

Analysis of 25-OH-Vitamin D₂ and 25-OH-Vitamin D₃ in Serum Using LC/MS/MS



Overview

A fast and sensitive LC/MS/MS method for quantitation of 25-OH-vitamin D₂ and 25-OH-vitamin D₃ in serum has been developed.

Introduction

Vitamin D consists of two compounds, vitamin D₂ and vitamin D₃, which differ in their side-chain structures (Figure 1). Vitamin D₃, or cholecalciferol, is formed in the skin upon exposure to sunlight or obtained from nutritional sources, especially fatty fish. Vitamin D₂, however, is obtained from irradiation of plant

sources. Vitamin D has no biological activity. Their hydroxy metabolites, 25-hydroxy-D₂ and 25-hydroxy-D₃, are converted to the biologically active 1,25-dihydroxyvitamin-D₂ and -D₃. It is this metabolite that is associated with calcium homeostasis and other functions, such as affecting immune response. A detailed description of vitamin D physiology can be found in reference 1. The levels of the monohydroxy metabolites have been determined to be the best indication of a person's vitamin D level.

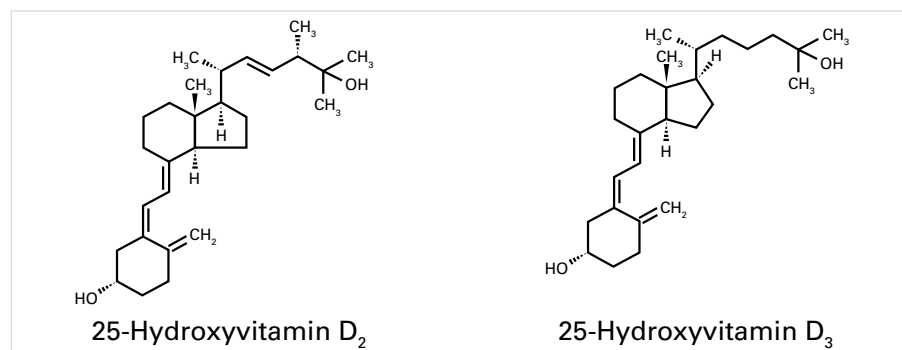


Figure 1. Structures of 25-OH-Vitamin D₂ and 25-OH-Vitamin D₃

Experimental

Because it is difficult to obtain biological matrices that are free of vitamin D and its metabolites, a 6% BSA* in a 0.01M PBS* solution was spiked with standards and extracted. For extraction, 100 μ L of a 100 ng/mL 25-OH-vitamin D₃-d6 internal standard solution was added to 250 μ L of spiked BSA. Then 1 mL of acetonitrile was added and the sample was vortexed, centrifuged, the supernatant removed and evaporated to dryness. The sample was then reconstituted in 100 μ L 50% acetonitrile solution and 25 μ L was injected for analysis.

Liquid Chromatography

Separation was carried out using a Shimadzu Prominence LC stack and a 2.1mm x 50 mm (5 μ m) Phenomenex Luna C8 column held at 45°C. Mobile phases A and B were water and acetonitrile, respectively, with 0.1% formic acid added to each. A fast gradient and flow rate of 0.700 mL/min were used. Total injection to injection time was 4 minutes.

Mass Spectrometry

An API 3200™ mass spectrometer operating in MRM mode was used for detection. One MRM transition for each analyte and internal standard was monitored. Positive mode atmospheric pressure chemical ionization (APCI) was used.

Analyte	MRM transition
25-OH Vit D ₂	395.4 – 209.1
25 OH Vit D ₃	383.4 – 211.1
ISTD OH-25 Vit D ₃ - d6	389.4 – 211.1

Results and Discussion

Sensitivity and Linearity

Figure 2 shows a representative chromatogram of BSA spiked at 10 ng/mL of each analyte, extracted, and analyzed. The LLOQs for 25-OH-vitamin D₂ and 25-OH-vitamin D₃ are 2 ng/mL and 1 ng/mL, respectively. Data at these levels are shown in Figures 3 and 4.

