IDENTIFICATION AND DETERMINATION OF 25-HYDROXYVITAMIN D₃ IN THE BLOOD AND LIVER OF VITAMIN D-DEFICIENT RATS IRRADIATED WITH ULTRAVIOLET LIGHT^{1,2}

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Summary The intestinal calcium transport activity and serum calcium and phosphorous concentrations of vitamin D-deficient rats were increased by irradiation with an ultraviolet (UV) lamp. The existence of 25-hydroxyvitamin D₃ (25-OH-D₃) in their bloods and livers was physicochemically confirmed by high-performance liquid chromatography (HPLC), gas-liquid chromatography (GLC) and mass fragmentography, whereas the compound could not be detected in the tissues of non-irradiated rats. The results strongly suggested that vitamin D₃ *in vivo* generated in irradiated rat skin might be normally metabolized and utilized to prevent rickets. The level of 25-OH-D₃ in the tissues was determined by a HPLC method.

It has been documented that irradiation of provitamin D (ergosterol or 7-DHC) in a test tube with UV light induces photochemical fission at the 9–10 linkage to produce previtamin D (previtamin D₂ or D₃) (1). On the other hand, the existence of 7-DHC (probably endogenous) in animal skin has been reported earlier (2–4) and we have also identified and determined the sterol in rat skin by physicochemical methods (5). By combining the observations, the preventive effect of sunlight on rickets in human beings and animals has been thought to be due to the photochemical conversion of 7-DHC to vitamin D₃ *via* previtamin D₃ in skin exposed to UV rays in sunlight, as shown in Fig. 1. However, the *in vivo* conversion had never directly been confirmed before our previous report (6), because the

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² Following abbreviations are used: 7-DHC, 7-dehydrocholesterol; 25-OH-D₃, 25-hydroxy-cholecalciferol; 1,25-(OH)₂-D₃, 1,25-dihydroxycholecalciferol; UV, ultraviolet; HPLC, high-performance liquid chromatography; HAP, hydroxyalkoxypropyl; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

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Fig. 1. Mechanism of the biogenesis and metabolism of vitamin D₃.

content of vitamin D₃ or previtamin D₃ in tissues is generally too small to be detected and determined. We solved this problem by using a HPLC method. Trace amounts of vitamin D₃ in rat skin irradiated with an UV lamp were identified and quantified by the method described in the previous paper (6). Recently, PETROVA *et al.* (7) and HOLICK *et al.* (8) reported results similar to ours. However, although our and their results directly realized the *in vivo* conversion, further investigations are necessary to confirm whether the vitamin formed in the skin is physiologically utilized to prevent rickets.

It is documented that vitamin D₃ is first metabolized to 25-OH-D₃ in the liver and subsequently to 1,25-(OH)₂-D₃ in the kidney, the active form of the vitamin showing the biological activity in stimulating intestinal calcium transport and bone mineral mobilization (9) (Fig. 1). Therefore, these active metabolites should be detected in the tissues of vitamin D-deficient rats exposed to UV light. We identified 25-OH-D₃ in the blood and liver of the rats by HPLC, GLC and mass fragmentography and also determined the levels of the compound in the tissues by a HPLC method.

EXPERIMENTAL

1. *Materials and reagents.* Crystalline 25-OH-D₃. This was kindly supplied by Eisai Co., Ltd. Other materials and reagents were used according to previous papers (5, 6).

2. *Animals and condition of UV irradiation.* Weanling male rats of the Wistar strain weighing about 40 g (3–4 weeks) were fed *ad libitum* a vitamin D-deficient diet (Ca, 0.47%; P, 0.3%) for 6 weeks and subsequently a low calcium, vitamin D-deficient diet (Ca, 0.02%; P, 0.3%) for 2 weeks (10). After shaving their backs with an electric shaver, the vitamin D-deficient rats were divided into irradiated and non-irradiated groups. The former group was irradiated with an UV lamp (National Erythema lamp, Matsushita Electric Co.; main wavelength, 280–310 nm; quantum of irradiated energy, 85 $\mu\text{W}/\text{cm}^2/\text{min}$) twice for 2 hr per day for 8 days, whereas the latter group was not irradiated as a control. All rats were fed the vitamin D-deficient diet during the irradiation experiment. They were immediately sacrificed after the final irradiation and the blood and liver were collected.

3. *Intestinal calcium transport assay.* Intestinal calcium transport assay using an everted sac and ⁴⁵Ca was performed according to the method of MARTIN and DELUCA (11).

4. *Serum calcium and phosphorous assay.* Serum obtained by centrifugation of the blood was diluted with either 0.2% strontium chloride in water (for calcium assay) or water alone (for phosphorous assay) to give a suitable concentration. Calcium was estimated with an Hitachi 508 atomic absorption spectrometer while phosphorous was estimated according to the result of CHEN *et al.* (12).

