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Development and Validation of a Robust RP-HPLC Method for the Simultaneous Analysis of Exemestane and Thymoquinone and Its Application to Lipid-Based Nanoformulations

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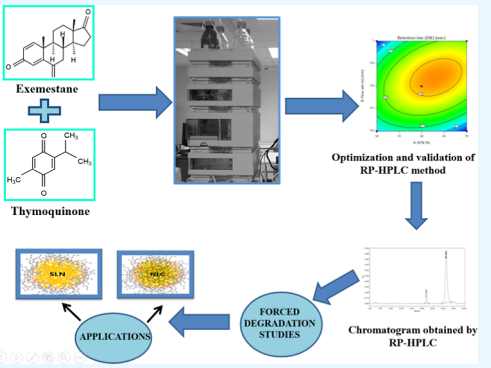
ABSTRACT: The present study describes the development and validation of a simple and rapid HPLC method for the simultaneous quantification of exemestane and thymoquinone. The separation of both compounds was performed on a 5 μ C-18 column utilizing phase A as water/methanol (45:5 v/v) and phase B as acetonitrile (50 v/v) (total ratio of A/B = 40:60 v/v) in isocratic elution mode as the mobile phase at a flow rate of 0.8 mL/min. Further, the Box−Behnken design was used for optimizing the analytical method. The proposed method was validated for various parameters, and all parameters were found to be within an acceptable range. The simultaneous detection of both drugs was monitored at 243 nm with a retention time of 5.73 and 6.93 min, respectively. Moreover, the forced degradation studies were conducted under various stress conditions, and the relevance of the validated RP-HPLC method was further explored for the estimation of drugs from lipid-based nanoformulation. Taken together, the study construed the development of an efficient and robust method that could be used for the quantification of these agents in various in vitro as well as in vivo models.



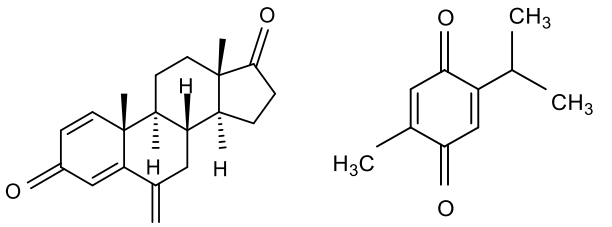
1. INTRODUCTION

Breast cancer has become the most commonly occurring type of cancer and is also considered the foremost cause of mortality in women worldwide.[1] In 2020, nearly 685,000 women died due to breast cancer, conforming to 16% or 1 in every 6 deaths caused due to cancer in women.[2] Among the various types of breast cancer that exist, hormone-dependent positive breast cancer is assigned as the predominant one, requiring estrogen synthesis for its progression.[3]

Exemestane (EXE), as illustrated in Figure 1a, is an oral aromatase inhibitor that was approved in 1999 by the USFDA for the treatment of estrogen-receptor positive breast cancer in postmenopausal women.[4] However, it is a BCS class IV drug and thus exhibits high first-pass metabolism, low bioavailability, high lipophilicity, and poor solubility, which subsequently results in limited clinical therapeutic efficacy.[5] Moreover, it has been reported to cause a loss in bone density at an accelerated rate, resulting in its demineralization and subsequently leading to fractures and osteoporosis.[6]

Thus, a combinatorial approach involving the codelivery of an herbal agent along with a synthetic chemotherapeutic agent has been suggested for overcoming the above-mentioned challenges by the authors. It would serve as an efficient strategy for breast cancer management since the natural phytocompound would augment the anticancer activity and also reduce the associated side effects of the conventional chemotherapeutic agent.

Thymoquinone (THY), as illustrated in Figure 1b, derived from the black seed oil of the plant *Nigella sativa*, is a naturally



(a) Exemestane **(b) Thymoquinone**

Figure 1. Chemical structures of (a) exemestane and (b) thymoquinone.

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occurring bioactive agent.[7] A large repertoire of studies has established its role in anticancer therapy and prevention,[8] additionally, it is also reported to exhibit marked chemosensitive activity.[9] It displays its significant anticancer action by suppressing the activation of Akt, NF-kB, and the extracellular signal-related kinase signaling pathways, thereby inducing apoptosis.[7] Furthermore, a plethora of studies have demonstrated the immense efficacy of THY in bone healing.[10,11] Unfortunately, THY also possesses low bioavailability, poor aqueous solubility,[12] extreme hydrophobicity, prompt metabolism, and a slow absorption rate.[13]

Thus, the authors have formulated a lipid nanodrug delivery system in which the codelivery of EXE and THY ultimately results in promising and accentuated management of breast cancer with minimal side effects.

Although the individual estimation of EXE and THY by various analytical methods has been very well reported in the literature. For instance, the quantification of EXE, viz., LC−MS,[14−16] UPLC,[17,18] and HPLC,[19−21] has been reported. Similarly, THY quantification by LC−MS[22] and HPLC[23,24] has been reported. However, to the best of our knowledge, the estimation of EXE and THY in combination with the RP-HPLC method has not been established so far. Henceforth, our group in this study has developed and validated a vital, efficient, simple, and rapid method for the simultaneous quantification of EXE and THY.

The individual quantification of EXE by the HPLC method has been reported at various wavelengths, such as 242, 247, 249, and 250 nm, in the literature.[25−28] Likewise, the individual quantification of THY has been reported at 250 and 254 nm.[29,30] In our method, during the scanning, the maximum wavelength of detection was found to be 244 nm for EXE and 253 nm for THY, respectively. Therefore, to mitigate the hurdles associated with the previously reported methods, we have developed an efficient and validated method as per the ICH guidelines for the simultaneous estimation of EXE and THY on 243 nm wavelength in considering to achieve optimum sensitivities for both compounds.

Apart from this, our study reports the utilization of the design of experiment (DoE) approach for facilitating the optimization step, which was considered owing to the consumption of less time and cost, as well. This serves as an effective approach by which the relation between the independent variables and the response is studied experimentally. Furthermore, DoE results in much fewer experimental runs, stressing its significant superiority over the OVAT approach.[31] Additionally, forced degradation studies under various stress conditions were performed, and the developed method was also explored for its application in lipid nanoformulation for the assessment of EXE and THY. The present analytical method will prove to be promising for the estimation of EXE and THY in batch products by various research groups.

