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Stability-Indicating RP-HPLC Method Development, Qbd Optimization and Validation for assessing Related Impurities in Labetalol Hydrochloride bulk and injectable form

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Abstract
The Current study aimed to develop and validate a reliable Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method for the quantification of Labetalol Hydrochloride and its related substances by using design of Experiments (DoE). The mobile phase A consists of Phosphate buffer (pH 3.7) (100 %) and the mobile phase B consist of Acetonitrile (100 %). The UV detector wavelength was 230 nm. The total run time was 60 minutes and flow rate was 0.8ml/min. Various parameters such as linearity, precision, accuracy, specificity, robustness, ruggedness, solution stability, Forced degradation, limit of detection, limit of quantification and system suitability were thoroughly evaluated according to USP guidelines. The method exhibited excellent linearity over the concentration range of 0.000258 to 0.003096 mg/ml with a correlation coefficient (r^2) of 0.999, The Method precision (% RSD) were found to be within acceptable limits. Accuracy studies revealed that the method was accurate with recovery values within the range of 99.6 % to 102.5 %. The method demonstrated high specificity, as indicated by the resolution of Labetalol Hydrochloride peak from potential impurities. Robustness and Ruggedness testing confirmed the method ability to produce consistent results under slight variations in chromatographic conditions. Furthermore, system suitability parameters such as resolution, tailing factor, and theoretical plates met the acceptance criteria, ensuring the method suitability for routine analysis. Overall, the developed RP-HPLC method proved to be accurate, precise, specific, and robust for the quantitative determination of Labetalol Hydrochloride in pharmaceutical formulations, thereby offering a valuable tool for quality control in pharmaceutical industries.

Key words: Reverse phase High Performance Liquid Chromatography, Labetalol Hydrochloride, Stability Indicating, Central Composite Design, Design of Experiments.

INTRODUCTION

The chemical name of Labetalol hydrochloride (Fig 1) is (RS)-2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl) amino] ethyl] benzamide monohydrochloride. Labetalol hydrochloride is a

combined alpha- and beta-adrenoceptor blocking agent for oral and intravenous use in the treatment of hypertension [1]. The impurities of Labetalol Hydrochloride include Labetalol Impurity-A, which is 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3phenylpropyl)amino]ethyl] benzoic acid (Fig 2), Labetalol Impurity-B, identified as methyl 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3phenylpropyl)amino]ethyl]benzoate (Fig 3) [2], Labetalol Impurity-C, recognized as 5-(N,N-dibenzylglycyl)-salicylamide (Fig 4), Labetalol Impurity-D, characterized as 5-[2-(3-cyclohexyl-1-methyl-propylamino)-1-hydroxyethyl]-2-hydroxybenzamide (Fig 5), and finally, 5-Hydroxy-2-Methyl Furaldehyde (Fig 6) [3]. The development of a stability-indicating RP-HPLC method involves the separation and quantification of Labetalol Hydrochloride from its degradation products under stressed conditions. By employing a suitable stationary phase, mobile phase composition, and detection wavelength, it is possible to achieve adequate resolution and sensitivity for the detection of impurities [4]. Validation of the RP-HPLC method is essential to ensure its reliability, accuracy, and reproducibility. Parameters such as specificity, linearity, precision, accuracy, robustness, and system suitability are evaluated during the validation process according to regulatory guidelines such as ICH (International Council for Harmonisation) [5] and USP (United States Pharmacopeia). Among the fractional factorial designs employed in the response surface model, the central composite design is most frequently utilized. A set of axial points referred to as star points is added to the center points in this design. To construct a second order (quadratic) model for the response variable without requiring the use of a full three-level factorial experiment, an experimental design known as a central composite design is helpful in response surface technique. [6]

