Development and Validation of HPLC Method for Determination of Benidipine Hydrochloride in Lipid Vesicles Formulations

Khater AL-Japairai[1], Syed Mahmood[2,*], Mohd Azmir Bin Arifin[1], Nadiya Abdul-Halim[1,*] and Samah Hamed Almurisi[3]

[1]*Department of Pharmaceutical Engineering, Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang, Gambang 26300, Malaysia*

[2]*Department of Pharmaceutical Technology, Faculty of Pharmacy, Universiti Malaya, Kuala Lumpur 50603, Malaysia*

[3]*Department of Pharmaceutical Technology, Kulliyyah of Pharmacy, International Islamic University Malaysia, Kuantan, 25200, Malaysia*

Abstract: In the current study, a novel high-performance liquid chromatographic method is being developed and validated to estimate benidipine hydrochloride in lipid-based pharmaceutical formulations including transfersome, ethosome, and transethosome. The chromatographic separation was accomplished on a ZORBAX Eclipse Plus C18 (4.6mm x 150mm) analytical column with a mobile phase consisting of a mixture of methanol and 50 mM phosphate buffer solution at a ratio of 70:30 (v/v). A standard calibration curve was used to quantitatively determine the medication at a UV wavelength of 237 nm. The limit of detection (LOD) and limit of quantitation (LOQ) were determined to be 0.005µg/ml and 0.015µg/ml, respectively. The relative standard deviation (%RSD) of the intra-day and inter-day studies for benidipine hydrochloride was less than 2%, and the percentage recovery of benidipine hydrochloride was found to be in the range of 98.57-100.27%. The method is specific, linear, accurate, precise, robust, and sensitive for its intended purpose, according to the results of the method validation. To determine the amount of benidipine hydrochloride and the effectiveness of drug entrapment in lipid-based formulated membrane like cell structures namely Transfersomes, Ethosomes, and Transethosomes the current method was successfully applied.

Keywords: Benidipine hydrochloride, HPLC, Lipid vesicles, Entrapment efficiency, Drug content.

1. INTRODUCTION

Benidipine HCL (BEN) is a potent and long-acting calcium channel blocker that works by blocking three kinds of calcium channels (L, N, and T). It is used for the treatment of hypertension and angina pectoris and has demonstrated renal protective effects [1, 2]. Additionally, because of increased nitric oxide generation and improved vascular selectivity, it had cardiac protective effects [3]. The chemical structure of BEN is presented in Figure **1**.

Transfersome, ethosome, and transethosome are examples of ultradeformable vesicles that have lately emerged as promising techniques for the creation of enhanced and novel transdermal therapeutics [4]. These vehicles deformable properties enhanced the efficiency of drug entrapment, permeation, and penetration of the drug through the skin membrane, and it was shown that they were more efficient than traditional drug delivery systems in these respects [5, 6].

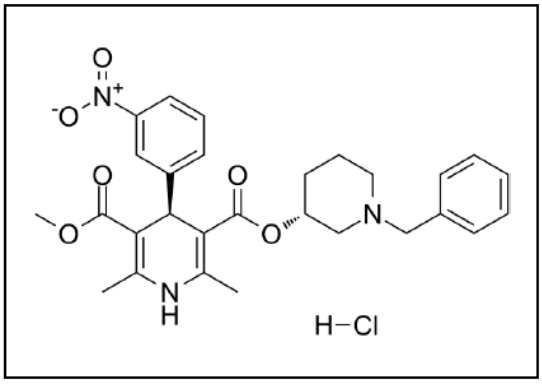

Figure1: Chemical structure of Benidipine HCl (BEN).

