The stereochemical configuration of the natural 23,25,26-trihydroxyvitamin D_3

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Four possible diastereoisomers of 23,25,26-trihydroxyvitamin D_3 were synthesized and compared with the natural metabolite. The 4 synthetic diastereoisomers could be separated into 4 peaks by high-performance liquid chromatography. The natural 23,25,26-trihydroxyvitamin D_3 comigrated with 23(*S*),25(*R*),26-trihydroxyvitamin D_3. This result unequivocally demonstrates that the stereochemistry of the natural 23,25,26-trihydroxyvitamin D_3 has the 23(*S*) and 25(*R*) configuration.

23,25,26-Trihydroxyvitamin D_3 *25-Hydroxyvitamin D_3-26,23-lactone* *Vitamin D_3 metabolite* *Stereochemistry* *Co-chromatography* *Metabolic pathway*

1. INTRODUCTION

It is now well established that vitamin D_3 requires metabolic activation before it can mediate the biochemical events that result in increased calcium and phosphate absorption by the intestine and bone mineral mobilization [1]. The 25-hydroxylation of vitamin D_3 takes place in liver [2,3] and subsequently the further hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) to 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) [4–6], 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) [7–9], 25,26-dihydroxyvitamin D_3 (25,26$(OH)_2D_3$) [10], 23,25-dihydroxyvitamin D_3 (23,25$(OH)_2D_3$) [11–13], and 25-hydroxyvitamin D_3-26,23-lactone (25-OH-D_3-26,23-lactone) [14–16] takes place in kidney and elsewhere.

A new metabolite of vitamin D_3 has been isolated from the incubation mixtures after 25-OH-D_3 was incubated with 1α,25$(OH)_2D_3$ supplemented chick kidney homogenates and from the serum of rats given large doses of vitamin D_3 and identified as 23,25,26-trihydroxyvitamin D_3 (23,25,26$(OH)_3D_3$) [17]. Moreover, this metabolite is an intermediate in the biosynthesis of 25-OH-D_3-26,23-lactone [16,18]. Although 23,25,26-$(OH)_3D_3$ has 4 possible stereochemical configurations at C-23 and C-25 positions, the stereochemistry of the natural 23,25,26$(OH)_3D_3$ is still unknown.

We now report the definite determination of the stereochemical configuration of the natural 23,25,26$(OH)_3D_3$ to be 23(*S*),25(*R*),26$(OH)_3D_3$.

2. MATERIALS AND METHODS

2.1. *Syntheses of 4 stereoisomers of 23,25,26$(OH)_3D_3$*

Four stereoisomers of 25-OH-D_3-26,23-lactone were synthesized as in [19]. 23(*S*),25(*R*),26$(OH)_3D_3$ was synthesized as follows. To 100 μg 23(*S*),25(*R*)-25-OH-D_3-26,23-lactone in 1 ml absolute tetrahydrofuran was added 1 mg $LiAlH_4$. The

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Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 23,25$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 25,26$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25-OH-D_3-26,23-lactone, 25-hydroxyvitamin D_3-26,23-lactone; 23,25,26$(OH)_3D_3$, 23,25,26-trihydroxyvitamin D_3

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reaction was stirred at room temperature for 60 min and then 2 ml water was added to terminate the reaction. The products were extracted with 2 ml ethylacetate twice and then were chromatographed on high-performance liquid chromatography (HPLC) using a 4.6 × 250 mm Zorbax Sil column (Du Pont) eluted with 15% isopropanol in *n*-hexane at 1 ml/min. The 23(*S*),25(*R*),26-$(OH)_3D_3$ peak was eluted at a retention time of 19.1 min. In the same manner, 100 μg of 23(*R*),25(*S*)-25-OH-D_3-26,23-lactone, 23(*S*),25(*S*)-25-OH-D_3-26,23-lactone and 23(*R*),25(*R*)-25-OH-D_3-26,23-lactone each were reacted with 1 mg $LiAlH_4$. According to these procedures, the total amounts of synthesized compounds were as follows: 23(*S*),25(*R*),26$(OH)_3D_3$, 19.6 μg; 23(*R*),25(*S*),26$(OH)_3D_3$, 18.4 μg; 23(*S*),25(*S*),26$(OH)_3D_3$, 20.4 μg; 23(*R*),25(*R*),26$(OH)_3D_3$, 20.6 μg.

