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DESIGN AND DEVELOPMENT OF OPTIMIZED BERBERINE CHLORIDE FORMULATION FOR TREATMENT OF DIABETES

Ms Priya Sharma[a], Dr Amarjeet Singh[b], Dr Navjot Singh Sethi[b]

[a*]Research Scholar, IEC School of Pharmacy, IEC University, Baddi, H.P., India
[b] Professor, IEC School of Pharmacy, IEC University, Baddi, H.P., India
[b] Associate Professor, IEC School of Pharmacy, IEC University, Baddi, H.P., India

***Corresponding Author:** Ms Priya Sharma
*Research Scholar, IEC School of Pharmacy, IEC University, Baddi, H.P., India

Abstract

Aim: To design and development of optimized berberine chloride formulation by using the phospholipid complexation and permeation enhancer for the treatment of diabetes.

Material and methods: The phospholipid complex was prepared by solvent evaporation method using the soya lecithin, Phospholipid 90G and lipoid S 100. The prepared phospholipid complex was evaluated for the physical appearance, FTIR, percentage of drug complexing efficiency, total drug content and percentage drug loading and *in-vitro* permeation study. The berberine loaded phospholipid complex using phenyl piperazine and methyl piperazine were used as permeation enhancer. The formulated complex was evaluated physical appearance, pH, FTIR, drug content, permeability study, *In-vitro* permeation study, stability study and *In-vivo* pharmacokinetic study.

Results: The preformulation study of Berberine HCl involved investigating various parameters such as organoleptic properties, melting point, partition coefficient, solubility, and absorption maxima. Berberine HCl exhibited a yellow color with a bitter taste, a melting point, partition coefficient. Solubility testing revealed its sparing solubility in methanol and slight solubility in ethanol, water, and phosphate buffer pH 6.8. FTIR analysis confirmed the presence of characteristic functional groups in Berberine HCl. Furthermore, the formation of a complex with phospholipids was indicated by shifts in characteristic peaks. Physical appearance assessments of the berberine HCl-phospholipid complex formulations showed a consistent yellow color. Percentage drug complexation ranged from 56.098% to 80.466%, with solubility in n-octanol and water varying across formulations. In vitro permeability studies demonstrated enhanced berberine HCl permeation from the complexed formulations, notably DBPC14. The stability study indicated no significant changes in physical appearance or drug content over three months. *In-vivo* pharmacokinetic analysis revealed improved bioavailability and absorption of berberine HCl from the phospholipid complex DBPC14 compared to the pure drug and a mixture with phenyl piperazine. These findings suggest the potential of berberine HCl-phospholipid complexes for enhanced therapeutic outcomes in the treatment of metabolic disorders such as diabetes mellitus.

Conclusion: From the present study, it can be concluded that the importance of optimization of formulation development using quality by design strategy to achieve phospholipid with consistent quality.

Keywords: Berberine HCl, Diabetes Mellitus, Permeation enhancer, Quality by design, Phospholipid complex.

1. Introduction

Diabetes mellitus (DM) is an endocrine system metabolic disorder. The International Diabetes Federation (IDF) reports that 8.3% of the global population between the ages of 20 and 79 had diabetes mellitus in 2013. In worldwide, 382 million patients were projected, with an estimated increase to almost 592 million by 2035 (Atlas 2015). The high prevalence of DM poses a serious threat to public health since it has a substantial effect on both the financial burden of the healthcare system and people's quality of life (Wang, Wang, and Chan 2013). Around 90 to 95% of the world's diabetic people suffer from type 2 diabetes mellitus (T2DM) (Xie, Zhao, and Zhang 2011; Zimmet, Alberti, and Shaw 2001). The Berberidaceae family includes a large number of woody trees spread out across the genus *Berberis*. *Berberis vulgaris L.*, the most well-known species, is grown in large numbers and used in food in the Middle East and Mediterranean. Berberine is a type of benzylisoquinoline alkaloids and is an ammonium salt. It is the primary active component of Berberis species and is typically administered orally for conditions such as diabetes (hyperglycemia), elevated cholesterol or other lipids in the bloodstream (hyperlipidemia), and hypertension.

