The Measurement of 3-Epimer 25-Hydroxyvitamin D by Mass Spectrometry in Clinical Specimens Detects Inconsequential Levels in Adult Subjects

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Background: Vitamin D is derived from dietary sources or from the action of ultraviolet light on 7-dehydrocholesterol and undergoes a number of enzymatic modifications that lead to the synthesis of active vitamin D metabolites or metabolites with reduced biological activity. Among these, epimerization at the 3-hydroxyl group leads to the synthesis of 3-epimer 25-hydroxyvitamin D (3EVD). Described first in biological system experiments using in vitro incubation of vitamin D in cell culture, this molecule has been reported as having distinct activities when compared with 25-hydroxy vitamin D (25OHVD). Measurements of vitamin D have been conducted using a variety of methodologies and have led to conflicting assessments of the quantities of $3EVD_3$ that are measured.

Method: The present article describes the development and use of a simple liquid chromatography–tandem mass spectrometry method validated by the Clinical Laboratory Improvement Amendments to quantitate $3EVD_3$ in 3528 subjects, including 309 children (162 are <2 years) and 232 pregnant women.

Results: Our findings demonstrate that, although $3EVD_3$ constitutes a significant proportion of measureable $25OHVD_3$ in subjects younger than 1 year, $3EVD_3$ levels are negligible in most subjects older than 1 year.

Conclusions: It is important to choose the correct 25OHVD assay dependent on the age of the patient. Patients younger than 1 year should be run on a liquid chromatography–tandem mass spectrometry assay proven to not have potential contributions from any 3EVD present in the sample.

Key Words: 25-hydroxy vitamin D, mass spectrometry, 3-epimer, pediatric, quantitative assay, adult, CLIA-validated assay

(*J Investig Med* 2014;62: 690–695)

Vitamin D has long been recognized to play a critical role in the development and maintenance of bone mineral density. Originally identified in the context of its role in the prevention and treatment of rickets, it has been recognized more recently that subtler forms of vitamin D deficiency are prevalent and represent a major factor in the development of osteopenia and osteoporosis in the adult population.[1–3] Furthermore, a number of studies have implicated vitamin D deficiency as contributing to the pathogenesis or progression of cardiovascular disease, cancer, multiple sclerosis, and abnormalities of the immune system.[4–9]

Vitamin D is derived from dietary sources (vitamin D_2 or D_3) or from the action of ultraviolet light on 7-dehydrocholesterol in the skin to form cholecalciferol (vitamin D_3). After transport to the liver, vitamin D_3 is hydroxylated at the 25 position by CYP2R1 to form 25-hydroxyvitamin D_3 ($25OHVD_3$).[10–13] Hydroxylation at the 1 position of 25OHVD occurs predominantly in the kidney and is responsible for the synthesis of the most active form of vitamin D, 1,25-dihydroxyvitamin D,[14] which exerts its effects by binding to the vitamin D receptor, a member of the nuclear receptor family.[15]

In addition to processes leading to the synthesis of active forms of vitamin D, additional enzymatic steps have been identified that further modify vitamin D. Hydroxylation at the 24 position leads to the synthesis of 24,25-dihydroxyvitamin D. The synthesis of 24,25-dihydroxyvitamin D is stimulated by increased levels of 1,25-dihydroxyvitamin D, and this enzymatic conversion is felt to serve as a mode of regulating circulating levels of 1,25-dihydroxyvitamin D. The 24,25-dihydroxyvitamin D lacks prototypical activity as vitamin D in many assays but may serve alternate roles in vitamin D signaling.[16,17]

In like fashion, the hydroxyl group at the C-3 position of vitamin D may be configured either in an R or S configuration. The formation of 3-epimer (3-epi) 25-hydroxyvitamin D (3EVD), which has the hydroxyl group at the 3 position in the S configuration, was originally identified in incubation studies using cultured neonatal keratinocytes but has subsequently been detected in a number of tissues and in clinical samples.[18–25] Although 3EVD can be converted to 3-epi 1,25-dihydroxyvitamin D, 3-epi 1,25-dihydroxyvitamin D lacks activity in some biological assays of vitamin D action.[26–28] As such, most recent attention has been focused on 3EVD as it pertains to its role as a potential confounder to the accurate determination of 25OHVD.

