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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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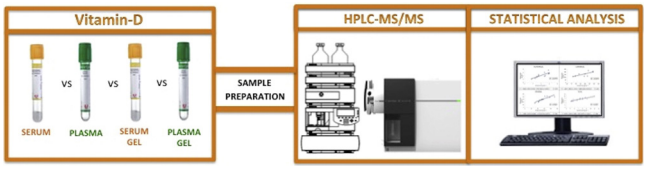
HIGHLIGHTS

- 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.
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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

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

Thirteen individuals (3 men and 10 women) gave their informed consent for an assistance study involving quantitation of vitamin D and its main metabolites. All steps from blood extraction to analysis were performed in compliance with the guidelines dictated by the World Medical Association Declaration of Helsinki (2004), which were supervised by the ethical review board of Reina Sofia University Hospital (Córdoba, Spain) that approved the experiments. Venous blood from each of the selected individuals was collected into four different Vacutainer® tubes (Becton Dickinson): plastic serum tubes with spray-coated silica (serum), plastic serum tubes with spray-coated silica and a polymer gel to favor serum separation (serum-gel), spray-coated silica tubes with heparin for plasma (plasma) and heparin tubes with polymer gel to favor plasma separation (plasma-gel). The gel forms a physical barrier between serum or plasma and blood cells during centrifugation, which allows setting a more efficient separation as compared to conventional tubes. All collection tubes were processed by centrifugation for 15 min at 1000 × *g* for conventional tubes for serum and plasma isolation and at 2000 × *g* for gel tubes. After that, the samples were placed in plastic ware tubes and stored at −80 °C until analysis. Serum and plasma pools were prepared by mixture of aliquots from the blood donors for optimization of the methods for analysis of vitamin D and metabolites in both types of samples.

2.4. Sample preparation procedure based on precipitation of proteins

A volume of 240 μL of serum or plasma in an amber glass vial was spiked with 10 μL of the deuterated working solution —final concentration: 25 ng mL^{-1} of vitamin D_3-d_6 and vitamin D_2-d_3, 25 ng mL^{-1} of 25(OH)D_3-d_6 and 25(OH)D_2-d_3 and 5 ng mL^{-1} of 24,25$(OH)_2D_3$-d_6 and 0.3 ng mL^{-1} 1,25$(OH)_2D_3$-d_6—, immersed in an ice bath and treated for deproteination with 500 μL of 0.1% (v/v) formic acid in methanol, one of the most common solvents used for this step [19]. The vial was shaken for 5 min and the precipitate removed after centrifugation for 5 min at 4 °C and 20,200 × *g*. The upper liquid phase was collected in a vial and evaporated; then, the dry residue was reconstituted with 30 μL methanol and placed in the LC autosampler for subsequent analysis.

2.5. Sample preparation procedure based on SPE

A volume of 240 μL of serum or plasma in an amber glass vial was spiked with 10 μL of the deuterated working solution —final concentration: 25 ng mL^{-1} of vitamin D_3-d_6 and vitamin D_2-d_3, 25 ng mL^{-1} of 25(OH)D_3-d_6 and 25(OH)D_2-d_3 and 5 ng mL^{-1} of 24,25$(OH)_2D_3$-d_6 and 0.3 ng mL^{-1} of 1,25$(OH)_2D_3$-d_6—, shaken and introduced into the autosampler. Supplementary Fig. 1A shows the instrumental arrangement used for analysis of vitamin D and its metabolites. The sample loop was filled with 0.2 mL from the sample vial refrigerated at 6 °C. The sequence of automatic operations followed in the procedure is described in Supplementary Table 1.

2.6. LC–MS/MS analysis

The LC–MS/MS method used in this study for analysis of vitamin D and metabolites was that developed by Mena-Bravo et al. [20]. The initial chromatographic mobile phase was 5 mM ammonium formate in 85:15 (v/v) methanol–water at a flow rate of 0.5 mL min^{-1}. The temperature of the analytical column compartment was set at 15 °C. A linear gradient was programmed from 2 to 5 min to obtain as final composition of the mobile phase 5 mM ammonium formate in methanol, which was kept for 10 min up to the end of the chromatographic step. The total analysis time was 15 min, 10 min being required for re-establishing and equilibrating the initial conditions. The chromatographic–detection step of one sample and the SPE step of the next sample were overlapped, thus improving the analysis frequency.

