Analytical measurement of serum 25-OH-vitamin D_3, 25-OH-vitamin D_2 and their C3-epimers by LC-MS/MS in infant and pediatric specimens

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ABSTRACT

Objectives: To develop a simple and sensitive LC-MS/MS procedure for quantification of serum 25-OH-vitamin D_3 (25-OH-D_3), 25-OH-vitamin D_2 (25-OH-D_2), and their C3-epimers.
Methods: Serum 25-OH-vitamin D metabolites were extracted with MTBE and quantified by LC-MS/MS. Commercially available calibrators and QC materials were employed. The ion-transition 401.2→365.2 was monitored for 25-OH-D_3 and C3-epi-25-OH-D_3, 407.2→371.3 for d_6-25-OH-D_3, 413.2→331.2 for 25-OH-D_2 and C3-epi-25-OH-D_2 and 419.2→337.1 for, d_6-25-OH-D_2. As a proof-of-principle, 25-OH-D_3 and C3-epi-25-OH-D_3 were quantified in 200 pediatric subjects (0-20 years of age). Cholecalciferol supplements were examined as a potential source of C3-epimer.
Results: The assay provided an LLOQ of ≤2.8 nmol/L for all 25-OH-D metabolites, with a linear response up to 400 nmol/L. The CV was <10% for 25-OH-$D_{2/3}$ and <15% for C3-epi-25-OH-D_3. C3-epi-25-OH-D_3 was quantified in all subjects, with higher concentrations observed in infants ≤1 year of age (11.44 nmol/L vs. 4.4 nmol/L; $p<0.001$). Within the first year of life, 25-OH-D_3 concentrations increased linearly, while C3-epi-25-OH-D_3 concentrations remained constant. At 12 months of age, C3-epi-25-OH-D_3 concentration dropped by almost 50% (11.4 nmol/L in infants ≤1 year of age vs. 5.4 nmol/L in infants 1-2 years of age; $p<0.001$). Liquid vitamin D_3 supplements did not contain appreciable amounts of C3-epi-D_3.
Conclusions: The proposed LC-MS/MS procedure is suitable for quantifying 25-OH-D_3 metabolites. Although the C3-epimer is present in all pediatric subjects, it is significantly elevated in individuals ≤1 year of age and drops at 12 months of age. Oral vitamin D supplements are unlikely to be a significant source of C3-vitamin D epimer.

INTRODUCTION

Quantitation of 25-hydroxyvitamin D by the clinical laboratory continues to receive considerable attention due to an ever-growing body of evidence suggesting it may be implicated in a number of disorders including osteoporosis, cancer, multiple sclerosis, diabetes and cardiovascular disease. In humans, cholecalciferol, or vitamin D_3, is the primary form of vitamin D, produced endogenously in the skin from 7-dehydrocholesterol in response to exposure to sunshine and ultraviolet light. Ergocalciferol, or vitamin D_2, is mainly derived from the diet in the form of supplements, fortified foods, and plants. Vitamin D_3 is hydroxylated in the liver to 25-OH-vitamin D_3 (25-OH-D_3) and is further transformed in the kidneys to produce the active metabolite 1α,25-dihydroxyvitamin D_3.

Various analytical techniques such as immunoassay, HPLC, and LC-MS/MS have been used for the purpose of quantifying 25-OH-D_3 and -D_2. Although tandem mass spectrometry offers good specificity and sensitivity, it is hampered by interferences such as isobaric or isomeric compounds that can affect measured values. In particular to 25-OH-D_3 quantitation, the C3-epimer of 25-OH-D_3, which is present in substantial amounts in some infants, co-elutes with 25-OH-D_3 in most HPLC conditions, potentially leading to an over estimation of 25-OH-D_3. To overcome this deficiency, a number of LC-MS/MS procedures have been described that employ chiral, CN or PFP HPLC columns and provide the necessary selectivity to separate the C3-epimer from 25-OH-D_3 and –D_2. Unfortunately, these prior procedures employ either complex extraction protocols or lengthy HPLC run times, limiting their application to a routine pediatric hospital where quantitation of C3-epi-25-OH-D_3 is recommended. In this report, we describe a simple, rapid, and sensitive LC-MS/MS procedure capable of quantifying 25-OH-D_3, 25-OH-D_2, and their C3-epimer isomers in 100 µL of serum. As a proof-of-principle, the assay is used to quantify 25-OH-D_3 and C3-epi-25-OH-D_3 in a pediatric cohort of 200 individuals. In addition, commonly available over-the-counter vitamin D_3 solutions are assessed for the presence of the C3-epimer. Importantly, this report provides enough analytical detail to permit other sites to adopt the methodology.