Controlling the ratio of free (not entrapped) to entrapped medication which is called entrapment efficiency (EE%) is crucial when creating this sort of formulation [7]. The degree of drug entrapment is a crucial property. Additionally, it is a regulatory requirement that EE% be evaluated [8, 9]. An analytical method that can be utilized to analyze the drug content and EE% is therefore urgently needed. Few analytical techniques have currently been published for the determination of BEN in formulations mostly bioanalytic methods containing BEN combined with another drug [10, 11]. The goal of the current work is to provide a rapid, sensitive, and accurate HPLC analytical

*Address correspondence to this author at the Department of Pharmaceutical Technology, Faculty of Pharmacy, Universiti Malaya, Kuala Lumpur 50603, Malaysia; Tel: +60102992943; E-mail: syedmahmood@um.edu.my

Department of Pharmaceutical Engineering, Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang, Gambang 26300, Malaysia; Tel: +60133372008; E-mail: nadiya@ump.edu.my

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approach that had been verified for the estimation and measurement of drug entrapment efficiency and drug content in existing formulations.

2. MATERIALS AND METHODS

2.1. Materials

Benidipine (BEN) was obtained from Hangzhou Hyper Chemicals Limited, China. Lipoid S75 was obtained from Lipoid GmbH, Nattermannallee, Switzerland, Sodium deoxycholate (SDC) (99.8%) was purchased from Sigma-Aldrich (St Louis, MO, USA), phosphoric acid and Potassium dihydrogen phosphate (KH_2PO_4) were purchased from Merck KGaA, Germany. Methanol, ethanol, and other HPLC-grade chemicals were purchased from Fisher Scientific Inc. (Malaysia).

2.2. Instrumentation and Chromatographic Conditions

The concentration of BEN was investigated using an HPLC system (HPLC 1260, Agilent) in conjunction with a UV detector, autosampler, and column oven. The procedure was conducted utilizing a stationary phase and a ZORBAX Eclipse Plus (C18 4.6mm x 150mm) column with a pore size of 5 µm (Agilent Technologies). The mobile phase contained a mixture of methanol and 50 mM phosphate buffer solution in a 70:30 (%v/v) (pH 4). Based on a preliminary investigation, the mobile phase and pH were chosen. Different ratios of mobile phases, including acetonitrile, methanol, and water, as well as pH-different buffers, were studied. Methanol was found to considerably improve peak symmetry and component resolution when added to the mobile phase. Additionally, the methanol ratio (60–75%, v/v) was studied. The BEN retention time shortened as the methanol ratio increased. However, when the methanol concentration was increased to over 75% (v/v), the BEN peak's resolution was poor. It was found that the sharp peak with the optimum retention period was created by methanol and phosphate buffer pH 4 at a 70:30 ratio. The column temperature was 25°C. The injection volume of the sample was 10 µl, and the mobile phase had a flow rate of 1 mL/min with a total run time of 8 minutes. The UV detector was used for drug detection at the wavelength of 237 nm which was earlier determined by the UV spectrophotometric method and the open lab control panel version A.01.05 software was used to obtain the chromatogram data (Agilent Corporation).

2.3. Preparation of BEN-Loaded Transfersome, Ethosome, Transethosome

The thin-layer rotary evaporation technique [12] was used to create transfersome, ethosome, or transethosome loaded with benidipine hydrochloride (BEN). The concentration of BEN in the pharmaceutical formulation was 1000 µg/ml. Transfersome was prepared using Lipoid S75 (300 mg) and Sodium deoxycholate SDC (30 mg) as surfactant. In a round-bottom flask, lipoid S75, BEN, and SDC were dissolved in a mixture of chloroform and methanol (2:1, v/v). At 40°C and reduced pressure, the organic solvent combination was evaporated using a rotary evaporator (IKA rotary evaporator). To get rid of any traces of solvent, the round-bottom flask was placed under a vacuum for the entire night. After the solvent had evaporated from the flask's inner surface, the lipid film layer was allowed to hydrate for one hour in distilled water (pH 6.67) at 41°C to 44°C and 120 rpm in an orbital shaker. Large multilamellar vesicles were produced by allowing the suspended vesicles to grow at room temperature for an additional two to three hours. To further minimize their size, these were sonicated then these small vesicles were passed through a 0.45 µm pore size nylon membrane. Similar steps were applied for ethosome, however in ethosome, the BEN and Lipoid S75 (300 mg) without SDC were the formulation and the hydration medium was ethanol and water in a ratio of (30:70, v/v). On the other hand, the transethosome is combined between both formulations whereas the formulation contained Lipoid S75 (300 mg), BEN, and SDC (30 mg) as transfersome and the hydration medium was ethanol and water in a ratio of (30:70, v/v) as ethosome.

