Biochemical Pharmacology, Vol. 58, pp. 1965–1973, 1999.

ELSEVIER

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00293-2

Isolation and Identification of 1α-Hydroxy-24-oxovitamin D_3 and 1α,23-Dihydroxy-24-oxovitamin D_3

METABOLITES OF 1α,24(*R*)-DIHYDROXYVITAMIN D_3 PRODUCED IN RAT KIDNEY

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ABSTRACT. 1α,24(*R*)-Dihydroxyvitamin D_3 [1α,24(*R*)$(OH)_2D_3$], a synthetic vitamin D_3 analog, has been developed as a drug for topical use in the treatment of psoriasis. At present, the target tissue metabolism of 1α,24(*R*)$(OH)_2D_3$ is not understood completely. In our present study, we investigated the metabolism of 1α,24(*R*)$(OH)_2D_3$ in the isolated perfused rat kidney. The results indicated that 1α,24(*R*)$(OH)_2D_3$ is metabolized in rat kidney into several metabolites, of which 1α,24(*R*),25-trihydroxyvitamin D_3, 1α,25-dihydroxy-24-oxovitamin D_3, 1α,23(*S*),25-trihydroxy-24-oxovitamin D_3, and 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 are similar to the previously known metabolites of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$]. In addition to these aforementioned metabolites, we also identified two new metabolites, namely 1α-hydroxy-24-oxovitamin D_3 and 1α,23-dihydroxy-24-oxovitamin D_3. The two new metabolites do not possess the C-25 hydroxyl group. Thus, the metabolism of 1α,24(*R*)$(OH)_2D_3$ into both 25-hydroxylated and non-25-hydroxylated metabolites suggests that 1α,24(*R*)$(OH)_2D_3$ is metabolized in the rat kidney through two pathways. The first pathway is initiated by C-25 hydroxylation and proceeds further via the C-24 oxidation pathway. The second pathway directly proceeds via the C-24 oxidation pathway without prior hydroxylation at the C-25 position. Furthermore, we demonstrated that rat kidney did not convert 1α-hydroxyvitamin D_3 [1α$(OH)D_3$] into 1α,25$(OH)_2D_3$. This finding indicates that the rat kidney does not possess the classical vitamin D_3-25-hydroxylase (CYP27) activity. However, from our present study it is apparent that prior hydroxylation of 1α$(OH)D_3$ at the C-24 position in the '*R*' orientation allows 25-hydroxylation to occur. At present, the enzyme responsible for the C-25 hydroxylation of 1α,24(*R*)$(OH)_2D_3$ is unknown. Our observation that the side chain of 1α,24(*R*)$(OH)_2D_3$ underwent 24-ketonization and 23-hydroxylation even in the absence of the C-25 hydroxyl group suggests that 1α,25$(OH)_2D_3$-24-hydroxylase (CYP24) can perform some steps of the C-24 oxidation pathway without prior C-25 hydroxylation. Thus, we speculate that CYP24 may be playing a dual role in the metabolism of 1α,24(*R*)$(OH)_2D_3$. BIOCHEM PHARMACOL **58**;12:1965–1973, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. 1α,25$(OH)_2D_3$; 1α,24(*R*)$(OH)_2D_3$; hydroxylation; kidney; metabolism; vitamin D_3

The main function of the secosteroid hormone 1α,25$(OH)_2D_3$¶ is to maintain calcium homeostasis through its actions in the classical target tissues, namely, intestine, bone, and kidney. 1α,25$(OH)_2D_3$, like other steroid hormones, functions through its binding to a specific nuclear receptor, which in turn modulates the transcription of various genes [1, 2]. The presence of vitamin D receptor (VDR) in several non-classical target tissues suggests that the hormone has additional functions beyond maintaining calcium homeostasis [3]. The original finding of Abe *et al.* [4] demonstrating that 1α,25$(OH)_2D_3$ modulates the growth and differentiation of myeloid leukemia cells *in vitro* led to therapeutic trials of using 1α,25$(OH)_2D_3$ in the treatment of leukemia. However, the development of hypercalcemia as a complication prevented the use of 1α,25$(OH)_2D_3$ as an antileukemic drug. As a result, efforts have been made to synthesize noncalcemic analogs of 1α,25$(OH)_2D_3$ that have equal or higher potency in effects against cell growth and cell differentiation, but much lower calcemic effects [5–8]. Recent studies suggest that some of the newly synthesized noncalcemic analogs of

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¶ *Abbreviations*: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,24(*R*)$(OH)_2D_3$, 1α,24(*R*)-dihydroxyvitamin D_3; 1α,24(*R*),25$(OH)_3D_3$, 1α,24(*R*),25-trihydroxyvitamin D_3; 1α,25$(OH)_2$-24-oxo-D_3, 1α,25-dihydroxy-24-oxovitamin D_3; 1α,23(*S*),25$(OH)_3$-24-oxo-D_3, 1α,23(*S*),25-trihydroxy-24-oxovitamin D_3; 1α,23$(OH)_2$-24,25,26,27-tetranor-D_3, 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 or C-23 alcohol; 1α(OH)-24-oxo-D_3, 1α-hydroxy-24-oxovitamin D_3; 1α,23$(OH)_2$-24-oxo-D_3, 1α,23-dihydroxy-24-oxovitamin D_3; and 25$(OH)D_3$, 25-hydroxyvitamin D_3.

Received 26 February 1999; accepted 17 May 1999.

1α,25(OH)$_2$D$_3$ can be used as potential drugs in the treatment of various cancers including leukemia and other, non-cancerous, hyperproliferative disorders such as psoriasis [6, 9, 10].

