

Rapid Analysis of Vitamin D in Serum Using Triple Quadrupole LC/MS

Application Note

Authors

M.P. George
Agilent Technologies, Inc.
Santa Clara, CA USA

Andre Szczesniewski
Agilent Technologies, Inc
Schaumburg, Illinois USA

Abstract

A rapid method for the quantification of 25-OH D₂ and 25-OH D₃ in serum was developed using the Agilent 1200 Series Rapid Resolution LC (RRLC) system coupled with an Agilent 6430 Triple Quadrupole LC/MS with multimode ionization (MMI). Solid phase extraction minimizes matrix interferences and ion suppression, while enabling automation.

Excellent linearity of quantification ($R^2 > 0.99$) was obtained in the range of 5 to 100 ng/mL, with run times of only 5 min, and day-to-day precision was well below 10%. The limits of detection (LOD) and limits of quantification (LOQ) were 2 ng/mL and 3 ng/mL respectively, in serum.

Introduction

Vitamin D regulates the calcium and phosphorus levels in the blood by promoting their absorption from food in the intestines, and by promoting reabsorption of calcium in the kidneys, which enables normal mineralization of bone. It is also necessary for bone growth and bone remodeling. 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 has been shown to induce cancer cell death *in vitro* and *in vivo*. Meta-analysis of epidemiological studies has demonstrated a positive correlation between intake of vitamin D and cancer prevention, with colon cancer risk being reduced 50% and breast and ovarian cancer risk down by 30%, with an intake of an additional 1,000 international units (25 mg) per day (1). Vitamin D deficiency is known to be associated with high blood pressure and increased cardiovascular disease risk, and a recent study has shown low serum levels of vitamin D (<17.8 ng/mL) to be linked to a higher incidence of peripheral artery disease (PAD) (2).

Vitamin D is produced in skin as D₃, and by plants as D₂, which is the form found in many nutritional supplements. Each form is metabolized by the liver to its 25-hydroxy derivative (25-OH) which is the active form. Both 25-OH D₂ and 25-OH D₃ are measured in blood and are collectively referred to as vitamin D in studies. It is critical that assays be capable of measuring total circulating vitamin D (D₂ and D₃). Traditionally, competitive binding experiments and immunoassays have been used to measure vitamin D. However, the cross-reactivity of the antibodies used in these assays can be less than 100%, and the results may not reflect total D₂ and D₃. More recently, LC/MS assays have gained acceptance as the methods of choice due to their ability to reliably quantify both vitamin D forms.



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This application note describes a method for the rapid, sensitive and accurate determination of total vitamin D in serum using the Agilent Triple Quadrupole LC/MS system with a multi mode (MMI) source in simultaneous ESI-APCI mode. The use of a solid phase extraction sample prep method reduces matrix effects and ion suppression, with no interferences observed in a large number of serum samples, while the MMI source minimizes degradation of the sample during analysis.

Experimental

Reagents and Standards

Stock solutions of 25-OH D₂, 25-OH D₃ and deuterated 25-OH D₃ (Sigma and Medical Isotopes) were prepared at 10 µg/mL in methanol (Fisher Scientific) and stored at ≤ 15°C. Calibration standard solutions were prepared from these stock solutions at 5, 10, 25, 50 and 100 ng/mL in 5% bovine serum albumin, and the deuterated 25-OH D₃ internal standard was diluted to 400 ng/mL with deionized water.

Instruments

This method was developed on an Agilent 1200 Series Rapid Resolution LC (RRLC) system coupled with an Agilent 6430 Triple Quadrupole LC/MS with multimode ionization (MMI). The instrument conditions are listed in **Table 1**.

Sample Preparation

Serum samples were treated using the Biochemical Diagnostics GV-65C solid phase extraction column and the following procedure:

1. Add 1 mL of acetonitrile (Fisher Scientific) with 2% formic acid to 250 µL of each sample to be tested, and then let them sit for 15 min.
2. Assemble the solid phase columns on the extraction vacuum box.
3. Centrifuge samples at 3,000 RPM for 10 min.

