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25-OHD3-26,23-LACTONE: A METABOLITE OF VITAMIN D3 THAT IS 5 TIMES MORE POTENT THAN 25-OHD3 IN THE RAT PLASMA COMPETITIVE PROTEIN BINDING RADIOASSAY

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SUMMARY

A new metabolite of Vitamin D3 (25-OHD3-26,23-lactone) has been found in the plasma of Vitamin D3-toxic pigs and cows. This metabolite is at least 5 times more potent than 25-OHD3 in the displacement of [^{3}H]-25-OHD3 from rat plasma protein binding sites under short-term incubation. This metabolite co-migrates with 24,25-(OH)2D3 on Sephadex LH-20 columns developed in chloroform:hexane 65:35 and with 25,26-(OH)2D3 on Sephadex LH-20 columns developed in hexane:chloroform:methanol 9:1:1. The presence of 25-OHD3-26,23-lactone represents a possible contaminant in the assay of 24,25-(OH)2D3 or 25,26-(OH)2D3 if only Sephadex LH-20 is used for pre-assay purification. 25-OHD3-26,23-lactone is, however, resolved from 24,25-(OH)2D3 by high pressure liquid chromatography (HPLC) using Zorbax Sil silicic acid columns developed in either isopropanol:hexane 8:92 or isopropanol:methylene chloride 3.5:96.5. We assayed for the presence of this new metabolite of Vitamin D3 and found it to be present in normal pig plasma and undetectable in normal cow plasma. Concentrations were elevated to 10-20 ng/ml following massive injection of Vitamin D3 to both species.

INTRODUCTION

Vitamin D3 has been shown to be converted in the liver to form 25-OHD3 (1). 25-OHD3 then can be converted in the kidney to 24,25-(OH)2D3, 25,26-(OH)2D3 and 1,25-(OH)2D3 (2,3). 25-OHD3, 24,25-(OH)2D3 and 25,26-(OH)2D3 have been shown to be equipotent in their competition for binding sites on the rat plasma Vitamin D binding protein (4,5). Vitamin D3 and 1,25-(OH)2D3, however, are 100 times less potent (6).

During the development of assays for the major forms of Vitamin D3 in animal plasma, a new metabolite that competed in the competitive protein binding for 24,25-(OH)2D3 was isolated from chick plasma (4). This metabo-

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lite was referred to as peak X and has now been identified at the University of Wisconsin as 25-OHD3-26,23-lactone (Wichmann, J. K., DeLuca, H. F., Schnoes, H. K., Horst, R. L., Shepard, R. M., and Jorgensen, N. A., Biochemistry, in press). This metabolite was named calcidiol lactone by Dr. DeLuca (DeLuca, H. F. and Schnoes, H. K., Proceedings of 4th Internatl. Workshop on Vitamin D, held in Berlin, Germany, Norman et al., eds., Degruter Publishing, in press) and has a molecular weight of 428. Our laboratory has isolated and purified this metabolite from Vitamin D_3-toxic pigs and found it to be 5-10 times more potent than 25-OHD_3 in its competition for binding on the plasma Vitamin D binding protein.

MATERIALS AND METHODS

Apparatus -- High pressure liquid chromatography (HPLC) was performed on a Model LC-204 fitted with a Model 6000A pumping system, U6K injection valve and a Model 440 ultraviolet fixed wavelength (254 nm) detector (all from Waters Associates, Milford, MA).

A variable wavelength Beckman DB spectrophotometer was used to scan the Vitamin D metabolites in solution. High resolution mass spectrometry was performed by Shrader Analytical and Consulting Labs, Inc., Detroit, MI.

Sterols -- [23,24-^{3}H]-25-OHD_3 (110 Ci/mmole) was purchased from Amershan Searle. Reference 24R, 25-$(OH)_2D_3$ was provided to us by Dr. M. Uskokovic of Hoffman-LaRoche (Nutley, NJ).