The recognition of the prevalence of vitamin D deficiency in the general population has resulted in a dramatic rise in the number of tests to measure 25OHVD that are performed annually in the United States and worldwide.[29] In concert with the rise in the numbers of tests performed is the recognition that tests to measure 25OHVD remain poorly standardized and that carefully characterized reference standards are essential.[30–32]

The recent application of liquid chromatography and tandem mass spectrometry (LC-MS/MS) has permitted the increasingly precise measurement of 25OHVD levels. Along with this precision, however, is the recognition that specific metabolites of vitamin D may not be measured owing to their distinct chemical properties. Finally, the application of other methods to the measurement of 25OHVD levels has led to some uncertainty as to whether metabolites, such as $3EVD_3$, constitute an important potential confounder to the measurement of vitamin D levels in distinct patient groups.

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Received October 17, 2013, and in revised form January 9, 2014.
Accepted for publication January 14, 2014.
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Supplemental digital content is available for this article. Direct URL citation appears in the printed text and is provided in the HTML and PDF versions of this article on the journal's Web site (www.jinvestigativemed.com).

ISSN: 1081-5589
DOI: 10.231/JIM.0000000000000067

MATERIALS AND METHODS

Standards/Calibrators, Internal Standard, and Controls

Standards for $3EVD_3$, $3EVD_2$, $25OHVD_3$, and $25OHVD_2$ were purchased from Isosciences (King of Prussia, PA). The calibrators and controls were spiked into vitamin D metabolite depleted diluent from SeraCare Life Sciences (Milford, MA).

Study Samples

Three different populations of samples were analyzed. Discarded samples submitted for the clinical analysis of 25OHVD were subjected to the measurement of $3EVD_2$ and $3EVD_3$, $25OHVD_2$, and $25OHVD_3$ using the assay described in this article. The analysis of discarded specimens was judged exempt by the Western Institutional Review Board. All pediatric samples and third trimester of pregnancy samples were obtained from patient discards 3 weeks after results had been provided to the ordering physician. Discarded samples from women in their first and second trimesters of pregnancy were purchased from Biotheme Research Solutions.

Sample Preparation

In both of the LC-MS/MS assays used in this study, patient samples were extracted using a direct-in-plate protein precipitation and filtration. This step was automated on a Perkin-Elmer Janus robot by adding 100 μL of patient serum to each well of a 96-well protein precipitation plate with a regular deep-well collection plate underneath it. The Impact protein precipitation plate from Phenomenex (Torrance, CA), together with the collection plate, was transferred to the Agilent Bravo System to dispense 400 μL of methanol containing the internal heavy isotope-labeled standard (D_3-$25OHVD_3$) to each well. It was then vortexed and spun down in a centrifuge until all supernatant settled in the collection plate and was then injected onto the LC-MS/MS system.

Liquid Chromatography-MS/MS

A Cohesive Technologies Aria TLX-4 System (ThermoFisher, San Jose, CA) was used for LC with separation accomplished using a 100 × 2.1-mm, 2.6-μm pentafluorophenyl solid-core high-performance LC column (Accucore from ThermoFisher Scientific, San Jose, CA). The mobile phase solvents were 0.1% aqueous formic acid and methanol, which were delivered in a gradient from 70% to 90% methanol during a period of 6 minutes 15 seconds (including column equilibration time).

Thermo Scientific TSQ Quantum Ultra MS/MS System with atmospheric pressure chemical ionization source was used as the detector for both assays used in this study. Mass spectrometer data were obtained in positive ion mode. Peak area ratios of the analytes and internal standards were used for calculations of concentrations. The precursor ions and its major fragment ions (*m/z*) are as follows: 395.3 → 179.1 + 209.1 + 251.1 for $25OHVD_2$ and $3EVD_2$ and 383.3 → 159.1 + 211.1 for $25OHVD_3$ and $3EVD_3$. The same fragments were used to measure $25OHVD_2$ and $25OHVD_3$ in both assays used in this study.

