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Simplified method for the determination of 25-hydroxy and 1α,25-dihydroxy metabolites of vitamins D_2 and D_3 in human plasma Application to nutritional studies

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Abstract

A simplified method for the determination of 25-hydroxy and 1α,25-dihydroxy metabolites of vitamins D_2 and D_3 in human plasma was developed. Plasma samples were deproteinizated and applied to a Bond Elut C_{18}OH cartridge to separate 25-hydroxyvitamin D (25-OH-D) and 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D] fractions. The 25-OH-D fraction was purified by a Bond Elut C_{18} cartridge and 25-OH-D_2 and 25-OH-D_3 were assayed by HPLC using a Zorbax SIL column. The 1,25$(OH)_2$D fraction obtained above was subsequently applied to HPLC using a Zorbax SIL column to separate 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ fractions which were determined by a radioreceptor assay (RRA) using calf thymus receptor. The method was applied to nutritional studies.

Keywords: Vitamins; 25-Hydroxyvitamin D_2; 1α,25-Dihydroxyvitamin D_2; 25-Hydroxyvitamin D_3; 1α,25-Dihydroxyvitamin D_3

1. Introduction

Vitamins D_2 and D_3 are metabolized to 25-OH-D_2 and 25-OH-D_3, respectively in the liver and subsequently to 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ in the kidney [1–3]. Since the photochemically biosynthesized vitamin D in the skin of mammals is vitamin D_3 and vitamin D_2 is not formed in the body [4], all of the vitamin D_2 and its metabolites detected in vivo are derived from exogenous sources, such as vitamin D_2 preparations, vitamin D_2 enriched foods and mushrooms. Therefore, separate determination of vitamin D_2 and D_3 metabolites in plasma is important in the field of nutritional and clinical studies. For instance, when vitamin D_2 is used for nutritional and/or clinical purposes, the assayed values of vitamin D_2 metabolites in plasma give direct information on the fate of exogenous vitamin D_2.

We previously reported a method for measuring vitamins D_2, D_3 and their metabolites in plasma [5]. However, it requires extensive procedures involving several steps of HPLC to completely eliminate concomitant impurities due to low specificity of the used chick intestinal receptor for 1,25$(OH)_2$D. Recently, it was found that the calf thymus receptor [6,7] has high sensitivity and specificity for

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$1,25(OH)_2D$ and therefore a simplified clean-up procedure using only a silica cartridge column (Bond Elut $C_{18}OH$) is sufficient to perform the assay of $1,25(OH)_2D$. Wei et al. [8] reported the determination of vitamin D metabolites in plasma or serum using a calf mammary gland receptor [9]. This receptor had a high sensitivity and specificity for $1,25(OH)_2D$ like that of the calf thymus, but the method does not include separate determination of the vitamin D_2 and D_3 metabolites. The systems using calf thymus or calf mammary gland receptor and Bond Elut $C_{18}OH$ cartridge can assay $1,25(OH)_2D$. We confirmed that a commercial kit using the calf thymus receptor is useful for the gross and separate determination of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ [10]. We extended this procedure to determine 25-OH-D_2, 25-OH-D_3, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ and applied it to two nutritional studies including vitamin D_2 supplementation.

2. Experimental

2.1. Materials, reagents, equipment and samples

2.1.1. Materials and reagents

Commercial grades (Solvay-Duphar, Weesp, Netherlands) of 25-OH-D_3 and $1,25(OH)_2D_3$ were used as the respective standard compounds. 25-OH-D_2 was obtained by the in vivo experiment as described previously [11]. $1,25(OH)_2D_2$ was kindly donated by Professor Hector F. DeLuca, University of Wisconsin. Commercial grades of [23,24(*n*)-^{3}H]-25-OH-D_3 (specific activity, 3.3 TBq/mmol) and [26,27-methyl-^{3}H]-$1,25(OH)_2D_3$ (specific activity, 6.7 TBq/mmol) were purchased from Amersham (Buckinghamshire, UK). Bond Elut C_{18} cartridges were purchased from Varian (Harbor City, CA, USA). A commercially available assay kit including the Bond Elut $C_{18}OH$ cartridge and calf thymus receptor was obtained from the Nichols Institute (Wijchen, Netherlands). Organic solvents of analytical grade were distilled before use. All other reagents were of the highest purity.

