Clinical Utility of Simultaneous Quantitation of 25-Hydroxyvitamin D and 24,25-Dihydroxyvitamin D by LC-MS/MS Involving Derivatization With DMEQ-TAD

Martin Kaufmann, J. Christopher Gallagher, Munro Peacock, Karl-Peter Schlingmann, Martin Konrad, Hector F. DeLuca, Rita Sigueiro, Borja Lopez, Antonio Mourino, Miguel Maestro, René St-Arnaud, Joel S. Finkelstein, Donald P. Cooper, and Glenville Jones

Department of Biomedical and Molecular Sciences (M.Ka., G.J.), Queen’s University, Kingston, Ontario, Canada K7L 3N6; Bone Metabolism Unit (J.C.G.), Creighton University School of Medicine, Omaha, Nebraska 68131; Indiana University School of Medicine (M.P.), Indianapolis, Indiana 46202–5250; Department of General Pediatrics, (K-P. S., M.Ko.) University Children’s Hospital, D-48149, Münster, Germany; Department of Biochemistry (H.F.D.), University of Wisconsin-Madison, Madison, Wisconsin 53706; Deparmento de Quimica Organica (R.S., B.L., A.M., M.M.), Laboratorio Ignacio Ribas, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain; McGill University and Shriners Hospital for Children-Canada (R.S.), Montréal, Québec, Canada H3G 1A6; Endocrine Unit (J.S.F.), Massachusetts General Hospital, Boston Massachusetts 02114; and Health Sciences Research, Waters Corporation (D.P.C), Stamford Avenue, Altrincham Rd, Wilmslow, SK9 4AX United Kingdom

Context: The discovery of hypercalcemic diseases due to loss-of-function mutations in 25-hydroxyvitamin D-24-hydroxylase has placed a new demand for sensitive and precise assays for 24,25-dihydroxyvitamin D \([24,25-(OH)2D]\).

Objective: We describe a novel liquid chromatography and tandem mass spectrometry-based method involving derivatization with DMEQ-TAD \(\{4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl-ethyl]-1,2,4-triazoline-3,5-dione}\) to simultaneously assay multiple vitamin D metabolites including 25-hydroxyvitamin D \(25-(OH)-D\) and 24,25-(OH)\(_2\)D using 100 \(\mu\text{L}\) of serum with a 5-minute run time.

Design: The assay uses a newly synthesized internal standard \(d_6-24,25-(OH)2D3\) enabling the quantitation of 24,25-(OH)\(_2\)D\(_3\) as well as the determination of the ratio of 25-OH-D\(_3\) to 24,25-(OH)\(_2\)D\(_3\), a physiologically useful parameter.

Setting: We report data on more than 1000 normal and disease samples involving vitamin D deficiency or hypercalcemia in addition to studies involving knockout mouse models.

Results: The assay showed good correlation with samples from quality assurance schemes for 25-OH-D (25-OH-D\(_2\) and 25-OH-D\(_3\)) determination (\(-2\%\) to \(-5\%\) bias) and exhibited low inter- and intraassay coefficients of variation (4\%–7\%) and lower limits of quantitation of 0.25–0.45 nmol/L. In clinical studies, we found a strong correlation between serum levels of 25-OH-D\(_3\) and 24,25-(OH)\(_2\)D\(_3\) \((r^2 = 0.80)\) in subjects over a broad range of 25-OH-D\(_3\) values and a marked lack of production of 24,25-(OH)\(_2\)D\(_3\) below 25 nmol/L of 25-OH-D. The ratio of 25-OH-D\(_3\) to 24,25-(OH)\(_2\)D\(_3\), which remained less than 25 in vitamin D-sufficient subjects (serum 25-OH-D \(> 50 \text{ nmol/L}\)) but was greatly elevated (80–100) in patients with idiopathic infantile hypercalcemia.

Conclusions: The new method showed good utility in clinical settings involving vitamin D deficiency; supplementation with vitamin D and idiopathic infantile hypercalcemia, as well as in animal models with ablation of selected cytochrome P450-containing enzymes involved in vitamin D metabolism. (J Clin Endocrinol Metab 99: 2567–2574, 2014)
Serum 25-hydroxyvitamin D (25-OH-D) is widely accepted as a biomarker of vitamin D status (1). Vitamin D plays important roles in the body including the regulation of calcium/phosphate homeostasis and the cell cycle (2). Over the past 10 years, many epidemiological studies have demonstrated an inverse correlation between serum 25-OH-D levels and a broad range of important disease states (3, 4), which has in turn emphasized the importance of adequate vitamin D status (5). Currently, serum 25-OH-D is routinely measured by methods involving RIA, HPLC, or liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based technologies (6).

