Rapid Analysis of 25-OH Vitamin D in Serum Using an Agilent Triple Quadrupole LC/MS System with Automated Online Sample Cleanup

Application Note

Clinical Research

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Abstract

A rapid and reliable LC/MS research method for accurate, sensitive, and reproducible quantification of 25-OH vitamin D in serum was developed using an Agilent 1260 Infinity LC coupled to an Agilent 6460 Triple Quadrupole Mass Spectrometer with Agilent JetStream (AJS). Sample preparation consists of a simple liquid-liquid extraction. Automated online sample cleanup further minimizes matrix interferences and ion suppression, which improves quantitation without increasing manual sample handling. Excellent linearity was observed from 1 to 250 ng/mL with run times of only 5 minutes and interday precision of well below 10 %.

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Introduction

Vitamin D exists in two forms (D2 and D3), each of which undergoes a similar metabolism to form 25-hydroxy vitamin D (25-OH D). Both 25-OH D2 and D3 are measured in blood and are often collectively referred to as vitamin D or 25-OH D. In order to accurately quantify 25-OH D levels, it is important to differentiate these metabolites. The ability to accurately distinguish these metabolites has helped LC/MS gain acceptance as the analytical research methodology of choice.

This application note describes a method for the rapid, sensitive and accurate determination of 25-OH vitamin D2 and D3 in serum using the Agilent 6460 Triple Quadrupole LC/MS system equipped with Agilent JetStream (AJS). The use of a simple liquid-liquid extraction sample preparation and automated online sample cleanup reduces matrix effects and ion suppression, with no interferences observed in a large number of serum samples.

Experimental

Reagents and Standards

Stock solutions of 25-OH D2, 25-OH D3 and their deuterated internal standards (IsoSciences) were prepared at 10 µg/mL in methanol (Honeywell) and stored at –20 °C. Calibration standard solutions were prepared from these stock solutions at 1, 25, 75, 125, and 250 ng/mL in pooled serum (Note: Pooled serum contains endogenous levels of vitamin D metabolites. To achieve calibration levels lower than the endogenous level, pooled serum was diluted with 5 % bovine serum albumin.) Deuterated 25-OH D2 and D3 internal standards were diluted to 1,000 ng/mL with methanol.

Automated Online Sample Cleanup

The HPLC used for this method was configured for automated sample cleanup using two binary pumps (Figure 1). Samples are loaded onto a trapping column where the analytes are retained and washed by the first pump. The wash is sent to waste, reducing the amount of matrix introduced into the mass spectrometer. Shortly before the analytes elute off of the trapping column, a valve is switched and the analytes are eluted onto an analytical column where further chromatography is performed using the second binary pump.

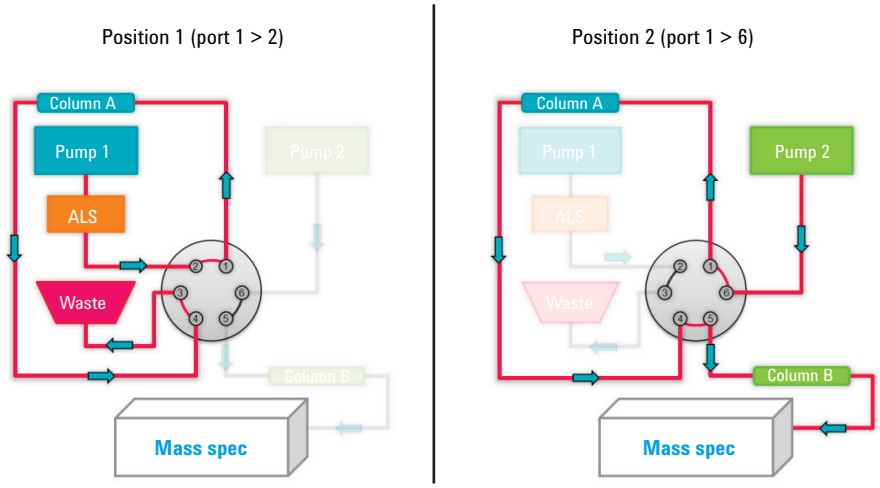

Figure 1. LC configuration for automated online sample cleanup.