2. MATERIAL AND METHODS

2.1. Reagents and Chemicals. EXE was obtained as a gift sample from Coral Pvt. Limited (New Delhi, India), and THY was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile, and water were obtained from Merck India. Compritol 888 ATO and Capryol 90 were obtained as kind gift samples from Gattefosse (Mumbai, India); Kolliphor P 188 (poloxamer 188) was gifted by BASF India Limited (Mumbai, India); and Tween 80 was purchased from SD Fine Chemical (Mumbai, India). All other chemicals used in the entire study were of analytical grade.

2.2. HPLC Instrumentation and Chromatographic Conditions. The RP-HPLC method was performed on a Waters 1525 instrument attached with a binary pump and a Waters 2998 PDA detector with EMPOWER software. The mobile phase was composed of water/methanol (45:5 v/v) as phase A and acetonitrile (50 v/v) as phase B. The total ratio of phase A: phase B used was 40:60 v/v with a flow rate of 0.8 mL/min in an isocratic elution mode. The successful elution of EXE and THY individually was carried out on the C18 column, 150 × 4.6 mm, with a pore size of 5 μ, and the ODS at their respective wavelengths of 244 and 253 nm. The absorption maxima of EXE and THY are depicted in Figure S1a,b. The simultaneous elution of both the analytes was monitored at 243 nm by a PDA detector.

2.3. Preparation of Standard and Working Solutions. The primary standard solution (1000 μg/mL individually) for both EXE (Analyte A1) and THY (Analyte A2) was prepared in HPLC-grade methanol. This was followed by the preparation of stock solutions of 100 μg/mL (stocks A1 and A2) from the standard solutions. The stock solutions were diluted further to obtain solutions with concentrations of 6, 9, 12, 15, 18, 21, and 24 μg/mL. In a similar manner, samples with concentrations of 6, 9, 12, 15, 18, 21, and 24 μg/mL were prepared for the EXE−THY combination using methanol. Further, the lower quality control, middle quality control, and higher quality control samples with concentrations of 10, 20, and 30 μg/mL, respectively, were prepared for both Analyte A1 and Analyte A2. All of the prepared samples were kept in light-resistant amber volumetric flasks and stored at 4 °C until their analysis. Additionally, all of the prepared samples were filtered by passing them through a 0.22 μm syringe filter (Millifilter, Milford, USA) before performing the HPLC analysis.

2.4. Optimization of the RP-HPLC Method. Box−Behnken design (BBD) was employed for the optimization of the proposed RP-HPLC method, in which three independent and six dependent variables were considered. It was chosen for the optimization since, in BBD, the studied factors are correlated with the obtained responses by second-order polynomial equations. Furthermore, in comparison to central composite design (CCD), BBD results in fewer experimental runs along with the generation of suitable mathematical models and is thus preferred as an alternative to CCD.[32] Design Expert software version 11 (Stat-Ease, USA) was used for carrying out the experimental designs. Factors namely % of acetonitrile (A), flow rate (mL/min) (B), and injection volume (C) were taken as independent factors, while retention time (RT) of EXE (Y1), THY (Y4), tailing factor of EXE (Y2), THY (Y5), and number of theoretical plates of EXE (Y3) and THY (Y6) were considered as dependent variables. The Design Expert software suggested a total of 17 runs (Table S1) that were conducted, and the effect of the dependent variables (Y) was studied by the generated polynomial equation.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A_2 + \beta_{22} B_2 + \beta_{33} C_2 \quad (1)$$

where, A, B, and C stand for independent variables; β_0 is an intercept; β_1, β_2, and β_3 are the linear coefficients; β_{12}, β_{13}, and β_{23} are the interaction coefficients; β_{11}, β_{22}, and β_{33} are the quadratic coefficients derived from experimental runs; AB, AC,

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BC, A_2, B_2, and C_2 represent the quadratic terms. Additionally, ANOVA was used as a statistical tool for validating the significance of the model. In the present study, we have chosen the range of independent variables as 50−70% acetonitrile concentration; the flow rate was increased from 0.6 to 1 mL/min; and the injection volume was specified between 15 and 25.

3. METHOD VALIDATION

A higher degree of assurance for a particular method is obtained that the procedure employed for the analytical process is appropriate for its intended use by performing the method validation process, which is a documented evidence procedure. The developed RP-HPLC method was validated according to the ICH Q2 (R1) guidelines.[33] They were validated for various parameters, namely, system suitability, specificity, linearity, accuracy, precision (both intraday and interday), limit of detection (LOD) and limit of quantification (LOQ), robustness, and ruggedness.

3.1. System Suitability. This parameter acts as an essential tool in the procedure of liquid chromatography that is used by the analysts for evaluating the reproducibility of the developed method as well as ensuring the suitability of the whole testing assembly for the proposed application. This was evaluated by six replicate injections of EXE and THY, wherein RT, theoretical plates, peak area, and tailing factor of both analytes at 243 nm were considered for estimating the results. As per the guidelines set by the US-FDA, the relative standard deviation (% RSD) for RT and peak area should be less than 2%,[34,35] for the tailing factor, it should not exceed by two, while it should be more than 2000 ($N > 2000$) for the theoretical plates of the column.[36]

3.2. Specificity. This characteristic feature of HPLC refers to the potential of the chromatography system in segregating the analyte from its mixture to ensure that the chromatograms of both drugs at their specific wavelengths were devoid of any interference from the solvent system. For the evaluation of specificity, individual, blank, and mixture chromatograms were compared independently at their MQC level. The blank solution was prepared in a manner similar to that of the sample solution, except that the addition of EXE and THY was not carried out in the preparation of the blank solution.

3.3. Linearity. If the obtained results are in correlation with the concentration tested within a specific range, then the analytically developed method is considered linear. The linearity of the method was analyzed by preparing nine mixed solutions ranging from 1 to 60 μg/mL with different concentrations. The calibration curves of EXE and THY were prepared individually by plotting their respective concentrations on the X-axis and peak areas on the Y-axis, followed by the derivation of the regression equation from them.

