Validation of a Bioanalytical Method for Serum Ergocalciferol and Cholecalciferol by LC-MS/MS

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Abstract

Summary
A simple, fast, LC-MS/MS method has been developed to allow quantitative measurements of the *precursors* of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 (Ergocalciferol; vitamin D2 and Cholecalciferol; vitamin D3) from serum or plasma.

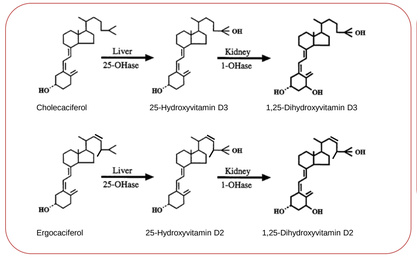
Introduction
Many different vitamin D metabolites in serum are measured in the clinical laboratory. The most commonly measured vitamin D test is 25-hydroxyvitamin D, which is a marker of vitamin D sufficiency. Another commonly ordered vitamin D test is 1,25-dihydroxyvitamin D, which can be useful in diagnosing rare disorders of calcium homeostasis. Other, less commonly ordered vitamin D tests are 24,25-dihydroxyvitamin D (a catabolic product in the vitamin D endocrine pathway that retains some biological activity) and the vitamin D that is made directly in the skin from ultraviolet light or is consumed through foods or dietary supplements (referred to as "calciferols"). This new method is specifically for measuring ergocalciferol and cholecalciferol (the class of vitamin D2 and vitamin D3 that has not yet been hydroxylated (other than carbon #3) by any tissues).

Methods
An analytical method was developed using a Thermo/Cohesive TX-4 HPLC system (Thermo-Fisher/Cohesive Technologies) with Agilent® 1200SL pumps (Agilent Technologies, Inc.) and an AB Sciex® 5000 (AB Sciex PTE. LTD.) triple quadrupole mass spectrometer. Independent calibration curves were prepared for Ergocalciferol (VD2) and Calciferol (VD3) in depleted serum (Golden West Biologicals). Sample preparation consisted of isotope dilution using a cocktail of both internal standards (IsoSciences) followed by protein precipitation and purification by phospholipid depletion (Phree). A Phenomenex® Synergi Max-RP® analytical column (50 x 2.1mm, 2.5um, 100A) was used with solvent gradient to achieve chromatographic separation. Positive mode atmospheric pressure chemical ionization (APCI) was used for detection in Multiple Reaction Monitoring (MRM) mode.

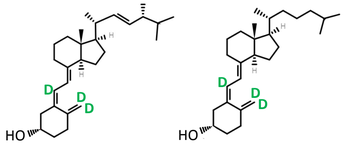
Validation Data
Analytical sensitivity was 1.0 ng/mL per analyte. Precision ranged from 3.8 – 11.4% (inter-assay). Accuracy ranged from 100.9% to 109.2%. Reference intervals were developed for total calciferols using discarded routine wellness screening specimens and found to be 0 – 50 ng/mL total calciferol.

Clinical Significance
The measurement of calciferol has historically been difficult owing to its very hydrophobic nature and complex biological matrices. Some clinical applications for measuring calciferol include:
1. *Compliance with oral supplement therapy*. Calciferol is normally converted within ~24hr to 25OHD. Measurement Ergocalciferol and Cholecalciferol can assist the ordering physician in determining whether supplements are being consumed (or UV therapy has been successful).
2. *Disorders of lipid absorption*. The absorption of fat-soluble vitamins is typically measured by stool fat analysis. Pre-treatment of a patient with high dose Ergocalciferol followed by Calciferol and 25OHD analysis could reduce or replace the high-fat diet required for stool fat analysis.
3. *Failure to hydroxylate*. The hydroxylation of Calciferols on carbon # 25 is almost exclusively performed in the liver. Patients with liver disease or those taking cytochrome P450 inhibitors (anticonvulsants) can show decreased hydroxylation, which can be better understood by measuring calciferols in addition to 25OHD.
4. *Assessment of acute toxicity*. Sporadic cases in the literature have identified cases of acute vitamin D intoxication, primarily by decreased PTH and rapidly increased 25OHD. Measurement of calciferols could be used to monitor the progress of therapy associated with vitamin D intoxication.
5. *First order kinetics*. The enzyme that mediates hydroxylation at carbon # 25 is best described as first order kinetics; Calciferols and 25OHD will reach equilibrium when concentration is equal. By measuring both classes of metabolites, enthusiasts can adjust their supplement dosage to optimize the Calciferol/25OHD ratio.

Fundamental Vitamin D Pathway

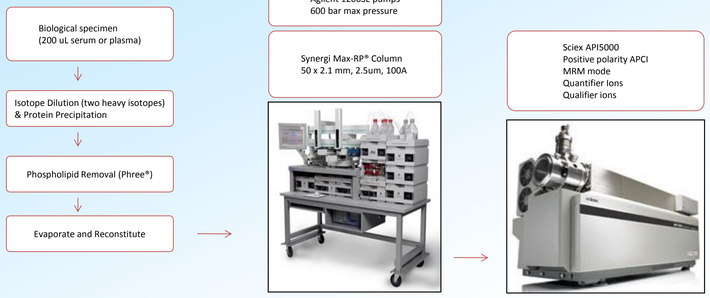


Analytes and Internal Standards



The green "D" letters indicate the position of the deuterated atoms in the corresponding internal standard.Heavy isotopes were purchased from IsoSciences (vitamin D_3-$^2H_3$ and vitamin D_2-$^2H_3$.

