Assessment of C3-Epi-25-OH vitamin D concentrations in adult serum: LC-MS/MS determination using [$^2H_3$] C3-epi-25OHD$_3$ internal standard and NIST traceable commercial 3-epi-25OHD calibrators

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Introduction

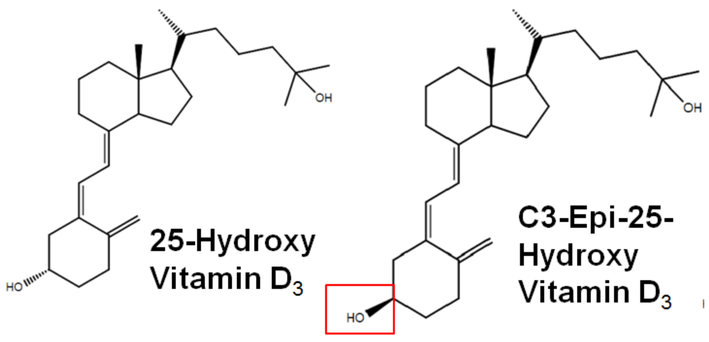


Figure 1 showing the structural configuration of the hydroxy group at the third carbon (C-3) position.

- LC-MS/MS is currently considered the gold standard method for the measurement of 25OHD. It is able to distinguish 25OHD$_3$ from 25OHD$_2$ providing a more accurate assessment of an individuals vitamin D status.
- Interferences from co-eluting isobaric compounds of identical elemental composition but of different structure can result in over estimation of total 25OHD.
- C-3 Epimer of 25-hydroxy vitamin D$_3$ and D$_2$ (C3-Epi-25OHD$_3$/D$_2$) differs from 25OHD in configuration of the hydroxyl group at the third carbon (C-3) position. It has been shown to be more prevalent in infants and in adults with specific disease states.
- Due to the similarity in mass, charge and ionisation characteristics, conventional mass spectrometric systems are unable to separate the epimer according to the MRM transitions.

Aims and Objectives

- To resolve and quantify C3-Epi-25(OH)D from 25(OH)D using LC-MS/MS technique.
- Analyse C3-Epi-25(OH)D$_3$/D$_2$ in patient samples received for 25(OH)D measurement at the Norfolk and Norwich University Hospital.

LC-MS/MS separations

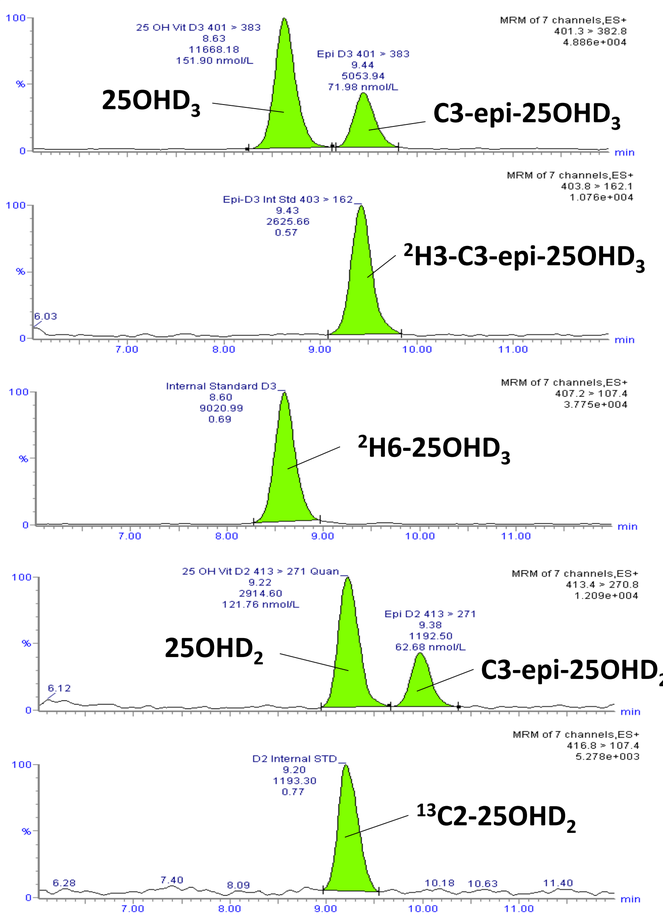


Figure 2: Chromatograms showing separation of C3-epimers from 25OHD.

