

**A high throughput LC-MS/MS method for the analysis of multiple vitamin D analytes in serum and solid tissues using supported liquid-liquid extraction.**

Carl Jenkinson, Angela Taylor, Radhika Susarla, Brian Keevil, Mark Kilby, Martin Hewison. CEDAM, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK, B15 2TT.

Demand for vitamin D (25-hydroxyvitamin D3 [25OHD3]) laboratory testing has increased dramatically over the last decade in response to greater awareness of 25OHD3-deficiency and associated clinical conditions. Although serum concentrations of 25OHD3 are routinely used to define patient vitamin D 'status', several other vitamin D metabolites, notably hormonal 1,25-dihydroxyvitamin D3 (1 $\alpha$ ,25(OH)2D3), also contribute to the physiological impact of vitamin D. This is particularly important for the many tissue-specific non-classical actions of vitamin D. Consequently, quantification of multiple vitamin D metabolites may provide a more accurate definition of vitamin D status for specific patients and disease scenarios. The aim of this study was therefore to develop an extraction and liquid chromatography-tandem mass-spectrometry (LC-MS/MS) method for multiple vitamin D metabolites that would: 1) ensure accurate 25OHD3 measurement by separating inactive C-3 epimers and isobar interference; 2) be applicable to conventional serum samples as well as target tissues for vitamin D such as the placenta.

Human samples were prepared for analysis by an optimised supported liquid-liquid extraction (SLE) method, which gave good recoveries and no observed matrix effects. LC-MS/MS analysis was carried out in multiple reaction monitoring (MRM) mode using a Waters AQUITY UPLC coupled to a XEVO TQ-S detector, and validated according to Food and Drug Administration (FDA) guidelines. The developed method was applied to the analysis of 14 normal pregnancy term placenta samples and matched sera.

Using a Phenomenex Lux-Cellulose-3 chiral column, LC-MS/MS separation of the following vitamin D metabolites, along with limits of detection [ng/mL], was achieved: 25OHD3 [0.063]; 25OHD2 [0.250]; 3-epi-25OHD3 [0.063]; 3-epi-25OHD2 [0.125], 1 $\alpha$ , 25(OH)2D3 [0.016]; 1 $\alpha$ ,25(OH)2D2 [0.063]; 23R,25(OH)2D3 [2.00]; 24R,25(OH)2D3 [2.00]; 24OHD2 [0.250]; 1 $\alpha$ ,24(OH)2D2 [0.031]. A C18 column, commonly used in clinical analysis, was

unable to separate the 3-epimers from 25OHD. The XEVO TQ-S detector enabled quantification of multiple vitamin D analytes in both serum and placenta tissue. The LC-MS/MS method had a 9 minute run time which separated 25OHD<sub>3</sub> from 3-Epi25OHD<sub>3</sub> and quantified D<sub>3</sub> and D<sub>2</sub> forms of vitamin D, minimizing misleading measurements of vitamin D in a routine clinical setting. The optimised SLE sample preparation method for vitamin D metabolites provides a simpler and less time consuming approach to sample preparation over commonly used liquid-liquid extraction methods.

To conclude, an LC-MS/MS method has been developed that can be applied for routine clinical analysis of vitamin D, enhancing the accuracy of 25OHD<sub>3</sub> measurements and reducing sample preparation time.