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METABOLISM OF 1,25(OH)$_2$16-ENE D$_3$ IN KIDNEY: INFLUENCE OF STRUCTURAL MODIFICATION OF D-RING ON SIDE CHAIN METABOLISM

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Abstract: Comparative metabolism studies between 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$-16-ene D$_3$ in rat kidney indicate that both compounds are metabolized into their respective 24 hydroxy and 24-oxo metabolites. While 1,25(OH)$_2$-24-oxo-D$_3$ is further metabolized into 1,23,25(OH)$_3$-24-oxo-D$_3$ in the usual fashion, 1,25(OH)$_2$-24-oxo-16-ene D$_3$ resists C-23 hydroxylation. Thus a minor change in D-ring structure appears to cause major changes in the side chain metabolism.

Introduction

It is now well established that the steroid hormone, 1,25(OH)$_2$D$_3$ produces its various biological effects via both genomic as well as nongenomic pathways.[1,2] **Only recently has it become possible to separate the genomic actions of 1,25(OH)$_2$D$_3$ from its rapid nongenomic actions such as transcaltachia due to the synthesis of a specific group of D-ring modified analogs with 16,17-double bond.** For example, 25(OH)-16,23E-diene D$_3$ binds to the vitamin D receptor 1000 times less than 1,25(OH)$_2$D$_3$, but stimulates intestinal calcium absorption 2½ times more than 1,25(OH)$_2$D$_3$. On the contrary, 1,25(OH)$_2$-16-ene D$_3$ binds to the vitamin D receptor 2½ times better than 1,25(OH)$_2$D$_3$, but causes no stimulation of intestinal calcium absorption.[3,4] Also, several studies have shown that analogs possessing a 16-ene-23-yne structural modification are very effective at inhibiting proliferation and inducing differentiation of HL-60 cells.[5,6] Thus, the various unique biological activities of the 16-ene analogs of 1,25(OH)$_2$D$_3$ can be due to several factors which include 1) changes in binding to the intracellular vitamin D receptor and the extracellular serum vitamin D binding protein (DBP), and 2) changes in their cellular metabolism and final inactivation. While it appears that different 16-ene analogs may have different affinities for vitamin D receptor and DBP, relatively little is known of how D-ring structural modification would affect further metabolism and final inactivation of these unique analogs.[4] Therefore, we compared the metabolic fate of 1,25(OH)$_2$-16-ene D$_3$, with that of 1,25(OH)$_2$D$_3$ in the isolated perfused rat kidney. **The results of our study for the first time indicate that a minor structural change such as insertion of a 16,17-double bond in the D-ring of 1,25(OH)$_2$D$_3$ can produce major changes in its side chain metabolism.**

Materials and Methods

General: High-performance liquid chromatography (HPLC) was performed with a Waters Model 600 equipped with a Model 990 photo diode array detector (Waters Associates, Milford, MA). Mass spectra (70 eV) were obtained on a Hewlett-Packard 5985 B mass spectrometer. Crystalline 25(S),26(OH)$_2$D$_3$,1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$-16-ene D$_3$ were synthesized at Hoffmann-La Roche, Inc., Nutley, NJ.

Study of 1,25(OH)$_2$-16-ene D$_3$ Metabolism Using the Technique of Kidney Perfusion

Isolated rat kidney perfusions were performed as described before.[7,8] Metabolism of 1,25(OH)$_2$-16-ene D$_3$ was studied by introducing cold 1,25(OH)$_2$-16-ene-D$_3$ (400 nmoles) into 100 mL of perfusate and the kidney perfusion was continued for 8 h. Lipid extraction of the final kidney perfusate was performed according to procedure of Bligh and Dyer.[10] Half of the bulk lipid extract obtained from the final perfusate of about 100 mL was then subjected directly to HPLC under the chromatographic conditions described in the legend from Figure 1. Fractions of each individual metabolite from the first HPLC run was pooled and subjected to a second HPLC run using the same Zorbax-SIL column eluted with methylene chloride:2-propanol (94:5). Each metabolite obtained from the second HPLC run was re-chromatographed twice using the first HPLC system. At this time, the purity of each metabolite was adequate for its structure identification process. A control perfusion was performed with cold 1,25(OH)$_2$D$_3$ in a similar fashion and the lipid extract of the final perfusate was analyzed using the same HPLC systems as above.

