THE ANALYSIS OF 25-HYDROXYVITAMIN D IN SERUM USING AUTOMATED SOLID-PHASE EXTRACTION AND LC/MS/MS

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INTRODUCTION

In recent years, demand for serum 25-hydroxyvitamin D (25OHD) analysis has increased considerably. In addition to the role vitamin D plays in bone metabolism, several studies now link vitamin D deficiency with increased risk of certain cancers, multiple sclerosis and heart disease.[1,2]

Serum 25OHD concentration is accepted as the clinical indicator for determining vitamin D status[3] and monitoring supplementation therapy, which is available in two forms, vitamin D2 and vitamin D3. Some immunoassays may under report the total 25OHD level for patients on vitamin D2 supplementation due to the cross-reactivity of the antibody for 25OHD2 being less than 100%.[4] Therefore, many clinical laboratories have now adopted LC/MS/MS based methods for measuring 25OHD to enable the reliable measurement of both 25OHD2 and 25OHD3. The analysis of 25OHD by LC/MS/MS requires sample pre-treatment to release 25OHD from the vitamin D binding protein and to minimize matrix effects. However, these steps are time consuming and sample transfer may be prone to human error.

This work describes an automated sample pre-treatment protocol, with sample tracking from the primary tube to processed results, using UPLC/MS/MS for the analysis of 25OHD.

METHODS

EXPERIMENTAL

An ACQUITY® TQD system (Waters Corporation, Milford, MA) operated in positive electrospray mode was used for all analyses.

The instrument was optimized to monitor 25(OH) D2, 25(OH) D3 and isotopically labeled internal standards by Multiple Reaction Monitoring (MRM).

Figure 1. System configuration of Waters® ACQUITY UPLC® TQ Detector.

UPLC Conditions

LC System:	Waters® ACQUITY UPLC® System
Column:	BEH Phenyl Column; 2.1 x 50 mm, 1.7µm
Column Temp:	35°C
Flow Rate:	450µL/min.
Mobile Phase A:	Water with 2mM ammonium acetate, 0.1% formic acid
Mobile Phase B:	Methanol with 2mM ammonium acetate, 0.1% formic acid
Gradient:	65–85% B over 3 minutes

Clinical Samples, Calibrators and Quality Controls

Calibrator and Quality controls (QCs) (Chromsystems, Germany) were used for quantitation in serum samples. Assay precision was determined using high and medium QCs (UTAK, USA) and a low QC was prepared by pooling 25OHD-deficient human serum and adding a known concentration of 25OHD2.

The method was compared by analyzing sixty-five annonymized clinical serum samples previously analysed at the University Hospital South Manchester (UHSM) by a hexane extraction LC/MS/MS assay.

Automated Sample Preparation

Unknowns, calibrators and QCs were placed on a robotic liquid-handling system (LHS; Tecan Freedom EVO®, Switzerland. Figure 2) and identified by a bar code reader for tracking throughout the extraction procedure.

The LHS transfers the samples (150µL) into a 96 deep well plate and adds the internal standard solution prior to protein precipitation with zinc sulphate and methanol.

Following centrifugation (off-line), the LHS transferred the supernatant to a conditioned Oasis® µElution solid-phase extraction (SPE) plate and the plate was washed with aqueous methanol.

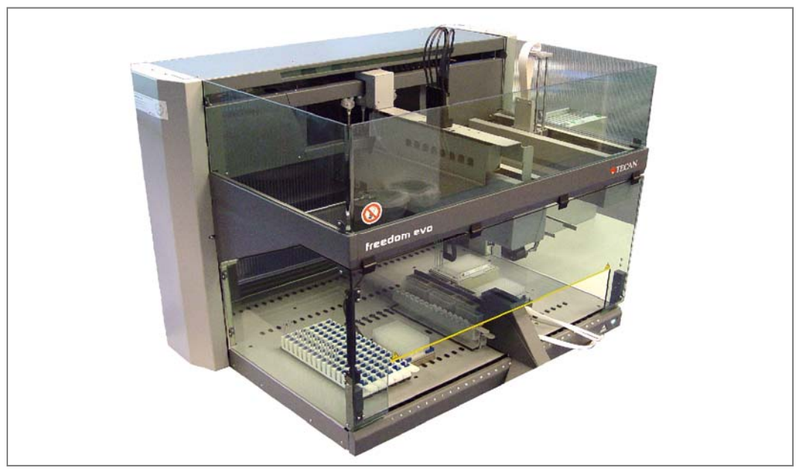

Figure 2. System configuration of the Tecan Freedom EVO® 100.

