Introduction

The effective metabolism of dietary and endogenous forms of vitamin D to their primary hydroxylated metabolites 25-OH-vitamin D2 and 25-OH-vitamin D3 respectively plays a vital role in the regulation of intestinal calcium and phosphate absorption thus protecting the human organism from rickets or osteomalacia.

Clinical research investigations have linked 25-OH-vitamin D3/D2 levels below 30 ng/ml to diabetes and hypertension and as such there is a continued interest in quantifying these metabolites in the clinical research setting.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) offers clinical researchers, a rapid and cost effective way to quantitatively determine the levels of these metabolites in serum over extended concentration ranges. Furthermore, unlike immunoassay, this technique also offers the capability to simultaneously determine the individual concentrations of the 25-OH D3 and 25-OH D2 metabolites. Here we report a novel approach using ion trap mass spectrometry for the rapid and accurate quantification of these metabolites in serum.

Prior to any analysis by LC-MS/MS the serum matrix which contains proteins, lipids and carbohydrates has to be removed. A simplified approach has been employed using the novel Tecan AC Extraction Plate™ to prepare 25-OH-vitamin D3/D2 serum samples for subsequent quantification by LC-MS/MS systems.

Application Note LCMS-95

Quantitative Determination of Serum 25-OH-vitamin D3/D2 using the Tecan AC Extraction Plate™ and the amaZon speed Ion Trap

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Keywords

Instrumentation and Software

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<td>Quantitation</td>
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The Tecan AC Extraction Plate

The Tecan AC Extraction Plate with TICE™ (Tecan Immobilized Coating Extraction) technology is a 96 deep-well microplate containing a proprietary coating which extracts small, apolar molecules from aqueous environment. (figure 1).

A modifier buffer supports analyte extraction by the coated phase. The early addition of D6-25-OH-vitamin D3 as internal standard compensates for any procedural variations. After a wash cycle both 25-OH-vitamin D3/D2 are retrieved from the coated wall, using an elution solvent. As a result proteins, lipids and carbohydrates are removed, and the clean extract can directly be used for LC-MS/MS analysis.

Employing the Tecan AC Extraction Plate eliminates the need for cumbersome precipitation, filtration, centrifugation or solvent evaporation steps thus simplifying and accelerating the entire sample preparation process.

For more information about the Tecan AC Extraction Plate, please consult following weblink: www.tecan.com/acplate.

Tecan AC Extraction Plate workflow

A mixture of modifier buffer and internal standard is dispensed into the TICE™ well of the AC Extraction Plate and 50 µl of serum sample is added. After mixing on a shaker the supernatant is discarded to waste. The analyte remains within the coated phase on the wall of the plate well. After a wash step the wells of the AC Extraction Plate are filled with elution solvent and shaken again. The elution solvent is then transferred to either HPLC vials or uncoated microplates and placed into an HPLC autosampler for injection (figure 2).

Experimental

Instruments

LC system: Dionex UltiMate 3000 RSLC (Thermo Fisher Scientific)
MS system: amaZon speed (Bruker Daltonik, Bremen)

HPLC parameter

Column: Waters Acquity BEH C18, 1.7 µm 2.1 x 100 mm
Eluents: A: 2.5 mM ammonium formate in H₂O
B: 2.5 mM ammonium formate in MeOH
Flow rate: 0.3 ml/min

Example of an AC Extraction Plate workflow

Figure 1: Tecan AC Extraction Plate
Figure 2: Tecan AC Extraction Plate Workflow
Column temperature: 50°C

Gradient:
- 0 min to 0.5 min: 60% B
- 0.5 min to 5 min: 60% to 99% B (linear)
- 5 min to 8 min: 99% B; re-equilibration

Injection volume: 20 µl

MS parameter

Ionization: APCI positive mode
Nebulizer: 3 bar
Vaporizer temp.: 470°C
Dry gas: 3 L/min @ 300°C
Corona Current: 4000 nA
Capillary voltage: -4 kV
Scan mode: XtremeScan (52.000 Da/s)
Scan range: m/z 100 to 425

MS/MS:
- Isolation width 2.5 m/z
- Broad band fragmentation (width 40 m/z)
- Fragmentation amplitude 2.1 V
- SmartFrag™ 90-110%

Internal standard

D₆-25-OH-vitamin D₃; 5 µg/ml in Ethanol (Cerilliant Corp., USA). From this original stock solution a working solution (50 ng/ml in 100% acetonitrile) was prepared.

