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IDENTIFICATION AND DETERMINATION OF 1α,25-DIHYDROXYVITAMIN D_3 IN RAT SKIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIORECEPTOR ASSAY

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SUMMARY

Endogenous 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in normal rat skin was identified by using thermal isomerization to convert the metabolite into its pre-isomer, high-performance liquid chromatography (HPLC) and displacement potency with a chick intestinal cytosol receptor. When the metabolite in normal rat skin was determined by a radioreceptor assay after purification by Sep-Pak silica cartridge column chromatography and HPLC, the concentration was 71.0 ± 6.6 pg/g of wet tissue (mean ± S.D.). It is also shown that [^{3}H]-1,25-$(OH)_2D_3$ and [^{3}H]-25-hydroxyvitamin D_3 administered intravenously in the mouse are located in the skin. These results suggest that the metabolite may play an important role in the skin.

INTRODUCTION

1α,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is the most active metabolite of vitamin D_3 and is responsible for important physiological functions in the target organs, i.e., intestine, kidney and bone. Recently, the metabolite has also been thought to play an important role in the skin. This idea has been supported by the findings that skin has a receptor with high specificity for 1,25-$(OH)_2D_3$ [1] and that differentiation and proliferation are regulated by the metabolite in cell culture [2–4]. Further, it is found that psoriasis can be cured by the metabolite [5,6]. Furthermore, the skin is now recognized not only as a site for vitamin D photobiogenesis but also as a target organ for 1,25-$(OH)_2D_3$. However, very few studies aimed at clarifying the biological mechanism have

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been reported, and even the existence of the metabolite itself in skin has not yet been demonstrated. In this study, the identification and determination of endogenous 1,25-$(OH)_2D_3$ in normal rat skin were performed by using physico-chemical methods. Further, we studied the transfer of intravenously administered [^{3}H]-1,25-$(OH)_2D_3$ and [^{3}H]-25-hydroxyvitamin D_3 ([^{3}H]-25-OH-D_3) into skin.

EXPERIMENTAL

Materials

1,25-$(OH)_2D_3$ was kindly donated by Chugai Pharmaceutical Co. (Tokyo, Japan). [26,27-^{3}H]-1,25-$(OH)_2D_3$ and [26,(27)-^{3}H]-25-OH-D_3, purchased from Amersham (Amersham, U.K.) (176 and 20.6 Ci/mmol, respectively), were purified by high-performance liquid chromatography (HPLC) before use. Chick embryonic intestinal receptor for 1,25-$(OH)_2D_3$ was purchased from Yamasa Shoyu (Chiba, Japan). All other chemicals were of analytical-reagent grade.

Animals

Male Wistar rats weighing about 150 g fed a normal diet (FW-1 diet containing 200 I.U. of vitamin D_3 per 100 g; Funahashi Noujyou, Shizuoka, Japan) were used. Male mice weighing about 20 g fed a normal diet (FW-1 diet) were also used.

Extraction of 1,25-$(OH)_2D_3$ from skin samples

After shaving dorsal hair with an electric animal clipper, the rats were killed and the dorsal skin was immediately removed and placed on a previously cooled glass plate. After removing the subcutaneous connective and adipose tissues, the skin was cut into pieces (6×8 cm^2). Ten pieces of the skin samples were polytron-homogenized with 300 ml of ethyl acetate for 30 s three times at 4°C. The homogenate was centrifuged at 105 000 *g* for 60 min at 4°C. The supernatant was filtered through a glass filter and the filtrate was evaporated under reduced pressure.

Clean-up with a Sep-Pak silica cartridge column and HPLC

The residue obtained above was applied to a Sep-Pak silica cartridge column. The column was washed repeatedly with 0.4% 2-propanol in *n*-hexane to eliminate large amounts of less polar contaminants and then the 1,25-$(OH)_2D_3$ fraction was eluted with 10 ml of 20% 2-propanol in *n*-hexane. The eluate was evaporated under reduced pressure and the resulting residue was further purified by successively employing the five HPLC procedures shown in Table I.

TABLE I

HPLC SYSTEMS

System	Column	Mobile phase
1	Zorbax SIL (250 mm×4.6 mm I.D.)	10% (v/v) 2-propanol in *n*-hexane
2	Nucleosil $5C_{18}$ (300 mm×5.0 mm I.D.)	50% (v/v) methanol in acetonitrile
3	Zorbax SIL (250 mm×4.6 mm I.D.)	5% (v/v) 2-propanol in *n*-hexane
4	Zorbax SIL (250 mm×4.6 mm I.D.)	Dichloromethane–tetrahydrofuran–2-propanol–*n*-hexane (25.24:1:50, v/v)
5	Zorbax SIL (250 mm×4.6 mm I.D.)	10% (v/v) 2-propanol in *n*-hexane