Recently some of us (7) showed that loss-of-function mutations of 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) in a group of German, Turkish, and Russian children are associated with the hypercalcemic condition, idiopathic infantile hypercalcemia (IIH). With this newly acquired knowledge about the importance of the catabolic enzyme CYP24A1 (8), there has developed renewed interest in measuring its main circulating product, 24,25-dihydroxyvitamin D₃ (24,25-(OH)₂D₃). Competitive binding assays based on the strong binding of 24,25-(OH)₂D₃ to the plasma vitamin D binding globulin allowed for early estimates of the levels of this catabolite (9). However, methods required precise chromatography steps to resolve 24,25-(OH)₂D₃ from its more abundant precursor, 25-OH-D₃ and from the other dihydroxyvitamin D metabolites including 25,26-dihydroxyvitamin D₃ and 25-OH-D₂-26,23-lactone, which are often increased in serum from hypervitaminotic D animals (10). In patients receiving vitamin D₂, the analytical problem of accurately assaying 24-hydroxylated vitamin D₃ and D₂ becomes even more complex and time consuming.

The emergence of LC-MS/MS as a tool to quantify a wide range of clinically important bioactive hormones and metabolites has been a major advance in clinical chemistry. Tandem mass spectrometry provides the ideal universal detector for vitamin D metabolites separated by conventional liquid chromatography techniques (11). Its only drawback has been the identification of interfering substances such as 3-epi-25-OH-D₃ in a 25-OH-D assay (12) and 4,25-(OH)₂D₃ in 1,25 dihydroxyvitamin D₃ [1,25-(OH)₂D₃] assay (13), which make the chromatographic step particularly important.

In this paper, we describe a sample preparation and derivatization method that allows the simultaneous assay in serum in both humans and mice of all abundant metabolites of both vitamin D₂ and D₃ and the accurate measurement of 24,25-(OH)₂D₃ and 25-OH-D₃ using a specific deuterated internal standard, whose synthesis is described. In addition, we demonstrate that simultaneous assay of multiple vitamin D metabolites including serum 24,25-(OH)₂D₃ and the 25-OH-D₃ to 24,25-(OH)₂D₃ ratio provide evidence for the presence of loss-of-function mutations of CYP24A1; provide evidence for an assessment tool for establishing the degree of vitamin D deficiency; and provide information on the catabolism of vitamin D during vitamin D supplementation.

Materials and Methods

Human and animal studies
Serum samples were analyzed from the following: 1) 163 healthy, white, postmenopausal women who were part of a 1-year randomized prospective, placebo-controlled VIDOS (Vitamin D Supplementation in Older Subjects) clinical trial approved by the Creighton University Institutional Review Board and published previously (14); 2) 110 healthy older African American women who were enrolled in a randomized, double-blind vitamin D supplementation trial at Creighton Medical Center and Indiana University Medical Center published previously (15); and 3) 198 healthy young women (119 Caucasian and 79 African American) enrolled in a 1-year prospective, randomized, placebo controlled vitamin D supplementation trial published previously (16). Serum samples from patients with idiopathic infantile hypercalcemia were provided by Schlingmann et al (7) as previously described. Mouse sera were provided by Dr Hector F. DeLuca and Dr René St-Arnaud; and all mice were managed in compliance with protocols approved by the University of Wisconsin Research Animal Resources Center or McGill University Animal Care Committee as previously described (17, 18).

Materials
25-OH-D₃ and 25-OH-D₂ calibrators (6PLUS1) were purchased from Chromsystems. A six-level calibrator set for 24,25-(OH)₂D₃ was generated in-house using a pool of human serum containing 6.5 ng/mL 25-OH-D₃ (courtesy of Dr Jackie Berry, University of Manchester, Manchester, United Kingdom). An artificial sample matrix was generated using 20% human serum in 0.1% BSA dissolved in PBS and supplemented with synthetic 24,25-(OH)₂D₃. Internal standards d₃-25-OH-D₃ and d₃-25-OH-D₂ were purchased from IsoSciences. The synthesis of d₆-24,25-(OH)₂D₃ is described below. All LC-MS/MS solvents, additives, and extraction solvents were Optima liquid chromatography-mass spectrometry grade and purchased from Fisher, with the exception of methyl tertiary butyl ether, which was purchased from Sigma. The Cookson reagent, DMEQ-TAD [4-[2-[6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl]ethyl]1,2,4-triazoline-3,5-dione] was purchased from Key Synthesis.

