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IDENTIFICATION AND QUANTIFICATION OF RELATED SUBSTANCES OF VITAMIND3 IN ITS PREPARTIONS BY RP-HPLC

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ABSTRACT

An analytical approach for quantifying vitamin D3 related substancesmethod was developed by using high-performance liquid chromatography (HPLC). The separation was achieved using a hypersil Silica, 250mm x 4.6mm, 5µm column, and a mobile phase composition of n-pentanol and n-hexane was used. The wavelength of the chromatogram was 265 nm at a temperature of 20°C, the flow rate was 2.0 mL/min. The method was specific, and linear within the range of 50%-150% and has demonstrated good recovery, inter day and intraday precision. As well as robustness, the detection and quantification limits were found to be within the limits at .001 and 0.0037 g/ml, respectively. The developed method ismore precise, accurate, specific and robust to determine the related substances of Vitamin D3 in its preparations and validated according to ICH guidelines Q2(R1).

Keywords: RP- HPLC, Cholecalciferol (CHL), Related Substances, Vitamind3, N-Pentanol

1. INTRODUCTION

Vitamin D3 (cholecalciferol) is produced endogenously from 7-dehydrocholesterol after UV irradiation or absorbed through the diet (1). It is also produced exogenously by irradiating ergosterol (2). Vitamin D is necessary for the growth and preservation of bones because it maintains appropriate levels of calcium and phosphorus in the blood (3). In addition to its connection with skeletal disorders, studies show that it has a substantial effect on cancer, heart disease, autoimmune illnesses, hypertension, and diabetes mellitus (4-6).

Vitamin D is not a single chemical but a group of molecules that work together to produce its effects (7). Its evaluation is essential as a clinical indicator of inadequate dietary vitamin D, which is one of the causes of osteoporosis (8). Current research suggests that vitamin D improves bone production, boosts bone protein concentration, and stimulates

osteoblastic gene expression (9). Along with vitamin D3, vitamin K2 has been shown to balance calcium levels for cardiovascular and bone health (10). Multivitamin formulations that contain vitamins D3 and K2 are essential for this purpose (11). However, there is currently no verified technique for determining the levels of vitamins K2 and D3 in supplements (12).

Sample size limitations present a barrier to clinical research, in contrast to other domains such as food analysis (13). Nevertheless, most available methods for quantifying vitamin D3 utilize LC-MS/MS (14).High-performance liquid chromatography (HPLC) is a fundamental technique in pharmaceutical analysis that offers accuracy, sensitivity, and effectiveness (15). Reversed-phase high-performance liquid chromatography (RP-HPLC) is especially suitable for identifying and measuring hydrophobic compounds like dutasteride (16). RP-HPLC verification ensures the reliability, precision, and consistency of analytical methods for assessing the potency and purity of pharmaceutical formulations (17).Given these factors, there is a need to develop a sensitive, easy, and inexpensive method for quantifying vitamin D3 using RP-HPLC (18). QbD (Quality by Design) principles offer a systematic approach, using statistical experimental designs to provide a design space for analytical techniques (19). These principles allow for a more robust method development process (20).

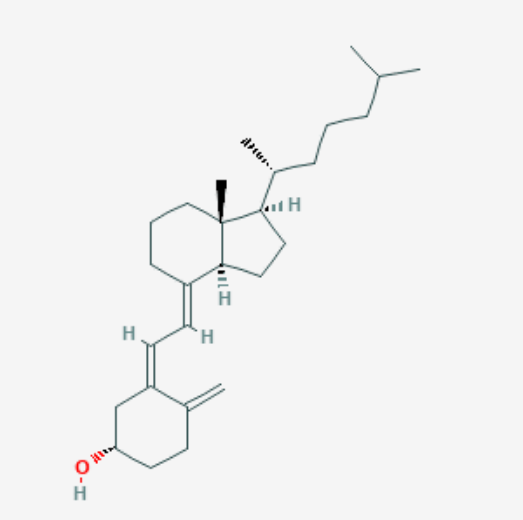


Figure1: Structure of vitamin D3

2. MATERIALS AND METHODS

2.1. INSTRUMENTATION:

Shimadzu High-Performance Liquid Chromatography systemequipped with aPDA detector and controlled by labs solution software was utilized for component separation.The system employed an isocratic elution mode and a 4.6mm x 250mm column packed with Cyanosilyl silica gel for chromatography (Zorbax SB-CN).

