

THE ABSOLUTE CONFIGURATION OF THE NATURAL 25,26-DIHYDROXYCHOLECALCIFEROL

J. REDEL, N. BAZELY, Y. TANAKA[†] and H. F. DELUCA[†]

Institut de Rhumatologie, Centre de Recherches sur les Maladies Ostéo-Articulaires (U. 5 INSERM, ERA 337 CNRS), Hôpital Cochin, 27, rue du Faubourg Saint-Jacques, 75674 Paris Cédex 14, France and [†]Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin-Madison, WI 53706, USA

Received 12 July 1978

1. Introduction

Two of us have reported [1,2] the synthesis of the metabolite of vitamin D₃ 25,26-dihydroxycholecalciferol (25,26(OH)₂D₃), 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)₂D₃ 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)₂D₃ with 25*R*,26(OH)₂D₃. 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)₂D₃.

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 mobile phase was *n*-hexane containing 2% (v/v) dichloromethane at a flow rate of 1 ml/min and a pressure of 42 kg/cm².

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)₂D₃

Vitamin D-deficient chicks were given an injection of 6.5 nmol vitamin D₃ 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-[23,24-³H]D₃ 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-D₃, 25-hydroxycholecalciferol; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; 25,26(OH)₂D₃, 25,26-dihydroxycholecalciferol; 24,25(OH)₂D₃, 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)₂D₃ from 1,25(OH)₂D₃ as in [9]. The 25,26(OH)₂D₃ was applied in total to a HPLC system equipped with a DuPont Zorbax-SIL column (4.6 mm × 25 cm) developed with a solvent system of 9% isopropanol in hexane at 7000 p.s.i. The purified 25,26(OH)₂D₃ 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)₂D₃

Mixture of synthetic [3] 25R,26- and 25S,26(OH)₂D₃ (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)₂D₃

In experiments with biologically generated 25,26(OH)₂-[23,24-³H]D₃, the radioactive material was added to the mixture of epimeric 25R,26- and

25S,26(OH)₂D₃ 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 25R,26- and 25S,26(OH)₂D₃ achieved by HPCL on high surface area silica. The retention time for the 25S epimer was 62 min while that for the 25R one was 65 min.

In experiments with biologically generated 25,26(OH)₂-[23,24-³H]D₃ the radioactivity migrates with the derivative of 25R,26(OH)₂D₃. Recovery for the radioactivity was 70%.

4. Discussion

Our results demonstrate clearly that the natural 25,26(OH)₂D₃ has a 25R configuration. This has been established first by preparing [3] synthetically the two C-25 epimers of 25,26(OH)₂D₃ 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

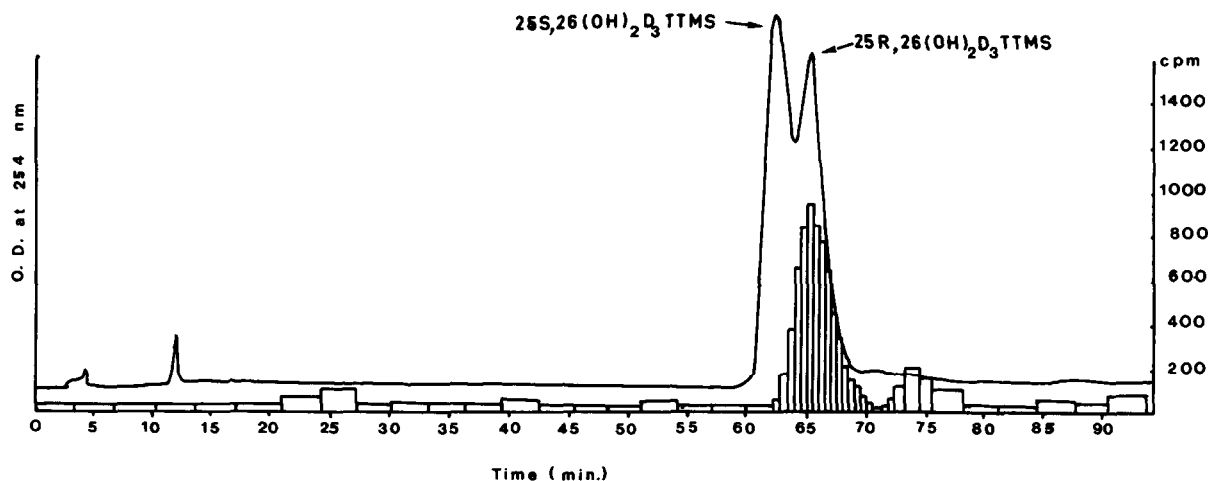


Fig.1. Separation of the Tris-TMS ethers of 25R,26- and 25S,26(OH)₂D₃ and co-chromatography of the Tris-TMS ether of radioactive biologically generated 25,26(OH)₂D₃ with the 25R,26(OH)₂D₃ derivative. The clear bars represent radioactivity in each fraction.

the derivative of $25,26(\text{OH})_2\text{D}_3$ of biological origin migrated with the $25R,26(\text{OH})_2\text{D}_3$ derivative.

It is of interest to note that another polar metabolite of vitamin D_3 , $24,25(\text{OH})_2\text{D}_3$ has been recently [7] identified with $24R,25(\text{OH})_2\text{D}_3$. But while the natural $24R,25(\text{OH})_2\text{D}_3$ was superior to the synthetic $24S$ epimer in elevating serum phosphorus and in the calcification of bone in rachitic rats [7], the natural $25R,26(\text{OH})_2\text{D}_3$ is surprisingly less effective [3] than $25S,26(\text{OH})_2\text{D}_3$ in this biological system. The natural $25,26(\text{OH})_2\text{D}_3$ isolated from porcine plasma has been found [5] inactive in the cure of rickets in rats.

References

- [1] Redel, J., Bell, P. A., Delbarre, F. and Kodicek, E. (1973) *Ct. Rd. Hebd. Seanc. Acad. Sci. Paris* 276 D, 2907–2910.
- [2] Redel, J., Bell, P. A., Bazely, N., Calando, Y., Delbarre, F. and Kodicek, E. (1974) *Steroids* 24, 463–475.
- [3] Redel, Y., Miravet, L., Bazely, N., Calando, Y., Carre, M. and Delbarre, F. (1977) *Ct. Rd. Hebd. Seanc. Acad. Sci. Paris* 285 D, 443–446.
- [4] Cesario, M., Guilhem, J., Pascard, C. and Redel, J. (1978) *Tetrahedron Lett.* 12, 1097–1098.
- [5] Suda, T., Deluca, H. F., Schnoes, H. K., Tanaka, Y. and Holick, M. F. (1970) *Biochemistry* 9, 4776–4780.
- [6] Tanaka, Y., Shepard, R. M., Deluca, H. F. and Schnoes, H. K. (1978) *Biochem. Biophys. Res. Commun* in press.
- [7] Tanaka, Y., Deluca, H. F., Ikekawa, N., Morisaki, M. and Koisumi, N. (1975) *Arch. Biochem. Biophys.* 170, 620–626.
- [8] Eisman, J. A., Hamstra, A. J., Kream, B. E. and Deluca, H. F. (1976) *Arch. Biochem. Biophys.* 176, 235–243.
- [9] Ribovich, M. L. and Deluca, H. F. (1978) *Arch. Biochem. Biophys.* 188, in press.