Thermal isomerization of purified 1,25-$(OH)_2D_3$ fraction and displacement potency to a chick embryonic intestinal cytosol receptor

The purified 1,25-$(OH)_2D_3$ fraction obtained from the fifth stage of the HPLC procedure was evaporated under reduced pressure and dissolved in 2 ml of aldehyde-free ethanol. The ethanolic solution was refluxed at 80°C for 2 h to obtain an equilibrium between 1,25-$(OH)_2D_3$ and its pre-isomer. The equilibrated solution was evaporated under reduced pressure and the resulting residue was applied to the fourth HPLC procedure shown in Table I. The fraction corresponding to the position of the pre-isomer on the chromatogram was collected and again refluxed as described above. After elution in the fourth HPLC system, the fraction corresponding to the position of authentic 1,25-$(OH)_2D_3$ was collected and measured by the radioreceptor assay (RRA) [7].

Radioreceptor assay

Various concentrations (2–64 pg per tube) of authentic 1,25-$(OH)_2D_3$ and the 1,25-$(OH)_2D_3$ fraction were incubated with chick embryonic intestinal cytosol receptor, after adding 10 000 dpm of [^{3}H]-1,25$(OH)_2D_3$, at 4°C for 3 h. Bound and free forms of the metabolite were separated by addition of dextran-coated charcoal. After centrifugation, the radioactivity of the receptor-bound [^{3}H]-1,25$(OH)_2D_3$ was measured with an Aloka LSC-700 liquid scintillation system.

Assay of 1,25-$(OH)_2D_3$ in rat skin

The dorsal skin of normal rat was removed from the centre area (2×3 cm^2). After removing the subcutaneous connective and adipose tissues and adding 2500 dpm of [^{3}H]-1,25-$(OH)_2D_3$ for measurement of recovery, the skin was extracted as described above. The resulting residue was dissolved in 2 ml of 0.4% 2-propanol in *n*-hexane and applied to a Sep-Pak silica cartridge column as described above. The 1,25-$(OH)_2D_3$ fraction was eluted with 10 ml of 20% 2-propanol in *n*-hexane and the eluate was evaporated under reduced pressure. The residue was applied to the first step of the HPLC procedure (Table I).

The fraction corresponding to the position of 1,25-$(OH)_2D_3$ on the chromatogram was collected and evaporated under reduced pressure and the 1,25-$(OH)_2D_3$ content was measured by RRA as described above.

Transfer of [^{3}H]-1,25-$(OH)_2D_3$ or [^{3}H]-25-OH-D_3 into mouse skin

[^{3}H]-1,25-$(OH)_2D_3$ or [^{3}H]-25-OH-D_3 (1 μCi) was applied intravenously to normal mice and the dorsal skin was removed after 5 h. After separating the epidermis and dermis, each lipid was extracted as described above and applied to the first step of the HPLC procedure. The eluate was collected as 1.8-ml aliquots in which the radioactivity was measured with an Aloka LSC-700 liquid scintillation system.

RESULTS

Identification of 1,25-$(OH)_2D_3$ extracted from rat skin

Fig. 1A and B show the profiles of authentic 1,25-$(OH)_2D_3$ and the 1,25-$(OH)_2D_3$ fraction obtained from a skin sample after purification with five steps of the HPLC procedure. The elution was carried out at a flow-rate of 1.8 ml/min and each 1.8 ml of the eluate was collected. The concentration of 1,25-$(OH)_2D_3$ in all the fractions was determined for 1,25-$(OH)_2D_3$ by RRA. The

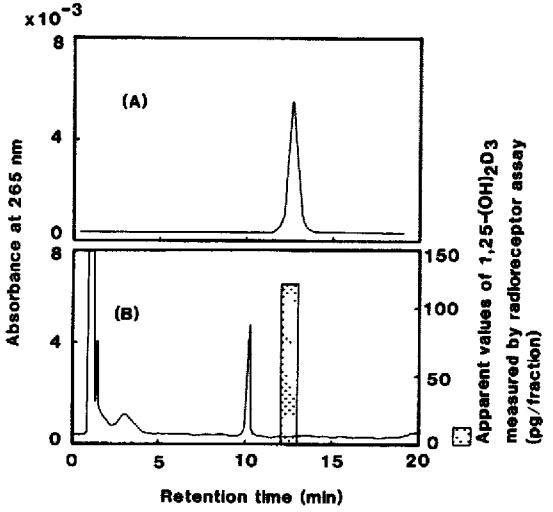

Fig. 1. HPLC profiles of (A) authentic 1,25-$(OH)_2D_3$ and (B) the 1,25-$(OH)_2D_3$ fraction obtained from normal rat skin after purification through the five HPLC systems. The conditions for the HPLC systems are shown in Table I. Each 1.8-ml fraction was collected and subjected to the RRA method for the assay of 1,25-$(OH)_2D_3$ in fractions. The stippled column indicates the apparent values of 1,25-$(OH)_2D_3$ measured by the method. A significant amount of 1,25-$(OH)_2D_3$ was observed only in the fraction corresponding to the position of the metabolite on the chromatogram as shown in B.

material assaying as 1,25-$(OH)_2D_3$ (shown in the stippled column) was observed only in the fraction corresponding to the position of authentic 1,25-$(OH)_2D_3$. No peak corresponding to the position of the metabolite was observed by UV absorbance because the amount of the metabolite in the fraction was too low for detection.

