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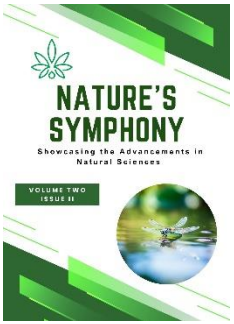

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Research Article

Development and Validation of RP-HPLC Method for The Analysis of Cholecalciferol in Injectable Products

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Cholecalciferol, Method validation, RP-HPLC, Vitamin D3, injectable products

ABSTRACT

This study focuses on the development and validation of a robust Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantification of cholecalciferol (vitamin D3) in commercially available pharmaceutical products. Vitamin D3 formulations, often produced in varying concentrations by pharmaceutical companies such as 1 mg/mL and 5 mg/mL, require precise dosage validation to ensure efficacy and safety. Accurate determination of cholecalciferol content is critical for quality control and compliance. In Pakistan, the most used method for estimating cholecalciferol (vitamin D3) is UV-visible spectroscopy, as recommended by the British Pharmacopoeia. This method involves the preparation of a diluent over 48 hours and the measurement of absorbance at 500 nm and 550 nm. However, it is prone to significant drawbacks, including time inefficiency and susceptibility to inaccuracies or losses during sample preparation. These limitations, coupled with potential interference from excipients overlapping with vitamin D3's absorption spectrum, make UV-visible spectroscopy an unreliable choice for routine analysis. To address these challenges, a more efficient and reliable Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated in this study. The method employed a C18 column and a photodiode array (PDA) detector, with detection set at 265 nm. The mobile phase consisted of methanol and water in a ratio of 98:2 (v/v), ensuring optimal separation and detection. The method demonstrated excellent linearity, accuracy, precision, and specificity, making it a reliable tool for routine quality control testing. The RP-HPLC technique was found to be efficient, sensitive, and less time-consuming, making it highly suitable for determining cholecalciferol concentrations in injectable vitamin D3 formulations and ensuring the quality and efficacy of these products in the market.

1. INTRODUCTION

Vitamin D is a fat-soluble vitamin that functions as a hormone and plays a crucial part in amendable the balance of calcium element and phosphorus in living organisms. It achieves this by enhancing and regulating the absorption of calcium and phosphorus in the intestines. Both vitamin D excess and deficiency have direct and indirect effects on human health. Vitamin D deficiency in the human body can lead to osteoporosis, tiredness, viral or auto-immune illnesses, diabetes, hypertension, and cardiovascular issues (Burt *et al.*, 2019; Eleni

and Panagiotis, 2020). Excessive use of vitamin D can cause an excessive uptake of calcium, which in turn increases the likelihood of developing hyperkalemia and renal issues (Arneson and Arneson, 2013; Da Silva *et al.*, 2021). Vitamin D can be gained from dietary sources or synthesized in the skin upon contact with UV-B light (Sarioglu *et al.*, 2001; Lu *et al.*, 2007). Vitamin D is primarily obtained from natural food and dietary supplements. Due to the demands of modern lifestyles, the human body cannot maintain the necessary levels of vitamins. Therefore, it is necessary to obtain them artificially from external sources. Vitamin D capsules and tablets provide a fixed dosage of vitamin D. With the widespread prevalence of vitamin D deficiency affecting approximately 1 billion people globally, research has increasingly highlighted the significance of cholecalciferol in preventing and managing various diseases, including osteoporosis, diabetes, cardiovascular disease, and certain types of cancer.

The production of pharmaceuticals with adequate quality, efficacy, and safety is the primary objective of the pharmaceutical industry. The development of a replacement medication product involves numerous pharmaceutical procedures, including analytical testing. The analytical data produced help to determine whether a therapeutic product should be released or support future decisions on how development should be pursued. Every such development or manufacturing process needs to be monitored and, if needed, continuously improved because the outcomes must be reliable and of consistent quality. One of the most crucial steps in the formulation and development of drugs is the use of analytical techniques. They are essential for development and production activities at every stage of the manufacturing of a pharmaceutical product. An analytical method must be dependable, accurate, and exact to be appropriate for the task at hand (Gaudin and Ferey, 2016; Parr and Schmidt, 2018).