1α,24(*R*)(OH)$_2$D$_3$, a synthetic analog of vitamin D$_3$, inhibits the growth of keratinocytes [11–13] and human leukemic cells [14] *in vitro* as effectively as 1α,25(OH)$_2$D$_3$. This *in vitro* activity of 1α,24(*R*)(OH)$_2$D$_3$ can be explained by the finding that both 1α,24(*R*)(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$ possess equivalent affinity for VDR [11, 12, 15]. However, 1α,24(*R*)(OH)$_2$D$_3$ induces less hypercalcemia than 1α,25(OH)$_2$D$_3$ [11]. Furthermore, the studies of Kato *et al*. [16] and Kragballe [17] indicated that 1α,24(*R*)(OH)$_2$D$_3$ is effective in the treatment of psoriasis. As a result, 1α,24(*R*)(OH)$_2$D$_3$ has been developed as a drug for topical use in the treatment of psoriasis. At present, the target tissue metabolism of this important vitamin D$_3$ analog is not understood completely. Therefore, we undertook the present study with the following aims: (i) to describe the metabolic pathway of 1α,24(*R*)(OH)$_2$D$_3$ in the kidney using isolated perfused rat kidney, and (ii) to identify the possible differences in the pathways of renal metabolism of 1α,24(*R*)(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$.

MATERIALS AND METHODS

Vitamin D Compounds

1α,25(OH)$_2$D$_3$, 24(*R*),25-dihydroxyvitamin D$_3$ [24(*R*),25(OH)$_2$D$_3$], and 25(OH)D$_3$ were synthesized at Hoffmann-La Roche. 1α,24(*R*)(OH)$_2$D$_3$ was synthesized at the Teijin Institute for Bio-Medical Research. 1α-Hydroxyvitamin D$_3$ [1α(OH)D$_3$] was a gift from Leo Pharmaceuticals. All the synthetic vitamin D compounds were found to be pure by HPLC analysis. All the known natural metabolites of 1α,25(OH)$_2$D$_3$, which include 1α,24(*R*),25(OH)$_3$D$_3$, 1α,25(OH)$_2$-24-oxo-D$_3$, 1α,23(*S*),25(OH)$_3$-24-oxo-D$_3$, 1α,23(OH)$_2$-24,25,26,27-tetranor-D$_3$ (C-23 alcohol), and 1α-hydroxy,23-carboxy-24,25,26,27-tetranorvitamin D$_3$ [1α,23COOH-24,25,26,27-tetranor-D$_3$ (C-23 acid)] were synthesized biologically in the rat kidney perfusion system as described previously [18, 19].

Solvents

All solvents were from Burdick & Jackson Laboratories

HPLC and Mass Spectrometry

HPLC was performed with a Waters System Controller (model 600E) equipped with a photodiode array detector (model PDA 990) to monitor UV absorbing material at 265 nm (Waters Associates). Mass spectra (70 eV) were obtained on a Hewlett Packard 5985B mass spectrometer. Samples of metabolites (~ 0.5 μg each) were introduced into the ion source maintained at 200° via a direct-insertion probe.

Animals

Male Sprague–Dawley rats (about 350 g), purchased from Taconic Laboratories, were fed a regular rodent diet, sufficient in calcium, phosphorus, and vitamin D. In our previous studies we demonstrated that the increase in activity of the enzymes involved in further metabolism of 1α,25(OH)$_2$D$_3$ can be induced in kidneys by pretreating rats with 1α,25(OH)$_2$D$_3$ [19, 20]. Therefore, in an analogous fashion in this study the rats were given an intracardiac injection of 1 μg of 1α,25(OH)$_2$D$_3$ in 50 μL of ethanol 6 hr prior to isolation of the kidney from the animal to increase the enzymatic activity required for further metabolism of both 1α,25(OH)$_2$D$_3$ and 1α,24(*R*)(OH)$_2$D$_3$.

Study of the Metabolism of 1α,24(R)(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$ Using the Technique of Kidney Perfusion

Kidney perfusions were performed as described before in detail [20]. Metabolism of 1α,24(*R*)(OH)$_2$D$_3$ was studied by introducing unlabeled 1α,24(*R*)(OH)$_2$D$_3$ (200 μg) into 100 mL of perfusate after a 5-min stabilization period, following isolation of the kidney. The perfusion was continued for 8 hr. The various further metabolites of 1α,24(*R*)(OH)$_2$D$_3$ and the remaining unmetabolized substrate in the lipid extract of a perfusate sample were analyzed and quantified by the technique of HPLC described later. Also, we performed another kidney perfusion using 1α,25(OH)$_2$D$_3$ (200 μg) as the substrate to identify the quantitative and qualitative differences between the metabolites formed from 1α,24(*R*)(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$. To monitor the viability of the kidney, we studied the metabolism of 24(*R*),25(OH)$_2$D$_3$ in the same kidneys that had been perfused earlier with either 1α,24(*R*)(OH)$_2$D$_3$ or 1α,25(OH)$_2$D$_3$. The details of this experiment are as follows: after the first 8-hr perfusion period, the same kidneys perfused earlier with either 1α,24(*R*)(OH)$_2$D$_3$ or 1α,25(OH)$_2$D$_3$ were washed with 200 mL of fresh perfusate, and then 100 mL of new perfusate was added to each perfusion system and the perfusions were continued for an additional 8-hr period. The second 8-hr kidney perfusions were performed by adding equal amounts of 24(*R*),25(OH)$_2$D$_3$ (200 μg) to both systems.

In this study, we also performed a control perfusion experiment in the absence of a kidney and demonstrated that there was no metabolism of 1α,24(*R*)(OH)$_2$D$_3$ in the absence of a kidney (data not shown).

Lipid Extraction

Lipid extraction of the kidney perfusate was performed according to the procedure of Bligh and Dyer [21], except that methylene chloride was substituted for chloroform.

