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ISOLATION AND IDENTIFICATION OF 23,25-DIHYDROXY-24-OXO-VITAMIN D_3: A METABOLITE OF VITAMIN D_3

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A new metabolite of vitamin D_3 has been isolated in pure form from incubations of rat kidney homogenates with 25-hydroxyvitamin D_3 [25-OH-D_3]. It was identified as 23,25-dihydroxy-24-oxo-vitamin D_3 [23,25$(OH)_2$-24-oxo-D_3] by means of ultraviolet absorption spectrophotometry and mass spectrometry. Also, 25-OH-D_3-26,23-lactone and 24R,25-dihydroxyvitamin D_3 were obtained from the same incubation mixtures. The enzyme activity responsible for the conversion of 25-OH-D_3 to 23,25$(OH)_2$-24-oxo-D_3 was induced by perfusion of the kidneys *in vitro* with 50 nM 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$].

Vitamin D_3 undergoes metabolic conversion before exerting its biological effects (1). It is first hydroxylated in the liver to form 25-hydroxyvitamin D_3 [25-OH-D_3]; subsequently it is further processed by the kidney to yield either 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] or 24R,25-dihydroxyvitamin D_3 [24R,25$(OH)_2D_3$]. 1,25$(OH)_2D_3$ is recognized as the biologically active form of vitamin D_3, whereas the biological role of 24R,25$(OH)_2D_3$ is still a matter of controversy (2). Several studies have demonstrated that 1,25$(OH)_2D_3$ can induce the 25-OH-D_3-24-hydroxylase both *in vivo* in kidneys (3) from vitamin D-depleted animals as well as in kidney cells grown in cell culture (4-6). Using isolated perfused

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kidneys from vitamin D-replete rats, we recently found (7) that exposure of the kidneys to $1,25(OH)_2D_3$ *in vitro* results in increased production of $24R,25(OH)_2D_3$ as well as another unknown vitamin D compound, which we have provisionally designated Peak X (7). Further, the induction of the enzyme(s) which convert 25-OH-D_3 to Peak X was completely abolished by the presence of actinomycin D or cycloheximide in the perfusate (7). We now report the isolation and chemical characterization of Peak X.

MATERIALS AND METHODS

Chemicals: 25-OH-D_3 was a gift from Upjohn Co. (Kalamazoo, MI); $24R,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were kindly provided by Dr. M. Uskoković, Hoffmann-LaRoche, Inc. (Nutley, NJ) and 25R-OH-D_3-26,23S-lactone was a gift from Dr. D. Williams, Cambridge, UK.

Animals: Male Wistar rats (300 g) were purchased from Harlam Industries Inc. (Indianapolis, IN) and fed a regular rodent diet (sufficient in calcium, phosphorus and vitamin D).

Kidney perfusion technique and incubation procedure: The isolated kidney perfusion technique was performed as described before in detail (7-9). The right kidneys of three rats were perfused for 15 min with a Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) containing bovine serum albumin (6 g/dl), glucose (5 mM), a mixture of L-amino acids and $1,25(OH)_2D_3$ (5×10^{-8} M). Using the same buffer without $1,25(OH)_2D_3$ included, the perfusion of the kidneys was contained for 4 h. Then, a 20% homogenate (w/v) was prepared in 10 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.2 M sucrose and 10 mM KCl. A crude mitochondrial fraction was prepared by centrifugation of the homogenates at 3,000 x g for 10 min. The pellet was transferred to a 500 ml Erlenmeyer flask containing 50 ml of 20 mM HEPES buffer (pH 7.4), 100 mM KCl, 2 mM $MgSO_4$ and 20 mM succinate. The flask was gassed for 30 s with 100 % oxygen and subsequently, the substrate 25-OH-D_3 (20 µg) dissolved in 100 µl ethanol was added. After incubation at 37°C for 30 min in a shaking water bath, the reaction was terminated by the addition of CH_2Cl_2/MeOH (1:2, v/v). The extraction was carried out as described by Lambert *et al.* (10).