To ensure that the choice of whether to release or discard a product is predicated on one or more types of control actions, the concept behind internal control is to examine and determine a true and appropriate product through a series of steps intended to prevent and identify errors at various production stages. Developing a straightforward and analytical procedure for a range of intricate formulations could be a critical area of study. The demand for brand-new analytical techniques within the pharmaceutical industry has rapidly increased due to the rapid growth of these industries and the continuous production of drugs across the globe. As a result, the development of analytical methods has become a fundamental activity of research in an extremely internal control laboratory (Ravisankar *et al.*, 2015). The following factors led to the development of novel drug analysis.

Pharmaceutical companies currently utilize several methods for quantifying cholecalciferol (vitamin D3) in formulations, including Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), Gas Chromatography-Mass Spectrometry (GC-MS), and Ultraviolet (UV) Spectrophotometry. While LC-MS/MS provides enhanced sensitivity and specificity through MS/MS transitions (e.g., 385.4 → 269.3 m/z), it involves complex purification steps and is prone to baseline drift or noise, which can impact accuracy and precision. GC-MS relies on the separation of cholecalciferol based on volatility, but its requirement for derivatization (e.g., silylation) and the thermal instability of vitamin D3 make it prone to degradation and unreliable results. UV Spectrophotometry, although simpler, faces significant interference from excipients, as their UV absorption at 550 nm overlaps with that of vitamin D3, leading to inaccurate quantification.

These existing methods are time-consuming,

expensive, labor-intensive, and susceptible to inaccuracies, necessitating the development of a simpler, more efficient alternative. In this study, a Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the accurate and precise quantification of cholecalciferol in pharmaceutical products. Using a C18 column and a photodiode array (PDA) detector at 265 nm with a mobile phase of methanol and water (98:2, v/v) eliminates the challenges associated with other techniques. The validation results demonstrated excellent precision, accuracy, and reproducibility, making this RP-HPLC method a superior choice for routine quality control of cholecalciferol-containing medicines.

2. MATERIAL AND METHODS

Cholecalciferol (Analytical Working Standard) was purchased from Duksan Pure Chemicals, Methanol from Sigma Aldrich, and water for injection from the WFI plant of the company. All other chemicals were provided by the pharmaceutical company.

2.1. Preparation of Standard Solution

In a 50-mL volumetric flask, 25 mg of the cholecalciferol working standard was dissolved in methanol. A 5 mL portion of this solution was then diluted to 25 mL using the mobile phase.

The final solution (0.1 mg/mL) was filtered through a 0.45 µm filter.

2.1.1. Preparation of Sample Solution

The injection volume corresponded to 5 mg of cholecalciferol (1 mL) and was diluted with 50 mL of mobile phase. The solution was filtered using a 0.45µm filter.

2.1.2. Preparation of Stock Solution

The stock solution was made by weighing 25 mg of cholecalciferol working standard and dissolving it in methanol in a 50 mL volumetric flask, then taking 5 ml of the aforesaid solution and diluting it to 25 ml with mobile phase. The material was totally dissolved after shaking in an ultrasonic bath. This stock solution was further diluted yielding solutions ranging from 0.02 mg/mL to 0.14 mg/mL.

The mobile phase consisted of methanol and water (98:02 v/v) supplied in a gradient at a rate of 2 ml/min with a retention time of 5.14 minutes. The appropriate retention period was obtained by adjusting the composition of the mobile phase; the above-mentioned composition yielded the best results. The results showed that the precision of this procedure was suitable. The novel approach's linearity, accuracy, precision, and specificity were verified in compliance with FDA draft guidance and ICH recommendations (Crumpler *et al.*, 2002; Bansal *et al.*, 2004; Bakhtiar *et al.*, 2007). The serial dilution approach was utilized to calculate the limit of detection (LOD), whereas American norms served as the basis for the limit of quantitation (LOQ) (Williams, 2006; Swartz and Krull, 2012).

