AppNote 4/2012

Automated Extraction of Vitamin D Metabolites from Serum

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Keywords
Automation, Clinical/Toxicology, Sample Preparation, Solid Phase Extraction, LC-MS, LC-MS/MS

Abstract
Testing for vitamin D deficiency is becoming increasingly important in settings such as research laboratories and hospital clinical laboratories, so a reliable, robust, high throughput method is needed to offer fast turnaround of samples. The automated extraction procedure described here features internal standard addition, protein precipitation, automated centrifugation and supernatant handling, and miniaturized solid phase extraction to eliminate sample matrix The prepared extracts are automatically introduced to the liquid chromatography - tandem mass spectrometry system for analysis.

This study focuses on the automated extraction of small sample volumes coupled to LC-MS/MS in order to provide high throughput analysis of both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2. A GERSTEL MultiPurpose Sampler (MPS) was used to automate all blood serum pre-treatment steps as well as automated solid phase extraction using a C8 solid phase extraction (SPE) sorbent in a miniaturized cartridge format referred to as Instrument Top Sample Preparation (ITSP). The resulting eluents from the automated SPE were then introduced into an Agilent 6460 LC/MS/MS instrument.

Introduction

Monitoring of vitamin D levels in patients is important for the prevention and control of disease. Vitamin D plays a critical role in regulating calcium and phosphorus levels in the body. If these levels are not adequately controlled, bone mineralization conditions, such as rickets in children or osteoporosis in adults may occur. Recent studies identifying a role for vitamin D in prevention of cancer and cardiovascular disease have generated renewed interest in monitoring vitamin D levels in serum. Vitamin D is a hydrophobic, fat soluble vitamin that is absorbed like a fat in the intestines. Vitamin D also promotes reabsorption of calcium in the kidneys. Vitamin D levels are commonly determined to diagnose conditions that interfere with fat absorption, such as Crohn‘s disease.

Automation of manual procedures enables scaling up of laboratory throughput, especially when sample preparation and SPE is performed automatically during chromatographic analysis of the preceding sample extract, reducing the total analysis time. Each sample extract is treated in exactly the same way and is prepared “just in time” for introduction to the analysis system when it reaches the ready state after the previous run. Identical treatment improves sample to sample reproducibility compared with manual batch processes. Improved data quality is achieved due to the consistency gained by automating liquid handling for addition of standards and reagents and due to the elimination of manual SPE procedures. In addition, sample sequence integration between the sample preparation system and the analysis system makes setup simpler and more efficient while also reducing the risk of transcription errors.

The data show that combining automated SPE with direct introduction to an Agilent 6460 LC/MS/MS instrument with APCI ionization source results in highly sensitive determination of Vitamin D3 and D2 metabolites in serum. Low limits of quantitation (5 ng/mL) were achieved along with good linearity for calibration curves. Correlation coefficients greater than 0.99 were obtained for both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 calibration curves and precision data of the automated method were found to be within typical acceptance ranges. Using the MAESTRO PrepAhead function, the automated system used for this work is capable of processing 96 samples within nineteen hours.

Experimental

Materials. 25-hydroxyvitamin D3 (cat.#H-083) and 25-hydroxyvitamin D2 (cat.#H-073) were purchased from Cerilliant. Intermediate analyte stock solutions in horse serum (Sigma cat.#HO146-10mL) were prepared by combining the analyte stock solutions at appropriate concentrations.

The deuterated analogue, d_6-25-hydroxyvitamin D3 (cat.#H-074), was purchased from Cerilliant. A working internal standard stock solution containing the deuterated internal standard was prepared in methanol at a concentration of 500 ng/mL.

Calibration standards and QC samples were prepared by making appropriate dilutions of the combined 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 intermediate stock solution using horse serum to give concentrations equivalent to 5, 10, 25, 50, 75, 100, and 150 ng/mL for the calibration standards and 12, 60, and 120 ng/mL for the QC samples.