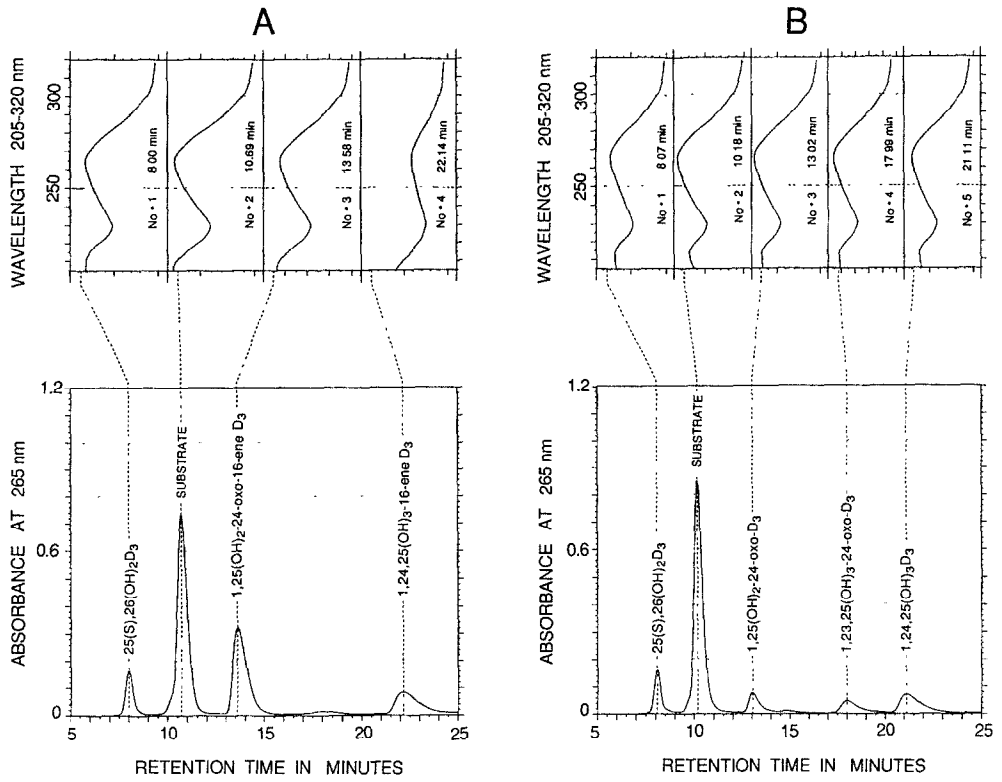

Figure 1: HPLC and UV analysis of the lipid extract of final perfusate obtained by perfusing (A) the experimental kidney with 1,25(OH)$_2$-16-ene D$_3$ and (B) control kidney with 1,25(OH)$_2$D$_3$. In each case HPLC analysis was performed on the lipid extract obtained from 50 mL of perfusate. Chromatographic conditions: Zorbax-SIL column (25 cm x 4.6 mm); Hexane:2-propanol (90:10), Flow rate: 2mL/min. A known amount of 25(S),26(OH)$_2$D$_3$ was added to the perfusate at the time of lipid extraction to assess extraction efficiency of vitamin D metabolites in general.

Results

From Figure 1A, it becomes obvious that $1,25(OH)_2$-16-ene D_3 (peak #2) is metabolized mainly into two metabolites represented by peaks #4 and #3. Evidence for correct structure assignment to each metabolite of $1,25(OH)_2$-16-ene D_3 purified from the peaks #3 and #4 was achieved through the following techniques. The two metabolites exhibited UV spectra with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm (Figure 1). This finding indicated that both the metabolites contained an intact 5,6-cis-triene chromophore.

