RESEARCH ARTICLE

Development and Validation of a Selective Reversed-phase High Performance Liquid Chromatography Method for the Quantification of 1α-Hydroxy Vitamin D2 and its Impurities

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ABSTRACT

A selective reversed-phase high-performance liquid chromatography (RP-HPLC) technique was developed for 1α-hydroxy vitamin D2 (Doxercalciferol) related substances analysis. The technique is proficient of eluting various impurities related to process and degradation. To separate both the vitamin D2 Analogs i.e., doxercalciferol and ergocalciferol. HPLC of Shimadzu, provided with autosampler, column oven, quaternary pump and UV detector, were used for development studies. The mobile phase used water and acetonitrile. The diluent used was methanol and detection was examined at wavelength 265 nm. The method is sensitive for doxercalciferol, ergocalciferol, impurity-A and impurity-B; the limit of detection (LoD), value is 0.01% and limit of quantitation (LoQ) value is 0.03%. Linearity of doxercalciferol and related impurities at various concentrations was performed and the obtained correlation coefficient for doxercalciferol is 0.995, ergocalciferol is 0.997, impurity-A is 0.990 and impurity-B is 0.993. The optimized method was validated and demonstrated that the technique is selective, precise, linear, sensitive and accurate. The method is simple, inexpensive and useful for routine testing of doxercalciferol API. In addition, the technique was effectively applied for the elution of both the vitamin D2 Analogs i.e., doxercalciferol and ergocalciferol. Further, the same method is applicable for the determination of assay for these two API's and the same assay method was validated.

Keywords: 1α-hydroxy vitamin D2 (Doxercalciferol), Ergocalciferol, Reversed-phase high-performance liquid chromatography, Validation and linearity.

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INTRODUCTION

The COVID-19 epidemic has triggered a public health emergency on a worldwide scale. The severity and risk of various acute respiratory tract infections, including COVID-19 are both decreased by vitamin D.[1] According to recent research, individuals with COVID-19 who were hospitalized had a lower chance of bad outcomes and mortality if their vitamin D levels were adequate. According to some studies, maintaining good vitamin D levels might help your immune system remain strong and may shield you from respiratory diseases.[2] Numerous vital jobs for vitamin D in the body of humans. Vitamin D is particularly crucial for the health of the immune system.

In general lack of exposure of human body to sunlight leads to vitamin D deficiency. In addition, low nutritional dietary intake also leads to deficiency of vitamin D. Around the globe, vitamin D deficiency is a serious issue that is becoming more widespread. Osteoporosis, muscle weakness, secondary hyperparathyroidism, and calcium malabsorption are symptoms.[3,4]

Vitamin D is crucial for boosting calcium absorption and healthy bone mineralization. Rickets in newborns and young children and osteomalacia in adults are both the results of severe vitamin D insufficiency.[5]

Additional benefits of vitamin D include boosting immune function, improving cardiovascular health,[6-8] diabetic nephropathy,[9] Blood pressure[10] and inhibiting growth of neuroblastoma.[11]

Numerous illnesses and conditions, such as immunological dysfunction, metabolic bone diseases, hyperproliferative diseases, and endocrine disorders, might benefit from the breadth and amplitude of vitamin D action.[12]

Patients with chronic renal illness may regulate their secondary hyperparathyroidism safely and effectively with

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doxercalciferol.[13-23] When given doxercalciferol, even juvenile patients with end-stage renal disease showed significant alterations in osteocytic protein expression, affecting bone mineralization and the skeletal response to parathyroid hormone (PTH).[24]

The synthetic vitamin D analog doxercalciferol, also known as 1α-hydroxy vitamin D2, is the subject of the current study. Both intravenous and oral formulations of doxercalciferol, also known as 1α(OH) D2 or 1α-hydroxyvitamin D2, have received approval from the US food and drug administration (FDA) to lower intact parathyroid hormone (iPTH) in hemodialysis patients with secondary hyperparathyroidism.[25,26]