5. *Isolation of 25-OH-D₃ from the blood and liver.* The blood (8–10 ml) or liver (7–10 g) collected from two or three rats of each group was used as a pooled

sample for the determination of 25-OH-D₃. Each sample was homogenized with 20 ml of 0.25 M sucrose solution for 3 min and then with 80 ml of chloroform-methanol (1:1)⁴ for 1 min. The homogenized mixture was extracted twice each with 50 ml of chloroform. The combined chloroform extracts were evaporated under reduced pressure. The resulting residue was saponified and the unsaponifiable matter was isolated according to our previous report (5). Exactly 100 ml of benzene was used to isolate the unsaponifiable matter and the benzene layer was filtered through a Whatman 1PS filter paper. Exactly 90 ml of the filtrate was placed in a round bottom flask and then evaporated to dryness under reduced pressure. The resulting residue was dissolved in 4 ml of a mixture of *n*-hexane-chloroform (8:2) and then applied to a glass column (44 × 2.1 cm *i.d.*) packed with 32 g of HAP Sephadex prepared according to ELLINGBOE *et al.* (13). The column was eluted with *n*-hexane-chloroform (8:2) with a flow rate of 1.7 ml/min and every 4 ml was fractionated. The 25-OH-D₃ fractions (fraction no. 55–66) were combined and evaporated to dryness under reduced pressure. The resulting residue was dissolved in 0.5 ml of a mixture of methanol-ethyl ether-*n*-pentane (2:2:96) and then applied to the following HPLC to determine 25-OH-D₃. However, if necessary, the residue was further purified by a preparative TLC before applying to the HPLC. The residue obtained from the liver usually needed TLC purification. The procedure of preparative TLC was as follows: The residue obtained above was dissolved in a mixture of *n*-hexane-acetone (7:3) and applied as a zone on a Kiesel gel GF₂₅₄ plate (250 μ-thick, 20 × 20 cm) activated at 110°C for 1 hr. After developing the plate with the same solvent mixture, the zone corresponding to 25-OH-D₃ (*R_f* value, 0.40–0.45) detected under 254 nm UV lamp was scraped. The scraped zone was extracted with acetone and filtered. The filtrate was evaporated to dryness under reduced pressure and the resulting residue was dissolved in 0.5 ml of a mixture of methanol-ethyl ether-*n*-pentane (2:2:96) to apply to the following HPLC.

6. *Determination of 25-OH-D₃ by HPLC.* A constant volume (15–25 μl) of the solution obtained above was applied to a Shimadzu-DuPont LC-2F high-performance liquid chromatograph equipped with an UV detector (254 nm). The operating conditions were as follows: column, stainless steel tubes (25 × 0.21 cm *i.d.*) packed with "Zorbax Sil" (DuPont Co.) for both analytical and reference columns; mobile phase, methanol-ethyl ether-*n*-pentane (2:2:96); pressure, 40 kg/cm² (flow rate, 0.7 ml/min). The solution (15–25 μl), taken with a microsyringe, was injected into the high pressure line by stop-flow technique. A chromatogram was continuously recorded by estimating the absorbances at 254 nm and the content of 25-OH-D₃ was determined by comparing the peak height of a sample to that of a standard 25-OH-D₃.

7. *Identification of 25-OH-D₃ in the blood and liver by GLC.* The 25-OH-D₃ fraction obtained from the above HAP Sephadex column chromatography was re-

⁴ Ratios of solvent mixtures are shown as volume ratios in this paper.

chromatographed with the same column. The resulting 25-OH-D₃ fraction was further purified by the preparative TLC described above followed by a preparative HPLC (the condition was the same as the analytical HPLC and the fraction corresponding to 25-OH-D₃ was collected.). After evaporating the fraction under reduced pressure, the resulting residue was dissolved in small quantities of acetone and then applied to the following GLC: apparatus, Shimadzu GC-4BPFE gas chromatograph equipped with a hydrogen flame ionization detector; column, a glass column (260 × 0.3 cm *i.d.*) packed with 1.5% OV-17 on Gas Chrom Q; column temperature, 250°C; injection port temperature, 270°C; detector temperature, 280°C; flow rate of carrier gas (nitrogen gas), 85 ml/min.

8. *Identification of 25-OH-D₃ in the blood and liver by mass fragmentography.* The 25-OH-D₃ fraction purified for GLC analysis was applied to mass fragmentography using the following apparatus and operating conditions: apparatus, JEOL JGC 20K-JMS 100 gas chromatograph-mass spectrometer system (Japan Electron Optics Lab.); column, a glass column (150 × 0.2 cm *i.d.*) packed with 3% OV-17 on Gas Chrom Q; column and injection port temperature, 290°C; pressure of carrier gas (helium gas), 3.8 kg/cm²; separator temperature, 300°C; ionizing current, 300 μA; ionizing voltage, 25 eV; multiple ion detector, (a) *m/e* 341 ($M^+ - C_3H_7O$), (b) *m/e* 367 ($M^+ - CH_3 - H_2O$), (c) *m/e* 400 (M^+).

