Pharmaceutica Analytica Acta
ISSN: 2153-2435

Kumar, Pharm Anal Acta 2015, 6:8
DOI: 10.4172/2153-2435.1000410

Research Article Open Access

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

Subodh Kumar[1], Diwesh Chawla[1] and Ashok Kumar Tripathi[2*]

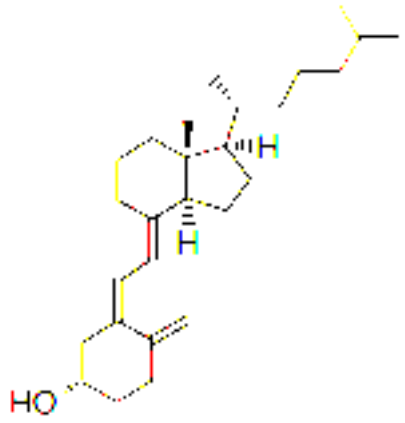
[1]*Central Research Laboratory, Multi-disciplinary Research Unit, University College of Medical Sciences, Delhi-110095, India*
[2]*Biochemistry and Immunology Laboratory, Department of Biochemistry, University College of Medical Sciences, Delhi-110095, India*

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 λ_{max} 265 nm. Retention time of vitamin D3 for method I and II was found to be 7.14 and 7.01 minutes, respectively, with R^2>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 D_3 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 D_3 estimation at laboratory scale.

Keywords: Vitamin D_3; RP-HPLC; Development and validation; DAD

Introduction



Vitamin D_3 (cholecalciferol)

India is well known for its traditional, cultural and lingual diversity. It is a vast tropical country extending from 8.4° N latitude to 37.6° N latitude. Majority of its population live in areas receiving abundant sunlight throughout the year and hence it was assumed that Vitamin D (Vit D) deficiency is uncommon in India [1] and globally [2-5]. However from various studies and data available in the published literature, Vit D deficiency is very common in India in all the age groups and both sexes across the country [6-8]. Hence, Vitamin D status screening is essential as it allows for monitoring a patient's response to Vitamin D therapy and also evaluation of treatment effect therefore, samples providing immediate and reliable results are highly desirable.

Vitamin D is very important fat soluble vitamin in human and animal diet. It exists in two forms "viz", Vitamin D_2 and D_3. Vitamin D_3 (cholecalciferol) is synthesized endogenously from 7-dehydrocholesterol after ultraviolet irradiation or is absorbed from the diet [9,10]; plant/yeast derived ergocalciferol (Vitamin D_2) 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 animportant 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 D_3 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 Extn 5210, +91- 9811259019; Fax: +91-11- 22590495; E-mail: aktripathiucms@gmail.com

Received December 10, 2014; **Accepted** August 13, 2015; **Published** August 17, 2015

Citation: Kumar S, Chawla D,Tripathi AK (2015) An Improved and Sensitive Method for Vitamin D3 Estimation by RP-HPLC. Pharm Anal Acta 6: 410. doi:10.4172/21532435.1000410

ultraviolet detection and reverse-phase separation. Recently, RP-HPLC methods for vitamin D_3 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 D_3 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 D_3 estimation.

Here, we have developed and described a comparative study between two methods for measurement of vitamin D_3 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 Fischer 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, Acclaim™ 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 D_3.

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.D_3 standard was obtained from Sigma Aldrich, Sweden.

Standard preparation

A stock solution mixture of vit.D_3 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 D_3 was prepared by dissolving 1mg Vit D_3 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 λ_{max}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 D_3 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

$LOD = 3 \times \sigma/S$ and

$LOQ = 10 \times \sigma/S$.

σ = Standard deviation, S = Slope

Efficiency

Efficiency was calculated by using the following formula;

$N = 16 (t_R/W_b)^2$

(N = Efficiency, t_R = Retention time, W_b= 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 upto1%.

Result and Discussion

In the present study we have developed HPLC method for sensitive and reliable detection of vitamin D_3 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 D_3 of method I and II was found 7.14 and 7.01 minutes respectively. Figure 1 shows the chromatogram of a standard Vit D_3 by method I and II, respectively.

