Changes in the Concentrations of Vitamin D and Its Metabolites in the Plasma of Healthy Subjects Orally Given Physiological Doses of Vitamin D₂ by Multivitamin or Vitamin D Preparations

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(Received March 30, 1989)

Summary Changes in the concentrations of vitamin D and its metabolites in plasma of healthy subjects orally given physiological doses of vitamin D₂ by multivitamin or vitamin D liquid preparations were determined and the bioavailability of vitamin D was studied. Separative assay on the D₂ and D₃ compounds of vitamin D, 25-hydroxyvitamin D (25-OH-D), 24R,25-dihydroxyvitamin D [24,25(OH)₂D], and 1α,25-dihydroxyvitamin D [1,25(OH)₂D] was performed in plasma of eight healthy male volunteers. When the concentrations of vitamin D and its metabolites in plasma of volunteers were assayed after daily oral administration of 400 IU of vitamin D₂ in a form of multivitamin tablet for 1 week, the variations of vitamin D₃ and its metabolites in plasma levels were very small. In contrast, the concentrations of 25-OH-D₂ and 1,25(OH)₂D₂ slightly increased after the administration, while neither vitamin D₂ nor 24, 25(OH)₂D₂ was detected. A single dose of 4,000 IU of vitamin D₂ was orally given to the volunteers in a form of a vitamin D liquid preparation and the hourly variations were observed during 24 h.

Abbreviations: 25-OH-D, 25-hydroxyvitamin D; 24,25(OH)₂D, 24R,25-dihydroxyvitamin D; 1,25(OH)₂D, 1α,25-dihydroxyvitamin D; DBP, vitamin D-binding protein; CPBA, competitive protein-binding assay; RRA, radioreceptor assay; RDA, recommended dietary allowance; HPLC, high-performance liquid chromatography.
These concentrations of vitamin D₂, 25-OH-D₂, and 1,25(OH)₂D₂ were slightly higher than those of the repeated doses. The result suggests that even the high dose of 4,000 IU has little effect on the plasma levels of vitamin D₂ and its metabolites by a single dose, indicating a low risk for hypervitaminosis D.

**Key Words** vitamin D, 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D, 1α,25-dihydroxyvitamin D, bioavailability

It has been well documented that vitamin D ["Vitamin D" is used as a general name for ergocalciferol (D₂) and cholecalciferol (D₃) and the same nomenclature system is also applied to the metabolites] is metabolized to 25-hydroxyvitamin D (25-OH-D) in the liver and subsequently to 1α,25-dihydroxyvitamin D [1,25(OH)₂D] in the kidney when serum calcium levels are lower than normal, while it is metabolized to 24R,25-dihydroxyvitamin D [24,25(OH)₂D] in the kidney when serum calcium levels are higher than normal. Since 1,25(OH)₂D has the most potent activity among the metabolites, it is known as an active form of vitamin D. There are two forms of vitamin D, namely D₂ and D₃, which are structurally different in the side chain but they are metabolized in the same way to show physiologically the same activity in mammals, including humans (1,2). Vitamin D₂ and its metabolites detected in human plasma are mainly derived from exogenous sources, such as multivitamin preparations and vitamin D-enriched milk. In contrast, vitamin D₃ and its metabolites found in the body are mainly derived from the endogenous vitamin D₃ which is photochemically biosynthesized in the skin with sunlight exposure, and the vitamin occurs in a few kinds of exogenous sources such as eel, skipjack, and Japanese pilchard but does not exist in other foods (3). Therefore, separative determination of the D₂ and D₃ compounds in plasma is very convenient to study the bioavailability of vitamin D to distinguish the origin of the exogenous D₂ from endogenous D₃ compounds. The authors have previously established a method for the separative determination of 25-OH-D₂ and 25-OH-D₃ in plasma using high-performance liquid chromatography (HPLC) (4). The proposed method has been applied to the plasma of mothers, neonates (5), and healthy adults (6). The results have shown that the seasonal variations showing higher values in summer and lower in winter were observed in the plasma levels of 25-OH-D₃ since few healthy Japanese adults are taking daily exogenous vitamin D₂ from multivitamin preparations or others. On the other hand, in bottle-fed neonates an increase in the plasma concentrations of 25-OH-D₂ has been observed (5). From these backgrounds, we have studied the bioavailability of vitamin D using healthy volunteers who received oral administration of 400 IU of vitamin D₂ per day for 1 week or a single oral dose of 4,000 IU of vitamin D₂. The concentrations of vitamin D₂, D₃, and their metabolites are assayed and the results are described in this paper.
EXPERIMENTAL

