Quantitative determination of 25-hydroxyvitamin D₃ in human plasma using Reversed Phase High Performance Liquid Chromatography (RP-HPLC): an easy and useful method for routine analysis

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Abstract

Determining human vitamin D levels by measuring 25(OH) D3 is challenging in various laboratories around the world. Our study aimed to develop and validate a reversed phase-high performance liquid chromatography (RP-HPLC) method characterized by accuracy, sensitivity, and ease of use to measure 25(OH) D3 in human blood plasma. The chromatographic separation was conducted using a reversed phase C18 column (250mm × 4.6 mm, 5 μm particle size) at 25°C with a mixture of acetonitrile and methanol (90:10, %v/v) as mobile phase in isocratic elution mode at a flow rate of 1 mL/min and photo diode array detection at 265 nm. The linearity of the method was excellent (R^2= 0.9999) over the concentration range of 10–100 ng/mL with lower limit of detection (LOD) of 0.5 ng/mL and lower limit of quantification (LOQ) of 1.5 ng/mL. The percentage coefficients of variation (%CV) for intraday and inter-day accuracy were ≤ 4.8 %, showing that the method provided good reproducibility and repeatability. The percentage recovery was 102.85%, indicating the precision and accuracy of the study. Values obtained through validation parameters conform to the Recommendations and Acceptance Criteria for Bioanalytical Method Validation by the US Department of Health Human Services Food and Drug Administration (FDA). Our method is characterized by a small plasma volume (200 µL), which is very practical, especially for pediatric application, in addition to the speed of the sample preparation phase based on protein precipitation and extraction by small amount of solvents that makes it low cost and less harmful to the environment.

Keywords: Vitamin D, 25(OH) D3, RP- HPLC, protein precipitation, blood plasma

1. Introduction

The measurement of vitamin D metabolites continues to be of great importance in many laboratories around the world [1]. Over the past years, this vitamin has been linked to human health and well-being. Vitamin D is a fat-soluble steroid hormone, it is found naturally in few foods, and it is also available as a dietary supplement [2]. Vitamin D deficiency is a common problem, especially for elderly people and people with severe liver and kidney disease. However, what is striking now is the exacerbation of the deficiency of this vitamin in the general population due to the lack of exposure to sunlight and poor nutritional status in addition to the excessive use of sunscreens [3]. In addition to the known associations of vitamin D with bone disorders, rickets and osteoporosis, its deficiency is related to other conditions such as high blood pressure, diabetes, cancer, multiple sclerosis, depression, and falls [4]. Vitamin D exists in humans in two forms, D3 (cholecalciferol), which is

produced in the skin by a photochemical reaction of the sun's ultraviolet rays with 7-dihydrocholesterol and D2 (ergocalciferol), which is found in plants and used as a food supplement. Several studies have been published that show the difference between the two forms, some of which indicated that cholecalciferol is more effective in humans than ergocalciferol [5]. D3 and D2 are metabolized to 25(OH) D3 and 25(OH) D2 in the liver, after which they are converted to the biologically active form 1, 25(OH)D2 in the kidneys [3]. For several reasons, 25(OH) D3 is the most useful marker for evaluating plasma vitamin D [1], first the half-life of 25(OH) D3 is long when compared to other metabolites as it is an accurate indicator of the vitamin D obtained, whether from ultraviolet radiation or food, over a certain period [6]. Second, the liver, a member that produces this metabolite, does not regulate the amounts excreted from it, as the resulting amount is determined by the concentration of the

substrate D3 cholecalciferol [6]. The process of measuring vitamin D and its metabolites has been described as complex, due to several obstacles, the most prominent of which is the hydrophobic nature of these molecules and their association with vitamin D binding proteins in addition to the effect of temperature and ultraviolet rays. The composition of biological samples and the similarity of metabolites in terms of structural composition is a major obstacle for the analysts [7]. In the past years, many different analytical methods have been developed to measure vitamin D, and we mention, for example, spectrophotometry [8], colorimetry [9], fluorimetry [10], capillary electrophoresis [11, 12], immunoassay and chromatographic methods [13]. If we examine most clinical laboratories, we find the most used are immunochemical methods, due to the simplicity of their use [14]. In contrast, it is not without flaws, as the interaction of antibodies in this technique cannot differentiate between the metabolites of 25(OH) D3 and 25(OH)D2 [1]. On the other hand, there are chromatographic methods such as liquid chromatography coupled with mass spectrometry (LC-MS/MS) that have shown more accuracy and reliability in their analytical performance compared to the previously mentioned immunological methods, but they still require a highly efficient staff during their use. Therefore, the need to develop an easy-to-use and highly sensitive method to measure 25(OH) D3 is of great scientific and clinical value. Our study aims to develop a RP-HPLC method for analyzing 25(OH) D3 in human blood plasma with one step protein precipitation method, using micro-volumes of both organic solvent mixture and plasma. This result is useful for clinical and research laboratory. In addition to make HPLC more expanding in clinical laboratories and especially in paediatric laboratory medicine, the present analytical method improves sample preparation process by using low-cost material and small plasma amount, which was provided by few previously published methods [15].