RESULTS

Assay Performance and Correlation With Existing Assay

The data collected during the Clinical Laboratory Improvement Amendments validation established a method for the determination of $25OHVD_3$ and $25OHVD_2$ as well as the $3EVD_2$ and $3EVD_3$ epimers. The assay was shown to be rapid and accurate, with a lower limit of quantitation (LOQ) of 3 ng/mL for $25OHVD_2$, $25OHVD_3$, $3EVD_2$, and $3EVD_3$. The data acquired also demonstrated the robustness of the assay and its reproducibility with coefficients of variation of 8% to 13% at the 3 concentration levels (15, 40, and 80 ng/mL) analyzed for all analytes.

A direct comparison of the values of 25OHVD was conducted in an adult subset constituting 2987 patients older than 18 years using both the existing assay for 25OHVD (in which 3EVD was not separated) and the new methodology reported in the present article (which permits the chromatographic separation and measurement of 3EVD levels). As depicted in Figure 1, these measurements showed excellent agreement. Supplemental Figures 1 and 2 (Supplemental Digital Content 1, http://links.lww.com/JIM/A20) show a bias plot and histogram, respectively, of the same analysis, providing further proof of the excellent agreement between the 2 protocols.

Levels of $3EVD_3$ Are Infrequently Measureable in Specimens From Subjects 18 Years or Older

The measurement of $25OHVD_2$, $25OHVD_3$, $3EVD_2$, and $3EVD_3$ revealed levels of $25OHVD_2$ greater than the limit of detection (1 ng/mL) in only a small number of individuals (n = 2), with no samples containing levels of $3EVD_2$ greater than the lower LOQ (3 ng/mL) in this age group.

The levels of $25OHVD_3$ in samples containing measureable $25OHVD_3$ were highly variable, ranging from 4 to 126 ng/mL. In the 2987 specimens analyzed from subjects 18 years or older, only 65 (2.2%) had measureable levels of $3EVD_3$ (>3 ng/mL), with only 15 (0.47%) having values of ng/mL or greater (Fig. 2).

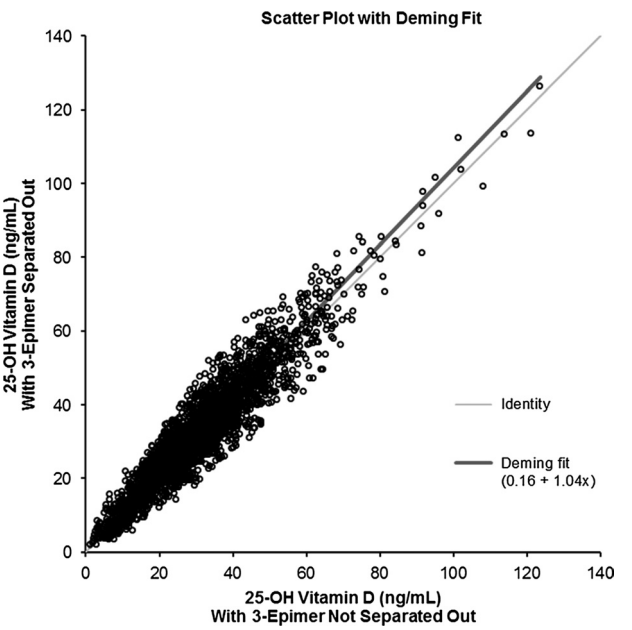

FIGURE 1. Comparison of the new and existing assays to measure 25OHVD. Individual adult samples (n = 2987) were assayed to measure 25OHVD using either the existing LC/MS method (*x* axis) or the method described in the present article (*y* axis), in which 3EVD is chromatographically separated from 25OHVD, permitting a quantitation of 25OHVD, without any potential contribution of 3EVD.