Survey on the literature reveals that few approaches were described for the determination of doxercalciferol and several studies present on the extraction of vitamin D from human blood samples, plasma, placebo etc. Few assay methods were also reported for vitamin D analogs[27-35] including the quantification of doxercalciferol degradation components by RP-HPLC and solid-phase extraction. In that study, the chromatographic parameters utilized for the estimation of doxercalciferol degradants/impurities with infusion volume 100 µL, the flow rate of 1.60 mL/min, water and acetonitrile are utilized as mobile solvent at a column temperature of 40ºC.[36]

A doxercalciferol related substance technique is reported in United States Pharmacopoeia (USP) with specified impurities. In this process, water and acetonitrile are utilized as mobile solvent with a flowing rate of 1.70 mL/min in a gradient separation approach at a temperature of 35ºC for column. During sample preparation, dissolve first in ethyl acetate using about 20% of the final volume with sonication in an ice water bath and dilute with acetonitrile to volume. (Analyte solution should be prepared fresh before injection and injected within 5 minutes of completing its preparation).

The reported USP monograph method and published method has small drawbacks. (i) the column oven temperature is 35ºC in USP and 40ºC in published paper since, 1α-hydroxy vitamin D_2 (Doxercalciferol) is a lower temperature storage/ cold storage condition product hence, there is a possibility of thermal degradation (ii) sample preparation is done first in ethyl acetate with sonication and followed by acetonitrile to volume. Sample solution should be prepared fresh before injection and injected with in 5 minutes of completion of preparation.

The present work describes a new selective RP-HPLC technique with column oven temperature at 25ºC (ambient temperature) capable of separating all specified impurities along with two additional impurities i.e., one process impurity and another is ergocalciferol. Sample preparation was done by using a single diluent i.e. in methanol, the product is easily soluble in methanol.

Ergocalciferol is the starting material for the manufacturing of doxercalciferol. To note, ergocalciferol is also one of the analogs of vitamin D and API. The literature review discloses that no reference present for the estimation of related substances of doxercalciferol along with Ergocalciferol and other process-related and degradation impurities.

Table 1: Gradient isolation program.

Time in min	*Pump A %*	*Pump B%*
0.01	15	85
15.00	15	85
20.00	0	100
35.00	0	100
40.00	15	85
50.00	15	85

Hence, it was felt necessary to develop the sensitive and selective HPLC technique for the estimation of 1α-hydroxy vitamin D_2 (Doxercalciferol) and related components. The technique was effectively validated as per the ICH guidelines. Further, the doxercalciferol and ergocalciferol assay method was also validated.

MATERIAL AND METHODS

Materials

Doxercalciferol and impurities samples were obtained from SLS pharma limited. Methanol HPLC and acetonitrile analytical grades were acquired from Merck life science PVT LTD. Purified water was taken from a Millipore Milli Q water purification system.

Instrumentation and Chromatographic conditions

Shimadzu' Liquid chromatography, model LC-2010 C HT, equipped with a quaternary pump, UV detector (UV, 'Shimadzu' # LC2010 detector), column oven and autosampler was utilized for development trainings. The signal output was examined and integrated by using 'LC Solution' software operated on a Microsoft windows XP professional.

The related substances isolation was processed on Thermo scientific Hypersil BDS (Base deactivate and silica) C18 150 x 4.6 mm, 3 µm, P/N: 28103-154630, S/N: 10138568 was found to be delivering good results during the development phase.

The mobile phase-A contains water and phase-B contains Acetonitrile. Mobile phases were filtered through 0.2 µ membrane filter. The mobile phase were forced with 1.5 mL/ min flow rate, by the gradient elution provided in Table 1. The column oven temperature was maintained at 25ºC (ambient temperature) and infusion volume has set at 20 µL. Detection was monitored at wavelength 265 nm. Methanol was utilized as a diluent.

