

THE CONFIGURATION AT C-25 OF HUMAN 25,26-DIHYDROXYCHOLECALCIFEROL

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1. Introduction

It is known that cholecalciferol (vitamin D₃) is first metabolized in the liver and the resulting 25-OH-D₃ is further hydroxylated in the kidney at either C-1, C-24 or C-26 position to give 1,25-, 24,25- and 25,26(OH)₂D₃, respectively. The latter metabolite, initially isolated [1] from hog's blood, has been reported [2] to stimulate selectively bone mineralization in vitamin D-deprived rats. Moreover, 25,26(OH)₂D₃ has been found to be a major dihydroxy-metabolite of cholecalciferol present in human plasma, apparently irrespective of vitamin D nutritional status and of serum calcium levels [3]. Its configuration at C-25, 25R or 25S, has remained unknown.

Recently, tritiated 25,26(OH)₂D₃ was generated enzymatically by chick renal homogenates and was shown [4] to be of 25R configuration by HPLco-C with synthetic C-25 epimers [5]. A similar identification of 25,26(OH)₂D₃ of human origin was highly desirable and depended upon the availability of a material with adequate radioactivity.

This report describes the preparation of tritiated human 25,26(OH)₂D₃ and its identity with synthetic 25R,26(OH)₂D₃ established by HPLco-C of their 3,25,26-Tris-TMS derivatives by a procedure described in [4].

Abbreviations: 25-OH-D₃, 25-hydroxycholecalciferol; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol; 24,25(OH)₂D₃, 24,25-dihydroxycholecalciferol; 25,26(OH)₂D₃, 25,26-dihydroxycholecalciferol; TMS, trimethylsilyl; HPL(co)-C, high pressure liquid (co-)chromatography

2. Experimental

2.1. Instruments

A Waters Associates HPLC Chromatograph with a 254 nm ultraviolet detector and a 30 cm × 4 mm i.d. column of μ Porasil was used. The mobile phase was *n*-hexane containing either 2.0% or 2.5% (v/v) dichloromethane at a 1 ml/min flow rate.

The radioactivity was detected 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 human radioactive 25,26(OH)₂D₃

Plasma (2.5 l) was obtained from a patient undergoing plasmapheresis for macroglobulinaemia. The patient had received 24 h previously an intravenous injection of 10 μ Ci [1,2-³H]vitamin D₃ (12.3 Ci/mmol, Radiochemical Centre, Amersham) in 1.0 ml ethanol, dispensed in 9.0 ml Intralipid (Vitrum, Stockholm). The nature of the investigation had been fully explained to the patient, who gave his informed consent to the procedure.

The plasma was extracted in aliquots using chloroform and methanol [6]; the extracts were combined and applied to columns of Sephadex LH-20 (1.4 × 50 cm) eluted with chloroform-*n*-hexane (13:7, v/v) [7]. The fractions eluted between 260 and 300 ml were collected, pooled and applied to a new column of the same type; the same fraction was collected. The residue from this fraction, which contained both 25,26(OH)₂D₃ and 1,25(OH)₂D₃, was further purified

by HPLC using a column of Spherisorb S 5W silica (0.45×10 cm, Anachem. Ltd), developed with a solvent mixture of dichloromethane–methanol (100:2, v/v) at a 1.3 ml/min flow rate. In this system 25,26(OH) $_2$ D $_3$ was eluted between 8 and 12 min and 1,25(OH) $_2$ D $_3$ between 12 and 16 min [8]. The purified 25,26(OH) $_2$ D $_3$ fraction from human plasma contained 6.7 nCi 3 H and was 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 $_3$

A mixture of synthetic [5] 25R,26- and 25S,26(OH) $_2$ D $_3$ (1.5 μ g each) was treated with trimethylsilylimidazol (4 μ l) in *n*-hexane (100 μ l) at 50°C for 30 min and left at room temperature for 48 h. The solution was then injected into HPL chromatograph.

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

In experiments with 25,26(OH) $_2$ -[1,2- 3 H]D $_3$ isolated from human plasma, the radioactive material was added to the mixture of epimeric 25R,26- and 25S,26(OH) $_2$ D $_3$ and treated as above. Fractions were

collected at 1 min intervals and the radioactivity determined in each fraction.