2.2. System Suitability

Five Standards were prepared independently each time (0.1 mg/ml). The chromatogram of each solution measured the responses in the form of a peak area. It is a critical component of validation that ensures the analytical system, including instruments, equipment, and software, is functioning correctly and producing reliable results. It is a test of the system's performance and stability, typically conducted at the beginning of each analytical run.

2.3. Method Validation

2.3.1. Linearity

Linearity concentration ranges of the test substance were studied by preparing a stock solution. In a 50-mL volumetric flask, 25 mg of

the cholecalciferol working standard was dissolved in methanol. A 5 mL portion of this solution was diluted to 25 mL with the mobile phase, and the resulting solution (0.1 mg/mL) was filtered through a 0.45 µm filter. The diluent was used as a blank. To prepare solutions with concentrations of 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.08 mg/mL, 0.10 mg/mL, 0.12 mg/mL, and 0.14 mg/mL, 2 mL, 3 mL, 4 mL, 5 mL, 6 mL, and 7 mL of the sample solution were transferred into separate 50-mL volumetric flasks, and the volume was adjusted with the mobile phase.

Each concentration was introduced into the HPLC system in triplicate. The reaction, expressed as peak area, was measured. A linear association between peak area and drug substance quantity (Cholecalciferol) was discovered.

2.3.2. Accuracy and Precision

The analytical method's accuracy was ascertained by using samples in triplicates. The media used to prepare QC standards was different than was used to prepare calibration curves. The precision of the analytical process was assessed and presented as %RSD for a statistical analysis. Three different drug concentrations were analyzed in triplicate, three times on the same day, to determine the intraday precision of the chosen method. The same sample analysis used for the intraday precision assay was also used to evaluate the intraday precision, and this process was done three days in a row.

2.3.3. Robustness

To check the robustness of the analytical method, an assay was performed to determine the percentage of the drug substance Cholecalciferol by making the following deliberate changes in the HPLC assay method.

± 2 mL/min in the flow rate; i.e., use a flow rate of 1.8 mL/min and 2.2 mL/min to perform the assay separately.

± 2nm in the detection wavelength, i.e., at 263nm and 267nm.

2.3.4. Specificity

The specificity was determined by spiking the sample with a placebo of excipient mixture

And diluent or mobile phase. The specificity of analytical methods was tested for both drugs when used simultaneously.

Placebo Dilution: Transfer a carefully weighed quantity of excipients that equals 25 mL of cholecalciferol (5 mg/mL injection), and then 1 mL of the above solution to a 50 mL volumetric flask. The mobile phase was used to make up the volume.

- Active ingredient (standard preparation as per the testing method).
- All excipients and active ingredients (single dilution containing all excipients and API as per the assay preparation described in the analytical method).

Each dilution was injected into the HPLC system in duplicate and checked for the clear separation of API Cholecalciferol from the bases (excipients) with acceptable resolution and at specific retention times.

3. RESULTS

3.1. Method Development

The liquid chromatographic system consists of a Waters Alliance 2695 HPLC system with a PDA (photo diode array) detector at wavelength 265 nm. At room temperature, an analysis was done using a C18 µ-bond pack column (4.6 x 250 mm) with 0.5 µm particle size. The material was added to the chromatographic apparatus using a 20 µL sample loop.

3.2. Method Validation

3.2.1. System Suitability

Before analyzing samples, the HPLC system underwent a system suitability test to confirm its functionality. Parameters tested were Resolution (R), Retention Time (RT), Peak Area (PA), Tailing Factor (TF).

