Isolation and Chemical Characterization of Two New Vitamin D Metabolites Produced by the Intestine

1,25-DIHYDROXY-23-OXO-VITAMIN D₃ AND 1,25,26-TRIHYDROXY-23-OXO-VITAMIN D₃*

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Two new vitamin D metabolites were isolated in pure form from incubations of 53 nm 1,25-dihydroxyvitamin D₃ with homogenates of small intestinal mucosa of vitamin D-replete chicks. The birds were injected intravenously with 8 to 9 nmol of 1,25-dihydroxyvitamin D₃/100 g body weight 5 to 8 h before death. The isolation involved methanol-chloroform extraction and four successive chromatographic procedures (Sephadex LH-20 and high performance liquid chromatography). Chemical structures of the metabolites are proposed on the basis of (a) their chromatographic behavior, (b) their mass spectra, and (c) ultraviolet absorption spectra. They are identified as 1α,25-dihydroxy-23-oxo-vitamin D₃ and 1α,25,26-trihydroxy-23-oxo-vitamin D₃. Neither of the two new metabolites is produced by the intestinal mucosa when 1,25S,26-trihydroxyvitamin D₃ is used as a substrate.

It has been firmly established that 1α,25-dihydroxyvitamin D₃ is a metabolite of vitamin D₃ active in intestinal calcium transport and bone mineral mobilization (2). Recent studies have revealed that 1,25(OH)₂D₃ may undergo further metabolism to several additional seco-steroids, including (i) 1α,24R,25-trihydroxyvitamin D₃ (3); (ii) side chain oxidation and cleavage to yield CO₂ (4) and a shortened side chain, C₂₃-COOH containing seco-steroid designated calcitroic acid (5); (iii) to 1,25,26-trihydroxyvitamin D₃ (6); or (iv) to 1,25-dihydroxyvitamin D₃-26,23-lactone (1). Another recently chemically characterized vitamin D metabolite is 23,25-dihydroxyvitamin D₃ (7) which also has been postulated to be an intermediate in the production of the 1,25(OH)₂D₃-26,23-lactone (8). Collectively, all these metabolites have been proposed as leading either to degradation and/or inactivation of the active form of vitamin D₃, 1,25(OH)₂D₃ (9-11).

Very recently, calcitroic acid was stated to be the major metabolite of 1,25(OH)₂D₃ present in the small intestine following the administration of 1,25(OH)₂D₃ to rats (12); these results suggested that the small intestine may have the complement of enzymes necessary for the production of calcitroic acid. It has been reported recently that the small intestinal mucosa can metabolize 1,25(OH)₂D₃ also to 1,25(OH)₂D₃-26,23-lactone and an unidentified metabolite designated Peak-X (13). This Peak-X metabolite has been demonstrated clearly to be a major metabolite in 1,25(OH)₂D₃ catabolism in the intestine; in vitro production of Peak-X was found to be markedly increased by predosing the animals with 1,25(OH)₂D₃. During the course of the present studies, another heretofore unidentified metabolite of 1,25(OH)₂D₃, designated 1,25-Prime, was also found to be produced by homogenates of chick intestinal mucosa. Thus, our present investigation has focused on both the in vitro production and the chemical identification of Peak-X and 1,25-Prime from 1,25(OH)₂D₃. We report the isolation and identification of two new metabolites of vitamin D₃ as well as 1,24,25(OH)₃D₃ obtained from the incubation of 1,25(OH)₂D₃ with homogenates of small intestinal mucosa from partially vitamin D-deficient chicks; the new compounds were determined to be 1,25-dihydroxy-23-oxo-vitamin D₃ and 1,25,26-trihydroxy-23-oxo-vitamin D₃.

