An Improved and Sensitive Method for Vitamin D3 Estimation by RP-HPLC

Subodh Kumar1, Diwesh Chawla1 and Ashok Kumar Tripathi2*

1Central Research Laboratory, Multi-disciplinary Research Unit, University College of Medical Sciences, Delhi-110095, India
2Biochemistry and Immunology Laboratory, Department of Biochemistry, University College of Medical Sciences, Delhi-110095, India

Abstract

Despite plenty of sunlight, vitamin D deficiency (VDD) in India is an epidemic. 50-90% populations among all age groups are associated with VDD. Among the common methods (RIA, immunoassay etc.) available for vitamin D estimation, the analytical method like HPLC is considering as a gold standard. In the proposed study, we have developed a RP-HPLC method for the estimation of vitamin D3 with greater precision and accuracy. Separation was achieved on C18 column in isocratic mode using two different mobile phases i.e. acetonitrile: methanol (method I) and methanol: water with 0.1% formic acid (method II). The column was maintained at 40 °C and the mobile phase was pumped at flow rate of 0.4 mL min⁻¹. The detection of eluent was carried out at λmax 265 nm. Retention time of vitamin D3 for method I and II was found to be 7.14 and 7.01 minutes, respectively, with R²=0.99. The standard curves were linear over the concentration range of 0.5-5 ng mL⁻¹. The LOD and LOQ values for vitamin D₃ for method I and II were found to be 1.64, 5.02 and 1.10, 3.60 ng mL⁻¹, respectively. The percentage recovery was found to be 69-79% and 75-87% for method I and II, respectively. The % RSD of intra and inter-day precision of method I was found <2 and <7%, whereas, for method II, <2 and <4% respectively. In conclusion, method II showed greater precision and accuracy and also cost effective, therefore, it can be used for vitamin D₃ estimation at laboratory scale.

Keywords: Vitamin D₃; RP-HPLC; Development and validation; DAD

Introduction

Vitamin D₃ (cholecalciferol) is synthesized endogenously from 7-dehydrocholesterol after ultraviolet irradiation or is absorbed from the diet [9,10]; plant/yeast derived ergocalciferol (Vitamin D₂) is formed exogenously by irradiation of ergosterol. Vitamin D plays an important role in the maintenance of normal levels of calcium and phosphorus in the blood stream and is essential for the proper development and maintenance of bone [11]. Scientific evidence revealed that, it is not only associated with skeletal disorder but also plays an important role in cancer, cardiovascular disease, autoimmune disease, hypertension, diabetes mellitus etc. [12-14]. Vitamin D is not a single compound but is a family of compounds that exhibit Vit D activity. Its measurement is important as a clinical indicator of nutritional vitamin D deficiency, which is one of the causes of osteoporosis.

Clinical laboratory scientists have a diverse selection of Vit D testing methods from which to choose. The routine methods for measurement of vitamin D₃ concentration in human plasma were based on competitive protein binding and used vitamin D-binding protein and a tritium-labelled tracer. These methods were replaced by a simpler, rapid RIA, and a radio iodinated tracer was incorporated into the RIA in 1993 [15]. Quantitative HPLC assays have been developed based on

*Corresponding author: AK Tripathi, Biochemistry and Immunology Laboratory, Department of Biochemistry, University College of Medical Sciences (University of Delhi) and G.T.B. Hospital, Dilshad Garden, Delhi-110095, India, Tel: +91-11-22582972-74 Ext 5210, +91- 9811259019; Fax: +91-11- 22590495; E-mail: aktripathicms@gmail.com

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ultraviolet detection and reverse-phase separation. Recently, RP-HPLC methods for vitamin D3 in human plasma have been developed [16].

In contrast, to other areas of research like food analysis, the major challenge in the clinical study is the limitation of sample size. In spite of that, most of the available methods for vitamin D3 estimation has been developed on LC-MS/MS which increases the per sample cost of analysis. Therefore, we need to develop a simple, sensitive and economic RP-HPLC method of vitamin D3 estimation.

Here, we have developed and described a comparative study between two methods for measurement of vitamin D3 through HPLC. The present study was designed to make vitamin D estimation process easy to use, sensitive, rapid and economical at laboratory scale.

