Vitamin D3 metabolites quantification; an example of where fast chromatography can lead to matrix effects.

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Today, mass spectrometry (MS) is commonly found in many analytical laboratories. Coupling MS to LC is regarded as an important achievement nowadays since it enables the analysis of non-volatile compounds which make up the vast majority of molecules. For most applications the quantification measurements made by MS are characterized by high sensitivity and high specificity. The latter feature enables the simplification of sample preparation before instrumental analysis.

LC-MS/MS (liquid chromatography-tandem mass spectrometry) is having a big impact in clinical laboratories where up to now most of the quantitative measurements are performed by immuno-assays (IAs). For some applications like steroid analysis, IAs have demonstrated a lack of specificity and therefore there is an advantage in switching to LC-MS/MS.

Measurement of Vitamin D

Amongst the large number of papers published by various authors on steroid analysis, we have recently presented a paper [1] dealing with the measurement of the 1,25 di-hydroxy-vitamin D3 ("DHVD3"), a secosteroid which presents several challenging features like a low propensity to be ionized. a very low concentration in plasma, and no straightforward and specific derivatization step for enhancing poor instrumental sensitivity.

By using a highly sensitive tandem mass spectrometer and a sophisticated two-dimension chromatography (2D-LC) we have been able to quantify such a challenging molecule at concentrations down to 15 pg/mL with an injection corresponding to 30 µL of the original plasma and with the sample pre-treatment limited to a protein precipitation step.

The method has been demonstrated to be robust and repeatable. Pivotal for success has been the LC strategy upstream of the tandem mass measurement. Due to very low concentration of the analyte itself, and the presence of a significant amount of matrix components which have similar structure and hydrophobicity, the injected sample is cleaned through a special perfusion column and an heart-cutting is performed for transferring the plug containing the analyte to a second column where the final chromatographic separation is accomplished.

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 trick for attaining adequate sensitivity has been to promote the Li-adduct formation of the analyte and adopt an MRM transition based on this adduct.

Whilst developing this strategy, the opportunity of including the quantification of the other Vitamin D3 metabolite, namely the 25, monohydroxy-vitamin D3 ("MHVD3") which is expected to be some 500 fold more concentrated, presented itself. Due to its higher concentration the task appears to be less challenging and indeed there are already several published protocols aiming at its quantification, one of the first is by S. Rainbow's group [2]. To enable simultaneous analysis with the DHVD3, it is necessary to address the higher relative hydrophobicity of the MHVD3 molecule. To this end, we have modified the heart-cutting parameters of the protocol published in reference [1] in order to trap it on the first-dimension and conveniently elute it through the second dimension as shown in the figure 1. Li-adduct formation has been exploited for both the analytes in addition to the extended sample clean-up as provided by the two-dimensional configuration.

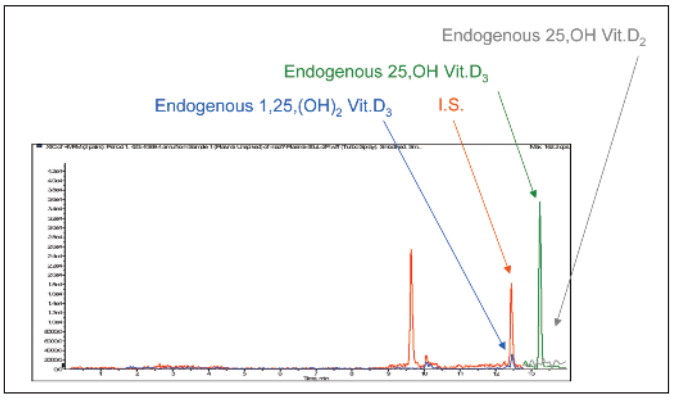


Figure 1. LC-MS/MS traces from the simultaneous measurement of 1,25 dihydroxy-vitamin D3, its deuterated internal standard, 25,monohydroxy-vitamin D3, 25,monohydroxy-vitamin D2 with a modified protocol based on reference (1). Due to the different concentrations, the traces related to the 1,25 dihydroxy-vitamin D3 and its deuterated internal standard have been magnified by a factor of x20.

Once this was achieved, a further opportunity presented itself namely the possibility of using the same configuration for quantifying solely the MHVD3 if requested, but reducing the analytical time. In fact it appeared by shortening the 2D-LC process and by keeping the Li-adduct formation strategy it should be possible to significantly reduce analytical run-time (target was 4 minutes).

