Analytical method development and validation by HPLC for estimation of Vitamin D-3

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ABSTRACT:

Using a high-performance liquid chromatograph (HPLC), an analytical method for quantifying Vitamin D-3 was designed. The vitamin D-3 was separated using a C-18 column. As a mobile phase 97:3 ratio of methanol and water was used. The chromatogram of Vitamin D3 was identified at a wavelength of 264 nm at a flow rate of 1.2mL/min. The method was validated in accordance with the ICH Q2 (R1) guidelines. Several runs were carried out to determine the optimum dilution range. The devised approach was found to be linear with a r^2 of 0.999 throughout a concentration range of 0.25-1.25µg/mL. At all levels, the mean percentage recovery of Vitamin D-3 were found to be between 95 and 105 percent, indicating that the approach was accurate. The percentage relative standard deviation was determined to be less than 2%, indicating that the approach was well-defined. For Vitamin D-3, the LOD and LOQ were determined to be 0.0001 and 0.0005, respectively. The approach was proven to be robust because there was no significant change in responses when the flow rate and mobile phase concentration were varied. The devised method passed all validation tests and may be used to quantify the amount of Vitamin D-3 in bulk as well as different pharmaceutical formulations

KEYWORDS: Vitamin-D3, HPLC, LOD; LOQ, accuracy, precision, linearity

Abbreviations

HPLC	High Performance Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification

RESEARCH HIGHLIGHTS

- This method is novel and accurate. Smallest amount of Vitamin D can be calculated by applying this method
- Developed method for estimation of Vitamin D is precise, sensitive and reproducible
- Developed method is easy, economical and robust

1. INTRODUCTION

Cholecalciferol is a steroid hormone produced in the skin when exposed to ultraviolet light or obtained from dietary sources.

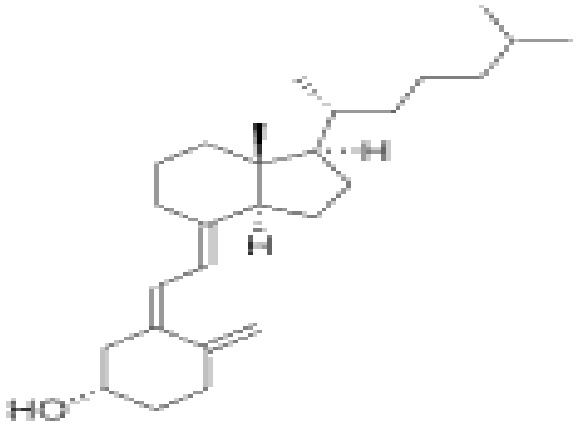

Fig. 1 : Vitamin D-3 chemical structure

The active form of Vitamin D-3 is 1,25-dihydroxycholecalciferol (calcitriol) it helps in maintaining calcium and phosphorus levels and also do mineralization of bone.1,25-Dihydroxyvitamin D_3, as well as is a major regulator of the expression of the cationic antimicrobial peptide cathelicidin, not only in monocytes but also in epidermal keratinocytes.(Bognar A. et al 1992). Vitamin D-3 helps in wound healing by production of cathelicidin, it is an important host defence peptide, which plays a good role in innate and adaptive immunity, it kills the microbes presesnt in sorrounding of wounds, modulate immune response and heals the wounds(Agier J. et al 2015) The of cathelicidin helps in wound healing and skin diseases such as as psoriasis, rosacea, and atopic dermatitis. (Segaert S 2008). Several analysis indicates that vitamin D-3 is more effective for increasing serum concentrations of active form of vitamin D-3 than vitamin D-2, and thus vitamin D3 could potentially become the preferred choice for further formulation development(Jafri L et 2011). It also remains in blood stream for longer time than vitamin d2(Keyfi F et al 2018).Viatmin D-3 is less potent in its pharmacological activity thanVitamin D-2, which makes vitamin D3 the preferred agent for medical use(Topcouglu C et al 2018). Vitamin D3 has antibacterial, anti inflammatory as well as wound healing activity(Razzagahi R et al 2017). Topical application of vitamin D3 gives good absorption and promote wound healing also prevents from several infections (Guttoff et al. 2015). There is no economical and simple method is available to quantify the vitamin D3 in formulations in very less quantity so fulfill criteria an analytical method has been developed (Kumar et al. 2021). In this method methanol and water was used in mobile phase, several runs were performed to get the serial dilutions range. Whole analytical method was developed performed according to ICH guidelines Q2 R1(Temova and Roskar 2011). The main aim of this newly developed analytical method was to obtain highest recovery amount of vitamin D-3 n formulations. Thus this method was produced the highest recovery amount of vitamin d-3 than previoulsly reported analytical methods and less standard deviation in all the validation parameters.

