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Accelerated Stability Indicating: Method Development of UV-Visible Spectrophotometric Analysis and its Validation for Vitamin-D3 Estimation in Tablets

Kashif Iqbal[1,2], Samra Tariq[1], Saad Salman[1], & Marvi[3]

1. Department of Pharmacy, The University of Lahore (Islamabad Campus), 44000, Islamabad
2. Faculty of Pharmacy IBADAT International University, Islamabad, Pakistan
3. Department of Pharmacology, Faculty of Pharmacy, university of Balochistan, Quetta

Abstract:
Purpose: A facile approach to develop and validate a precise accurate, and stable method over UV-visible spectrophotometer (UV-vis) for Vitamin D3 (VD3) tablets. Methods: In this study, three brands of VD3 tablets from various companies were employed. UV-vis was used to test the short-term stability research of vitamin D3. In short-term stability research, an assay of vitamin D3 is evaluated on UV-vis after one month of their manufacturing that is known as zero month then after 3 months and lastly after 6 months. Conditions of short-term stability study for six months consists of temperature 40 °C within upper-lower limit of 2 % and moisture content of 75 % within upper-lower limit of 5 %. For identification of VD3 in tablet dosage form, a simple UV-vis procedure was formulated that had an appropriate susceptibility according to accuracy, precision, and linearity. Guidelines of International Council of Harmonization of technical requirements for pharmaceuticals of Human use and United States Pharmacopeia were followed for validation of these parameters. Results: Out of three brands, one brand of vitamin D3 (VD3) tablet dosage form by UV-vis method was found to be stable at the appropriate temperature and moisture content in comparison to other two brands of VD3 that started to lose their efficiency at elevated temperature and moisture content from the normal range. VD3 shows its absorbance in deionized water at the wavelength of 265 nm, linearity range of 0.65-9.94 µg ml-1, molar absorptivity of 4.32 x 104 L/molcm-1 with a correlation coefficient equation of y=0.0503×-0.4988 and regression of R2= 0.9991. Conclusion: It was found that VD3 demonstrated the best result in climate atmosphere as compared to an accelerated parameters which lead to the breakdown of VD3 when tested with the help of UV-vis method.

Keywords: Vitamin-D3, drug degradation, accelerated stability study, UV-spectrophotometric method, Validation and calibration.

INTRODUCTION

Cholecalciferol, or Vitamin D3 (VD3) is also known as sunshine vitamin because it is obtained directly from the sunlight (Hoffmann-La Roche 1989). VD3 plays a fundamental role for healthy bone by the breakdown of calcium and its absorption in the body. Calcium and phosphorous that are involved in biological processes are sustained by VD3. The more stable form of VD3 is produced by giving an appropriate temperature (Holick *et al.*, 2000, ICH 2003). The tropical zones with high temperatures are not good for pharmaceutical products (Belsey, *et al.*, 1974). When pharmaceuticals are dispersed in these areas, the test results turned out to be lowered due to the breakdown of active pharmaceutical ingredients at high temperatures that affect their assay

(Brander *et al.*, 1991, Connors *et al.*, 1986). Physical properties of a product are of prime importance that are altered due to improper handling as well as manufacturing. Variability in temperature and humidity of the environment will affect the activity due to the lowering of active constituents of drug product. All these environmental changes should be checked through stability analytical methods (Dean *et al.*, 2000, Grady *et al.*, 1980).

Humid air has more susceptibility for VD3 (ICH 2003). The storage temperature for VD3 is 56 °C at high temperature while with its breakdown occurs (Iqbal 2011). Our aim was to explore the breakdown of the samples of VD3 tablets through a new UV-visible spectrophotometric (UV-vis) method with quick, precise, accuracy and robustness. In previous findings, VD3 was analyzed by thin layer Chromatography, Liquid Chromatography (LC), Liquid extraction, and Reverse-Phase High-Performance Liquid-Chromatography (RP-HPLC) methods (Iqbal 2011, Merck 1976). Herein, we took the VD3 commercial tablets from various companies that subjected them to stability testing to analyze its breakdown at elevated temperatures and correlated it with VD3. USP (USP 2004) and the ICH (Q_2 (R_1)- 2005) has provided specification and master formulae for method validation, so all the analytical results were validated according to their recommendations.