The eluate from the chromatographic column was monitored by MS/MS in SRM mode. The flow and temperature of the drying gas (N_2) were 9 L min^{-1} and 350 °C, respectively. The nebulizer pressure was 50 psi, and the capillary voltage 4750 V in positive ionization mode. The SRM parameters are specified in Supplementary Table 2 for each analyte monitored in this study as well as the isotopic standards.

2.7. Data treatment

Quantitation was carried out using the ratio between the peak area of each analyte and that of the corresponding isotopic standard. Calibration models were developed for each analyte using a pool of serum or plasma, which was spiked with different concentrations of standard solutions of the target analyte and with constant concentrations of the deuterated standards. Also, aliquots of serum or plasma spiked only with deuterated standards was prepared to correct the endogenous concentration of the target analytes in the serum or plasma pool.

3. Results and discussion

3.1. Comparison of the two sample preparation procedures

Protein precipitation and SPE were compared in terms of sensitivity, linear calibration range and matrix effects for analysis of vitamin D and its metabolites. For this purpose, four calibration models were built for each analyte using serum and plasma and the two sample preparation methods. The calibration models were prepared by using pools of both biofluids from donors, which were spiked with the target analytes at different concentrations. Table 1 lists the main parameters of the resulting calibration models, the calibration ranges of which were defined according to the normal blood levels of each analyte described in the literature [18]. The upper value of the linear dynamic range was the same for both sample preparation alternatives, 500 ng mL^{-1} for vitamin D_2 and D_3 and 250 ng mL^{-1} for the two monohydroxy metabolites. Nevertheless, the lower limits of the linear dynamic ranges were clearly influenced by the sample preparation approach. Thus, protein precipitation both in plasma and serum led to higher limits of quantitation than those provided by SPE. The most compromised situation was found for the three dihydroxymetabolites —1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$— since the protein precipitation method provided limits of quantitation (LOQs) from 0.1 to 0.15 ng mL^{-1}, considerably above the typical levels of these metabolites in blood. On the other hand, the LOQs were 15 pg mL^{-1} for the two dihydroxymetabolites of vitamin D_3, while for 1,25$(OH)_2D_2$ the LOQ was 50 pg mL^{-1}. As can be checked, vitamin D_2 and its metabolites gave higher LOQs than their vitamin D_3 analogues. This different sensitivity should be attributed to the better electrospray ionization efficiency for vitamin D_3 and its metabolites that could be explained by the higher hydrophobic character of vitamin D_2 and its metabolites. Fig. 1 shows the SRM chromatograms obtained by analysis of the pools of serum and plasma spiked with the target analytes at intermediate concentrations [18] by protein precipitation and SPE–LC–MS/MS.

According to the obtained LOQs and linear calibration ranges, deproteination can only be implemented in methods targeted at

Table 1
Features of the calibration models for analysis of vitamin D and metabolites in serum and plasma as a function of sample preparation.

