The Isolation and Identification of 5,6-trans-25-Hydroxyvitamin D₃ from the Plasma of Rats Dosed With Vitamin D₃

EVIDENCE FOR A NOVEL MECHANISM IN THE METABOLISM OF VITAMIN D₃

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We isolated 5,6-trans-25-hydroxyvitamin D₃ from the plasma of vitamin D-deficient rats which had received 5,6-cis-vitamin D₃ orally. The relative amounts of 5,6-trans-25-hydroxyvitamin D₃ and 5,6-cis-25-hydroxyvitamin D₃ in plasma were 1:9. Control experiments in which 5,6-cis-25-hydroxyvitamin D₃ was added to plasma or water showed that less than 0.5% of the added 25-hydroxyvitamin D₃ was converted to 5,6-trans-25-hydroxyvitamin D₃ during the isolation procedure (p < 0.001, control versus experimental). We synthesized 5,6-trans-25-hydroxyvitamin D₃ from 25-hydroxyvitamin D₃ and demonstrated that authentic 5,6-trans-25-hydroxyvitamin D₃ co-eluted with the isolated putative 5,6-trans-25-hydroxyvitamin D₃ on a high performance liquid chromatography system. In addition, the isolated metabolite demonstrated mass spectral, ultraviolet absorption, and protein-binding properties similar to those of synthetic 5,6-trans-25-hydroxyvitamin D₃. As 5,6-trans-25-hydroxyvitamin D₃ binds more efficiently to intestinal cytosol-binding protein than 25-hydroxyvitamin D₃, this observation could explain, in part, how vitamin D₃ and 25-hydroxyvitamin D₃ are effective in large doses when administered to anephric patients or patients with advanced renal failure or hypoparathyroidism. This observation may also hold true for other analogs of vitamin D. Further, our observation provides strong evidence for the existence of 5,6-cis-trans isomerization in vivo. Whether this process is enzymatic or nonenzymatic is unknown, and the site of formation of the 5,6-trans metabolite has not been determined.

Vitamin D₃ plays an important role in normal calcium and phosphorus physiology (1). D₃ is metabolized in the liver to

25(OH)D₃; the latter is metabolized in the kidney to either 1,25(OH)₂D₃ or 24,25-dihydroxyvitamin D₂ (2-4). The regulation of 1,25(OH)₂D₃ synthesis, its metabolism, and its mode of action have been studied in detail (1, 5-8). We report the isolation and identification of a major new metabolite of D₃ from the plasma of D-deficient hypocalcemic rats administered D₃ orally, and suggest a possible role for it in the metabolism of vitamin D.

MATERIALS AND METHODS

General—All solvents were distilled prior to use. UV spectra of D₃ analogs were recorded in ethanol with a Beckman model 35 spectrophotometer (Beckman Instruments, Palo Alto, CA). Mass spectra were obtained at 70 eV on a Kratos MS-50 mass spectrometer/computer system (Kratos Instruments, United Kingdom) at a source temperature of 200°C. Sephadex LH-20 was obtained from Pharmacia (Piscataway, NJ). HPLC was performed on a Beckman Isocratic liquid chromatograph model 330 with a UV detector set at 254 nm.

Synthesis of 5,6-trans-9,10-Secocholesta-5,7,10(19)-trien-3β,25-diol—This was performed using an improved synthetic method (9). To a solution of 25(OH)D₃ (1 mg; 2.5 nmol) in diethyl ether (3 ml), 10 μg of diphenylisobenzene (Aldrich) was added; the reaction mixture was irradiated, under argon, with a 200-Watt tungsten lamp for 15 min. 5,6-trans-25(OH)D₃ was isolated using a HPLC system (pPorasil column (0.4 x 30 cm); solvent, 1.25% 2-propanol in hexane; flow rate, 2 ml/min; 750 psi). 5,6-trans-25(OH)D₃ eluted between 38 and 42 min. (UV absorbance λmax, 273 nm, ε₂73 = 22,700; mass spectrum (Fig. 1B) m/z 400 (M⁺; C₂₆H₄O₂), 382 (M⁺; H₂O, 271 (M⁺ - side chain), 253 (271-H₂O), 136 (A ring and C₆) and 118 (136-H₂O); the diMe₃Si ether derivative produced ions at m/z 544 (M⁺; C₆H₉O₃Si), 529 (M⁺-CH₃), 131 (fragmentation between C₆ and C₆), and 118 (A ring and C₆; C₆Me₃SiOH); all assignments were confirmed by accurate mass measurements.)