2.1.2. Equipment

Shimadzu LC-6A high-performance liquid chromatographs (Kyoto, Japan) were equipped with Shimadzu SPD-6A detectors (265 nm, AUFS 0.001) and fraction collectors with a drop counter (Gilson, Middleton, WI, USA) were used.

2.1.3. Plasma samples

Heparin-anticoagulated blood was collected by venepuncture and centrifuged to prepare plasma samples. Intra- and inter-assay variation was examined in plasma samples obtained from healthy male volunteers before vitamin D_2 supplementation in the following experiment described as the Application 1 (see Section 2.4.1, vitamin D_2 supplementation studies). The study was approved by the hospital ethical committee and informed consent from the volunteers was obtained.

2.2. Analytical methods for the determination of 25-OH-D_2, 25-OH-D_3, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ in plasma or serum samples

The flow scheme of the proposed method is summarized in Fig. 1.

2.2.1. Deproteinization and isolation of 25-OH-D and $1,25(OH)_2D$ with the Bond Elut $C_{18}OH$ cartridges

Plasma or serum samples (0.5 ml) are placed in glass centrifuge tubes (10 ml) with a screw cap, then [^{3}H]-25-OH-D_3 (2000 dpm) and [^{3}H]-$1,25(OH)_2D_3$ (2000 dpm) are added and the mixture is stirred. One

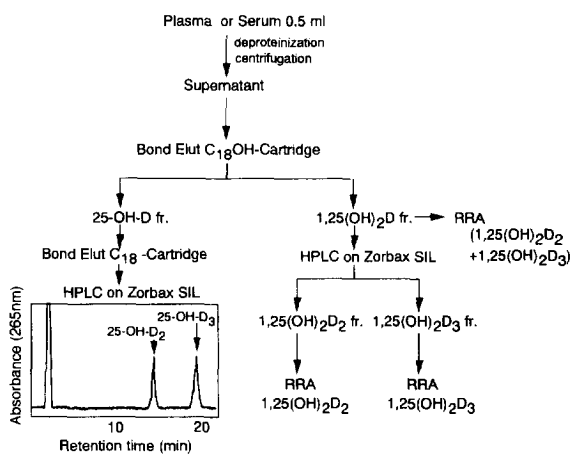

Fig. 1. Flow scheme for the determination of 25-OH-D and $1,25(OH)_2D$.

ml of acetonitrile is added and the tube is centrifuged. The deproteinizated supernatant is transferred to another tube. After adding 1 ml of a phosphate buffer solution (pH 10.5) and centrifugation, the supernatant is applied to a Bond Elut C_{18}OH cartridge. After subsequent washing with 5 ml of water, 5 ml of 70% methanol in water and 1 ml of hexane, the 25-OH-D fraction is collected by elution with 10% dichloromethane in hexane. After washing with 1% isopropanol in hexane, the $1,25(OH)_2D$ fraction is collected by subsequent elution with 5% isopropanol in hexane. Each fraction is evaporated to dryness under reduced pressure.

2.2.2. Further purification of 25-OH-D and quantification of 25-OH-D_2 and 25-OH-D_3

The residue of the 25-OH-D fraction is dissolved in 1 ml of 70% methanol in water and further purified by a Bond Elut C_{18} column. After subsequent washing of the column with 15 ml of 70% methanol in water and 1 ml of 20% methanol in acetonitrile, the purified 25-OH-D fraction is collected by subsequent elution with 4 ml of 20% methanol in acetonitrile.

The purified 25-OH-D fraction is subjected to the following HPLC conditions to quantify 25-OH-D_2 and 25-OH-D_3 by UV detection. Column, Zorbax SIL (250×4.6 mm); mobile phase, 2% isopropanol in hexane; flow-rate, 2.1 ml/min (80~90 kg/cm^2); retention time, 14.8 min for 25-OH-D_2 and 19.2 min for 25-OH-D_3.