2.4. Preparation of Standard Stock Solutions and Working Standard Solutions

About 50 mg of BEN was precisely weighed and diluted up to 50 ml with Methanol to prepare a standard stock solution of BEN with a concentration of 1000 µg/ml. The appropriate volume of standard stock solutions diluted with mobile phase to prepare concentrations ranging from 0.01 to 400 µg/ml of BEN using in the validation of HPLC method.

2.5. BEN HPLC Method Validation

Based on ICH Q2 (R1) guidelines, the HPLC technique for the BEN analyses was validated in terms of specificity, selectivity, LOD, LOQ, linearity, precision and accuracy, and robustness [13]. A harmonized

guideline known as ICH Q2 defines the validation criteria required for a range of drug analysis laboratory techniques and offers helpful tips on how to explore specificity, linearity, LOD, LOQ, precision, accuracy, and robustness [14, 15]. Accordingly, it serves as a broad, generally recognized basis for the validation of analytical procedures.

2.5.1. System Suitability

The system suitability was assessed by analyses of BEN (15 µg/ml) for six replicates. This validation test was conducted to determine the suitability of the chromatographic system for analysis and its reproducibility [16]. The method's suitability has been determined based on the theoretical plates, the relative standard deviation of the areas of each consecutive set of six standards (not more than 2%), and the acceptable chromatographic parameters of the produced peaks in terms of the tailing factor (acceptance limit is less than 2).

2.5.2. Specificity and Selectivity

To ensure that the excipients used in the formulations did not interfere, the specificity of the HPLC method was assessed. The specificity was studied by injecting the pure drug (BEN) and various excipients including SDC, mobile phase, lipoid S75, and ethanol. These excipients were used in the preparation of transfersome, ethosome, transethosome. The requirement for specificity acceptance is that no excipient peak should show at the typical peak of drug (BEN). Other peaks that may have appeared in the chromatogram must be separated from the drug peak (BEN) in order to avoid interfering with it.

2.5.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

For determining the LOD and LOQ, the peak area was plotted against concentrations using six points at low concentration levels (0.01, 0.05, 0.075, 0.1, 0.15, and 0.2 µg/ml) to create a calibration curve for BEN. LOD is the lowest concentration in a sample that can be detected but not always measured while the lowest concentration of an analyte that may be identified with reasonable accuracy and precision is known as the LOQ. The LOD and LOQ for the analyte concentration were quantitatively calculated from the curve using the following formulae:

$$\mathrm{LOD} = \frac{3.3\times \sigma\ (\text{Standard error of the response})}{\text{S (Slope of the regression line)}} \quad (1)$$

$$\mathrm{LOQ} = \frac{10 \times \sigma\ (\text{Standard error of the response})}{\text{S (Slope of the regression line)}} \quad (2)$$

The prepared concentration values of both limits were injected for six repeat measurements. The LOD and LOQ are accepted if the % RSD is less than 33% in LOD, and less than 10% in LOQ [17]. The equation below was used to calculate the relative standard deviation (% RSD) for both LOD and LOQ:

$$\%\mathrm{RSD} = \frac{\text{SD (standard deviation)}}{\text{M (mean value of six replicate measurements)}} \times 100 \quad (3)$$

2.5.4. Linearity and Range

The capacity to give test findings that are inversely proportional to the analyte concentration is known as linearity. Standard concentrations (1, 10, 50, 100, 200, 300, and 400 µg/mL) of BEN were prepared from the stock solution (1000 µg/mL) using the mobile phase as the diluent. The standard curve was plotted in terms of the peak area against the concentration of the prepared standards. The linearity of the standard curve was tested by determining the correlation coefficient value (R^2). This value was reported and should not be less than 0.999 for a valid analysis.

