thermo scientific

POSTER NOTE 64923

Development of Microflow LC-MS/MS Method for Vitamins and Steroids in Complex Matrix for Research Purposes

Authors

Ed Goucher,[1] and
Kerry M. Hassell[2]
[1]Thermo Fisher Scientific,
Somerset, New Jersey, USA
[2]Thermo Fisher Scientific,
San Jose, CA, USA

ABSTRACT

Utilizing the Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano system configured with a capillary flow meter with the Thermo Scientific™ TSQ Quantiva™ triple quadrupole mass spectrometer to quantitate vitamin D, its metabolites, and testosterone in a complex matrix with minimal sample preparation for research purposes.

INTRODUCTION

Analyzing vitamins and steroids in complex matrices has shown to be an analytical challenge. The ionization of these compounds are not very efficient and the chemical structure similarities prove to be difficult to separate chromatographically. While LC/MS provides the sensitivity and selectivity needs, past methods require extensive derivatization or sample preparation. Advancements in UHPLC systems, has systems operating at lower flow rates and supporting smaller columns. Nanoflow provides the highest level of sensitivity but generally provides limited robustness and often requires user intervention. Capillary (1-10 µL/min) provides both the sensitivity and robustness required for bioanalytical laboratories. This work investigates the ability to use UHPLC and triple quadrupole mass spectrometry to quantitate vitamins and steroids in a complex matrix.

MATERIALS AND METHODS

Sample Preparation

Standards were spiked into human donor serum and precipitated with a 1:2 ratio of acetonitrile, the supernatant was directly injected into the instrument.

LC/MS

For LC/MS analysis a flow rate of 5 µL/min was delivered from a UltiMate 3000 RSLCnano pump configured with a capillary flow meter. The LC flow was directed to the Quantiva triple quadrupole mass spectrometer shown in Figure 1. Timed SRM transitions allowed for sufficient data points under the chromatographic peak. An additional ionization source was not needed to support the lower flow rate; instead a smaller diameter capillary needle was inserted in the HESI II ionization source. The smaller capillary provided higher sensitivity than the larger bore capillary. Similar LC conditions were used for the analysis of vitamin D and testosterone found in Table 1, with ammonium formate and formic acid in the mobile phases.

ThermoFisher SCIENTIFIC

Start Time (min)	Flow Rate (uL/min)	% Pump A	% Pump B
0.00	5.0	40	60
2.00	5.0	40	60
9.00	5.0	1	99
12.0	5.0	1	99
12.1	5.0	40	60
15	5	40	60

Table 1. Table of liquid chromatography method for Vitamin D on the RSLCnano Pump.

PRELUDE SPLC System

A multi-channel LC was also tested with this method at 50 uL/min using the same mobile phase conditions.

DATA ANALYSIS

Quantitation data was processed in Thermo Scientific™ TraceFinder™ 4.1 software, and SRM chromatograms were created using Freestyle™.

Figure 1. UltiMate 3000 RSLCnano system and TSQ Quantiva mass spectrometer.

RESULTS

Optimization of all the compounds were performed to obtained maximum sensitivity. The water loss of the compounds for vitamin D gave the most analytical selectivity and sensitivity as seen in Figure 2.

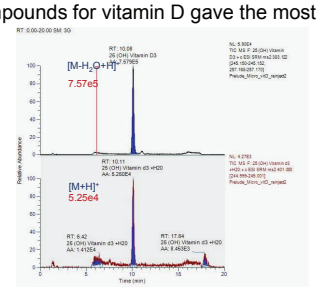

Figure 2. SRM chromatograms comparing $[M+H]^+$ to $[M-H_2O+H]^+$

Final SRM transitions, collision energy and RF lens are found in Table 2. Transitions can be timed in the TSQ software to provide optimal points under the peak.

