SHIMADZU

WP 042

Automated Clinical Sample Preparation and Triple Quadrupole LC-MS/MS Analysis via Reverse Trap-and-Elute Chromatography of Serum Vitamin D Metabolites

Stanislaw Krzyzanowski[1], Praveen K. Alla[2], Milaan Thirukumaran[1], Alyssa S. Aebersold[2], Mathew S. Hautman[1], Mohamed Nazim Boutaghou[1]

1 Shimadzu Scientific Instruments, Columbia, MD,USA 2 CompuNet Clinical Laboratory, Dayton, OH USA

1. Overview

Development and validation of a fully automated sample preparation and subsequent LC-MS/MS with trap-and-elute chromatography assay analyzing 25-hydroxyvitamin-D2 and D3.

2. Introduction

Over the past two decades, the demand for vitamin D metabolite testing has risen significantly, driven by greater awareness of vitamin D deficiency and the investigation of how vitamin D metabolism influences physiological processes beyond calcium absorption.

Vitamin D status is determined by measuring the concentrations of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in serum.[1,2] These primary hydroxylated metabolites serve as biomarkers for vitamin D status because they are the major circulating forms of vitamin D, making them clinically measurable. With a half-life of approximately 2-3 weeks, these metabolites represent both dietary intake and production from sun exposure.[2]

Although LC-MS/MS is regarded as the gold standard for accurately quantifying 25-hydroxyvitamin D, many laboratories continue to utilize automated immunoassays. The automation provided by immunoassays reduces labor while increasing throughput and revenue.

To combine the accuracy of LC-MS/MS with the advantages of automation, a fully automated and integrated LC-MS/MS workflow (Figure 1) is introduced. This workflow includes integrated protein precipitation and online solid-phase extraction (SPE) steps. By utilizing this automated platform, technicians can significantly reduce their active time in the sample preparation workflow, focusing primarily on sample registration and results review.

Figure 1: CLAM-2040 module coupled with a Nexera 40 series Liquid Chromatograph tandem LCMS-8060 triple quadrupole mass spectrometer.

3. Methods

Analysis of vitamin D metabolites from serum was performed using a CLAM-2040 online with a Nexera 40 series LC configured for online SPE and an LCMS-8060.

Protocol for sample preparation on the CLAM-2040 prior to automatic transfer of sample extract to the autosampler is described in Figure 2. LC, MS interface, and MRM conditions for optimized method are described in Table 1, Table 2, and Table 3, respectively. Figure 3 illustrates LC pump set up for on-line trap and elute.

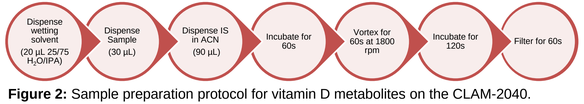

Figure 2: Sample preparation protocol for vitamin D metabolites on the CLAM-2040.

Table 1: LC conditions

Trap Column	Shim-pack MAYI-C4
Guard Column	Shim-pack Velox PFPP (2.7 µm, 3.0 x 5 mm)
Analytical Column	Shim-pack Velox PFPP (2.7 µm, 3.0 x 100 mm)
Column Oven Temperature	50°C
Injection Volume	20 µL
Mobile Phase A	0.1% Formic acid
Mobile Phase B	Methanol
Mobile Phase C	80:10:10:0.1 (H_2O:Methanol:Acetonitrile:formic acid, v:v:v:v)

Table 2: MS interface conditions

Ionization	ESI (positive mode)
Interface Temperature	300°C
Desolvation Line Temperature	250°C
Heat Block Temperature	400°C
Heating Gas Flow	20 L/min
Drying Gas Flow	10 L/min
Nebulizing Gas Flow	3 L/min

Table 3: MRM transitions and retention time (RT) for analytes and internal standard.

Analyte	RT (min)	Precursor (m/z)	Product (m/z)	Q1 PreBias (V)	CE	Q3 PreBias (V)
25-hydroxyvitamin D2	3.04	**395.3**	**269.3**	**-20**	**-17**	**-18**
		395.3	209.1	-20	-26	-21
25-hydroxyvitamin D3	2.97	**383.3**	**257.3**	**-22**	**-22**	**-29**
		383.3	211.2	-21	-28	-24
D_6-25-hydroxyvitamin D3	2.97	**389.3**	**263.3**	**-30**	**-19**	**-29**
		389.3	211.2	-15	-28	-10

A dwell time of 32 ms was used for all transitions. Bolded transitions were used for quantitation.

