

Measurement of 25-OH-vitamin D₃ and 3-epi-25-OH-vitamin D₃ by LC-MS/MS

Using the SCIEX Triple Quad™ 4500 LC-MS/MS System

Because of the similarity in chemical structure (see Figure 1), 25-OH-vitamin D₃ and 3-epi-25-OH-vitamin D₃ display very similar chromatography and produce the same fragment ions when subjected to collisionally-induced dissociation (CID) in a triple quadrupole mass spectrometer. Since it is not possible to distinguish these epimers by tandem mass spectrometry alone, it is necessary to resolve these epimers chromatographically, to avoid the overestimation of 25-OH-vitamin D₃ levels when analysis is performed by LC-MS/MS.

We have developed an LC-MS/MS method utilizing a Phenomenex Kinetex PFP column that achieves baseline separation of 25-OH-vitamin D₃ and 3-epi-25-OH-vitamin D₃ in a total run time of 6 minutes, employing a shallow gradient consisting of water and methanol with formic acid. 'Blank' stripped serum and spiked serum samples have been analyzed to determine LOD and LOQ for the method.

The method developed here allowed the independent quantification of both 25-OH Vitamin D₃ and 3-epi-25-OH Vitamin D₃ in a single LC-MS/MS analysis. The estimated limit of quantification was approximately 2 ng/mL for each analyte, and the method displayed good accuracy, reproducibility, and linearity over a concentration range from 1-100 ng/mL.

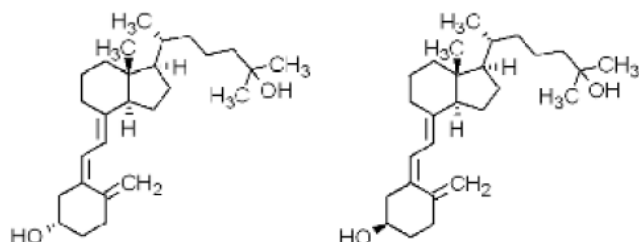
25-OH-Vitamin D₃3-epi-25-OH-Vitamin D₃

Figure 1. Structures of 25-OH-vitamin D₃ epimers.



SCIEX Triple Quad™ 4500 LC/MS/MS System

Experimental

Sample Preparation: The sample preparation consisted of a simple liquid-liquid extraction, using hexane, followed by dry-down and reconstitution of the sample:

- 200 µL of plasma was measured into a 1.5mL polypropylene microcentrifuge tube.
- 200 µL of internal standard solution (25-OH-vitamin D₃-d₆) was added to each tube, which was then vortex mixed for 15 seconds to precipitate proteins
- 1 mL of hexane was added to each sample, which was vortex mixed for 15 seconds
- The samples were centrifuged at 14,000 rpm for approximately 15 minutes, until the upper hexane layer was free of particulates.
- 800 µL of the supernatant was transferred into a clean 1.5 mL microcentrifuge tube and dried down under nitrogen gas at 45°C.
- The dried sample was reconstituted using 175 µL of methanol:water

HPLC conditions: Chromatographic separation was accomplished using a Phenomenex Kinetex PFP analytical

column (100 x 3.0 mm, 2.6 μ m, 100A) with a SecurityGuard C18 cartridge (4 x 3 mm).

The UHPLC method employed a binary gradient, at a flow rate of 400 μ L / minute. Mobile phase A consisted of water with formic acid, and mobile phase B consisted of methanol with formic acid.

MS/MS conditions: MS/MS detection was accomplished using the SCIEX Triple Quad™ 4500 LC/MS/MS System, equipped with Turbo V™ Ion Source, operated in positive electrospray ionization (ESI) mode. The multiple reaction monitoring (MRM) mode of operation was used, with 2 MRM transitions monitored per analyte. The deuterated analogue of 25-OH-vitamin D₃ was used as an internal standard for all the analytes.

Results

A rapid, 6-minute LC-MS/MS method has been developed for the quantification of 25-OH-vitamin D₃, 3-epi-25-OH-vitamin D₃, and 25-OH-Vitamin D₂ in a single analysis, using the SCIEX Triple Quad™ 4500 LC/MS/MS system. Chromatographic separation was accomplished using a Phenomenex Kinetex PFP (2.6 μ m, 100 x 3mm) analytical column. Using this binary flow gradient, it was possible to reduce the total run-time of the method to just 6 minutes, while still achieving baseline separation of 25-OH-vitamin D₃ and 3-epi-25-OH-vitamin D₃. This method also provided much narrower peak widths (approximately 0.1 minutes), resulting in improved S/N, and therefore more sensitive detection of the target analytes. An example chromatogram of the separation achieved is shown in Figure 2.

