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Isolation and Identification of 24(*R*)-Hydroxyvitamin D_3 from Chicks Given Large Doses of Vitamin D_3†

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ABSTRACT: A new metabolite of vitamin D was isolated from the blood plasma of chicks given large doses of vitamin D_3. The isolation involved methanol–chloroform extraction and four column chromatographic steps. The metabolite was identified by high- and low-resolution mass spectroscopy, chemical derivatization, and comigration with authentic standard as 3β,24(*R*)-dihydroxy-9,10-seco-5,7,10(19)-cholestatriene [24(*R*)-hydroxyvitamin D_3]. No detectable 24(*R*)-hydroxyvitamin D_3 was recovered from 16 L of plasma from chicks receiving physiologic levels of vitamin D_3.

The toxicity of vitamin D in mammals when administered at elevated levels is well established (DeLuca, 1978). The mechanism of this toxic effect, however, has received little attention and is poorly understood.

Gross changes in the plasma concentrations of the known vitamin D metabolites in rats have been shown to occur on administration of toxic and subtoxic amounts of vitamin D_3 and 25-hydroxyvitamin D_3 (25-OH-D_3)[1] (Shepard & DeLuca, 1980). Use of this fact led to the recent identification of 25-OH-D_3-26,23-lactone (Wichmann et al., 1979). This metabolite is present in plasma at <1 ng/mL under physiologic conditions; however, at elevated vitamin D_3 levels, it becomes one of the major circulating metabolites.

The work described here was initiated to determine if other vitamin D_3 metabolites which normally are not present or are present at nondetectable levels are found in plasma of animals receiving large amounts of vitamin D_3. We wish to report the isolation and identification of 24(*R*)-hydroxyvitamin D_3 [24(*R*)-OH-D_3] from plasma of chicks receiving massive doses of vitamin D_3. This metabolite was not detected in plasma of chicks receiving physiologic levels of vitamin D_3.

Materials and Methods

Plasma Procurement and Extraction. Plasma Sample from Chicks Given High Doses of Vitamin D_3. Eighty 12-week-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, WI) raised on standard chicken mash were dosed intramuscularly with 10^5 IU of vitamin D_3 (Aldrich Chemicals, Milwaukee, WI) in 50 μL of ethanol daily for 3 days. The purity of the vitamin D_3 was found to be at least 99% by high-performance LC. On the fourth day, each chick received a total of 10^7 IU of vitamin D_3 dissolved in 500 μL of ethanol in four

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 24(*R*)-OH-D_3, 24(*R*)-hydroxyvitamin D_3; 24(*S*)-OH-D_3, 24(*S*)-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; LC, liquid chromatography.

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intramuscular doses. Six days after this dose, blood was collected by cardiac puncture with use of heparin to prevent clotting. The blood was immediately centrifuged, yielding 1.4 L of plasma. The plasma was extracted for 16 h at 4 °C with 2:1 (v/v) methanol–chloroform as described by Blunt et al. (1968). The chloroform phase was concentrated, dried by ethanol azeotrope, and used for chromatography.

Plasma Sample from Chicks Given a Physiologic Level of Vitamin D_3. Twenty-four liters of blood was obtained by decapitation of 8-week-old chicks (A-G Coop, Arcadia, WI). These chicks had received a maintenance amount of vitamin D_3 in their feed (1000 IU/pound of feed). The blood was separated, yielding 16 L of plasma, and extracted as described by Wichmann et al. (1979).

Chromatographic Purification. Both samples were first chromatographed on a 3 × 30 cm Sephadex LH-20 column eluted with 9:1:1 hexane–methanol–chloroform. The plasma sample from chicks given large doses of vitamin D_3 was chromatographed in one run while the plasma sample from normal chicks was chromatographed in three equal batches. In all cases, 50 20-mL fractions were collected. A 1-mL plasma equivalent aliquot from each fraction (high dose) and a 10-mL plasma equivalent aliquot from each plasma fraction (normal plasma) was used to detect metabolites by use of the competitive protein binding assay of Haddad et al. (1977). In all cases the competitive binding activity eluting between 200 and 500 mL was pooled and concentrated. Both samples were then chromatographed on a 2 × 58 cm Lipidex 5000 column eluted with 92:8 hexane–chloroform. A 3-mL plasma equivlanet aliquot (high dose) or a 10-mL plasma equivalent aliquot (normal plasma) from each fraction was assayed as above. In both cases the competitive binding peak eluting from 520 to 760 mL was pooled and concentrated.