**Material and Methods**

**Apparatus**

The HPLC system used throughout this study consisted of a quaternary pump (Ultimate 3000, Thermo Fischer Scientific, USA), a manual sample injector with a 20 μL sample loop, a degasser and photodiode array detector (Thermo Fisher Scientific, USA). The evaluation and quantification of output signals were made on Chromelion software version 6.80 which controls the whole liquid chromatographic system. The column used was a reversed phase, AcclaimTM 300, C18 column (150 x 4.6 mm i.d., 3μm particle size, 300 Å diameter), procured from Thermo Fischer Scientific, USA. Two different mobile phases were used in the study, which were acetonitrile (pH 5.19)/methanol (pH 4.7) (95:5%) and methanol with 0.1% formic acid (pH 3.0)/water with 0.1% formic acid (pH 2.83) (95:5%) respectively. The samples were passed at a flow rate of 0.4 mL min−1 mobile phase with a time window of 10 min. The column temperature was held constant at 40°C. The results were monitored at 265 nm wavelength for vitamin D3.

**Materials**

Chemicals and solvents used throughout this study such as methanol (Sigma Aldrich, Sweden) and acetonitrile (Sigma Aldrich, Sweden) were of high quality and HPLC grade. Vit.D3 standard was obtained from Sigma Aldrich, Sweden.

**Standard preparation**

A stock solution mixture of vit.D3 standard was prepared (1 mg mL−1) in methanol and stored at -20°C. Working solution mixture was prepared by diluting the stock solution in acetonitrile.

**Vitamin D extraction**

Vitamin D extraction was done by slight modification in the method of Turpeinen et al. [17]. To 0.5 ml of sample/matrix, we added 350 μl of methanol and 2-propanol in the ratio of 80:20 (v/v). The contents were mixed in a vortex mixer for 30s. Vitamin D was extracted by mixing two times (60 s each time) with 1 ml of hexane. The phases were separated by centrifugation, and, the upper organic phase was transferred to a conical tube and dried under nitrogen. The residue was dissolved in appropriate volume of mobile phase.

**Linearity**

A stock solution of 1mg mL−1 Vit D3 was prepared by dissolving 1mg Vit D3 lyophilized standard in 1mL of methanol and then solutions of different concentrations (0.5-5 ng mL−1) for construction of calibration plots were prepared from this stock solution. The mobile phase was filtered through a 0.45 µm membrane filter and passed through column at 0.4 mL min−1 for column equilibration; the baseline was monitored continuously during this process. Detection was carried out at λmax 265 nm. The prepared dilutions were injected in series, peak area was calculated for each dilution, and concentration was plotted against peak area.

**Accuracy**

Accuracy was determined by the standard addition method. Matrix (1X PBS + 0.1% BSA, pH 7.2) was spiked with 3.5 and 15 ng mL−1 standard and the mixtures were analysed by the proposed method. The experiment was performed in triplicate. Recovery (%), RSD (%), and standard error (SE) were calculated for each concentration.

**Precision**

Precision was determined as both repeatability and intermediate precision, in accordance with ICH recommendations. Repeatability of sample injection was determined as intra-day variation and intermediate precision was determined by measurement of inter-day variation. For intra and inter-day variation study, we were chosen 0.5 and 2 ng mL−1 and 0.5-5 ng mL−1 of Vit D3 standards, respectively.

**Limit of detection (LOD) and Limit of quantification (LOQ)**

LOD and LOQ were determined by the standard deviation (σ) method. LOD and LOQ were determined from the slope, S, of the calibration plot, S y/x, by use of the formulae

\[
\text{LOD} = 3 \times \sigma /S \text{ and}
\]

\[
\text{LOQ} = 10 \times \sigma /S.
\]

σ = Standard deviation, S = Slope

**Efficiency**

Efficiency was calculated by using the following formula;

\[
N = 16 \left( \frac{t_s}{W_b} \right)^2
\]

\[(N = \text{Efficiency}, \ t_s = \text{Retention time, } W_b = \text{Peak Width})\]

**Robustness**

The robustness of the method was determined to assess the effect of small but deliberate variation of the chromatographic conditions of the analyte and was determined by changing the flow rate and concentration of mobile phase up to 1%.

**Result and Discussion**

In the present study we have developed HPLC method for sensitive and reliable detection of vitamin D3 by using two different mobile phases according to ICH guideline [18]. The results are divided into sub sections which are as shown below.

**Optimization of the chromatographic separation**

Separation was achieved on C18 column (150 X 4.6 mm i.d., 3 μm) in isocratic mode using two different mobile phases i.e acetonitrile: methanol (method I) and methanol: water with 0.1% formic acid (method II), in the ratio of 95:5 (v/v), respectively. Mobile phase was pumped into the column at flow rate of 0.4 mL min−1 and the detection of eluent was carried out at 265 nm. The total run time was 10 min and the column was maintained at 40°C. The retention time of vitamin D3 of method I and II was found 7.14 and 7.01 minutes respectively. Figure 1 shows the chromatogram of a standard Vit D3 by method I and II, respectively.
System suitability
The results (Mean ± %RSD of six replicates) of the chromatographic parameters are shown in Table 1, indicating the good performance of the system.

