FEBS Letters 448 (1999) 278–282 FEBS 21845

1α,25-Dihydroxy-3-epi-vitamin D_3: In vivo metabolite of 1α,25-dihydroxyvitamin D_3 in rats

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Received 1 November 1998; received in revised form 2 March 1999

Abstract We recently identified 1α,25-dihydroxy-3-epi-vitamin D_3 as a major in vitro metabolite of 1α,25-dihydroxyvitamin D_3, produced in primary cultures of neonatal human keratinocytes. We now report the isolation of 1α,25-dihydroxy-3-epi-vitamin D_3 from the serum of rats treated with pharmacological doses of 1α,25-dihydroxyvitamin D_3. 1α,25-dihydroxy-3-epi-vitamin D_3 was identified through its co-migration with synthetic 1α,25-dihydroxy-3-epi-vitamin D_3 on both straight and reverse phase high performance liquid chromatography systems and by mass spectrometry. Along with 1α,25-dihydroxy-3-epi-vitamin D_3, other previously known metabolites, namely, 1α,24(*R*),25-trihydroxyvitamin D_3, 1α,25-dihydroxy-24-oxo-vitamin D_3 and 1α,25-dihydroxyvitamin D_3-26,23-lactone, were also identified. Thus, our study for the first time provides direct evidence to indicate that 1α,25-dihydroxy-3-epi-vitamin D_3 is an in vivo metabolite of 1α,25-dihydroxyvitamin D_3 in rats.

Key words: Rat; In vivo metabolism; 1α,25-dihydroxyvitamin D_3; 1α,25-dihydroxy-3-epi-vitamin D_3; 1α,24(*R*),25-trihydroxyvitamin D_3; 1α,25-dihydroxyvitamin D_3-26,23-lactone

1. Introduction

It is now well established that the secosteroid hormone, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) is metabolized in its various target tissues, such as the kidney [1,2], intestine [3–5], keratinocytes [6,7], bone [8–14] and cartilage [8,16], through both C-24 and C-23 oxidation pathways. The C-24 oxidation pathway, initiated by C-24 hydroxylation, leads to the conversion of 1α,25$(OH)_2D_3$ into calcitroic acid [2,17], while the C-23 oxidation pathway, initiated by C-23 hydroxylation, leads to the conversion of 1α,25$(OH)_2D_3$ into calcitriol lactone [1,4,18,19]. It is now well accepted that the C-24 oxidation pathway plays an important role in the final inactivation of the hormone in its various target tissues [17]. The significance of the C-23 oxidation pathway remains to be elucidated. Recently, the production of 1α,25-dihydroxy-3-epi-vitamin D_3 (1α,25$(OH)_2$-3-epi-D_3), through the C-3 epimerization pathway as a novel metabolite of 1α,25$(OH)_2D_3$, was identified in human keratinocytes incubated with both pharmacological and physiological concentrations of 1α,25$(OH)_2D_3$ [20,21].

Even though 1α,25$(OH)_2$-3-epi-D_3 was identified as one of the major in vitro metabolites of 1α,25$(OH)_2D_3$, it still remains to be determined whether this novel in vitro metabolite is also a circulating metabolite in intact animals. Therefore, in our present study, we investigated the in vivo metabolism of 1α,25$(OH)_2D_3$ in rats. We successfully isolated a metabolite less polar than 1α,25$(OH)_2D_3$ in the pure form from the serum of rats treated with pharmacological doses of 1α,25$(OH)_2D_3$. This metabolite exhibited chromatographic and mass spectral properties identical to the synthetic standard of 1α,25$(OH)_2$-3-epi-D_3. Thus, for the first time, we provide evidence indicating that 1α,25$(OH)_2$-3-epi-D_3 is one of the in vivo metabolites of 1α,25$(OH)_2D_3$, detectable in rats treated with pharmacological doses of 1α,25$(OH)_2D_3$, and that the C-3 epimerization pathway is operative in vivo.