In order to determine extraction recovery at a concentration equivalent to an extracted 60 ng/mL QC sample, an extraction recovery standard was prepared by combining the analyte stock solutions and deuterated analogue stock solution with combined eluents from five native, horse serum samples previously extracted without the addition of internal standard.

The 0.2 M zinc sulfate solution used during protein precipitation was prepared by combining 50 mL of a 2.0 M zinc sulfate solution (Sigma cat.#83265-250mL-F) with 450 mL of LCMS grade water (Sigma cat.# 270733-4L). All other reagents and solvents used were reagent grade.

Instrumentation. All automated PrepSequences and injections were performed using a Dual Head MultiPurpose Sampler (MPS XL) equipped with Anatune CF-100 Centrifuge Option, MicroLiter ITSP Option and Active WashStation as shown in Figure 1. All analyses were performed using an Agilent 1290 HPLC with a Zorbax Eclipse Plus C18 column (2.1 x 100 mm, 1.8 µm, 600 bar) and an Agilent 6460 Triple Quadrupole Mass Spectrometer with APCI source. Sample injections were made using a 6 port (0.25 mm) Cheminert C2V injection valve fitted with a 20 µL stainless steel sample loop.

Figure 1. MultiPurpose Sampler (MPS XL) with CF-100 Centrifuge and ITSP Options.

Serum Sample Pretreatment:

- Pipette 200 µL of horse serum sample into a clean autosampler vial and seal with a magnetically transportable screw cap.

Figure 2 shows the MAESTRO Prep Sequence used during the automated sample preparation and extraction process. The automated preparation and extraction steps used for this method consisted of the following:

Automated 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 Prep Sequence - Internal Standard Addition and Protein Precipitation:

1. The MPS adds 50 µL of the 500 ng/mL d_6-25-hydroxyvitamin D3 solution into the sample vial.
2. The MPS adds 200 µL of the 0.2 M zinc sulfate solution into the sample vial.
3. The MPS adds 500 µL of 100 % methanol into the sample vial.
4. Agitate the sample vial using the Anatune CF-100 centrifuge for 30 seconds.
5. Centrifuge the sample vial at 575 g for 3 minutes using the Anatune CF-100 centrifuge.

