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STEREOCHEMISTRY OF NATURALLY-OCCURRING 25-HYDROXYVITAMIN D_3-26,23 LACTONE AS DETERMINED BY RADIOLIGAND BINDING ANALYSIS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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The four stereoisomers of 25-hydroxyvitamin D_3-26,23 lactone (25-OHD_3-26,23 lactone) were tested against in vivo 25-OHD_3-26,23 lactone to determine their relative competition in the radioligand binding assays for 25-OHD_3 and 1,25-$(OH)_2D_3$. The 25R-OHD_3-26,23S lactone and in vivo 25-OHD_3-26,23 lactone behaved identically in the radioligand binding assay for 25-OHD_3 and were ~5-fold more potent than 25-OHD_3 at displacing 25-OH[^{3}H]D_3. The 25S-OHD_3-26,23S lactone was the poorest competitor in this assay, requiring a 10-fold excess relative to 25-OHD_3 to displace 50% of the 25-OH[^{3}H]D_3. The order of competition in the 25-OHD_3 radioligand binding assay was 25R-OHD_3-26,23S lactone = in vivo 25-OHD_3-26,23 lactone >> 25S-OHD_3-26,23R lactone > 25-OHD_3 >> 25R-OHD_3-26,23R lactone > 25S-OHD_3-26,23S lactone. The order of competition in the 1,25-$(OH)_2D_3$ cytosol receptor assay was essentially reversed from the competition in the 25-OHD_3 assay and was 25S-OHD_3-26,23S lactone > 25-OHD_3 >> 25S-OHD_3-26,23R lactone > 25R-OHD_3-26,23S lactone = in vivo 25-OHD_3-26,23 lactone. When tested in a high-performance liquid chromatographic system which separates all four stereoisomers, the in vivo 25-OHD_3-26,23 lactone comigrated with synthetic 25R-OHD_3-26,23S lactone. These data firmly establish that the naturally-occurring 25-OHD_3-26,23 lactone has the 25R, 23S stereochemistry. In addition, these data are the first to demonstrate that the four stereoisomers of 25-OHD_3-26,23 lactone have different affinities for the plasma vitamin D binding protein and the 1,25-$(OH)_2D$ cytosol receptor.

INTRODUCTION

In 1979, a naturally-occurring substance which competed in the high-performance liquid chromatography (HPLC)-competitive protein binding analysis

Abbreviations used: 25-OHD_3-26,23 lactone, 25-hydroxyvitamin D_3-26,23 lactone; 25-OHD_3, 25-hydroxyvitamin D_3; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3.

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[24,25-$(OH)_2D_3$] was demonstrated (1). This compound was isolated and identified as 25-OHD_3-26,23 lactone (2) which has been shown to circulate normally in chicks and pigs (3) and during vitamin D_3 toxicity in cows (4). Horst (4) has shown that 25-OHD_3-26,23 lactone competes five times better than 25-OHD_3 for binding sites on the 4.2S rat plasma vitamin D binding protein. In addition, Horst and Littledike (5) have demonstrated the importance of the kidney in the formation of 25-OHD_3-26,23 lactone, suggesting that either 25-OHD_3-26,23 lactone itself or an intermediate to its synthesis is produced by the kidney. Napoli et al. (6) have shown that supplying nephrectomized animals with 23S,25-$(OH)_2D_3$ results in 25-OHD_3-26,23 lactone synthesis comparable to intact animals given the same metabolite. The 23-hydroxylation of 25-OHD_3 by the kidney, therefore, appears to be a limiting step in the formation of 25-OHD_3-26,23 lactone biosynthesis.

Wichmann et al. (7) have recently demonstrated the synthesis of all four possible diasteriomers of 25-OHD_3-26,23 lactone, but did not establish the C-23 and C-25 stereochemistries. Morris et al. (8) have recently demonstrated the synthesis and stereochemical identification of all four 25-OHD_3-26,23 lactone stereoisomers. Using these four stereoisomers and *in vivo* 25-OHD_3-26,23 lactone in the competitive protein binding assays for 25-OHD_3 and 1,25-$(OH)_2D_3$ in addition to comigration on HPLC has enabled us to conclusively demonstrate that the naturally-occurring 25-OHD_3-26,23 lactone has the 25R, 23S configuration. We will also show that 25S-OHD_3-26,23S lactone has a relatively high affinity for the 1,25-$(OH)_2D$ cytosol receptor compared to the other 25-OHD_3-26,23 lactone stereoisomers and 25-OHD_3.

MATERIALS AND METHODS

All HPLC was done on a Waters Model ALC/GPC 204 liquid chromatograph equipped with a Model 6000A solvent delivery system, Model 440 ultraviolet detector and Model U6K injector. All analytical (0.45 x 25 cm) microparticulate silica gel (normal phase) columns were purchased from DuPont. All vitamin D_3 compounds were detected at 254 nm. All solvents were distilled in glass and purchased from Burdick and Jackson (Muskegon, MI). Ultraviolet spectra of the vitamin D_3 derivatives in ethanol were recorded on a Beckman Model 25 recording spectrophotometer using a molar extinction coefficient (Σ_{264}) of 18,200 m^{-1} cm^{-1}.