Compounds and reagents. Among the standard compounds for the assay, commercially available crystalline vitamin D$_2$, vitamin D$_3$, 25-OH-D$_3$, 24,25(OH)$_2$D$_3$, and 1,25(OH)$_2$D$_3$ (Duphar Co., Weesp, The Netherlands) were used. Purified compounds of 25-OH-D$_2$ and 1,25(OH)$_2$D$_2$ were obtained by the in vivo and in vitro experiments according to Okano et al. (7) and Horiuchi et al. (8), respectively, while the 24,25(OH)$_2$D$_2$ chemically synthesized by ours (9) was used. These compounds were dissolved in absolute ethanol with a stream of argon gas to make appropriate concentrations and stored at $-20^\circ$C until use. Commercial grades of $[26,27$-methyl-$^3$H]$\cdot$1,25(OH)$_2$D$_3$ (specific activity, 183 Ci/mmol) and $[23,24(n)$-$^3$H]$\cdot$24,25(OH)$_2$D$_3$ (specific activity, 64 Ci/mmol) were purchased from Amersham Co. (UK). The 2,5-diphenyloxazole, 1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene, Triton X, and toluene for liquid scintillator were purchased from Nakarai Chem. Co. (Japan).

The rat plasma vitamin D-binding protein (DBP) solution was prepared by diluting the vitamin D-deficient male rat plasma (1:70,000) in 3.5 mM barbiturate buffer solution (pH 8.6) containing 0.1% ovalbumin. Commercial grades of chick embryonal intestinal receptor (Yamasa Shoyu Co., Japan) were used. The powdered receptor (25 mg) was dissolved in 50 ml of 0.05 M phosphate buffer solution including 0.3 M KCl (0.5 ml). All organic solvents of analytical grades (Nakarai Chem. Co.) were distilled before use. Other reagents as described in the previous papers (10) were used.

Vitamin preparations. Multivitamin tablet: A commercially available multivitamin tablet (commercial name: Panvitan Hi, Takeda Chem. Ind.) containing 100 IU of vitamin D$_2$ per tablet was used. The tablet contains thiamin tetrahydrofurfuryldisulfide, vitamin B$_2$, B$_6$, B$_{12}$, and C, nicotinamide, calcium pantothenate, retinyl palmitate, dl-$\alpha$-tocopheryl acetate, calcium, and magnesium, beside vitamin D$_2$. Vitamin D liquid preparation: A commercially available vitamin D liquid preparation containing 16,000 IU of vitamin D$_2$ per ml, but not containing other vitamins and minerals, was used. When the concentrations of vitamin D$_2$ in both preparations were determined by the method previously reported (3), both of them were between 105 and 110% of the respective guaranteed values.

Subjects. Six healthy male volunteers, 20–25 years old, participated in the study. None of them showed HBs antigen positive, history of drug allergy, daily intake of vitamin preparations, and abnormality in a medical examination. All of them showed the values within the respective normal ranges on blood pressure, physiological tests (body temperature, respiratory rate, and pulse), and the laboratory tests including routine hematological and urinary examinations. These medical checks and the laboratory tests were performed before and after the experiments. In addition, two other male volunteers, 30 and 40 years of age, participated in a preliminary experiment under the same condition as described above. This study was approved by the Medical Ethics Committee of the Faculty of
Protocols for the experiments. 1) Experiment 1 (Daily administration of 400 IU of vitamin D$_2$ by a multivitamin tablet for 1 week): All of the volunteers stayed in a hotel in Kyoto and took the same meals at fixed times during the period from one day before the experiment to the final day. They were allowed to smoke as usual if they were smokers, but neither soft drinks, orange juice, nor alcoholic beverages were allowed during the experiment. Since Shiitake mushroom (Lentinus edodes) contains rather large amounts of vitamin D$_2$ (3), intake of the mushroom was restricted during the experiment. Their daily activities were not restricted, unless these were unusual.