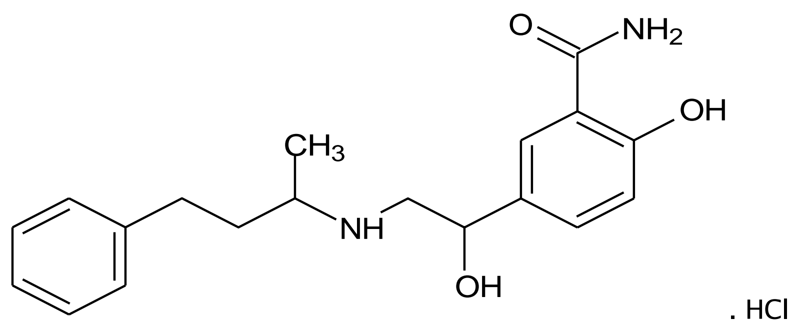

Figure 1: Structure of Labetalol hydrochloride

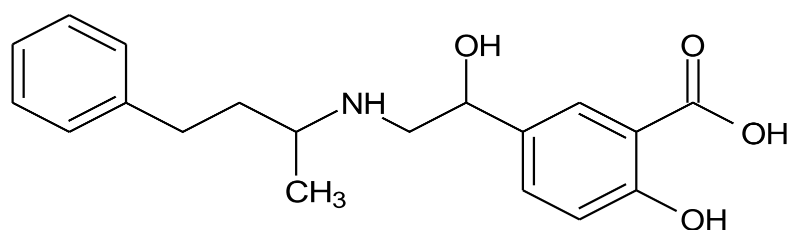

Figure 2: Structure of Labetalol Impurity- A

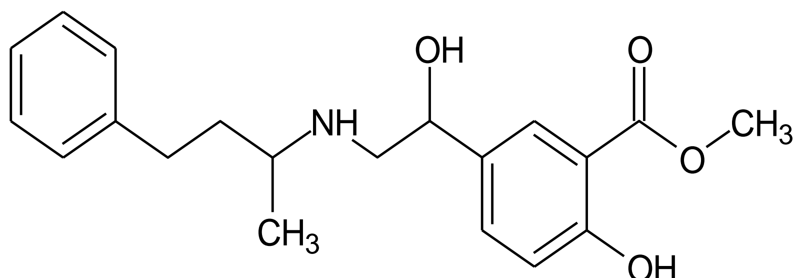

Figure 3: Structure of Labetalol Impurity- B

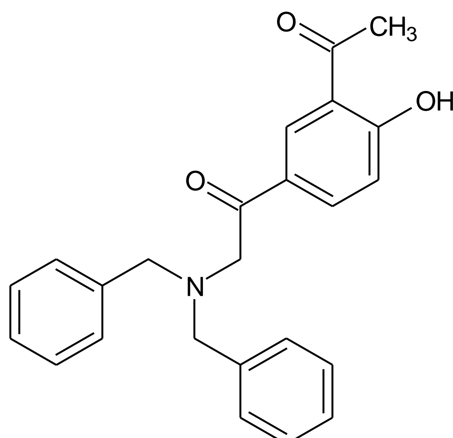

Figure 4: Structure of Labetalol Impurity- C

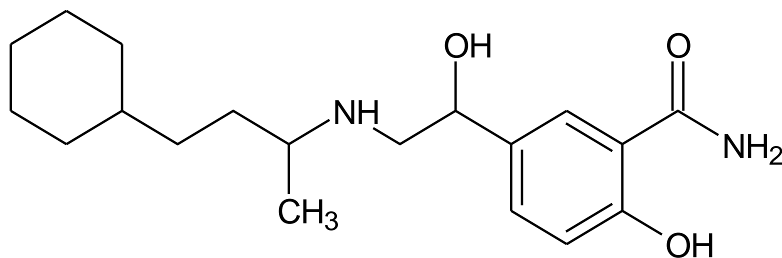

Figure 5: Structure of Labetalol Impurity- D

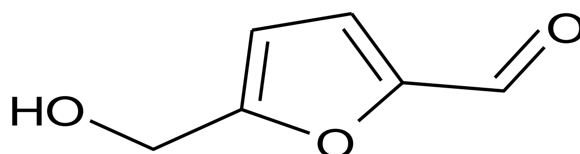

Figure 6: Structure of 5- Hydroxy-2-Methyl Furaldehyde

Literature review revealed method development and Validation of Labetalol Hydrochloride by RP-HPLC method (Simultaneous estimation) [7] , Stability indicating by UV method [8], LC-MS method by using Human plasma [9], Stability indicating by NP-HPLC method [10], Fluorimetry method [11], Spectrofluorimetry and Spectrophotometric method [12], Determination of Labetalol by Spectrophptometric method [13], Determination of Labetalol by UV method [14], Spectrophotometric method by using urine and blood samples [15], Spectrofluorimetry by using urine samples [16], Recaemate ratio by HPLC method [17], Determination Labetalol, sotalol, oxprenalol in combined form by UV method using human serum [18], TLC densitometric method [19], Electrochemical quantitative assessment of labetalol [20], Spectrofluorimetric method by using biological fluids [21] has been reported. From the literature there was no method reported for the stability indicating method development and validation of Labetalol Hydrochloride and its related

substances by RP-HPLC method using quality by design. So an attempt was made to develop, optimize and validation by using RP-HPLC (QbD).

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile (HPLC grade) , Sulfuric acid, 1- Pentane sulfonic acid sodium salt monohydrate and Sodium dihydrogen phosphate monohydrate (analytical grade) Merck brand were obtained from Caplin steriles Pvt. Ltd, Chennai. The Labetalol hydrochloride (API), Labetalol Impurities-A, B, C, D and 5-HMF and Milli-Q water was procured from Caplin steriles Pvt, Ltd, Chennai.

Instruments and Chromatographic conditions

Water's 2689 series with UV detector HPLC instrument, Analytical, precision balance (RADWAG), Vacuum oven (Thermo Lab) and Ultra sonicator (Labman) instruments were used. The High Performance Liquid Chromatography with UV detector (Water's 2689 series) was used to achieve the chromatographic separation. The mobile phase A consists of Phosphate buffer (pH 3.7) (100 %) and the mobile phase B consist of Acetonitrile (100 %). The mobile phase was filtered through 0.45 µm membrane filter and degassed. Cosmosil MS-II C18 (250 X 4.6 mm), 5 µm Part No. 38020-41 column was used as stationary phase at a flow of 0.8 ml\min. The UV detector wavelength was 230 nm.The total run time was 60 minutes.

Preparation of Buffer

About 38.571g of 1- Pentane Sulfonic acid Sodium salt monohydrate and 34.347 g of Sodium dihydrogen phosphate monohydrate were weighed and transferred into a 1000 ml water containing beaker, then it was dissolved well and pH was adjusted to 3.71 with 1N sulfuric acid solution. Then the solvent was through 0.45µ membrane filter.

Preparation of Mobile Phase

Mobile phase consist of phosphate buffer (pH 3.7) was used as an Mobile phase A and 100 % acetonitrile was used as Mobile phase B. Phosphate buffer pH (3.7) was used as a diluent and blank

Preparation of Labetalol Standard Stock Solution (100 ppm)

About 10.0 mg of Labetalol HCl standard was weighed and transferred into a 100 ml volumetric flask. Then, 60 ml of diluent was added, and the solution was sonicated until dissolved. Finally, the volume was made up to the desired level with diluent and mixed thoroughly.

Preparation of Labetalol Sensitivity Solution (0.25 ppm)

From 0.25 ml of Labetalol Standard Stock Solution, it was transferred into a 100ml volumetric flask, and 60 ml of diluent was added. The solution was sonicated until dissolved well. Finally, the volume was made up to the desired level with diluent and mixed thoroughly.

Preparation of Identification solution (Labetalol 500 ppm and 0.5 ppm of each impurities A, B, C, D)

About 10.0mg of Labetalol Hydrochloride standard were weighed and transferred into a 20ml volumetric flask and 10ml of diluent, sonicated and dissolve. 0.1ml of each Labetalol impurity A, B, C, D stock solution was added. Then made upto the volume with diluent and mixed well.

Preparation of Spiked Sodium chloride Sample solution (500 ppm Labetalol and 0.5 ppm of Impurity A, B, C, D spiked)

20 ml of sodium chloride sample solution into a GC vial was transferred, pipette 10ml solution and transferred into 20 ml volumetric flask. Then 1ml of each Labetalol Impurity A, B, C and D stock solution was added. Then made upto the volume with diluent and mixed well.

