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Isolation and identification of 4,25-dihydroxyvitamin D_2: a novel A-ring hydroxylated metabolite of vitamin D_2

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Abstract

Vitamin D_2 is less toxic in rats when compared to vitamin D_3. Our laboratory has been involved in research projects which were directed towards identifying the possible mechanisms responsible for the toxicity differences between vitamins D_2 and D_3 in rats. The present research project was designed to isolate and identify new metabolites of vitamin D_2 from serum of rats which were fed toxic doses of vitamin D_2. Hypervitaminosis D_2 was induced in 30 rats by feeding each rat with 1000 nmol of vitamin D_2/day × 14 days. The rats were sacrificed on the 15th day and obtained 180 ml of serum. The lipid extract of the serum was directly analyzed by a straight phase HPLC system. The various vitamin D_2 metabolites were monitored by their ultraviolet (UV) absorbance at 254 nm. One of the UV absorbing peaks did not comigrate with any of the known vitamin D_2 metabolites. This unknown metabolite peak was further purified by HPLC and was then subjected to UV absorption spectrophotometry and mass spectrometry. The structure assignment of the new metabolite was established to be 4,25-dihydroxyvitamin D_2 [4,25$(OH)_2D_2$] by the techniques of UV absorption spectrophotometry and mass spectrometry and by the new metabolite's susceptibility to sodium metaperiodate oxidation. At present the biological activity of this unique 'A-ring' hydroxylated vitamin D_2 metabolite is not known. As this new metabolite is isolated from the serum of rats intoxicated with vitamin D_2, we speculate that 4,25$(OH)_2D_2$ may be playing an important role in the deactivation of vitamin D_2.

1. Introduction

Vitamin D_3 (cholecalciferol) is the form of vitamin D that is synthesized by vertebrates, whereas vitamin D_2 (ergocalciferol) is the major naturally occurring form of the vitamin D in plants [1]. It has been known that there are differences between both forms of the vitamins in terms of their toxicity in mammalian species [2–7]. However, the reasons for the lesser toxicity of vitamin D_2 are not fully understood. The presence of a double bond at C-22 and an extra methyl group at C-24 pos-

Abbreviations: 1α(OH)D_2, 1α-hydroxyvitamin D_2; 2α(OH)D_2, 2α-hydroxyvitamin D_2; 24OHD_2, 24-hydroxyvitamin D_2; 25OHD_2, 25-hydroxyvitamin D_2; 1α,24$(OH)_2D_2$, 1α,24-dihydroxyvitamin D_2; 1α,25$(OH)_2D_2$, 1α,25-dihydroxyvitamin D_2; 1α,25$(OH)_2$-3-epi-D_3, 1α,25-dihydroxy-3-epi-vitamin D_3; 4,25$(OH)_2D_2$, 4,25-dihydroxyvitamin D_2; 2,25$(OH)_2D_2$, 2,25-dihydroxyvitamin D_2; 24(R),25$(OH)_2D_2$, 24(R),25-dihydroxyvitamin D_2; 24,26$(OH)_2D_2$, 24,26-dihydroxyvitamin D_2; 25,28$(OH)_2D_2$, 25,28-dihydroxyvitamin D_2; 24(R),25,26$(OH)_3D_2$, 24(R),25,26-trihydroxyvitamin D_2; 24(S),25,28$(OH)_3D_2$, 24(S),25,28-trihydroxyvitamin D_2.

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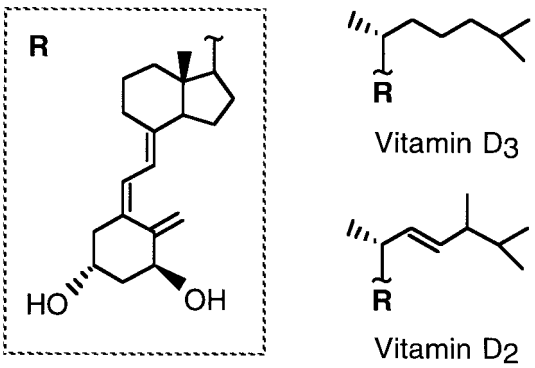

Fig. 1. Chemical structures of vitamins D_3 and D_2.

ition, of vitamin D_2 side chain (Fig. 1) is responsible for the differences from the oxidative processes known to occur on the side chain of vitamin D_3 [5,8]. As a result, several differences in the pathways of side chain metabolism of these two vitamins are noted.