The peak heights of 25-OH-D_2 and 25-OH-D_3 in the chromatogram are compared with those of the respective authentic compounds. The 25-OH-D_3 fraction is collected and the radioactivity is measured to calculate the recovery. The same value of recovery can be used for 25-OH-D_2.

2.2.3. Separation and quantification of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$

The $1,25(OH)_2D$ fraction is subjected to the following HPLC conditions to separate $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. Column, Zorbax SIL (250×4.6 mm); mobile phase, 5% isopropanol in dichloromethane; flow-rate, 2.0 ml/min (100~110 kg/cm^2); collection of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ fractions, 10~11.5 min for $1,25(OH)_2D_2$ and 12~14 min for $1,25(OH)_2D_3$.

The separated $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ fractions are applied to the procedure using a commercial assay kit with the calf thymus receptor [10]. When separate determination of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ is unnecessary, the $1,25(OH)_2D$ fraction obtained from the Bond Elut C_{18} column can be directly applied to the radioreceptor assay (RRA).

2.2.4. Measurement of total $1,25(OH)_2D$

When separate determination of $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ is unnecessary, the $1,25(OH)_2D$ fraction obtained from the Bond Elut C_{18}OH cartridge is directly applied to the RRA.

2.3. Measurement of calcium, phosphorus, parathyroid hormone and alkaline phosphatase

Calcium (Ca) and inorganic phosphorus (Pi) in plasma are determined by atomic absorption spectrophotometry as described previously [12] and by the colorimetric method of Chen et al. [13], respectively. High-sensitive parathyroid hormone (HS-PTH) in plasma is determined by radioimmunoassay using a commercial kit (Yamasa Shoyu, Tokyo, Japan), which recognizes the inclusive PTH (44–68). Alkaline phosphatase activity (AlP) in plasma is determined by a commercial kit (Wako Pure Chemical Industries, Osaka, Japan).

2.4. Application to nutritional studies

2.4.1. Application 1: Vitamin D_2 supplementation studies

Healthy Japanese volunteers including ten young males (average age: 27.6 years), ten young females (average age: 23.5 years), ten institutionalized elderly males (average age: 77.5 years) and ten institutionalized elderly females (average age: 80.2 years) were enrolled in this study. They were living with normal ambulation, without receiving excess sunlight exposure or especially unusual results in examination and screening tests. No-one had received pharmaceuticals containing vitamin D for several months before the study. They received successive oral administrations of a multivitamin preparation containing 200 IU/day

of vitamin D_2 for 4 weeks from May to June in 1993. Heparin-anticoagulated blood collected by venepuncture from the volunteers on the first day before taking the multivitamin preparation and after 4 weeks' supplementation was centrifuged to prepare plasma samples. All of the plasma was stored at −20°C until assay. The study was approved by the hospital ethical committee and informed consent from the volunteers was obtained.

2.4.2. Application 2: Studies on a patient with xeroderma pigmentosum

A male infant (normal delivery and fed naturally) was diagnosed as having xeroderma pigmentosum at 7 months of age and protected from sunlight to cut off the ultraviolet (UV) rays above 290 nm for 3 months. He was diagnosed as vitamin D deficient and treated with 1α-hydroxyvitamin D_3 (1α-OH-D_3) and vitamin D_2.

2.5. Statistics

Student's *t*-tests were used for confirming significant differences between the levels before and after vitamin D_2 supplementation as paired tests and between those of males and females or between those of elderly and young subjects as unpaired tests. Values of $p<0.05$ were considered significant.

Table 1
Analytical data obtained by the proposed method

	n	25-OH-D_3	1,25$(OH)_2D_3$
Sensitivity		0.5 ng	1.25 pg
Overall recovery(%)		94.1	105.2
Intra-assay			
Mean±S.D.	6	32.6±1.3 ng/ml	38.0±3.1 pg/ml
C.V. (%)		4.0	8.2
Inter-assay			
Mean±S.D.	6	24.2±0.2 ng/ml	6.5±0.7 pg/ml
C.V. (%)		0.9	10.3

