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A NEW IN VIVO METABOLITE OF VITAMIN D_3: 1,25,26-TRIHYDROXYVITAMIN D_3

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SUMMARY

A new metabolite of vitamin D_3 was detected with a modified receptor-binding assay in plasma of cows given large parenteral doses of vitamin D_3. This metabolite has been identified as 1,25,26-trihydroxyvitamin D_3 by comigration of the in vivo-isolated metabolite with synthetic 1,25(S),26-trihydroxyvitamin D_3 on two different high-pressure liquid chromatography systems and by mass spectroscopy.

INTRODUCTION

Metabolism of vitamin D_3 to more polar and active metabolites has been well documented (1). Several new metabolites of vitamin D_3 have recently been described in detail (1-4); however, it has been pointed out that

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Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,24(R),25-$(OH)_3D_3$, 1,24(R),25-trihydroxyvitamin D_3; 1,25,26-$(OH)_3D_3$, 1,25,26-trihydroxyvitamin D_3; HPLC, high-pressure liquid chromatography.

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several metabolites remain to be identified and chemically characterized (1).

During the development of a receptor assay for 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] in plasma, we found an unidentified vitamin D_3 metabolite that was more polar than 1,25-$(OH)_2D_3$ or 1,24,25-$(OH)_3D_3$ in plasma of cows receiving large parenteral doses of vitamin D_3. The compound was isolated in pure form, and we report here the structure of this new polar vitamin D_3 metabolite to be 1,25,26-$(OH)_3D_3$.

MATERIALS AND METHODS

Animals -- Jersey cows were treated as described (5), and blood was collected after 4 weeks of treatment. Blood obtained from untreated cows served as controls.

Apparatus -- High-pressure liquid chromatography (HPLC) was performed on a Model LC-204 HPLC (Waters Associates, Milford, MA). Mass spectra were obtained using a solid probe at 54 eV with a Finnigan Model 4021 spectrometer at 150°C above ambient.

Sterols -- Synthetic 1,25-$(OH)_2D_3$, 1,24(R),25-$(OH)_3D_3$, and 1,25(S),26-$(OH)_3D_3$ were provided by Dr. M. Uskokovic of Hoffman-LaRoche (Nutley, NJ). 1,25-Dihydroxy[^{3}H-26,27]vitamin D_3 (160 Ci/mmole) was purchased from New England Nuclear (Boston, MA). 25,26-Dihydroxy-[^{3}H-23,24]vitamin D_3 was prepared as previously described (6). 1,25,26-Trihydroxy[^{3}H-23,24]vitamin D_3 was biosynthesized in vitro from 25,26-dihydroxy[^{3}H-23,24]vitamin D_3 using a procedure described for the 1α-hydroxylation of 25-hydroxyvitamin D_3 (7).

Receptor-binding assay -- The assay was performed as previously described (7), using chick intestinal cytosol and 1,25-$(OH)_2$[^{3}H-26,27]-vitamin D_3 with the following modifications. The cytosol was prepared in a buffer consisting of 50 mM Tris-HCl, 300 mM KCl, 1.5 mM EDTA, 5 mM dithiothreitol and glycerol 10% (v/v), pH 7.5. Changes in this buffer's KCl concentration are noted in succeeding steps. The 1,25-$(OH)_2D_3$ receptor was precipitated from crude cytosol with 40% $(NH_4)_2SO_4$. The pellet obtained after centrifugation (16,000 x g) was dissolved in the buffer described (25 mM KCl) and chromatographed on a 2 x 5 cm column of Blue Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ). The column containing the 1,25-$(OH)_2D_3$ receptor was washed with 100 ml of the 25 mM KCl buffer. The 1,25-$(OH)_2D_3$ receptor was then eluted with 50 ml of buffer (0.6 M KCl). This receptor fraction was used in the assay.

