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Original Research Article

Simple Extraction and Method Validation for the Analysis of Vitamin D3 In Fortified Full Cream Milk Powder by High Performance Liquid Chromatography (HPLC)

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Abstract

This paper proposes a simple HPLC method for the identification of vitamin D3 in fortified milk powder. The method was applied to the determination of the vitamin D3 (as cholecalciferol) concentration in commercial products containing known amounts of vitamin D, under the following chromatographic conditions: C 18, 5 µrn, 120 A, 4.6 x 150 mm column, a mobile phase consisted of methanol and UV (DAD) set at 265 nm. The linearity range was established between 0.01-0.2 µg/ml of cholecalciferol prior to the analysis. The extraction was performed with dichloromethane: methane 1:1 (v/v) prior to the analysis. The limit of quantification was 50 ng/ml. Rapid determination of vitamin D through the chromatographic method represents a good solution for its quantification in the fortified milk powder.

Keywords: vitamin D3, analysis, HPLC, Fortified Milk.

INTRODUCTION

Vitamin D is essential for the maintenance of calcium homeostasis and bone mineralisation. Extreme deficiency of vitamin D in adults causes osteomalacia. Vitamin D deficiency is common in adult Bangladeshi women of different physiological statuses [1]. Therefore, fortification of vitamin D in some dietary sources becomes necessary. Fortification markedly improves the availability of vitamin D through diet [2].

As evidenced from many studies in Europe and other countries, vitamin D deficiency in pregnant women may have adverse effects on fetal growth, bone ossification, tooth enamel formation, and neonatal calcium homeostasis [1]. Vitamin D fortification and supplementation strategies implemented in the USA and Canada have significantly improved the vitamin D status in these nations [3]. Women in Bangladesh are at risk of hypovitaminosis D [1]. Very few studies have so far been done in this country on quality of powdered milk.

Vitamins accounts for a large percentage of all dietary/nutritional supplements purchased and consumed. Vitamin D is a fat-soluble vitamin that has been known for its ability to enhance intestinal absorption of calcium, iron, magnesium, phosphate, and zinc [4]. The most important vitamin D compounds are ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3) [4].

Despite abundant sunlight in Bangladesh, the prevalence of vitamin D deficiency among adults due to lack of awareness about the importance of sun exposure [2]. Vitamin D food fortification could be the most appropriate way of improving vitamin D intake and status in the general population in order to meet dietary vitamin D recommendations [5]. The aim of this work is the development and the validation of a fast and selective method for the determination of vitamin D3 in fortified milk through HPLC method using the UV detection system with diode-array detection (DAD) and the validation of the working conditions.

Test procedures for assessment of the quality levels of product are subject to various requirements. Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirement for the intended analytical applications. Validation of an analytical procedure is performed in order to demonstrate that the procedure is suitable for

its intended use and show that the result(s) generated by a particular analytical procedure are reliable and accurate.

The need for label claim accuracy is both a quality and a safety concern. The use of chromatography via HPLC-UV analysis is a great tool to accurately identify and quantify the amounts of specific vitamins to ensure what is reported on the label [6].

In order to analyze vitamin D3 the compound must be extracted from the matrix before it can be analyzed using RPHPLC. Here in this work a rapid and simple extraction procedure has been developed for HPLC analysis.

MATERIALS AND METHODS

Chromatographic Conditions

Column	**:**	Acclaim C 18, 5 µrn, 120 A, 4.6 x 250 mm *(PIN 059133)*
Mobile phase	**:**	Filtered and degassed Methanol.
Flow rate	**:**	1.0 ml per minute.
Detector	**:**	UV
Wavelength	**:**	265 nm
Injection volume	**:**	20 µl
Column oven temperature	**:**	25 °C
Run Time	**:**	About 17 minutes

Standard Solution

Accurately weighed 10.0 mg of vitamin D3 was transferred into a 15 ml falcon tube. 5 ml of Dichloromethane was added followed by sonication for 3 minutes with intermittent shaking. Then 5 ml Methanol added and sonicated for the same duration. The tubes were allowed to vortex for 10 minutes. Standard solution was filtered through 0.20 µm PTFE membrane filter before injecting into vial.

Sample Extraction

2.00 g of sample weighed into a 15 ml Falcon tube and 5 ml of Dichloromethane added followed by sonication for 3 minutes. 5ml Methanol was added and centrifuged for 10 minutes at 4000 rpm. Supernatant was filtered through 0.20 µm PTFE membrane filter before injection.