MATERIALS AND METHODS

RESULTS

Isolation of the Metabolites—A flow chart of the procedures used in the isolation of the three metabolites is presented in Fig. 1. By these procedures, in the first isolation trial, approximately 1.7 pg, 2.3 pg, and 11 pg of 1,25-Prime, Peak-X, and an unidentified metabolite designated Peak-X (13). This Peak-X metabolite has been demonstrated clearly to be a major metabolite in 1,25(OH)₂D₃ catabolism in the intestine; in vitro production of Peak-X was found to be markedly increased by predosing the animals with 1,25(OH)₂D₃. During the course of the present studies, another heretofore unidentified metabolite of 1,25(OH)₂D₃, designated 1,25-Prime, was also found to be produced by homogenates of chick intestinal mucosa. Thus, our present investigation has focused on both the in vitro production and the chemical identification of Peak-X and 1,25-Prime from 1,25(OH)₂D₃. We report the isolation and identification of two new metabolites of vitamin D₃ as well as 1,24,25(OH)₃D₃ obtained from the incubation of 1,25(OH)₂D₃ with homogenates of small intestinal mucosa from partially vitamin D-deficient chicks; the new compounds were determined to be 1,25-dihydroxy-23-oxo-vitamin D₃ and 1,25,26-trihydroxy-23-oxo-vitamin D₃.

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Fig. 1. Flow chart of procedures employed for the isolation of 1,25(OH)2-23-oxo-D3, 1,25,26(OH)3-23-oxo-D3, and 1,24,25(OH)3D3. All UV measurements were made in ethanol.

Fig. 2. Ultraviolet spectra of isolated vitamin D metabolites. A, 1,25(OH)2-23-oxo-D3; B, 1,25,26(OH)3-23-oxo-D3; C, 1,24,25(OH)3D3. All UV measurements were made in ethanol.

Fig. 3. Proposed structures of the three isolated vitamin D metabolites.
migrated with 1,24,25(OH)3-D3 during Sephadex LH-20 chromatography, but was separated from it by HPLC. As shown in Fig. 5C, Peak-X migrated as a single peak on HPLC developed with isopropyl alcohol:dichloromethane (1:10) (v/v). However, when this pure peak (based on the UV tracing of the chromatogram as well as the UV absorption spectroscopic analysis (Fig. 2B)) after being heated to 50-60 °C was rechromatographed on HPLC with isopropyl alcohol:dichloromethane (1:10) (v/v) a "split" peak was observed (Fig. 6A). In the process of warming the pure sample (Fig. 5C) to change solvents for the UV absorption spectrophotometric analysis, the UV form of the Peak-X metabolite apparently underwent racemization to yield a mixture of the syn (Peak-X) and anti (Peak-X-Prime) forms of 1,25,26(OH)3-23-oxo-D3. These two anomeric forms could then be separated from one another by HPLC with isopropyl alcohol:hexane (1:19) (v/v) (Fig. 6B). As shown in Figs. 8, A and B, and the analysis of the fragmentation patterns (Figs. 10 and 11), this apparently anomalous chromatographic behavior and the very close similarity of the mass spectra of Peak-X and Peak-X-Prime metabolites could be explained by assignment of the syn (Peak-X) and anti (Peak-X-Prime) forms of the hemiketal ring of 1,25,26(OH)3-23-oxo-D3. The introduction of the hydroxyl on C-26 of 1,25,26(OH)3-23-oxo-D3 makes C-25 asymmetric; the absolute configuration at C-25 is as yet not known. However, several studies on 25(OH)D3-26,23-lactone (6, 8, 18) showed that the C-25 hydroxyl has the R orientation, it might be proposed that this is also the case in 1,25,26(OH)3-23-oxo-D3.

The isolation and characterization of 1,24,25(OH)3-D3 from homogenates of the chick intestinal mucosa incubated with 1,25(OH)2-D3 was also straightforward. This report is the second to describe the characterization of this metabolite from the intestine; the other report describes its isolation from incubations of rat intestinal homogenates (17).

The enzymatic complement of the chick intestinal mucosa to effect metabolic transformations of the hormonally active form of vitamin D, namely 1,25(OH)2-D3, is impressive. The intestinal mucosa can convert 1,25(OH)2-D3 to 1,24,25(OH)3-D3 (17, and this report), to 1,25(OH)2-23,25-oxo-D3 (13), to 1,25(OH)2-24-25-oxo-D3 (this report), and to 1,25,26(OH)3-23-oxo-D3 (this report). The biological activity of 1,25(OH)2-23-oxo-D3 and 1,25,26(OH)3-23-oxo-D3, as well as the physiological regulation of the enzymes necessary for their production from 1,25(OH)2-D3 will be described in another report.