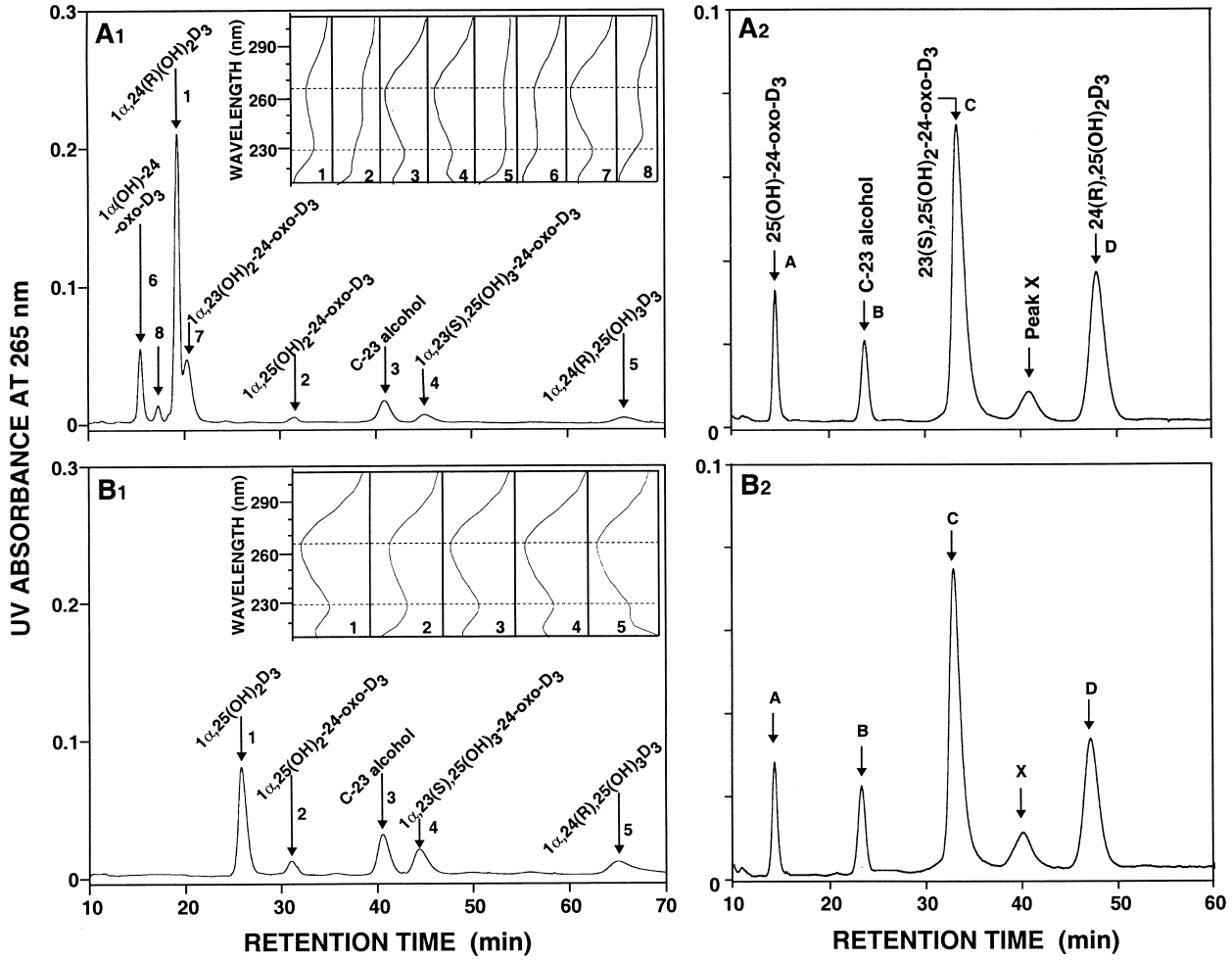

FIG. 1. HPLC profiles of lipid extracts of the perfusate samples (20 mL) obtained by perfusing a kidney for 8 hr with 200 μg of 1α,24(*R*)(OH)$_2$D$_3$ (A1) or 200 μg of 1α,25(OH)$_2$D$_3$ (B1) in 100 mL of perfusate. HPLC was performed using a Zorbax-SIL column (25 cm × 4.6 mm) eluted with hexane:2-propanol (94:6) at a flow rate of 2 mL/min. The various metabolites of 1α,24(*R*)(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$ were identified by monitoring their UV absorbance at 265 nm. The amounts of the various metabolites identified are given in Table 1. Panels A2 and B2: HPLC profiles of lipid extracts of the perfusate samples (20 mL) obtained by perfusing kidneys for 8 hr with 200 μg of 24(*R*),25(OH)$_2$D$_3$. The lipid extracts were subjected to HPLC under the same chromatographic conditions as described earlier. The kidneys were previously perfused for 8 hr with either 200 μg of 1α,24(*R*)(OH)$_2$D$_3$ (A2) or 200 μg of 1α,25(OH)$_2$D$_3$(B2) in 100 mL of perfusate.

Isolation and Purification of the Various Metabolites of 1α,24(R)(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_3$ Produced in Rat Kidney

Bulk lipid extract obtained from the final perfusate of ~100 mL, belonging to the kidney perfusion with 1α,24(*R*)(OH)$_2$D$_3$ as the substrate, was divided into five portions. Then each lipid portion belonging to 20 mL of perfusate was separately subjected directly to HPLC under the same chromatographic conditions described in the legend for Fig. 1 without overloading the column with lipid. Fractions of each individual metabolite from the first five HPLC runs were pooled and subjected to a second HPLC run with the same Zorbax-SIL column (25 cm × 4.6 mm) eluted with a methylene chloride:2-propanol (96:4) solvent mixture at a flow rate of 2 mL/min. Finally, each metabolite obtained from the second HPLC run was purified further with a third HPLC system eluted with hexane:2-propanol (93:7). At this point, the purity of each metabolite was adequate for its structure identification process. The final perfusate of about 100 mL belonging to the kidney perfusion with 1α,25(OH)$_2$D$_3$ as the substrate also was treated similarly as described above, and the metabolites of 1α,25(OH)$_2$D$_3$ were identified by their comigration with known authentic standards.