Vit-D_3	Sample	Calibration range	R^2	Equation
SPE	Serum	1 pg mL^{-1}–500 ng mL^{-1}	98.75%	$y = 1.276x - 1.5581$
	Plasma	1 pg mL^{-1}–500 ng mL^{-1}	99.72%	$y = 1.3693x - 0.8741$
Protein precipitation	Serum	1.5 ng mL^{-1}–500 ng mL^{-1}	99.52%	$y = 0.1122x - 0.1758$
	Plasma	1.5 ng mL^{-1}–500 ng mL^{-1}	98.82%	$y = 0.1096x - 0.0339$
Vit-D_2	Sample	Calibration range	R^2	Equation
SPE	Serum	50 pg mL^{-1}–500 ng mL^{-1}	99.23%	$y = 0.1716x - 0.4733$
	Plasma	50 pg mL^{-1}–500 ng mL^{-1}	99.90%	$y = 0.1662x + 0.2503$
Protein precipitation	Serum	1.5 ng mL^{-1}–500 ng mL^{-1}	98.96%	$y = 0.0731x - 0.2493$
	Plasma	1.5 ng mL^{-1}–500 ng mL^{-1}	96.31%	$y = 0.06x + 0.0738$
25(OH)D_3	Sample	Calibration range	R^2	Equation
SPE	Serum	1 pg mL^{-1}–250 ng mL^{-1}	98.08%	$y = 0.5148x - 0.4278$
	Plasma	1 pg mL^{-1}–250 ng mL^{-1}	99.87%	$y = 0.1542x + 0.4620$
Protein precipitation	Serum	0.75 ng mL^{-1}–250 ng mL^{-1}	98.63%	$y = 0.0285x + 0.032$
	Plasma	0.75 ng mL^{-1}–250 ng mL^{-1}	98.34%	$y = 0.0331x + 0.0483$
25(OH)D_2	Sample	Calibration range	R^2	Equation
SPE	Serum	0.25 ng mL^{-1}–250 ng mL^{-1}	98.66%	$y = 0.6219x - 0.5481$
	Plasma	0.25 ng mL^{-1}–250 ng mL^{-1}	99.72%	$y = 0.3908x + 0.7616$
Protein precipitation	Serum	0.75 ng mL^{-1}–250 ng mL^{-1}	98.96%	$y = 0.019x + 0.1157$
	Plasma	0.75 ng mL^{-1}–250 ng mL^{-1}	98.97%	$y = 0.0261x - 0.0293$
24,25$(OH)_2D_3$	Sample	Calibration range	R^2	Equation
SPE	Serum	15 pg mL^{-1}–50 ng mL^{-1}	99.59%	$y = 1.7943x - 0.564$
	Plasma	15 pg mL^{-1}–50 ng mL^{-1}	99.38%	$y = 3.913x - 0.8856$
Protein precipitation	Serum	0.15 ng mL^{-1}–50 ng mL^{-1}	96.96%	$y = 0.1013x + 0.0852$
	Plasma	0.15 ng mL^{-1}–50 ng mL^{-1}	96.84%	$y = 0.1918x + 0.1188$
1,25$(OH)_2D_2$	Sample	Calibration range	R^2	Equation
SPE	Serum	50 pg mL^{-1}–50 ng mL^{-1}	99.10%	$y = 2.0654x - 0.4258$
	Plasma	50 pg mL^{-1}–50 ng mL^{-1}	99.73%	$y = 2.8137x + 1.7614$
Protein precipitation	Serum	0.15 ng mL^{-1}–50 ng mL^{-1}	98.31%	$y = 0.1603x + 0.2784$
	Plasma	0.15 ng mL^{-1}–50 ng mL^{-1}	96.47%	$y = 0.2676x + 0.1188$
1,25$(OH)_2D_3$	Sample	Calibration range	R^2	Equation
SPE	Serum	15 pg mL^{-1}–5 ng mL^{-1}	99.16%	$y = 0.0013x + 0.1445$
	Plasma	15 pg mL^{-1}–5 ng mL^{-1}	98.89%	$y = 0.0043x - 0.09$
Protein precipitation	Serum	0.1 ng mL^{-1}–5 ng mL^{-1}	96.49%	$y = 0.1835x + 0.0116$
	Plasma	0.1 ng mL^{-1}–5 ng mL^{-1}	94.04%	$y = 0.0002x + 0.1976$

the analysis of vitamin D and its metabolites, except 1,25$(OH)_2D_3$. The differences in sensitivity can be justified by the volume of sample injected on-column in each approach and, thus, by the preconcentration effect: 41.6 μL in protein precipitation (by considering all the steps involved in this sample preparation procedure) *versus* 200 μL for the SPE-based method, 4.8 times higher in SPE.

3.2. Differences between serum and plasma for SPE–LC–MS/MS analysis of vitamin D

Once the best performance of the SPE method for analysis of vitamin D was assured, the influence of the type of sample, plasma or serum, on the optimum values of the variables that affect the analysis of vitamin D and metabolites was assessed. The chemical composition of serum and plasma allowed establishing differences in the loading of the sample and the wash of the SPE cartridge. Thus, the composition of the loading solution for serum and plasma was the same: 25% (v/v) ACN in water acidified with formic acid, but the concentration of the acid was 0.7% (v/v) for serum and 0.5% (v/v) for plasma. Concerning the cartridge washing, a slight difference was also observed in the used solution, 30% (v/v) ACN in water for serum and 20% (v/v) for plasma. Differences in the loading and washing steps of the SPE process should be strongly associated to the matrix composition of both samples. The major difference between plasma and serum is the removal of fibrinogen and associated proteins by the coagulation process. The absence of these proteins allows to increase the concentration of organic solvent and formic acid in the SPE process for analysis of serum, while these concentrations enhance protein precipitation in plasma.

Concerning the calibration models built for serum and plasma, particular differences were found in the slopes of the calibration equations for some metabolites, as Table 1 shows. These differences allow establishing a comparison in terms of sensitivity of the method. Thus, the sensitivity was significantly better for the dihydroxymetabolites in plasma as compared to serum; while the opposite effect was observed for the two monohydroxymetabolites. The variability in sensitivity associated to the type of sample could be attributed to matrix interferents that could exert influence on each at a particular retention time.