The known metabolites of vitamin D_2 in mammals receiving dietary vitamin D_2 are depicted in Fig. 2. Vitamin D_2 is metabolized *in vivo* to both 25-hydroxyvitamin D_2 ($25OHD_2$) and 24-hydroxyvitamin D_2 ($24OHD_2$), the major circulating metabolites of vitamin D_2 in rats and humans [9–12]. Both $25OHD_2$ and $24OHD_2$ are subsequently hydroxylated at C-1 position to form 1α,25-dihydroxyvitamin D_2 [1α,25$(OH)_2D_2$] and 1α,24-dihydroxyvitamin D_2 [1α,24$(OH)_2D_2$] respectively [13,10]. Further 1α,25$(OH)_2D_2$ is metabolized into 1α,24(R),25-trihydroxyvitamin D_2 [1α,24(R),25$(OH)_3D_2$], 1α,24(R),25,26-tetrahydroxyvitamin D_2 [1α,24(R),25,26$(OH)_4D_2$] and 1α,24(S),25,28-tetrahydroxyvitamin D_2 [1α,24(S),25,28$(OH)_4D_2$] respectively [8] whereas, 1α,24$(OH)_2D_2$ is converted into 1α,24(S),26-trihydroxyvitamin D_2 [1α,24(S),26$(OH)_3D_2$] [14]. It is important to understand the deactivation pathways of $25OHD_2$ and $24OHD_2$ especially during hypervitaminosis D_2, a condition not uncommon in clinical medicine, as vitamin D_2 is used routinely as a therapeutic agent. The inactivation of $24OHD_2$ occurs through its conversion to 24(S),26-dihydroxyvitamin D_2 [24(S),26$(OH)_2D_2$] [15], and the inactivation of $25OHD_2$ occurs through its further metabolism into 24(R),25-dihydroxyvitamin D_2 [24(R),25$(OH)_2D_2$] [9,16]. We demonstrated that 24(R),25$(OH)_2D_2$ is further metabolized into 24(S),25,28-trihydroxyvitamin D_2 [24(S),25,28$(OH)_3D_2$] and 24(R),25,26-trihydroxyvitamin D_2 [24(R),25,26$(OH)_3D_2$] in the isolated perfused rat kidney and these two metabolites circulate in vitamin D_2-intoxicated rat, suggesting that the formation of the two trihydroxylated metabolites of vitamin D_2 plays an important role in the deactivation of $25OHD_2$ [11].

During the course of a pilot study designed to identify the circulating vitamin D_2 metabolites in a vitamin D_2-intoxicated rat using radiolabeled vitamin D_2 we found a major radiolabeled peak which did not comigrate with any of the known metabolites of vitamin D_2 on a straight phase HPLC system. The metabolite peak was more polar than the standard 24(R),25$(OH)_2D_2$ but less polar than the standard 1α,25$(OH)_2D_2$. It migrated just prior to the standard 25,28-dihydroxyvitamin D_2 [25,28$(OH)_2D_2$] [11]. We designed the present study to isolate and identify this unknown circulating vitamin D_2 metabolite from the serum of vitamin D_2-intoxicated rats. We identified this new vitamin D_2 metabolite as 4,25-dihydroxyvitamin D_2 [4,25$(OH)_2D_2$]. The isolation and identification of this new A-ring hydroxylated metabolite of vitamin D_2 is described in this paper.

2. Materials and methods

2.1. Vitamin D compounds

Vitamin D_2 was purchased from Sigma Chemical

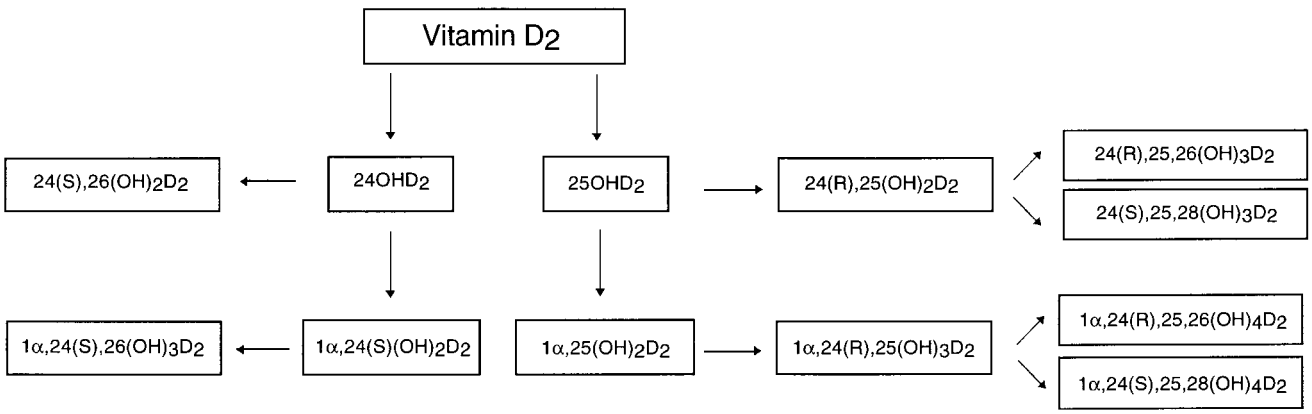

Fig. 2. Pathways of vitamin D_2 metabolism through side chain modification.

