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Measurement of 25-hydroxyvitamin D3 and C3-epi-25-hydroxyvitamin D3 using UPLC/MS/MS in the adult population

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INTRODUCTION

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 now link vitamin D deficiency with increased risk for certain cancers, multiple sclerosis and heart disease.[1,2]

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 D3 (25OHD3) differs only in the asymmetrical arrangement of a hydroxyl group in position C3, as shown in Figure 1, making chromatographic separation difficult. In 2006, Singh *et al* described a chiral chromatography method to partially separate these compounds.[3] The study concluded that the C3-epimer was primarily detected in infants and not adults. More recently, NIST 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 25OHD3.[4] This report also described the detection of C3-epimer in some adult serum samples, however, concentrations were not given.

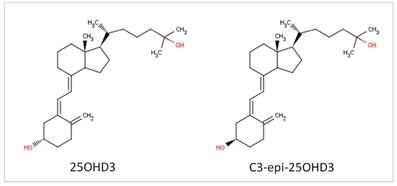

Figure 1. Structures of 25OHD3 and the optical isomer C3-epi-25OHD3.

In this initial study we have developed a UPLC reverse-phase chromatographic separation of 25OHD3 from C3-epi-25OHD3 to determine the C3-epi-25OHD3 concentrations in an adult population. The aim of this study was to develop a shorter analysis time for the chromatographic separation. This was achieved by using the Waters UPLC chromatography system enabling the use of 1.8µm particle size column packing material. The method describes a semi-automated sample pre-treatment protocol, with sample tracking from the primary tube to processed results, using the ACQUITY UPLC TQ detector system (Figure 2).

Figure 2. System configuration of Waters® ACQUITY UPLC® TQ Detector.

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.
- C3-epi-25-hydroxyvitamin D3 (IsoSciences) and NIST SRM 972 were used to confirm the retention time and concentrations of the three analytes.
- Tri-deuterated 25OHD2 and tri-deuterated 25OHD3 purchased from IsoSciences were used as the internal standards.

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

- Primary serum samples and calibrators were placed on a Tecan Freedom EVO 100 liquid-handling system and identified by bar code to be tracked throughout the extraction procedure.
- Tri-deuterated 25OHD2 was used as the internal standard for C3-epi-25OHD3, as it eluted closer to the retention time compared with the 25OHD3 internal standard, to compensate for any matrix effects upon the ionisation of the molecule.
- The sample preparation uses only 150µL of serum and the time to prepare 96 samples is approximately two hours involving minimal manual intervention.
- The internal standards and precipitation reagents were added to the dispensed samples. Following centrifugation (off-line), the supernatant was transferred to a conditioned Oasis® µElution SPE plate and washed. The retained analytes were eluted by the liquid-handling system.
- The eluant was chromatographed using a Waters ACQUITY UPLC™ with a Zorbax SB-CN column (2.1x50mm, 1.8µm) employing a water/methanol/ammonium acetate gradient over 12.5mins.
- A Waters TQD mass spectrometer was used to quantify 25OHD3 and C3-epi-25OHD3, monitoring two transitions for each analyte. Levels of 25OHD2 were quantified separately to provide a Total 25OHD concentration for each sample.

Compound	MRM Transition (m/z)	Cone (V)	Collision (eV)
25OHD3	401.35 > 159.1	24	28
25OHD3*	401.35 > 383.3	24	10
d_3-25OHD3	404.35 > 162.1	24	28
25OHD2	413.35 > 355.3	26	10
25OHD2*	413.35 > 83.1	26	22
d_3-25OHD2	416.35 > 358.3	26	10

Table 1. The tuning parameters used when monitoring for 25OHD2 or 25OHD3 and the internal standards. C3-epi-25OHD3 was monitored using the same transition as 25OHD3.
** denotes optional qualifier ion*

RESULTS

Linearity

- The assay was linear over the range 0.76-37.7ng/mL for C3-epi-25OHD3 and over the range 4-100ng/mL for 25OHD2 and 25OHD3 with all coefficient of determinations (R^2) > 0.996.

