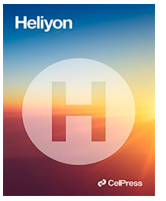
Heliyon 10 (2024) e25598

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Simultaneous quantification of 4-hydroxytamoxifen and hesperidin in liposomal formulations: Development and validation of a RP-HPLC method

Cynthia Lizzie Lobo, Manohar M, Amitha Shetty, Ananya S, Pallavi K, Akhilesh Dubey *

Nitte (Deemed to be University), NGSM Institute of Pharmaceutical Sciences, Department of Pharmaceutics, Mangaluru, India

ARTICLE INFO

Keywords:
4-Hydroxytamoxifen
Hesperidin
Reverse-phase high-performance liquid chromatography
Box-Behnken design
Simultaneous quantification
Breast cancer

ABSTRACT

Breast cancer treatment options are diverse, with tamoxifen commonly used as a selective estrogen receptor modulator (SERM) for hormone receptor-positive breast cancer. However, tamoxifen can have adverse systemic effects. Local transdermal therapy offers a potential solution by delivering the drug directly to the breast and minimizing systemic exposure. Hesperidin, a flavonoid, exerts synergistic effects when combined with anticancer agents. This combination therapy may be a more effective approach to breast cancer management. Analytical methods have been developed to quantify 4-Hydroxytamoxifen (4-HT) and hesperidin separately; however, no method currently exists for their simultaneous quantification in pharmaceutical formulations. This study aimed to develop and validate a reverse-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous quantification of 4-HT and hesperidin in liposomal formulations. A Design of Experiments (DoE) approach was employed using a Box-Behnken design (BBD) to optimize the RP-HPLC method. BBD allowed for a reduction in the number of required tests by creating a statistical model to estimate the significance of various factors and interactions. The methanol concentration, flow rate, and injection volume were considered as independent variables for optimization. A mobile phase (90:10 ratio of methanol: 0.1% v/v orthophosphoric acid) with a flow rate of 0.4 mL/min, and an injection volume of 10 μL was selected as optimized chromatographic condition. 4-HT showed a retention time (Rt) of 5.05 min and hesperidin showed an Rt of 7.11 min using an optimized analytical method and was detected at 275 nm. The developed RP-HPLC method was validated according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines, confirming its accuracy, precision, linearity, selectivity, and robustness. The validated method was then successfully applied to determine the entrapment efficiency and permeation of 4-HT and hesperidin into loaded liposomes. This study fills a gap in the literature by providing a simple and reliable RP-HPLC method for the simultaneous quantification of 4-HT and hesperidin in liposomal formulations.

* Corresponding author. Department of Pharmaceutics NGSM Institute of Pharmaceutical Sciences Nitte (Deemed to be University) Mangaluru, 575018, Karnataka, India.
E-mail address: akhilesh@nitte.edu.in (A. Dubey).

https://doi.org/10.1016/j.heliyon.2024.e25598
Received 23 August 2023; Received in revised form 30 January 2024; Accepted 30 January 2024
Available online 20 February 2024

1. Introduction

Breast cancer is a complex disease with several treatment options. Tamoxifen is a selective estrogen receptor modulator (SERM) commonly used in hormone receptor-positive breast cancer. SERMs interact with estrogen receptors, acting as agonists or antagonists depending on the tissue and hormonal environment. Tamoxifen, a first-generation SERM, has been widely used to treat hormone receptor-positive breast cancer. Although it effectively inhibits estradiol-induced cell proliferation, it may also hinder the proliferation of normally proliferating breast cells during the menstrual cycle. Its active metabolite, 4-Hydroxytamoxifen (4-HT), exhibits a higher binding affinity to breast estrogen receptors than tamoxifen [1,2]. However, tamoxifen and 4-HT can have adverse effects such as uterine cancer, blood clots, and stroke [3]. These systemic effects pose challenges to long-term treatment. However, local transdermal therapy offers a potential solution by delivering the drug directly to the breast, resulting in high local drug concentrations with minimal systemic exposure. This approach may help overcome the systemic adverse effects associated with tamoxifen therapy [4].

Hesperidin, a flavonoid commonly found in fruits and vegetables, has anti-inflammatory, antioxidant, and potent anticancer properties. Studies have demonstrated its ability to inhibit proliferation and induce apoptosis in breast cancer cells, particularly MCF7 cells. Furthermore, Hesperidin has synergistic effects when combined with anticancer agents, including tamoxifen [5]. The combination of tamoxifen and hesperidin has shown promising results in enhancing antiproliferative effects on breast cancer cells. Studies have reported that combination treatment exhibited a higher antiproliferative effect on MCF7 and T47D cells than individual treatments [6,7]. This combination may provide a more effective therapeutic approach for breast cancer treatment. Additionally, Hesperidin pretreatment and doxorubicin treatment showed less toxicity to vital organs due to its anti-inflammatory and antioxidant effects [8]. Therefore, combination therapy containing 4-HT and hesperidin can improve the management of breast cancer.

For visual illustration, Fig. 1a and b provide graphical depictions of the chemical structures of 4-HT and hesperidin, respectively. Various analytical methods have been employed to quantify 4-HT and hesperidin. These methods include LC-MS/MS [9,10], gas chromatography/negative chemical ionization mass spectrometry [11], high-performance liquid chromatography (HPLC) [12] for 4-HT, and spectroscopy [13], high-performance thin-layer chromatography (HPTLC) [14], reverse-phase high-performance liquid chromatography (RP-HPLC) [15], and LC-MS/MS [16,17]. However, no method has yet been reported for the simultaneous quantification of 4-HT and hesperidin in pharmaceutical formulations. Analyzing drugs individually consumes time and resources, hinders pharmaceutical development, and leads to sample wastage, which is a critical concern for limited or precious samples. In the method development process, we encountered challenges involving hesperidin solubility, which we addressed by dissolving hesperidin in a solution of NaOH and methanol, as well as HPLC peak overlap, which we resolved by adjusting the mobile phase flow rate.

Design of Experiments (DoE) is a valuable tool for optimizing analytical techniques, allowing for a reduction in the number of required tests. By creating a statistical model, DoE enables the estimation of the statistical significance of various factor effects and the interactions between responses [18]. In chromatography, particularly in HPLC, the Box-Behnken design (BBD) has been widely employed to optimize the chromatographic conditions [19]. Building on this, the present study aimed to develop and validate a simple and reliable RP-HPLC method for the simultaneous quantification of 4-HT and hesperidin in liposomal formulations. To achieve this, a preliminary method development work was conducted, and BBD was utilized for method optimization. The independent variables considered for optimization included methanol concentration, flow rate, and injection volume. Using this design, the optimal conditions for simultaneous quantification of 4-HT and hesperidin were determined. Subsequently, the developed method was used to quantify 4-HT and hesperidin in the liposomal formulations.

2. Materials and methods

2.1. Materials and reagents

4-Hydroxytamoxifen, Hesperidin, and L-α Phosphatidylcholine were procured from Sigma-Aldrich (St. Louis, Missouri, USA). Cholesterol, Tween 80, sodium deoxycholate, diethyl ether, Chloroform, Hydrochloric acid, and orthophosphoric acid for HPLC and HPLC grade methanol were obtained from Loba Chemie Pvt., Ltd. Mumbai, India. Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, sodium hydroxide, hydrogen peroxide, and Triton X-100 were purchased from HiMedia Laboratories (Maharashtra, India). Milli-Q water was prepared in the laboratory by using a Milli-Q filtration system (Evoqua, USA).

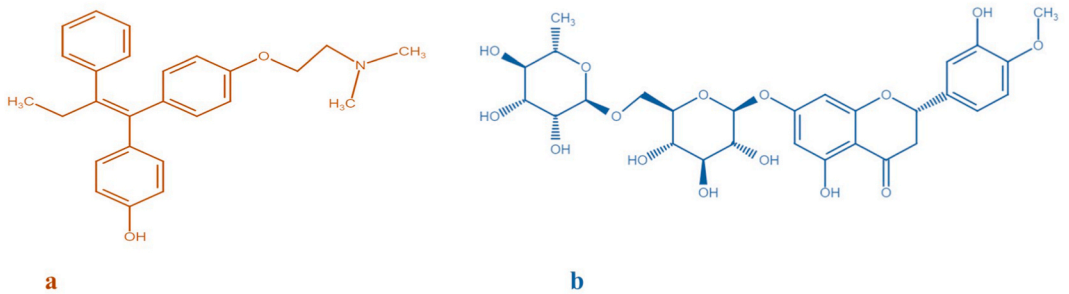


Fig. 1. Structure of a) 4-HT b) Hesperidin.

2.2. Instruments

The RP-HPLC system (Shimadzu, LC-20AD, Japan) was equipped with an autosampler (SIL-20ACHT), column oven (CTO-10ASvp), and a photodiode array detector (SPD-M40). The LabSolutions software was used to record and integrate the chromatograms. Vesicle size reduction was achieved using a probe sonicator (Sonics Vibra cell, CV18), whereas the separation of liposomes and free drugs was performed using a cold centrifuge (Remi, C-24 Plus), and *ex vivo* drug permeation studies were conducted using a vertical Franz diffusion cell (Electrolab, EDC-07).

2.3. Chromatographic conditions

The mobile phase consisted of methanol (organic phase) and Milli-Q water with 0.1 % v/v orthophosphoric acid (aqueous phase) at a ratio 90:10. Prior to use, the solvents were filtered using a 0.45 μm membrane filter and degassed using an ultra-bath sonicator. The stationary phase employed was a Shim-Pack Solar C18 column (5 μm, 4.6 × 250 mm). The analysis was conducted under isocratic conditions, with a flow rate of 0.4 mL/min and an injection volume of 10 μL. The detector wavelength used for detection and quantification was 275 nm.

2.4. Preparation of standard solutions

Individual primary stock solutions of the drugs were prepared. First, 10 mg of 4-HT was accurately weighed and dissolved in 10 mL of methanol to obtain a concentration of 1 mg/mL. Next, 10 mg of hesperidin was dissolved in 0.1 M NaOH and further diluted with methanol to a final volume of 10 mL, achieving a concentration of 1 mg/mL. Subsequently, equal volumes of the prepared solutions of both the drugs were mixed in a 1:1 ratio. The resulting mixture was further diluted with methanol to obtain working stock solutions ranging from to 1–100 μg/mL.