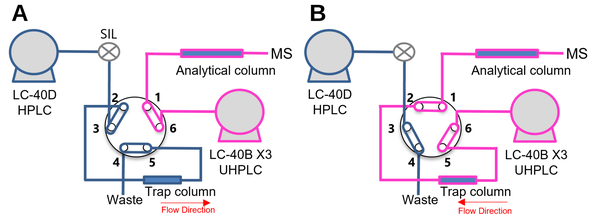

Figure 3: LC pump configuration for online-SPE. **A.** the valve positioning used at the beginning and end of each chromatographic run to load the trap column with sample and re-equilibrate both the trap and analytical column, respectively. **B.** the valve positioning used to elute analyte off the trap column and perform the analytical separation.

4. Results

The optimized method had an injection-to-injection time of 5.0 min. A six-point matrix matched calibration curve with internal standard correction was constructed to measure 25-hydroxyvitamin D2 and D3 between 5 – 130 µg/L (Figure 4).

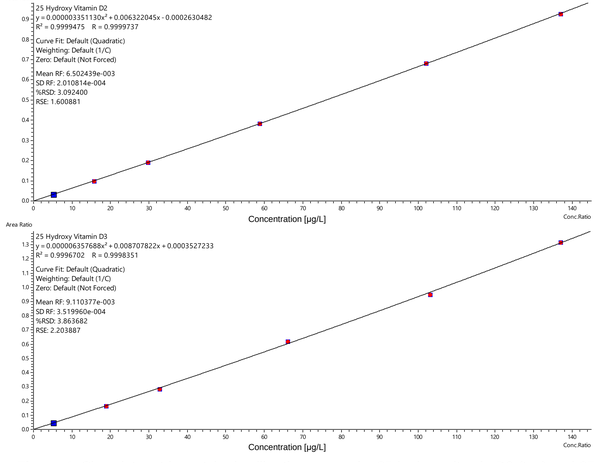

Figure 4: Six-point matrix-matched calibration curves for 25-hydroxyvitamin D2 (top) and 25-hydroxyvitamin D3 (bottom).

Intra and inter day accuracy and precision studies were conducted using three QC levels (n=5); low, medium, and high. Data for 25-hydroxyvitamin D2 is presented in Table 4 while data for 25-hydroxyvitamin D3 is presented in Table 5.

Table 4: Intra and inter day accuracy and precision data for 25-hydroxyvitamin D2

	Intraday		Interday	
QC Level	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Low	94.9	6.4	97.1	5.9
Medium	105.5	5.7	97.4	16.9
High	105.7	5.6	85.0	2.7

Table 5: Intra and inter day accuracy and precision data for 25-hydroxyvitamin D3

	Intraday		Interday	
QC Level	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
Low	95.5	7.2	96.0	8.0
Medium	105.8	2.8	95.0	8.3
High	94.6	5.7	96.3	2.2

Matrix effect was measured by comparing the back calculated concentration of water spiked with 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 against calibrator material of the same concentrations. Matrix effects were found to be within 80 – 120%.

Carryover tests were conducted by running a blank sample after the analysis of the highest calibrator. Blank samples did not show carryover with area counts being under 10% of the area counts for the lowest calibrator.

5. Conclusion

Shimadzu CLAM-2040 automated sample preparation module was used online with a Shimadzu triple quadruple LCMS-8060 to quantify serum 25-hydroxyvitamin D via electrospray ionization. The implementation of reverse trap elute chromatography combined with the Shimadzu CLAM-2040 automated sample preparation system enabled robust, high-throughput analysis of vitamin D metabolites with no carryover. By automating and optimizing the workflow, this method supports efficient and reproducible quantification.

6. References

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2. *Giustina A. et al. Consensus Statement on Vitamin D Status Assessment and Supplementation: Whys, Whens, and Hows. Endocrine Reviews. 2024 Apr 27; 45(5): 625-654.*

3. *Miyamoto et. Al. Determination of a Serum 25-Hydroxyvitamin D Reference Ranges in Japanese Adults Using Fully Automated Liquid Chromatography– Tandem Mass Spectrometry. The Journal of Nutrition. 153 (2023)1253–1264*

The authors declare no competing financial interest.

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