Optimising the Derivatisation of Vitamin D Metabolites Within Clinical Research Using Amplifex™ Diene Reagent

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An increased understanding of the importance of vitamin D has led to a growing interest in the analytical methods used in research for the detection of vitamin D and its metabolites. Here a new derivatisation process using Amplifex™ diene reagent is described, which allows the challenges associated with the measurement of vitamin D and analogues to be overcome.

Introduction

In recent years more researchers have been investigating the role of vitamin D and its metabolites with a view to characterise its metabolic pathways, and to identify and measure its biologically significant metabolites.

The blood levels of 25-hydroxyvitamin D (25-OH-D) are the most appropriate indicator of vitamin D status. The biologically active form of vitamin D, DHVD, is produced when 25-OH-D is hydroxylated and is usually present in the plasma in low pg/ml ranges. Two major forms of vitamin D can be found in the body, these include vitamin D_3, which is endogenously produced, and vitamin D_2, which is only present when it has been given in food or as a supplement [1].

There are several challenges that can make accurate measurement of 25-OH vitamin D difficult. Immunoassays are commonly used in clinical research laboratories for the analysis of 25-OH vitamin D. This is because they are relatively simple to perform and allow a high throughput of samples, however, some immunoassays cannot accurately detect 25-OH vitamin D_2 [2]. Mass spectrometry on the other hand can be used to accurately measure 25-OH vitamin D_2 and 25-OH vitamin D_3, giving separate values for both forms of vitamin D and their metabolites.

Despite the greater sensitivity of mass spectrometry for the detection of 25-OH vitamin D_2 and 25-OH vitamin D_3, there are factors that make accurate measurement of vitamin D difficult. For example, vitamin D in its active form ($DHVD_3$) is low in abundance (<100 pg/ml) and it has a neutral charge making it difficult to ionise for mass spectrometry based methods. $DHVD_3$ is also dehydration prone, and thermally and photochemically labile. The different metabolites of Vitamin D can be similar in structure and molecular weight to $DHVD_3$ causing interference in the accurate identification and quantification of metabolites by mass spectrometry (or immunoassay). The different isobaric metabolites have to be separated for accurate measurement of $DHVD_3$ levels. It is also essential to improve the ionisation efficiency and stability of these compounds via derivatisation prior to the mass spectrometry analysis for a highly selective and reliable detection.

