

C-3 Epimers Can Account for a Significant Proportion of Total Circulating 25-Hydroxyvitamin D in Infants, Complicating Accurate Measurement and Interpretation of Vitamin D Status

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Context: We have recently introduced liquid chromatography-tandem mass spectrometry (LC-MS/MS) for 25-hydroxyvitamin D₂ (25OHD₂) and 25OHD₃ testing. During subsequent clinical use, we identified significantly elevated results in some infants. We hypothesized this might represent assay interference caused by C-3 epimers of 25OHD₂ or 25OHD₃.

Objective: Our aims were to 1) determine the prevalence of C-3 epimers of 25OHD₂ or 25OHD₃ in human serum, and 2) identify the patient populations that might be affected.

Study Design: We modified our LC-MS/MS method to allow detection of C-3 epimers. We retested specimens from four patient groups with the new method and an extracted RIA: 1) children less than 1 yr old, 2) children 1–18 yr old, 3) adults aged 20–87 yr with liver disease, and 4) adults aged 19–91 yr without liver disease.

Results: In 172 children from group 1 with detectable 25OHD₂ or 25OHD₃, we identified C-3 epimers in 39 (22.7%). The epimers contributed 8.7–61.1% of the total 25-OHD. There was an inverse relationship between patient age and epimer percentage ($r = 0.48$; $P < 0.002$). The RIA gave accurate 25-OHD results that correlated with the modified LC-MS/MS method. No C-3 epimers were detected in any of the other groups.

Conclusions: Significant concentrations of C-3 epimers of 25OHD₂ or 25OHD₃ are commonly found in infants. This can lead to overestimation of 25-OHD levels. Measurements in children less than 1 yr should therefore be performed with an assay that allows accurate detection of 25-OHD in the presence of its C-3 epimers. (*J Clin Endocrinol Metab* 91: 3055–3061, 2006)

VITAMIN D LABORATORY testing has increased significantly during the last decade because of an increasing awareness that vitamin D deficiency is very common and can increase fracture and, possibly, cancer risk (1–6). Measurement of total 25-hydroxyvitamin D (25-OHD; sum of 25OHD₂ and 25OHD₃) is the preferred test for assessing vitamin D status, because it has a long serum half-life and its concentration is considered to be in equilibrium with vitamin D body stores (7–10).

Unfortunately, there are substantial discrepancies between test results obtained with different 25-OHD assays. Most 25-OHD assays are competitive immunoassays or competitive assays based on vitamin D binding proteins (11, 12). For such assays, 25-OHD is a difficult analyte because of its hydrophobicity and relatively low serum concentrations (10, 12). This often necessitates sample extraction and concentration before analysis, potentially increasing assay variability. Furthermore, equal detection of 25OHD₂ and 25OHD₃ represents a challenge, in particular for assays based on vitamin D binding protein, because binding proteins from

many species show higher affinity for 25OHD₃ than for 25OHD₂ (13). As a consequence of all these factors, only 50–60% of the approximately 100 laboratories that participate in the international quality assessment scheme for vitamin D metabolites (DEQAS), meet performance criteria consistently, and the results obtained for the same sample can differ up to 2- to 4-fold, sometimes even for the same assay, when performed in different laboratories (11).

In an attempt to overcome these problems, we replaced our previous 25-OHD extracted RIA with a candidate reference method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) (14, 15). The method uses deuterated 25OHD₃ as an internal standard in each sample, thereby enabling correction of extraction variability and allowing accurate, separate measurement of 25OHD₂ and 25OHD₃. In our assay validation, it agreed well with our previous RIA, which some authors have considered a gold standard (Fig. 1) (12, 16).

In subsequent practice, we became aware of a small number of unexpectedly high 25-OHD results in infants. We became concerned that these might result from assay interference. Although most natural vitamin D metabolites and vitamin-D-related drugs are either too different in chemical structure to be a potential interferent or were excluded as such during our assay validation, some isomers of 25-OHD could potentially interfere. Epimers in particular have identical chemical structures except for a single site of molecular

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Abbreviations: CV, Coefficients of variation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; 25-OHD, 25-hydroxyvitamin D; VDR, vitamin D receptor.