Table 1. Parameters and Condition of RP-HPLC

Parameters	Conditions
Method	Reversed phase high performance liquid chromatography
Mobile phase	Gradient elution water-methanol (02:98)
Column	C18 µ-bond pack column (4.6 x 250mm) with 0.5 µm particle size
Flow rate	Flow rate 02 ml/min
Detection	PDA-detector, 265 nm
Column temp	Ambient temperature
Injection	20µl for assay and compatibility

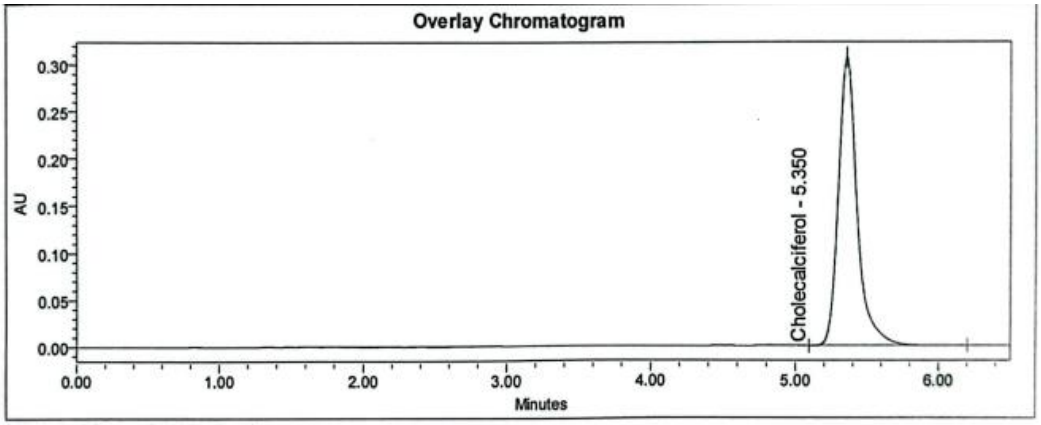

Figure 1. System Suitability spectrums of cholecalciferol working standard

Table 2. System Suitability of Cholecalciferol

System Suitability				
	Plate count	Tailing	Retention Time	Peak Area
Standard 1	8859.31	1.39	5.35	2815990
Standard 2	8846.33	1.38	5.35	2830636
Standard 3	8870.72	1.40	5.35	2817316
Standard 4	8870.83	1.39	5.35	2821113
Standard 5	8858.14	1.39	5.35	2821494
Mean	8861.1	1.39	5.35	2821309.77
Std.	10.21	0.01	0.00	5729.34
%CV	0.12	0.51	0.00	0.20

The system suitability test results in Figure 1 showed that the HPLC system was acceptable for analysis, with all test parameters meeting the acceptance requirements.

3.2.2. Linearity

The linearity test was used to determine the linearity of the HPLC technique for measuring cholecalciferol. We got a linear graph as shown in Figure 2 of these concentrations and in Table 3. The correlation coefficient was found to be r^2=0.9994. Table data showed all the results were in acceptance limit as the acceptance limit was RSD (r^2 ≥0.99) and we got a linear graph of the injected concentrations.

3.2.3 Accuracy

We ran samples with 80%, 100%, and 120% concentrations. Accuracy tests were carried out to show that the assay could determine the known quantity of Cholecalciferol. Table 4 showed outcomes that were within acceptable limits.

Table 3. Linearity of Cholecalciferol

Sr.#	AVG AUC of Standard for 4 injections = 2783795.17				Correlation Coefficient (r^2) and Y-intercept
	Conc. of Cholecalciferol Injection		Area under curve (AUC)	% Results	
	%age	mg/ml			
1	20	0.02	589235.40	21.17	r^2= 0.9994 Y=2817x+3025
2	60	0.06	1654903.26	59.45	
3	80	0.08	2223463.24	79.87	
4	100	0.1	2782860.18	99.97	
5	120	0.12	3387901.75	121.70	
6	140	0.14	3968266.32	142.54	