Method validation

Method validation parameters were assessed according to the USP, FDA guidelines

System suitability studies

The system suitability studies conceded as per USP guidelines. The parameters like capacity Factor, Tailing factor, asymmetry factor, and number of theoretical plates were calculated.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Specificity was checked by comparing the retention time of the analyte against blank, placebo & known impurities. A Standard solution of Labetalol HCl and Impurities A, B, C, and D at concentrations of 100 ppm for each solution were prepared. Additionally, a peak identification solution was prepared with Labetalol at 500 ppm and 1 ppm of each impurity. Furthermore, a 5-HMF solution was prepared at a concentration of 1125 ppm, and a sample solution containing 500 ppm of Sodium chloride was also prepared. Finally, 10 ml of the Sodium chloride sample solution (500 ppm) was mixed with 0.2 ml of each impurity (100 ppm) in a 20 ml volumetric flask, and the volume was adjusted with diluent. A chromatogram was then recorded after injecting a 20μl solution into an HPLC instrument.

Method Precision

Six homogeneous samples was prepared as per the test method procedure (100 % concentration) Method precision was checked by using this test solution. 10 ml of sodium chloride sample was transferred into six 20 ml volumetric flask. Then 1 ml of Impurity-A stock solution (10 ppm) and 0.8 ml of 5-HMF stock solution (225 ppm) was added and made upto the volume with diluent. 20 μl of each sample were injected into an HPLC system and the resulting chromatogram was recorded.

Linearity (Preparation of Calibration graph)

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The linearity range of analyte and known impurities for LOQ (25 %), 50 %, 80 %, 100 % , 150 %, 200 % and 300 % level of 7 different standard concentration was prepared. 5 ml of Labetalol HCl standard stock solution (100 ppm), was pipetted out and transferred into a 50 ml volumetric flask. The flask was then filled up to the mark with diluent and thoroughly mixed (10 ppm). The aliquots ranging from 0.5 ml, 1.0 ml, 1.6 ml, 2.0 ml, 3.0 ml, 4.0 ml, 3.0 ml of the 10 ppm solution, they were pipetted out separately and transferred into seven 20 ml volumetric flasks and made upto the volume with diluent. 1 ml of the 5-HMF impurity stock solution (225 ppm) was pipetted out and the final concentrations of Labetalol Impurity-A (0.000258, 0.000516, 0.000826, 0.001032, 0.001548, 0.002064, 0.00309 mg/ml) were placed into a 10 ml volumetric flask. After adding diluent to the flask to the mark, it was well mixed (22.5 ppm). The aliquots ranging from 0.4 ml, 0.5 ml, 0.8 ml, 0.2 ml, 0.4 ml, 0.8 ml, 0.8 ml of the 10 ppm solution, they were pipetted out separately and

transferred into seven 20 ml volumetric flasks. This resulted in final concentrations ranging from 0.000463, 0.005782, 0.009251, 0.011563, 0.023126, 0.046253, 0.092506 mg/ml, 20 µl of this solution was injected into an HPLC system and the resulting chromatograms were recorded.

LOD and LOQ

LOD is the minimum concentration of an analyte within a test sample that can be readily distinguished from zero. The lowest concentration of an analyte that can be found under test conditions with appropriate precision (repeatability) and accuracy is known as the limit of quantification (LOQ). In LOQ Solution, Separately 0.5ml of Labetalol HCl, Labetalol Impurity-A (10 ppm / 0.000258 mg/ml), and 0.4 ml of 5-HMF (22.5 ppm/ 0.000463 mg/ml) was transferred into 20 ml volumetric flask and made upto the volume with diluent. (Note: Labetalol HCl and Labetalol Imp-A were 25 % and 5-HMF were 4% of LOQ solutions). In LOD Solution, 3ml of LOQ solution was pipetted and transferred into 10 ml volumetric flask. Then it was made upto the volume with diluent. This solution was injected into an HPLC system at 20 µl, and the resulting chromatograms were recorded.

Accuracy

Accuracy refers to the degree of closeness of a measured value to a standard or true value. Accuracy was performed at three levels i.e., LOQ, 100 % and 200 %. Each level was prepared thrice and average recovery was calculated. In LOQ level, 10ml of sample was transferred in 20 ml volumetric flask, then 0.25 ml of Labetalol Impurity A (20 ppm) stock solution and 0.4 ml of 5-HMF (22.5 ppm) stock solution was added, finally made upto the volume with diluent and mixed well. In 100 % level, 10 ml of sample was transferred in 20 ml volumetric flask, then 1ml of Labetalol Impurity A (20 ppm) stock solution and 0.8ml of 5-HMF (225 ppm) stock solution was added, finally made upto the volume with diluent and mixed well. In 200% level, 10ml of sample was transferred in 20 ml volumetric flask, then 2ml of Labetalol Impurity A (20 ppm) stock solution and 1.6 ml of 5-HMF (225 ppm) stock solution was added, finally made upto the volume with diluent and mixed well. Finally, this solution was injected into an HPLC system at 20 µl, and the resulting chromatograms were recorded. (the same procedure was followed for each accuracy level).

Robustness

The Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. In this parameter should adjust column temperature at 40℃ (± 5) and pH at 3.7 (± 1). For 10ml of sample was transferred into 20ml volumetric flask, then added 2ml of Labetalol Imp-A (10 ppm) and 0.8 ml of 5-HMF (225 ppm) and made upto the volume with diluent. (Similarly for change the pH preparation). Finally, this solution was injected into an HPLC system at 20 µl, and the resulting chromatograms were recorded.

Ruggedness

In analytical chemistry, ruggedness refers to the robustness and reliability of an analytical method or instrument to produce consistent and accurate results despite variations in ensures the method’s ability to withstand everyday variations without compromising the quality of analysis. For Peak identification weighed 10.58 g of Labetalol Standard and transferred into a 20 ml volumetric flask, then add 0.2 ml of each impurity A, B, C, D stock solution (100 ppm) and made upto the volume with added diluent. Finally, this solution was injected into an HPLC system at 20 µl, and the resulting chromatograms were recorded.

Solution Stability

The main purpose of Solution Stability is identification of conditions necessary to form a stable solution study. In 10 ml of sample solution was transferred into 20 ml volumetric flask, then 1ml of Labetalol impurity- A (10 ppm) and 0.8 ml of 5-HMF (22.5 ppm) stock solution was added, finally, made upto the volume with diluent and mixed well. This solution was injected into an HPLC system at 20 μl, and the resulting chromatograms were recorded.