Mobile phase

Water – Mobile phase A
Acetonitrile – Mobile phase B
Hypersil BDSC18 15 x 4.60 mm, 3 µ : Column.
UV Detector: 265 nm
Flowing rate: 1.50 mL /min.
Diluent: Methanol
Running time: 50.0 minutes
Volume of infusion: 20.0 µL

Method Development

The HPLC method for quantitative analysis of 1α-hydroxy vitamin D2 (Doxercalciferol) and its impurities was developed

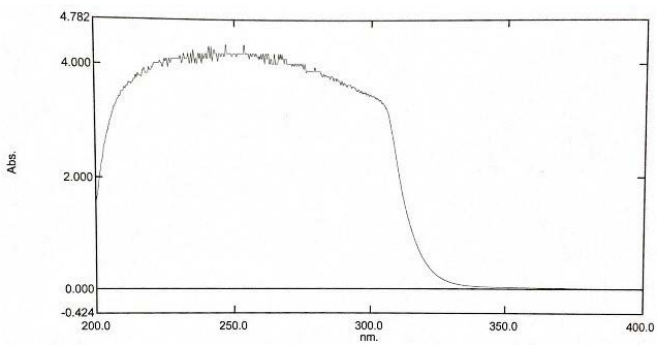


Figure 1: UV spectrum of Doxercalciferol scanned from 200 to 400nm.

by optimization of the stationary phase, column temperature, mobile phase, gradient program and flow rate.

Main focused areas during the development are (i) the development of a single technique for elution of doxercalciferol, its process impurities, degradation impurities and ergocalciferol (ii) reduction of the column oven temperature (iii) ease of sample preparation.

During the method development initially, doxercalciferol, ergocalciferol, impurity-A and impurity-B were checked for solubility in different solvents. Based on the observations in solubility a common solvent, methanol was chosen for solubility of doxercalciferol, its impurities and ergocalciferol. Hence, methanol is used as a diluent in sample preparation.

UV spectrum is checked for doxercalciferol, impurity-A, impurity-B and ergocalciferol on UV Spectrometer using methanol as blank and scanned the samples from a wavelength range of 200 to 400 nm. Maximum absorbance was observed around 265 nm. Therefore, wavelength was chosen has 265 nm from the obtained UV spectrums and doxercalciferol UV spectrum is provided in Figure 1.

The initial trails were performed by an isocratic method flow on a Shimadzu' Liquid chromatography choosing a C18 column with 25 to 27°C column oven temperature, flow rate 2.0 mL/min, injection volume of 20 µL, UV detection at a wavelength of 265 nm, with a run time 90 minutes and injected the blank, doxercalciferol, ergocalciferol, impurity-A, impurity-B individual solutions and spike solution. Observed the elution of peaks, doxercalciferol at 18.84 RT, impurity-A at 17.66 RT, ergocalciferol at 62.12 RT and impurity-B peak is not eluted.

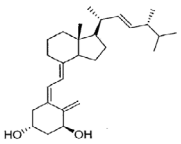
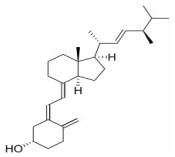
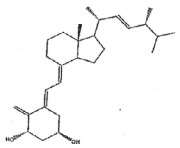
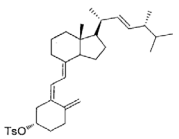
Therefore, a single step gradient method is selected to elution impurity-B and decrease the runtime for ergocalciferol, doxercalciferol and impurity-A. The single-step gradient progamme (i.e., mobile solvent A: H_2O and mobile solvent B: acetonitrile), reverse phase C18 column with 25°C column oven temperature, flowing rate 1.50 mL/min, infusion volume of 20 µL, UV detection at a wavelength of 265 nm and sequence was run for 60 minutes run time. It was observed that ergocalciferol peak is eluting at 45 RT in spike solution but impurity-B was not eluting. Further, the method was developed by changing single step gradient programme (i.e. changing the proportion of mobile solvent-A and mobile solvent-B) and observed the impurity-B peak elution at 26.18 RT, ergocalciferol peak elution runtime is decreased from 45 to 38 RT and finally to 23.56 RT, doxercalciferol peak elution runtime is decreased from 18.84 RT to 10.72 RT and impurity-A peak elution runtime is decreased from 17.66 RT to 10.03 RT.