LC configuration and conditions

Configuration	
2 x Agilent 1260 Infinity Binary Pump (G1312B)	
1 x High Performance WP Sampler (G1367E)	
1 x Sampler Thermostat (G1330B)	
1 x Thermostated Column Compartment (G1316C with option #058)	
1 x 2 Pos/6 port valve-head (G4231A)	
Trapping column	Agilent ZORBAX Eclipse Plus C18 Guard column, 2.1 × 12.5 mm, 5 µm
Analytical column	Agilent Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 µm
Column temperature	50 °C
Injection volume	10 µL
Needle wash	flush port, 50:25:25 IPA:MeOH:H_2O, 5 seconds
Mobile phase A	H_2O + 0.1 % Formic acid
Mobile phase B	MeOH + 0.1 % Formic acid
Flow rate	0.5 mL/min
Stop time	5.0 minutes

Loading (Pump 1) gradient		Eluting (Pump 2) gradient		Valve timing	
Time	**B %**	**Time**	**B %**	**Time (min)**	**Position**
0.00	50	0.00	85	0.00	1
1.00	90	3.20	85	1.90	2
2.30	90	3.21	98	2.30	1
2.31	98	3.80	98		
3.30	98	3.81	85		
3.31	50				

MS/MS configuration and conditions

Configuration	
Agilent 6460 Triple Quadrupole LC/MS equipped with Agilent JetStream	
Ion mode	Positive
Drying gas temperature	250 °C
Drying gas flow	5 L/min
Nebulizer pressure	45 psi
Sheath gas temperature	325 °C
Sheath gas flow	11 L/min
Capillary voltage	5,000 V
ΔEMV	200 V
Nozzle voltage	1,500 V

MRM transitions					
Compound	**Precursor**	**Product**	**Frag (V)**	**Dwell (msec)**	**CE (V)**
25-OH Vitamin D3	401.3	383.2	106	50	4
25-OH Vitamin D3	401.3	365.3	106	50	4
25-OH Vitamin D2	413.3	395.3	106	50	4
25-OH Vitamin D2	413.3	355.2	106	50	4
25-OH Vitamin D3-d3	404.3	386.3	106	50	4
25-OH Vitamin D2-d3	416.4	398.3	106	50	4

Sample Preparation

Serum samples were prepared with a simple liquid-liquid extraction:

1. Combine 15 µL of 1,000 ng/mL internal standard solution, 150 µL of serum, and 150 µL of acetonitrile.
2. Vortex for 30 seconds and let stand for 15 minutes at room temperature.
3. Add 750 µL of heptane and vortex for 30 seconds.
4. Centrifuge at 13,000 rpm for 5 minutes.
5. Transfer organic layer (top) to a clean tube.
6. Evaporate to dryness with nitrogen at room temperature.
7. Reconstitute in 200 µL of 75:25 methanol:0.1% formic acid in water

Results and Discussion

The rapid analysis of 25-OH D2 and D3 has been achieved in less than 5 minutes (Figure 2). Calibration curves were constructed for 25-OH vitamin D2 and D3 using concentrations of 1, 25, 75, 125, and 250 ng/mL. Figure 3 illustrates the excellent linearity obtained for these analytes, with R^2 values ≥ 0.995. The interday coefficients of variation (CV's) for quantification of these vitamin D metabolites in standard reference material were also excellent, never exceeding 10 % (Table 3).

Table 3. CVs for various levels of 25-OH vitamin D2 and D3.