Preparation of calcidiol lactone (in vivo) -- Calcidiol lactone was prepared according to the scheme of Fig. 1. The preparation involved a lipid extraction (7) of 8 liters of plasma from Vitamin D_3-toxic pigs. The lipid extract was placed on a 2.2 x 60 cm Sephadex LH-20 column developed in 65:35 chloroform:hexane. The region corresponding to the elution of 24,25-$(OH)_2D_3$ (370-460 ml) was collected and rechromatographed on a 2.2 x 60 cm Sephadex LH-20 column developed in 9:1:1 hexane: chloroform:methanol. The region corresponding to the elution of 25,26-$(OH)_2D_3$ (400-600 ml) was collected and chromatographed by HPLC or a Zorbax Sil silicic acid column developed in isopropanol:methylene chloride 3.5:96.5. The major UV peak (Fig. 2), which also contained the binding activity, was collected and chromatographed by HPLC on a Zorbax ODS column developed in water:methanol 22:88. The major UV peak (20-26 ml) was collected and chromatographed on a Zorbax Sil silicic acid column 8:92 isopropanol:hexane. Calcidiol lactone eluted at 20-22 mls (Fig. 3). The peak was recycled through the system 2 times to assure UV homogeneity. The concentration of this purified preparation in 100% ethanol was determined by UV absorbance (ε = 18,200).

Preparation of [^{3}H]-calcidiol lactone (in vitro) -- One-day-old cockerels were raised on a Vitamin D-deficient diet for 7 days. The

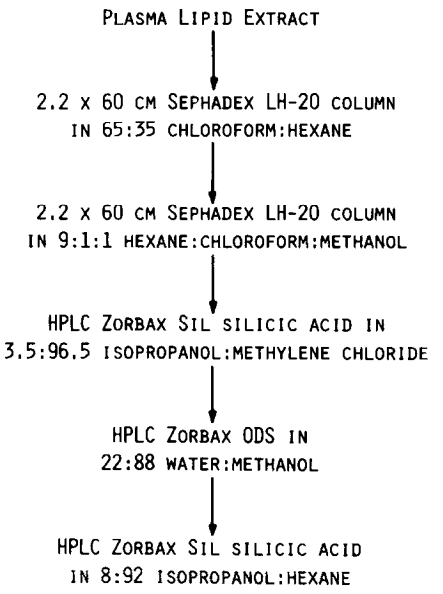

Figure 1. Purification scheme for calcidiol lactone.

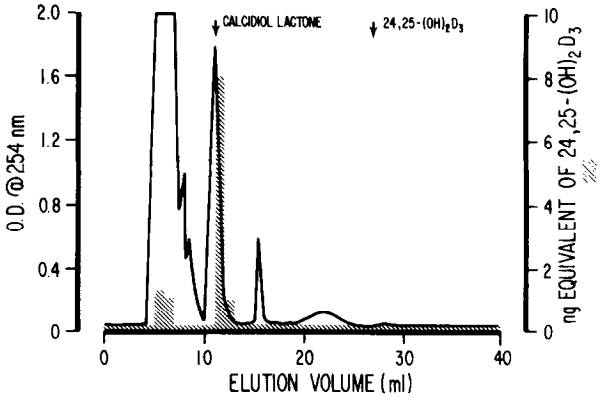

Figure 2. HPLC UV and binding activity profile of the calcidiol lactone isolate after purification on Sephadex LH-20. The profile represents the elution of calcidiol lactone and 24,25-$(OH)_2D_3$ on a Zorbax Sil silicic acid column developed in 3.5:96.5 isopropanol:methylene chloride.

chicks received an injection of 250 ng of 1,25-$(OH)_2D_3$ on day 6 (24 hr before sacrifice) and again 6 hours before sacrifice. The kidneys were harvested, homogenized and incubated with [23,24-^{3}H]-25-OHD_3 according to the procedure of Tanaka et al. (7) for the preparation of 24,25-$(OH)_2D_3$. The lipid extract was purified as described in the preparation of calcidiol lactone *in vivo*. This incubation gave a radioactive peak which was homogeneous and co-migrated with calcidiol lactone in 5 chromatographic systems and, therefore, was assumed to be calcidiol lactone. This purified preparaton was used to monitor the recovery of calcidiol lactone during the assay of this metabolite.

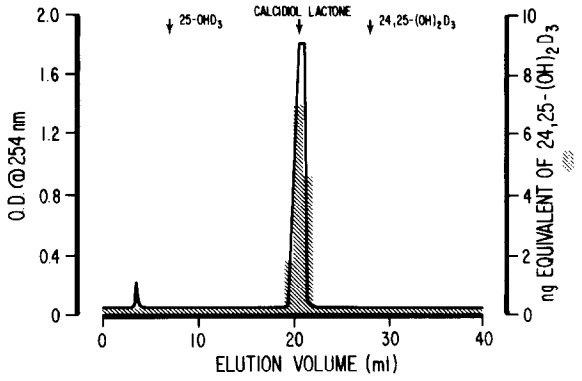

Figure 3. Elution of calcidiol lactone on a Zorbax Sil silicic acid column developed in 8:92 isopropanol:hexane.