2.5. Spectral analysis

To determine the absorption maximum, solutions of 4-HT and hesperidin were prepared at a concentration of 10 μg/mL. The solutions were then scanned using a UV–visible Jasco V-630 Spectrophotometer (Japan) in the wavelength range of 200–400 nm. Methanol was used as a blank reference for the measurements.

2.6. Optimization of RP-HPLC method

A BBD was employed to optimize the proposed method, which allowed for the assessment of three independent and seven dependent variables. BBD offers the advantage of obtaining higher-order responses of independent variables with fewer experimental runs than the standard factorial approach. The independent variables considered in this study were the% methanol concentration (A) (88%–92%), flow rate (B) (0.3–0.5 mL/min), and injection volume (C) (8–12 μL). The dependent variables included Rt of 4-HT (Y_1) and hesperidin (Y_2), peak area of 4-HT (Y_3), hesperidin (Y_4), tailing factor (Tf) of 4-HT (Y_5), hesperidin (Y_6), and resolution (Y_7). The experimental conditions were optimized according to the runs generated by Design Expert® Software (Version 13.0.9.0; Statease,

Table 1
Experimental conditions for BBD with values of Observed Responses.

Run order	Independent variables			Responses						
	A: Methanol concentration (%)	B: Flow rate (mL/min)	C: Injection volume (μL)	Rt of 4-OHT (min)	Rt of Hesperidin (min)	Peak area of 4-OHT (cm^2)	Peak area of Hesperidin (cm^2)	Tf of 4-OHT	Tf of Hesperidin	Resolution
1	88	0.3	10	6.82	9.37	1027470	568276	1.36	1.49	6.34
2	92	0.3	10	6.74	9.25	1048390	603872	1.28	1.55	6.51
3	88	0.5	10	3.84	5.71	1058290	628468	1.47	1.38	4.79
4	92	0.5	10	4.02	5.83	1013760	603723	1.83	1.94	4.82
5	88	0.4	8	4.93	7.08	1005670	579439	1.24	1.43	5.73
6	92	0.4	8	4.87	7.03	1012360	590274	1.27	1.64	5.81
7	88	0.4	12	4.82	7.06	1053930	617623	0.93	1.36	6.02
8	92	0.4	12	4.9	7.12	1063540	613298	1.35	1.68	6.17
9	90	0.3	8	6.52	9.37	1210260	817618	1.17	1.25	6.46
10	90	0.5	8	3.96	5.93	1193750	683796	1.67	1.73	4.83
11	90	0.3	12	6.61	9.45	1310260	837618	1.09	1.38	6.73
12	90	0.5	12	3.91	5.71	1222940	730222	1.78	1.98	4.98
13	90	0.4	10	4.97	7.12	961747	608819	0.95	1.3	6.08
14	90	0.4	10	4.99	7.12	954888	609231	1.03	1.31	5.97
15	90	0.4	10	5	7.12	972865	608792	1.06	1.31	5.7
16	90	0.4	10	5	7.12	976121	610918	1.06	1.31	6.14
17	90	0.4	10	5	7.12	975675	609464	1.07	1.31	6

Minneapolis, MN 55413, USA). A total of 17 experimental runs were performed to optimize the method parameters based on the responses obtained (Table 1). An optimized formulation was achieved by a numerical optimization technique using the desirability function approach. Table 2 lists the selection of the appropriate target levels (constraints) to attain the desired responses and the optimized method parameters.

2.7. Validation of the method

The RP-HPLC method developed for the simultaneous quantification of 4-HT and hesperidin was validated according to the ICH Q2 (R1) guidelines. The following parameters were evaluated during the validation process to ensure the reliability and robustness of the method [20].

2.7.1. System suitability

The suitability of the developed RP-HPLC method was assessed to ensure that the analytical background was appropriate for the intended application, and to verify the reproducibility of the method. This evaluation involved analysis of six replicates of a mixture containing 4-HT and hesperidin at a known concentration of 40 μg/mL. The suitability of the method for the intended purpose was determined by examining the consistency and reproducibility of the results.

2.7.2. Specificity

To evaluate the specificity of the RP-HPLC method, the potential interference between the diluent and placebo was examined at the Rt of the 4-HT and hesperidin peaks. This assessment involved injecting triplicate samples of diluent, placebo, drug solution (4-HT and hesperidin), and liposomal formulation into the HPLC system. By analyzing these injections, any observed interference or overlapping peaks at the specific retention times of 4-HT and hesperidin were identified.

2.7.3. Linearity

The linearity of RP-HPLC was evaluated by preparing a series of seven concentrations of a mixed standard solution containing 4-HT and hesperidin. The concentration range selected for the calibration curves was 1–100 μg/mL for both the analytes. Individual calibration curves were constructed by plotting the peak area of each analyte on the y-axis against their respective concentrations along the x-axis. Regression equations were generated from these calibration curves, allowing for quantification of 4-HT and hesperidin based on the peak areas obtained during analysis.

2.7.4. Sensitivity

To assess the sensitivity of the RP-HPLC method, the limits of detection (LOD) and quantification (LOQ) were determined for both analytes. The LOD and LOQ were calculated using the following formulas:

$LOD = 3.3\ \sigma/s.$

$LOQ = 10\ \sigma/s.$

In these equations, σ represents the standard deviation of the y-intercepts of the regression line and s represents the slope of the calibration curve. The LOD indicates the lowest concentration of an analyte that can be reliably detected using this method, whereas the LOQ is the lowest concentration that can be quantified with acceptable accuracy and precision [21].

2.7.5. Precision and accuracy

To assess the precision of the RP-HPLC method, intra-day (same day) and inter-day (different days) precision measurements were conducted. Six injections of 40 μg/mL solutions of 4-HT and hesperidin were performed on the HPLC system to evaluate the consistency of the results obtained within the same day and across different days. To evaluate the accuracy of the method, the recoveries of 4-HT and hesperidin were determined at three different concentrations (80%, 100%, and 120%). Standard was added to the sample solution (40 μg/mL) at 80%, 100%, and 120% of the 4-HT and hesperidin content. Each concentration level was analyzed in triplicate.

Table 2
Criteria for numerical optimization and optimized method parameters.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
A: Methanol	is in range	88	92	1	1	3
B: Flow rate	is in range	0.3	0.5	1	1	3
C: Injection volume	is in range	8	12	1	1	3
Rt of 4-HT	is in range	4.8	5.2	1	1	3
Rt of Hesperidin	is in range	6.9	7.3	1	1	3
Peak area of 4-HT	is in range	954888	980424	1	1	3
Peak area of Hesperidin	is in range	588276	627618	1	1	3
Tf of 4-HT	is in range	0.85	1.25	1	1	3
Tf of Hesperidin	is in range	1.15	1.51	1	1	3
Resolution	is in range	5.5	6	1	1	3
Optimized method parameters	Methanol	Flow rate		Injection volume		Desirability
	90.00	0.40		10.00		1.00

The recovery percentage was calculated to demonstrate the accuracy of the analyte quantification method. The percent recovery was calculated as follows: recovery (%) = (amount detected/amount spiked) × 100 [22].

2.7.6. Robustness

The robustness of the developed RP-HPLC method was evaluated by intentionally introducing deliberate variations in chromatographic conditions, as outlined in Table 3. This assessment aimed to determine the ability of the method to remain unaffected by small changes in key parameters such as methanol concentration, flow rate, column temperature, and injection volume. By systematically altering these parameters within the specified limits, the effects on the retention time and peak area of 4-HT and hesperidin were observed. The introduced variations were designed to simulate potential real-world fluctuations that may occur during routine analysis.

2.7.7. Sample solution stability and mobile phase stability

To assess sample solution stability, triplicate injections of 24-h-old samples were compared with freshly prepared samples [23]. The purpose of this evaluation was to determine any potential changes in the sample over time that could affect the accuracy and reliability of the analytical method. To quantitatively measure the similarity between old and freshly prepared samples, a similarity index was calculated using the following formula:

$$\text{Similarity Index} = \frac{\text{Peak area}_{\text{old std}} \text{ X } \text{Amt}_{\text{new std}}}{\text{Average peak area}_{\text{new std}} \text{ X } \text{Amt}_{\text{old std}}}$$

In this equation, the peak area of the old standard represents the area obtained after 24 h, whereas the average peak area of the new standard corresponds to the peak area of freshly prepared samples. Additionally, $\text{Amt}_{\text{new std}}$ and $\text{Amt}_{\text{old std}}$ denote the amount of drug present in the fresh and old samples, respectively.

To evaluate the stability of the mobile phase, both 4-HT and hesperidin were eluted using a 24-h-old mobile phase and a freshly prepared mobile phase. The purpose of this assessment was to examine any potential changes in the mobile phase composition over time that could impact the accuracy and reliability of the analytical method. To quantitatively assess the similarity between the old and freshly prepared mobile phases, the similarity index was calculated using the following formula:

$$\text{Similarity Index} = \frac{\text{Avg. peak area}_{\text{old mobile phase}} \text{ X } \text{Amt}_{\text{fresh mobile phase}}}{\text{Average peak area}_{\text{fresh mobile phase}} \text{ X } \text{Amt}_{\text{old mobile phase}}}$$

in this equation, the average peak area of the old mobile phase corresponds to the peak area obtained using the 24-h-old mobile phase, whereas the average peak area of the fresh mobile phase represents the peak area of the freshly prepared mobile phase. By calculating the similarity index, any changes or degradation in the mobile phase composition over time can be identified and evaluated. This assessment provides insights into the stability of the mobile phase and its suitability for consistent and accurate analysis using the developed RP-HPLC method.

Table 3
Results of validation parameters.