3.4. Robustness. The suitability of the method was determined by this parameter by making minor variations in the detection wavelength of EXE (241−247 nm), THY (250−256 nm), flow rate (0.6−1.0), and composition of the mobile phase (50:50−40:60). Furthermore, the results were analyzed after evaluating the effects that were caused by variations. The % RSD of the mean peak area and the mean % recovery of both EXE and THY were determined to investigate the robustness of the method, which should not surpass the 2% limit. Additionally, the theoretical plates, RT, and tailing factors were also estimated.

3.5. Accuracy. The accuracy of an analytical method refers to the closeness of the estimated values to the actual values obtained. The accuracy of the developed method was determined by determining the % recovery of both analytes. It was estimated by spiking known concentrations of EXE and THY at three concentration levels, including low, medium, and high (80, 100, and 120%), and from each concentration, the samples were injected in triplicate. The following formula was used for determining the % recovery of EXE and THY, wherein the accepted range for it is 90−110% while it should not be more than 2% for % RSD and SE.

$$\%\ \text{Recovery} = [(\text{recovered concentration}/\text{injected concentration}) \times 100] \quad (2)$$

3.6. Precision. The extent to which a technique is used in a repetitive manner for investigating several replicates in different instances is referred to as "precision". For calculating precision, intraday as well as interday precision was estimated, and the results were reported as % RSD. Additionally, the repeatability of the developed method was verified for both analytes under the same experimental conditions. It was performed by preparing and injecting six solutions of 10 g/mL, followed by the determination of the concentration found. For intraday precision, three different concentrations, including low, medium, and high, were analyzed on the same day at three different time points. While estimating interday, both samples were analyzed on three consecutive days. The acceptable range for the calculated % RSD must not exceed 2%.

3.7. Ruggedness. Ruggedness refers to the ability to obtain reproducible results under various conditions that include the use of varied instruments and analysts. In the present study, the ruggedness was determined by considering three different samples exhibiting 10, 20, and 30 μg/mL as different concentrations. The analysis was done by two different analysts in the same as well as different laboratories on identical HPLC instruments. The % recovery was then determined for both analytes, followed by the comparison of the obtained results with each other for the evaluation of the ruggedness of the proposed method.

3.8. Sensitivity. LOD and LOQ were determined for estimating the sensitivity of the proposed method. In the case of LOD concentration, the ratio of signal-to-noise is approximately 3:1, while for LOQ concentration, a signal-to-noise ratio of approximately 10:1 with a % RSD of less than 10% ($n = 3$) is required. The formula mentioned below was used for calculating the LOD and LOQ of both the analytes.

$$A = k\sigma/s$$

where A stands for the LOD/LOQ value, σ is the standard deviation of the response peak area, s stands for the slope obtained from the calibration curve, and k is 3.3 and 10 for the LOD and LOQ, respectively.

4. STABILITY STUDIES

For assessing the stability of the EXE−THY solution, these studies were conducted at low, high, and medium concentrations (10, 20, and 30 μg/mL, respectively) in triplicate under different stability conditions, namely, short-term, long-term, and freeze−thaw. For evaluating the short-term stability of the solutions, the mixtures were kept at room temperature and analyzed for 12 h, while the long-term stability studies were carried out for 3 weeks at −30 °C. Furthermore, the

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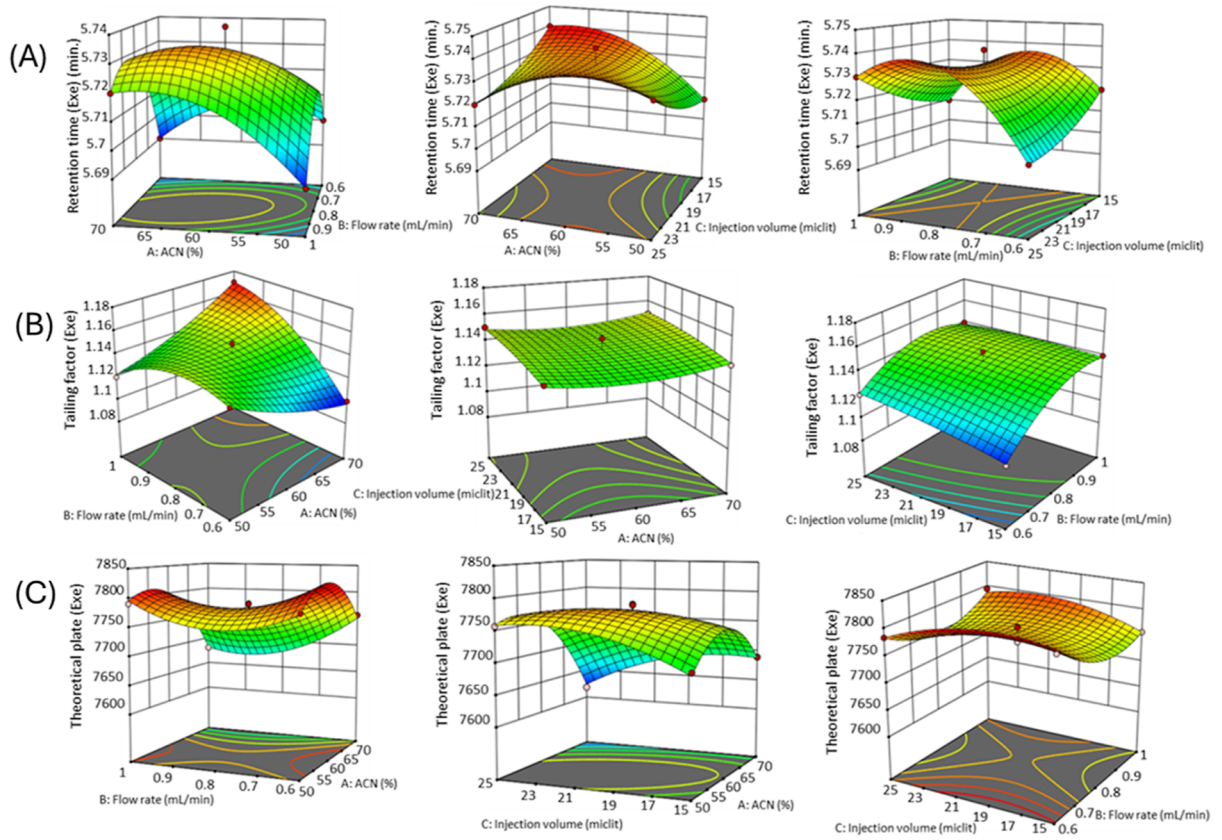

Figure 2. 3D response surface plots illustrating the effect of independent variables on (A) RT (B) tailing factor and (C) theoretical plates of EXE.

samples were kept at −20 °C for 24 h and then thawed at room temperature in an unassisted manner, wherein the results of the experiments were interpreted in terms of the % recovery of the samples.