* BSA = Bovine Serum Albumin; PBS = Phosphate Buffered Saline

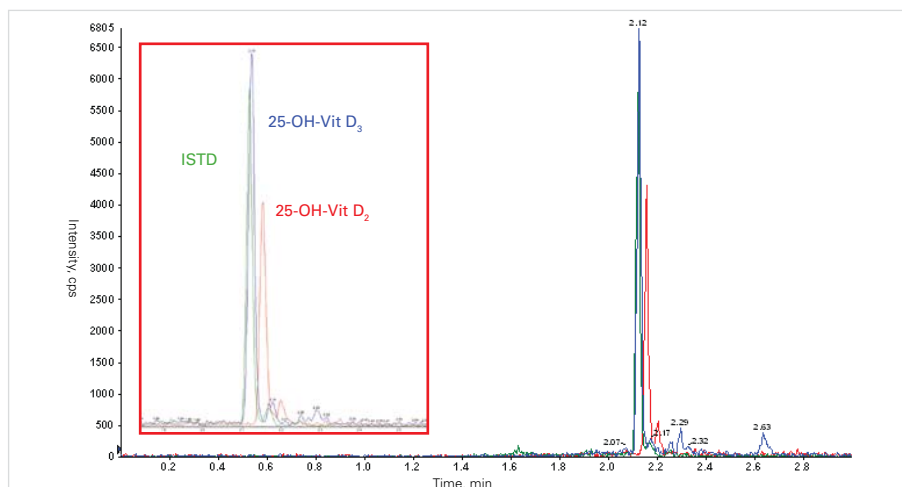


Figure 2. Extracted ion chromatograms for a 10 ng/mL extracted calibrator.

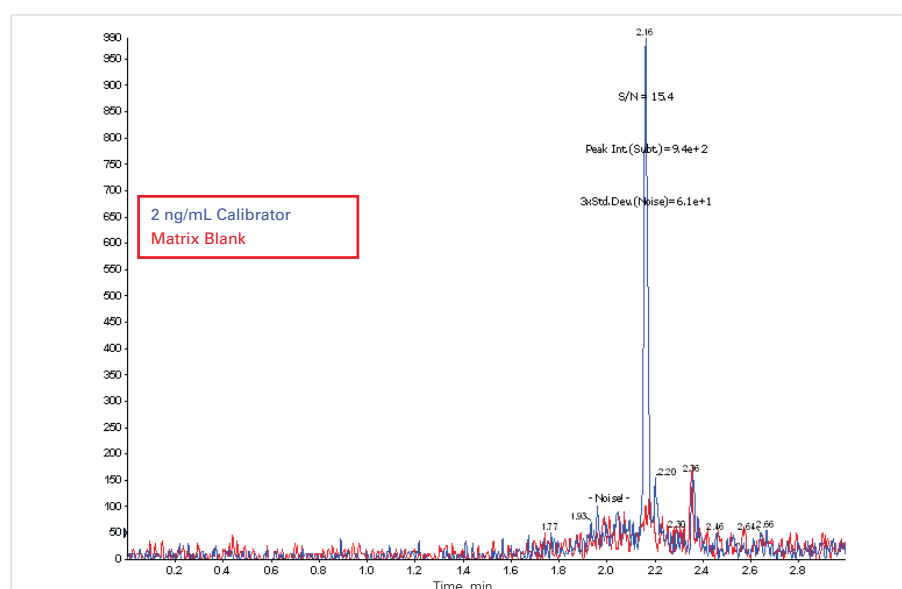


Figure 3. Data from a 2 ng/mL calibrator of 25-OH-Vitamin D₂, which represents the LLOQ for this analyte. A 3-point smooth was applied to the chromatogram and the signal-to-noise calculated using 3 times the standard deviation.