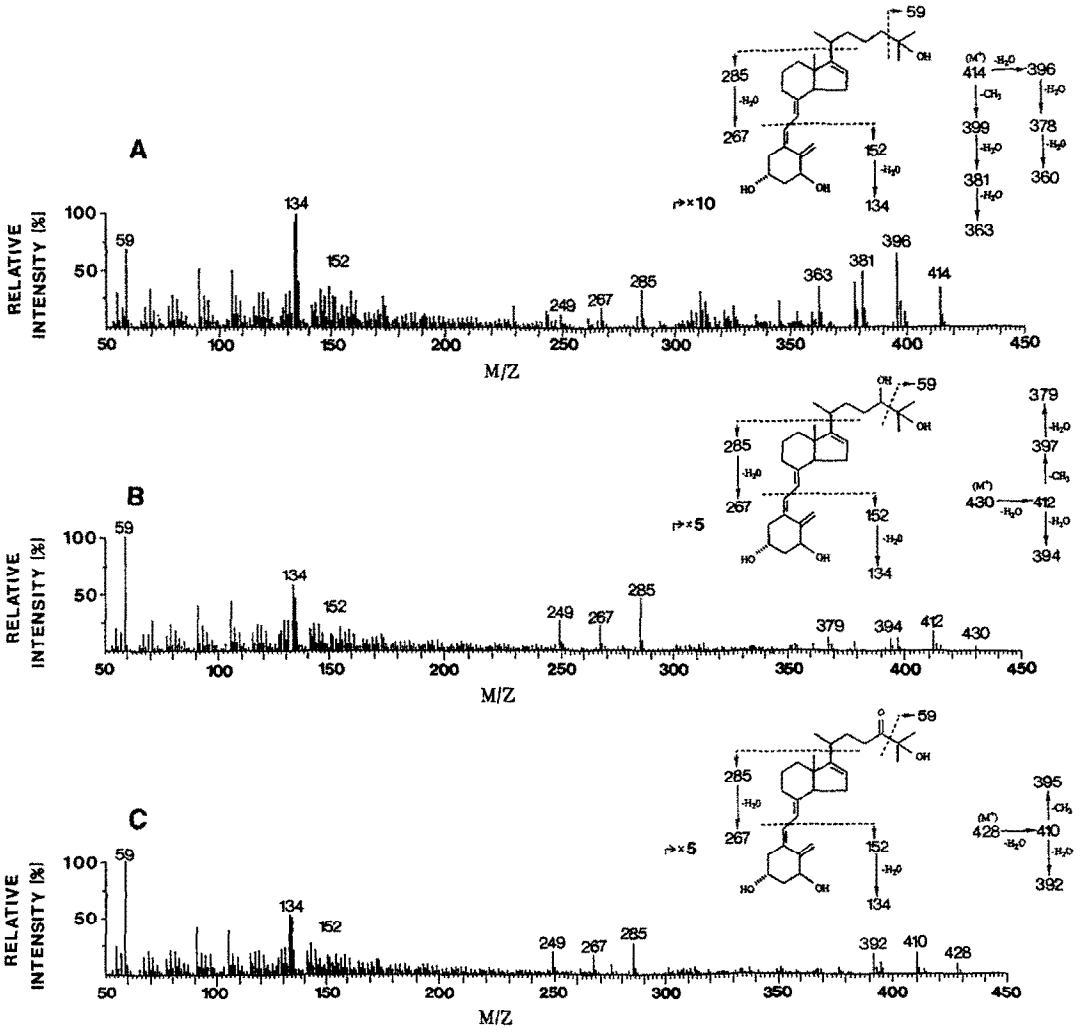

Figure 2: Mass spectra of $1,25(OH)_2$-16-ene D_3 (A); $1,24,25(OH)_3$-16-ene D_3 (B) and $1,25(OH)_2$-24-oxo-D_3 (C)