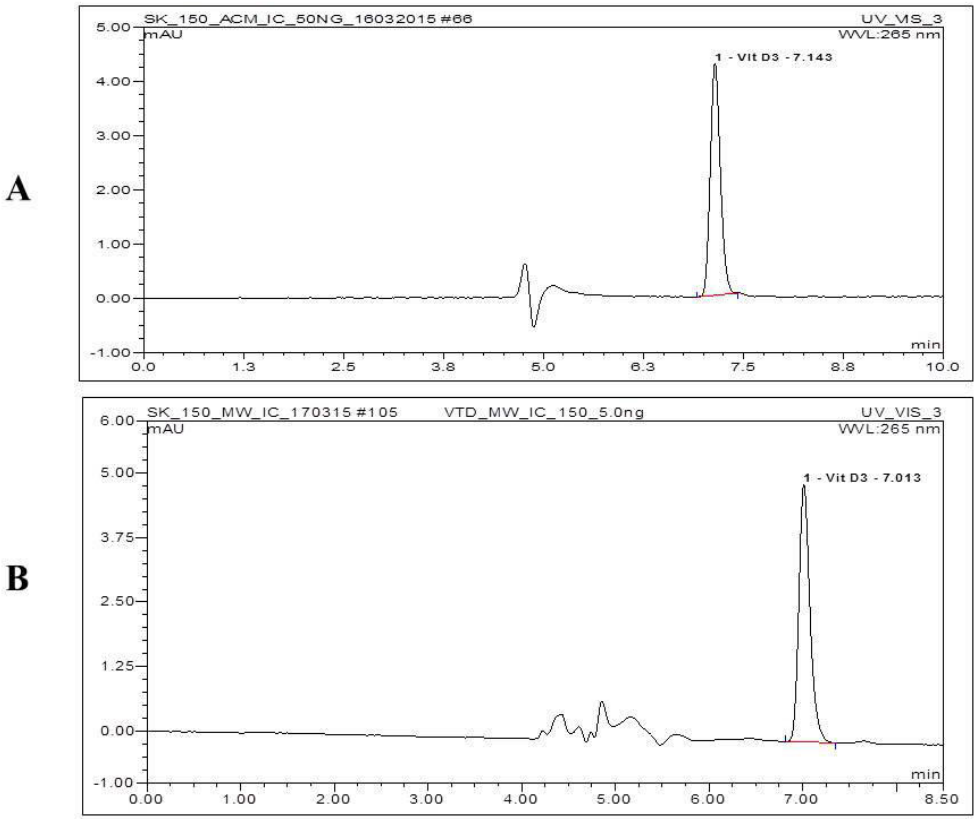


Figure 1: Chromatogram of a standard Vitamin D_3 using mobile phases (A) Acetonitrile : Methanol (95:5 v/v) (Method I) and (B) Methanol: Water with 0.1% formic acid (95:5 v/v) (Method II).

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 D_3 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 6.80.Calibration Curve was prepared by plotting peak area (y) versus Vit D_3 concentrations (ng mL^{-1}) (x) for both the methods (Figure 2). The regression line (r^2> 0.99) demonstrates the excellent relationship between peak area and Vit D_3 concentration in both the methods, over a concentration range of 0.5-5 ng mL^{-1} (Table 1). Turpeinen et al. reported that HPLC was preferred in terms of accuracy and precision in comparison to RIA and Diasorin [17].

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 D_3 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 D_3, due to small amount in circulation.

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	0.1389x – 0.0262	0.138x – 0.0025
Correlation Coefficient (R^2)	0.9929	0.996
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 1: Chromatographic characteristics of system suitability, linearity and sensitivity.

	Matrix Spiked with Vit D_3 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

Table 2: Recovery analysis of vitamin D_3.