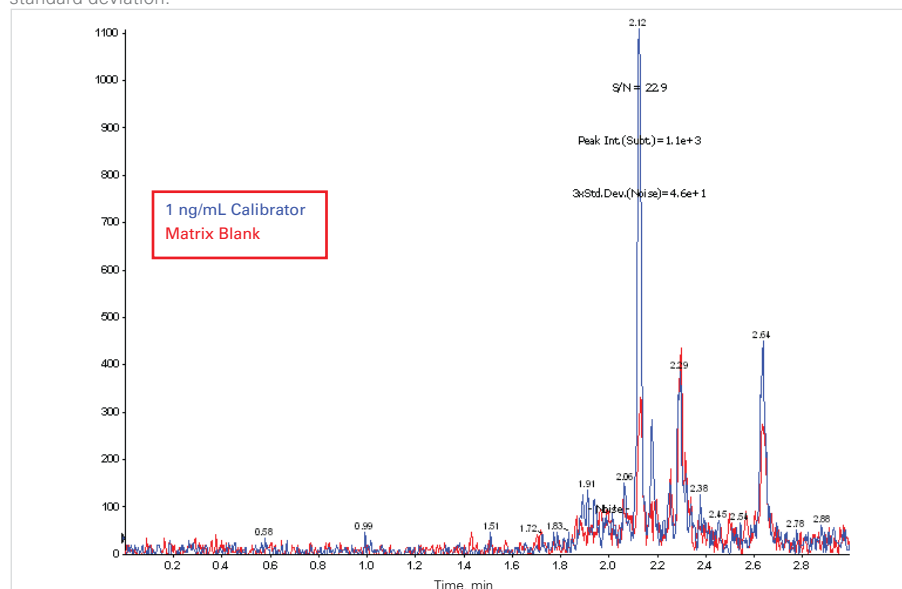


Figure 4. Data from a 1 ng/mL calibrator of 25-OH-Vitamin D₃, which represents the LLOQ for this analyte. A 3-point smooth was applied to the chromatogram and the signal-to-noise calculated using 3 times the standard deviation.

Linearity of the method covered at least 2.5 orders of magnitude, from the LLOQ up to 500 ng/mL for both analytes. Precision and accuracy were typically better than 10%. Calibration curves demonstrating the precision, accuracy, and linearity for each analyte are shown in Figures 5 and 6. Analysis of standards suggests linearity extends beyond 500 ng/mL; however, concentrations above 100 ng/mL are considered extremely high and there is no analytical reason to quantify above 500 ng/mL. According to the literature^{2,3}, the cutoff level indicating a sufficient level of vitamin D is between 12-30 ng/mL (30-80 nmol/L). With an LLOQ of ≤ 2 ng/mL, this method had sufficient sensitivity.

Reproducibility and Ruggedness

Assay ruggedness was evaluated by performing 100 replicate injections of vitamin D in extracted human serum. A graph of peak areas vs. injection number shows no degradation of data quality across the data set (Figure 7). CVs of the peak areas were $< 10\%$.

