Automated, high-throughput workflow for the analysis of 25-hydroxyvitamin $D_{2/3}$ and 3-*epi*-25-hydroxyvitamin D_3 by Transcend TLX-2 liquid chromatography-tandem mass spectrometry system.

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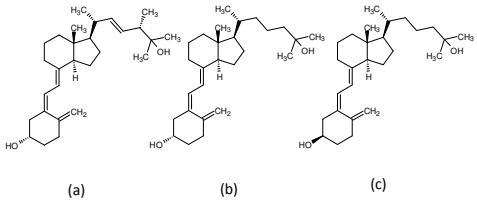
Introduction

- Analysis of total serum 25-hydroxyvitamin D_2/D_3 ($25OHD_{2/3}$ – Figure 1) is routinely carried out in many research laboratories for assessing vitamin D status.
- Demand for this analysis continues to grow and analysis by LC-MS/MS is increasingly used for this purpose.
- For 25OHD, TurboFlow technology has previously been shown to significantly improve the removal of matrix components compared with alternative sample preparation techniques.[2]
- However, there are still some pre-analytical steps that must be performed:
  - Complete removal of the analytes from the endogenous vitamin D binding protein.
  - Addition of isotopically-labelled internal standard(s) for quantitation.
- Performing these steps manually, prior to TurboFlow LC-MS/MS analysis can contribute extensively to the total analysis time (approximately 2 hrs for a manually prepared batch of 96 samples).
- We therefore present a workflow using a liquid handling system that prepares a 96-well plate for analysis in under 20 minutes.

Figure 1. Structural formulae of (a) $25OHD_2$, (b) $25OHD_3$ and (c) 3-*epi*-$25OHD_3$



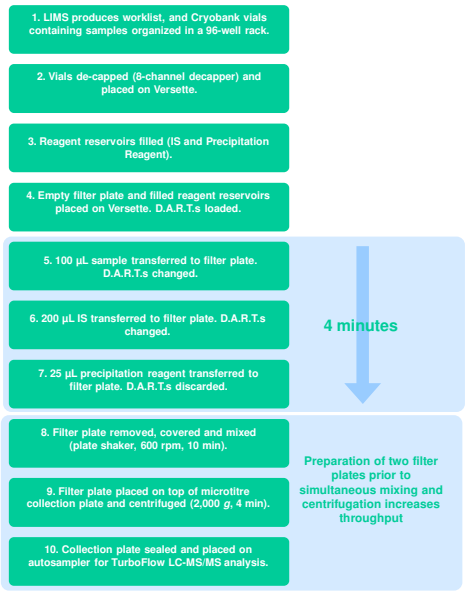
(a) (b) (c)

- Furthermore, MS/MS alone is an achiral technique. This can be problematic for some isobaric 25OHD metabolites, notably 3-*epi*-25-hydroxyvitamin D_3 (3-*epi*-$25OHD_3$. - Figure 1c).
- For accurate analysis of $25OHD_3$, LC-MS/MS should resolve 3-*epi*-$25OHD_3$, but this requires extended chromatographic analysis times, which also impacts method throughput.[3]
- We have therefore included a chromatographic method for the analysis of serum $25OHD_2$ and $25OHD_3$, which resolves the interfering 3-*epi*-$25OHD_3$, and uses multiplexing technology to maximize throughput without compromising method selectivity.

Methods

- All liquid handling was carried out using a Thermo Scientific Versette™ automated liquid handling system (Figure 3), fitted with a 96-channel pipetting head, using 300 µL extended-tip disposable pipette tips.
- Calibration standards and quality controls (both Chromsystems MassChrom 25-hydroxyvitamin D2/3 kit) and samples (100 µL) were transferred from de-capped 1 mL Thermo Scientific Nunc Cryobank storage vials (Figure 3), locked into a 96-well rack, to a 96-well filter plate.
- Internal standard solution (25 µL, $^2H_6$-$25OHD_3$) and precipitation reagent (200 µL) were then separately added from reagent reservoirs. 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 the Transcend for analysis.