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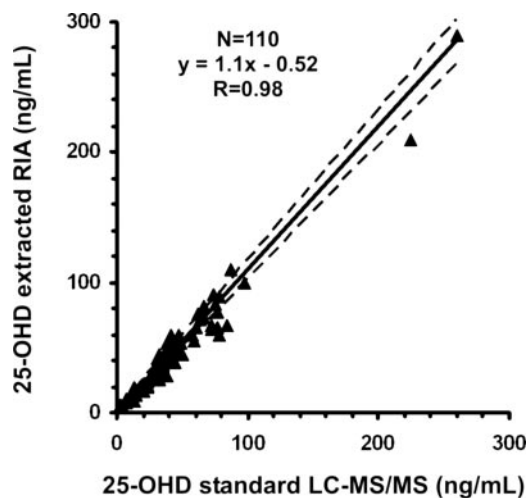


FIG. 1. Comparison of total 25-OHD measurements by standard LC-MS/MS and extracted RIA by scatter plot of total serum 25-OHD concentrations measured in 110 subjects by standard LC-MS/MS (*abscissa*) and extracted RIA (*ordinate*), with Passing-Bablok linear fit with 95% CI superimposed.

asymmetry and might therefore display very similar chromatography and give rise to the same MS/MS ion pairs. The C-3 epimers of 25OHD₂ and 25OHD₃, 3-epi-25OHD₂ and 3-epi-25OHD₃, caught our attention specifically, because C-3 epimerization of 25OHD₃ and its downstream metabolite, 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], have been reported in several human and animal cell lines (Fig. 2) (17–21). Furthermore, 3-epi-1,25-OHD has some unique properties of potential clinical significance. It is nearly as potent as 1,25-OHD in suppressing PTH secretion (22, 23) but has significantly reduced calcemic effects (24, 25). Distinguishing be-

tween 25-OHD and 3-epi-25-OHD could therefore be of relevance. The C-3 epimers can be converted to their respective 3-epi-1,25-OHD metabolites, and their presence might also be an indicator of elevated C-3 epimerization at the level of 1,25-OHD synthesis.

However, it is currently unknown whether C-3 epimers of 25-OHD can be found in humans in significant circulating concentrations. We hypothesized that this might be the case in some young children. Immaturity of, possibly hepatic, vitamin D metabolism in some infants could favor formation of C-3 epimers. In particular, enzymes from the cytochrome P450 family, notably CYP24, CYP27A1, and CYP27B1, are known to be involved in various facets of vitamin D metabolism and are expressed at particularly high levels in the liver. We therefore modified our existing LC-MS/MS method to also allow detection of 3-epi-25OHD₂ and 3-epi-25OHD₃ and studied a series of pediatric samples, normal adult samples, and adult samples from patients with impaired hepatic function.

Patients and Methods

The studies were approved by the Mayo Clinic institutional review board.

Our study aims were to 1) determine the prevalence of detectable 3-epi-25OHD₂ or 3-epi-25OHD₃ in human serum, and 2) determine the patient populations that might be affected.

The samples for this study were consecutive waste specimens from routine testing received in our laboratory, selected based only on age and, for adult samples, the likely presence or absence of hepatic dysfunction. They included samples from four groups of subjects: group 1, children less than 1 yr old (n = 183; 116 males and 67 females); group 2, children 1–18 yr old (n = 47; 19 males and 28 females); group 3, adults aged 20–87 yr with liver disease (hepatoma surveillance patients with elevated serum α -fetoprotein concentrations; n = 53; 25 males and 28