2.2. PREPARATION OF MOBILE PHASE:

Prepare a mixture of n-pentanol and n-hexane in ratio of 3:997. Mix the mobile phase for 10minutesona magneticstirreranddegasfor10minutes.

Diluent: Isooctane

2.3. PREPARATION OF BHT(1G/100ML):

The BHT should be weighed into a 100 mL volumetric flask, dissolved, and made up with diluent to the required volume.

2.4. PREPARATION OF BLANKSOLUTION:

Dilute1mL of BHT in100mLvolumetric flask with diluent.

2.5. PREPARATION OF RESOLUTIONSOLUTION:

Weigh about 12.5 mg of Cholecalciferol WS/RS in to a 100 ml volumetric flask, add 1ml of BHT solution shake well to disperse and dilute to volume with diluent. Dilute 2mL of above solution into 10mL volumetric flask, add 1ml of BHT solution shake well to disperse and dilute to volume with diluent. Dilute 5ml of above solution into a 25ml volumetric flask, add 5ml of mobile phase and make upto its volume with diluent. Heat in a water bath at 60°C for 1hour and cool.

2.6. PREPARATION OF DILUTED STANDARD SOLUTION (10IU/ML):

In a 100 ml volumetric flask, weigh approximately 12.5 mg of cholecalciferol WS/RS. Then, add 1 ml of BHT solution, shake well to scatter, and dilute with diluent to volume. Take a 10 mL volumetric flask, add 2 mL of the aforementioned solution, shake well to distribute, and then dilute with diluent to the level required. Using a pipette, transfer 1 ml of the above standard to a 100 ml volumetric flask, and then dilute the volume to the required amount.

2.7. PREPARATION OF SAMPLESOLUTION (1000IU/ML):

Cut twenty capsules open. Place the medication in a dry, clean Petri dish and stir thoroughly. Fill a 100 mL volumetric flask with approximately 416.7 mg of medication (or 100,000 IU), add 1 mL of BHT solution, mix thoroughly, and dilute to fill the flask to the full capacity.

2.8. PREPARATION OF PLACEBOSOLUTION:

Cut twenty Placebo capsules open. Place the medication in a dry, clean Petri dish and stir thoroughly. About 414.0 mg of medication should be weighed into a 100 ml volumetric flask. 1 ml of BHT solution should then be added, shaken thoroughly, and filled to capacity with diluent.

2.9. PROCEDURE:

Measure the responses and record the chromatograms after injecting 200 µL of diluent as blank, one injection of Placebo, Resolution solution, six replicate injections of diluted standard preparation, two replicate injections of diluted check standard preparation, each injection of sample preparation, and one injection of bracketing standard separately. Record your usage of the instrument and column in the log book, then compute using the results.

Table1: Chromato graphic conditions

Instrument	HPLCwithUV/PDADetector
Column	ThermoScientific,HypersilSilica, 250X4.6mm, 5µm
Flow rate	2.0ml/min
Oventemperature	25°C
Injectionvolume	200µl
Wavelength	265nm
Runtime	30min
Retentiontime	17-21in

3. RESULTS AND DISCUSSION:

In order to obtain the intended Limit of detection (LOD)and precision at the limit of quantification (LOQ), accuracy, linearity, method precision, robustness, specificity, and force degradation studies, the analytical technique has been Optimized and Validated incompliance with current ICH guidelines.

3.1. SYSTEM SUITABILITY:

Six distinct preparations of the standard solution were used to evaluate system suitability in order to guarantee the robustness and dependability of the analytical technique.

Table 2: system suitability results

S.NO	NAME	AREA	RETENTION TIME	THEORETICAL PLATE	TAILING FACTOR
1	Cholecalciferol	67967	20.1	13583	1.0

3.2 SPECIFICITY:

The capacity to definitively evaluate the analyte in the presence of elements that might be anticipated, such as matrix, degradants, contaminants, etc." which is meant to be intended as specificity, according to ICH recommendations.

The outcome demonstrates that the RT of cholecalciferol-related substances in cholecalciferol 60000 IU Capsules is free from interference from blank and placebo peaks. The cholecalciferol related substances peak purity index values for the standard and sample solutions of cholecalciferol 60000 IU Capsules fall within the acceptable range.

Hence it is concluded that the method is specific to estimate the amount of CholecalciferolRelated Substances in Cholecalciferol 60000 IU Capsules without the interference of blankandplacebopeaks.