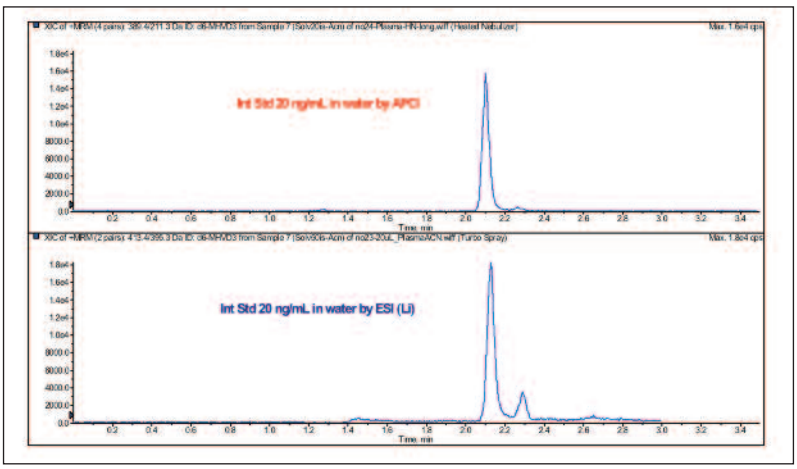


Figure 2. LC-MS/MS traces collected for the short-runtime method for 25,monohydroxy-vitamin D3. A methanol-water solution of d6-25,monohydroxy-vitamin D3 has been injected. The upper trace refers to the signal obtained by the APCI source and the lower one by the ESI source with the Li-adduct formation. By coincidence the signal intensities are very similar.

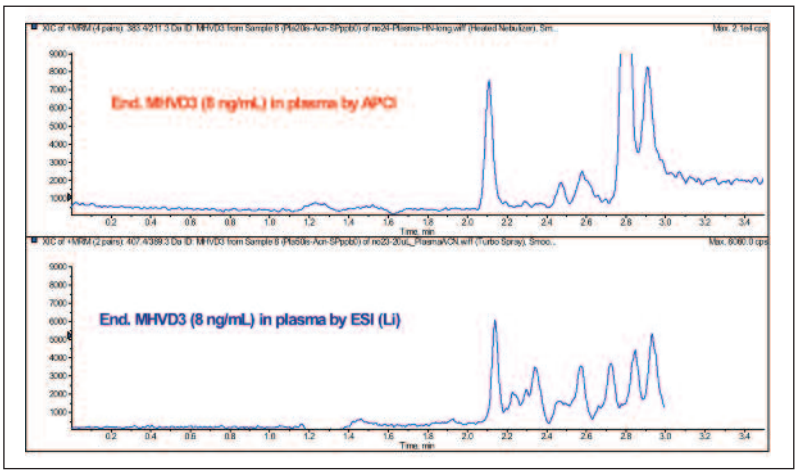


Figure 3. LC-MS/MS traces collected for the short-runtime method for 25,monohydroxy-vitamin D3 on a real-life sample. The upper trace refers to the signal obtained by the APCI source and the lower one by the ESI source with the Li-adduct formation. The APCI trace shows both a cleaner baseline and higher signal intensity.

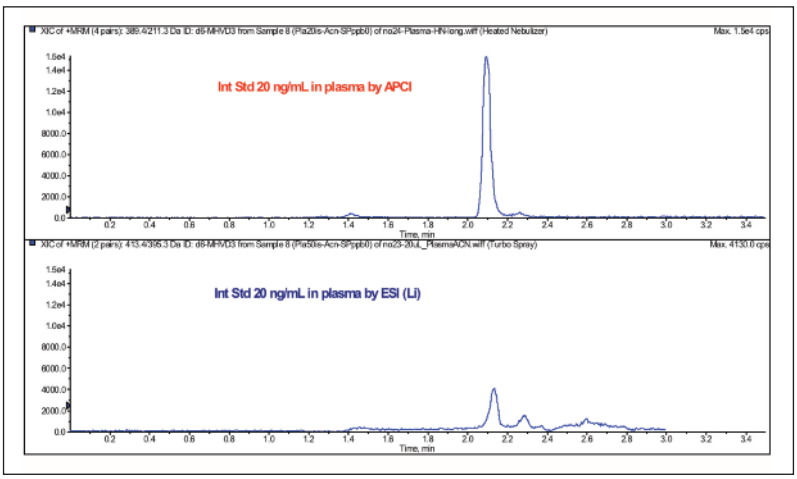


Figure 4. LC-MS/MS traces collected for the short-runtime method for 25,monohydroxy-vitamin D3 on a real-life sample by monitoring the deuterated internal standard (d6-25,monohydroxy-vitamin D3). The upper trace refers to the signal obtained by the APCI source and the lower one by the ESI source with the Li-adduct formation. Due to likely ion suppression effects (still under investigation), the ESI trace looks to be significantly affected.