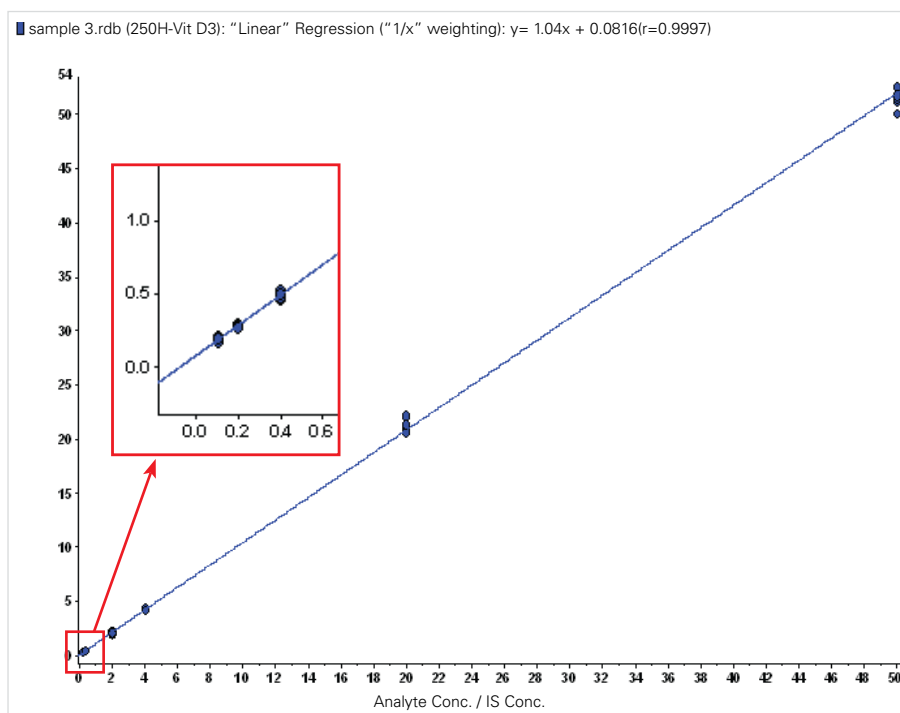


Figure 5: Calibration curve for 25-OH-vitamin D₃. Precision (%CV) and accuracy were typically within 5% and 10%, respectively, across the analytical range.

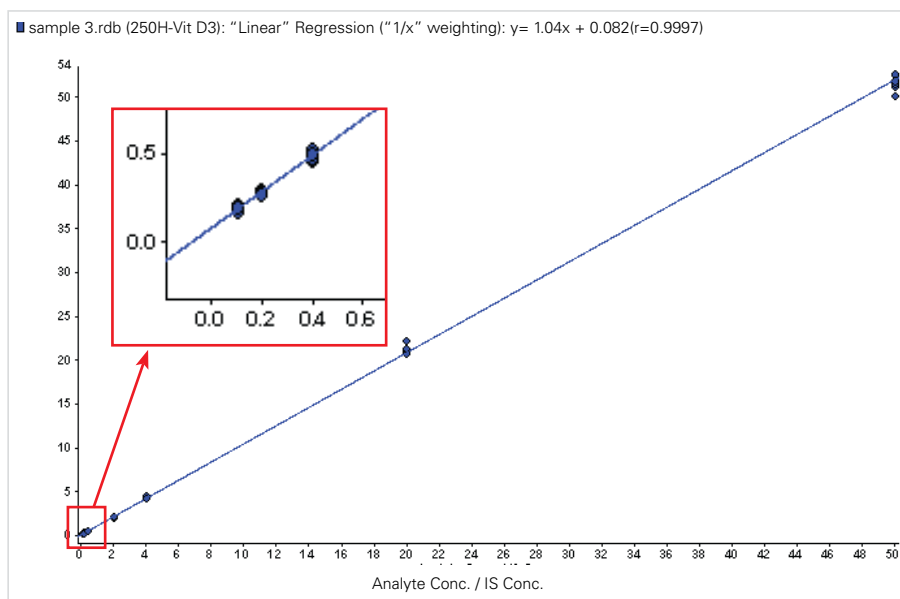
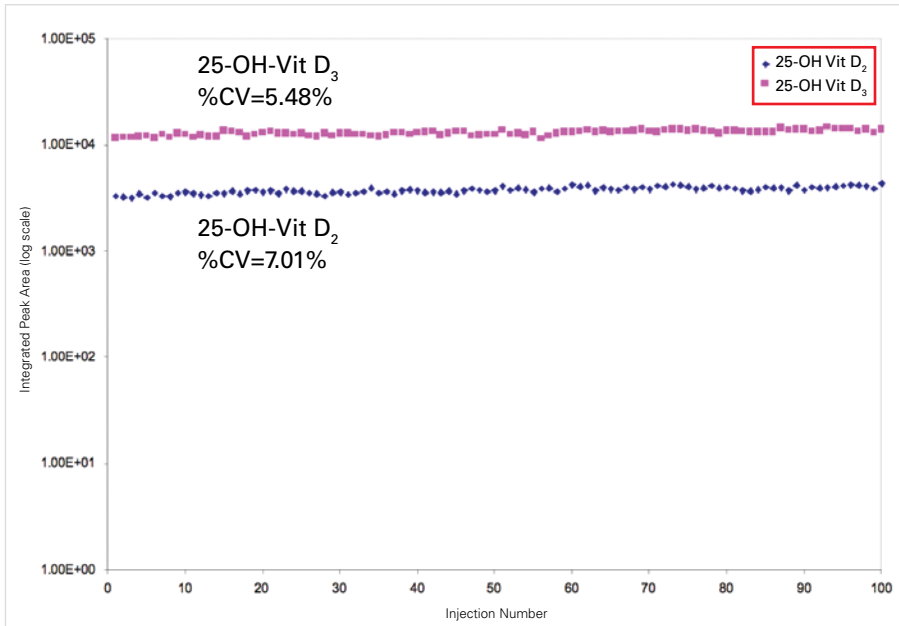