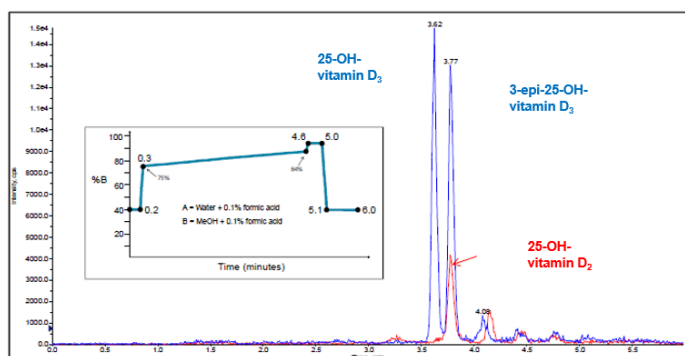


Figure 2. Separation of 25-OH-vitamin D₃ and 3-Epi-25-OH-Vitamin D₃ using a 6-minute binary flow gradient

The performance of the method was evaluated using spiked serum samples covering a concentration range of 1-100 ng/mL. Representative calibration curves for the 3 analytes are shown in Figure 3.

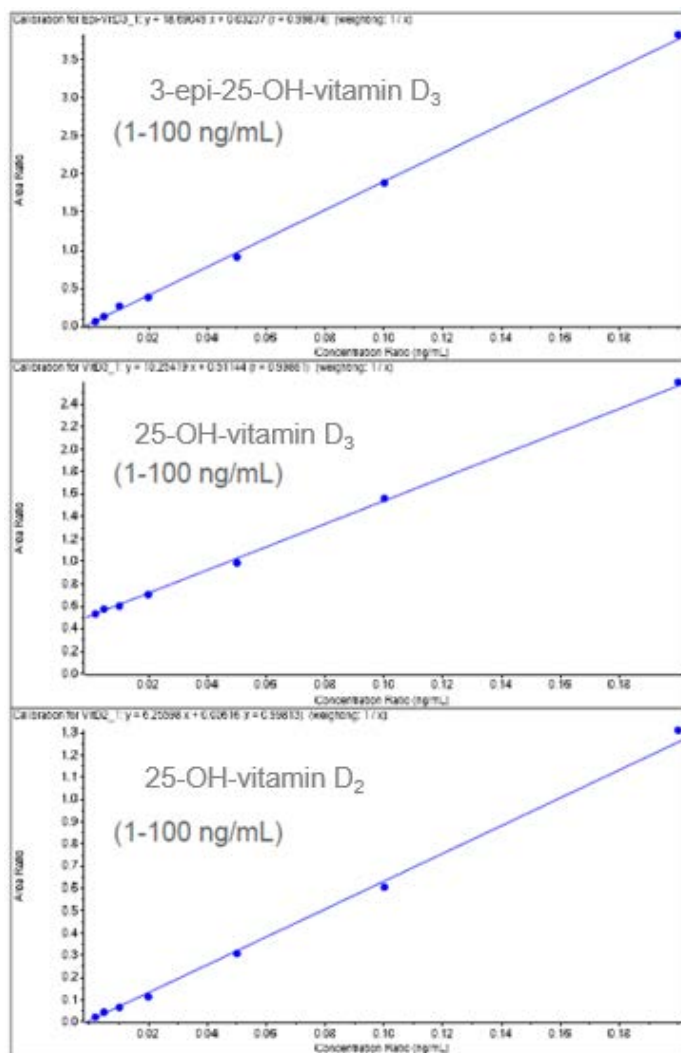


Figure 3. Calibration curves for 3-epi-25-OH-vitamin D₃, 25-OH-vitamin D₃, and 25-OH-Vitamin D₂.

The lower limits of quantification (LLOQ) for the method were determined to be approximately 1ng/mL, 2ng/mL, and 1ng/mL, for 3-epi-25-OH-vitamin D₃, 25-OH-vitamin D₃, and 25-OH-Vitamin D₂, respectively. The accuracy of the measurements for each analyte ranged from 83-119% over the entire concentration range, including at the LLOQ. The method displayed excellent linearity over the concentration range from 1-100 ng/mL, with $r > 0.998$ for each analyte. Levels for these same samples had been determined previously using an established LC-MS/MS method, thus allowing for a direct comparison of the two analytical techniques.

Conclusions

A rapid LC-MS/MS method has been developed to allow the simultaneous, and independent quantification of 25-OH-vitamin D₃, 3-epi-25-OH-vitamin D₃, and 25-OH-Vitamin D₂, with a run-time of only 6 minutes. Liquid-liquid extraction with hexane is used to clean up the serum samples prior to analysis using the SCIEX Triple Quad™ 4500 LC-MS/MS System.

Chromatographic separation of the 25-OH-vitamin D₃ epimers is accomplished using a Phenomenex Kinetex PFP analytical column, employing a simple binary gradient at a flow rate of 400 µL/min.

The SCIEX clinical diagnostic portfolio is For In Vitro Diagnostic Use. Rx Only. Product(s) not available in all countries. For information on availability, please contact your local sales representative or refer to <https://sciex.com/diagnostics>. All other products are For Research Use Only. Not for use in Diagnostic Procedures.

Trademarks and/or registered trademarks mentioned herein are the property of AB Sciex Pte. Ltd. or their respective owners in the United States and/or certain other countries.

© 2020 DH Tech. Dev. Pte. Ltd. RUO-MKT-02-10905-A. AB SCIEX™ is being used under license.



Headquarters

500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales

For our office locations please call the division headquarters or refer to our website at sciex.com/offices