Synthesis of d₆-24,25-(OH)₂D₃
The synthesis of the deuterated internal standard, 24R,25-dihydroxyvitamin D₃ (26,26,27,27-d₆) (Figure 1) (1, seocalciferol-d₆) starts with inhoffen-lythgoe diol (2), which can be prepared from vitamin D₂ (19) and converted to the acetate 3 by...
Serum preparation for ultraperformance liquid chromatography (UPLC)-tandem mass spectrometry

In microcentrifuge tubes, 100-μL aliquots of test serum or calibrator were diluted with 200 μL of water and supplemented with the following internal standards: 80 ng/mL d5-25-OH-D3, 65 ng/mL d3-25-OH-D2, and 6 ng/mL d6-24,25-(OH)2D3. A 100-μL volume of 0.1 M HCl was added, and protein precipitation was carried out by adding 0.2 M zinc sulfate and 450 μL of methanol, with vortexing after addition of each component. The mixture was centrifuged at 12 000 g for 10 minutes, and the supernatant was transferred to borosilicate glass tubes. Organic extraction was carried out by adding 700 μL of hexane and 700 μL of methyl tertiary butyl ether, with vortexing after the addition of each component. The upper organic phase was transferred to LC-MS/MS sample vials and dried under a stream of purerified N2 at 37°C. Samples were derivatized by redissolving the dry residue in 25 μL of ethyl acetate and incubating for 30 minutes at room temperature in the dark. A second aliquot of DMEQ-TAD was added and allowed to incubate for an additional 60 minutes (21, 22). A 40-μL volume of ethanol was added, and the derivatized extract was dried and redissolved in 60 μL of 60:40 (vol/vol) methanol/water running solvent.

Results

Optimization and performance of the simultaneous vitamin D metabolite assay

Derivatization of vitamin D metabolites with DMEQ-TAD offers the advantage of improving ionization efficiency relative to native metabolites and increasing molecular mass by 363 Da, to a region of the mass spectrum in which there is reduced background. The major characteristic ions for derivatized 25-OH-D3, 25-OH-D2, and 24,25-(OH)2D3 were their molecular ions [M+H]+ at a mass/charge (m/z) ratio of 746.6, 758.6 and 762.6 respectively, and when subjected to collision-induced dissociation under optimized conditions they yield an A-ring/DMEQ-TAD fragment (m/z 468) and a DMEQ-TAD fragment (m/z 247) as the major products. The fragmentation pattern for the 24,25-(OH)2D3 adduct is shown in Figure 2, A and B. The m/z 468 was selected as the fragment ion for multiple reaction monitoring (MRM) analysis because of greater specificity and lower background as compared with the fragment at m/z 247. DMEQ-TAD adducts of the target analytes consisted of 6R and 6S isomers, of which the more abundant 6S isomer was used for quantitation. In a representative serum sample, the 6S isomers of the DMEQ-TAD adducts of 25-OH-D3, 25-OH-D2, and 24,25-(OH)2D3 eluted at 3.80, 4.02, and 2.30 minutes, respectively, as shown in Figure 2B. The peak at 3.58 minutes comigrated with synthetic 3-epi-25-OH-D3, characterized by a single broad peak, suggesting coelution of the 6R and 6S isomers for this analyte. 3-epi-25-OH-D3 is a known isomer of 25-OH-D3. Because these two analytes were chromatographically resolved, the presence of 3-epi-25-OH-D3 did not confound the 25-OH-D3 measurement using the current assay.