2. Materials and methods

2.1. Vitamin D compounds

Synthetic standards of 1α,25$(OH)_2D_3$, 1α,25$(OH)_2$-3-epi-D_3, 1α,24(*R*),25-trihydroxyvitamin D_3 (1α,24(*R*),25$(OH)_3D_3$) and 1α,25-dihydroxyvitamin D_3-26,23-lactone (1α,25$(OH)_2D_3$-lactone) were synthesized at Hoffmann-La Roche (Nutley, NJ, USA). 1β,25$(OH)_2D_3$ and 1β,25$(OH)_2$-3-epi-D_3 were synthesized using the procedure described previously [22]. The authentic standard of 1α,25-dihydroxy-24-oxo-vitamin D_3 (1α,25$(OH)_2$-24-oxo-D_3) was produced enzymatically using the isolated rat kidney perfusion system as described before [2]. All the vitamin D_3 compounds were quantified by UV spectroscopic analysis assuming an extinction coefficient (λ_{265} nm) of 18 300 dm^3/mol/cm. 1α,25$(OH)_2D_3$ was dissolved in ethanol and injected in vehicle consisting of 50% normal rat serum in a saline solution.

2.2. Rats

Male Sprague-Dawley rats (Taconic Laboratories, Germantown, NY, USA), weighing approximately 350 g, were used in all experiments, following adaptation to laboratory conditions for at least 5 days. They were housed two per cage with free access to food and water in a regulated environment, with a 12 h light-dark cycle.

2.3. In vivo metabolism of 1α,25(OH)$_2$D$_3$ in rats

In vivo metabolism studies were performed using two protocols. The first protocol was designed to find out the right time point to kill the rats in order to obtain the best information about the various circulating metabolites of 1α,25$(OH)_2D_3$. In this protocol, four rats were used. Each rat was given a bolus dose of 500 μg of 1α,25$(OH)_2D_3$ intravenously. The rats were killed at different time points (1, 2, 4 and 8 h) following the bolus dose. The blood was collected from each rat by catheterization of the aorta. The blood samples were immediately centrifuged to obtain 5–7 ml of serum from each rat. Based on the information obtained from the analysis of the metabolism data of the first protocol, the second protocol was designed. In this protocol, five rats were used. One rat was treated with vehicle solution alone, as the control. Each of the remaining four

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PII: S0014-5793(99)00377-4

rats received respectively, a single bolus dose of 50, 100, 250 or 500 μg of 1α,25(OH)$_2$D$_3$ intravenously. 4 h after dosing, each rat was killed and the blood, collected by catheterization of the aorta, was centrifuged immediately. About 5–7 ml of serum was obtained from each rat and the serum was stored at −20°C for future analysis.

2.4. Lipid extraction

Lipid extraction of serum was initiated by first adding two volumes of methanol to each serum sample. The protein precipitate was separated from the supernatant by centrifuging at 3000 rpm in a refrigerated centrifuge at 4°C for 15 min. The supernatant was mixed with four volumes of dichloromethane in a separatory funnel. The lower organic phase was collected and dried at 50°C under nitrogen gas. After reconstitution in 10% isopropanol/hexane, the lipid extract was directly subjected to analysis by high performance liquid chromatography (HPLC). In these experiments, we performed the lipid extraction of serum samples without an internal standard and therefore, the results should be considered semi-quantitative. However, in a separate experiment, we calculated the recovery of extraction of 1α,25(OH)$_2$D$_3$ from rat serum. The extraction efficiency was 74.6 ± 0.7% (mean ± S.D., $n = 5$), with an intra assay coefficient of variation of 1.0%. These results indicate that the efficiency of our lipid extraction procedure should not change significantly from sample to sample.

2.5. HPLC

HPLC analysis was performed with a Waters system controller (Millennium 32) equipped with a photodiode array detector (Model PDA 996) to monitor the UV absorption at 265 nm. A Zorbax SIL 9.4×250 mm column (Dupont, Wilmington, DE, USA) was used for all straight phase systems and a Zorbax ODS 4.6×250 mm column (Dupont, Wilmington, DE, USA) was used for the reverse phase system.

