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Automated Sample Preparation for Analysis of 25-OH-Vitamin D3 and 25-OH-Vitamin D2 by LC/MS/MS

Beckman Coulter Biomek NX^{P} Workstation and SCIEX 3200™ QTRAP® LC/MS/MS system

Michael Kowalski and Ruth Zhang | Beckman Coulter Life Sciences, Indianapolis, IN, USA

Yun Yun Zou and Michael Jarvis | SCIEX, Concord, ON, Canada

Abstract

In this work, we demonstrate the feasibility of automating the sample preparation for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis of 25-OH-Vitamin D2 and 25-OH-Vitamin D3.

Introduction

LC/MS/MS technology provides research laboratories with a powerful tool for robust, accurate, sensitive detection of a wide variety of analytes. However, the preparation of samples for LC/MS/MS analysis can be both time-consuming and prone to human error. Automation of the sample preparation procedure can improve reproducibility while reducing both human error and active bench-time.

In this work, we have utilized a Biomek NX^{P} Workstation to automate the liquid handling steps of 25-OH-Vitamin D extraction from serum, coupled with off-line centrifugation and evaporation to dryness. A comparison is made between the manual and automated sample preparation procedures.

Materials and Methods

Samples consisted of commercially available serum calibrators and controls, containing 25-OH-Vitamin D2 and 25-OH-Vitamin D3 across a concentration range from 10 to 73 ng/mL. These were processed in a tube-based format using a liquid-liquid extraction protocol. Deuterated internal standard (25-OH vitamin D3-d6, part number H-074) was obtained from Cerilliant Corporation (Round Rock, TX). In brief, barcoded sample tubes were scanned and sampled using the Biomek Automated Tube Bar Code Reader (ATBCR). Internal standard solution was then added to the serum samples, followed by a liquid-liquid extraction using hexane. Following mixing and centrifugation, the organic layer was transferred to a clean tube and dried down under nitrogen, and the analytes were reconstituted in 50% methanol, prior to analysis by LC/MS/MS. The detailed sample preparation protocol is described in Table 1.

Step 1	Barcoded sample tubes automatically scanned by ATBCR device on the Biomek NX^{P} system.	Automated
Step 2	200 µL of serum (calibrator, QC or unknown) samples loaded into 1.5 mL Eppendorf tubes.	Automated
Step 3	200 µL of 25-OH-Vitamin D3 (26,26,26,27,27,27-d^{6}) internal standard solution added to each tube, and mixed.	Automated
Step 4	1000 µL of HPLC-grade hexane added to each tube, and mixed.	Automated
Step 5	Samples centrifuged at maximum rpm, for 20 minutes.	Offline with option to automate
Step 6	800 µL of clean, precipitate-free upper organic layer removed from each tube, and transferred to a clean tube.	Automated
Step 7	Samples evaporated to dryness under N_2 gas, or using a speed vacuum concentrator, at 35°C.	Offline
Step 8	Samples re-constituted with 150 µL of 50% methanol solution, and sonicated as needed.	Automated
Step 9	Samples transferred to HPLC vials.	Automated
Step 10	Analysis by LC/MS/MS system.	

Table 1. Sample Preparation Protocol for Human Serum Samples.

The described sample preparation was performed either manually or using a Biomek NX^{P} Workstation with Span-8 Pipettors (Figure 1). For the automated sample preparation on the Biomek NX^{P} Workstation, centrifugation and sample dry-down steps were performed off-line. However, these steps can be automated with appropriate device integration, which adds additional functionality to the base Biomek Workstation described. Figure 2 shows the labware and the locations on the deck of the instrument. The final samples were transferred to HPLC vials, which were placed in the autosampler for injection onto the LC/MS/MS system. Replicates of each calibration standard were prepared using both automated and manual methods to compare the accuracy, reproducibility, and extraction efficiency of each method. For each concentration level, 8 replicates were prepared using the automated sample preparation procedure on the Biomek NX^{P} system, and 3 additional replicates were prepared manually.

Figure 1A. Beckman Coulter Biomek NX^{P} Workstation.

Figure 1B. AB SCIEX 3200™ QTRAP® LC/MS/MS System.

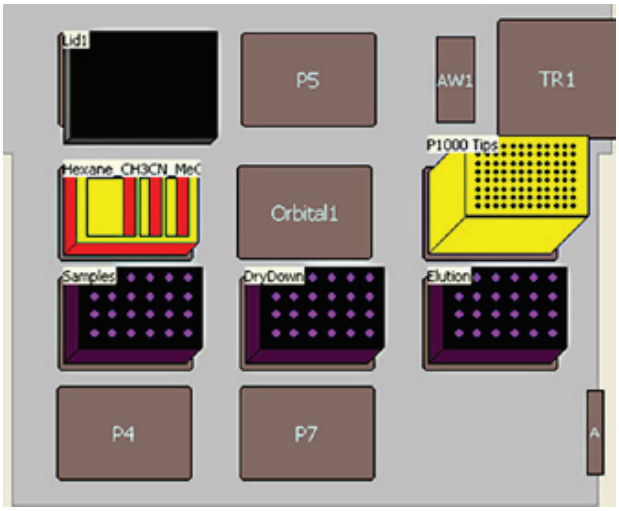

Figure 2. Deck layout on the Biomek NX^{P} Workstation configured for preparation of serum samples, prior to the analysis of 25-OH-Vitamin D2 and D3 by LC/MS/MS.