Analysis of 5,6-trans-25(OH)D₃ from plasma—The plasma or water showed that less than 0.5% of the added 25-hydroxyvitamin D₃ was converted to 5,6-trans-25-hydroxyvitamin D₃ during the isolation procedure (p < 0.001; control versus experimental). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: D₃, vitamin D₃; 9,10-secocholesta-5,7,10(19)-trien-3β,25-diol (5Z or 5,6-cis isomer); 25(OH)D₃, 25-hydroxyvitamin D₃; the latter is metabolized in the kidney to either 1,25(OH)₂D₃ or 24,25-dihydroxyvitamin D₂ (2-4). The regulation of 1,25(OH)₂D₃ synthesis, its metabolism, and its mode of action have been studied in detail (1, 5-8). We report the isolation and identification of a major new metabolite of D₃ from the plasma of D-deficient hypocalcemic rats administered D₃ orally, and suggest a possible role for it in the metabolism of vitamin D.
experimental rats were killed by decapitation and their blood was collected in 10 heparinized tubes. Plasma from control rats was isolated in 10 separate heparinized tubes. A group of rats received 190,000 cpm/rat of [1,2-3H]vitamin D₃. The plasma was purified as shown in Fig. 2.

RESULTS
The HPLC profile of the plasma from experimental animals is shown in Fig. 3. The UV spectrum of the peak eluting between 38 and 42 min shows a λ_max at 272-273 nm (Fig. 4). Comparison UV spectra of authentic cis and trans metabolites are plotted on the same figure. Following HPLC on a reversed-phase column, the UV spectrum displayed the same features as shown in Fig. 4. The mass spectrum of the material isolated after reversed-phase HPLC (Fig. 1A) demonstrates a molecular ion at m/z 400 (C_{27}H_{46}O_{2}), with prominent ions at m/z 382 (M⁺ - H₂O), 136 (A ring and C₆, C₁₀), and 118 (136-H₂O). These assignments were confirmed by accurate mass measurements. Ions m/z 271 (M⁺ - side chain) and 253 (m/z 271-H₂O) in the spectrum were clearly identified by examination of the accurate mass data. The mass spectrum of the isolated material and the authentic compound are virtually identical. The mass spectrum of the diMe₃Si ether of the isolated compound clearly revealed a molecular ion at m/z 544 (C_{33}H_{54}O_{2}Si₃), and ions at m/z 529 (M⁺ - CH₃), 131 (fragment formed as a result of cleavage between C_{25} and C_{24}) and 118 (A ring and C₆, C₇-Me₃SiOH). These assignments were also confirmed by accurate mass measurement. HPLC of plasma from D-deficient rats demonstrated no peak at this elution position. When 25(OH)D₃ was carried through the extraction procedure in the absence or presence of plasma, the amount of 5,6-trans-25(OH)D₃ formed was less than 0.5% (p < 0.001) (Table I).

