# VALIDATION AND EVALUATION OF AN HPLC METHOD FOR QUANTIFICATION OF VITAMIN D3 IN PHARMACEUTICAL FORMULATIONS

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**ABSTRACT:** In accordance with the guidelines provided by ICH Q2 (R1), the analytical procedure that you have described for quantifying vitamin D-3 through the use of HPLC is quite comprehensive and has been thoroughly validated. The apparatus appears to be reliable, as it consists of a C-18 column, a mobile phase consisting of methanol and water, and detection at 264 nm, which is an adequate wavelength for vitamin D-3 identification. The flow rate of 1.2 millilitres per minute appears to be well optimised, and it is encouraging to observe that the method is linear (r2 = 0.999) between 0.25 and 1.25 microgrammes per millilitre across the concentration range. The accuracy of the procedure, which is proved by the recovery percentage that falls between 95 and 105%, in conjunction with a relative standard deviation (RSD) that is less than 2%, suggests that the method is both exact and trustworthy. The low limit of detection (LOD) of 0.0001 µg/mL and the low limit of quantification (LOQ) of 0.0005 µg/mL indicate that the method is sufficiently sensitive to identify low concentrations of Vitamin D-3 in samples. The robustness of the approach, which does not exhibit any substantial variations in reaction when the flow rate and mobile phase composition are changed, exemplifies the method's capabilities of adaptability and dependability in a variety of circumstances. Furthermore, on the basis of these validation results, it would appear that this HPLC approach is suited for routine measurement of vitamin D-3 in pharmaceutical formulations as well as bulk quantities. It would be interesting to gather information about whether or not the approach has been tested on a variety of formulations or matrices, as well as whether or not any difficulties were encountered throughout the testing process.

**KEYWORDS**: LINEARITY, ACCURACY, PRECISION, VITAMIN-D3, HPLC,LOD

**INTRODUCTION:**

Calcitas-D3 is a dietary supplement that mixes calcium and vitamin D3, both of which are necessary for the maintenance of healthy bones. Calcium and vitamin D3 are needed for maintaining healthy bones. The absorption of calcium by the body is facilitated by vitamin D3, which in turn encourages the production of healthy bones and ensures their strength. Calcium is an essential mineral that plays a role in how well bones and teeth are able to keep their integrity. Moreover, vitamin D3 facilitates the body's mineral absorption. Calcitas-D3 is a steroid hormone that can be created in the skin when it is exposed to UV radiation or acquired through the consumption of food. Both of these processes are natural processes. The Calcitas-D3 supplement is meant to promote bone health, particularly in persons who may be weak in calcium or vitamin D3. In individuals who are at risk of developing osteoporosis, those who have limited sun exposure (due to the fact that vitamin D is produced by the skin when it is exposed to sunlight), and those who have dietary restrictions that limit their intake of calcium and vitamin D, the use of this supplement is among the most common types of people who take it [1]. A representation of the chemical structure of vitamin D3 may be found in Figure 1. Calculitriol, which is the active form of calcitriol, is the hormone that plays a significant part in the process of bone mineralisation as well as the regulation of calcium and phosphorus levels in the blood. Furthermore, it has a significant impact on the expression of the cationic antimicrobial peptide cathelicidin in monocytes as well as epidermal keratinocytes. This is a significant influence. Cathelicidin is a vital host defence peptide that makes a significant contribution to both innate and adaptive immunity. One of the tasks that vitamin D-3 plays in the process of wound healing is the creation of cathelicidin because it is an essential component of the immune system. It modulates the immune response and enhances healing, according to germs that are positioned in close proximity to wounds. Additionally, it allows for faster recovery. As a result of the role, that cathelicidin plays in the healing of wounds and the treatment of a variety of skin conditions, including psoriasis, rosacea, and atopic dermatitis, it is possible that new avenues for the application of vitamin D in dermatology could be opened up. The findings of a number of meta-analyses indicate that vitamin D3 is more effective than vitamin D2 in boosting serum 25(OH) D concentrations. This is the conclusion reached by numerous researchers. As a result of this discovery, vitamin D3 has the potential to emerge as the more preferred option for the formulation development of future goods [2]. It also stays in the bloodstream for a longer period of time than vitamin D2 does, which is another characteristic of this substance.Vitamin D3 is the preferable agent for usage in medical applications because ergocalciferol is pharmacologically less effective than cholecalciferol. This is because vitamin D3 is more effective than cholecalciferol. According to the findings of the research that was carried out, vitamin D3 not only has antibacterial properties but also anti-inflammatory properties and wound healing properties. The administration of vitamin D3 topically has been demonstrated to be useful in a number of ways, including the promotion of efficient absorption, the enhancement of wound healing, and the provision of protection against a wide range of injuries and illnesses. An analytical approach has been devised because there is currently no method that is simultaneously economical and easy for testing vitamin D3 in small quantities [3]. As a result, this method has been developed. For the purpose of this process, the mobile phase consisted of methanol and water, and a number of tests were carried out in order to ascertain the range of serial dilutions. It should be stated that the entire procedure was carried out in a manner that was in accordance with the recommendations of the ICH Q2 R1. In order to get the greatest possible recovery of vitamin D3, the primary objective of this freshly developed analytical method was to acquire the highest possible recovery from the vitamin. In light of this, the approach that was utilised resulted in a greater recovery of vitamin