LC/MS/MS Analysis

A Shimadzu Prominence HPLC system was used, with a Luna C18, 50x2.1 mm, 3 µm analytical column (Phenomenex, Torrance, CA). An AB SCIEX API 3200™ LC/MS/MS system (Figure 1), equipped with Turbo V™ ionization source, was used in positive Atmospheric Pressure Chemical Ionization (APCI) mode. The following source settings were employed: nebulizer current = 5; temperature = 240°C; Gas1 = 50; interface heater = on; curtain gas = 25.

Two MRM transitions were used to monitor each analyte, and a single MRM transition was used to monitor the internal standard, 25-OH-Vitamin D3-d6. The MRM conditions are summarized in Table 2 (next page).

	Q1	Q3	CE (V)
25-OH-Vitamin D2 (quantifier)	395	269	26
25-OH-Vitamin D2 (qualifier)	395	119	32
25-OH-Vitamin D3 (quantifier)	383	211	32
25-OH-Vitamin D3 (qualifier)	383	229	27
25-OH-Vitamin D3-d6	389	211	32

Table 2. MS/MS Conditions for the Analysis of 25-OH-Vitamin D2 and 25-OH-Vitamin D3.

Integration of Automated Sample Preparation

Once the automated sample preparation protocol was completed, a worklist file containing sample properties was automatically created, and this was uploaded into the Cliquid® mass spectrometer control software (Figure 3) during batch submission to the LC/MS/MS system. The LC/MS/MS data acquisition, processing, and reporting were performed using the Cliquid® software.

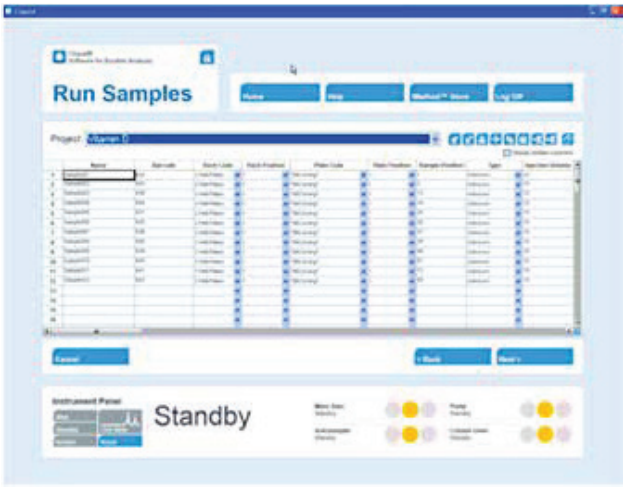

Figure 3. Cliquid® mass spectrometer control software. Upon completion of the automated sample preparation method, a worklist file containing sample properties was automatically created, and this was uploaded into the Cliquid® mass spectrometer control software.

Results

Serum calibrators and controls containing concentrations of 25-OH-Vitamin D3 and D2 ranging from 10 to 73 ng/mL were processed and analyzed. Representative chromatograms are shown in Figure 4 for a sample containing 10 ng/mL of 25-OH-Vitamin D3, demonstrating that the sample extraction efficiency was equivalent whether the sample preparation was performed in an automated fashion using the Biomek NX^{P} Workstation (Figure 4a), or performed manually (Figure 4b)

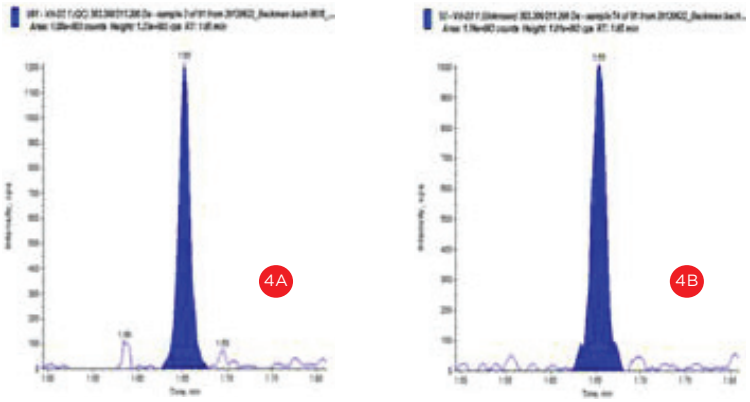

Figure 4A and 4B. Representative chromatograms for serum standards containing 10 ng/mL of 25-OH-Vitamin D3. A: Automated sample preparation using Biomek NX^{P} Workstation. B: Manual sample preparation.

