

Improved Automated Sample Preparation for the Analysis of 25-OH-Vitamin D3 by LC/MS/MS



Abstract

In this work, we demonstrate an improved automated sample preparation for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis of 25-OH-Vitamin D₃. By replacing hexane extraction with zinc sulfate precipitation, we reduce the reagent hazards, remove the need for dry down and resuspension steps, and have the capability to automate the workflow without user intervention.

Introduction

LC/MS/MS technology provides research laboratories with a powerful tool for robust, accurate and 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 salt-assisted liquid-liquid extraction of 25-OH-Vitamin D from serum. This report builds on our previous work in which automated liquid handling was coupled with off-line centrifugation and evaporation to dryness to process hexane extraction of 25-OH-Vitamin D. This new process provides numerous advantages. By eliminating the use of hexane, the reagent hazards are reduced. Secondly, the use of the LC-friendly acetonitrile as the organic phase removes the need for dry down and resuspension of the samples. In addition, the greater compatibility of the reagents with plastics allows processing the samples in plates, thereby increasing the throughput potential. Finally, this process is

compatible with lower speed centrifugation that allows the sample plates to be spun on an integrated centrifuge, thereby enabling complete walkaway automation.

Materials and Methods

Samples consisted of commercially available serum calibrators and controls, containing 25-OH-Vitamin D3 across a concentration range from 10 to 73 ng/mL. Deuterated internal standard (25-OH Vitamin D3-d6, part

number H-074) was obtained from Cerilliant Corporation (Round Rock, TX). In brief, samples were transferred from barcoded tubes to a 96-well deepwell plate for processing. Acetonitrile containing the internal standard was added to the serum samples and samples were mixed using an orbital shaker. This was followed by two additions of a saturated zinc sulfate solution with subsequent orbital and tip mixing. Samples were then centrifuged at 4,500 X g for 20

Table 1. Sample preparation protocol for human serum samples

| | | |
|--------|---|-----------------------------------|
| Step 1 | Barcoded sample tubes automatically scanned by ATBCR device on the Biomek NXP system | Automated |
| Step 2 | 300 µL of serum (calibrator, QC or unknown) samples loaded into a 96-well deep well plate | Automated |
| Step 3 | 300 µL of 25-OH-Vitamin D3 (26,26,26,27,27,27-d6) internal standard solution (200 ng/mL) added to each sample and the plate is mixed on orbital shaker for 1 min at 1,200 rpm | Automated |
| Step 4 | 300 µl saturated ZnSO4 solution (70 g/mL) added to each sample | Automated |
| Step 5 | Samples mixed on orbital shaker for 2 min at 1,200 rpm | Automated |
| Step 6 | 300 µL saturated ZnSO4 solution added to each sample and mixed by pipetting, followed by orbital shaking for 2 min at 1,000 rpm | Automated |
| Step 7 | Samples centrifuged at 4,500 X g for 20 min | Offline (with option to automate) |
| Step 8 | 180 µl supernatant transferred to a 96-well plate | Automated |
| Step 9 | Analysis by LC/MS/MS system | Offline |



Fig. 1. Beckman Coulter Biomek NX^P sample preparation workstation (left) and AB SCIEX 3200 QTRAP[®] LC/MS/MS system (right).

min. 180 μ l of the supernatant were then transferred to a 96-well plate for analysis by LC/MS/MS. The detailed sample preparation protocol is described in Table 1.

The sample preparation was performed using a Biomek NX^P Workstation with Span-8 Pipettors (Figure 1), with an integrated Biomek ATBCR (Automated Tube Bar Code Reader). The method utilized a simple user interface to input the number of samples and this information is used to automate the creation of a balance plate of water to utilize in the centrifugation step. This centrifugation step was performed offline, however, this step can be automated with an integrated centrifuge, which provides additional functionality to the base Biomek platform described. Figure 2 shows the labware and the locations on the deck of the instrument.

