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High throughput LC–MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum

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ABSTRACT

Recent studies suggest that vitamin D-deficiency is linked to increased risk of common human health problems. To define vitamin D 'status' most routine analytical methods quantify one particular vitamin D metabolite, 25-hydroxyvitamin D3 (25OHD3). However, vitamin D is characterized by complex metabolic pathways, and simultaneous measurement of multiple vitamin D metabolites may provide a more accurate interpretation of vitamin D status.

To address this we developed a high-throughput liquid chromatography-tandem mass spectrometry (LC–MS/MS) method to analyse multiple vitamin D analytes, with particular emphasis on the separation of epimer metabolites. A supportive liquid-liquid extraction (SLE) and LC–MS/MS method was developed to quantify 10 vitamin D metabolites as well as separation of an interfering 7α-hydroxy-4-cholesten-3-one (7αC4) isobar (precursor of bile acid), and validated by analysis of human serum samples.

In a cohort of 116 healthy subjects, circulating concentrations of 25-hydroxyvitamin D3 (25OHD3), 3-epi-25-hydroxyvitamin D3 (3-epi-25OHD3), 24,25-dihydroxyvitamin D3 (24R,25$(OH)_2$D3), 1,25-dihydroxyvitamin D3 (1α,25$(OH)_2$D3), and 25-hydroxyvitamin D2 (25OHD2) were quantifiable using 220 μL of serum, with 25OHD3 and 24R,25$(OH)_2$D3 showing significant seasonal variations.

This high-throughput LC–MS/MS method provides a novel strategy for assessing the impact of vitamin D on human health and disease.

1. Introduction

In the last decade, studies of the prevalence of vitamin D deficiency and its clinical implications have increased demand for laboratory testing to determine vitamin D 'status' [1]. The most common approach to this has been the measurement of serum levels of 25-hydroxyvitamin D3 (25OHD3). Compared to other vitamin D metabolites, 25OHD3 levels are significantly higher in serum and it has a relatively long serum half-life, making it an ideal marker for monitoring short and long-term changes in vitamin D status [1,2]. Analytical methods detecting only 25OHD3 metabolite can be automated, allowing laboratories with large sample demands to perform high throughput measurements of vitamin D [2].

Vitamin D occurs as two forms, D3 and D2, with approximately 95% of 25OHD circulating as the D3 form. Without dietary supplementation, only a small proportion of 25OHD is comprised of the D2 form [3,4]. Contradictory data exists as to whether the D2 form, through supplementation, has less or equal effectiveness at maintaining vitamin D status and action in comparison to the D3 form [3]. The biologically active vitamin D metabolite, 1α,25$(OH)_2$D, is formed by hydroxylation of 25OHD in the kidney [5]. The 1α,25$(OH)_2$D3 metabolite is present in serum at low picomolar concentration ranges with a short half life time of 4 h. These low concentration ranges have proved challenging for the development of analytical methods that can accurately measure this metabolite [6], particularly using less sensitive older generation LC–MS/MS instruments. Chiral metabolites 23R,25$(OH)_2$D3 and 24R,25$(OH)_2$D3 can also be converted from 25OHD3, however unlike 1α,25$(OH)_2$D3, these chiral metabolites are thought to be non-active [5].