Forced Degradation

Forced degradation studies are carried out to establish degradation pathways of drug substances and drug products. They are processed by Acid, Base, Peroxide, Thermal and photolytic degradation process. In acid stress, 10 ml of sample was transferred into 20 ml volumetric flask and add 1ml of 5N HCl then made upto the volume with diluent and kept in a water bath for 4hrs at 80℃. In base stress, 10 ml of sample was transferred into 20 ml volumetric flask and add 1ml of 5N NaOH then made upto the volume with diluent and kept in a room temperature for 24 hrs.

In peroxide stress, 10 ml of sample was transferred into 20 ml volumetric flask and add 2 ml of 30 % H_2O_2 then made upto the volume with diluent and kept in a room temperature for 24 hr in dark area. In thermal stress, 40 ml of sample solution was transferred into a glass vial were exposed to 105℃ temperature for 24hrs and 48hrs in vacuum oven. In photolytic stress, Sample solution was exposed to 1.2 million Lux hour in normal light and 200 watt hour/sq meter in UV light.

RESULT AND DISCUSSION:

Chromatographic method optimization

The successful utilization of selectivity in chromatographic variables, including analyte separation, simultaneous optimization of retention time and tailing factor, response surface design using RRT chemometric technique, and Derringer's desirability function, was achieved. The central composite design could be used to enhance the quality of the separation and help build a better understanding of how various chromatographic parameters interact with one another. Through a central composite design experiment, the key chromatographic factors in this work were chosen and optimized. Risk assessment guided the selection and optimization of the factors. Creating a methodical procedure, obtaining data from many sources, utilizing tools and techniques to identify risks, recording those risks, and evaluating the process' efficacy are the processes in risk assessment. After developing a method, it is imperative to build a control plan. This means creating an analytical target profile to guide the strategy's development. Analytical processes, fitness for purpose, and risk management form the basis of the predetermined controls in the analytical control approach. All of these components work together to guarantee that the process runs smoothly and produces results that are in line with the specified analytical goal profile. According to the reference, the technique incorporates controls for measurement, replication, and sample preparation. [22]

A critical quality attribute (CQA) is any physical, chemical, biological, or microbiological property or characteristic that must fall within an appropriate range, limit, or distribution in order to ensure the desired level of product quality, according to the Quality by Design ICH Q8 (R2) guidance document. [23,24] In order to evaluate CQAs, it is important to first establish the necessary product attributes for that performance and take other information sources into consideration. This is done by creating a quality target product profile, or QTPP. [25,26] The variability of each identified CQA within the production process needs to be assessed in order to establish acceptance criteria and an

efficient control plan. This ties the problem to the characterization of the process even more. The objective of the CQA assessment is to identify the quality traits that require oversight.

The factors selected for optimization process were Acetonitrile concentration (A), phosphate buffer pH (B) and Flow rate (C). Acetonitrile concentration (45-55% v/v), buffer pH (3.5-3.9), and flow rate (0.6-1.0 ml/min) were the ranges of parameters used. The retention time of third eluted peak of Labetalol Hydrochloride (Std) (Rt_3), the Tailing factor of second peak of Labetalol Impurity-A (Tf_2) and Relative retention time of first peak of 5-Hydroxymethyl-2- furaldehyde were selected as responses (Table 1).

All experiments were conducted in randomized order to minimize the effects of uncontrolled variables that might introduce a bias on the measurements. Replicates (n = 6) of the central points were performed to estimate the experimental error. For an experimental design with the three factors, including linear, quadratic and cross terms, the model can be expressed as

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X2\ X_3 + \beta_{11} X_{12} + \beta_{22} X_{22} + \beta_{33} X_{32}$

Where β is the regression coefficient, Y is the response to be modeled, and X1, X2, and X3 stand for factors A, B, and C, respectively.

Table 1: Responses and Central Composite Arrangement

Std	Run	Space Type	Factor1 A: ACN %v/v	Factor 2 B: Phosphate buffer pH	Factor 3 C: Flow rate ml/min	Response 1 Retention time	Response 2 Tailing Factor	Response 3 RRT
17	2	Center	50	3.7	0.8	17.906	0.76	1.44
19	3	Center	50	3.7	0.8	17.906	0.76	1.44
20	4	Center	50	3.7	0.8	17.906	0.76	1.44
18	12	Center	50	3.7	0.8	17.906	0.76	1.44
16	13	Center	50	3.7	0.8	17.906	0.76	1.44
15	20	Center	50	3.7	0.8	17.906	0.76	1.44
14	1	Axial	50	3.7	1.13636	18.907	0.55	1.23
12	6	Axial	50	4.03636	0.8	19.234	0.83	1.67
11	8	Axial	50	3.36364	0.8	18.11	1.02	1.54
9	10	Axial	41.591	3.7	0.8	20.678	0.93	1.39
10	16	Axial	58.409	3.7	0.8	19.567	0.98	1.17
13	17	Axial	50	3.7	0.463641	15.678	1.11	1.34
4	5	Factorial	55	3.9	0.6	21.65	1.15	1.23
6	7	Factorial	55	3.5	1	18.5	1.09	1.53
3	9	Factorial	45	3.9	0.6	19.45	0.95	1.25
8	11	Factorial	55	3.9	1	17.53	0.63	1.45
2	14	Factorial	55	3.5	0.6	15.995	0.71	1.56
1	15	Factorial	45	3.5	0.6	16.761	0.83	1.26
7	18	Factorial	45	3.9	1	20.134	0.95	1.33
5	19	Factorial	45	3.5	1	21.145	1.15	1.47

ANOVA-derived statistical characteristics for the simplified models (Table 2). To create a straightforward and practical model, the unimportant terms ($p > 0.05$) were removed from the model using the backward elimination procedure. In statistical modeling, the adjusted R^2, which accounts for the number of regressor variables, is typically chosen because R^2 always drops when a regressor variable is removed from a regression model. The adjusted R^2 values demonstrated an

excellent fit between the experimental data and second order polynomial equations, falling well within the allowed bounds of $R^2 \geq 0.80$. A p value of less than 0.05 was found for each of the simplified models, indicating their significance. The ratio of signal (response) to noise (deviation) is measured by the appropriate precision value. It is preferred to have a ratio higher than 4. The model was important for the separation process since the ratio was found to be within the acceptable signal range of 4.8360 - 14.7245. The coefficient of variation (C.V) is a measure of reproducibility of the model and as a general rule a model can be considered reasonably reproducible if it is less than 10 %. [27,28]