Chemical Modification of the Metabolites

SODIUM BOROHYDRIDE (NABH$_4$) REDUCTION OF 1α (OH)-24-OXO-D$_3$. 1α(OH)-24-oxo-D$_3$ (1 μg) was dissolved in 50 μL of ethanol containing 1 mg of NaBH$_4$. After 30 min at 25°, the reaction product was dried under nitrogen and dissolved in 2 mL of a methylene chloride:2-propanol

mixture (96:4). The sample was filtered through a syringe fitted with a Swinney filter holder containing a 0.45-μm Teflon filter (Millipore) and was concentrated under nitrogen to a volume of 100 μL. Then the sample was chromatographed on a Zorbax-SIL column eluted with methylene chloride:2-propanol (96:4) at a flow rate of 2 mL/min. The purified $NaBH_4$ reduction product eluting in the expected elution position of 1α,24(*R*)$(OH)_2D_3$ was then subjected to mass spectrometry.

SODIUM METAPERIODATE ($NAIO_4$) OXIDATION. The susceptibility of 1α,23$(OH)_2$-24-oxo-D_3 to $NaIO_4$ oxidation was tested. 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 1 μg of 1α,23$(OH)_2$-24-oxo-D_3 was dissolved in 15 μL of methanol and was allowed to react with 10 μL of 5% aqueous $NaIO_4$. After 30 min at 25°, the reaction product was dried under nitrogen gas and subjected to HPLC using a Zorbax-SIL column eluted with a methylene chloride:2-propanol (96:4) solvent system at a flow rate of 2 mL/min. The purified cleavage product was then subjected to mass spectrometry.

Study of 1α(OH)D_3 Metabolism by the Perfused Kidney

To study the metabolic conversion of 1α(OH)D_3 into 1α,25$(OH)_2D_3$, a kidney perfusion was performed with unlabeled 1α(OH)D_3 (1 μM) in 100 mL of the perfusate for a period of 4 hr. A control perfusion was performed in an identical fashion without the kidney in order to demonstrate the absence of metabolism of 1α(OH)D_3 in the perfusion apparatus in the absence of the kidney. This experiment was repeated thrice. Lipid extracts obtained from the perfusate were analyzed under the HPLC conditions as described in the legend for Fig. 3.

RESULTS

Metabolites of 1α,24(R)$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ Produced by the Perfused Rat Kidney

Figure 1 shows the HPLC profiles and UV spectra of substrate and metabolites produced in kidneys perfused with either 1α,24(*R*)$(OH)_2D_3$ (panel A1) or 1α,25$(OH)_2D_3$ (panel B1). From Fig. 1 (panel A1), it becomes obvious that 1α,24(*R*)$(OH)_2D_3$ was metabolized by the isolated perfused rat kidney into seven metabolite peaks, which exhibited UV spectra (maxima at 265 nm and minima at 228 nm) characteristic of an intact 5,6-*cis*-triene chromophore of vitamin D compounds. Metabolite peaks 2, 3, 4, and 5 (panel A1) comigrated with the known metabolites of 1α,25$(OH)_2D_3$, namely, 1α,25$(OH)_2$-24-oxo-D_3, C-23 alcohol, 1α,23(*S*),25$(OH)_3$-24-oxo-D_3, and 1α,24(*R*),25$(OH)_3D_3$ (peaks 2, 3, 4 and 5, panel B1), respectively. Furthermore, all the aforementioned metabolites of 1α,24(*R*)$(OH)_2D_3$ also were shown to comigrate with the corresponding known metabolites of 1α,25$(OH)_2D_3$ on a second HPLC system using a methylene chloride:2-propanol (96:4) mixture as the solvent system. The final identification of all the metabolites was obtained through mass spectrometry (data not shown). Thus, the last four metabolites of 1α,24(*R*)$(OH)_2D_3$ in Fig. 1 (panel A1, peaks 2, 3, 4, and 5) were proven to correspond to the known metabolites of 1α,25$(OH)_2D_3$ (panel B1, peaks 2, 3, 4, and 5).

In addition to the metabolites similar to the ones produced from 1α,25$(OH)_2D_3$, two new metabolite peaks of 1α,24(*R*)$(OH)_2D_3$ (Fig. 1, panel A1, peaks 6 and 7) were also obtained from the kidney perfusate. These two new metabolites required structural identification. A minor metabolite represented by peak 8 was not identified in this study.

Structural Identification of the New Metabolites of 1α,24(R)$(OH)_2D_3$

The correct structure assignment to the two new metabolites of 1α,24(*R*)$(OH)_2D_3$ was achieved by the techniques of UV absorption spectrometry and mass spectrometry and through specific chemical modifications of the metabolites.

UV SPECTRAL FINDINGS. The new metabolites purified from kidney perfusate exhibited UV spectra with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm. This finding indicated that the two new metabolites contained an intact 5,6-*cis*-triene chromophore, characteristic of the D vitamins.

MASS SPECTRAL FINDINGS. Figure 2 shows the mass spectra of both the new metabolites (panels B and C) and their parent, 1α,24(*R*)$(OH)_2D_3$ (panel A). The mass spectrum of 1α,24(*R*)$(OH)_2D_3$ yielded a molecular ion of *m/z* 416. All three vitamin D_3 compounds exhibited a peak at *m/z* 287, which is due to the side-chain cleavage from the rings structure (C-17/C-20 cleavage). The peaks at *m/z* 269 and 251 were a result of two sequential losses of water from the peak at *m/z* 287. The cleavage between carbons 7 and 8 resulted in the peak at *m/z* 152, which represents the A ring plus the carbon 6 and 7 fragment. Loss of water from the peak at *m/z* 152 resulted in the base peak at *m/z* 134. All the aforementioned fragment ions at *m/z* 287, 269, 251, 152, and 134 in both the new metabolites indicated that the secosteroid nucleus of their parent, 1α,24(*R*)$(OH)_2D_3$, had remained unchanged. Thus, the new metabolites of 1α,24(*R*)$(OH)_2D_3$ were formed as a result of changes occurring only on the side chain of their parent, 1α,24(*R*)$(OH)_2D_3$. A definite structure assignment of each new metabolite is as follows:

1α(OH)-24-oxo-D_3

The mass spectrum of the new metabolite yielded a molecular ion of *m/z* 414 (M^+) (Fig. 2B), which suggests that the new metabolite has a keto group on the side chain when compared with its parent, 1α,24(*R*)$(OH)_2D_3$ (Fig. 2A). As

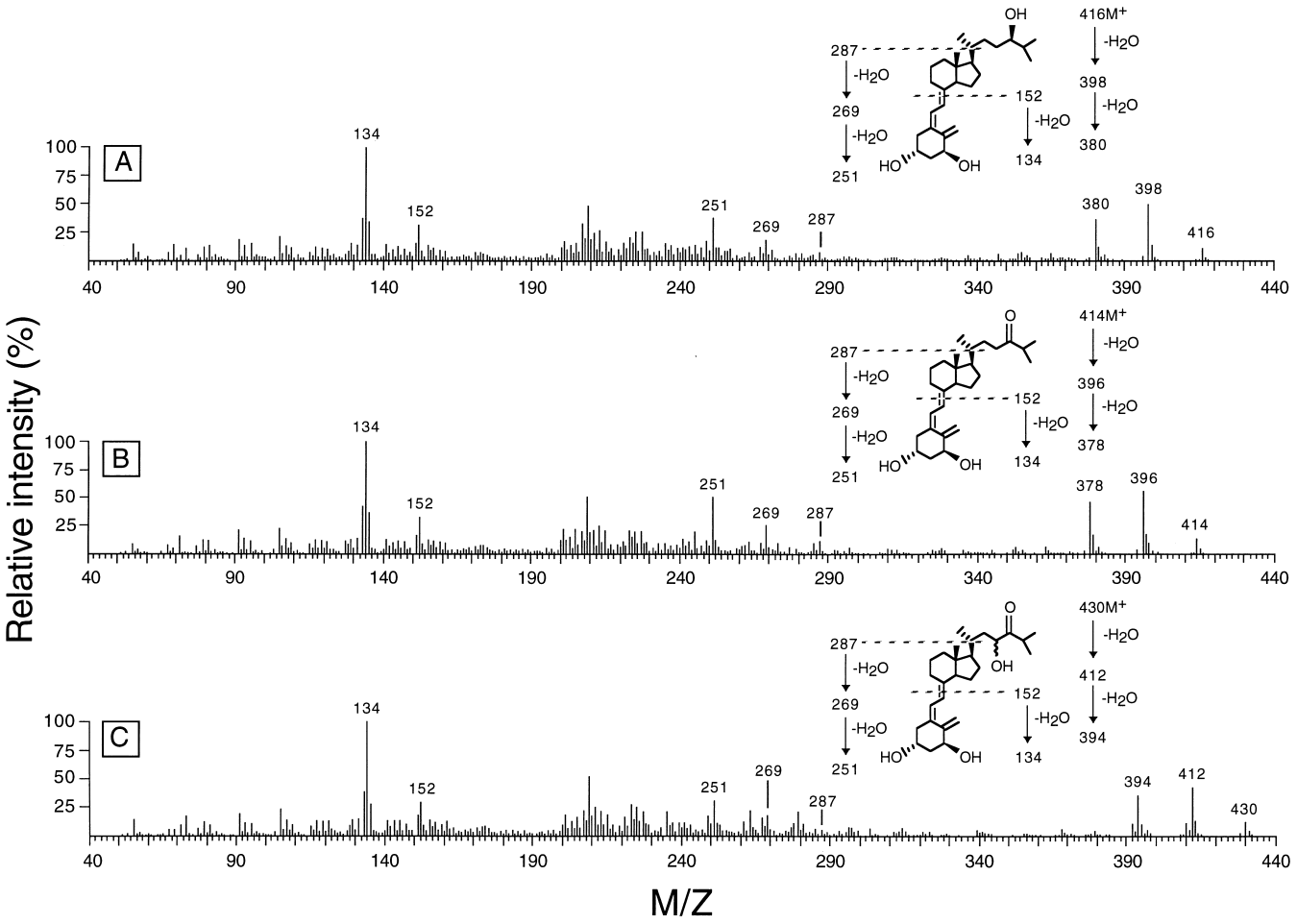

FIG. 2. Mass spectra of 1α,24(R)$(OH)_2D_3$ (A) and its two new metabolites, 1α(OH)-24-oxo-D_3 (B) and 1α,23$(OH)_2$-24-oxo-D_3 (C) produced in the rat kidney.

expected, borohydride reduction of the metabolite yielded the parent compound 1α,24(*R*)$(OH)_2D_3$, which exhibited a mass spectrum identical to that of 1α,24(*R*)$(OH)_2D_3$. The reduced product also co-chromatographed with authentic 1α,24(*R*)$(OH)_2D_3$ on HPLC (data not shown). From this evidence we concluded that the keto functionality was located on carbon 24. Thus, the new metabolite can be identified as 1α(OH)-24-oxo-D_3.

1α,23$(OH)_2$-24-oxo-D_3

The mass spectrum of the second new metabolite of 1α,24(*R*)$(OH)_2D_3$ showed a molecular ion at *m/z* 430 (M^+) (Fig. 2C), which suggested that two oxygen atoms and one degree of unsaturation were present in the side chain of the metabolite. When this metabolite was subjected to $NaIO_4$ cleavage, the product formed was identical to 1α-hydroxy-23-oxo-24,25,26,27-tetranorvitamin D_3 (C-23 aldehyde), which showed an identical mass spectrum and co-chromatographed with the authentic compound (data not shown). From the above data, we deduced that a hydroxyl group and a keto functionality were located on carbons 23 and 24, respectively. Thus, the structure of this new metabolite of 1α,24(*R*)$(OH)_2D_3$ is assigned as 1α,23$(OH)_2$-24-oxo-D_3.

