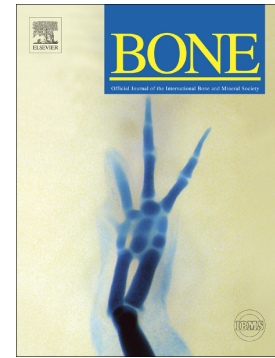


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Comparison of the Effect of Daily Versus Bolus Dose Maternal Vitamin D₃**Supplementation on the 24,25-dihydroxyvitamin D₃ to 25-hydroxyvitamin D₃ Ratio**

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Abbreviated title: 25(OH)D₃ to 24,25(OH)₂D₃ ratio during lactation

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Abstract

Objective: Supplementing lactating mothers with high doses of vitamin D₃ can adequately meet vitamin D requirements of the breastfed infant. We compared the effect of bolus versus daily vitamin D₃ dosing in lactating mothers on vitamin D₃ catabolism. We hypothesized that catabolism of 25(OH)D₃ to 24,25(OH)₂D₃ would be greater in the bolus than in the daily dose group.

Design, Setting and Patients: Randomized controlled trial (clinicaltrials.gov NCT01240265) in 40 lactating women.

Interventions: Subjects were randomized to receive vitamin D₃ orally, either a single dose of 150,000 IU or 5000 IU daily for 28 days. Vitamin D metabolites were measured in serum and breast milk at baseline, 1, 3, 7, 14 and 28 days.

Main Outcome Measure: Temporal changes in the serum 24,25(OH)₂D₃/25(OH)D₃ ratio.

Results: The concentration of serum 24,25(OH)₂D₃ was directly related to that of 25(OH)D in both groups ($r^2=0.63$; $p<0.001$). The mean (\pm SD) 24,25(OH)₂D₃/25(OH)D₃ ratio remained lower at all time points than baseline values in the daily dose group (0.093 \pm 0.024, 0.084 \pm 0.025, 0.083 \pm 0.024, 0.080 \pm 0.020, 0.081 \pm 0.023, 0.083 \pm 0.018 at baseline, 1, 3, 7, 14, and 28 days, respectively). In the single dose group, the increase in 24,25(OH)₂D₃ lagged behind that of 25(OH)D, but the 24,25(OH)₂D₃/25(OH)D₃ values (0.098 \pm 0.032, 0.067 \pm 0.019, 0.081 \pm 0.017, 0.092 \pm 0.024, 0.103 \pm 0.020, 0.106 \pm 0.024, respectively) exceeded baseline values at 14 and 28 days and were greater than the daily dose group at 14 and 28 days ($P=0.003$). The 24,25(OH)₂D₃/25(OH)D₃ ratio remained in the normal range with both dosing regimens. Greater breast milk vitamin D₃ values in the single dose group were inversely associated with the 24,25(OH)₂D₃/25(OH)D₃ ratio ($r^2 = 0.14$, $p<0.001$), but not with daily dosing.

Conclusions: After a 14-day lag, a single high dose of vitamin D led to greater production of 24,25(OH)₂D₃, presumably via induction of the 24-hydroxylase enzyme (CYP24A1), relative to the 25(OH)D₃ value than did daily vitamin D supplementation, and this effect persisted for at least 28 days after vitamin D administration. A daily dose of vitamin D may have more lasting effectiveness in increasing 25(OH)D₃ with lesser diversion of 25(OH)D₃ to 24,25(OH)₂D₃ than does larger bolus dosing.

Key words: vitamin D metabolism; catabolism; nutrition; LC-MS/MS; lactation; safety.