Table 2 Reduced Response Surface Models and Statistical parameters obtained from ANOVA

Responses	Regression model	Adjusted R^2	Model p value	C.V (%)	Adequate Precision
Retention Time (RT)	$+17.9-0.4162A+0.6043B+0.6505C+0.3759AB-0.8354AC-1.29BC+0.8157A^2+0.3029B^2-0.1848C^2$	0.8863	<0.0001	2.88	14.7245
Tailing Factor (Tf_2)	$+0.7593-0.0158A-0.0307B-0.0558C+0.0075AB-0.0575AC-0.1525BC+0.0733A^2+0.0627B^2+0.0291C^2$	0.4145	<0.0001	5.27	5.6466
RRT	$+1.44+0.0066A-0.0250B+0.0216C-0.0325AB-0.0125AC+0.0150BC-0.0570A^2+0.0579B^2-0.0552C^2$	0.1768	<0.0001	8.30	4.8360

In order to gain a better understanding of the results, the predicted models were presented in the form of perturbation plots (Figure 7,9,11) and 3D response surface plots (figure 8,10,12). Variables giving quadratic and interaction terms with the largest absolute coefficients in the fitted models were chosen for the axes of the response surface plots. In table 2 the interaction with the largest absolute coefficients among the fitted model was AC (-0.8354) of retention time (Rt). The Negative interaction between A and C was statistically significant <0.0001 for Rt. With all factors maintained constant at the reference value, the perturbation plot offered shadow views of the response surface plots that demonstrated how the response varies as each factor moves away from the selected reference point. The response's sensitivity to a particular element was represented by the steepest slope or curve. Figure 7 indicates that the most significant factor influencing retention time (Rt_3) was acetonitrile concentration (factor A). Acetonitrile organic solvents having more polar nature it tends to decrease retention time for polar analyte (Labetalol HCl), because they have compete more effectively with the polar stationary phase for the analyte (Labetalol HCl). Flow rate (factor C) had effect on Retention time (Rt_3), then followed by the rest of the factor had significant effect on Buffer pH (factor B) on tailing factor Tf_2. Factor A and Factor B had little significant effect on relative retention time for RRT_1 . It was observed that labetalol has pKa value of 10.1. In spite of the fact that in the reversed-phase separations, pH of selected buffer should have the pH from the pKa values of the analyte [29], the selection of buffer with proper pH leads to ionization of analyte which consequences the sharp and symmetric peak shapes and reproducible retention times (RTs). Increasing the proportion of organic solvent in mobile phase generally reduces the retention time. Analyzing the optimization models perturbation and response plots showed that factors A and B significantly impacted the analyte ability to separate [30, 31]. In the current study, the global optimization of three replies and the selection of several optimal conditions for the formulation analysis were conducted using Derringer's desirability function. Peak height, elution time, and

resolution between peaks were determined as the optimization criteria. The geometric mean of each of the various desirability functions, whether weighted or not, is known as the Derringer's desirability function, or D. The following expression describes the desirability function of Derringer:

$$D = [d_1^{p2} \times d_2^{p2} \times d_3^{p2} \times \ldots \times d_n^{pn}]^{1/n}$$

where d1 is the unique desirability function of each response, n is the number of responses, and pi is the weight of the response. The values of the desirability function (D) range from 0 to 1. The range of weights is 0.1 to 10. The goal is given more weight when its weight exceeds 1, and less weight when its weight is less than 1. The standards for optimizing every single response (Table 3).

Table 3: Criteria for the optimization of the individual responses

Response	Lower limit	Higher limit	Criteria / Goal
Rt_3	15.678	21.65	Minimize
Tailing factor(Tf_2)	0.55	1.15	Minimize
RRT	1.17	1.67	Minimize

In criteria, the responses Retention time (Rt_3) for Labetalol Hydrochloride (Std) third peak was minimize in order to shorten the analysis time, Tailing factor (Tf_2) Labetalol Impurity-A second eluting peak was minimized and the Relative retention time (RRt_1) for 5-HMF first eluting peak was minimized. Following the conditions and restrictions above, the optimization procedure was carried out. The 3D surface obtained for the Derringer's desirability function was presented in figure 13.

It could be concluded that there was a set of coordinates producing high desirability value (D = 0.707) were Mobile phase-A Acetonitrile concentration (55%),Mobile phase-B Phosphate buffer (pH 3.8) flow rate 1.0 ml/min and wavelength 230 nm. The optimized assay conditions were Mobile phase-A Acetonitrile concentration (55 %),Mobile phase-B Phosphate buffer (pH 3.8) flow rate 1.0 ml/min The predicted response values corresponding to the later value of D were Rt_3=17.866 min, Tf_2 =0.638 and RRT_1 =1.343 min. Within a 4.5 % variation, the observed variations between the experimental and anticipated responses were found to be in good agreement. The percentage of prediction error was calculated by using the following equation (Table 4). The optimzed chromatograms for standard and sample were shown in figure 14,15.

Average error = Experimental- predicted/ predicted X 100

Table 4: Comparing the predicted and experimental values of various functions under ideal circumstances

Optimum conditions	ACN Comp (% v/v)	Phosphate Buffer pH	Flow rate (ml/min)	Retention time (Rt_3)	Tailing factor (Tf_2)	RRT_1
Predictive	55	3.8	1.0	17.866	0.63	1.343
Experimental	55	3.8	1.0	17.801	0.612	1.381
Average error				3.6	4.07	2.8
Desirability value (D) =0.707						