System suitability	Parameters	Acceptance criteria		Observed	
				4-HT	**Hesperidin**
	RSD of peak area (n = 6)	RSD <2.0%		0.90	0.13
	Tf	<1.5		0.95	1.30
	NTP	>2000		4158	5778
Linear regression data	Linearity (μg/ml) (n = 3)			1–100	
	Slope			53738	36032
	Y-intercept			37485	12972
	R^2			0.9994	0.9991
Precision	% RSD for intraday	<2.0%		0.12	0.19
	% RSD for interday	<2.0%		0.31	0.40
Accuracy	Initial concentration (μg/mL)	Observed mean concentration (μg/mL) n = 3		Mean recovery (%)	
		4-HT	**Hesperidin**	**4-HT**	**Hesperidin**
	32	31.09	30.43	97.16	95.12
	40	41.06	40.64	102.65	101.61
	48	47.63	47.00	99.23	97.92
LOD (μg/mL)				0.06	0.04
LOQ (μg/mL)				0.19	0.13
Robustness	Flow rate, mobile phase ratio, column temperature, injection volume	<2.0%		1.28	1.51
Stability	Sample solution (24 h)	RSD		0.94	0.93
		Similarity Index		1.00	1.00
	Mobile phase (24 h)	RSD		1.06	0.61
		Similarity Index		0.99	0.99

2.7.8. Stress studies

To investigate the degradation behaviour of 4-HT and hesperidin, the compounds were subjected to various stress conditions. Acid hydrolysis was conducted using 0.1 N and 1 N hydrochloric acid (HCl), while alkaline hydrolysis was performed using 0.1 N and 1 N sodium hydroxide (NaOH). Both hydrolysis reactions were carried out at 60 °C for 24 and 12 h, respectively. For oxidative degradation, the drug solutions were exposed to a 3 % w/v hydrogen peroxide (H_2O_2) solution for duration of 24 h. Photolytic degradation was accomplished by exposing the drug to direct sunlight for 24 h. Thermal degradation was induced by storing the drugs in an oven set at 60 °C for 24 h [24], To ensure the neutralization of the samples prior to HPLC injection, they were treated to attain a neutral pH. Subsequently, the samples were diluted and analyzed using HPLC.

2.8. Evaluation of greenness of HPLC method

The greenness of the proposed chromatographic method was evaluated using the Analytical Eco-Scale Assessment (ESA) approach. In this work, the environmental impact of the newly developed chromatographic method was considered for all steps, including sample preparation, method, development, analysis solvents, and the waste produced [25,26].

2.9. Application of the validated analytical method

The validated HPLC method was further applied to the simultaneous quantification of 4-HT and hesperidin in liposomal formulations. Drug entrapment efficiency (%EE) and cumulative drug permeation studies were performed using the developed liposomal formulation.

2.9.1. Preparation of 4-Hydroxytamoxifen and hesperidin loaded liposomal formulation

To prepare dual drug-loaded liposomal formulations, L-α-phosphatidylcholine, cholesterol, and Tween 80 were accurately weighed and dissolved in a solvent mixture of diethyl ether and chloroform (3:1 ratio). Subsequently, the drug solutions of 4-HT and hesperidin were added to the mixture, which was then maintained at room temperature to form a thin film. The film was hydrated using phosphate-buffered saline pH 7.4 containing sodium deoxycholate. The desired vesicle size was achieved by ultrasonic probe sonication [27].

2.9.2. % Drug entrapment efficiency

The percentage drug entrapment efficiency was determined using a direct method. The dual drug-loaded liposomal formulation was centrifuged at 12000 rpm at 4 °C for 15 min to obtain a white pellet. The pellet was treated with Triton X-100 (2% v/v) and vortexed thoroughly to ensure complete lysis of the liposomes and release of the drug. The lysed samples were diluted with methanol for drug extraction, followed by centrifugation at 12000 rpm at 4 °C for 15 min [5]. The drug extraction process was performed in triplicate, and all supernatants were pooled together for analysis of the entrapped amounts of 4-HT and hesperidin using the optimized and validated RP-HPLC method. Drug entrapment efficiency was determined using the following formula:

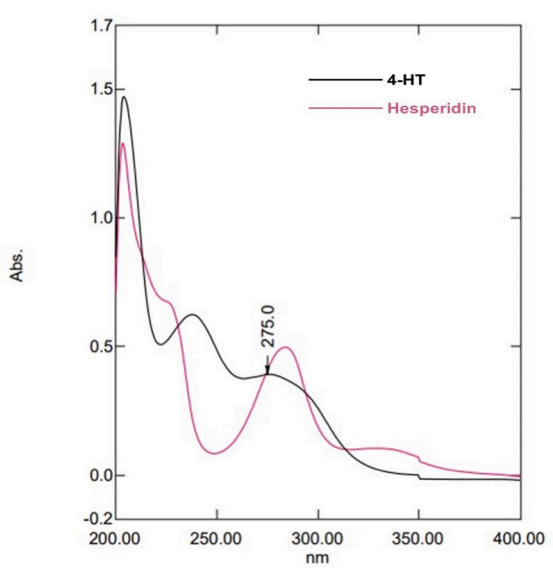
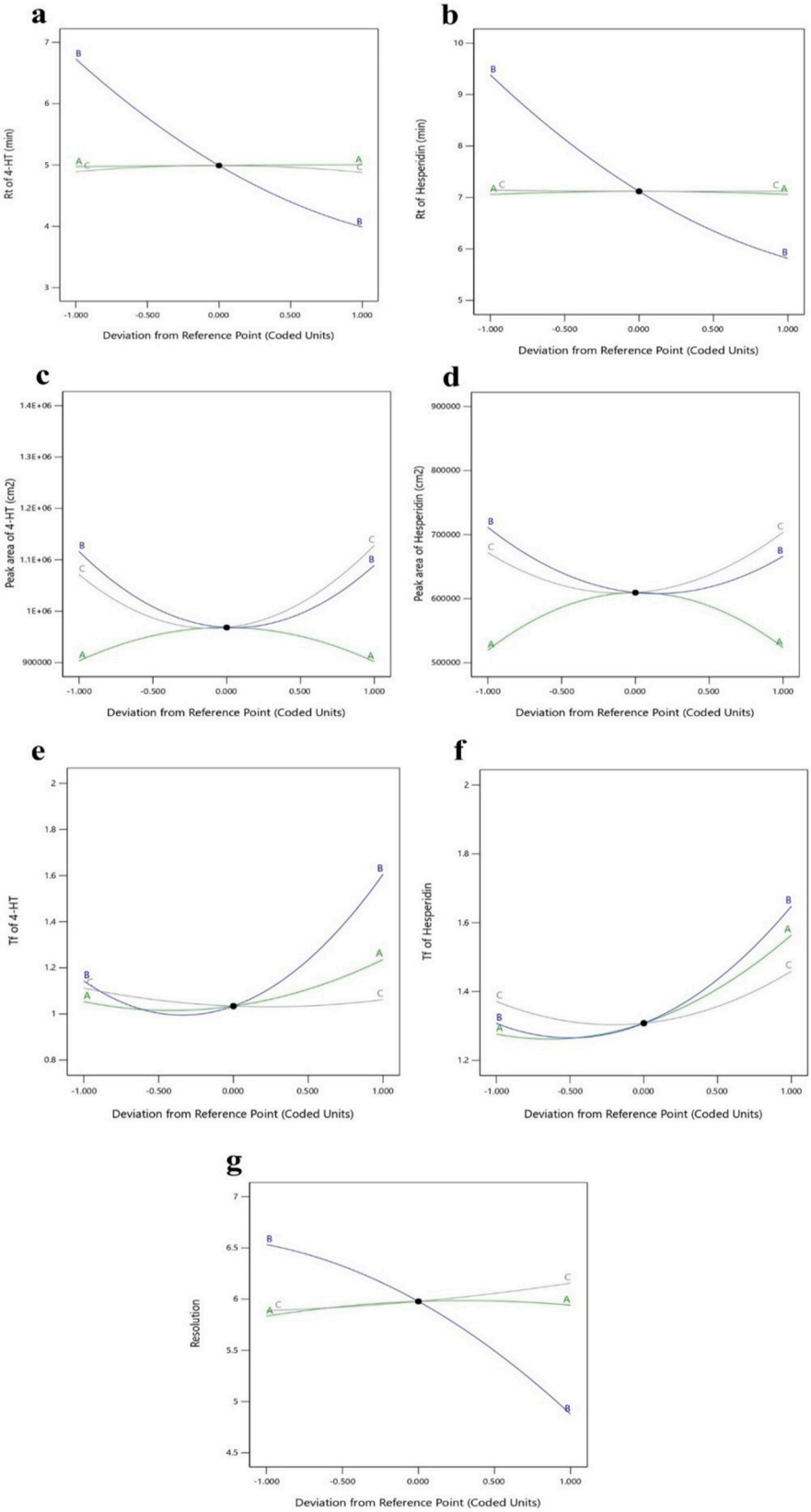


Fig. 2. Isosbestic point of 4-HT and Hesperidin.



(caption on next page)

Fig. 3. Pertubation plots (a and b) retention time (Rt); (c and d) peak area, (e and f) tailing factor (Tf) and (g) resolution. Note: A, methanol concentration (%); B, flow rate (mL/min); C, injection volume (μL). a, c, and e represent responses for 4-HT; b, d, and f represent responses for Hesperidin.

$$\%\ \text{Drug entrapment efficiency} = \frac{\text{Amount of entrapped drug}}{\text{Total amount of drug}}\ \text{X}\ 100$$

The obtained values reflect the proportion of drugs effectively entrapped within liposomes [5].

2.9.3. Ex vivo drug permeation study

Ex vivo skin permeation studies were conducted on porcine skin using a Franz diffusion cell apparatus (Electrolab EDC-07). Skin was obtained from pig ears, separated from the cartilage, and cleaned to remove fat. The skin was mounted in a Franz diffusion cell, where phosphate-buffered saline (pH 7.4) served as the receptor medium, stirred at 50 rpm, and maintained at 37 ± 0.2 °C [12]. The drug mixture and liposomal formulation containing 1000 μg of 4-HT and hesperidin were placed in the donor compartment. Samples were withdrawn at predetermined time intervals. Sink conditions were maintained throughout the investigation by replacing the withdrawn sample with an equal volume of the receptor medium. The samples were suitably diluted with methanol and analyzed using a validated RP-HPLC method for the simultaneous quantification of 4-HT and hesperidin at 275 nm.