Chromatographic procedures: The concentrated lipid extracts were first chromatographed on Sephadex LH 20 columns (0.6 x 12 cm), which were eluted with n-hexane:chloroform:methanol (9:1:1, v/v). The fraction from 11 to 20 ml was evaporated under a stream of nitrogen. Further purification was achieved by high performance liquid chromatography (Waters Model 6000A equipped with a detector, Model 440, monitoring UV-absorbing material at 254 nm, Waters Associates, Milford, MA). A µPorasil column (0.39 x 30 cm, Waters) was equilibrated and eluted with hexane:isopropyl alcohol (98:2, v/v) at a flow rate of 2 ml/min (Figure 1). Peak X was eluted from 46 to 53 ml, $24R,25(OH)_2D_3$ from 72 to 84 ml. Another compound (Peak Z) was eluted sharply after Peak X (54 to 60 ml). Peak X as well as Peak Z were further purified by reverse phase HPLC using a µBondapak column (0.39 x 30 cm), which was eluted with water: methanol (35:65, v/v) at a flow rate of 2 ml/min. Peak X was eluted from 38 to 46 ml, Peak Z from 48 to 56 ml. Further purification was achieved by the straight phase HPLC system described above. Finally, the compounds were chromatographed on a Zorbax SIL column (0.41 x 25 cm, Du Pont, Wilmington, DE) which was equilibrated and eluted with dichloromethane:isopropyl alcohol (98:2, v/v) at a flow rate of 2 ml/min. Peak X as well as Peak Z were separately eluted at 14 to 15 ml, while authentic 25-OH-D_3 eluted at 11 to 11.5 ml.

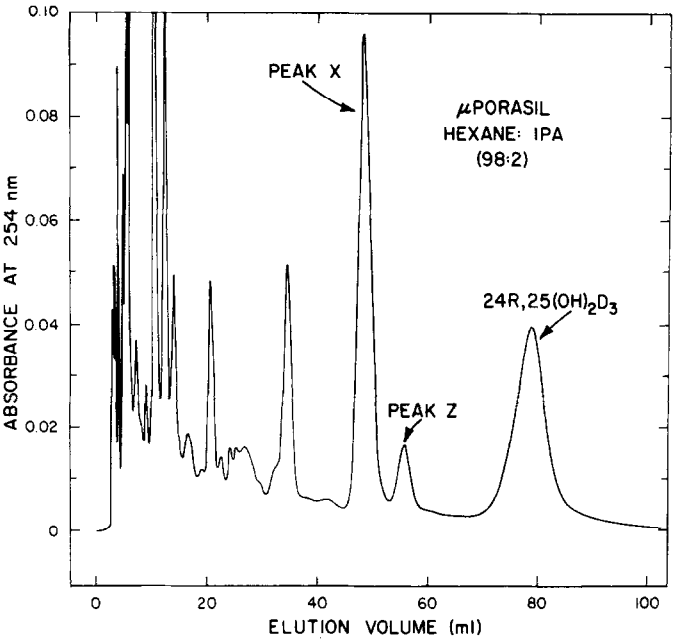

Figure 1: Purification of Peak X [23,25(OH)$_2$-24-oxo-D$_3$] by high performance liquid chromatography. A μPorasil column was eluted with hexane: isopropyl alcohol (98:2, v/v) at a flow rate of 2 ml/min.

RESULTS AND DISCUSSION

The ultraviolet absorption spectra of Peak X, Peak Z and putative 24R,25(OH)$_2$D$_3$ showed a λ_{max} at 265 nm and a λ_{min} at 228 nm demonstrating the presence of the 5,6-cis-triene-chromophore characteristic for the D vitamins. The overall yield from incubations with the kidney homogenates of 24 rats in total was 4.8 μg of Peak X, 0.7 μg of Peak Z and 6 μg of putative 24R,25(OH)$_2$D$_3$ after 5 chromatographic steps. The mass spectrum of Peak X is shown in Figure 2. Major ions, relative intensities and structural assignments are as follows: m/e 430, 22, M^+; 397, 6, M^+-H_2O-CH_3; 372, 6, M^+-C_3H_6O; 271, 8, M^+-side chain; 253, 12, 271-H_2O; 136, 85, (A ring + C_6 + C_7)$^+$; 118, 100, 136-H_2O. Characteristic vitamin D cleavage ions at m/e 271, 253, 136 and 118 indicate that the seco-steroid nucleus of the molecule has remained unchanged and that the metabolic alterations have been introduced in the side chain. The apparent molecular ion at m/e 430 suggests that three oxygens and one degree of unsaturation are present in the side chain of the 25-hydroxylated vitamin D molecule. To localize the hydroxyl functions in the metabolite, Peak X was converted to a tris-trimethyl-