2. Materials and methods

2.1. Materials and reagents

The 25(OH) D3 standard was purchased from Sigma-Aldrich. Acetonitrile (ACN) and methanol (MeOH) HPLC grade were purchased from BIOCHEM Chemo pharma. All other reagents were A.R. grade. Milli-Q water was used.

2.2. Instruments and HPLC conditions

A Shimadzu (Prominence liquid chromatograph LC-20AD) series HPLC system equipped with PDA detector (Prominence SPD-M20A) was employed for the chromatographic analysis. An instrument control CBM-20A communication bus module was utilized. For analytes separation Shim-pack RP-C18 analytical column 4.6×250 mm, 5.0 µm particle size (Japan), the temperature of separation was controlled using a column Oven (Prominence CTO-20AC). The mobile phase composed of 90 % acetonitrile and 10 % of methanol was used in isocratic elution mode at 25°C with a flow rate of 1 mL/min. The detection was carried out at 265 nm. Data collection and quantification were interpreted using LC solution software.

An auto sampler (Prominence SIL-20AC) was used for the injection of 100 µL of the final extracts samples.

2.3. Procedure

Stock standard solution of 25(OH) D3 was prepared in mobile phase and stored at -20°C. Working standards were prepared from single standard solution with appropriate dilution in the same solvents mixture. To prepare sample, 200 µL of plasma was placed into Eppendorf tube (1.5 mL), then 300 µL acetonitrile-methanol (9:1, v/v) solvents mixture was added for both the precipitation and extraction. After vortex mixing for 90s, the solution was centrifuged at 12000 rpm for 10 min. The supernatant was separated and filtered, then placed into auto-sampler glass vial for HPLC analysis.

3. Results and Discussions

3.1. Chromatographic Conditions Optimization

With regard to the effect of the mobile phase composition, it was observed that the ratio acetonitrile and methanol (70:30, v/v) provided overlapping chromatographic peaks, which means a bad separation of the compounds. Reducing the methanol content in the mobile phase to 20 % led to more resolution and clarity for the separated compounds, but with a longer retention time. Finally, acetonitrile and methanol (90:10, v/v) mobile phase composition was chosen to obtain perfect chromatogram with a clear 25(OH) D3 peak shape and shorter retention time. This is explained by a higher elution strength and selectivity of acetonitrile than methanol for 25(OH) D3 molecules in reversed phase chromatography. Since there is a proportional relationship between the flow and the column backpressure, we have selected a mobile phase flow of 1 mL/min and a column temperature of 25°C to match it with a moderate value of the column backpressure and separation conditions.

3.2. Extraction Procedure Optimization

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are the most common and prominent methods used to extract 25(OH) D3 from blood plasma. Although SPE is an effective and very practical to separate interfering compounds in the plasma, but the high cost is an impediment to routine use. The use of the liquid-liquid extraction method using micro-volumes of samples and reagents is an effective solution to separate target compounds from plasma matrix. A few amount of organic solvents such acetonitrile, methanol are used to extract the target compounds from the mixture. According to the previous studies completed, there is a difference in the volume of plasma used, the composition of the solvents selected, and its volume. Three different solvents mixtures have been tested in addition to diverse volumes of plasma and added extraction solvents. These multiple tests aim to obtain the optimal method of extraction, taking into account both the amount of 25(OH) D3 extracted, the volume of solvent used, time and costs. The results obtained from this study showed that the use of the (acetonitrile- methanol) solvents composition gave a high peak area value compared to the other tested mixtures (Table 1), this is due to the elution force and selectivity of extraction with methanol. An increase in the peak area value