3. Results & discussion

3.1. Selection of UV wavelength

During the analysis, it was observed that 4-HT exhibited an absorption maximum at 238 nm, while hesperidin showed an absorption maximum at 284 nm. Both drugs displayed an isosbestic point at 275 nm (Fig. 2). Based on this information, method development and subsequent validation for the quantification of both drugs were performed at a common wavelength of 275 nm. This wavelength selection allowed for the accurate and simultaneous determination of 4-HT and hesperidin in the developed RP-HPLC method.

3.2. Method optimization using BBD

A BBD was used in this study, and 17 trials were conducted. The experimental details of each trial are presented in Table 1. Analysis of variance (ANOVA) test was performed to assess the significance of the chosen independent variable model. An effect analysis was conducted to investigate the impact of each independent variable and its interaction with the various responses measured in this study. Fig. 3a-g illustrates the pertubation plots obtained from the BBD model, which visually depict the influence of the independent variables on the observed responses. These plots provide valuable insights into the magnitude and direction of the effects, enabling a better understanding of how changes in the independent variables affect the measured responses.

Based on the ANOVA results, the equation obtained using % methanol concentration (A), flow rate (B), and injection volume (C) as independent factors with Rt of 4-HT (Y_1) was:

$$Y_1 = 4.99 + 0.0150A - 1.37B - 0.0050C + 0.0650AB + 0.0350AC - 0.0350BCE - 0.0035A^2 + 0.3665\ B^2 - 0.1085C^2 \quad (1)$$

The ANOVA analysis indicated that the independent variable, flow rate (B), had a significant effect on the Rt of 4-HT (Y_1) ($p < 0.0001$), while the effect of % methanol concentration (A) and injection volume (C) had less significant influence on the p values ($p = 0.5201$ and $p = 0.8279$, respectively). The adjusted R^2 value was 0.9960, indicating a high level of goodness-of-fit for the model. Quadratic EQ. [1] indicates that the flow rate (B) has a negative effect on the Rt of 4-HT (Y_1). This can be attributed to the decrease in the interaction time between the analyte and stationary phase, resulting in a reduced retention time. Changes in the flow rate have a definite effect on Rt, as it defines the time of interaction between the stationary phase and the analyte [28].

The equation for the Rt of Hesperidin (Y_2) was obtained as follows:

$$Y_2 = 7.12 + 0.0013A–1.78B – 0.0088C + 0.0600AB + 0.0275AC – 0.0750BCE – 0.0613A^2 + 0.4812B^2 + 0.0138C^2 \quad (2)$$

The statistical parameter Y_2 revealed an adjusted R^2 value of 0.9994, suggesting a good fit of the model.

A quadratic Eq. [2] obtained by ANOVA analysis indicated that flow rate (B) had a significant effect on the Rt of Hesperidin (Y_1) ($p < 0.0001$), while the effect of % methanol concentration (A) and injection volume (C) had less significant influence as p values ($p = 0.9127$ and $p = 0.4524$, respectively). As the flow rate increased, the Rt of Hesperidin decreased. The effect analysis further demonstrated the interaction between the factors % methanol concentration (A) and flow rate (B), which showed a positive effect on the Rt of 4-HT.

Eqs [3,4] show the response of the peak areas of 4-HT (Y_3) and BD (Y_4). The statistical results revealed an adjusted R^2 value of 0.9757 for 4-HT and 0.7417 for hesperidin, indicating a good model fit.

$$Y_3 = 968300–915.12A - 13456.12B + 28578.75C–16362.50AB + 729.75AC - 17702.25BCE - 65850.85A^2 + 134600B^2 + 131500C^2 \quad (3)$$

$$Y_4 = 609400 + 2170.12A - 22646.88B + 15954.25C - 15085.25AB - 3790.00AC + 6606.50BCE - 87757.53A^2 + 79397.48B^2 + 78471.23C^2 \quad (4)$$

The effect of the independent variable, injection volume (C), was more significant ($p = 0.0018$) on the peak area of 4-HT, whereas that of % methanol concentration (A) and flow rate (B) was less significant ($p = 0.8804$ and $p = 0.0551$, respectively). An increase in the peak area of 4-HT was observed with an increase in the injection volume (C), as indicated by Eq. [3]. Theoretically, with an increase in injection volume, the number of moles of analyte available to emit the signal increases [29]. This can be attributed to an increase in the response Y_3.

A quadratic Eq. [4] obtained by ANOVA analysis indicated that all three independent variables (A, B, and C) had a quadratic effect on the peak area of hesperidin with a less significant effect ($p = 0.8832$, $p = 0.1557$, and $p = 0.2995$, respectively).

Eq. [5] explains the correlation between the Tf response of 4-HT and the methanol concentration, flow rate, and injection volume. The statistical results show an adjusted R^2 value of 0.8947, suggesting a high level of goodness-of-fit for the model.

$$Y_5 = 1.03 + 0.0913A + 0.2313B - 0.0250C + 0.1100AB + 0.0975AC + 0.0475BCE + 0.1105A^2 + 0.3405B^2 + 0.0530C^2 \quad (5)$$

The effect of the independent variables % methanol concentration (A) and flow rate (B) was more significant ($p = 0.0242$ and $p = 0.0002$ respectively) on the Tf of 4-HT, while that of injection volume (C) was less significant ($p = 0.4586$). Both independent variables A and B had positive effects on the Tf of 4-HT, as represented in Eq. [5]. This can be attributed to the increased interaction between the analyte and stationary phase, leading to stronger retention and broader peaks [30].

Similarly, Eq. [6] explains the response of Tf of Hesperidin to varying methanol concentrations, flow rates, and injection volumes. The results of ANOVA revealed an adjusted R^2 value of 0.7189, suggesting a good fit of the model.

$$Y_6 = 1.31 + 0.1438A + 0.1700B + 0.0438C + 0.1250AB + 0.0275AC + 0.0300BCE + 0.1122A^2 + 0.1697B^2 + 0.1073C^2 \quad (6)$$

The effects of the independent variables % methanol concentration (A) and flow rate (B) on the Tf of Hesperidin were significant ($p = 0.0120$ and $p = 0.0053$, respectively), whereas volume (C) was less significant ($p = 0.3397$). An increase in the methanol concentration (A) and flow rate (B) had a significant positive effect on response Y_6 as shown in Eq. [6].

Eq. [7] corresponds to the response of the resolution to varying methanol concentrations, flow rates, and injection volumes. The results of the ANOVA analysis revealed a relatively high level of goodness-of-fit of the model, with an adjusted R^2 value of 0.9540.

$$Y_7 = 5.98 + 0.0538A - 0.8275B + 0.1338C - 0.0350AB + 0.0175AC - 0.0300BCE - 0.0903A^2 - 0.2728B^2 + 0.0448C^2 \quad (7)$$

The resolution is the difference between the elution times of the two adjacent peaks. The effects of the independent variables, flow rate (B), and injection volume (C) were more significant on response Y_7 ($p \leq 0.0001$ and $p = 0.0248$, respectively), while that of methanol concentration (A) was less significant ($p = 0.2903$). An increase in the flow rate (B) had a significant negative effect on response Y_7 while the effect of the injection volume (C) had a positive effect, as shown in Eq. [7]. The negative effect of the flow rate on resolution can be attributed to the reduced separation efficiency and decreased analyte retention, leading to broad peaks and poor resolution. However, the positive effect of injection volume on resolution can be explained by the increased sample loading and improved separation between 4-HT and hesperidin [29].

Numerical point prediction was employed to select the optimal parameters. The model demonstrated a desirability value of 1, indicating its overall effectiveness in achieving desired outcomes. Based on the results obtained, it was found that the levels of methanol concentration, flow rate, and injection volume at 90 %, 0.4 mL/min, and 10 μL, respectively, yielded satisfactory outcomes in terms of Rt of 4-HT (5.05), Rt of Hesperidin (7.11), peak area of 4-HT (968259.20), peak area of hesperidin (609444.80), Tf of 4-HT (1.034), Tf of Hesperidin (1.308), and resolution (5.978). Analysis of the model using ANOVA confirmed the statistical significance of model parameters.

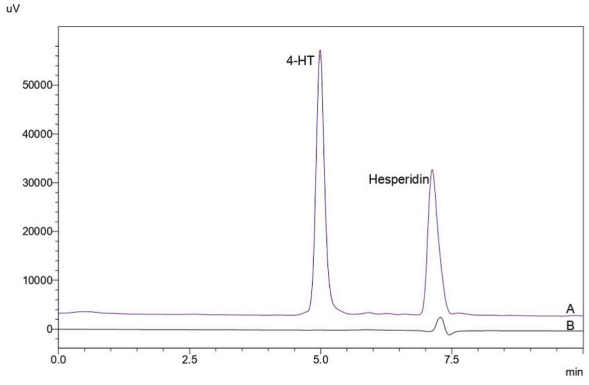


Fig. 4. Optimized HPLC chromatogram of 4-HT (10 μg/mL) and Hesperidin (10 μg/mL).

3.3. Chromatographic separation

During the chromatographic analysis, 4-HT exhibited Rt of 5.05 min, clearly distinct from the peak corresponding to Hesperidin, which had a Rt of 7.11 min. This clear separation of the two compounds can be observed in Fig. 4, which shows the complete chromatogram spanning a duration of 10 min, displaying distinct peaks corresponding to both drugs. The developed method effectively separated 4-HT and hesperidin, two basic drugs that were well-resolved using an acidic mobile phase.

3.4. Analytical method validation

3.4.1. System suitability

System suitability is a critical aspect of analytical method validation, particularly in RP-HPLC, to ensure the suitability and reliability of the chromatographic system for the simultaneous quantification of 4-HT and hesperidin. It assesses the system performance and verifies its capability to generate accurate and precise results [31]. The validation results are listed in Table 3. The relative standard deviation (RSD) values for the peak area, Tf, and the number of theoretical plates (NTP) were evaluated to assess the precision and reproducibility of the method. The obtained percentage RSD values for peak area were found to be less than 2.0%, indicating a high level of precision in the measurement of peak areas. This indicates that the method consistently and reliably quantifies analyte concentrations. A low Tf value indicates symmetrical and well-shaped peaks, suggesting the absence of peak distortion or broadening. The NTP values, which represent the efficiency of chromatographic separation, were greater than 2000. Higher NTP values indicate a higher number of theoretical plates, implying improved separation efficiency and resolution of the analytes. This demonstrated the effectiveness of the RP-HPLC method in resolving 4-HT and hesperidin, ensuring accurate and reliable quantification.

