The Technical and Clinical Benefits from Measuring 25 OH Vitamin D by LC- MS/MS

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A dramatic explosion of interest in the clinical effects of Vitamin D and its metabolites in the past 5-10 years has stimulated a massive increase in requests for the measurement of 25 Hydroxy Vitamin D (25 OH D) in Clinical Chemistry laboratories throughout the world. Vitamin D can play an important role not only in the classical and well described area of bone health, where the re-emergence of rickets in population subgroups within the UK is a worrying problem and the role of 25 OH D in prevention of osteoporotic fractures is a major issue, but also in other disease areas where epidemiological and some outcome data suggests a strong association of low vitamin D status and disease such as diabetes, cancer, multiple sclerosis, infection (immune status), allergy, asthma and cardiac function [1]. Supplementation studies have resulted in variable outcomes with the suggestion that the dose of vitamin D, change and final concentration of 25 OH D may be insufficient to result in the required beneficial effects. The ability to measure 25 OH D with precision and accuracy is therefore of paramount importance, to ensure that the clinical recommendations that are made regarding vitamin D measurement and subsequent supplementation are both correct and achievable.

Measurement of 25 OH D: The Problem

25 OH D has a half life in the circulation of around three weeks, which is one of the reasons it is the best measurement to reflect the Vitamin D status of an individual. The majority of circulating 25 OH D is derived from the effects of sunlight on the skin resulting in the generation of 25 OH D_3 (Cholecalciferol). Dietary sources of vitamin D are mainly from fish and dairy products supplying vitamin D_3 but also include plants and fungi which contain vitamin D_2 and so generate 25 OH D_2 (ergocalciferol).

Many supplements contain 25 OH D_2 and so it is important to be able to measure both 25 OH D_2 and 25 OH D_3 to correctly assess an individual's vitamin D status. Immunoassays have variable cross-reactivity with 25 OH D_2 [2] and also vary in their ability to detect ingested 25OH D_2 [3] with the result that some methods have significantly underestimated the true vitamin D status of subject when they are receiving or are inadvertently taking 25 OH D_2.

Standardisation of 25 OH D assays has been a problem that has been highlighted recently and attempts have been made to improve the performance of participants in the vitamin D External Quality Assurance Scheme (DEQAS), with some success(4) but problems with the approaches adopted still exist [5]. A new international standard produced by the National Institute of Standards and Technology (NIST) may help overcome some of these issues.

The dramatic increase in demand also means that any technology adopted to generate clinical results must not only meet the quality standards that are required for service provision, but also that the turnaround time for each measurement is relatively short (within a working week) and that instrument downtime is minimal.

It is within this background that Tandem MS/MS measurement of 25 OH D has been developed to meet the exacting demands of the clinical service and the research needs of clinical investigator.

Measurement of 25 OH D by Tandem MS/MS

The introduction of LC-MS/MS into routine clinical laboratories coupled with the availability of a deuterated stable isotope (d6 25 Hydroxy vitamin D_3) internal standard, resulted in a significant increase in the numbers of investigators wanting to employ this 'new' technology.

As previously stated, the early performance of this technique in the international proficiency testing scheme was disappointing to say the least. There are however, a significant number of variables that could account for this. They include sample pre treatment, calibration, mode of chromatographic separation, potential ion suppression, mode of ionisation and subsequent transition selection.

Leaving the issue of calibration to one side, the two most fundamental choices that need to be considered are the mode of ionisation and the pre analytical treatment of the plasma sample. In routine clinical laboratories, the issue of ionisation mode is really a straight forward choice between electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI).

Sample pre treatment is a choice between protein precipitation (Ppt), liquid/liquid extraction (LLE) or solid phase extraction (SPE). Whilst it is quick, simple and cheap, a 'protein crash' can have the fundamental drawbacks of sample dilution and potential ion suppression of a poorly ionisable analyte present in low nanomolar concentrations.

LLE and SPE, whilst more labour intensive and costly, do offer the opportunity of a degree of selectivity and the potential for pre analytical concentration, whilst removing the chromatographically harmful proteins and lipids. This has lead to a number of manual, semi automated and fully automated SPE procedures.

Variations of semi automated off and on-line methods, utilising robotic sample processors, such as those available from TECAN or Gilson have been used extensively. More recently however, with the continued growth in requesting Vitamin D measurements, we are seeing a growing interest in fully automated on-line processing of tens of thousands of samples annually.

These technologies can now offer a range of automation that includes everything from primary sample tube recognition, addition of precipitation reagents and internal standards, mixing, centrifugation, solid phase extraction and trace enrichment. Single use cartridges, as found in the Spark Holland Symbiosis or the multiple use TurboFlow Technology from ThermoFisher Scientific and Chromsystems' 'trap column' kit approach, are attracting a great degree of interest. This is despite the additional financial outlay required for the additional hardware. This can be off set, over a number of years, by significant savings in operator time.