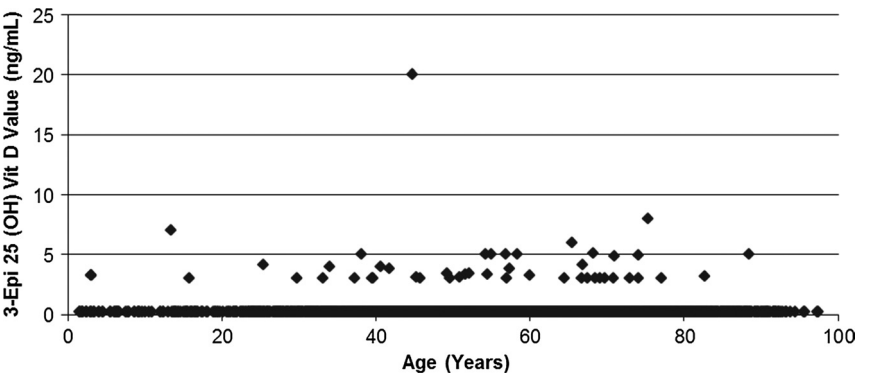

FIGURE 2. Absolute 3EVD values in subjects 2 to 97 years old (n = 3134).

Pediatric Samples Have an Increased Proportion of Samples With Measureable Levels of $3EVD_3$, Which Declines With Age

In contrast to results obtained using samples obtained from older subjects, levels of $3EVD_3$ were measureable at a much higher frequency in the 309 pediatric specimens tested (<18 years old). This statement relates not only to the absolute levels of $3EVD_3$ detected (Fig. 3) but also to the frequency with which samples contained measureable $3EVD_3$ (supplemental Figure 3, http://links.lww.com/JIM/A20). Comparison of Figure 3 and Supplemental Figure 3, http://links.lww.com/JIM/A20, demonstrates that the proportion of samples in which $3EVD_3$ is detectable is less than 15% after age of 1 year (with that percentage dropping with increasing age) and that the concentrations measured in subjects older than 1 year are near the limits of detection (3 ng/mL; Table 1).

Pregnancy

The demonstration that young children have a higher incidence of measureable levels of 3EVD and that, when measureable, have values that are more frequently substantial prompted us to examine the measured levels of 3EVD in pregnant women at various stages in their pregnancy. Of the 232 samples assayed, 44 of 232 subjects (19%) had detectable $3EVD_3$ concentrations (mean, 5.1 ng/mL; range, 3–8). Notably, although these levels are low and relatively infrequent, 24 of these measurements were in individuals with low levels of measured $25OHVD_3$ (5 ng/mL of $3EVD_3$ with 35 ng/mL of $25OHVD_3$; 8 ng/mL of $3EVD_3$ with 35 ng/mL of $25OHVD_3$; 3 ng/mL of $3EVD_3$ with 20 ng/mL of $25OHVD_3$; 3 ng/mL of $3EVD_3$ whit 25 ng/mL of $25OHVD_3$), in which the levels of $3EVD_3$ detected could cloud the interpretation of the measured $25OHVD_3$ concentrations.

Relationship Between Levels of 3EVD and Levels of 25OHVD

Although the bulk of our attention was drawn to the changes that are evident in the different populations that we examined (young pediatric subjects, older pediatric subjects, adults, pregnant women), we noted that the proportion of $3EVD_3$ detected exhibited a crude, inverse relationship to the measured levels of $25OHVD_3$. The largely unchanging levels of measured $3EVD_3$ would support that this relationship is a reflection of changes in the measured $25OHVD_3$. These relationships are depicted in Figures 4A and B.

DISCUSSION

These data establish a rapid method for the separation and measurement of $25OHVD_3$ and $25OHVD_2$ along with their epimers $3EVD_3$ and $3EVD_2$. This assay is rapid and accurate with an LOQ of 3 ng/mL. The data presented here demonstrate the robustness of the assay and its reproducibility with