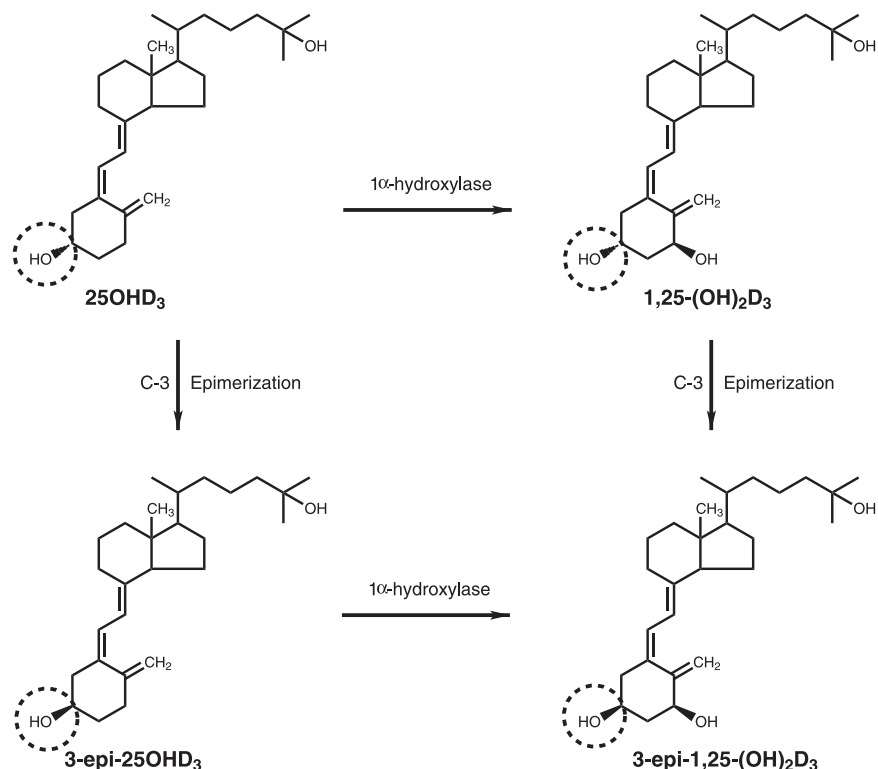


FIG. 2. C-3 epimerization of 25-OHD and 1,25-OHD. The epimerization of 25-OHD and 1,25-OHD at carbon number 3 (C-3) is shown using 25OHD₃ and 1,25(OH)₂D₃ as examples. Both 25-OHD and 1,25-OHD can be epimerized at the C-3 position (*dashed-line circles*). The only difference between the epimers is whether the bond between the C-3 and the attached hydroxyl group points upward or downward. 3-Epi-25-OHD can also be converted to 3-epi-1,25-OHD by 1 α -hydroxylase in renal and extrarenal tissues.

females); and group 4, adults 19–91 yr of age without biochemical evidence of liver disease (n = 147; 35 males and 112 females).

Standards/calibrators, internal standard, and controls

25OHD₂ and 25OHD₃ standards/calibrators and 25OHD₃-d₆ internal standard were purchased from Sigma Chemical Co. (St. Louis, MO) and As Vitas (Oslo, Norway), respectively. Manufacturer-stated concentrations were confirmed by UV spectrophotometry. The 3-epi-25OHD₃ standards were prepared and verified by one of the authors (G.S.R.) as described previously (21). Stock solutions of all standards were stored at –20 C.

Three pools of control samples each were prepared for 25OHD₂ and 25OHD₃. Control target values of 25OHD₂ were established at 4.2, 17, 42, and 110 ng/ml (10.2, 41.3, 101.9, and 267 nmol/liter). Target values of 25OHD₃ were established at 1.7, 24, 55, and 132 ng/ml (4.25, 60, 137.5, and 330 nmol/liter).

Sample preparation

Twenty-five microliters of working internal standard at a concentration of 200 ng/ml (492.6 nmol/liter) were added to each 200 μl of patient samples, controls, or calibrators. The mixtures were incubated for 15 min at room temperature to allow the internal standard to equilibrate with binding proteins. Proteins were precipitated by addition of 200 μl of acetonitrile and centrifugation. The supernatant was transferred to 96-well plates for analysis.

LC-MS/MS

For the standard 25-OHD method, online extraction and HPLC chromatography of the supernatants were performed using a TX4 Turbo Flow system (Cohesive Technologies, Franklin, MA) with 1.0 × 50 mm Cyclone extraction columns and 3.3 cm × 4.6 mm, 3-μm LC-18 (Supelco, St. Louis, MO) analytical columns. After online extraction, the analytes were eluted onto the analytical column for 90 sec with a mobile phase of 39.5% vol/vol methanol, 0.005% vol/vol formic acid. There was a step gradient to 87% vol/vol methanol, 0.005% vol/vol formic acid for the analytical column. The analytes then entered an API 4000 triple-quadrupole mass spectrometer (ABI-Sciex, Toronto, Canada) and were ionized in an atmospheric-pressure chemical-ionization source and detected by multiple reaction monitoring of the following ion pairs: *m/z* 413.0/395.3 for 25OHD₂, *m/z* 401.4/383.3 for 25OHD₃, and *m/z* 407.4/389.5 for 25OHD₃-d₆. The raw signals of 25OHD₂ and 25OHD₃ in the calibrators, controls, and samples were normalized to their respective internal standard 25OHD₃-d₆ signals, and concentrations in the samples and controls were calculated off the normalized six-point calibration curves [0–200 ng/ml (0–500 nmol/liter)]. Samples with concentrations that exceeded the highest calibrator were diluted and run again. The total 25-OHD concentrations of each control and sample were calculated by summing the measured values of 25OHD₂ and 25OHD₃.