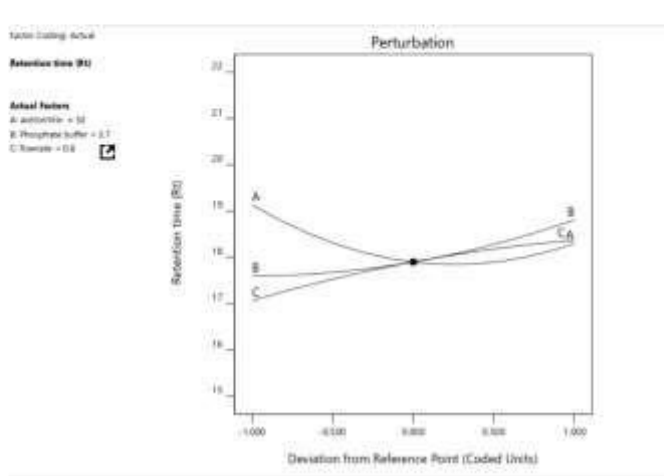

Figure 7: Perturbation Plot for RT

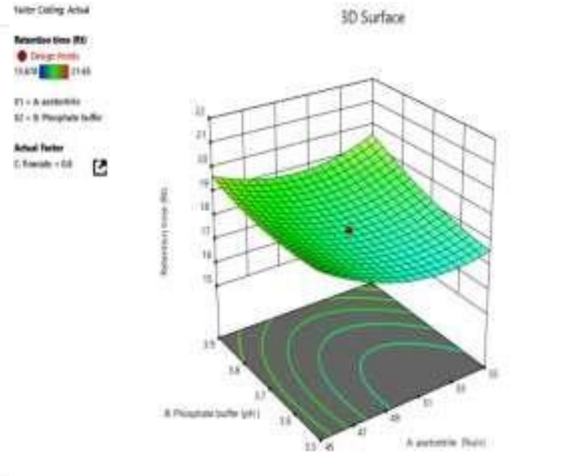

Figure 8: 3D Surface for RT

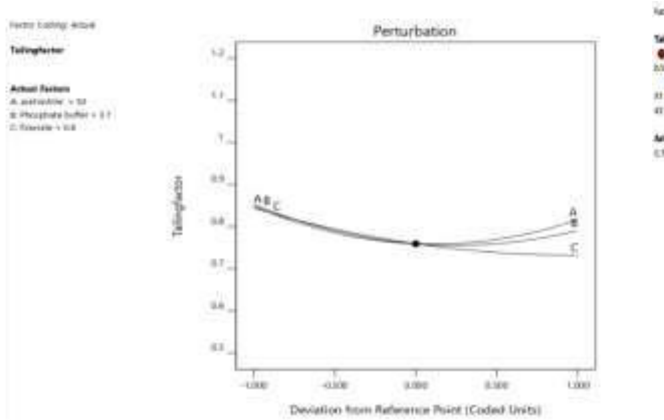

Figure 9: Perturbation Plot for Tailing factor

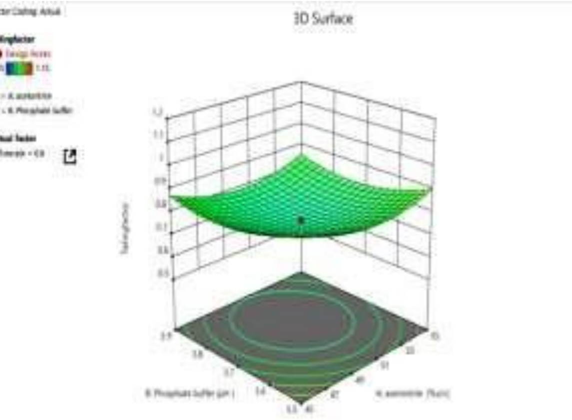

Figure 10: 3D Surface for Tailing factor

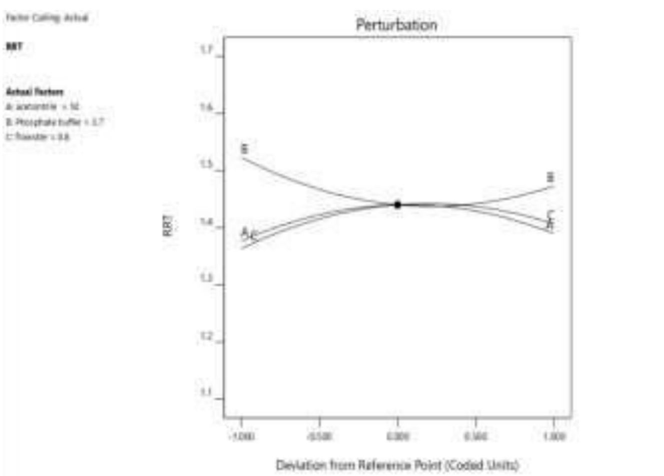

Figure 11: Perturbation Plot for RRT

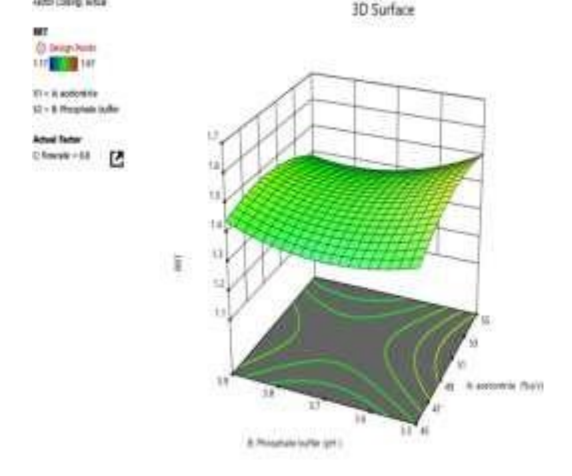

Figure 12: 3D Surface for RRT

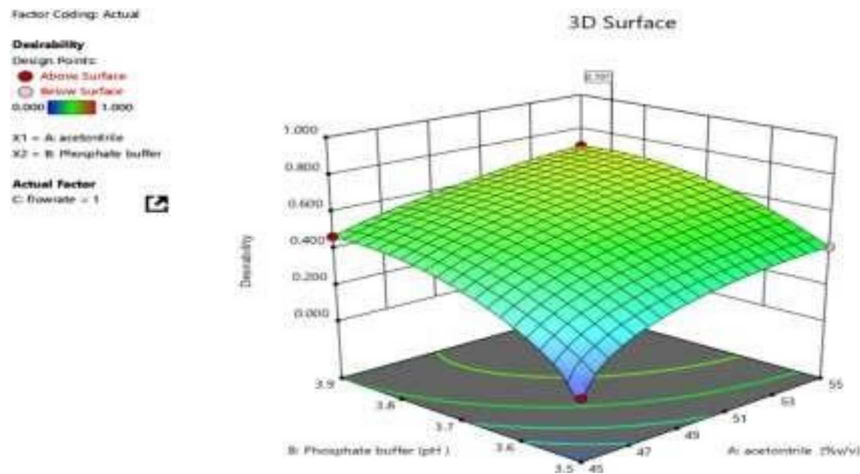

Figure 13: 3D plots for Derringer's Desirability function

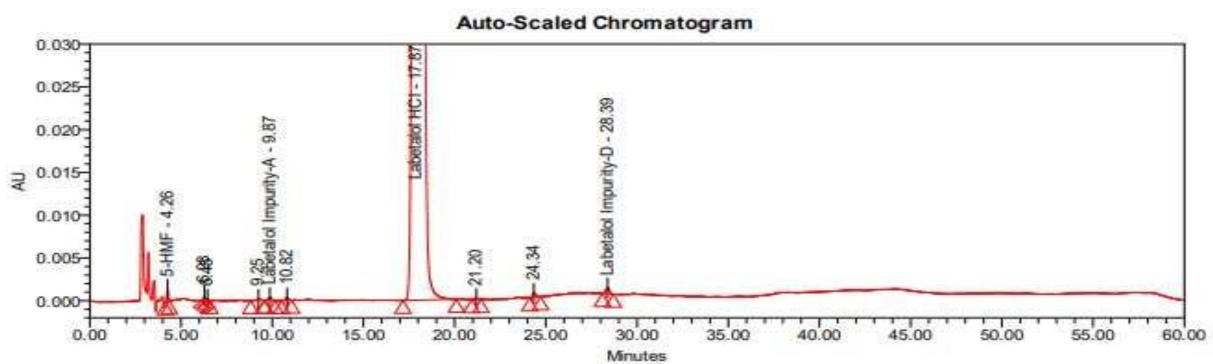

Figure 14: Optimized Chromatogram for Sample

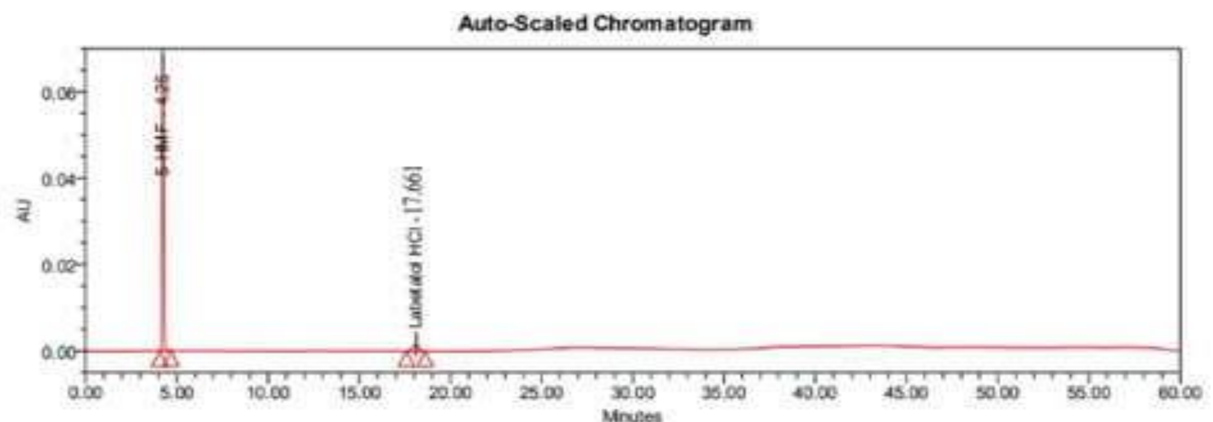

Figure 15: Optimized Chromatogram for Standard

Method Validation [32]

System Suitability

The System suitability test offers additional reassurance that the method is yielding precise and accurate outcomes on a particular instance. Each test results are compared to predefined acceptance criteria; if they satisfy these requirements, the procedure is deemed adequate for that particular instance. The Tailing Factor for Standard and 5-HMF should not exceed 0.8 to 1.8, and the Acceptance Criteria for System Suitability for % RSD should not exceed 5.0.The reports were Shown in table:5

Specificity

The Peak purity angle value for Impurity A, B, C, D, 5-HMF and Labetalol HCl Std were below their respective impurity threshold values. Consequently, no interference was detected at the retention time of these impurities from the blank and placebo. Therefore, the method demonstrated specificity and for quantifying the related substance of Labetalol HCl. The reports were shown in the table: 5

