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Quantitative analytical method to evaluate the metabolism of vitamin D ☆

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ABSTRACT

A method for quantitative analysis of vitamin D (both D_2 and D_3) and its main metabolites — monohydroxylated vitamin D (25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3) and dihydroxylated metabolites (1,25-dihydroxyvitamin D_2, 1,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3) in human serum is here reported. The method is based on direct analysis of serum by an automated platform involving on-line coupling of a solid-phase extraction workstation to a liquid chromatograph–tandem mass spectrometer. Detection of the seven analytes was carried out by the selected reaction monitoring (SRM) mode, and quantitative analysis was supported on the use of stable isotopic labeled internal standards (SIL-ISs). The detection limits were between 0.3–75 pg/mL for the target compounds, while precision (expressed as relative standard deviation) was below 13.0% for between-day variability. The method was externally validated according to the vitamin D External Quality Assurance Scheme (DEQAS) through the analysis of ten serum samples provided by this organism. The analytical features of the method support its applicability in nutritional and clinical studies targeted at elucidating the role of vitamin D metabolism.

1. Introduction

The vitamin D endocrine system (VDES) plays an essential role in human health beyond the regulation of calcium homeostasis and bone mineralization [1]. Vitamin D is obtained as vitamin D_3 (cholecalciferol) mainly derived from photosynthesis in the skin, after conversion of 7-dehydrocholesterol by ultraviolet irradiation. Small amounts of vitamin D can also be obtained by nutritional intake of either vitamin D_3 or by foods fortified with vitamin D_2 (ergocalciferol). Both forms undergo a substrate-dependent liver hydroxylation to 25-hydroxyvitamin D [25(OH)D_2 and 25(OH)D_3], the major circulating form of vitamin D considered a reliable indicator of its nutritional status [2], despite the recognized lack of assay standardization for its determination [3]. To be fully active, 25(OH)D must further be converted to 1,25-dihydroxyvitamin D [1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$], the hormonally active form of VDES, and under whose control is considered to be around 3% of the human genome [4].

The recent growing interest in VDES is mainly due to its multiple roles in global health maintenance. Apart from classical diseases such as rickets, osteomalacia and osteoporosis, vitamin D insufficiency has been associated to several chronic diseases (e.g. arthritis, cardiovascular, cancer, diabetes, multiple sclerosis, psychiatric illnesses) [5], thus increasing nearly exponentially over the last decade the demand from basic and clinical scientists for tests intended to quantify vitamin D and metabolites [5]. The essential question of how much vitamin D is needed for optimal bone and global health remains unsolved [6,7].

Abbreviations: 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; 24,25$(OH)_3$D, 24,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; ACE, automatic cartridge exchange; CLIA, chemiluminescence immunoassays; CPB, competitive protein binding assay; DEQAS, vitamin D external quality assurance scheme; ESI+, electrospray ionization in positive mode; ELISA, enzyme-linked immuno-sorbent assay; EIC, extracted ion chromatogram; IDS, immuno diagnostic systems; IS, internal standard; LOD, limit of detection; LOQ, limit of quantitation; LC–MS/MS, liquid chromatography–tandem mass spectrometry; RIA, radioimmunoassay; RP-LC, reversed-phase liquid chromatography; RSD, relative standard deviation; SRM, selected reaction monitoring; SIL-IS, stable isotopic labeled-internal standard; SPE, solid phase extraction; VDES, vitamin D endocrine system.

☆ Contribution to the overall work: A. Mena-Bravo, C. Ferreiro-Vera, F. Priego-Capote and M. D. Luque de Castro developed and applied the method, organized and wrote the manuscript. J. M. Quesada-Gómez proposed applications and actively participated in the discussion of the manuscript. M.A. Maestro and A. Mouriño synthesized and provided the deuterated standards.

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Despite vitamins D_2 and D_3 are generally considered to be equivalent in humans, there are evidences that vitamin D_3 is substantially more efficient than vitamin D_2 to rise serum 25(OH)D concentrations [8,9].

The analysis of vitamin D is complicated by the structural similarity of metabolites, hydrophobic nature and thermal and UV-light instability [10]. In fact, there are over 40 identified vitamin D (D_2/D_3) metabolites [11], among which, the targets of clinical analyses are $1,25(OH)_2D_3$ and $25(OH)D_3$ as indicators of vitamin D nutritional status [2,6–9,12]. Measurement of $1,25(OH)_2D_3$ is more challenging than that of $25(OH)D_3$ as the former is more unstable and it is present in serum/plasma at the pg/mL level versus ng/mL level for $25(OH)D_3$.

Current methods for determination of 25(OH)D and $1,25(OH)_2D$ include competitive protein binding assay (CPB) [13], Radioimmunoassay (RIA) [14,15], Enzyme-Linked Immuno-Sorbent Assay (ELISA) [16], and Chemiluminescence Immunoassays (CLIA) [13], which have demonstrated cross-reactivity for $25(OH)D_3/25(OH)D_2$ and $1,25(OH)_2D_3/24,25(OH)_2D_3$ pairs [5,17]. Despite liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods are at present considered the "gold standard" [3,18–20], they have already aroused controversy [21,22], which requires in depth research to achieve more consistent results [3].