D3 in comparison to analytical processes that had been reported in the past, as well as a decreased standard deviation across all validation criteria [4,5].

## MATERIALS AND METHODS:

In order to conduct the study, we used HPLC-quality water and methanol, whereas the other components that were utilised were of an analytical grade. Vitamin D-3 was also utilised. The Waters High-Performance Liquid Chromatography (HPLC) model number L185CH361G was employed, which has a pore size of 0.35µ and a flow rate of 1 ml/min. In order to validate the results, the octadecylsilane column was selected [6]. A flow rate of 1 ml/min was utilised to inject the sample, and the volume of the sample was 50 µL. The injection was carried out for a length of 15 minutes. There is a UV detector for the purpose of analyte detection that is included in the Waters HPLC. For the purpose of validating the Vitamin D-3 approach, the meteorological conditions were suitable [7]. For the purpose of obtaining a linear graph or straight line equation, the C18 column was selected as the stationary phase, while methanol and HPLC-grade water were selected in various quantities to serve as the mobile phase. Vitamin D-3 was found to have a maximum wavelength of 264 nanometres (nm)[8]. Following a series of experiments, a standard curve was produced using concentrations of 0.25, 0.5, 0.75, 1.0, and 1.25 µg/mL of Vitamin D-3. This curve demonstrated linearity with an R2 value of 0.999, and hence, these dilutions were selected forfuture investigation [9].

### Method Validation:

Standard parameters such as linearity, range accuracy, precision, robustness, limit of detection, limit of quantification, peak purity index, tailing factor, and relative time were calculated [10].

### Selection and Preparation of different Quality Control Standard for Method Validation:

A concentration of 0.75µg/mL was chosen as the Medium Quality Control (MQC). Based on this concentration, two more dilutions were generated. These dilutions were as follows: 80% of the MCQ would be 0.6µg/mL (LQC), and 120% of the MCQ would be 9µg/mL (HQC). The abbreviation MQC refers to mid-level quality control, while the abbreviation HQC refers to a higher quality control standard. For these three concentrations, all of the other parameters were carried out[11]

### Linearity and Range:

The calibration curve for vitamin D3 was plotted, and a straight-line equation was devised for the purpose of estimating the regression line [12]

### Accuracy:

Through the process of recovering the medication from the quality control standard solutions, the accuracy of the procedure was analyzed and determined [13]. A total of five injections of each of the three-quality control standard solutions were made into the HPLC, and the mean response of the system was recorded [14]. By applying a method, which consisted of dividing the actual amount of drug with their theoretical concentrations and then multiplying the result by one hundred, we were able to calculate the percentage of drug that was recovered. Following that, the mean of the responses and the relative standard deviation were computed [15].