The pathways (6) for the metabolism of vitamin D are becoming increasingly complex. To date, some 16 metabolites of vitamin D have been isolated and chemically characterized (1-10). The three major organs responsible for these metabolic transformations are the liver, kidney, and intestine; however, there is increasing evidence that under some physiological conditions, other tissues such as the placenta (19) and bone (20) may also effect metabolic transformation of one or other D-metabolites. A recent evaluation of the literature (21) suggests that there are an additional 15 to 20 unidentified peaks on various chromatograms which await eventual purification and chemical characterization.

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REFERENCES
Isolation and Functional Characterization of New Vitamin D Metabolites Produced by the Interleukin-1,25-Dihydroxyvitamin D3 and 1,25,26-Dihydroxyvitamin D3

M. A. F. Hamadeh, J. R. Krome, C. G. H. Cooper and A. W. Howells

Vitamin D and Its Metabolites

Vitamin D is a hormone that plays a critical role in health. It is produced by the body and can be consumed through diet. A vitamin D deficiency can lead to health problems, including bone disease and increased risk of certain cancers. The vitamin D system includes two main forms: vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). Vitamin D3 is produced in the skin when it is exposed to sunlight. It is then converted into its active form, 1,25-dihydroxyvitamin D3 (vitamin D3), which is essential for calcium absorption and bone health. Vitamin D2 is produced by plants and fungi and is not as effective as vitamin D3. Vitamin D is also present in some foods, such as fatty fish, egg yolks, and fortified milk.

Figure 1: HPLC purification of a new vitamin D metabolite designated Peak-1. (A) Chromatographic profiles of authentic vitamin D3, Peak-1, and 1,25(OH)2D3. (B) HPLC chromatogram showing the retention times of authentic vitamin D3 and Peak-1. (C) Mass spectrum of Peak-1. (D) Characterization of Peak-1 by NMR spectroscopy. (E) Structure of Peak-1. (F) Biological activity of Peak-1. (G) Structure of 1,25(OH)2D3.

The figure shows the purification and characterization of a new vitamin D metabolite, Peak-1, which was isolated from a variety of sources, including human and animal tissues. The metabolite was purified using high-performance liquid chromatography (HPLC) and mass spectrometry. The mass spectrum and NMR spectra of Peak-1 were consistent with a 24-oxo-vitamin D3 metabolite. The biological activity of Peak-1 was determined using the calcium mobilization assay, which showed that Peak-1 had a similar effect to 1,25(OH)2D3.

References


After obtaining a UV spectrum (see Figure 7), the elution fraction was dried under a stream of N₂ gas to a water bath at 30 to 40 °C, then chromatographed again on the column with a mixture of isopropyl alcohol and dichloromethane (1:19, v/v) (Fig. 8). Since the HPLC profile showed two peaks with retention times of 8.5 and 15.3 min, these peaks were combined and applied to a column developed with isopropanol–acetic anhydride (11:9, v/v). The first peak which migrated between 9.5 and 15.5 min was collected and designated as Peak-1. The second peak which migrated between 15.5 and 23 min was collected and designated as Peak-2. The UV spectra of these two fractions were compared with those of 1,25(OH)₂D₃ and the results were stored at -20 °C before more experimental procedures.

In separate experiments 1,25(OH)₂D₃ (0.6 mg), was also included with the initial sections of homogenate. The purpose of these injections was to determine whether the new metabolites, 1,25(OH)₂D₃ and Peak-2 could be produced from 1,25(OH)₂D₃. The incubation conditions were similar to those given in Figure 3, but the 1,25(OH)₂D₃ was added to each reaction mixture (10 ml) at 20 ml of ethanol at the beginning of the incubation and agitated at 5 and 10 min after the incubation started. The metabolic profile of the 1,25(OH)₂D₃ incubated with homogenate at 50 °C for 10 min with a 10 times higher sensitivity than the sensitivity used for monitoring the kinetics of the metabolism observed when 1,25(OH)₂D₃ was the substrate. As evident in Figure 9A, a metabolite Peak-2 was formed from 1,25(OH)₂D₃ (0.6 mg) as the substrate. This indicates that 1,25(OH)₂D₃ is not likely as intermediate between 1,25(OH)₂D₃ and Peak-2.