2.2. *Preparation and purification of 23,25,26-$(OH)_3D_3$ in rat serum*

Male weanling Wistar rats were fed a normal vitamin D_3-containing diet (Nippon Clea Corp. CE-2; Ca, 1.0%; P, 1.0%; vitamin D_3, 2000 IU/kg) for 8 weeks ad libitum. The 150 rats were then dosed intramuscularly, each with 4 × 10^5 IU vitamin D_3 in 100 μl ethanol, and after 3 days, they were again dosed intramuscularly, each with 4 × 10^5 IU vitamin D_3 in 100 μl ethanol. Three days after this dose, the rats were anesthetized with ether and their blood was withdrawn from the abdominal aorta. The serum (600 ml) was diluted with the same volume of water and extracted with 2 vol. in vitro of chloroform:methanol (1:1). The chloroform extract of serum was chromatographed on a Sephadex LH-20 column (1.5 × 25 cm) eluted with chloroform:*n*-hexane (65:35). The 23,25,26$(OH)_3D_3$ fraction was isolated and purified by HPLC equipped with a Zorbax Sil column (4.6 × 250 mm) eluted with 20% isopropanol in *n*-hexane as in [17].

2.3. *Preparation and purification of radioactive 23,25,26(OH)$_3$D$_3$* from in vitro incubation of chick kidney homogenates

One-day-old White Leghorn cockerels were fed a vitamin D-deficient diet [20] for 6 weeks, and then they were given orally 2 μg 1α,25$(OH)_2D_3$ daily for 10 days. The chicks were sacrificed, their kidneys were taken and 10% tissue homogenate in 0.25 M sucrose was prepared with the aid of a Potter-Elvehjem homogenizer fitted with a Teflon pestle. To 24 ml homogenate (13 mg protein/ml) in a 300 ml flask was added 56 ml reaction mixture containing 30 mM Tris–HCl (pH 7.4), 3.6 mM $MgCl_2$, 50 mM sucrose and 20 mM sodium succinate as in [21]. The incubation was initiated by addition of 30 μCi [26,27-*methyl*-^{3}H]25-OH-D_3 (9.7 Ci/mmol) in 0.5 ml ethanol. The incubation was carried out at 37°C for 60 min with shaking. Chloroform:methanol (1:1, v/v, 160 ml) was added to terminate the reaction. The metabolite 23,25,26$(OH)_3D_3$ was separated and purified by Sephadex LH-20 column chromatography and HPLC as in [17].

2.4. *Compounds*

We synthesised the 4 stereoisomers of 25-OH-D_3-26,23-lactone and 1α,25$(OH)_2D_3$ as in [19,22]. Vitamin D_3 was obtained from Sigma Chemicals (Chicago IL). [26,27-*methyl*-^{3}H]25-OH-D_3 (spec. act. 9.7 Ci/mmol) was purchased from Amersham (Bucks).

2.5. *Instruments*

HPLC was performed with a Hitachi Model 635A equipped with a 264 nm ultraviolet detector and a 4.6 × 250 mm Zorbax Sil column. The mobile phase was 15% isopropanol in *n*-hexane at 1 ml/min. The radioactivity was measured with a Packard Tri-Carb liquid scintillation counter Model 3255 using an external standard.

3. RESULTS

The resolution of 4 diastereoisomers of 23,25,26$(OH)_3D_3$ was performed by HPLC on a Zorbax Sil column using a mobile phase of 15% isopropanol in *n*-hexane. The retention time was 14.8 min for 23(*R*),25(*R*),26$(OH)_3D_3$, 16.0 min for 23(*R*),25(*S*),26$(OH)_3D_3$, 17.1 min for 23(*S*),25(*S*),26$(OH)_3D_3$ and 19.1 min for 23(*S*),25(*R*),26$(OH)_3D_3$, respectively (fig.1A). The same retention times were also recorded when injecting the 4 diastereoisomers separately. The 23,25,26$(OH)_3D_3$ isolated from the serum of rats clearly comigrated with the authentic 23(*S*),25(*R*),26$(OH)_3D_3$ (fig.1B). The [26,27-*methyl*-^{3}H]23,25,26$(OH)_3D_3$ generated by in vitro incubation of [26,27-*methyl*-

