Fast Separation of 25-Hydroxyvitamin D_3 from 3-Epi-25-Hydroxyvitamin D_3 in Human Serum by Liquid Chromatography–Tandem Mass Spectrometry: Variable Prevalence of 3-Epi-25-Hydroxyvitamin D_3 in Infants, Children, and Adults

To the Editor:

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is becoming increasingly popular for the measurement of 25-hydroxyvitamin D [25(OH)D] in human serum. A limitation of most LC-MS/MS methods is the potential interference from coeluting isomeric compounds having identical elemental composition but different structure, leading to overestimation of true 25(OH)D concentrations. Of particular interest is the C3 epimer of 25(OH)D, 3-epi-25(OH)D, which can be present at relatively high concentrations in sera from infants, but can also be found in sera from adults, albeit at lower concentrations *(1, 2)*. To separate 3-epi-25(OH)D from 25(OH)D, current procedures require lengthy chromatographic run times varying from 12 to 40 min *(3)*, which makes these methods unsuitable for clinical laboratories that must deal with increasing numbers of vitamin D requests.

We describe a modification of an established LC-MS/MS method for measurement of $25(OH)D_3$ and $25(OH)D_2$ *(4)* that allows fast separation of $25(OH)D_3$ from 3-epi-$25(OH)D_3$ in human serum. Sample preparation, assay calibration, and instrument operation were carried out as described *(4)*, with minor modifications. $25(OH)D_3$, $25(OH)D_2$, and 3-epi-$25(OH)D_3$ were from Sigma Aldrich, and the internal standard (IS) 26,27-hexadeuterium–labeled $25(OH)D_3$ was from Synthetica AS.

Calibrators, controls, and patient sera were treated with sodium hydroxide to release vitamin D metabolites from the binding protein before protein precipitation. Subsequent off-line solid-phase extraction was followed by chromatographic separation performed by use of a pentafluorophenyl-propyl (PFP) column (Acquity UPLC CSH™ fluoro-phenyl 1.7 μm, 2.1 × 100 mm; Waters). The relative rigidity of the fluorinated bonded phase provides enhanced shape selectivity *(5)*. Mobile phases A and B consisted of 1 mL/L formic acid in ammonium acetate (2 mmol/L), and 3 mL/L formic acid in methanol, respectively. A flow rate of 0.35 mL/min was used, with reduction to 0.30 mL/min in the final step, by using a gradient to 85% B (0–5 min), 85% B rinse (5.0–5.3 min), and reversion to 50% B (5.3–5.4 min), followed by 50% B (5.4–6.5 min). Detection was by registration *m/z* transitions 401.5→159.2 for $25(OH)D_3$ and 3-epi-$25(OH)D_3$, 413.4→159.2 for $25(OH)D_2$, and 407.5→159.2 for the IS. The percentage of 3-epi-$25(OH)D_3$ was calculated relative to the total $25(OH)D_3$ content. Interassay CVs for 3 concentrations of $25(OH)D_3$ control sera (39, 92, and 127 nmol/L; Chromsystems) (n = 4) were 4.2%, 3.5%, and 2.8%, respectively. $25(OH)D_3$ and IS eluted at about 4.32 min, with 3-epi-$25(OH)D_3$ eluting at 4.42 min, near base-line separation from $25(OH)D_3$ (Fig. 1). $25(OH)D_2$ eluted at 4.42 min (result not shown).