### Precision:

Two of the most important aspects of precision data are described as repeatability and intermediate precision. Repeatability was determined by injecting samples of all three quality control standards—LQC, MQC, and HQC—on the same day and under the same experimental settings. This allowed for the calculation of repeatability. For the purpose of evaluating the intermediate precision, the 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. Meanwhile, the experimental conditions remained the same, and the mean of response and percentage relative standard deviation were calculated [16, 17].

### Robustness:

The investigation was carried out by adjusting the flow rate (0.8, 1.0, and 1.2 mL/min) and the ratio of mobile phase accordingly. This was done in order to examine the effect that minor adjustments have on the robustness of the method that was created. We injected five replicates of the medium concentration, which was 0.75µg/mL. We observed and documented the effect of these medium concentrations on the area of the peak, recovery time, and retention time. Additionally, we recorded the mean of the response [18].

**Estimation of LOD and LOQ:**

In order to determine the LOD and LOQ, the standard deviation of the response (sigma) and the slope of the calibration curve (S) were utilized. The Y intercepts of the regression line were utilized as the standard deviation for the calculation of the standard deviation [19].

**System suitability**:

For the purpose of determining the characteristics of the system's applicability, five samples were injected into the high-performance liquid chromatography (HPLC) system, and the data was calculated. [20]

**RESULT& DISCUSSION:**

**Selection of Mobile Phase:**

For the purpose of determining the appropriate mobile phase, a number of experiments have been carried out. These experiments have included the use of methanol, methanol and water in varying proportions, dimethyl sulfoxide (DMSO), and phosphate buffer. Then, methanol and water in a ratio of 97:3 were chosen as the solvents of choice, taking into consideration parameters such as peak sharpness in the HPLC chromatogram, retention duration of vitamin D-3, and resolution. Additionally, the mobile phase that was chosen had a high resolution of vitamin D-3, improved peak sharpness, and a chromatogram that was of high quality. There was no peak in any of the mobile phases of the experiment; however, the solution of methanol and water in the ratio of 97:3 produced satisfactory findings. To proceed with the analysis, a solution consisting of methanol and water was chosen.

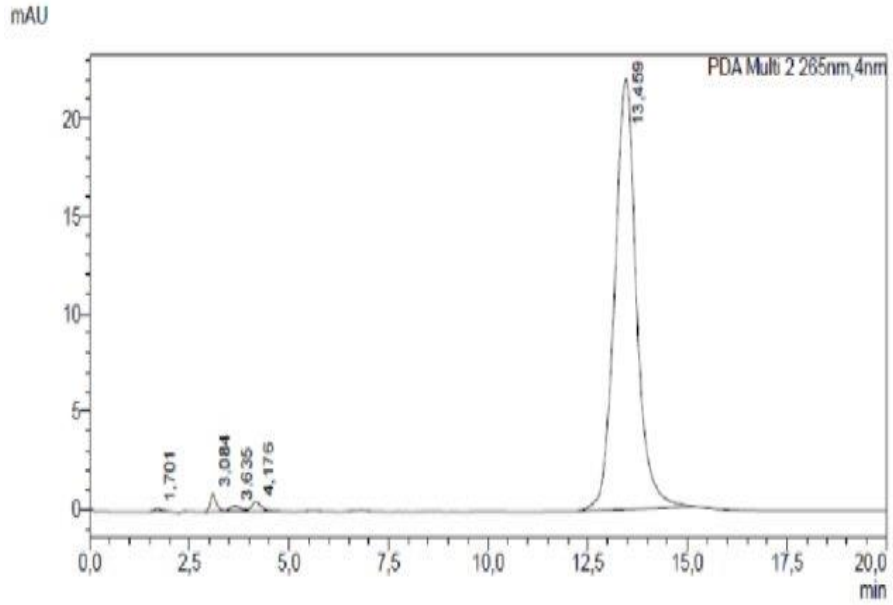


**Figure2: Chromatogram of Vitamin D-3 Peak**

**Preparation of Stock Solution and standard curve:**

At a volumetric flask with a capacity of 50 ml, 25 mg of vitamin D-3 was added, and the remaining volume was filled with a mixture of methanol and water at a ratio of 97:3. Then,

using the same solvent, various dilutions such as 0.25, 0.5, 0.75, 1.0, and 1.25µg/mL were made after the initial preparation.