Co. (St. Louis, MO). $25OHD_2$ was a gift from Dr. J.A. Campbell and Dr. J. Babcock (Upjohn, Kalamazoo, MI). $24(R),25(OH)_2D_2$ was a gift from Dr. T. Kobayashi, Department of Hygienic Sciences, Kobe Pharmaceutical University, Kobe, Japan. All the various synthetic standards of vitamin D_2 metabolites used in this study were a gift from Hoffmann-La Roche, Nutley, NJ. Authentic $24OHD_2$ was isolated from the serum of vitamin D_2-intoxicated rats, and the structure of biologically produced $24OHD_2$ was verified by mass spectrometry as described before [9].

2.2. Solvents

All solvents were from Burdick and Jackson Laboratories, Muskegan, MI.

2.3. High performance liquid chromatography (HPLC) and mass spectrometry

Ultraviolet (UV) absorbance spectra were taken in 2-propanol with a Beckman DU 8 recording spectrophotometer. HPLC was performed with a Waters Model 600 equipped with a detector (Model 440) to monitor UV absorbing material at 254 nm (Waters Associates, Milford, MA). Mass spectra (70 eV) were obtained on a Hewlett-Packard 5985 B mass spectrometer. Sample of the metabolite (~0.5 μg) was introduced into the ion source maintained at 200°C via a direct insertion probe.

2.4. Animals

Male Sprague–Dawley rats (about 300 g) were purchased from Zivic-Miller Laboratories, Inc., Allison Park, PA, and placed in individual hanging wire cages. The rats were fed a vitamin D-deficient diet containing adequate calcium and phosphorus [17] for the duration of the experiment.

2.5. Study of in vivo metabolites of vitamin D_2 in vitamin D_2-intoxicated rats

This experiment was designed to study the circulating vitamin D_2 metabolites in hypervitaminosis. On the basis of the information given in a previous study by Shepard and DeLuca [18], we first estimated 1000 μg of vitamin D_2 as a safe total dose that can intoxicate a rat without causing death. After feeding vitamin D-deficient diet to the rats for 6 weeks, we noted that all the rats had undetectable circulating levels of $25OHD_3$ and $25OHD_2$, indicating that the rats were vitamin D-deficient. We then induced hypervitaminosis D_2 in these rats ($n = 30$) by administering 1000 nmol of vitamin D_2 (oral dose in 0.1 ml of Wesson oil) each day for a period of 14 days. At the end of the 15th day, for hypervitaminosis D_2, the serum calcium concentrations in rats were determined. As expected the rats were hypercalcemic, the serum calcium levels of the rats were found to be 13.1 ± 0.5 (mean $\pm$ SE), when compared to control rats, 10.3 ± 0.1. Twenty four hours following the final dose, all the rats were sacrificed by exsanguination and obtained 180 ml of serum. The various metabolites of vitamin D_2 in the lipid extract of the serum samples were analyzed and quantified by HPLC.