2.5.5. Precision

The precision of the assay was determined by intra-day (repeatability) and inter-day (intermediate precision). Repeatability refers to the application of the analytical process in a brief period of time (same day). On the other hand, comparing the experiments performed on various days allowed us to evaluate intermediate precision [18]. The average percentage of RSD was calculated after the evaluation of three quality control (QC) samples at concentrations of 75, 150, and 350 µg/ml, which represented the low, medium, and high concentrations.

2.5.6. Accuracy

The proximity of the data arrangement between the theoretical value and experimental value is how the methods' accuracy is expressed. Accuracy is typically expressed as a percentage of recovering the known amount of analyte. BEN was utilized in three different concentrations: 75, 150, and 350 µg/ml. The percentage of the experimental concentration to the theoretical concentration was used to compute the sample recovery. To be acceptable, the mean recovery must be between 98% and 102%.

2.5.7. Robustness

According to the ICH, robustness is the ability of analytical procedures to be unaffected by minor and intentional changes [19, 20]. Robustness was evaluated on the developed HPLC method by running the QC samples at flow rates of 0.9 ml/min and 1.1 ml/min, the column oven temperature was changed to ±2°C from 25°C in the original method, and the pH of the buffer used in the mobile phase was changed by ± 0.2. These variations were evaluated in terms of theoretical plate count, asymmetry factor, and assay (%).

2.6. Determination of Drug Entrapment Efficiency (EE%) and Drug Content (%)

An indirect method was used to assess the effectiveness of drug entrapment in the transfersome, ethosome, and transethosome formulations. To separate the vesicles from the free medication, the formulations were centrifuged at 20,000 rpm for an hour at 4°C. To further remove any unentrapped medication that was still attached to the separated vesicles, they were rinsed with distilled water once more. After that, the liquid supernatant was analyzed using the aforementioned HPLC procedure. The following equation was used to compute the drug entrapment efficiency:

$$EE\% = \frac{\text{Amount of drug added} - \text{amount of drug determined in the supernatant}}{\text{amount of drug added}} \times 100 \quad (4)$$

For drug content, about 1 mL of transfersome, ethosome, and transethosome formulations equivalent to 1000 µg of BEN was properly diluted with methanol to reach a final concentration of 100 µg/ml. The HPLC technique was used to evaluate the drug content in samples using the following equation [21]:

$$\%\ \text{Drug content} = \frac{\text{Measured concentration}}{\text{Theoretical concentration}} \times 100 \quad (5)$$

2.7. Statistical Analysis

All measurements were made in triplicate. The data were statistically analysed and presented as mean ± standard deviation using Minitab 17 software (Minitab Inc., State College, PA, USA).

3. RESULTS AND DISCUSSION

3.1. HPLC Method Validation

3.1.1. System Suitability

The standard BEN solution (15 µg/ml) was analyzed in 6 replicates as part of a system suitability test, as shown in Figure **2**. A representative chromatogram was subjected to the system suitability test to examine several parameters, including the retention time, which was discovered to be 6.045 min, theoretical plates, which were 5263, and tailing factor, which was 1.33, and percent RSD of six replicate injections, which was 0.52. Additionally, the run time is rather quick and ideal for analysis. The low run time can be attributed to the mobile phase's organic (methanol) content [22] as well as the peak's excellent peak shape and absence of tailing.

3.1.2. Specificity and Selectivity

The proposed HPLC technique was found to be selective and specific. Figure **3** depicts the chromatograms of the mobile phase, Lipoid S75, SDC,