The protocol for experiment 1 was set as follows: The volunteers took breakfast at $8:30$ and subsequently 4 tablets of a multivitamin preparation (400 IU of vitamin D$_2$) at $9:30$ according to a medical doctor's direction every morning. They took lunch at noon and dinner at $6:30$ every evening. Blood samples were collected just before (0h) and 2, 4, 6, 8, 10, 12, 24, 48, 96, 144, 146, 148, 150, 152, 154, 156, and 168h after the first administration. The samples were immediately heparinized, centrifuged to separate the plasma, and stored at $-20^\circ$C with a stream of argon in sealed test tubes until use. The blood samples were similarly taken from the two volunteers of the preliminary experiment, while the 24-h urine samples were collected from these two volunteers once at the last morning. Separative assay on the concentrations of the D$_2$ and D$_3$ compounds of vitamin D, 25-OH-D, and 1,25(OH)$_2$D in all the plasma and urine samples was performed, while those of 24, 25(OH)$_2$D in the plasma samples obtained just before (0h) and 24, 144, and 168h after the first administration were determined. The preliminary experiment and the experiment 1 were performed in August, 1987, and in December, 1987, respectively.

2) Experiment 2 (Single administration of 0.25ml of a vitamin D liquid preparation equivalent to 4,000 IU of vitamin D$_2$): The three volunteers controlled to experiment 2 were from the five volunteers participating in experiment 1 (A volunteer who showed abnormally high levels of 1,25(OH)$_2$D was omitted). The protocol for experiment 2 was set as follows: The volunteers subsequently stayed in the same hotel for a week after finishing experiment 1. Their living conditions were the same as described above, but they were not allowed to take vitamin D to wash out the vitamin D$_2$ and metabolites administered by experiment 1. After taking breakfast at $8:30$ in the morning of the 8th day, the volunteers were orally given 0.25ml of a vitamin D liquid preparation (4,000 IU of vitamin D$_2$) by a medical doctor and maintained the same living conditions for the subsequent one day. Blood samples were collected from the volunteers just before (0h) and 2, 4, 6, 8, 10, 12, and 24h after the first administration. The samples were similarly treated as mentioned above and separative assay on the concentrations of the D$_2$ and D$_3$ compounds of vitamin D, 25-OH-D, 1,25(OH)$_2$D, and 24,25(OH)$_2$D in all the plasma samples was performed.

Simultaneous determination of the D$_2$ and D$_3$ compounds of vitamin D and 25-OH-D in plasma. Exactly 1.0ml of a plasma sample was taken, and vitamin D$_2$,
vitamin D₃, 25-OH-D₂, and 25-OH-D₃ were simultaneously assayed by the method previously reported (10). The detection limits were 0.5 ng/ml for vitamin D₂ and vitamin D₃, and 1 ng/ml for 25-OH-D₂ and 25-OH-D₃.

Simultaneous determination of 24,25(OH)₂D₂, 24,25(OH)₂D₃, 1,25(OH)₂D₂, and 1,25(OH)₂D₃ in plasma. 1) Extraction of lipid. Exactly 1.5 ml of plasma sample was placed in a 10 ml glass centrifuge tube with a screw cap and then each 25 µl of the radioactive standard solutions (2,500 disintegrations per minute (dpm) in 25 µl of ethanol) of [23,24(n)-³H]-24,25(OH)₂D₃, and [26,27-methyl-³H]-1,25(OH)₂D₃ ethanolic solution was added for the measurement of recovery. After allowing these to stand for 1 h at room temperature, 0.75 ml of tetrahydrofuran (THF) was added dropwise with stirring and then 4.5 ml of ethyl acetate was added. The extraction of lipid was performed by vortex-mixing for 2 min, standing for 10 min, and again vortex-mixed for a further 2 min. After centrifugation at 3,000 rpm for 10 min, the separated ethyl acetate layer was taken in another 10 ml centrifuge tube and the remaining water layer was similarly extracted once more with 3.0 ml of ethyl acetate. The combined ethyl acetate extract was dehydrated with anhydrous sodium sulfate and centrifuged at 3,000 rpm for 5 min. The separated ethyl acetate layer was evaporated under reduced pressure.

2) Separation of 24,25(OH)₂D and 1,25(OH)₂D fractions. The residue thus obtained was dissolved in 2.0 ml of 20% (v/v) isopropanol in n-hexane and then filtered through a membrane filter (Kurabo Co., Japan) to eliminate the remaining sodium sulfate completely. The filtrate was evaporated under reduced pressure and the resulting residue was dissolved in 200 µl of the same solvent mixture. The solution was applied to the preparative HPLC [I] as described in the following to collect the fractions of 24,25(OH)₂D and 1,25(OH)₂D:

Preparative HPLC [I]
Column: Zorbax SIL (4.6 × 250 mm, DuPont Co., USA)
Mobile phase: 20% (v/v) isopropanol in n-hexane
Flow rate: 1.0 ml/min (40 kg/cm²)
Collection: 24,25(OH)₂D fraction, 370-470 drops (retention time, 6.5-8.3 min); 1,25(OH)₂D fraction, 490-620 drops (retention time, 8.6-10.9 min).