**Linearity and Range:**

In order to make a comparison between the concentration of the samples and the area that was recorded in the HPLC, the standard curve was plotted using the area that was recorded. It was observed that the value of R2 was 0.999, and it was also discovered that the curve was linear at concentration ranges ranging from 0.25 to 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 |

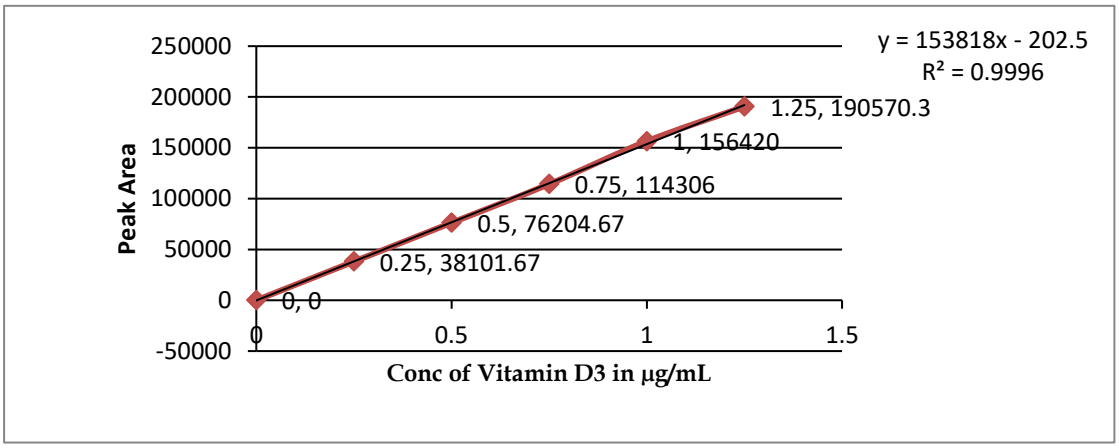


**Fig.3: Standard curve of Vıtamin D3**

**Accuracy:** It was determined whether or not the developed approach was accurate by determining the percentage of drug recovery, which was then followed by the relative standard deviation.The acceptable limit for the percentage of drug recovery is between 95 and 105%, while the tolerance for RSD is below 2%. Through the review of these data, the method was validated, and all of the readings fell within the acceptable range. All of the information is presented in the table that follows.

**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 |

**Table6: 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 |

**Robustness:**

All of the data were within the specified limit, which supported the validation, and the robustness was tested in terms of changes in flow rate as well as changes in the ratio of mobile Phase. Additionally, the percentage relative standard deviation was calculated.

**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 |

**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: 10 System Suitability Parameters:**

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

**CONCLUSION:**

Vitamin D-3 is superior to vitamin D-2 because it is able to persist in the systemic circulation for a much longer period of time. Cathelicidin is an important host defence peptide that plays

a good role in both innate and adaptive immunity. It kills the bacteria that are present in the vicinity of wounds, modulates immune response, and heals wounds. It makes wound healing possible through the creation of cathelicidin. A new analytical approach was created in the current study by employing methanol and water as the solvent system. This method was designed for the purpose of estimating or quantifying the amount of vitamin D3 present in a number of pharmaceutical formulations, as well as in a number of nutrition supplements and neutraceuticals.The method that was created was precise and accurate, and it produced the maximum recovery of vitamin D-3 compared to any other approach that was tested thus far. This is due to the fact that the limit of detection was discovered to be 0.0005µg. In addition to being accurate, sensitive, and reproducible, the method that was developed for estimating vitamin D-3 is also simple, cost-effective, and well-established.

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