Table 3: Specificity data of blank, placebo, standard and sample,

SpecificityParameter	Blank	Placebo	Resolution			DilutedStandard	Sample	Acceptance criteria
			Precholecalciferol	Transcholecalciferol	Cholecalciferol			At the designated wavelength, there shouldn't be any interference from blank and placebo peaks with the main analyte signal.
Interference	NIL	NIL	29765	2497	1300149	61584	7699926	
Retentiontime	NA	NA	10.8	11.7	20.4	20.1	20.2	
Peak PurityIndex	NA	NA	1.00	1.00	1.00	1.00	1.00	**NLT0.99**

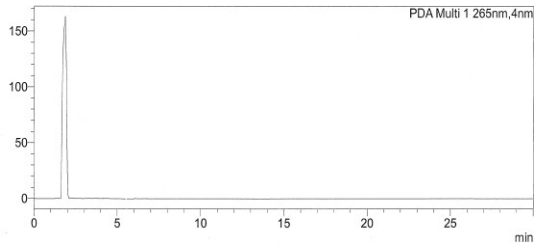


Figure 2: Specificity Blank Chromatogram

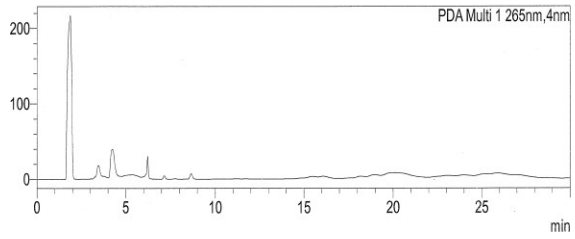


Figure3: Specificity Placebo Chromatogram

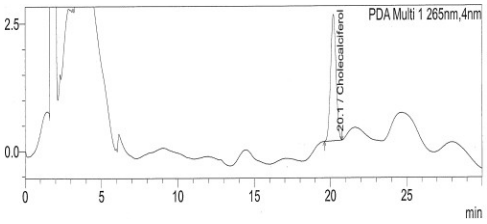


Figure 4: Specificity Standard chromatogram

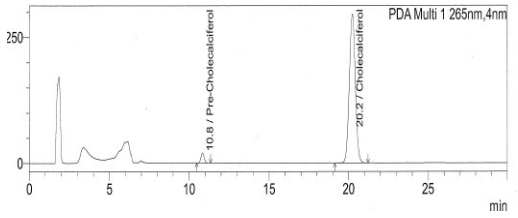


Figure 5: Specificity Sample Chromatogram

3.2. ACCURACY:

The measure of accuracy is how closely the test findings produced by the procedure match the actual value. A common way to describe accuracy in an analyte assay is as a percentage of recovery. A measure of an analytical method's exactness is its accuracy. The "4" concentration (LOQ solution, 50%, 100%, and 150%) will be used to evaluate accuracy. Standard and placebo solutions are made with concentrations of LOQ, 50%, 100%, and 150% in accordance with the protocol. Based on the area obtained for each concentration, % of Recovery is calculated. The details are given below.

Table4: Accuracy results,

Sample	AmountAdded(ppm)	Amountrecovered(ppm)	Area	%Recovery	Average	%RSD
LOQ-1	0.0004	0.0005	1359	125.5371	122.4	2.2
LOQ-2		0.0005	1300	120.0870		
LOQ–3		0.0005	1317	121.6574		
50%-1	0.0123	0.0103	28172	83.6501	83.2	0.4
50%-2		0.0102	27997	83.1305		
50%-3		0.0102	27907	82.8633		
100%-1	0.0246	0.0214	58802	87.2074	88.0	0.8
100%-2		0.0217	59502	88.2455		
100%-3		0.0218	59748	88.6104		

150%-1	0.0368	0.0327	89604	88.8806	88.4	0.5
150%-2		0.0326	89311	88.5900		
150%-3		0.0323	88578	87.8629		

3.3. LINEARITY:

Six test concentrations, ranging from 50.0% to 150% of working concentrations, are used to determine the method's linearity in accordance with protocol. The concentrations of LOQ, 50%, 75%, 100%, 125%, and 150% with regard to 100% working concentration were used to prepare the standard solutions. Three duplicate injections and six duplicates for both higher and lower concentrations. Aspirate current concentrations into the HPLC system using injections.
Based on the average areaobtained with each concentration, a graph is plotted between Area and Concentration. The details are given below.