Linearity
The standard having concentration 0.5, 1, 2, 3, 4, 5 ng mL⁻¹ Vit D₃ were prepared by diluting the stock solution (1 mg mL⁻¹) with mobile phase. Each of these standard solutions were injected three times into the HPLC-column and the peak area was calculated using Chromelion software version 6.8.0. The regression line (r²> 0.99) demonstrates the excellent relationship between peak area and Vit D₃ concentration in both the methods, over a concentration range of 0.5-5 ng mL⁻¹ (Table 1).

Accuracy
The recovery of the methods, were determined by spiking matrix with different concentration of standard solution, by comparing the peak area obtained from the standard and from the spiked matrix. The value of Recovery (%), RSD (%) and SE indicate that method II is better than method I (Table 2). Formic acid in the mobile phase is known to improve peak shapes of the resulting separation. The other study used solid phase extraction (SPE) for recovery of Vit D₃, and reported 55-85% recovery [19] whereas we used liquid-liquid extraction (LLE) and found the same, as LLE is less specific in comparison to SPE in case of Vit D₃, due to small amount in circulation.

Table 1: Chromatographic characteristics of system suitability, linearity and sensitivity.

| Parameter                  | Method I     | Method II    |
|----------------------------|--------------|--------------|
| Peak Area (Mean±%RSD)      | 0.6369±1.504 | 0.6753±1.037 |
| Theoretical plates (EP) (Mean±%RSD) | 15472.83±0.3301 | 17800±0.3628% |
| Retention time (Mean±%RSD) | 7.134±0.0289 | 7.003±0.1474 |
| Linearity (ng mL⁻¹)        | 0.5–5        | 0.5–5        |
| Regression equation        | 0.1369x–0.0262 | 0.138x–0.0025 |
| Correlation Coefficient (R²)| 0.9999  | 0.9999  |
| LOD (ng mL⁻¹)              | 1.643        | 1.100        |
| LOQ (ng mL⁻¹)              | 5.026        | 3.600        |

*Indicates mean of three replicates, RSD= Relative standard deviation, SE= Standard error

Table 2: Recovery analysis of vitamin D₃.

| Matrix Spiked with Vit D₃ standard | Method I | Method II |
|-----------------------------------|----------|-----------|
|                                   |          | 3 ng mL⁻¹ | 5 ng mL⁻¹ | 15 ng mL⁻¹ | 3 ng mL⁻¹ | 5 ng mL⁻¹ | 15 ng mL⁻¹ |
| % Recovery                        | 68.87    | 78.74     | 69.91     | 75.18      | 81.49     | 87.43     |
| % RSD                             | 3.16     | 9.30      | 0.77      | 4.22       | 3.81      | 2.78      |
| SE                                | 0.004    | 0.035     | 0.189     | 0.007      | 0.012     | 0.746     |

85% recovery [19] whereas we used liquid-liquid extraction (LLE) and found the same, as LLE is less specific in comparison to SPE in case of Vit D₃, due to small amount in circulation.
Precision

The intra and inter-day precision were carried out for various concentration of standard in three replicates at different time intervals in the same day and at the same time in different days respectively. The % RSD and SE were calculated for both the methods and the low value of RSD (%) of method II showed good precision in comparison to method I (Table 3). The other reported HPLC methods developed for vitamin D₃ estimation showing within-batch and between-batch precision ranged from 0.83 to <10% and 1.8 to <12% respectively [20,21].

Injection precision

Injection precision was determined by injecting 5 ng mL⁻¹ of Vit D₃ standard six times continuously and the injection precision repeatability of both the methods showed that the assay is repeatable (Table 4). The peak resolution of method II was found better (>2) than method I (<2).

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) for Vit D₃ were evaluated by determining the average concentration that gives the signal three and ten times the background noise and was found 1.643 (0.64), 5.02 (2.0) and 1.1 (0.44), 3.60 (1.44) ng mL⁻¹ (nmol L⁻¹) of method I and II, respectively (Table 1). In contrast to this the other HPLC methods reported the LOD ranged between 3 and 7.5 nmol/L while the LOQ ranged between 10 and 17.5 nmol/L [22-26].

Efficiency

The efficiency was determined as mentioned in methodology section and it was found 15415 (N) and 17827 (N) for method I and II, respectively. The Chromlieon software generated number of theoretical plates also correlates with the efficiency data which reconfirms that method II was better competent than method I (Table 1).

