The C-3 Epimer of 25-Hydroxyvitamin D₃ Is Present in Adult Serum

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Context: Epimers have identical molecular structure but differ in stereochemical configuration. It is widely believed that the C-3 epimer of 25-hydroxyvitamin D₃ [3-epi-25(OH)D₃] is found only in neonates. However, this epimer was recently detected in a limited number of adults. The physiological importance of 3-epi-25(OH)D₃ is uncertain but might affect 25-hydroxyvitamin D test results and thereby reliability of the 25-hydroxyvitamin D₃ [25(OH)D₃] measurement.

Objective: This project describes development of a highly sensitive method for 3-epi-25(OH)D₃ measurement and establishes the prevalence of this epimer in adult clinical serum specimens.

Design, Setting, Participants, and Main Outcome Measure: Serum 25(OH)D₃, 3-epi-25(OH)D₃, and 25(OH)D₂ concentrations were determined in a cohort of patients (n = 214; age neonate to 80+ yr). High-performance liquid chromatography with ultraviolet detection and high-performance liquid chromatography tandem mass spectrometry with atmospheric pressure chemical ionization equipped with cyanopropyl analytical columns were used to baseline separate and quantitate 25(OH)D₃, 3 epi-25(OH)D₃, and 25(OH)D₂.

Results: The C-3 epimer was detected in 212 of 214 (99%) of samples. Concentrations ranged from 1 to 93 ng/ml for 25(OH)D₃ and 0.1 to 23.7 ng/ml for 3-epi-25(OH)D₃. The relative amounts of epimer to 25(OH)D₃ ranged from 0 to 25.5% (mean 4.75%). The epimer amount increased as 25(OH)D₃ increased in a nonlinear mode. In sera with approximately the same 25(OH)D₃ concentration, the ratio of epimer to 25(OH)D₃ varied, e.g. at 25(OH)D₃ values of 20–22 ng/ml, the ratio varied from 2–8.5%.

Conclusion: 3-Epi-25(OH)D₃ is present in the majority of human serum specimens. Although this concentration is generally low, further work must investigate the impact of 3-epi-25(OH)D₃ on the various 25-hydroxyvitamin D assays and ultimately what information, if any, C-3 epimer measurement can provide clinically. (J Clin Endocrinol Metab 97: 163–168, 2012)

Measurement of circulating 25-hydroxyvitamin D [25(OH)D] is currently accepted as the best measure of an individual’s vitamin D status (1, 2). In the past, substantial laboratory and methodological variability confounded measurement of this analyte (3–5). More recently assay improvements and development of standards have enhanced agreement between laboratories and methodologies (6–9). Despite these improvements, it remains probable that currently unappreciated factors continue to confound 25(OH)D measurement. Because the C-3 epimer of 25(OH)D₃ has been reported in human sera, (10, 11), it is possible that this epimer is one such confounder. This is clearly the case for those high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) methods that do not separate this metabolite. Some chromatographic methods, and importantly, the Diasorin methodology, are not affected by the 3 epimer (10). Whether other immunoassays cross-react with the 3 epimer remains to be clarified.

Abbreviations: 3-Epi-25(OH)D₃, C-3 epimer of 25-hydroxyvitamin D₃; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry; LC-MS/MS-APCI, LC-MS/MS with an atmospheric pressure chemical ionization; m/z, mass to charge ratio; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D₂, 25-hydroxyvitamin D₂; 25(OH)D₃, 25-hydroxyvitamin D₃.
Epimers are compounds that have identical structure with the exception of a stereochemical difference at one site. Given the identical structure (and therefore identical molecular weight, Fig. 1), it is likely that 25(OH)D epimers complicate assessment of vitamin D status with some methods. In fact, the C-3 epimer of 25(OH)D confounds 25(OH)D measurement with some liquid chromatography-tandem mass spectrometry methods, the methodology that some argue is currently the most specific methodology (12). Prior work found the C-3 epimer of 25-hydroxyvitamin D3 \[3-\text{epi-25(OH)D}3\] to occur only in infants and not adults (10). However, a recent report described the detection of the C-3 epimer in some adult sera, although the concentration is not listed (11).

