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Development and validation of a stability-indicating RP-HPLC method of cholecalciferol in bulk and pharmaceutical formulations: Analytical quality by design approach

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ABSTRACT

The present article utilized analytical quality by design (AQbD) methodology to optimize chromatographic conditions for the routine analysis of Cholecalciferol (CHL). Taguchi orthogonal array design and Box–Behnken design were employed to screen and optimize critical method parameters for augmenting the method performance. The optimal chromatographic separation was attained on Eurosphere® 100-5, C8 (250 × 4.6 mm i.d., 5 µm) column in an isocratic elution mode using methanol:acetonitrile (50:50, % v/v) as mobile phase at a flow rate of 1.0 ml/minutes and photodiode array detection at 265 nm. The optimized chromatographic method was successfully validated as per International Council for Harmonisation Q2 (R_1) guidelines. The method was found to be linear (r^2 = 0.9993) in the range of 20–100 IU/ml. Limit of detection and limit of quantitation were found to be 10 and 20 IU/ml. The precision, robustness, and ruggedness values were within the acceptance limits (relative standard deviation < 2). The percent recovery of in-house developed 400 IU mouth dissolving tablets and marketed Tayo 60k tablets were found to be 99.89% and 101.46%, respectively. The forced degradation products were well resolved from the main peak suggesting the stability-indicating the power of the method. In conclusion, the AQbD-driven method is highly suitable for analysis of CHL in bulk and pharmaceutical formulations.

INTRODUCTION

During product development, quality assurance of pharmaceutical molecules is a matter of great concern in the pharmaceutical industry. Analytical methods are critical elements in product development due to their roles in assisting with process development and product quality control. Poor analytical methods can lead to inaccurate results, resulting in misleading information that may be detrimental to the drug development program. In an endeavor to address such plausible crucial issues, different Pharma regulatory agencies, such as International Council for Harmonisation (ICH) and U.S. Food and Drug Administration, have been transforming by adopting quality by design (QbD) principles to circumvent these quality crises. Recently, ICH has announced new guideline ICH Q14 on analytical procedure development and revision of Q2 (R_1) analytical Validation Q2 (R_2)/Q14 (ICH Assembly, Kobe, Japan, June 2018).

The traditional liquid chromatographic method development for any drug molecule was performed by a trial and error approach, for example, by varying one-factor-at-a-time and examines the resolution of the result until the best method was found. It is a time-consuming process and required a large amount of manual data interpretation. This approach requires typically some experimental trials, and in some circumstances, the established method requires further modification in method or a supplementary purification stage when scaled up, consequently slackening the drug development process (Monks *et al*., 2011; Peraman *et al*., 2015). Moreover, this type of method development provides a limited understanding of a method's capabilities and robustness. This can be overcome by applying QbD principles to the analytical method development as it uses a statistical experimental design to

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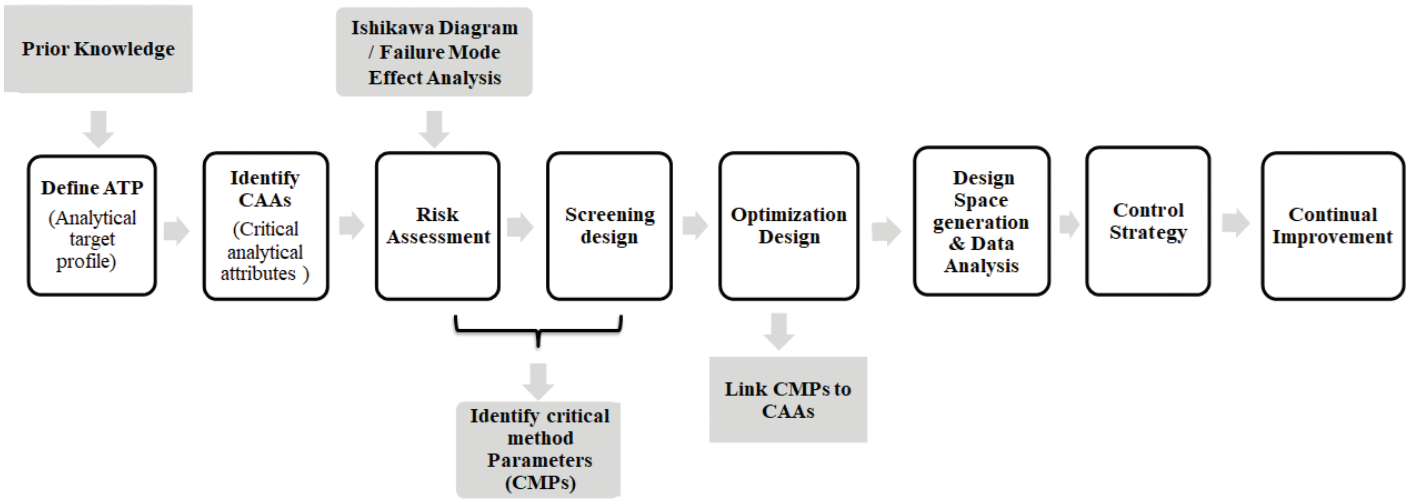

Figure 1. A complete flow layout of AQbD scheme.

generate a "method operable design space" of a robust analytical method (Peraman *et al*., 2015). The method operable design space outlines the experimental operable region in which variations to method parameters will not considerably influence the quality and results of the method. Therefore, it will be technically essential to understand if a method operable design space for variations in high-performance liquid chromatography (HPLC) method parameters can be obtained to assist the development of a robust and rugged analytical method (Rozet *et al*. 2013). Various research scientists have started to adopt the QbD principles and methodology to chromatographic analysis (Awotwe-Otoo *et al*., 2012; Bossunia *et al*., 2017; Garg *et al*., 2015; Ganorkar *et al*., 2017; Panda *et al*., 2017; Thakur *et al*., 2017).

Rational and systematic adoption of quality by design (QbD) elements to analytical method development to achieve optimal method performance is termed as analytical QbD (AQbD) (Jayagopal and Shivashankar, 2017; Reid *et al*., 2013b). This approach guarantees the high quality and reliability of the analytical method and diminishes the risk of failure in the validation phase and routine practice. It is a scientific and risk-based approach for the understanding of the critical analytical attributes (CAAs) and influential independent factors impacting the method performance. Instituted on the doctrines of risks assessment and design of experiments, AQbD offers the in-depth knowledge about the possible risks and connected interactions between the method variables (Borman *et al*., 2010; Jayagopal and Shivashankar, 2017; Karmarkar *et al*., 2011; Reid *et al*., 2013a; 2013b). Besides, AQbD helps in reducing and controlling the source of variability to gain in-process information for taking control decisions promptly. Figure 1 portrays a complete flow layout of AQbD scheme.