A characteristic of the present LC–MS/MS methods for vitamin D analysis including $1,25(OH)_2D$ as target analyte when applied to serum or plasma is the time-consuming and tedious sample preparation step. Preparation of serum/plasma samples for determination of the dihydroxymetabolite makes mandatory its preconcentration and removal of interferents. Automated solid phase extraction (SPE) constitutes an excellent tool to facilitate and speed up the steps involved in sample preparation. In addition, the system can be on-line coupled to LC equipment thus allowing monitoring of the entire SPE process with recovery estimation [20]. Apart from automation, minimization of matrix effects and other variability sources is crucial to improve the overall method. The use of stable isotopic labeled internal standards (SIL-ISs) [23–25] with physico-chemical properties identical or similar to the target analytes is critical to overcome variability sources [26]. Proficiency testing programs have been created to assess comparative performances and improve the accuracy of total 25(OH)D and $1,25(OH)_2D$ methods [3,27]. Among them, the Vitamin D External Quality Assessment Scheme (DEQAS) is presently considered an accuracy-based program for selected vitamin D metabolites [28].

Despite present methods based on LC–MS/MS are most times endowed with excellent sensitivity and selectivity, there is a demand for more robust, fully automated platforms that can meet the need for throughput, precision and accurate testing of vitamin D and metabolites with special emphasis on the dihydroxymetabolites due to their low physiological concentrations. The present research was aimed at validating an automated SPE–LC–MS/MS platform with optimum analytical features for determination of vitamin D and the most important metabolites in the clinical and nutritional fields. Apart from vitamins D_2 and D_3, the method was targeted at the monohydroxymetabolites of the two forms of vitamin D as well as the three dihydroxymetabolites: $1,25(OH)_2D_2$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$. The inclusion of vitamin D_2 and metabolites in analytical methods is not usual as this form is practically not detected in humans unless it is administered as supplement, which is a common practice in USA. It is worth emphasizing that the development of the method was supported on the use of SIL-IS for the different analytes and the validation was carried out with an external quality assessment (DEQAS).

2. Experimental

2.1. Chemicals and reagents

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

Metabolites $1,25(OH)_2D_2$, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $25(OH)D_2$, $25(OH)D_3$, vitamin D_2 and vitamin D_3 were from Sigma. Stable isotopic standards $1,25(OH)_2D_3$–D_6, $24,25(OH)_2D_3$–D_6, $25(OH)D_3$–D_6, and vitamins D_3–D_6 were synthesized by A. Mouriño and M. A. Maestro (vitamin D research group). The $25(OH)_2D_2$–D_3 and vitamins D_2–D_3 isotopic standards 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 used for spiking the biological samples (either serum or plasma) 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 for dihydroxymetabolites $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$; 1 μg/mL for $24,25(OH)_2D_3$; 5 μg/mL for $25(OH)D_3$ and $25(OH)D_2$; and 10 μg/mL for vitamin D_2 and vitamin D_3. Other solution was prepared with each isotopic standard — 7.5 ng/mL for isotopic standard $1,25(OH)_2D_3$–D_6 and 125 ng/mL $24,25(OH)_2D_3$–D_6; 625 ng/mL for $25(OH)D_3$–D_6 and $25(OH)D_2$–D_3; and 625 ng/mL for vitamins D_2–D_3 and vitamins D_3–D_6.

2.2. Instruments and apparatus

The analyses were performed by reversed-phase LC (RP-LC) separation followed by electrospray ionization in positive mode (ESI+) and MS/MS detection. 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 analyses. 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 tubing of 0.25 mm i.d. (VICI, Houston, Texas, USA) was used to connect all valves of the Symbiosis unit and LC–MS/MS modules. A stainless tube of 1.0 mm i.d. and 130 cm length, 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 particle size, 5.0 × 2.1 mm i.d.), also from Agilent, was used to preserve the integrity of the analytical column.

2.3. Sampling, sample collection and storage

Ten serum samples were provided by DEQAS for analysis of 25(OH)D and $1,25(OH)_2D$ using the proposed method. These samples were prepared from individual blood donations and were sterilized by filtration through 0.2 μm microbiological grade filters by the Oncology/Endocrinology Laboratory of Charing Cross Hospital (Fulham Palace Rd, London W6 8RF, UK) [28].

Three serum pools were prepared by spiking the target analytes at low, intermediate and high concentrations according to the physiological levels described in the literature [29]. The concentrations used for preparation of each pool are listed in Supplementary Table 1. The pools were used for evaluation of analytical features such as precision, recovery factor for the SPE step and accuracy of the method.

2.4. Proposed procedure

A volume of 240 μL of serum in an amber glass vial was spiked with 10 μL of the deuterated working solution — final concentration in serum: 25 ng/mL of vitamins D_3–D_6 and vitamins D_2–D_6, 25 ng/mL of $25(OH)D_3$–D_6 and $25(OH)D_2$–D_6, and 5 ng/mL of $24,25(OH)_2D_3$–D_6