The Elution Profile of Vitamin D₂ Metabolites from Sephadex LH20 Columns

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A metabolite of vitamin D₂ in the tissues of primates fed this vitamin co-migrates to a significant extent with 24,25-dihydroxyvitamin-D₃ on columns of Sephadex LH20, with chloroform/n-hexane (60/40 by vol) as eluent. In individuals receiving vitamin D₂ and in individuals with scant exposure to sunlight this metabolite, which we believe to be 25,26-dihydroxyvitamin-D₂, may yield falsely higher concentrations of apparent 24,25-dihydroxyvitamin-D₃ if separation on LH20 is used.

Two assays (1,2) are currently used to measure 24,25-(OH)₂D₃,³ one of the metabolites of D₃ produced in the kidney. In both methods Sephadex LH20 chromatography is used to separate the vitamin and its metabolites. There are two important differences between the methods: Taylor et al. (1) used 30-cm columns and two chromatographic steps, whereas Haddad et al. (2) used 15-cm columns and one chromatographic step.

Measured values of 24,25(OH)₂D₃ reported by these two groups differ by a factor of two. Taylor (3) reports a mean ± SE for 24,25(OH)₂D₃ of 1.63 ± 0.19 μg/L (range, 0.44–3.39) for 19 normal subjects. Haddad et al. (2), however, found the normal value in a sample of 42 healthy subjects to be 3.7 ± 0.2 μg/L. In addition, Haddad et al. (2) claim that anephric patients have serum 24,25(OH)₂D₃ concentrations of 3.0 ± 0.7 μg/L, whereas Taylor (3) was unable to detect the presence of this metabolite in anephric kidneys. The kidney is known to produce 24,25(OH)₂D₃ (4); however, in view of their findings Haddad et al. (2) claim that some extrarenal synthesis of this metabolite must occur. De Luca (5) supports this claim; he reports a value of 0.76 ± 0.18 μg of 24,25(OH)₂D₃ per liter in anephric patients. Taylor (3), on the other hand, suggests that this metabolite is only produced in the kidney.

Interest in this dihydroxy metabolite of D₃ is increasing and it has been suggested recently (6) that 24,25(OH)₂D₃ may be a calcium-regulating hormone in man.

In the course of an investigation of D₂ metabolism in New World primates, we observed a phenomenon which might reconcile these conflicting claims. We report here that if chloroform/hexane (60/40) is used as the solvent for Sephadex LH20 chromatography, D₂ metabolites migrate more rapidly than do their corresponding D₃ analogs.

The difference in elution profiles may help explain the higher 24,25(OH)₂D₃ values reported by Haddad et al. (2).

Materials and Methods

Animals

Eight female Capuchin monkeys (Cebus apella), previously maintained on a diet (7) deficient only in vitamin D, received oral doses of isotopically labeled D₂ and/or D₃. Four animals received ³H- and ¹⁴C-D₃; two received ³H-D₂; and the remaining two received both ¹⁴C-D₂ and ³H-D₃. The D₂ and D₃ isotopes were fed on an equivalent weight/weight basis, which was equivalent to 0.51 μg of vitamin D per kilogram of body weight.

Radiochemicals

[⁴⁺⁻¹⁴C]Vitamin D₃ (36 Ci/mol), [1α,2α-³⁷H]vitamin D₃ (12.3 kCi/mol), and 25-hydroxy[26,27-³H]vitamin D₃ (11.9 kCi/mol) were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. 24,25-Dihydroxy[26,27-³H]vitamin D₃ and 1,25-dihydroxy[26,27-³H]vitamin D₃ were gifts from Drs. E. B. Mawer and C. Taylor, Department of Medicine, University of Manchester, U.K.

[³α,⁻³βH]Vitamin D₂ was synthesized by the sodium boro[³H]hydride (11.25 kCi/mol) reduction of 3α-acetoxyergosta-3,5,7,22-tetraene (8, 9).

Plasma and Tissue Preparation

The animals were bled at 2, 4, 8, 24, and 48 h after dosing and the plasma (heparinized) was obtained. Liver, kidney, intestine, muscle, and bone were removed from animals killed by intravenous administration of a barbiturate (Euthanal; May & Baker, Dagenham, U.K.) 48 h after receiving the isotopes. The tissues were washed, frozen for periods of one to two weeks at −20 °C, then homogenized in phosphate buffer (pH 7.6, 20 mmol/L) and the radioactivity was extracted by the method of Bligh and Dyer (10).

Chromatography

Tissue lipid extracts were chromatographed on Sephadex LH20 columns (15, 30, and 42 × 0.9 cm), with chloroform/ hexane (60/40 by vol) as eluent.

