Evidence for a Monoglucuronide of 1,25-Dihydroxyvitamin D₃ in Rat Bile*  

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We have isolated and characterized a monoglucuronide fraction of 9,10-secocholesta-5,7,10(19)triene-1α,3β,25-triol, 5,6-cis isomer (1,25-dihydroxyvitamin D₃) from rat bile. Polar radioactive metabolites of 1,25-dihydroxyvitamin D₃ were purified by a sequence of chromatographic procedures which utilized Amberlite XAD-2, diethylaminohydroxypropyl Sephadex LH-20, liquid-liquid partition on paper, and reverse phase chromatography on C-18 microparticulate columns. A purified radioactive substance showed maximal absorbance at 264 nm, indicating the presence of a triene in the 5,6-cis configuration. Mass spectrometry by fast atom bombardment of the product demonstrated an ion at m/z 637 atomic mass units that is consistent with a natriated sodium salt of a monoglucuronide of 1,25-dihydroxyvitamin D₃ (5αMNa·Na⁺). Following methylation of the carboxylic acid group and formation of trimethylsilyl ethers of the hydroxy groups, the fragmentation pattern of the product was compatible with that of a monoglucuronide of 1,25-dihydroxyvitamin D₃. The intact metabolite was treated with β-glucuronidase and the aglycon was isolated by chromatography on microparticulate silica. The aglycon co-migrated with authentic 1,25-dihydroxyvitamin D₃ during chromatography and it gave a mass fragmentation pattern consistent with 1,25-dihydroxyvitamin D₃. The aglycon was bound by an intestinal cytosol receptor with essentially the same affinity as 1,25-dihydroxyvitamin D₃. These findings indicate that bile contains a monoglucuronide of 1,25-dihydroxyvitamin D₃.

Vitamin D₃ plays an important role in the transport of calcium in the intestine and in the normal mineralization of bone. The active metabolite of vitamin D₃, 1,25-dihydroxyvitamin D₃, is metablized rapidly in experimental animals and in man (1–3). The sterol undergoes side chain oxidation to calcitroic acid in the liver and intestine; 24-hydroxylation occurs in the kidney and intestine, and the hormone and its metabolites are excreted in the bile, feces, and urine (4–15). Metabolites which appear in bile, feces, and urine after the administration of [1H]25-dihydroxyvitamin D₃ are much more polar than the parent sterol, and when bile which contained such radiolabeled metabolites was treated with β-glucuronidase there was an increase in the amount of radioactivity which co-migrated with 1,25-dihydroxyvitamin D₃ (13), a finding which suggested the presence of a glucuronide of 1,25-dihydroxyvitamin D₃. We now report the isolation and characterization of a fraction from rat bile which contains a monoglucuronide of 1,25-dihydroxyvitamin D₃.

EXPERIMENTAL PROCEDURES

General—All solvents were distilled prior to use or were ultraviolet spectral grade. Ultraviolet spectra were obtained in ethanol or methanol on a Beckman 35 recording spectrophotometer (Beckman Instruments). Mass spectra were obtained at 70 eV on a Kratos M-50/DS-55 high resolution mass spectrometer/computer system. The source temperature was 200 °C and the probe temperature was programmed from ambient to 350 °C. Mass spectrometry by fast atom bombardment was performed on a Kratos M-50/DS-55 mass spectrometer/computer system equipped with a fast atom bombardment source (Middle Atlantic Mass Spectrometry Laboratory, Baltimore, MD, Dr. C. Fenselau, Director). Monothioglycerol was used as a solvent. High performance liquid chromatography was performed using Waters Associates chromatograph (Waters Associates, Inc., Milford, MA) equipped with two 6000 A pumps, a model 660 gradient programmer, a model 450 variable absorbance detector set at 265 nm, and a Hewlett Packard 3380A integrator (Hewlett-Packard Co., Palo Alto, CA). Radioactivity was measured with a Beckman LS-9000 liquid scintillation counter using Budget-solve (RPI, E7 Grove, IL) as scintillant (13).

