Automated, High-Throughput LC-MS/MS Workflow for the Analysis of 25-Hydroxyvitamin $D_{2/3}$ and 3-*epi*-25-Hydroxyvitamin D_3

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Application Note 584

Key Words
Vitamin D, 25-hydroxyvitamin $D_{2/3}$, 3-*epi*-25-hydroxyvitamin D_3, TurboFlow method, Transcend, TSQ Vantage, Versette, LC-MS/MS

Goal
Develop an automated, high-throughput LC-MS/MS workflow for the analysis of 25-hydroxyvitamin $D_{2/3}$ and 3-*epi*-25-hydroxyvitamin D_3 in human serum for research laboratories.

Introduction
Analysis of total serum 25-hydroxyvitamin D_2/D_3 (25OHD, shown in Figure 1) is performed routinely in many research laboratories. Demand for this analysis continues to grow, and liquid chromatography with tandem mass spectrometry (LC-MS/MS) is increasingly used for this purpose.[1]

Compared with other sample preparation techniques, Thermo Scientific™ TurboFlow™ technology has been shown to significantly improve the removal of matrix components prior to LC-MS/MS analysis of 25OHD.[2] However, there are additional pre-analytical steps that must be performed, the most important of which are the complete removal of the analytes from the endogenous vitamin D binding protein and the addition of isotopically-labeled internal standards for quantitation. When performed manually, these steps can increase the total analysis time by approximately two hours for a batch of 96 samples. This application note presents a workflow that uses an automated liquid handling system to reduce the time required to prepare a 96 well plate for analysis to less than 20 minutes.

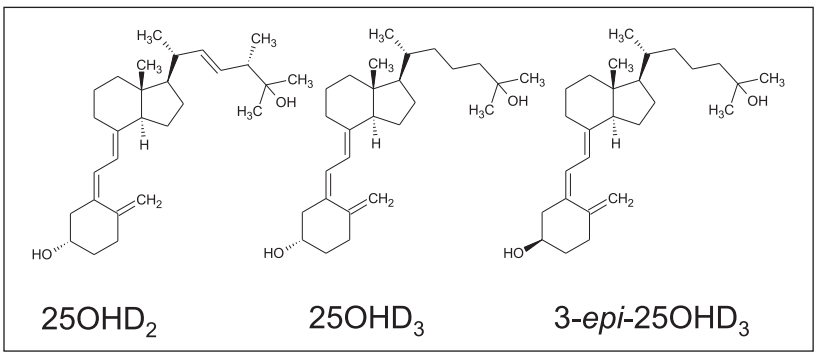

Figure 1. Structures of 25-hydroxyvitamin D_2/D_3

Further, MS/MS alone is an achiral technique. This can be problematic for some isobaric 25OHD metabolites, notably 3-*epi*-25-hydroxyvitamin D_3 (shown in Figure 1 as 3-*epi*-25OHD$_3$). For accurate LC-MS/MS analysis of 25OHD$_3$, LC-MS/MS extended chromatographic analysis times are needed to resolve 3-*epi*-25OHD$_3$. In this application note, multiplexing technology is used to maximize throughput of the chromatographic method used to resolve interfering 3-*epi*-25OHD$_3$.

Experimental
Sample Preparation
Human serum samples from the international Vitamin D External Quality Assessment Scheme (DEQAS, samples 404 and 405) were used for the analysis.

All liquid handling was carried out using a Thermo Scientific™ Versette™ automated liquid handling system. An overview of the liquid handling procedure is shown in Figure 2. The Versette system was fitted with a 96 channel pipetting head and Thermo Scientific™ D.A.R.T.'S™ 300 µL extended-tip disposable pipette tips. Calibration standards and quality controls (both from the Chromsystems MassChrom® 25-hydroxyvitamin $D_{2/3}$ kit) and samples (100 µL) were transferred from decapped 1 mL Thermo Scientific™ Nunc™ Cryobank storage vials to a 96 well filter plate. Internal standard solution (25 µL, $^2H_6$-25OHD$_3$) and precipitation reagent (200 µL), both from Chromsystems, were then added separately from the reagent reservoirs. The filter plates were covered and mixed on a plate shaker (600 rpm, 10 min). Supernatents were collected into a microtitre plate by centrifugation (200 *g*, 3 min). The plate was sealed with an adhesive plate seal and transferred to a Thermo Scientific™ Transcend™ TLX-2 system for analysis. All of the consumables utilized in the process are listed in Table 1.