3) Competitive protein-binding assay (CPBA) for 24,25(OH)₂D₂ and 24,25(OH)₂D₃. The collected 24,25(OH)₂D fraction was evaporated under reduced pressure and the resulting residue was dissolved in 200 µl of 20% (v/v) methanol in acetonitrile to apply successively to the following preparative HPLC [II] for further clean-up.

Preparative HPLC [II]
Column: Nucleosil 5C₁₈ (7.5 × 300 mm, Nagel Co., Federal Republic of Germany)
Mobile phase: 20% (v/v) methanol in acetonitrile
Flow rate: 1.5 ml/min (40 kg/cm²)
Collection of 24,25(OH)₂D fraction: 800-1,050 drops (retention time,
The collected 24,25(OH)₂D fraction was evaporated under reduced pressure and the resulting residue was dissolved in 200 µl of 5.5% (v/v) isopropanol in n-hexane to apply successively to the following preparative HPLC [III] for separating the fractions of 24,25(OH)₂D₂ and 24,25(OH)₂D₃.

**Preparative HPLC [III]**
- **Column**: Zorbax SIL (4.6 × 250 mm)
- **Mobile phase**: 5.5% (v/v) isopropanol in n-hexane
- **Flow rate**: 1.1 ml/min (40 kg/cm²)
- **Collection of 24,25(OH)₂D₂ fraction**: 1,150–1,300 drops (retention time, 15.0–17.0 min); 24,25(OH)₂D₃ fraction, 1,300–1,450 drops (retention time, 17.0–19.0 min).

Each of the collected fractions was evaporated under reduced pressure and each resulting residue was dissolved in each 650 µl of ethanol and applied to a CPBA method, a modification of Belsey's 25-OH-D₃ assay (11). Exactly 100 µl of the aliquot of 24,25(OH)₂D₃ fraction was taken for measuring the recovery. The value was also applied to that of 24,25(OH)₂D₂.

Each standard solution of 24,25(OH)₂D₂ or 24,25(OH)₂D₃ was prepared by dissolving each standard compound in ethanol to make the concentrations of 0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 512 ng/100 µl, respectively. The [23,24(n)-³H]-24,25-OH-D₃ (15,000 dpm/50 µl) was put in each test tube and the aliquot obtained above (100 µl) or one of the standard solutions (100 µl) was added to it. After adding the rat plasma DBP solution (1 ml) to each tube and allowing this to stand for 1 h in ice water, a 0.025% dextran–0.25% activated-charcoal suspended solution (0.5 ml) was added with stirring. After allowing this to stand for 10 min in ice water, it was centrifuged at 3,000 rpm for 10 min. A portion of the supernatants (1 ml) was placed in a vial tube followed by adding the liquid scintillator (3 ml) and then the radioactivity was measured by a liquid scintillation counter (Aloka LSC-700, Aloka Co., Japan). The counting efficiency was approximately 45%. A calibration curve was described by the data of standard solutions. A concentration of 24,25(OH)₂D₂ or 24,25(OH)₂D₃ (ng/ml) was calculated by the following formula:

\[
\text{Concentration of 24,25(OH)₂D₂ or 24,25(OH)₂D₃ in plasma (ng/ml)} = A \times \frac{0.65}{0.10} \times \frac{1}{R} \times \frac{1}{V} \times 100;
\]

- **A**: Calculated value by the data on the sample and the calibration curve (ng/tube)
- **R**: Recovery (%)
- **V**: Taken volume of a sample solution (ml).

4) **Radioreceptor assay (RRA) for 1,25(OH)₂D₂ and 1,25(OH)₂D₃**. The 1,25(OH)₂D fraction collected from the preparative HPLC [I] was evaporated under reduced pressure and the resulting residue was successively applied to the following preparative HPLC [IV] for further clean-up.
Preparative HPLC [IV]
Column: Nucleosil 5C18 (7.5 × 300 mm)
Mobile phase: 20% (v/v) methanol in acetonitrile
Flow rate: 1.5 ml/min (40 kg/cm²)
Collection of 1,25(OH)₂D fraction: 800–1,050 drops (retention time, 9.6–12.6 min).

