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Development and Validation of RP-HPLC Method for Simultaneous Quantification of Tacrolimus (TAC) and Thymoquinone (THQ)

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Abstract

The development and validation of analytical methods are essential for ensuring the quality of pharmaceutical formulations. This study focuses on the development of a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous quantification of tacrolimus (TAC) and thymoquinone (THQ) in combined formulations. A robust method was optimized using a C18 column with a mobile phase enabling efficient separation and quantification. The method was validated following ICH guidelines, assessing parameters such as linearity, accuracy, precision, robustness, and specificity. The results demonstrated excellent linearity for both TAC and THQ over a wide concentration range, with correlation coefficients exceeding 0.999. Recovery studies confirmed the method's accuracy, showing a recovery rate within 98–102%. Precision studies indicated low intra- and inter-day variability, while robustness tests highlighted the method's reliability under minor experimental variations.

The validated RP-HPLC method was successfully applied to quantify TAC and THQ in pharmaceutical formulations, offering a simple, rapid, and reliable tool for quality control and routine analysis. This work provides a significant contribution to pharmaceutical research, facilitating the development of combination therapies involving TAC and THQ for inflammatory and autoimmune disorders.

Keywords: RP-HPLC, tacrolimus, thymoquinone, simultaneous quantification, method validation, pharmaceutical analysis, ICH guidelines, combination therapy, quality control, C18 column.

Introduction

The simultaneous quantification of multiple drugs in combination therapies is critical for pharmaceutical development, ensuring efficacy, stability, and safety. Tacrolimus (TAC), a macrolide immunosuppressant, and thymoquinone (THQ), a natural compound with anti-inflammatory and antioxidant properties, have shown significant therapeutic potential in inflammatory conditions such as psoriasis [1,2]. Developing a robust analytical method to simultaneously estimate these compounds is crucial for optimizing their formulation and therapeutic monitoring [3].

High-performance liquid chromatography (HPLC) is widely used due to its precision, sensitivity, and ability to analyze complex matrices [4]. Reverse-phase HPLC (RP-HPLC) has gained popularity in pharmaceutical analysis for its ability to separate hydrophobic compounds effectively [5]. Method validation in compliance with International Council for Harmonisation (ICH) guidelines ensures the reliability of analytical results by addressing specificity, linearity, accuracy, precision, and robustness [6].

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Although several HPLC methods exist for individual drugs, the simultaneous quantification of TAC and THQ in combined formulations remains underexplored [7]. This study aims to develop and validate a novel RP-HPLC method tailored to their combined estimation, emphasizing pharmaceutical quality control and therapeutic applications [8-10].

The current research provides a framework for accurate and reproducible quantification, contributing to the development of TAC and THQ-based therapies [11-15].

Material and Method

UV Spectrophotometric methods

Preparation of calibration curve of THQ in different solvents

Calibration curve of tacrolimus (TAC) and thymoquinone (THQ) was prepared in different solvents like methanol, octanol, water and phosphate buffer saline pH 7.4, which was used for determining drug con- centration in various samples. Stock solution of TAC was prepared by separately dis- solving 5 mg of drug in 50 mL of solvent (methanol, octanol, water and phosphate buffer saline pH 7.4) in a volumetric flask to obtain a concentration of 100 µg/mL. From this primary stock solution (100 µg/mL), aliquots were taken and suitably diluted with the same solvent to obtain a concentration ranging from 0.5-10 µg/mL. The absorption of these dilutions was taken at drug absorption maxima using a UV-visible spectrophotometer (Shimadzu Corp, Kyoto, Japan). Finally, a calibration curve was plot- ted between concentration (x-axis) and absorbance (y-axis).

RP-HPLC method development

The RP-HPLC method was developed and validated for simultaneous estimation of tacrolimus (TAC) and thymoquinone (THQ) during in-vitro and ex-vivo analysis.