The development achieved by changing the parameters such as mobile phase from isocratic to gradient elution, run time, flow rate, column oven temperature and concentration of mobile solvent-A and mobile solvent-B in gradient separation etc.

For better, more precise, and more reliable results, the gradient elution programme has been designed and modified. Thus, a selective RP-HPLC technique was developed which can usefully identify the combination of doxercalciferol, its impurities and ergocalciferol.

The developed method is capable of detecting the doxercalciferol impurities such as, ergocalciferol which is the starting material, impurity-A is process related as well as degradation impurity, impurity-B is one of the process related impurity. Doxercalciferol and impurities names and chemical structure are provided in Table 2. The developed method is

Table 2: Chemical structure and name of Doxercalciferol and impurities. (A): Doxercalciferol; (B): Ergocalciferol; (C): Impurity-A; (D): Impurity-B.

Fig.	*Name*	*Chemical structure*	*Chemical name*
A	Doxercalciferol		(5Z, 7E, 22E)-9,10-Secoergosta-5, 7, 10(19), 22-tetraene-1α, 3β-diol
B	Ergocalciferol		(3β, 5Z, 7E, 22E) -9, 10-secoergosta-5, 7, 10(19), 22-tetraen-3-ol
C	Impurity-A		(1R, 3S, E) -5- ((E)-2- ((7aR)-1-((2R, 5R, E)-5, 6-dimethylhept-3-en-2- yl)- 7a-methyl dihydro-1H -inden-4 (2H, 5H, 6H, 7H, 7aH) -ylidene) ethylidene) -4-methylcyclo hexane-1,3-diol
D	Impurity-B		(R,3Z)-3-((7E)-2-((7aR) -hexahydro-7a-methyl-1- ((E, 2R, 5R)-5, 6-dimethylhept-3- en-2-yl) -1H-inden-4 (7aH) -ylidene) ethylidene) -4-methylenecyclohexyl 4-methyl benzene sulfonate.

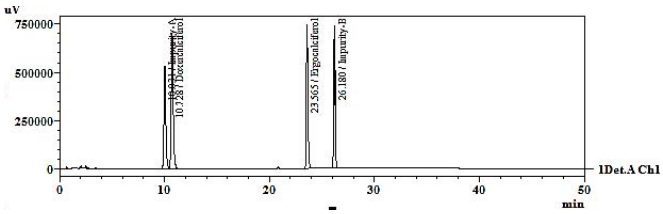


Figure 2: Selectivity chromatogram of Impurity –A, Doxercalciferol, Ergocalciferol and Impurity –B.

validated for doxercalciferol and the impurities ergocalciferol, impurity-A and impurity-B.

RESULTS AND DISCUSSION

Method Validation

The technique underwent the validation procedure after attaining the best chromatographic elution in order to demonstrate its dependability and appropriateness for the intended purpose. The validation process was carried out in accordance with ICH Q2 requirements. The described method was extensively validated in terms of selectivity, solution stability, method precision, accuracy, system precision, linearity, LOD and LoQ.

Selectivity

Selectivity was performed by injecting individual injections of the doxercalciferol, its impurities ergocalciferol, impurity-A, impurity-B and spiked solution using the developed method conditions. Observed the component and impurities are eluted with good peak shapes and with better resolution in the spiked solution. The spiked solution results were presented in the Table 3 and the chromatogram were displayed in Figure 2.