Sample Preparations

1. 100µL of sample/Std/QC.
2. Add 100µL of 0.1M Zinc Sulphate.
3. Add 200µL of acetonitrile containing internal standards.

Gradient Timetable

Flow rate: 0.4 mL/min
(A)Water : (B)methanol (both contains in 0.1% formic acid)
0 – 9.0 min 25% A : 70% B
9.0 – 10.0min 100%B
10.0 – 11.0 min 25% A : 70% B

HPLC Column

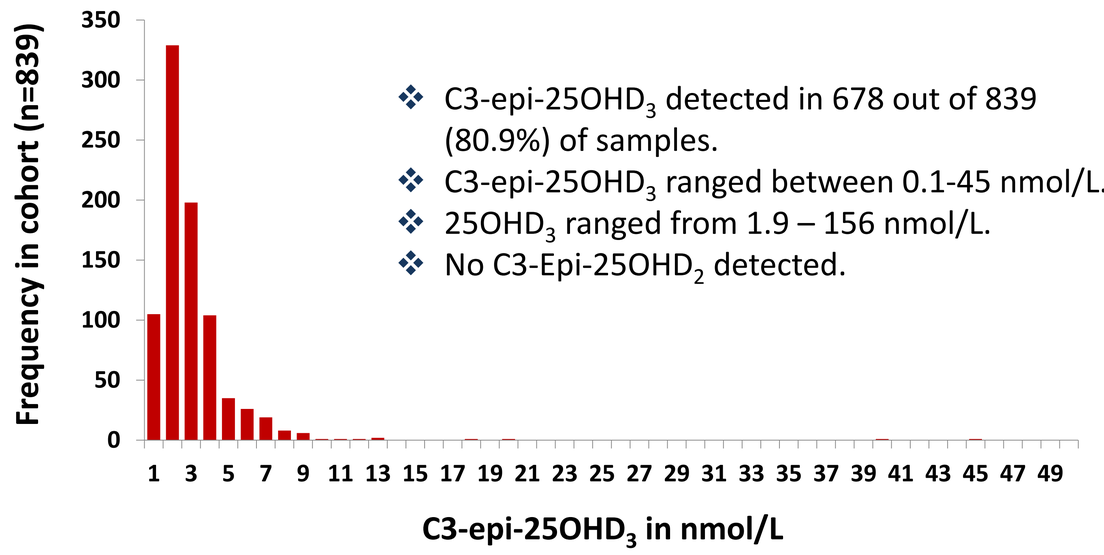
Thermo Accucore 2.6µm 100 x 2.1mm I.D. pentoflurorphenyl solid core particle column.

LC-MS/MS system

Micromass Quattro Ultima triple quadrupole tandem mass spectrometer.