Table 1. LC and Mass Spectrometer Conditions

LC Run Conditions	
Column	Zorbax Eclipse Plus C-18, 2.1 x 50 mm, 1.8 µm (P/N)
Column temperature	50°C
Injection volume	5 µL
Autosampler temperature	6°C
Needle wash	Flush port (50:25:25 H ₂ O, IPA:MeOH:H ₂ O, 5 sec)
Mobile phase	A = H ₂ O + 0.1% Formic Acid B = Methanol + 0.1% Formic Acid
Flow rate	0.5 mL/min
Isocratic analysis	A=13%; B=87%
Analysis time	3.0 min
MS Conditions	
Ion mode	Positive, ESI+APCI multimode ionization
Drying gas temperature	250°C
Vaporizer temperature	170°C
Drying gas flow	5 L/min
Nebulizer pressure	60 psi
Capillary voltage	4000 V
Corona current	2 µA
Charging voltage	2000 V
MRM acquisition	Q1 peak and Q2 peak widths = 0.70 m/z
Delta EMV	200 V

4. Sequentially wash each extraction column with 1 x 1 mL methanol, 1 x 1 mL methanol: deionized water (50:50), using gravity flow only.
5. Transfer the clear layer of the sample to the preconditioned column.
6. Sequentially wash the column with 1x1 mL methanol : water (50:50), then 1x1 mL methanol : water (75:25), using gravity flow.
7. Turn the vacuum on for 30 sec, and then add 200 µL of n-heptanes (Scientific Products) to each column. Let the n-heptanes sit for 2 min, then apply full vacuum for 5-7 min.
8. Elute with 250 µL of methanol under gravity flow only for 2 min, followed by 250 µL of deionized water.

Analysis Parameters

The parameters used in the analysis of vitamin D₂ and D₃, as well as the deuterated internal standard (IS), are shown in **Table 2**.

Table 2. Analysis Parameters

Compound	RT (min)	MRM Transition	Fragmentor Voltage	Dwell (msec)	Collision Energy (EV)
25-Hydroxy Vitamin D ₃	0.99	401.3 > 383.3	100	150	3
25-Hydroxy Vitamin D ₂	1.12	413.3 > 395.3	100	150	3
IS-25-Hydroxy Vitamin D ₃ -d6	0.98	407.3 > 389.3	100	150	3

Results and Discussion

Solid Phase Extraction

Several sample preparation methods are available for the analysis of vitamin D in plasma and serum samples. However, liquid-liquid extraction methods require a drying step and subsequent reconstitution in an LC compatible solvent. Multiplexed, offline solid phase extraction methods offer simplicity and high throughput. The method used here reduces the matrix effect and ion suppression, resulting in clean chromatograms in which vitamin D₂ and D₃ are the only major components (Figure 1).

Accurate, Reproducible Quantification

Calibration curves were constructed for 25-OH vitamin D₂ and 25-OH D₃, using concentrations of 5, 10, 25, 50 and 100 ng/mL.

Figure 2 illustrates the excellent linearity obtained for both forms of vitamin D, with R² values very close to 1.000. The day-to-day (inter-assay) coefficients of variation (CV's) for quantification of each of the calibration standards were also excellent, falling as low as 1.7%, and never exceeding 10% (Table 3).

High Sensitivity with Real-World Samples

The method was validated with a set of 66 fresh serum samples that had previously been quantified using another LC/MS procedure. The correlation of quantification with the previous method was quite good, giving an R value of 0.979. The LOD and LOQ were 2 ng/mL and 3 ng/mL, respectively, for both forms of vitamin D, and no interferences were observed in any of the samples. It should be noted that the method described in this application note is not a diagnostic solution for vitamin D testing. Fresh serum samples have been used in the verification of the method to illustrate its performance in the detection and quantification of vitamin D.