ng/mL	D2	D3
10	2.8	3.3
30	2.7	2.7
70	8.0	7.8

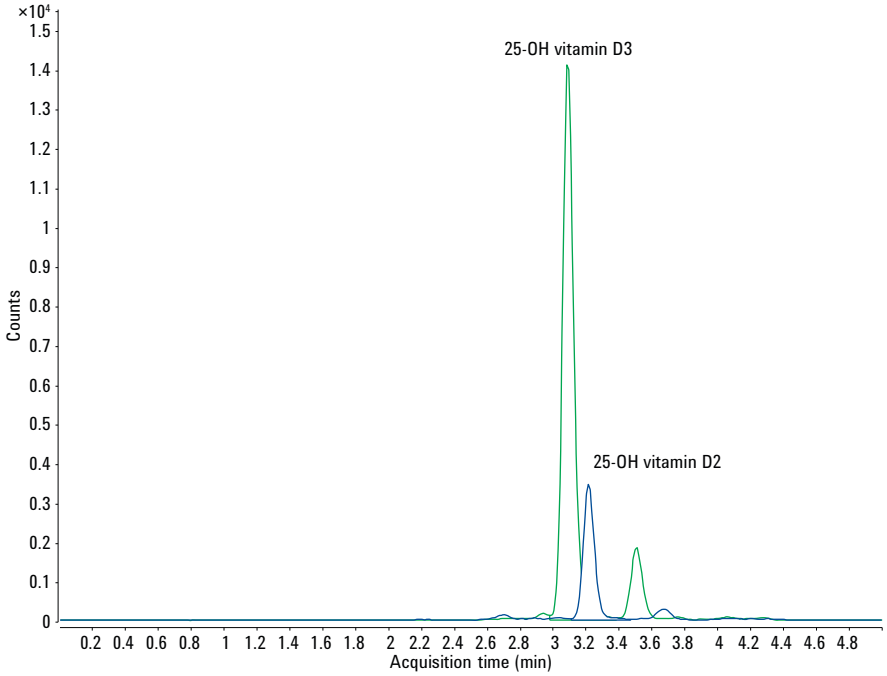

Figure 2. Chromatography for 25-OH vitamin D2 and D3.

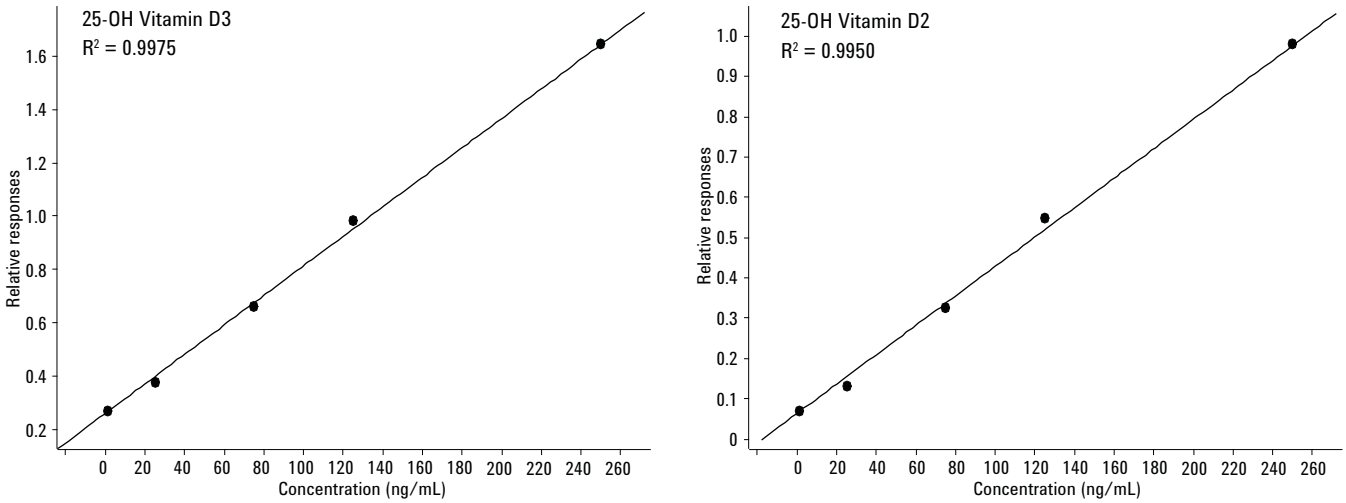

Figure 3. Calibration curves for 25-OH vitamin D2 and D3 from 1 to 250 ng/mL.

Conclusions

A rapid and reliable method has been developed for the accurate, sensitive and reproducible detection of 25-OH vitamin D2 and D3 in serum using an Agilent 6460 Triple Quadrupole LC/MS. Excellent linearity has been demonstrated over the range of 1 to 250 ng/mL. A simple liquid-liquid extraction followed by automated online sample cleanup minimizes the matrix effect and ion suppression due to lipids and other biological compounds present in serum. Analysis can be performed in less than 5 minutes.

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