Method Summary

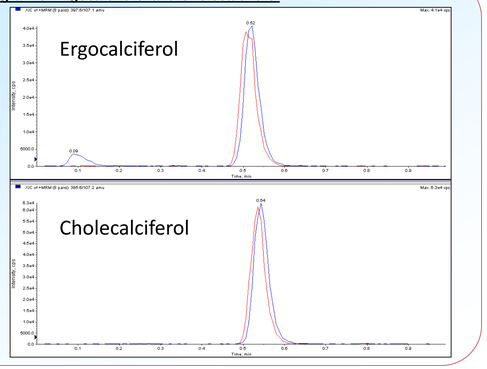


Analytical Performance

Mass Chromatogram of Ergocalciferol and Cholecalciferol

Sample mass chromatogram:
(25 ng/mL each analyte and IS)
Blue traces: Analytes
Red traces: Internal Standard

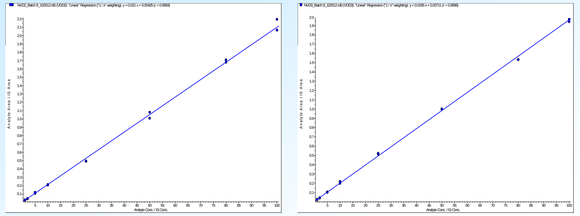
Peak	Ion	Ergo-calciferol	Chole-calciferol
Analyte	Quantifier	397.6 – 107.1	385.6 – 107.2
	Qualifier	397.6 – 271.4	385.6 – 259.5
Internal Standard	Quantifier	400.6 – 110.1	388.6 – 110.2
	Qualifier	400.6 – 271.4	388.6 – 259.5



Calibration Curves for Ergocalciferol and Cholecalfierol

Duplicate Calibration curves are obtained for each analyte:

Cal Matrix: Golden West Biologicals MSG-1000



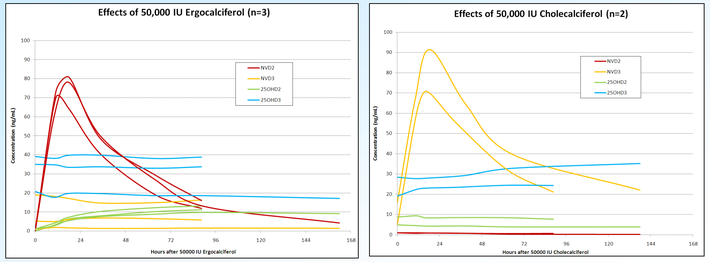
Ergocalciferol
1.0 – 100 ng/mL

Cholecalciferol
1.0 – 100 ng/mL

Sensitivity defined as concentration with 80-120% accuracy and <20% CV (true LLOQ; not LLOD)

Time course – Calciferol Supplementation

Volunteers consumed 50000 IU of over the counter supplements of ergocalciferol or cholecalciferol and blood specimens were collected over the following week. The peak calciferol concentration was obtained within 12-24 hours.



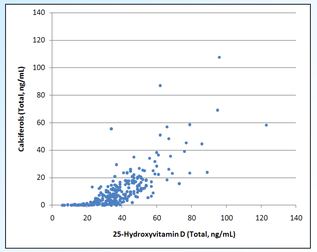
Analytical Performance

Precision and Accuracy (Inter-Assay, n=18, six replicates on three separate days), ng/mL

	Ergocalciferol								Cholecalciferol							
	Precision				Accuracy				Precision				Accuracy			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Average	1.05	2.18	53.9	105	1.05	2.18	53.9	105	1.07	2.02	51.0	101	1.07	2.02	51.0	101
CV%	11.4	9.9	3.8	5.0	105.2	109.2	107.9	105.2	9.6	6.1	4.0	3.8	106.8	100.9	102.0	101.0

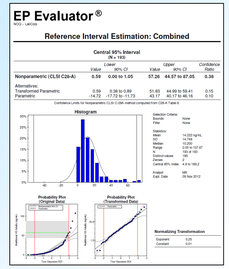
Relationship between Calciferols & 25OHVD in Routine Subjects

Calciferol and 25-Hydroxyvitamin D from 286 routine specimens were measured by LC-MS/MS. The absence of patients with low 25OHD and detectable calciferol, coupled with the presence of patients with high normal 25OHD and calciferol, suggests that this method would be a valuable tool for monitoring absorption and/or patient compliance.



Reference Interval

Out of 286 specimens analyzed, 193 were normal for 25OHD and used to generate a reference interval of <57 ng/mL total Calciferol. Only seven of the 193 patients used had ergoalciferol >10 ng/mL



Summary

Results and Discussion

1. A fast, simple 1-D bioanalytical HPLC-MS/MS method has been developed to measure Ergocalciferol and Cholecalciferol from serum or plasma.
2. Analysis of native molecules rather than employing Diels-Alder derivatization resulted in a simpler workflow and reduced instrument downtime.
3. Very little calciferol was found when total 25-Hydroxyvitamin D was below 20 ng/mL.
4. Oral supplementation experiments verified the data from existing literature regarding 25-Hydroxylation rate.
5. This method will enable further insight into understanding the metabolism of dietary or ultraviolet vitamin D supplementation.

References and Acknowledgements

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Thank you to the staff members who volunteered to have blood drawn for the time course study.

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