Animals—Two hundred g male, Sprague-Dawley rats were obtained from the Institute Hills Farm, Rochester, MN. Each animal was a gift of 2.3 or 4.6 μmol of 1,25-dihydroxyvitamin D₃ and [26,27-3H]25-dihydroxyvitamin D₃ (965,000 dpm/rat) intravenously as described previously (13). Bile was collected for 24 h in glass tubes kept at 4 °C. Immediately following collection, α-tocopherol and ascorbic acid (0.001%, w/v) were added to the bile.

Vitamin D₃, Metabolites and Steroid Glucosiduronates—1,25-Dihydroxyvitamin D₃ was a gift of Dr. M. Uskokovic (Hoffmann-LaRoche). 11-Deoxycorticosterone 21-glucoisuronic acid, which was used to calibrate chromatographic columns, was synthesized by a previously described procedure (16).

Purification and Chromatography—Bile obtained from rats which had been injected with 1,25-dihydroxyvitamin D₃ was diluted with water and extracted twice with an equal volume of peroxide-free diethyl ether. The aqueous phase was diluted with 200 ml of 0.1 M acetic acid and chromatographed on a column (3 × 22 cm) of Amberlite XAD-2 (Rohn and Haas Co., Philadelphia, PA); elution was performed by using 0.1 M acetic acid, water, and methanol in succession (15).

Diethylaminohydroxypropyl Sephadex LH-20 was prepared as described by Setchell et al. (15). The residue of the methanolic eluate from the Amberlite XAD-2 column was applied to a column (0.9 × 19.5 cm) of Diethylaminohydroxypropyl Sephadex LH-20 and the column was eluted with successive 90-ml portions of 72% ethanol, 0.25 M acetic acid in 72% ethanol, 0.25 M formic acid in 72% ethanol, 0.25 M acetic acid-potassium acetate (pH 6.3) in 72% ethanol, and 0.5 M potassium acetate (pH 10) in 72% ethanol.

Paper chromatography was performed by descending flow on sheets of Schleicher and Schuell paper (2043A) with a solvent (19) consisting of 1,2-dichloroethane-butyl alcohol-water-acetic acid (9:1:7.3, v/v). The radioactive material which migrated at Rf 0.59 was eluted with methanol, dried, and processed as described below.

Liquid chromatography was performed using four separate syst-
Derivatization of Metabolite—The metabolite (2 μg) was esterified with excess diazomethane and converted to the appropriate trimethylsilyl ether using trimethylsilyl chloride and hexamethyldisilazane as described previously (21).

RESULTS

A total of 34 μmol of 1,25-dihydroxyvitamin D₃ was administered to nine rats. Of this, 11.3 μmol (radioactivity = 2.74 × 10⁶ dpm) were recovered in the bile. Upon extraction with ether, 8% of the radioactivity remained in the ether phase. The remaining water-soluble radioactivity (~10.4 μmol) was applied to an Amberlite XAD-2 column in two batches. Eighty-six per cent of the applied radioactivity (~9 μmol of material) was eluted with methanol. When the radioactive material from the Amberlite XAD-2 column was applied to a Diethylaminohydroxypropyl LH-20 Sephadex column, 53% (~4.8 μmol) of the radioactivity was eluted with 0.25 M formic acid in 72% aqueous ethanol, a solvent system which normally elutes steroid glucuronides (18). Seventeen per cent, 6%, 9%, and 1.5% of the applied radioactivity was eluted with 72% aqueous ethanol, 0.25 M acetic acid in 72% ethanol, 0.3 M acetic acid-potassium acetate (pH 6.3) in 72% ethanol, and 0.5 M potassium acetate (pH 10) in 72% ethanol, respectively. The material which was eluted with 0.25 M formic acid in 72% aqueous ethanol was chromatographed on paper. Eight per cent (0.38 μmol) of the radioactive material applied to the paper migrated at RF 0.58; a sample of deoxycorticosterone glucosiduronic acid run in parallel had RF 0.52. The zone corresponding to RF 0.58 was eluted from the paper with methanol and processed further on reversed phase high performance liquid chromatography systems as described under “Experimental Procedures” and in Fig. 1. Following gradient elution high performance liquid chromatography on a Micropak reverse phase MCH 10 column, the material which was eluted at 33–39 min was chromatographed again on the same column using isotropic conditions. The peak which emerged between 50 and 60 min was collected and chromatographed on a µBondapak column as shown in Fig. 1. The material which emerged between 45 and 50 min was collected and recycled 10 times on a C-18 µBondapak column utilizing a solvent system of ~70% methanol, 30% water, and 0.1% acetic acid. The ultraviolet-absorbing peak was essentially symmetrical on the first recycle, noticeably unsymmetrical on the fifth recycle and appeared to be separating into two zones on the tenth recycle at which time the band had spread until it