2.6. Isolation and identification of various metabolites of 1α,25(OH)$_2$D$_3$ from rat serum

The initial identification and quantification of all the in vivo metabolites were performed using the straight phase HPLC system I consisting of a Zorbax-SIL column eluted with 10% isopropanol/hexane at a flow rate of 2 ml/min. The data shown in Table 1 and Figs. 1 and 2 were generated using only the HPLC system I. For the final identification of the metabolites, the total HPLC eluates obtained from the analysis of all the individual serum samples were pooled together and re-subjected to the same straight phase HPLC system I. The HPLC fractions 20–28 min, 38–44 min and 51–62 min containing the putative 1α,25(OH)$_2$-3-epi-D$_3$ (retention time (R.T.) 22.7 min), 1α,24(*R*),25(OH)$_3$D$_3$ (R.T. 42.9 min) and 1α,25(OH)$_2$D$_3$-lactone (R.T. 59.3 min), respectively, were collected manually. In order to separate the 1α,25(OH)$_2$-3-epi-D$_3$ metabolite from the contaminant J1 and the substrate, fraction 20–28 min was further analyzed using a straight phase HPLC system consisting of 6% isopropanol/hexane at a flow rate of 2 ml/min (HPLC system II). The 1α,25(OH)$_2$-3-epi-D$_3$ metabolite was further purified on a reverse phase HPLC system using 20% water/methanol at a flow rate of 1 ml/min (HPLC system III). After purification, the putative 1α,25(OH)$_2$-3-epi-D$_3$ was identified by its co-elution with the synthetic standard of 1α,25(OH)$_2$-3-epi-D$_3$ using two different HPLC systems (HPLC systems I and III), (Table 2). The putative 1α,24(*R*),25(OH)$_3$D$_3$ was identified by its co-elution with the synthetic standard of 1α,24(*R*),25(OH)$_3$D$_3$ on a straight phase HPLC system using 6% isopropanol/dichloromethane at a flow rate of 2 ml/min (HPLC system IV). The putative 1α,25(OH)$_2$D$_3$-lactone was identified by its co-elution with the synthetic standard of 1α,25(OH)$_2$D$_3$-lactone on a straight phase HPLC system using 10% isopropanol/dichloromethane at a flow rate of 2 ml/min (HPLC system V). The final amounts of all the metabolites obtained in pure form for gas chromatography/mass spectrometry (GC/MS) analysis from 70 ml of serum are as follows: 1α,25(OH)$_2$-3-epi-D$_3$, 1 μg; 1α,24(*R*),25(OH)$_3$D$_3$, 2 μg and 1α,25(OH)$_2$D$_3$-lactone, 4 μg.