Robustness

There were no significant change in the retention time of analyte was found when flow rate of the mobile phase has been changed (Table 5).

Conclusion

India is a developing country which is socio-economically poor. Although, plenty of sunlight is available throughout year, recent studies reported the Vitamin D deficiency in Indian population among all age groups. The person who is Vitamin D deficient is more prone to develop either non-communicable disease or auto-immune diseases. The available methods of Vit D₃ diagnosis are very costly and most of them can't afford this. Therefore, we need to develop the simple, reliable and cost effective method of Vit D₃ estimation. It is very well documented that analytical methods are sensitive and cost-effective in comparison to RIA or ELISA based method [17]. The separation of Vitamin D₃ under different chromatographic conditions has been developed but the best separation was achieved on octadecyl-bonded stationary phase and the DAD detector at λmax265 nm. We have checked different ratios of mobile phase combination and got good result at ACN: MeOH (95:5) and MeOH: H₂O (95:5) with 0.1% FA. When we were added FA to the mobile phase (method II) then we got better resolution in comparison to method I. The LOD and LOQ were also found relatively low in comparison to other existing methods, developed on HPLC. The developed RP-HPLC method for the determination of Vit D₃ is simple,
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| Concentration (ng mL⁻¹) | Method I | Method II |
|-------------------------|----------|-----------|
|                         | Repeatability (intra-day precision)* | Intermediate Precision (inter-day precision)* | Repeatability (intra-day precision)* | Intermediate Precision (inter-day precision)* |
|                         | %RSD | SE | %RSD | SE | %RSD | SE | %RSD | SE |
| 0.5                     | 1.509 | 0.0004 | 5.605 | 0.0015 | 1.358 | 0.0004 | 3.624 | 0.0012 |
| 1.0                     | 6.766 | 0.0035 | 1.314 | 0.0073 | 1.707 | 0.0024 | 2.251 | 0.0027 |
| 2.0                     | 2.127 | 0.0040 | 2.665 | 0.0162 | 2.657 | 0.0066 |
| 3.0                     | 5.467 | 0.0162 | 3.589 | 0.0105 |
| 4.0                     | 6.265 | 0.0162 | 3.784 | 0.0067 |
| 5.0                     | 6.766 | 0.0162 | 2.657 | 0.0066 |

*Indicates mean of three replicates, RSD= Relative standard deviation, SE= Standard error

Table 3: Precision analysis of method I and II.

| Concentration (ng mL⁻¹) | RT (min) | Area | EP | RT (min) | Area | EP |
|-------------------------|----------|------|----|----------|------|----|
| 5.0                     | 7.133    | 0.6390 | 15430 | 6.99 | 0.6718 | 17877 |
| 7.133                   | 0.6417 | 15506 | 6.99 | 0.6799 | 17733 |
| 7.133                   | 0.6354 | 15430 | 7.01 | 0.6837 | 17866 |
| 7.133                   | 0.6399 | 15468 | 7.01 | 0.6714 | 17722 |
| 7.137                   | 0.6190 | 15559 | 7.01 | 0.6651 | 17801 |
| 7.137                   | 0.6469 | 15444 | 7.01 | 0.6800 | 17801 |
| Mean                    | 7.134 | 0.0289 | 0.0008 | 15472 | 0.3301 | 20.85 |
| %RSD                    | 0.6369 | 1.504 | 0.0039 | 0.6753 | 0.1474 | 0.0042 |
| SE                      | 0.0008 | 0.0147 | 0.0028 | 0.0362 | 0.0008 | 0.0008 |

RT= Retention time, EP = Theoretical plates, RSD= Relative standard deviation, SE= Standard error

Table 4: Result analysis of injection precision repeatability of method I and method II.

| Parameter | Normal Condition | Change Condition | Change in %RSD |
|-----------|------------------|------------------|---------------|
| Flow Rate |                  |                  |               |
| 0.4 mL min⁻¹ | 0.396 mL min⁻¹ | 0.1239 | Method I    |
|           | 0.404 mL min⁻¹ | 0.1281          |               |
| 0.4 mL min⁻¹ | 0.396 mL min⁻¹ | 1.5127 | Method II   |
|           | 0.404 mL min⁻¹ | 0.0312          |               |

Table 5: Robustness of the experiment.

precise, accurate, reproducible, cost effective and validated according to ICH guidelines [18]. Hence, both the methods can be used for the routine determination of vitamin D₃ but method II is comparatively better than method I because formic acid improves the peak shape. Also, method I uses the acetonitrile in a major proportion whereas method II uses methanol, is a cheaper solvent in comparison to acetonitrile.

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Conflict of Interest
The authors declare that there is no conflict of interest.

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