System Precision

The system precision was studied by injecting one blank and six standard preparations at test concentration i.e. 1-mg/ mL of impurity-A, ergocalciferol, impurity-B for six times into chromatography using the established method conditions. The response from these runs was subjected to the calculation of standard deviation and %RSD (depicted in Table 4.). The %RSD was observed to be less than 5.0% which apparently showed a good system precision. System precision chromatogram of six standard preparations is shown in Figure 3.

Method precision

The method precision was studied by injecting one blank and six standard preparations of impurity-A, ergocalciferol, impurity-B, spiked in doxercalciferol for 6 times into a chromatograph using the developed method conditions. The method is precise across the range for impurities: impurity-A, ergocalciferol, impurity-B, and doxercalciferol. Method precision chromatogram is shown in Figure 4 and % RSD is represented in Table 5.

Linearity

The response obtained for LOQ linearity of doxercalciferol and related impurities at various concentrations (i.e., 50, 75, 100, 125 and 150%) was plotted by considering % concentration on x-axis and peak response on the y-axis. The observed correlation coefficient for doxercalciferol is 0.995, ergocalciferol is 0.997, impurity-A is 0.990 and impurity-B is 0.993. The graphical representation of LoQ linearity is depicted in Figure 5 to 8.

Table 3: Selectivity data /order of elution of Doxercalciferol and its impurities.

Name	*Retention time (RT)*	*RRT (versus Doxercalciferol)*
Doxercalciferol	~10.72 min	1.00
Impurity –A	~10.03 min	0.94
Ergocalciferol	~23.56 min	2.19
Impurity –B	~26.18 min	2.44

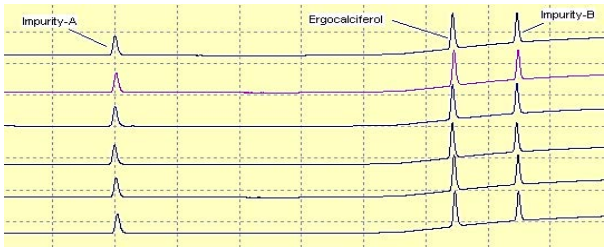


Figure 3: System precision chromatogram of six standard preparations of Impurity-A, Ergocalciferol and Impurity-B.

Table 4: % RSD of System Precision of Impurity-A, Ergocalciferol and Impurity-B.

	System Precision					
Impurity-A			*Ergocalciferol*		*Impurity-B*	
	RT	Area	RT	Area	RT	Area
1	10.00	76397	23.57	109207	26.16	84333
2	10.05	76397	23.62	107398	26.20	84609
3	10.00	76130	23.57	108817	26.15	83834
4	9.98	76239	23.55	108277	26.14	83407
5	10.04	76033	23.62	106565	26.20	83470
6	10.09	76103	23.66	104377	26.22	83735
%RSD	0.41	0.20	0.18	1.66	0.12	0.58

LoD and LoQ

The LoD and LoQ were determined for doxercalciferol and its impurities. The method is sensitive for doxercalciferol, ergocalciferol, impurity-A and impurity-B; the LOD value is 0.01%. The LoQ for doxercalciferol, ergocalciferol, impurity-A and impurity-B is 0.03%.

Accuracy

The accuracy of the projected technique was assessed by spiking with known amounts of impurities to the test sample at the level of LoQ, 50, 100 and 150%. Each level of the sample solution was processed in triplicate. The recovery of percent values was calculated for all related substances and those within the acceptance criteria limit.

Precision study was performed at the LoQ concentration level by injecting six individual preparations of doxercalciferol and its impurities and calculated the % RSD for the areas of

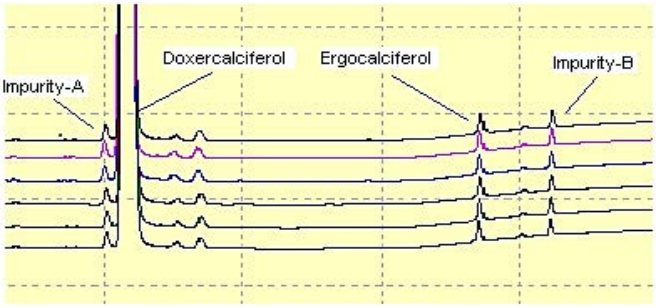


Figure 4: Method precision chromatogram of Impurity-A, Ergocalciferol, Impurity-B along with Doxercalciferol.