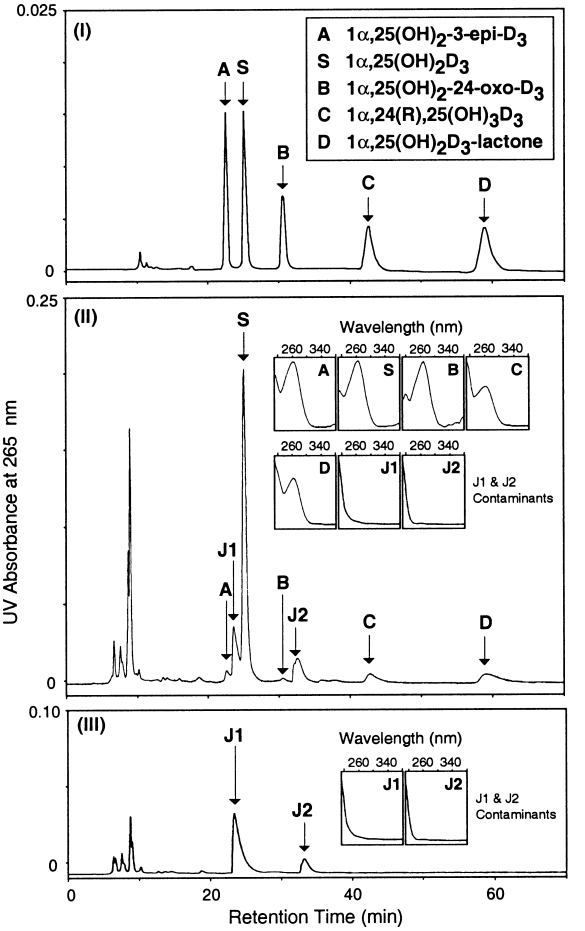

Fig. 1. The HPLC profile of various metabolites of 1α,25(OH)$_2$D$_3$ in rat serum. I: HPLC profile of synthetic standards of vitamin D$_3$. II: HPLC profile and UV spectra of the vitamin D$_3$ metabolites in the lipid extract of 7 ml of serum obtained from a rat which received 500 μg of 1α,25(OH)$_2$D$_3$ intravenous, 4 h prior to be killed. III: HPLC profile and UV spectra of the lipid contaminants in 7 ml serum obtained from the control rat, treated with only vehicle solution intravenous, 4 h prior to be killed. HPLC was performed using a Zorbax-SIL column (9.4×250 mm) eluted with 10% isopropanol/hexane at a flow rate of 2 ml/min.

2.7. GC/MS

Identification of all the metabolites of 1α,25(OH)$_2$D$_3$ was performed using a Hewlett-Packard GC-MSD system, composed of a

Table 1
The serum concentration of 1α,25(OH)$_2$D$_3$ and its metabolites following a single intravenous dose (500 μg) of 1α,25(OH)$_2$D$_3$, given to various rats, killed at different time points

	Concentration (ng/ml)			
	1 h	2 h	4 h	8 h
1α,25(OH)$_2$D$_3$	2232	1975	1745	515
1α,25(OH)$_2$-3-epi-D$_3$	10	18	34	15
1α,24(R),25(OH)$_3$D$_3$	12	34	152	48
1α,25(OH)$_2$-24-oxo-D$_3$	9	10	33	8
1α,25(OH)$_2$D$_3$-lactone	ND*	17	120	143

*ND, not detectable.

Table 2
Retention times of 1α,25(OH)$_2$D$_3$, its three diastereomers and peak A

	A-ring hydroxy orientation	Retention time (min) Straight phase HPLC	Reverse phase HPLC
1α,25(OH)$_2$D$_3$	1α, 3β	24.91	24.78
1β,25(OH)$_2$-3-epi-D$_3$	1β, 3α	25.01	24.88
1β,25(OH)$_2$-D$_3$	1β, 3β	22.87	26.49
1α,25(OH)$_2$-3-epi-D$_3$	1α, 3α	22.50	24.49
Peak A		22.57	24.26

5890 series II chromatograph, a 5971 mass selective detector and a 7673 GC autosampler. Synthetic standards and metabolites of 1α,25(OH)$_2$D$_3$ were dried down under nitrogen, reconstituted in 15 μl of acetonitrile and trimethylsilylated by the addition of 15 μl of Power SIL-Prep (Alltech Associates, Deerfield, IL, USA). The samples were incubated at 70°C for 15 min to ensure the complete derivatization. Derivatives were then transferred to autosampler vials and analyzed on a HP-5 capillary column (30 m×0.25 mm×0.25 mm, 5% phenyl methyl silicone), using UHP helium as a carrier gas. The oven temperature program was as follows: 150°C for 6 min, then increasing at 10°C/min until reaching a final temperature of 300°C which was held for 10 min. Full scan spectra were acquired over a mass range of m/z 50–650 and published spectra were averaged and background-subtracted.