Sterols -- Synthetic 25-OHD_3 was a gift of Upjohn Company (Kalamazoo, MI). The 25R-OHD_3-26,23S lactone, 25S-OHD_3-26,23R lactone, 25R-OHD_3-26,23R lactone, and 25S-OHD_3-26,23S lactone were synthesized according to the procedure of Morris et al. (8). In vivo 25-OHD_3-26,23 lactone was isolated from the plasma of pigs receiving large doses of vitamin D_3 (4). All the 25-OHD_3-26,23 lactone stereoisomers and other vitamin D_3 metabolites were purified by HPLC just prior to use in the competitive protein binding assays. Purity of the compounds was assessed by inspection of the HPLC profiles generated during their purification and the ultraviolet absorbance spectrum obtained following HPLC purification. All the compounds had a λ_{max} at 264 nm and a λ_{min} at 228 nm. The ratios of $\lambda_{max}/\lambda_{min}$ ranged between 1.6 and 1.8.

Radioligand binding assays -- The comparison of the relative binding affinity of the lactone isomers to in vivo lactone and 25-OHD_3 was accomplished using two sources of vitamin D binding proteins: 1) rat plasma vitamin D binding protein diluted in 0.05 M potassium phosphate buffer containing 0.01% gelatin (3); and 2) the 1,25-$(OH)_2D_3$-specific cytosol receptor with the isolation and assay conditions repeated precisely as described by Reinhardt et al. (9).

RESULTS

Comparisons were made of the quantities of 25-OHD_3-26,23 lactone stereoisomers which competitively inhibited the binding of 25-OH[^{3}H]D_3 to the rat plasma vitamin D binding protein or 1,25-$(OH)_2$[^{3}H]D_3 to 1,25-$(OH)_2$D-specific cytosol receptor (Table I). The 25-OHD_3-26,23 lactone synthesized in vivo and the synthetic 25R-OHD_3-26,23S lactone competed identically in both assays. They were the best competitors of the compounds tested for binding by the rat plasma vitamin D binding protein and the poorest competitors for binding by the 1,25-$(OH)_2$D cytosol receptor. The 25S-OHD_3-26,23S lactone, on the other hand, was the poorest competitor for the rat plasma vitamin D binding protein and the best competitor for the 1,25-$(OH)_2$D-specific cytosol receptor. The 25R-OHD_3-26,23R lactone bound poorly in both assays, but the 25S-OHD_3-26,23R lactone bound ~1.8 times better than 25-OHD_3 to the rat plasma vitamin D binding protein.

Figure 1A shows the HPLC chromatogram of the four stereoisomers of 25-OHD_3-26,23 lactone following HPLC using a silicic acid column developed in 4/96 isopropanol/hexane. When 100 ng of in vivo lactone was added to the same mixture, the 25R-OHD_3-26,23S lactone peak tripled in peak height (Fig. 1B).

TABLE I

ABILITY OF 25-OHD_3 AND THE 25-OHD_3-26,23 LACTONE STEREOISOMERS TO COMPETITIVELY INHIBIT BINDING OF 25-OH[^{3}H]D_3 TO THE 4.2S RAT PLASMA VITAMIN D BINDING PROTEIN ($K_i{}^a$) OR COMPETITIVELY INHIBIT 1,25-$(OH)_2$[^{3}H]D_3 BINDING TO THE 3.7S 1,25-$(OH)_2$D CYTOSOL RECEPTOR ($K_i{}^b$)†

Compound	($K_i{}^a$)*		($K_i{}^b$)†	
	-nM-		-nM-	
25-OHD_3	5.0	(0.59)	40	(8.0)
in vivo 25-OHD_3-26,23 lactone	1.1	(0.13)	1904	(380)
25*R*-OHD_3-26,23*S* lactone	1.0	(0.13)	1920	(383)
25*S*-OHD_3-26,23*R* lactone	2.7	(0.32)	235	(47)
25*R*-OHD_3-26,23*R* lactone	41.6	(4.9)	563	(113)
25*S*-OHD_3-26,23*S* lactone	54.3	(6.4)	27	(5.4)

*The number in parentheses is the amount (ng) of compound which results in 50% competition for 25-OH[^{3}H]D_3 binding sites.

†The number in parentheses is the amount (ng) of compound which results in 50% competition for 1,25-$(OH)_2$[^{3}H]D_3 binding sites.