5. FORCED DEGRADATION STUDIES

For determining the degradation behavior, these studies were performed by exposing the EXE and THY samples to various stress conditions, such as acidic, alkaline, oxidative, and photolytic, as recommended by the ICH guidelines. For acid−base degradation, to a 1 mL mixture of EXE and THY, approximately 1 mL of 1 N HCl and 1 mL of 1 N NaOH were separately added. These solutions were then placed in amber-colored bottles, sealed successfully, and heated at 80 °C for an hour. Both samples were neutralized before the analysis. For studying oxidative stress, 1 mL of 30% H_2O_2 was mixed with 1 mL of the drug mixture. In the study of photolytic degradation, to 1 mL of the drug mixture was added 10 mL of mobile phase in a transparent 10 mL volumetric flask. The volumetric flask was sealed and exposed to direct sunlight for a period of 30 min to investigate the photochemical stability. In all the studies that have been mentioned above, a mobile phase of up to 10 mL was utilized for adjusting the final volume. All of the samples were filtered by a 0.2 μm filter before being injected into the HPLC chromatographic system for further analysis.

6. APPLICATION OF THE SIMULTANEOUS DEVELOPED METHOD TO LIPID-BASED NANOFORMULATIONS

The developed and validated analytical method was further applied for the quantification of EXE and THY in liposomes, solid lipid nanoparticles (SLNs), and nanostructured lipid carriers (NLCs). The amount of entrapped drugs in the mentioned nanoformulations were determined by the method developed.

6.1. Preparation of EXE−THY−SLNs. EXE−THY−SLNs were prepared by an ultrasonication technique utilizing Compritol 888 ATO as the solid lipid. For the preparation of the lipid phase, the solid lipid was melted 5−10 °C above the melting point of Compritol 888 ATO. This was followed by the addition of accurately weighed EXE and THY in the lipid phase. Simultaneously, the aqueous phase was prepared by dispersing Poloxamer 188 in milli-Q water, and it was maintained at the same temperature as that of the lipid phase. The melted lipid phase was then added to the aqueous surfactant solution under constant stirring for 40 min at 800 rpm, which further resulted in the formation of a primary emulsion. After that, the obtained primary emulsion was sonicated using a probe sonicator for a duration of 2 min followed by its immediate immersion in a water bath stirred further at 200 rpm that generated the EXE−THY's−SLNs. Further, the SLNs thus obtained were then centrifuged at a temperature of 4 °C for 20 min at 15,000 rpm resulting in the separation of a supernatant which was discarded, and the pellet obtained was washed with milli-Q water for the estimation of percent entrapment efficiency.[37]

6.2. Preparation of EXE−THY−NLCs. The ultrasonication technique was employed for the preparation of NLCs coloaded with EXE and THY utilizing Compritol 888 ATO and Capryol 90 as the solid lipid and liquid lipid, respectively. Briefly, Compritol 888 ATO and Capryol 90, both weighed accurately, were heated above the 5−10 °C melting point of the solid lipid. Then, accurately weighed amounts of both of

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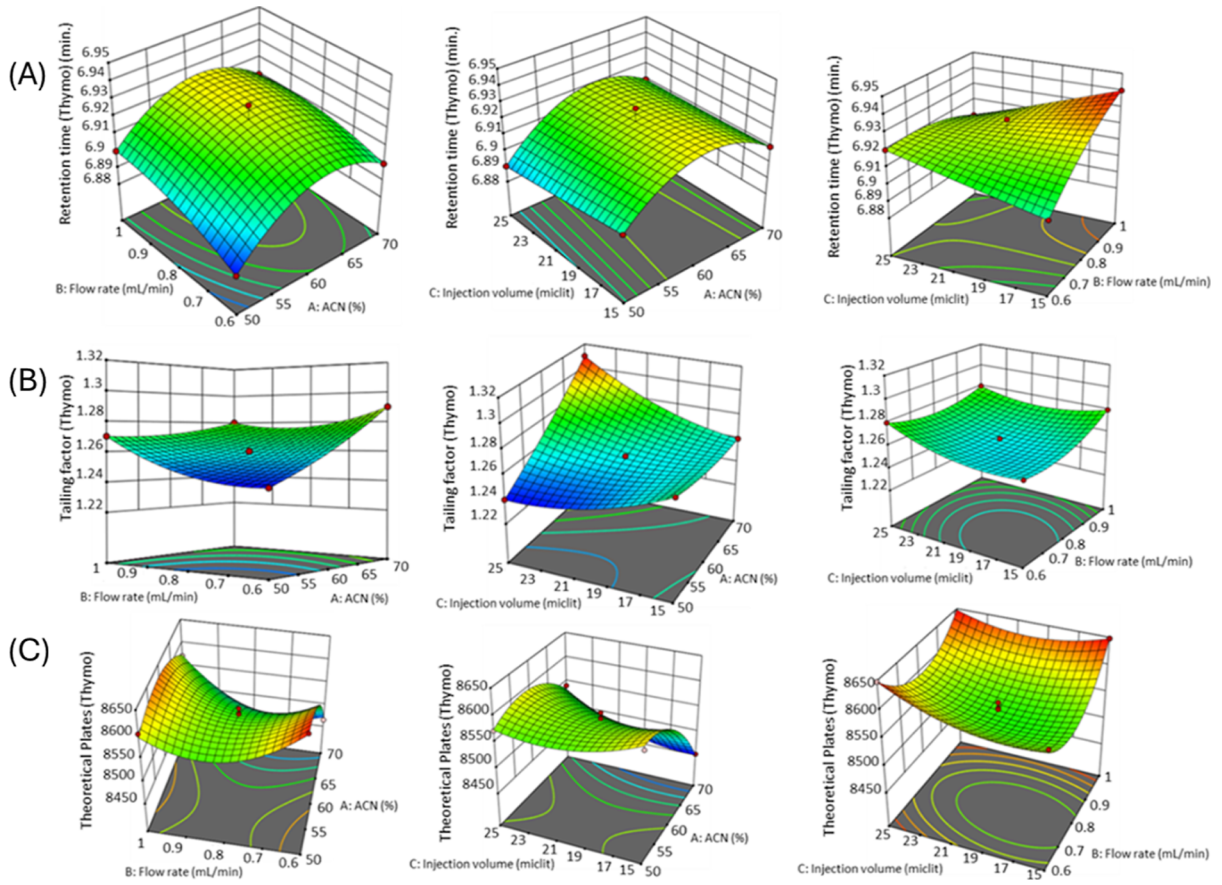