For separation of epimers, the standard LC-18 column was replaced with a longer 5-dinitrobenzoyl-(R)-phenylglycine column (Chirex-PGLY and DNB 250 × 4.6 mm; Phenomenex, Torrance, CA) and 100 μl

of the supernatant was injected. The step gradient extends only up to 67% vol/vol methanol, 0.005% vol/vol formic acid at an analytical column flow rate of 0.9 ml/min. The mass spectrometer settings remained unchanged. The concentrations of 25OHD₂, 25OHD₃, and 25-OHD were calculated as above. The concentrations of any detected C-3 epimers of 25OHD₂ or 25OHD₃ were also calculated off the normalized 25OHD₂ and 25OHD₃ calibration curves, and the total 3-epi-25-OHD concentration is the sum of 3-epi-25OHD₂ and 3-epi-25OHD₃ concentrations.

Assay performance parameters

The performance of the two LC-MS/MS methods was similar for 25OHD₂ and 25OHD₃. Interassay coefficients of variation (CV) for the 25OHD₂ controls were 14, 5, 7, and 6%, respectively. Corresponding interassay CV for 25OHD₃ controls were 13, 8, 8, and 6%. The recovery of analyte spiked into patient samples was 82–115% (mean, 102%) of predicted for 25OHD₂ and 88–115% (mean, 103%) for 25OHD₃. The 25-OHD concentrations obtained by the two LC-MS/MS methods matched each other closely (Fig. 3).

Extracted RIA

In the 25-OHD RIA (Diasorin, Stillwater, MN), samples were extracted with acetonitrile, followed by a competitive RIA. According to the Diasorin packet insert, the RIA uses ¹²⁵I-labeled 25OHD₃ and a goat polyclonal antibody specific to the 25-OH-containing side chain; in the original published assay description, a rabbit-derived antibody was used (26, 27). A secondary antibody against the primary antibody was used as a precipitating reagent.

Interassay CV for this assay range from 8.5–14.4% across the reportable range. The assay compares closely with the LC-MS/MS standard method (Fig. 1).

Testing of study samples

All study samples were assayed by the standard and the modified LC-MS/MS method. In addition, all samples with total 25-OHD concentrations more than 100 ng/ml (250 nmol/liter) underwent confirmatory retesting. Chromatograms obtained with the modified method were inspected for the presence of C-3 epimer peaks. All detectable 25OHD₂, 25OHD₃, 3-epi-25OHD₂, and 3-epi-25OHD₃ peaks were quantitated, as described above, and the total 25-OHD and 3-epi-25-OHD concentrations were calculated. For every sample with detectable C-3 epimer peaks, we calculated the percent contribution of total 3-epi-25-OHD to the corresponding total 25-OHD concentration.

We also assayed all samples with epimer peaks and sufficient residual sample volume with the Diasorin RIA. The results were compared with the total 25-OHD values obtained by the modified LC-MS/MS assay.

Data analysis

The frequencies of occurrences of C-3 epimers were tabulated for the different study groups and compared with each other by χ^2 -analysis,

