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Effect of Vitamin D supplement use on serum concentrations of total 25OHD levels in elderly women ☆

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Abstract

Vitamin D_2 and D_3 are generally considered equipotent in humans. As Vitamin D_2 supplements are commonly used by elderly in United States, we determined the contribution of 25OHD$_2$ to the total serum 25OHD levels by HPLC in elderly women who reported taking Vitamin D_2 supplements ($n = 56$) and also in a group of randomly selected unsupplemented women ($n = 60$). In addition, we compared the total serum 25OHD measured by HPLC with competitive protein-binding assay (CPBA), a method routinely employed to measure Vitamin D status. A correlation of 0.91 ($P < 0.001$) was observed between the two methods for the serum total 25OHD measurement. The mean serum 25OHD level in Vitamin D_2 supplemented group was significantly higher than in unsupplemented group measured by HPLC (32 versus 28 ng/ml) and marginally higher measured by CPBA (33 vs. 31 ng/ml). Seventy eight percent of women taking Vitamin D_2 supplements had appreciable amounts of circulating 25OHD$_2$, which constituted about 25 percent of their total serum 25OHD. It is also interesting to note that Vitamin D deficiency was less prevalent in elderly women taking Vitamin D_2 supplements (1.8%) compared to women not taking any supplements (12%).
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1. Introduction

Vitamin D exists in two forms, cholecalciferol (Vitamin D_3) synthesized endogenously from 7-dehydrocholesterol in the skin by the action of UV radiation and, plant derived ergocalciferol (Vitamin D_2) formed exogenously by irradiation of ergosterol. Vitamin D supplements use either cholecalciferol or ergocalciferol as their source of Vitamin D. Some of the commonly used vitamin supplements in United States like Centrum, Walgreens, Osco multivitamin etc. use Vitamin D_2 as their source of Vitamin D.

In early 1990's, both Vitamin D_2 and D_3 were considered equipotent. However, later research in different animal species showed differences in response to Vitamin D_2 and D_3 [1–3]. In humans, it was originally thought that both Vitamin D_2 and D_3 follow the same metabolic pathway and are equally bioactive in normal subjects [4,5]. Later studies suggested differential response to Vitamin D_2 and D_3 in humans as well [6–8].

In United States, about 50% of women above the age of 50 years use vitamin and mineral supplements [9]. As some of the Vitamin D supplements commonly used by the elderly have Vitamin D_2 as their source of Vitamin D, in the present study, we estimated the contribution of 25OHD$_2$ to the total serum 25OHD levels by HPLC in elderly women taking Vitamin D_2 supplements. In addition, in women taking Vitamin D_2 supplements and in a randomly selected group of women not taking any vitamin supplements, we compared the total serum 25OHD levels measured by HPLC with that measured by competitive protein-binding assay (CPBA), a routinely employed method to measure circulating 25OHD levels.

2. Materials and methods

Serum samples were obtained from a study population consisting of 489 elderly women, age range 65–77 years, enrolled in an osteoporosis intervention trial, Sites Testing Osteoporosis Prevention/Intervention Treatment (STOP IT), intended to test the efficacy of three therapies in reversing bone loss in proximal femur and spine compared with placebo. The subjects were ambulatory, free-living, healthy volunteers. The information about the multivitamin supplement use was obtained by a questionnaire administered to

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the subjects by the study personnel. The form of Vitamin D (either D_2 or D_3) used in the supplements was obtained from the package inserts. Of the total of 489 women, 307 were not taking any Vitamin D supplements and 182 were taking a Vitamin D supplement, of which 56 were taking a Vitamin D_2 supplement. Women taking a Vitamin D_2 supplement were taking about 400 IU/day. 25OHD measurements by HPLC and CPBA were done on a total of 116 subjects, a randomly selected group of unsupplemented women ($n = 60$) and in women taking Vitamin D2 supplements ($n = 56$). The protocol was approved by Creighton University Institutional Review Board.

3. 25OHD measurement

Fasting blood samples were collected from the subjects, allowed to clot, centrifuged at 4 °C for 15 min at 2056 × g to separate serum. The serum was stored frozen at −70 °C until analysis.

The total 25OHD measurement was performed both by CPBA and HPLC after precipitating plasma proteins with acetonitrile.

3.1. Competitive protein-binding assay

Total serum 25OHD was measured by CPBA [10] after extraction and purification of serum on Sep-Pak C-18 and silica cartridges (Waters Associates, Milford, MA) [11]. Briefly, after precipitating plasma proteins using acetonitrile, the supernate was back washed with potassium phosphate, 0.4 M (pH 10.5) to enhance the solubilization of lipids. The samples were then extracted on a reverse phase Sep Pak C18 columns. The acetonitrile fraction containing the Vitamin D metabolites was taken through a normal phase extraction with a silica Sep Pak cartridge, where the 25OHD was eluted with 96:4 hexane: *iso*-propanol. 25OHD was quantitated by CPBA employing normal rat serum as the source of binding protein. The minimum detection limit for the assay was 12.5 nmol/l (5 ng/ml) and the inter-assay variation was 5%.