3.4.2. Specificity

Specificity is an essential parameter in method development as it determines the ability of the method to accurately differentiate and quantify the target analytes in the presence of potential interference from other components in the sample. In this study, the specificity of the developed method for the simultaneous estimation of 4-HT and hesperidin was assessed by comparing the chromatograms of various samples, including the diluent, placebo, drug solution (4-HT and hesperidin), and liposomal formulation (Fig. 5). Upon examination of the chromatograms obtained from the various samples, it was observed that there were no interferences at the Rt of either drug. Furthermore, the chromatograms of the diluent and placebo samples, which represent blank matrices without the presence of target analytes, did not show any peaks at the respective retention times of 4-HT and hesperidin. This confirmed that there were no endogenous components or matrix interference that could interfere with the quantification of the analytes. Overall, the obtained chromatograms clearly demonstrated the specificity of the developed method for the simultaneous estimation of 4-HT and hesperidin.

3.4.3. Linearity

The linearity of an analytical method is a measure of its ability to establish a linear correlation between the analyte concentration and the corresponding detector response. In this study, the linearity of a chromatographic method developed for the simultaneous quantification of 4-HT and hesperidin was evaluated. By plotting the mean peak area against the concentrations of 4-HT (Fig. 6a) and hesperidin (Fig. 6b), it was observed that the chromatographic method exhibited a linear relationship over a concentration range of 1–100 μg/mL for both analytes. The linearity of the method was further confirmed by calculating the coefficient of determination (R^2), which represents the goodness-of-fit of the calibration curve. The obtained R^2 values of 0.9994 for 4-HT and 0.9991 for hesperidin indicated a high degree of linearity, suggesting a strong correlation between analyte concentration and detector response.

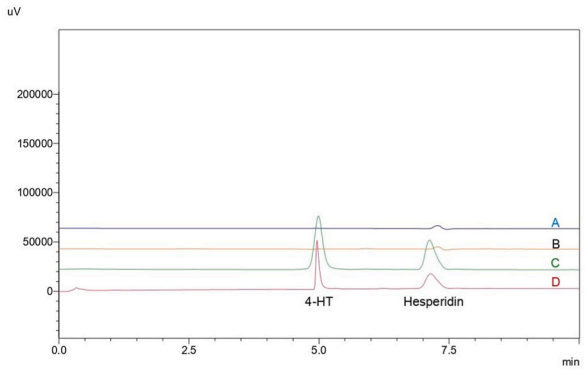
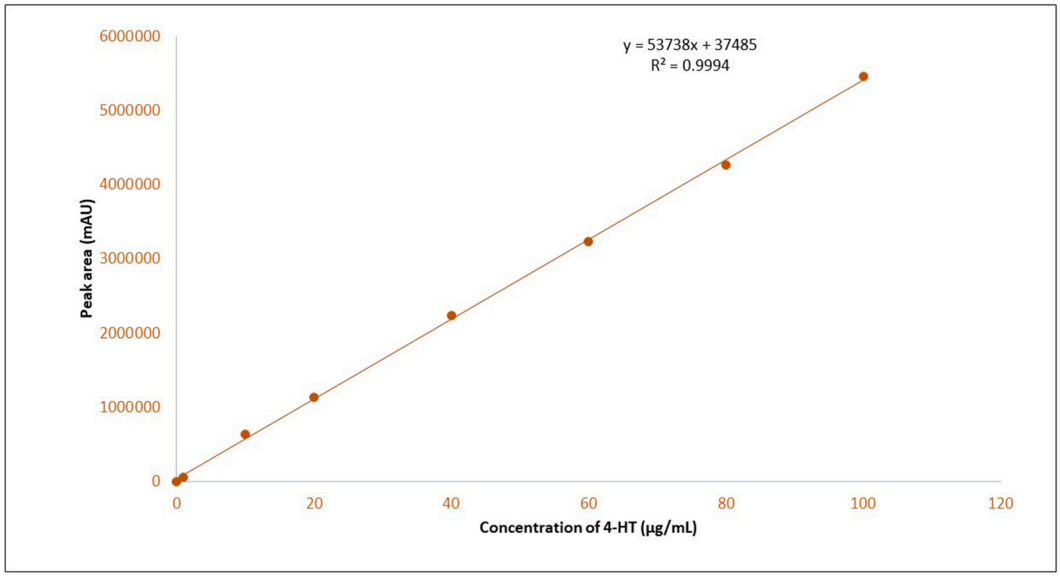


Fig. 5. Overlay chromatograms (A) Diluent, (B) Placebo liposomal formulation, (C) Standard 4-HT (10 μg/mL) and Hesperidin (10 μg/mL) solution, (D) Liposomal formulation loaded with 4-HT and Hesperidin.



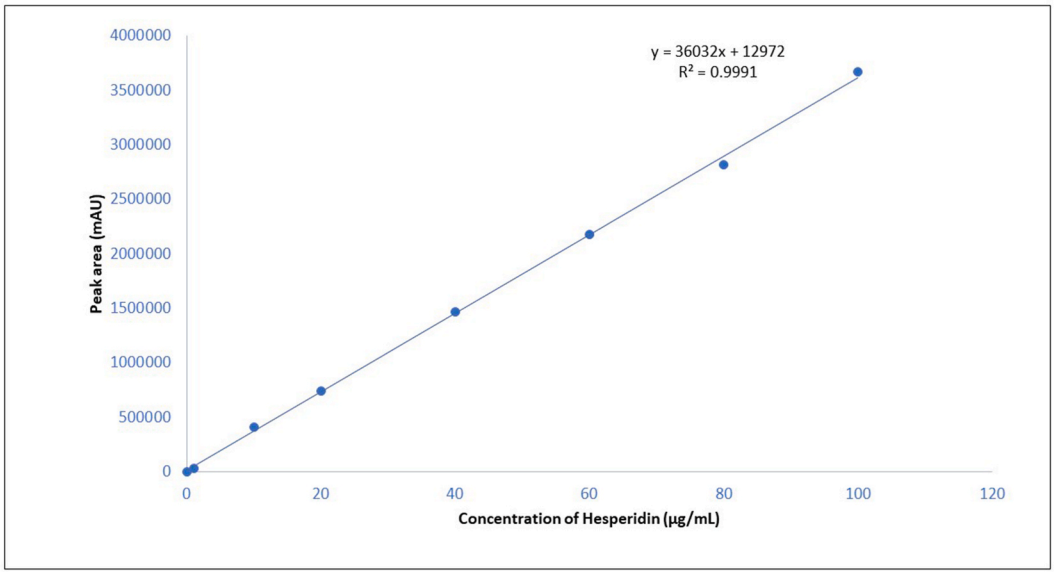


Fig. 6. Fig. 6a. Linearity of 4-Hydroxytamoxifen, **Fig. 6b.** Linearity of Hesperidin

3.4.4. Sensitivity

Sensitivity, as determined by the LOD and LOQ, plays a crucial role in analytical method validation for the simultaneous quantification of 4-HT and hesperidin using RP-HPLC. The evaluation of LOD and LOQ ensures that the method possesses the necessary sensitivity to detect and quantify drugs at appropriate levels, providing a measure of its overall sensitivity [20]. In this study, for 4-HT, the LOD was determined to be 0.06 µg/mL, indicating that this method can reliably detect 4-HT at concentrations as low as 0.06 µg/mL. The LOQ value for 4-HT was found to be 0.19 µg/mL, which represents the minimum concentration that can be accurately quantified using this method. Similarly, for Hesperidin, the LOD was calculated to be 0.04 µg/mL, demonstrating the method's ability to detect Hesperidin at concentrations as low as 0.04 µg/mL. The LOQ value for hesperidin was determined to be 0.13 µg/mL, which signifies the minimum concentration that can be accurately quantified using this method. The low LOD values indicate that the method is highly sensitive and capable of reliably detecting trace amounts of the analytes. Similarly, low LOQ values reflect the method's ability to accurately quantify analytes at low concentrations [32].

3.4.5. Precision and accuracy

Accuracy assesses the proximity of the obtained results to the true or accepted values, whereas precision evaluates the consistency and reproducibility of the results. The % recovery values of the added standard amounts were calculated. The accuracy was assessed by

evaluating the percentage recovery of the analyte, as shown in Table 3. The accuracy of the method was demonstrated by the drug recovery results, which ranged between 95 % and 102 % at low, medium, and high concentration levels. Three measurements were performed for each level. The precision of the method was confirmed by % RSD, Tf < 2 %, and NTP not less than 2000. These findings demonstrate that the developed RP-HPLC method is accurate and precise for simultaneous quantification of 4-HT and hesperidin. The close agreement between the obtained results and the true or accepted values indicates the accuracy of the method, whereas the low % RSD values reflect the high precision and reproducibility of the analytical measurements [24,33].

3.4.6. Robustness

To determine the suitability of a method for routine analysis, its robustness is essential, as it assesses the reliability and performance under minor variations in chromatographic conditions [34]. We investigated the effects of varying the flow rate, column temperature, mobile phase ratio, and injection volume using the one factor at a one-factor-at-a-time (OFAT) approach [35]. By quantifying the results with %RSD, we could express the method's consistency in response to small deviations in chromatographic conditions, considering the peak area, Rt, Tf, and NTP (Table 3), which shows that the developed method is robust for the simultaneous quantification of 4-HT and hesperidin.