Instrumentation and chromatographic conditions

A RP-HPLC (Shimadzu, SPD-M20A, Tokyo, Japan) system, equipped with a photo- diode array (PDA) detector was employed for analysis. For chromatographic separation of drugs, RP-HPLC apparatus with a C-18 column (Shimadzu, 250 x 4.6 mm dimension, 5µm) was used. The whole system was kept at ambient conditions. Chromatographic conditions was optimized by exploring various combinations of methanol: water and ACN : water (10:90 to 90:10) as mobile phases, along with adjustments in flow rates ranging from 0.4 to 0.8 mL/min to assess their impact on retention time and peak shapes. The sample volume was kept at 20 µL with a total run time fixed for this elution process at 20 min. Prior to analysis; the mobile phase and samples were degassed and passed through a (0.2 µ) nylon filter. The detection wavelength was set at 254 nm for THQ respectively.

Preparation of standard stock and working solution

The standard stock solution of THQ (500 µg/mL for each) was prepared by dissolving accurately weighed 5 mg of drug in 10 ml of methanol in a volumetric flask. The standard stock solution (500 µg/mL) of both drugs was mixed in equal ratios (1:1) to get working standard solution (250 µg/mL). From this working standard solution (250 µg/mL), different dilutions were prepared for the validation of the RP- HPLC method for the analysis of samples.

Preparation of calibration standard

The standard calibration plot of the samples was prepared in the concentration range of1-80 µg/mL. The different concentration used for plotting calibration standards were 1, 5, 10, 20, 40, 80 µg/mL. These different concentrations were prepared by suitably diluting working standard solution (250µg/mL) with methanol. Finally, the calibrationcurve of the sample was prepared in the concentration range of 1-80 µg/mL, with concentration on x-axis and peak area on y-axis. The samples were filtered through a 0.22 µm nylon filter before analysis.

Method validation

The developed RP-HPLC method was validated in accordance with the ICH Q2 (R1) guidelines. The various parameters used for validating developed HPLC technique arelinearity, accuracy, precision, the limit of detection (LOD), the limit of quantification (LOQ), and robustness (Mangla et al., 2020, Ahmad et al., 2013).

Linearity and range

The linearity of the drug was determined by preparing a serial dilution of the working standard solution prepared in methanol (250 µg/mL) over the concentration range of 1- 80 µg/mL and

plotting the graph between the concentration of drug on x-axis and area of peak on y-axis. The sample was injected in triplicate under the respective chromatographic condition.

Accuracy as recovery

The accuracy of the developed HPLC method was validated by the addition of drugs solution at three different levels (50%, 100%, and 150%) to the previously analyzed samples. Accuracy was determined by amount of drug recovered as compared to the amount added and is expressed in terms of % recovery. The samples was analysed in triplicate.

Precision

The precision of the developed RP-HPLC method was carried out to determine the intra-day (repeatability) and inter-day (over three consecutive days) variation at three concentration levels (10, 40 and 80 µg/mL). The analysis of samples was carried out intriplicate.

Robustness

The robustness of the developed RP-HPLC method was validated by making slight variations in chromatographic conditions at three different levels at a fixed drug con- centration (40 µg/mL). The robustness of the samples was analyzed by changing flow rate and ratio of mobile phase. The % RSD of the samples was calculated to assess the robustness of the method. The samples were analyzed in triplicate under the respective chromatographic condition.

Limit of detection (LOD) and Limit of quantification (LOQ)

The LOD and LOQ are the minimum quantity of drug that can be detected and quantified respectively using the developed analytical method. The LOD and LOQ of the developed RP-HPLC method was calculated from the slope (S) of the calibration plot and standard deviation (σ) of the response.

Preparation of calibration curve in phosphate buffer saline (PBS) pH 7.4

The standard stock solution of THQ (500 µg/mL for each) was prepared by dissolving accurately weighed 5 mg of drug in 10 mL of PBS pH 7.4 in a 10 mL volumetric flask. The standard stock solution (500 µg/mL) of both drugs was mixed in equal ratios to get working standard solution of 250 µg/mL for each drug. From this working standard solution (250 µg/mL), different dilutions of 1, 5, 10, 20, 40, 80 µg/mL were prepared by suitably diluting working standard solution with PBS pH 7.4. Finally, the calibration curve of the samples was prepared in the concentration range of 1-80 µg/mL, with concentration on x-axis and peak area on y-axis. The samples were filtered through a 0.22 µm nylon filter before analysis by developed RP- HPLC method.