Over the calibration range of 25-OH-D3 (10–370 nmol/L), 25-OH-D2 (12–291 nmol/L), and 24,25-(OH)2D3 (1–28 nmol/L), the method was shown to be linear for each analyte giving representative r2 values of at least 0.997 (Supplemental Table 3). Lower limits of quantification as defined by signal to noise ratios of 10 or greater were estimated to be within the 0.1- to 0.2-ng/mL range and lower limits of detection (signal to noise ratio ≥ 3) were estimated to be as low as 0.04 ng/mL. Intraassay and interassay imprecision was determined by analyzing five replicates of a serum sample containing 55 nmol/L 25-OH-D3, 83 nmol/L 25-OH-D2, and 6 nmol/L of 24,25-
determined by the simultaneous vitamin D metabolite assay agreed well with the values previously published using the Diasorin RIA method ($n = 289; r^2 = 0.817; \text{slope} = 0.902; \text{y intercept} = 17.3 \text{nmol/L}$) (13). In the complete set of approximately 672 serum samples from three published studies (13–15), the relationship between serum 25-OH-D$_3$ and serum 24,25-(OH)$_2$D$_3$ (Figure 3A) covered a range for serum 25-OH-D from 12.5 nmol/L to 200 nmol/L, reflecting the range of vitamin D supplementation and resulting in serum 24,25-(OH)$_2$D levels in the range of 0.25–30 nmol/L. There was a strong correlation between serum 25-OH-D and 24,25-(OH)$_2$D$_3$ ($n = 672, r^2 = 0.80$, slope = 0.13, x-intercept = 25.6 nmol/L), a relationship previously observed by others (23, 24). The intercept on the x-axis was 25 nmol/L, demonstrating that serum 24,25-(OH)$_2$D$_3$ is zero in individuals with serum 25-OH-D levels less than 25 nmol/L, a value considered vitamin D deficient by current public health criteria (5). The relationship between the molar ratio of serum 25-OH-D to 24,25-(OH)$_2$D to serum 25-OH-D is shown in Figure 3B and indicates that a ratio below 20 in a population corresponds to vitamin D sufficiency and a ratio above 20 suggests vitamin D insufficiency (25). Although less than

Utility of simultaneous vitamin D metabolite assay in women supplemented with vitamin D

The simultaneous vitamin D metabolite assay method was used to analyze serum 25-OH-D and 24,25-dihydroxyvitamin D [24,25-(OH)$_2$D] in a total of 694 samples from healthy women and in black and white postmenopausal osteopenic women before and after oral supplementation with between 0 and 4800 IU vitamin D$_3$ for 1 year (13–15). In samples collected from white postmenopausal osteopenic women (13), serum 25-OH-D values

Figure 2. A, Structure and fragmentation of DMEQ-TAD adduct of 24,25-(OH)$_2$D$_3$ used for detection in the LC-MS/MS assay. B, Mass spectrum of the DMEQ-TAD adduct of 24,25-(OH)$_2$D$_3$, indicating that a $m/z$ of 468 is a major fragment of the molecular ion $m/z$ 762. C, Composite chromatogram from superimposition of MRM at 746–>468 for 25-OH-D$_3$ and 3-epi-25-OH-D$_2$, at 758–>468 for 25-OH-D$_2$ and at 762–>468 for 24,25-(OH)$_2$D$_3$ in a serum extract.

Figure 3. A, Plot of serum 24,25-(OH)$_2$D$_3$ vs serum 24,25-(OH)$_2$D$_3$ in 694 serum samples from three different clinical trials determined by LC-MS/MS. B, Lowess plot (Prism; GraphPad) of the ratio of serum 25-OH-D$_3$ to 24,25-(OH)$_2$D$_3$ vs serum 25-OH-D$_3$ in 694 serum samples from three different clinical trials.
20% of these women exhibited measurable serum 25-OH-D$_2$ at baseline, this metabolite was negligible (<5 nmol/L) after vitamin D$_3$ supplementation for 1 year. 3-epi-25-OH-D$_3$ was measurable in most patients and averaged 6% of the serum 25-OH-D$_3$ value, in agreement with data from others (26, 27).