is observed as the greater amount of plasma used as shown in (Table 2). There are two reasons for choosing 200 µL as a plasma volume. First to minimize the interference from plasma matrix with 25(OH) D3 during chromatographic separation and second for the possibility to apply the method in clinical laboratories and especially in Paediatric Laboratory Medicine. Using the volume of extraction solvent (acetonitrile - methanol) 300 µL, it was observed that the amount of extracted 25(OH) D3 was high when compared to the same solvents mixture ratio at a quantity of 200 µL, which is explained on the basis of the insufficiency in the amount of solvents needed for extraction. So, adding more solvent mixture make the extraction more efficient which mean that more 25(OH) D3 molecules were attracted to the acetonitrile-methanol phase. The decrease in the amount of 25 (OH) D3 extracted using 400 µL of solvents mixture (Table 3) is explained with the increase in methanol amount, which leads to the formation of finer precipitates that affect negatively the filtration step in the extraction process [16].

3.3. Method Development and Validation

Typical chromatogram of 25(OH) D3 plasma sample is shown in figure 1. The retention time for 25(OH) D3 was 6.4 min and the total analysis time was 20 min typical for RP-HPLC assay. No other interference was observed with the retention time of 25(OH) D3. For the evaluation of analytical performance, we selected the following validation parameters: Precision data, linearity, limit of detection (LOD), limit of quantification (LOQ) and average recovery for 25(OH) D3.

3.4. Linearity and Range

The linearity of the method was determinate using seven 25(OH) D3 standards solutions in the range of 10-100 ng/mL to match the 25(OH) D3 range in human blood plasma (Fig. 2). Table 4 shows the calibration curve parameters. The calibration curve was linear over the studied range, the equation y= 211.36x + 24.76 was obtained with a correlation coefficient of $r^2 = 0.9999$ indicating the good linearity of the developed method.

3.5. Accuracy and Recovery

The analytical accuracy was estimated at 86.5 ng/mL, and the coefficient of variation (CV) was 3.7 %. For the recovery study, an amount of 25(OH) D3 standard was added to six plasma samples replicas with the same initial concentration. The recoveries mean was 102.8 % in a range from 99.4 % to 109.9 % with a standard deviation (SD) and CV of 3.8 % and 3.7 % respectively, which indicates the efficiency of the extraction method used and its applicability (Table 4).

3.6. Precision

The intraday data precisions were calculated from five (n = 5) determinations of plasma samples performed under the same experimental conditions and on the same day. For the estimation of analysis variations, the inter-day precisions were performed using ten plasma (n = 10) replicas on different days. The precision results were given as SD and CV in Table 5. The percentage coefficients of variation for intraday and inter-day accuracy were ≤ 4.8 %, showing that the method provided good reproducibility and repeatability.

3.7. Limits of Detection (LOD) and Quantification (LOQ)

LOD and LOQ were calculated using equations (1) and (2) respectively:

$$LOD = 3.3 \times \frac{SD}{S} \qquad (1)$$
$$LOQ = 10 \times \frac{SD}{S} \qquad (2)$$

where SD is the standard deviation of calibration curves intercepts and S is the slope of the calibration curve. The LOD and LOQ for 25(OH) D3 in the developed method were 0,5 ng/mL and 1,5 ng/mL respectively (Table 6). Over the past years, there have been many cases of vitamin D deficiency in the world, both in adults and children. This matter required many doctors and researchers to evaluate its levels [17], especially with the emergence of many methodologies and explanations associated with the apparent spread of vitamin D tests [18]. The difficulty in measuring vitamin D metabolites in plasma is due to several factors, the nature of the lipophilic molecules, the association with DBP and also the low concentration [19, 20]. We have developed an RP-HPLC method characterized by the speed in both the sample preparation and analysis time, in addition to the high sensitivity for accurate measurement of 25(OH) D3 metabolite in blood plasma at nanogram levels. At present, the widespread use of immunochemical assays tests can be explained by their low cost and speed [21]. The LC-MS/MS methods have imposed their use as a reference tool in several laboratories [18], but the primary explanation for the not use of it in clinical laboratories is the high cost of this technology and the need for highly experienced specialists to run it. Hence, HPLC is the most suitable tool for its technical simplicity and relatively lower cost when compared to LC-MS/MS [5]. With the preparation of the plasma sample by precipitating the protein followed by the extraction process and using HPLC equipment, measuring 25(OH) D3 becomes easier and more practical for the routine [17]. Based on this, the need arose for a method characterized by simplicity and accuracy in measuring 25(OH) D3, in addition to the possibility of applying it between different laboratories. Our internally developed method is based on simple and more practical steps that rely on a small amount of 200 µL of plasma with the addition of both protein precipitation and extraction factor in order to obtain an extract ready for analysis in a record time of 20 min. The volume of the plasma sample used in our analytical method is small compared to previous studies [5, 15, 22], and this feature recommends it for convenient use in pediatrics. By comparing the analytical performance of our method in terms of accuracy, linearity, LOD and LOQ, we find that it is compatible with other scientific publications [14]. This method provides a good recovery, precision, limit of detection and quantification and complies with standards and recommendations approved by the Bioanalytical Method Validation by the US Department of Health Human Services Food and Drug Administration [23]. Currently, the use of RP-HPLC technique for analyzing vitamins in different types of matrices is rapidly and clearly due to its selectivity, its speed and accuracy in separating compounds, in addition to its high analytical efficiency.

