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Review

Determination of human reference values for serum total 1,25-dihydroxyvitamin D using an extensively validated 2D ID-UPLC–MS/MS method

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ABSTRACT

Background: To assess a patient's vitamin D status the precursor metabolite 25-hydroxyvitamin D can be determined. However, measurement of 1,25-dihydroxyvitamin D is required when disorders of 1a-hydroxylation, extrarenal 1a-hydroxylation, or vitamin D receptor defects are suspected.
Methods: The aim of this study was to determine reference values for 1,25-dihydroxyvitamin D_3 and D_2 using a 2D ID-UPLC–MS/MS method.
Results: The LC–MS/MS method, able to measure picomolar concentrations of both 1,25-dihydroxyvitamin D_3 and D_2 in human serum, was extensively validated. Intra-assay variations were <5% and 8.5% and <7.5% and 11%, for 1,25-dihydroxyvitamin D_3 and D_2, respectively, over the whole dynamic range (3.1–376 and 3.1–652 pmol/L). Limit of quantitation was 3.4 pmol/L for both compounds. Our method correlated well with a published LC–MS/MS method ($r = 0.87$) and with the average 1,25-dihydroxyvitamin D_3 results of the vitamin D External Quality Assessment Scheme (DEQAS) determined with LC–MS/MS ($r = 0.93$). Reference ranges, determined in 96 plasma samples of healthy volunteers were 59–159 pmol/L and <17 pmol/L for respectively 1,25-dihydroxyvitamin D_3 and D_2. The female part of the reference group showed a statistically significant decrease of 1,25-dihydroxyvitamin D_3 concentrations with age. The presence of significantly higher average 1,25-dihydroxyvitamin D_3 levels in premenopausal women taking oral contraceptive pills compared to postmenopausal women suggests that this effect is estrogen-related, as estrogens lead to a higher vitamin D binding protein.
Conclusions: The major finding of the present study is a reference interval of 59–159 pmol/L for 1,25-dihydroxyvitamin D_3 determined with a highly sensitive and precise LC–MS/MS method.

Contents

Abbreviations: DEQAS, vitamin D External Quality Assessment Scheme; 25OHD, 25-hydroxyvitamin D; 24,25diOHD, 24,25-dihydroxyvitamin D; 1,25diOHD, 1,25-dihydroxyvitamin D; VDR, vitamin D receptor; RIA, radioimmunoassay; 2D ID-UPLC–MS/MS, two-dimensional isotope dilution ultra pressure liquid chromatography–tandem mass spectrometry; FA, formic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PTAD, 4-phenyl-1,2,4-triazole-3,5-dione; CV, coefficient of variation; LOQ, limit of quantitation; SNR, signal-to-noise ratio; OCP, oral contraceptive.

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1. Introduction

Considering the importance of vitamin D and its derivatives in calcium homeostasis and bone metabolism, it is pivotal to be able to accurately measure circulating vitamin D metabolites. These include 25-hydroxyvitamin D (25OHD), 24,25-dihydroxyvitamin D (24,25diOHD) and 1,25-dihydroxyvitamin D (1,25diOHD). 25OHD is the main circulating metabolite and the latter two are its metabolites. 1,25diOHD is the hormonally active form of vitamin D, while there is also evidence about the activity of 24,25diOHD [1–3].

1,25diOHD is known to circulate in two forms, 1,25diOHD$_3$ and 1,25diOHD$_2$, which only differ in a double bond and a methyl group and both hail from their corresponding, unhydroxylated, vitamin D precursors. Vitamin D$_2$ originates from plants, whereas vitamin D$_3$ derives from animal material or is synthesized by the body itself converting 7-dehydrocholesterol upon UVB radiation. The two 1,25diOHD forms can both bind the vitamin D receptor (VDR), although 1,25diOHD$_3$ has proven to be significantly more potent [4].

Preferably, one would measure 1,25diOHD when calcium concentrations are altered significantly and measurement of merely PTH does not provide a proper diagnosis. This indicates the presence of altered 1a-hydroxylase activity. This enzyme, expressed mainly in the kidney and which hydroxylates 25OHD to form 1,25diOHD, might be absent or malfunctioning, like in renal failure [5], vitamin D-dependent rickets type I [6] and hypophosphatemic rickets [5], or is expressed extrarenally, as can be the case in sarcoidosis [7], tuberculosis [8], inflammatory bowel disease [5], rheumatoid arthritis and lymphoproliferative disease [9]. In these conditions extrarenal hydroxylation occurs in macrophages or granulomas, and is not tightly regulated by feedback control. Moreover, one might wish to measure if 1,25diOHD concentrations have risen when vitamin D resistance or insensitivity is suspected. Finally, inborn errors of the VDR, like vitamin D-dependent rickets type 2 [5], cause extremely high concentrations of 1,25diOHD.

On the contrary, measuring 1,25diOHD to assess a patient's vitamin D status is unsuited, as serum 1,25diOHD levels are tightly regulated by hormonal control as long as possible. This means that 1,25diOHD concentrations can be within the reference range even in the case of vitamin D deficiency [5].

1,25diOHD can be measured using several methods. Today, serum 1,25diOHD is often measured using a radioimmunoassay (RIA). These assays can be very sensitive; yet often lack specificity, as they may suffer from cross reactivity by analogous compounds, like 24,25diOHD and 25OHD or need extensive and time consuming sample pretreatment [10]. Previous studies, however, have demonstrated the possibility to specifically and accurately quantify the very low concentrations of 1,25diOHD in human serum by using very sensitive LC–MS/MS systems, albeit derivatization is still commonly used to increase sensitivity further [11–14]. Here we describe the analytical characteristics of a two-dimensional isotope dilution ultra pressure liquid chromatography tandem mass spectrometry (2D ID-UPLC–MS/MS) method, the comparison with another published LC–MS/MS method for the measurement of both 1,25diOHD$_3$ and 1,25diOHD$_2$ and the comparison with the average 1,25-dihydroxyvitamin D results of the vitamin D External Quality Assessment Scheme (DEQAS). Additionally, we have established reference values for 1,25diOHD$_3$ and 1,25diOHD$_2$ in 96 healthy individuals.

2. Materials and methods

2.1. Chemicals and reagents

1,25diOHD$_3$, 1,25diOHD$_2$, 1,25diOHD$_3$-d6 and 1,25diOHD$_2$-d6 were purchased from Sigma, Cayman Chemical Toronto Research and Santa Cruz Biotech, respectively. Ethanol was purchased from Emsure HPLC grade methanol, acetonitrile and ammonium acetate (Biosolve) formic acid (FA) (Sigma–Aldrich) and reagent grade deionised water (Milli-Q; Millipore) were used to make eluent compositions. 6% Bovine serum albumin (BSA) in phosphate-buffered saline (PBS, B. Braun Meslungen AG) was prepared by dissolving 15 g of BSA in 250 mL PBS and pH was set to 7.3. 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD) was purchased from Sigma and prepared by dissolving 40 mg in 50 mL acetonitrile. Immunoaffinity columns were purchased from Immundiagnostik AG.

2.2. Laboratory procedure

Stock concentrations were prepared by dissolving 1 mg of 1,25diOHD$_3$ or 1,25diOHD$_2$ in 25 mL of ethanol and checked by