The SPE–LC–MS/MS methods for analysis of vitamin D and metabolites in serum and plasma were compared in terms of recovery, accuracy and precision. These analytical features were calculated by using pools of serum and plasma spiked at three concentrations representing low, intermediate and high concentrations of the target analytes, as Table 2 shows, according to the literature [18]. The configuration of the SPE approach used for the recovery estimation was based on the coupling of two cartridges, as Supplementary Fig. 1B illustrates [21–23]. The average values obtained for both types of samples at the three spiked

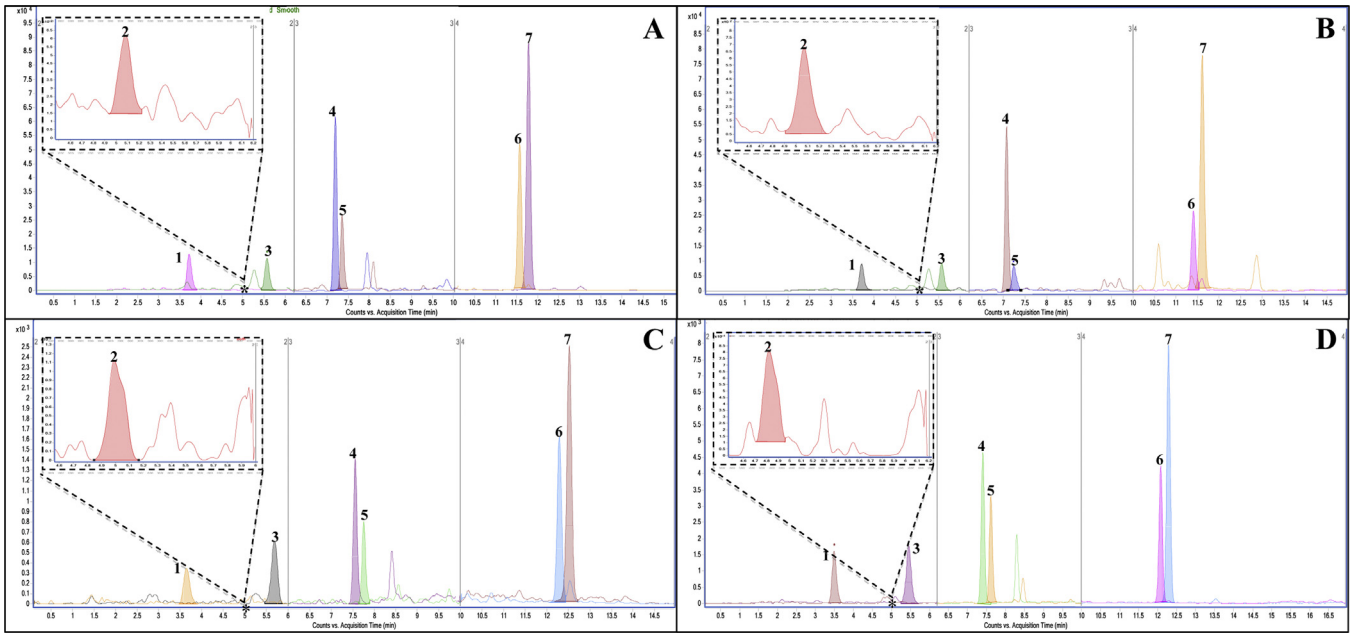

Fig. 1. SRM chromatograms obtained by analysis of (A) serum with the SPE–LC–MS/MS method, (B) plasma with the SPE–LC–MS/MS method, (C) serum with protein precipitation prior to LC–MS/MS and (D) plasma with protein precipitation prior to LC–MS/MS spiked with the target analytes at intermediate concentrations. (1) 24,25 $(OH)_2D_3$ (10 ng mL^{-1}), (2) 1,25$(OH)_2D_3$ (0.1 ng mL^{-1}), (3) 1,25$(OH)_2D_2$ (10 ng mL^{-1}), (4) 25$(OH)D_3$ (50 ng mL^{-1}), (5) 25$(OH)D_2$ (50 ng mL^{-1}), (6) vitamin D_2 (50 ng mL^{-1}) and (7) vitamin D_3 (50 ng mL^{-1}).