1. Introduction

Low levels of vitamin D in breast milk (20-80 IU/L)^{1,2} increase the risk of nutritional rickets in exclusively breast fed infants compared with infants who are formula fed, supplemented with vitamin D or who are breastfed by mothers taking high dose vitamin D supplements.^{3,4} Supplemental vitamin D 400 IU/d has been recommended for breastfed infants by the American Academy of Pediatrics and Institute of Medicine.⁵ Several studies have investigated the optimal maternal vitamin D dose as an alternative to direct supplementation of the infant. However, vitamin D supplementation of lactating mothers with doses of 4000-6400 IU/day effectively enriches the breast milk with appropriate levels of vitamin D to satisfy the breastfed infant's requirements,^{3,4,6} and this approach is often preferred by mothers over infant supplementation.⁷ Cholecalciferol (not 25(OH)D or 1,25(OH)₂D), which crosses readily from maternal circulation into breast milk, is the form available to the infant.

Vitamin D differs in its side chain substitution based on the vitamin's source wherein the plant derived form is called vitamin D₂ and mammalian form is called vitamin D₃. Metabolism of both forms are follow the same biochemical route in humans.⁸ Vitamin D and parathyroid hormone (PTH) are essential for normal calcium and phosphorus homeostasis.⁹ Vitamin D₃ is synthesized in the skin by ultraviolet-light mediated photo-isomerization of 7-hydrocholesterol^{9,10} and is metabolized in the liver to 25(OH)D₃ by the 25-hydroxylase CYP2R1. Calcium and phosphorus demands regulate the synthesis of 1,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) by the 25(OH)D₃-1 α -hydroxylase CYP27A1. The 25(OH)D₃ metabolite can alternatively be catabolized to an inactive metabolite, 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), by the 25(OH)D-24-hydroxylase CYP24A1.^{8,9,11-14} A dose dependent linear correlation exists between serum 25(OH)D₃ and 24,25(OH)₂D₃ at normal 25(OH)D₃ levels. The effect of a single large dose

vs daily low dose vitamin D₃ on temporal changes in the vitamin metabolites including 25(OH)D₃, 1,25(OH)₂D₃ in the supplemented mother, in the breast milk and the exclusively breast fed infant has been studied.⁴ While the 24,25(OH)₂D₃/25(OH)D₃ ratio has been proposed as an indicator of response to vitamin D₃ supplementation,¹⁵ changes in 24,25(OH)₂D₃/25(OH)D₃ with different vitamin D₃ dosing regimens in breast feeding mothers have not been reported. Our study aimed to investigate the effect of bolus versus daily vitamin D₃ dosing regimens on temporal changes in 25(OH)D₃/24,25(OH)₂D₃ ratio in a group of breast feeding mothers who were randomized to receive either a single large dose (150,000 IU) or a daily dose (5000 IU) of vitamin D over 28 days. We sought to investigate if catabolism of 25(OH)D₃ to 24,25(OH)₂D₃ would be greater in the bolus than the daily dose group, its correlation with breast milk vitamin D₃ concentration, and whether it can be used as a safety marker for high dose vitamin D₃ supplementation in lactating women.

2. Methods and Materials

Subjects and samples: The study was a randomized controlled trial in 40 lactating females, ages 24-40 years, with a singleton infant between the ages of 1 and 6 months. Subjects were randomized to receive vitamin D₃ as a single oral dose of 150,000 IU (N = 20), or 5000 IU daily (N = 20) for 28 days. Additional details regarding the study design have been previously described.⁴ The Mayo Clinic Institutional Review Board approved the study.

We measured serum vitamin D₃, 25(OH)D₃, 24,25(OH)₂D₃, 1,25(OH)₂D₃, and breast milk vitamin D₃ at baseline and 1, 3, 7, 14 and 28 days following vitamin D₃ administration. All vitamin D₃ metabolites were measured at Mayo Clinic by previously established liquid chromatography mass spectrometry based methods.¹⁶⁻¹⁸ The Mayo 25(OH)D test is calibrated against NIST and has been successfully passing the DEQAS-PT program.