Both samples were then subjected to high-performance liquid chromatography on a Waters Model ALP/GPC 204 instrument equipped with a Model 440 absorbance detector (Waters Associates, Milford, MA). The samples were chromatographed on a 0.45 × 25 cm microparticulate silica column eluted with 2% 2-propanol in hexane at a flow rate of 2 mL/min. Thirty 2-mL fractions were collected for each sample and assayed as above with use of 3-mL plasma equivalent aliquot (high dose) or a 50-mL plasma equivalent aliquot (normal plasma). The fractions eluting from 30 to 34 mL had binding activity and were pooled (high dose). The normal plasma sample showed no binding activity in this region; however, the fractions eluting from 30 to 34 mL were pooled for further chromatography. Both samples were rechromatographed in the same high-performance LC system. The material eluting from 28 to 39 mL was recycled, and the fraction eluting from 67.5 to 72 mL was collected for both samples. This fraction was concentrated and used for spectral analysis.

Spectroscopy. Ultraviolet absorption spectra were recorded from an ethanol solution by use of a Beckman Model 24 spectrophotometer.

Low- and high-resolution mass spectra were obtained with an AEI 902 mass spectrometer (Associated Electrical Industries, Ltd., Manchester, England) interfaced with a DS-50 data system (Data General Corp., Southboro, MA). All spectra were run at 70 eV at a source temperature between 90 and 115 °C above ambient.

Trimethylsilyl Derivative. The compound isolated from chicks given the high dose of vitamin D_3 (400 ng) was reacted with 25 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylsilyl chloride in 30 μL of pyridine at 55 °C for 45 min. Reagents were removed under N_2 and the product was dissolved in 50 μL of hexane. The product was purified by high-performance LC on a 0.45 × 25 cm microparticulate silica column eluted with 0.15% ethyl acetate in hexane at a flow rate of 2 mL/min. The major 254-nm absorbing peak eluting between 13.5 and 16 mL was collected and used for mass spectrometry.

Determination of Stereochemistry. Authentic samples of 24(*R*)- and 24(*S*)-OH-D_3 (Ikekawa et al., 1975) were chromatographed (high-performance LC) on a 0.45 × 25 cm microparticulate silica column eluted with 2% 2-propanol in hexane. This system allowed better than 80% base-line resolution of the two compounds. One-hundred nanograms of the metabolite from the plasma sample from the chicks given large doses of vitamin D_3 was cochromatographed on this system with 100 ng of 24(*R*)-OH-D_3.

Results

For space conservation, column profiles from the purification procedures are not shown. In all cases, column fractions were assayed by use of the competitive protein binding assay of Haddad et al. (1977). On both the Sephadex LH-20 and Lipidex 5000 columns, the compound comigrated with 25-OH-D_3. Complete resolution of these two compounds was easily achieved on high-performance LC. In the plasma sample from chicks given large amounts of vitamin D_3, a binding peak for the new compound was found with all columns used. Normal plasma showed no binding peak for this compound on the high-performance runs; however, the elution region for the unknown component was collected.

The ultraviolet absorption spectrum of the isolated compound exhibits the typical vitamin D triene absorbance with λ_{max} 265 nm, λ_{min} 227 nm and $OD\lambda_{max}/OD\lambda_{min}$ = 1.80 (DeLuca, 1978). A total recovery of 1.85 μg was calculated for the plasma sample, assuming an ϵ of 18 600 and a molecular weight of 400. This corresponds to a plasma level of approximately 1.3 ng/mL.

The ultraviolet absorption spectrum of the corresponding region from the normal plasma sample was taken in 200 μL of ethanol in a cell having a 1-cm path length. This spectrum showed no detectable absorbance in the 230–270-nm region.

The low-resolution mass spectrum from a 400-ng (high dose) sample is presented in Figure 1. Major ions, relative intensities, and structural assignments are as follows (Blunt et al., 1968; Holick et al., 1972; DeLuca & Schnoes, 1976; Jones et al., 1980): m/z 400, 22, M^+; m/z 382, 3, $M^+ - H_2O$; m/z 271, 5, M^+ – side chain; m/z 253, 7, M^+ – side chain – H_2O; m/z 136, 100 (A ring + C-6 and C-7)$^+$; m/z 118, 79, 136 – H_2O. The apparent molecular ion at m/z 400 is consistent with a monohydroxylated vitamin D_3 derivative. Diagnostic ions at m/z 271, 253, 136, and 118 indicate that the compound is a vitamin D_3 derivative with an intact secosteroid nucleus. These ions also show that the 3β-hydroxy group is present in the A ring and that the additional hydroxyl group is on the side chain.