MATERIAL AND METHODS

Chemicals and Reagents

VD3 tablet dosage form of three different companies were used as a sample. Formic acid, Methanol, Glacial Acetic Acid, Dinitrophenylhydrazine, and Distilled water were purchased from BDH/Lab Scan Asia/RCI and were of analytical grade**s.**

Instrumentation

Shimadzu-1600, Shimadzu-1800 and Hitachi-UV were supplied by LabX, Canada. UV-vis was used to measure VD3 solution samples. For 6 months, vitamin D3 was kept at humidity of 75 percent ± 5 percent and temperature 40 ± 2 °C in the climatic chamber. By using the UV-vis procedure that is currently developed and not mentioned in official pharmacopeias, the test result of active pharmaceutical substance (VD3) was accomplished for different brands. The maximum wavelength for VD3 is 265 nm and test results were performed thrice and average was calculated (Temora *et al.*, 2016).

Standard Preparation

Standard solution of VD3 was prepared by taking 100 mg of VD3 and 10 ml of formic acid in 50 ml of the volumetric flask by placing it in Sonicator for some time while methanol was used as a blank. After, the addition of methanol, 2 ml from the stock solution was taken into a 100 ml volumetric flask while 1 ml of 2, 4 dinitrophenyl hydrazine compound was added to it and volume was made up to the mark to get absorbance at 265 nm.

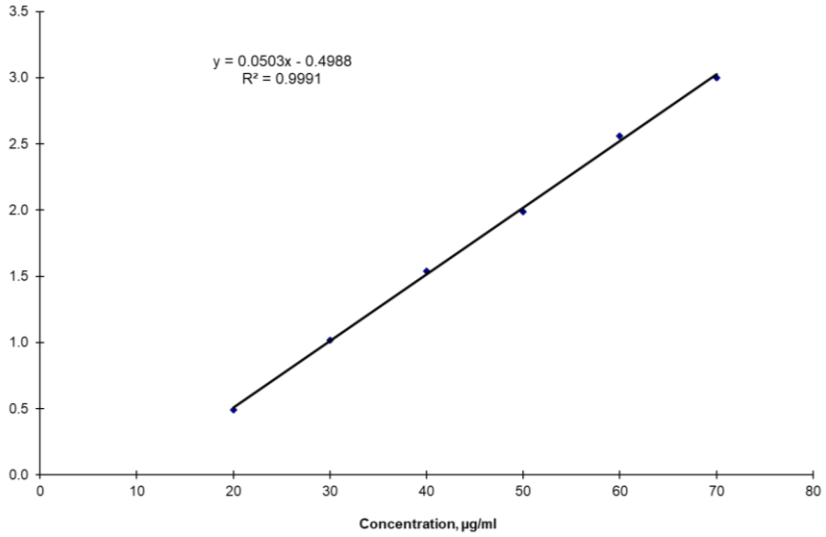
Sample Preparation

The sample solution was prepared by taking 100 mg of VD3 and 10 ml of formic acid in 50ml of the volumetric flask by placing in Sonicator while methanol was used as Blank. After filtration (0.45-µm) process, 2 ml of filtrate solution and 2, 4 dinitrophenyl hydrazine compound was added into 100 ml volumetric flask while volume was made up to the mark and absorbance was measured at 265 nm with the help of UV-vis spectrophotometer. VD3 tablet samples were taken at regular intervals for the accelerated stability study.