METHODS

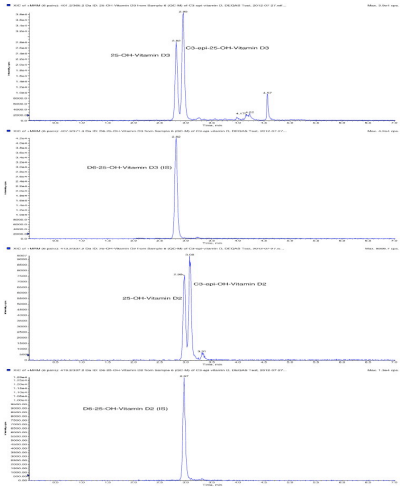
A volume of 100 µL serum was spiked with 25 µL internal standard (D_6-25-OH-D3 and D_6-25-OH-D2, 80 nmol/L final concentration). Samples were vortexed, incubated for 15 min/RT, and extracted with 1 mL of methyl-tert-butyl ether. Extracts were evapourated to dryness under N_2 gas and the residue was re-dissolved in 50 µL of methanol/water. A 20 µL aliquot was analyzed by LC-MS/MS. The LC-MS system consisted of an Agilent 1200 series HPLC and an API4000 QTRAP mass spectrometer. Vitamin D metabolites were separated on a Phenomenex Kintetex PFP column (100×3.0 mm, 2.6 µm). Mass spectrometry was conducted in positive APCI mode. One ion-transition was selected for each metabolite, as follows: 401.2→365.2, 407.2→371.3, 413.2→331.2, and 419.2→337.1 for 25-OH-D_3 and C3-epi-25-OH-D_3, D_6-25-OH-D_3, 25-OH-D_2 and C3-epi-25-OH-D_2, and D_6-25-OH-D_2 respectively. The source temperature was set at 300°C and the nebulizing gas at 40 units. Data acquisition and quantification was performed using the Analyst software and the Analyst Auto-Quant feature. The signal from the unknown analyte was measured against the calibration curve to obtain the concentration. Commercially available calibrators (3PLUS1 multilevel serum calibrator set, Chromsystems) and quality control material (tri-level vitamin D Plus serum control, UTAK Laboratories) were employed.

RESULTS

25-OH VITAMIN D LC-MS/MS METHODOLOGY

The LC-MS/MS spectra profile of serum spiked with C3-epi-25-OH-D metabolites is displayed in Figure 1. The extraction procedure and HPLC protocol provided adequate separation of the vitamin D metabolites with a run time of 7 min.

Figure 1. Representative LC-MS/MS spectra of serum spiked with C3-25-OH-D metabolites.



LIMIT OF QUANTITATION

The limit of quantitation was determined from four replicate measures of serum pools diluted with water. The cut-off criteria for LLOQ was either a CV of 20% or a non-linear response.

Table 1. Serum 25-OH-D metabolite limit of quantitation

Analyte	LLOQ (nmol/L)	SD (nmol/L)	%CV
25-OH-D_3	2.1	0.1	2.5
C3-epi-25-OH-D_3	2.0	0.1	5.4
25-OH-D_2	2.8	0.1	2.8
C3-epi-25-OH-D_2	2.4	0.2	8.5

IMPRECISION

Within-run (n=20) and between-day (n=10) imprecision were determined using UTAK Tri-Level control material.