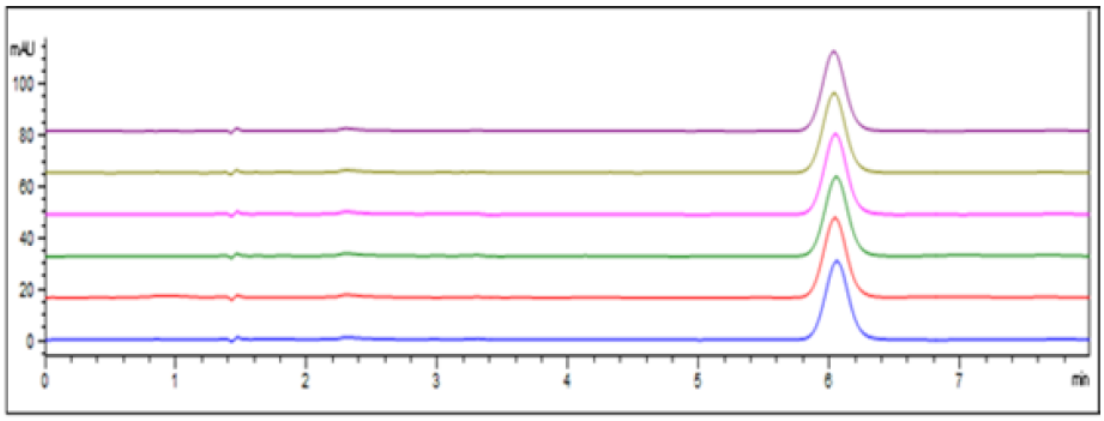

Figure 2: The chromatogram of BEN standard solution (15 µg/ml) with 6 replicates for system suitability test.

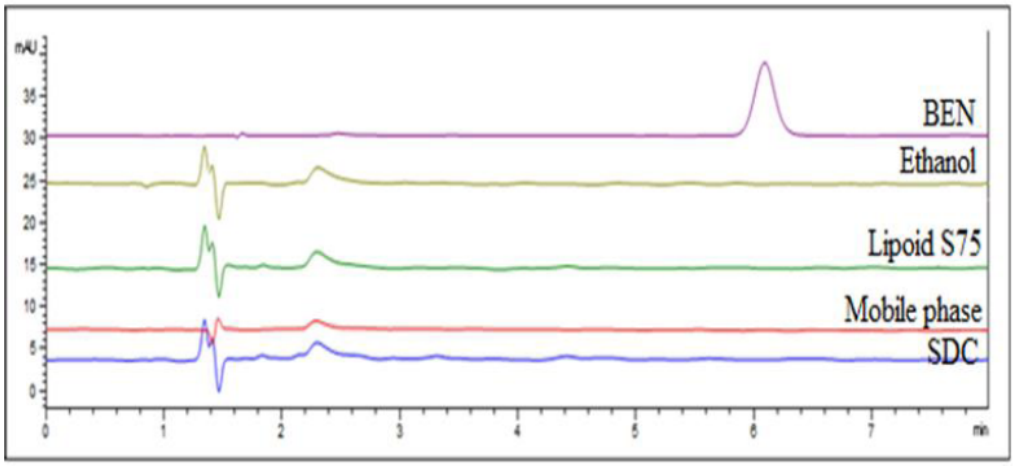

Figure 3: Typical chromatogram of SDC, mobile Phase, lipoid S75, ethanol, and BEN.

ethanol, and BEN. At the retention time of BEN, which was around 6.045 min, no interference was seen. The approach can therefore be described as selective and specific for the detection of BEN in both raw drugs and formulations.

3.1.3. Determination of (LOD) and (LOQ)

LOD and LOQ of pure BEN were calculated using equations 1 and 2 mentioned in the methodology based on the low concentration linearity curve (Figure **4**). The LOD and LOQ were predicted as 0.005µg/ml and 0.015µg/ml, respectively. Confirmation of derived LOD and LOQ concentrations was done by calculating %RSD (relative standard deviation) between areas of 6 injections of LOD and LOQ concentrations. The %RSD for LOD and LOQ were 6.56 and 4.84, respectively, which were within the acceptable limit of %RSD of 33% and 10% for LOD and LOQ, respectively. Low LOD and LOQ stand for the high sensitivity of the method [23].

3.1.4. Linearity & Range

The approach was found to be linear for BEN concentrations between 1 and 400 µg/ml with a regression equation of Y=27.294X+21.669 and an excellent correlation coefficient of 0.9998, as shown in Figure **5**. The axes Y and X, respectively, represent for the area under the curve and the BEN concentration in µg/ml.