3.4.7. Sample solution stability and mobile phase stability

Sample solution stability refers to the capacity of prepared sample solutions to maintain their chemical integrity and concentration over a designated period. It is crucial to assess the stability of sample solutions to ensure the reliability and accuracy of measurements obtained using a given method, even when samples are stored or analyzed over an extended duration [24]. One way to evaluate sample solution stability is to utilize a similarity index, which measures the similarity between freshly prepared sample solutions and those that have been stored. This index provides insight into the ability of the method to consistently produce reliable results over time, thereby establishing its suitability for practical applications. In our study, we evaluated the stability of the sample solution over a 24-h period. The stability of the sample solution was determined by analyzing two key parameters: the recovery and RSD. The recovery values obtained for the stored sample solutions ranged from 98 % to 102 %, indicating that the method maintained the concentration and chemical integrity of the samples effectively (Table 3). This high recovery percentage suggested that the prepared sample solutions remained stable and reliable throughout the specified timeframe.

Mobile phase stability is a critical factor in chromatographic methods, as it directly affects the consistent and reliable separation of analytes. This refers to the ability of the mobile phase composition to remain unchanged over time, ensuring consistent performance of the method. It is crucial to monitor and maintain the stability of the mobile phase in order to achieve accurate and reproducible results. In our study, we assessed mobile phase stability over a 24-h period. The stability of the mobile phase was evaluated by analyzing two key parameters: recovery and RSD. The recovery values obtained for the analytes using the mobile phase were in the range of 97 %–102 %. This indicates that the mobile phase composition remained constant and unchanged, allowing for accurate and efficient separation of the analytes. The high recovery values suggested that the mobile phase remained stable and reliable throughout the designated timeframe. Additionally, the RSD values associated with the measurements obtained using the mobile phase were below 2.0 %. The low RSD values indicated minimal variability in the analytical results, reinforcing the stability of the mobile phase composition and its ability to consistently deliver reliable separation and quantification of the analytes (Table 3).

3.4.8. Stress studies

Table 4 presents the results of stress-induced degradation studies conducted on the compounds of interest. These degradation studies are crucial for establishing the specificity of stability indicating methods. They not only provide insights into the degradation pathways and products that may form during storage but also assist in formulation development. The rationale behind conducting force degradation studies lies in the inherent dissimilarities in the chemistry of active pharmaceutical ingredients (APIs) and the formulations of each compound [36]. Fig. 7(I-VII) shows the forced degradation chromatograms for 4-HT and hesperidin. Under acidic stress conditions, 4-HT and hesperidin exhibited greater stability than under alkaline conditions. When 4-HT is exposed to hydrochloric acid (HCl), acid-catalyzed reactions occur. Protonation of the hydroxyl group (-OH) by HCl leads to the formation of positively charged species, resulting in alterations in the chemical properties of 4-HT [37]. Another possible reaction is hydrolysis facilitated by the acid, which cleaves the ether bond present in 4-HT. Similarly, when treated with HCl, hesperidin underwent acid-catalyzed reactions that resulted in the hydrolysis of the glycosidic bond, leading to the release of hesperetin and rhamnose. Acidic conditions also

Table 4
Results of Stress studies for 4-HT and Hesperidin.

Stress type	Stress condition	% Degraded	
		4-HT	Hesperidin
Acid hydrolysis	0.1 N HCl, 60 °C, 24 h	67.65 ± 0.06	65.82 ± 0.02
	1 N HCl, 60 °C, 12 h	73.58 ± 0.40	92.12 ± 0.09
Base hydrolysis	0.1 N NaOH, 60 °C, 24 h	71.34 ± 0.21	78.68 ± 0.01
	1 N NaOH, 60 °C, 12 h	95.86 ± 0.03	92.39 ± 0.06
Oxidation	3% w/v H_2O_2, RT, 24 h	68.57 ± 2.48	58.28 ± 0.34
Photolysis	Under direct sunlight, 24 h	48.64 ± 0.03	47.54 ± 0.06
Thermal	60 °C, 24 h	18.60 ± 0.23	25.51 ± 0.04

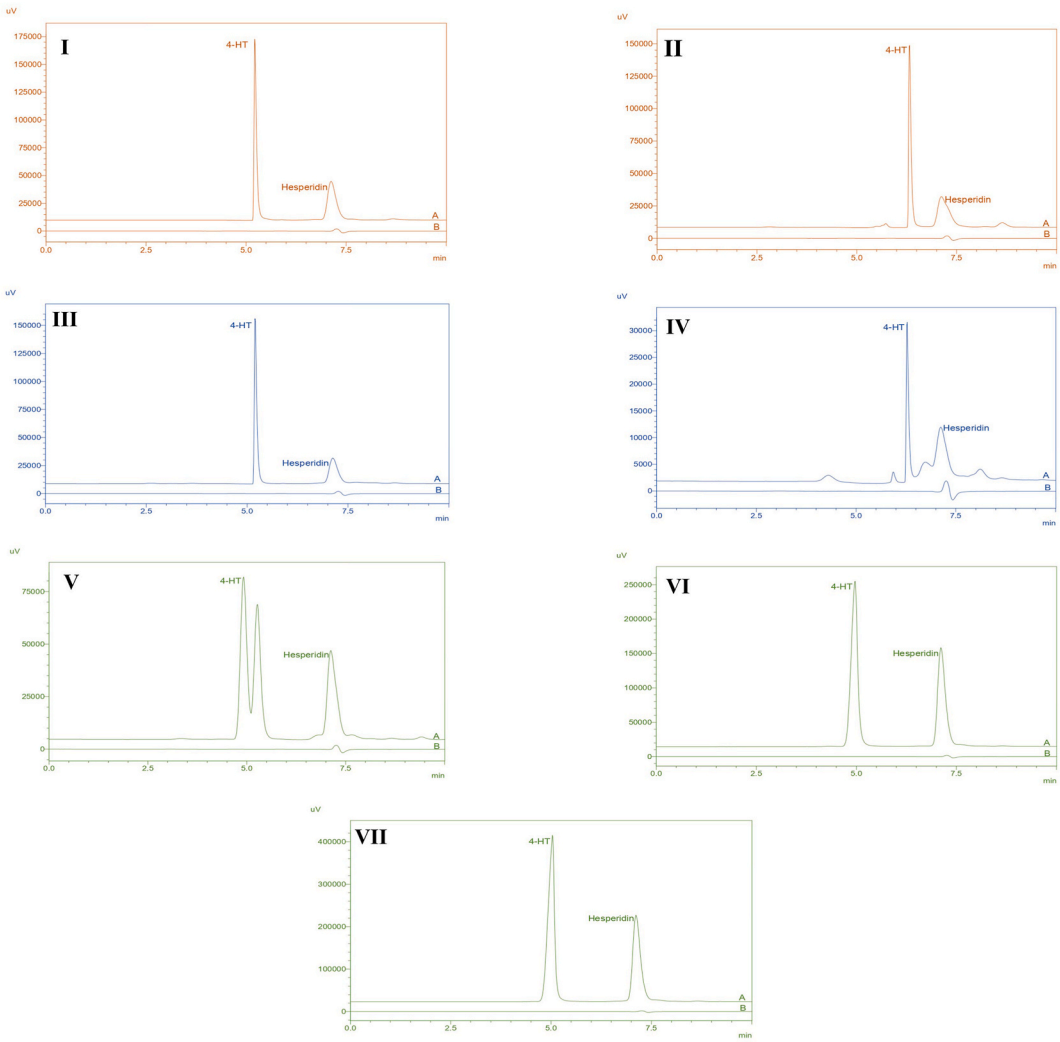


Fig. 7. Forced degradation chromatograms of 4-HT and Hesperidin obtained with (**I**) 0.1 N HCl – (A) Sample, (B) Blank, (**II**) 1 N HCl – (A) Sample, (B) Blank, (**III**) 0.1 N NaOH – (A) Sample, (B) Blank, (**IV**) 1 N NaOH – (A) Sample, (B) Blank, (**V**) 3% H_2O_2 – (A) Sample, (B) Blank, (**VI**) Photolytic degradation – (A) Sample, (B) Blank, (**VII**) Thermal degradation – (A) Sample, (B) Blank.

induce transformations in the hydroxyl groups of hesperidin, including protonation and esterification [38].

In contrast, when 4-HT is subjected to sodium hydroxide (NaOH), base-catalyzed reactions occur. The phenolic hydroxyl group in 4-HT was deprotonated by NaOH, generating a negatively charged phenoxide ion. This deprotonation significantly affects the reactivity and chemical properties of 4-HT [37]. Additionally, the reaction with NaOH results in the formation of a sodium salt by replacing the proton on the phenolic hydroxyl group. Similarly, when Hesperidin is exposed to NaOH, base-catalyzed reactions occur, leading to the deprotonation of hydroxyl groups and potential hydrolysis of the glycosidic bond [38]. The alkoxide ions formed through

Table 5
The penalty points of the proposed HPLC method according to analytical Eco-Scale.

Reagents/Instrument	Penalty points
Methanol	6
Water	0
Orthophosphoric acid	2
Occupational hazard	0
Waste	5
Energy	1
Total penalty	14
Eco-scale	86

deprotonation can engage in nucleophilic substitution or condensation reactions, leading to the formation of new products.

Furthermore, oxidative reactions using 3 % hydrogen peroxide caused significant degradation of both 4-HT and hesperidin, with degradation percentages of 68.57 % and 58.28 %, respectively. The hydroxyl group in 4-HT can be oxidized to form a ketone upon treatment with hydrogen peroxide. Similarly, Hesperidin undergoes oxidation reactions with hydrogen peroxide, resulting in the formation of various products including aldehydes, ketones, and other oxidized derivatives. In photolytic degradation studies, 48.64 % of 4-HT and 18.60 % of hesperidin were degraded. In case of thermal degradation studies, 47.54 % of 4-HT and 25.51 % of hesperidin were degraded. These degradation processes occurred because of the effects of light and heat, which led to the breaking of chemical bonds and the subsequent deterioration of 4-HT and hesperidin [39].

3.5. Greenness assessment of HPLC method

Each greenness assessment method has its own benefits and drawbacks, as well as its own procedures. The greenness evaluation of the proposed chromatographic methods is presented in the form of the penalty points and shown in Table 5. It shows that the proposed HPLC method achieved a score of 86 which is higher than 75, thus it can be considered as an excellent green analysis method [40]. Greener here means that the method is more eco-friendly as it avoids usage of toxic solvent and reagent and switch to more benign chemicals. In general, greater the number of steps involved in the procedure, the less the greenness as there will be an increased in the energy consumption and the volume of waste produced. The higher the score, the greener the developed method.