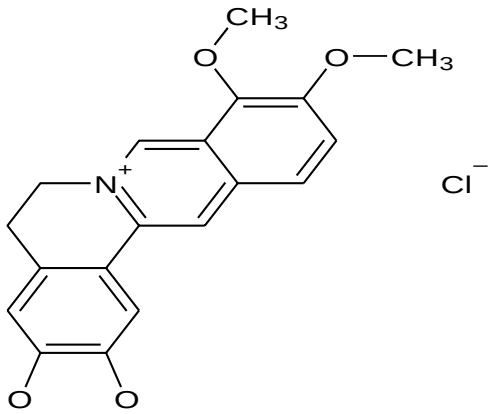

Figure 1: Chemical structure of berberine HCl.

2. Materials and Methods

2.1 Materials

Berberine HCl was obtained from Yucca enterprises, Mumbai. Phenyl piperazine was obtained from Synova chemicals, Mumbai. Phospholipid 90G and Lipoid S100 were purchased from Lipoid. Methanol, Potassium dihydrogen orthophosphate and Sodium hydroxide were purchased from the Finar Ltd. Company. All other chemicals and reagents used were of analytical reagent grade.

2.2 Methodology

2.2.1 Pre-formulation studies

2.2.1.1 Organoleptic Properties

Visual observations were employed to perform the properties associated with sensations like color, Odour, and taste. The current investigation was performed by adding a small amount of the test samples berberine HCl over the piece of butter paper and investigating in a well-lit area (Thumma et al. 2008).

2.2.1.2 Melting point

The capillary tube method was employed to assess the melting point of the berberine HCl via a melting point apparatus (Thumma et al. 2008).

2.2.1.3 Solubility studies

The solubility character of the berberine HCl was investigated in a variety of solvents, including ethanol, methanol, phosphate buffer pH 6.8, and water separately. The saturated solution of berberine HCl in each solvent was developed by adding the surplus amount of the berberine HCl in a tube with

2 ml of each solvent separately. The suspension containing vails was transferred into the maintained water bath shaker, the vials holding the resulting solutions at 100rpm and 37°C, and the suspension for 24 hours in triplicate. Each sample was withdrawn from the water bath shaker, and rotated at 10000 rpm and 20 minutes at 37°C, the supernatant was filtered and suitably diluted with methanol. The samples were scanned at a range of between 200nm-400nm employing the UV spectrophotometer for quantitive estimation of berberine HCl in each sample separately (Elsheikh et al. 2018; Thumma et al. 2008).

2.2.1.4 Fourier transform Infrared spectroscopy (FTIR)

The FT-IR spectra of pure berberine HCl and phospholipid (Lipoid S 100) using the KBr pellet method in the region of 400-4000 cm^{-1} (Dora et al. 2017).

2.2.1.5 Partition coefficient studies

The investigation related to the partition coefficient property of berberine HCl was performed by employing a mixture of the n-octanol and water. Both solvents were mutually saturated in a 1:1 ratio before conducting the tests. The excess amount of berberine HCl was mixed with a 4ml solution comprising n-octanol and water solvents separately to create a saturated solution. Mix the solution uniformly for 15 min to disperse the drug molecule into the mixture of the solvents for 15min and transfer the resultant solution into the separating funnel and keep the funnel for 24 hours at 25°C. After 24 hours, separate both layers, centrifuge both layers at 5000rpm at 25°C, filter the supernatant, and suitably dilute with methanol resultant samples were charged into the UV-VIS spectrophotometer to determine the concentration of berberine HCl in n-octanol and water separately (Elsheikh et al. 2018; Thumma et al. 2008). The partition coefficient of the berberine HCl in a mixture of the n-octanol and water was determined by employing the following equations (1):

$$\textbf{Log p}=\frac{Concentration of drug\ in\ nonaqueous\ phase}{Concentration\ of\ drug\ in\ aqueous\ phase} \quad \textbf{(1)}$$

2.2.2 Spectrophotometric Measurements

2.2.2.1 Selection of Wavelength

Stock solutions of berberine HCl containing 0.1mg/ml concentration in methanol were prepared. Weighed 10mg of berberine HCl was added into the 100ml of the volumetric flask containing 50ml of methanol, sonicated for 1 min, and volume was made up to the mark with the methanol, resulting in a solution with a concentration of 100 µg/ml labeled as stock solution.