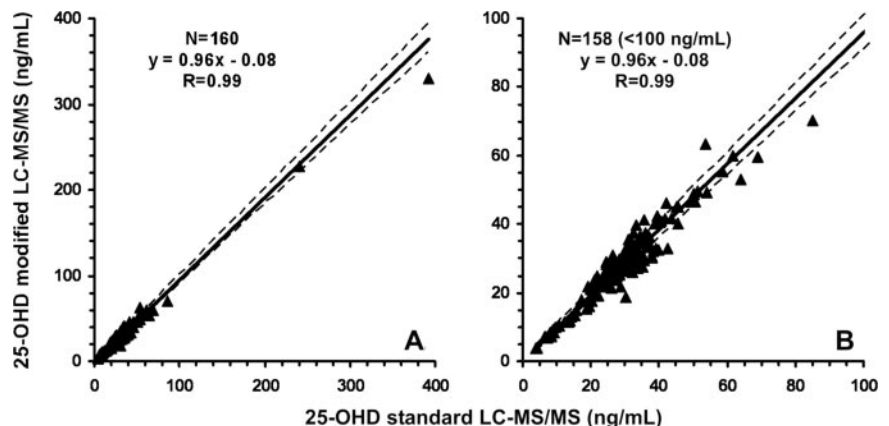


FIG. 3. Comparison of total 25-OHD measurements by standard LC-MS/MS and by modified LC-MS/MS. A, Scatter plot of total serum 25-OHD concentrations measured in 160 subjects by the standard LC-MS/MS (*abscissa*) as well as the modified LC-MS/MS method (*ordinate*) that allows detection of C-3 epimers of 25OHD₂ and D₃. Passing-Bablok linear fit with 95% CI is superimposed. B, The same dataset after removal of data points more than 100 ng/ml.

using appropriate degrees of freedom. In each group, χ^2 testing was also used to compare the likelihood for the presence of C-3 epimer peaks depending on whether patients had detectable 25OHD₂ or 25OHD₃ as well as to determine whether there was a relationship between patient gender and the presence of detectable C-3 epimers in any of the groups. Yates correction was used for all single degree of freedom χ^2 tests.

Regression analysis and ANOVA, respectively, were applied to all cases with detectable C-3 epimers to determine whether there was any relationship between the percent contribution of the C-3 epimers to the total 25-OHD values and the patient's age or gender. Statistical comparisons between different analytical methods were performed using Passing-Bablok linear regression.

For all statistical tests, a *P* value of <0.05 was considered significant, corrected if necessary for degrees of freedom or multiple comparisons.

Results

In group 1 (children <1 yr old), the total 25-OHD concentrations (including the contribution of C-3 epimers) ranged from 0–188 ng/ml [0–470 nmol/liter; median, 29.3 ng/ml (73.2 nmol/liter), mean, 34.2 ng/ml (85.5 nmol/liter)], with 172 of the 183 children having detectable levels of 25OHD₂ or 25OHD₃. We detected 25OHD₃ in 163 of these 172. Thirty children also had detectable levels of 25OHD₂. An additional nine children had detectable 25OHD₂ but no detectable 25OHD₃. We did not detect any 25OHD₃ or 25OHD₂ in 11 subjects.

We found 3-epi-25OHD₂ (*n* = 2) or 3-epi-25OHD₃ (*n* = 38) in 39 of the 172 children (22.7%) with detectable 25OHD₂ or 25OHD₃ (Fig. 4). All subjects with detectable C-3 epimers also had detectable 25OHD₂ or 25OHD₃. Among the 39 epimer-positive children, 31 had only detectable 25OHD₃, one had only detectable 25OHD₂, and seven had detectable 25OHD₂ and 25OHD₃. Of the eight children in this subgroup with detectable 25OHD₂, two, including the one who only had 25OHD₂, had 3-epi-25OHD₂ peaks. 3-Epi-25OHD₃ was detected in all 38 children with detectable 25OHD₃ peaks.