The mass spectra of 1,25$(OH)_2$-16-ene D_3 and its two metabolites are shown in Figure 2. All the three spectra exhibit a peak at m/z 285, which is due to the side-chain cleavage from the main steroid molecule (C-17/C-20 cleavage). The peaks at m/z 267 and 249 are as a result of two sequential losses of water from the peak at m/z 285. The peak at m/z 152 represents the A ring plus the carbon 6 and 7 fragment. Loss of water from the peak at m/z 152 results in the base peak at m/z 134. Collectively the presence of the common peaks at m/z 285, 267, 249, 152, and 134 in the mass spectra of 1,25$(OH)_2$-16-ene D_3 and its two metabolites indicate that the secosteroid nucleus has remained unchanged and that the two new metabolites are formed as a result of changes occurring only on the side chain of 1,25$(OH)_2$-16-ene D_3. A definite structure assignment of each metabolite is as follows.

Structure Identification of 1,24,25(OH)$_3$-16-ene D$_3$: The molecular ion at m/z 430 (M^+) in the mass spectrum (Figure 2B) suggests that this new metabolite is a trihydroxy metabolite and is formed due to addition of a hydroxy group to the side chain of 1,25$(OH)_2$-16-ene D_3. The peak at m/z 59 indicates that this metabolite contains an intact C-25 hydroxy group with no hydroxylations occurring on C-26 or C-27. Also, this metabolite is susceptible to periodate oxidation and this finding suggests that the additional hydroxy group on the side chain of this new metabolite is adjacent to the C-25 hydroxy group. As we have already demonstrated that no hydroxylations occurred at carbons C-26 and C-27, it can be concluded that the extra hydroxy group is at C-24. Collectively, from the above data, this new metabolite is identified as 1,24,25$(OH)_3$-16-ene D_3.

Structure Identification 1,25(OH)$_2$-24-oxo-16-ene D$_3$: The molecular ion at m/z 428 (M^+) in the mass spectrum (Figure 2C) suggests that this new metabolite is formed as a result of addition of an oxo functionality to the side chain of 1,25$(OH)_2$-16-ene D_3. The peak at m/z 59 indicates that this metabolite also like 1,24,25$(OH)_3$-16-ene-D_3 contains an intact C-25 hydroxy group with no changes occurring on C-26 or C-27. Also, this metabolite on sodium borohydride reduction is converted into a product which comigrated with 1,24,25$(OH)_3$-16-ene D_3 on HPLC. Collectively, from the above data this new metabolite is identified as 1,25$(OH)_2$-24-oxo-16-ene D_3.

From Figure 1B, it can be seen that 1,25$(OH)_2D_3$ is metabolized mainly through C-24 oxidation pathway shown in Figure 3 and produced the following metabolites: 1,24,25$(OH)_3D_3$ (peak #5), 1,25$(OH)_2$-24-oxo-D_3 (peak #3), 1,23,25$(OH)_3$-24-oxo-D_3 (peak #4). The pattern of metabolism is identical to the one previously described from our laboratory.[8,9] The following Table 1 compares the amounts of 24-hydroxy and 24-oxo metabolites of 1,25$(OH)_2$-16-ene D_3 produced in the experimental kidney with those of 1,25$(OH)_2D_3$ produced in the control kidney.