Serum samples

Three lyophilized serum calibrators and a low level calibrator (Cal0) containing only a marginal concentration of 25-OH-vitamin D₃ and no 25-OH-vitamin D₂ as well as two serum control levels (Chromsystems, Munich, Germany) containing both D₃ and D₂ were reconstituted according to the manufacturer’s instructions (table 1).

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>25-OH-vitamin D₃ [ng/ml]</th>
<th>25-OH-vitamin D₂ [ng/ml]</th>
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<tbody>
<tr>
<td>Cal0</td>
<td>4.2</td>
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<td>Cal1</td>
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<td>Control Level 2</td>
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</table>

Table 1: Concentrations of serum calibrators and serum controls after reconstitution

Extraction reagents

- Modifier buffer: 0.2 M sodium carbonate/sodium hydrogen carbonate 1:1 (v/v) in water/acetonitrile 95/5 (v/v)
- Wash solution: water/methanol 90:10 (v/v)
- Elution solvent: water/methanol 10:90 (v/v)

Sample preparation and analysis

The modifier buffer was mixed with the internal standard working solution (50 ng/ml) at a ratio of 2:1 (v/v) and 150 µl of this mixture were dispensed into a well. 50 µl of reconstituted serum calibrator or control were added. Each sample was extracted in one well and injected five times.

MS/MS fragments used for quantification:

- 25-OH-vitamin D₃:
  - EIC (158.8; 210.8; 257.0; 323.2)±0.3 +MS²(383.4)
- 25-OH-vitamin D₂:
  - EIC (208.8; 226.8; 251.0; 269.0; 325.2; 335.2)±0.3 +MS²(395.4)
- D₆-25-OH-vitamin D₃:
  - EIC (263.1; 329.2)±0.3 +MS²(389.4)

Sensitivity

Figure 3 depicts the MS/MS extracted ion chromatograms (EIC) of 25-OH-vitamin D₃ extracted from Cal0 0 (4.2 ng/ml) and of the Internal Standard D₆-25-OH vitamin D₃ (50 ng/ml). 25-OH-vitamin D₃ yields a signal at retention time 6.2 min which is clearly quantifiable.

![MS/MS extracted ion chromatograms (EIC) of 25-OH-vitamin D3](image)

After further optimization of the HPLC conditions the retention times were 5.5 min for 25-OH-vitamin D₃ and 5.6 min for 25-OH-vitamin D₂ (figure 4).
Conclusion

The Tecan AC Extraction Plate combines a fast and easy-to-use approach for analytical sample preparation with minimal sample pretreatment while at the same time demanding only low volumes (50 µl) of sample. No precipitation, filtration, centrifugation or evaporation is required thus simplifying and accelerating the entire sample preparation workflow. The extraction method can be performed manually or be fully automated on a liquid handling platform. The Bruker ion trap mass spectrometer amaZon speed is capable of quantifying 25-OH-vitamin D3 and 25-OH-vitamin D2 from serum after sample preparation with the AC Extraction Plate in the relevant concentration range. 25-OH-vitamin D3 is detected even in the low level calibrator at 4.2 ng/mL with sufficient sensitivity. Serum calibrators show excellent linearity and both control levels show satisfactory precision and accuracy for a rapid analytical set-up requiring low sample volume and minimal sample preparation.

Linearity and accuracy

The calibration curve for 25-OH-vitamin D3 shows excellent linearity (figure 5). The signals from quality controls (level 1 and level 2) are depicted in blue. The calibration curve for 25-OH-vitamin D2 yields very good linearity as well (figure 6). For quality controls of 25-OH-vitamin D2 a small bias towards higher concentrations is observed.

Precision

Quality control level 1 and level 2 were separately extracted in four wells. The eluates from each well were injected five times and the concentrations were determined using the calibration curve based on peak area ratios of analyte/internal standard (table 2).

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