The retained analytes were eluted by the LHS in a two-step elution protocol to match the organic strength of the initial chromatographic conditions. The elution plate was sealed, transferred to the ACQUITY autosampler and 20µL was injected onto the UPLC/MS/MS system using the load-ahead feature of the ACQUITY Sample Manager resulting in an injection-to-injection time of 4.9 minutes. The sample preparation time for 96 samples is approximately 1.75 hours with minimal manual intervention.

RESULTS

Linearity

Assay linearity was determined by spiking horse serum (Sigma-Aldridge, UK) over the concentration range 2.5–220ng/mL. Linearity was determined daily for five days with a coefficient of determination (r^2) for 25OHD3 >0.998 and for 25OHD2 >0.997. Calculated concentrations for the calibrators were within 10% of the assigned values, a deviation of ±15% of the nominal value was accepted at the limit of quantification for each analyte.

Precision

The intra-assay precision was determined by analyzing five replicates of each QC level. Coefficients of variation (CV) for 25OHD2 and 25OHD3 were ≤7.7%. The inter-assay precision was determined over five consecutive days analyzing five replicates of all QC samples with CVs <12% for both analytes.

Accuracy

Assay accuracy for 25OHD3 was determined by analyzing sixteen external quality control samples from the international Vitamin D External Quality Assessment Scheme (DEQAS; www.deqas.org). The Chromsystems calibration curve was used to calculate the DEQAS sample concentrations. All results were within 10.8% of the 25OHD3 LC/MS method mean.

Recovery

The recovery of 25OHD2 and 25OHD3 was >80% (analyte response to blank spiked horse serum pre- and post-extraction expressed as a percentage) over the analytical range of the assay.

Ion Suppression

Ion suppression was investigated during the development of the chromatographic conditions. The effects of phospholipids, plasticizers and release agents from labware and blood collection devices were evaluated and minimized.

Sensitivity and Specificity

A chromatogram of a patient sample with a calculated concentration of 4.7ng/mL for 25OHD3 is shown in Figure 3. The quantification transition (*m/z* 401.35>159.1) is free from interference, enabling reproducible peak integration and the detection of 25OHD in samples from severely deficient patients.

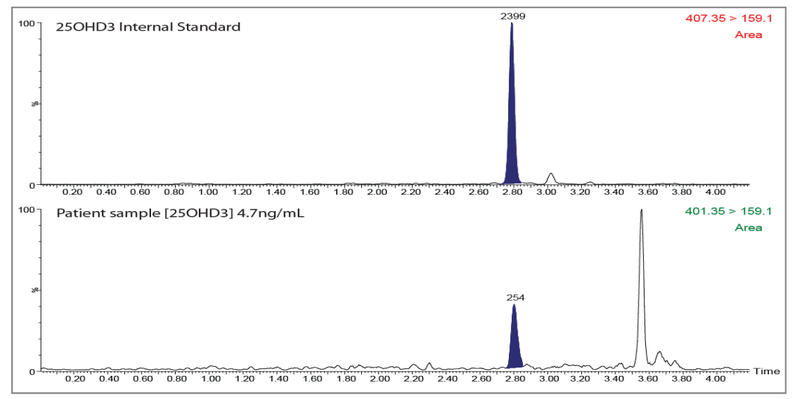

Figure 3. Chromatogram of a 25OHD deficient patient.

Sample Analysis

Sixty five anonymized patient samples from UHSM were analyzed and the total 25OHD concentrations determined. Regression analysis was calculated using the Passing and Bablok method and agreement was assessed using the Bland–Altman method (Microsoft Office Excel 2003 with Add-In Analyse-It version 1.73).[5,6] There was minimal difference between the methods (Waters=UHSM-1.0ng/mL) when analyzed using the Bland–Altman difference plot.

The overall regression line comparing the two methods was Waters = 0.98(UHSM)-0.65 (r^2 =0.90).

DISCUSSION

LC/MS/MS is used in many laboratories for vitamin D measurement, however, many of these methods require manual sample pre-treatment usually requiring experienced laboratory staff.

This assay demonstrates excellent linearity (r^2 > 0.997) with good accuracy and precision over five consecutive days and correlates well with a routine LC/MSMS assay.

The liquid handling and sample tracking capabilities of the Tecan Freedom EVO 100 reduces manual steps, operator variability and generates reproducible results. Oasis µElution plate technology eliminates the need for solvent evaporation and reconstitution steps.

The described method overcomes many of the limitations of current LC/MS/MS methodology. In particular, several time consuming and labour intensive manual sample pre-treatment steps have been eliminated. This will enable a wider range of laboratories to implement UPLC/MS/MS methodology for 25OHD analysis.

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