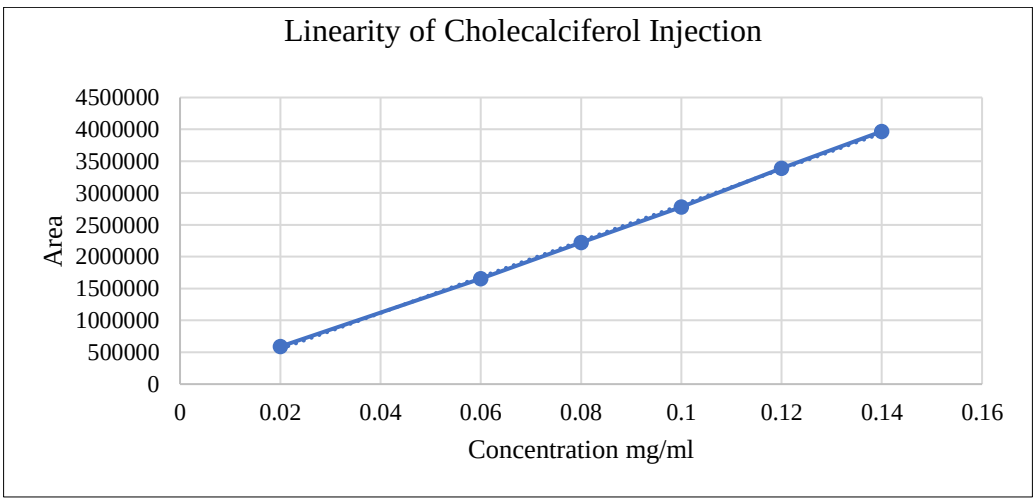

Figure 2. Graphical Representation of Linearity

Table 4. Accuracy of Cholecalciferol

Sr. No.	AVG AUC of Standard for three injections = 2805979				
	Conc. of Silodosin		Area under Curve (AUC)	Mean	% Results
	% age	mg/ml			
1	80%	0.08	2298120	2294808	81.78 %
			2302336		
			2283967		
2	100%	0.1	2771937	2772929	98.82 %
			2775630		
			2771221		
3	120%	0.12	3358770	3359784	119.74 %
			3356963		
			3363619		

Table 5. Intermediate Precision of Cholecalciferol

					Intra -Assay		Inter-Assay			
					Prep-to-Prep n=9		Day-to-Day n=27		Analyst-to Analyst n=54	
PeakArea	Samples	Inj. 1	Inj. 2	Inj. 3	Mean	%CV	Mean	%CV	Mean	%CV
Analyst 1 Instrument 1 Day 1	Prep-1	2858915	2845985	2862728	2849096	0.28	2800270	1.32	2799825	1.52
	Prep-2	2846355	2848208	2854617						
	Prep-3	2839845	2839545	2845669						
Analyst 1 Instrument 1 Day 2	Prep-1	2794604	2773345	2781664	2769143	0.48				
	Prep-2	2759730	2771199	2772989						
	Prep-3	2753424	2758659	2756680						
Analyst 1 Instrument 1 Day 3	Prep-1	2805653	2784439	2778658	2782571	0.33				
	Prep-2	2778938	2780444	2781114						
	Prep-3	2781668	2779756	2772472						
Analyst 2 Instrument 1 Day 1	Prep-1	2862290	2871346	2858064	2855904	0.28	2799379	1.59		
	Prep-2	2852350	2857612	2858612						
	Prep-3	2849928	2845419	2847516						
Analyst 2 Instrument 1 Day 2	Prep-1	2772608	2768826	2768387	2753343	0.53				
	Prep-2	2748113	2735724	2734014						
	Prep-3	2741127	2752415	2758874						
Analyst 2 Instrument 1 Day 3	Prep-1	2788903	2780653	2782849	2788892	0.18				
	Prep-2	2787143	2794621	2794542						
	Prep-3	2791902	2786147	2793271						

3.1.1. Intermediate Precision

The intermediate precision of Cholecalciferol was evaluated by analyzing three replicates of a standard solution on three different days by two different analysts. The results are presented in Table 5. Data from three days analyzed by two different analysts were shown, all chromatograms are present in supplementary data. All the results from both analysts fall within acceptance limits. The RSD for all preparations is NMT 2% and has proven the method effective and correct.

3.2.3. Robustness

Robustness was carried out by changing the different parameters described above to check the variation in the assay. The resilience test was done by altering the flow rate and wavelength.