Figure 3. 3D response surface plots illustrating the effect of independent variables on (A) RT (B) tailing factor and (C) theoretical plates of THY.

the drugs, EXE and THY, were added to the clear, oily, melted lipid phase. Concurrently, a surfactant solution comprising Poloxamer 188 and Tween 80 was prepared by adding them to distilled water, which was also heated to the same temperature as that of the lipid phase. Then the hot surfactant solution was added dropwise to the lipid blend with constant stirring at 800 rpm for a duration of 40 min, which resulted in the generation of a primary emulsion. The generated primary emulsion was ultrasonicated for 2 min via a probe sonicator. Finally, the dispersion was cooled to room temperature, wherein the coloaded NLCs were formed by the solidification of lipids.[5] The obtained EXE−THY−NLCs were then centrifuged at a temperature of 4 °C for 20 min at 15,000 rpm, resulting in the separation of a supernatant, which was discarded, and the pellet obtained was washed with milli-*Q* water for the estimation of percent entrapment efficiency.[38]

7. RESULTS AND DISCUSSION

7.1. Development and Optimization of the Method.

The method was optimized by considering the simultaneous detection of EXE and THY for further application in various lipid-based nanoformulations. While developing this analytical method, the desired wavelength for simultaneous estimation was obtained by adjusting the PDA detector at various wavelengths in the UV range. It was observed that at 243 nm both EXE and THY showed appropriate peak area and height with optimum sensitivity for the simultaneous estimation of both analytes.

For the optimization of the proposed HPLC analytical method, the BBD was selected. Table S2 illustrates the various parameters considered in the optimization of the HPLC method. BBD model with quadratic response that generated 17 runs was used to optimize the analytical method. The evaluation of acetonitrile (%), flow rate (mL/min), and injection volume (μL) was done against retention time, tailing factor, and theoretical plates, and the results were analyzed wherein significant differences were revealed between the values. Tables S3−S9 illustrates the predicted and actual R^2 values for all the dependent variables. The adjusted and predicted r^2 values were found to be in close proximity to each other, signifying the exceptional prognostic efficiency of the experimental design for the response variables. Additionally, a good correlation between the experimental data and the fitted data was revealed by the higher value of the adjusted r^2. The complex interaction between the variable and the responses studied was predicted by the polynomial equations generated by the software. As is evident from eqs 3−8, both positive and negative signs exist. The positive sign indicates that the process of optimization is favored, whereas the negative sign denotes the inverse relationship that exists between the various factors. From Figures 2A and 3A and eqs 3 and 4, it was inferred that the concentration of acetonitrile and flow rate had a prominent effect on the retention time, whereas the injection volume exhibited a limited effect. It was observed that the retention time increased with an increase in the acetonitrile concentration since in RP-HPLC, a nonpolar stationary phase is utilized; thus, upon increasing the polarity of the mobile phase, the affinity of both the nonpolar analytes toward the stationary phase increases, resulting in the retention of the analytes on the column for a longer time.

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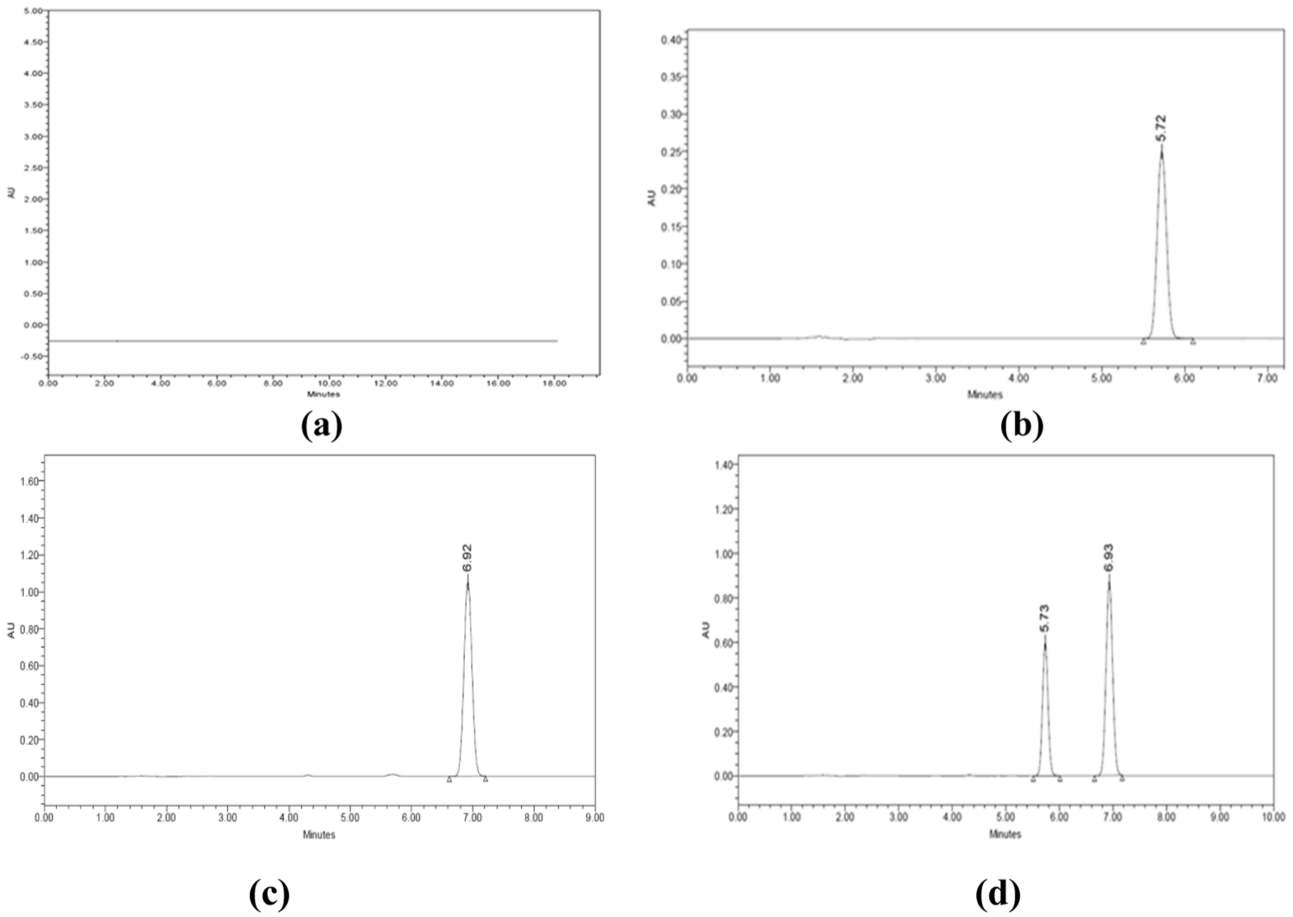