3. Results and discussion

3.1. Determination of 25-OH-D_2, 25-OH-D_3, 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$ in plasma

3.1.1. Elution profiles of 25-OH-D and 1,25$(OH)_2$D through Bond Elut C_{18}OH and of 25-OH-D through Bond Elut C_{18}

The elution profile of [^{3}H]-25-OH-D_3 and [^{3}H]-1,25$(OH)_2D_3$ through a Bond Elut C_{18}OH cartridge is shown in Fig. 2A. Using the solvent mixtures described in the text (Section 2.2), [^{3}H]-25-OH-D_3 and [^{3}H]-1,25$(OH)_2D_3$ were eluted by 10% dichloromethane in hexane and 5% isopropanol in hexane, respectively. The overall recoveries of the both compounds by this procedure were over 90% as shown in Table 1. The elution profile of [^{3}H]-25-OH-D_3 through a Bond Elut C_{18} cartridge is shown

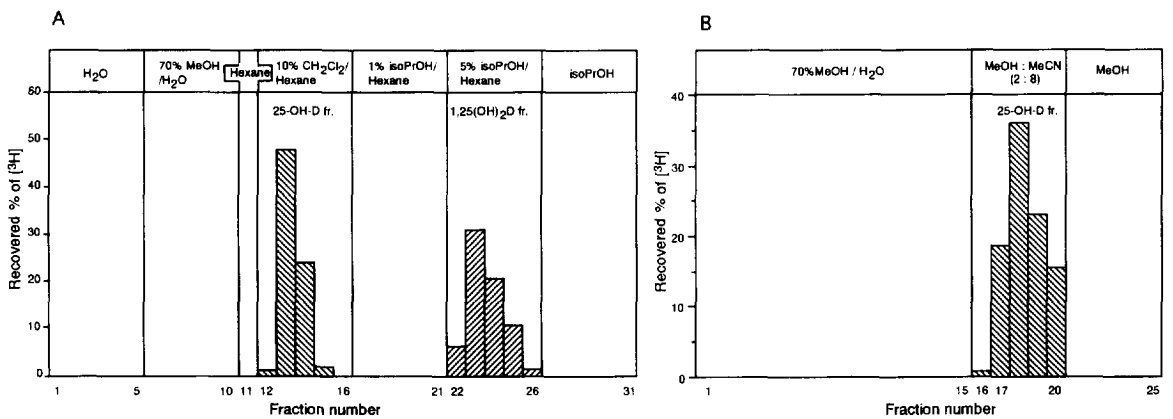

Fig. 2. (A) Elution profile of [^{3}H]-25-OH-D_3 and [^{3}H]-1,25$(OH)_2D_3$ through a Bond Elut C_{18}OH cartridge; 1-ml fractions were collected and the radioactivity was measured. (B) Elution profile of [^{3}H]-25-OH-D_3 through a Bond Elut C_{18} cartridge; 1-ml fractions were collected and the radioactivity was measured.

in Fig. 2B. The recovery of the compound by this procedure was over 90%. 25-OH-D_2/D_3 or $1,25(OH)_2D_2/D_3$ were eluted without separation through these cartridges.

3.1.2. Determination of 25-OH-D and $1,25(OH)_2D$ in plasma

Since 25-OH-D_2 and 25-OH-D_3 were separated by HPLC as shown in Fig. 1, the two compounds were easily determined by measuring the respective peak heights in the chromatogram. Fig. 3 shows the chromatographic profiles of authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. Since the two compounds were separated, the respective fractions were collected and applied to the RRA using the calf thymus receptor. As reported previously [5], since both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ gave practically the same calibration curve using the receptor, $1,25(OH)_2D_3$, which is easily obtained from commercial sources, can be used as a standard compound for the RRA of both compounds. To check sensitivity and reproducibility, we examined pooled plasma samples of healthy subjects (vitamin D_2 metabolites were undetectable in the samples). Table 1 shows that sensitivity and reproducibility were satisfactory.