Total 3-epi-25-OHD concentrations ranged from 5–92

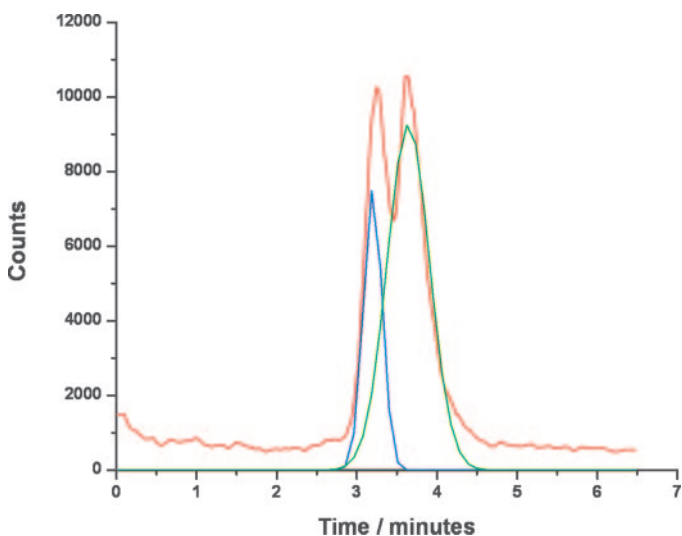


FIG. 4. Chromatographic separation of 3-epi-25OHD₃ from 25OHD₃. The LC-MS/MS chromatogram depicts a 25OHD₃ standard (blue line), a 3-epi-25OHD₃ standard (green line), and a patient sample containing approximately 50% each of 25OHD₃ and 3-epi-25OHD₃ (red line). This sample does not contain 25OHD₂, but similar separation of 25OHD₂ from its C-3 epimer was achieved in the two study samples that contained 3-epi-25OHD₂.

ng/ml [12.5–230 nmol/liter; median, 9 ng/ml (22.5 nmol/liter); mean, 16.1 ng/ml (40.2 nmol/liter)], with the C-3 epimers contributing 8.7–61.1% to the total 25-OHD concentration (median, 24%; mean, 27.8%). There was no correlation between 3-epi-25-OHD concentrations and 3-epi-25-OHD as a percentage of total 25-OHD.

There was sufficient sample volume for RIA testing in 34 of the 39 children with detectable 3-epi-25OHD₂ or 3-epi-25OHD₃. The standard LC-MS/MS method and the Diasorin RIA were in good agreement in this group with a Passing-Bablok regression slope of 1.059, an intercept of –5.8, and a correlation coefficient of 0.8.

There was a greater likelihood of detectable 3-epi-25OHD₃ than 3-epi-25OHD₂, with 38 of 163 25OHD₃-positive individuals having 3-epi-25OHD₃ peaks, whereas only two of 39 patients with detectable 25OHD₂ showed 3-epi-25OHD₂ peaks [odds ratio = 5.62; confidence interval (CI) = 1.24–35.40; χ^2 = 5.46; *P* < 0.02]. In the patients with detectable C-3 epimer peaks, regression analysis revealed an inverse relationship between patient age and percentage of total 3-epi-25-OHD detected, with a linear fit resulting in a correlation coefficient 0.48 (*P* < 0.002), whereas an exponential decay function fit resulted in a marginally higher correlation coefficient of 0.49 (*P* < 0.007) (Fig. 5).

Gender was not related to the presence or absence of C-3 epimer peaks, nor did it correlate with total 3-epi-25-OHD concentrations or percentages.

In group 2 (children 1–18 yr of age), total 25-OHD concentrations were 0–58 ng/ml [0–145 nmol/liter; median, 34 ng/ml (85 nmol/liter); mean, 33 ng/ml (82.5 nmol/liter)]. Thirty-nine children had only detectable 25OHD₃, six had detectable 25OHD₂ and 25OHD₃, one had only 25OHD₂, and one had neither 25OHD₂ nor 25OHD₃. We did not detect any C-3 epimer peaks in any of these 47 older children.

Total 25-OHD concentrations in group 3 (adults with compromised liver function, 20–87 yr old) were 0–57 ng/ml

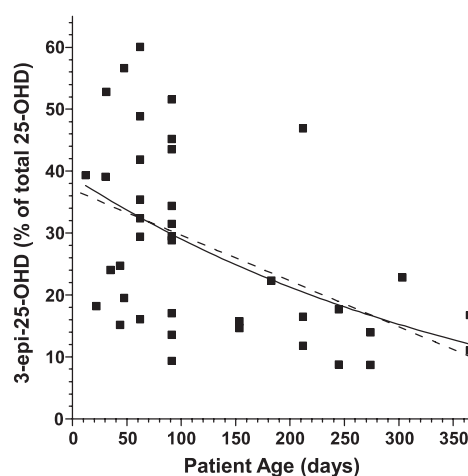


FIG. 5. Relative 3-epi-25-OHD concentrations and patient age. In this scatter plot, the percent contribution of total 3-epi-25-OHD to the total 25-OHD (ordinate) is plotted against the patient age in days (abscissa) for all samples with detectable 3-epi-25OHD₂ or 25OHD₃ peaks. There was an inverse relationship between age and percentage of total 3-epi-25-OHD. The linear fit (dashed line; $y = -0.07x + 37.02$; $r = 0.48$) and an exponential decay model (solid line; $y = 44.57e^{(-x/393.72)} - 5.55$; $r = 0.49$) are superimposed.

