Synthesis and Biological Activity of Vitamin D₃-Sulfate*

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Vitamin D₃-3β-sulfate has been synthesized using pyridine sulfur trioxide as the sulfate donor. It has been shown to be pure by high performance liquid chromatography and spectral methods. Unlike previous reports, the product has been identified unambiguously as the 3β-sulfate ester of vitamin D₃ by its ultraviolet, nuclear magnetic resonance, infrared, and mass spectra. The biological activity of vitamin D₃-sulfate was then determined in vitamin D-deficient rats. Vitamin D₃-sulfate has less than 5% of the activity of vitamin D₃ to mobilize bone calcium and approximately 1% of the ability of vitamin D₃ to stimulate calcium transport, elevate serum phosphorus, or support bone calcification. These results disprove previous claims that vitamin D₃-sulfate has potent biological activity, and they further do not support the contention that vitamin D₃-sulfate represents a potent water-soluble form of vitamin D in milk.

In recent years it has been suggested that conjugation of vitamin D with sulfate (SO₄) (Fig. 1) plays an important role in vitamin D metabolism. Higaki et al. (1) reported the in vitro formation of vitamin D₂-SO₄ by rat liver homogenates. They further suggested that vitamin D₂-SO₄ has approximately the same antirachitic potency as vitamin D₂ itself (2) but is less toxic when administered in large amounts (3). Unfortunately the authors failed to provide convincing evidence that the vitamin D₂-SO₄ had been prepared nor was satisfactory evidence of purity offered, casting doubt on the reported biological activity. Miravet et al. (4) repeated the experiments of Higaki using vitamin D₃, but they also failed to provide evidence of purity or physical evidence that the correct product was obtained. Thus, the reported potent biological activity of the vitamin D₂-3β-sulfate remained in considerable question.

The sulfate ester of vitamin D has also been isolated from the urine of both rabbits (1) and rats (5) after oral administration of massive amounts of the vitamin. High levels of a water-soluble form of vitamin D activity have been reported to occur in milk. Sahashi et al. (6) found cows’ milk to contain 240 IU of vitamin D activity per liter, of which 80% was claimed to be water soluble. In addition, these authors report that human milk contains 965 IU of vitamin D activity per liter. Similar findings have been reported by Lakhdawala and Widdowson (7). Both studies infer that this water-soluble form of vitamin D is the sulfate conjugate. Since it is generally believed that even fairly minor structural changes greatly affect the biological activity of analogs of vitamin D (8), it was of great importance to determine if vitamin D₂-SO₄ has biological activity and if it acts upon all of the target organs of vitamin D. The results of this study demonstrate that vitamin D₂-SO₄ has little or no biological activity, in contrast to previous reports.

Materials and Methods

Vitamin D₃ was purchased from the Thompson-Hayward Chemical Co., Kansas City, KS. Ultraviolet absorption spectra were recorded in HPLC grade methanol (Fisher Chemical Co., Chicago, IL) with a Beckman model 24 recording spectrophotometer. Nuclear magnetic resonance spectra were obtained with a Bruker 270-MHz spectrometer using CD₂OD as solvent; infrared absorption spectra were obtained with a Beckman model 4230. Mass spectrometry was performed with an AEI MS-9 mass spectrometer at 70 eV. using a direct probe for introduction of the sample. HPLC was performed with a Waters model ALC/GPC 204 liquid chromatograph equipped with a Waters model 440 absorbance detector operating at 254 nm. A Packard 10 ODS-2 semipreparative column (0.94 x 25 cm) was used. Serum calcium concentrations were determined using a Perkin-Elmer model 402 atomic absorption spectrophotometer. Radioactivity (¹⁴C) was measured by liquid scintillation counting with a Packard model 3255 counter. Samples were counted in a solution of toluene containing 0.2% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Synthesis of Vitamin D₃-SO₄—Vitamin D₃-SO₄ was synthesized according to a procedure similar to that of Sobel and Spoerri (9). Vitamin D₃ (1.0 g) and 1.0 g of pyridine sulfur trioxide were suspended in 6 ml of pyridine. Solution was added to the mixture was heated to 56°C; 0.4 ml of triethylamine was added and the reaction mixture held at 56–58°C for 20 min. At this time thin layer chromatography developed with methanol:CHCl₃ (1:4) indicated the disappearance of vitamin D₃ (Rₛ= 0.8) and the appearance of the product (Rₛ = 0.4). The solvent was removed by flash evaporation at a temperature of 40–45°C. The residue was dissolved in CH₃OH/CHCl₃ (1:9) and applied to a silica column (1.5 × 25 cm) eluted with the same solvent. A broad UV-absorbing peak eluting at approximately 5 column volumes was collected. A preliminary NMR spectrum identified it as the triethylamine salt of vitamin D₃-SO₄. In order to convert this product to a 1 The abbreviations used are: vitamin D₂-SO₄, vitamin D₃-3β-sulfate; HPLC, high performance liquid chromatography.