Vitamin D3-dosed animals -- The pigs and cows used in this experiment were maintained on a diet adequate in energy, protein and Vitamin D3. The cows were injected with 15 x 10^6 IU of Vitamin D3 in 100% ethanol. The pigs were injected initially with 5 x 10^6 IU of Vitamin D3 followed 1 week later by 30 x 10^6 IU of Vitamin D3 in 100% ethanol. Sixty mls of blood were collected from all the animals in heparinized syringes (10 U/ml blood) 20 days following the initial Vitamin D3 injection. The plasma was harvested and stored frozen until analyzed.

Assays for Vitamin D and its metabolites -- Vitamin D and its metabolites were measured according to modifications to the procedure of Horst et al. (4). The 24,25-$(OH)_2D_3$ and calcidiol lactone are poorly resolved by HPLC on the Zorbax Sil silicic acid column developed in 10:90 isopropanol:hexane described by Horst et al. (4). Therefore, the 24,25-$(OH)_2D_3$-calcidiol lactone isolate from this column was reapplied to a Zorbax Sil silicic acid column developed in 3.5:96.5 isopropanol:methylene chloride to achieve baseline resolution of these two metabolites (refer to Fig. 2 for profile). Other modifications involved the addition of 0.01% gelatin to the 24,25-$(OH)_2D$ binding assay buffer to achieve higher specific binding with minimal changes in non-specific binding.

RESULTS

Spectroscopy -- The ultraviolet spectra of the calcidiol lactone in 100% ethanol is shown in Fig. 4. The spectrum displays the cis-triene ultraviolet spectrum (absorption maximum at 264 nm and minimum at 228 nm $\lambda_{max}:\lambda_{min}$ ~1.8:1) which is typical of Vitamin D_3 and its metabolites.

High resolution mass spectrometry of 5000 ng of calcidiol lactone (Fig. 5) yielded a molecular weight of 428.2936 (calculated m^+ = 428.2926)

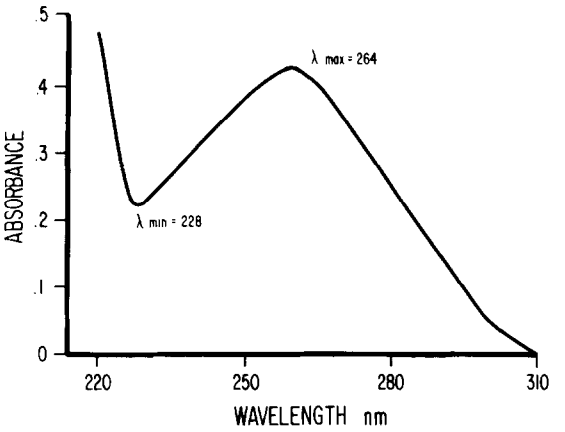

Figure 4. UV spectra of calcidiol lactone purified from pig plasma.

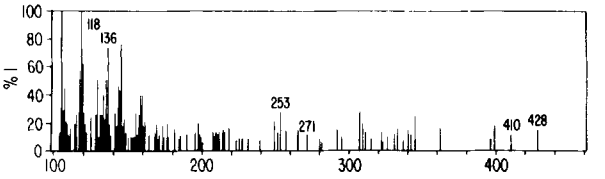

Figure 5. High resolution mass spectra of calcidiol lactone. The spectra includes the metabolite plus the calibration compound perfluorokerosene (pfk).

requiring a molecular formula $C_{27}H_{40}O_4$ which corresponds with the spectrum obtained by Wichmann. The ions of m/e 271 ($C_{19}H_{27}O$) and 253 ($C_{19}H_{25}$) which represent the steroid nucleus establish the presence of 3 oxygen atoms on the side chain.

Competition in the rat plasma competitive protein binding assay -- The calcidiol lactone prepared from the Vitamin D_3-toxic pig plasma was compared against chemically synthesized 24,25-$(OH)_2D_3$ and 25-OHD_3 and 25,26-$(OH)_2D_3$ (Fig. 6). The calcidiol lactone was at least 5 times more potent than either 25-OHD_3, 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ at displacing [^{3}H]-25-OHD_3 from rat plasma protein binding sites.