[0–142.5 nmol/liter; median, 17 ng/ml (42.5 nmol/liter); mean, 19 ng/ml (47.5 nmol/liter)]. Three patients had no detectable circulating 25-OHD, 39 had detectable 25OHD₃, 10 had detectable 25OHD₂ and 25OHD₃, and one had only 25OHD₂. No patient had any detectable C-3 epimer peaks.

In group 4, the 147 adults aged 19–91 yr, without known liver disease, total 25-OHD levels were 4–330 ng/ml [10–825 nmol/liter; median, 28.5 ng/ml (71.2 nmol/liter); mean, 31.3 ng/ml (78.2 nmol/liter)]. All patients had detectable 25OHD₃, and 47 also had detectable 25OHD₂. No C-3 epimer peaks were detected.

The differences in C-3 epimer detection rates between the four groups were highly significant with an overall χ^2 (three degrees of freedom) of 60.81 ($P < 0.00001$), whereas individual paired comparisons of group 1 against groups 2, 3, and 4 yielded χ^2 values of 11.21 ($P < 0.00082$; multicomparison corrected $P < 0.00246$), 12.23 ($P < 0.00047$; multicomparison corrected $P < 0.00141$), and 35.89 ($P < 0.00001$; multicomparison corrected $P < 0.00003$), respectively. There were no significant differences between groups 2, 3, and 4.

Discussion

Our study shows that C-3 epimers of 25OHD₂ or 25OHD₃ can be found in significant concentrations in a sizable minority of very young children who undergo clinical 25-OHD testing. Although we detected both 3-epi-25OHD₃, presumably endogenous, as well as 3-epi-25OHD₂, most likely from supplements, our data suggest that 25OHD₃ may be more likely to undergo C-3 epimerization than 25OHD₂.

Within the limitations of our study, the phenomenon seems to be confined to children under the age of 1 yr. Furthermore, within this group, it is inversely correlated with age (Fig. 5). This suggests that high rates of C-3 epimerization might be a function of immaturity of vitamin D metabolism. It also suggests that C-3 epimerization could be a major metabolic pathway for 25-OHD under certain circumstances. In fact, recent *in vitro* studies have hinted at the possibility that C-3 epimerization of 25-OHD could play an equal or more important role than epimerization of 1,25-OHD. Microsomal enzyme systems from a variety of cell lines show greater specificity and substrate conversion rates for the C-3 epimerization of 25-OHD than for the corresponding conversion of 1,25-OHD (28). However, the exact nature of the conditions, which might favor C-3 epimerization of 25-OHD, remains to be determined. Our studies seem to exclude deranged hepatic metabolism, despite the fact that hepatic microsomal cytochrome enzymes are known to play a major role in vitamin D metabolism and the associated clinical observation that liver patients often have biochemical evidence of disturbed vitamin D metabolism. This is consistent with *in vitro* experiments that have failed to identify the enzyme involved in C-3 epimerization of vitamin D metabolites among a group of known enzymes in the vitamin D pathway, including CYP24, CYP27A1, CYP27B1, and 3(α - β)-hydroxysteroid epimerase (28). It therefore appears that although microsomal enzyme systems seem to play a role in C-3 epimerization of vitamin D metabolites, the actual enzymes involved are distinct from the classical hepatic enzyme systems of vitamin D metabolism.

