THE ABSOLUTE CONFIGURATION OF THE NATURAL 25,26-DIHYDROXYCHOLECALCIFEROL

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1. Introduction

Two of us have reported [1,2] the synthesis of the metabolite of vitamin D_3 25,26-dihydroxycholecalciferol (25,26$(OH)_2D_3$), which led to a mixture of the C-25 epimers and more recently [3] the resolution of these latter by HPLC. The absolute configuration of the epimers could be established [4] by X-ray diffraction analysis. One of these epimers must be identical with the natural metabolite 25,26$(OH)_2D_3$ isolated from porcine plasma [5] and more recently generated biologically [6]. Its configuration at C-25 remained unknown.

The present report describes the identification of the natural 25,26$(OH)_2D_3$ with 25*R*,26$(OH)_2D_3$. It was achieved by HPL co-C of their 3,25,26-Tris–TMS derivatives by a procedure similar to that used in [7] for the identification of the natural 24,25$(OH)_2D_3$.

2. Experimental

2.1. *Instruments*

HPLC was performed with a Waters Associates chromatograph equipped with a 6000A pump, a U6K injector, a 254 nm ultraviolet detector and a 30 cm × 4 mm i.d. column of Microporasil. The mobil phase was *n*-hexane containing 2% (v/v) dichloromethane at a flow rate of 1 ml/min and a pressure of 42 kg/cm^2.

The radioactivity was determined using a counting solution (Liquid Scintillator Unisolve 1, Koch-Light Lab.) and a liquid scintillation counter (Intertechnique Model SL 40).

2.2. *Preparation and purification of biological radioactive 25,26(OH)$_2D_3$*

Vitamin D-deficient chicks were given an injection of 6.5 nmol vitamin D_3 in 0.05 ml ethanol subcutaneously 48 h before they were killed by decapitation. Kidneys were removed, placed in ice-cold sucrose (0.25 M) and homogenized to yield a 20% homogenate (w/v) in 0.19 M sucrose containing 15 mM Tris–acetate (pH 7.4) and 1.9 mM magnesium acetate. An aliquot of the homogenate containing 200 mg kidney tissue was incubated in a 25 ml Erlenmeyer flask in a final 1.5 ml. The reaction mixture ultimately contained 0.19 M sucrose, 15 mM Tris–acetate (pH 7.4), 1.9 mM magnesium acetate and 25 mM succinate. The contents of the flask were flushed for 30 s with 100% oxygen and stoppered. The substrate (2.6 nmol 25-OH-[23,24-^{3}H]D_3 dissolved in 0.02 ml 95% ethanol) was added to each vessel. The reaction mixtures were incubated at 37°C for 10 min. The reaction was stopped by the addition of 10 ml dichloromethane. Extraction was carried out by the dichloromethane extraction procedure in [8]. The dichloromethane extracts were applied directly to a Sephadex LH-20 column (2 × 34 cm) eluted with

Abbreviations: 25-OH-D_3, 25-hydroxycholecalciferol; 1,25$(OH)_2D_3$, 1,25-dihydroxycholecalciferol; 25,26$(OH)_2D_3$, 25,26-dihydroxycholecalciferol; 24,25$(OH)_2D_3$, 24,25-dihydroxycholecalciferol; TMS, trimethylsilyl; HPL(co-)C, high pressure liquid (co-)chromatography; TLC, thin layer chromatography

hexane:chloroform:methanol (9:1:1) to resolve 25,26(OH)$_2$D$_3$ from 1,25(OH)$_2$D$_3$ as in [9]. The 25,26(OH)$_2$D$_3$ was applied in total to a HPLC system equipped with a DuPont Zorbax-SIL column (4.6 mm X 25 cm) developed with a solvent system of 9% isopropanol in hexane at 7000 p.s.i. The purified 25,26(OH)$_2$D$_3$ was then used for co-chromatography with the synthetic material as described in sections 2.3 and 2.4.

2.3. *Preparation of 3,25,26-Tris–TMS derivatives of 25,26(OH)$_2$D$_3$*

Mixture of synthetic [3] 25*R*,26- and 25*S*,26(OH)$_2$D$_3$ (1.5 μg each) were treated with trimethylsilylimidazol (4 μl) in *n*-hexane (100 μl) at 50°C for 30 min and left at room temperature for 12 h. The structure of 3,25,26-Tris-TMS derivatives was confirmed by TLC [2]. The solvent was evaporated under nitrogen, the residue dissolved in *n*-hexane containing 2% (v/v) dichloromethane, filtered and injected into the HPLC system.

