Determination of $1\alpha,25(OH)_2$-Vitamin D_3 (Calcitriol) in Human Plasma using the AB SCIEX API 4000™ System

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Introduction

The routine adoption of liquid chromatography-tandem mass spectrometry (LC/MS/MS) for clinical research is rapidly gaining momentum. In order to realize the full benefits of adopting LC/MS/MS for analytical measurement, the sample preparation procedure needs to be inexpensive and simple to implement. For challenging applications such as the quantitative measurement of low-level steroids, immunoassay-based technologies have demonstrated insufficient specificity, resulting in poor accuracy and precision. Therefore, there are considerable advantages to be gained by switching to LC/MS/MS as the technique of choice.

Among the large number of papers published by various authors on steroid analysis, we have recently presented a paper[1] on the measurement of the secosteroid $1\alpha,25(OH)_2$-vitamin D_3 (calcitriol). Although $1\alpha,25(OH)_2$-vitamin D_3 is not a good indicator of overall vitamin D status in humans, it is useful for the differential assessment of hyper and hypocalcemia and for distinguishing between vitamin D-dependent and vitamin D-resistant rickets. It is also used to monitor the vitamin D status of patients with chronic renal failure and for assessing compliance of $1\alpha,25(OH)_2$-vitamin D_3 therapy. The determination of this analyte presents several analytical challenges including:

- Very low ionization efficiency resulting in an intrinsically low response when using tandem mass measurement
- The absence of a straight-forward and specific derivatization step for the enhancement of ionization efficiency
- Intrinsically ultra-low concentration levels (low pg/mL) in plasma
- The presence of significant amounts of isobaric and isomeric endogenous species in real life plasma samples, causing chemical interferences during the analysis

By using a highly sensitive tandem mass spectrometer and a sophisticated two-dimensional chromatography (2D-LC) set up, we were able to quantify $1\alpha,25(OH)_2$-vitamin D_3 at levels down to 15 pg/mL[1]. The injection volume used corresponded to only 30 µL of the original plasma. In addition, the sample pre-treatment stage was reduced to a simple protein precipitation step.

The method has proven to be both robust and reproducible. Pivotal to the success of the method was the LC strategy employed upstream of the tandem mass measurement. To account for the very low intrinsic concentration of this analyte in plasma, and the presence of a significant amount of matrix components with similar structure and hydrophobicity, the sample was first extracted and cleaned online. The online process was achieved by injecting the protein-precipitated serum onto a special POROS™ perfusion column in the first LC dimension, prior to being automatically transferred to a second column where the final chromatographic separation is accomplished in the second dimension.

Using this approach, the analyte can be selectively separated from the other matrix components with negligible interferences (e.g. ion suppression) in the ion source. The final step for attaining sufficient sensitivity has been to promote lithium adduct (Li+) formation of the analyte and to monitor the associated MRM transition using the high-end AB SCIEX Triple Quad™ 5500 system.

This technical note details how this proven methodology has been transferred to the AB SCIEX API 4000™ system using the same 2D-LC configuration and a larger injection volume to compensate for its lower sensitivity. Embedding a higher injected sample volume in this application is not trivial based on:

- The maximum injection volume for most autosamplers is about 100 µL
- The programming for the sample loading, extraction and cleanup steps on the POROS™ perfusion column need to be revised due to the significant analyte displacement in the column resulting from a larger injection volume

The following strategy has been devised for addressing these issues.

Experimental Method

The layout of the plumbing for the measurement of 1α,25(OH)$_2$-vitamin D$_3$ is shown in Figure 1, and the method described in reference [1] is modified using the following protocol:

1. 200 µL of serum are added to 20 µL of the internal standard solution containing 20 ng/mL of d6-1α,25(OH)$_2$-vitamin D$_3$. After vortex mixing, 400 µL of acetonitrile is added for the protein precipitation.
2. After further vortex mixing and centrifugation at 12,000 x g for 5 minutes, 500 µL of the supernatant are collected and put into an autosampler vial for a subsequent double injection comprising two 100 µL aliquots.

The two-dimensional liquid chromatographic (2D-LC) process consists of the following steps:

1. After the first injection of 100 µL, sample is delivered to the perfusion column (POROS™ R1/10, 4.6 x 50 mm – Life Technologies) by the "loading" pump, at a flow of 0.25 mL/min for 1 minute, with a solvent composition of 98% Eluent A (water) and 2% Eluent B (3:1 methanol-acetonitrile mixture).
2. After the second injection of 100 µL, the sample is cleaned by washing the perfusion column for 2 minutes at a flow of 1.5 mL/min, with a solvent composition of 98% Eluent A (water) and 2% Eluent B (3:1 methanol-acetonitrile mixture).
3. At two minutes, the eluent composition is changed to 70% Eluent B and a linear gradient initiated, increasing to 90% in 1 minute with a flow rate of 1.15 mL/min.
4. With activation of the switching valve at 3 minutes, the perfusion column is placed in-line with two serially-connected monolithic columns (Phenomenex Onyx monolithic C18, 3 x 100 mm) and flushed for 1.5 minutes by the "loading" pump at 250 µL/min, with 100% Eluent B. Downstream of the perfusion column and through a Lee mixer, an aqueous solution containing 0.5 mM lithium acetate is added to the eluent. This solution (Eluent A) is supplied by the second binary pump (the "separation" pump) at 250 µL/min, prior to entering the monolithic columns.
5. When the valve switches back to its original position, the perfusion column is cleaned through the "loading" pump with 100% Eluent B and is subsequently equilibrated for the next injection with a 2% Eluent B composition.