The immediate clinical consequence of our findings lies in the potential for inaccurate measurement of 25-OHD in young children. In our laboratory, we have changed our practice based on this study. We now use the alternative LC-MS/MS method for all 25-OHD measurements in children under the age of 1 yr. It is interesting to speculate how other LC-MS/MS or HPLC-UV detection methods in other laboratories might be performing with regard to C-3 epimer separation. Similarly, for the most part, it remains to be determined whether the various assays based on vitamin D binding protein and immunoassays are able to distinguish 25-OHD from its C-3 epimers. Cross-reactivity of 25-OHD assays with one or several of the over 40 known natural vitamin D metabolites is common (10, 29). For example, the Diasorin RIA cross-reacts with 24,25-(OH)₂D₃, 25,26-(OH)₂D₃, and 25OHD₃-26,23-lactone (26, 27). Traditionally, these and similar cross-reactivities in other 25-OHD assays have been regarded as clinically irrelevant because the serum concentrations of the cross-reactants are between one and two orders of magnitude lower than those of 25-OHD. However, as we have shown, 3-epi-25OHD₂ and 3-epi-25OHD₃ concentrations are much higher and could represent a relevant interference. Reassuringly, the Diasorin extracted RIA does not appear to cross-react with 3-epi-25OHD₂ or 3-epi-25OHD₃, giving accurate total 25-OHD results that correlate well with our modified LC-MS/MS method. However, we did not study any of the other 25-OHD assays that are currently in clinical use in the United States or Europe, and some of these might display cross-reactivity with 3-epi-25OHD₂ or 3-epi-25OHD₃.

Another issue that needs to be discussed is whether distinguishing between 25-OHD and its C-3 epimers is of clinical importance. Although the phenomenon is fairly prevalent among young infants and significant concentrations of 3-epi-25-OHD are found in affected children, the biological consequences depend on whether 25-OHD and its C-3 epimers differ in their physiological effects. This question can be separated into two parts. Because 25-OHD is a prohormone that needs to be converted into 1,25-OHD, we need to determine first whether 3-epi-25-OHD is converted to 3-epi-1,25-OHD and then to dissect the differential biological effects of 1,25-OHD *vs.* 3-epi-1,25-OHD.

With regard to 3-epi-25-OHD conversion to downstream metabolites, the literature indicates that it is a substrate for 1 α -hydroxylase and is converted into 3-epi-1,25-OHD (21). In addition, the conditions that favor C-3 epimerization of 25-OHD probably also favor the same metabolic pathway for 1,25-OHD, possibly involving extrarenal tissues (18–21, 30, 31) (Fig. 2). It therefore seems highly probable that children with detectable 3-epi-25-OHD might also have 3-epi-1,25-OHD. Final proof, however, will have to await the development of a method for measurement of serum 3-epi-1,25-OHD.

The issue of the bioactivity of 1,25-OHD *vs.* that of 3-epi-1,25-OHD is more complex. 3-Epi-1,25-OHD can stimulate gene transcription through the vitamin D receptor (VDR) despite the fact that it appears to have weaker binding affinity to the VDR than 1,25-OHD (32, 33). The lower receptor binding affinity does not translate into universally reduced biological effects of 3-epi-1,25-OHD in all vitamin-D-respon-

sive tissues. The transcriptional, as well as the ultimate physiological, response to 3-epi-1,25-OHD has been found to be highly variable for different VDR-regulated genes in different tissues (20, 22, 23, 25, 30, 31, 34, 35). These differences may in part relate to the longer half-life of 3-epi-1,25-OHD (30) but could also reflect selective partial agonistic-antagonist effects, such as have been described for a number of synthetic vitamin D analogs (36). Potential examples of apparently contradictory biological effects include the C-3 epimers' reduced calcemic properties and less potent gene-regulatory effects on some VDR-responsive genes involved in bone metabolism, such as osteocalcin (18, 24, 25, 34), whereas, on the other hand, suppression of gene transcription of PTH, antiproliferative effects in epithelial cells, and induction of surfactant gene transcription in pulmonary type II alveolar cells are comparable to 1,25-OHD (22, 23, 30, 35).

In conclusion, significant serum concentrations of 3-epi-25-OHD are commonly found in infants. Although the biological consequences of this phenomenon remain uncertain, in clinical practice, it can lead to overestimation of serum 25-OHD levels. Because the calcemic effects of the active downstream metabolite 3-epi-1,25-OHD are low, this might result in inappropriate reduction or omission of 25-OHD treatment in some children or unjustified anxiety about possible 25-OHD overdosing or toxicity in other children. Serum 25-OHD in children below the age of one should therefore be measured with an assay that either does not cross-react with 3-epi-25-OHD or allows unequivocal separation of 3-epi-25-OHD from 25-OHD. Currently, the only assays that we have verified to fulfill these requirements are our modified LC-MS/MS assay and the extracted Diasorin RIA. Laboratories that use other assays should evaluate whether their assays measure 25-OHD accurately in the presence of 3-epi-25-OHD.

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