Results and Discussion

UV Spectrophotometric methods

Calibration curve of TAC and THQ was plotted in different solvents like methanol, octanol, water and phosphate buffer saline pH 7.4, which were used for determining drug concentration in various samples. The calibration curve was found to be linear over the concentration range of 5- 50 µg/mL for TAC and 0.5- 10 µg/mL for THQ in all solvents. The calibration plots of TAC and THQ in all solvents are shown in (**Table1- 4)** and (**Figure 1-4).**

Table 1: Calibration plot of THQ in methanol (n=3) at λmax 253

Concentration (µg/mL)	Mean Absorbance ± SD
0.5	0.081 ± 0.0169
1	0.152 ± 0.0158
2	0.279 ± 0.0082
4	0.544 ± 0.0156

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6	0.829 ± 0.0270
8	1.137 ± 0.0458
10	1.405 ± 0.0600

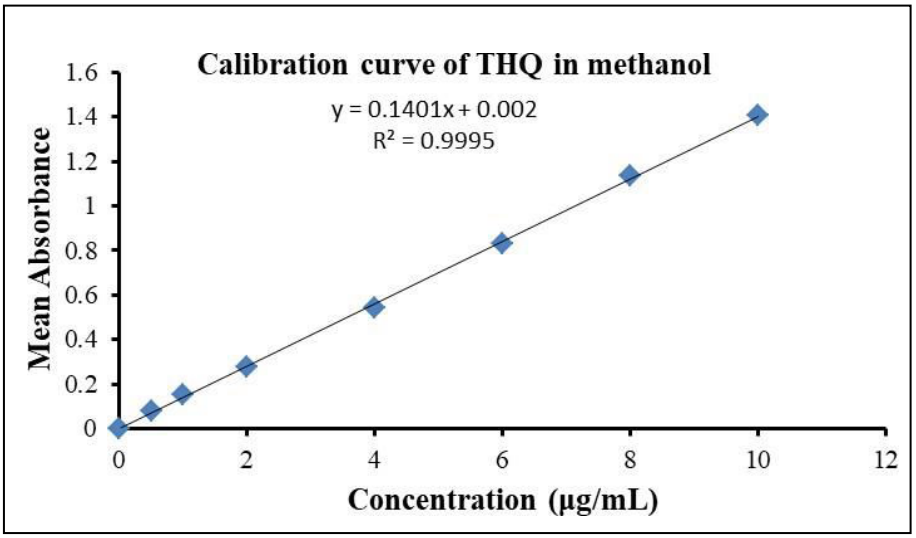

Figure 1: Calibration curve of THQ in methanol at λmax 253 Table 2: Calibration plot of THQ in octanol (n=3) at λmax 253.5

Concentration (µg/mL)	Mean Absorbance ± SD
0.5	0.063 ± 0.0027
1	0.125 ± 0.0125
2	0.253 ± 0.0018
4	0.498 ± 0.0046
6	0.733 ± 0.0024
8	0.954 ± 0.0029
10	1.179 ± 0.0072

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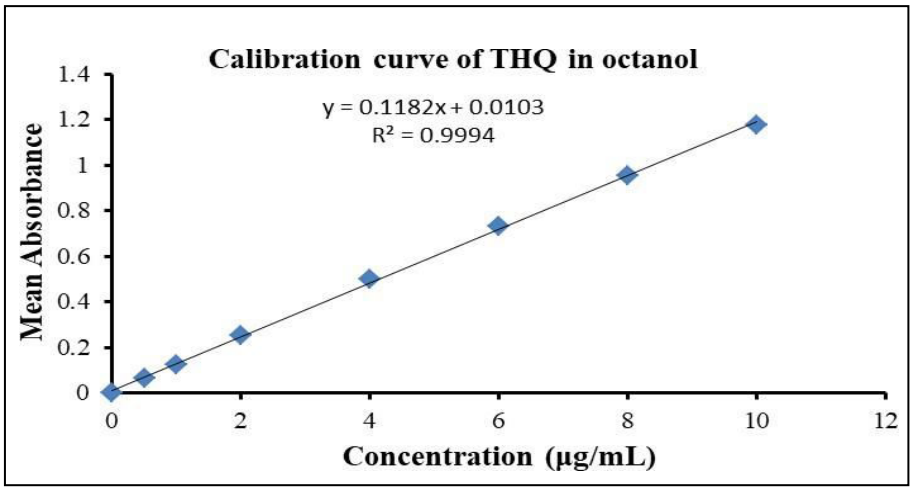