3. Results

3.1. In vivo metabolites of 1α,25(OH)$_2$D$_3$ in rats

Fig. 1I illustrates the chromatographic profile of the not metabolized 1α,25(OH)$_2$D$_3$ (peak S) and four of its metabolites detected in 7 ml of serum obtained from a rat treated with 500 μg of 1α,25(OH)$_2$D$_3$. All of the four metabolites possessed the characteristic vitamin D *cis*-triene chromophore and the initial identification of each metabolite was achieved by HPLC via co-chromatography with known authentic standards (Fig. 1.II). The less polar metabolite eluting before the substrate (peak A) was identified as the putative 1α,25(OH)$_2$-3-epi-D$_3$, which had been previously observed in in vitro studies [3,7,15,20,24,25]. The more polar metabolites were identified as 1α,25(OH)$_2$-24-oxo-D$_3$ (peak B), 1α,24(*R*),25(OH)$_3$D$_3$ (peak C) and 1α,25(OH)$_2$D$_3$-lactone (peak D). None of the metabolites of 1α,25(OH)$_2$D$_3$ were detected in 7 ml of the serum from the rat treated with vehicle alone (Fig. 1.III). The serum from the control rat contained only peaks J1 and J2, which were considered to be contaminants based on their UV spectra. The contaminants did not exhibit the UV spectral characteristics which are typical to a vitamin D *cis*-triene chromophore. The final identification of all the afore-mentioned metabolites, with the exception of 1α,25(OH)$_2$-24-oxo-D$_3$, was obtained through GC/MS as described later. The results of the time course study are presented in Table 1. At 1 h, only 1α,25(OH)$_2$-3-epi-D$_3$, 1α,24(*R*),25(OH)$_3$D$_3$ and 1α,25(OH)$_2$-24-oxo-D$_3$ could be detected in the serum. The concentration of these metabolites reached a maximum at 4 h and started to decrease at 8 h. On the contrary, 1α,25(OH)$_2$D$_3$-lactone was detected at 2 h after dosing and its concentration was the highest at 8 h.

The results of the dose-response study are summarized in Fig. 2. The concentration of unmetabolized substrate, 1α,25(OH)$_2$D$_3$ (S) and the concentration of the metabolites 1α,25(OH)$_2$-3-epi-D$_3$ (A), 1α,24(*R*),25(OH)$_3$D$_3$ (C), 1α,25(OH)$_2$-24-oxo-D$_3$ (B) and 1α,25(OH)$_2$D$_3$-lactone (D), detected in the rats dosed with 100, 250 and 500 μg, are illustrated in II, III and IV, respectively. None of the above metabolites were detected by HPLC in the rat treated with 50 μg of 1α,25(OH)$_2$D$_3$ (Fig. 2.I). The concentration of 1α,25(OH)$_2$-3-epi-D$_3$ increased in a dose-dependent manner, as did the metabolites derived from the C-24 and C-23 oxidation pathways. At all doses, the relative concentrations of the metabolites at 4 h were as follows: 1α,25(OH)$_2$D$_3$-lactone > 1α,24(*R*),25(OH)$_3$D$_3$ > 1α,25(OH)$_2$-3-epi-D$_3$ > 1α,25(OH)$_2$-24-oxo-D$_3$.

3.2. Final identification of the in vivo metabolites of 1α,25(OH)$_2$D$_3$

All the metabolites isolated from the serum were purified using the various HPLC systems described in Section 2. The initial identification of the previously known metabolites (1α,24(*R*),25(OH)$_3$D$_3$, 1α,25(OH)$_2$-24-oxo-D$_3$ and 1α,25-

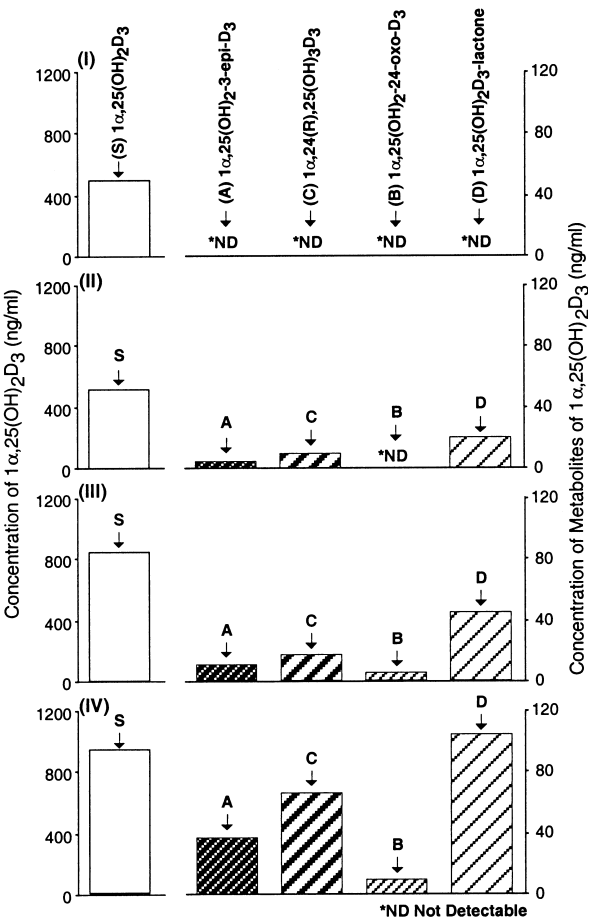

Fig. 2. The serum concentration of 1α,25(OH)$_2$D$_3$ and its metabolites in rats, 4 h after intravenous treatment with 50 μg (I), 100 μg (II), 250 μg (III) and 500 μg (IV) of 1α,25(OH)$_2$D$_3$. The amounts of 1α,25(OH)$_2$D$_3$ and its metabolites were quantitated using the HPLC system described in Fig. 1.