Cholecalciferol (CHL), renowned as vitamin D_3, is the most widely prescribed drug for vitamin D_3 deficiency. Vitamin D_3 deficiency is associated with osteoporosis and osteomalacia in adults and rickets in children (Holick and Chen, 2008). CHL plays a critical role in calcium and phosphorus homeostasis and skeletal mineralization (Gueli *et al*., 2012). In common medical practice, vitamin D_3 deficiency is normally treated with CHL ranging from 400 to 1,000 IU/day. Recent studies of the physiologic effects of vitamin D_3 suggest its role in autoimmune diseases like cancers, type 1 diabetes mellitus, hypertension, multiple sclerosis, Alzheimer's disease, and cognitive impairment (Marques *et al*., 2010). Chemically, it is (3β, 5Z, 7E)-9, 10-secocholesta-5,7,10(19)-trien-3-ol. CHL exists as a white, odorless needle-like crystalline powder, soluble in ethanol, benzene, acetone, chloroform, and fatty oils but practically insoluble in water. The log P of the drug substance is 10.24 at 20°C and pH 7. The molecular weight of CHL is 384.64 g/mol and formula is $C_{27}H_{44}O$. Figure 2 depicts the chemical structure of the CHL.

The USP analytical method is the only reliable method for CHL estimation, but suffers from various disadvantages of having complicated, tedious, multiple extraction steps that make it as time-consuming method. Also, the majority of published HPLC-UV methods of vitamin D_3 have limited application as they have complex mobile phase composition, no stability indicating capability, longer retention time, i.e., more than 10 minutes and mostly followed by time consuming and complicated sample

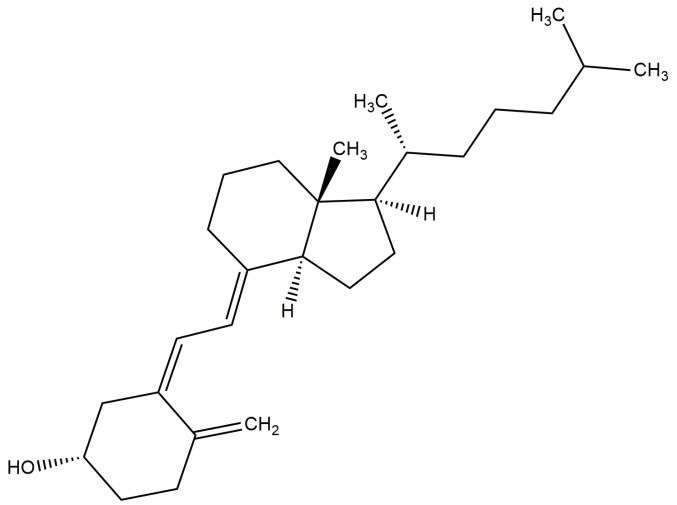

Figure 2. Chemical structure of Cholecalciferol.

preparation for instance solid phase extraction or supercritical fluid extraction (Al-Qadi *et al*., 2010; Gamiz-Gracia *et al*., 2000; Kienen *et al*., 2008; Klejdus *et al*., 2004; Kucukkolbasti *et al*., 2013; Luque-Garcia and de Castro, 2001; Moreno and Salvado, 2000; Sarioglu *et al*., 2001). Moreover, scientific and risk-based AQbD-oriented approach to reversed-phase HPLC (RP-HPLC) method development of CHL has not been widely discussed till date. Therefore, there is an unmet need for the development of robust, simple, and highly sensitive HPLC method of CHL using AQbD principles to overcome the problems as mentioned above and to ensure the quality of the method throughout the material lifecycle.

In this research article, the ultimate goal of present work was to develop simple, rapid, sensitive, robust, effective, and reliable stability-indicating HPLC method by applying AQbD principles and methodology for assessment of CHL in bulk drug and pharmaceutical drug products, i.e., CHL 400 IU mouth dissolving tablets and marketed 60000 IU chewable (Tayo 60k) tablets.

MATERIALS AND METHODS

Experimental

Chemicals and reagents

CHL (purity 99.7%, 40,000 IU/mg) was obtained as a gift sample from Fermenta Biotech Ltd, Mumbai, India; used as a reference standard. HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Avantor Performance Materials India Ltd, Thane, India. The mobile phase was filtered using 0.45-µm nylon membrane filters made by Pall India Pvt Ltd, Mumbai, India, was sonicated, and degassed using sonicator. In-house 400 IU vitamin D_3 mouth dissolving tablets (MDTs) and marketed cholecalciferol 60,000 IU chewable tablets (Tayo 60k manufactured by Eris Lifesciences Pvt. Ltd.) were analyzed for assay by use of the established RP-HPLC method.

Instrumentation and chromatographic conditions

The HPLC method development of CHL was performed on Jasco AS-2055 plus (Tokyo, Japan) containing a system controller, quaternary gradient pump, mobile phase degasser, autoinjector (injection volume ranging between 5 and 100 µl) and photodiode array (PDA) detector. Chromatographic separation was achieved on a reversed-phase C8 column, Eurosphere® 100-5 C8 (M/S KNAUER Wissenschaftliche Gerate GmbH, Berlin, Germany) with a dimension of 250 mm × 4.6 mm and particle size 5 µm, at the room temperature. Isocratic elution was employed with ACN and MeOH (50:50, % v/v) as mobile phase and PDA detection was carried out at 265 nm. Before the chromatographic analysis of test solutions, the column was saturated with the mobile phase for 60 minutes. The 50 µl of sample was injected for the quantification of CHL. The run time of all the test samples was kept 10 minutes. The run time for forced degradation test samples was extended up to 20 minutes to estimate probable co-eluting degradation products. The data acquisition, analysis, and storage were performed by using Jasco ChromNAV software.