Table 2
Concentrations of the target analytes in three spiked serum and plasma pools (low level, intermediate level and high level in concentration terms) for average estimation of the recovery factor and the accuracy.

Analyte	Low level (ng mL^{-1})	Intermediate level (ng mL^{-1})	High level (ng mL^{-1})
Vit D_3	15	50	100
Vit D_2			
25$(OH)D_3$	15	50	100
25$(OH)D_2$			
24,25$(OH)_2D_3$	3	10	20
1,25$(OH)_2D_2$			
1,25$(OH)_2D_3$	0.05	0.1	0.2

concentrations are shown in Table 3. The recovery factor was practically 100% for the three monitored dihydroxymetabolites either in serum or plasma. A slight decrease in the recovery factor was observed for the monohydroxymetabolites as their retention time increased, which was more significant in the case of 25$(OH)D_2$ when determined in plasma. Thus, the recovery factor was 98.8 and 97.2% for 25$(OH)D_3$ in serum and plasma, respectively; while this parameter was 97.2 and 83.0% for 25 $(OH)D_2$ in serum and plasma, respectively. The decrease of the recovery factor as the retention time increased was more significant for vitamins D_2 and D_3, as this parameter was around 60% both in serum and plasma. The higher polarity of the didydroxymetabolites as compared to the monohydroxymetabolites and the two forms of vitamin D allows obtaining an efficient retention of the dihydroxylated forms, which is of paramount importance to address their quantitative analysis taking into account their low levels in biological samples.

The accuracy and potential matrix effects were studied by the configuration described in Supplementary Fig. 1A. The average accuracy values were above 89% for all analytes in the case of serum, except for vitamin D_2 that yielded 83.3% (see Table 3). For plasma, the accuracy values ranged were above 90.1%. In general, the accuracy calculated for most analytes was slightly better in plasma than in serum, except for vitamin D_3.

Table 3
Average values of recovery factor and accuracy estimated in serum and plasma pools spiked at three concentrations (Table 2) by the SPE–LC–MS/MS methods.

Sample	Recovery factor (%)						
	Vit-D_3	Vit-D_2	25$(OH)D_3$	25$(OH)D_2$	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	60.1	59.0	98.8	97.2	100	100	99.8
Plasma	58.6	56.4	97.2	83.0	100	100	99.7
Sample	Accuracy (%)						
	Vit-D_3	Vit-D_2	25$(OH)D_3$	25$(OH)D_2$	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	93.5	83.3	96.3	89.3	89.3	95.9	90.9
Plasma	90.1	104.1	96.4	95.6	96.9	97.5	105.1

Table 4
Precision study by estimation of within-day and between-days variability (%) in serum and plasma spiked with the target analytes at intermediate levels by application of the SPE–LC–MS/MS methods.

Sample	Within-day (%)						
	Vit-D_3	Vit-D_2	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	2.1	6.0	7.2	4.4	3.2	3.9	6.4
Plasma	3.9	11.5	4.5	2.0	1.5	2.9	8.0
Sample	Between-days (%)						
	Vit-D_3	Vit-D_2	25(OH)D_3	25(OH)D_2	24,25$(OH)_2D_3$	1,25$(OH)_2D_3$	1,25$(OH)_2D_2$
Serum	11.6	8.9	9.9	5.3	8.2	7.8	11.3
Plasma	7.1	12.9	8.3	3.5	2.6	9.1	8.9

The precision for each type of sample was evaluated under two experimental conditions: within-day variability and between-day variability. For this purpose, a single experimental set-up with duplicate analysis per day was carried out with pools of serum and plasma spiked with the target analytes at intermediate levels (see Table 2) for 7 days. The results thus obtained are summarized in Table 4, showing that the precision was below 12.9% and, this analytical feature was not influenced by the type of sample: serum or plasma.

3.3. Influence of the tube used for blood collection

The influence of the blood collection tube on the determination of vitamin D and metabolites was evaluated by analysis of serum and plasma samples collected in conventional tubes (spray-coated silica tubes) and in polymer-gel tubes. The concentrations of vitamin D and metabolites found in the four types of samples obtained from the target cohort are listed in Table 5. A paired *t*-test (95% confidence level) was applied to check the existence or absence of statistical differences between levels of the target analytes in serum or plasma collected in conventional and gel tubes. It is worth mentioning that this study was based on the determination of vitamin D_3 and metabolites —25OHD_3, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$— since vitamin D_2 and metabolites were not detected in the volunteers of the cohort as they were not receiving vitamin D supplement. In the case of serum, conventional and gel tubes could be indistinctly used for quantitative analysis of vitamin D. On the contrary, plasma analysis led to detect statistical differences in the analysis of vitamin D_3 depending on collection, which is currently out of the scope of clinical tests. Fig. 2 shows that the levels of vitamin D_3 in plasma tubes were always higher than in plasma-gel tubes. Therefore, the use of plasma and plasma-gel tubes is not critical for quantitative determination of vitamin D metabolites, which are the main objective from a clinical perspective.