However, we observed that by speeding up the chromatographic process (in both dimensions), the matrix components were no longer separated and lead to aberrant results for the MHVD3 measurements.

After extensive investigation, it appeared that Li-adduct formation using electrospray ionization, which enabled the sensitive measurement of the DHVD3, was more prone to matrix effects for MHVD3. The solution was to use Atmospheric Pressure Chemical Ionization (APCI) without any adduct formation, which would not be expected with this technique.

Using fast 2D-LC parameters, as described below, a comparison has been made between the ESI measurement through the Li-adduct formation ("Li-ESI") and the APCI measurement through the "classical" MRM transitions ([M-H2O+H+]+ > m/z 211.2 or m/z 229.2) either on the investigated analyte (MHVD3) and on its deuterated internal standard (d6-MHVD3).

Figure 2 shows when MHVD3 is injected from a pure methanol-water solution, similar sensitivity (expressed in signal intensity) is obtained for the Li-ESI and for the APCI.

However, when the MHVD3 is accompanied by the matrix, as shown in figure 3, the Li-ESI trace shows both a lower sensitivity, and a higher number of isobaric interferences shown by the presence of matrix components existing in proximity of the analyte retention time. This matrix effect is also responsible for another phenomenon: the deuterated internal standard intensity is significantly reduced with Li-ESI measurement (see Figure 4), much more than the analyte itself (compare Figure 3 & 4). This results in poor accuracy and precision when the quantification is performed with Li-ESI and use of a deuterated internal standard.

The above observations demonstrate the importance of separating the target analytes from the hidden matrix components and the effect that they can have on MS detection. For this application, the following strategy has been devised.

Experimental

For the simultaneous measurement of DHVD3 and MHVD3, the method of reference [1] is slightly modified and results in the following protocol:

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 D3 and 200 ng/mL of d6-25(OH)-Vitamin D3. After vortex-mixing, 400 µL of Acetonitrile is added for the protein precipitation.

After further vortex-mixing and centrifugation at 12,000 x g for 5 minutes, 500 µL of the supernatant is collected and put in an autosampler vial for a subsequent injection of a 90 µL-aliquot.

The two dimensional-liquid chromatographic (2D-LC) process is run through the following steps.

- Upon sample injection, analytes are trapped on a Perfusion column (POROS R1/10, 4.6 x 50 mm –Applied Biosystems-) with an aqueous solution containing 98 % water (Eluent A) and 2 % of a mixture Methanol-Acetonitrile (3:1, Eluent B) and delivered by one binary pump (the "LOADING" pump) at 1.5 mL/min for 2 minutes.
- At two minutes, the eluent composition is quickly changed to 70 % of Eluent B and a gradient initiated which moves up to 90 % in 1 minute .
- With activation of the switching valve at 3 minutes, the Perfusion column is connected to two serially connected Monolithic columns (Onyx Monolithic C18, 3 x 100 mm columns – Phenomenex) where the Perfusion column is flushed by the "LOADING"

pump at 250 µL/min at 90 % Eluent B for 3.5 minutes. Downstream of the Perfusion column and through a Lee mixer, 250 µL/min of an aqueous solution containing Lithium Acetate at 0.5 mM (Eluent A) supplied by the second binary pump (the "SEPARATION" pump) and before entering the Monolithic columns is added to the eluent.

- With the switching–back of the valve, Perfusion column is cleaned through the "LOADING" pump with 100 % of Eluent B and subsequently equilibrated for the next injection with the 2 %-Eluent B composition.
- With the same switching-back of the valve, "SEPARATION" pump flow-rate is increased to 500 µL/min with a composition of 70 % of Eluent B (Methanol containing 0.5 mM Lithium Acetate) with a gradient moving to 100 % Eluent B in 9 minutes is activated.
- "SEPARATION" pump composition is left at 100 % Eluent B until the next injection is activated where the composition is switched back to 100 % Eluent A.

The AB Sciex 5500 tandem mass spectrometer is run with the Electrospray probe on the Turbo-V source and in Multiple Reaction Monitoring (MRM) mode by exploiting the transition m/z 423/369 for the 1α,25(OH)2-Vitamin D3, the transition m/z 429/393 for the internal standard d6-1α,25(OH)2-Vitamin D3, the transition m/z 407/389 for the 25(OH)2-Vitamin D3 and the transition 413/395 for the internal standard d6-25(OH)-Vitamin D3.