Accuracy, reproducibility, and extraction efficiency for Vitamin D3 standards were determined. For each concentration level, at least 3 samples were prepared using the automated sample preparation procedure on

the Biomek NX^P system and injected into the LC/MS/MS in two replicates

LC/MS/MS Analysis

Shimadzu Prominence HPLC system was used with Luna C18, 50x2.1 mm, 3 μ m analytical column (Phenomenex, Torrance, CA). 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 Multiple Reaction Monitoring (MRM) transitions were used to monitor each analyte and a single MRM transition was used to monitor the internal standard, 25-OH-Vitamin

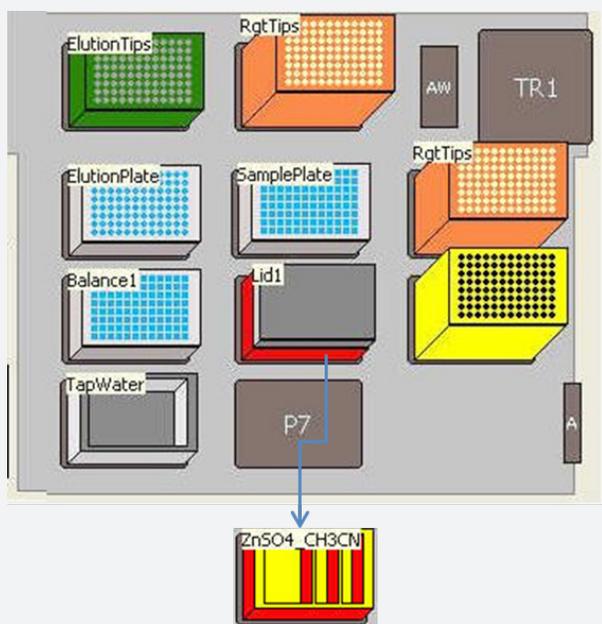


Fig. 2. Deck layout on the Biomek NX[®] Workstation, configured for preparation of serum samples prior to the analysis of 25-OH-Vitamin D3 by LC/MS/MS. Sample tubes are presented to the instrument using the automated tube barcode reader (deck position "A"). A plate to balance the centrifuge is filled with water based on the number of samples being processed.

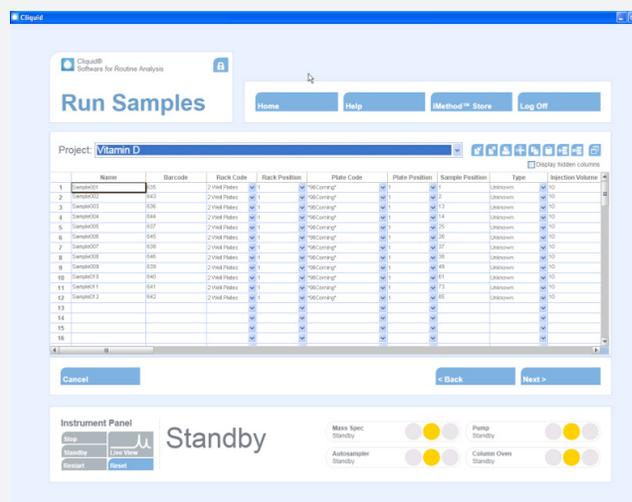


Fig. 3. Cliquant[®] mass spectrometer control software.

Table 2. MS/MS Conditions for the analysis of 25-OH-Vitamin D3.

| | Q1 | Q3 | CE (V) |
|-------------------------------|-----|-----|--------|
| 25-OH-Vitamin D3 (quantifier) | 383 | 211 | 32 |
| 25-OH-Vitamin D3 (qualifier) | 383 | 229 | 27 |
| 25-OH-Vitamin D3-d6 | 389 | 211 | 32 |

D3-d6. The MRM conditions are summarized in Table 2. The LC/MS/MS data acquisition, processing, and reporting were performed using Cliquant[®] software.

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 Cliquant[®] mass spectrometer control

software (Figure 3) during batch submission to the LC/MS/MS system.