RESULTS AND DISCUSSION

1. Serum calcium and phosphorous concentrations and intestinal calcium transport assay

When the rats were housed with the vitamin D-deficient diet for 6 weeks and subsequently with the low calcium, vitamin D-deficient diet for 2 weeks, the assayed values of calcium and phosphorous concentrations in the serum were 5.9 and 3.8 mg/100 ml, respectively, which showed typical vitamin D deficiency. These increased to 9.2 and 5.1 mg/100 ml, respectively, with the UV irradiation experiment for 8 days.

The results of the intestinal calcium transport assay are shown in Table 1. The values of the irradiated group were significantly higher than those of the control. These results strongly suggested that UV irradiation of rat skin restored the vitamin D deficiency.

Table 1. Intestinal calcium transport response of UV-irradiated and non-irradiated (control) vitamin D-deficient rats.

Group	No of trials	⁴⁵ Ca transport (serosal/mucosal)
UV-irradiated	4	1.59 ± 0.09 ^a
Non-irradiated	4	1.06 ± 0.03 ^b

Each value is shown as M ± S.D.

The value of ^a is significantly higher than that of ^b (*p* < 0.01).

2. Determination of 25-OH-D₃ in the blood and liver of rats

The high-performance liquid chromatograms of the 25-OH-D₃ fractions obtained from the blood and liver of the irradiated group are shown in Fig. 2. Many unknown peaks were observed besides that corresponding to 25-OH-D₃ in the chromatograms of Fig. 2A, but most could be eliminated by purification with

Fig. 2. High-performance liquid chromatograms of the 25-OH-D₃ fractions obtained from the blood and liver of the irradiated group. The chromatograms (A) are those of the 25-OH-D₃ fractions after purification with HAP Sephadex column chromatography, while the chromatograms (B) are those of the fractions after further purification with preparative TLC. The symbol * indicates the peak corresponding to 25-OH-D₃. The purification procedures of the fractions and the operating conditions of HPLC are shown in the EXPERIMENTAL Section.

preparative TLC as shown in Fig. 2B. 25-Hydroxyvitamin D₃ in the blood could be determined without TLC, because the peak was not disturbed by the others. On the other hand, the purification procedure was necessary for the determination of 25-OH-D₃ in the liver, because tailing due to irrelevant peaks was too large to perform normal chromatography although the peak of 25-OH-D₃ was not disturbed by the

Table 2. Assayed values of 25-OH-D₃ in the blood and liver of vitamin D-deficient rats.

Tissue	UV irradiation	No of trials ^a	25-OH-D ₃ (ng/g of tissue) ^b
Blood	(+)	4 (2 or 3)	37.2 ± 8.0
	(-)	1 (2)	N.D.
	(-)	1 (6)	N.D.
Liver	(+)	4 (2 or 3)	22.7 ± 3.1
	(-)	1 (2)	N.D.
	(-)	1 (6)	N.D.

^a The number in parenthesis means that of the rats used for one assay.

^b Each value is shown as M ± S.D.

others. The peak corresponding to 25-OH-D₃ was observed in the chromatograms of the blood and liver of the irradiated group, whereas it could not be detected in those of the non-irradiated group.

When co-chromatography on the HPLC was performed by mixing the sample solution obtained from the blood and liver with an authentic 25-OH-D₃, the peak corresponding to the compound indicated a mono peak with increase of peak height. When recovery tests then were performed by mixing the lipid extracts of the tissues with 10 ng/g of authentic 25-OH-D₃, satisfactory data were obtained (more than 90%). It was concluded from the results that the proposed method was useful for the determination of 25-OH-D₃ in the tissues. The assayed values of 25-OH-D₃ are shown in Table 2.

3. Identification of 25-OH-D₃ in the blood and liver of rats

The gas chromatogram of the sample solutions obtained from the blood or liver of the irradiated group gave two peaks whose retention times agreed with those of 25-hydroxypyrovitamin D₃ and 25-hydroxyisopyrovitamin D₃ as shown in Fig. 3. Furthermore, the results of mass fragmentography showed that the retention times and relative intensity of the three ions of each sample agreed with the respective data of an authentic 25-OH-D₃.

Fig. 3. Gas-liquid chromatograms of the 25-OH-D₃ fractions obtained from the blood and liver of the irradiated group. (1) 25-Hydroxypyrovitamin D₃; (2) 25-hydroxyisopyrovitamin D₃. The purification procedure of the fractions and the operating conditions are shown in the EXPERIMENTAL Section.

Identification of 1,25-(OH)₂-D₃ was not successful, because the content of the compound in rat tissues was too small to be detected and quantified. However, the biological response of the serum calcium and phosphorous concentrations and the intestinal calcium transport assay strongly suggested that the 25-OH-D₃ generated *in vivo* might be metabolized to 1,25-(OH)₂-D₃ to show the physiological effects.

Fig. 4. Mass fragmentograms of the 25-OH-D₃ fractions obtained from the blood and liver of the irradiated group. The panel (A), (B) and (C) are the mass fragmentograms of authentic 25-OH-D₃ and the fractions of the blood and liver, respectively. The marks of a, b and c mean the detected ions of m/e 341, 367 and 400 (M^+), respectively. The purification procedure of the fractions and the operating conditions of mass fragmentography are shown in the EXPERIMENTAL Section.

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