Radioactivity

Total radioactivity of the column eluent was measured with an Intertechnique SL/30 Liquid Scintillation Spectrometer and a scintillant consisting of 4.0 g of 2,5-diphenyloxazole, 0.1
Results

Elution profiles representative of the lipid extracts of the livers of two Capuchin monkeys are shown in Figure 1. One primate received $^3$H-D$_3$ and $^{14}$C-D$_3$, the other $^3$H-D$_2$. The Figure shows the elution profile of only $^1$C for the first animal and $^3$H for the second, 48 h after the radioisotope dose. The dihydroxy metabolites of the vitamins were present in greater proportions in the liver extracts, thus we chose this tissue to illustrate their migration on Sephadex LH 20.

The metabolites of D$_3$ were identified by using pure specimens of biosynthesized material. Because 25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$ co-migrate on Sephadex LH 20 (1), we used the elution profile of $^3$H-1,25(OH)$_2$D$_3$ for the location of both metabolites. Identification of D$_2$ metabolites was more complicated. D$_2$ itself was identified by using a prepared standard and was observed to elute earlier than D$_3$. The first peak appearing after D$_2$ was assumed to be 25(OH)D$_2$. When we analyzed 2-mL plasma samples taken from this and other animals 2, 4, 8, 24, and 48 h after administration of the isotope, we found that the proportion of radioactivity in this peak increased as that in D$_3$ decreased. Eventually the peak identified as 25(OH)D$_3$ contained most of the circulating radioactivity, and it can be seen from the Figure that it, too, migrates more rapidly than 25(OH)D$_3$. No peaks other than those shown in the Figure were observed in any of the plasma or tissue samples analyzed.

As for the other two major $^3$H-containing peaks derived from $^3$H-D$_2$, no positive identification was possible. 24,25(OH)$_2$D$_2$ has only recently been identified as a biological metabolite, and no standard was available at the time of this study (11). 25,26(OH)$_2$D$_2$ has not yet been identified as a major biological metabolite of D$_2$. We believe, for the following reasons, that it is reasonable to assume that the peaks identified in the Figure as A and B are probably 24,25(OH)$_2$D$_2$, and 25,26(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_2$, respectively. The peaks labeled A and B were only significant in blood samples taken 8 h after isotopically labeled D$_2$ was fed to the animals. The same was true for the peaks produced by plasma samples from animals receiving labeled D$_3$ and which co-chromatographed with 24,25(OH)$_2$D$_3$, and 25,26(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$. We were able to establish that D$_2$ and 25(OH)D$_2$ migrate more rapidly than the corresponding D$_3$ analogs. Our peaks A and B, if they follow a similar elution sequence to the D$_3$ metabolites, would seem to fit the criteria for the D$_2$ analogs of 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$. Peak B could also contain 1,25(OH)$_2$D$_2$, and positive identification of these metabolites is currently in progress.

Similar elution profiles to those shown in Figure 1 were obtained in the analysis of the other tissues, although the proportion of the metabolites varied from one tissue to another. The detailed results of the tissue analysis will be reported elsewhere.

Discussion

The Figure makes it quite clear that the peak we believe may contain 25,26(OH)$_2$D$_2$ overlaps with that of 24,25(OH)$_2$D$_3$, even on a column 42 cm long. Shortening the column had the effect of increasing the overlap. Accordingly, we believe that the two principal assays (1,2) in use for measuring
24,25(OH)2D3 are inadvertently measuring 25,26(OH)2D2 as well.

In the U. K., where food fortification with D2 is not common practice, the D2 metabolite may be of little consequence. In the United States, however, where food fortification with D2 is widespread, inclusion of 25,26(OH)2D2 in the assay for 24,25(OH)2D3 might yield higher concentrations for the D3 metabolite. Mawer (12) has shown that 24,25(OH)2D3 and 25,26(OH)2D2 concentrations are comparable in the serum of vitamin D-replete man. It would thus seem reasonable to assume that in the United States 25,26(OH)2D2 could be present in significant quantities in human serum. If this D2 metabolite were present in sufficient quantity, it could account for the higher 24,25(OH)2D3 values reported by Haddad et al. (2) for U. S. subjects as compared with the lower U. K. value recorded by Taylor (3). It may also help explain the ratio of 25(OH)D2:24,25(OH)2D3 which Haddad et al. (2) claim is 5:1, whereas Taylor (3) suggests it is 10:1.

Finally, Haddad et al. (2) and De Luca (5) claim that there is extrarenal production of 24,25(OH)2D3, whereas Taylor (3) says there is none. If 25,26(OH)2D2 is present in the assay of Haddad et al. (2) it may account for the 24,25(OH)2D3 (3.0 ± 0.7 μg/L) value reported for anephrics. Taylor (3) could find no 24,25(OH)2D3 in these subjects. We would suggest that it is essential to identify the contribution 25,26(OH)2D2 may make to 24,25(OH)2D3 concentrations where D2 food fortification is practiced, and that this should be done before extrarenal production of the dihydroxy metabolite of D2 is assessed.

References