The physiological importance of 3-epi-25(OH)D3 is uncertain. It is a substrate for the 1\(\alpha\)-hydroxylase enzyme, thus being converted to 3-epi-1, 25 dihydroxyvitamin D\(_3\) and is capable of binding to the vitamin D receptor (13–17). The physiological effects of 3-epi-1, 25 dihydroxyvitamin D\(_3\) appear to be variable (14). It has been reported that the 1, 25 dihydroxymetabolite of this epimer is relatively inactive at \textit{in vitro} osteocalcin mRNA stimulation (18) and does not raise serum calcium but does suppress circulating PTH concentration in rats (19). It has been suggested that this greater PTH suppression by 3-epi-25(OH)D\(_3\) may reflect slower metabolism of this epimer (20). Although the physiological relevance of 3-epi-25(OH)D\(_3\) remains to be clarified, of direct and current clinical relevance is the possibility that the 3 epimer could be a confounder of 25(OH)D measurements. If this is the case, it could contribute to confusion surrounding the 25(OH)D relationship with clinical outcomes, for example, PTH, and thereby enhance uncertainty surrounding definition of an optimal 25(OH)D value.

We recently observed a peak consistent with 3-epi-25(OH)D3 in serum of adults tested with a HPLC method (Fig. 2) (21). Thus, as a quality improvement exercise, we developed a HPLC-MS/MS method capable of quantifying 3-epi-25(OH)D3 and evaluated the prevalence and concentration of this analyte in patient sera sent for analysis to our clinical laboratory.

**Materials and Methods**

**Samples**

Residual serum from 214 clinical specimens on which 25(OH)D measurement was ordered for clinical care were analyzed. These specimens were not subjected to freeze/thaw cycles and were taken from the routine clinical workload, regardless of whether a 3-epi-25(OH)D3 peak was observed on HPLC. In many cases no C-3 epimer of 25(OH)D peak was observed by HPLC, but the epimer was subsequently demonstrable using HPLC-MS/MS. The only unrandomness in the sample selection process was that one author (G.L.) made sure that the 25(OH)D3 concentration of these specimens included a fairly broad range of 25(OH)D3. Given that this method development was performed as a quality assurance/improvement project, institutional review board oversight was not required. Patient age for all of these specimens was known; gender was available in 191 (146 females and 45 males) of 214 specimens. No information allowing identification of an individual patient was retained during the 3-epi-25(OH)D3 analysis.
LC-MS/MS methodology

We used a modification of our original HPLC methodology for this study (21). In our earlier report, we described a peak that elutes and separates adjacent to 25(OH)D₃ and was tentatively identified as 3-epi-25(OH)D₃ by matching retention time with authentic C-3 epimer supplied by Dr. G. S. Reddy (personal communication). Subsequent HPLC-MS/MS with an atmospheric pressure chemical ionization (HPLC-MS/MS-APCI) analysis confirmed the identity of the C-3 epimer peak (see below).

For the current study, 300 µl of patient serum was deproteinated with 0.78 ml of a mixture of acetonitrile/water/zinc sulfate in water (0.35 mg/ml); [870:130 (vol)] in a 96-well Strata-impact protein precipitation plate (Phenomenex, Torrance, CA). After 15 min of allowing the mixture to sit, an additional 0.35 ml of acetonitrile was added and vortexed.

The resulting supernatant was applied to a Strata octyldecyl (C₁₈E) (100 mg) of extraction sorbent in a 96-well plate (Phenomenex) to retain the vitamin D metabolites. The sorbent was washed with 1.0 ml of acetonitrile/water [45:55 (vol)]. The compounds were eluted with 1.3 ml acetone/acetonitrile (20:80), which was subsequently evaporated to dryness at 35°C under a gentle stream of nitrogen. The dry residue was reconstituted with 150 µl of acetonitrile/water (60:40), and a 50-µl portion was injected onto a Zorbax stable bond cyanopropyl HPLC column (4.6 × 100 mm; 3.5 µm particle from Agilent Technologies, Santa Clara, CA) at 48°C with isocratic mobile phase of methanol/water/formic acid [670:330:2 (vol)] flow rate 1.2 ml/min and UV detection at 275 nm. A Zorbax guard column (Stable-Bond cyanopropyl, 4.6 × 12.5 mm, 5 µm particle) and a silica saturating column (4.6 × 30 mm; Phenomenex) were also part of the HPLC system. With this analysis, we obtained the concentrations for 25(OH)D₃ and 25(OH)D₂.