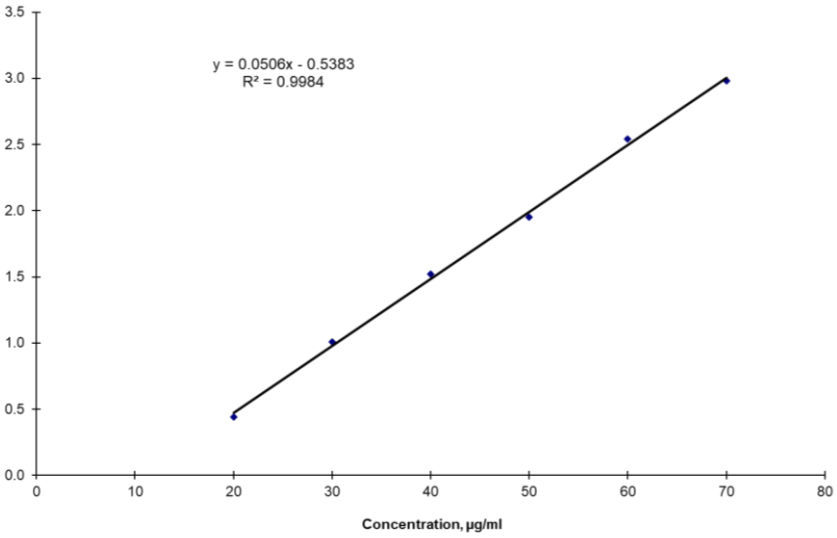
UV-vis spectrophotometer and climatic chamber were used in this procedure. At a given temperature of 40 °C and moisture Content of 75 %, the stability of VD_3 was tested by UV-vis at 1st month of manufacturing of the drug, then after three months, and lastly at six months after manufacturing. Five samples of VD_3 ranging from concentration 20-60 µg/ml were prepared and checked by the developed analytical procedure. These samples were used for preparing the calibration curve and the regression equation was calculated. Six samples were prepared to check the precision of the proposed developed procedure. The ruggedness of the analytical procedure was demonstrated by employing minor changes (solvent composition) in developed analytical procedure. Three brands of VD_3 were analyzed on different UV-vis models, performed by different analysts, on different days and time and results were calculated to demonstrate the robustness.

Preparation of Calibration Curve

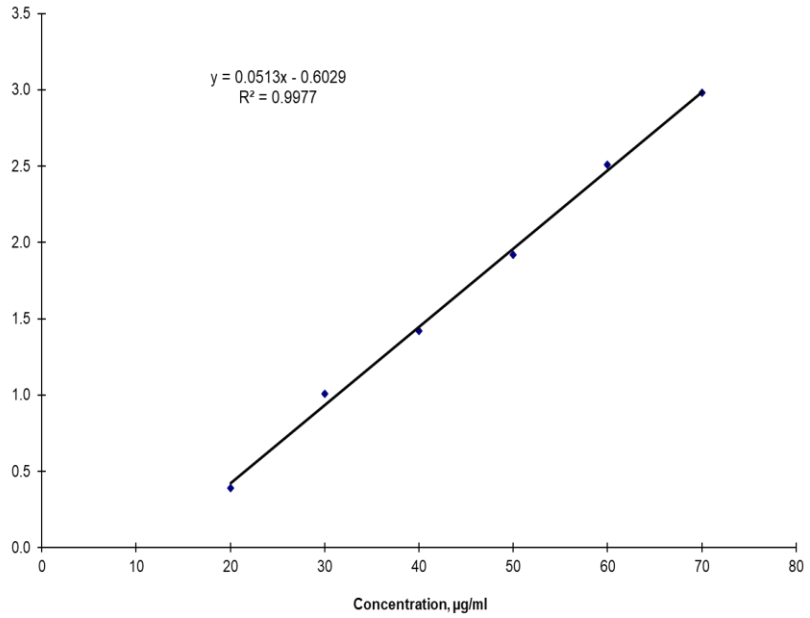
By plotting the absorbance against the concentration, the calibration curve of VD_3 of three different brands were generated. The concentration range of VD_3 for the calibration curves were 20-60 µg/ml.



Calibration Curve of brand 1



Calibration Curve of brand 2



Calibration Curve of Brand 3

Fig. 1: A calibration curve of VD3.

Linearity:

The linearity was estimated by analyzing the three different brands of VD3 at the different concentrations of VD3 in tablet dosage form. The Beer–Lambert's concentration range was found to be 20-60 µg/ml. The linearity of the relationship between absorbances and conc. was determined by plotting the calibration curve for VD3 as shown in the above Figure. Five different conc. of each brand was taken and measure at their absorbance at the respective wavelengths. Then mean, standard deviation, correlation coefficient, and slope were calculated.