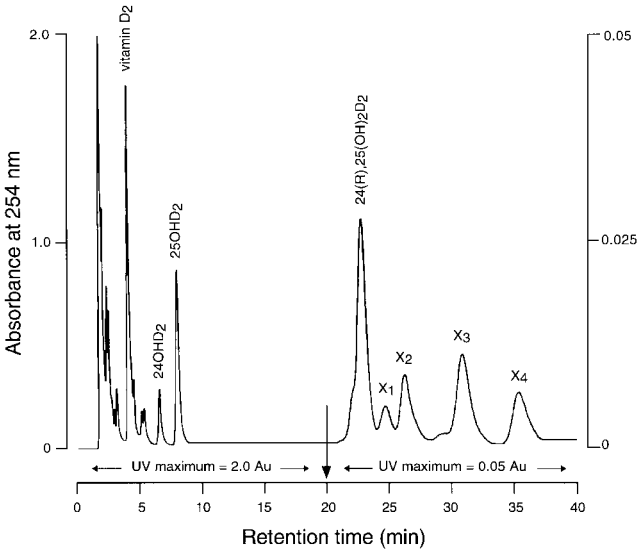

Fig. 3. HPLC profile of the various metabolites of vitamin D_2 obtained from the serum of 30 vitamin D_2-intoxicated rats. Each rat was given 1000 nmol of vitamin D_2/day for a period of 14 days. The lipid extract of the serum sample containing the various vitamin D_2 metabolites was analyzed by HPLC under the following chromatographic conditions: HPLC was performed on a Zorbax-SIL column (25 cm × 4.6 mm) eluted with hexane–2-propanol (97.5:2.5) at a flow rate of 2 ml/min. The various metabolites of vitamin D_2 were identified by monitoring their UV absorbance at 254 nm. The peaks eluting within the first 40 ml elution volume were monitored at UV maximum 2.0 and the next 40 ml were monitored at UV maximum 0.05. Peak X_1, X_2, X_3 and X_4 represent unknown peaks. Peaks X_1, X_2 and X_3 were not identified in this study.

2.6. Lipid extraction

Lipid extraction of the serum sample was performed according to the procedure of Bligh and Dyer [19], except that methylene chloride was substituted for chloroform.

2.7. Isolation and purification of the various metabolites of vitamin D_2 from serum samples for their structure identification

The lipid extract obtained from 180 ml of serum, was divided into 9 portions. Each lipid portion was subjected directly to HPLC under the same chromatographic conditions described in the legend for Fig. 3.

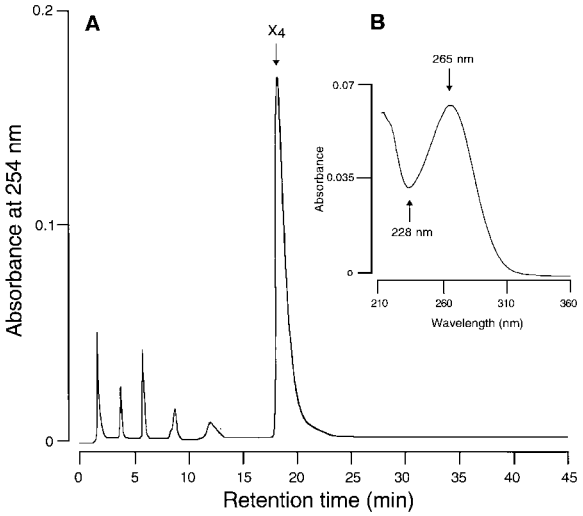

Fig. 4. HPLC profile of the unknown metabolite (peak X_4) obtained from the serum of vitamin D_2-intoxicated rats re-analyzed using hexane–methylene chloride mixture (99:1) as the solvent system (panel A). HPLC was performed under the same chromatographic conditions described in the legend for Fig. 2. Panel B: UV absorption spectrum of the unknown metabolite of vitamin D_2 exhibiting a UV maximum at 265 nm and a UV minimum at 228 nm, a characteristic for D vitamins.

We traced the various metabolites of vitamin D_2 by monitoring their UV absorbance at 254 nm.

We have found a peak (represented as X_4) in the region corresponding to synthetic standard $25,28(OH)_2D_2$ in the first HPLC run (Fig. 3). Therefore, we collected the fractions eluting between 34 and 37 min during the first HPLC run and subjected to a second HPLC system with the same Zorbax-SIL column (25 cm × 4.6 mm) eluted with hexane–2-propanol mixture (98.25:1.75) at a flow rate of 2 ml/min. Further, the UV peak of the unknown metabolite of vitamin D_2 (peak X_4) obtained from the second HPLC run was subjected to a third HPLC run using hexane–methylene chloride mixture (99:1) as the solvent system. Finally, the UV peak of the unknown vitamin D_2 metabolite (peak X_4) obtained from the third HPLC run (Fig. 4, panel A) was further purified with a fourth HPLC system eluted with hexane–2-propanol (90:10). At this point, the purity of the UV peak was tested by obtaining the UV spectra. The UV absorbing material from the unknown peak, exhibited a UV spectrum that is characteristic for all D vitamins (Fig. 4, panel B). The vitamin D_2 metabolite obtained from peak X_4 was then subjected to mass spectrometry and sodium metaperiodate ($NaIO_4$) oxidation in order to identify its chemical structure.