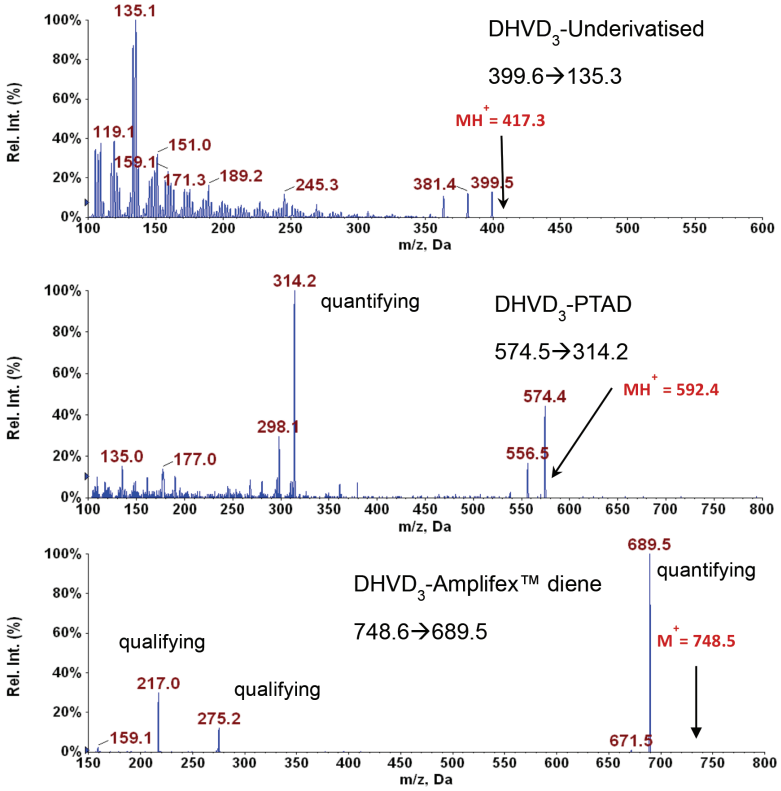


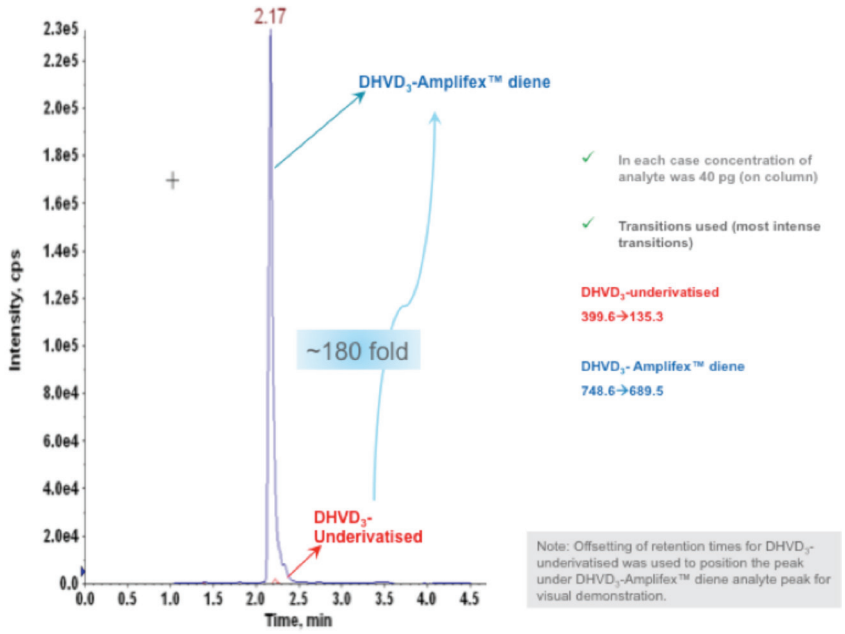
Figure 1. Fragmentation patterns observed for PTAD or Amplifex™ diene derivatised 1α, 25-di-OH-vitamin-D $(DHVD)_3$. Amplifex™ diene improved the fragmentation pattern of the analyte.

Experimental Methods

The sample preparation, derivatisation and analysis of $DHVD_3$ can be made simpler, faster and more affordable by using one-step SPE with two inexpensive cartridge and solvents, followed by a one-step derivatisation process.

Traditionally, Cookson-type reagents (PTAD, MBOTAD, DMEQTAD and similar molecules)

2(A)



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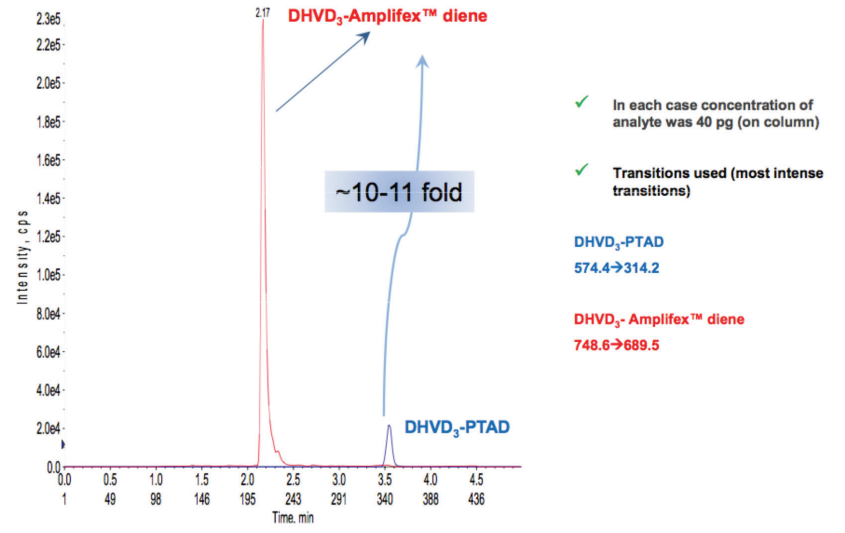