Table 2. Between-day imprecision (n = 10)

Analyte	Concentration (nmol/L)	SD (nmol/L)	%CV
25-OH-D_3			
High	165.7	8.9	5.4
Medium	63.3	5.5	8.7
Low	21.7	1.6	7.4
C3-epi-25-OH-D_3			
High	207.1	13.9	6.7
Medium	100.3	8.2	8.2
Low	15.2	2.2	14.4
25-OH-D_2			
High	139.9	7.6	5.4
Medium	55.7	5.2	9.4
Low	19.0	1.7	9.1
C3-epi-25-OH-D_2			
High	145.6	7.8	5.3
Medium	70.2	6.6	9.5
Low	10.1	1.8	17.4

LINEARITY

Linearity was evaluated by diluting a serum pool spiked with 25-OH-D metabolites (600 nmol/L) with increasing volumes of charcol-stripped serum. Eleven dilutions covering 0-100% of the spiked serum pool were analyzed in duplicate. A linear response was maintained for all analytes up to 400 nmol/L, with a LLOQ of 2.8 nmol/L or less.

ACCURACY

Accuracy was established by quantifying vitamin D external quality assessment scheme (DEQAS) materials and NIST vitamin D SRM 972. The DEQAS405 specimen contained 25-OH-D_3 and C3-epi-25-OH-D_3. The DEQAS410 specimen contained both 25-OH-D_3 and 25-OH-D_2 and the sum of both metabolites was in close agreement with the reported all methods mean.

Table 3A. Analysis of vitamin D external quality assessment material

Specimen	25-OH-D_3 (nmol/L)		
	Proposed method	MS methods mean	All methods mean
DEQAS401	79.0	85.7	79.0
DEQAS402	28.0	29.2	26.6
DEQAS403	63.7	65.5	62.2
DEQAS404	51.1	50.9	45.9
DEQAS405[a]	52.0 (C3-epi-25-OH-D_3 = 69.0)	111.2	51.2
DEQAS406	22.1	24.6	25.5
DEQAS407	46.9	51.8	45.4
DEQAS408	64.2	80.0	71.2
DEQAS409	62.6	69.5	65.9
DEQAS410[b]	14.3 (25-OH-D_2 = 28.0)	55.1	41.8

Table 3B. Analysis of vitamin D NIST standards

Specimen	25-OH-D_3 (nmol/L)		C3-epi-25-OH-D_3 (nmol/L)	
	Proposed method	Certified value	Proposed method	Certified value
NIST SRM972-1	60.8	59.6 ± 2.1		
NIST SRM972-2	29.4	30.8 ± 1.5		
NIST SRM972-3	42.4	46.2 ± 2.8		
NIST SRM972-4	77.0	82.3 ± 2.0	91.1	94.1

[a] DEQAS405 contained both 25-OH-D_3 and C3-epi-25-OH-D_3.
[b] DEQAS410 contained both 25-OH-D_3 and 25-OH-D_2.

INTERFERENCES

Matrix effect and ion suppression were investigated by analyzing serum extracts during post-column infusion of a mixture of D_6-25-OH-D_3 and D_6-25-OH-D_2 (400 nmol/L). Aside from the solvent front (0.6 min), no disturbance in baseline recordings was observed. Interference by 1α-OH-D_3 was assessed by analyzing a solution of 1α-OH-D_3 and spiked serum. 1α-OH-D_3 eluted at 3.84 min and therefore does not contribute to 25-OH-D quantitation. Interference from hemolysis, lipemia, and icterus was assessed by analyzing three serum samples (low, medium, and high 25-OH-D concentration) spiked with human hemoglobin (5 g/L), triglyceride (17 mmol/L), or total bilirubin (500 µmol/L).