2.8. $NaIO_4$ oxidation of the new metabolite of vitamin D_2

The susceptibility of the new metabolite of vitamin D_2 to $NaIO_4$ oxidation was tested in order to locate the exact position of the hydroxyl groups in the new metabolite. It is well known that $NaIO_4$ cleaves the bond between two carbons when each carbon bears a hydroxyl group or one carbon bears a hydroxyl group and the other bears a keto group. A total of 0.5 μg of new metabolite or $1\alpha,25(OH)_2D_2$ was dissolved in 15 μl of methanol and was allowed to react with 10 μl of 5% aqueous $NaIO_4$ for 5 min. After 5 min at 25°C, the reaction products were separately dried under nitrogen gas, and were subjected to HPLC under the same chromatographic conditions described in the legend for Fig. 6.

3. Results

3.1. Metabolites of vitamin D_2 isolated from the serum of vitamin D_2-intoxicated rats

Fig. 3 shows the HPLC profile of the parent substrate and the various metabolites produced in vitamin D_2-intoxicated rats. From Fig. 3 it becomes obvious that, vitamin D_2 is metabolized into several metabolites as represented by the UV absorbing peaks. The identities of the known metabolites of vitamin D_2 i.e. $24OHD_2$, $25OHD_2$ and $24(R),25(OH)_2D_2$ were confirmed by their comigration with the corresponding authentic cold standards on HPLC. In addition to the aforementioned metabolites, we have found four unknown peaks, represented as X_1, X_2, X_3 and X_4 (Fig. 3). Among these four peaks, peak X_4 was found in the region corresponding to the elution volume of synthetic standard $25,28(OH)_2D_2$ during the first HPLC run. We were interested in identifying this unkown vitamin D metabolite peak. The amounts of $24OHD_2$, $25OHD_2$, $24(R),25(OH)_2D_2$ and peak X_4 in the serum of vitamin D_2-intoxicated rats are in the ratio of about 5:15:3:1. We further purified peak X_4 using three different HPLC systems as described in Materials and methods. At this point, the purity of peak X_4 was adequate for its structure identification process.

3.2. Structure identification of the new metabolite of vitamin D_2 (peak X_4)

The correct structure assignment to the unknown metabolite of vitamin D_2 was achieved by the techniques of UV absorption spectrophotometry and mass spectrometry.

3.3. UV and mass spectral findings of the new metabolite and its $NaIO_4$ cleavage product

The new metabolite purified from the serum of vita-

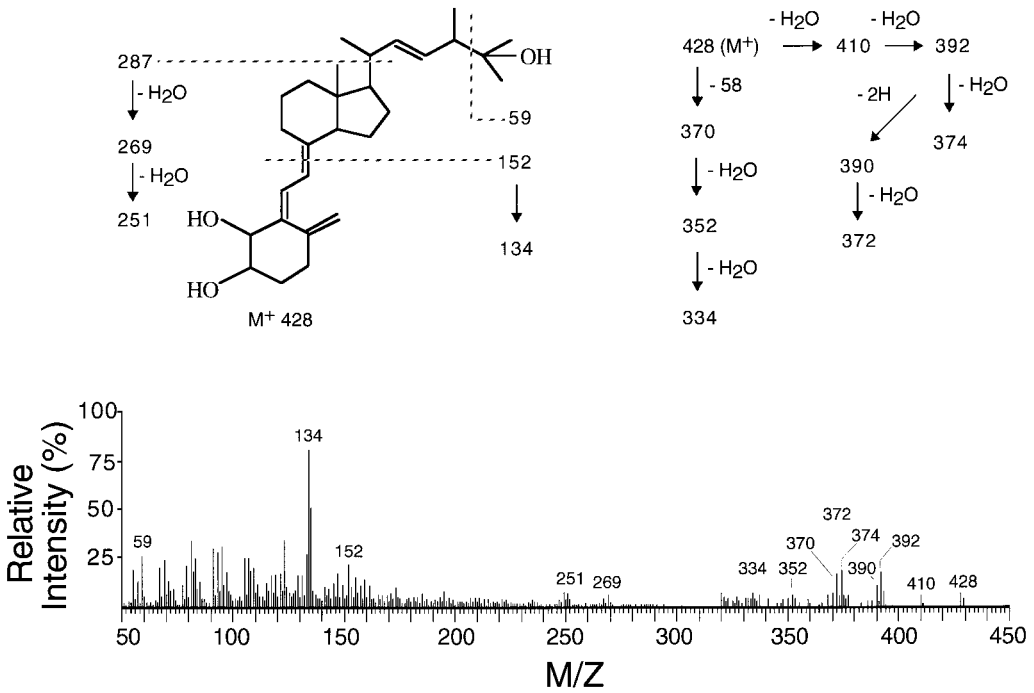

Fig. 5. Mass spectrum and proposed structure of the new metabolite of vitamin D_2, $4,25(OH)_2D_2$.

min D_2-intoxicated rats exhibited UV spectra with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm (Fig. 4, panel B). This finding indicated that the new metabolite contained an intact 5,6-*cis*-triene chromophore, characteristic of the D vitamins.