Brand 1		**Brand 2**		**Brand 3**	
Conc.	**Abs**	**Conc.**	**Abs**	**Conc.**	**Abs**
20	0.950	20	0.952	20	0.911
30	1.425	30	1.421	30	1.418
40	1.900	40	1.807	40	1.904
50	2.375	50	2.370	50	2.369
60	2.850	60	2.850	60	2.811
Mean	1.900	Mean	1.880	Mean	1.88
SD	0.75	SD	0.75	SD	0.75
R^2	0.9991	R^2	0.9984	R^2	0.9977

Conc. (µg /ml), D (Day) Abs (Absorbance), Standard Deviation (SD), Correlation Co-efficient R^2

Precision:

The Inter-day precision was identified for a solution of conc. (40 µg/ml) of three brands of VD3 tablet and were analyzed for the three times on a different day. (Day 1, Day 2, Day 3). Average, SD, %RSD were calculated and the % RSD was not more than 2 %.

Results of Method Precision (Inter-day) of Different Brands of VD3:

Brand 1				Brand 2				Brand 3			
Conc.	**Day1**	**Day 2**	**Day 3**	**Conc.**	**Day 1**	**Day 2**	**Day 3**	**Conc**	**Day 1**	**Day 2**	**Day 3**
40	1.89	1.90	1.91	40	1.90	1.91	1.91	40	1.89	1.9	1.88
Avg.	1.90			Avg.	1.90			Avg.	1.89		
SD	0.01			SD	0.005			SD	0.01		
%RSD	0.52			%RSD	0.26			%RSD	0.52		

Conc. (µg /ml), D (Day) Abs (Absorbance), Average (avg.), Standard Deviation (SD)

In the intra-day variation, the study was determined for a solution of conc.40 µg/ml of three brands of VD3 tablet and was investigated thrice on the same day at different timings. Mean, Standard deviation, & % RSD were calculated as shown in the below tab

Result of Method Precision (Intra-day) of Different Brands of VD3:

Brand 1				Brand 2				Brand 3			
Conc.	**Abs 1**	**Abs 2**	**Abs 3**	**Conc.**	**Abs 1**	**Abs 2**	**Abs 3**	**Conc.**	**Abs 1**	**Abs 2**	**Abs 3**
40	1.91	1.88	1.90	40	1.92	1.90	1.91	40	1.89	1.92	1.89
Avg.	1.89			Avg.	1.91			Avg.	1.90		
SD	0.01			SD	0.01			SD	0.01		
% RSD	0.52			%RSD	0.52			% RSD	0.52		

Conc. (µg /ml), Abs (Absorbance), Average (avg.)

Range

Brands	**Range (µg/ml)**	**Regression equation**	**R^2**
Brand 1	20-60	0.0503×-0.4988	0.9991
Brand 2	20-60	0.0506×-0.5383	0.9984
Brand 3	20-60	0.0513×-0.6029	0.9977

Accuracy (% Recovery):

A standard addition procedure was used for the determination of accuracy study. The 40 µg/ml sample solution of each brand of VD3 was spiked with an extra 50, 100 and 150 % of standard concentration of VD3. Absorbance was measured at 265 nm and the concentration of the drug was determined. The experiment for each brand was performed three times. Amount recovered, % recovery, average recovery, and % RSD were calculated as shown in the below Table.

Brand	**Initial Amount (µg)**	**Level (%)**	**Amount Added (µg)**	**Amount recovered (µg)**	**% Recovery**	**Average Recovery**	**%RSD**
Brand 1	40	50	20	19.95	99.75	99.21	0.85
		100	40	39.87	99.67		
		150	60	58.94	98.23		
Brand 2	40	50	20	19.98	99.90	99.60	0.51
		100	40	39.95	99.89		
		150	60	58.81	99.01		
Brand 3	40	50	20	19.99	99.95	99.94	0.02
		100	40	39.97	99.92		
		150	60	59.89	99.96		

Amount

Optical Characteristics of VD3

Beer's law limit (µg/ ml)	20-60
Correlation Coefficient	0.9991

Regression equation	0.0503×-0.4988

Robustness:

The robustness of the method was determined by carrying out the analysis by taking the standard concentration from the three brands of VD3. The assay was performed by different analysts, on different instruments, and on different day/time.

