Baseline 25-hydroxyvitamin D concentrations were not significantly different among groups (one-way ANOVA).

²Significantly different from baseline, *P* < 0.02 (paired *t* test).

³Significantly greater change than for vitamin D₂, *P* = 0.03 (unpaired *t* test).

the same. There were no significant differences between men and women in terms of basal serum 25(OH)D concentrations or in the changes observed with vitamin D dosing. Both the vitamin D₂ and vitamin D₃ supplements significantly increased serum 25(OH)D (*P* < 0.02; **Table 1**). The vitamin D₂ supplement increased 25(OH)D by 13.7 nmol/L whereas the vitamin D₃ supplement increased it by 23.3 nmol/L. The mean difference between the increases was 9.6 nmol/L, and this had a 95% CI of 1.4 and 17.8 nmol/L. There was no change in 25(OH)D concentration during the study period in untreated subjects.

The concentration of 1,25(OH)₂D was not affected by either supplement, and there were no differences between group means (*P* > 0.35). For the vitamin D₂ group, serum 1,25(OH)₂D concentrations were 90.7 ± 23.6 pmol/L at baseline and 93.3 ± 25.4 pmol/L after the end of the protocol; for the vitamin D₃ group the corresponding before and after values were 84.5 ± 30.1 and 85.9 ± 20.9 pmol/L.

The plot of basal 25(OH)D concentration against the increase in 25(OH)D for the vitamin D₃-treated group showed a significant inverse linear correlation (*r* = -0.472, *P* < 0.001; **Figure 1**).

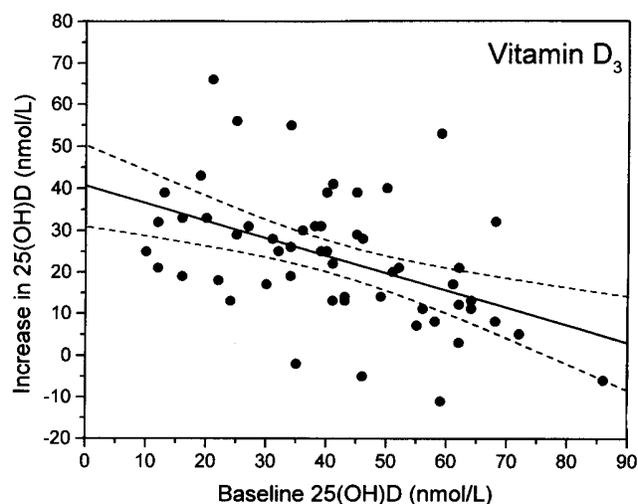


FIGURE 1. A plot of baseline 25-hydroxyvitamin D [25(OH)D] concentrations versus the increase in 25(OH)D concentrations after vitamin D₃ supplementation in healthy volunteers. The data showed a significant inverse relation (*r* = -0.472, *P* < 0.001). Dotted lines indicate the 95% CI of the mean.

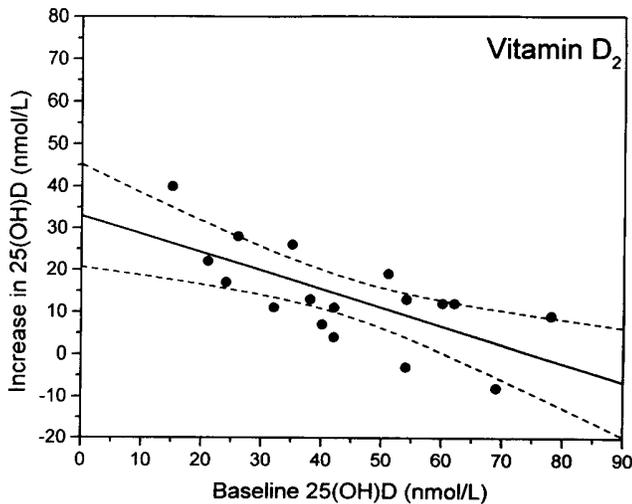


FIGURE 2. Baseline 25-hydroxyvitamin D [25(OH)D] concentrations versus the change in 25(OH)D concentrations after vitamin D₂ supplementation. The data showed an inverse relation similar to that of the vitamin D₃-supplemented group ($r = -0.681$, $P = 0.003$).

