STEREOCHEMICAL CONFIGURATION AT C-24 OF 24,25-DIHYDROXYCHOLECALCIFEROL OF HUMAN ORIGIN

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1. Introduction

Vitamin D_3 (cholecalciferol) is first hydroxylated in the liver to form 25-OH-D_3 before it is further metabolized in the kidney to either 1,25$(OH)_2D_3$ or to 24,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$, respectively. 24,25$(OH)_2D_3$ is the major dihydroxy form of cholecalciferol appearing in the plasma of normal rats [1]. It is also found in sizeable quantities in normal man and in much smaller amounts or not at all, in anephric subjects [2,3]. The stereochemical configuration of 24,25$(OH)_2D_3$ at C-24, 24*R* or 24*S*, remained unknown until the tritiated metabolite was enzymatically generated by chick renal homogenates and identified with synthetic 24*R*,25$(OH)_2D_3$ by HPLco-C of their 3,24, 25-Tris-TMS-derivatives [4].

Similar identification of 24,25$(OH)_2D_3$ from human origin was of great interest more especially since the determination [5] of the stereochemical configuration at C-25 of 25,26$(OH)_2D_3$ isolated from human plasma.

This paper reports the preparation of tritiated human 24,25$(OH)_2D_3$ and its identification with 24*R*,25$(OH)_2D_3$ in a straight-phase HPLco-C on silica of high surface area.

2. Experimental

2.1. *Instruments*

Analytical HPLC was effected with a Waters Associates chromatograph equipped with a 254 nm ultraviolet detector and a 30 cm × 4 mm i.d. column of μ Porasil. The mobile phase was dichloromethane containing 1% (v/v) methanol at a 1 ml/min flowrate.

The radioactivity was determined using a toluene counting solution containing 0.9% 2,5-diphenyloxazole and 0.02% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene and a liquid scintillation counter (Intertechnique model SL 40).

2.2. *Preparation and purification of human radioactive 24,25$(OH)_2D_3$*

Plasma (2.5 l) was obtained during plasmapheresis of a patient suffering from macroglobulinaemia. Between the 14th and 7th days before donating plasma, the patient received 900 IU (22.5 μg) calciferol daily; this was to ensure a state of vitamin D repletion, the condition necessary for formation of 24,25$(OH)_2D$ [6]. On the last day of this pretreatment, when the serum level of 25-OH-D was 16.8 ng/ml, the patient was given an intraveneous injection of 8 μCi 25-hydroxy-[26,27-^{3}H]cholecalciferol (12 Ci/mmol, Radiochemical Centre, Amersham) in 1.0 ml ethanol dispersed in 9.0 ml Intralipid (Vitrum, Stockholm). Plasma was collected 7 days later, at which time the concentration of 24,25$(OH)_2$ [^{3}H]D_3 is maximal [7]. The patient consented voluntarily to the procedure after a full explanation had been given.

The plasma was extracted in batches, with chloroform and methanol and was chromatographed on columns of Sephadex LH20 eluted with chloroform–*n*-hexane (13:7, v/v) [5]. Material eluting between 150–250 ml was collected, the batches were pooled and applied to a new column of the same type, from which the fraction eluted between 140–200 ml was

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

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retained. After evaporation of solvent this fraction was purified by HPLC using a column of Zorbax-SIL (Du Pont) with 12% isopropanol in *n*-hexane, at a 1.5 ml/min flowrate, as mobile phase. The fraction eluting between 9 and 10 min, corresponding to standard 24,25$(OH)_2D_3$, was collected; the HPLC step was repeated and this purified fraction used for identification by analytical HPLC (section 2.4).

2.3. *Resolution of a mixture of epimeric 24R, 25- and 24S,25(OH)$_2$D$_3$ by HPLC*

A mixture of synthetic [8] 24*R*,25$(OH)_2D_3$ (1.7 μg) and 24*S*,25$(OH)_2D_3$ (0.85 μg) was dissolved in dichloromethane containing 1% (v/v) methanol and injected into the chromatograph. Fig.1 illustrates the resolution of the epimers.

2.4. *Co-chromatography with radioactive human 24,25(OH)$_2$D$_3$*

In experiments with 24,25$(OH)_2$-[26,27-^{3}H]D_3 from human plasma, the radioactive material was added to the above-mentioned mixture of epimeric 24*R*,25- and 24*S*,25$(OH)_2D_3$ and submitted to HPLC. Fractions were collected at 1 min intervals, the solvent was evaporated, the counting solution was added and the radioactivity was determined in each fraction (fig.1).

3. Results and discussion

Resolution of 24*R*,25- and 24*S*,25$(OH)_2D_3$ was performed by HPLC on μ Porasil using a mobile phase 1% methanol–dichloromethane. The retention time was 42 min for the 24*R* epimer and 45 min for the 24*S* epimer (fig.1). Similar retention time was also recorded when injecting the epimers separately. It is noteworthy that as the free form the 24*R* epimer was less polar than the 24*S* epimer, while with the 3,24,25-Tris-TMS-derivatives the reverse sequence of elution has been observed [4] and confirmed by us.

In experiments with 24,25$(OH)_2$-[26,27-^{3}H]D_3 the radioactivity clearly migrated with the less polar 24*R*,25$(OH)_2D_3$ (fig.1). Recovery of the radioactivity was 74%.

Our experiments demonstrate that 24,25$(OH)_2D_3$ from human plasma and synthetic 24*R*,25$(OH)_2D_3$ comigrate by HPLC with a solvent system which clearly resolves C-24 epimers. This result establishes unequivocally that configuration of human 24, 25$(OH)_2D_3$ is 24*R* and is identical with that found for biosynthetically generated 24,25$(OH)_2D_3$ [4].

It should be emphasised that in the case of 25, 26$(OH)_2D_3$, another dihydroxy metabolite of vitamin D_3 found in man, the configuration at C-25 was determined [5] as 25*S* for material of both human and biosynthetic origin.

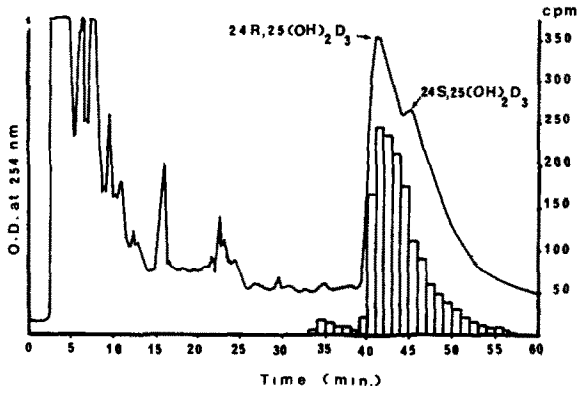

Fig.1. Separation of 24*R*,25- and 24*S*,25$(OH)_2D_3$ and co-chromatography of human radioactive 24,25$(OH)_2D_3$ with 24*R*, 25$(OH)_2D_3$. Chromatography was realized with 1% methanol–dichloromethane. The clear bars represent radioactivity of each fraction. O.D. = absorbance at 254 nm.

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