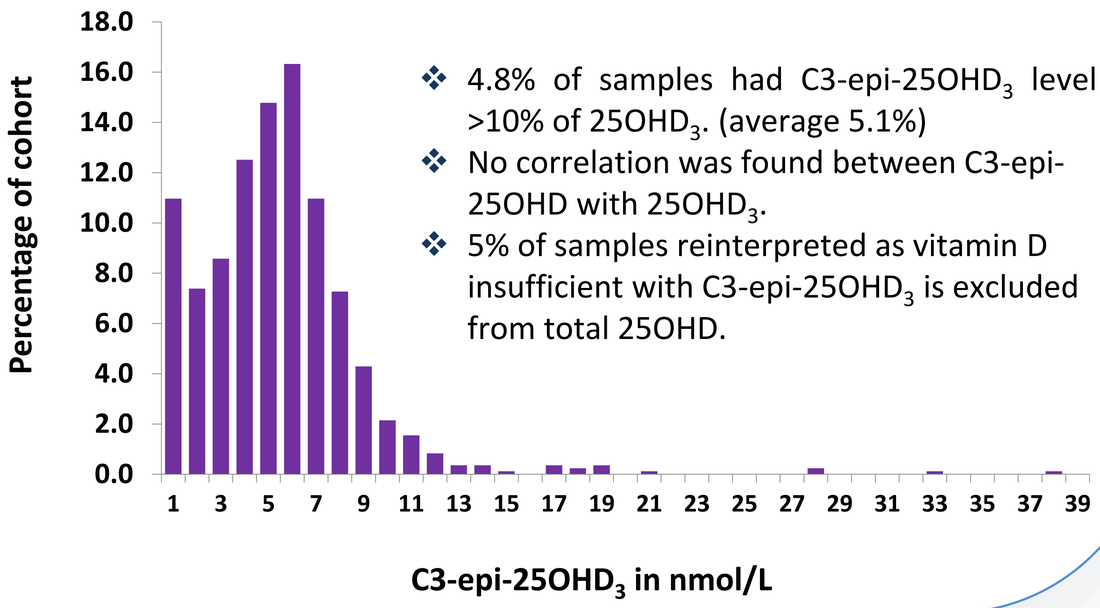
C3-Epi-25OHD$_3$ – Prevalence and concentrations

Distribution of C3-epi-25OHD$_3$ concentration in cohort of 839 adult samples.



- C3-epi-25OHD$_3$ detected in 678 out of 839 (80.9%) of samples.
- C3-epi-25OHD$_3$ ranged between 0.1-45 nmol/L.
- 25OHD$_3$ ranged from 1.9 – 156 nmol/L.
- No C3-Epi-25OHD$_2$ detected.

Percentage of C3-epi-25OHD$_3$ in relation to 25OHD$_3$.



- 4.8% of samples had C3-epi-25OHD$_3$ level >10% of 25OHD$_3$. (average 5.1%)
- No correlation was found between C3-epi-25OHD with 25OHD$_3$.
- 5% of samples reinterpreted as vitamin D insufficient with C3-epi-25OHD$_3$ is excluded from total 25OHD.

Assay characteristics

Assay imprecision

	n	$\bar{x}$	*SD*	CV%
Intra-assay imprecision	10	3.5	0.2	6.6
	10	42.4	3.0	7.1
	10	64.8	6.4	9.9
Inter-assay imprecision	12	3.4	0.2	6.4
	12	23.6	2.3	9.7
	12	109.9	10.4	9.4

- Linear calibration from 2.5 – 180nmol/L
- Typical linear regression analysis with internal standard $r^2 = 0.995$.
- Lower limit of quantification (LLOQ): 2.5nmol/L (S:N 10:1).

Recovery efficiency

	Endogenous C3-Epi-25OHD$_3$ present (nmol/L)	Spiked (nmol/L)	*Measured value (nmol/L)*	% Recovery
Sample 1	15	50	67	97.0
Sample 2	33.2	50	81	102.7
Sample 3	19.2	100	112	106.4
Sample 4	34.8	100	126	107

Conclusions

- C3-epi-25OHD$_3$ was found in the majority of our sample cohort, but prevalence was low.
- C3-epi-25OHD$_3$ contributed to the overestimation of 25OHD3, resulted in misinterpretation of total vitamin D status.
- High prevalence in infant. Separation of epimer in neonatal samples is essential.
- DEQAS LC-MS/MS method group using NIST-aligned standards showed a positive bias against ALTM. NIST assay can resolve C3-epi-25OHD.
- Biological activity and clinical utility of C3-epi-25OHD remains to be elucidated.

References:

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[4] Schleicher, R.L., et al., *Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum.* Clinica chimica acta; international journal of clinical chemistry, 2011. **412**(17-18): p. 1594-9.