

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

J. REDEL, E. B. MAWER\*, J. T. HANN\* and N. BAZELY

*Institut de Rhumatologie, Centre de Recherches sur les Maladies Ostéo-Articulaires (U5 INSERM, ERA 337 CNRS),  
Hôpital cochin, 27, rue du Faubourg Saint-Jacques, 75674 Paris Cedex 14, France and \*Department of Medicine,  
University of Manchester, Medical School, Oxford Road, Manchester M 13 9PL, England*

Received 28 May 1980

### 1. Introduction

Vitamin D<sub>3</sub> (cholecalciferol) is first hydroxylated in the liver to form 25-OH-D<sub>3</sub> before it is further metabolized in the kidney to either 1,25(OH)<sub>2</sub>D<sub>3</sub> or to 24,25(OH)<sub>2</sub>D<sub>3</sub> and 25,26(OH)<sub>2</sub>D<sub>3</sub>, respectively. 24,25(OH)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> 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)<sub>2</sub>D<sub>3</sub> by HPLco-C of their 3,24,25-Tris-TMS-derivatives [4].

Similar identification of 24,25(OH)<sub>2</sub>D<sub>3</sub> from human origin was of great interest more especially since the determination [5] of the stereochemical configuration at C-25 of 25,26(OH)<sub>2</sub>D<sub>3</sub> isolated from human plasma.

This paper reports the preparation of tritiated human 24,25(OH)<sub>2</sub>D<sub>3</sub> and its identification with 24*R*,25(OH)<sub>2</sub>D<sub>3</sub> in a straight-phase HPLco-C on silica of high surface area.

### 2. Experimental

#### 2.1. Instruments

Analytical HPLC was effected with a Waters Asso-

*Abbreviations:* 25-OH-D<sub>3</sub>, 25-hydroxycholecalciferol; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxycholecalciferol; 24,25(OH)<sub>2</sub>D<sub>3</sub>, 24,25-dihydroxycholecalciferol; 25,26(OH)<sub>2</sub>D<sub>3</sub>, 25,26-dihydroxycholecalciferol; TMS, trimethylsilyl; HPL(co-)C, high pressure liquid (co-)chromatography

ciates chromatograph equipped with a 254 nm ultra-violet detector and a 30 cm × 4 mm i.d. column of  $\mu$  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)<sub>2</sub>D<sub>3</sub>

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  $\mu$ g) calciferol daily; this was to ensure a state of vitamin D repletion, the condition necessary for formation of 24,25(OH)<sub>2</sub>D [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 intravenous injection of 8  $\mu$ Ci 25-hydroxy-[26,27-<sup>3</sup>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)<sub>2</sub> [<sup>3</sup>H]D<sub>3</sub> 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

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)<sub>2</sub>D<sub>3</sub>, 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)<sub>2</sub>D<sub>3</sub> by HPLC

A mixture of synthetic [8] 24R,25(OH)<sub>2</sub>D<sub>3</sub> (1.7 μg) and 24S,25(OH)<sub>2</sub>D<sub>3</sub> (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)<sub>2</sub>D<sub>3</sub>

In experiments with 24,25(OH)<sub>2</sub>-[26,27-<sup>3</sup>H]D<sub>3</sub> from human plasma, the radioactive material was added to the above-mentioned mixture of epimeric 24R,25- and 24S,25(OH)<sub>2</sub>D<sub>3</sub> 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 24R,25- and 24S,25(OH)<sub>2</sub>D<sub>3</sub> was