The collected 1,25(OH)₂D fraction was evaporated under reduced pressure and the resulting residue was dissolved in 200 μl of 5% (v/v) isopropanol in dichloromethane to apply successively to the following preparative HPLC [V] for separating the fractions of 1,25(OH)₂D₂ and 1,25(OH)₂D₃.
Preparative HPLC [V]
Column: Zorbax SIL (4.6 × 250 mm)
Mobile phase: 5% (v/v) isopropanol in methylene chloride
Flow rate: 1.1 ml/min (40 kg/cm²)
Collection of 1,25(OH)₂D₂ fraction: 1,060–1,220 drops (retention time, 10.6–12.2 min); 1,25(OH)₂D₃ fraction: 1,250–1,410 drops (retention time, 12.4–14.2 min).

The 1,25(OH)₂D₂ and 1,25(OH)₂D₃ fraction collected from the preparative HPLC [V] was evaporated under reduced pressure and each resulting residue was dissolved exactly in 80 μl of ethanol (sample solution) to apply to a RRA method modified from Eisman et al. (12). Exactly 20 μl of the aliquot of 1,25(OH)₂D₃ fraction was taken for measuring the recovery. The value was also applied to 1,25(OH)₂D₂. The ethanol solutions containing 0, 2, 4, 8, 16, 32, 64, 128, and 5,000 pg per 50 μl of 1,25(OH)₂D₂ or 1,25(OH)₂D₃ were prepared as the standard solutions. After taking [26,27-methyl-³H]-1,25(OH)₂D₃ ethanolic solution (10,000 dpm/100 μl) in each test tube, exactly 50 μl of either the sample or standard solutions and 0.5 ml of the receptor solution were added to them with mixing and the solutions were allowed to stand for 3 h at 4°C. Then, a cooled 0.006% dextran–0.06% activated-charcoal suspended solution (0.125 ml) was added to them with vigorous shaking and the solutions were allowed to stand for 30 min in ice water. After centrifugation at 3,000 rpm for 10 min, a portion of the supernatants (0.5 ml) was taken in a vial tube and the liquid scintillator (3 ml) was added to them to measure the radioactivity. The counting efficiency was approximately 45%. A calibration curve was described by the data of standard solutions. A concentration of 1,25(OH)₂D (pg/ml) was calculated by the following formula:

Concentration of 1,25(OH)₂D₂ or 1,25(OH)₂D₃ in plasma (pg/ml)
\[
A = A \times \frac{0.08}{0.05} \times \frac{1}{R} \times \frac{1}{V} \times 100;
\]

where:
- \( A \): Calculated value by the data on the sample and the calibration curve (pg/tube)
- \( R \): Recovery (%)
- \( V \): Taken volume of a sample solution (ml).
The detection limits were 0.2 ng/ml for 24,25(OH)₂D₂ and 24,25(OH)₂D₃, and 8 pg/ml for 1,25(OH)₂D₂ and 1,25(OH)₂D₃, respectively. The ND (not detected) means that the values were below the respective lower limits.

Assay of vitamin D and its metabolites in urine. Exactly 50 ml of a urine sample was taken and lyophilized, saponified with alkali, and the unsaponifiable matter was extracted according to the conventional method, which was applied to the assay of vitamin D and its metabolites in the plasma.

Biochemical measurements. Biochemical measurements were performed by Nichiyaku Medical Laboratories Co. (Japan) according to the conventional methods.

RESULTS

1. Medical examinations for volunteers

   The medical examinations described in the EXPERIMENTAL section were performed three times—before, during, and after the experiments—in the eight volunteers including the two who were enrolled in a preliminary experiment. All data showed that all of them were healthy, and neither hypervitaminosis nor hypovitaminosis was observed in the volunteers.

2. Results on the preliminary experiment

   A preliminary experiment on experiment 1 (repeated administration of 400 IU of vitamin D₂ per day through a multivitamin tablet for 1 week) was performed on the volunteers according to the EXPERIMENTAL section, and the changes in the plasma concentrations of vitamin D and its metabolites are shown in Table 1. When the concentrations of vitamin D and its metabolites in the urine during 24 h at the 7th day were also assayed on the two volunteers, neither vitamin D (D₂ and D₃) nor the metabolites was detected in the urine of volunteers. Therefore, we decided that the collection of urine samples would not be performed in experiments 1 and 2.