Statistical Validation for Robustness studies of VD3

Sr. No	Parameter	Brand 1	Brand 2	Brand 3
1	System	Shimadzu-1600	Hitachi-UV	Shimadzu-1800
2	Sample	Brand 1	Brand 2	Brand 3
3	Date	7/12/2020	8/12/2020	9/12/2020
4	Day	Monday	Tuesday	Wednesday
5	Time	11:00 AM	12:00 PM	1:00 PM
6	Analyst	X	Y	Z
7	Sample	40 µg/ml	40 µg/ml	40 µg/ml
8	Absorbace	1.901	1.892	1.894
9	Assay	101.11%	99.84%	99.57%

Ruggedness:

The Ruggedness was carried out to calculate the effect of small changes in the spectrophotometric conditions for the determination of VD3 in different brands. By changing the composition of solvent, taking the sample concentration 40 µg/ml, the absorbance, standard deviation, %RSD, and %assay was calculated.

Statistical Validation for Ruggedness Studies of VD3.

Sr. No	Brands	Wavelength(nm)	Conc. (µg/ml)	Abs.	Avg. Abs.	STD	%RSD	%Assay
1	Brand 1	259	40	1.869	1.853	0.009	0.48	98.56
2		259	40	1.850				
3		259	40	1.855				
1	Brand 2	265	40	1.917	1.900	0.01	0.52	99.63
2		265	40	1.895				
3		265	40	1.913				
1	Brand 3	270	40	1.953	1.970	0.017	0.86	99.39
2		270	40	1.987				
3		270	40	1.976				

Limit of Detection (LOD) and Limit of Quantification (LOQ):

Limit of detection (LOD) is the minor concentration of the sample that can be identified. LOD was determined by equation LOD- 3.3 s/m where S is the standard deviation and m is the slope of the calibration curve. Limit of Quantification (LOQ) is the lowest concentration of sample that can be quantitatively identified by suitable precision and accuracy. LOQ was determined by equation LOQ-10 s/m.

Specificity:

In specificity, by comparing the spectrum of tablet solution with that of the standard solution was determined. Then the spectrum of sample was checked if there is any interference of excipients.

Statistical Analysis

By applying suitable analytical tests, analytical analysis can be done, e.g., p values observed level of significance of rejection of more than 0.25 to the coefficient of the regression and zero-time intercepts for the single batches. Statistical software Minitab 15 (Two-way analysis) was applied to get accurate and precise results of the given method.

RESULT

In this study, three different brands of VD3 tablets i.e., (a) Brand 1, (b) Brand 2, and (c) Brand 3 containing 125, 125, and 400 IU respectively of VD3 were used. The brands of VD3 tablets were found to meet the Pharmacopoeial requirements of method validation as shown in the Table. At a recommended temperature and moisture content, batch 1,2, and 3 of brand 1 of VD3 were tested and their stability study was performed at the initial month, then at the third month, and six months of their manufacturing. Accelerated stability study data shows that assay results of VD3 of brand 1 of batch 1, batch 2 batch 3, were reduced from 100.87 % to 100.02 %,102.89 % to 101.32 %, and 100.54 % to 98.38 %. The p-value is not more than 0.05 according to this standard out of two "p" value,1 "p" value of brand 1 tablet was not lie in range.

At a recommended temperature and moisture content, batch 1, 2, and 3 of brand 2 of VD3 were tested and their stability study was performed at the initial, third, and six months of their manufacturing. Accelerated stability study data shows that assay result of VD3 of brand 2 of batch 1, batch 2, batch 3 was reduced from 105.60% to 98.11%,107.71% to 97.48%, and 110.42% to 104.65% in the first six months. The "p" value is not more than 0.05 according to this standard, out of two "p" values, 1 "p" value of brand 2 tablets was not in range.

Table: Parameters of the analytical analysis of results in the determination of assay content of VD3 tablets during accelerated stability study conditions.