Procedure

The column was equilibrated with mobile phase for 30 minutes. 20 µl of standard solution was injected until the relative standard deviation of areas of the peaks of five consecutive chromatograms were within 2.0%

Calculation

Content of vitamin D3 per ml:

$$= \frac{PT \times WS \times 10 \times 100 \times 100 \times P \times 10^6}{PS \times 50 \times 50 \times WT \times 10 \times 1000} \times wt/ml$$

= IU per ml of vitamin D3

Where,

PT=Peak area of vitamin D3 obtained from the chromatogram of the test solution.
PS=Average area of peaks of vitamin D3 obtained from the chromatogram of the standard solution.
WS=Weight of vitamin D3 working standard in mg.
WT=Weight of sample in g.
P=Potency of vitamin D3 working standard in million IU per g.
Wt/ml=Weight per milliliter of sample.

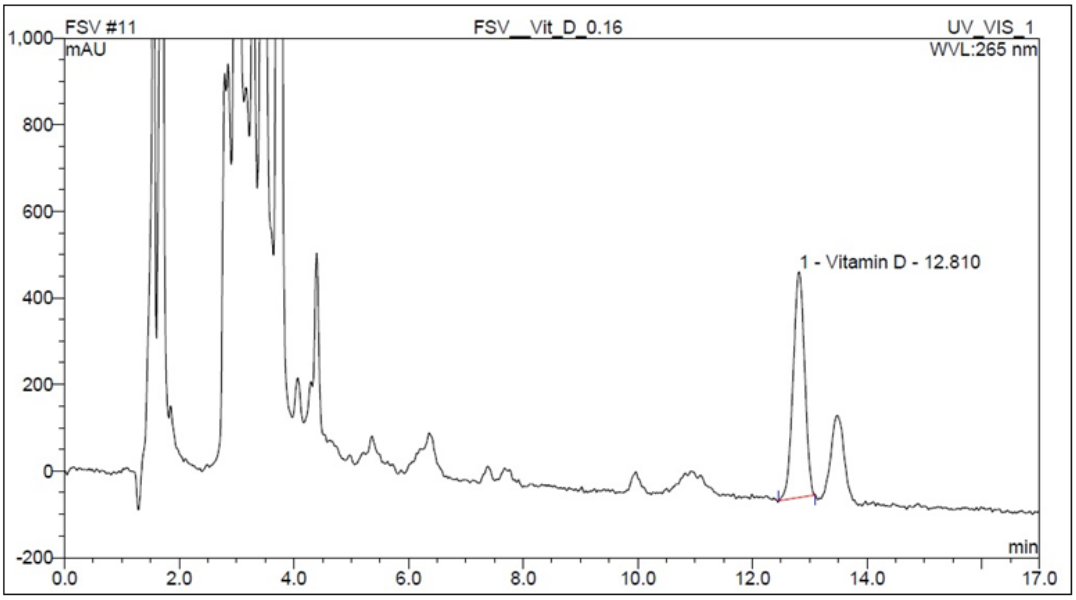

Fig-1: Chromatogram of vitamin D3 at 265 nm

VALIDATION PARAMETERS

The method was validated according to the ICH guidelines Q2 (R1) in terms of selectivity, linearity, repeatability, precision, accuracy, limit of detection (LOD), quantification of limit (LOQ) and sample stability.

RESULTS AND DISCUSSION

The validity of the methods for the assay of vitamin D3 was examined by determining precision and accuracy [7]. Precision was measured in terms of repeatability of application and measurement date. Repeatability by injecting standard for six times in a same batch. Precision is usually expressed as the relative standard deviation (co-efficient of variation).

System Precision/System Suitability

System suitability was determined by measuring the retention time, peak area, theoretical plates and asymmetry of standard solution containing 100% working concentration for six times . It was calculated on the basis of % RSD for retention time (Not more than 1.0%), peak areas (Not more than 2.0%), average value of theoretical plates (Not less than 2000), Average asymmetry factor (Not more than 2.0%). The results are summarized in Table-1:

Table-1: Data for System Precision

Standard Concentration (µg/ml)	No. of Measurement	Retention Time	Peak Area	Theoretical Plates	Asymmetry Factor
0.04	06	12.83	31.21	19208	0.79
		12.77	29.69	22116	0.99
		12.72	30.27	19361	1.02
		12.66	30.33	17072	1.00
		12.63	31.21	18835	1.16
		12.58	30.49	17727	1.03
Average	-	-	-	19048	0.99
% RSD	-	0.712%	0.915%	-	-

From above data, % RSD 0.0.712% (not more than 1%) for retention time, 0.0.915% (not more than 2%) for peak areas, 19048 average theoretical plates (not less than 2000) and 0.99 average asymmetry factor (not more than 2.0) are found for six replicate measurements of standard solution, which indicates that the system is precise to analyze cholecalciferol (as vitamin D3).