Figure 2: Calibration curve of THQ in octanol at λmax 253.5

Table 4: Calibration plot of THQ in PBS (n=3) at λmax 254

S.no	Concentration (µg/mL)	Mean Absorbance ± SD
1	0.5	0.046 ± 0.0009
2	1	0.118 ± 0.0039
3	2	0.237 ± 0.0033
4	4	0.458 ± 0.0126
5	6	0.685 ± 0.0049
6	8	0.887 ± 0.0046
7	10	1.084 ± 0.0030

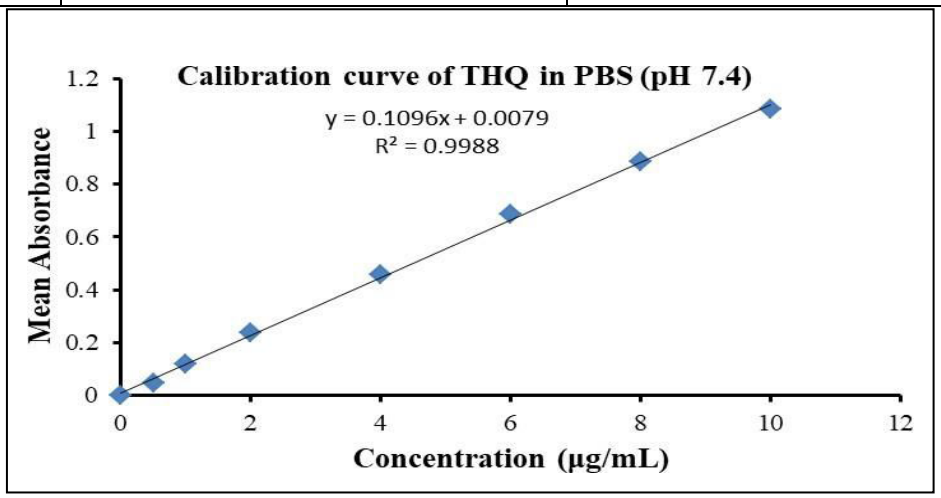

Figure 4 : Calibration curve of THQ in PBS (pH 7.4) at λmax 254
RP-HPLC method development for estimation of TAC and THQ

The RP-HPLC method was developed and validated according to ICH guidelines for simultaneous estimation of TAC and THQ during in-vitro and ex-vivo analysis.

Chromatographic conditions

The optimization of a chromatographic method involves modifying various parameters of the mobile phase to achieve the best separation and resolution of drugs. The choice of the mobile phase was established by conducting an initial experiment with varying ratios of mobile phase (methanol: water and ACN: water). The most effective combination was determined to be a 90:10 v/v ratio of ACN: water, providing appropriate retention and resolution of THQ. Further, a flow rate of 0.6 mL/min gave better resolution and symmetrical peaks for both drugs (THQ). In optimized chromatographic condition, as described in **Table 5**, both drugs showed well defined peaks. The representative HPLC chromatogram of THQ and a mixture of THQ are shown in **Figure 5.**

Table 5: Optimized chromatographic condition of the developed RP-HPLCmethod for the simultaneous estimation of THQ.

S. No	Process parameter	Optimized value
1	Mobile phase	Acetonitrile and Water
2	Ratio (ACN: water)	90:10 (v/v)
3	Flow rate	0.6 mL/min
4	Run time	20 min
5	Retention time (minutes)	8.0 (THQ)
6	Column	C-18 (Shimadzu, 250 x 4.6 mm, 5µm)
7	Detector	PDA
8	Detection wavelength (nm)	254 (THQ)
9	Temperature	25 ± 0.5 °C
10	Injection volume	20 µL