The isolated peak eluted in a manner similar to synthetic 5,6-trans-25(OH)D₃ (Fig. 3). We examined the binding of 5,6-
ever, no 5,6-trans-25(OH)D₃ was detectable in the plasma of rats administered -0.01 mol of vitamin D₃. How-
dosed with D₃. The data show that 1) the metabolite has a molecular weight of 400 (C₂₇H₄₄O₂), suggesting the presence of one additional hydroxyl group in the D₃ molecule. 2) The diMe₃Si ether derivative has a molecular weight of 544 (C₇₀H₄₀O₂S₁₂), suggesting the presence of two hydroxyl groups in the molecule. 3) A peak at m/z 271 (C₂₉H₄₆O₂) in the mass spectrum of the underivatized metabolite localizes the extra hydroxyl group in the side chain, since the fragment at m/z 271 arises by loss of side chain through cleavage of the C₁₇-20 bond. 4) A prominent peak at m/z 131 (C₁₂H₁₂O₂S) in the mass spectrum of the diMe₃Si ether derivative suggests fragmentation between C₆ and C₁₉, localizing the new hydroxyl group to the C₁₉ position. 5) There is a prominent ion in the mass spectrum of the diMe₃Si derivative at m/z 118 (C₁₉H₂₂O₂S), confirming that the extra hydroxyl group is not in the A ring. The mass spectral data are compatible with the structure of a 9,10-secocholesta-5,7,10(19)-triene-3β,25-diol. No information relative to the nature of the 5,6 double bond can be obtained from the mass spectrum. 5,6-cis and trans isomers behave in a similar fashion on mass spectrometry. 6) A λₘₚ at 272-273 nm appears on UV spectrometry, that is characteristic of a 5,6-trans or 5E configuration. The metabolite also has 7) chromatographic and mass spectral characteristics that are identical with synthetic 5,6-trans-25(OH)D₃ and 8) a similar B₀ value when compared with that of synthetic 5,6-trans-25(OH)D₃. On the basis of the
above observations, we conclude that the new metabolite is 5,6-trans-25(OH)D$_3$ (9,10-secocholesta-5,7,10(19)-triene-3β,25-diol,5E isomer). It is unlikely that the compound is formed during the isolation procedure, because appropriate controls show only trace amounts of the 5,6-trans isomer. The formation of 5,6-trans-25(OH)D$_3$ may explain, in part, why large doses of 25(OH)D$_3$ or D$_3$ are effective in anephric or hypoparathyroid individuals. Holick et al. (11), Lawson and Bell (12), and Kraft et al. (13) have previously shown that the 5,6-trans isomers of vitamin D analogs are biologically active as trans isomers because rotation of the “A” ring of the secosteroid by 180° provides the correct stereochemical features necessary for binding to the intestinal cytosol receptor. We speculate that when other analogs of D$_3$ (such as 24,25-dihydroxyvitamin D$_3$) are administered in large doses, they may undergo similar isomerization in vivo and thus become active. The site of formation of this new metabolite and the mechanism by which it is formed are unknown at present.

Our observations strongly support the existence of a heretofore unknown metabolic route in vitamin D$_3$ metabolism, namely, the conversion of 5,6-cis compounds to 5,6-trans compounds. The mechanism of this transformation is not clear at present; it could be enzymatic or nonenzymatic. The site of formation of 5,6-trans-25-hydroxyvitamin D$_3$ is unknown.

In summary, when rats are administered large doses of vitamin D$_3$, 5,6-trans-25(OH)D$_3$ is found in plasma. This unique process of 5,6-cis-trans isomerization may be of biological significance, particularly when 1α-hydroxylase activity is absent or greatly reduced.

REFERENCES

1. DeLuca, H. F. (1979) *Nutr. Rev.* 37, 161-193
2. Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968) *Biochemistry* 7, 3177-3222
3. Fraser, D. R., and Kodicek, E. (1970) *Nature (Lond.*) 228, 764-766
4. Holick, M. F., Schnoes, H. K., DeLuca, H. F., Gray, R. W., Boyle, I. T., and Suda, T. (1972) *Biochemistry* 11, 4251-4255
5. Boyle, I. T., Gray, R. W., and DeLuca, H. F. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 2131-2134
6. Tanaka, Y., and DeLuca, H. F. (1973) *Arch. Biochem. Biophys.* 154, 566-574
7. Kumar, R., Harnden, D.H., and DeLuca, H. F. (1976) *Biochemistry* 15, 2420-2423
8. Kumar, R., Nagubandi, S., Mattox, V. R., and Londowski, J. M. (1980) *J. Clin. Invest.* 65, 277-284
9. Barrett, A. G. M., Barton, D. H. R., Johnson, G., and Nagubandi, S. (1978) *Synthesis* 16, 74
10. Kumar, R., Cohen, W. R., Silva, P., and Epstein, F. H. (1979) *J. Clin. Investig.* 63, 342-344
11. Holick, M. F., Garabedian, M., and DeLuca, H. F. (1972) *Biochemistry* 11, 2715-2719
12. Lawson, D. E. M., and Bell, P. A. (1974) *Biochem. J.* 142, 37-46
13. Kraft, D., Schaefer, K., Grigoleit, H.-G., Offerman, G., von Herrath, D., and Delling, G. (1979) *Dtsch. Med. Wochenschr.* 104, 1705-1710