An Automated Liquid Handling and Sample Preparation Workflow for LC-MS/MS Analysis of Vitamin D Metabolites, Testosterone, and Cortisol for Research Use

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Overview

Purpose: To evaluate the Thermo Scientific Platemate 2x3 automated liquid handling system to remove manual pippetting from the analysis of vitamin D metabolites, testosterone, and cortisol. The accuracy and precision data of the Platemate™ Pipetting Workstation was measured following regulatory guidelines for validation.

Methods: The Platemate system was used for aliquotting samples, internal standards, and protein precipitation reagents. A new Thermo Scientific Transcend system was equipped with a Thermo Scientific TurboFlow Cyclone-P column (50 x 0.5 mm) and a Thermo Scientific Accucore PFP analytical column (50 x 2.1 mm, 2.6 µm). The detector for the system was a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer with a HESI-II source.

Results: The Platemate system is an automated liquid handling solution for accurate sample preparation which saves both time and resources. The system met regulatory guidelines for the validation acceptance criteria for the methods tested.

Introduction

The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis of cortisol, testosterone, and vitamin D metabolites is well established in the literature. Here we present data using an automated liquid handling system to eliminate the need for manual pipetting. In this work, we evaluated the accuracy and precision of 25-hydroxy vitamin D_2, 25-hydroxy vitamin D_3, testosterone, and cortisol using the Platemate system (seen in Figure 1) for sample preparation.

In order for the Platemate workstation to be evaluated it was subjected to a number of bioanalytical validation requirements. The data generated must fall within a set of parameters outlined by standard operating procedures (SOP). Briefly summarized for validation, these parameters include, but are not limited to:

1) The lower limit of quantitation (LLOQ) and low quality control need to be ±20% of the expected concentration.
2) All of the remaining calibrators and controls need to be ±15%.
3) In order to determine both inter- and intra-day accuracy and precision, these requirements must be met for three consecutive days.

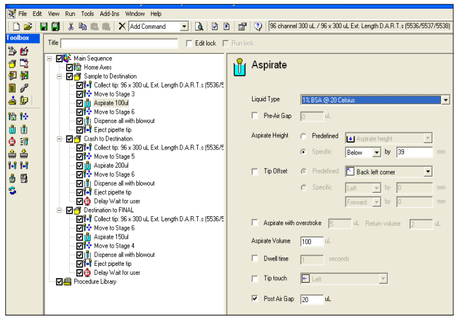
Methods

Sample Preparation: 25-hydroxy Vitamin D_2, 25 hydroxy Vitamin D_3, Testosterone, and Cortisol

1. Create and design optimum protocol for each method using the Thermo Scientific ControlMate OLE Software Module (shown in Figure 2).
2. Place glass or plastic sample containing plate onto Versette stage
3. Aliquot 100 µL of each sample vial into a 2 ml deep, 96-well plate
4. Add 200 µL of crashing reagent containing internal standard to each sample
5. Vortex for 30 seconds
6. Centrifuge at 4500 rpm for 3 minutes
7. Transfer 200 µL of supernatant into a clean plate for injection

FIGURE 1. The Platemate System (left) and 96-well head with tips (right) used for the sample preparation of Vitamin D, testosterone, and cortisol.

Figure 2. ControlMate Software



LC System: A new Transcend™ system that maximizes efficiency and minimizes solvent consumption.

Mobile Phases:

1) A: aqueous phase, 10 mM ammonium formate, 0.01% formic acid in water
2) B: organic phase, 10 mM ammonium formate, 0.01% formic acid in methanol
3) C: column wash, 45% isopropanol, 45% acetonitrile and 10% acetone

Needle washes:

1) 60% water, 40% methanol, and 0.5% formic (aqueous)
2) 45% isopropanol, 45% acetonitrile, and 10% acetone (organic)

Columns:

TurboFlow™ Cyclone-P column (50 x 0.5 mm)
Accucore™ PFP analytical column (50 x 2.1 mm, 2.6 µm) encased in a 70 ºC column heater

MS System: TSQ Vantage™ triple stage quadrupole mass spectrometer equipped with a heated electrospray ionization (HESI II) probe in positive ion mode.

Results

Vitamin D

The analytical measurement range for 25-hydroxy vitamin D_2 and 25-hydroxy vitamin D_3 is 2.0 ng/mL to 100 ng/mL. The multiple reaction monitoring (MRM) chromatograms at the LLOQ and the calibration curves for 25 hydroxy-vitamin D_2 and D_3 are shown in Figures 3 and 4 respectively. The accuracy and precision data for the quality controls for the three days of validation for 25-hydroxy vitamin D_2 and D_3 are listed in Tables 1 and 2 respectively. The coefficient of variability (r^2) for all three days for both analytes ranged from 0.9918 to 0.9980.