Table 4. Effect of hemolysis, lipemia, and icterus on 25-OH-D metabolite quantitation

	Percent recovery compared to control serum		
	Hemoglobin (5 g/L)	Bilirubin (500 µmol/L)	Triglycerides (17 mmol/L)
High (139 nmol/L)			
25-OH-D_3	108.1	98.3	98.8
C3-epi-25-OH-D_3	107.4	98.7	99.0
25-OH-D_2	109.1	95.7	107.1
C3-epi-25-OH-D_2	112.7	96.6	105.9
Medium (79 nmol/L)			
25-OH-D_3	99.8	98.2	93.8
C3-epi-25-OH-D_3	96.1	102.8	92.1
25-OH-D_2	96.9	100.3	101.1
C3-epi-25-OH-D_2	98.5	100.0	103.0
Low (26 nmol/L)			
25-OH-D_3	100.5	102.4	98.5
C3-epi-25-OH-D_3	94.5	105.4	98.2
25-OH-D_2	102.3	101.0	101.2
C3-epi-25-OH-D_2	94.5	109.8	99.1

SERUM CONCENTRATIONS OF 25-OH-D

Previous studies are discrepant as to whether C3-epi-25-OH-D_3 concentrations are elevated within the first year of life. Therefore, 25-OH-D3 and C3-epi-25-OH-D3 were quantified in 63 children ≤1 year of age and 137 children >1 year of age.

Figure 2. Serum concentrations of 25-OH-D3 (A) and C3-epi-25-OH-D3 (B) in children ≤ 1 year of age and children >1 year of age (C,D)

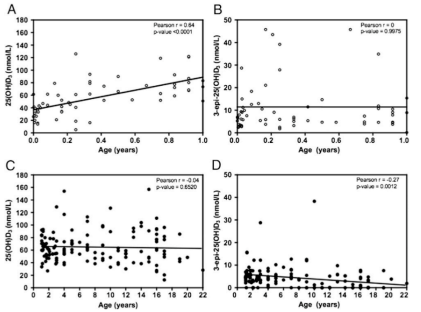
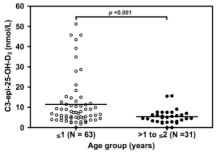


Figure 3. Serum concentrations of C3-epi-25-OH-D3 drop precipitously between the first and second year of life



LIQUID VITAMIN D SUPPLEMENTS

Liquid vitamin D supplements are commonly prescribed to infants <1 year of age. Four over-the-counter liquid vitamin D_3 supplements were examined to address whether they contain appreciable amounts of C3-epi-25-OH-D_3. All supplements displayed a single prominent peak at all ion transitions monitored for cholecalciferol (vitamin D_3) and a small peak consistent with C3-epi-D_3. However, the relative abundance of this peak (2% versus vitamin D_3) failed to explain entirely the elevated C3-epi-25-OH-D_3 concentrations seen in infants.

CONCLUSION

The proposed LC-MS/MS is suitable for routine use in a clinical laboratory as it provides the following:

- Adequate separation of vitamin D metabolites and C3-epimers with a simple extraction procedure and short analytical run time
- A limit of detection and linear range suitable for clinical samples (2.8 – 400 nmol/L)
- Acceptable imprecision, even for C3-epimers (<20% for low C3-epimer concentrations)
- Accuracy consistent with other 25-OH-vitamin D methodologies
- No observed matrix effects or ion suppression
- Minimal interference by hemolysis, lipemia, and icterus

By applying this assay to the quantitation of 25-OH-D_3 and C3-epi-25-OH-D_3 in infants and children we demonstrated the following:

- 25-OH-D_3 concentrations increase within the first year of life and stabilize after 1 year of age
- C3-epi-25-OH-D_3 concentrations remain constant until 1 year of age, when they drop abruptly, and continue to fall gradually throughout childhood
- Liquid vitamin D supplements are unlikely to be a significant source of C3-epimers

If LC-MS/MS methodologies fail to differentiate 25-OH-D from C3-epi-25-OH-D, pediatric subjects may be misclassified as vitamin D sufficient. Therefore, pediatric laboratories should invest in a method, such as this one, capable of discriminating between 25-OH-vitamin D and the epimeric forms.