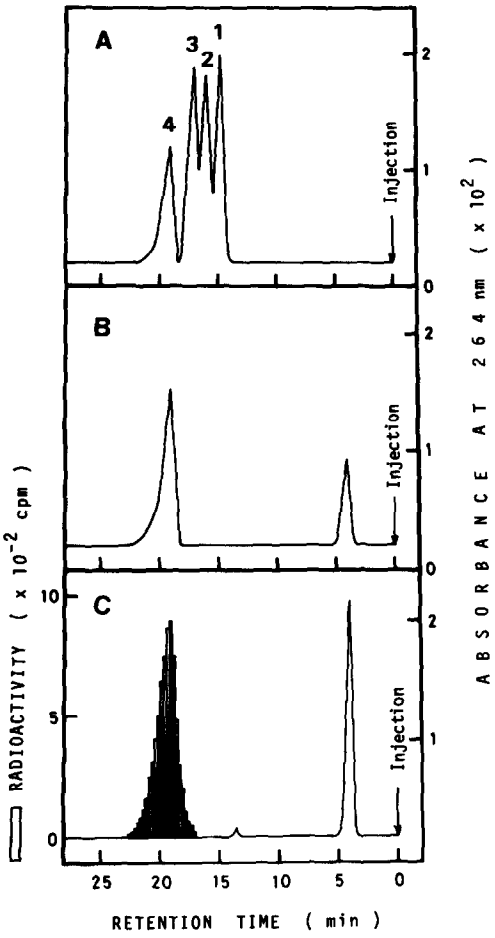

Fig.1. High-performance liquid chromatographic profiles of diastereoisomers of synthetic 23,25,26(OH)$_3$D$_3$ and natural 23,25,26(OH)$_3$D$_3$: (A) 4 diastereoisomers of synthetic 23,25,26(OH)$_3$D$_3$; (B) natural 23,25,26(OH)$_3$D$_3$ from rat serum; (C) natural [26,27-*methyl*-^{3}H]23,25,26(OH)$_3$D$_3$ generated by in vitro incubation of [26,27-*methyl*-^{3}H]25-OH-D$_3$ with 1α,25(OH)$_2$D$_3$-supplemented chick kidney homogenates. 23,25,26(OH)$_3$D$_3$ was subjected to high-performance liquid chromatography on a 4.6 × 250 mm Zorbax Sil column eluted with 15% isopropanol in *n*-hexane at 1 ml/min. Arabic numerals in (A) indicate elution positions of 4 isomers of authentic 23,25,26(OH)$_3$D$_3$: (1) 23(*R*),25(*R*),26(OH)$_3$D$_3$; (2) 23(*R*),25(*S*),26(OH)$_3$D$_3$; (3) 23(*S*),25(*S*),26(OH)$_3$D$_3$; (4) 23(*S*),25(*R*),26(OH)$_3$D$_3$. The bars in the figure represent the radioactivity of each fraction.

^{3}H]25-OH-D$_3$ with 1α,25(OH)$_2$D$_3$-supplemented chick kidney homogenates also comigrated with the authentic 23(*S*),25(*R*),26(OH)$_3$D$_3$ (fig.1C). Recovery for the radioactivity was 86%.

4. DISCUSSION

To prove unequivocally the absolute configuration of the natural 23,25,26(OH)$_3$D$_3$, we compared the HPLC elution times of the natural metabolite and the synthetic 4 diastereoisomers of 23,25,26(OH)$_3$D$_3$. Our data unquestionably demonstrate that the naturally-occurring stereoisomer of 23,25,26(OH)$_3$D$_3$ has the 23(*S*) and 25(*R*) configuration. These data are further substantiated in [16,18] where 23,25,26(OH)$_3$D$_3$ was metabolized to 23(*S*),25(*R*)-25-OH-D$_3$-26,23-lactone.

It is possible that the 23,25,26(OH)$_3$D$_3$ can arise from 23,25(OH)$_2$D$_3$ [11] or 25,26(OH)$_2$D$_3$ [23], a metabolite of vitamin D$_3$. The stereochemistry at the C-23 position of the natural 23,25(OH)$_2$D$_3$ was determined to be the 23(*S*) configuration [12,13], and 23(*S*),25(OH)$_2$D$_3$ is metabolized to 23,25,26(OH)$_3$D$_3$ [18]. On the other hand, 25,26(OH)$_2$D$_3$ possesses the 25(*S*) absolute configuration [24]. With 25(*S*),26(OH)$_2$D$_3$ as a precursor, stereochemical inversion of the hydroxyl group at the C-25 position must occur during the course of C-23 hydroxylation. Thus it might be more reasonable to consider that 23(*S*),25(*R*),26(OH)$_3$D$_3$ is produced from 23(*S*),25(OH)$_2$D$_3$.

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