Cortisol

The analytical measurement range for cortisol is 3.62 ng/mL – 362 ng/mL. The MRM chromatogram at the LLOQ and the calibration curve for cortisol is shown in Figure 5. The accuracy and precision data for the quality controls for the three days of validation for cortisol is listed in Table 3. The r^2 values for all three days ranged from 0.9971 to 0.9988.

Testosterone

The analytical measurement range for testosterone is 0.020 ng/mL – 10 ng/mL The MRM chromatogram at the LLOQ and the calibration curve for testosterone is shown in Figure 6. The accuracy and precision data for the quality controls for the three days of validation for testosterone is listed in Table 4. The r^2 values for all three days ranged from 0.9978 to 0.9985.

Figure 3. MRM Chromatogram of 25-hydroxy Vitamin D_2 at the LLOQ and an Example Calibration Curve

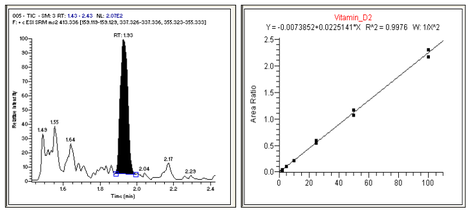


Figure 4. MRM Chromatogram of 25-hydroxy Vitamin D_3 at the LLOQ and an Example Calibration Curve

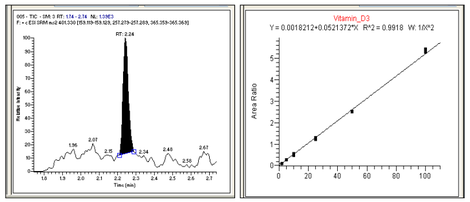


Table 1. Accuracy and Precision Data for 25-hydroxy Vitamin D_2

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
6.0	6.26	4.3	4.93	-17.8	6.79	13.2
6.0	6.73	12.1	5.46	-9.1	5.18	-13.6
6.0	5.50	-8.4	6.01	0.1	5.30	-11.7
6.0	5.46	-9.1	6.77	12.9	6.12	2.0
6.0	6.00	-0.1	6.43	7.2	5.03	-16.2
Average	5.99	-0.2	5.92	-1.4	5.68	-5.3
SD	0.53		0.74		0.75	
%RSD	8.9		12.5		13.2	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
40.0	34.9	-12.8	38.7	-3.4	37.0	-7.5
40.0	38.7	-3.2	41.2	3.1	37.0	-7.6
40.0	36.5	-8.7	39.4	-1.5	38.5	-3.7
40.0	35.0	-12.4	39.2	-2.0	37.9	-5.2
40.0	40.4	0.9	36.7	-8.2	39.6	-1.0
Average	37.1	-7.2	39.1	-2.4	38.0	-5.0
SD	2.38		1.62		1.11	
%RSD	6.4		4.1		2.9	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
80.0	72.3	-9.6	85.1	6.4	80.3	0.4
80.0	75.6	-5.5	80.7	0.9	77.9	-2.7
80.0	74.7	-6.6	81.9	2.3	80.5	0.7
80.0	78.6	-1.8	81.7	2.1	85.6	7.0
80.0	82.5	3.1	84.0	5.0	80.8	1.0
Average	76.7	-4.1	82.7	3.3	81.0	1.3
SD	3.91		1.81		2.82	
%RSD	5.1		2.2		3.5	

Table 2. Accuracy and Precision Data for 25-hydroxy Vitamin D_3

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
6.0	6.24	4.1	6.30	4.9	6.09	1.4
6.0	6.00	-0.1	5.83	-2.8	6.16	2.7
6.0	5.49	-8.5	5.81	-3.2	6.65	10.9
6.0	5.98	-0.4	6.77	12.9	6.65	10.8
6.0	5.35	-10.9	5.63	-6.3	6.49	8.1
Average	5.81	-3.1	6.07	1.1	6.41	6.8
SD	0.38		0.47		0.27	
%RSD	6.54		7.74		4.21	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
40.0	35.6	-11.00	41.9	4.81	39.2	-2.04
40.0	38.6	-3.46	40.3	0.7	37.4	-6.50
40.0	39.0	-2.60	37.7	-5.65	39.0	-2.57
40.0	36.0	-9.97	38.8	-3.08	40.8	1.97
40.0	41.2	2.92	38.7	-3.14	38.1	-4.79
Average	38.1	-4.8	39.5	-1.3	38.9	-2.8
SD	2.29		1.64		1.28	
%RSD	6.01		4.15		3.29	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
80.0	78.5	-1.8	81.2	1.5	81.7	2.1
80.0	83.5	4.4	79.2	-1.0	81.4	1.7
80.0	79.2	-1.0	79.3	-0.9	77.7	-2.9
80.0	82.0	2.5	77.9	-2.7	80.9	1.1
80.0	80.3	0.4	78.2	-2.2	84.1	5.2
Average	80.7	0.9	79.2	-1.1	81.2	1.4
SD	2.04		1.28		2.32	
%RSD	2.5		1.62		2.86	