Figure 4. Representative chromatogram of (a) blank λ_{max} of 243 nm, (b) EXE at λ_{max} of 244 nm, (c) THY at λ_{max} 253 nm, and (d) EXE and THY at λ_{max} of 243 nm.

On the other hand, upon increasing the flow rate, a decrease in RT was observed since RT refers to the time of interaction between the analyte and the stationary phase. Thus, as the flow rate increases, the interaction time of the analyte with the stationary phase decreases owing to the comparatively shorter time the analyte takes for propelling through the column, thereby causing the expected effect on RT.

Additionally, from Figure 2B and eq 5, it was concluded that the response variable tailing factor for EXE was significantly influenced by acetonitrile concentration and flow rate, while it was negligibly influenced by injection volume. It was observed that the tailing factor increased with an increase in the acetonitrile concentration. On the contrary, as portrayed in Figure 3B and eq 6, the tailing factor for THY was influenced by all three studied factors; however, acetonitrile concentration showed a marked effect compared to the other two factors. It was concluded that as the acetonitrile concentration increased, the tailing factor increased too, whereas it was slightly increased with an increase in flow rate and injection volume. Increasing the mobile phase has an influential effect on the tailing factor, attributed to the secondary interactions between the polar mobile phase and the residual ionic silanol groups present on the silica surface of the column that finally result in peak tailing. Furthermore, some of the samples may lag behind at high flow rates owing to the adsorption of the analyte to the stationary phase, subsequently generating a broadening of peaks.

Figure 2C and eq 7 show that all three factors influenced the theoretical plates of EXE. The graphs depicted that with an increase in all three factors, the theoretical plates decreased, with the effect of acetonitrile being the prominent one. The no. of theoretical plates defines the efficiency of the column since the higher the no. of the theoretical plates, the greater the column efficiency. With increasing the concentration of acetonitrile, the no. of theoretical plates decreased, which was evident by the nonefficient resolution of the peaks, resulting in the generation of merged peaks. However, the no. of theoretical plates was still within the acceptable limit of <12,000.

Furthermore, at high flow rates, mass transfer increases, which often leads to a decrease in the column efficiency. Also, peak fronting is observed due to the injection of sample in large volume, which in turn results in poor resolution and decreased retention time, consequently degrading the column efficiency.

In contrast, as evident from Figure 3C and eq 8, with increasing both the factors, i.e., flow rate and injection volume, the theoretical plates of THY increased, whereas they decreased with increasing the amount of acetonitrile. Although changes were observed, the no. of theoretical plates were within the acceptable limit of <12,000, and thus, it was inferred that there was no significant deviation.

Lastly, based on all the observations and considering the optimum conditions for chromatography, the analysis for both

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drugs was carried out, wherein excellent separation of both analytes was achieved on the C18 chromatographic column.

$$\text{RT (EXE)} = +5.73 + 0.0050A + 0.0050B + 0.0000C + 0.0100AB - 0.0100AC + 0.0100BC - 0.0110A^2 - 0.0210B^2 + 0.0040C^2 \quad (3)$$

$$\text{RT (THY)} = +6.92 + 0.0075A + 0.0075B - 0.0025C - 0.0025AB + 0.0025AC - 0.0125BC - 0.0198A^2 - 0.0048B^2 + 0.0003C^2 \quad (4)$$

$$\text{tailing factor (EXE)} = +1.14 + 0.0013A + 0.0137B + 0.0050C + 0.0225AB + 0.0000AC - 0.0050BC + 0.0052A^2 - 0.0147B^2 - 0.0022C^2 \quad (5)$$

$$\text{tailing factor (THY)} = +1.26 + 0.0150A + 0.0050B + 0.0050C - 0.0100AB + 0.0200AC - 0.0050BC + 0.0035A^2 + 0.0085B^2 + 0.0085C^2 \quad (6)$$

$$\text{theoretical plate (EXE)} = +7774.00 - 36.12A - 14.12B - 0.7500C - 20.50AB - 31.25AC + 16.25BC - 58.00A^2 + 37.00B^2 - 24.25C^2 \quad (7)$$

$$\text{theoretical plate (THY)} = +8558.00 - 48.75A + 12.62B + 12.88C + 38.25AB + 28.75AC - 13.00BC - 40.25A^2 + 54.00B^2 + 23.00C^2 \quad (8)$$

7.2. Method Validation. The primary aim of the method validation process is to ensure that the developed method is reliable and acceptable for its assigned purpose. The proposed analytical method was validated for various parameters, as per the ICH guidelines.