Product Name	Parameters for Accelerated Stability	Batch 1	Batch 2	Batch 3	p-Value	
					Months	Batches
Brand 1	Initial month	100.87 %	102.89 %	100.54 %	0.014	0.003
	Three months	100.43 %	101.61 %	99.31%		
	six months	100.02 %	101.32 %	98.38 %		
Brand 2	Initial month	105.60 %	107.71 %	110.42 %	0.019	0.096
	Three months	102.75 %	105.63 %	105.18 %		
	six months	98.11 %	97.48 %	104.65 %		
Brand 3	Initial month	104.59 %	102.64 %	103.59 %	0.012	0.367
	Three months	102.62 %	98.13 %	98.52 %		
	six months	96.21 %	97.22 %	98.19%		

At a recommended temperature and moisture content, batch 1, 2 and 3 of brand 3 of VD3 were tested and their stability study was performed at the initial month, then at the third month and six months of their manufacturing accelerated stability study data shows that assay result of VD3 of brand 3 of batch 1, batch 2, batch 3 was reduced from 104.59% to 96.21%, 102.64% to 97.22% and 103.59% to 98.19%. The "p" value was not more than 0.05 according to this standard out of two "p" value,1 "p" value of brand 2 tablets was not in range. The method was found linear in a range of 20 to 60 µg ml^{-1} with a good correlation of 0.99 to lower % RSD (below 2 %) of % assay values, observed during replicate analysis of different tablets as part of precision, indicate the suitability of the method. For chamber saturation time, the procedure was found rugged.

DISCUSSION

During accelerated stability study, VD3 tablet dosage form of brand 1 was provided with the temperature of 40 ±2 °C and moisture content of 75 % ±5%. During this heat and humidity, VD3 remains unchanged. At the same time, during the storage of the other two brands of VD3, heat and moisture content were increased from the normal range and its lea breakdown (Iqbal 2011). When VD3 Brand 1 tablet having 100%-100.89 % result was compared to LC, liquid extraction and RP-HPLC methods having recovery 93%-102 %, 98%-100.9 %, and 94.43% respectively, it showed confirmation of previous findings and precision and accuracy of the new method (Tiles 1994 – Staff et al., 2003). Nature of VD3 breakdown is unidentified, but after passing some time the decrease in the test result of VD3 is due to the oxidative breakdown of VD3 during its storage.

Under the selected elaborated conditions i-e at appropriate temperature and humidity, after storage VD3 test was performed for stability purpose. (Hoffmann-La Roche 1989). From our research, it is verified that VD3 does not maintain its stability at high heat and moisture content and starts breakdown and loss efficiency at increased heat and moisture content from the normal level. (Connors *et al*., 1986, Grady *et al*., 1980, Merck 1976). When both heat and moisture contents are increased from their normal range at the same time, VD3 starts to breakdown most rapidly where all the test samples of VD3 are losing their efficacy after few days of their storage during accelerated stability test conditions.

CONCLUSION

Hence a precise, accurate, and simple UV-vis spectrophotometric method was developed for the analysis of VD3. It was concluded that elevated heating and moisture content are two important factors that affect the stability of VD3 tablets of different brands so VD3 found susceptible to both of these factors. Recently formulated procedure for the determination of stability of VD3 tablet under different circumstances/environmental conditions produced high-quality outcomes. We analyzed three tablet dosage forms of VD3 of different manufacturers. The stability of the brand 1 tablet dosage form of VD3 is long-lasting as compared to the other two tablet dosage form brands of VD3. Brand 1 of VD3 is kept at an appropriate temperature and moisture content so it didn't degrade and remained stable during its shelf life. But in case of other two brands of VD3, during their storage temperature and humidity was increased from their normal level so VD3 does not remain stable and loose efficacy before their shelf life.

SUGGESTIONS

Tablet dosage form is one of the complicated preparations that comprises minerals and vitamin rich nutritional compounds. VD3 is sensitive to high temperature and humidity, so while their preparation, deposit and distribution when it come in contact with high temperature and moisture content it started degradation and lost functional activity.

Assessment of stability features of cholecalciferol are exceptionally so hard, due to susceptibility of VD3, and its sensitivity towards high temperature and high moisture content and formulations that contain more than one vitamin.

This may be used to conduct further systematic studies on the evaluation of VD3 stability in tablet mixtures.