A similar inverse relation was also found in the vitamin D₂-treated group ($r = -0.681$, $P = 0.003$; **Figure 2**). For the vitamin D₃-treated group, the regression equation between the change in 25(OH)D (change) and the baseline value (baseline) was as follows: change = $-0.418(\text{baseline}) + 40.6$; for the vitamin D₂-treated group: change = $-0.440(\text{baseline}) + 33.0$.

On the basis of the subjects baseline 25(OH)D concentrations, data from the vitamin D₃-treated group was divided into tertiles to test for the effect of prior vitamin D nutrition on the response to vitamin D supplementation. The first (lowest) tertile had the largest increase in 25(OH)D concentration whereas the third tertile showed less than one-half of that increase (**Table 2**). One-way ANOVA and Tukey's HSD test indicated that the increase in 25(OH)D in the third tertile was smaller than the increase seen in the first or second tertile.

Because the increase in 25(OH)D after dosing was affected by baseline concentration, the baseline concentration was used as a covariate in the ANCOVA to adjust for the slight differences between the 2 vitamin D-treated groups. After accounting for the slight differences in baseline concentrations between the vitamin D₃- and D₂-supplemented groups, the increase in 25(OH)D with vitamin D₃ supplementation remained significantly greater than that for vitamin D₂ ($P = 0.03$).

DISCUSSION

As expected, vitamin D₂ and vitamin D₃ both elevated serum 25(OH)D concentrations. With vitamin D₃, the increase in 25(OH)D was 70% greater (1.70 times) than the increase obtained with vitamin D₂. At first glance, this difference in the 25(OH)D response may seem modest. However, in studies in which the same form of vitamin D was given at doses that differed by 2- to 5-fold, the differences in 25(OH)D response have been even smaller and more difficult to measure. Van Der Klis et al (15) found no difference in the final serum 25(OH)D concentration achieved after 1 mo of 400 or 800 IU vitamin D₃. Davie et al (16) treated subjects with 400 or 1000 IU vitamin D₃/d and reported that the higher

TABLE 2
Increase in serum 25-hydroxyvitamin D in vitamin D₃-treated group stratified into tertiles by baseline 25-hydroxyvitamin D concentration¹

Tertile	Baseline	Increase ²
	nmol/L	
1, 10–34 nmol/L ($n = 19$)	22.3 ± 7.9	30.6 ± 16.2
2, 35–49 nmol/L ($n = 18$)	41.1 ± 4.1	25.5 ± 11.7
3, 50–86 nmol/L ($n = 18$)	61.5 ± 8.5	13.3 ± 13.9^3

¹ $\bar{x} \pm \text{SD}$.

²Significant differences according to tertile of baseline 25-hydroxyvitamin D concentration, $P = 0.002$ (one-way ANOVA).

³Significantly less than for tertile 1, $P = 0.001$, and tertile 2, $P = 0.03$ (Tukey's honestly significant-difference test).

dose resulted in an increase in 25(OH)D that was only 17% more than that achieved with the lower dose. Lips et al (17) compared 400 and 800 IU vitamin D₃/d and reported that the higher dose increased 25(OH)D by 30% more than that achieved with the lower dose. Recently, Francis et al (18) gave subjects vitamin D₂ in doses of 500 or 1000 IU/d; the higher dose increased the 25(OH)D concentration by an insignificant 2 nmol/L more than that with the lower dose. In a study using moderately higher doses, Stamp et al (19) compared 1800 with 10000 IU vitamin D₂/d (dose ratio of 5.5) and reported an increase in 25(OH)D with the higher dose that was 56% more than that seen with the lower dose. According to data in the literature, more than a 5-fold increment in vitamin D dose would be required to achieve the 70% difference in 25(OH)D response that we observed between the same dose of vitamin D₃ and vitamin D₂.