Table 5: % RSD of Method Precision of Impurity-A, Ergocalciferol and Impurity-B.

Method Precision						
Impurity-A			*Ergocalciferol*		*Impurity-B*	
	RT	Area	RT	Area	RT	Area
1	10.05	57793	23.62	50263	26.19	42425
2	10.00	61823	23.59	51844	26.16	42747
3	10.01	58024	23.57	50718	26.14	41119
4	10.09	57145	23.60	51692	26.15	40888
5	10.12	58321	23.58	52524	26.12	39553
6	10.10	57361	23.55	49452	26.10	39672
%RSD	0.49	2.95	0.10	2.23	0.12	3.25

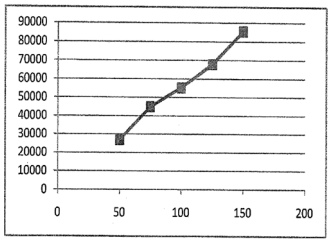


Figure 5: Doxercalciferol

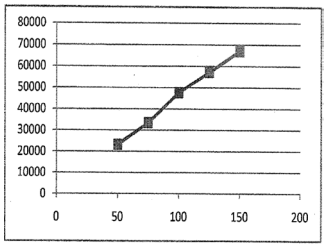


Figure 6: Ergocalciferol

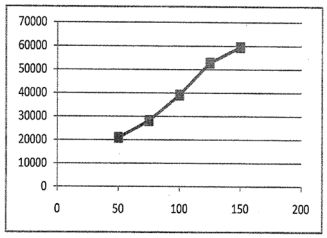


Figure 7: Impurity-A

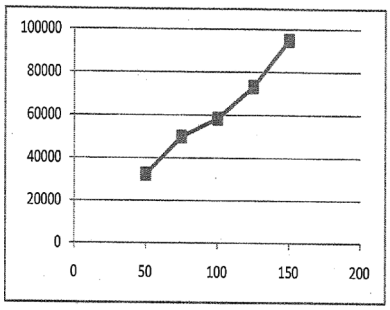


Figure 8: Impurity-B

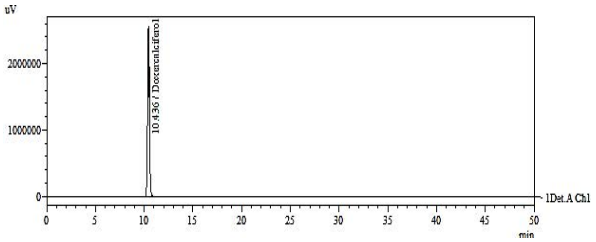


Figure 9: Chromatogram of Doxercalciferol Assay.

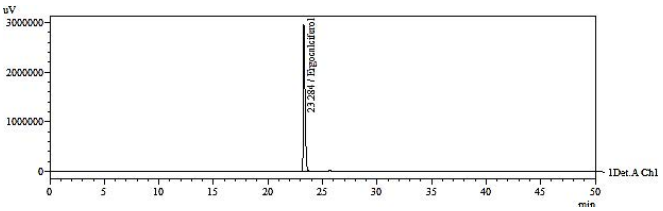


Figure 10: Chromatogram of Ergocalciferol Assay.