2. MATERIALS AND METHODS

Vitamin D-3 and all materials were used of analytical grade for conduction of validation HPLC grade water and methanol was used for analysis. Waters HPLC model no L185CH361G having pore size 0.35µ with flow rate 1ml/min was used. Octadecylsilane column was selected for validation. Sample was injected with flow rate 1ml/min, sample volume of 50µL for run time of 15 minutes. Waters HPLC contains UV detector for detection of analyte. Atmospheric condition was suitable for method validation of Vitamin D-3. C18 column was selected as stationary phase and for mobile phase methanol and HPLC grade water was selected in different ratio to get the linear graph or straight line equation. ƛmax of Vitamin D-3 was found to be 264 nm and after several trial standard curve was prepared with 0.25, 0.5, 0.75, 1.0 and 1.25µg/mL concentration of Vitamin D-3 these concentrations were showing linearity and R^2 value 0.999 that's why these dilutions were selected for further analysis.

2.1 Method Validation

Standard parameters for analytical method development was used like linearity, range accuracy, precision, robustness, Limit of detection (LOD), Limit of Quantification (LOQ), peak purity index, tailing factor and Relative retention time(RRT).

2.2 Selection and Preparation of different Quality Control Standard for Method Validation

0.75µg/mL was selected as MQC(Medium quality Control) according to that concentration another two dilutions were prepared i.e 80% of MCQ would be 0.6µg/mL(LQC) and 120% of MCQ would be 9µg/mL(HQC). MQC stands for medium quality control and HQC stands for higher quality control standard. All other parameters were performed for these three concentrations.

2.3 Linearity and Range

Standard curve was plotted for Vitamin D-3 and straight-line equation was developed for estimation of regression line.

2.4 Accuracy

The accuracy of the method was calculated in the terms of recovery of the drug from the quality control standard solutions. All three quality control standard solutions were injected to HPLC five times and its mean of response was recorded. Percentage recovery was calculated by applying formula i.e dividing the actual amount of drug with their theoretical concentrations and multiplying them by hundred. Then mean of response and relative standard deviation was calculated

2.5 Precision

Repeatability and intermediate precision are two main components of precision data. On the same day and under same experimental conditions repeatability was calculated through injecting the samples of all three quality control standards.. For evaluation the intermediate

precision LQC, MQC and HQC were injected five times in HPLC on each of three different days (inter-day) as well as by other two analysts and under the experimental conditions remain constant, mean of response and percentage relative standard deviation was calculated.

2.6 Robustness

In order to check the effect of small changes on robustness of the developed method, the study was carried out by varying the flow rate of sample from column (0.8, 1.0 and 1.2 mL/min) as well as ratio of mobile phase respectively. Five replicates of medium concentration (0.75µg/mL) were injected and their effect on area of the peak, recovery and retention time was observed and mean of response was recorded.

2.7 Estimation of LOD and LOQ:

Limit of detection (LOD) and Limit of quantification (LOQ) were estimated by using standard deviation of response (sigma) and slope of calibration curve (S). Standard deviation of Y intercepts of regression line was used as standard deviation.

LOD = 3.3 σ/S

LOQ = 10 σ/S

2.8 System suitability:

For determination of system suitability parameters 5 times sample were injected into HPLC and data was calculated.

3. RESULTS

3.1Selection of Mobile Phase

Several trials have performed for detection of suitable mobile phase for example methanol, methanol and water in different ratios, dimethyl sulfoxide (DMSO) and phosphate buffer. Then according to parameter like peak sharpness in HPLC chromatogram, retention time of vitamin D-3 and resolution, methanol and water in 97:3 ratios was selected. The selected mobile phase was showing high resolution of vitamin D-3 better peak sharpness and also a good chromatogram. In several mobile phases, there was no peak but in the solution of methanol and water in ratio, 97:3 gave good results. So methanol and water solution selected for further analysis