$(OH)_2D_3$-lactone) was achieved through their co-chromatography with the known synthetic standards on two different HPLC systems. The final identification of two of the metabolites (1α,24(*R*),25$(OH)_3D_3$ and 1α,25$(OH)_2D_3$-lactone) was also achieved by GC/MS (data not shown). The initial identification of the new metabolite obtained in pure form from peak A (putative 1α,25$(OH)_2$-3-epi-D_3) was achieved first through its co-chromatography with synthetic 1α,25$(OH)_2$-3-epi-D_3 on both straight and reverse phase HPLC systems. The retention times of peak A, the synthetic standards of 1α,25$(OH)_2D_3$ and its three diastereomers on both straight and reverse phase HPLC systems are given in Table 2. The final identification of peak A as 1α,25$(OH)_2$-3-epi-D_3 was achieved through GC/MS. GC/MS analysis of the trimethylsilylated standard, 1α,25$(OH)_2$-3-epi-D_3, yielded a major chromatographic peak at 25.32 min. The resulting mass spectrum, shown in Fig. 3I, exhibits many of the ion fragments typical to the dihydroxylated vitamin D_3 side-chain. By comparison, the trimethylsilyl derivative of 1α,25$(OH)_2D_3$ (the 1α,3β-diastereomer) elutes at a retention time of 23.94 min (data not shown) and exhibits noticeably lower relative intensities for the m/z 217 and m/z 501 ion fragments. Analysis of peak A, isolated from rat serum, yielded a retention time of 25.31 min and a mass spectrum (Fig. 3.II) which is effectively identical to that of 1α,25$(OH)_2$-3-epi-D^3 (Fig. 3.I). Thus, based on both the GC retention time data and the mass spectral analysis, the identity of the metabolite in peak A isolated from rat serum is unequivocally confirmed as 1α,25$(OH)_2$-3-epi-D_3.

4. Discussion

The identification of 1α,25$(OH)_2$-3-epi-D_3 as a novel in vitro metabolite of 1α,25$(OH)_2D_3$ produced in primary cultures of human keratinocytes [20] led us to perform the present study. In rats treated with 100–500 μg of 1α,25$(OH)_2D_3$, we detected a metabolite which is less polar than 1α,25$(OH)_2D_3$ on a straight phase HPLC system. The metabolite co-migrated with the synthetic standard of 1α,25$(OH)_2$-3-epi-D_3 on both straight and reverse phase HPLC systems and its final unequivocal identification was achieved by GC/MS. The concentration of 1α,25$(OH)_2$-3-epi-D_3 increased in a dose-dependent manner. Along with 1α,25$(OH)_2$-3-epi-D_3, we also isolated from the serum other previously known metabolites, namely, 1α,24(*R*),25$(OH)_3D_3$, 1α,25$(OH)_2$-24-oxo-D_3 and 1α,25$(OH)_2D_3$-lactone. Earlier, in vivo studies by Ishizuka et al. [23] in dogs treated with a total of 3 mg of 1α,25$(OH)_2D_3$, also identified 1α,24(*R*),25$(OH)_3D_3$, 1α,25$(OH)_2$-24-oxo-D_3 and 1α,25$(OH)_2D_3$-lactone as the circulating metabolites of 1α,25$(OH)_2D_3$. Our time course study results showed that the 1α,25$(OH)_2$-3-epi-D_3 production reached a maximum at 4 h and decreased at 8 h, suggesting that 1α,25$(OH)_2$-3-epi-D_3 was further metabolized. Results from kidney perfusion experiments with 1α,25$(OH)_2$-3-epi-D_3 revealed that 1α,25$(OH)_2$-3-epi-D_3 is also metabolized through the C-24 oxidation pathway (Reddy, G.S. et al., unpublished observation), which would account for the decrease in 1α,25$(OH)_2$-3-epi-D_3 concentration at 8 h.