The LC/MS/MS method enabled quantification of 25-OH-Vitamin D3 and D2 at concentrations as low as 5 ng/mL in human serum, using both the automated and manual extraction protocols. Calibration curves are shown in Figures 5 and 6, and display excellent linearity, accuracy and precision across the concentration range covered.

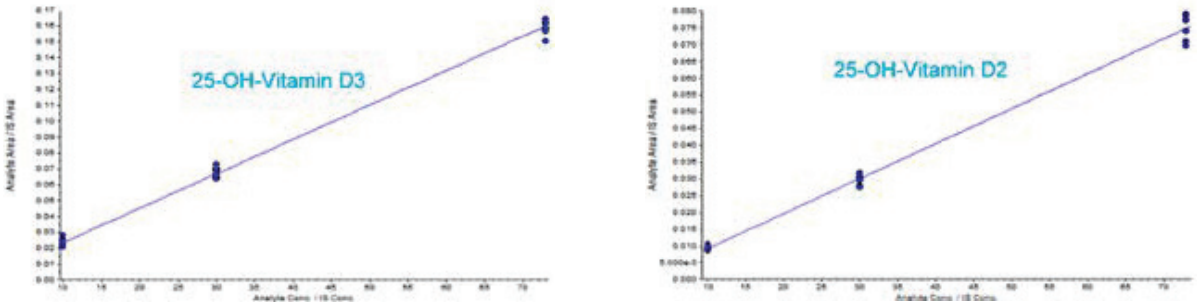

Figure 5. Calibration curves for 25-OH-Vitamin D3 and D2, with automated sample preparation using the Biomek NX^{P} Workstation.

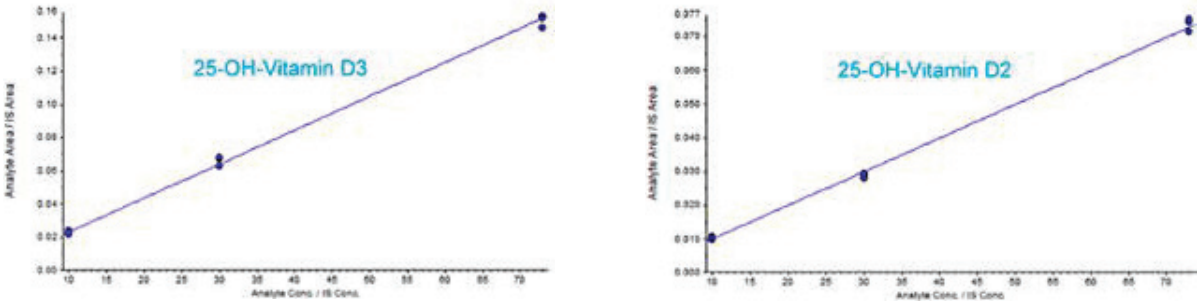

Figure 6. Calibration curves for 25-OH-Vitamin D3 and D2, with manual sample preparation.

The accuracy (%) and CV (%) values for automated and manual sample preparation methods are summarized in Tables 3A and 3B for the analyte 25-OH-Vitamin D3, which clearly demonstrates that the two sample preparation methods provide comparable performance. Similar results were obtained for the analyte 25-OH-Vitamin D2 (data not shown).

	Number of Samples	Mean Conc. (ng/mL)	Accuracy (%)	cv (%)
10 ng/mL	8	9.89	98.9	11.8
30 ng/mL	8	30.5	101.6	4.6
73 ng/mL	8	72.6	99.5	2.8

Table 3A. Statistical Summary for the Quantitation of 25-OH-Vitamin D3 by LC/MS/MS, Using Automated Sample Preparation on the Biomek NX^{P} Workstation.

	Number of Samples	Mean Conc. (ng/mL)	Accuracy (%)	cv (%)
10 ng/mL	3	9.79	97.9	6.5
30 ng/mL	3	30.9	103.1	4.5
73 ng/mL	3	72.3	99.0	2.5

Table 3B. Statistical Summary for the Quantitation of 25-OH-Vitamin D3 by LC/MS/MS, Using Manual Sample Preparation.

Summary

Here we have demonstrated the ability to automate the liquid handling steps in a liquid-liquid extraction of vitamin D from serum for analysis by LC/MS/MS, using the Beckman Coulter Biomek NX^{P} Workstation.

Automation reduced the active bench-time required for sample preparation, by eliminating all manual pipetting and mixing steps. Automation of the sample preparation also reduced the opportunity for pipetting errors, while aiding sample tracking by using automated barcode-reading, and through the creation of sample worklists that were compatible with the Cliquid® mass spectrometer control software.

The accuracy, precision, linearity and recovery of the automated sample preparation method compared favorably to the manual sample preparation method. For the 3 concentration levels measured here, the average accuracies for the manual sample preparation method ranged from 97.9% to 103.1%, while the average accuracies for the automated sample preparation method ranged from 98.9% to 101.6%. The % CV ranged from 2.5% to 6.5% for the manual sample preparation method, and from 2.8% to 11.8% for the automated sample preparation method.

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