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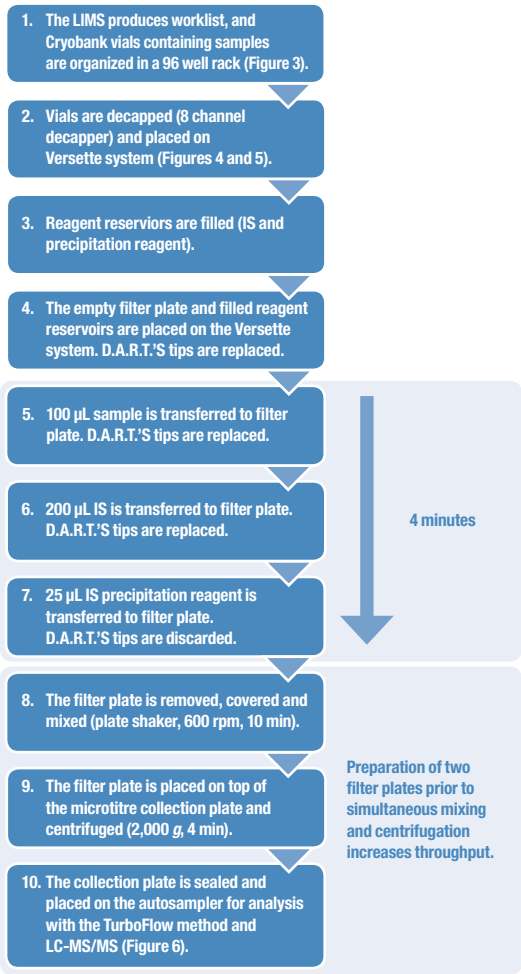

Figure 2. Liquid handling procedure

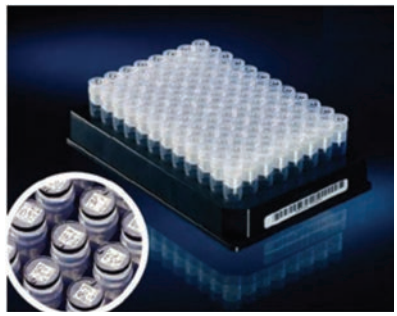

Figure 3. Cryobank vials

Figure 4. 8 Channel handheld screw cap capper/decapper

Figure 5. Versette liquid handling system and one rack of D.A.R.T.'S tips

Figure 6. Transcend system and TSQ Vantage mass spectrometer

Table 1. Parts and consumables required for specific steps in the liquid handling procedure

Step Number	Description	Part Number
1	1 mL Cryobank vials (96 tubes per rack)	374088
2	8 channel handheld screw cap capper/decapper with stand for Thermo Scientific Nunc-style tubes	4105NUN
3	Thermo Scientific™ Matrix™ 96 deep-well automation reservoir	1064-15-6
4	Filter plate	Chromsystems
2–8	Thermo Scientific Versette system	
	- Base unit	650-01-BS
	- 96 and 384 channel pipetting module	650-02-NTC
	- 6 position stage	650-03-SPS
	- 96 channel air disp. pipetting head, volume 5–300 µL	650-06-96300
4–7	Tips – 300 µL extended-length D.A.R.T.'S	5536
8	Titer plate shaker	4625-1CEQ
9	Thermo Scientific™ Heraeus™ Labofuge™ 400 with microplate rotor package	75008371
10	Nunc pierceable 96 well cap mats	276002
	Nunc polypropylene microplate - 96 well (1.0 mL)	260252

Liquid Chromatography

Sample supernatants (100 µL) were injected onto a TurboFlow XL C18 column (50 x 0.5 mm i.d.) under turbulent flow conditions (2 mL/min). Retained analytes were back-flushed from the TurboFlow column using elution solvent stored in a holding loop and focused onto a Thermo Scientific™ Accucore™ PFP analytical column (2.6 µm particle size, 50 x 2.1 mm i.d.) maintained at 40 °C. During isocratic elution (0.40 mL/min) from the analytical column, the TurboFlow column was back-flushed with eluent C and the elution solvent loop refilled. Eluent flow was diverted to waste for 8 minutes following each injection onto the TurboFlow columns. The system was then re-equilibrated prior to the next injection.