Table 5: Linearity data for Cholecalciferol related substances,

%Conc.	Cholecalciferolstandard stocksolution	Volume of stocksolutiontaken inmLwithdiluent	Further Dilute (mL)withdiluent	Potencyin %	Conc. inppm
LOQ	12.467mg ↓100mL	5mL ↓100mL	0.3mL → 100	99.7	0.0037
50			2.0mL → 100		0.1243
75			3.0mL → 100		0.1864
100			4.0mL → 100		0.2486
125			5.0mL → 100		0.3107
150			6.0mL → 100		0.3729

3.5 PRECISION

3.5.1. SYSTEMPRECISION

Six repetitions of a standard Cholecalciferol solution were made for this investigation and added to the HPLC apparatus. The system suitability parameter was ascertained in accordance with the protocol, and the tabulated results are shown in the table below.

Table 6: System precision results

S.NO	Systemprecision
1	68029
2	69483
3	67605
4	67067
5	67920
6	67696
Mean	67967
SD	815
%RSD	1.1

3.6. ROBUSTNESS

The ability to accurately measure the amount of cholecalciferol in Cholecalciferol 60000 IU capsules is proven by carefully adjusting the column oven temperature, flow rate, and wavelength.

3.6.1. EFFECT OF VARIATION IN FLOW RATE (±0.2ML/MINUTE)FOR CHOLECALCIFEROL:

The protocol-adherent little fluctuations in the flow rate (1.8, 2.0, and 2.2 mL/min) show the analytical method's resilience. In accordance with the procedure, the standard and sample solutions were made and injected into the HPLC.

Table7:Changeofflowrate

System Suitability Parameter	ObservedValue			AcceptanceCriteria
	1.8 mL/min	2.0 mL/min	2.2 mL/min	
The Tailing factor for Cholecalciferol peak from first injection of Diluted standard Solution.	1.0	1.0	1.0	**NMT2.0**
System Suitability Parameter	**ObservedValue**			**AcceptanceCriteria**
	1.8 mL/min	**2.0 mL/min**	**2.2 mL/min**	
The Theoretical plate count for CholecalciferolpeakfromfirstinjectionofDilutedstandardsolution	13192	13196	12495	**NLT2000**
The %RSD for RT of Cholecalciferol peak from 6 replicate injections of Diluted standardsolution	0.1	0.1	0.1	**NMT1.0**
The % RSD for Peak responses of Cholecalciferol peak from 6 replicate injections of Diluted standard solution	0.7	0.5	0.2	**NMT5.0**
The Relative retention time of Trans cholecalciferol with respect to cholecalciferol	0.6	0.6	0.6	**0.6**

3.6.2. EFFECT OF VARIATION IN WAVELENGTH (±2NM) FOR CHOLECALCIFEROL:

The analytical method's resilience is proved by tiny fluctuations in the wavelengths (263 nm, 265 nm, and 267 nm) according to protocol. The standard and sample solutions were produced and injected into the HPLC according to the technique.

Table 8: Change of wavelength,

SystemSuitability Parameter	ObservedValue			Acceptance Criteria
	263nm	265nm	267nm	
The Tailing factor for Cholecalciferol peak from First injection of Diluted standard solution.	1.0	1.0	1.0	**NMT2.0**
The Theoretical plate count for Cholecalciferol peak from first injection of Diluted standard solution	13194	13196	13203	**NLT2000**
The %RSD for RT of Cholecalciferol peak from 6 Replicate injections of Diluted standard solution	0.1	0.1	0.1	**NMT1.0**
SystemSuitability Parameter	**ObservedValue**			**Acceptance Criteria**
	263nm	**265nm**	**267nm**	
The %RSD for Peak responses of Cholecalciferol peak from 6 replicate injections of Diluted standard Solution	0.5	0.5	0.5	**NMT5.0**
The resolution between the peaks due to pre Cholecalciferol and Trans-cholecalciferol in the resolution standard	2.026	2.033	2.032	**NLT1.3**

The Relative retention time of Trans Cholecalciferol with respect to cholecalciferol	0.6	0.6	0.6	**0.6**
The %Recovery between diluted standard and Diluted check standard preparations	99.8	99.7	99.8	**90.0to110.0**

3.7. STABILITY OF ANALYTICAL SOLUTIONS:

By injecting standard and sample solutions for up to 48 hours, the stability of the solution is shown. The calculation of the percentage RSD of the standard and sample solutions is displayed below.