Preparation of plasma for assay -- Plasma (5-10 ml) from normal and vitamin D_3-treated cows was extracted as described (8). This extract was chromatographed on a 0.6 x 15 cm Sephadex LH-20 column developed in hexane:chloroform:methanol (9:1:1). The initial 18 ml of column solvent were discarded, and the solvent was changed to hexane:chloroform:methanol (8:2:2). The next 10 ml were collected, dried under N_2, and subjected to

HPLC on a Zorbax Sil column 0.46 x 25 cm using hexane:isopropanol (83:17) at a flow rate of 2 ml/min. One-min (2 ml) fractions were collected, dried, and assayed using the receptor assay. Regions of binding were located, and a duplicate plasma extract was purified using the Zorbax Sil column above. The individual peak areas were isolated and rechromatographed on a 0.46 x 25 cm Zorbax ODS column developed in methanol:H_2O (80:20). Fractions (2 ml) again were collected and assayed for binding. This was done to confirm the homogeneity of the isolated compounds and their comigration with authentic standards.

Isolation of new vitamin D_3 metabolite -- Plasma (16 l) from vitamin D_3-treated cows was extracted (9), dried under vacuum, labeled with 1,25,26-$(OH)_3$[^{3}H-23,24]vitamin D_3 (200,000 cpm), and chromatographed on a 4.0 x 60 cm Sephadex LH-20 column with 1.5 l of chloroform:hexane:methanol (50:48:2). The column contents were removed and extracted 2 times with methanol. The extract was dried under vacuum and rechromatographed on a 1.8 x 25 cm Sephadex LH-20 column in chloroform:hexane:methanol (75:23:2). The region corresponding to the elution of the 1,25,26-$(OH)_3$[^{3}H-23,24]vitamin D_3 was collected (260-380 ml) and chromatographed by HPLC on a 0.46 x 25 cm Zorbax ODS column using methanol:H_2O (80:20). The 1,25,26-$(OH)_3D_3$ region (24-30 ml) was rechromatographed on a 0.46 x 25 cm Zorbax Sil column using methylene chloride:isopropanol (93:7), and the 1,25,26-$(OH)_3D_3$ region (22-26 ml) from this column was chromatographed on a 0.46 x 25 cm Zorbax Sil column in hexane:isopropanol (83:17). The sole 254 nm absorbing peak was recycled twice to confirm homogeneity and then collected for structural analysis. Five hundred ng of the pure compound were isolated.

RESULTS AND DISCUSSION

The HPLC receptor binding profiles of the purified plasma extracts from vitamin D_3-treated cows showed three distinct peaks of binding activity (Fig. 1). The new metabolite observed comigrated with synthetic 1,25(S),26-$(OH)_3D_3$ (Fig. 1). The two additional peaks comigrated with synthetic 1,25-$(OH)_2D_3$ and 1,24(R),25-$(OH)_3D_3$. The absence of the new metabolite in plasma extracts from control cows precluded further study of the controls (Fig. 1).

When the 1,25,26-$(OH)_3D_3$ isolate from the Zorbax Sil column (Fig. 1) was rechromatographed on a reverse-phase Zorbax ODS column and fractions were analyzed, the binding activity was found to be homogeneous and comigrate with synthetic 1,25(S),26-$(OH)_3D_3$ (Fig. 2). Both the 1,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ isolates from the Zorbax Sil column (Fig. 1) were found to be homogeneous and to comigrate with their

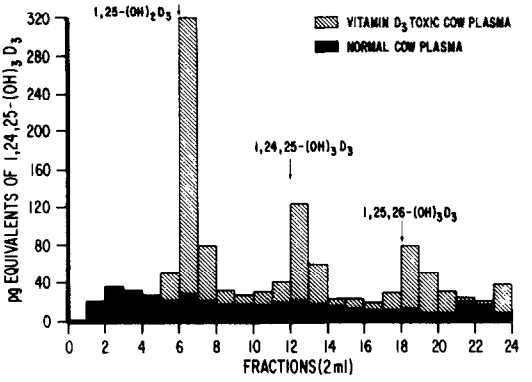

Figure 1. HPLC receptor binding profile of plasma extracts from normal and vitamin D_3-treated cows after purification on Sephadex LH-20. The profile represents elution from a Zorbax Sil column developed in hexane:isopropanol (83:17). Arrows indicate the elution positions of authentic standards.

respective synthetic standards on the Zorbax ODS column (data not shown). The comigration of the receptor binding activities with synthetic 1,25(S),26-$(OH)_3D_3$ on HPLC columns with different selectivities suggested that this new vitamin D_3 metabolite was 1,25,26-$(OH)_3D_3$.