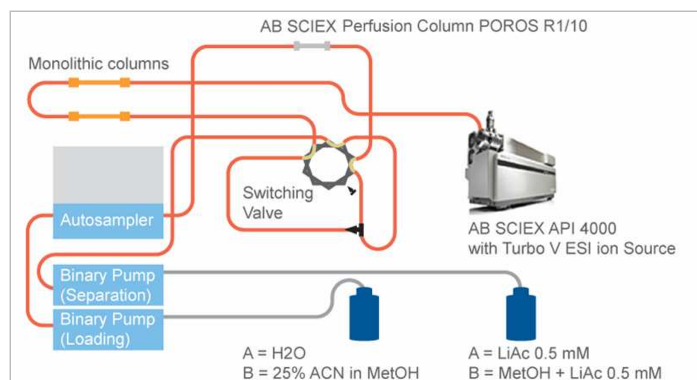

Figure 1. 2D-LC configuration for the measurement of 1α,25(OH)$_2$-vitamin D$_3$ on the AB SCIEX API 4000™ System.

6. With the same switch-back of the valve, the "separation" pump flow-rate is increased to 500 µL/min with a composition of 70% Eluent B (methanol containing 0.5 mM lithium acetate), with a linear gradient increasing to 100% Eluent B in 9 minutes.
7. The "separation" pump composition is maintained at 100% Eluent B until the next injection is initiated, at which time the composition reverts back to 100% Eluent A.

The API 4000™ tandem mass spectrometer is operated using the Turbo V™ source in Positive Ion Electrospray mode. Multiple Reaction Monitoring (MRM) of the transition m/z 423/369 for the 1α,25(OH)$_2$-vitamin D$_3$ and the transition m/z 429/393 for the internal standard d6-1α,25(OH)$_2$-vitamin D$_3$ was used throughout.

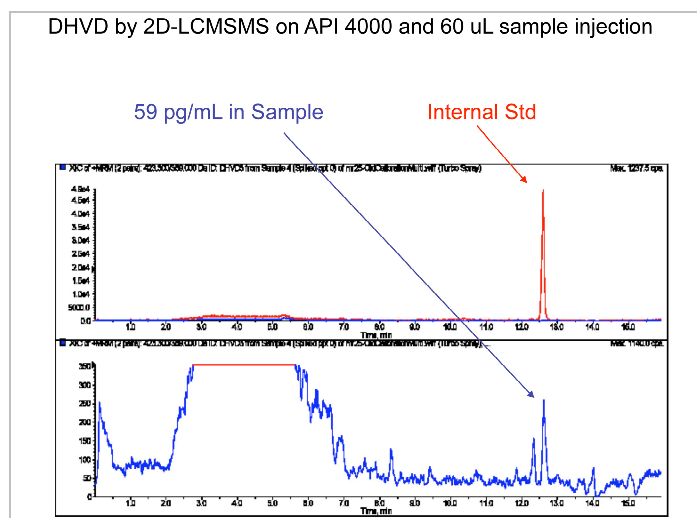

Figure 2. LC/MS/MS traces from the measurement of 1α,25(OH)$_2$-vitamin D$_3$ and its deuterated internal standard, obtained running a real-life sample containing 59 pg/mL on the AB SCIEX API 4000™ system

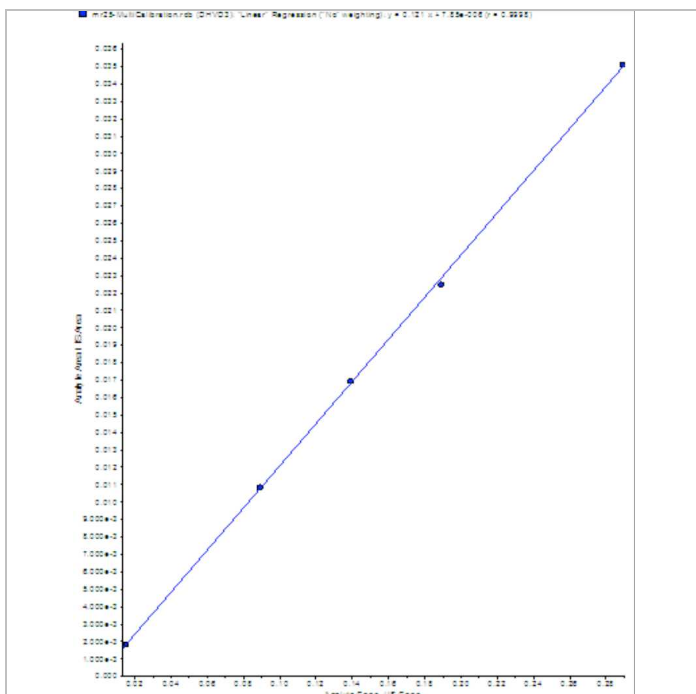

Figure 3. Linearity obtained in the lower region of the determined range: 0, 50, 100 and 200 pg/mL of 1α,25(OH)$_2$-vitamin D$_3$

Conclusions

In the original published paper[1], the analytical performance presented for the determination of 1α,25(OH)$_2$-vitamin D$_3$ was achieved using the AB SCIEX Triple Quad™ 5500 system. In this technical note we have achieved similar performance levels using an API 4000™ system by modifying both the 2D-LC chromatographic steps and increasing the injection volume to 66 µL of the original sample.

Further studies are planned to validate this method, however preliminary estimates indicate that a limit of quantification (LOQ) of 15 to 20 pg/mL is achievable.

References

[1]. B. Casetta, I. Jans, J. Billen, D. Vanderschueren, R. Bouillon, European Journal of Mass Spectrometry 16 (2009) 81

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