When [^{3}H]-25-OHD_3 was replaced with [^{3}H]-calcidiol lactone, the binding assay was less sensitive to both calcidiol lactone and 24,25-$(OH)_2D_3$

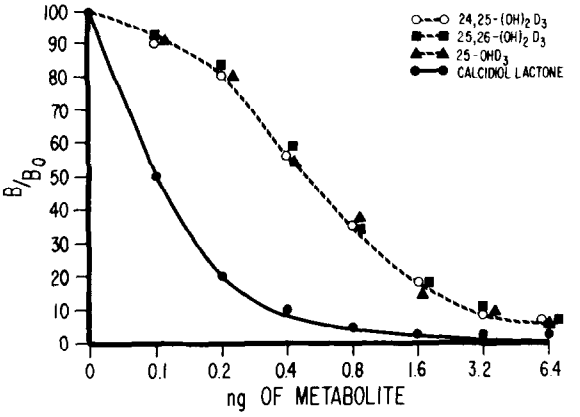

Figure 6. Competitive displacement of [23,24-^{3}H]-25-OHD$_3$ by 24,25-(OH)$_2$D$_3$ o—o, 25,26-(OH)$_2$D$_3$ ■—■, 25-OHD$_3$ ▲—▲ and calcidiol lactone ●—●.

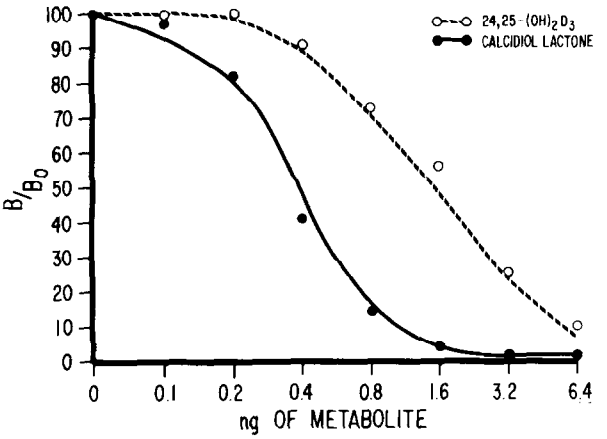

Figure 7. Competitive displacement of [^{3}H]-calcidiol lactone by 24,25-(OH)$_2$D$_3$ o—o and calcidiol lactone ●—●.

(Fig. 7). The calcidiol lactone still, however, remains the more competitive of the two metabolites. Less sensitivity would be expected with the [^{3}H]-calcidiol lactone as the tracer, since presumably it is equally as competitive for binding sites as the cold calcidiol lactone.

Using [^{3}H]-25-OHD$_3$ as tracer and calcidiol lactone as standard in the rat plasma competitive protein binding assay, we measured the amount of calcidiol lactone in normal and Vitamin D$_3$-dosed cows and pigs. The results demonstrate very low to nondetectable levels in plasma from normal

TABLE 1. Vitamin D_3, 25-OHD_3 and Calcidiol Lactone in Pigs and Cows Dosed with Vitamin D_3 (n = 2)

	D_3	25-OHD_3	Calcidiol Lactone
	ng/ml		
Cow (Control)	3.5	38.3	< .05
(+ D_3)	38.0*	247.9*	21.2*
Pig (Control)	12.0	52.3	1.0
(+ D_3)	270.0*	815.7*	12.0*

*Massive injection of Vitamin D_3 caused significant ($p < .05$) elevations of the metabolites compared to controls.

cows with 2-3 ng/ml appearing in normal pig plasma. A marked elevation occurred in animals receiving large doses of Vitamin D_3 (Table 1). Plasma Vitamin D_3 and 25-OHD_3 levels were likewise elevated in pig and cow plasma as a result of the Vitamin D_3 dosing.

DISCUSSION

Our laboratory has demonstrated for the first time a metabolite of Vitamin D_3 (calcidiol lactone) which is more competitive than 25-OHD_3 for binding sites on the plasma Vitamin D binding protein. We have also found that this metabolite co-migrates with 24,25-$(OH)_2D_3$ on Sephadex LH-20 columns developed in 65:35 chloroform:hexane. Therefore, using Sephadex LH-20 to purify plasma extracts for the quantitation of 24,25-$(OH)_2D$ (as described by Haddad et al., 1977) could result in an overestimation of the plasma 24,25-$(OH)_2D_3$ concentration, especially if high levels of Vitamin D_3 or 25-OHD_3 are given.

The physiological importance of our observations to the role of calcidiol lactone in Vitamin D toxicity will have to await an exciting investigational period.

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