1. In the tablet dosage form of VD3 recognition of unfamiliar aerophilic and deterioration product.

2. Stability- Ascertained procedures for test results of VD3 in the existences of deteriorated compounds were generated.
3. For the time, assay of VD3 in tablet dosage form, connection of speed of compound breakdown with UV-vis procedure were studied.

REFERENCES

Ballard JM, Zhu L, Nelson ED, Seberg RA. degradation of vitamin D_3 in a stressed formulation: The identification of esters of vitamin D_3 formed by transesterification with triglycerides. Journal of Pharmaceutical and Biomedical Analysis. 2007; (43): 142-150

Belsey R, Clark MB, Bernat M, Glowacki J, Holick MF, Deluca HF, Potts JT Jr. Physiologic significance of plasma transport of vitamin-d and metabolites Am. J. Med. 1974; **57**: 50-56.

Brander GC, Pugh DM, Bywater RJ, Jenkins WL. Veterinary Applied Pharmacology and Therapeutic, 5th Edn. ELBS with Bailliere Tindall, London, 1991. 240, 250 p.

Connors KA, Amidon GL, Stella VJ. Chemical Stability of Pharmaceuticals, A handbook for Pharmacists.2nd Edn. John Wiley and Sons, Inc. 1986. 351,352 p.

Dean DA, Carstensen JT, Rhodes CT, (Eds). Drug Stability, Principles, and Practices, Marcel Decer, Inc., New York, 2000.

Desai ND, Singh PP, Amin PD, Jain SP. Stability indicating LC method for the Assay of Cholecalciferol. Chromatographia. 2009; 69 (3-4): 385-388.

Grady LT, Thakker KD. Stability of Solid drugs: degradation of ergocalciferol (Vitamin D_2) and Cholecalciferol (Vitamin D_3) at high humidities and elevated temperatures. J. Pharm. Sci. 1980; 69: 1099-1102.
Hoffmann-La Roche FAG. Drug master file, Cholecalciferol, 1989.

Holick MF, Song WO, Beecher GR, Eitenmiller RR, Wineforner JD, (Eds). Modern Analytical methodologies in Fat- and Water-Soluble Vitamins, (Chemical Analysis: A Series of Monographs on Analytical Chemistry and its Applications, vol. 154), John Wiley and Sons, Inc., New York, 2000. 51 p.

ICH Harmonized Tripartite Guidelines. Q1F: Stability Data Package for Registration Applications in Climatic Zones III and IV, London, 2003.

ICH Guidance for Industry. Q1A (R2): Stability Testing Guidelines (ICH Step 5) Note for guidance on stability testing: Stability testing of new drug substance and products (Revision 2) London, 2003.

Iqbal K. Accelerated stability study of different brands of Cholecalciferol tablets and Injections, M-Phil. Thesis, Gomal University, Dera Ismail Khan, 2011.

Martindale the Extra Pharmacopoeia. 26th Edn. The Pharmaceutical Press, London, 1973. 1973, 1949, 1946 p.

Mauldin RE, Johnston JJ. An improved method for analysis of Cholecalciferol-Treated Baits. Journal of AOAC International. 1999; 82 (4): 792-798.

Q_2 (R_1)- Validation of Analytical method, text, and methodology, International Conference on Harmonization of technical requirements for registration of pharmaceuticals for human use (2005).

Staff A, Nyman A. Determination of Cholecalciferol (Vitamin D_3) in selected foods by Liquid Chromatography. NMKL Collaborative Study. Journal of AOAC International. 2003; 86 (2): 400-406.

The Merck Index. 9th Edn. Merck and Company, INC Rahway, N.J., U. S. A, 1976. 9679 p.

Temora Z, Roskar R. Stability – Indicating HPLC-UV method for vitamin D_3 determination in solutions, nutritional supplements and Pharmaceuticals. Journal of Chromatographic Science. 2016; 54 (7): 1180-1186.

Tiles S, Hasil Y. The determination of vitamin D_3 by high-pressure liquid chromatography (HPLC). GIDA. 1994; 19(16): 377-379.

United States Pharmacopoeia, Validation of Compendial methods (Pharmacopoeial Convention Inc., Rockville, MD) 2004: 2622-2625.