Figure 2. Signal intensity of 1α, 25-di-OH-vitamin-D ($DHVD_3$) produced following derivatisation with Amplifex™ diene or PTAD using the following high flow conditions: 2.1 x 100mm, C18, 1.7 µm, low particle size column e.g. Phenomenex Kinetex or similar, flow rate 0.7 ml/min, column temp 40°C, injection volume 20µl, mobile phase A: 0.1 (v/v) formic acid in HPLC water, mçobile phase B: 0.1 (v/v) formic acid in acetonitrile. A) $DHVD_3$ derivatised with Amplifex™ diene compared to underivatised analyte. B) $DHVD_3$ derivatised with PTAD compared to Amplifex™ diene derivatised analyte.

have been used for the derivatisation of $DHVD_3$ [3]. These reagents improve the stability and ionisation efficiency of the analyte compared with underivatised samples, however, the fragmentation pattern could be made cleaner and ionisation efficiency improved. A cleaner fragmentation pattern and better ionisation efficiency would increase the accuracy of separation of the different vitamin D analogues, and allow them to be reliably identified and quantified [4].

The new Amplifex™ diene reagent is based on established 1,2,4-Triazoline-based Cookson-type reagents, but the inclusion of a quaternary ammonium group and other modifications gives an improved mass spectrometry response and more controlled multiple reaction monitoring (MRM) fragmentation, resulting in enhanced analytical detection limits. The Amplifex™ diene reagent can be synthesised in high yields with an ionisation enhancing group, the reagent is also very hydrophilic (which is beneficial for liquid chromatography (LC) methods) but soluble in organic solvent. Amplifex™ diene reacts with vitamin Ds (including vitamin D_3, vitamin D_2 and their respective metabolites) within minutes and unreacted reagents elute very early in reversed phase LC.

A major advantage of the one step derivatisation process using Amplifex™ diene is that it takes 5 min to perform instead of 30 min, and can be automated and carried out using a 96-well plate. The Amplifex™ diene solution is prepared from dried material, added to the prepared (dried) serum samples (50 µl per sample) and incubated. After a short incubation the reaction is quenched by the addition of water.

Results

Here we show that the LC-MRM analysis can be performed using a 20 µl injection, a standard water/acetonitrile (0.1% formic acid) gradient and a 0.7 ml/min flow rate. The QTRAP® 5500 LC/MS/MS system with electrospray ion source in MRM mode was used to quantify vitamin D levels following derivatisation.

Figure 1 displays the fragmentation patterns (measured using the QTRAP 5500 system) of underivatised $DHVD_3$ compared with PTAD derivatised or Amplifex™ diene derivatised $DHVD_3$.

A cleaner and more defined fragmentation pattern was observed for $DHVD_3$ derivatised with the Amplifex™ diene reagent compared with the PTAD reagent, with most of the ion current funnelled to the quantifying transition. The whole analyte was included (structure specific) and was practically free of dehydration. In contrast, $DHVD_3$-PTAD and its fragment ion underwent dehydration and the quantifying ion carried only a small portion of the analyte. Derivatisation using PTAD would therefore make it difficult to separate isomers that have the Q3 ion in common (for example, $DHVD_2$). The qualifier ion for PTAD derivatised sample was 298.1 or the water loss at 556.5, showing poor specificity using the PTAD reagent.

Figure 2 (A) demonstrates that the Amplifex™ diene reagent increased the signal intensity for $DHVD_3$ approximately 180-fold compared with the underivatised analyte. The same concentration of analyte (40 pg) was used for derivatised and underivatised preparations. The transitions for underivatised $DHVD_3$ were 399.6 – 135.3 compared with transitions of 748.6 – 589.5 after derivatisation with Amplifex™ diene. Figure 2 (B) shows the transitions for $DHVD_3$-PTAD were closer in range to the underivatised analyte i.e. 574.4 – 314.2 than with Amplifex™ diene. The limits of detection of $DHVD_3$ and $DHVD_2$ in stripped serum after derivatisation with Amplifex™ diene have also been determined using the QTRAP 5500 system. The assay requires 200 µl