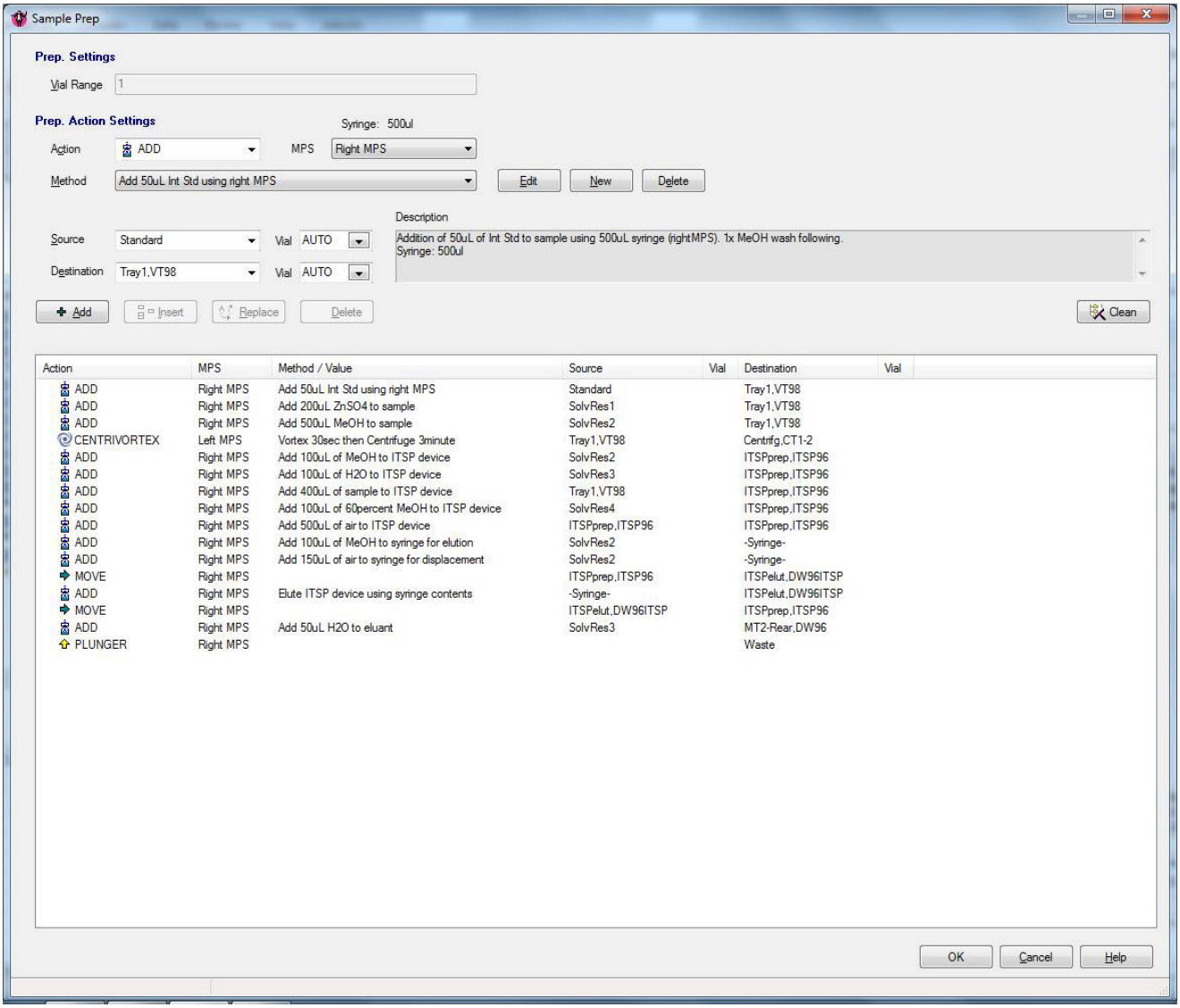


Figure 2. MAESTRO PrepSequence for the sample preparation and extraction process.

Solid Phase Extraction using ITSP:

6. The MPS conditions the C8 ITSP device by adding 100 µL of methanol.
7. The MPS conditions the C8 ITSP device by adding 100 µL of water.
8. The MPS adds 400 µL of the supernatant from the previously centrifuged serum sample into the C8 ITSP device.
9. The MPS washes the C8 ITSP device by adding 100 µL of 60 % methanol in water followed by 500 µL of air for positive displacement.
10. The MPS moves the ITSP device to a clean, 96 position, deep well plate and elutes the analytes using 100 µL of methanol followed by 150 µL of air for positive displacement.
11. The MPS dilutes the final extract by adding 50 µL of water.
12. The MPS injects 20 µL of the final extract into the LC/MS/MS system using the loop-overfill technique.

Analysis conditions LC.

Pump:	gradient (650 bar), flowrate = 0.4 mL/min	
Mobile Phase:	A - 0.1 % acetic acid in water	
	B – 0.1 % acetic acid in MeOH	
Gradient:	Initial	80 % B
	2.0 min	90 % B
	2.5 min	90 % B
	2.6 min	80 % B
	5.0 min	80 % B
Run time:	5.0 minutes	
Injection volume:	20 µL (loop over-fill technique)	
Column temp.:	50°C	

Analysis conditions MS.