There is other work consistent with our findings. In an earlier study by Chapuy et al (20), 800 IU vitamin D₂/d was used to treat postmenopausal women. This raised 25(OH)D concentrations from the initial mean of 43 to 71 nmol/L in 6 mo. In a subsequent study by the same group (21), the same dose of vitamin D₃ was used instead because of the report by Tjellesen et al (14) that vitamin D₂ was less effective at raising 25(OH)D concentrations in premenopausal women. In the later Chapuy et al study, the basal 25(OH)D concentration was 40 nmol/L and it reached 100 nmol/L by 6 mo. We recognize that there are difficulties in comparing results across studies, but the doses, subject groups, and treatment durations were similar and the studies were carried out by the same researchers. The 2-fold difference in the rise in serum 25(OH)D between the protocols using equal vitamin D₂ and D₃ doses by Chapuy et al (20, 21) is consistent with what we observed here.

Previous reports comparing efficacy of vitamins D₂ and D₃ in humans may have been influenced by several factors (12, 13, 22). Particularly troublesome is the stability of the vitamin D preparations used. Before carrying out the present study, we tested the vitamin D preparations made for us by the pharmacy departments of 2 local hospitals. At both institutions it was conventional to prepare the vitamin D in "simple syrup," an aqueous sugar solution in which the vitamin D broke down within days. This breakdown was particularly striking for vitamin D₂. The peak ultraviolet absorbance at 265 nm was distinct in the preparations initially, but decreased significantly within days. Within weeks, the characteristic vitamin D absorption peak and valley at 265 and 220 nm, respectively, had disappeared completely from the preparations of the 2 hospital pharmacies.

Consistent with our observation of vitamin D breakdown, Whyte et al (13) determined the potency of intramuscular vitamin

D₂ and D₃ preparations by bioassay and biochemical methods. Surprisingly, they found that the vitamin D content differed significantly from the manufacturer's labeled claim, in some cases by as much as 50%. Vitamins D₂ and D₃ have long been known to degrade differently, particularly when exposed to varying temperatures, humidity, or even storage in different containers (23). Moreover, different theoretically inert constituents in vitamin D formulations can substantially affect vitamin D stability (24). There is no indication in earlier studies comparing vitamins D₂ and D₃ in humans that vitamin D stability was controlled or monitored. We prepared our own vitamin D doses in USP-grade ethanol in collaboration with the hospital pharmacy and validated both vitamin D preparations before and after the dosing period to verify that there was no change in vitamin D dosage.

Endogenous production of vitamin D₃ through ultraviolet light exposure could have confounded earlier studies. In one study the time of dosing was neither considered nor specified (12). Two studies specified early summer or "from April to November" (13, 22), when solar exposure would have increased endogenous production of vitamin D. We conducted our study between February and early May, when the basal concentration of 25(OH)D would have been at its annual nadir. Our untreated subjects showed no change in serum 25(OH)D, indicating that endogenous production of vitamin D did not influence the outcome. Finally, previous studies were hampered by insufficient statistical power because all groups had ≤ 10 subjects (12, 13, 22) and degrees of freedom for *t* tests were < 20 . In the present study, the statistical degree of freedom was 70.

According to experiments in rats, hormones may influence vitamin D-25-hydroxylase (calciferol 25-hydroxylase; 25). If the same effect were to occur in humans, it is unlikely to have affected the present findings because the ratios of men to women were essentially identical in the groups. Furthermore, there were no significant differences in the 25(OH)D results between sexes.

We found that the increase in serum 25(OH)D after vitamin D supplementation was dependent on prior vitamin D nutrition. Above 50 nmol basal 25(OH)D/L, the effect of vitamins D₂ and D₃ at increasing serum 25(OH)D concentrations diminished progressively. We may have been able to detect the inverse relation between basal 25(OH)D and the rise in 25(OH)D because we carried out the dosing at the annual nadir for 25(OH)D when the contribution of endogenous vitamin D production is minimal. In addition, these Toronto subjects had 25(OH)D concentrations (mean: 41 nmol/L) that were more similar to European values than they were to the mean of 75 nmol/L reported for US cities (26). Comparison between our results and the US results is valid because both laboratories now use the same method to measure 25(OH)D and both participate in the External Quality Assessment Scheme proficiency survey, sharing samples. At the higher basal 25(OH)D concentrations in US cities, the inverse relation shown in Figures 1 and 2 and Table 2 could go undetected unless normal subjects were preselected for lower 25(OH)D concentrations. With basal 25(OH)D concentrations > 50 nmol/L, the phenomenon must approach a plateau that is not quite evident from the data presented in Figures 1 and 2 because it is unlikely that extra vitamin D would ever cause a decrease in 25(OH)D concentration.