3.1.5. Precision and Accuracy

The percent relative standard deviations of the peak area were used to express intra-day and inter-day precision. Table **1** reports the %RSD of the peak region of the intra-day and inter-day precision results. Both the intra-day %RSD value (all three concentrations) and

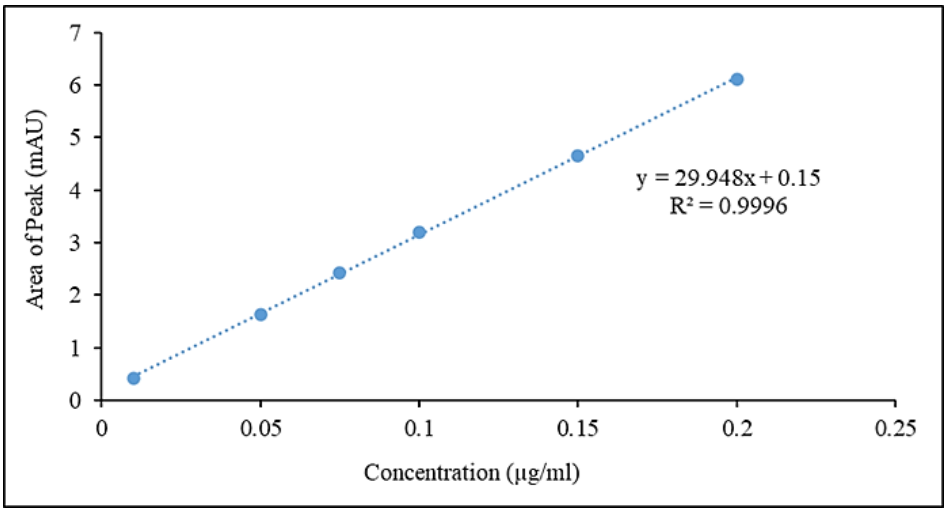

Figure 4: Low concentrations calibration curve of BEN (0.01-0.2 µg/ml).

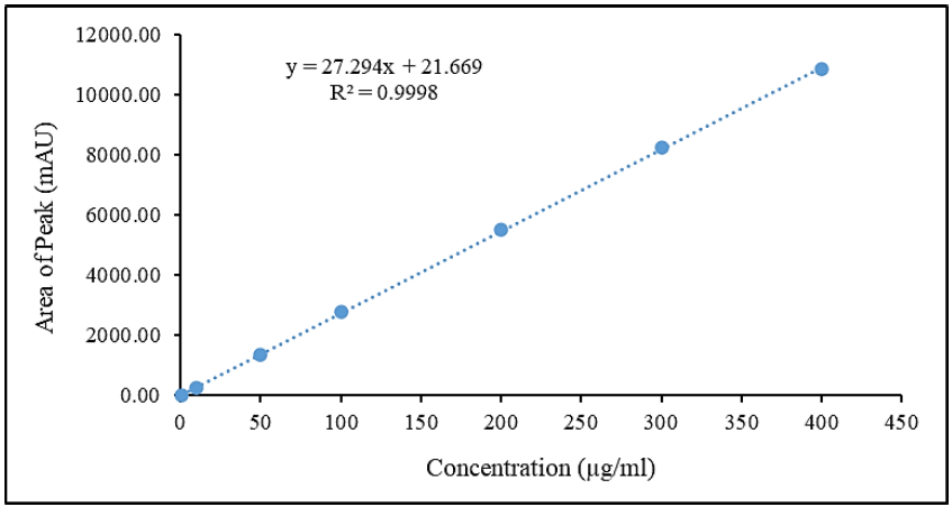

Figure 5: Standard curve of Linearity for BEN.