7.2.1. System Suitability. The authenticity of the developed method was investigated by performing the analysis of RT and the peak area repetitively. The average RT for EXE and THY was 5.72 ± 0.024 and 6.92 ± 0.021 min with % RSD of 0.42 and 0.31, respectively. The average peak area for EXE and THY was observed to be 19,55,360.50 ± 5087.779 with a % RSD of 0.26 and 0.02, respectively. In contrast, the average theoretical plates for EXE and THY were 7716.83 ± 75.539 and 8551.16 ± 71.878 with % RSD of 0.97 and 0.84, respectively. Finally, the average tailing factor for EXE and THY was 1.14 ± 0.012 and 1.26 ± 0.011 with % RSD of 1.07 and 0.92, respectively. The % RSD obtained for RT and peak area was found to be less than 2% (Tables S10 and S11), suggesting the suitability of our novel and rapid analytical method in generating reproducible results.

7.2.2. Specificity. To determine the specificity of the prepared HPLC method, the chromatograms of the standard solution of EXE and THY and the EXE−THY mixture were compared with the blank solution chromatogram (Figure 4a). The chromatograms were generated and analyzed after injecting a volume equivalent to 10 μL. It was inferred that the individual solutions of EXE and THY were eluted at the retention time of 5.72 and 6.92 min, respectively (Figure 4b,c). In comparison, their combination displayed well-separated peaks at 5.73 and 6.93 min (Figure 4d). As per the results obtained, it was revealed that there was no variation in the RT of the combination and individual solutions, indicating the specificity of the developed method.

7.2.3. Linearity. The calibration curve was utilized to estimate the linearity of the newly developed analytical method. The calibration curve obtained by plotting the mean peak area of EXE and THY against the different concentrations of the drugs depicted linearity for both the analytes with R^2 values of $y = 2{,}19{,}100x + 46{,}782$, 0.9995 and $y = 2{,}50{,}310x + 98{,}795$, 0.9997 for EXE and THY, respectively (Figures S2 and S3). Thus, the obtained results successfully depicted the linearity of the developed method over the measured concentration range.

7.2.4. Robustness. The method's robustness was measured by making deliberate changes in wavelength, flow rate, and mobile phase composition and studying their effect on the theoretical plates, RT, and tailing factor. The results, as shown in Table S12, revealed no significant change in the theoretical plates, RT, or tailing factor that subsequently suggests the robustness and reliability of the developed analytical method.

7.2.5. Accuracy. In accuracy studies, the percent recoveries were found to be in the range of 98.12−101.82% for EXE whereas it was found to be in the range of 99.18−101.61% for THY (Table S13). Furthermore, the SD for all of the studied samples was found to be within the range of 2%. It was observed that the developed method exhibited high accuracy for all of the concentration levels studied.

7.2.6. Precision. In intraday precision, the % RSD ranged from 0.56 to 1.38 for EXE and 0.88−1.4 for THY, whereas for interday precision, the % RSD ranged from 1.06−1.77 and 1.06−1.94 for THY.

A good precision of the method was thus obtained, confirmed by the % RSD values for intraday and interday precision which was found to be <2%. Furthermore, the low variability between the results demonstrated good precision of the developed method. The precision values obtained have been depicted in Table S14, indicating the reliability and repeatability of the method.

7.2.7. Ruggedness. To assess the reproducibility of the developed method, a ruggedness test was performed on the same HPLC instrument with different analysts and on different HPLC instruments with the same analysts. The results suggested an insignificant change in the % recoveries for both drugs. Hence, the results obtained (Tables S15 and S16) demonstrated the remarkable reproducibility of our developed analytical method, further indicating its ruggedness.

7.2.8. Sensitivity. In the present study, the LOD and LOQ values for EXE were found to be 2.99 and 9.05 μg/mL, respectively, whereas 0.29 and 0.87 μg/mL were for THY, respectively. Based on the results obtained, it was inferred that the developed method was highly sensitive and thus can be used in various studies such as percent entrapment efficiency, in vitro and ex vivo release studies, and in vivo studies for detecting and quantifying less amount of the drugs.

8. STABILITY STUDIES

The stability studies were conducted at low, medium, and high concentration levels (10, 20, and 30 μg/mL) for EXE and THY. The predicted concentrations for short -term, long-term,

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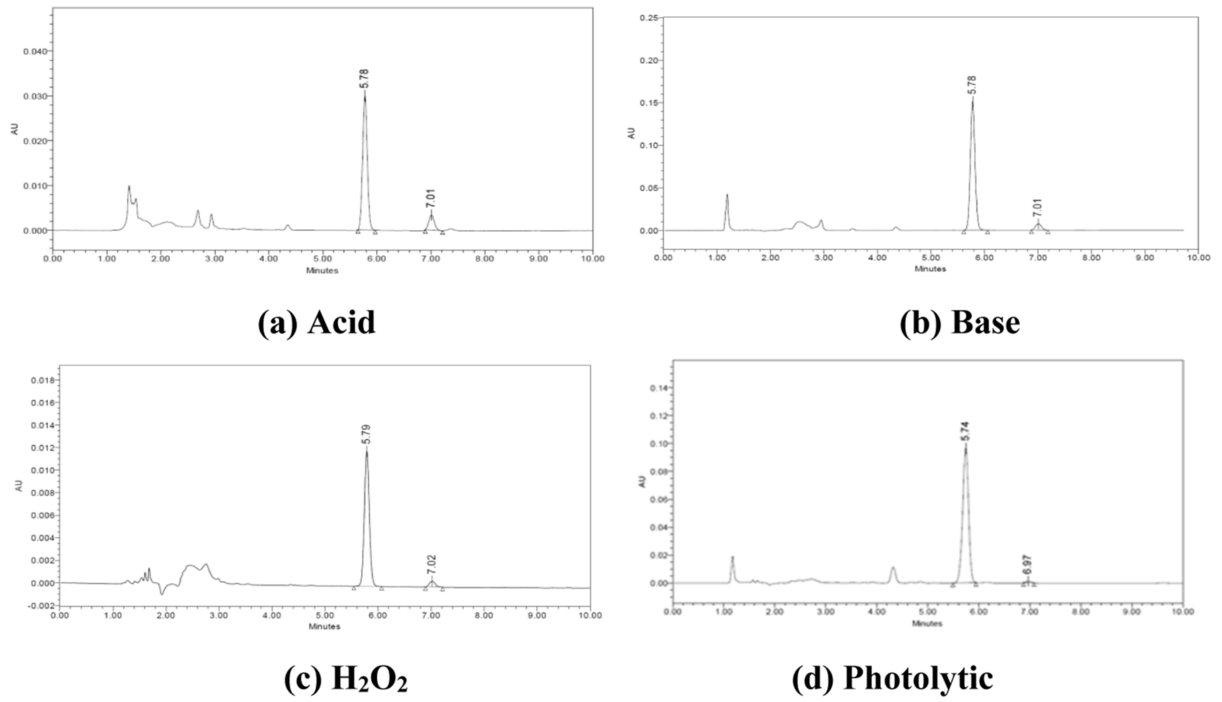

Figure 5. Forced degradation chromatograms analyzed at λ_{max} of 243 nm for (a) acidic, (b) basic, (c) oxidative, and (d) photolytic conditions.