Relative Amounts of the Unmetabolized Substrates 1α,24(R)$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ and their Metabolites Produced in the Isolated Perfused Kidney

The comparative study of the metabolism of 1α,24(*R*)$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ was performed using the data generated from HPLC analysis of the final perfusates (Fig. 1). The relative amounts of unmetabolized substrate and the metabolites of both 1α,24(*R*)$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ calculated from Fig. 1 are given in Table 1. The concentration of each metabolite was quantitated by measuring its peak area and comparing it with the corresponding peak area in a standard curve produced by 1 μg of each metabolite. The rate of metabolism of 1α,24(*R*)$(OH)_2D_3$ when compared with that of 1α,25$(OH)_2D_3$ appeared to be slow, as the amount of unmetabolized 1α,24(*R*)$(OH)_2D_3$ at the end of an 8-hr perfusion was about 1.7 times higher than that of 1α,25$(OH)_2D_3$.

TABLE 1. Metabolites of 1α,24(*R*)$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ present in the final kidney perfusate after 8 hr of kidney perfusion

Vitamin D metabolites	Kidney perfusion with 1α,24(R)$(OH)_2D_3$	Kidney perfusion with 1α,25$(OH)_2D_3$
Unmetabolized substrate	6.59	3.97
1α(OH)-24-oxo-D_3	1.54	
1α,23$(OH)_2$-24-oxo-D_3	2.17	
1α,24(*R*),25$(OH)_3D_3$	0.42	0.53
1α,25$(OH)_2$-24-oxo-D_3	0.07	0.43
1α,23(*S*),25$(OH)_3$-24-oxo-D_3	0.25	1.41
C-23 alcohol	1.13	2.00

Each value represents the amount of the metabolite (in μg) in 20 mL of lipid extract of the final kidney perfusate.

The concentrations of 1α(OH)-24-oxo-D_3 and 1α,23$(OH)_2$-24-oxo-D_3 were about 3.5 and 1.5 times higher than those of the corresponding 25-hydroxylated metabolites [1α,25$(OH)_2$-24-oxo-D_3 and 1α,23(*S*),25$(OH)_3$-24-oxo-D_3] produced from 1α,25$(OH)_2D_3$. Furthermore, the levels of 1α,24(*R*),25$(OH)_3D_3$, 1α,25$(OH)_2$-24-oxo-D_3, and 1α,23(*S*),25$(OH)_3$-24-oxo-D_3 formed from 1α,24(*R*)$(OH)_2D_3$ were quite low when compared to the corresponding metabolites generated from 1α,25$(OH)_2D_3$. These findings suggested that C-25 hydroxylation probably plays a minor role in the metabolism of 1α,24(*R*)$(OH)_2D_3$. We also noted that the amount of C-23 alcohol formed from 1α,25$(OH)_2D_3$ was around twice that formed from 1α,24(*R*)$(OH)_2D_3$. It is possible that the rate of conversion of 1α,23$(OH)_2$-24-oxo-D_3 into C-23 alcohol also may be slow compared with the rate of conversion of 1α,23(*S*),25$(OH)_3$-24-oxo-D_3 into C-23 alcohol. However, it still remains to be determined whether the side chain cleavage of 1α,23$(OH)_2$-24-oxo-D_3 can occur without prior C-25 hydroxylation (see Fig. 4).

Kidney Viability

The HPLC profiles of the substrate and the metabolites produced in kidneys perfused with 24(*R*),25$(OH)_2D_3$ are shown in Fig. 1 (panels A2 and B2). The concentrations of the 24(*R*),25$(OH)_2D_3$ metabolites [25-hydroxy-24-oxovitamin D_3, 23(*S*),25-dihydroxy-24-oxovitamin D_3, and 23-hydroxy-24,25,26,27-tetranorvitamin D_3 (23(OH)-24,25,26,27-tetranor-D_3 or C-23 alcohol)] and unmetabolized substrate were found to be similar in both the perfusion systems, i.e. kidneys perfused earlier with either 1α,24(*R*)$(OH)_2D_3$ (panel A2) or 1α,25$(OH)_2D_3$ (panel B2). This finding indicates that both the kidneys are viable not only during the first 8 hr of kidney perfusion, but also during the second 8 hr of perfusion. Thus, the differences between metabolism of 1α,24(*R*)$(OH)_2D_3$ and 1α,25$(OH)_2D_3$ noted in our present study were not a result of possible differences in the viability of the kidneys.