**Utility of simultaneous vitamin D metabolite assay in patients with IIH due to loss-of-function CYP24A1 mutations**

The simultaneous assay was used to measure vitamin D metabolite levels in serum samples from two patients previously diagnosed with IIH due to CYP24A1 mutations (16) (Table 1). Both patients with loss-of-function mutations of CYP24A1 had virtually undetectable levels of serum 24,25-(OH)$_2$D$_3$. Whether these measurable traces of 24,25-(OH)$_2$D$_3$ in IIH patients (Figure 4A) are small amounts of the metabolite made by other cytochrome P450s or represent other interfering dihydroxyvitamin D metabolites with the same retention time and mass spectral characteristics is unknown at this time (28, 29). Over the years, there have been reports that cytochrome P450s, other than CYP24A1, can generate 24,25-(OH)$_2$D$_3$, but those reports are mainly from in vitro studies (8, 28) and it is difficult to judge whether this can occur in vivo. On the other hand, if the trace metabolite is not 24,25-(OH)$_2$D$_3$ but an interfering metabolite, it is not 1,25-(OH)$_2$D$_3$, which runs with a different retention time on liquid chromatography [DMEQ adducts of 1,25-(OH)$_2$D$_3$ = 2.43 and 2.81 min; DMEQ adducts of 24,25-(OH)$_2$D$_3$ = 2.30 and 1.6 min]. Considering that the normal production of 24,25-(OH)$_2$D$_3$ is down-regulated completely when serum 25-OH-D$_3$ levels fall into the vitamin D deficient range, we propose that the ratio of 25-OH-D$_3$ to 24,25-(OH)$_2$D$_3$ is a more accurate parameter to use to express the absence of 24,25-(OH)$_2$D$_3$ in patients with IIH, especially because some of these patients have low vitamin D status (16). In both cases, the ratio of 25-OH-D$_3$ to 24,25-(OH)$_2$D$_3$ was in the 90–120 range, whereas normal values for this ratio rarely rise above 20.

**Utility of simultaneous vitamin D metabolite assay in patients with hypervitaminosis D**

The simultaneous vitamin D metabolite assay was used to assess a patient with hypercalcemia due to an excessive intake of vitamin D$_3$ (30). The 78-year-old patient pre-

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**Table 1. LC-MS/MS Analysis of Vitamin D Metabolites in Patients With Hypercalcemia**

<table>
<thead>
<tr>
<th>Patient</th>
<th>25-OH-D$_3$, nmol/L</th>
<th>24,25-(OH)$_2$D$_3$, nmol/L</th>
<th>25-OH-D$_3$ to 24,25-(OH)$_2$D$_3$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIH1</td>
<td>94.8</td>
<td>0.9</td>
<td>98.7</td>
</tr>
<tr>
<td>IIH2</td>
<td>81.2</td>
<td>0.7</td>
<td>112.8</td>
</tr>
<tr>
<td>Hypervitaminosis D</td>
<td>420.0</td>
<td>34.0</td>
<td>12.35</td>
</tr>
<tr>
<td>Control</td>
<td>53.5</td>
<td>5.0</td>
<td>10.6</td>
</tr>
</tbody>
</table>

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**Figure 4.** A. MRM of 24,25-(OH)$_2$D$_3$ in a serum extract from a patient with IIH due to loss-of-function mutation in CYP24A1 as compared with an unaffected sibling. B–D, MRMs of 25-OH-D$_3$, 24,25-(OH)$_2$D$_3$, and 25-OH-D$_3$-26,23-lactone in a serum extract from a hypervitaminotic D patient as compared with a normal vitamin D control. Note that all three metabolites are elevated in the hypervitaminotic D patient. MRM for 25-OH-D$_3$-26,23-lactone uses m/z 774->468.
sented with severe hypercalcemia (total calcium = 3.95 mM) and serum 25-OH-D of 788 nmol/L (normal range 82.5–250 nmol/L) and 1,25-(OH)2D of 18.3 pM (normal range 45–160 pM), by hospital liquid chromatography-mass spectrometry and RIA methods, respectively, suggesting hypervitaminosis D. Analysis of a subsequent serum sample using the simultaneous LC-MS/MS assay gave a 25-OH-D3 value of 420 nmol/L; a 24,25-(OH)2D3 value of 34 nmol/L (Figure 4, B and C) and a molar ratio of 25-OH-D3 to 24,25-(OH)2D3 of 12.35, suggesting no defect in CYP24A1 activity. Consistent with previous animal studies of hypervitaminosis D (31), the hypervitaminotic D patient also showed detectable amounts of the 25-OH-D3-26,23-lactone, another metabolic product of CYP24A1 (Figure 4D). The ability to detect multiple metabolites simultaneously is a major advantage of the use of DMEQ-TAD (21, 22).