DISCUSSION

In vivo 25-OHD_3-26,23 lactone is known to be a more potent competitor than 25-OHD_3 for binding sites on the plasma vitamin D binding protein. However, early experiments demonstrating this relationship were done without knowledge of the C-23 and C-25 stereochemistries. During the period of time when synthesis and stereoconfirmation of the four possible 25-OHD_3-26,23 lactone stereoisomers was being accomplished, Hollis et al. (10) reported that 25-OHD_3-26,23 lactone could be synthesized *in vitro* from 25,26-$(OH)_2D_3$ isolated from cow plasma. Since 25,26-$(OH)_2D_3$ has the 25*S* configuration (11), the naturally-occurring isomer of 25-OHD_3-26,23 lactone was hypothesized to be 25*S*-OHD_3-26,23*R* lactone (8). However, in view of the similar binding characteristics in two different radioligand binding assays and confirmation of its comigration with 25*R*-OHD_3-26,23*S* lactone synthetic standard, our data unquestionably demonstrate that the naturally-occurring stereoisomer of 25-OHD_3-26,23 lactone has the 25*R*, 23*S* configuration. These data are further

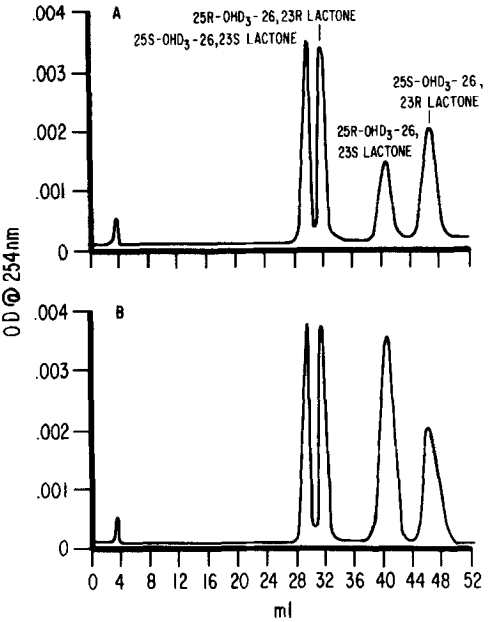

Figure 1. (A) UV chromatogram of the four stereoisomers of 25-OHD_3-26,23 lactone following HPLC on a silicic acid column developed in 4/96 isopropanol/hexane at a flow rate of 2 ml/min. (B) UV chromatogram of the same mixture described in (A) with the addition of 100 ng of *in vivo*-generated 25-OHD_3-26,23 lactone.

substantiated by the recent experiment of Napoli et al. (6) who showed that 23S,25-$(OH)_2D_3$ rather than 25S,26-$(OH)_2D_3$ was a biologic precursor to 25-OHD_3-26,23 lactone. In addition, Ishizuka et al. (12) demonstrated co-migration of *in vivo* 25-OHD_3-26,23 lactone with 25R-OHD_3-26,23S lactone in an HPLC system that separates the 25R, 23S and 25S, 23R stereoisomers of 25-OHD_3-26,23 lactone. The compound 25S-OHD_3-26,23S lactone, however, was not available to Ishizuka et al. (12) for HPLC comigration studies and, therefore, unambiguous evidence of the 25-OHD_3-26,23 lactone stereochemistry was lacking in their studies.

The superior binding (relative to 25-OHD_3) of 25S-OHD_3-26,23S lactone to the 1,25-$(OH)_2D$-specific cytosol receptor was unexpected in view of the poor competition provided by the other stereoisomers. The current paucity of knowledge with regard to the cytosol receptor ligand binding interactions circumvents the formation of an intelligible hypothesis to explain this interaction. However, 1α-hydroxylation of the 25S, 23S stereoisomer may result in a compound with a high binding affinity for the 1,25-$(OH)_2D$ cytosol receptor.

REFERENCES

1. Horst, R. L., Shepard, R. M., Jorgensen, N. A., and DeLuca, H. F. (1979) J. Lab. Clin. Med. 93, 277-285.
2. Wichmann, J. K., DeLuca, H. F., Schnoes, H. K., Horst, R. L., and Shepard, R. M. (1979) Biochemistry 18, 4775-4780.
3. Horst, R. L., Littledike, E. T., Gray, R. W., and Napoli, J. L. (1981) Anal. Biochem. 116, 184-203.
4. Horst, R. L. (1979) Biochem. Biophys. Res. Commun. 89, 286-293.
5. Horst, R. L., and Littledike, E. T. (1980) Biochem. Biophys. Res. Commun. 93, 149-154.
6. Napoli, J. L., Pramanik, B. C., Partridge, J. J., Uskoković, M. R., and Horst, R. L. (1982) J. Biol. Chem. (in press).
7. Wichmann, J. K., Paaren, H. E., Fivizzani, M. A., Schnoes, H. K., and DeLuca, H. F. (1980) Tetrahedron Lett. 21, 4667-4668.
8. Morris, D. S., Williams, D. H., and Norris, A. F. (1981) J. Chem. Soc. Commun. 9, 424-425.
9. Reinhardt, T. A., Horst, R. L., Littledike, E. T., and Beitz, D. C. (1982) Biochem. Biophys. Res. Commun. 106, 1012-1018.
10. Hollis, B. W., Roos, B. A., and Lambert, P. W. (1980) Biochem. Biophys. Res. Commun. 95, 520-528.
11. Partridge, J. J., Shiuey, S.-J., Chadha, H. K., Baggiolini, E. G., Blount, J. E., and Uskoković, M. R. (1981) J. Am. Chem. Soc. 103, 1253-1255.
12. Ishizuka, S., Yamaguchi, H., Yamada, S., Nakayama, K., and Takayama, H. (1981) FEBS Lett. 134, 207-211.