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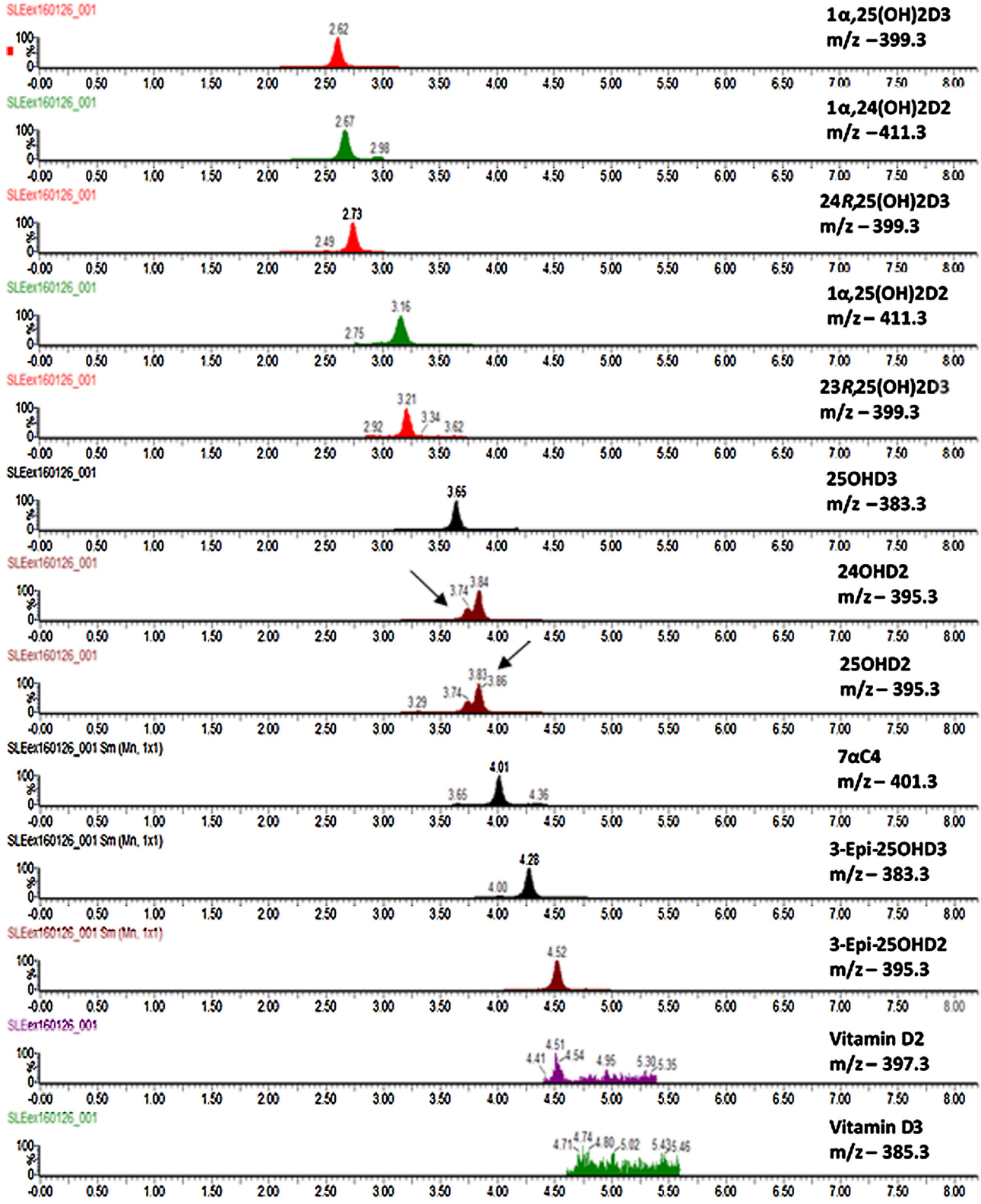

Fig. 1. Chromatogram of vitamin D analytes extracted from a spiked serum quality control standard. All vitamin D metabolites could be quantified except vitamin D2, vitamin D and vitamin D2-d3 which could not be quantified from the SLE extraction.

Epimerisation of 25OHD2 and 25OHD3 form the C3-epimers, 3-epi-25OHD2 and 3-epi-25OHD3 respectively [1]. The site of epimerisation occurs at the third carbon atom of 25OHD, altering the position of the hydroxyl group at this site. The structures of 25OHD and 3-epi-25OHD are identical apart from the position of epimerisation [7]. 3-Epi-25OHD metabolites are hydroxylated to form 3-epi-1α,25$(OH)_2$D, in the same manner of 25OHD is hydroxylated to form 1α,25$(OH)_2$D. The physiological role of 3-epi-1α,25$(OH)_2$D remains unclear, although it is capable of binding to the vitamin D receptor with reduced physiological effect