Figure 5. MRM Chromatogram of Cortisol at the LLOQ and an Example Calibration Curve

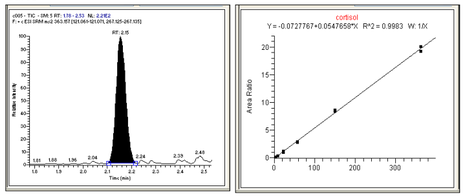


Table 3 Accuracy and Precision Data for Cortisol

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
10.0	10.9	9.3	9.3	-7.1	10.4	3.5
10.0	10.2	2.3	9.5	-5.5	9.8	-1.6
10.0	9.5	-4.7	9.8	-1.7	10.6	6.2
10.0	9.7	-3.4	9.6	-3.6	11.0	9.6
10.0	na	na	10.7	7.2	10.6	6.1
Average	10.09	0.9	9.79	-2.1	10.48	4.8
SD	0.64		0.56		0.42	
%RSD	6.34		5.72		4.01	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
145.0	136	-6.52	132	-8.67	141	-2.93
145.0	138	-4.70	128	-12.0	144	-0.64
145.0	130	-10.25	138	-4.94	148	2.25
145.0	137	-5.50	141	-2.98	140	-3.43
145.0	134	-7.70	132	-9.14	144	-0.76
Average	134.9	-6.9	134.1	-7.5	143.4	-1.1
SD	3.14		5.17		3.27	
%RSD	2.33		3.86		2.28	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
290.0	278	-4.1	286	-1.4	313	7.9
290.0	298	2.7	395	36.4	318	9.5
290.0	317	9.5	275	-5.3	334	15.1
290.0	311	7.3	291	0.3	315	8.7
290.0	310	6.8	300	3.5	317	9.3
Average	302.8	4.4	309.5	6.7	319.3	10.1
SD	15.54		48.96		8.34	
%RSD	5.1		15.82		2.61	

Figure 6. MRM Chromatogram of Testosterone at the LLOQ and an Example Calibration Curve

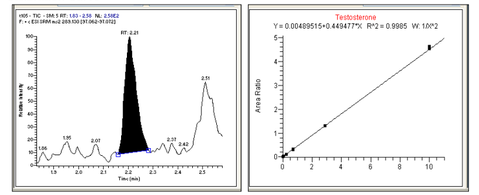


Table 4. Accuracy and Precision Data for Testosterone

Low QC	Day 1		Day 2		Day 3	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
0.06	0.064	6.18	0.054	-10.06	0.054	-10.42
0.06	0.058	-3.44	0.063	5.50	0.061	1.13
0.06	0.061	1.84	0.058	-3.95	0.058	-2.52
0.06	0.063	4.91	0.064	5.99	0.057	-5.31
0.06	0.059	-1.18	0.059	-1.30	0.062	3.40
Average	0.061	1.66	0.059	-2.45	0.059	-2.74
SD	0.002		0.004		0.003	
%RSD	3.28		6.72		5.09	
Mid QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
0.45	0.438	-2.58	0.434	-3.53	0.413	-8.20
0.45	0.450	0.05	0.422	-6.23	0.435	-3.32
0.45	0.468	4.05	0.456	1.33	0.422	-6.23
0.45	0.461	2.54	0.439	-2.34	0.436	-3.06
0.45	0.435	-3.36	0.451	0.23	0.415	-7.87
Average	0.45	0.14	0.44	-2.11	0.42	-5.74
SD	0.014		0.014		0.011	
%RSD	3.11		3.18		2.59	
High QC	**Day 1**		**Day 2**		**Day 3**	
Theoretical Concentration (ng/mL)	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference	Measured Concentration (ng/mL)	%Difference
8	8.04	0.54	7.89	-1.35	7.85	-1.82
8	7.88	-1.49	7.84	-2.02	8.18	2.24
8	7.89	-1.41	8.07	0.91	8.12	1.44
8	7.95	-0.68	8.04	0.55	8.18	2.25
8	7.84	-2.00	7.96	-0.46	7.98	-0.28
Average	7.92	-1.01	7.96	-0.47	8.06	0.77
SD	0.079		0.099		0.142	
%RSD	1.00		1.24		1.76	

Conclusions

- The Platemate Pipetting Workstation is equally as accurate and precise as a manual pipette, and is much more efficient. All methods were validated with the r^2 values ranging from 0.9918 to 0.9988.
- The Platemate workstation offers 90% time savings and passes all acceptance criteria for the compounds tested.
- Approximate sample preparation time for these methods when performed manually ranges from 2 - 3 hours, whereas the Platemate sample preparation time is about 10 - 15 minutes. This system minimizes technician sample handling and saves time, money, and resources.
- The Platemate, in combination with TurboFlow technology and the TSQ Vantage mass spectrometer, allows for complete online preparation of samples for LC-MS/MS analysis.