24,25(OH)₂D₃ Measurement: Briefly, 500 μ L of calibrator, quality control or patient serum was placed in a glass test tube to which 50 μ L acetonitrile solution of the internal standard (1.25 ng deuterated 24,25(OH)₂D₃-d₆, Toronto Research Chemicals, Canada) was added. The contents of the test tube were mixed using a vortex mixer, and the samples were incubated at room temperature for 15 minutes. Then, 500 μ L aqueous hydrochloric acid solution (0.2 M) was added. The samples were vortex mixed and incubated at room temperature for 15 minutes. The contents of the test tube were transferred into a solid phase extraction cartridge (Bond-Elut (C18, 250 mg, 6 mL), Varian Instruments). The solid phase extraction cartridges containing the sample were placed on a positive pressure manifold, and the contents of the cartridge were passed through the resin by applying 3-5 psi pressure. The cartridge was washed once with 2 mL of 70:30 methanol/water (vol/vol) and once with 2 mL of 90:10 hexane/methylene chloride (vol/vol). Vitamin D metabolites were eluted with 2 mL of 90:10 hexane/isopropyl alcohol (vol/vol). Eluents were dried and derivatized with 4-phenyl-1,2,4,-triazoline-3,5-dione (PTAD) (Sigma; 250 μ L of 200 μ g/mL solution in acetonitrile). The derivatized vitamin D metabolites were separated by liquid chromatography at a on an Agilent XDB-C8, 2.1x50-mm column with a methanol-H₂O-ammonium formate (1 mM) linear gradient (60%–95%) and analyzed on a AB Sciex 5500 mass spectrometer with Analyst 1.6.2 software (AB Sciex) for data acquisition and analysis.

For 25(OH)D quantification, to a 100 μ L serum sample 2.5 ng deuterated internal standard solution (25(OH)D₃-d₆) was added, and the sample was equilibrated on an orbital shaker for 15 min at room temperature. 100 μ L acetone, followed by 450 μ L ethyl acetate were added. After the solutions had settled, the organic top layer was transferred to a different sample tube. The extraction was repeated 4 times. The samples were subjected to PTAD derivatization

as described above. Reaction mixtures were dried under a N₂ stream, and the residue was reconstituted in 150 µL of 50:50 methanol: water mixture. The reconstituted residue was then separated on an analytical LC column and analyzed on an AB Sciex 4500 mass spectrometer.

The normal range for serum 24,25(OH)₂D₃/25(OH)D₃ ratio was determined using serum samples from 91 healthy individuals were collected between May and August 2012, and 24,25(OH)₂D₃ and 25(OH)D were measured by the methods described above.¹⁷ All patients included in the reference range study were screened by a detailed chart review to ascertain that none of the individuals from whom samples were obtained were taking drugs known to affect mineral metabolism or had systemic conditions known to affect mineral metabolism. They were not taking vitamin D or calcium supplements, or if they were, were taking no more than 1000 mg calcium per day and 1000 IU vitamin D₃ per day.

Statistical analysis: Data were entered in and analyzed with Excel 2010 (Microsoft Corp., Redmond, WA). The Student t test was used to compare continuous variables between the two treatment groups. P values less than 0.05 were considered significant.

3. Results

Baseline characteristics of the study participants are shown in **Table 1**. The two study groups had no statistically significant differences. Alterations in serum concentration of 24,25(OH)₂D₃ and 24,25(OH)₂D₃/25(OH)D₃ ratio in the single dose and daily dose are shown in **Figure 1**. Following a single oral dose of 150,000 IU of vitamin D₃, serum 24,25(OH)₂D₃ increased by day 3 to 4.17 ± 1.46 ng/mL (45% increase) from a basal value of 2.88 ± 1.20 ng/mL (p<0.001) and remained elevated for the subsequent 28 days compared to the baseline values. On day 28, while the serum 24,25(OH)₂D₃ concentration was not significantly different between the two groups (p = 0.20), the 24,25(OH)₂D₃/ 25(OH)D₃ ratio was significantly greater in the single

dose group than the daily dosing group. Serum 25(OH)D₃ increased by day 1 to 43.05 ± 10.15 ng/mL (48 % increase) from a basal value of 29.06 ± 7.75 ng/mL in the single dose group. With daily administration of vitamin D₃ (5000 IU/day), no increase in serum 24,25(OH)₂D₃ was noted until day 14 (Figure 1a), with a value at day 14 of 3.34 ± 1.35 ng/mL compared with a baseline value of 2.87 ± 1.40 ng/mL (p = 0.03). In the daily dose group, serum 24,25(OH)₂D₃ did not reach the same final concentration as the single dose group. Compared with the corresponding basal value, the day 28 serum 24,25(OH)₂D₃ concentration was 52% higher in the single dose group and 32% greater in the daily dosing group.