Table 9: Stabilitydata

Timeinterval	Dil.StdArea	SampleArea
Initial	63418	7174659
After12 Hours	55031	7044506
After24 Hours	62638	7104756
After36 Hours	58570	6544715
After48 Hours	63370	7023809
Average	60605	6978489
SD	3704.794	249498.5
Cumulative%RSD	6.1	3.6

3.8. FORCED DEGRADATION STUDY:

Forced degradation is a method that breaks down drug ingredients and drug products under more adverse conditions than under accelerated conditions. This produces degradation products that may be analyzed to find out how stable a molecule is Stress testing, according to the ICH guidelines Is meant to confirm the stability indicating methodologies that have been employed and to recognize the anticipated breakdown products, which help define degradation routes and ascertain the chemical's intrinsic stability. Types of forced degradation study includes; Acid, base, oxidation, UV, thermal, humidity and light.

3.8.1. ACID DEGRADATION:

For acid degradation, 30ml of a 0.1N Hcl degradant solution was added, followed by the additionof 30ml of neutralizing solution (0.1 N NaOH). The sample was then exposed to benchtopconditionsfor1hrat25ºC.

3.8.2. BASED DEGRADATION:

Base degradation is doneby, mixing30ml of a0.1NHcl degradant solution and 30ml of neutralizing solution (0.1NNaOH).The sample was then exposed to benchtop conditions for 1hr at25ºC.

3.8.3. PEROXIDE OR OXIDATION DEGRADATION:

Oxidation degradation can be assessed by adding 10 mL of 1% degradant solution to a 10 mL of neutralizing solution. The sample was then exposed to benchtop conditions for 1 hr at25ºC.

3.8.4. THERMAL DEGRADATION:

Thermal degradation was carried out by preparing the drug samples and keeping it in oven at105°Cforaperiodof6hrs.

3.8.5. HUMIDITY:

The sample was subjected to 90% relative humidity during the humidity test, with a maximum temperature of 25°C being maintained. Seven days were spent doing the study in a desiccator.

3.8.6. PHOTOLYTIC DEGRADATION (UV):

Exposure: 200wattshr/m^2inaphotostabilitychamber.

3.8.7. PHOTOLYTIC DEGRADATION (LIGHT):

Exposure: 1.2 million lux hours in a photo stability chamber

Table10: Forced degradation study results

StressedConditions	Contentin %	% Degradation	Peakpurityindex	%ofTranscholecalciferol	%ofUnknown	MassBalance
Unstressedsample	101.0	NA	1.00	ND	0.367	ND
AcidHydrolysisSample	102.2	1.2	1.00	ND	0.671	101.2%
BaseHydrolysisSample	99.3	1.7	1.00	ND	0.687	98.3%
Humidity sample	98.7	3.3	1.00	ND	ND	97.7%
UV Sample	99.1	1.9	1.00	ND	ND	98.1%
Thermal stressed Sample(Heat)	99.4	1.6	1.00	ND	0.703	98.4%
Photolytic Light sample	99.4	1.6	1.00	ND	ND	98.4%
Peroxide Oxidation Sample	102.2	1.2	1.00	ND	0.694	101.2%

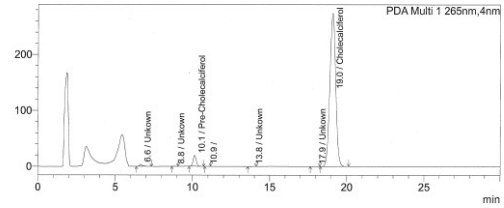


Figure6: Acid degradation

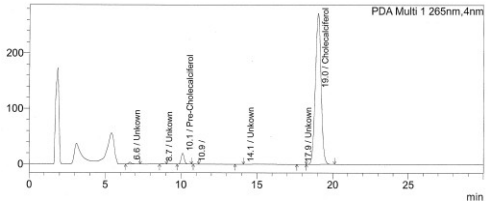
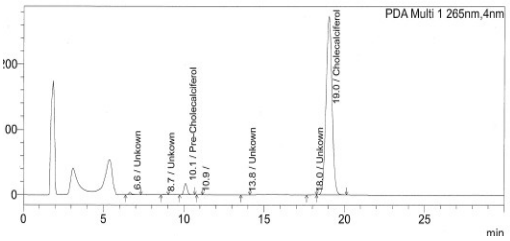