Fig. 1. Structures of vitamin D₂ and vitamin D₃-SO₄.
Vitamin D Sulfate

to the sodium salt, the solvent was evaporated and the residue dissolved in distilled water. Saturated NaCl was added dropwise until a white precipitate formed. The mixture was then centrifuged and the supernatant removed. The precipitate was redissolved in CH$_2$OH/CHCl$_3$ (1:9), applied to a silica column (1.5 x 25 cm), and eluted with the same solvent. A very broad UV-absorbing peak eluting at approximately 8 column volumes was collected. The solvent was evaporated, and the resulting compound was characterized by UV, NMR, and mass spectrometry. A second portion of the product was chromatographed on DEAE-Sephadex eluted first with methanol and then with methanol containing 0.4 M ammonium acetate. An additional portion of the compound was subjected to reversed phase HPLC, eluted with water:CH$_3$OH (1:4) containing 10 mM ammonium bicarbonate. A white precipitate formed. The mixture was then centrifuged, and calcium determinations were carried out on 0.10 ml of serum diluted with 0.1% lanthanum chloride.

**Assay for Antirachitic Activity**—The radii and ulnae were stained in 1.5% silver nitrate solution. New calcification on the epiphyseal plate was scored visually according to the U. S. Pharmacopoeia (14).

**RESULTS**

The 3β-sulfate ester of vitamin D$_3$ was produced under mild conditions using pyridine sulfur trioxide as the sulfate donor. The product of the reaction was converted to the sodium salt and purified by chromatography on a silica column. It was then eluted from reversed phase HPLC as a single peak at approximately 5 column volumes (Fig. 3A). The UV absorption spectrum of the reaction product indicates a maximum absorbance at $\lambda = 265$ nm and a minimum at $\lambda = 227$ nm (Fig. 2), which is also characteristic for vitamin D. However, vitamin D$_3$ is eluted from a DEAE-Sephadex column in approx-
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Effect of vitamin D₃-SO₄ on bone calcium mobilization

Vitamin D-deficient, calcium-depleted rats were dosed daily for 1 week. The data are expressed as the mean value of six animals ± S.E.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (nmol/day)</th>
<th>Serum calcium concentration (mg/100 ml ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene glycol</td>
<td>0.065</td>
<td>4.3 ± 0.1</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>0.65</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>Vitamin D₃-SO₄</td>
<td>6.5</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

* Compound administered by intubation; all others were injected intraperitoneally.

DISCUSSION

The biological activity of vitamin D₃ is reduced to less than 5% by conjugation of the 3β-hydroxyl with sulfate. This very low biological activity indicates that vitamin D₃-SO₄ has little or no activity itself. In addition, the sulfate ester is evidently stable under physiological conditions, and very little vitamin D is released in vivo from the substrate. This might be expected, since at room temperature vitamin D₃-SO₄ is stable in base and is only slowly hydrolyzed to release vitamin D in acid. It is likely that vitamin D₃-SO₄ is hydrolyzed slowly in the stomach when the compound is administered orally. This would account for the slightly higher biological activity observed under these conditions.

The present results are in direct contrast to reports of high biological activity of vitamin D₃-SO₄ (3, 13) and vitamin D₂-SO₄ (4). In neither of those cases did the authors provide adequate evidence that the vitamin D-sulfate was obtained under sufficient purity for meaningful biological evaluation. As a result of those and other (1-7) similar reports there has been the widespread belief among nutritionists that vitamin D-sulfate has potent biological activity. The present study clearly dispels this belief.

The results of the present study also do not support the idea that vitamin D-sulfate is a potent form of vitamin D in

Fig. 6. Infrared spectrum of vitamin D₃-SO₄. Peaks at 1240 cm⁻¹ and 1062 cm⁻¹ are absent in the spectrum of vitamin D₃, and can be assigned to the sulfate ester function.