Method Precision

Method precision was established by analyzing the % RSD the content of vitamin D3 (as cholecalciferol) in fortified full cream milk powder from six separate samples at 100% of the working concentration of the same batch. Percent of result was calculated against calculating the percent recovery.

Table-2: Data for Method Precision in Day-1

Sample No.	Weight in g	Amount added (µg/ml)	Amount Recovered in (µg/ml)	% Recovery	% RSD (Relative standard deviation)
S-01	2.0	0.08	0.0789	98.62	1.45%
S-02.	2.0	0.08	0.0788	98.50	
S-03.	2.0	0.08	0.0779	97.35	
S-04.	2.0	0.08	0.0767	95.87	
S-05.	2.0	0.08	0.0801	100.13	
S-06.	2.0	0.08	0.0786	98.25	

The relative standard deviation of assay results of six separate samples from a single batch was found 1.45% (not more than 2.0%), which indicate that the method was precise to analyze vitamin D3 (as cholecalciferol) fortified milk powder.

Intermediate Precision

Intermediate precision was established by analyzing the content of vitamin D3 (as cholecalciferol) from six separate samples at in different day by different analyst. Percent of RSD was calculated against the percent of recovery.

Table-3: Data for Precision in Day-2

Sample No.	Weight in g	Amount added in (µg/ml)	Amount Recovered in (µg/ml)	% Recovery	% RSD (Relative standard deviation)
S-01	2.0	0.08	0.0779	97.38	1.14%
S-02	2.0	0.08	0.0790	98.75	
S-03	2.0	0.08	0.0784	98.00	
S-04	2.0	0.08	0.0789	98.62	
S-05	2.0	0.08	0.0795	99.38	
S-06	2.0	0.08	0.0805	100.63	

Table-4: Data for Intermediate Precision

Sample No.	% Recovery	
	Day-1	Day-2
S-01	98.62	97.38
S-02	98.50	98.75
S-03	97.35	98.00
S-04	95.87	96.37
S-05	100.13	99.38
S-06	98.25	100.63
% RSD	1.45%	1.14%
% RSD of 12 samples	1.29%	

The relative standard deviation (%RSD) of assay results of 12 separate samples was found 1.29%, As % RSD is less than 2.0%, it can be inferred that the method is precise to analyze vitamin D3 (as cholecalciferol) fortified milk powder.

Accuracy

Accuracy was established by analyzing nine sample solutions of vitamin D3 (as cholecalciferol) at three (3) working concentration (three replicates for each concentration) into a placebo mixture and calculating the percent recovery of active ingredient from the placebo solution. The percent recovery at each level should be within 95.0% to 105.0%. A linear curve was prepared by plotting amount added vs amount recovered and correlation co-efficient was calculated.

Table-5: Data for Accuracy

Sample No.	Amount added in (µg/ml)	Amount Recovered in (µg/ml)	% Recovery
S-01	0.025	0.0258	103.2
S-02	0.025	0.0248	99.2
S-03	0.025	0.0249	99.6
S-01	0.05	0.0502	100.4
S-02	0.05	0.0525	105.0
S-03	0.05	0.0497	99.4
S-01	0.1	0.0973	97.3
S-02	0.1	0.1004	100.4
S-03	0.1	0.1040	104.0

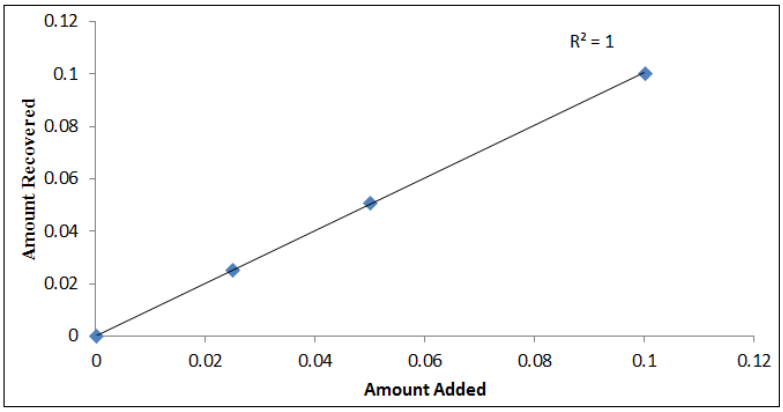

Fig-2: Graphical representation for accuracy of vitamin D3 (as cholecalciferol) placebo matrix

The percent recovery was calculated for nine determinations and found within range (97.3% to 105%). A graphical representation between amount added vs amount recovered also showed linearity. Thus the method was accurate to analyze the vitamin D3 (as cholecalciferol) fortified full cream milk powder.