Fig. 1. Scheme used to isolate the biliary metabolite of 1,25-dihydroxyvitamin D₃, DEAP, diethylaminohydroxypropyl; FAB, fast atom bombardment; MS, mass spectrometry; HPLC, high performance liquid chromatography.
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FIG. 3. Mass spectrum (electron impact) of the methyl ester pentatrimethylsilyl derivative of the biliary metabolite.

FIG. 4. Upper, mass spectrum of aglycon of isolated metabolite. Lower, mass spectrum of authentic 1,25-dihydroxyvitamin D₃.

was not feasible to recycle further. The scarcity of the metabolite (~4.8 µg) precluded further purification on other chromatography systems. Thus, we characterized the product by the following techniques.

The material was analyzed by fast atom bombardment-mass spectrometry. Initially it showed an ion at m/z 615 (consistent with [MNa][H⁺]) which rapidly disappeared and was replaced by an ion at m/z 637 (Fig. 2) which is compatible with a natriated sodium salt of a monoglucosiduronate of 1,25-dihydroxyvitamin D₃ ([MNa][Na⁺]). When the fraction was methylated with diazomethane, treated with trimethylsilyl chloride and hexamethyldisilizane, and analyzed by electron impact-mass spectrometry it showed the following fragmentation pattern (Fig. 3): m/z 966 (M⁺⁻), 951 (M⁺⁻CH₃), 876 (M⁺⁻(CH₃)₃SiOH), 559 (M⁺⁻407), 542 (966-glucuronide-OH), and 452 (542-(CH₃)₃SiOH). Ions at m/z 407, 317, 217, and 204 were observed. An ion of significant intensity at m/z 131 (cleavage between C-24 and C-25 on the aglycon) was not found. No ion was seen at m/z 431 (966- (side chain + C-25 glucuronide)) or at m/z 785 (966-side chain). However, an ion at m/z 675 (M⁺⁻side chain-(CH₃)₃SiOH) was noted.

Hydrolysis of the conjugate by treatment with β-glucuronidase liberated a sterol moiety that co-migrated with 1,25-dihydroxyvitamin D₃ on a µPorasil column. The aglycon was bound by a specific intestinal cytosol receptor with an affinity similar to that of authentic 1,25-dihydroxyvitamin D₃. When analyzed by mass spectrometry the aglycon (Fig. 4, upper) gave prominent ions at m/z 416 (M⁺⁻), 398 (M⁺⁻H₂O), 380 (M⁺⁻2H₂O), 362 (M⁺⁻3H₂O), 289 (M⁺⁻side chain-H₂O), 251 (269-H₂O), 136 (C₆H₅ ring + C₆H₇ with protons)-H₂O, a pattern identical with that of authentic 1,25-dihydroxyvitamin D₃ (Fig. 4, lower). Both the intact glucuronide and the isolated aglycon absorbed maximally at 264 nm, the wavelength of maximal absorption for vitamin D₃ in the 5,6-cis configuration.