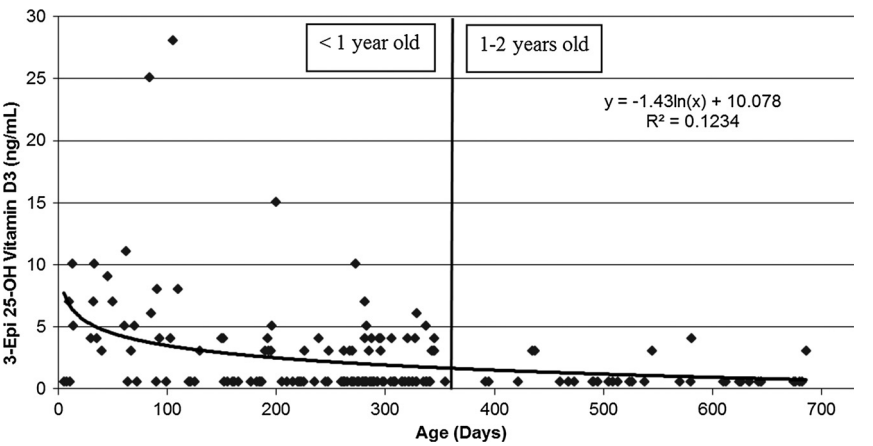

FIGURE 3. Absolute level of epimer D_3 in children younger than 2 years (n = 162). The absolute level of $3EVD_3$ measured (*y* axis) is plotted versus the age of the subject (in days) on the *x* axis. Although measurable at a low level in children between 1 and 2 years old, all measured levels of 3-epi were less than 4 ng/mL.

TABLE 1. Distribution of Measureable Values of $3EVD_3$ in Subjects Aged 0 to 6 Years

	Measurable/ Analyzed, n (%)	Mean of Measureable Values, ng/mL	Range, ng/mL
<2 mo	10/13 (77)	6.6	3–10
2 and 3 mo	11/15 (73)	17.9	3–28
4 and 5 mo	3/12 (25)	3.6	3–4
6 and 7 mo	8/20 (40)	5	3–15
8 and 9 mo	13/39 (33)	4.3	3–10
10 and 11 mo	9/29 (31)	4	3–6
12–24 mo	6/34 (17)	3.1	3–4
24–36 mo	5/26 (19)	3	3–3
36–48 mo	2/19 (10)	3	3–3
48–60 mo	2/37 (5)	3.5	3–4
60–72 mo	1/21 (5)	3	3–3
72–84 mo	4/44 (9)	4	3–5

coefficients of variation of 8% to 13% for 25OHVD and 3EVD in the 3 concentration ranges tested.

Application of this assay demonstrates that $3EVD_3$ and $3EVD_2$ concentrations were measureable in only 65 of 3134 samples (2.2%) obtained from nonpregnant subjects 2 years and older (Fig. 2). This low frequency of measurable values is paralleled by the consistently lower levels detected in this group, with only 18 samples (0.57%) having measureable values of 5 ng/mL or greater. Further breakdown of this group to adults only (≥18 years old, n = 2987) showed that, although most values of 5 ng/mL or greater (n = 15) were in this age range, this did not affect the rate of positivity (0.47%) because the bulk of the patients fell in this age range.

The distribution of results from adult patients was in stark contrast to the measurements performed in specimens obtained from the younger subjects. The frequency of measureable levels of 3EVD was observed to be substantially higher in subjects younger than the 2 years (Fig. 3). In this age range, both the frequency of measureable values and the absolute levels of 3EVD measured increased significantly. These findings are broken down by chronological age of the pediatric patients in Table 1. Of note, although measurable at a low frequency in children older than 1 year, all measured levels of 3EVD were less than 5 ng/mL in subjects older than 1 year.

Finally, in pregnancy, it was noted that a significant portion of the samples tests (44/232, 19%) had detectable levels of 3EVD with the mean value being 5.1 ng/mL. However, 24 of those 44 samples (24/232, 10%) had values high enough that they could affect the clinical definition of the patient's total vitamin D status. Because of this, it may be advisable to use a test such as the one detailed in this report to measure vitamin D status in pregnant women to ensure accuracy.