Specificity

The retention time of the major peak in the sample chromatogram is concordant with that of the standard chromatogram.

Placebo Interference

Placebo solution was prepared in the same manner as standard and sample preparation. No interference of placebo was found.

Linearity

Linearity was evaluated based on standards in the concentration range from 0.01 to 0.16 µg/ml. The procedure was repeated three times, using different working standard solution.

Five different standard solutions were prepared of vitamin D3 (as cholecalciferol) and all peaks areas were recorded. A linear curve was prepared by plotting actual concentration (µg/ml) vs peak area and correlation co-efficient was calculated. The results obtained correlate with the concentrations resulting in the following calibration curve.

Table-6: Data for Linearity

Concentration (µg/ml)	Peak Area	Correlation co-efficient
0.01	5.1360	0.999
0.02	13.0351	
0.04	26.6566	
0.08	55.6046	
0.16	111.9378	

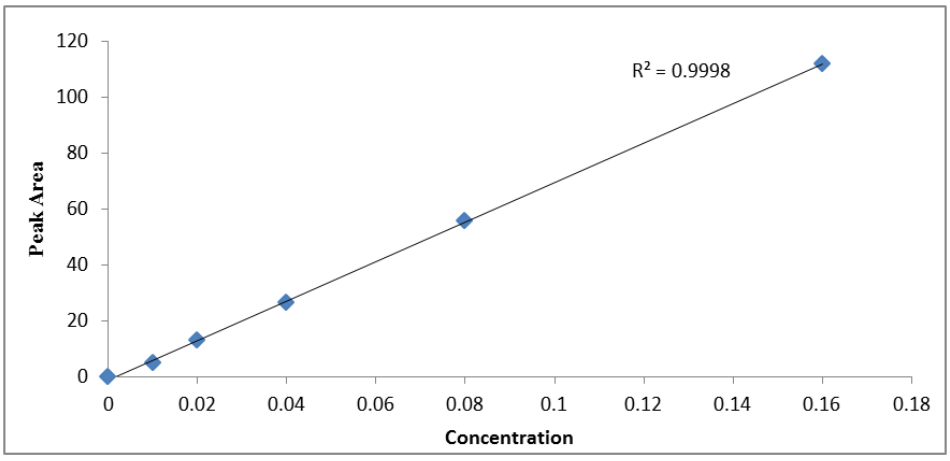

Fig-3: Graphical Representation of Linearity for Cholecalciferol (as vitamin D3)

The correlation co-efficient was found 0.999, which was within the limit (limit: NLT 0.990). Thus the graph confirming the linearity of the method.

The linearity for the vitamin D3 was determined by using of 0.01-.16 µg/ml of standard

solution of vitamin D3 prepared from working standard. The linear regression data for the calibration curves indicated that the response was linear over the concentration range studied with coefficient of correlation (r) value as 0.999. Results are summarized in Table-6.

Table-7: Summary of assay method validation of vitamin D3 in Fortified milk powder

Sl. No	Test Parameters	Acceptance Criteria	Results
1.0	System Suitability		
	I) Retention Time	Relative standard deviation: Not more than 1%.	0.712%
	II) Peak Area	Relative standard deviation: Not more than 2%.	0.915%
	III) Theoretical Plate	Average theoretical plates: Not less than 2000.	19048
	IV) Asymmetry Factor	Average value of asymmetry: Not more than 2.00	0.99
2.0	Method Precision	Relative standard deviation of assay: Not more than 2%.	1.45%
3.0	Intermediate Precision	Relative standard deviation of assay: Not more than 2%.	1.14%
4.0	Accuracy	Between 95% and 105%.	(97 - 105)%
5.0	Specificity	Interference of placebo/diluent/mobile phase.	No interference
6.0	Linearity	Regression Value: Not less than 0.990	0.999

There are some reports in the literature on the recovery of fat-soluble vitamins [8], in which the authors have reported similar results; however, their evaluations were carried out by different extraction methods.

CONCLUSION

The developed method in this work can be an alternative of traditional methods for routine analysis of the quantification of vitamin D3 in fortified full cream powder samples in Bangladesh. From the above data it was observed that all validation parameters (such as system suitability, method precision, intermediate precision, accuracy, specificity, linearity) met the predetermined acceptance criteria. In the present research, simple extraction and accurate, precise, and linear stability-indicating HPLC method has been developed and validated for vitamin D3 and hence it can be employed for routine quality control analysis. The analytical method conditions and the mobile phase solvents provided good resolution for vitamin D3.

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