Method Precision

Method precision was done for the six replicate analysis of Labetalol IMP-A and 5-HMF Solution. The %RSD of Labetalol IMP- A was found to be 0.7 and %RSD of 5-HMF was found to be 0.9. Therefore, the results showed %RSD value was found to be within the limit of the acceptance criteria should Less Than 2.0. Hence, it concluded and the method was précised for the determination of Labetalol HCl. The Statistical data reports were shown table: 5

Linearity

The linearity range of 25 % to 300 % for Labetalol IMP-A and Labetalol HCl Std and 4 % to 800 % for 5-HMF. The correlation Coefficient value for Labetalol IMP-A and 5-HMF Std was found to be within the limit of not less than 0.990. So the method was linear from the above said range. The results were shown in table: 5

LOD and LOQ

For LOQ of Labetalol Hydrochloride, Labetalol Impurity- A and 5- Hydroxy-2- Methyl Furaldehyde % RSD was calculated and it should be within the limit. The report indicates the Labetalol HCl and Labetalol IMP-A and 5-HMF peaks were visible at the LOD concentration. So it should be the method were sensitive for the determination of related substances of Labetalol HCl, Statistical data results were shown in table: 5

Accuracy

The accuracy assessment involved testing the concentration range from the limit of quantification (LOQ) up to 200 % for Labetalol IMP-A and 5-HMF. The mean percentage recovery was determined to be 99.6 % for Labetalol IMP-A and 102.0 % for 5-HMF. The percentage relative standard deviation (% RSD) was calculated to be 1.7 for both Labetalol IMP-A and 5-HMF. Consequently, all results fell within the acceptable limits. Statistical data reports were Shown in table: 6