Resolve Vitamin D₂ and D₃ with minimal interferences and matrix effects

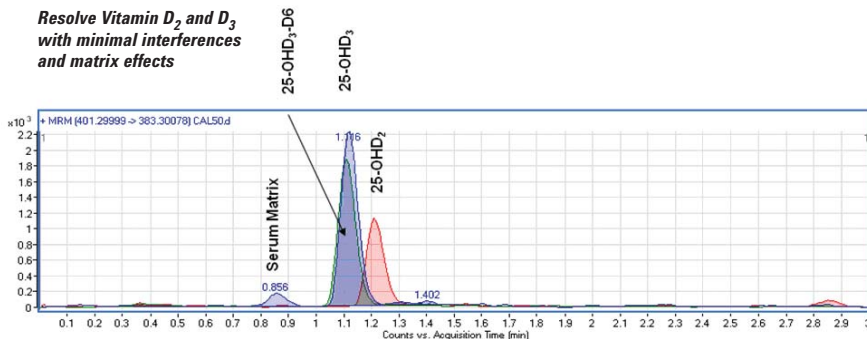
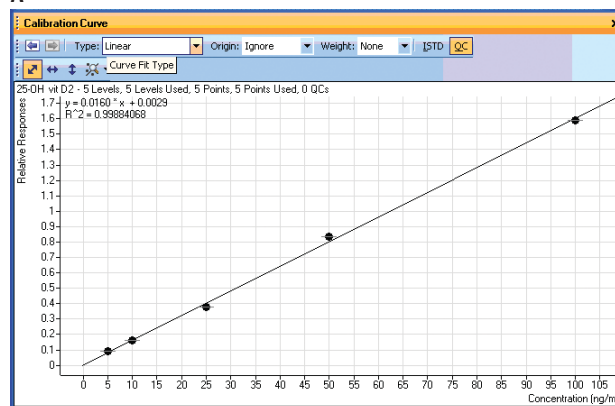


Figure 1. Total ion current chromatogram of a representative serum sample containing Vitamin D₂ and D₃, as well as the internal standard.

Accurately quantify D₂ and D₃ from 5 to 100 ng/mL

A



B

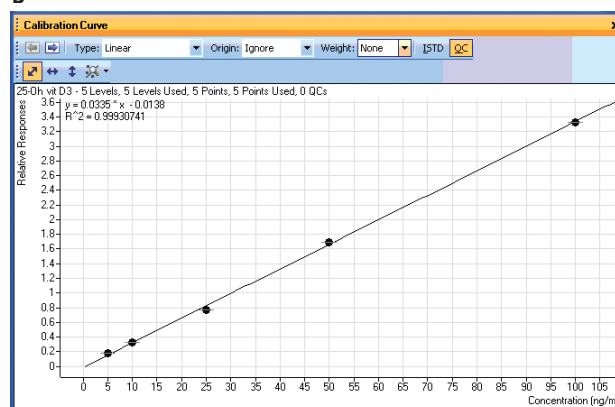


Figure 2. Calibration curves for 25-OH D₂ (A) and 25-OH-D₃ (B) in 5% bovine serum albumin.

Table 3. Inter-Assay Precision of Quantification (Coefficient of Variation)

Concentration (ng/mL)	25-OH D ₂ (%)	25-OH D ₃ (%)
5	7.03	6.74
10	5.73	3.18
25	4.46	3.53
50	3.81	3.34
100	2.04	1.70

Conclusions

A method has been demonstrated for the accurate, sensitive and reproducible detection of 25-OH vitamin D₂ and D₃ in serum, using Triple Quadrupole LC/MS. The LOD is 2 ng/mL and the LOQ is 3 ng/mL. Solid phase extraction sample preparation minimizes the matrix effect and ion suppression due to lipids and other biological compounds present in serum. The use of an MS multi mode source in simultaneous ESI-APCI mode minimizes degradation of the sample (loss of the 25-OH group). Analysis can be performed in less than 5 min, and can be automated using standard solid phase sample preparation automation systems.

Acknowledgements

The authors gratefully acknowledge the material assistance of Biochemical Diagnostics, Edgewood, New York (SPE columns), and Frank Crow, David Barnidge, Ravinder Singh and James Bruton at the Mayo Clinic, Rochester, Minnesota for providing the 66 samples used for the verification of this method.

References

1. E. D. Gorham, Garland F. C., C. F. Garland, W. B. Grant, S. B. Mohr, M. Lipkin, H. L. Newmark, E. Giovannucci, M. Wei, M. F. Holick. "Optimal vitamin D status for colorectal cancer prevention: a quantitative meta analysis", *Am J Prev Med.* **32**, 210-216 (2007).
2. M. L. Melamed, P. Muntner, E. D. Michos, J. Uribarri, C. Weber, J. Sharma, P. Raggi. "Serum 25-Hydroxyvitamin D Levels and the Prevalence of Peripheral Arterial Disease. Results from NHANES 2001 to 2004". *Arterioscler. Thromb. Vasc. Biol.* **28**, 1179-1185 (2008).

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Published in the U.S.A. September 14, 2009
5990-4549EN



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