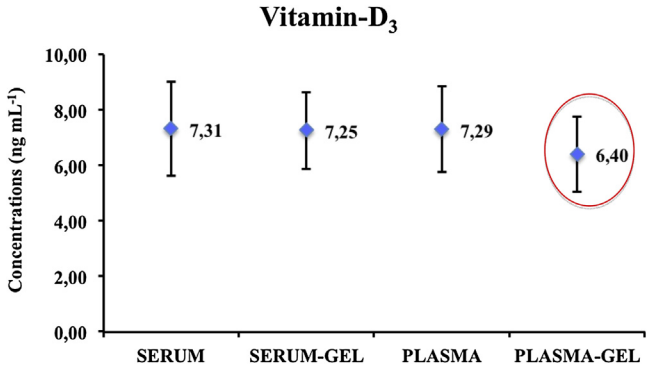

Fig. 2. Concentration of vitamin D_3, expressed as ng mL^{-1}, in samples from the selected cohort collected by using the four types of tubes.

Table 5
Minimum, maximum and mean concentrations of the target analytes found in four types of samples (collected in serum, serum-gel, plasma and plasma-gel tubes) from the selected cohort.

Analyte (serum tube)	Minimum	Maximum	Mean
Vitamin D_3 (ng mL^{-1})	4.9	11.4	7.3
25(OH)D_3 (ng mL^{-1})	13.8	45.9	29.2
24,25$(OH)_2D_3$ (ng mL^{-1})	2.0	7.9	4.3
1,25$(OH)_2D_3$ (pg mL^{-1})	50.3	111.6	103.7
Analyte (serum-gel tube)	Minimum	Maximum	Mean
Vitamin D_3 (ng mL^{-1})	5.0	9.5	7.3
25(OH)D_3 (ng mL^{-1})	20.6	46.4	30.0
24,25$(OH)_2D_3$ (ng mL^{-1})	1.3	6.7	4.2
1,25$(OH)_2D_3$ (pg mL^{-1})	51.7	174.4	109.2
Analyte (plasma tube)	Minimum	Maximum	Mean
Vitamin D_3 (ng mL^{-1})	4.7	10.3	6.9
25(OH)D_3 (ng mL^{-1})	15.9	49.2	31.5
24,25$(OH)_2D_3$ (ng mL^{-1})	1.5	6.4	4.3
1,25$(OH)_2D_3$ (pg mL^{-1})	160.5	228.1	160.3
Analyte (plasma-gel tube)	Minimum	Maximum	Mean
Vitamin D_3 (ng mL^{-1})	4.3	9.4	6.4
25(OH)D_3 (ng mL^{-1})	15.4	41.3	31.5
24,25$(OH)_2D_3$ (ng mL^{-1})	1.9	7.9	4.3
1,25$(OH)_2D_3$ (pg mL^{-1})	73.4	235.0	152.8