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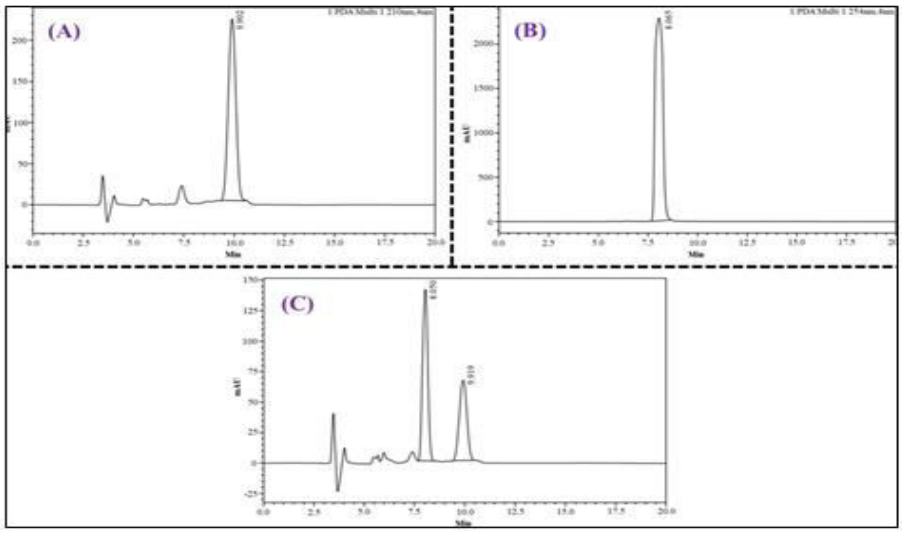

Figure 5: Representative RP-HPLC chromatogram of (A)TAC (B) THQ (C) THQ for simultaneous estimation.

Method validation

The developed HPLC method for simultaneous estimation of THQ was validated for various parameters like linearity, precision, accuracy, robustness, LOD and LOQ.

Linearity and range

The peak area vs. drug concentration calibration curve of THQ over the concentration range of 1-80 µg/mL was observed to be linear. The regression equation and coefficientof regression of calibration plot for THQ the regression equation and coefficient of regression was found to be y = 376604x + 412563 and 0.9998, respectively as shown in **Figure 6**.

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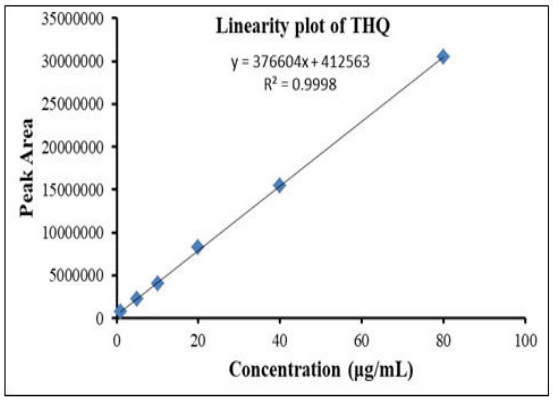

Figure 6: Linearity plot of THQ in methanol by RP-HPLC method

Accuracy as recovery

The developed HPLC method was validated for accuracy in terms of % recovery for THQ, employing the method outlined in the experimental section. The recovery process involved spiking with an extra addition of standard drug solution at three concentration levels: 50%, 100%, and 150% to the pre-analyzed samples. The calculated recovery, based on this approach, ranged from (THQ 98.87 - 100.27 %). Further in each caseall the value of % RSD was found to be less than 2 % which indicates the accuracy of the proposed method. The experimental data, including % recovery, % RSD are detailedin **Table 6.**

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Table 6: Recovery data for the validation of HPLC method

Drug	Excess drug added to analyte (%)	Theoreticalcontent (µg/mL)	Observed Conc. (µg/mL) ± SD	covery(%)	RSD (%)
	50	15	14.77 ± 0.116	98.44	0.78
THQ	100	20	19.95 ± 0.202	99.73	1.01
	150	25	25.04 ± 0.226	100.16	0.90

Precision

The study assessed the precision of a developed HPLC method for quantifying THQ through inter-day and intra-day variations, with results expressed as %RSD. The precision analysis was performed at concentrations of 10, 40, and 80 µg/mL for both THQ, and the outcomes are detailed in **Table 7.** Lower % RSD values indicate the precision and accuracy of the developed method. The data affirm the system's good repeatability, affirming its suitability for THQ quantification in samples.