Preparation of standard stock solution

The stock solution of CHL was prepared by dissolving accurately weighed 25 mg of the drug in 50 ml of MeOH. The drug solution was sonicated to dissolve the drug, and then 1 ml of this stock solution transferred into 100 ml of amber colored volumetric flask, and it was diluted up to 100 ml with HPLC grade MeOH (final concentration, 200 IU/ml, knowing that 1 IU of vitamin D_3 = 0.025 µg). It was used for both screening and optimization experiments. The working standard solutions of CHL were prepared by subsequent dilutions of the stock solution. The series of dilutions were done using HPLC grade MeOH and filtered using a 0.45 syringe filter. Then, these dilutions were transferred to vials before chromatographic analysis.

Defining the method goals, i.e., analytical target profile (ATP)

The AQbD-based methodology defines and proposes vital elements of ATP for the stepwise, scientific development of the analytical method. The method goals cover a possible summary of the quality features of the analytical method. Table 1 depicts vital elements of ATP framework for obtaining an efficient HPLC method for CHL.

Critical analytical attributes (CAAs)

To achieve the anticipated ATP, various CAAs were recognized and explored. These are peak area, retention time, theoretical plates, and peak tailing factor.

Table 1. Vital elements of ATP for HPLC method of CHL.

ATP elements	Target	Justification
Target Analyte	CHL	HPLC method development of CHL is needed for drug content analysis in pharmaceutical drug products and stability samples.
Chromatographic mode	RP-HPLC	Commonly, RP-HPLC has the advantage of good retention of the lipophilic drug molecules as it consists of hydrophobic stationary phase. CHL exhibits high lipophilicity (log P 10.24). Thus, reverse-phase method would be more accurate and reliable for this purpose.
Instrument necessity	Quaternary pump	Quaternary Pump delivers a precise and efficient mixing of solvents of mobile phase as compared to the binary pump.
Sample state	Liquid	In RP-HPLC, analyte should be in a liquid state for its miscibility with mobile phase
Standard preparation	Standard dilutions of CHL	Standard dilution of the drug is generally prepared in MeOH for proper separation
Sample preparation	Handling, weighing, sampling, admixing with solvents	Sample preparation is generally carried out by weighing the accurate quantity of CHL, mixing with Sample solution to get a stock solution, followed by sonication and appropriate dilutions.
Method application	For assay of CHL	The method has applicability for assay of CHL and its degradation product in bulk drugs and pharmaceutical drug products.

Risk assessment studies

Risk assessment studies were performed to identify the critical method parameters (CMPs), which are high-risk factors and have a critical impact on the CAAs. In the risk assessment plan, Ishikawa fishbone diagram was constructed to identify potential risk factors that may have an effect on method performance and corresponding causes. This could be method factors like extraction method, extraction time, extraction solvent, etc. and instrumental settings such as chromatographic mode, mobile phase ratio, flow rate, injection volume, etc. From this, high-risk method variables were shortlisted based on criticality and impact on the method CAAs and exposed to further analysis by applying suitable screening and experimental optimization design.

Taguchi orthogonal array screening study design

Taguchi orthogonal array (TOA) design is a multifactorial two-level design that can be applied for identification and control of the main effect independent variables with a minimum number of experiment runs from various suspected independent factors (Dash *et al*., 2016; Sahu *et al*., 2017). Therefore, this experimental design was generally employed for identification of independent factors that could be fixed or eliminated in further study.

The TOA design was employed in this study for screening studies to recognize the CMPs censoriously influencing the method CAAs using the following polynomial model as shown in the following equation:.

$$Y = A_0 + A_1X_1 + A_2X_2 + A_3X_3 + A_4X_4 + A_5X_5 + A_6X_6 + A_7X_7 \quad (1)$$

where, Y is the response variable, A_0 is the constant, and A_1, A_2, A_3, A_4, A_5, A_6, and A_7 are the regression coefficients of the independent factors.

Table 2 represents the TOA design layout enlisting the different factors with respective low (−1) and high levels (+1) and their studied responses (theoretical plates and peak tailing factor). Standard Pareto charts were drawn to illustrate the effect of each independent factor on the specified responses. Then, the critical factors were recognized and further employed for Box–Behnken design.

Box–Behnken optimization study design

The optimization of chromatographic conditions was performed by employing three factors, three levels Box–Behnken design to estimate the main, interaction and quadratic effects of critical factors on the specified response variables (Ahmad *et al*., 2016; Beg *et al*., 2012; Ferreira *et al*., 2007; Sahu *et al*., 2015; 2017; Wani and Patil, 2017). In the present study, the Box–Behnken experimental design, comprising 15 experiment runs with 12 factorial points and three center points, was employed to get design space for attaining the desired ATP. The polynomial quadratic equation is generated by this design as shown in the following equation:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{23}X_2X_3 + B_{13}X_1X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 \quad (2)$$

where Y is the response variable, B_0 is the constant, and B_1, B_2, and B_3 are the regression coefficients of the linear terms of X_1, X_2, and X_3, respectively. B_{12}, B_{23}, and B_{13} are the regression coefficients for the interaction terms of X_1X_2, X_2X_3, and X_1X_3,

Table 2. Chromatographic factors and response variables for Taguchi experimental design.

	Low level (−1)	High level (+1)
Independent factors		
X_1: Mobile Phase ratio (% v/v)	40:60	60:40
X_2: Flow rate (ml/minutes)	0.8	1.2
X_3: Injection volume (µl)	10	30
X_4: Mode of Flow	Isocratic	Gradient
X_5: Column type	C_8	C_{18}
X_6: Column length (mm)	150	250
X_7: Column temperature (°C)	25	30
Dependent factors (responses)		
Y_1: Theoretical plates		
Y_2: Peak tailing factor		

Taguchi design layout (seven-factor eight-run)							
Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7
1	−1	+1	+1	+1	+1	−1	−1
2	+1	−1	+1	+1	−1	+1	−1
3	+1	−1	+1	−1	+1	−1	+1
4	−1	+1	+1	−1	−1	+1	+1
5	−1	−1	−1	−1	−1	−1	−1
6	+1	+1	−1	−1	+1	+1	−1
7	−1	−1	−1	+1	+1	+1	+1
8	+1	+1	−1	+1	−1	−1	+1

respectively. B_{11}, B_{22}, and B_{33} are the regression coefficients for the squared terms of X_{12}, X_{22}, and X_{32}, respectively.