DISCUSSION

We have isolated and partially purified a metabolite of 1,25-dihydroxyvitamin D₃ from the bile of rats dosed with 1,25-dihydroxyvitamin D₃. This material contains a monoglucuronide of 1,25-dihydroxyvitamin D₃ as indicated by its mass spectrum, ultraviolet spectrum, chromatographic behavior, and hydrolysis with β-glucuronidase. On fast atom bombardment-mass spectrometry (Fig. 2) the ion at m/z 637 is consistent with a natriated sodium salt of the monoglucuronide of 1,25-dihydroxyvitamin D₃ ([MNa][Na⁺]), a type of ion common in fast atom bombardment-mass spectrometry. The electron impact mass spectrum of the derivatized glucuronide revealed (Fig. 3) a molecular ion at m/z 966 which is consistent with the proposed structure. The ions at m/z 407, 317, 217, and 204 are characteristic of glucuronides (22). The ion at m/z 542 represents the aglycon with (CH₃)₃SiOH groups intact. The ion at m/z 432 represents 542-(CH₃)₃SiOH. The ion at

² C. Fenselau, personal communication.
m/z 131, which represents cleavage between C-24 and C-25 with loss of the fragment (CH₂)₅C═O-Si(CH₃)₃, was not of sufficient intensity to establish unambiguously the presence of a nonconjugated C-25 hydroxyl group. The absence of ions at m/z 431 (M⁺-conjugated side chain) and at m/z 765 (M⁺-side chain) does not allow differentiation between C-1, C-3, and C-25 glucuronides. The ion at m/z 675 (765-(CH₃)₃SiOH) suggests that the compound is an 'A' ring glucuronide of 1,25-dihydroxyvitamin D₃. However, this assignment must be regarded as provisional.

The aglycon liberated by β-glucuronidase treatment of the intact metabolite has been identified as 5,6-cis 1,25-dihydroxyvitamin D₃ by its chromatographic properties, binding characteristics to a specific receptor protein, mass spectrum, and ultraviolet spectrum. The mass spectrum of the aglycon (Fig. 4 upper) reveals a molecular ion at m/z 416 and ions at m/z 398, 380, and 362 which represent successive loss of water from the molecule. Ions were also observed at m/z 269 (416-129 side chain-18) and 251 (269-18). This fragmentation pattern is characteristic of 1,25-dihydroxyvitamin D₃. Ions at 559, 542, and 452 in the mass spectrum of the derivatized glucuronide (Fig. 3) also support the contention that the aglycon is 1,25-dihydroxyvitamin D₃.

Taken in their entirety, our data indicate that we have isolated a monoglucuronide of 1,25-dihydroxyvitamin D₃. The product which we isolated was not completely resolved from a coeluting impurity. The impurity may be an unrelated substance; alternatively, two monoglucuronides with similar chromatographic and mass spectral properties (e.g. the C-1 monoglucuronide and the C-3 monoglucuronide) may be present in the isolated material. However, the conclusion that the product contains a monoglucuronide of 1,25-dihydroxyvitamin D₃ is unequivocal.

Vitamin D₃ and its metabolites are excreted in bile and some of them undergo an enterohepatic recirculation (23-26). The presence of polar radioactive biliary material that liberates 1,25-dihydroxyvitamin D₃ upon β-glucuronidase hydrolysis has been reported (13). In addition, it has been shown that vitamin D₃-glucuronide and 25-hydroxyvitamin D₂-glucuronide are excreted in bile (23-27). In physiological situations, biliary glucuronides of 1,25-dihydroxyvitamin D₃ may represent up to 15% of the total metabolites of 1,25-dihydroxyvitamin D₃ (13). Calcitriol acid may represent another 15% (28). The biological activity and the physiological relevance of the monoglucuronide of 1,25-dihydroxyvitamin D₃ are not known. However, it is known that vitamin D₃ 3β-glucuronide, which lacks hydroxyl groups at C-1 and C-25, is biologically active after in vitro hydrolysis and that the free sterol can be reutilized by the organism (29). It seems possible that a similar phenomenon might occur with monoglucuronides of 1,25-dihydroxyvitamin D₃. If this reutilization should occur as a normal process, failure by the organism to reutilize biliary glucuronides of 1,25-dihydroxyvitamin D₃ could result in depletion of 1,25-dihydroxyvitamin D₃. Such a mechanism could be operative in malabsorption syndromes due to diverse causes.

REFERENCES