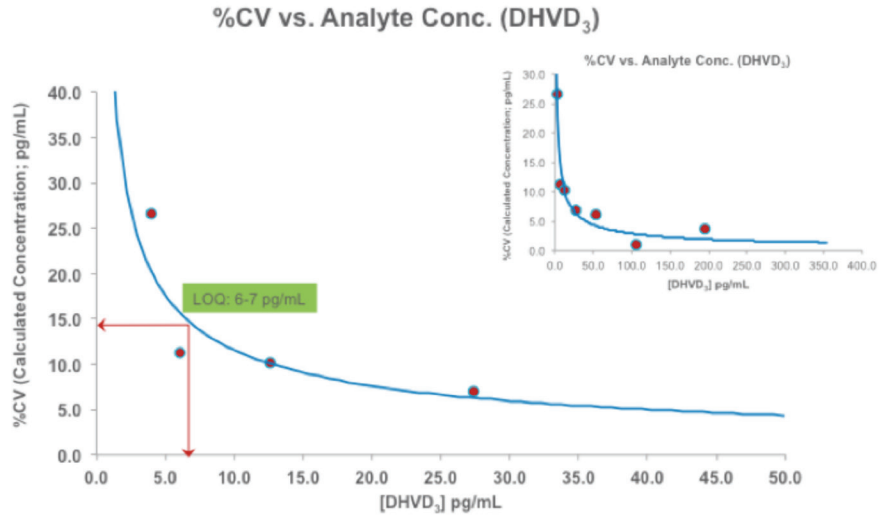

Figure 3. Limit of quantification for 1α, 25-di-OH-vitamin-D ($DHVD_3$) measured using the QTRAP 5500 following derivatisation with Amplifex™ diene.

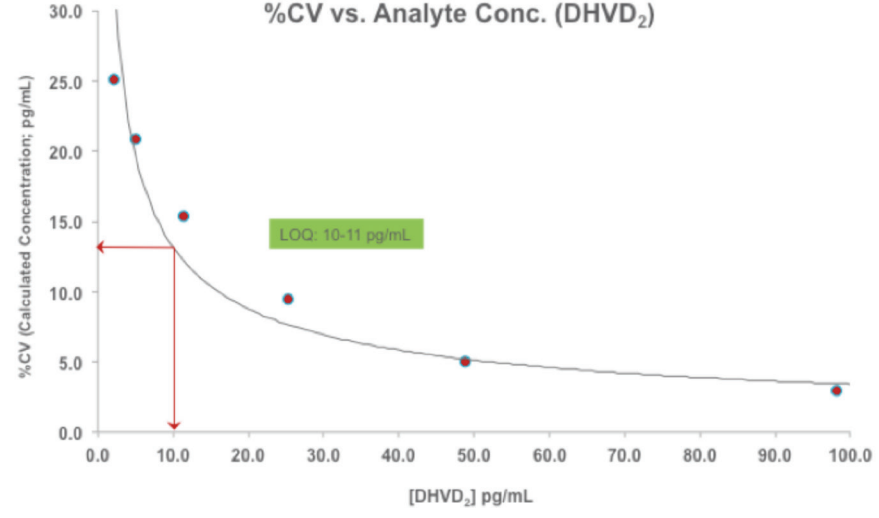

Figure 4. Limit of quantification for 1α, 25-di-OH-vitamin-D ($DHVD_2$) measured using the QTRAP 5500 following derivatisation with Amplifex™ diene.

of sample, which is quite standard for a clinical laboratory. The limit of quantification for detection of $DHVD_3$ was approximately 6 pg/ml (Figure 3) whereas the limit of quantification for $DHVD_2$ was slightly higher at approximately 10 pg/ml (Figure 4). These detection limits for active vitamin D (DHVD) relate back to the absolute concentration of the sample and are lower than for more traditional methods using Cookson-type reagents.

Conclusions

The Amplifex™ diene process overcomes many of the difficulties associated with accurate measurement of 1α, 25-di-OH-vitamin-D_3 such as dehydration, low ionisation efficiency, low structural stability and interference from isobaric isomers. This novel derivatisation reagent can be used to simplify and speed up the chromatography of 1α, 25-di-OH-vitamin-D_3. Analytes derivatised using Amplifex™ diene have an improved fragmentation pattern, which leads to enhanced sensitivity and lower detection limits compared to more traditional methods. The advantages of using Amplifex™ diene over more traditional derivatisation methods or immunoassays for the detection of 1α, 25-di-OH-vitamin-D_3 may help to encourage an increase in the use of mass spectrometry methods for this clinical research application.

References

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