3.2. HPLC

Total serum 25OHD was estimated by HPLC after pre-purification of the acetonitrile extract of serum by solid phase extraction using "Bond-Elut LRC" C18/OH cartridges and Sep-Pak Silica cartridges (Waters Associates, Milford, MA) [12]. HPLC of the purified extract was performed using a Shimadzu LC-10 system with Shimadzu LC-10AT pump, GT-104 degasser, Sil 10A Injector (autosampler) with sample cooler, CTO-10A column oven and SCL-10A system controller. The separation of $25OHD_2$ and $25OHD_3$ was achieved on a 0.45 i.d. × 25 cm (5 μm) Zorbax SIL column using hexane/*iso*-propanol (97/3) at a flow rate of 2 ml/min. $25OHD_2$ and $25OHD_3$ were detected using a Shimadzu SPD-10A UV-Vis detector and the data was analyzed using the CLASS-VP chromatography data system. The total 25OHD was computed by measuring both $25OHD_2$ and $25OHD_3$. The minimum detection limit for the assay was about 2.5 ng/ml and the inter-assay variation was less than 1%.

Table 1
Characteristics of the subjects in unsupplemented and Vitamin D_2 supplemented women

Variable	Unsupplemented	Vitamin D_2 supplemented
N	60	56
Age (years)	71.8 ± 0.45	71.9 ± 0.42
Height (cm)	159.5 ± 0.82	159 ± 0.89
Weight (kg)	67.7 ± 1.70	66.9 ± 1.67
Dietary calcium intake (mg/day)	735 ± 35.5	806 ± 35[a]
Dietary Vitamin D intake (IU/day)[b]	148.7 ± 11.5	529.9 ± 9.9[a]

[a] $P < 0.05$ compared to unsupplemented group.
[b] Includes dietary and supplemental Vitamin D.

4. Statistical analysis

All analyses were done using SPSS for windows (Version 11.0, SPSS, Chicago, IL). The characteristics and serum 25OHD levels between the unsupplemented and Vitamin D_2 supplemented women were compared using Student's t-test. Pearson's correlation coefficient and simple linear regression methods were used to assess the relation between the two methods of 25OHD estimation.

5. Results and discussion

The characteristics of the subjects of unsupplemented and Vitamin D_2 supplemented groups are given in Table 1. There were no significant differences between the two groups with respect to age and weight. Dietary calcium intake and Vitamin D intake were significantly higher in Vitamin D_2 supplemented women compared to unsupplemented group (Table 1). The mean serum 25OHD measured by CPBA in Vitamin D_2 group was 33.0 ± 1.6 ng/ml and in the unsupplemented group the levels were 31.2 ± 1.3 ng/ml (Table 2). The

Table 2
Mean serum 25OHD concentrations in unsupplemented and Vitamin D_2 supplemented women

Variable	Unsupplemented	Vitamin D_2 supplemented
N	60	56
Total 25OHD (CPBA) (ng/ml)	31.2 ± 1.3	33.0 ± 1.6
Total 25OHD (HPLC) (ng/ml)	27.7 ± 1.2	31.6 ± 1.4[a]
$25OHD_3$ (HPLC) (ng/ml)	26.8 ± 1.2	23.2 ± 1.6
$25OHD_2$ (HPLC) (ng/ml)	0.9 ± 0.40	8.4 ± 0.80

Values are mean ± S.E.M.
[a] $P < 0.05$ compared to unsupplemented group.

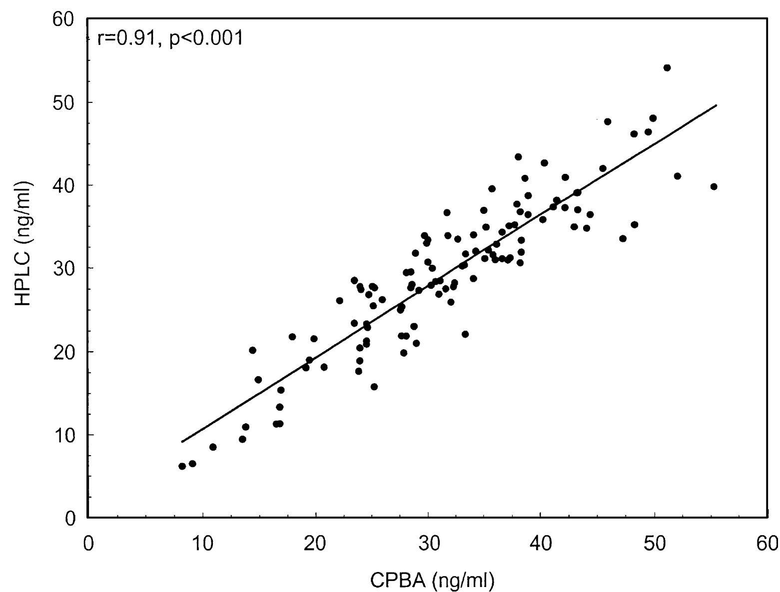

Fig. 1. Correlation between competitive binding assay (CPBA) and HPLC methods for serum total 25OHD measurement.