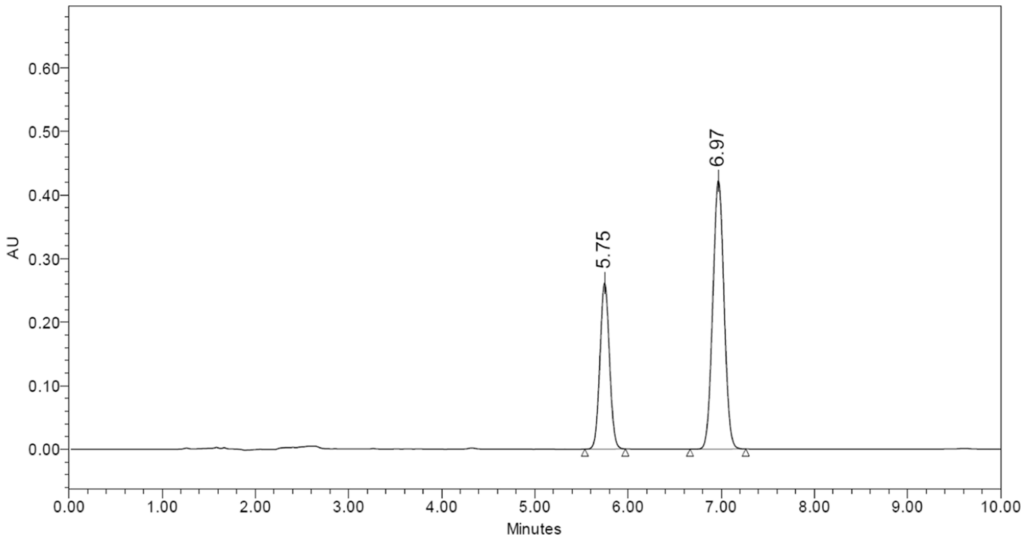

Figure 6. HPLC chromatogram of EXE−THY−NLCs at an λ_{max} of 243 nm.

and freeze−thaw stability studies, as shown in Table S17, were in the accuracy range of 90.4−105.9% for EXE and 92.5−104.1% for THY, respectively. It was revealed from the results that both drugs were stable and did not undergo degradation during the experimental study.

9. FORCED DEGRADATION STUDIES

For studying the forced degradation of both samples, the ICH guidelines were followed: acidic, basic, peroxide, and photolytic. The approvability of the method was revealed by the well-separated peaks of the degradation products from the peaks of the analytes, as illustrated in Figure 5a−d. It was observed that some of the degradation peaks were formed with EXE, while the peak intensity of THY was also significantly diminished in all the conditions. It was inferred that the integrity of peaks for both the sample analytes was maintained under all the circumstances, and there was an adequately visible separation between the drug peaks and their formed degraded products, indicating the selectivity and specificity of the developed method.

10. APPLICATION OF THE DEVELOPED METHOD TO LIPID-BASED NANOFORMULATIONS

The developed analytical method was successfully applied for the determination of % EE of EXE and THY coloaded in SLNs and NLCs at a wavelength of 243 nm. The percent entrapment efficiency of EXE and THY from SLNs was found to be 74.5 and 72.1%, wherein it was found to be 78.6 and 77.8%, respectively (Figure 6). The results suggested that the entrapment efficiency of EXE−THY−NLCs was maximum

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when compared to EXE−THY−SLNs. The increased percent entrapment from NLCs could be attributed to the presence of both solid and liquid lipids that further aid in the formation of a matrix system at the same time augmenting the solubility of the drugs. Additionally, it was inferred that the developed method was robust and highly specific in determining the percent entrapment efficiency since there was no significant change in the retention time and peak areas of both the analytes.

11. CONCLUSIONS

This study has reported for the first time a novel, simple, and efficient analytical method for the simultaneous estimation of EXE and THY with a specific application to lipid-based nanoformulations. Despite the extreme differences between the analytes, their well-resolved separation was achieved within less than 10 min at a single wavelength, making it more economical and improvised. Additionally, the developed method depicted excellent validation parameters fulfilling all of the criteria as proposed by the ICH guidelines. Furthermore, the developed method adequately estimated the amount of EXE and THY entrapped in the NLCs and the degradation products, demonstrating the method's applicability. Overall, the method developed was found to be reliable and rapid for the analysis of both the drugs at various platforms in future correlation studies, including combined nanoformulations, in vitro, and in vivo models.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c08078.

Absorption maxima of EXE; absorption maxima of THY; independent and dependent variables chosen for optimization of RP-HPLC by BBD; 3^3 Box−Behnken design outcomes with measured responses utilized for optimization study; data of fit summary for EXE and THY RT; sequential model sum of squares for EXE and THY RT; data of fit summary for EXE and THY tailing factor; sequential model sum of squares for EXE and THY tailing factor; data of fit summary for EXE and THY theoretical plates; sequential model sum of squares for EXE and THY theoretical plates; values of correlation coefficient r^2 of dependent variables; system suitability test for EXE; System suitability test for THY; calibration curve of EXE; calibration curve of THY; robustness evaluation of developed HPLC method for EXE and THY; accuracy of developed HPLC method for EXE and THY; Repeatability of EXE and THY; intraday precision of EXE and THY (n = 3); interday precision of EXE and THY (n = 3); different HPLC instruments with same analyst; same HPLC instruments with different analysts; and stability of EXE and THY at different conditions of storage (n = 3) (PDF)

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Notes

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