3.2. Application to nutritional studies

3.2.1. Application 1: Vitamin D_2 supplementation studies for normal subjects

The plasma levels of 25-OH-D_2, 25-OH-D_3, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ were assayed by the proposed method. As shown in Fig. 4, the plasma 25-OH-D levels significantly increased due to the increase of exogenous 25-OH-D_2 after 4 weeks of vitamin D_2 supplementation in both young and elderly people. On the other hand, although exogenous $1,25(OH)_2D_2$ levels increased in both young and elderly people, there was no significant increase in the plasma $1,25(OH)_2D$ ($1,25(OH)_2D_2$+$1,25(OH)_2D_3$) levels because of the well-known feed-back regulation of the kidney [1–3]. Plasma levels of HS-PTH in all the subjects including those before and after vitamin D_2 supplementation showed a significant inverse relation with the levels of 25-OH-D (25-OH-D_2+25-OH-D_3) but not with those of $1,25(OH)_2D$. Krall et al. [14], Eastell et al. [15] and Hegarty et al. [16] also observed the inverse relation between serum 25-OH-D and PTH levels. Krall et al. [14], Eastell et al. [15] and Dubbelman et al. [17] observed that serum PTH levels increased with age. Van der Klis et al. [18] reported that postmenopausal Dutch women had a higher PTH concentration in plasma than premenopausal Dutch women. These results suggest that the plasma levels of 25-OH-D and PTH are useful for evaluating vitamin D nutri-

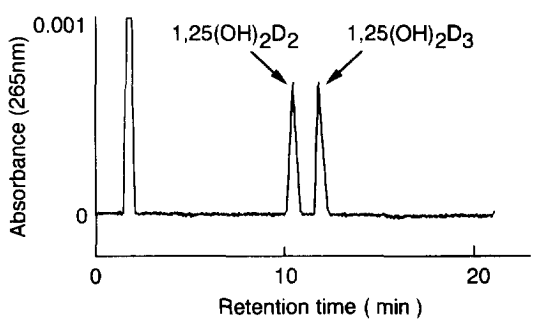

Fig. 3. HPLC profiles of authentic $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. Column, Zorbax SIL (250×4.6 mm); mobile phase, 5% isopropanol in dichloromethane; flow-rate, 2.0 ml/min (100–110 kg/cm^2); retention time, 10.8 min for $1,25(OH)_2D_2$ and 12.9 min for $1,25(OH)_2D_3$.

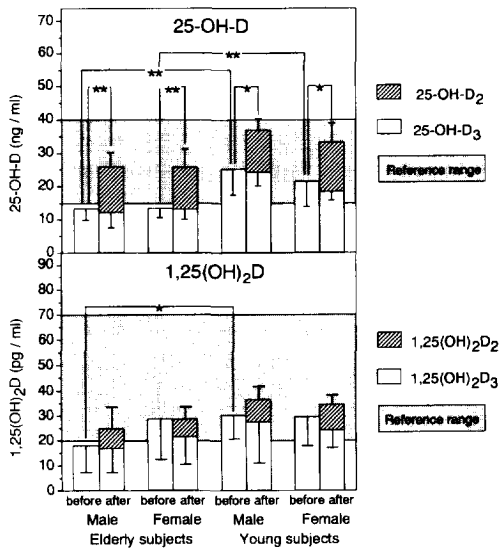

Fig. 4. Changes in the plasma levels of 25-OH-D_2, 25-OH-D_3, $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ in normal young and elderly people before and after daily administration of 200 IU of vitamin D_2 for 4 weeks. Values are expressed as means±S.E.; $*p<0.05$, $**p<0.01$.

tional status. The presently proposed method for the separate assay of vitamins D_2 and D_3 metabolites is especially useful, because the increase in the levels of vitamin D_2 metabolites can be evaluated as originated from the orally administered vitamin D_2.

Our results in this paper indicated that successive supplementation of 200 IU/day of vitamin D for 4 weeks improved vitamin D nutritional status. Dawson-Hughes et al. [19] also reported that vitamin D intake of 200 IU/day reduced bone loss in healthy postmenopausal women residing at latitude 42°N. Van der Klis et al. [18] reported that oral vitamin D_3 administration (either 400 or 800 IU/day) for 4–5 weeks to women living at northern latitudes in late winter/early spring increased plasma 25-OH-D levels to the baseline levels of elderly people living in the tropics. Chapuy et al. [20] reported that supplementation of 800 IU/day of vitamin D and 1200 mg/day of calcium for 18 months to elderly women significantly increased the femoral bone mineral density and significantly decreased the number of hip fractures. These results suggest that successive supplementation of vitamin D and calcium can not only improve the vitamin D nutritional status but also prevent osteoporosis.