The independent variables selected were mobile phase [ACN:MeOH] ratio (X_1), flow rate (X_2), and injection volume (X_3), whereas peak area (Y_1), retention time (Y_2), theoretical plates (Y_3), and peak tailing factor (Y_4) were selected as the dependent responses. The Box–Behnken optimization study design layout is shown in Table 3.

Analytical method validation

The optimized chromatographic method was validated as per the ICH Q2 (R_1) guidelines for specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), robustness, and ruggedness (Guideline ICH, 2005).

Forced degradation studies

Forced degradation of CHL was executed to deliver a sign of the stability indicating properties and specificity of the established method (Blessy *et al*., 2014; Krishna *et al*., 2016). PDA detection was employed to analyze the purity of degraded test samples. The stress conditions used for the degradation study included acid hydrolysis (1 N HCl), base hydrolysis (1 N NaOH), All the test samples were filtered using a 0.45 µm nylon membrane filter and analyzed to estimate the percent degradation of CHL.

Application of the analytical method for analysis of CHL in tablet dosage form

The established and validated analytical method for CHL was applied for its determination in in-house CHL 400 IU mouth dissolving tablets (50% overages) and marketed 60,000 IU (Tayo 60k) chewable tablets. Vitamin D_3 MDTs (50% overages) were manufactured by blending stabilized CHL (100 IU/mg) with other excipients by direct compression technique. To determine the content of CHL in the developed MDTs and marketed chewable tablets, 20 tablets were weighed and finely powdered with the help of mortar and pestle. The required quantity of powder was accurately weighed and transferred to a volumetric flask containing HPLC grade MeOH and sonicated for 30 minutes, for complete extraction of the drug to take place.Finally, this prepared test sample was filtered through 0.45 µm nylon membrane before using it for analysis. The analysis was performed in five replicates.

RESULT AND DISCUSSION

Preliminary method development studies

The preliminary studies were performed according to previously reported literature for the development of HPLC

Table 3. Chromatographic factors and response variables for Box–Behnken optimization design.

	Low level (−1)	Medium level (0)	High level (+1)
Independent f**actors**			
X_1: Mobile Phase ratio (% v/v)	40:60	50:50	60:40
X_2: Flow rate (ml/minutes)	0.8	1.0	1.2
X_3: Injection volume (µl)	10	20	30
Dependent factors (r**esponses**)			
Y_1: Peak area			
Y_2: Retention times (minutes)			
Y_3: Theoretical plates			
Y_4: Peak tailing factor			

Box–Behnken optimization design layout

Run	Coded level pattern (X_1 X_2 X_3)	X_1: Mobile phase ratio (% v/v)	X_2: Flow rate (ml/minutes)	X_3: Injection Volume (µl)
1	0−1+1	50:50	0.8	30
2	+1+10	60:40	1.2	20
3	000	50:50	1	20
4	−1+10	40:60	1.2	20
5	−10−1	40:60	1	10
6	+10+1	60:40	1	30
7	−1−10	40:60	0.8	20
8	+1−10	60:40	0.8	20
9	000	50:50	1	20
10	+10−1	60:40	1	10
11	0+1+1	50:50	1.2	30
12	−10+1	40:60	1	30
13	000	50:50	1	20
14	0−1−1	50:50	0.8	10
15	0+1−1	50:50	1.2	10

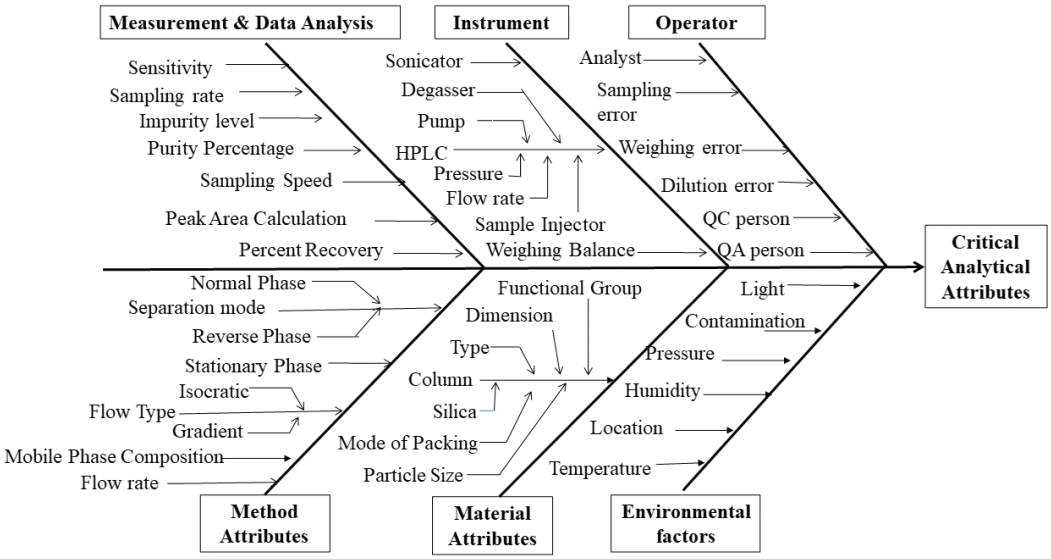

Figure 3. Ishikawa Fish-bone diagram illustrating the influence of possible factors on CAAs of analytical method of CHL.

method for the estimation of CHL in pharmaceutical dosage forms. The RP-HPLC method was successfully employed for evaluation of CHL. First, several combinations of mobile phase were tried by using ACN and MeOH at a variable flow rate between 0.8 and 1.2 ml/minutes. From preliminary studies, it was found that the selection of ACN and MeOH as a mobile phase composition showed excellent chromatographic resolution with low peak tailing factor.

Risk assessment studies

Risk assessment studies as per ICH Q9 guidelines were performed with an objective to get all the possible high impact factors which will be subjected to the design of experiment study to establish method operable design region. Ishikawa fish-bone was used for risk identification and risk assessment. Figure 3 depicts the effect of possible key factors such as method & material attributes, environmental factors, operator, instrument requirements, and measurement & data analysis affecting on method performance. It illustrates the cause and effect relationship between method parameters and CAAs of the analytical method of CHL. Risk assessment studies identified seven high potential risk factors such as X_1: mobile phase ratio (% v/v), X_2: flow rate (ml/minutes), X_3: injection volume (µl), X_4: mode of flow, X_5: column type, X_6: column dimension (mm), and X_7: column temperature (˚C). These factors have potential impact on critical analytical attributes (CAAs), i.e., theoretical plates (Y_1) and peak tailing factor (Y_2). These seven factors would be used for further screening study to get the major factors affecting selected CAAs by Taguchi orthogonal array (TOA) design.