2.4. *Co-chromatography with radioactive 25,26(OH)$_2$D$_3$*

In experiments with biologically generated 25,26(OH)$_2$-[23,24-^{3}H]D$_3$, the radioactive material was added to the mixture of epimeric 25*R*,26- and 25*S*,26(OH)$_2$D$_3$ and treated as above. Fractions were collected at 30 s intervals and the radioactivity determined in each fraction.

3. Results

Figure 1 illustrates the separation of the Tris–TMS derivatives of 25*R*,26- and 25*S*,26(OH)$_2$D$_3$ achieved by HPCL on high surface area silica. The retention time for the 25*S* epimer was 62 min while that for the 25*R* one was 65 min.

In experiments with biologically generated 25,26(OH)$_2$-[23,24-^{3}H]D$_3$ the radioactivity migrates with the derivative of 25*R*,26(OH)$_2$D$_3$. Recovery for the radioactivity was 70%.

4. Discussion

Our results demonstrate clearly that the natural 25,26(OH)$_2$D$_3$ has a 25*R* configuration. This has been established first by preparing [3] synthetically the two C-25 epimers of 25,26(OH)$_2$D$_3$ and assigning [4] their respective configurations by X-ray studies. Then a method for separating the epimers as Tris–TMS ethers by HPLC was elaborated and it was found that

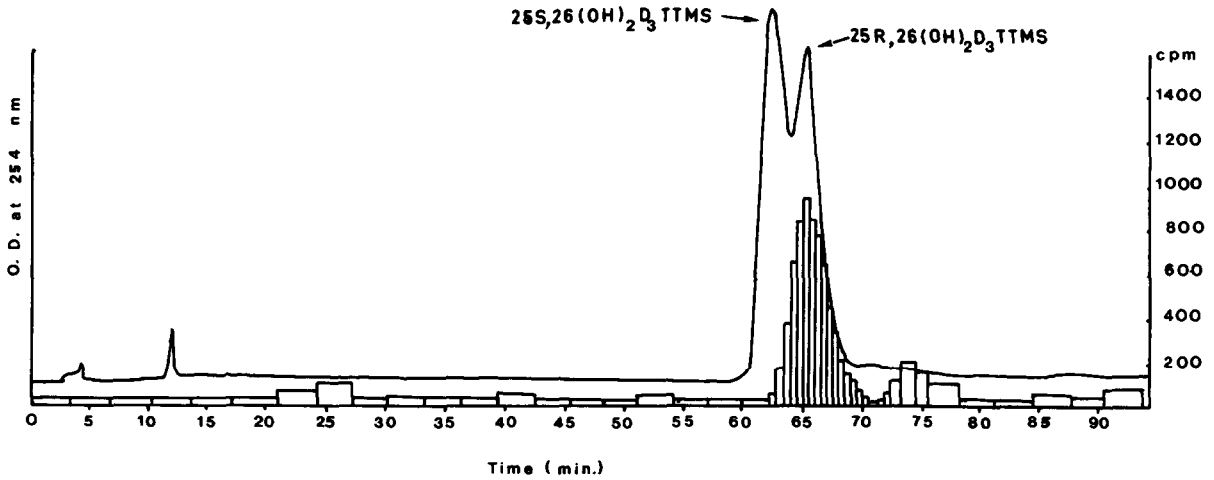

Fig.1. Separation of the Tris–TMS ethers of 25*R*,26- and 25*S*,26(OH)$_2$D$_3$ and co-chromatography of the Tris–TMS ether of radioactive biologically generated 25,26(OH)$_2$D$_3$ with the 25*R*,26(OH)$_2$D$_3$ derivative. The clear bars represent radioactivity in each fraction.

the derivative of 25,26$(OH)_2D_3$ of biological origin migrated with the 25*R*,26$(OH)_2D_3$ derivative.

It is of interest to note that another polar metabolite of vitamin D_3, 24,25$(OH)_2D_3$ has been recently [7] identified with 24*R*,25$(OH)_2D_3$. But while the natural 24*R*,25$(OH)_2D_3$ was superior to the synthetic 24*S* epimer in elevating serum phosphorus and in the calcification of bone in rachitic rats [7], the natural 25*R*,26$(OH)_2D_3$ is surprisingly less effective [3] than 25*S*,26$(OH)_2D_3$ in this biological system. The natural 25,26$(OH)_2D_3$ isolated from porcine plasma has been found [5] inactive in the cure of rickets in rats.

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