1,25-di-hydroxy Vitamin D analysis by LC-MS: Optimization for sample prep automation and medium throughput lab

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Introduction

Currently we run a manual RIA assay for 1,25di-OH Vitamin D (1,25DiOHVitD) from serum, which takes 2 days and has a limited throughput. Our goal is to establish a LC-MS based workflow that significantly reduces hands on time (manual work), shortens the time to results and enables us to analyze the samples on our existing LC-MS system, without influencing already established routine applications.

For extraction of the target analyte we choose a 96-well plate suitable for easy automation (AC Extraction Plate™, Tecan, Switzerland) with a protocol for 25OH Vitamin D slightly modified to efficiently extract the more hydrophilic di-OH metabolite. A signal enhancement for 1,25DiOHVitD with lithium adducts in the ionization source of the MS system was not an option with our lab settings. Therefore, we tested and compared several derivatization methods (with PTAD reagent, DAPTAD reagent, Amplifex chemistry; Cookson type reagents) to achieve sufficient sensitivity and selectivity for the 1,25DiOHVitD analysis. The liquid chromatography was initially set up towards high selectivity to ensure sufficient chromatographic resolving power for isobars from derivatization and for potential interferences from sample matrix. Analysis of 46 patient samples with the LC-MS based assay was compared with the RIA assay. For the LC-MS assay we also compared the quantitative values of the 2 isobares formed by the derivatization and determined the reproducibility of the extraction efficiency.

Methods

- Extraction
  - As described in the Application Note for VitD on AC Extraction Plate [1] with the following modifications: i) internal standard (IS) in 75% ACN, ii) prepare extraction mix with 2.33 mL modifier buffer (carbonat buffer) and 1mL IS, iii) use 200µL of extraction mix per well, iv) add 100µL matrix (serum sample, control, calibrator or Blank.) to accommodate for the more hydrophilic character of the 1,25DiOHVitD and its low concentration; v) elution with 100% acetonitrile in preparation of the following derivatization (dry down under nitrogen at 40°C)
- Derivatization
  - PTAD reagent, DAPTAD reagent, Amplifex chemistry was prepared and/or used according to the literature or the manufacturer description in case of Amplifex (AB Sciex, Toronto, CA); reconstitution in 50µL ACN/Water (20/80)
- LC-MS analysis –
  - LC-MS/MS system: Waters TQ-S in positive ESI mode
  - LC Gradient of high purity water and acetonitrile (both containing 2mM $CH_3\text{-}COO^-NH_4^+$ and 0.1% formic acid), run time 27.5min
  - Column: Cortex UPLC C18+, 1.6 µm, 2.1 mm X 100 mm

Results

The derivatization with DAPTAD reagent showed highest sensitivity and best signal to noise values on our LC-MS system although 2 isobars were produced. The calibration curve for standard materials (taken from the RIA assay kit) was linear for the concentration range of 5 to 160 pg mL^{-1} (12 to 400 pmol mL^{-1}). Extraction efficiency was 84% at 100pg mL^{-1}, with a high reproducibility of the signal response (area / IS area) for replicate extractions (1.6%, n=5). The calculated concentration for the 46 serum samples showed a significant bias (+25%) compared to the concentration measured with the RIA assay as determined with a Bland-Altman plot. Potential reasons for limited comparability of the assays will be evaluated as soon as appropriate reference material is available and/or an option for comparison with another LC-MS based method is found.

The currently long chromatography showed an additional peak for patient samples which were not in the calibrator or QC material (Figure 1). An interference of this peak with the first isobar peak of the analyte shall be avoided when shortening the chromatographic run time during further method optimization.

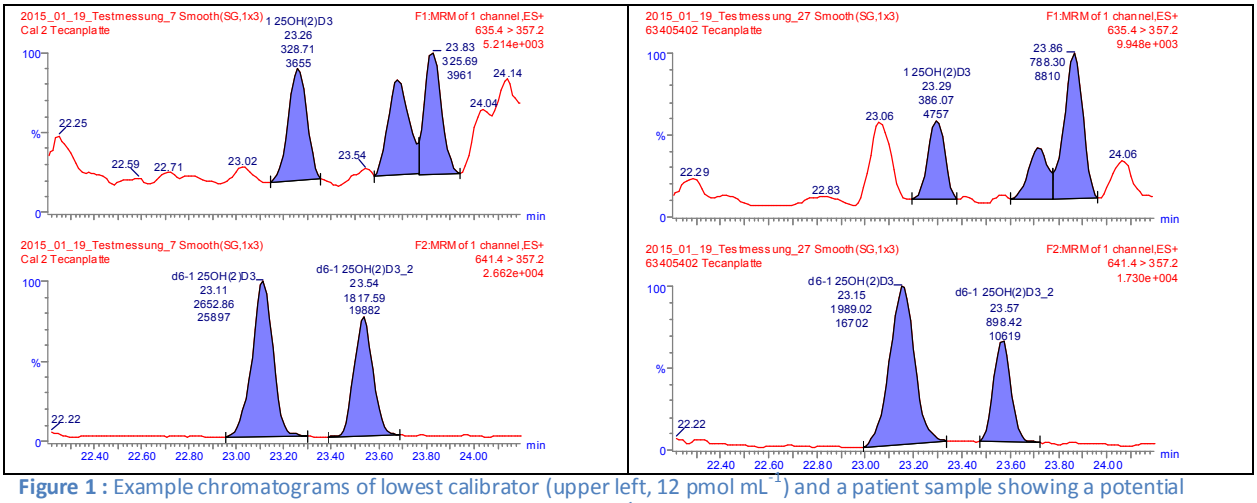

Figure 1 : Example chromatograms of lowest calibrator (upper left, 12 pmol mL^{-1}) and a patient sample showing a potential interference in front of the first isobar (upper right, 24 pmol mL^{-1}). Chromatograms below show the isobar peaks of the derivatized internal standard, respectively.

Conclusions

Extraction of 1,25-di-hydroxy Vitamin D with the easy to automate AC Extraction Plate followed by derivatization with DAPTAD and analysis by LC-MS showed a good extraction efficiency and appears to be very reproducible. Accuracy of the LC-MS assay (in comparison to the RIA) is to analyze with certified sample materials. LC- run time needs to be shortened significantly without compromising the selectivity.

However, the run time for the whole assay is already significantly reduced at our medium sample throughput. Automation of the sample preparation and LC-MS measurements can be added without comprising the already established applications running in routine.

[1] Automated Sample Preparation of 25-OH-Vitamin D3/D2 from Serum with the AC Extraction Plate and the Tecan Freedom EVO® for the quantification by LC-MS/MS, 2014, Doc No. 398079 V1.0 (www.tecan.com)