Fig. 2A shows the separation of thermally equilibrated authentic 1,25-$(OH)_2D_3$ and its pre-isomer using the fourth step of the HPLC procedure. Both the authentic compounds were clearly separated and it was therefore concluded that this HPLC system could be used to collect 1,25-$(OH)_2D_3$ and its pre-isomer from the eluate separately. Fig. 2B shows the chromatogram of the 1,25-$(OH)_2D_3$ fraction obtained from a skin sample after thermal equilibration in the same way. About 75% of purified 1,25-$(OH)_2D_3$ was recovered in the fraction corresponding to the authentic 1,25-$(OH)_2D_3$. The fraction cor-

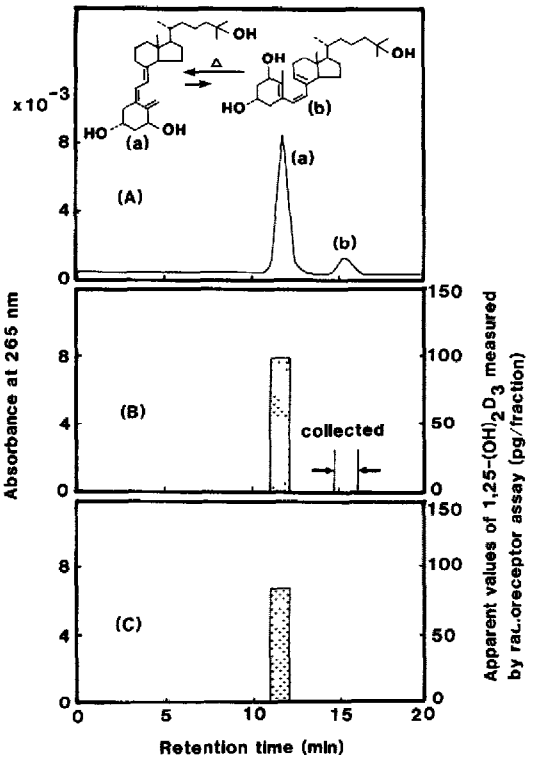

Fig. 2. HPLC profiles of (A) thermally equilibrated authentic 1,25-$(OH)_2D_3$ and its pre-isomer and (B) the thermally equilibrated solution of the 1,25-$(OH)_2D_3$ fraction obtained from normal rat skin. The HPLC conditions used are those for the fourth HPLC system in Table I. The stippled columns indicate the apparent values of 1,25-$(OH)_2D_3$ measured by the RRA method. (C) HPLC profile after thermal equilibration of the pre-isomer fraction collected as shown in B; the stippled column showns assayable 1,25-$(OH)_2D_3$.

responding to the pre-isomer was collected and refluxed again for 2 h at 80°C as described above so as to reconvert some of it to 1,25-$(OH)_2D_3$. The solvent was evaporated under reduced pressure and the resulting residue was applied to the same HPLC system. Fig. 2C shows the HPLC profile of the thermally equilibrated solution of the pre-isomer fraction obtained from the HPLC step shown in Fig. 2B. The 1,25-$(OH)_2D_3$ fraction reacted with the receptor as shown in the stippled column in Fig. 2C. The results confirm the presence of 1,25-$(OH)_2D_3$ in the skin sample indirectly by the thermal isomerization technique.

Determination of 1,25-$(OH)_2D_3$ in rat skin

Fig. 3 shows the displacement curves of authentic 1,25-$(OH)_2D_3$ and the fraction of the metabolite obtained from a skin sample after purification with the five steps of the HPLC procedures. The two curves are nearly identical. The concentration of 1,25-$(OH)_2D_3$ in normal young rat skin assayed by the proposed method was 71.0 ± 6.6 pg/g of wet tissue (mean ± S.D.).

Location of intravenously administered 1,25-$(OH)_2D_3$ in skin

Fig. 4A and B show the HPLC profiles of the lipid extracts of skin obtained from mice that had received intravenous doses of [^{3}H]-1,25-$(OH)_2D_3$ and [^{3}H]-25-OH-D_3, respectively. The results show that the compounds were rapidly transferred to the skin as the respective intact forms. With [^{3}H]-25-OH-D_3, a minor peak corresponding to 1,25-$(OH)_2D_3$ was observed, as shown in Fig. 4B, but it is not clear whether this peak resulted from metabolism in the skin or elsewhere.