Results

Serum calibrators and controls containing concentrations of 25-OH-Vitamin D3 ranging from 10 to 73 ng/mL were processed and

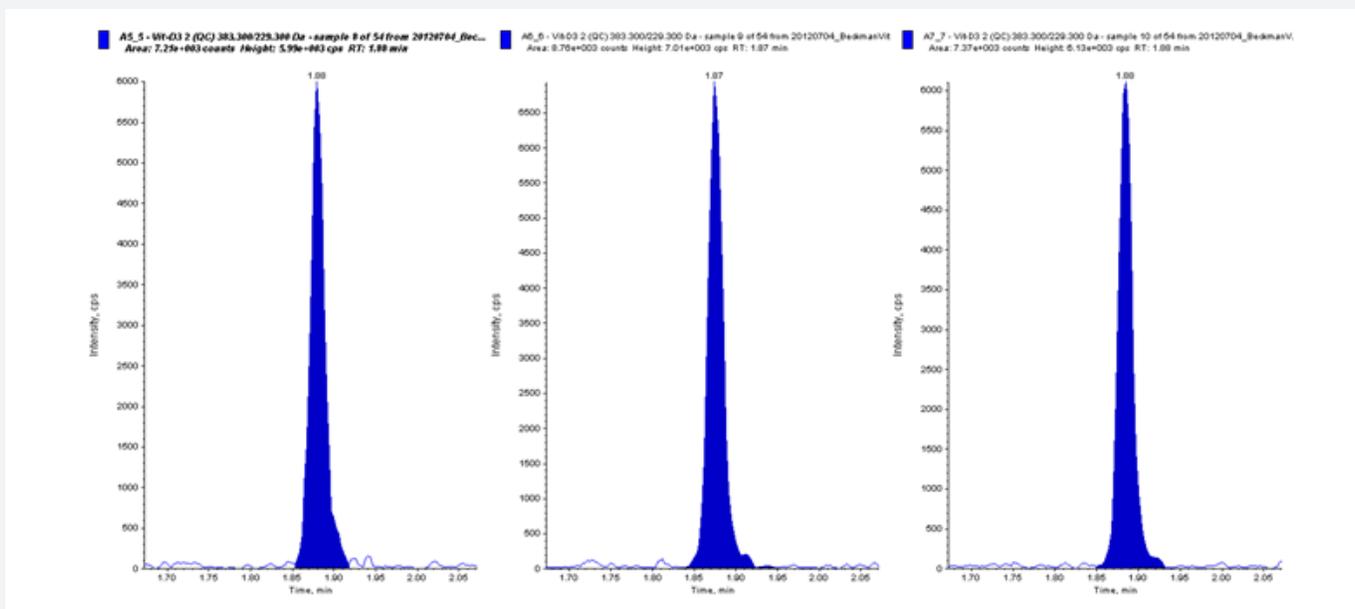


Fig. 4. Representative chromatograms for serum standards containing 30 ng/mL of 25-OH-Vitamin D3.