3.6. Application of the validated analytical method

Liposomes are spherical vesicles composed of lipid bilayers and offer several advantages for topical drug delivery, including enhanced skin penetration, prolonged drug release, and improved stability of encapsulated drugs [41]. Successful loading of both 4-HT and hesperidin into a liposomal formulation was achieved using the reverse phase evaporation method.

3.6.1. % Drug entrapment efficiency

The analysis of 4-HT and hesperidin within the liposomal formulations was performed without any interference from formulation excipients (Fig. 5). The entrapment efficiency of the liposomal formulations was determined to be 68.42 ± 1.26 % for 4-HT and 93.51 ± 1.02 % for hesperidin. The entrapment efficiency represents the percentage of loaded drugs that were successfully incorporated into the liposomal formulation. Additionally, it is a crucial parameter for ensuring liposome quality and is considered a regulatory requirement [42].

3.6.2. Ex vivo drug permeation study

Ex vivo drug permeation studies were performed with a standard drug mixture solution and a dual-drug-loaded liposomal formulation containing 4-HT and hesperidin. The chromatogram for the drug permeation study of dual-drug-loaded liposomes is shown in Fig. 8. At the end of 8 h, 30.78 ± 1.69 % of 4-HT and 28.78 ± 2.45 % of hesperidin permeated from the pure drugs through the skin. Whereas 60.95 ± 3.84 % in 4-HT and 62.34 ± 2.87 %, Hesperidin had permeated from the liposomal formulation through the skin as shown in Fig. 9. A significantly greater amount of drug permeation was observed in the case of liposomes compared to the pure drugs, as liposomes containing the drugs, 4-HT, and hesperidin entrapped within a lipid bilayer that resembles the stratum corneum of the skin. This structural similarity allows liposomes to interact with skin and penetrate more easily. The validated RP-HPLC method demonstrated excellent performance in separating and accurately quantifying all the individual peaks observed in the *ex vivo* drug

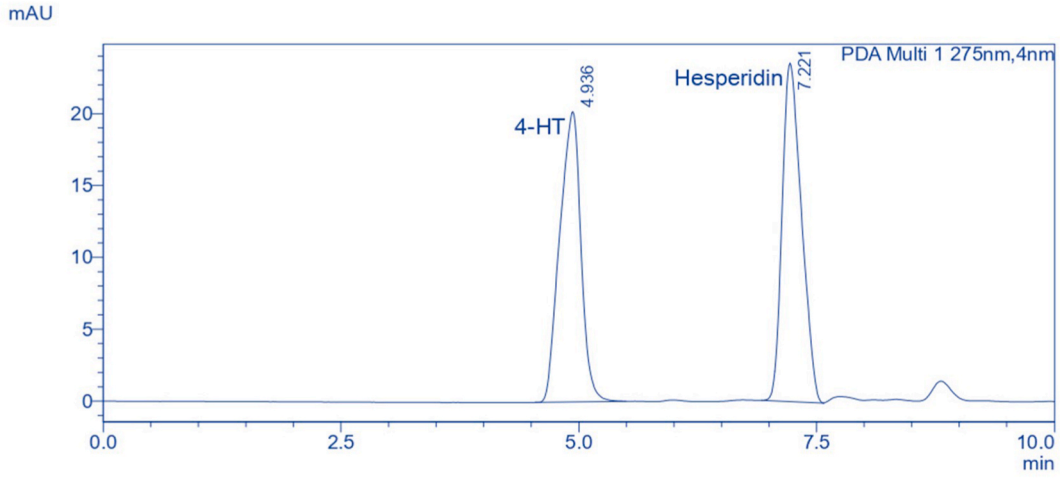


Fig. 8. Chromatogram of 4-HT and Hesperidin from skin permeation study of liposomal formulation loaded with 4-HT and Hesperidin.

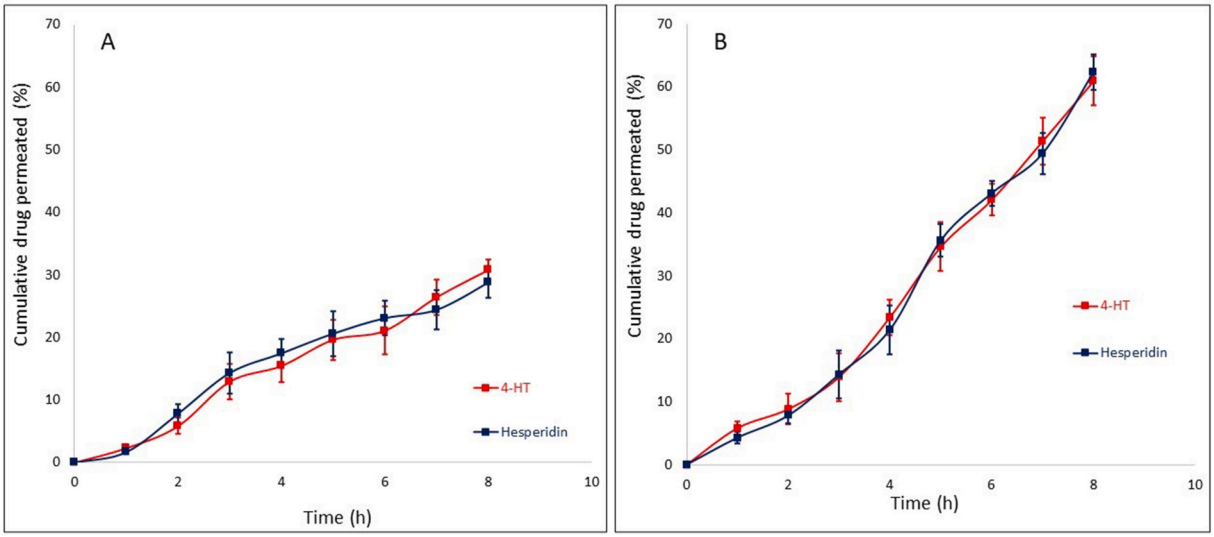


Fig. 9. Cumulative % permeation of 4-HT and Hesperidin from (A) Pure drug solution mixtures and (B) Liposomal formulation through validated simultaneous RP-HPLC method.

permeation study.

4. Conclusion and future scope

This study introduces a robust and reliable RP-HPLC method for the simultaneous quantification of 4-HT and hesperidin in liposomal formulations. The method optimization employed a Box-Behnken design (BBD), and the results were statistically analyzed using analysis of variance (ANOVA) and response surface analysis. Successful validation of the RP-HPLC method demonstrated compliance with the guidelines established by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). The validation process confirmed the accuracy, precision, linearity, selectivity, and robustness of the method, ensuring its suitability for the quantitative analysis of 4-HT and hesperidin in liposomal formulations. The validated method was effectively applied to analyze 4-HT and hesperidin in liposomal formulations under investigation. Determination of drug entrapment efficiency and calculation of the amount of entrapped drug provided valuable insights into the extent of drug incorporation into liposomal formulations, enabling accurate determination of drug content. Additionally, the developed method assessed the cumulative % permeation of 4-HT and hesperidin from the liposomal formulation through porcine skin. The dual-drug-loaded liposomes exhibited significantly higher permeation than the mixture of pure drugs. Hence, this analytical method is highly suitable for conducting comprehensive physicochemical evaluations of liposomes or any other pharmaceutical formulation containing a combination of 4-HT and hesperidin, offering valuable insights for research and development purposes. The use of less toxic and bio-accumulative reagents, along with the substitution of acetonitrile with the more environmentally friendly solvent methanol in the mobile phase, represents a step toward a more eco-friendly approach.

CRediT authorship contribution statement

Cynthia Lizzie Lobo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Manohar M:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis. **Amitha Shetty:** Writing – review & editing, Supervision, Software. **Ananya S:** Writing – review & editing, Visualization, Resources, Formal analysis. **Pallavi K:** Writing – review & editing, Validation, Software, Methodology. **Akhilesh Dubey:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Akhilesh Dubey reports financial support and article publishing charges were provided by NITTE Gulabi Shetty Memorial Institute of Pharmaceutical Sciences. Akhilesh Dubey reports a relationship with NITTE Gulabi Shetty Memorial Institute of Pharmaceutical Sciences that includes: employment. Cynthia Lizzie Lobo, Manohar M, Amitha Shetty, Ananya S, Pallavi K reports a relationship with NITTE Gulabi Shetty Memorial Institute of Pharmaceutical Sciences that includes: employment. Akhilesh Dubey has

patent pending to Akhilesh Dubey. No conflict of interest If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] C. Fabian, L. Tilzer, L. Sternson, Comparative binding affinities of tamoxifen, 4-hydroxytamoxifen, and desmethyltamoxifen for estrogen receptors isolated from human breast carcinoma: correlation with blood levels in patients with metastatic breast cancer, Biopharm Drug Dispos 2 (4) (1981) 381–390.

[2] PubChem. Tamoxifen | C26H29NO, PubChem 13 (2022) [cited 2023 Jan 9]. pp. 1–52. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/tamoxifen#section=Molecular-Formula.

[3] K.M. Schweikart, S.R. Eldridge, S.L. Safgren, T. Parman, J.M. Reid, M.M. Ames, et al., Comparative uterotrophic effects of endoxifen and tamoxifen in ovariectomized sprague-dawley rats, Toxicol. Pathol. 42 (8) (2014) 1188–1196.

[4] O. Lee, S.A. Khan, Novel routes for administering chemoprevention: local transdermal therapy to the breasts, Semin. Oncol. (2016 Feb 1) [cited 2022 Sep 11];43 (1):107–15. Available from, https://www.scholars.northwestern.edu/en/publications/novel-routes-for-administering-chemoprevention-local-transdermal-.

[5] R. Devulapally, T.V. Sekar, R. Paulmurugan, Formulation of anti-miR-21 and 4-hydroxytamoxifen co-loaded biodegradable polymer nanoparticles and their antiproliferative effect on breast cancer cells, Mol Pharm [Internet 12 (6) (2015), 2080–92. Available from: https://pubs.acs.org/sharingguidelines.