For the fast measurement of the MHVD3 only, as shown on Figure 5 the AB Sciex 5500 built-in switching valve is plumbed with a Perfusion column POROS R1/20 2.0 x 30 mm (Applied Biosystems – Foster City CA, USA) for the on-line sample cleanup and a C18 column (Acclaim C18 120, 3 µm 3 x 33 mm -Dionex-) kept at 50°C for the second dimension chromatography. The devised strategy is as follows:

200 µL of serum are added to 20 µL of the internal standard solution containing 200 ng/mL of d6-25(OH)-Vitamin D3. After a vortex-mixing, 400 µL of Acetonitrile are added for the protein precipitation.

After a further vortex-mixing and centrifugation at 12,000 x g for 5 minutes, 500 µL of the supernatant are collected and put in an autosampler vial for a subsequent injection of a 20 µL-aliquot.

The two dimension-liquid chromatographic (2D-LC) protocol is as follows:

- Upon sample injection, the analytes are trapped on a Perfusion column (POROS R1/20, 2.0 x 30 mm –Applied Biosystems-) with an aqueous solution containing 10 % Acetonitrile and delivered by one pump (the "LOADING" pump) at 2.5 mL/min for 0.8 minute.
- With the activation of the valve at 0.8 minute, the Perfusion column is connected in backflush mode to a C18 column (Acclaim C18 120, 3 µm 3 x 33 mm -Dionex-) kept at 50°C and flushed by the second pump (the "SEPARATION" pump) at 500 µL/min with an initiatal composition of 90 % Eluent B (a mixture Methanol-Acetonitrile 3:1) and 10 % Eluent A (water). A mobile phase gradient is activated on switching. moving to

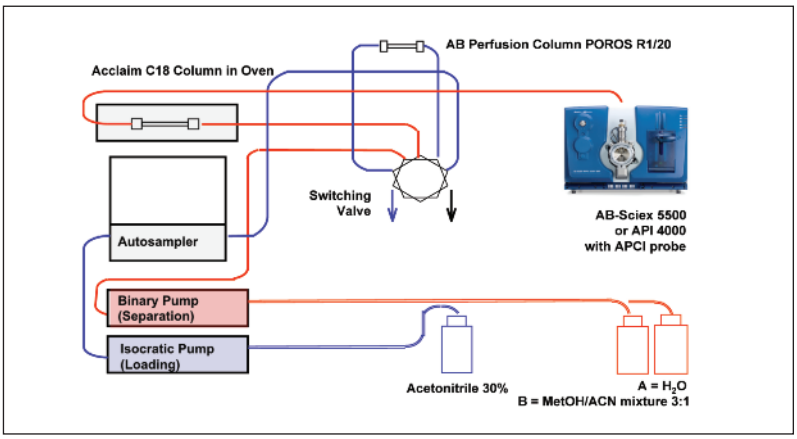


Figure 5. Hardware configuration for the high-throughput quantification of 25,monohydroxy-vitamin D3.

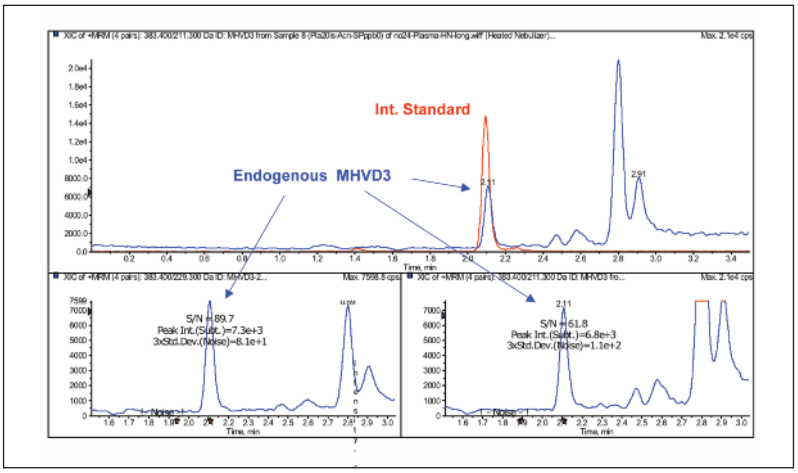


Figure 6. LC-MS/MS traces for the deuterated internal standard (red trace) and the endogenous 25,monohydroxy-vitamin D3 (blue trace) measured at 8 ng/mL.
The lower panels show the S/N ratio performances for the two transitions explored (m/z 383/229 and m/z 383/211).