3.2.4. Specificity

A reverse-phase HPLC method was developed for the analysis of Cholecalciferol using a C18 column, with a mobile phase consisting of acetonitrile and water, and UV detection set at 265 nm. Individual impurity solutions were prepared and introduced in specified quantities to the test solution. The specificity test was performed by injecting the extracted placebo to confirm the absence of interference. When Cholecalciferol was diluted and injected, a split peak was detected with a retention duration of around 5.14. No impurities or degradation products interfered with the Cholecalciferol peak.

Table 6. Robustness at Maximum Flow Rate

Maximum Flow Rate			
Conditions	Peak Area	Mean, actual	% Results
	2438305		
Standard at 2.2ml flow rate	2445616	2442053	
	2442237		99.54 %
	2426209		
Sample at 2.2ml flow rate	2433337	2430894	
	2433137		

Table 7. Robustness at Minimum Flow Rate

Minimum Flow Rate			
Conditions	Peak Area	Mean, actual	% Results
	3081138		
Standard at 1.8ml flow rate	3116504	3124140	
	3174778		99.40%
	3129105		
Sample at 1.8ml flow rate	3090027	3105482	
	3097315		

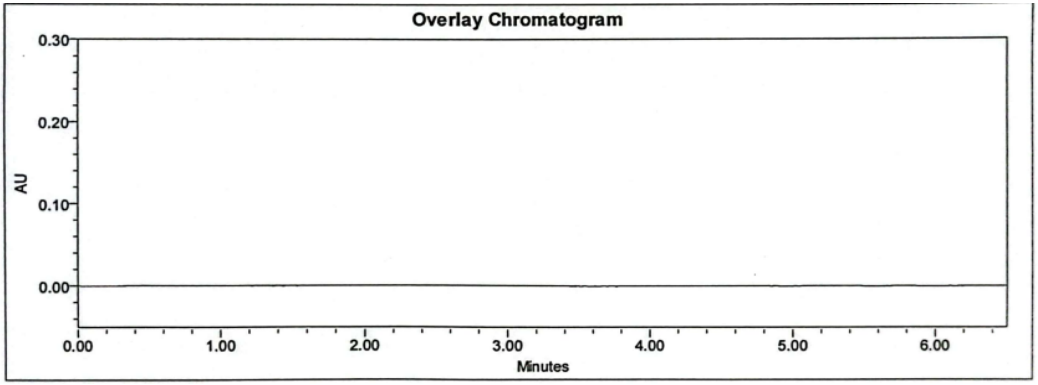

Figure 3. Placebo spectrum of Placebo

Table 8. Specificity of Cholecalciferol

Sr. No.	Avg AUC of 100 % STD	AUC of 100 % SMP	Results	Percentage Placebo
1	2813346.88	2809458	99.86 %	No interference of excipients
2		28081 00	99.81 %	
3		2812256	%	

4. DISCUSSION

The quality control of pharmaceuticals and healthy supplements containing vitamin D3 could be achieved through distributed HPLC-UV strategies, which were limited in their ability to indicate stability. These strategies often required longer run times than 10 minutes and were typically preceded by extremely complex and time-consuming test planning methods like solid-phase or supercritical liquid extraction. (Temova and Roškar, 2016). On the other hand, our validated method yielded results in just 7 minutes, providing precise and accurate linearity, accuracy, precision, robustness, and specificity of the test method for the quantitative determination of Cholecalciferol.

The British Pharmacopoeia stated that cholecalciferol was quantified using UV visible spectroscopy analysis. 48 hours before the test, antimony trichloride was produced as a diluent for the Cholecalciferol 1, 2 dichloromethane assays. The sample solution was added to the diluent in 90 to 120 seconds, and UV-visible spectroscopy was used to measure the absorbance of the solution at 500 and 550 nm. If our investigation revealed that our solution was not precisely prepared or that there was an accidental loss, we would have needed another 48 hours to complete the analysis. To obtain the highest yield, the vitamin D3 testing procedure needed to be improved to save analysis time and expense (Da Silva *et al.*, 2021). On the other hand, Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) was a technology that was applied and validated to solve the aforementioned difficulties.