Table 1 Nanomoles of Metabolite Produced During 8 Hour Perfusion

Substrate	24-hydroxy metabolite	24-oxo metabolite	23-hydroxy - 24-oxo metabolite
1,25$(OH)_2$-16-ene D_3	38	90	--
1,25$(OH)_2$ D_3	30	15	16

Discussion

During the past decade from the work of ours and others, it appears that target tissue metabolism of $1,25(OH)_2D_3$ occurs mostly through three different pathways as shown in Figure 3. Out of the three, C-24 oxidation pathway appears to be physiologically significant. However, it is important to note that all the three pathways are interconnected. For example, 23-hydroxylase, the first enzyme involved in C-23 oxidation pathway also participates in C-24 oxidation pathway. In the same way C-26 hydroxylase participates in both C-26 and C-23 oxidation pathways. Relatively little is known about the characteristics of these various enzymes involved in the side chain metabolism of $1,25(OH)_2D_3$. Only recently, with the purification of 24-hydroxylase and with the availability of cDNA probes for this enzyme[11], there has been renewed interest in studying the properties and regulation of the various enzymes involved in the side chain metabolism of $1,25(OH)_2D_3$. Also, with the discovery that the structural alteration of $1,25(OH)_2D_3$ can result in dissociation of its calcemic actions from its actions on cell growth and differentiation, there has been a major interest in the synthesis of various novel analogs of $1,25(OH)_2D_3$ with altered side chain structures[4]. Recently, it has been shown that a specific structural change on the side chain can alter the side chain metabolism of that particular compound and this in turn appears to have an impact on its biological activity.[12] **However, with the synthesis of new class of analogs with modifications of main molecule, it remained to be determined whether structural modification of the main molecule would have any effect on their side chain metabolism and their subsequent inactivation.**

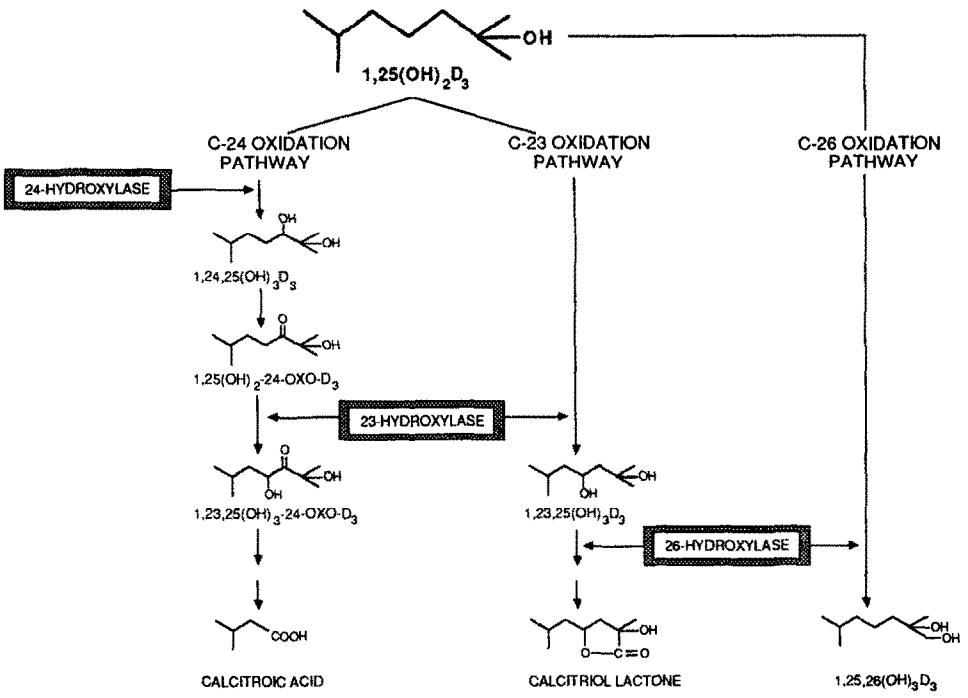

Figure 3: Established pathways of target tissue metabolism of $1,25(OH)_2D_3$.