The biological importance of 3EVD remains to be clarified. Although 3-epi 1,25-dihydroxyvitamin D has been shown to be capable of modulating some biological effects of vitamin D, it does not possess all attributes associated with 1,25-dihydroxyvitamin D. As a result, the focus of most

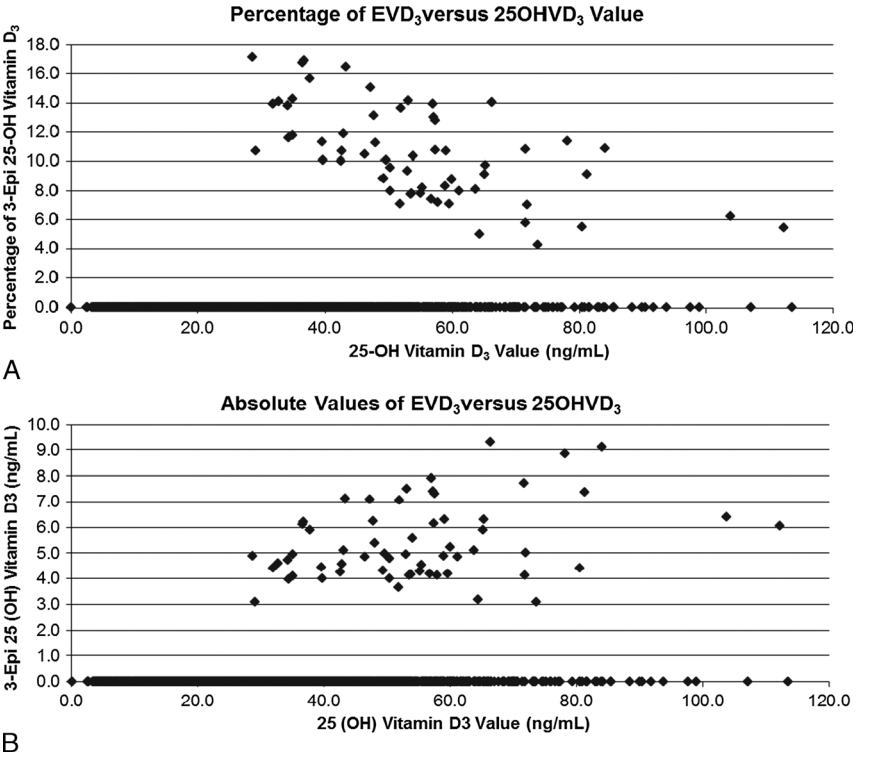

FIGURE 4. Relationship between the measured levels of $25OHVD_3$ and EVD_3 for all patients older than 18 years (n = 2987). Measured levels of $3EVD_3$ are displayed on the *y* axis as a percentage of the measured $25OHVD_3$ (shown on the *x* axis). In this representation, most individuals have levels of EVD_3 below the limits of assay detection. An inverse relationship is evident between the levels of $25OHVD_3$ and the percentage of EVD_3 in individuals in whom EVD_3 levels are measureable. Figure 4B shows the same representation, but as absolute values rather than percentages.

investigations relating to 3EVD has centered on the recognition that this molecule could represent a significant confounder in the measurement of 25OHVD$_3$. This issue has been examined in a number of studies.

Singh et al.[33] used an LC/MS assay to examine levels of 3EVD in 439 individuals, including 183 individuals younger than 1 year and 47 individuals aged 1 to 18 years. This group demonstrated that subjects younger than 1 year often had measureable levels of 3EVD. In this series, 3EVD was not detected in patients older than 1 year (n = 256).

In a limited study, Stepman et al.[34] used an ultra performance liquid chromatography-MS/MS assay to examine specimens from 6 infants aged between 1 and 4 months and 32 samples from adults. This analysis detected 3EVD in all 6 infants, comprising 15% to 44% of the 25OHVD measured. The 3EVD$_3$ was detected in all 32 adult specimens assayed, but at lower levels compared with the infant specimens (between 2.5% and 17% of the 25OHVD$_3$ measured).

In a letter to the editor, van den Ouwelanc and colleagues[35] used an LC-MS/MS method to separate and quantitate the levels of 3EVD$_3$ in 51 subjects younger than 1 year, 74 subjects aged 1 to 10 years, and 104 individuals 18 years or older. These investigators were able to detect 3EVD$_3$ in all sera from infants and in three fourths of adult samples. In this assay, the proportion of the total 25OHVD$_3$ contributed by 3EVD$_3$ ranged from a median of 11.1% in the youngest group, to 6.2% in the children, and to 3.5% in the adults.