Numerous techniques have been documented for determining cholecalciferol, the present study is compared with the other methods presently used by pharmaceutical companies, a method that includes utilizing the surfactant

Table 9. Summary of Parameters and Acceptance Criteria

Validation Parameters		Acceptance Criteria	Results	Pass/ Fail
System Suitability		RSD of Retention time: NMT 2 RSD of Theoretical plates: Limit of Theoretical plates: NLT2000 Tailing: NMT 2 RSD of Peak Area: NMT 2	0.00% 0.12% 8861.10 1.39 0.20%	Pass
Specificity		No interference	Complies	Pass
Precision-Repeatability		RSD of Peak Area< 2.0 % RSD of Retention Time< 2.0%	0.07% 0.00%	Pass
Intermediate Precision		RSD < 2.0 %	1.52%	Pass
Accuracy		78.0-82.0% 98.0-102.0% 118.0-122.0%	81.78% 98.82% 119.74%	Pass
Linearity		$r^2 \geq 0.990$	r^2=0.9994	Pass
Robustness	Flow Rate	Flow Rate + 0.2ml (2.2ml) Flow Rate - 0.2ml (1.8ml)	99.54% 99.40%	Pass
	Wavelength	Wavelength - 2nm (263nm) Wavelength + 2nm (267nm)	100% 99.37%	Pass

solution as the mobile phase and modifying a C18 column to contain an aqueous solution of sodium dodecyl sulphate (SDS) at a concentration of 3.00% (w/v) at pH 7. The organic solvent modifier added to the mixture is 15.0% (v/v) butyl alcohol (Kienen *et al.*, 2008). Another method that combines linear gradient elution with isocratic elution, the mobile phase was composed of methanol (solvent B) flowing at a rate of 0.7 ml min−1 and 0.010% trifluoroacetic acid (solvent A) with a pH of 3.9. For the first four minutes of the separation, the linear gradient profile (A: B), which began at 95:5, remained constant. Subsequently, it decreased linearly to 2:98 during the following six minutes, stayed at that level for the next twenty minutes, and in the last five minutes of the separation, it grew linearly to a 95:5 ratio of water phase (Klejdus *et al.*, 2004). On the other hand, our validated method was simple and easy to handle which consisted of only methanol and water as a mobile phase with a constant flow rate of 02 ml/min. The area under curve of cholecalciferol was constant throughout the analysis, there was no interference of any diluent or any active ingredient.

It was a highly effective, sensitive, and time-efficient method. This approach used a PDA detector, which was more sensitive and effective at detecting several wavelengths, in conjunction with a C18 column. It was capable of detecting any unknown concentration that fell within the 200–800 nm range. This method was prepared quickly and was cost-effective because it simply used water and methanol as the mobile phase. The validated HPLC approach is simple, selective, specific, fast, sensitive, repeatable, precise, and accurate for estimating cholecalciferol in pharmaceutical formulations. Furthermore, the verified approach is cost-effective.

It has been determined that the verified HPLC method is easy to use, fast, sensitive, repeatable, precise, and accurate for estimating

cholecalciferol in pharmaceutical formulations. Furthermore, because the validated method uses methanol and water in the mobile phase rather than pricy chemicals, it is inexpensive (economical method) and environmentally benign. Therefore, it is simple and convenient to use this approach for regular quality control analysis of pharmaceutical formulations and bulk cholecalciferol.

Peak identification on the chromatogram of the cholecalciferol sample occurred at the same retention time as the cholecalciferol standard, representing another qualitative feature. By guaranteeing the outcome of identification and writing an essay. It can be concluded based on the explanation above, that the active ingredient (cholecalciferol) in the injectable dosage form can be identified and its assay tested using an analytical method.

5. CONCLUSION

This validation study successfully demonstrated the system applicability, specificity, repeatability, accuracy, precision, linearity, and robustness of the test method for quantitative cholecalciferol measurement. The study's objectives were met by verifying the test process for quantitative measurement of cholecalciferol. According to the validation studies, the test method's intended use for quantitative measurement of cholecalciferol is appropriate.

Supplementary Data

All chromatograms and relevant data are present as supplementary data.

Conflict of Interest

The authors declare no conflict of interest.

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