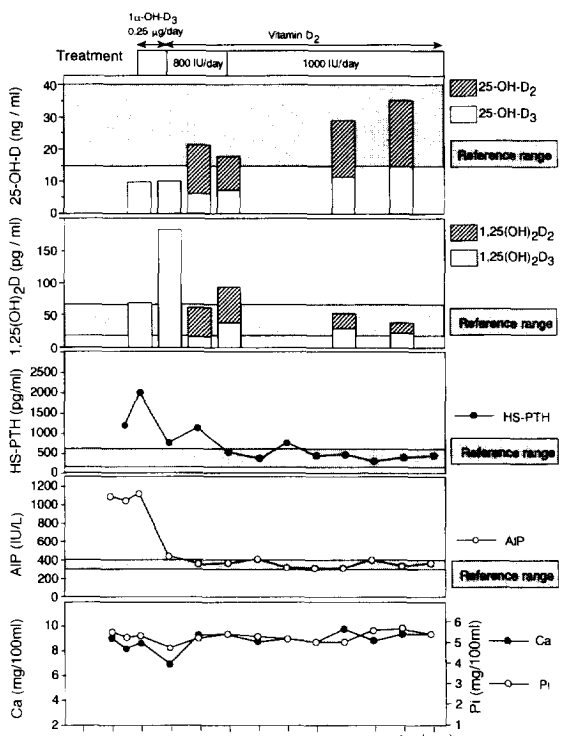

Fig. 5. Changes in the plasma levels of 25-OH-D_2, 25-OH-D_3, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, HS-PTH, AlP, Ca and Pi in a patient with xeroderma pigmentosum during therapy.

3.2.2. Application 2: Studies on a patient with xeroderma pigmentosum

Since the patient had abnormal high values of plasma AlP and HS-PTH with low plasma 25-OH-D levels, he was diagnosed as being vitamin D deficient. He was given oral administration of 0.25 μg/day of 1α-OH-D_3 for 2 months followed by 800 IU/day of vitamin D_2 for 4 months and 1000 IU/day of vitamin D_2 for 20 months since reaching the age of 12 months under protection from sunlight. Fig. 5 shows changes in the plasma levels of AlP, HS-PTH, Ca, Pi and 25-OH-D_2, 25-OH-D_3, 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$, respectively. An abnormal value in the plasma 1,25$(OH)_2D_3$ level, (183.7 pg/ml; 440.9 pmol/l) higher than the reference range (20~70 pg/ml; 48.0~168.0 pmol/l) [5] was observed during treatment with 1α-OH-D_3. Although the ratio of urine Ca/creatinine was 0.03 (reference range: below 0.26) and other hypervitaminosis symptoms were not observed, the treatment was changed to the oral administration of vitamin D_2 to avoid the risk of hypervitaminosis. Plasma 25-OH-D levels (sum of 25-OH-D_2 and 25-OH-D_3) which were mainly derived from exogenous 25-OH-D_2 increased to the normal range (15~40 ng/ml; 37.4~99.8 nmol/l) [5] upon the oral administration of vitamin D_2, while the 1,25$(OH)_2D$ levels (sum of 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$) rapidly decreased to the normal range (20~70 pg/ml; 48.0~168.0 pmol/l), although the 1,25$(OH)_2D_2$ derived from the exogenous vitamin D_2 significantly increased. However, since the HS-PTH levels were above the upper limit of the reference range (180~560 pg/ml), the dose was increased to 1000 IU/day of vitamin D_2 for 20 months. As shown in Fig. 5, all of the plasma levels including HS-PTH remained within the respective reference ranges without showing any symptoms of hypervitaminosis. Since the infant was completely protected from sunshine during treatment, we concluded that at least 1000 IU/day of exogenous vitamin D supplementation is necessary to prevent vitamin D-deficiency in infants without cutaneously

biosynthesized vitamin D_3. These results suggest that the proposed method can help to evaluate the effects of therapy.

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