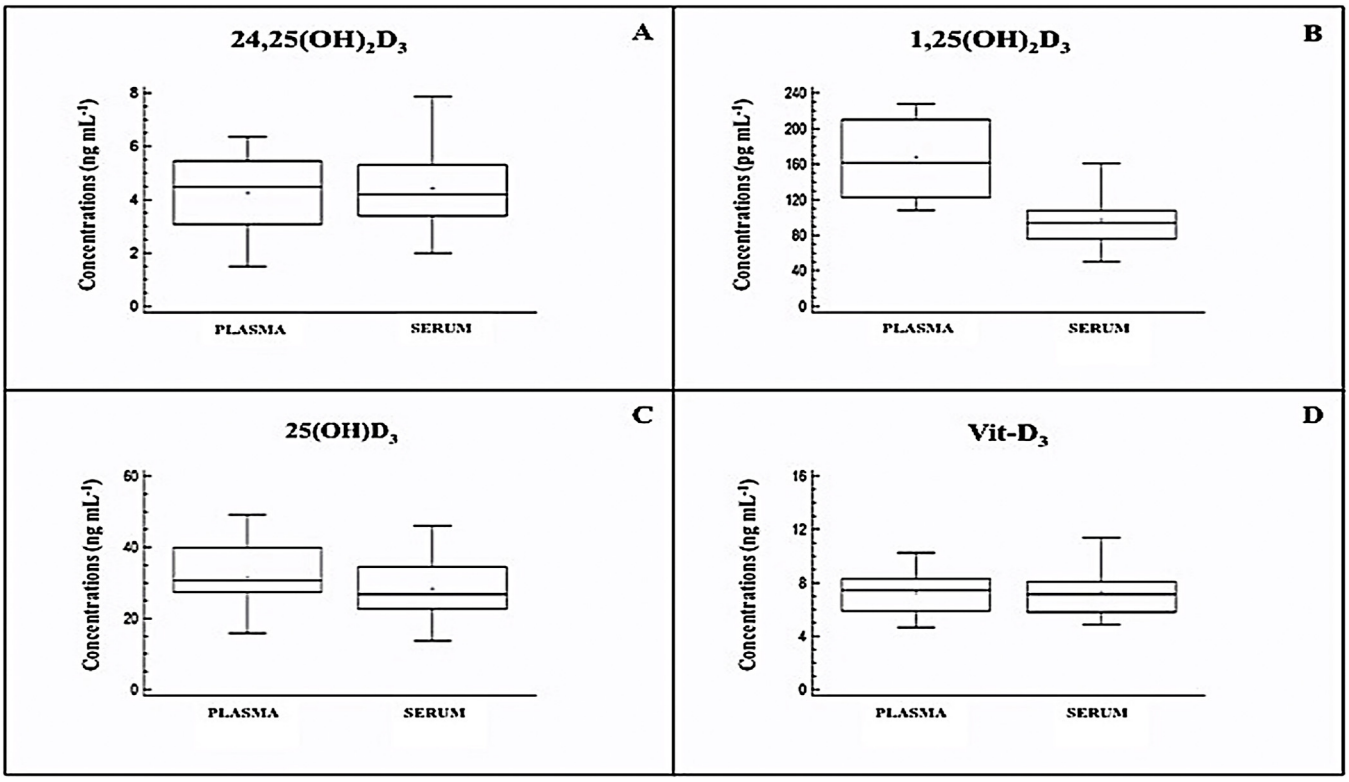

Fig. 3. Box-and-whisker plots presenting the concentrations of (A) $24,25(OH)_2D_3$, (B) $1,25(OH)_2D_3$, (C) $25OHD_3$ and (D) vitamin D_3 found in plasma and serum from the cohort of volunteers.