High-resolution mass spectrometry on 900 ng (high-dose sample) revealed a molecular weight of 400.3336 and formula of $C_{27}H_{44}O_2$ (calcd for $C_{27}H_{44}O_2$, 400.3341). This spectrum also confirmed composition of the fragments assigned to the ions in the lower-resolution spectrum. A low-resolution mass spectrum was run on the sample from normal plasma. This spectrum contained no detectable ions at m/z 400, 382, 271, or 253 and only background intensities at m/z 136 and 118. The instrument gave a recognizable spectrum on 100 ng of standard 25-OH-D_3; thus less than 100 ng of the metabolite was present in the normal plasma sample.

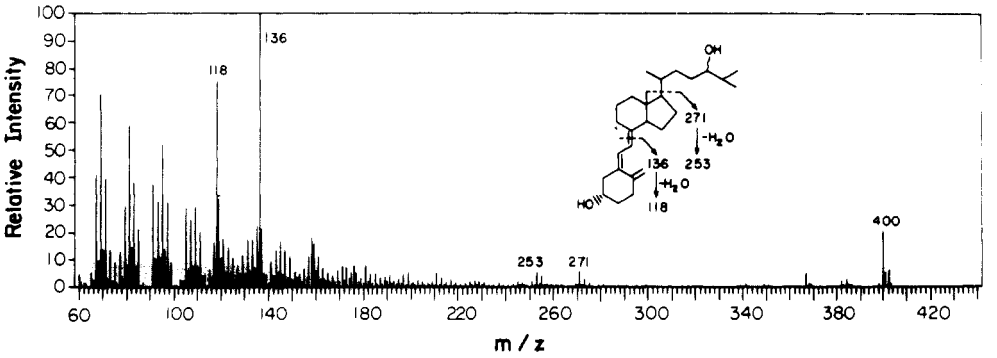

FIGURE 1: Mass spectrum of 24(*R*)-OH-D_3 isolated from the plasma sample obtained from chicks given large doses of vitamin D_3.

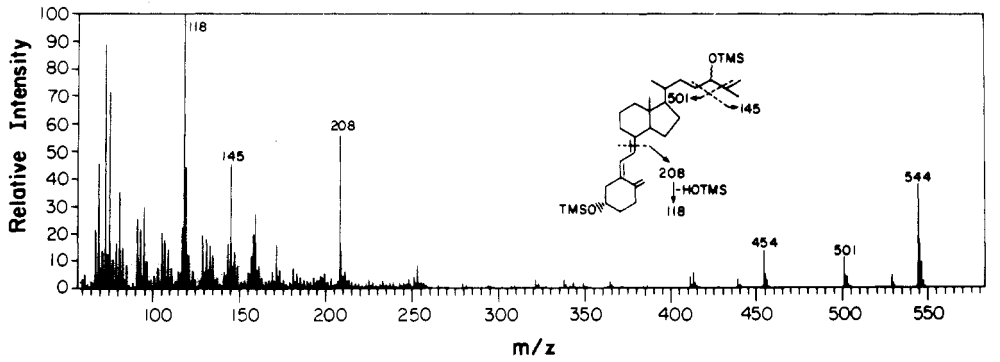

FIGURE 2: Mass spectrum of the $(Me_3Si)_2$ derivative of 24(*R*)-OH-D_3 from Figure 1.

The low-resolution mass spectrum of the Me_3Si derivative of 400 ng of high-dose plasma sample is presented in Figure 2. Major ions, relative intensities, and structural assignments are as follows: *m/z* 544, 45, M^+; *m/z* 529, 6, $M^+ - CH_3$; *m/z* 501, 13, $M^+ - C_3H_7$; *m/z* 454, 16, $M^+ - HOSiMe_3$; *m/z* 208, 67, (A ring + C-6 and C-7)$^+$; *m/z* 145, 52, $(CH_3)_3SiO^+$=CHCH$(CH_3)_2$; *m/z* 118, 100, 208 – $HOSiMe_3$. The presence of two hydroxyl groups on the molecule is apparent from the molecular ion at *m/z* 544 corresponding to a $(Me_3Si)_2$ derivative. Ions at *m/z* 208 and 118 reconfirm the 3β-hydroxyl group as the only functionality on the A ring. The position of the second hydroxyl group is apparent from the ions at *m/z* 501 and 145. The *m/z* 501 ion arises from cleavage between C-24 and C-25 for loss of C_3H_7 from the molecular ion. The alternative α cleavage between C-23 and C-24 gives rise to the ion at *m/z* 145. Thus the hydroxyl group on the side chain is on C-24 and the compound is 3β,24-dihydroxy-9,10-seco-5,7,10(19)-cholestatriene.