Utility of simultaneous vitamin D metabolite assay in rodent serum samples
Rodent studies are often limited by the volumes of serum/plasma available for analysis and often necessitate sample pooling to allow for detection of vitamin D metabolites. The simultaneous vitamin D metabolite assay was used to analyze 100-μL samples from individual mice in a metabolic study involving wild-type, CYP2R1 knockout, and CYP2R1/CYP27A1 double-knockout mice (17) (Table 2). The simultaneous vitamin D metabolite assay gave serum 25-OH-D3 results in line with the other methods used, but, in addition, the simultaneous vitamin D metabolite assay provided serum 24,25-(OH)2D3 and 25-OH-D3-26,23-lactone levels. In a further study, analysis of serum from CYP24A1 knockout mice and their heterozygous littermates showed widely different levels of 25-OH-D3, 24,25-(OH)2D3, and 25-OH-D3-26,23-lactone in the two genotypes (Table 2). Homozygous CYP24A1 knockout mice exhibited 6-fold higher levels of 25-OH-D3 than littermates and virtually undetectable levels of serum 24,25-(OH)2D3, similar to the values observed in human IIH patients with genetic blocks in CYP24A1. The small residual 24,25-(OH)2D3 peak evokes the same questions raised for IIH of whether this is an interfering substance or evidence of a non-CYP24A1 vitamin D 24-hydroxylase. These rodent studies confirmed earlier findings (10) that the normal mouse exhibits a different ratio of 25-OH-D3 to 24,25-(OH)2D3 compared with the human and a significantly increased production of 25-OH-D3-26,23-lactone.

Discussion
We describe here a novel, highly-sensitive LC-MS/MS-based method to simultaneously assay six different vitamin D metabolites including 25-OH-D3, 25-OH-D2, 3-epi-25-OH-D3, 24,25-(OH)2D3, 24,25-(OH)2D2, and 25-OH-D3-26,23-lactone in triplicate in 100-μL serum samples of human or animal origin. The new method has been tested on approximately 1000 clinical samples, and the method for 25-OH-D shows good correlation with the National Institute for Standards and Technology and DEQAS samples for 25-OH-D (25-OH-D2 and 25-OH-D3) determination, which is the only parameter with good external standardization available. Data for the accurate determination of serum 24,25-(OH)2D3 were made possible by the novel synthesis of d6-24,25-(OH)2D3 by our organic chemistry collaborators, and this can also now be used to estimate serum 24,25-(OH)2D2 and 25-OH-D3-26,23-lactone concentrations as well as the ratio of 25-OH-D3 to 24,25-(OH)2D3, which is a pathophysiologically useful ratio. The clinical utility of the new method is illustrated in several situations such as the following: 1) its usefulness as a novel approach for predicting vitamin D deficiency by an elevated 25-OH-D3 to 24,25-(OH)2D3 ratio, which is in our opinion is at least equivalent to the PTH to 25-OH-D3 plots commonly used; 2) its usefulness in aiding in the diagnosis of IIH due to loss-of-function CYP24A1 mutations; and 3) its usefulness in distinguishing CYP24A1 defects from hypervitaminosis D during vitamin D intoxication. Furthermore, we also show the value of the new method to study the vitamin D metabolite

Table 2. LC-MS/MS Analysis of Serum Vitamin D Metabolites in Several CYP Knockout Mice

<table>
<thead>
<tr>
<th>Cyp2r1/Cyp27a1/ CYP24a1</th>
<th>25-OH-D3, nmol/La</th>
<th>24,25-(OH)2D3, nmol/La</th>
<th>25-OH-D3-26, 23-Lactone, nmol/La</th>
<th>25-OH-D3 to 24, 25-(OH)2D3 ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ +/+ +/+ +</td>
<td>42.2 ± 3.4</td>
<td>28.9 ± 2.2</td>
<td>13.5 ± 1.4</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>+/−/+ +/+ +/+</td>
<td>34.7 ± 3.3</td>
<td>25.5 ± 2.6</td>
<td>9.2 ± 2.1</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
<td>−/+ +/+ +/+</td>
<td>16.8 ± 2.5</td>
<td>13.5 ± 1.6</td>
<td>4.0 ± 0.9</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>+/+−/− +/+ −/−</td>
<td>176.3 ± 28.3</td>
<td>107.7 ± 6.4</td>
<td>42.1 ± 6.9</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>−/+ +/− +/− −/−</td>
<td>28.1 ± 3.8</td>
<td>25.8 ± 5.1</td>
<td>5.9 ± 0.9</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>+/+ +/ +/+ −/−</td>
<td>37.5 ± 3.6</td>
<td>19.2 ± 2.2</td>
<td>9.5 ± 0.8</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>+/+ +/+ −/− /−</td>
<td>256.6 ± 23.1</td>
<td>2.9 ± 0.3</td>
<td>0.3 ± 0.0</td>
<td>87.6 ± 9.2</td>
</tr>
</tbody>
</table>

*a Mean ± SD, n = 3–8 animals.
profile in the small blood volumes available in small animal models such as the knockout mouse.