In the single dose group, the rise in concentration of 25(OH)D₃ preceded the increase in 24,25(OH)₂D₃, accounting for a decline in the 24,25(OH)₂D₃/ 25(OH)D₃ ratio on day 1. The 24,25(OH)₂D₃/ 25(OH)D₃ ratio in the single dose group gradually attained a significantly greater value than the daily dose group after day 7 (Figure 1c), and the final value was greater than the baseline value in the single dose group. In contrast, the 24,25(OH)₂D₃/ 25(OH)D₃ ratio in the daily dose group initially declined and remained relatively stable and lower than the baseline value throughout the duration of treatment in the daily dosing group.

Serum 25(OH)D₃ and 24,25(OH)₂D₃ over all time points were strongly correlated (r²=0.63; p < 0.0001) in both groups (Figure 2a). Serum 24,25(OH)₂D₃/25(OH)D₃ and serum 25(OH)D₃ showed a significant direct correlation in the single dose group (r²=0.13, p<0.001) but not in the single dose group (r²=0.025; p=0.09; Figure 2b). Serum 24,25(OH)₂D₃/25(OH)D₃ and breast milk vitamin D₃ were inversely correlated in the single dose group (r² = 0.14, p<0.001) but not in daily dose group (r² = 0.03, p=0.07; Figure 2c). An inverse relationship was observed between breast milk vitamin D₃ and 24,25(OH)₂D₃ in the single dose group (r²=0.05, p=0.01) whereas an opposite (direct) correlation (r²=0.13, p<0.001; Figure 2d) was observed in the daily

dose group. We did not observe a significant reciprocal relationship between 24,25(OH)₂D₃ and 1,25(OH)₂D₃.

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4. Discussion

We found evidence of greater production of 24,25(OH)₂D₃ resulting from a single, high dose bolus of vitamin D than with a daily dose of vitamin D over the course of 28 days. The concentration of serum 24,25(OH)₂D₃ was directly related to that of 25(OH)D in both groups, but the increase in 24,25(OH)₂D₃ lagged behind that of 25(OH)D in the single dose group. The 24,25(OH)₂D₃/25(OH)D₃ ratio was significantly correlated with the serum 25(OH)D₃ in the daily dose group but not in single dose group, which likely reflects the lag time in activation of the CYP24A1 enzyme.

Use of a high-dose bolus vitamin D was historically used for treatment of rickets in the setting of vitamin D deficiency. Contemporary studies have evaluated bolus cholecalciferol and ergocalciferol dosing in several clinical settings where potential benefit of modulating the vitamin D metabolic pathway is hypothesized including osteoporosis, cystic fibrosis, chronic kidney disease and inflammatory diseases. Results from studies on the effect of high-dose vitamin D supplementation in populations with high risk of fracture have been mixed. A paradoxical increase in risk of fracture found in some studies has been hypothesized to be caused by an up-regulation of CYP24A1 with high bolus doses of ergocalciferol or cholecalciferol, causing increased catabolism of 1,25(OH)₂D. Turner et al. reported an increase in serum 1,25(OH)₂D₂ concentration three months after a bolus dose of 300,000 IU of ergocalciferol was administered to vitamin D deficient patients.¹⁹ In our study, while we were unable to determine if the greater production of 24,25(OH)₂D₃ in the single dose group was due to induction of the *CYP24A1* gene or due to increased substrate concentrations, the data raises an important point of whether an assessment of serum vitamin D metabolites over a longer time-frame might have shown differences in serum metabolic patterns consistent with other reports. Of note,