SRM Table — Import Export

	Compound	Start Time (min)	End Time (min)	Polarity	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)	Source Fragmentation (V)
1	1,25 (OH) Vit D2	0	9.2	Positive	399.4	151.1	18	72	10
2	1,25 (OH) Vid D2	0	9.2	Positive	399.4	287.25	14	72	10
3	1,25 (OH) Vid D2	0	9.2	Positive	399.4	363.3	13	72	10
4	1,25 (OH) Vid D3	0	9.2	Positive	411.3	285	16	75	10
5	1,25 (OH) Vid D3	0	9.2	Positive	411.3	375	16	75	10
6	25 (OH) Vitamin D3	9.2	15	Positive	383.122	245.151	14.854	80	10
7	25 (OH) Vitamin D3	9.2	15	Positive	383.122	257.169	15.562	80	10
8	Vitamin D2	9.2	15	Positive	385.1	259.4	15	65	0
9	25 (OH) Vitamin D2	9.2	15	Positive	395.243	269.111	14	85	10
10	25 (OH) Vitamin D2 1	9.2	15	Positive	395.243	377.208	14	85	10
11	Vitamin D3	9.2	15	Positive	397.3	271.2	15	70	0
12	25 (OH) Vitamin d3 +H20	9.2	15	Positive	401	245	14	85	0
13	25 (OH) Vitamin D2 +H2O	9.2	15	Positive	413	269	16	87	0

Table 2. SRM chromatograms comparing $[M+H]^+$ to $[M-H_2O+H]^+$

Calibration curves of neat samples were performed on both forms of Vitamin D2 and D3, the metabolites 25-OH-Vitamin D, and 1,25-OH Vitamin D. The fifteen minute method gave baseline resolution of all isomers, SRM chromatograms can be seen in Figure 3. Calibration curves of concentrations from 0.05 to 100 ng/mL were performed and illustrated in Figure 4. Accuracy and precision data were collected in neat matrix**.** The analytical performance gave RSD values less than 15.0% for all compounds tested. Additionally, accuracy was within 15.0% of the theoretical value for all the assays. The correlation coefficient values for all the compounds were greater than 0.990, demonstrating the linearity of detection across all concentrations and analytes.

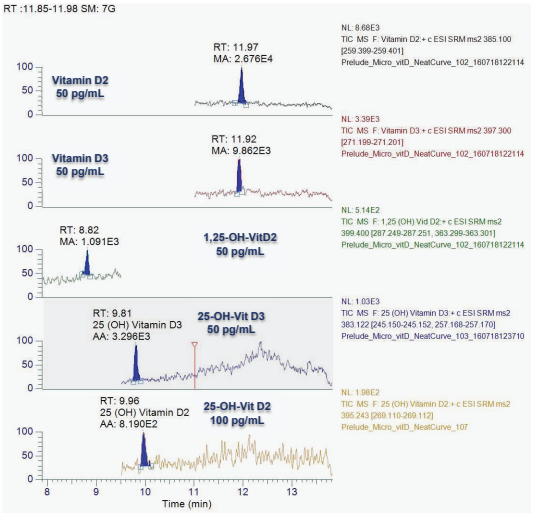

Figure 2. SRM chromatograms of Vitamin D compounds at LLOQ

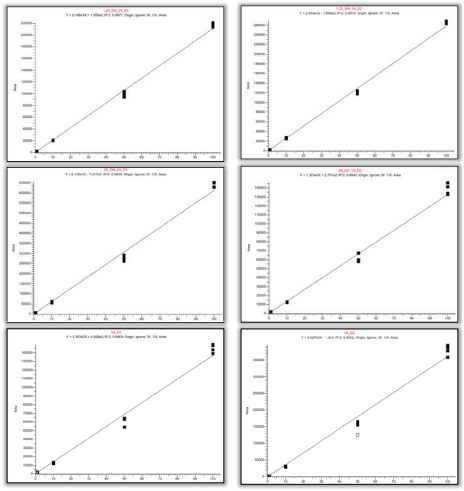

Figure 3. Calibration curve for vitamin D compounds in serum

For serum samples, the LLOQ was 250 pg/mL, using a 5 µL injection volume. SRM chromatograms for 25-OH-Vitamin D3 are presented in Figure 4 and all the compounds at their upper limit of detection in Figure 5. Tracefinder software was used to detect peak area and for quantitation analysis.

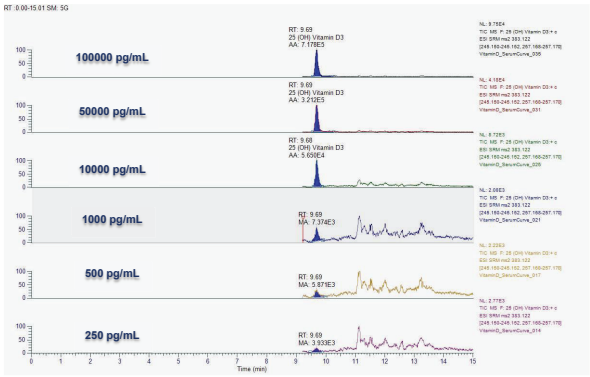

Figure 4. SRM chromatograms of 25-OH Vitamin D3 for the calibration curve.