The mass spectrum of the new metabolite shown in Fig. 5 exhibited a molecular ion (M^+) at *m*/*z* 428, which indicates that two hydroxyl groups are added to the parent vitamin D_2. The prominent mass fragments at *m*/*z* 152 and 134, similar to that observed in all 1-hydroxylated vitamin D_2 and D_3 metabolites suggests that one of the hydroxyl group is located on the A-ring. The mass fragments at *m*/*z* 269 and 251, which are formed from the cleavage of bond between C-17 and C-20 with the subsequent losses of water molecules, are consistent with the location of the second hydroxyl group on the side chain. The fragment at *m*/*z* 59, which is formed from the cleavage of bond between C-24 and C-25, is typical of vitamin D compounds with a 25-hydroxyl group.

Even though the mass spectrum of this metabolite is similar to that derived from $1\alpha,25(OH)_2D_2$ [13], it is less polar when compared to synthetic standard $1\alpha,25(OH)_2D_2$ (Fig. 6) on a straight phase HPLC system. This finding indicates that the new vitamin D_2 metabolite is not hydroxylated at C-1 position, and leaves only possibility of hydroxylation at either C-2 or C-4. Further, this conclusion of a vicinal-diol structure was confirmed by the sensitivity of the metabolite to $NaIO_4$ oxidation (Fig. 6, panel C). The new metabolite was highly susceptible to $NaIO_4$ oxidation, and we noticed that during an incubation of 5 min this metabolite was almost completely converted to its corresponding periodate cleavage product (Fig. 6 panels B and C). As expected $1\alpha,25(OH)_2D_2$ was not susceptible to $NaIO_4$ oxidation (Fig. 6, panel A). The purified cleavage product was then collected and subjected to UV spectrophotometry and mass spectrometry for structure identification.

The first clue for assignment of the metabolite structure as $4,25(OH)_2D_2$ instead of 2,25-dihydroxyvitamin D_2 [$2,25(OH)_2D_2$] is obtained by the UV absorption spectrum of the periodate cleavage product of the metabolite. As shown in Fig. 6 (panel D), the UV absorption maximum of the cleavage product is shifted to 300 nm from the usual 265 nm, typical of a *cis*-triene structure of a vitamin D compound. This shift in absorption maximum indicates that an additional conjugation of double bond has been created by the reaction. Only the 4-hydroxyl group substitution in the A-ring would produce this additional double bond conjugation upon periodate cleavage. Both aldehyde groups derived from the cleavage of a 2-hydroxyl metabolite are not conjugated to the original triene structure. Fieser's rule on enone absorption [20] predicts that the addition of such an additional conjugated double bond would increase the absorption maximum of a *cis*-triene to about 290 to 300 nm.

The second clue for the assignment of the metabolite structure as $4,25(OH)_2D_2$ is obtained by the mass spectrum of the $NaIO_4$ cleavage product. The mass spec-

