Measurement of 25-hydroxyvitamin D3 and C3-epi-25-hydroxyvitamin D3 using UPLC/MS/MS in the adult population.
In recent years the demand for serum 25-hydroxyvitamin D (25OHD) analysis has increased considerably. In addition to the role vitamin D plays in bone metabolism, several clinical studies have shown that vitamin D deficiency is associated with increased risk for certain cancers, multiple sclerosis and heart disease.

The C3-epimer of 25OHD has been identified as a potential interference in the assessment of vitamin D sufficiency, although the clinical significance remains unclear. The C3-epimer of 25-hydroxyvitamin D (25OHD) offers only in the assay for quantification of 25-hydroxyvitamin D. The study concluded that the C3-epimer was primarily detected in infants and not adults. More recently, NST described a candidate reference procedure for the measurement of 25OHD in serum using extended reverse-phase HPLC tandem mass spectrometry that demonstrated baseline resolution of the C3-epimer from 25OHD. This report also described the detection of C3-epimer in some adult serum samples, however, concentrations were not given.

In this initial study we have developed a UPLC reverse-phase chromatographic separation of 25OHD2, 25OHD3 and its C3-epimer using the Waters® ACQUITY™ UPLC system and identified by bar code to be tracked throughout the extraction procedure.

The internal standards and precipitation reagents were added to the deproteinized serum. A liquidliquid extraction was followed by centrifugation (off-line), the supernatant was transferred to a UPLC plate and washed. The retained analytes were eluted by the liquid-handling system and monitored by using the Waters ACQUITY™ UPLC system with a Zorbax SB-chrom column (2.1x50mm, 1.8µm) employing a water/methanol/ammonium acetate gradient over 12.5min.

A Waters TQ mass spectrometer was used to quantify 25OHD2 and 25OHD3, monitoring a [M-H]- transition as [25OHD]+. The C3-epimer was detected in 90% of the adult population tested. For this cohort of samples the mean Total 25OHD concentration for each sample was determined by extracting and quantifying five concentrations were 11.9ng/mL and 0.63ng/mL respectively. On average the C3-epimer contributed to 4.34% of the Total 25OHD concentration. At the 95% interval the lower and upper limits were 0.073ng/mL and 2.85ng/mL respectively.

The relationship of C3-epi-25OHD to 25OHD3 is shown in Figure 6. The concentration of C3-epimer generally increases with 25OHD3 levels.

**METHODS**

**Standards, Samples and Calibrators**

156 anonymized adult serum samples from the North West of England (53° North) were analysed to determine 25OHD2, 25OHD3 and its C3-epimer using the method outlined below. Samples were anonymized following advice from the UK National Research Ethics Service.

**Semi-Automated Sample Preparation and UPLC/MS/MS Conditions**

Primary serum samples and calibrators were placed on a Skymed Freedom EVO 100 liquid-handling system and identified by bar code to be tracked throughout the extraction procedure.

The concentration of C3-epimer for this population ranged from 0 (undetectable) to 3.48ng/mL. On average the C3-epimer contributed to 4.34% of the Total 25OHD concentration.

**RESULTS**

**Linearity**

The assay was linear over the range 0.76-77.0ng/mL for C3-epi-25OHD3 and over the range 4-100ng/mL for 25OHD3 and 25OHD2 with all coefficient of determinations (R²) > 0.996.

**Precision**

The inter-assay precision for 25OHD2 and 25OHD3 was determined by extracting and quantifying five replicates of the same serum on 3 different days and over five consecutive days. The coefficients of variation were 8.6% and 10.4% for 25OHD2 and 25OHD3 respectively. The inter-assay precision of C3-epi-25OHD3 at 37.7 and 1.89ng/mL was 8.95% and 9.15% respectively.

**Sensitivity and Specificity**

Figure 3 shows the chromatographic separation of 25OHD3 from its C3-epimer in the NIST SRM972. The chromatogram for serum sample 152 represents serum levels of 25OHD3 at 18ng/mL and relatively high C3-epimer levels for this population. The LOD (S:N 3:1) was determined using six individual serum samples to be 0.49ng/mL.

**Sample Analysis**

The calculated mean Total 25OHD concentration for the 156 serum samples was 14.7ng/mL (range 0.37-74.7ng/mL). 78.2% of the population were insufficient (Total 25OHD <12ng/mL).

Figure 4 shows the calculated C3-epi-25OHD levels ranged from 0.4ng/mL (mean 0.66ng/mL) with the relative amount of C3-epimer to 25OHD3 ranging from 0-16.24% (mean 4.8%), as shown in Figure 5.

**DISCUSSION**

For this cohort of samples the mean Total 25OHD concentration was 14.7ng/mL representing a deficient population.

The concentration of C3-epimer for this population ranged from 0 (undetectable) to 3.48ng/mL. On average the C3-epimer contributed to 4.34% of the Total 25OHD concentration.

Further work includes improvement in the LLOQ and LOD to enable precise measurement at the low levels of C3-epi-25OHD present.

**REFERENCES**