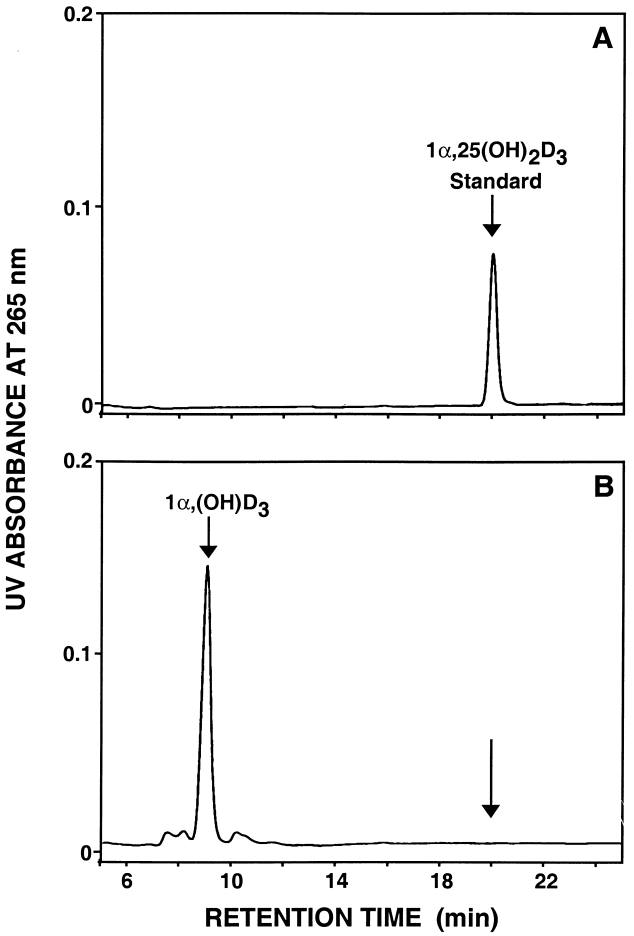

FIG. 3. HPLC profile of lipid extract of the perfusate sample (10 mL) obtained by perfusing a kidney for 4 hr with 1 μM 1α(OH)D_3 (B) in 100 mL of perfusate. HPLC was performed using a Zorbax-SIL column (25 cm × 4.6 mm) eluted with hexane:2-propanol (98:2) at a flow rate of 2 mL/min. The elution position of synthetic standard 1α,25$(OH)_2D_3$ is shown in panel A.

Metabolism of 1α(OH)D_3 by Perfused Kidney

Since the results of the previous experiment indicated that the rat kidney hydroxylates 1α,24(*R*)$(OH)_2D_3$ at the C-25 position, we next examined the enzymatic ability of the rat kidney to metabolize 1α(OH)D_3. From the results of Fig. 3, it is clear that 1α(OH)D_3 (panel B) was not metabolized in the rat kidney. At the end of the 4-hr period, only the substrate peak was observed in the chromatogram. The elution position of the 25-hydroxylated synthetic standard is shown in panel A. These results indicate that the rat kidney does not hydroxylate 1α(OH)D_3 at the C-25 position.

DISCUSSION

The results of our present study clearly demonstrated that the rat kidney has the enzymatic ability to metabolize 1α,24(*R*)$(OH)_2D_3$ via the C-24 oxidation pathway. The various metabolites of 1α,24(*R*)$(OH)_2D_3$ in the kidney perfusate were traced by subjecting the lipid extract of the

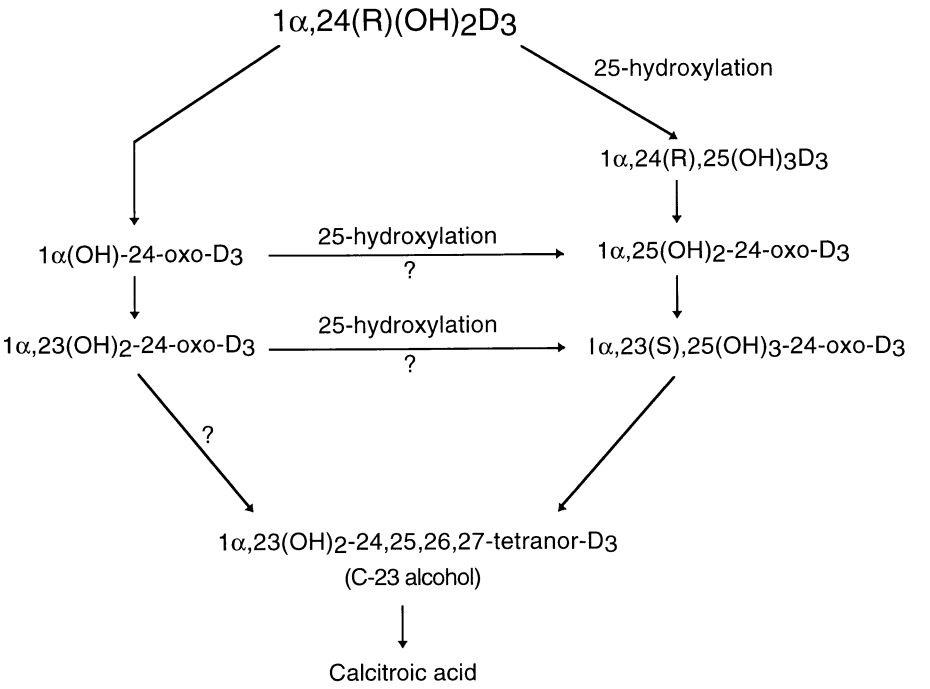

FIG. 4. Metabolic pathways of 1α,24(R)$(OH)_2D_3$ to calcitroic acid in the rat kidney.

perfusate directly to the HPLC column and by monitoring the UV absorbance (at 265 nm) of lipids eluting out of the HPLC column. The metabolites of 1α,24(*R*)$(OH)_2D_3$ can be divided into two groups: (i) previously known metabolites, which are the same as metabolites of 1α,25$(OH)_2D_3$, and (ii) unknown metabolites. The previously known metabolites are 1α,24(*R*),25$(OH)_3D_3$, 1α,25$(OH)_2$-24-oxo-D_3, 1α,23(*S*),25$(OH)_3$-24-oxo-D_3, and C-23 alcohol. In addition to the known metabolites, we also identified two new metabolites, 1α(OH)-24-oxo-D_3 and 1α,23$(OH)_2$-24-oxo-D_3. The identities of these two metabolites of 1α,24(*R*)$(OH)_2D_3$ were confirmed by mass spectrometric analysis and specific chemical reactions. These two new metabolites differ from the known metabolites by the absence of a C-25 hydroxyl group. Earlier studies showed that calcitroic acid is the end product of the C-24 oxidation pathway, and C-23 alcohol is its immediate precursor [18, 22]. Thus, we assume calcitroic acid to be formed from C-23 alcohol during 1α,24(*R*)$(OH)_2D_3$ metabolism. This assumption is further confirmed by our finding of calcitroic acid glucuronide in the bile of rats to which 1α,24(*R*)$(OH)_2D_3$ was administered (Ishizuka S, unpublished observations).