Figure 2. Liquid handling procedure flow chart



Methods (cont.)

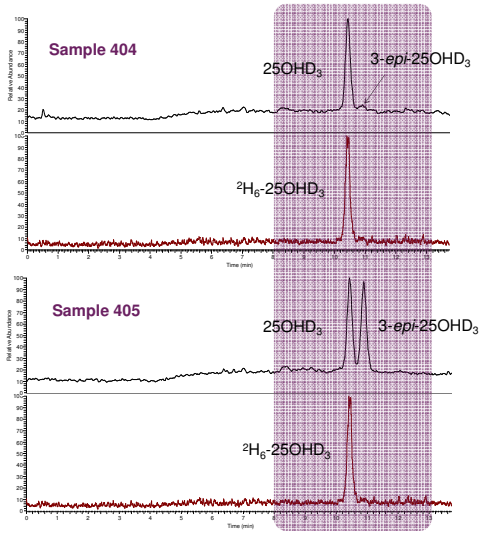
- A Thermo Scientific Transcend TLX-2 system was used (Figure 3).
- Sample supernatants (100 µL) were injected onto a TurboFlow XL C18 column (50 x 0.5 mm i.d.) under turbulent flow conditions (2 mL/min).
- Retained analytes were back-flushed from the TurboFlow column using elution solvent stored in a holding loop and focused onto a Thermo Scientific Accucore™ PFP analytical column (total 2.6 µm aps, 50 x 2.1 mm i.d.) maintained at 40 °C.
- During elution (0.40 mL/min) from the analytical column, the TurboFlow column was back-flushed with Eluent C and the elution solvent loop re-filled. The system was then re-equilibrated prior to the next injection.
- Mass spectrometry was carried out in positive ionization mode using APCI on a Thermo Scientific TSQ Vantage triple quadrupole Mass Spectrometer. SRM transitions did not include water-loss fragmentations.[3]

FIGURE 3. Instrumentation workflow.

Results

- Retention times were 10.94 min, 11.47 min and 11.82 min for $25OHD_3$, 3-*epi*-$25OHD_3$ and $25OHD_2$, respectively (Figure 4). Total analysis time was 13 min, including column re-equilibration.
- Eluent flow was diverted to waste for 8 min following each injection onto the TurboFlow columns and MS/MS data were acquired for 5 min per analysis to allow multiplexing (analysis time with multiplexing 7 min per sample).
- Human serum samples from the international Vitamin D External Quality Assessment Scheme (DEQAS, samples 404 and 405) were analyzed. Sample 405 was correctly found to contain 3-*epi*-$25OHD_3$, which would have been misidentified as additional $25OHD_3$ using our previous LC-MS/MS method (C18 analytical column).

Figure 4. Chromatograms showing resolution of 3-*epi*-$25OHD_3$ from $25OHD_3$ in DEQAS Samples 404 and 405. Highlighted area corresponds to data window for multiplexing.



Conclusions

- The automated workflow described allows for the resolution of 3-*epi*-$25OHD_3$, a known interferent in most LC-MS/MS 25OHD methods, without significant decrease in throughput because of multiplexing the method.
- The inclusion of the Versette automated liquid handler within the workflow reduced sample preparation time from 2 hrs for 96 samples, to less than 20 mins. It reduced the number of manual pipetting steps from as many as 864 steps to 0.
- The result is a process that (i) minimizes manual errors, (ii) increases method precision and (iii) removes the risk of repetitive strain injury for laboratory researchers.

References

1. El-Kouhry, J.M.; Reineks, E.Z. and Wang, S. *Clin. Biochem.* **2011**, *44*, 66-76.
2. Thermo Scientific Application Note 526: Quantitative analysis of 25-OH vitamin D_3/D_2 comparing 1D chromatography, 2D chromatography and automated online separation. Leaver, N.; Chihoho, B.; Robinson, S. **2010**.
3. Couchman, L.; Benton, C.M. and Moniz, C.F. *Clin. Chim. Acta* **2012**, *413*, 1239-1243.
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Acknowledgement

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