3. Results

Separation of the Tris-TMS derivatives of 25S,26- and 25R,26(OH) $_2$ D $_3$ was carried out by HPLC on high surface area silica with *n*-hexane containing either 2% or 2.5% dichloromethane. The retention time for the 25S and 25R epimers, respectively, was 91 and 95 min with the former, and 54 and 56 min with the latter, solvent mixtures. The resolution of epimers was better with 2% dichloromethane–*n*-hexane (fig.1B), but was distinctly apparent using 2.5% dichloromethane–*n*-hexane (fig.1A).

In experiments with human 25,26(OH) $_2$ -[1,2- 3 H]D $_3$ the radioactivity clearly migrated with the derivative of 25R,26(OH) $_2$ D $_3$ (the more polar peak) using both solvent systems (fig.1A,B). Recovery for the radioactivity was 71%.

4. Discussion and conclusion

The TMS-derivatives of human 25,26(OH) $_2$ -[1,2- 3 H]D $_3$ co-migrated by HPLC with the more polar C-25 epimer: 25R,26(OH) $_2$ D $_3$ -Tris-TMS in two

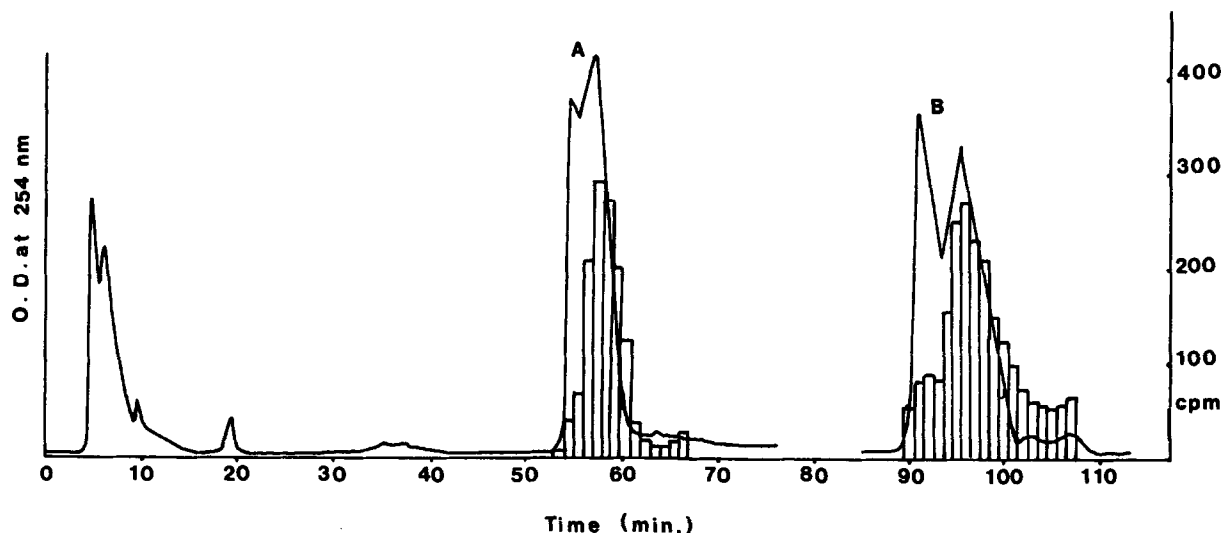


Fig.1. Separation of the Tris-TMS ethers of 25R,26- and 25S,26(OH) $_2$ D $_3$, and co-chromatography of the Tris-TMS ether of human radioactive 25,26(OH) $_2$ D $_3$ with the 25R,26(OH) $_2$ D $_3$ derivative (the more polar peak). Chromatography was performed with: (A) 2.5% dichloromethane–*n*-hexane; (B) 2% dichloromethane–*n*-hexane. The clear bars represent radioactivity in each fraction. OD = absorbance.

solvent systems: 2% dichloromethane-*n*-hexane and 2.5% dichloromethane-*n*-hexane. Since this identity was recorded with two mobile phases of various capacities, the retention time increasing by ~70% with the less polar solvent, an artefact due to isotopic separation of the tritiated compound by HPLC [9] appears unlikely.

We have shown conclusively, that 25,26(OH)₂D₃ isolated from human plasma and 25,26(OH)₂D₃ biosynthetically generated by chick renal homogenates are identical and have a 25*R* configuration.

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