We have developed an on-line SPE trace enrichment method, using a Waters 2777 auto sampler working in conjunction with a Knauer Smartline 1000 quaternary pump and 6300 Sample Preparation Unit, fitted with a Knauer 10µm C18 guard column. One part of serum is mixed with 2 parts of acetonitrile and centrifuged. This allows 100µl of precipitate to be trace enriched prior to chromatographic separation on a Waters Sunfire 3.5µm 2.1mm x 50mm C18 column.

Detection of 25OH vitamin D_2 and D_3 is achieved using a Micromass Quatro Ultima Pt fitted with a Z Spray ESI inlet. Utilisation of the on line SPE and excellent chromatographic resolution of the peaks of interest have enabled us to use the primary daughter ions from the water loss transitions, seen in *Figure 1*.

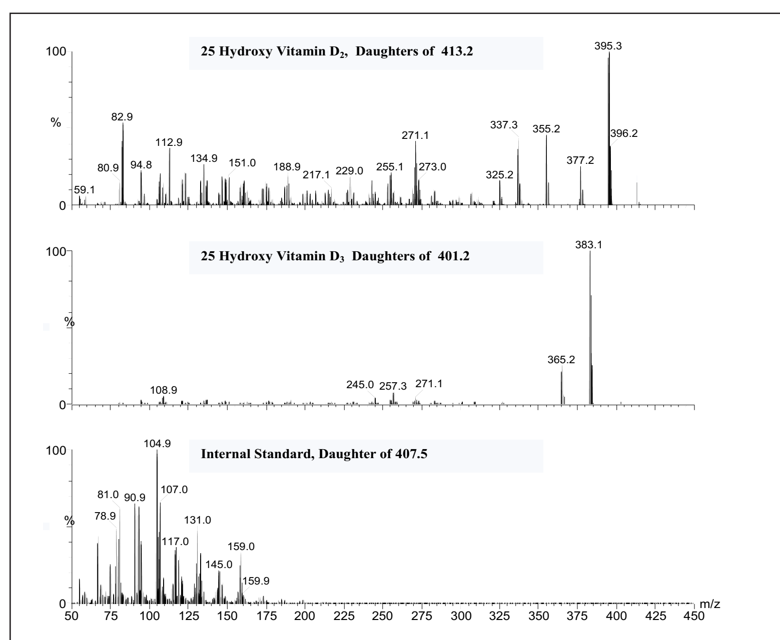
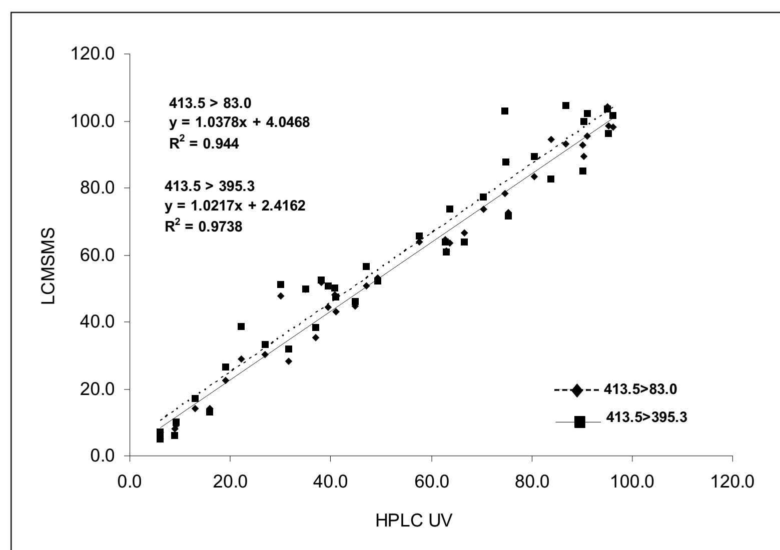


Figure 1. Daughter Scans

Conclusion

We have developed a robust, cost effective automated on line sample preparation method for the sensitive simultaneous measurement of 25 Hydroxy vitamin D_2 and D_3, on 50 µl of serum. The required limit of detection of 1ng/mL is easily achieved. It has the potential to analyse 300 samples in a twenty four hour period. Sample preparation time takes approximately 60 mins and post analytical processing can be completed in 30 mins. Analysis using both a primary quantifier ion and a secondary qualifier ion *(Figures 2 and 3)*, when compared to an established HPLC method showed excellent agreement.