Table 7: Intra-day and inter-day precision of the method (THQ)

Conc. (µg/mL)	Intra-day precision		Inter-day precision	
	Mean area ± SD	RSD (%)	Mean area ± SD	RSD (%)
10	4058641 ± 39543	0.97	4056282 ± 39996	0.99
40	15447034 ± 135450	0.88	15439934 ± 136493	0.88
80	30503368 ± 289386	0.94	30491180 ± 289886	0.95

Robustness

The validation of the HPLC method included an assessment of its robustness by making slight adjustments to system parameters, such as the mobile phase ratio (88:12 and 92:08) and flow rate (0.4 and 0.8 mL/min). The resulting data, detailed in **Table 8,** involved noting the (%RSD)

Table 8: Robustness of the method (THQ)

Parameters	Study conditions		Mean area ± SD	RSD	RT ± SD	RSD
	Original	Used	(%)		(%)	
		88:12	15421382 ± 141913	0.92	8.21 ± 0.017	0.21
Mobile phase	90:10	90:10	15447034 ± 135450	0.88	8.06 ± 0.010	0.10

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		92:08	15466795 ± 139455	0.90	7.92 ± 0.032	0.41
		0.4	15460058 ± 141425	0.91	8.19 ± 0.021	0.25
Flow rate	0.6	0.6	15447034 ± 135450	0.88	8.06 ± 0.010	0.10
		0.8	15437811 ± 136239	0.88	7.87 ± 0.018	0.23

of both peak area and retention time of THQ. The observed low %RSD values signify that the method remains robust, producing nearly ideal outcomes despite variations in analytical conditions. This suggests a consistent and reliable performance of the HPLC method, even when subjected to minor alterations in system parameters.

Limit of detection (LOD) and limit of quantification (LOQ)

In an analytical process, it's important to establish the LOD and LOQ to figure out the smallest detectable and quantifiable amount of a drug. By following the ICH guidelines and utilizing the standard deviation and slope of the plot, the calculated while for THQ,the LOD and LOQ were observed to be 0.021 µg/mL and 0.062 µg/mL, respectively. These figures indicate that the method is adept at identifying and measuring the THQ even at low concentrations in samples.

Calibration curve of THQ in PBS pH 7.4 using HPLC

Calibration curve of THQ was plotted on phosphate buffer saline (PBS) pH 7.4 which was used for determining drug concentration in various samples during in- vitro and ex- vivo studies. The peak area on x-axis vs. drug concentration on y-axis, calibration plot was found to be linear over the concentration range of 1-80 µg/mL for TAC and THQ. The regression equation and coefficient of regression of calibration plot for TAC was found to be y = 333990x + 5458 and 0.9984, respectively as shown in **Figure 7**, and for THQ the regression equation and coefficient of regression was found to be y = 369782x + 341099 and 0.9996, respectively as shown in **Figure 7**. The optimal outcome in a linear regression suggests that the variables, concentration and peak exhibit a perfect linear relationship.

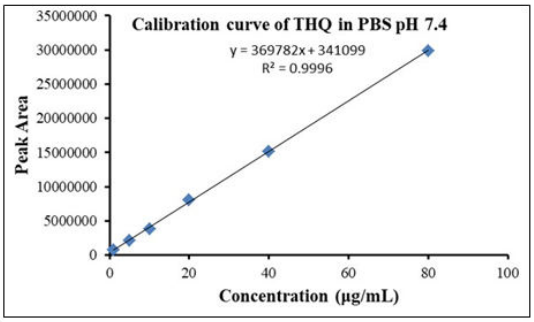

Figure 7: Calibration curve of THQ in PBS pH 7.4 by RP-HPLC method

Conclusion

The calibration curve of drugs was prepared by UV spectrophotometer in methanol, octanol, water and PBS for routine analysis. The calibration curve was found to be linear between absorbance and drug concentration with regression coefficient close to one in all solvents. A RP-HPLC method was developed for simultaneous quantification of and THQ. It showed a linear relationship (1-80 µg/mL) and was validated for linearity, precision, accuracy, robustness, LOD, and LOQ, with all parameters within acceptable limits (<2% RSD). Thus, the method was found to be sensi- tive, precise, accurate, specific, and robust.

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