Operation:	APCI positive mode
Gas temperature:	350°C
APCI heater:	250°C
Gas flow (N2):	5 L/min
Nebulizer pressure:	25 psi
Capillary voltage:	3000 V
Delta EMV:	+700 V

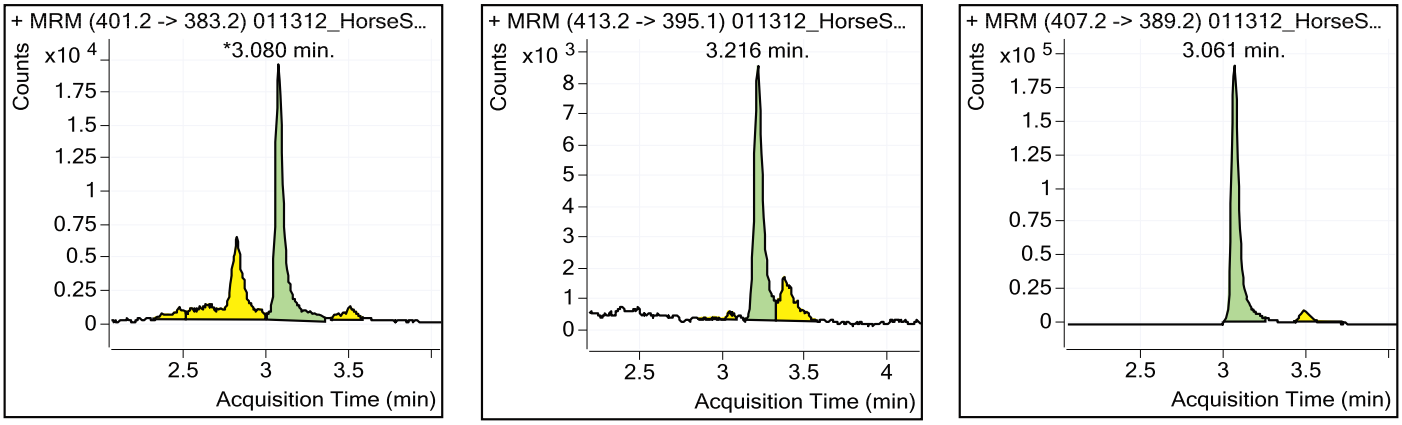
The mass spectrometer acquisition parameters and the qualifier ions used are shown in Table 1.

Table 1. Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]		CE [V]		Ret Time [min]
25-hydroxyvitamin D3	401.2	383.2	158.9	2	25	3.07
25-hydroxyvitamin D2	413.2	395.1	158.9	2	25	3.21
d_6-25-hydroxyvitamin D3	407.2	389.2		2		3.07

Results and Discussion

Figure 3 shows representative mass chromatograms for 25-hydroxyvitamin D3, 25-hydroxyvitamin D2, as well as d_6-25-hydroxyvitamin D3, from an extracted low QC sample.



25-hydroxyvitamin D3 | 25-hydroxyvitamin D2 | d_6-25-hydroxyvitamin D3

Figure 3. Representative mass chromatograms for low QC sample.

The lower limits of quantitation of this method were found to be 5 ng/mL for both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2. Representative calibration curves are shown in Figure 4. Regression analysis for both analytes resulted in R^2 values of 0.99 or greater.

