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Study of blood collection and sample preparation for analysis of vitamin D and its metabolites by liquid chromatography–tandem mass spectrometry

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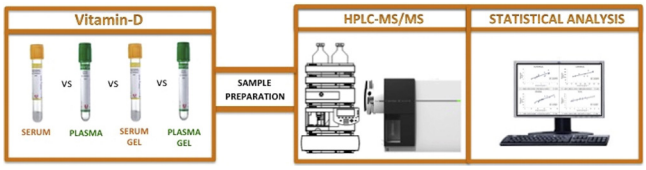
HIGHLIGHTS

- Sample preparation using SPE provides better sensitivity than deproteination.
- Serum and plasma provided different levels of 1,25-dihydroxyvitamin D_3.
- Plasma is more suited for quantitative analysis of 1,25-dihydroxyvitamin D_3.
- Sample collection and treatment were significant in the analysis of vitamin D.

GRAPHICAL ABSTRACT



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ABSTRACT

The analysis of vitamin D status, with special emphasis on 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, is gaining interest in clinical studies due to the classical and non-classical effects attributed to this prohormone. In this research, the influence of the two steps preceding determination (*viz.* sample collection and preparation) on the quantitative analysis of vitamin D and its more important metabolites has been studied. Two preparation approaches, deproteination and solid-phase extraction (SPE), have been evaluated in terms of sensitivity to delimit their application, thus establishing that detection of 1,25-dihydroxyvitamin D cannot be addressed by protein precipitation. Concerning sample collection, serum and plasma reported high accuracy (above 83.3%) for vitamin D and metabolites, while precision, expressed as relative standard deviation, was below 12.9% for all analytes in both samples. Statistical analysis revealed that serum and plasma provided similar physiological levels for vitamin D_3, 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3, while significantly different levels were obtained for 1,25-dihydroxyvitamin D_3, always higher in plasma than in serum. Sample collection and treatment have proved to be significant in the analysis of vitamin D and its relevant metabolites.

Abbreviations: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; ACE, automatic cartridge exchange; CLIA, chemiluminescence immunoassays; CPB, competitive protein binding assay; ESI+, electrospray ionization in positive mode; ELISA, enzyme linked immuno sorbent assay; LOQs, limits of quantitation; LC–MS/MS, liquid chromatography–tandem mass spectrometry; RIA, radioimmunoassay; RP-LC, reversed-phase liquid chromatography; SRM, selected reaction monitoring; SPE, solid phase extraction.

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1. Introduction

Clinical testing for vitamin D has increased exponentially in the past decade. In the United States, requests to clinical laboratories for analysis of this vitamin have increased at a rate of 80–90% per year [1]. This growing demand is a consequence of the recognition of both a high prevalence of deficiency in vitamin D in diverse social sectors [2] and the decisive role of vitamin D in multiple physiological functions. Thus, vitamin D deficiency or insufficiency has been associated to skeletal diseases such as rickets, osteomalacia and osteoporosis, but also to several non-skeletal chronic diseases including cardiovascular diseases, certain types of cancer, diabetes and psychiatric illness, among others [3].

Currently, vitamin D metabolites determined with clinical purposes are 25(OH)D (known as the circulating form of vitamin D), and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], known as the most physiologically active form of vitamin [4]. The analysis of both metabolites can provide information on the status of vitamin D and its availability for the organism [5]. Other less studied metabolite such as 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], produced by vitamin D catabolism, is receiving growing attention from clinicians as an increase in the production of this metabolite could pinpoint an optimum balance of vitamin D [6].

The analysis of vitamin D and its metabolites is characterized by a high complexity owing to their instability in the presence of heat or UV light, their hydrophobic nature, the high affinity for vitamin D binding proteins, the structural similarity to other in circulation metabolites, and the composition of biological samples [7,8]. All these factors make the accurate measurement of vitamin D a challenging task. Semiautomated and fully automated immunoassay methods have been reported; most of them based on competitive protein-binding assay (CPB) [9] —radioimmunoassay (RIA) [10,11], enzyme-linked immuno-sorbent assay (ELISA) [12], and chemiluminescence immunoassays (CLIA) [9]—, and endowed with good accuracy, mainly for 25(OH)D measurement. However, interferences caused by cross-reactivity for monohydroxy and dihydroxy metabolites have been described [7]. The high selectivity and sensitivity of mass spectrometers allow differentiating 25$(OH)D_3$ from 25$(OH)D_2$, and also make possible quantitation of dihydroxymetabolites 1,25$(OH)_2$D and 24,25$(OH)_2$D, present in blood at pg mL^{-1} and ng mL^{-1} levels, respectively [6,7].