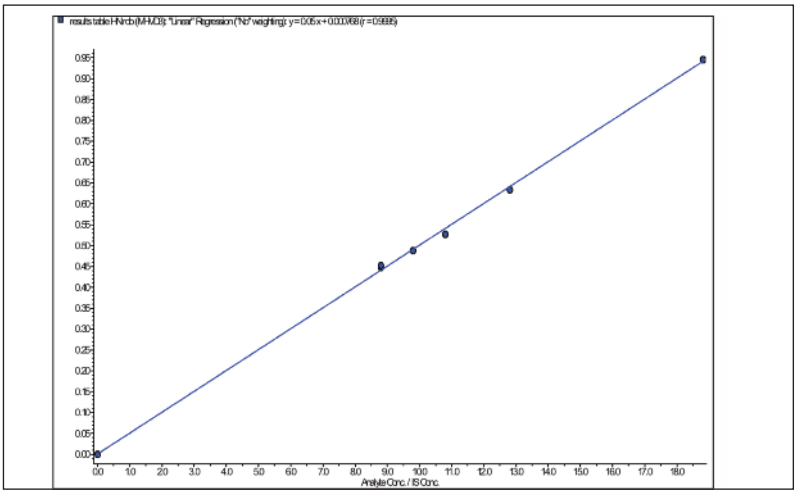


Figure 7. Calibration curve obtained in the lower region by running some in-house prepared calibrators at concentrations of 8.8, 9.8, 10.8, 12.8 and 18.8 ng/mL by using the method devised for the fast quantification of 25,monohydroxy-vitamin D3.

100 % Eluent B in 1 minute, then leaving the composition of 100 % Eluent B until the end of the running (3.5 minutes).

- With the switching–back of the valve at 2.8 minutes, Perfusion column is equilibrated with the aqueous solution containing 10 % Acetonitrile.

The tandem mass spectrometer (either an AB Sciex 5500 or a AB Sciex API 4000) is run with the APCI probe on the Turbo-V source and in Multiple Reaction Monitoring

(MRM) mode by using the transitions m/z 383/211 (quantifier) and m/z 383/229 (qualifier) for the 25(OH)2-Vitamin D3 and the transition m/z 389/211 for the internal standard d6-25(OH)-Vitamin D3.

Figure 6 shows a representative ion chromatogram for a sample containing 8 ng/mL, on the API 4000. The same figure shows as the S/N ratios for both the monitored transitions (m/z 383/211 and m/z 383/229) are very similar.

This method is going to be formally validated, however, preliminary estimates show that the within-day precision for replicate samples to be around 1 % CV and the achievable limit of quantification (LOQ) is expected to be lower than 0.5 ng/mL: Figure 7 shows the linearity obtained in the lower region by running some in-house prepared calibrators at concentrations below 20 ng/mL.

Conclusion

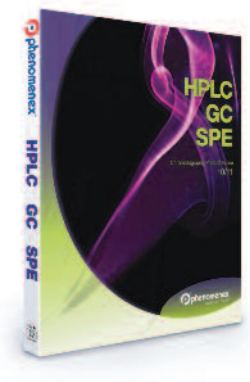
This method demonstrates that for some specific and quite challenging analytes, changes in the chromatography, e.g. shortening the running time, can result in a radical change in the mass spectrometer response due to matrix interference. For this application, when MHVD3 is required alone, speeding up the chromatorgraphy also requires the use of different ion source parameters to ensure accuracy, precision and sensitivity of detection are optimised. However, the effort and ease of swapping from one hardware configuration to the other is compensated for by the improved quality of results and increased speed of analysis.

References

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

[2] Z. Maunsell, D.J. Wright, S.J. Rainbow, Clinical Chemistry 51 (2005) 1683.

New Chromatography product guide launched



Phenomenex announces the release of its updated 2010/2011 product guide. The new guide is available in print as well as in interactive digital format. Two product sections have been added for this edition: Chiral LC and Supercritical Fluid Chromatography (SFC).

The 2010/2011 guide, with more than 400 pages of Phenomenex chromatography products, accessories and equipment, is organized with industry-specific icons that guide customers in choosing the most suitable solutions for their applications. The catalogue is available, in print or digital versions, by request at www.phenomenex.com/iselect.

The digital version of the product guide allows users to filter products by industry, search key words and bookmark frequently referenced pages. Interactive tools help users select the right product according to the samples they are analyzing.

"In response to evolving analytical trends, Phenomenex has expanded its product offerings to serve customers who are adopting new techniques including chiral and SFC," said Karen Brauneck, Corporate Communications Manager. Email international@phenomenex.com.