Robustness

The robustness study indicated that the factors selected remaining unaffected by small variation of Column temperature (± 5℃) and pH (± 0.10). The system suitability was should be within the limit, Hence the method was robust for the quantification of Related substances in Labetalol HCl. The reports of robustness were shown in table 6

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and analyst from analyst. The analyst-I was observed poor resolution between impurity A and unknown impurity. Therefore, the Analyst-II was developed and validated completely, From the below data, it clearly shows that the both analyst results were precise. Hence the method was precise. Statistical data was Shown in below table: 6

Solution Stability

For 80 Hours Standard at 25°C observed stable, the % RSD of Labetalol HCl was found to be 3.1 and RSD of 5-HMF was found to be 1.3. For sample at room tempertaure 60 hrs, the % difference of Labetalol impurity-A was 5.22% and 5-HMF was-0.41%. The Acceptance criteria of % RSD in area response obtained from standard solution between initial and different time intervals should be NMT 5.0. The % Difference in area response of impurities (More than LOQ level) between initial and different time intervals should be ±10.0 % , Statistical data was Shown in the table: 6

Forced Degradation

In the forced degradation study, it was imperative to maintain a mass balance within the range of 95 % to 105 %. The methods employed for all stressed conditions met this criterion, ensuring the reliability of the results. Upon comparison of the percentage degradation obtained from stressed samples under various conditions using both assay and related substances procedures, it was noted that degradation was approximately 9 % under acid stress conditions. In contrast, minimal degradation was observed under base and thermal stress conditions. This indicates a clear and organized presentation of results. Statistical data for degradation study was shown in table: 6

Table 5: Method Validation Data

Test	Details	Acceptance Criteria	Results					
			Labetalol Std	Imp-A	Imp-B	Imp-C	Imp-D	5-HMF
System Suitability	Tailing Factor	For Std, Tailing factor NMT 0.8 to 1.8 and % RSD NMT 5 %	0.94	1.0	0.99	0.95	1.0	1.14
	% RSD		1.7	1.9	1.6	1.5	1.9	0.2
Specificity	RT	Purity angle value of main peak & known impurities observed less than Purity Threshold value	21.406	11.931	29.98	51.713	30.723	4.205
	Purity Angle		2.435	3.154	2.25	4.773	4.264	0.041
	Purity Threshold		3.424	4.129	3.116	5.676	5.468	0.275
Method Precision	% RSD	The % RSD of impurities values obtained from Six samples preparation should NMT 5.0 %	NA	0.7	NA			0.9
Linearity (LOQ to 300 % level)	Correlation Co-efficient	The correlation coefficient should NLT 0.990	0.999	0.999	NA			0.999
	Slope		33661998.7	31930158.5				32920681.08
	Intercept		171.8	-46.848				10103
	Regression Equation (Y= mx+c)		y= 3661998.7x + 171.8	y = 31930158.5x - 46.848				y = 32920681.8x + 10103
Limit of Quantification (LOQ)	% RSD	The % RSD is NMT 10.0 and S/N ratio should NLT 10.0	0.6	0.8	NA			0.4
	S/N ratio		121	130				580
Limit of Detection (LOD)	% RSD	The % RSD is NMT 10.0 and S/N ratio should NLT 3.0	2.0	1.0	NA			0.1
	S/N ratio		42	48				205

NLT- Not Less Than, NMT-Not More Than, NA- Not Applicable, RSD- Relative Standard Deviation, Rt-Retention Time, S/N - Signal/Noise

Table 6: Method Validation data

Accuracy (Recovery)	LOQ	The % recovery of individual preparation for LOQ level should b/w 70 % to 130 % and other level should b/w 80% to 120 %	NA	98.9	NA			103.9
	100 %			99.1				100.3
	200 %			100.7				103.1
	Overall mean			99.6				102.5
	% RSD			1.7				1.7
Robustness (for CT and pH)	RT (High)	No interference should be observed at RT of main peak & impurity Peak	19.745	10.829	NA			4.325
	RT (Low)		20.892	11.685				4.380
	Test Condition		20.249	11.179				4.340
Ruggedness (Total Impurities)	Analyst-1	There should not be any impact on previous analyst results	NA	0.061	NA		0.07	NA
	Analyst-2			0.056			0.071	
Solution Stability	% RSD	The % RSD NMT 5.0 % and % Difference in area of impurity should be ±10.0 %	3.1	NA	NA			1.3
	% Difference		NA	5.22				-0.41
Forced Degradation (mass balance %)	Control sample	Mass Balance ratio should be in the range of 95% to 105 % in all stressed condition	100.0	100.0	100.0	100.0	100.0	100.0
	Acid		98.2	98.5	98.7	98.4	98.1	98.6
	Base		101.4	101.8	101.2	101.7	101.0	101.4
	Peroxide		98.9	98.3	98.9	98.2	98.4	98.7
	Thermal		98.1	97.3	98.6	97.7	98.1	98.0
	Photolytic		98.0	97.98	98.9	98.2	98.5	98.1

NA-Not Applicable

Conclusion

The current research aimed to develop and validate a robust reverse-phase high-performance liquid chromatography (RP-HPLC) method using Quality by Design (QbD) principles. This method was designed to meet the stringent requirements outlined by the US Pharmacopeia (USP).The developed RP-HPLC method was validated and found to comply with USP guidelines, demonstrating simplicity, precision, cost-effectiveness, and accuracy based on the HPLC reports. The study employed a systematic approach by utilizing Design of Experiments (DoE), specifically the Central Composite Design (CCD) technique, to identify significant factors influencing the chromatographic performance. Furthermore, Derringer's desirability function was employed to simultaneously optimize these factors, reducing the overall assay development time. This methodology allowed for the exploration of interaction effects among chromatographic factors on key separation attributes, enhancing method efficiency and effectiveness. In conclusion, the developed RP-HPLC method can be successfully employed for the routine analysis of Labetalol Hydrochloride in its injection form. The systematic application of QbD principles, along with advanced statistical techniques like CCD and desirability function, ensured a robust and reliable analytical method suitable for pharmaceutical quality control laboratories.

Conflict of interest

The authors declare no Conflict of interest.

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