Precision

- The inter-assay precision for 25OHD2 and 25OHD3 was determined by extracting and quantifying five replicates of the tri-level QC material from UTAK over five consecutive days. The coefficients of variation (CV) for 25OHD2 and 25OHD3 were ≤10.4% and ≤8.6% respectively. The inter-assay precision of C3-epi-25OHD3 at 37.7 and 1.06ng/mL was ≤8.5%CV.

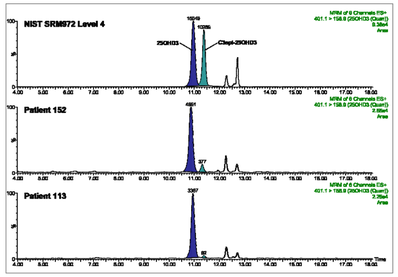

Figure 3. Chromatographic separation of 25OHD3 from the corresponding C3-epimer.

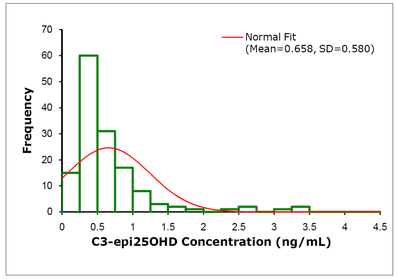

Figure 4. Distribution of C3-epi25OHD3 in the adult population (n=156).

Sensitivity and Specificity

- Figure 3 shows the chromatographic separation of 25OHD3 from it's C3-epimer in the NIST SRM972 level 4 at 36.9ng/mL and 37.7ng/mL respectively.
- The chromatogram for serum sample 152 represents serum levels of 25OHD3 at 18ng/mL and relatively high C3-epimer levels for this population at 2.5ng/mL.

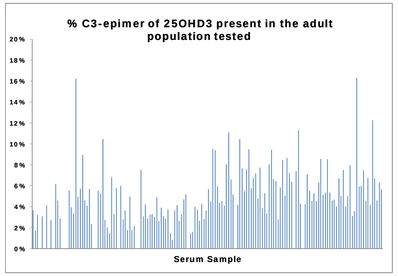

Figure 5. Relative amount of C3-epi-25OHD3 to 25OHD3 in the adult population (n=156) expressed as a percentage.

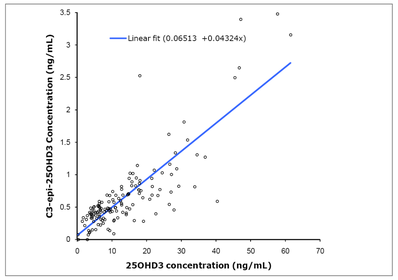

Figure 6. Relationship of C3-epi-25OHD3 to 25OHD3 concentrations (R^2=0.69).

- The chromatogram for serum sample 113 represents typical serum levels of 25OHD3 and C3-epimer for this population. Calculated concentrations were 11.9ng/mL and 0.63ng/mL respectively.
- 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 0.37-64.7ng/mL). 78.2% of the population were insufficient (Total 25OHD <20ng/mL).
- Figure 4 shows the calculated C3-epi-25OHD concentrations ranged from 0-3.48ng/mL (median 0.49ng/mL, mean 0.66ng/mL) with the relative amount of C3-epimer to 25OHD3 ranged from 0-16.24% (mean, 4.8%), as shown in Figure 5.
- Using non-parametric statistics (CLSI C28-A) a reference range was established for this population. At the 95% interval the lower and upper limits were 0.073ng/mL and 2.851ng/mL respectively.
- The relationship of C3-epi-25OHD3 to 25OHD3 concentration is shown in Figure 6. The concentration of C3-epimer generally increases with 25OHD3 levels.

DISCUSSION

- For this cohort of samples the mean Total 25OHD concentration was 14.7ng/mL representing a deficient population.
- C3-epimer was detected in 90% of the adult samples tested.
- 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.

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