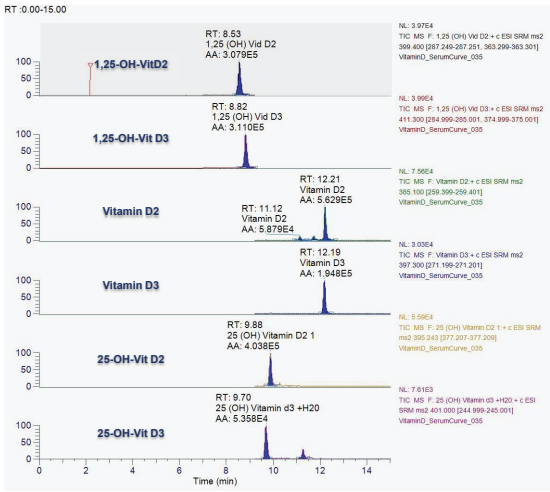

Figure 5. SRM chromatograms of Vitamin D compounds at LLOQ in serum at the ULOQ.

A feature in the software producing a graph showing reproducible retention times even at lower flow rates, Figure 6 represents the retention times for some of the compounds.

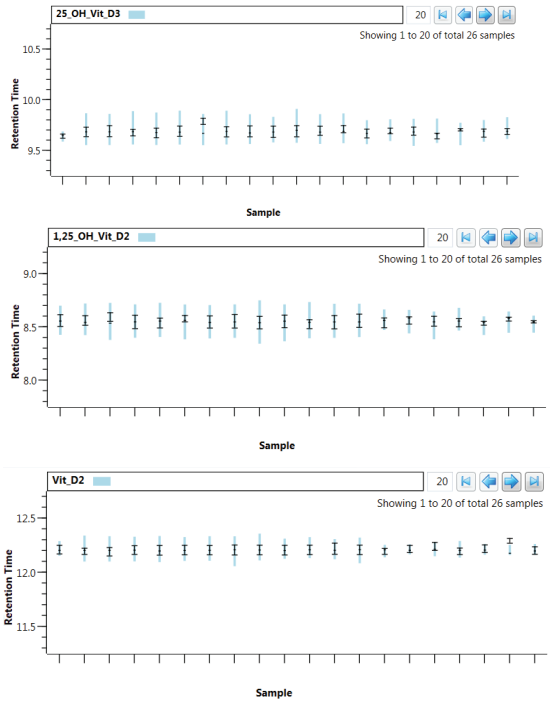

Figure 6. Retention time comparisons for the first twenty samples run in the serum calibration curve.

TESTOSTERONE

For testosterone, neat samples were made from 0.01 to 100 ng/mL, having great analytical performance as mentioned above, the chromatograms can be found in Figure 6. One transition was monitored and based on the analytical performance at 10 pg/mL, reliable signal could be detected at lower concentrations.

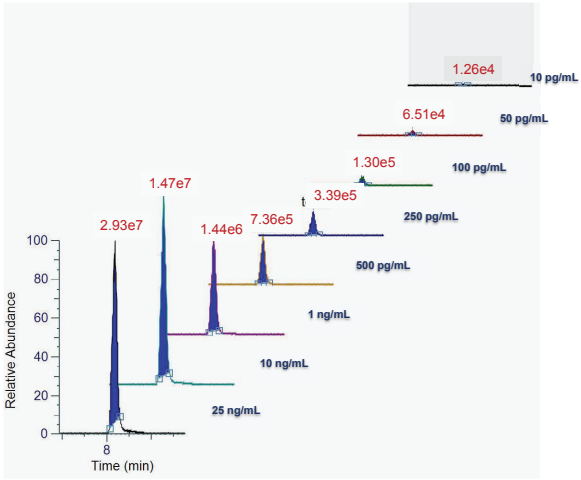

Figure 6. SRM chromatograms of Testosterone for the calibration curve.