Next, a second 30 µl portion of the purified sample extract was injected into the HPLC-MS/MS equipped with the same type of Zorbax stable bond cyanopropyl column used with HPLC with UV detection as noted above. Positive identification and improved sensitivity for quantitation of 3-epi-25(OH)D₃ was achieved.

The HPLC consisted of a model 1200 binary pump, vacuum degasser, thermostated column compartment held at 60.0°C, and a model 1100 thermostated autosampler held at 40.0°C, all from Agilent Technologies. The HPLC was coupled directly to a model API 4000 triple quadrupole mass spectrometer equipped with a Turbo V atmospheric pressure ionization source fitted with the heated nebulizer probe, held at 475°C, from MDS Sciex (Concord, Ontario, CA). A 200- × 4.6-mm SB CN (Agilent Technologies), 3.5-µm HPLC column was the analytical column. The mobile phase solvents were: A) Millipore type 1 water, and B) HPLC-grade methanol. The solvents were mixed and delivered isocratically (33% A/67% B) at 1000 µl/min for 10.5 min. Mass spectrometry data were obtained in positive ion mode. Multiple reaction monitoring transitions were mass to charge ratio ($m/z$) 395.3 → $m/z$ 209.1 for 25(OH)D₂ and $m/z$ 383.3 → $m/z$ 211.1 for 25(OH)D₃ and 3-epi-25(OH)D₃ (Fig. 3). Instrument parameters were curtain gas 11 U, gas1 20 U, collision gas 6 U, interface heater on, nebulizer current 5.00, declustering potential 63 V, exit potential 14 V, collision exit potential 15.5 V, collision energy 37.5 V, dwell 300 msec.

Authentic C-3 epimer substance kindly supplied by Dr. G. S. Reddy and National Institute of Science and Technology SRM 972 reference material were extracted to validate the identity and concentration of 25(OH)D₃, 3 epi-25(OH)D₃, and 25(OH)D₂ chromatographic peaks. In addition, using the HPLC-MS/MS, we confirmed that the C-3 epimer peak is real and not an artifact of the analytical process by demonstrating that no C-3 epimer was detected in the reagent blank, serum calibrators, or commercial serum controls that we routinely processed in the same manner patient serum samples are assayed.

Between-run precision for the assays was less than 7%; the assays were linear up to at least 300 ng/ml. The lower limit of
detection for 25(OH)D$_3$ was 0.1 ng/ml and for the C-3 epimer was 0.1 ng/ml.

Data analysis

Descriptive statistics were calculated and linear regression analysis of the relationship of 3-epi-25(OH)D$_3$ concentration relative to 25(OH)D$_3$ concentration as well as with age were performed using Statview software (SAS Institute, Cary, NC).

Results

3-Epi-25(OH)D$_3$ prevalence and concentration

25(OH)D$_3$ was present in all 214 sera [mean (SD) 29.7 (15.3) ng/ml, range 1–93 ng/ml; Fig. 4A). A measurable concentration of 3-epi-25(OH)D$_3$ was present in 212 of 214 of these sera. The mean (SD) 3 epi-25(OH)D$_3$ concentration was 1.5 (1.9) ng/ml with epimer values ranging from 0.1 to 23.7 ng/ml (Fig. 4B). Concentration of 3-epi-25(OH)D$_3$ was often low; in 92% of these specimens, it was 3.0 ng/ml or lower. The highest 3-epi-25(OH)D$_3$ value was observed in a 64-yr-old in whom no 25(OH)D$_2$ was detected and the 25(OH)D$_3$ was 93 ng/ml.

3-Epi-25(OH)D$_3$ relationship with 25(OH)D$_3$

A greater amount of the 3 epimer was observed at higher concentrations of 25(OH)D$_3$ (Fig. 5). However, at a given 25(OH)D$_3$ concentration, the 3 epimer concentration was variable. As an example, for those specimens with a 25(OH)D$_3$ concentration from 28 to 32 ng/ml, the 3-epi-25(OH)D$_3$ concentration ranged from 0.5 to 3.7 ng/ml. In the entire cohort, the 3 epimer concentration ranged from 0 to 25.5% of the 25(OH)D$_3$ concentration.