3. Results on experiment 1

   Experiment 1 (daily administration of 400 IU of vitamin D₂ by a multivitamin tablet for 1 week) was performed on the six volunteers according to the protocol. Since one of the six volunteers showed abnormally high values of 1,25(OH)₂D₂ and 1,25(OH)₂D₃ in the plasma levels at the all stages after administration, all the data on this volunteer were excluded from the statistical analysis. The results on the remaining five volunteers are shown in Figs. 1 and 2, which stress mainly the daily and hourly variations of the plasma levels, respectively.

4. Results on experiment 2

   Experiment 2 (single administration of 0.25 ml of a vitamin D liquid preparation equivalent to 4,000 IU of vitamin D₂) was performed in the three volunteers according to the protocol. The hourly variations in the plasma levels of vitamin D₂
Table 1. Assayed values on the preliminary experiment.

<table>
<thead>
<tr>
<th>Time</th>
<th>Vitamin D&lt;sub&gt;2&lt;/sub&gt; (ng/ml)</th>
<th>25-OH-D&lt;sub&gt;3&lt;/sub&gt; (pg/ml)</th>
<th>24,25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt; (pg/ml)</th>
<th>24,25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt; (pg/ml)</th>
<th>25(OH)&lt;sub&gt;2&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt; (pg/ml)</th>
<th>24,25(OH)&lt;sub&gt;3&lt;/sub&gt;D&lt;sub&gt;3&lt;/sub&gt; (pg/ml)</th>
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<td>0h</td>
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<tr>
<td>4h</td>
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Note: The values are the means of each data on the two volunteers.
Fig. 1. Daily variation in the concentrations of vitamin D and its metabolites in the plasma of healthy subjects after daily oral administration of 400 IU of vitamin D2. (○) D2 compounds; (●) D3 compounds. The data are shown as M ± SEM.

and D3 and their metabolites are shown in Fig. 3.

DISCUSSION

Studies on the bioavailability of vitamin D were previously reported by Whyte et al. (13), Hartwell et al. (14), and Tjellesen et al. (15). Whyte et al. (13) observed the changes in plasma levels of 25-OH-D after administration of vitamin D2 or vitamin D3 to healthy subjects orally, intramuscularly, or intravenously. However, since the dose was extremely high, i.e., 100 μg/kg (4,000 IU/kg, equivalent to 240,000 IU for a person weighing 60 kg), the results could not be taken into consideration in a study of bioavailability at a physiological dose. On the other hand, Hartwell et al. (14) administered orally 4,000 IU of vitamin D2 or vitamin D3 per day to healthy premenopausal women aged from 22 to 49 years old for 8 weeks, and reported the changes in plasma levels of 1,25(OH)2D2 and 1,25(OH)2D3. The results showed that the plasma 1,25(OH)2D2 levels increased with a decrease of 1,25(OH)2D3 levels to keep a constant level of the sum of 1,25(OH)2D2 and 1,25(OH)2D3.
1,25(OH)\textsubscript{2}D\textsubscript{3} with regard to the administration of vitamin D\textsubscript{2}, while the opposite results were obtained by the administration of vitamin D\textsubscript{3}. The results are very interesting because it is likely that the strict feedback regulation of 1\alpha-hydroxylation occurs in the process of conversion from 25-OH-D to 1,25(OH)\textsubscript{2}D in the kidney without distinguishing the D\textsubscript{2} and D\textsubscript{3} compounds.

The recommended dietary allowances (RDA) of vitamin D for adults are 100 IU/day in Japan, 200 IU/day in USA, and 400 IU/day in France, respectively. To our knowledge, there have been no reports on bioavailability with the RDA levels; thus, this study was conducted. The results on the preliminary experiment showed that the reflection of vitamin D\textsubscript{2} administration on the plasma levels of the metabolites was not so high (Table 1) even when the highest RDA dose (400 IU/day) was chosen. Therefore, we chose daily oral administration of 400 IU for 1 week in experiment 1, whereas the single oral dose of 4,000 IU was chosen in experiment 2 to compare with experiment 1.