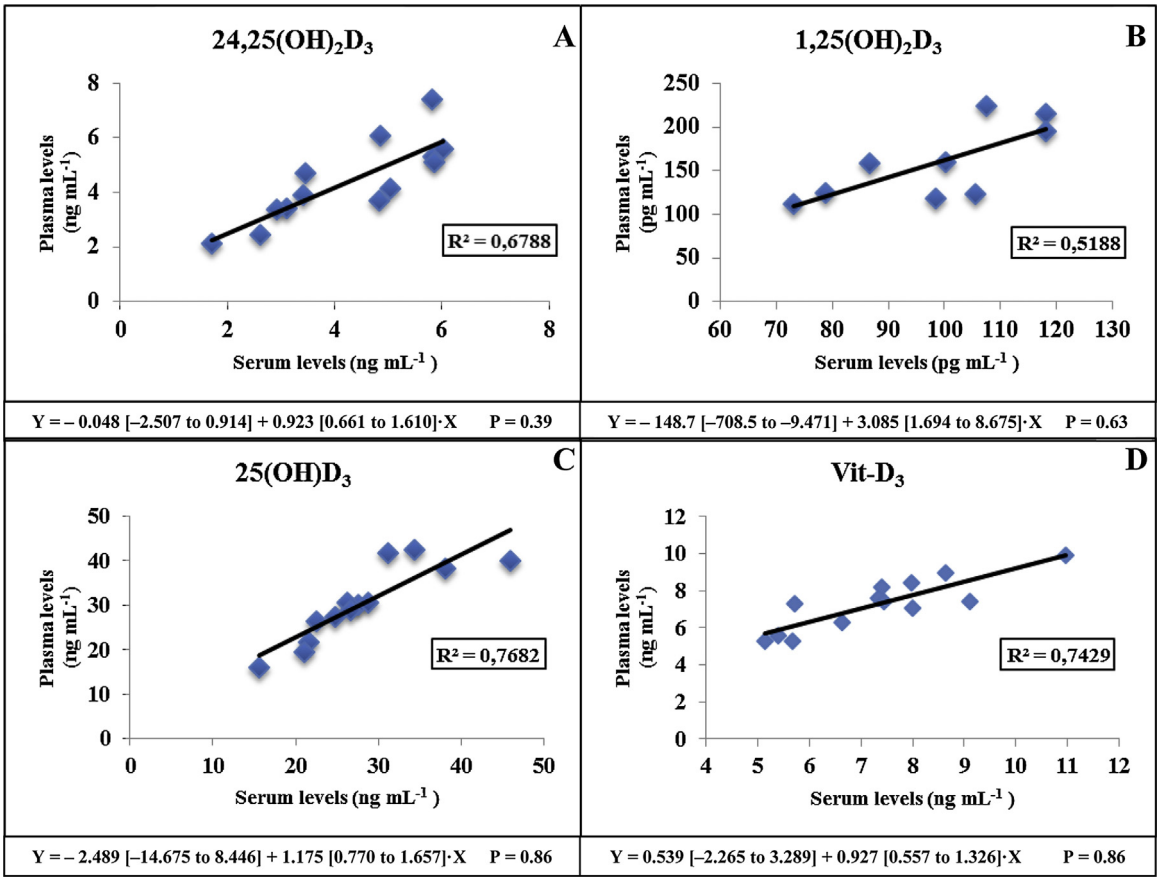

Fig. 4. Passing–Bablok regression analysis for levels of (A) $24,25(OH)_2D_3$, (B) $1,25(OH)_2D_3$, (C) $25OHD_3$ and (D) vitamin D_3 obtained in serum and plasma of the volunteers.

3.4. Comparison of the use of plasma or serum for quantitative analysis of vitamin D

The levels of vitamin D_3 and metabolites in plasma and serum samples of the cohort were also statistically compared by the paired *t*-test (95% confidence level). Fig. 3 shows the concentration ranges of vitamin D_3 and metabolites —particularly, $25OHD_3$, $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$— found in the cohort of volunteers. The *t*-test allowed detecting no statistical differences between serum and plasma levels of vitamin D_3, $25OHD_3$ and $24,25(OH)_2D_3$ metabolites, but statistical differences between levels of $1,25(OH)_2D_3$ measured in serum and plasma. Thus, the concentration of this metabolite, which is characterized by lower concentrations than the resting metabolites, varied from 111.5 to 226.2 pg mL^{-1} in plasma; while its range was quite lower in serum: from 52.7 to 152.6 pg mL^{-1}. Therefore, plasma seems to be more suited than serum for quantitative analysis of $1,25(OH)_2D_3$, which is important taking into account that quantitation of this dihydroxymetabolite is the most limiting aspect of methods for assessment of vitamin D status.

A Passing–Bablok regression analysis [24] for each analyte was carried out with the levels measured in serum and plasma of the volunteers. Fig. 4 illustrates the regression graphs thus obtained, as well as the values of the main statistical parameters. The regression coefficients ranged from 0.518 for $1,25(OH)_2D_3$ to 0.768 for $25(OH)D_3$. The validity of the linear models was studied by the Cusum test, which reported no significant deviation from linearity for vitamin D_3 and its three metabolites (*p*-value of 0.39 for $24,25(OH)_2D_3$ and above 0.86 for the rest of the analytes).

4. Conclusions

The developed research has allowed elucidation of key aspects on collection and preparation of blood for analysis of vitamin D and its metabolites. Thus, sample preparation based on SPE provides lower quantitation limits for all the target analytes than deproteination.

The type of sample influences the sensitivity of the method since plasma is better for determination of the dihydroxymetabolites, while the two monohydroxymetabolites are determined with better sensitivity in serum.

Statistical comparison of the use of plasma or serum as target sample has shown that the concentration of $1,25(OH)_2D_3$ in plasma was higher than that in serum. This finding is important taking into account that quantitation of this dihydroxymetabolite is the most limiting aspect of methods for assessment of vitamin D status. The use of collection tubes with or without coating gel shows significant statistical differences only for vitamin D_3 in plasma, which yielded a higher concentration when the sample was collected in conventional plasma tubes as compared with plasma-gel tubes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.03.012.

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