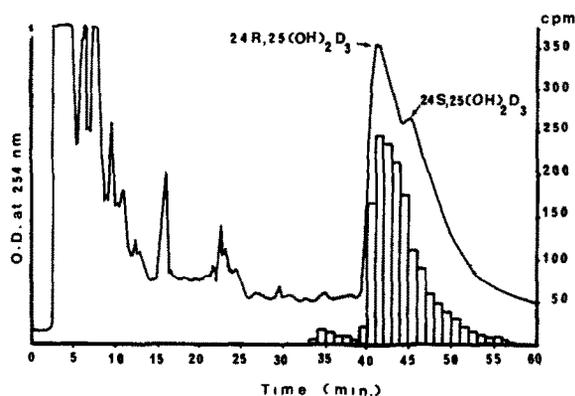


Fig.1. Separation of 24R,25- and 24S,25(OH)<sub>2</sub>D<sub>3</sub> and co-chromatography of human radioactive 24,25(OH)<sub>2</sub>D<sub>3</sub> with 24R,25(OH)<sub>2</sub>D<sub>3</sub>. Chromatography was realized with 1% methanol-dichloromethane. The clear bars represent radioactivity of each fraction. O.D. = absorbance at 254 nm.

performed by HPLC on μ Porasil using a mobile phase 1% methanol-dichloromethane. The retention time was 42 min for the 24R epimer and 45 min for the 24S epimer (fig.1). Similar retention time was also recorded when injecting the epimers separately. It is noteworthy that as the free form the 24R epimer was less polar than the 24S 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)<sub>2</sub>-[26,27-<sup>3</sup>H]D<sub>3</sub> the radioactivity clearly migrated with the less polar 24R,25(OH)<sub>2</sub>D<sub>3</sub> (fig.1). Recovery of the radioactivity was 74%.

Our experiments demonstrate that 24,25(OH)<sub>2</sub>D<sub>3</sub> from human plasma and synthetic 24R,25(OH)<sub>2</sub>D<sub>3</sub> comigrate by HPLC with a solvent system which clearly resolves C-24 epimers. This result establishes unequivocally that configuration of human 24,25(OH)<sub>2</sub>D<sub>3</sub> is 24R and is identical with that found for biosynthetically generated 24,25(OH)<sub>2</sub>D<sub>3</sub> [4].

It should be emphasised that in the case of 25,26(OH)<sub>2</sub>D<sub>3</sub>, another dihydroxy metabolite of vitamin D<sub>3</sub> found in man, the configuration at C-25 was determined [5] as 25S for material of both human and biosynthetic origin.

## Acknowledgements

A programme grant from the Medical Research Council to Professor S. W. Stanbury was used to support work performed in the Department of Medicine, University of Manchester. We should like to thank the Staff of the Department of Clinical Haematology, Manchester Royal Infirmary for preparation of the plasma, and Darrel Bayley and Joanne Howarth for their skilled technical assistance.

## References

- [1] Boyle, I. T., Gray, R. W. and DeLuca, H. F. (1971) Proc. Natl. Acad. Sci. USA 68, 2131-2134.
- [2] Horst, R. L., Shepard, R. M., Jorgensen, N. A. and DeLuca, H. F. (1979) J. Lab. Clin. Med. 93, 277-285.
- [3] Taylor, C. M., Mawer, E. B., Wallace, J. E., St. John, J., Cochran, M., Russel, R. G. G. and Kanis, J. A. (1978) Clin. Sci. Mol. Med. 55, 541-547.
- [4] Tanaka, Y., DeLuca, H. F., Ikekawa, N., Morisaki, M. and Koisumi, N. (1975) Arch. Biochem. Biophys. 170, 620-626.

- [5] Redel, J., Bazely, N., Mawer, E. B., Hann, J. and Jones, F. S. (1979) *FEBS Lett.* 106, 162–164 and correction (1980) *FEBS Lett.* 113, 345.
- [6] Mawer, E. B., Backhouse, J., Hill, L. F., Lumb, G. A., De Silva, P., Taylor, C. M. and Stanbury, S. W. (1975) *Clin. Sci. Mol. Med.* 48, 349–365.
- [7] Mawer, E. B. (1980) in: *Vitamin D: Molecular biology and clinical nutrition* (Norman, A. W. ed) pp. 149–196, Dekker, New York.
- [8] Redel, J., Bazely, N., Delbarre, F. and Calando, Y. (1976) *Ct. Rd. Hebd. Seanc. Acad. Sci. Paris* 283D, 857–860.