Taguchi orthogonal array screening study design

Ideally, screening designs are applied when numerous independent factors expected to have an impact on a specific response. The objective of this study was to identify the most significant factors influencing the CAAs by using TOA screening study design. TOA design was used to estimate the main effects of seven independent factors on selected CAAs. Figure 4 shows the standard Pareto charts illustrating the impact of method parameters on the CAAs of the method. The standard Pareto ranking analysis presented that the factors, such as mobile phase ratio, flow rate, and injection volume, had a significant impact on method CAAs. Hence, these factors were selected as CMPs for further analytical optimization study employing Box–Behnken design.

The standard Pareto charts were derivative of multivariate regression analysis and the length of each bar in the Pareto chart is equal to the magnitude of the regression coefficient of that factor. It was observed that little change in mobile phase ratio, flow rate, and injection volume resulted in a pronounced change in CAAs. Henceforth, these factors needed to be strictly controlled while the effect of mode of flow, column type, column length, and column temperature were found to be statistically insignificant. Based on the desirability function of Taguchi screening design, mobile phase ratio, flow rate, and injection volume were optimized further by Box–Behnken optimization design to detect main, interaction and quadratic effects of these factors on peak area, retention time, theoretical plates, and peak tailing factor.

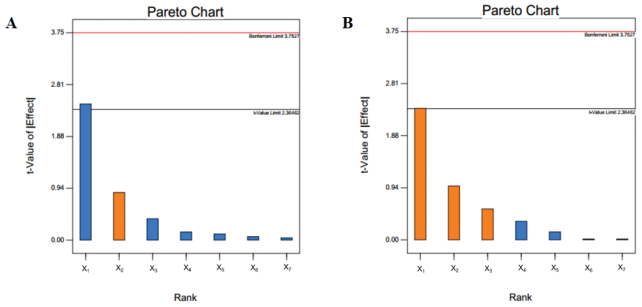

Figure 4. Standard Pareto charts showing effects of independent variables on analytical method CAAs. (A) Theoretical plates and (B) peak tailing factor of CHL during the screening.

Box–Behnken optimization study design

This study aimed at detecting the main, interactions and quadratic effects of mobile phase ratio, flow rate, and injection volume on peak area (Y_1), retention time (Y_2), theoretical plates (Y_3), and peak tailing factor (Y_4). The 15 experimental runs were performed, and obtained results were statistically analyzed using Design expert software version 7.0.0 (Stat-Ease Inc., Minneapolis, MN). The software performs response surface methodology, which includes the multiple regression analysis (MRA), analysis of variance (ANOVA), and statistical optimization. The classical polynomial quadratic equation in terms of coded factors for each selected CAAs estimating regression coefficients are shown in the following equations:

$$\text{Peak area } (Y_1) = 11744.33 - 319.75X_1 - 14.88X_2 + 226.88X_3 - 44.00X_1X_2 + 95.50X_2X_3 + 204.25X_1X_3 - 3260.04X_1^2 - 3059.79X_2^2 - 3375.29X_3^2 \quad (3)$$

$$\text{Retention time } (Y_2) = 5.03 + 0.088X_1 - 0.11X_2 + 0.025X_3 + 0.100X_1X_2 - 0.025X_2X_3 + 0.025X_1X_3 - 0.092X_1^2 - 0.49X_2^2 - 0.32X_3^2 \quad (4)$$

$$\text{Theoretical plates } (Y_3) = 11442.00 + 500.12X_1 - 34.13X + 88.50X_3 - 50.25X_1X_2 + 124.00X_2X_3 - 70.50X_1X_3 - 2164.13X_1^2 - 1249.62X_2^2 - 1279.38X_3^2 \quad (5)$$

$$\text{Peak tailing factor } (Y_4) = 1.00 - 0.094X_1 - 0.059X_2 - 0.023X_3 + 0.045X_1X_2 + 0.052X_2X_3 + 0.052X_1X_3 + 0.22X_1^2 + 0.17X_2^2 + 0.19X_3^2 \quad (6)$$

The ANOVA results for each CAAs were shown in Table 4. The ANOVA with its significance method for all CAAs proves that the relationship between response and variables is statistically significant ($p < 0.05$). The value of correlation coefficient (R^2) for all CAAs indicates a perfect fit of the model. This implies that the model is valid. The adjusted *R*-squared was a more valuable marker of the variation in response variables, while predicted *R*-squared indicated how well the model could predict future data, relatively high values of adjusted and predicted *R*-squared inferred that the applied statistical model effectively predicted the response. The main effects (B_1, B_2, and B_3) signify the average response of varying one factor at a time from its low to high level. The interaction term (B_{12}, B_{23}, and B_{13}) shows how the response changes when two factors are concurrently altered. The polynomial quadratic terms (B_{11}, B_{22}, and B_{33}) were added to examine nonlinearity. The Polynomial quadratic equations were employed to conclude after considering the magnitude of coefficients and the mathematical sign it carries, i.e., positive or negative.

From Equation (3), it was observed that factors X_1 and X_2 have a negative effect, while X_3 has a positive effect on the peak area. Negative value coefficients of X_1 and X_2 factor indicate that peak area increases with a decrease in the mobile phase ratio and flow rate, whereas positive sign of coefficients of X_3 terms indicates that low to medium level of injection volume favors the increased peak area. When the coefficient values of independent key variables (X_1, X_2, and X_3) compared, the coefficient value of variable X_3 (226.88) was found to be higher, and hence injection volume was considered to be a major contributing factor for incredible effect on peak area (Y_1).

From Equation (4), variation in mobile phase ratio significantly affects the retention time as the coefficient value of X_1 was found to be maximum among all independent factors. The polynomial equation for retention time suggests that factor X_1 has a positive effect on Y_2, up to a particular concentration. After a particular concentration factor, X_1 has a negative effect on Y_2 as indicated by the negative sign of the coefficient of X_1.