Table I

Effect of vitamin D₃-SO₄ on duodenal calcium transport

Pats which had received the vitamin D-deficient, low calcium diet for 3 weeks were divided into groups of six animals and dosed as indicated for 7 days. Calcium transport was measured by the everted duodenal sac method. Values are mean ± S.E.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (nmol/day)</th>
<th>Calcium transport (S/M ± S.E.)</th>
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<tbody>
<tr>
<td>Propylene glycol</td>
<td>0.026</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>0.65</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Vitamin D₃-SO₄</td>
<td>1.3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2.6</td>
<td>3.1 ± 0.2*</td>
</tr>
</tbody>
</table>

* Differs from control at P < 0.005.


approximately 1 column volume of methanol (Fig. 3B). In contrast, the reaction product fails to elute from the same column in methanol and is eluted only with 0.4 M ammonium acetate in methanol. This indicates that the synthesized compound is charged and, therefore, cannot be vitamin D itself.

The NMR spectrum (Fig. 4) shows the typical pattern expected for vitamin D (15) except that the 1-proton multiplet which corresponds to the 3a-proton is shifted from δ 3.9 to 4.0 to δ 4.7. The spectrum confirms an unaltered cis-trans chromophore, and the shift of the 3a-proton is in the direction and magnitude expected for the conversion of hydroxy to an sulfate substituent to give the base peak at m/e 384. From the physical data obtained, it is evident that the Na salt of vitamin D₃-SO₄ has been formed.

The mass spectrum (Fig. 5) shows the expected elimination of the sulfate substituent to give the base peak at m/e 386 [M⁺-SO₄-H] with additional fragments at m/e 253 (366-side chain) and m/e 118 (dehydro ring A plus carbon 6 and carbon 7). In contrast, the fragments characteristic of vitamin D₃ at m/e 384, m/e 271, and m/e 136 are very weak. Both the NMR and mass spectra are consistent with a vitamin D derivative containing an electronegative substituent on the 3β-hydroxyl position. The infrared spectrum of the synthesized compound (Fig. 6) contains absorption bands at 1240 and 1062 cm⁻¹ which are not present in the infrared spectrum of vitamin D₃ itself (16) and may be compared to organic sulfate (17-19). From the physical data obtained, it is evident that the Na salt of vitamin D₃-SO₄ has been synthesized.

Vitamin D₃-SO₄ exhibits very low activity in all of the classical assays for the functions of vitamin D. The data in Table I demonstrate that vitamin D₃-SO₄ retains less than 1% of the ability of vitamin D₃ to stimulate duodenal calcium transport. No stimulation of calcium mobilization from bone could be detected even when 6.6 nmol/day of vitamin D₃-SO₄ was administered intraperitoneally.

Table II

Relative effectiveness of vitamin D₃-SO₄ on endochondral calcification and maintenance of serum phosphorus concentration in rachitic rats

Groups of six vitamin D-deficient, phosphorus-depleted rats were given the indicated dose for 7 days. Serum phosphorus concentrations are expressed as the mean ± S.E.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (nmol/day)</th>
<th>Serum phosphorus (mg/100 ml ± S.E.)</th>
<th>Calcification score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propylene glycol</td>
<td>0.026</td>
<td>2.3 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>0.065</td>
<td>4.3 ± 0.4</td>
<td>4-5</td>
</tr>
<tr>
<td>Vitamin D₃-SO₄</td>
<td>0.65</td>
<td>4.3 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>4.2 ± 0.3</td>
<td>2</td>
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* Differs from control at P < 0.005.


As indicated for 7 days. Calcium transport was measured by the everted duodenal sac method. Values are mean ± S.E.

Table I

Effect of vitamin D₃-SO₄ on duodenal calcium transport

Pats which had received the vitamin D-deficient, low calcium diet for 3 weeks were divided into groups of six animals and dosed as indicated for 7 days. Calcium transport was measured by the everted duodenal sac method. Values are mean ± S.E.

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<td>2.6</td>
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milk (6, 7); if the sulfate-ester is in fact present in milk, it has little or no biological activity. Furthermore, the methods used in previous studies to assess the presence of vitamin D-sulfate in milk are questionable, making the results difficult to evaluate. This question must, therefore, be re-examined.

The question of whether vitamin D-sulfate is an important excretory form of the vitamin has not been addressed in the present study. The significance of earlier reports of vitamin D3-SO4 as an excretory product in animals (1, 4) is difficult to assess, in view of the massive amounts of vitamin D3 administered in these studies and the very low abundance of sulfate ester recovered. This question should be examined more closely before its role as an excretory product can be assessed. At present, however, the concept of a highly biologically active vitamin D-sulfate can be discarded.

REFERENCES