Introduction

The analysis of 25-Hydroxy-Vitamin D (25(OH)D) and its different forms (D3 and D2) can be easily and rapidly measured by LC-MS/MS with high sensitivity and specificity using various column types and solvent combinations.

In this case, we developed LC/MS/MS analytical methods on a QQQ and QTOF and evaluated the mass spectrometer capabilities of each platform in order to demonstrate their suitability for the analysis of 25(OH)D3 and D2 in serum. Liquid Liquid extraction was the sample preparation of choice due to its cleanliness and ease of use and the intact 25(OH)D2 and D3 were analyzed. An Agilent 1260 HPLC and 6460 Mass Spectrometer QQQ using MRM acquisition with Agilent Jet Stream-ESI and 6430 Mass Spectrometer were used. The analysis involved a 5 minute analytical method in positive ionization mode.

Experimental

Reagents, Standards, Calibrators and Controls

25-Hydroxy-Vitamin D3 Stock: 50µg/ml in Ethanol (Isosciences)
25-Hydroxy-Vitamin D2-Stock: 50µg/ml in Ethanol (Isosciences)
25-Hydroxy-Vitamin D2-H3-ISTD: 50µg/ml in Ethanol (Isosciences)
25-Hydroxy-Vitamin D3-H3-ISTD: 50µg/ml in Ethanol (Isosciences)

UTAK:
Recipe:
Level L and 1 and 2
Methanol:
Honeywell
Heptane:
Honeywell
Formic Acid:
Sigma Aldrich
Zinc Sulphate:
Sigma Aldrich

All in-house calibrators were prepared in Vitamin D Free Serum or VD-DC Mass Spec Gold Serum or DC Mass Spec Gold Serum (Golden West Biological, Inc.)

Sample Preparation

Liquid-Liquid Extraction

• 150 µl of Serum sample, calibrators, controls were taken. 150 µl of 0.2M Zinc Sulphate, 300 µl of Methanol and 7.5 µl ISTD at 1000 ng/ml were added to each and vortexed for 1 min
• 1.2 ml HPLC grade Heptane was added to each tube and vortexed for 1 min prior to centrifugation for 10 minutes at 3000 rpm
• Organic layer (Upper) was transferred to another clean tube and then dried down under nitrogen at room temperature
• Samples, calibrators, controls and blanks were reconstituted in 120 µl 75% Methanol:25% 0.1% Formic Acid in water and transferred to an MS vial or 96 well plate for analysis.

Method

HPLC Conditions

Agilent 1260 Infinity HPLC series binary pump, well plate, thermostat column compartment

Column Temperature: 50 °C
Injection Volume: 10 µl
Autosampler Temperature: 4 °C
Needle Wash: Flush port (50%Methanol:50%Water) 5 seconds
Flow Rate: 0.5 mL/min
Gradient:
0 mins- 20%A:80%B
2.3 mins- 20%A:80%B
3 mins- 2%A:98%B
3.9 mins- 2%A:98%B

Run time: 5 minutes
Column: Poroshell 120 EC-C18 2.1 x 50 mm, 2.7 µm
Mobile Phase A: 0.1% Formic Acid in Water
Mobile Phase B: 0.1% Formic Acid in Methanol

Results and Discussion

Linearity/Sensitivity

The linear range of 25-hydroxy-Vitamin D2 and D3 was from 1 to 500 ng/ml in Serum. The linearity was determined in triplicate over 5 days and the results are shown with LOD and LOQ being determined as 3:1 and 10:1 of signal to noise respectively where possible and the mean coefficient of determination (R²) > 0.99 for each matrix and the %CV for each calibration point were all <10%.

Accuracy

The accuracy was determined by the analysis of the Tri-level UTAK QC and the Bi-Level Recipe QC control material as the percentage deviation from the targeted mean and the results were < 10% for all levels in each matrix. The mean of the Utak QC's were Utak L- 9.56 ng/mL, Utak 1- 33.2 ng/mL and Utak 2- 76.3 ng/ml and for Recipe QC’s were Level 1- D3- 24.6 ng/mL, D2- 19.1 ng/ml and Level 2- D3- 89.4 ng/ml and D2- 94.3 ng/ml. Therefore, the analytical method in positive mode can achieve the required levels for the analysis of 25-Hydroxy-Vitamin D2 and D3 using a QQQ and a QTOF.

Precision

The intra-assay precision (%CV) of 25(OH)D3 and 25(OH)D3 were determined by extracting and quantifying five replicates of the Tri-level QC material from UTAK and the Bi-Level QC material from Recipe and the intra-assay precision was determined over 5 consecutive days and was found to be less than 10% for each analyte and by each mode. MRM mode on the QQQ resulted in the most accurate %CV’s.

Other data

Analyses of 25-OHVD2 and D3 was also carried out on a 6550 QTOF and better sensitivity was achieved with LOD’s for both analytes being less than 5 ng/ml in both MS and MS/MS mode. The interferences were still present and so the hydrosyl ion of 25-OHVD2 and D3 were analyzed as well resulting in even lower LOD with little interfering compounds and was comparable to the results achieved by the 6460 QQQ. Data not shown.

Conclusions

• Good LOD/LOQ was achieved on both QQQ and QTOF platforms but the enhanced sensitivity was achieved with the 6460 QQQ.
• The LC conditions were the same on both mass spectrometer platforms and baseline separation was achieved in 5 minutes.
• Demonstrated that any Mass Spectrometer platform can be used for the analysis of 25-OHVD2 and D3 the
• Excellent linearity of calibration curves with acceptable accuracy, precision and reproducibility in positive mode was achieved in all matrices <10% for %CV.