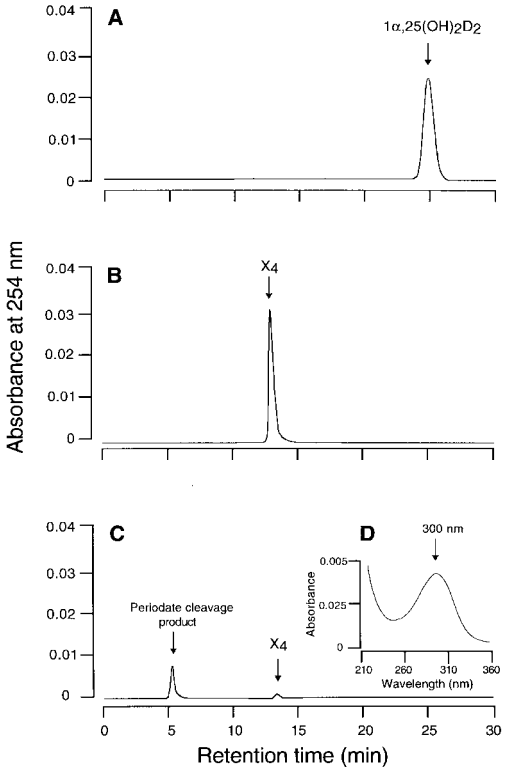

Fig. 6. HPLC analysis of the reaction product obtained by treating 0.5 μg of the new metabolite of vitamin D_2 or $1\alpha,25(OH)_2D_2$ with $NaIO_4$ for 5 min: new metabolite (panel C); $1\alpha,25(OH)_2D_2$ (panel A); new metabolite untreated with $NaIO_4$ (panel B). HPLC was performed on a Zorbax-SIL column (25 cm × 4.6 mm). The column was eluted with hexane–2-propanol (95:5) at a flow rate of 2 ml/min to elute the periodate cleavage product of the new metabolite and $1\alpha,25(OH)_2D_2$ and the untreated parent metabolites. The UV absorption spectrum of the $NaIO_4$ cleavage product of the new metabolite is shown in panel D.

trum of the $NaIO_4$ cleavage product (Fig. 7) exhibited a molecular ion at *m/z* 426, and is consistent with the cleavage of a cyclic vicinal diol to form a dialdehyde. Furthermore, the lack of the characteristic fragments at *m/z* 134 and 152 as in the precursor molecule confirmed that the A-ring structure has been altered. Either a 2-hydroxyl or a 4-hydroxyl group substitution would produce a dialdehyde consistent with these mass fragmentation and chemical properties. However, a significant mass fragment occurred at *m/z* 383 indicating that the precursor is most likely a 4-hydroxyl substitution. This mass fragment is formed from the loss of 43 neutral fragment from the molecular ion, *m/z* 426. This type of fragmentation is consistent with a β-cleavage in aldehyde compounds with the loss of CH_2CHO. A β-cleavage is more likely to occur in dialdehyde structure formed from 4-hydroxyl metabolite than that from the corresponding 2-hydroxyl metabolite. For the latter compound, the β-cleavage to lose 43 would involve the cleavage of a vinylic bond (a single bond immediately connected to a double bond), which is energetically unfavored. In addition, the mass fragment at *m/z* 368 is derived from the loss of mass fragment 58 from the molecular ion. This type of fragmentation is derived from the McLafferty Rearrangement involving a 25-hydroxyl group and the 22–23 double bond in vitamin D_2. This evidence further supports that the side chain hydroxyl group is at the C-25 position.

4. Discussion

We report the isolation and identification of $4,25(OH)_2D_2$ a novel A-ring hydroxylated metabolite of vitamin D_2 in vitamin D_2-intoxicated rats. Our finding of A-ring hydroxylation of $25OHD_2$ is not surprising. In a previous study, Thierry-Palmer et al. [21] demonstrated for the first time the possibility of A-ring hydroxylation of $25OHD_3$ by rat renal microsomes in vitro. This finding was based on the mass spectrometric analysis of the metabolite and its sensitivity to $NaIO_4$, The mass spectrum of the metabolite indicated that the metabolite is a dihydroxylated metabolite with one hydroxyl group at C-25 and the other in the A-ring. The sensitivity of the metabolite to $NaIO_4$ indicated that the hydroxyl group in the A-ring is vicinal to the 3-hydroxyl group. These findings together suggested that the metabolite has to be either $2,25(OH)_2D_3$ or $4,25(OH)_2D_3$. Unlike the study of Thierry-Palmer et al. [21], we were able to obtain both UV as well as mass spectra of the $NaIO_4$ cleavage product of the unknown metabolite. This allowed us to locate the exact position of the hydroxyl group in the A-ring of the metabolite and to assign a definite structure to the metabolite. Furthermore, our study also provided the new information to indicate that the A-ring hydroxylated metabolite is one of the major circulating metabolites in vitamin D_2-intoxicated rats.

The 'A-ring' hydroxylations are known to have a profound influence on the biological activity of the vitamin D compounds. For example, the binding affinity of synthetic $2,25(OH)_2D_3$ to chick intestinal cytosolic receptor was found to be less than 1/1000 when compared to $1\alpha,25(OH)_2D_3$. The ability of $2,25(OH)_2D_3$ to induce HL-60 cell differentiation (ATCC cells) was also reduced in comparison to $1\alpha,25(OH)_2D_3$ [22]. Further, synthetic 2α-hydroxyvitamin D_3 [$2\alpha(OH)D_3$] did not exhibit any significant biological activity as determined by its ability to induce intestinal calcium transport and bone calcium resorption in vivo in rats when compared to 1α-hydroxyvitamin D_3 [$1\alpha(OH)D_3$]. The biological activity of $2\alpha(OH)D_3$ was estimated to be less than 1/1000 times that of $1\alpha(OH)D_3$ [23]. It was also shown that vitamin D_3 itself was several times more potent than $2\alpha(OH)D_3$ in inducing intestinal calcium transport ac-