3-Epi 25(OH)D$_3$ relationship with age

No relationship between age and 3-epi-25(OH)D$_3$ concentration was observed (Fig. 6). Specifically, there was no evidence for lower 3 epimer concentration among older adults. However, it must be noted that in this cohort only two individuals were under age 1 yr, and only an additional four were less than 20 yr of age.

Discussion

In this report, we describe a methodology for the HPLC-MS/MS measurement of 3-epi-25(OH)D$_3$ in serum. Importantly, this epimer is present in virtually all samples tested. The source of and/or reason for the presence of 3-epi-25(OH)D$_3$ in serum is not known; future work is
needed to clarify the source of this epimer. Although the 3 epimer concentration is often low, this likely contributes to noise in some currently available assays. For example, because the molecular weight of 3-epi-25(OH)D₃ is identical with 25(OH)D, it seems likely that the presence of this epimer contributes to the slightly higher results sometimes observed with HPLC-MS/MS methodology that does not chromatographically separate the two forms. Importantly, given the high specificity of some HPLC-MS/MS methods, this analytical technique is emerging as the preferred reference for 25(OH)D measurement. As such, the importance of confounders of this methodology, such as 3-epi-25(OH)D₃, is apparent. Moreover, although the 3 epimer concentration is generally low, in some individuals the concentration is substantial and may contribute up to 25% of the total 25(OH)D measured.

These results differ from a prior publication in which 3-epi-25(OH)D₃ was not observed in adults. It is likely that this apparent difference between the prior study and our current report is assay sensitivity. Specifically, perhaps reflecting the concentration step used in our study, the lower limit of detection for 3-epi-25(OH)D₃ is 0.1 ng/ml. In contrast, the report of Singh et al. (10), whereas not directly specifying a lower detection limit, reports 3-epi-25(OH)D₃ concentrations ranging from 5–92 ng/ml. If one were to apply a 5 ng/ml detection level to the current study results, 3-epi-25(OH)D₃ would have been detectable in fewer than 3% (six of 214) of sera.

Although measurement of 25(OH)D concentration is currently accepted as the best way to evaluate an individual’s vitamin D status (2), assay standardization continues to evolve and remains challenging (8, 12). Moreover, the relationship of measured circulating 25(OH)D concentration, with a physiological end point, PTH concentration, shows much between individual variability. It seems likely that currently unappreciated confounders to 25(OH)D measurement, for example, 3-epi-25(OH)D₃ are contributing to the observed variability. Anecdotally, we have observed an occasional patient who has up to 50% of their total 25(OH)D in the 3 epimer form; it is plausible that this has biological activity, thereby suppressing PTH. From a clinical perspective, the presence in variable amounts of 3-epi-25(OH)D₃ identifies an additional confounder to 25(OH)D measurement. This epimer may explain some of the variability noted in the PTH/25(OH)D relationship. Further work to elucidate the clinical importance of 3-epi-25(OH)D₃ is required. For example, understanding why some individuals have a high circulating 3-epi-25(OH)D₃ concentration, and the physiological importance of the 3 epimer (if any), needs clarification. The identification of 3-epi-25(OH)D as a potential confounder by methodology similar to that described here will facilitate such understanding. Importantly, the influence of 3-epi-25(OH)D₃ on 25(OH)D test results obtained with various immunochemical and chromatographic assays and ultimately on the reliability/validity of 25(OH)D measurement remains to be defined.

Limitations of this study include a relatively small sample size, performance at only a single clinical site and inclusion of few young individuals. Future work will need to include more individuals across the life span, evaluation of effects of race on 3-epi-25(OH)D₃ concentration and exploration of the effects of vitamin D supplementation on 3-epi-25(OH)D₃ concentration. We acknowledge the possibility that what we are identifying as 3-epi-25(OH)D₃ could plausibly be 25-OH-previtamin D₃. However, we believe this unlikely given that the peak we label as 3-epi-25(OH)D₃ is identical to what the National Institute of Science and Technology has identified as 3-epi-25(OH)D₃ based on our ability to recover the values of 3-epi-25(OH)D₃ in the human serum reference material.

In conclusion, we describe a method for the sensitive detection of 3-epi-25(OH)D₃ in human sera. Measurable amounts of this epimer are demonstrable in virtually all human sera tested. The physiological importance of this, if any, and the effect of this epimer on current measures of 25(OH)D need to be clarified.

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References

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