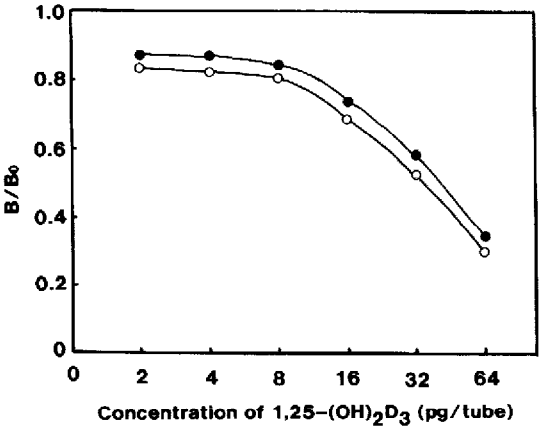

Fig. 3. Displacement curves for (○) authentic 1,25-$(OH)_2D_3$ and (●) the 1,25-$(OH)_2D_3$ fraction obtained from normal rat skin with a chick embryonic intestinal cytosol receptor.

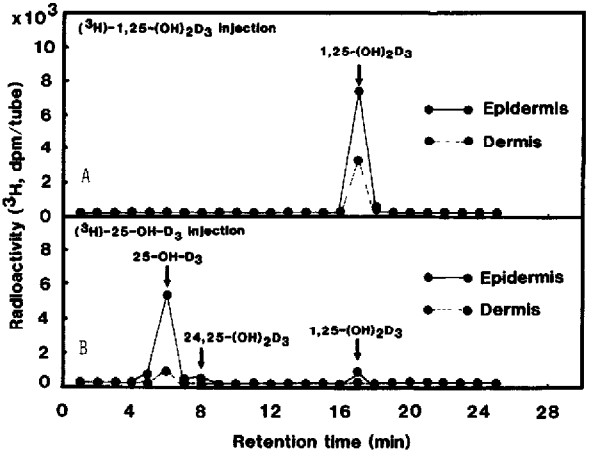

Fig. 4. HPLC profiles of lipid extracts of normal mouse skin after intravenous administration of (A) [^{3}H]-1,25-$(OH)_2D_3$ or (B) [^{3}H]-25-OH-D_3. Almost all the radioactivity applied to the HPLC system was recovered in the same chemical forms that were administered to the mice.

DISCUSSION

This study has clearly identified endogenous 1,25-$(OH)_2D_3$ in normal rat skin by methods including thermal isomerization, HPLC and displacement potency with a chick embryonic intestinal cytosol receptor. Although the existence of 1,25-$(OH)_2D_3$ receptor in rat skin has been reported [1], the presence of the endogenous metabolite itself in animal skin has not been described. The demonstration in this study is a new finding. The novel aspect of this investigation was the use of the thermal isomerization technique to convert putative 1,25-$(OH)_2D_3$ to its pre-isomer, which does not react with the intestinal receptor. The assumed pre-isomer, having been isolated by HPLC, was then re-equilibrated by heat treatment to yield 1,25-$(OH)_2D_3$, which was again isolated by HPLC and shown to react with the receptor. Having established the identity of 1,25-$(OH)_2D_3$ extracted from skin, we then proceeded to determine its concentration. The average concentration of the metabolite in normal rat skin was 71.0 pg/g of wet tissue, which was close to the plasma levels of normal rats.

Recently, Bikle et al. [8] reported that human neonatal foreskin keratinocytes produced significant amounts of 1,25-$(OH)_2D_3$ in a serum-free culture. This result suggests that the production of the metabolite, which is normally synthesized in the kidney, may be occurring in the epidermis. As both skin fibroblasts and keratinocytes are known to have the receptor for 1,25-$(OH)_2D_3$ [9,10], the claim that the metabolite is synthesized in skin cells suggests that the metabolite may serve an autocrine or paracrine function to regulate cell differentiation and proliferation in skin. However, Holick et al. [11] reported

that neither human neonatal nor adult keratinocytes cultured in a serum-free medium showed 1α-hydroxylase activity, and this discrepancy has not yet been resolved.

In order to clarify the origin of 1,25-$(OH)_2D_3$ in skin, we examined the distribution of the tritiated metabolite in normal mice and showed that intravenously administered [^{3}H]-1,25-$(OH)_2D_3$ was transferred into skin as in the intact form. The results suggest that most of the 1,25-$(OH)_2D_3$ in skin may be derived from the circulation even if skin fibroblasts and/or keratinocytes may have an enzyme system to produce the metabolite in an autocrine manner.

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