Equation (5) showed that the positive value of coefficients of X_1 and X_3 factor indicates that theoretical plates increase with the increase in the mobile phase ratio and injection volume up to medium level. After a medium level factor, X_1 and X_3 have a negative effect on Y_3 as indicated by the negative sign of the coefficient of X_1 and X_3. Mobile phase ratio was considered to be an influential factor to have a major impact on theoretical plates as the coefficient value of variable X_1 was found to be higher.

From Equation (6), it was observed that the negative sign of coefficients of X_1, X_2, and X_3 terms indicates that low to medium level of independent factors favors the lesser tailing factor. From medium to high level of factors displays increased peak tailing factor. As compared to the magnitude of all factor coefficients, injection volume showed the influential impact on peak tailing factor, while the flow rate was found to have relatively less impact on the peak tailing factor. Minimum values were observed at the intermediate level.

Table 4. ANOVA results for each CAAs.

ANOVA Parameters	Y_1: Peak area	Y_2: Retention time	Y_3: Theoretical plates	Y_4: Peak tailing factor
R-squared	0.9965	0.9728	0.9698	0.9588
Adjusted ***R***-squared	0.9901	0.9237	0.9156	0.9145
Predicted ***R***-squared	0.9541	0.8978	0.8855	0.8986
Standard deviation	268.63	0.089	415.93	0.065
C.V.%	4.09	1.94	4.65	4.97
PRESS	4.697E+006	0.54	7.301E+006	0.33
F-value	156.92	19.83	17.87	12.92
p-value	< 0.0001	0.0021	0.0027	0.0058

R-squared = Coefficient of determination.
F-value = Value on the F distribution.
p-value = Probability of falsely detecting a significant effect.
PRESS = Predicted errors sum of squares.
C.V.% = Percent Coefficients of variance.

Three-dimensional surface and 2-D contour plots were also analyzed to define design space and to visualize the effect of independent factors and their interactions on the concerned response variables. Since the proposed model has more than two independent factors, one factor was kept constant for each plot. These plots were found to agree with MRA and ANOVA parameters. Figure 5 portrays 3D surface plot, and it's corresponding 2D contour plot depicting the effects of mobile phase ratio, flow rate and injection volume on peak area, retention time, theoretical plates, and peak tailing factor.

Identification for optimum method conditions

Identification for optimum method conditions was performed by numerical optimization simply by trading off different CAAs to gain anticipated targets, i.e., maximum theoretical plates, peak area, retention time around 5 minutes, and minimum peak tailing factor (about 1.0). Desirability function close to one was selected as the optimum solution. The optimized method conditions were found to be mobile phase ratio of 50:50 (%v/v) ACN and MeOH, the injection volume of 20 µl, and flow rate of 1 ml/minutes with the desirability of 0.958 (as shown in Fig. 6). The graphical optimization was found to agree numerical optimization and estimated optimized solution was found within operable analytical design space. The yellow region in an overlay plot indicated optimum method conditions suggested by design expert software to obtain method targets as shown in Figure 6.

Analytical method validation

Figure 7 showed the chromatogram of placebo mixture, standard drug solution, and developed 400 IU MDTs. The placebo does not show any drug peak in the chromatogram. Figure 7B shows the standard chromatogram of 40 IU/ml of CHL with Rt 5.0 minutes. Figure 7C shows the chromatogram

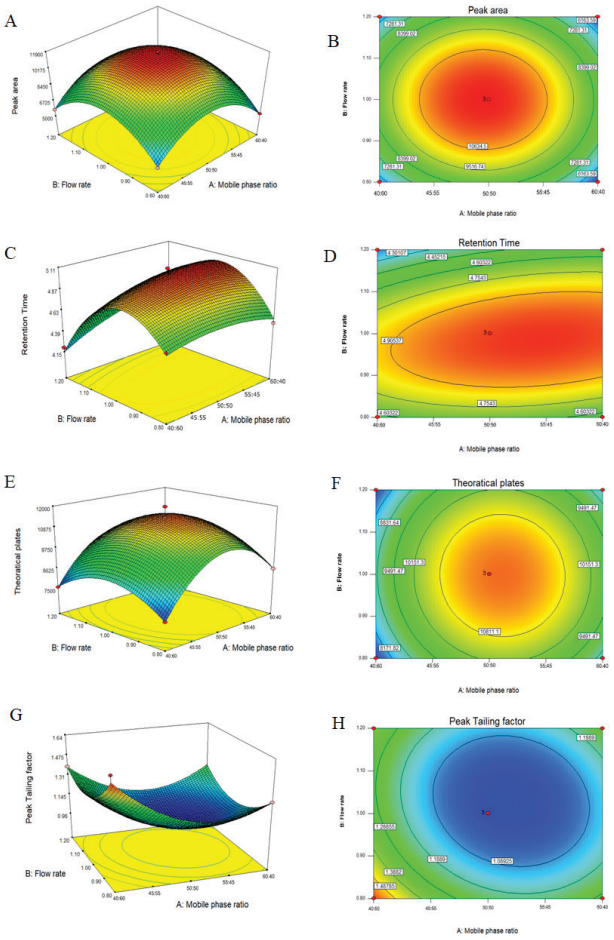

Figure 5. 3D surface and 2D Contour plots depicting the effect of (A and B) mobile phase ratio and flow rate on peak area, (C and D) retention time, (E and F) theoretical plates, and (G and H) peak tailing factor.

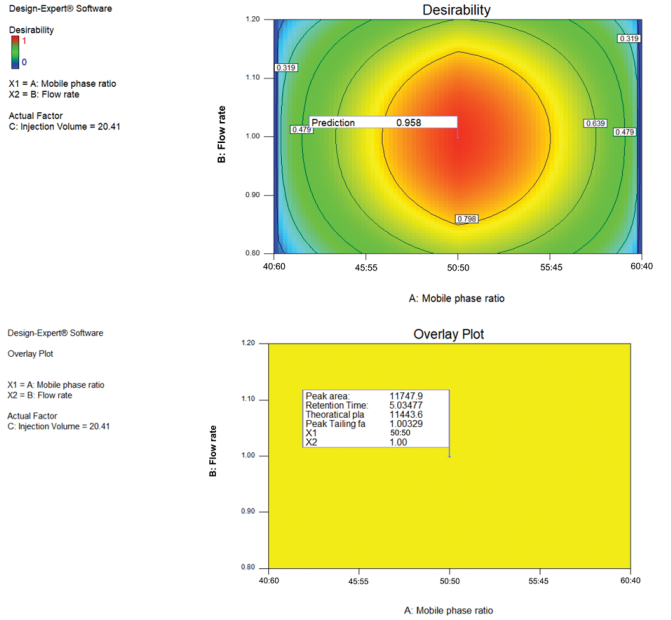

Figure 6. Desirability and overlay contour plot showing optimum method operable design region.