Since the discovery of 1α,25$(OH)_2$-3-epi-D_3 as a major metabolite of 1α,25$(OH)_2D_3$ in primary cultures of keratinocytes [20], the in vitro production of 1α,25$(OH)_2$-3-epi-D_3 was also confirmed in several cell lines such as human colon adenocarcinoma cells (Caco-2 cells) [3], rat osteosarcoma cells (UMR 106 and Ros 17/2.8) [24,25] and primary cultures of bovine parathyroid cells [15]. Siu-Caldera, M.-L. et al. also observed that 1α,25$(OH)_2$-3-epi-D_3 was produced in ROS 17/2.8 cells which do not possess the C-24 oxidation pathway [26]. Thus, it appears that the metabolism of 1α,25$(OH)_2D_3$ into 1α,25$(OH)_2$-3-epi-D_3 can also occur in some tissues in which the C-24 oxidation pathway is absent. It is reported that the genomic activities (intestinal calcium transport and bone calcium mobilization) of 1α,25$(OH)_2$-3-epi-D_3 when compared to 1α,25$(OH)_2D_3$ are significantly reduced. Furthermore, the binding affinity of 1α,25$(OH)_2$-3-epi-D_3 to the vitamin D receptor is only 24% of that of 1α,25$(OH)_2D_3$ [25]. Therefore, the C-3 epimerization pathway, like the previously well established C-24 oxidation pathway, appears to play an important role in inactivating 1α,25$(OH)_2D_3$. Furthermore, C-3 epimerization might even become the alternate pathway for the inactivation of 1α,25$(OH)_2D_3$ in vitamin D target tissues, especially in which the C-24 oxidation pathway is absent.

In summary, the results of our study indicate for the first time that 1α,25$(OH)_2$-3-epi-D_3 is a circulating metabolite of 1α,25$(OH)_2D_3$ in rats treated with pharmacological doses of 1α,25$(OH)_2D_3$ and that the C-3 epimerization pathway is operative in vivo. The formation of 1α,25$(OH)_2$-3-epi-D_3 in these rats may play an important role in reducing the toxicity of 1α,25$(OH)_2D_3$.

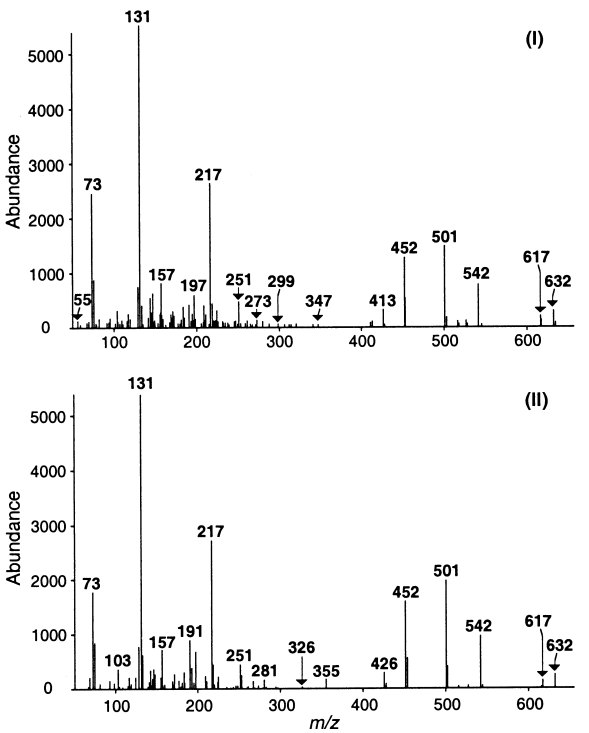

Fig. 3. Mass spectra of trimethylsilylated derivatives of (I) synthetic 1α,25$(OH)_2$-3-epi-D_3, GC retention time 25.32 min. (II) Peak A obtained from rat serum, GC retention time 25.31 min.

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