The observation that the increase in serum 25(OH)D relates inversely to basal 25(OH)D concentrations has been made before. In subjects exposed to ultraviolet light treatment, Mawer et al (27), Large et al (28), and Snell et al (29) showed figures illustrating similar results. MacLennan and Hamilton (30) described a

similar response to vitamin D treatments, in which 25(OH)D increased more in those with lower initial 25(OH)D concentrations. All of these studies attributed the phenomenon to product inhibition of liver vitamin D-25-hydroxylase. In rats, vitamin D supplementation was shown to have a marked lowering effect on vitamin D-25-hydroxylase both in vitro and in vivo (31). Our results show that the same apparent product inhibition applies to vitamin D-25-hydroxylase of both vitamin D₂ and vitamin D₃ in humans. Feedback inhibition of vitamin D-25-hydroxylase would account for the difficulty in showing the vitamin D-dose-related responses in serum 25(OH)D discussed above (15-19).

Several mechanisms could contribute to the greater capacity of vitamin D₃ to increase 25(OH)D concentration. We did not determine the intestinal absorption of vitamin D. Studies of tritium-labeled vitamin D₂ and vitamin D₃ in healthy subjects found similar fecal recoveries after oral dosing (13), and suggest that different intestinal absorption is not the reason. The relative affinity for vitamin D-binding protein (DBP) and substrate affinity for vitamin D₃ by vitamin D-25-hydroxylase should also be considered. Nilsson et al (32) measured vitamin D affinity for purified human DBP and reported higher association constants for vitamin D₃ than for vitamin D₂, 2.8×10^8 and 1.3×10^8 L/mol, respectively. After measuring vitamin D and its metabolites, Hollis and Frank (33) compared human milk and plasma concentrations by regression analysis. They found higher quantities of vitamin D₂ and its major metabolite 25(OH)D₂ than of vitamin D₃ and its metabolite 25(OH)D₃ in milk. This suggests that vitamin D₂ and 25(OH)D₂ have lower affinity for DBP, and thus exist in relatively greater free amounts available for transport into milk. In rats, vitamin D-25-hydroxylase is known to exist in both microsomal and mitochondrial fractions. In humans, mitochondrial vitamin D-25-hydroxylase converts vitamin D₃ to 25(OH)D₃ 5 times as fast as it does vitamin D₂ to 25(OH)D₂; the human microsomal fraction hydroxylates vitamin D₃ somewhat but shows no detectable vitamin D-25-hydroxylase of vitamin D₂ (34). Similarly, transfected human liver P-450 hydroxylase metabolized vitamin D₃ but showed no vitamin D₂ hydroxylating ability (35). Taken together, the most likely explanation for the difference between vitamin D₂ and vitamin D₃ is that the higher affinity for DBP should reduce the clearance rate of vitamin D₃ compared with that of vitamin D₂. The more efficient 25-hydroxylation by the mitochondrial fraction should increase the production rate of 25(OH)D₃ over that of 25(OH)D₂.

Perhaps it should not be surprising that vitamin D₂ is less effective per mole than is vitamin D₃. Vitamin D₂ is not a natural part of human biology (4). Vitamin D₂ can be manufactured through the ultraviolet radiation of lipid extracted from yeast (5, 36). Its existence in our food supply is due to artificial supplementation with a product that exists because of synthetic convenience.

In summary, we showed that on a per mole basis, vitamin D₃ is more effective at raising serum 25(OH)D concentrations than is vitamin D₂. The long-standing assumptions concerning the equivalence of vitamin D₂ and D₃ (7, 8) are based on 60-y-old studies whose experimental endpoint was the antirachitic action in infants, which is difficult to ascertain (5). Since then, differences between these forms of vitamin D have been widely recognized for all species except humans. Our results emphasize that like other primates (10), the physiologic compound vitamin D₃ is preferable to vitamin D₂. Care should be taken to specify the type of vitamin D used for nutritional studies. The assumption of vitamin D₂ and D₃ equivalence used to express vitamin D

nutrition is probably wrong by a large margin and should be reconsidered. 

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