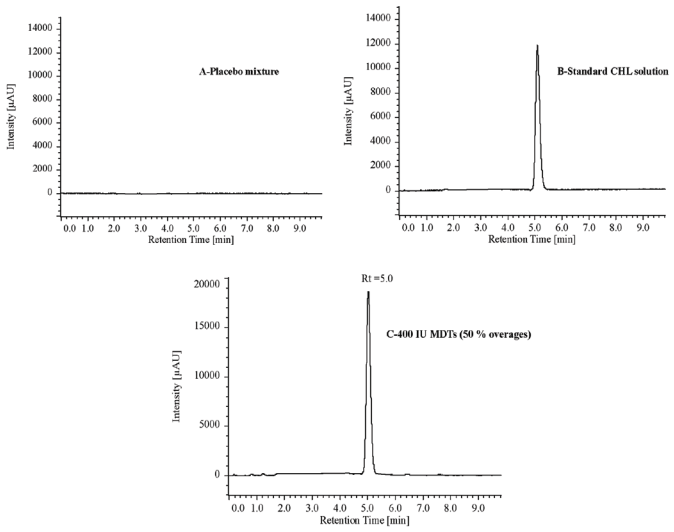

Figure 7. Chromatograms depicting (A) placebo mixture, (B) standard CHL solution, and (C) CHL in the developed formulation, i.e., 400 IU (50% overages) MDTs.

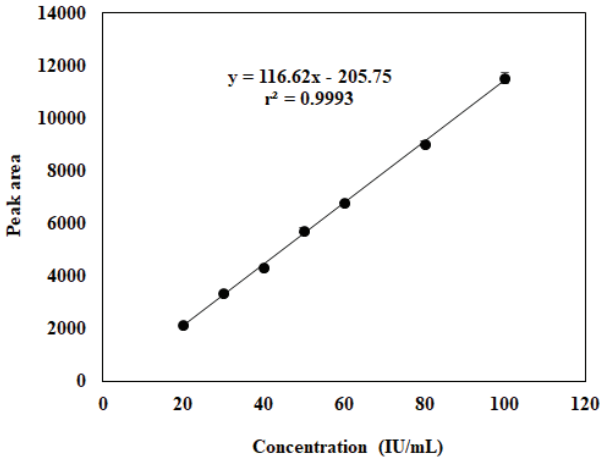

Figure 8. Calibration curve of vitamin D_3 in MeOH.

of the drug in a formulation, which confirms no change in the chromatogram of a drug in the presence of other ingredients. There was no interference observed at the retention time of drug peak; thus, method was found to be specific for CHL. Standard calibration curve of CHL in MeOH was plotted in the range of 20–100 IU/ml (Fig. 8). The method was found to be linear in the selected concentration range with a regression coefficient of 0.9993.

Data represented in Table 5 summarise the system, intraday and interday precision, solution stability, robustness, and ruggedness of established HPLC method. It can be observed from the table that relative standard deviation % relative standard deviation (RSD) of each parameter for validation falls within the limit, i.e., not more than 2%.

The LOD and LOQ concentrations were found to be 10 and 20 IU/ml, respectively. The stability of the drug solution of concentration 100 IU/ml up to 24 hours was estimated by using the established HPLC method. Vitamin D_3 was found to be stable in the MeOH. No peaks corresponding to degradation products were observed. The robustness of the projected method was estimated by altering mobile phase composition from Acetonitrile: Methanol 48:52–52:48 v/v, varying the injection volume from 19.5 to 20.5 ml and changing the flow rate from 0.9 to 1.1 ml/minutes. Acceptable %RSD values were obtained after making small deliberate changes in the ratio of mobile phase, injection volume, and flow rate. This indicates that the method is robust for the envisioned purpose. The ruggedness of the analytical method by different analysts and different instrument confirms the reliability of the analytical method for ruggedness in the chromatographic conditions.

An excellent recovery with %RSD of less than 2% was obtained from all three levels, i.e., 70%, 100%, and 130%. The %RSD of all the three levels is shown in Table 6. The developed method did not include any pre-treatment, complicated-tedious stages for extraction of CHL. Hence, there was not any recovery problem with this method.

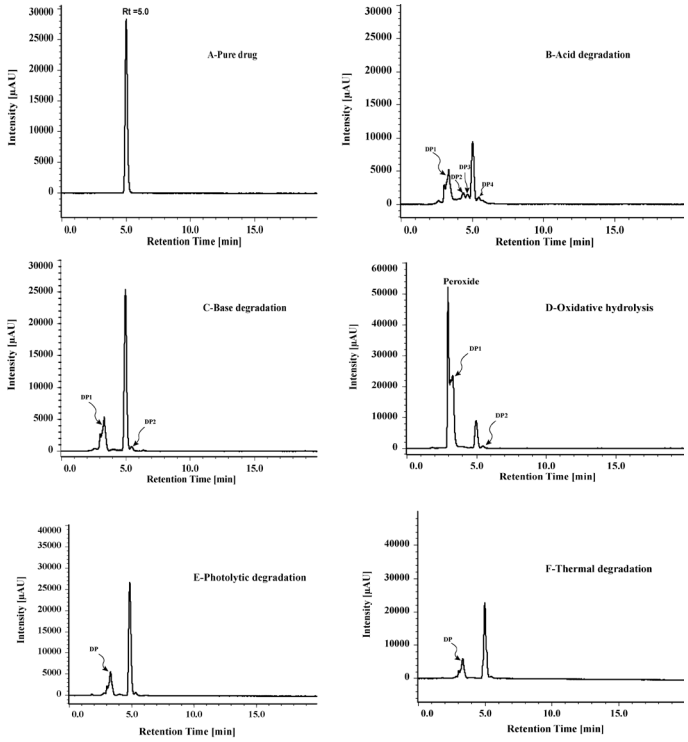

Figure 9. Chromatograms depicting CHL and its DPs under stress conditions (A) Pure drug (B) Acidic (C) Basic (D) Oxidative hydrolysis/Peroxide (E) Photolytic (F) Thermal degradation

Forced degradation studies

The Forced degradation studies were carried out by analyzing the test samples under all stress conditions and computed the percent degradation of CHL for each stress condition as shown in Table 7. In short, the forced degradation studies revealed that the established method was sensitive enough for the determination of DPs.