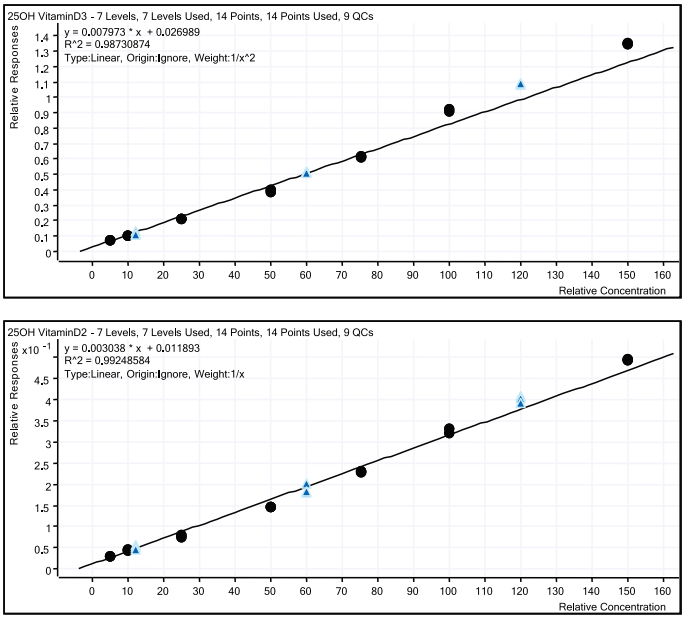


Figure 4. Representative calibration curves.

The accuracy and precision of the method were determined for both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 using QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for both analytes. For 25-hydroxyvitamin D3, accuracy data averaged 101 % (range: 92.4 - 111 %) and precision data averaged 5.70 % RSD (range: 0.292 -15.9 %). For 25-hydroxyvitamin D2, accuracy data averaged 104 % (range: 96.9 - 107 %) and precision data averaged 7.43 % RSD (range: 2.12 -14.4 %).

Table 2. QC samples accuracy and precision data.

Compound	QC1 12 ng/mL	QC2 60 ng/mL	QC3 120 ng/mL
25OH Vitamin D3			
mean	11.1	60.9	133
SD	1.77	0.547	0.388
% CV	15.9	0.899	0.292
Ave.% Accuracy	92.4	101	111
25OH Vitamin D2			
mean	12.8	58.1	128
SD	1.84	3.36	2.72
% CV	14.4	5.78	2.12
Ave.% Accuracy	107	96.9	107

Extraction recovery for both 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 was assessed by comparing the average peak area ratios from the extracted mid-level QC samples prepared in horse serum to the average peak area ratios of recovery standards prepared at a concentration equivalent to an extracted mid-level QC sample. As shown in Table 3, the recovery for 25-hydroxyvitamin D3 was found to be 98.5 % and the recovery for 25-hydroxyvitamin D2 was found to be 102 %.

Table 3. Extraction % recovery data.

	25-OH Vitamin D3 Resp Ratio	25-OH Vitamin D2 Resp Ratio
Extr. QC		
	0.5081	0.1836
	0.5121	0.1817
	0.5168	0.2002
mean	0.512	0.188
SD	0.00436	0.0102
% CV	0.851	5.41
N	3	3
Recovery Std.		
	0.5221	0.1795
	0.5161	0.1864
	0.5298	0.1897
	0.5276	0.1885
	0.5163	0.1881
	0.5091	0.1801
mean	0.520	0.185
SD	0.00783	0.00443
% CV	1.50	2.39
N	6	6
% Recovery	98.5	102

Conclusions

As a result of this study, we were able to show:

- 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 can be successfully extracted from blood serum samples and determined using automated centrifugation and miniaturized solid phase extraction followed by LC/MS/MS determination using an Agilent 6460 Triple Quadrupole Mass Spectrometer.
- This preparation and extraction method proved to be rapid and readily automated using the Dual Head GERSTEL MultiPurpose Sampler (MPS XL).
- Linear calibration curves resulting in R^2 values 0.99 or greater were achieved with limits of quantitation of 5 ng/mL for both analytes.
- The complete extraction and LC/MS/MS analysis method proved to be accurate and precise. For 25-hydroxyvitamin D3, accuracy data averaged 101 % (range: 92.4 - 111 %) and precision data averaged 5.70 % RSD (range: 0.292 -15.9 %). For 25-hydroxyvitamin D2, accuracy data averaged 104 % (range: 96.9 - 107 %) and precision data averaged 7.43 % RSD (range: 2.12 -14.4 %).
- Extraction recovery data was found to be 98.5 % for 25-hydroxyvitamin D3 and 102 % for 25-hydroxyvitamin D2.

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