Samples were spiked into human serum and precipitated and the supernatant was injected directly into the LC/MS instrument. The calibration curve for the serum samples were made from 50 pg/mL to 25 ng/mL, shown in Figure 7. The LLOQ for testosterone was 100 pg/mL at a 2 uL injection in serum and 50 pg/mL for limit of detection found in Figure 8. The limitation in signal is due to the high background in the serum samples. High resolution of the quadrupole (0.2-0.4 m/z) will be investigated to see if the noise in the testosterone transitions can be decreased.

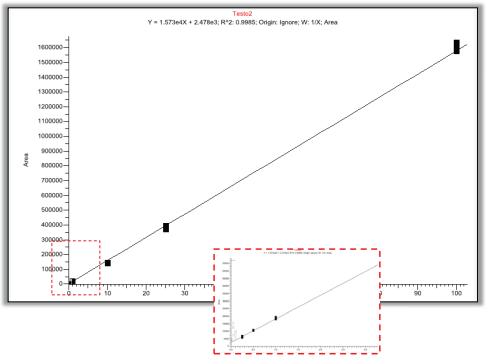

Figure 7. Calibration curve for testosterone in serum.

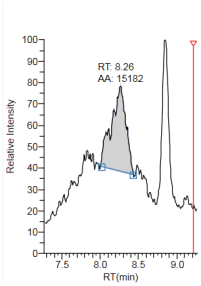

Figure 8. LOD of testosterone in serum.

Figure 9 exemplifies the retention times for testosterone compound in serum.

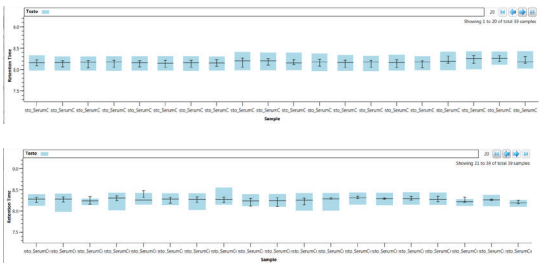

Figure 9. Retention times for forty samples of testosterone in serum.

PRELUDE - MICROFLOW

The Thermo Scientific™ Prelude™ SPLC liquid chromatography instrument is a multi-channel instrument with syringe based pumps. This instrumentation set up is beneficial for a clinical research environment which necessitates high throughput analysis. Proof of principle work was done to see if the traditional high flow pumps could run at microflow, 50 uL/min flow rates.

Reproducibility of running vitamin D at 50 uL/min on the Prelude SPLC instrument was very good. The instrument method had to be extended because of the void volume in the system. Unlike the nanoflow pump that has lower diameter tubing, the Prelude SPLC system is built for high flow use.

SRM chromatograms of Vitamin D2 and D3 are found in Figure 10. Compared to 400 uL/min run on the Prelude there was about a 3-4 times improvement in signal.

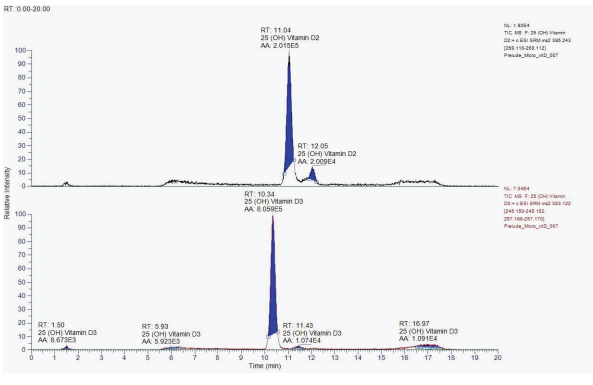

Figure 10. SRM chromatograms of Vitamin D2 and D3 on the Prelude platform.

CONCLUSIONS

- The UltiMate 3000 RSLCnano pump configured with a capillary flow meter yield reproducible and accurate results for Vitamin D and testosterone for clinical research.
- Analytical performance for %RSD is under 15% and for percent accuracy under 15% as well.
- Minimal sample preparation was needed to inject serum samples into the LC/MS.

FUTURE PROJECTS

Utilizing this LC/MS low flow platform for research into clinically relevant small molecules. Perform robustness studies to verify sample clean up does not hinder analysis over time.

For research use only. Not for use in diagnostic procedures.

Find out more at **thermofisher.com**

ThermoFisher SCIENTIFIC