Figure 9: HPLC chromatography of purified vitamin D metabolites. Peak 2 (subsequently identified as 1,25,26(OH)₃D₃) was detected by HPLC of the column. (Panel A) Amino acid system that shows an increase in the retention time of the metabolites. The split peak eluting at 8.9-9.3 min was designated Peak-2. The first peak eluted at 23-23.5 min is the 1,25(OH)₂D₃ used as the substrate. The second peak eluted at 24-26.5 min and is designated as Peak-2.

Data Spectrometry - The mass spectra of the isolated 1,25(OH)₂D₃ is shown in Figure 7. Major ions, product ion fragments and structural assignments are: m/z 430, 62, 64, 412, 313, 357 (59%), 296, 249, 195, 189, 186, 154, 129, 118, 108, 83, 129, 69, 58, 40, 39, 38, 37, 29, 27, 25, 19, 17, 11, 9, 8, 6, 4, 3, 2, 1. Product ion fragments were selected for mass spectrometry. The major fragment ions at m/z 118 and 357 suggest that the metabolite is a dioxo vitamin D which is consistent with the UV spectra. The major fragment ions at m/z 118 and 357 suggest that the metabolite is a dioxo vitamin D which is consistent with the UV spectra.
This idea can be further supported by the following analysis. 1,25,26(OH)_{2}-23-oxo-vitamin D_{3} has two configurations: the 1α and 25α forms. Since the C-3 and C-25 configuration are both asymmetric, the naturally occurring 23-hydroxy compound has a fixed configuration in either the 1α- or 25α-form. Further, this naturally occurring 23-oxy compound will readily undergo racemization upon heating to produce a mixture of the 1α and 25α forms. It is reasonable to expect that the 1α form of the 23-oxy compound, which makes a hydrogen bond between the hydroxy groups attached to C-23 and C-24, will have a higher stability against the electron impact ionization than the corresponding 25α form which cannot form a hydrogen bond. Therefore it is possible to propose that 1α Peak-5 and 25α Peak-3 are the 1α and 25α form of the 23-oxy and 24-hydroxyl of 1,25,26(OH)_{2}-23-oxo-vitamin D_{3}, respectively, and that Peak-8 cannot give a molecular ion at m/z 466 due to its high inherent stability to electron impact ionization. This speculation can be supported by the presence of trace amounts of 1α- and 25α-fragments in the high-resolution mass spectrum (see Figure 6). The reactivity of 1α- and 25α-fragments is also shown in Figure 3.B, peak 1 and peak 7-8 show almost the same retention time as the column when 23-hydroxy cholesterol (3-10, m/z 586) was used, while peak 1-2 shows a peak prior to peak 6, when the column was eluted with 25α-cholesterol (11.69, m/z 586). Since the hydrogen bond cannot be formed in 25α-cholesterol or even if hydrogen bond is formed in cholesterol, the different behavior of the 23-oxy compound on the same column is probably due to a difference in the interaction of the hydroxy group attached to C-23 and C-24 position of the sterol with the stationary phase.

This idea was further supported by the analysis of the high-resolution mass spectrum of the positive-ion spectrum of 1α,25,26(OH)_{2}-23-oxo-vitamin D_{3} as shown in Figure 5. The molecular ion at m/z 466 and the series of 1α-fragments from m/z 386, 358, 328, 308, 286, 260, 244 and 230 are not present in the mass spectrum of 1α,25,26(OH)_{2}-23-oxo-vitamin D_{3}, while the 25α-fragments are present. Based on these considerations, it can be understood that the loss which could be observed in the spectrum is due to the cleavage of 1α-hydroxyl and the 25α-hydroxyl at m/z 466, 434, 412, 390 as well as the loss (m/z 586, 522) produced via hemiester cleavage from the ion at m/z 586 interchangeably. This suggests that Peak-8-Prime has a similar chemical structure to Peak-6, probably the 1α,25,26(OH)_{2}-23-oxo-vitamin D_{3} hemiester but with a different configuration with respect to the hydroxy group attached to C-23 from that of the hydroxy on C-23 of the hemiester of Peak-6.
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