In our present study we investigated the metabolism of 1,25(OH)$_2$-16-ene D$_3$ which differed from 1,25(OH)$_2$D$_3$ only in the structure of D-ring. From the results, it is obvious that both 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$-16-ene D$_3$ are metabolized through the C-24 oxidation pathway and are converted into their respective C-24 hydroxy and C-24 oxo metabolites. However, as expected, while 1,25(OH)$_2$-24-oxo-D$_3$ is further metabolized into 1,23,25(OH)$_3$-24-oxo-D$_3$ as a result of C-23 hydroxylation, 1,25(OH)$_2$-24-oxo-16-ene D$_3$ appears to resist C-23 hydroxylation. As a result 1,25(OH)$_2$-24-oxo-16-ene D$_3$ accumulates in increasing amounts when compared to 1,25(OH)$_2$-24-oxo-D$_3$ (Table 1). **Thus, it appears that a minor modification in the D-ring structure such as insertion of a 16,17-double bond can produce a conformational change in the side chain, which is acceptable to 24-hydroxylase, but not to 23-hydroxylase.** Even though at present it is not clear whether 24 and 23 hydroxylases are different enzymes or a single enzyme,it is tempting to hypothesize basing on the results of our present study that 24 and 23 hydroxylases are probably different enzymes. Studies are in progress in our laboratory to test this hypothesis. Also, it is not clear whether the block in side chain metabolism can be responsible for some of the unique biological actions of 1,25(OH)$_2$-16-ene D$_3$ analogs. To address this issue, we are presently comparing the biological activity of the 1,25(OH)$_2$-16-ene D$_3$ with its further metabolites 1,24,25(OH)$_3$-16-ene D$_3$ and 1,25(OH)$_2$-24-oxo-16-ene D$_3$.

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References

1. Reichel, H.; Koeffler, H. P.;Norman A. W. *N. Engl. J. Med.* **1989**, 320, 980.
2. Lowe, K.E.; Maiyar, A.C.; Norman A.W. Crit. Rev. *Eukaryot. Gene Expr.* 1992, 2, 65.
3. Uskokovic, M.R.; Baggiolini, E.; Shiuey, S.-J.; Iacobelli, J.; Hennessy, B.; Kiegiel, J.; Daniewski, A.R.; Pizzolato, G.; Courtney, L.F.; Horst, R.L. in *Vitamin D: Gene regulation structure-function analysis and clinical application (Norman, A.W.; Bouillon, R.; Thomasset, M. eds.)* Walter de Gruyter, Berlin **1991**, 139.
4. Norman, A.W.; Koeffler, H.P.; Bishop, J.E.; Collins, E.D.; Sergeev, I.; Zhou, L.X.; Nemer, I.; Zhou, J.; Henry, H.L.; Okamura, W.H. in *Vitamin D: Gene regulation structure-function analysis and clinical application (Norman, A.W.; Bouillon, R.; Thomasset, M. eds.)* Walter de Gruyter, Berlin **1991**, 146.
5. Norman, A.W., Zhou, J.Y., Henry, H.L., Uskokovic, M.R. and Koeffler, H.P. *Cancer Res.* 1990, 50, 6857-6864.
6. Zhou, J-Y., Norman, A.W., Chen, D-L., Sun, G-W., Uskokovic, M. and Koeffler, H.P. *Proc. Natl. Acad. Sci. USA* 1990, 87(10) 3929-3932.
7. Reddy, G.S., Jones, G., Kooh, S.W. and Fraser, D. *Am. J. Physiol.* 1982, 243, E265-E271.
8. Reddy, G.S., Tserng, K-Y., Thomas, B.R., Dayal, R. and Norman, A.W. *Biochemistry* 1987, 26, 324-331.
9. Reddy G.S. and Tserng K-Y. *Biochemistry* **1989**, 28,1763.
10. Bligh, E.G. and Dyer, W.J. *Can. J. Biochem. Physiol.* 1959, 37, 911-917.
11. Ohyama, Y., Noshino, M., Okuda, K. *FEBS Lett* 1991, 278, 195.
12. Brown, A.J.; Berkoben, M.; Ritter, C.; Kubodera, N; Nishii, Y.; Slatopolsky, E. *Biochem. Biophys. Res. Commun.* 1992, 189, 759.