1,25(OH)₂D₃ concentrations were not significantly different in the two dosing groups in our study.⁴

Whereas the ratio of 24,25(OH)₂D₃/25(OH)D₃ initially declined and remained relatively constant below the baseline value with daily supplementation, this ratio was significantly greater in the single dose group and greater than the baseline value after 7 days. This suggests that the single high dose of vitamin D led to greater induction of the 24-hydroxylase CYP24A1 relative to the 25(OH)D₃ value than daily vitamin D supplementation, and this effect persisted for at least 28 days after vitamin D administration. The high single dose may produce more prolonged catabolic activation of CYP24A1 than the smaller daily dose. The implication of this is that smaller daily doses of vitamin D would have a more favorable effect of increasing 25(OH)D with lesser activation of CYP24A1 catabolism.

The 24,25(OH)₂D₃/25(OH)D₃ ratio remained in the normal range with both dosing regimens and supports the safety of high dose vitamin D₃ supplementation used in this study, irrespective of the lag time to reach catabolic activity. Sub-optimal CYP24A1 activity may exacerbate the risk of toxicity in mothers receiving a high dose vitamin D₃ supplement. The correlation between breast milk vitamin D₃ content and maternal serum 24,25(OH)₂D₃/25(OH)D₃ differed between the single and daily dose groups. The inverse relationship between breast milk vitamin D₃ and 24,25(OH)₂D₃ after the single large dose of vitamin D₃ likely reflects the fact that initially high concentrations of vitamin D₃ in breast milk in the first 3 days preceded the delayed increase in 24,25(OH)₂D₃ concentrations. Because of the 14-day lag in the increase of the 24,25(OH)₂D₃/25(OH)D₃ ratio, higher values of breast milk vitamin D₃ were associated with a lower 24,25(OH)₂D₃/25(OH)D₃ ratio. The direct relationship in the daily dose group reflects breast milk vitamin D₃ increasing over the 28 days of

supplementation, while the $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ ratio remained relatively unchanged. Given the nearly identical increment of serum $25(\text{OH})\text{D}$ in breast fed infants of these two groups of women,⁴ it is unlikely that differences in $25(\text{OH})\text{D}$ catabolism between these two dosing groups, reflected in the $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ ratio, have an impact on vitamin D_3 availability in breast milk.

One limitation of our study is that we did not measure 3-epi- $25(\text{OH})\text{D}_3$ in mother's serum or breast milk. In adults, the concentration of 3-epi- $25(\text{OH})\text{D}_3$ is generally very low, but production of 3-epi- $25(\text{OH})\text{D}_3$ might be predicted to be higher in the high dose group. If this were the case, the changes in $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ ratios could be even more extreme or the level of $25(\text{OH})\text{D}_3$ at the end of the study might differ between groups.

An average fetus requires approximately 30 g of calcium for bone mineralization.²⁰ An increase in serum $1,25(\text{OH})_2\text{D}_3$ in pregnant females has been demonstrated.²¹ In mouse models of high bone density, a change in CYP24A1 activity lower $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ compared to wild type mice has been demonstrated as the cause of increased calcium accretion.²² However, the effect of increased calcium demand during lactation on CYP24A1 activity and $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ is largely unexplored. $24,25(\text{OH})_2\text{D}_3$ is a major catabolic product of $25(\text{OH})\text{D}_3$. While its biological role is debated, its ratio with its precursor, $25(\text{OH})\text{D}_3$ is well established as a biomarker of abnormal CYP24A1 enzyme function.^{17, 23-27} Additionally, $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ functions as an index of $25(\text{OH})\text{D}_3$ clearance in healthy adult male and female subjects.¹⁵ During 8 weeks of 28,000 IU/wk vitamin D_3 supplementation, $24,25(\text{OH})_2\text{D}_3$ was directly correlated to $25(\text{OH})\text{D}_3$. Evidence of a lag in 24-hydroxylation was similarly found during the early phase of supplementation. This was attributed to the slower reaction kinetics of CYP24A1 (24-hydroxylase) compared to CYP27A1 (25-hydroxylase).²⁸ The effect of a greater

24,25(OH)₂D₃/25(OH)D₃ ratio was to blunt the increase in 25(OH)D₃ with vitamin D₃ supplementation. In this sense, the 24,25(OH)₂D₃/25(OH)D₃ ratio could be considered a functional marker of vitamin D sufficiency, reflecting increased catabolism of 25(OH)D as the concentration of 25(OH)D increases.