Table 1. Optimization of extraction solvents composition with ACN, ACN-H_2O and ACN- MeOH.

Plasma volume (µL)	Extraction solvents mixtures	Ratio (v/v)	25(OH) D3 peak area (AU)
200	ACN	-	522
200	ACN – H_2O	9:1	1710
200	ACN - MeOH	9:1	1933

Table 2. Peak area of 25(OH) D3 extracted with ACN - MeOH (9:1 v/v) from different plasma volumes.

Plasma volume (µL)	Volume of the extraction solvents mixtures ACN - MeOH (µL)	Peak area (AU)
100	300	601
150	300	1332
200	300	1602

Table 3. Optimization of extraction solvents volume with ACN - MeOH (9:1 v/v)

Plasma volume (µL)	Volume of the extraction solvents mixtures ACN - MeOH (µL)	25(OH) D3 peak area (AU)
200	200	1572
200	300	1752
200	400	1203

Table 4. Recovery of 25(OH) D3 in spiked plasma sample

Plasma samples replicas (45 ng/mL)	Recovery			
	% Recovery	Mean	SD	%CV
1	100.05	102.85	3.85	3.74
2	109.94			
3	101.10			
4	99.41			
5	102.72			
6	103.86			

Table 5. Intra- and inter-day precision for plasma

25(OH) D3 plasma samples concentration (ng/mL)	Intraday			Inter-day		
	n	SD	%CV	n	SD	%CV
46.5	5	2.25	4.8	10	2.06	4.42

Table 6. Regression analysis of 25(OH) D3 calibration curve

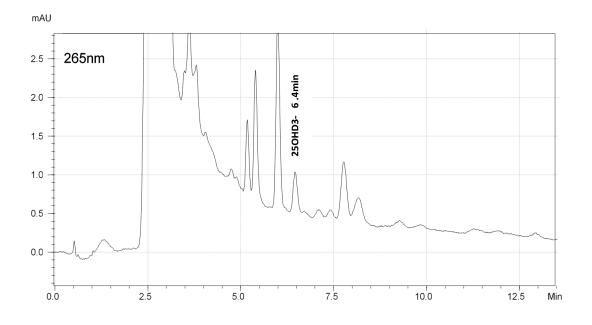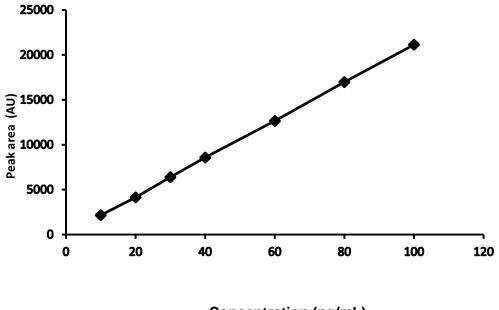| Parameters	Result
Number of concentrations per curve	7
Linearity range	10-100 ng/mL
Regression equation	y= 211.36x + 24.76
Slope	211.36
Intercept	24.76
Regression coefficient r^2	0.9999
Limit of detection (LOD) (ng/mL)	0.5
Limit of quantification (LOQ) (ng/mL)	1.5

Figure 1. Representative RP-HPLC-chromatogram of extracted plasma sample

Figure 2. Calibration curve of 25(OH) D3 standard solutions (y= 211.36x + 24.76, $r^2 = 0.9999$)

4. Conclusions

The proposed method is able to analyze 25(OH) D3 in blood plasma in a short time, accurately, and especially at a lower cost, and this makes it suitable for daily use in clinical and research analyzes.

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