Schleicher et al.[36] described an ultra performance liquid chromatography-MS/MS method for simultaneous measurement of 25OHVD$_3$, 25OHVD$_2$, and 3EVD$_3$ in human serum. These investigators applied this method to 2 convenience samples composed of 98 blood donors and 35 pregnant women. In these samples, 3EVD$_3$ was detectable in 43% of random blood donors and 80% of pregnant women. Of note, these investigators suggested that the concentrations of 3EVD$_3$ were correlated with the concentrations of 25OHVD$_3$.

Strathmann et al.[37] measured concentrations of 25OHVD$_3$ and 3EVD$_3$ in 125 subjects (aged 3 months to 1 year) and 626 patients aged 1 to 94 years undergoing routine 25OHVD testing. These investigators found higher proportion of 3EVD$_3$ in the youngest age groups with a trend to decreasing proportion of 3EVD$_3$ compared with the total with increasing age, which they attributed to constant levels of 3EVD$_3$ in the face of gradually increasing 25OHVD$_3$. Of note, their analysis suggests that ~24% of children and 3% of adults would be misclassified on the basis of the bias introduced by the measured levels of 3EVD$_3$.

Lensmeyer et al.[38] reported the application of an LC-MS/MS to measure the levels of 25OHVD$_3$ and 3EVD$_3$ in 214 clinical specimens from neonates to subjects 80 years and older. The 3EVD$_3$ epimer was detected in 212 of 214 (99%) of samples. Concentrations ranged from 0.1 to 23.7 ng/mL for 3EVD$_3$. Of note, in this assay, a clear relationship of 3EVD$_3$ to 25OHVD$_3$ was noted, with higher amounts of 3EVD$_3$ measured in samples with higher 25OHVD$_3$ concentrations.

Baecher et al.[39] reported the application of a high-performance LC-MS/MS method to measure 25OHVD$_3$, 25OHVD$_2$, 3EVD$_3$, and 24R,25-dihydroxyvitamin D$_3$ in human serum. In these specimens (no clinical data available), the concentration of 3EVD$_3$ was, on the average, approximately 7% (ranging from 4% to 20%) of the concentration of 25OHVD$_3$.

Bailey et al.[40] used an LC-MS/MS method to measure 25OHVD$_3$ and 3EVD$_3$. In all subjects, 3EVD$_3$ was measured, with higher concentrations observed in infants. Within the first year of life, 25OHVD$_3$ concentrations increased linearly, whereas 3EVD$_3$ concentrations remained constant. At 12 months old, 3EVD$_3$ concentration dropped by almost 50%.

The results of the current study support and extend various items identified in prior reports.

First, our findings indicate that the proportion of subjects with measureable levels of 3EVD$_3$ is greatest at ages younger than 1 year.

Second, this larger sample set permits a clearer definition of the changes that occur at older ages: the proportion of subjects with measurable levels decreasing progressively and the absolute levels progressively declining.

Third, the high concordance seen between the currently offered 25OHVD LC-MS/MS assay and the method detailed in this article, coupled with the low prevalence of detectable 3EVD concentrations in the general population studied, gives further evidence of the negligible contribution of either 3EVD to the currently reported 25OHVD LC-MS/MS assays.

Finally, in contrast to the report of Lensmeyer, a crude inverse relationship between the levels of 25OHVD$_3$ and the levels of 3EVD$_3$ was apparent in the patient population analyzed.[38]

Overall, the results of this study reinforce the notion that, in very young pediatric patients (<1 year old), it is important to account for the potentially high level of 3EVD$_3$ that may be present in this specific age group. To that end, we have developed a highly selective and specific mass spectrometry–based assay that does not experience interference posed by this vitamin D metabolite. Furthermore, the highly powered analysis of the age range of 2 to 97 years shows negligible contributions to the overall 25OHVD measurement, whether the 3-epi is separated chromatographically by the LC-MS/MS assay. From these data, it would therefore seem advisable for ordering physicians to consider the use of an LC-MS/MS method capable of separating the 3-epi metabolite away from the 25OHVD analytes in pediatric patients younger than 1 year. On the basis of our study, the same concern does not seem warranted for patients older than 1 year.

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