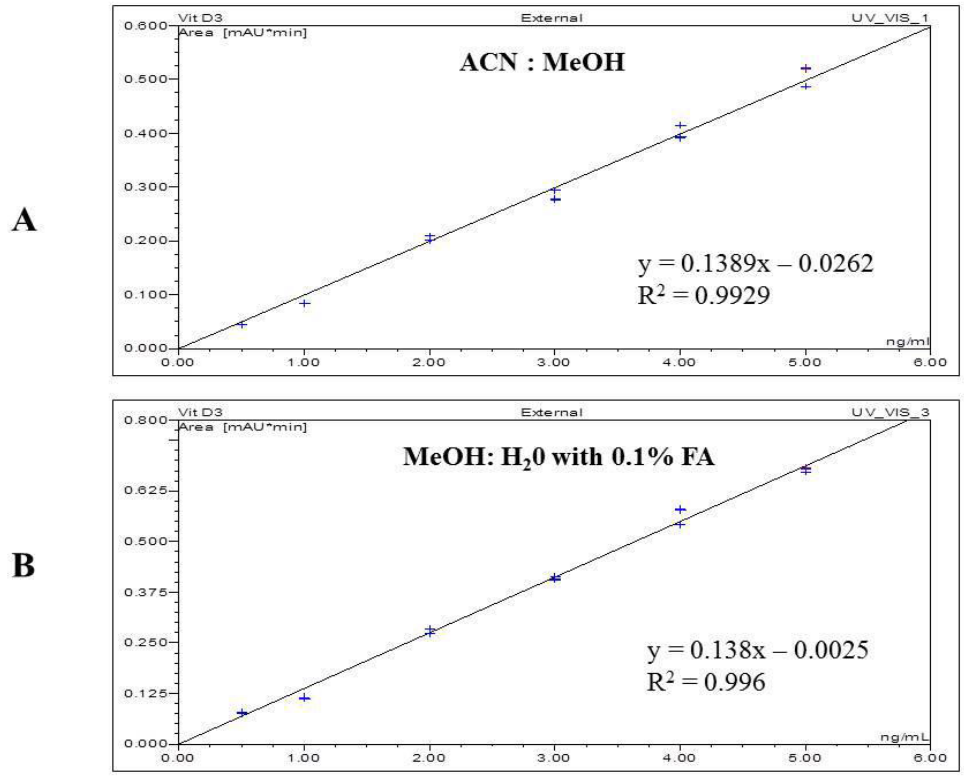


Figure 2: Calibration curve of Vitamin D_3 standard (A) Method I and (B) Method II.

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 D_3 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 D_3 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 D_3 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 D_3 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 D_3 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 D_3 under different chromatographic conditions has been developed but the best separation was achieved on octadecyl-bonded stationary phase and the DAD detector at λ_{max}265 nm. We have checked different ratios of mobile phase combination and got good result at ACN: MeOH (95:5) and MeOH: H_2O (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 D_3 is simple,

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			3.239	0.0016
2.0	0.896	0.0011	5.814	0.0073	1.707	0.0024	2.251	0.0027
3.0			2.127	0.0040			3.784	0.0067
4.0			6.265	0.0162			2.657	0.0066
5.0			5.467	0.0162			3.589	0.0105

*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})	**Method I**			**Method II**		
	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 %RSD SE	7.134 0.0289 0.0008	0.6369 1.504 0.0039	15472 0.3301 20.85	7.003 0.1474 0.0042	0.6753 1.037 0.0028	17800 0.3628 26.36

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}	0.1293	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 D_3 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.