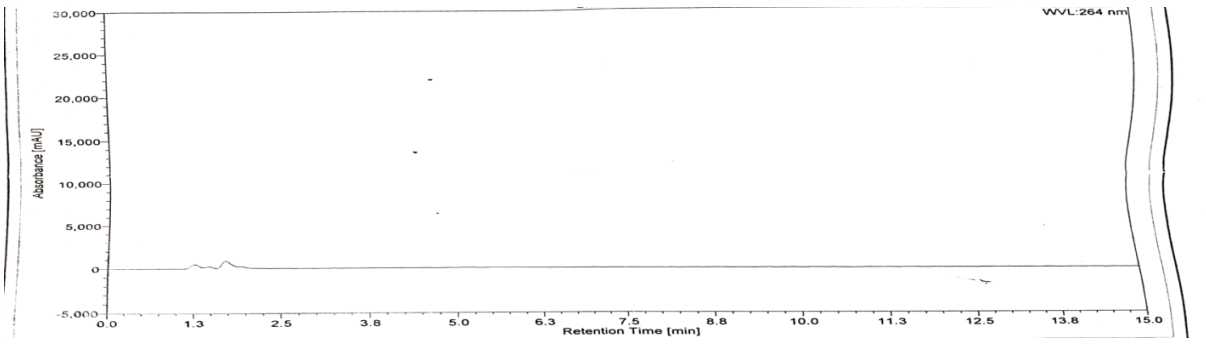

Fig. 2 : Chromatogram showing no peak

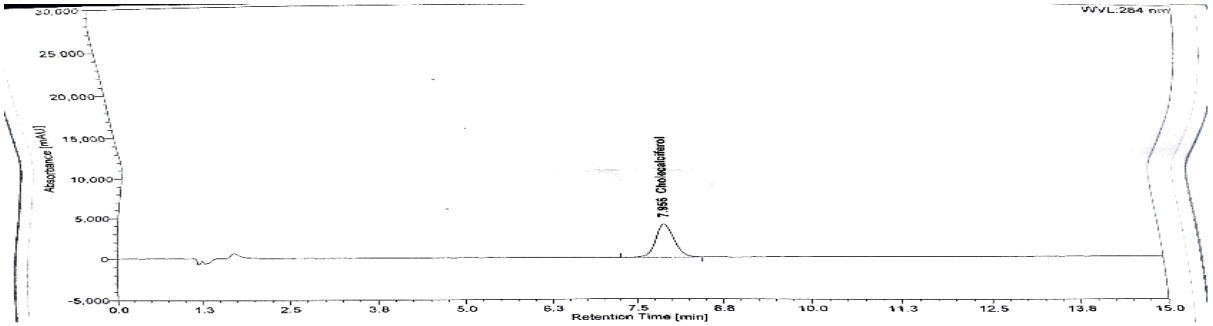

Figure 3: Chromatogram of Vitamin D-3 Peak

3.2 Preparation of Stock Solution and standard curve

25 mg of Vitamin D-3 was added in 50 ml of volumetric flask and made up the 50 ml volume with methanol and water (97:3) ratio. then different dilutions like 0.25, 0.5, 0.75, 1.0 and 1.25µg/mL were prepared by using same solvent

3.3 Linearity and Range

Standard curve was plotted by using area recorded in HPLC against concentration of the samples. R^2 value was found to be 0.999 as well as curve was linear at the concentration ranges between 0.25-1.25µg/mL

Table 1: Standard Curve data of Vitamin D-3

CONC.(µg/mL)	PEAK AREA			AVG	SD
0	0	0	0	0	0
0.25	38102	38101	38102	38101.67	0.471405
0.5	76205	76204	76205	76204.67	0.471405
0.75	114307	114306	114305	114306	0.816497
1	156420	156421	156419	156420	0.816497
1.25	190570	190571	190570	190570.3	0.471405

Fig. 4: Standard curve of Vıtamin D3

3.4Accuracy: The accuracy of developed method was checked by in terms of percentage drug recovery followed by relative standard deviation.for detection of these parameters all three quality control standards i.e LQC, MQC and HQC were injected three times in HPLC and data was recoreded. Limit accepted for percentage drug recovery in 95-105% as well as for % RSD is below 2%. Method was verified by evaluation these data, all the readings were in accepted range. All data is given below in table

Table:2 Repeatability data for MQC

CONC µg/mL	**PEAK AREA**			**AVG**	**SD**
0.75	114307	114306	114305	114306	0.816497

Table: 3 Accuracy Data

Conc µg/mL	Area			Mean	S.D.	%RSD	Conc			Mean %Recovery	
0.6	91445	91443	91449	91445.67	3.05505	0.002	0.592	0.591	0.595	0.592 98.7	
0.75	114301	114305	114307	114303.3	4.93288	0.002	0.747	0.749	0.748	0.746 99.7	
0.9	137161	137154	137165	137160	5.56776	0.001	0.896	0.897	0.892	0.895 99.4	

Table: 4 Precision Data (Intra-day)

Conc. µg/mL	PEAK AREA					MEAN	SD	%RSD
0.6	91442	91441	91449	91439	91446	91443.4	3.61109	0.004
0.75	114306	114309	114300	114308	114311	114306.8	3.76297	0.003
0.9	137155	137162	137159	137154	137158	137158.0	2.87054	0.002