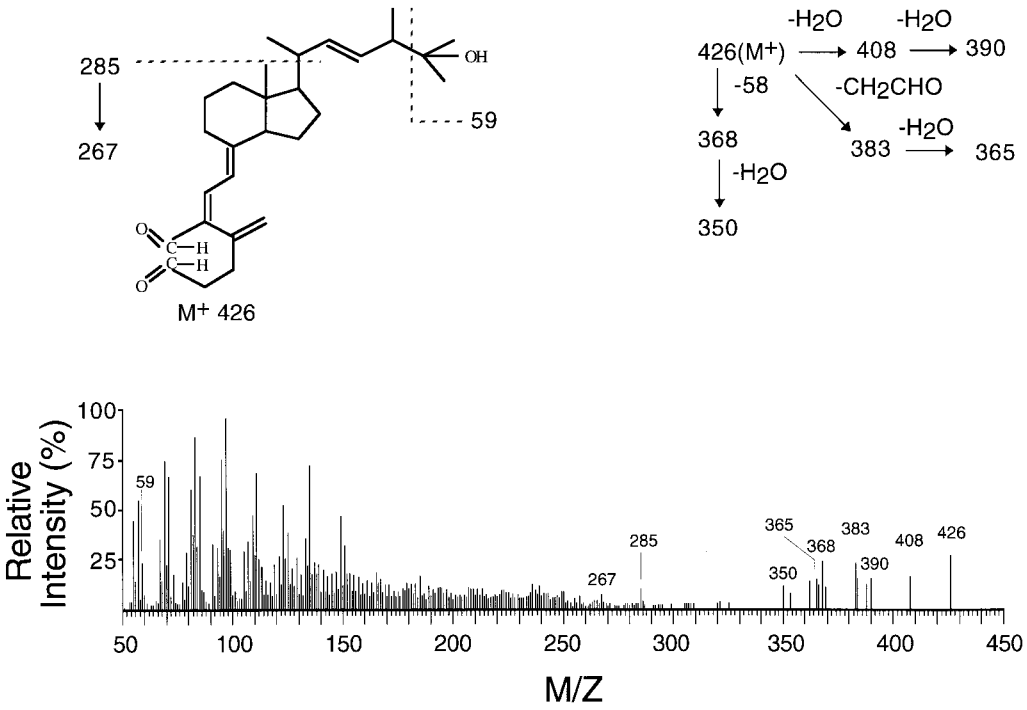

Fig. 7. Mass spectrum and proposed structure of the periodate cleavage product of the new metabolite of vitamin D_2.

tivity [23]. Thus, all these above findings suggest that the 2α-hydroxyl function leads to inactivation of the vitamin D compounds. At present no information is available with regard to the biological activity of vitamin D compounds with a hydroxyl group at C-4 position.

Up to now, it is known that the side chain metabolism through C-24 oxidation pathway is the only inactivation pathway for vitamin D_2 [24]. But now the isolation and identification of $4,25(OH)_2D_2$ as a major circulating metabolite in rats intoxicated with vitamin D_2 suggests that 'A-ring' hydroxylation may become an alternative pathway for the deactivation of vitamin D_2. Further, when we compared the amounts of the major side chain dihydroxylated metabolite of vitamin D_2 i.e. $24(R),25(OH)_2D_2$ with the A-ring hydroxylated metabolite in the circulation of vitamin D_2-intoxicated rats, we found that $4,25(OH)_2D_2$ was about 1/3 the concentration of $24(R),25(OH)_2D_2$ suggesting that 'A-ring' modification also appears to be a significant metabolic pathway for vitamin D_2. However, it remains to be determined whether C-4 hydroxylation of vitamin D_2 occurs directly or prior C-25 hydroxylation is essential for C-4 hydroxylation to occur. Also it will be of interest to determine whether C-4 hydroxylation of $1\alpha,25(OH)_2D_2$ occurs.

In conclusion, we have identified for the first time $4,25(OH)_2D_2$, a novel 'A-ring' hydroxylated vitamin D_2 metabolite, as one of the major circulating metabolite in vitamin D_2-intoxicated rats. As this new metabolite is isolated from the serum of rats intoxicated with vitamin D_2, we speculate that 'A-ring' hydroxylation may be playing an important role in the deactivation of vitamin D_2 compounds and protect the animal from the toxic effects of vitamin D_2.

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