[6] T. Tanaka, T. Tanaka, M. Tanaka, T. Kuno, Cancer chemoprevention by citrus pulp and juices containing high amounts of β-cryptoxanthin and hesperidin, J. Biomed. Biotechnol. 2012 (2012).

[7] A.A.A. Khamis, E.M.M. Ali, M.A.A. El-Moneim, M.M. Abd-Alhaseeb, M.A. El-Magd, E.I. Salim, Hesperidin, piperine and bee venom synergistically potentiate the anticancer effect of tamoxifen against breast cancer cells, Biomed. Pharmacother. 105 (2018) 1335–1343.

[8] P. Patel, J. Shah, Protective effects of hesperidin through attenuation of Ki67 expression against DMBA-induced breast cancer in female rats, Life Sci. (2021) 285.

[9] S.P. Singh, Wahajuddin, M.M. Ali, K. Kohli, G.K. Jain, Liquid chromatography-mass spectrometry method for the quantification of tamoxifen and its metabolite 4-hydroxytamoxifen in rat plasma: application to interaction study with biochanin A (an isoflavone), J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 879 (27) (2011) 2845–2851.

[10] U. Sundralingam, S. Chakravarthi, A.K. Radhakrishnan, S. Muniyandy, U.D. Palanisamy, Efficacy of emu oil transfersomes for local transdermal delivery of 4-oh tamoxifen in the treatment of breast cancer, Pharmaceutics 12 (9) (2020) 1–19.

[11] J. Girault, B. Istin, J.B. Fourtillan, Quantitative measurement of 4-hydroxy tamoxifen in human plasma and mammary tumours by combined gas chromatography/negative chemical ionization mass spectrometry, Biol. Mass Spectrom. 22 (7) (1993).

[12] U. Sundralingam, S. Muniyandy, A.K. Radhakrishnan, U.D. Palanisamy, Ratite oils for local transdermal therapy of 4-OH tamoxifen: development, characterization, and ex vivo evaluation, J. Liposome Res. 31 (3) (2021 Sep) 217–229.

[13] S.S. Chimagave, S.S. Jalalpure, A.K. Patil, B.K. Kurangi, Development and validation of stability indicating UV-spectrophotometric method for the estimation of hesperidin in bulk drugs, plant extract, ayurveda formulation and nanoformulation, Indian J Pharm Educ Res 56 (3) (2022) 865–872.

[14] P. Alam, A. Alam, M.K. Anwer, S.I. Alqasoumi, Quantitative estimation of hesperidin by HPTLC in different varieties of citrus peels, Asian Pac. J. Trop. Biomed. 4 (4) (2014) 262–266.

[15] S.S. Chimagave, S.S. Jalalpure, A.K. Patil, B.K. Kurangi, Development and validation of stability indicating RP-HPLC method for estimation of hesperidin in nanotransferosome and Madhiphala rasayana—an Ayurvedic marketed product, J. Appl. Pharmaceut. Sci. 13 (2) (2022).

[16] L. Ding, X. Luo, F. Tang, J. Yuan, Q. Liu, S. Yao, Simultaneous determination of flavonoid and alkaloid compounds in Citrus herbs by high-performance liquid chromatography-photodiode array detection-electrospray mass spectrometry, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 857 (2) (2007) 202–209.

[17] J.T. Lee, L.H. Pao, C Da Hsieh, P.W. Huang, O.Y.P. Hu, Development and validation of an LC-MS/MS method for simultaneous quantification of hesperidin and hesperetin in rat plasma for pharmacokinetic studies, Anal. Methods 9 (22) (2017) 3329–3337.

[18] S. Beg, G. Sharma, O.P. Katare, S. Lohan, B. Singh, Development and validation of a stability-indicating liquid chromatographic method for estimating olmesartan medoxomil using quality by design, J. Chromatogr. Sci. 53 (7) (2015) 1048–1059.

[19] S. Bandopadhyay, S. Beg, O.P. Katare, T. Sharma, B. Singh, Integrated analytical quality by design (AQbD) approach for the development and validation of bioanalytical liquid chromatography method for estimation of valsartan, J. Chromatogr. Sci. 58 (7) (2020) 606–621.

[20] INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED TRIPARTITE GUIDELINE VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY Q2(R1).

[21] N.A. Bhaskaran, L. Kumar, M.S. Reddy, G.K. Pai, An analytical "quality by design" approach in RP-HPLC method development and validation for reliable and rapid estimation of irinotecan in an injectable formulation, Acta Pharm. 71 (1) (2021) 57–79.

[22] I.C. Pinto, C. Cerqueira-Coutinho, Z.M.F. De Freitas, E.P. Dos Santos, F.A. Do Carmo, E. Ricci Junior, Development and validation of an analytical method using high performance liquid chromatography (HPLC) to determine ethyl butylacetylaminopropionate in topical repellent formulations, Brazilian J Pharm Sci 53 (2) (2017).

[23] S. Bajaj, D. Singla, N. Sakhuja, Stability testing of pharmaceutical products, J. Appl. Pharmaceut. Sci. 2 (2012) 129–138.

[24] P. Mullick, S.P. Mutalik, A.R. Hegde, A. Pandey, P.C. Jagadish, S.G. Kini, et al., Simultaneous estimation of apremilast and betamethasone dipropionate in microsponge-based topical formulation using a stability indicating RP-HPLC method: a quality-by-design approach, J. Chromatogr. Sci. 59 (10) (2021) 928–940.

[25] E.Y. Santali, I.A. Naguib, A.M. Alshehri, Y.A. Alzahrani, A.E. Alharthi, T.S. Alosaimi, et al., Greenness assessment of chromatographic methods used for analysis of empagliflozin: a comparative study, Separations 9 (10) (2022) 275.

[26] N.S. Abdelwahab, M.M. Abdelrahman, Appraisal of the greenness profile of a chromatographic method for the simultaneous estimation of carbamazepine and oxcarbazepine, along with two potential impurities and three formulation excipients, RSC Adv. 11 (14) (2021) 7790–7800.

[27] J. Malakar, S.O. Sen, A.K. Nayak, K.K. Sen, Formulation, optimization and evaluation of transferosomal gel for transdermal insulin delivery, Saudi Pharm J 20 (4) (2012) 355–363.

[28] D.V. McCalley, Effect of temperature and flow-rate on analysis of basic compounds in high-performance liquid chromatography using a reversed-phase column, J. Chromatogr. A 902 (2) (2000) 311–321.

[29] D.B. Ren, Z.H. Yang, Y.Z. Liang, W. Fan, Q. Ding, Effects of injection volume on chromatographic features and resolution in the process of counter-current chromatography, J. Chromatogr. A 1277 (2013) 7–14.

[30] R. Tsao, R. Yang, Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography, J. Chromatogr. A 1018 (1) (2003) 29–40.

[31] Y. Harahap, N. Leony, L.M.C. Silalahi, R. Andalusia, Simultaneous quantification of tamoxifen and 4-hydroxy-n-desmethyltamoxifen levels in human plasma by liquid chromatography-tandem mass spectrometry: development and application in breast cancer patients, Int. J. Appl. Pharm. 10 (Special Issue 1) (2018) 370–375.

[32] N.A. Abdallah, M.E. Fathy, M.M. Tolba, A.M. El-Brashy, F.A. Ibrahim, A quality-by-design eco-friendly UV-HPLC method for the determination of four drugs used to treat symptoms of common cold and COVID-19, Sci. Rep. 13 (1) (2023) 1–14, https://doi.org/10.1038/s41598-023-28737-3.

[33] R. Khursheed, S.K. Singh, B. Kapoor, M. Gulati, S. Wadhwa, S. Gupta, et al., Development and validation of RP-HPLC method for simultaneous determination of curcumin and quercetin in extracts, marketed formulations, and self-nanoemulsifying drug delivery system, ReGEN Open 1 (1) (2021) 43–52.

[34] V.B. Subramanian, N.K. Katari, V. Ponnam, N. Konduru, T. Dongala, V.M. Marisetti, et al., Stability-indicating reversed-phase-HPLC method development and validation for sacubitril/valsartan complex in the presence of impurities and degradation products: robustness by quality-by-design approach, Biomed. Chromatogr. 36 (1) (2022).

[35] T. Tol, N. Kadam, N. Raotole, A. Desai, G. Samanta, A simultaneous determination of related substances by high performance liquid chromatography in a drug product using quality by design approach, J. Chromatogr. A 1432 (2016) 26–38.

[36] M. Blessy, R.D. Patel, P.N. Prajapati, Y.K. Agrawal, Development of forced degradation and stability indicating studies of drugs - a review, J Pharm Anal 4 (3) (2014 Jun) 159–165.
[37] B. Mangla, K.S. Patel, P. Kumar, K. Kohli, Validation of forced degradation and stability indicating studies of Tamoxifen in nanoformulation using spectroscopic technique, Mater Today Proc [Internet 26 (2019) 3265–3270, https://doi.org/10.1016/j.matpr.2020.02.910.
[38] S.S. Chimagave, S.S. Jalalpure, A.K. Patil, B.K. Kurangi, Development and validation of stability indicating RP-HPLC method for estimation of hesperidin in nanotransferosome and Madhiphala rasayana—an Ayurvedic marketed product, J. Appl. Pharmaceut. Sci. 13 (2) (2023) 39–48.
[39] K.A. Gaidhani, M. Harwalkar, P.S. Nirgude, World journal of pharmaceutical ReseaRch SEED EXTRACTS, World J Pharm Res 3 (3) (2014) 5041–5048.
[40] A. Gałuszka, Z.M. Migaszewski, P. Konieczka, J. Namieśnik, Analytical Eco-Scale for assessing the greenness of analytical procedures, TrAC - Trends Anal Chem. 37 (July) (2012) 61–72.
[41] N. Kothalawala, T.K. Mudalige, P. Sisco, S.W. Linder, Novel analytical methods to assess the chemical and physical properties of liposomes, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 1091 (2018) 14–20.
[42] C. Ran, D. Chen, M. Xu, C. Du, Q. Li, Y. Jiang, A study on characteristic of different sample pretreatment methods to evaluate the entrapment efficiency of liposomes, J. Chromatogr., B: Anal. Technol. Biomed. Life Sci. 1028 (2016) 56–62.