The formation of both 25-hydroxylated and non-25-hydroxylated metabolites during the metabolism of 1α,24(*R*)$(OH)_2D_3$ suggests that 1α,24(*R*)$(OH)_2D_3$ is metabolized through two pathways in the rat kidney (Fig. 4). One pathway is initiated by C-25 hydroxylation first and proceeds further via C-24 oxidation (pathway I). The second pathway directly proceeds via the C-24 oxidation pathway without prior hydroxylation at the C-25 position. Thus, the rat kidney possesses the enzymatic ability to hydroxylate 1α,24(*R*)$(OH)_2D_3$ at the C-25 position. Pathway I, in which the 25-hydroxylation of 1α,24(*R*)$(OH)_2D_3$ occurs prior to alterations at C-24 and C-23, appears to play a minor role when compared with pathway II, in which C-24 ketonization and C-23 hydroxylation proceed effectively even without prior 25-hydroxylation.

In our present study, we also demonstrated that 1α(OH)D_3 was not metabolized to 1α,25$(OH)_2D_3$, suggesting that the rat kidney does not appear to possess the classical vitamin D_3-25-hydroxylase (CYP27), which hydroxylates vitamin D_3 and 1α(OH)D_3 at the C-25 position [23]. Unlike the results of our present study in rat kidney, earlier *in vitro* studies of Tucker *et al.* [24] have shown that the chick kidney has 25-hydroxylase activity. Thus, there appear to be species differences in the expression of 25-hydroxylase activity. However, recent studies demonstrated the presence of CYP27 mRNA in rat kidney [25]. The expression of CYP27 mRNA in rat kidney in the absence of classical 25-hydroxylase activity indicates that the active enzyme is not produced. This might be due to a translational block or a post-translational modification inhibiting the production of active CYP27. However, from our present study it is apparent that prior synthetic hydroxylation of 1α(OH)D_3 at the C-24 position with an '*R*' orientation allows 25-hydroxylation to occur in the rat kidney. At present, the enzyme responsible for the C-25 hydroxylation of 1α,24(*R*)$(OH)_2D_3$ in the rat kidney is not known. The enzyme 1α,25$(OH)_2D_3$-24-hydroxylase (CYP24) is responsible for C-24 hydroxylation, the first step in the inactivation of 1α,25$(OH)_2D_3$ and 25(OH)D_3 [26–28]. It was found that CYP24 was capable of performing various steps in the C-24 oxidation pathway, suggesting that the enzyme has multicatalytic functions [28, 29]. More recent studies showed that CYP24 hydroxylates the C-26 of the vitamin D analog 24,24-difluoro-1α,25$(OH)_2D_3$ [30]. Thus, it is likely that CYP24 also may be responsible for the C-25

hydroxylation of 1α,24(*R*)$(OH)_2D_3$. Furthermore, our study demonstrated for the first time that the side chain of 1α,24(*R*)$(OH)_2D_3$ undergoes 24-ketonization and 23-hydroxylation even in the absence of the C-25 hydroxyl group. This observation suggests that CYP24 can perform some steps of the C-24 oxidation pathway without prior C-25 hydroxylation. Thus, we speculate that CYP24 may be playing a dual role in the metabolism of 1α,24(*R*)$(OH)_2D_3$. This study raises the possibility that CYP24 also has the capability to hydroxylate carbons other than C-23, C-24, and C-26. Further studies using COS cells transfected with a plasmid expressing CYP24 would lead to a more complete understanding of the involvement of this enzyme in C-25 hydroxylation.

When 1α,24(*R*)$(OH)_2D_3$ is administered systemically, its metabolism is known to occur in the liver, where the drug is readily metabolized into 1α,24(*R*),25$(OH)_3D_3$ by the classical CYP27 [31]. 1α,24(*R*)$(OH)_2D_3$ has been developed for topical use in psoriasis. Calcipotriol (MC903), another analog of vitamin D, has been approved for topical use in psoriasis and is currently being used worldwide for the successful control of this skin lesion [9]. However, skin irritation (in about 20% of treated patients) is an important local side-effect seen with calcipotriol therapy. Since 1α,24(*R*)$(OH)_2D_3$ does not exhibit this side-effect, it also appears to be a promising drug for the treatment of psoriasis. Recent studies have shown the conversion of 1α(OH)D_3 to 1α,25$(OH)_2D_3$ in keratinocytes, indicating that they do possess 25-hydroxylase activity [32]. Studies of Chen *et al*. [33] have reported the expression of 24-hydroxylase in human keratinocytes. It is likely that 1α,24(*R*)$(OH)_2D_3$ may be metabolized in keratinocytes at a faster rate than in the kidney because the keratinocytes possess the classical 25-hydroxylase, unlike the kidney. Thus, the rate of 1α,24(*R*)$(OH)_2D_3$ metabolism in a given tissue can be slow or fast depending on the presence of classical 25-hydroxylase activity in that tissue. This, in turn, can result in tissue-specific differences in the levels of 1α,24(*R*)$(OH)_2D_3$ and its metabolites, resulting in tissue-specific differences in the potency of 1α,24(*R*)$(OH)_2D_3$.

In conclusion, we have identified 1α(OH)-24-oxo-D_3 and 1α,23$(OH)_2$-24-oxo-D_3, two new metabolites of the antipsoriatic drug 1α,24(*R*)$(OH)_2D_3$. Additional studies examining the cell differentiating and calcemic activities of these two new metabolites may give further insight into our understanding of the mechanisms responsible for the ability of 1α,24(*R*)$(OH)_2D_3$ to generate *in vitro* cell differentiation activities equal to 1α,25$(OH)_2D_3$ without generating significant *in vivo* calcemic effects. At present these studies are in progress in our laboratory.

This work was supported, in part, by a research grant from Hoffmann-La Roche, Inc. to G. S. R. We gratefully acknowledge Mrs. Kursheed Wankadia for expert technical assistance.

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