mean serum 25OHD determined by HPLC in women receiving Vitamin D_2 supplement (31.6 ± 1.4 ng/ml) was significantly higher when compared to the mean 25OHD in women not receiving any Vitamin D supplement (27.7 ± 1.21 ng/ml) (Table 2). The 25OHD measurements done by CPBA were not different from that measured by HPLC. The mean concentrations of serum 25OHD observed in the present study agree with previously reported values determined by HPLC or CPBA [13] in normal subjects [14]. We observed a good correlation between CPBA and HPLC for serum 25OHD measurement. The overall correlation between CPBA and HPLC for the total serum 25OHD was about 0.91 ($P < 0.001$) (Fig. 1). Other studies comparing HPLC and CPBA showed a correlation of 0.89 [15] and 0.94 [16] between HPLC and CPBA for total serum 25OHD measurement, which is similar to our observation. In a recent study conducted to assess the inter-laboratory variation for the measurement of serum 25OHD, Lips et al. [17] reported that the mean serum 25OHD was about 80% higher when measured by CPBA than by HPLC. From our results, it appears that serum 25OHD measured by CPBA with prior chromatography step gives a fairly accurate measurement comparable to HPLC.

In the present study, about 78% of women taking a Vitamin D_2 supplement had appreciable amounts of serum $25OHD_2$ (Fig. 2), while subjects not taking any Vitamin

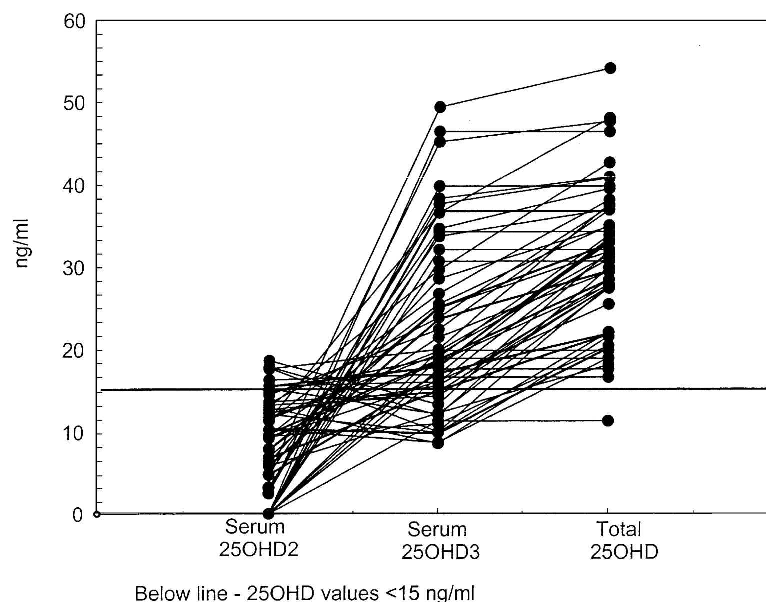

Fig. 2. Serum $25OHD_2$, $25OHD_3$, and total 25OHD measured by HPLC in women taking Vitamin D_2 supplements.

D supplement had negligible amounts. In women taking Vitamin D_2 supplements, approximately 25% of the total serum 25OHD was contributed by $25OHD_2$. About 20% of Vitamin D_2 supplemented women would have been classified as Vitamin D deficient if their serum $25OHD_2$ levels were not accounted for the total 25OHD content (Fig. 2).

Vitamin D deficiency is a significant risk factor for bone loss and subsequent fracture. In the present study carried out in Omaha (41° N), Vitamin D deficiency (<15 ng/ml) was less prevalent (1.8%) in Vitamin D_2 supplemented group (Fig. 2). However, in elderly subjects not taking any vitamin supplements, about 12% had serum 25OHD levels below 15 ng/ml. In Boston (42.2° N), it has been reported that in elderly nursing home residents about 40% had serum 25OHD levels below 10 ng/ml and about 80% had below 15 ng/ml in the winter time [18]. Seasonal variation in serum 25OHD levels has also been reported in our study population [19]. Another recent study reported that about 13.6% of the population (age range 18–86 years) attending a Boston outpatient clinic had serum 25OHD levels less than 16 ng/ml in winter time [20]. They also reported that Vitamin D supplementation is a positive determinant of serum 25OHD concentration with about 65% of those taking a Vitamin D supplement having serum 25OHD levels as high as 32 ng/ml.

In conclusion, in women taking Vitamin D_2 supplements about 25% of their serum total 25OHD is contributed by $25OHD_2$ and about 20% would have been classified as Vitamin D deficient if their $25OHD_2$ levels were not accounted for the total Vitamin D content. Further, though HPLC is advantageous to know the relative contribution of diet/Vitamin D supplement and exposure to UV light to the total 25OHD pool, to assess the general Vitamin D status, CPBA with prior chromatography step gives a fairy accurate Vitamin D status of an individual.

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