A wide variety of biological samples have been tested for analysis of vitamin D and its metabolites. A sample with low clinical interest such as saliva has been used to determine 25(OH)D and 1,25$(OH)_2$D metabolites [13]. Cerebrospinal fluid has also reported detectable levels of vitamin D metabolites, particularly 25(OH)D [14,15]. Dried blood spots have provided good accuracy and precision for quantitation of 25(OH)D [16,17]. Despite the studies carried out with all these samples, serum and plasma persist as the two most common for determination of vitamin D and its metabolites because both can be easily obtained and contain the most important metabolites at measurable levels. Additionally, sample preparation protocols are properly known. Protein precipitation has been mainly carried out by methanol, acetonitrile or mixtures of both solvents, followed by a separation step based on either liquid–liquid extraction (by heptane, hexane, ethyl acetate or ethyl-*tert*-butyl ether as extractants) or by solid-phase extraction (SPE) [18].

The principal aim of this research was to compare the influence of the procedure for blood collection on the determination of vitamin D and its metabolites by SPE–LC–MS/MS. With this aim, the study was focused on evaluation of two aspects that could exert a significant influence on the blood levels of vitamin D found. The first was selection of the analytical sample used for quantitative analysis: serum or plasma; the second aspect was the effect of the gel present in the blood collection tubes, which is used to favor separation of serum or plasma from blood cells, on the analysis of vitamin D and its metabolites. A cohort formed by thirteen volunteers was selected for blood sampling using four different tubes (plasma, plasma-gel, serum and serum-gel). The resulting samples were analyzed by an isotopic dilution SPE–LC–MS/MS method for absolute quantitation of vitamin D as well as its main metabolites with clinical interest. Prior to analysis, protein precipitation and SPE using an automated system were evaluated as sample preparation alternatives.

2. Experimental

2.1. Chemicals and reagents

LC–MS grade solvents were used in this research. Ammonium formate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) and acetonitrile (ACN), formic acid and methanol from Scharlab (Barcelona, Spain) were used from preparation of chromatographic mobile phases and solutions for sample preparation.

Vitamins D_2 and D_3, the monohydroxy metabolites 25$(OH)D_2$ and 25$(OH)D_3$, and dihydroxy metabolites 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ were from Sigma. Stable isotopic standards 1,25$(OH)_2D_3$-d_6, 24,25$(OH)_2D_3$-d_6, 25$(OH)_2D_3$-d_6, and vitamin D_3-d_6 were provided by A. Mouriño (Department of Organic Chemistry, University of Santiago, Santiago de Compostela, Spain) and M.A. Maestro (Department of Fundamental Chemistry, University of La Coruña, La Coruña, Spain), while 25$(OH)D_2$-d_3 and vitamin D_2-d_3 were from Sigma. Individual standard solutions were prepared by dissolving 1 mg of each analyte or isotopic standard in 10 mL of methanol, from which two solutions were prepared by dilution of the appropriate volume in methanol. One multistandard working solution was prepared with the target analytes at different concentrations: 100 ng mL^{-1} for dihydroxymetabolites 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$; 1 μg mL^{-1} for 24,25$(OH)_2D_3$; 5 μg mL^{-1} for 25$(OH)D_3$ and 25$(OH)D_2$; and 10 μg mL^{-1} for vitamin D_2 and vitamin D_3. Other solution was prepared with each isotopic standard —7.5 ng mL^{-1} for 1,25$(OH)_2D_3$-d_6 and 125 ng mL^{-1} for 24,25$(OH)_2D_3$-d_6; 625 ng mL^{-1} for 25$(OH)D_3$-d_6 and 25$(OH)D_2$-d_3; and 625 ng mL^{-1} for vitamin D_2-d_3 and vitamin D_3-d_6. Both solutions were used for optimization, characterization and validation of the analytical methods.

2.2. Instruments and apparatus

The analyses involved reversed-phase LC (RP-LC) separation followed by electrospray ionization in positive mode (ESI+) and MS/MS detection in selected reaction monitoring (SRM). Chromatographic separation was carried out with an Agilent (Palo Alto, CA, USA) 1200 Series LC system coupled to an Agilent 6410 triple quadrupole mass spectrometer. The data were processed using MassHunter Workstation Software (V-B.05) for qualitative and quantitative analysis. Hyphenated SPE was performed by a Symbiosis system (Spark Holland, Emmen, The Netherlands). This commercial equipment comprises a unit for automatic cartridge exchange (ACE), an autosampler (Reliance) furnished with a 0.2 mL sample loop and two high-pressure syringe dispensers (HPDs) for SPE solvent delivery. Peek tube of 0.25 mm i.d. (VICI, Houston, Texas, USA) was used to connect all valves of the Symbiosis unit and LCKMS/MS modules. Peek tubing of 1.0 mm i.d. and 130 cm length, and about 1 mL volume, was used to connect the Reliance to the ACE unit for mixing the serum sample and loading solution. A 10 × 2 mm cartridge packed with Hysphere C8 (Spark Holland) as sorbent material was used for SPE. The analytical column was a Poroshell 120 EC-C18 (2.7 μm particle size, 50 × 4.6 mm i.d.) from Agilent, while a guard column (2.7 μm