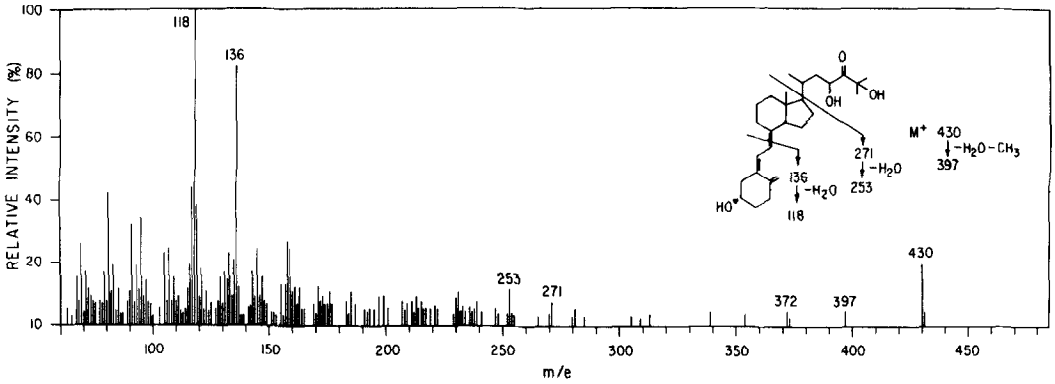

Figure 2: Mass spectrum of Peak X [23,25(OH)$_2$-24-oxo-D$_3$].

silylether derivative and then analyzed by mass spectrometry. Major ions, relative intensities and structural assignments are as follows: m/e 646, 8, M^+; 556, 1, M^+-HOTMS; 515, 1, M^+-C_3H_6-OTMS; 487, 3, M^+-$C_4O_2H_6$ TMS; 397, 2, 487-HOTMS; 307, 2, 397-HOTMS; 208, 7, (A ring + C_6 + C_7)$^+$; 131, 100, $C_3H_6OTMS^+$; 118, 19, 208-HOTMS. The apparent molecular ion at m/e 646 demonstrates that three hydroxyl groups are present as trimethylsilylether derivatives in the molecule. The ion at m/e 487 strongly suggests the presence of a hydroxyl group at C-23 (11, 12) and also the likelihood that there are no further alterations below C-23. The cleavage peak at m/e 131 (base peak) illustrates that no further modifications at carbon 26 and/or carbon 27 have occurred. Therefore, the structure of the new vitamin D metabolite is proposed to be 23,25(OH)$_2$-24-oxo-D$_3$.

Peak Z comigrated exactly on three different HPLC systems with authentic 25R-OH-D$_3$-26,23S-lactone. Further evidence for the structure of Peak Z was obtained by mass spectrometry. Major ions, structural assignments and relative intensities were as follows: m/e 428, 16, M^+; 410, 16, M^+-H_2O; 136, 76, (A ring + C_6 + C_7)$^+$; 118, 100, 136-H_2O. The mass spectrum of Peak Z is consistent with the structure of 25-OH-D$_3$-26,23-lactone as described by Wichmann et al. (13).

The putative 24R,25(OH)$_2$D$_3$ comigrated on HPLC in all three systems studied with authentic 24R,25(OH)$_2$D$_3$. The mass spectrum of the compound as well as the tris-trimethylsilyl-ether derivative demonstrated its identity with

authentic 24R,25(OH)$_2$D$_3$ (data not shown). Using the isolated kidney perfusion technique, we reported previously that 1,25(OH)$_2$D$_3$ induces in rat kidney the production of 24R,25(OH)$_2$D$_3$ and another metabolite which comigrated with authentic 25-OH-D$_3$-26,23-lactone on both HPLC systems applied (14). However, more rigorous chromatography revealed that this compound is, in fact, not identical with authentic 25-OH-D$_3$-26,23-lactone; it was designated as Peak X. The production of 25-OH-D$_3$-26,23-lactone was not detectable in these experiments where 25-OH-[26,27-^{3}H]-D$_3$ was employed as substrate, possibly due to isotope effects (15). It will be interesting to learn by further experiments whether 24R,25(OH)$_2$D$_3$ can serve as a precursor of 23,25(OH)$_2$-24-oxo-D$_3$. It was shown before that 24R,25(OH)$_2$D$_3$ can be oxidized to 25-OH-24-oxo-D$_3$ by chick kidney homogenates (16). It is feasible that 25-OH-24-oxo-D$_3$ might undergo 23-hydroxylation to yield 23,25-(OH)$_2$-24-oxo-D$_3$. Also, the biological activity of this new metabolite is of interest. Further studies on these issues are now underway in our laboratories.

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