System eluents (Fisher Chemical™ brand) were as follows:

Loading and Eluting Pumps A:	0.1 % (v/v) aqueous formic acid
Loading and Eluting Pumps B:	0.1 % (v/v) formic acid in methanol
Loading Pump C:	Acetone/2-propanol/acetonitrile (1:2:2 v/v/v)

Mass Spectrometry

Mass spectrometry was carried out in positive ionization mode using atmospheric pressure chemical ionization (APCI) on a Thermo Scientific™ TSQ Vantage™ triple-stage quadrupole LC-MS/MS system. Selected-reaction monitoring (SRM) transitions did not include water-loss fragmentations.[3] MS/MS data were acquired for 5 minutes per analysis to allow multiplexing. Total LC time was 14 minutes, as shown in Figure 7, which means the TLX-2 system could do one analysis every 7 minutes).

Results and Discussion

As shown in Figure 7, retention times were 10.94, 11.47, and 11.82 minutes for $25OHD_3$, 3-*epi*-$25OHD_3$, and $25OHD_2$, respectively. Total analysis time was 14 minutes, including column re-equilibration. Sample 405 was correctly found to contain 3-*epi*-$25OHD_3$. This compound would have been misidentified as additional $25OHD_3$ if a C18 analytical column, which does not resolve the epimer well, if at all, had been used as part of the LC-MS/MS method.

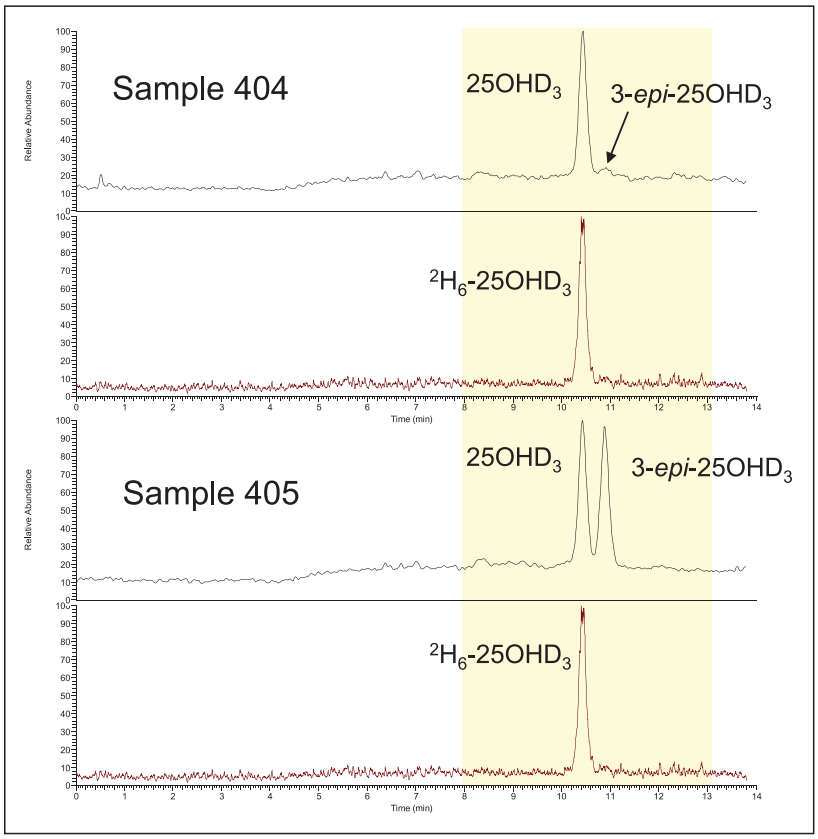

Figure 7. Chromatograms showing resolution of 3-*epi*-$25OHD_3$ from $25OHD_3$ in DEQAS samples 404 and 405. $25OHD_2$ chromatograms not shown. Sample 405 was prepared by addition of 3-*epi*-$25OHD_3$ to sample 404.[4] Yellow highlighted area corresponds to data window for multiplexing.

Conclusion

The Versette automated liquid handling system reduced the time required to prepare 96 samples from two hours to less than 20 minutes – a dramatic reduction of 80%. In addition, the Versette system reduced the number of manual pipetting steps from as many as 864 to none. The workflow minimized manual errors, increased method precision, and reduced the risk of repetitive strain injury in research laboratories.

Using multiplexing on the Transcend TLX-2 and an analytical column using a pentafluorophenyl stationary phase, the method resolved 3-*epi*-25OHD$_3$, an interferent in most LC-MS/MS 25OHD$_3$ methods, without significantly decreasing chromatographic throughput.

The same basic workflow employing automated liquid handling and automated online sample preparation can be used by research laboratories for the analysis of other compounds including mass spectrometric immunoassay-selective (MSIA) assays to measure parathyroid hormone (PTH) and vitamin D-binding protein.

Acknowledgements

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