Our data in vitamin D₃ supplemented breast feeding mothers, in whom the calcium demands are high, confirms the dependence of serum concentrations of 24,25(OH)₂D₃ upon the concentrations of its precursor, 25(OH)D₃. While changes in serum 24,25(OH)₂D₃ occur in parallel with changes in serum 25(OH)D₃, concentrations of 1,25(OH)₂D₃ did not change,⁴ demonstrating that the production of 1,25(OH)₂D₃ is substrate independent, and is regulated by other factors, such as serum calcium, parathyroid hormone, and phosphate. With low 25(OH)D₃ concentrations (<20 ng/mL), the 24,25(OH)₂D₃/25(OH)D₃ ratio declines to low normal ranges in healthy subjects,^{17, 26} likely due to favored biosynthesis of the bioactive 1,25(OH)₂D₃ during stages of 25(OH)D₃ depletion.²⁹

Conclusions

A single large dose of vitamin D₃ produced evidence of greater production of the catabolic product of 24-hydroxylase CYP24A1 activity than daily dosing of vitamin D. Evidence of increased 24,25(OH)₂D₃ production relative to serum 25(OH)D₃ values persisted for at least 28 days following a single large dose of vitamin D₃, but remained within the normal range. Daily vitamin D₃ supplementation may provide more predictable effects on vitamin D status due to the greater stability of the 24,25(OH)₂D₃/25(OH)D₃ ratio compared with bolus dosing.

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Table 1: Baseline Characteristics of Study Subjects

Characteristic	Daily Dose 5000 IU (n=20)	Single Dose 150,000 IU (n=20)
Maternal age (years)	30.3 ± 2.9	30.1 ± 4.0
Infant age (weeks)	13.7 ± 7.3	11.0 ± 5.6
Infant gestation at birth (weeks)	39.9 ± 1.3	39.5 ± 0.9
Maternal race (% white)	95%	95%
Maternal weight (kg)	72.7 ± 10.6	67.6 ± 12.1
Maternal height (cm)	165.6 ± 5.5	163.8 ± 4.2
Maternal BMI (kg/m ²)	26.5 ± 4.0	25.2 ± 4.7
Maternal serum 25(OH)D (ng/mL)	28.8 ± 9.2	29.3 ± 7.5
Maternal serum 24,25(OH) ₂ D ₃ (ng/mL)	2.87 ± 1.40	2.88 ± 1.20
Maternal serum 1,25(OH) ₂ D ₃ (pg/mL)	60.4 ± 24.9	52.0 ± 10.7
Enrollment date		
January-March	9 (45%)	8 (40%)
April-July	11 (55%)	12 (60%)

Figure 1: Alterations in (a) serum concentration of 24,25(OH)₂D₃, (b) serum concentration of 25(OH)D₃, (c) ratio of 25(OH)D₃/24,25(OH)₂D₃, and (d) serum concentration of cholecalciferol in single dose (open circle) and daily dose (black triangles) groups. The error bars represent standard deviations. P values are for comparison of values in daily and single dosing groups at corresponding time points.