Figure 6: Calibration curve for 25-OH-vitamin D₃. Precision (%CV) and accuracy were typically within 5% and 10%, respectively, across the analytical range.



Summary

A fast, sensitive, and rugged method for analysis of 25-OH-vitamin D₂ and 25-OH-vitamin D₃ in serum has been developed. The linear quantifiable range was from ≤ 2 ng/mL to 500 ng/mL, which was more than sufficient to cover the concentration levels of interest. Precision and accuracy were better than 5% for most concentration levels and better than 15% for all concentration levels. No observable degradation of data quality occurred over the course of 100 injections, demonstrating the stability and ruggedness of the method.

¹ Lips, P., *Progr Biophys Mol Bio*, 92 (2006) 4-8.

² Mawer, E. B., Davies, M., *Rev Endocr Metab Disord*, 2 (2001) 153-64.

³ Saenger, A. K., et. al., *Am J Clin Pathol*, 125 (2006) 914-920.

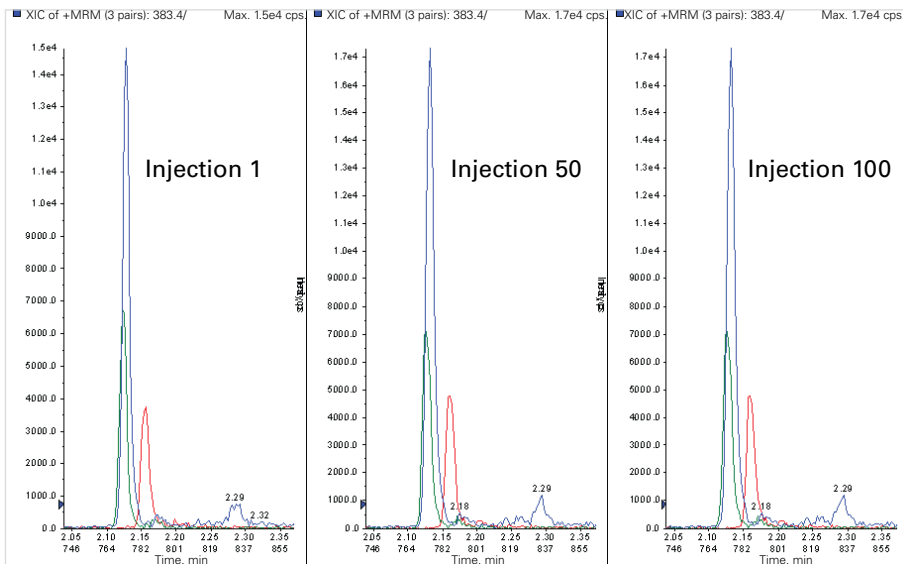


Figure 7: A ruggedness test of 100 injections showed peak area CVs of <10%.

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