In experiment 1, the protocol was designed to study both daily and hourly variations in the plasma levels of vitamin D and metabolites. Figure 1 shows the daily variation. As mentioned above, vitamin D\textsubscript{3} and its metabolites are mainly derived from the photobiogenesis in the skin with sunlight exposure. With regard to the changes in the plasma levels of endogenous vitamin D\textsubscript{3} and its metabolites, the plasma levels of vitamin D\textsubscript{3} itself always showed very low values below 3.5 ng/ml, which were lower than those of 25-OH-D\textsubscript{3} (25–33 ng/ml) as shown in Fig. 1. The results suggest that the photosynthesized vitamin D\textsubscript{3} in the skin is rapidly
transported and metabolized to 25-OH-D₃ in the liver. It has been reported that the major circulating metabolite of vitamin D₃ is 25-OH-D₃ and its plasma concentration directly reflects the nutritional status of vitamin D, especially endogenous vitamin D₃ (1). The levels of 25-OH-D₃ gave fairly constant values between 25 and 33 ng/ml. Since experiment 1 was carried out in the winter season to avoid the seasonal variation and the subjects were instructed to conduct a usual life, relatively constant levels within the normal range (10-40 ng/ml) were obtained. Therefore, the data suggested that this vitamin D₃ is normally photosynthesized in the skin with appropriate exposure to sunlight. It is well documented that the metabolism of 25-OH-D to 1,25(OH)₂D in the kidney is strictly regulated by a feedback system to give a constant level of 1,25(OH)₂D in plasma (1). Our data on the plasma levels of 1,25(OH)₂D₃ also showed the regulation system because all of the values were approximately constant within the normal range (35-65 pg/ml) as shown in Fig. 1. The results are similar to those reported by Hartwell et al. (14). The plasma levels of 24,25(OH)₂D₃ showed only a small change of about 1 ng/ml, being within the normal range (1-4 ng/ml).

With regard to the changes in the plasma levels of exogenous vitamin D$_2$ and its metabolites in experiment 1, as shown in Fig. 1, both the levels of vitamin D$_2$ and 24,25(OH)$_2$D$_2$ remained below the detectable limits during the experiment period, respectively. On the other hand, although the levels of 25-OH-D$_2$ and 1,25(OH)$_2$D$_2$ showed a slight increase and the undetectable levels in the first half of the experiment period, respectively, minor increases in both compounds were observed in the latter half. Therefore, it is suggested that a significant increase in 25-OH-D$_2$ may occur when an experiment with a daily dose of 400 IU of vitamin D$_2$ is conducted for a term longer than 1 week (i.e., longer than 6 months). Figure 2 shows the hourly changes in the plasma levels of 25-OH-D and 1,25(OH)$_2$D by the daily oral administration of 400 IU of vitamin D$_2$. When the changes were observed at short time intervals after the administration, no special pattern was obtained; more long-term administration in the experiments in thus required. We have already started a long-term experiment with daily oral administration of 400 IU of vitamin D$_2$/day for one year and the results will be reported.

Figure 3 shows the hourly variation in the plasma levels after a single dose of 4,000 IU of vitamin D$_2$. The plasma levels of vitamin D$_3$ and its metabolites were not specifically affected by the administration of vitamin D$_2$, which were within the respective normal ranges. On the other hand, the single dose of vitamin D$_2$ (4,000 IU) effected a significant increase in the plasma levels of vitamin D$_2$ itself although these were not so large. The results suggest that a significant increase of the D$_2$ compounds in plasma may be observed by single dose of 4,000 IU of vitamin D$_2$. However, since the administration of vitamin D$_2$ results in the small increase of plasma 25-OH-D$_2$ levels, a single dose of 4,000 IU of vitamin D$_2$ may be still insufficient to give positive effects on the plasma levels. The plasma levels of 1,25(OH)$_2$D$_2$ were slightly increased within 24 h after the administration, and those of 24,25(OH)$_2$D$_2$ were below the detectable limit. The results also suggest that the single dose may be insufficient to increase the plasma levels of 1,25(OH)$_2$D and 24,25(OH)$_2$D. However, the single dose of 4,000 IU of vitamin D$_2$ (which is 40 times higher than the RDA in Japan) may not give a risk of hypervitaminosis D.

We wish to thank Prof. H. Morii of Osaka City University for his helpful discussion. We are also indebted to A. Morita of Osaka City University, and A. Takeuchi and N. Tsugawa of Kobe Women’s College of Pharmacy for their technical assistance.

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Vol. 35, No. 4, 1989