Table: 5 Precision Data (Inter-day)

	Conc. µg/mL	PEAK AREA					MEAN	SD	%RSD
DAY 1	0.6	91443	91442	91439	91432	91443	91439.8	4.1	0.0045
	0.75	*114309*	114307	114299	114301	114298	114302.8	4.4	0.0038
	0.9	137154	137160	137165	137162	137159	137160.0	3.6	0.0026
DAY 2	0.6	91439	91443	91432	91431	91443	91437.6	5.2	0.005
	0.75	*114302*	114306	114300	114305	114299	114302.4	2.7	0.002
	0.9	137166	137160	137164	137162	137158	137162.0	2.8	0.002
DAY 3	0.6	91442	91440	91430	91433	91442	91437.4	4.9	0.0054
	0.75	*114308*	114308	114298	114306	114299	114303.8	4.4	0.0038
	0.9	137166	137169	137164	137168	137160	137165.4	3.2	0.0023

Table 6: Inter day precision by changing analyst

Conc. µg/mL		PEAK AREA					MEAN	SD	%RSD
ANALYST 1	0.6	91441	91443	91439	91432	91443	91439.6	4.079216	0.0044
	0.75	*114304*	114301	114299	114306	114298	114301.6	3.006659	0.0026
	0.9	137154	137160	137165	137162	137159	137160.0	3.63318	0.0026
ANALYST 2	0.6	91445	91441	91432	91435	91440	91438.6	4.586938	0.005
	0.75	*114307*	114305	114297	114302	114299	114302.0	3.687818	0.003
	0.9	137152	137162	137164	137161	137150	137157.8	5.670979	0.004

3.5 Robustness

Robustness is evaluated in terms of change in flow rate as well as change in ratio of mobile Phase and percentage relative standard deviation was calculated, all the data were in specified limit and justified the validation.

Table: 7 Robustness data by change in Flow rate

Flow rate	Conc. µg/mL	Retention time			Mean	SD	%RSD
0.8	0.75	8.1	8.2	7.9	8.066667	0.152753	1.89362
1	0.75	7.92	7.95	7.9	7.943333	0.020817	0.26206
1.2	0.75	7.12	7.16	7.22	7.166667	0.050332	0.70231

Table: 8 Change in mobile phase ratio

		Retention time					
Mobile phase	Conc. µg/mL	I	II	III	Mean	SD	%RSD
95:5	0.75	8.12	8.2	8.1	8.14	0.052915	0.650062
97:3	0.75	7.92	7.95	7.9	7.943333	0.020817	0.262065

Table: 9 Effect on peak area on Mobile Phase ratio

Mobile Phase	Conc. µg/mL	Peak Area					SD	Mean	%RSD
95:5	0.75	76267	76144	76243	76157	76155	57.2643	76193.2	0.075157
97:3	0.75	76167	76188	76175	76167	76177	8.671793	76174.8	0.011384

3.6 System Suitability

For the system suitability tailing factor, Peak purity index relative retention time and theoretical plate parameters were estimated and it was found that developed method is suitable for validation.

Table: 8 System Suitability Parameters

PARAMETERS	VALUE
Theoretical plate	4362
Tailing factor	1.45
Peak Purity index	1.00
R.R.T	1.00

4. CONCLUSION

Vitamin D-3 is better than vitamin D-2 because its remain in systemic circulation for longer time. İt helps in wound healing by cathelicidin production, it is an important host defence peptide, which plays a good role in innate and adaptive immunity, it kills the microbes presesnt in sorrounding of wounds, modulate immune response and heals the wounds. In present study new analytical method was developed by using methenol and water as solvent system for estimation or quantification of Vitamin D3 in several pharmaceutical formulations as well as in several nutrition supplement and neutraceuticals.The developed method was accurate and précised and produce highest recovery of Vitamin D-3 than any other reporetd method. This method is novel and accurate because here methenol and water

used as solvent. İn comparison to other developed method it was found that smallest amount of Vitamin D-3 can be calculated by applying this method because the limit of detection was found to be 0.0005µg Developed method for estimation of Vitamin D-3 is precise, sensitive and reproducible and it is also easy, economical and robust.

The summary of all the validation parameters is given below:

Table: 10 Validation Parameters

PARAMETERS	VALUE
Linearity and Range	Linear curve with r^2 value 0.999
Accuracy	%RSD was below 2%
Precision	%RSD was below 2%
Robustness	%RSD was below 2%
LOD	0.0001
LOQ	0.0005
Theoretical Plate	4362
Peak purity index	1.00
Tailing factor	1.45
RRT	1.00

Conflict of interest : The authors declared no conflict of interest" in the manuscript

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