Five hundred ng of this new metabolite of vitamin D_3 were isolated from 16 l of plasma from vitamin D_3-treated cows and subjected in part to mass spectrometric analysis. The mass spectrum of this new metabolite is characteristic of a trihydroxylated vitamin D_3 metabolite (Fig. 3). The molecular ion at m/e 432 and the three sequential losses of H_2O at m/e 414, 396, and 378 indicate the presence of 3 hydroxyl groups in addition to the 3β-hydroxyl group. The peak at m/e 269 arises from side-chain cleavage and loss of H_2O from the remaining fragment. Loss of a second H_2O molecule provides the peak at m/e 251. The peak at m/e 152 arises from formal cleavage between carbons 7 and 8 to give rise to the A ring, plus carbons 6 and 7 fragment, containing 2 hydroxyl groups. Loss of H_2O from m/e 152 provides the base peak at m/e 134. The fragments at m/e 152 and 134 illustrate that the secosteroid nucleus of the vitamin has remained unchanged.

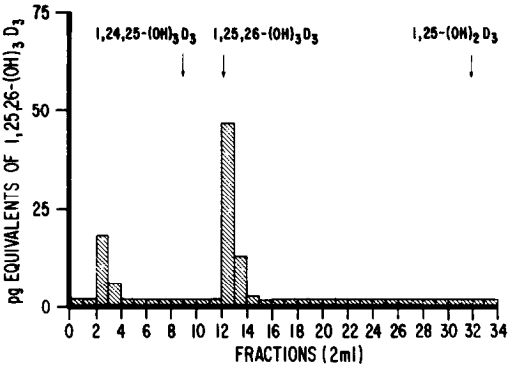

Figure 2. HPLC receptor binding profile of the 1,25,26-(OH)$_3$D$_3$ isolate, after purification on the Zorbax Sil column (Fig. 1), and re-chromatographed on a Zorbax ODS column developed in methanol:H$_2$O (80:20). Arrows indicate the elution positions of authentic standards.

A recent study (10) has concluded that kidney 1α-hydroxylation probably is required for the expression of biological activity of 25,26-(OH)$_2$D$_3$ in vitamin D-deficient rats. To date, no such metabolite of vitamin D$_3$ has been isolated or identified. In the present communication, we have isolated and identified 1,25,26-(OH)$_3$D$_3$ from the plasma of vitamin D$_3$-treated cows. The cochromatography of this new in vivo metabolite of vitamin D$_3$ with synthetic 1,25(S),26-(OH)$_3$D$_3$ on HPLC

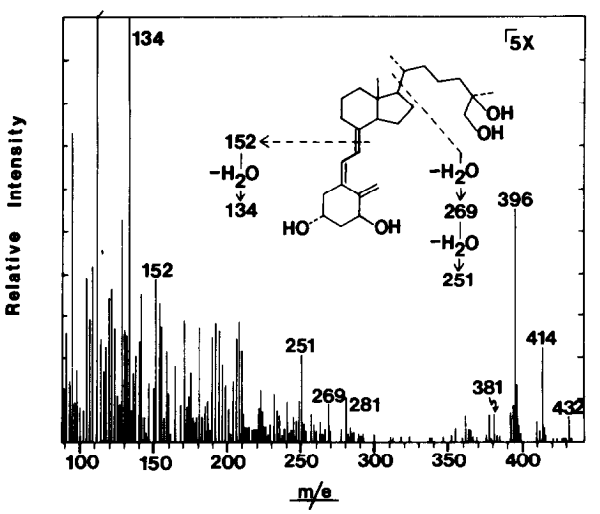

Figure 3. Mass spectrum of 1,25,26-(OH)$_3$D$_3$ isolated from vitamin D$_3$-treated cows.

systems with different selectivities and the mass spectrometric data strongly suggest that this newly discovered metabolite of vitamin D_3 is 1,25,26-$(OH)_3D_3$.

It may be significant that 1,25,26-$(OH)_3D_3$ has been detected and isolated only from cows treated with large doses of vitamin D_3 because cows are the only animals that have been shown to increase the production of 1,25-$(OH)_2D_3$ following the administration of large doses of vitamin D_3 (5, 11). This suggests that the 1,25,26-$(OH)_3D_3$ found may reflect the degradation of abnormally elevated levels of 1,25-$(OH)_2D_3$ in these cows.

Further structural and biological investigations are in progress in our laboratory.

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