Acknowledgment

This work is supported through funds provided by Department of Health Research - Indian Council of Medical Research, Government of India, New Delhi under Multi-disciplinary research scheme.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Mehlawat U, Singh P, Pande S (2014) Current status of Vitamin-D deficiency in India. IPP (2): 328-335.
2. Luque de Castro MD, Fernández-Romero JM, Ortiz-Boyer F, Quesada JM (1999) Determination of vitamin D3 metabolites: state-of-the-art and trends. J Pharm Biomed Anal 20: 1-17.
3. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357: 266-281.
4. Kennel KA, Drake MT, Hurley DL (2010) Vitamin D deficiency in adults: when to test and how to treat. Mayo Clin Proc 85: 752-757.
5. Rosen CJ (2011) Clinical practice. Vitamin D insufficiency. N Engl J Med 364: 248-254.
6. Harinarayan CV (2005) Prevalence of vitamin D insufficiency in postmenopausal south Indian women. Osteoporos Int 16: 397-402.
7. Marwaha RK, Sripathy G (2008) Vitamin D & bone mineral density of healthy school children in northern India. Indian J Med Res 127: 239-244.
8. Harinarayan CV, Joshi SR (2009) Vitamin D status in India--its implications and remedial measures. J Assoc Physicians India 57: 40-48.
9. Holick MF (1995) Environmental factors that influence the cutaneous production of vitamin D. Am J Clin Nutr 61: 638S-645S.
10. Holick MF (2004) Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. Am J Clin Nutr 79: 362-371.
11. Weaver CM, Fleet JC (2004) Vitamin D requirements: current and future. Am J Clin Nutr 80: 1735S-9S.
12. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP (2007) Vitamin D and calcium supplementation reduces cancer risk: results of a randomized trial. Am J Clin Nutr 85: 1586-1591.
13. Garland CF, Gorham ED, Mohr SB, Garland FC (2009) Vitamin D for cancer prevention: global perspective. Ann Epidemiol 19: 468-483.
14. Stechschulte SA, Kirsner RS, Federman DG (2009) Vitamin D: bone and beyond, rationale and recommendations for supplementation. Am J Med 122: 793-802.

15. Terry AH, Sandrock T, Meikle AW (2005) Measurement of 25-hydroxyvitamin D by the Nichols ADVANTAGE, DiaSorin LIAISON, DiaSorin RIA, and liquid chromatography-tandem mass spectrometry. Clin Chem 51: 1565-1566.

16. Wallace AM, Gibson S, de la Hunty A, Lamberg-Allardt C, Ashwell M (2010) Measurement of 25-hydroxyvitamin D in the clinical laboratory: current procedures, performance characteristics and limitations. Steroids 75: 477-488.

17. Turpeinen U, Hohenthal U, Stenman UH (2003) Determination of 25-hydroxyvitamin D in serum by HPLC and immunoassay. Clin Chem 49: 1521-1524.

18. (1996) Validation Of Analytical Procedures: Text And Methodology, ICH Harmonized Tripartite guidelines.

19. Ding S, Schoenmakers I, Jones K, Koulman A, Prentice A, et al. (2010) Quantitative determination of vitamin D metabolites in plasma using UHPLC-MS/MS. Anal Bioanal Chem 398: 779-789.

20. Glendenning P, Taranto M, Noble JM, Musk AA, Hammond C, et al. (2006) Current assays overestimate 25-hydroxyvitamin D3 and underestimate 25-hydroxyvitamin D2 compared with HPLC: need for assay-specific decision limits and metabolite-specific assays. Ann Clin Biochem 43: 23-30.

21. Mata-Granados JM, Quesada Gómez JM, Luque de Castro MD (2009) Fully automatic method for the determination of fat soluble vitamins and vitamin D metabolites in serum. Clin Chim Acta 403: 126-130.

22. Alvarez JC, De Mazancourt P (2001) Rapid and sensitive high-performance liquid chromatographic method for simultaneous determination of retinol, alpha- tocopherol, 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human plasma with photodiode-array ultraviolet detection. J Chromatogr B Biomed SciAppl 755: 129-135.

23. Brunetto MR, Obando MA, Gallignani M, Alarcón OM, Nieto E, et al. (2004) HPLC determination of Vitamin D(3) and its metabolite in human plasma with on-line sample cleanup. Talanta 64: 1364-1370.

24. Roth HJ, Schmidt-Gayk H, Weber H, Niederau C (2008) Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography-tandem mass spectrometry as a reference. Ann Clin Biochem 45: 153-159.

25. Jakobsen J, Bysted A, Andersen R, Bennett T, Brot C, et al. (2009) Vitamin D status assessed by a validated HPLC method: within and between variation in subjects supplemented with vitamin D3. Scand J Clin Lab Invest 69: 190-197.

26. Narayanaswamy R, Iyer V, Khare P, Bodziak ML, Badgett D, et al. (2015) Simultaneous determination of oxysterols, cholesterol and 25-hydroxy-vitamin D3 in human plasma by LC-UV-MS. PLoS One 10: e0123771.