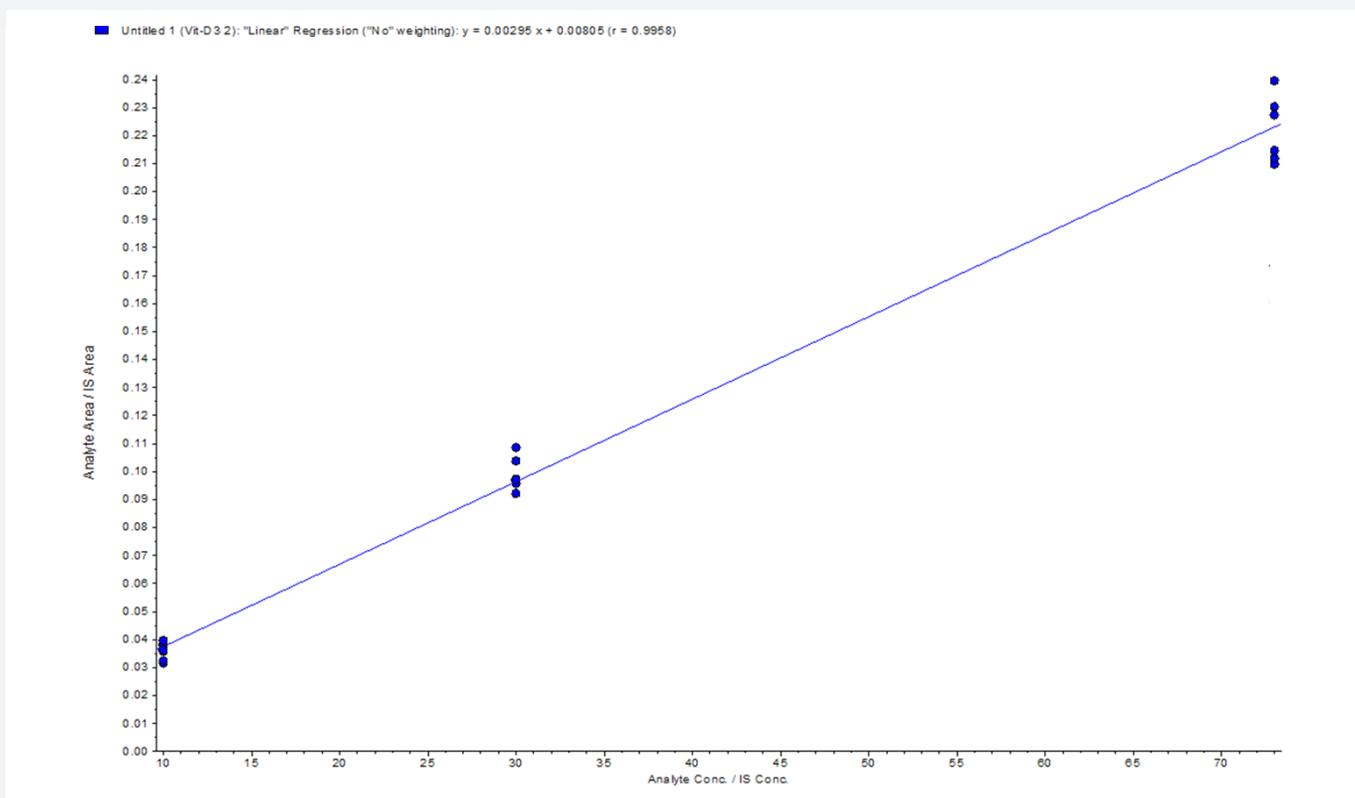


Fig. 5. Calibration curve for 25-OH-Vitamin D3 with automated sample preparation using the Biomek NX[®] workstation (concentrations used: 10 ng/mL, 30 ng/mL and 73 ng/mL).

Table 3. Statistical summary for the quantitation of 25-OH-Vitamin D3 by LC/MS/MS, using automated sample preparation on the Biomek NXP workstation.

| | Number of samples | Mean Conc. (ng/mL) | Accuracy (%) | CV (%) |
|----------|-------------------|--------------------|--------------|--------|
| 10 ng/mL | 8 | 9.34 | 93.4 | 10.1 |
| 30 ng/mL | 8 | 31.35 | 104.5 | 5.7 |
| 73 ng/mL | 6 | 73.66 | 100.9 | 5.6 |

analyzed. Representative chromatograms are shown in Figure 4 for samples containing 30 ng/mL of 25-OH-Vitamin D3.

The LC/MS/MS method enabled quantification of 25-OH-Vitamin D3 at concentrations as low as 10 ng/mL in human serum. In addition, the calibration curve shown in Figure 5 displays excellent linearity, accuracy and precision across the concentration range covered (Table 3).

Summary

This study demonstrates the ability to automate the liquid handling steps in a salt-assisted liquid-liquid extraction of 25-OH-Vitamin D3 from serum for analysis by LC/MS/MS, using the Beckman Coulter Biomek NXP^P Workstation. While a relatively low number of control samples were tested for this workflow, the estimated time to process 96 samples using this automated method is 75 minutes.

Automation of this sample processing provides numerous advantages over manual preparations. First, the active bench-time required for sample preparation is reduced by eliminating all manual pipetting and mixing

steps. Automation of the sample preparation also reduced the opportunity for pipetting errors while aiding sample tracking through automated barcode-reading and the creation of sample worklists that were compatible with the Cliquant[®] mass spectrometer control software. Finally, the excellent assay accuracy and precision seen here can be achieved while minimizing the variability that typically arises from multiple users performing sample preparation. These properties make the Biomek workstation an ideal solution for processing samples for analysis by mass spectrometry.

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AAG-499APPO9.14-A