The initial application of the new method was in the simultaneous determination of 25-OH-D3 and 24,25-(OH)2D3 in normal individuals over a wide range of vitamin D3 intakes (13–15). We chose to study a population of osteopenic women receiving vitamin D3 supplementation to gauge the importance of 24-hydroxylation in the catabolism of 25-OH-D3 and to relate the serum 25-OH-D levels, previously determined using a RIA (13–15). Results obtained using the RIA method correlated well with the LC-MS/MS technology. This was true both before and after supplementation with oral vitamin D3. The data, which include values for normal unsupplemented younger women, suggest that the new method works well over a wide serum 25-OH-D range from 12.5 nmol/L to 200 nmol/L. The ratio of 25-OH-D3 to 24,25-(OH)2D3 proved to be a valuable parameter in predicting vitamin D deficiency and showed consistent performance over a wide range of 25-OH-D values.

The new LC-MS/MS vitamin D metabolite assay was primarily developed for use in the diagnosis of IIH (16), in which the simultaneous analysis of 25-OH-D and 24,25-(OH)2D3 on small infant serum samples is critical in pinpointing defective CYP24A1 activity without the complications of vitamin D deficiency. The method works well in this application, giving absolute serum 24,25-(OH)2D3 values, as well as the ratio of 25-OH-D3 to 24,25-(OH)2D3, which together indicate the likelihood of IIH due to loss-of-function CYP24A1 mutation, which can be diagnosed only by expensive genetic testing. We conclude that 25-OH-D3 to 24,25-(OH)2D3 ratios greater than 80 are indicative of IIH due to inactivating mutations in CYP24A1. Furthermore, use of this method and determination of the 25-OH-D3 to 24,25-(OH)2D3 ratio allowed us to rule out CYP24A1 mutations in a patient with hypercalcemia resulting from vitamin D intoxication rather than an inability to catabolize 1,25-(OH)2D3 (7, 30). Taken together, the simultaneous assay of 25-OH-D3 and 24,25-(OH)2D3 is a valuable screening tool for patients presenting with hypercalcemia.

We show here the utility of the new method for the analysis of vitamin D metabolites including 24,25-(OH)2D3 in rodent sera (17, 18). The example involving mouse sera shows that LC-MS/MS, unlike certain commercially available RIAs (32), is not sensitive to the presence of blood proteins from certain species. Also unlike commercially available RIAs, LC-MS/MS can resolve or improve the complications caused by the presence of high levels of 24,25-(OH)2D3 and 25-OH-D3-26,23-lactone found in serum from wild-type mice (9). The rodent studies using CYP24A1 reinforce the current dogma that this cytochrome P450 is responsible for the production of many side-chain hydroxylated metabolites including 24,25-(OH)2D3 and 25-OH-D3-26,23-lactone (7). The examples here show that in addition to the advantage the LC-MS/MS method measures multiple vitamin D metabolites, it is sensitive enough to allow convenient analyses on individual animals as opposed to pooled samples. Animal-to-animal variability in mice with a similar genotype and diet is small and allows for more rigorous statistical analysis.

LC-MS/MS methodology continues to improve in terms of sensitivity, and the addition of a derivatization step with DMEQ-TAD improves this by at least 10-fold over conventional LC-MS/MS using the native vitamin D metabolites. LC-MS/MS methods, including the current simultaneous assay, have proven to be superior to immunoassays in their detection and accurate measurement of 25-OH-D2 that is present at high concentrations in vitamin D2-treated patients (33, 34). Part of the utility of the LC-MS/MS method for 24,25-(OH)2D3 is that it uses much smaller aliquots of serum than competitive-binding assays that preceded it. In addition, because there are a variety of Cookson reagents/dienophiles now becoming available (35, 36), we can expect this technology to become even more sensitive in the years to come and these improvements will eventually allow for the routine inclusion of 1,25-(OH)2D3 in the metabolites that can be measured using a 100-μL serum aliquot.

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Address all correspondence and requests for reprints to: Glenville Jones, BSc (Hons), PhD, Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, Ontario, Canada K7L 3N6. E-mail: gj1@queensu.ca.

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