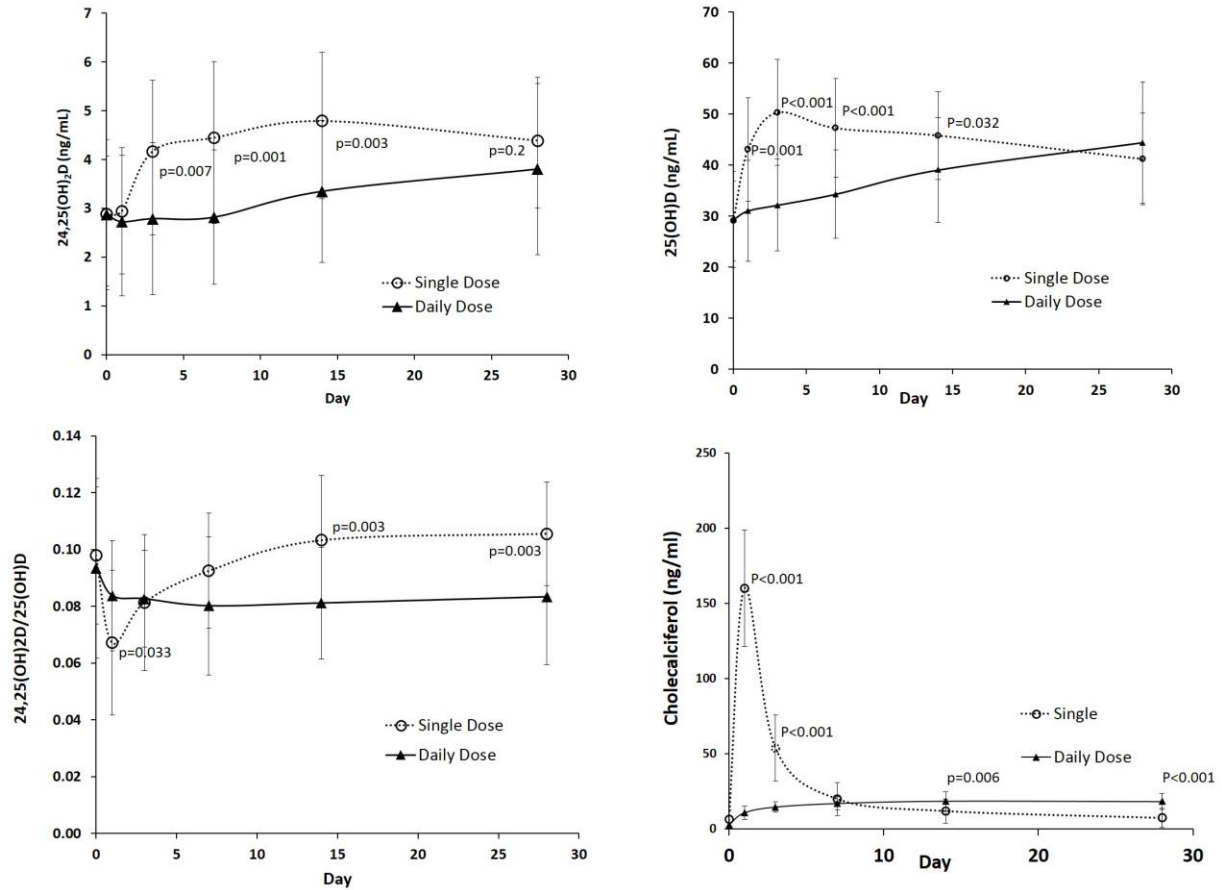
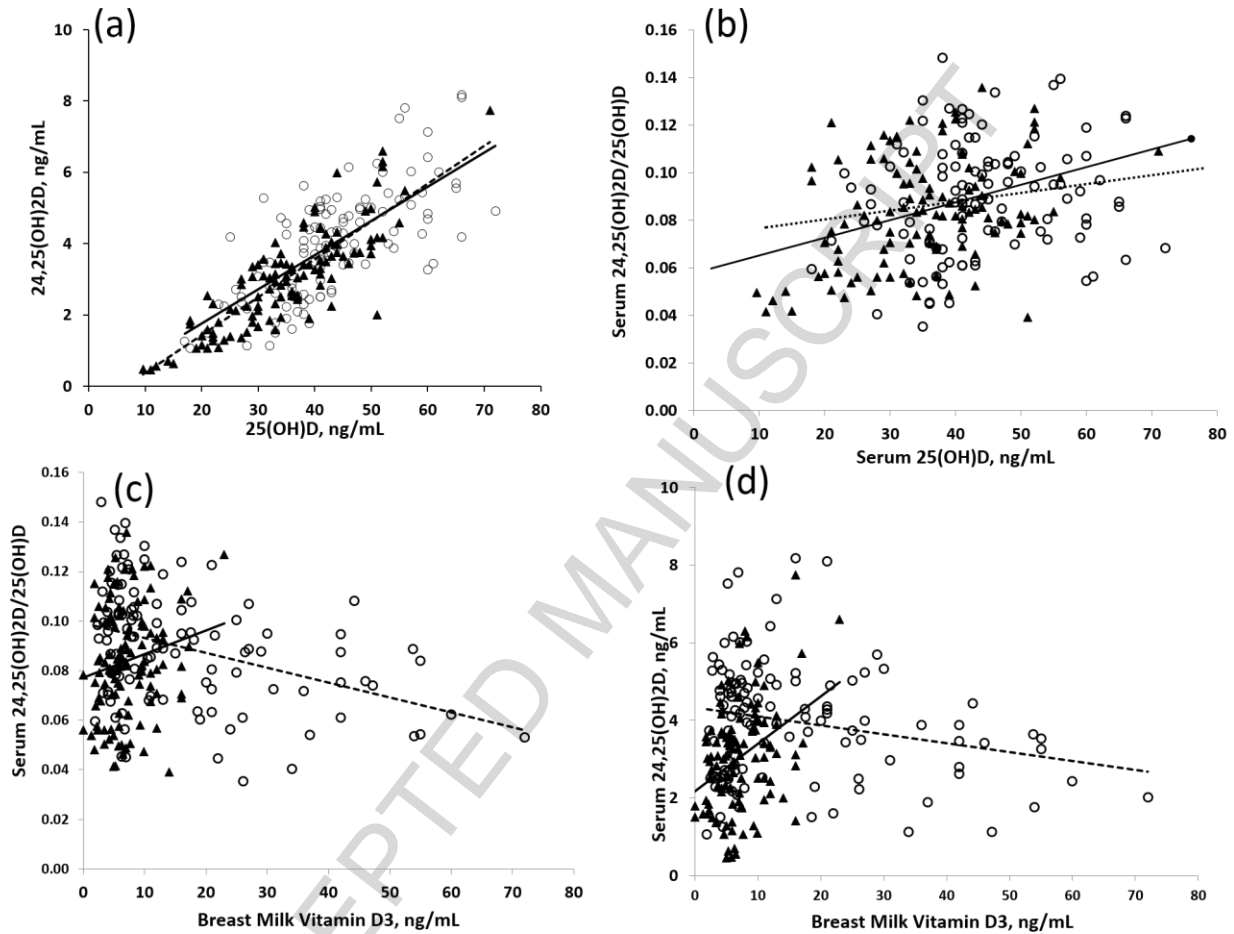


Figure 2: Correlations between serum concentration of (a) 25(OH)D₃ and 24,25(OH)₂D₃ ($r^2=0.63$; $p < 0.0001$), (b) serum 24,25(OH)₂D₃/25(OH)D₃ and serum 25(OH)D₃ ($p < 0.001$), (c) serum 24,25(OH)₂D/25(OH)D₃ and breast milk vitamin D₃ and (d) serum 24,25(OH)₂D and breast milk vitamin D₃ in single dose (open circle) and daily dose (black triangles) groups. The solid line represents the linear trend in the daily dose group and the dotted line the linear trend in single dose group.



Highlights

- Bolus high-dose vitamin D produced more 24,25(OH)₂D than daily supplementation
- After bolus vitamin D, the increase in 24,25(OH)₂D lagged behind that of 25(OH)D
- Daily vitamin D may increase 25(OH)D more effectively than larger bolus dosing

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