We further investigated the prevalence of the 3-epi-$25(OH)D_3$ in leftover serum samples from infants (<1 year of age, n = 51), children (1–10 years of age, n = 74), and adults (>18 years of age, n = 104). The samples were treated in agreement with local ethics guidelines. $25(OH)D_3$ concentrations ranged from 4.3 to 300 nmol/L. No relevant concentrations of $25(OH)D_2$ were measured in these samples. We could detect the presence of 3-epi-$25(OH)D_3$ in all sera from infants and children and in 75% of sera from adults. The mean (median; range) percentages were 11.1% (9.3%; 2.3%–49.2%) in infants, 6.2% (5.7%; 2.5%–20.0%) in children, and 3.5% (3.1%; <2%–10.6%) in adults. No correlation was found between the relative content of 3-epi-$25(OH)D_3$ and the absolute amount of $25(OH)D_3$. Percentages of 3-epi-$25(OH)D_3$ exceeding 10% were mainly found in 18 (39%) of 46 infants <3 months of age, consistent with previous findings *(1)*, although higher percentages (10%–20%) were found in 4 (5.4%) of 74 children and 1 (1.0%) of 104 adults as well, confirming recent findings of considerable amounts of 3-epi-$25(OH)D_3$ in adults *(2)*. In adult sera, the new LC-MS/MS method gives a mean 4% lower concentration for $25(OH)D_3$ compared with our previous method, for which we used C18 as stationary phase *(4)* [Passing and Bablock regression: PFP LC-MS/MS = 0.94 (95% CI: 0.92–0.97) × C18 LC-MS/MS + 0.31 (95% CI: −0.42 to 0.96); r = 0.995; n = 104] due to exclusion of 3-epi-$25(OH)D_3$. The comeasurement of 3-epi-25(OH)D is likely to contribute to the positive bias of many current LC-MS/MS assays compared to the NIST candidate reference measurement procedure *(3)*. Evidently, further investigations are needed to elucidate the biological significance of the 3-epi-25(OH)D metabolites, the conditions that favor C3 epimerization of 25(OH)D, and to what extent separate reporting of 3-epi-$25(OH)D_3$ might be of clinical relevance.

In conclusion, the presence of 3-epi-$25(OH)D_3$ in nearly all human sera necessitates the use of an LC-MS/MS method that separates 3-epi-$25(OH)D_3$ from $25(OH)D_3$ for accurate detection of $25(OH)D_3$. By using a PFP column, $25(OH)D_3$ and the 3-epi-$25(OH)D_3$ can be separated within a total run time of 6.5 min, making this method fast and attractive for

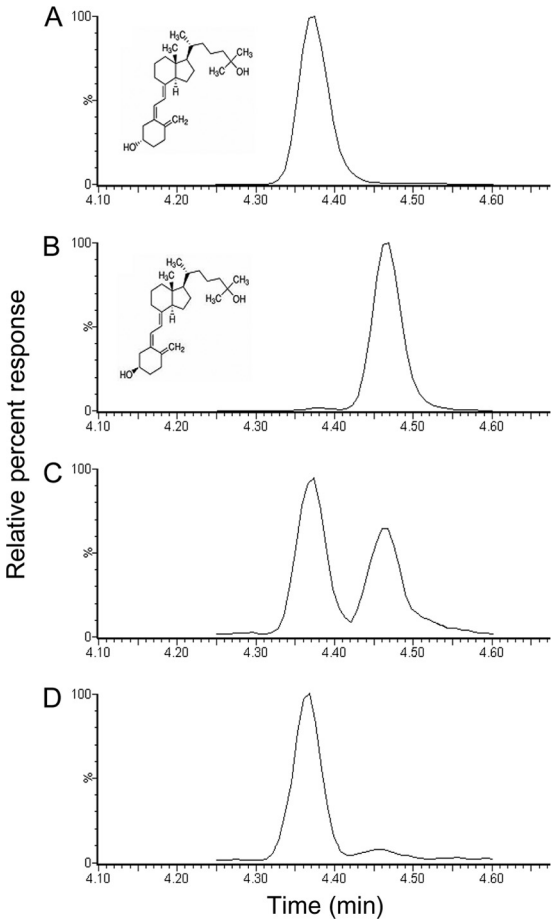

Fig. 1. Chromatographic separation of 3-epi-25(OH)D_3 from 25(OH)D_3.
LC-MS/MS chromatograms of injections of (A), pure 25(OH)D_3 standard; (B), pure 3-epi-25(OH)D_3 standard; (C), infant serum sample containing 68.5 nmol/L 25(OH)D_3 and 44.6% relative 3-epi-25(OH)D_3 concentration; and (D), adult serum sample containing 79.0 nmol/L 25(OH)D_3 and 4.2% relative 3-epi-25(OH)D_3 concentration. Note the subtle difference in orientation of the C3-hydroxylgroup between 25(OH)D_3 (β-position) and 3-epi-25(OH)D_3 (α-position).

routine measurement of 25(OH)D in clinical laboratories.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

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Previously published online at
DOI: 10.1373/clinchem.2011.170282