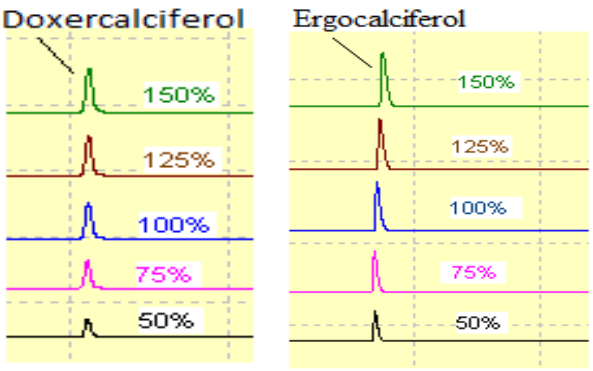


Figure 11: Chromatograms of Doxercalciferol and Ergocalciferol at Linearity Concentrations (50, 75, 100, 125 and 150%).

each peak. Accuracy at LoQ level was verified by injecting three individual preparations of doxercalciferol spiked with impurities at LoQ level. Thus, the method was considered precise and accurate at the LoQ level.

Assay by HPLC of Doxercalciferol and Ergocalciferol

The same method is used for determination of Assay by HPLC validation of doxercalciferol and ergocalciferol. Assay of doxercalciferol by HPLC method is depicted in the below Table 6. Assay of ergocalciferol by HPLC method is depicted in the below Table 7. Doxercalciferol and ergocalciferol assay chromatogram were displayed in Figures 9 and 10 (Table 6).

The method was proven selective, precise (system precision % RSD for doxercalciferol is 0.38%, ergocalciferol is 0.18%

Table 6: Doxercalciferol Assay by HPLC.

Preparation	*Doxercalciferol Assay by HPLC*
1	99.38
2	99.43
3	99.71
4	99.12
5	99.67
6	99.17
Average	99.41

Table 7: Ergocalciferol Assay by HPLC.

Preparation	*Ergocalciferol Assay by HPLC*
1	99.95
2	99.56
3	99.25
4	99.12
5	99.38
6	99.16
Average	99.40

and method precision % RSD for doxercalciferol is 0.25%, ergocalciferol is 0.31%). Linear across the range and the method is accurate.

The linearity of the method was studied at various concentrations between 50 to 150% and observed correlation coefficient is 0.952 for doxercalciferol, 0.997 for ergocalciferol. Linearity chromatograms at different concentrations (50, 75, 100, 125 and 150%) as shown in Figure 11.

Solution Stability

Solution stability is performed for the doxercalciferol and ergocalciferol at initial (0 hour),

12 and 24 hours. The prepared test sample solution was kept constant during the study period. No significant changes in the amounts of doxercalciferol and ergocalciferol were observed during the study. The results at initial, 12 and 24 hours confirmed that sample solutions were stable up to 24 hours at ambient temperature (25°C).

The developed method was validated according to ICH Q2 for selectivity, system precision, linearity, range, accuracy and observed that the developed method is sensitive, selective and repeatable for vitamin D2 Analogs API's doxercalciferol and ergocalciferol.

CONCLUSION

A new selective RP-HPLC method was developed for the determination of 1α-hydroxy vitamin D_2 (Doxercalciferol) and its impurities. The critical parameters like stationary phase, mobile phase and flow rate were effectively optimized. The focused areas during the development are achieved, developed method is capable for the elution of doxercalciferol, its impurities and ergocalciferol, with a column oven temperature at 25°C (ambient temperature) and easy sample preparation using methanol as diluent.

Developed method is simple, inexpensive, reliable and selective for quantification of doxercalciferol and its impurities. The two vitamin D_2 analogs i.e., doxercalciferol, ergocalciferol and doxercalciferol impurities are eluting in single method. The developed method was validated as per the ICH guidelines and found to be selective, precise, accurate and linear. The developed method was selective than the published method and USP method. The developed method can be conveniently used for quality control testing of doxercalciferol API for related substances by HPLC, assay by HPLC of doxercalciferol, assay by HPLC of ergocalciferol and the same can be used for testing of cleaning samples.

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