Table 1: Intra-Day and Inter-Day Precision and Accuracy of BEN

Conc. µg/mL	Intra-Day Precision				Inter-Day Precision			
	Measured Conc. µg/mL	SD	Precision % RSD	Accuracy % recovery	Measured Conc. µg/mL	SD	Precision % RSD	Accuracy % recovery
75	75.009	0.60	0.47	100.013	74.91	0.35	0.47	99.89
150	149.94	0.57	0.38	99.96	148.26	1.44	0.977	98.84
350	349.83	1.77	0.507	99.95	348.55	1.11	1.11	99.58

the inter-day %RSD value (all three concentrations) were determined to be less than 1% and 2%, respectively. The percent recovery ranged from 98.57 to 100.27% for these three distinct concentration levels. An analytical approach is considered to be more accurate, according to ICH Q2R1 (2005), when the recovery is between (98-102%) and the relative standard deviations were less than 2%. The recovery (%) does not have to be 100 % and in most cases the recovered values are within the range of 98-102% [24, 25]. The percentage of the recovery results may be influenced by the concentration of analyte present in the matrix [26].

3.1.6. Robustness

The approach is robust to tiny deliberate modifications applied to the flow rate, column temperature, and pH of the buffer utilized because no significant changes were identified when small variations to the chromatographic conditions were made. The percentage assay value of the injected solution was unaffected by the modest variations in the

Table 2: Robustness Data of the Proposed HPLC Method

Parameter	Level	Assay (%)	Theoretical Plate Count	Tailing Factor
Flow rate of mobile phase (mL/min)	0.9	100.5 ± 0.12	3516	1.28
	1.1	100.4 ± 0.25	3348	1.25
Column oven temperature (°C)	23	98.8 ± 0.72	3528	1.23
	27	99.2 ± 0.51	3191	1.21
pH of buffer used in the mobile phase	3.8	98.78 ± 0.91	6990	1.33
	4.2	100.5 ± 0.26	7235	1.31

various chromatographic settings because BEN peaks were always symmetric (tailing factor less than 2). The robustness investigation also indicated that the system appropriateness characteristics were adequate.

3.2. Determination of Drug Entrapment Efficiency (EE%) and Drug Content (%)

The entrapment efficiency and drug content for each formulation were calculated and summarised in Table **3**. The ethosome had the lowest EE% whereas the transferosome had the highest. That's because ethanol causes the vesicle membrane to become more permeable, which causes the entrapment efficiency to drop [27]. On the other hand, the drug content of formulations was found in the range of 98-101% and these values are within the acceptable range. The good EE% and drug content results were observed in the study conducted by Mayangsari *et al*., [28] to prepared berberine loaded transfersomes formulations, the EE% was in ranging from 88.64 ± 1.31 to 93.97 ± 0.31 and the drug content was found to be ranged from 98.34 to 105.87%. On the other hand, in the study conducted by Dol *et al*., [29] for preparing ranolazine loaded ethosome formulation, the EE% was in the range of 57.57 ± 1.08 to 87.29 ± 1.23 and the drug content of the optimized formulation was 100 ± 1.82%. The EE% ethosomes formulation decreased from 87.29 to 57.57% due to an increase in ethanol percentage from 25 to 45%. By comparing with our results, the EE% of BEN loaded ethosomes was in a higher range that might be attributed to lower ethanol content (30%).

Table 3: The Result of (%EE) and Drug Content (%) of BEN in Three Different Formulations

Formulation Type	%EE	Drug Content (%)
Transfersome	98.29 ± 0.52	101.19 ± 0.31
Ethosome	88.96 ± 0.91	98.69 ± 0.52
Transethosome	92.41 ± 0.66	99.5 ± 0.27

CONCLUSION

The HPLC method was developed for estimating benidipine HCL (BEN) in pharmaceutical formulations of transfersome, ethosome, and transethosome. Additionally, this HPLC method was validated in terms of system suitability, specificity, LOD, LOQ, linearity, precision, accuracy, and robustness in accordance with ICH criteria to confirm the suitability of this method for the routine analysis of BEN.

The Method found to be specified for estimating the BEN with existing with other excipients. Also, all the results were shown to be within limits with Low %RSD values for validation such as accuracy, precision, and linearity, which suggests that the approach is accurate and sensitive. Also, the HPLC method is robust with minor chromatographic condition modifications. Therefore, this HPLC method is applicable to laboratory work.

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DECLARATION OF COMPETING INTEREST

The authors report no conflict of interest.

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