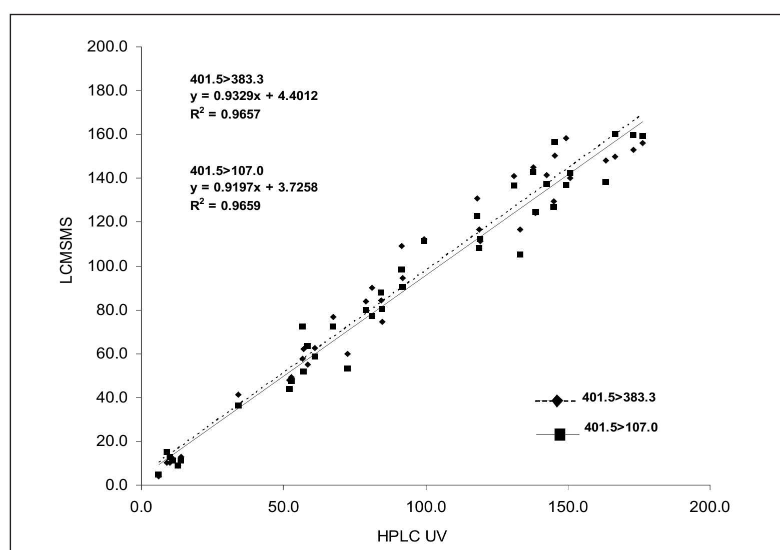


Figure 2. (top) and 3 (bottom): Ion ratios of primary and secondary transitions indicated excellent selectivity for both 25(OH)D_2 and 25(OH)D_3, as indicated in Figures 2 and 3.

The accuracy of the assay was assessed using sixteen DEQAS samples and comparing them to the consensus method means *Figure 4*. Two hundred patient samples were also compared to an established HPLC method with UV detection *Figure 5*.

Instrument imprecision, determined by repetitive injection of the same sample (n=8) showed a CV of 5.1% for 25(OH)D_2 at a concentration of 2.3ng/mL and a CV of 2.5% for 25(OH)D_3 at a concentration of 4.7ng/mL.

Mean recovery of the sample extraction at 5ng/mL and 50 ng/mL of 25(OH)D_2 was 91.2% and that of 25(OH)D_3 was 90.4% when compared to equivalent methanolic non-extracted standards at equivalent concentrations. Between batch (n=12) CVs for 25(OH)D_2 were 5.5% and 4.2% at concentrations of 1.4 and 13.5 ng/mL respectively. Between batch (n=12) CVs for 25(OH)D_3 were 4.5% and 5.7% at concentrations of 4.9 and 19.7ng/mL respectively.

This technology and application moves the measurement of 25 OH D forward in the clinical arena to meet the stringent demands of both physicians investigating and treating patients and researchers requiring consistent measurement of large numbers of samples.

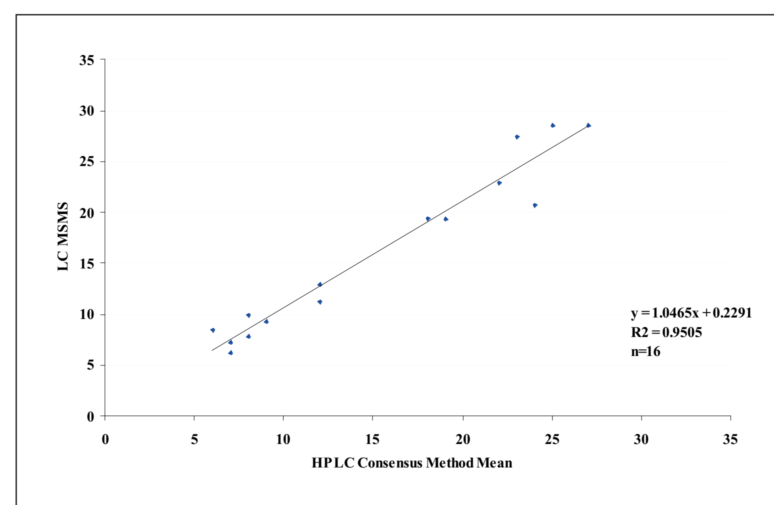


Figure 4. Comparison with HPLC DEQAS Consensus Method Mean

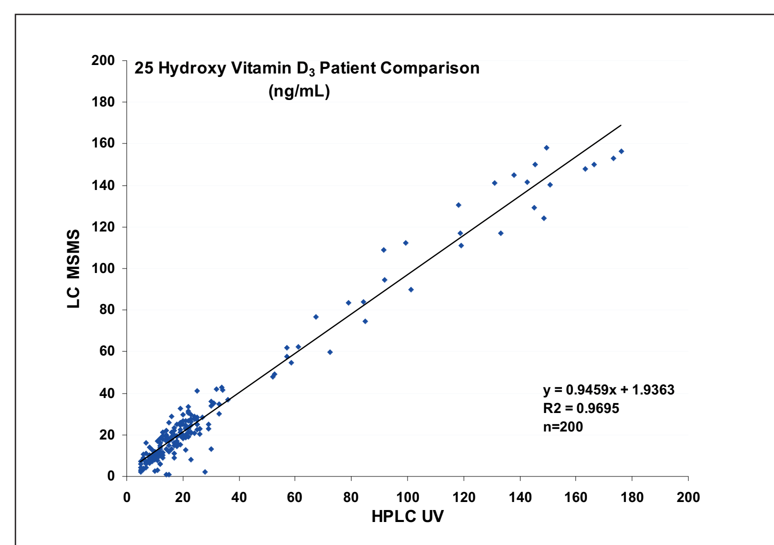


Figure 5. Comparison of LCMSMS method Vs established HPLC UV method

References

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