Application of the analytical method for analysis of CHL in tablet dosage form

The chromatographic analysis of 400 IU MDTs and marketed Tayo 60k chewable tablets showed excellent recovery on the assay. The percent recovery of in-house developed 400 IU MDTs and marketed Tayo 60 K chewable tablets were found to be 99.89% and 101.46%, respectively. Moreover, the retention time of CHL in tablet formulation showed unchanged with respect to standard CHL solution. Nevertheless, the theoretical factors and peak tailing factor were found to be within the acceptable limits. All test samples showed no unwanted extra peaks in chromatograms that suggested there was no inference of other tablet excipients with CHL. This confirmed a high degree of ability of established method for routine analysis of CHL in bulk and pharmaceutical dosage forms.

Future Research Plan

The degradation pathway of Cholecalciferol can be further elucidated by using of liquid chromatography-tandem mass spectrometry technique.

Table 5. Analytical method validation of cholecalciferol.

System precision data of Cholecalciferol			Intraday precision data of Cholecalciferol		Interday precision data of Cholecalciferol		
Injection number	**Retention time (minutes)**	**Concentration (IU/ml)**	**Sample**	**Concentration (IU/ml)**	**Injection number**	**Day 1 concentration (IU/ml)**	**Day 2 concentration (IU/ml)**
1	5.00	38.57	1	38.36	1	38.57	37.36
2	4.99	37.52	2	39.22	2	37.32	37.50
3	5.03	38.09	3	38..41	3	38.09	36.28
4	5.05	37.96	4	37.26	4	37.96	37.12
5	5.04	38.22	5	38.70	5	38.19	37.18
6	5.03	38.82	6	39.12	6	37.70	38.46
Mean	5.02	38.16	Mean	38.36	Mean	37.97	37.32
SD	0.02	0.52	SD	0.71	SD	0.43	0.71
%RSD	0.47	1.37	%RSD	1.84	%RSD	1.13	1.89
						Pooled data for 12 values	
						Mean	37.64
						SD	0.46
						%RSD	1.23

Stability of Cholecalciferol in MeOH		Robustness Results Data					Ruggedness Results Data	
Time (hours)	**Concentration (IU/ml)**	**Parameter**	**Method condition**	**% RSD of concentration (IU/ml)**	**Instrument-1, Analyst-1**		**Instrument-2, Analyst-2**	
					Sample	**Concentration (IU/ml)**	**Sample**	**Concentration (IU/ml)**
0	104.02	Mobile Phase ratio (% v/v)	48:52	1.73	1	37.63	1	37.43
2	103.47		50:50	0.56	2	38.11	2	38.09
4	103.43		52:48	1.89	3	38.44	3	39.12
6	102.85	Injection volume (µl)	19.5	1.68	4	37.02	4	37.86
12	102.07		20	1.17	5	37.86	5	37.14
24	100.51		20.5	1.61	6	38.54	6	37.40
Mean	102.73	Flow rate (ml/ minutes)	0.9	1.91	**Mean**	37.93	**Mean**	37.84
SD	1.27		1.0	0.61	**SD**	0.57	**SD**	0.72
%RSD	1.24		1.1	1.79	**%RSD**	1.49	**%RSD**	1.90

SD = Standard deviation.
% RSD = Percent relative standard deviation.

Table 6. Recovery studies of Cholecalciferol formulations.

Level	Sample	Recovery (%)	Mean recovery (%)	%RSD
70%	S1	101.09	101.80	0.92
	S2	101.44		
	S3	102.85		
100%	S1	100.78	100.37	0.82
	S2	99.43		
	S3	100.90		
130%	S1	101.24	101.05	0.18
	S2	101.04		
	S3	100.88		

% Recovery = Percent recovery.
% RSD = Percent relative standard deviation.

Table 7. Forced degradation studies of Cholecalciferol**.**

Condition of degradation	Percentage of degradation	Degradation products retention time (Rt)
Acid	75.12%	DP_1 at Rt 3.0, DP_2 at Rt 3.8, DP_3 at Rt 4.7 and DP_4 at Rt 5.4
Base	19.32%	DP_1 at Rt 3.0 and DP_2 at Rt 5.4
Peroxide	71.02%	DP_1 at Rt 3.0 and DP_2 at Rt 5.4
Thermal	22.15%	DP_1 at Rt 3.0
Photolytic	20.82%	DP_1 at Rt 3.0

CONCLUSION

The present article successfully demonstrates the effectiveness of quality by design concept to optimize the HPLC chromatographic method for CHL analysis with a better understanding of the critical factor-response relationship for augmenting the method performance. This AQbD-driven HPLC method development of CHL ensured robustness of the analytical method before validation studies. This novel approach helps the analyst to define control strategies to decrease the undesirable effect of these CMVs on method performance. The validation studies confirmed excellent linearity, accuracy, precision with a high degree of specificity, sensitivity, robustness, and ruggedness. The forced degradation studies on established HPLC method of CHL easily identified degradation products while exposing to a variety of stress conditions. It was found that CHL was rapidly degraded under oxidative, hydrolytic (acid and alkali), and photolytic conditions. The developed, validated method further used for the analysis of in-house developed vitamin D_3 (400 IU) MDTs and marketed chewable tablets Tayo 60k (60,000 IU) to ratify the applicability of the method. Overall, practicing the AQbD approach for estimation of CHL in tablet dosage form ensured stepwise, scientific, risk-based method development where quality assurance will be guaranteed.

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ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of variance
AQbD	Analytical quality by design
ATP	Analytical target profile
BBD	Box–Behnken design
CAAs	Critical analytical attributes
CHL	Cholecalciferol
CMPs	Critical method parameters
DPs	Degradation products
ICH	International Council for Harmonization
LC-MS	Liquid chromatography-tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MDTs	Mouth dissolving tablets
MeOH	Methanol
MRA	Multiple regression analysis
PDA	Photodiode array
RP-HPLC	Reversed-phase high-performance liquid chromatography
RSM	Response surface methodology
TOA	Taguchi orthogonal array
USFDA	United States Food and Drug Administration

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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