The stereochemistry of the 24-hydroxyl group was determined by high-performance LC comparison of the metabolite with known 24(*R*)- and 24(*S*)-OH-D_3 standards. Figure 3a shows the elution positions and separation obtained from cochromatography of 100 ng of both 24(*R*)-OH-D_3 and 24(*S*)-OH-D_3. Figure 3b is the chromatogram obtained when 100 ng of 24(*R*)-OH-D_3 and 100 ng of metabolite from plasma (high dose) were cochromatographed on the same system. The presence of a single peak in Figure 3b compared to the resolution of the two isomers in Figure 3a proves that the compound isolated from the plasma sample is 24(*R*)-OH-D_3 (see Figure 4).

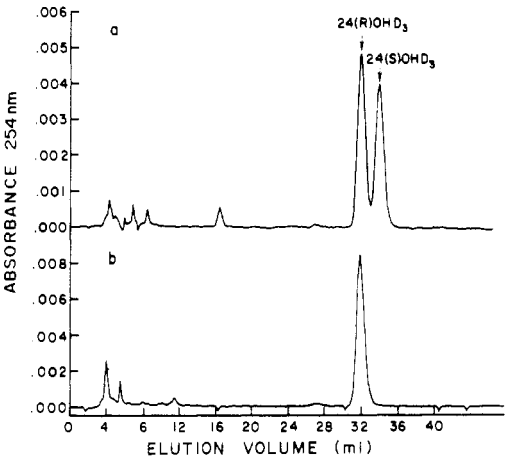

FIGURE 3: (a) High-performance LC chromatogram of 100 ng each of 24(*R*)-OH-D_3 and 24(*S*)-OH-D_3. (b) High-performance LC chromatogram of 100 ng of 24(*R*)-OH-D_3 and 100 ng of the compound isolated from plasma sample from chicks given high doses of vitamin D_3.

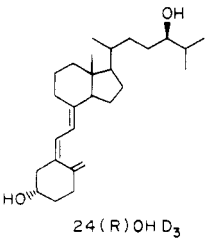

FIGURE 4: Structure of 24(*R*)-OH-D_3.

Discussion

The presence of 24(*R*)-OH-D_3 in plasma of chicks dosed with large amounts of vitamin D_3 is unexpected. Although it can easily be argued that the renal 24(*R*)-hydroxylase system is so highly induced under these conditions that low levels of nonspecific substrates are hydroxylated, no 24(*R*)-hydroxylation of vitamin D_3 in vitro has been observed (Tanaka et al., 1976). On the other hand, 24(*R*)-hydroxyvitamin D_2 has been isolated and identified (Jones et al., 1980).

The mechanism of vitamin D_3 toxicity is not understood. Recent work of vitamin D_3 toxicity in rats indicates that 1,25-dihydoxyvitamin D_3 [1,25-$(OH)_2D_3$] is not responsible for vitamin D_3 toxicity (Shepard et al., 1980; Hughes et al., 1976). At near-lethal levels of vitamin D_3, rats showed a

marked decrease in plasma levels of 1,25-$(OH)_2D_3$ (Shepard et al., 1980). Other metabolites of the vitamin such as 25-OH-D_3, 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$], and 25-OH-D_3-26,23-lactone are present at greatly elevated levels under this condition. One possible mechanism of toxicity is that at such high concentrations metabolites may interfere with the normal control mechanism of the vitamin D_3 system, for example, by substituting for 1,25-$(OH)_2D_3$ at the receptor level, or at other key points of metabolic control. Another possibility is that the presence of compounds such as 24-(*R*)-OH-D_3 and other metabolites yet to be identified may be responsible for vitamin D_3 toxicity, and not the high levels of the normal metabolites of vitamin D_3. The plasma level of 24(*R*)-OH-D_3 in the plasma of chicks given high doses of vitamin D_3 fell in the physiological range of most vitamin D_3 metabolites under normal dose conditions, i.e., 1–30 ng/mL. It is possible that the presence abnormal vitamin D_3 metabolites such as 24(*R*)-OH-D_3 and others yet to be identified, circulating at levels approaching those of 25-OH-D_3, are responsible for the toxic effect of vitamin D_3. These abnormal metabolites could interfere with normal vitamin D_3 feedback controls, resulting in a disruption of calcium and phosphorus homeostasis.

The present results illustrate that metabolites of vitamin D_3 not normally encountered in most plasma samples may complicate measurement of normal metabolites and interpretation of data obtained with nonspecific detectors such as the plasma transport protein. Great care must be exercised when samples taken from animals or patients given large amounts of vitamin D are measured. High resolution using more than one high-performance system is at present the only way to be certain of the metabolite in question. Clearly there is great need for more specific detectors for vitamin D metabolites, as, for example, the intestinal cytosol receptor for 1,25-$(OH)_2D_3$ (Kream et al., 1977).

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