Figure7: Base degradation



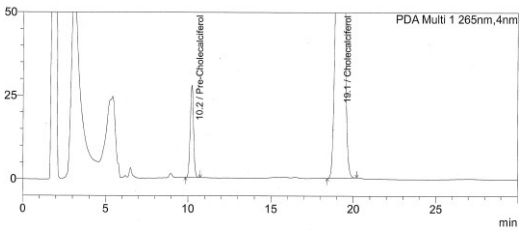
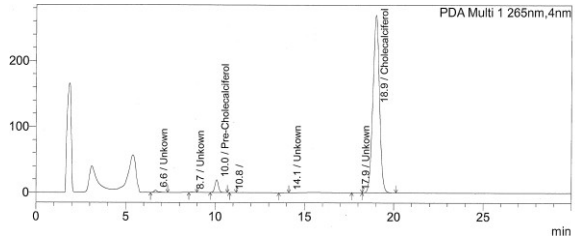


Figure 8: Peroxide degradation

Figure 10: Humidity



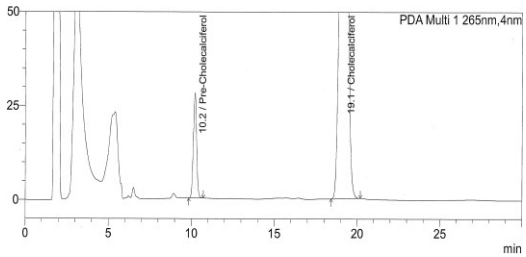


Figure 9: Thermal degradation

Figure 11: Photolytic degradation-UV

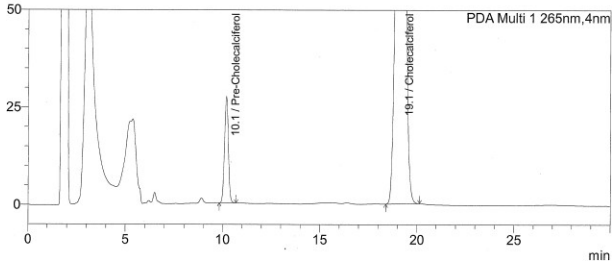


Figure12: Photolytic degradation-Light

3.8.8. FORCED DEGRADATION STUDY CONCLUSION:

The chromatograms of the sample, placebo, and stressed blank solutions demonstrate that there is no interference from the peaks of degradants, blanks, or placebos during the cholecalciferol retention period. Both the standard and sample

solutions' peak purity index values fall within the Acceptance requirements. Thus, it can be said that the method is stability indicating and specific for estimating the amount of cholecalciferol without interference from blank, placebo, or degradant peaks.

4. SUMMARY

A simultaneous assessment of calcitriol and cholecalciferol in vitamin D3-containing dietarysupplements. Numerous target analytes can be rapidly quantified and separated with a singleinjection. For each of the target nutritional components, this validated technique showed stronglinearity, good recovery, low level of Detection and Limit of Quantification (D3), great selectivity, and Specificity. This article shows how to enhance HPLC by better understanding the factor-response connection, which is essential to quality by design, using HPLC chromatography for CHL analysis. Before conducting validation trials, the analytical technique's robustness was ensured via CHL's AQbD-driven approach to method development. With the use of this noveltechnology, the analyst can more effectively create control measures to mitigate the unwanted influence of these CMVs on method performance. High levels of linearity ,precision ,and accuracy were confirmed by the validation studies.

5. CONCLUSION

The majority of them are unable topay the high cost of the existing vitamin D diagnostic procedures. As a result, we must create a straight forward, trustworthy, and affordable technique of estimating vitamin D. Analytical techniques are more sensitive and economical than RIA- orELISA-based procedures, as is widely proven. Vitamin D2 has been separated using a variety of chromatographic settings, but the best separation was eventually accomplished.

This new technique aids in the analyst's creation of control measures to lessen these CMVs' undesired impact on method performance. Highdegrees of precision, accuracy, and linearity were validated by the validation investigations. Because methanol and water are utilized as the solvents

in this procedure, it is both unique and accurate. Comparing this approach to other developedmethods, it was discovered that the least quantity of Vitamin D-3 could be computed since thedetection limit was determined to be 0.0005µg. The method for estimating vitamin D-3 that has been developed is simple, affordable, robust, sensitive, and repeatable.

The Related Substances method for the cholecalciferol in the cholecalciferol 6000 IU Capsules by HPLC technique is appropriate, linear, range, precise, accurate, specific, and robust, according to the results of the analytical method validation. As a result, this approach is deemed validated and suitable for ongoing investigation. At room temperature, the Standard and Sample were stable for a full 48 hours.

CONFLICT OF INTERESTS

None.

ACKNOWLEDGMENTS

None.

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