Aliquots of 0.5ml from the stock solution were withdrawn, and transferred to the 10ml volumetric flask containing a small amount of the methanol. The volume of volumetric flask was made up to mark with methanol resulting in a working solution with a concentration of 5µg/ml. The UV spectrum of the working solution was recorded using UV spectroscopy. The working solution was transferred to the cuvette and transferred to the UV spectrophotometer. The working solution was scanned in the UV range of 200-400 against methanol solvent as the reference spectrum of berberine HCl samples was recorded (Li et al. 2020; Mendez, Steppe, and Schapoval 2003).

2.2.2.2 Preparation of calibration curve

Stock solutions of berberine HCl containing 100 µg/ml concentration in methanol were prepared by solubilizing accurately weighed 10mg of berberine HCl and transferred into the 100ml of the volumetric flask containing 50ml of methanol, sonicated it for 5 min, finally, volume was made up to the mark with the methanol to make 100µg/ml stock solution. Aliquots accurately measure volumes of 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml. 0.5 ml, 0.6 ml, 0.7 ml, 0.8ml, 0.9 ml, 1.0 ml, and 1.1 ml from the stock solution were withdrawn individually in 10ml volumetric flask and volume was made up to mark with methanol to give the working solutions concentration of 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml, 5µg/ml, 6µg/ml, 7µg/ml, 8µg/ml, 9µg/ml, 10µg/ml, and 11µg/ml respectively. It was scanned in the

UV range (200-400 nm) in a 1.0 cm cell against the solvent blank, the spectrum of berberine HCl samples was recorded.

2.2.2.3 Methods validation

Developed UV method for the estimation of berberine HCl was validated in terms of parameters like linearity, precision, robustness, ruggedness, accuracy, limit of quantification (LOQ) and limit of detection (LOD) using predefined calibration standards as portrayed below.

2.2.2.3.1 Linearity

The linearity of an analytical method is its ability to elicit that test results are proportional to the concentration of drug in samples within a given range. The linear range of the method must be determined regardless of the stage of the formulation during drug development. Linearity was evaluated by regression analysis of the berberine HCl standard solution measured in triplicate at 11 different concentrations ranging from 1 to 11µg/ml. Measurements were taken in triplicates (n = 3). The values are reported as the mean ± S.D. of the calibration curves (AM Fiorentino and RN Salgado 2012; Ich 2005; Li et al. 2020).

2.2.2.3.2 Accuracy

Accuracy was determined using recovery experiments. A solution containing known concentration of berberine HCl was used for this purpose. From the absorbance at the selected wavelength potency was calculated. The accuracy was assessed from the test results as the percentage of the drug recovered by the assay at 3 levels. The method accuracy was determined by measuring the reference standard recovery in triplicate at three levels from 80 to 120% of the method concentration (6 µg/ml), according to ICH recommendations. A standard stock solution of 100µg/ml of berberine HCl was prepared. In volumetric flasks of 10 ml, aliquots of 0.4ml, 0.6ml, and 0.8 mL of the stock solution were diluted up to 10ml volumes with methanol. Therefore, the final concentrations were 4µg/ml, 6µg/ml, and 8µg/, which correspond to 80, 100, and 120% of the target concentration, respectively. The mean recoveries of berberine HCl, expressed in terms of the percent recovery and relative standard deviation (R.S.D.), were determined (AM Fiorentino and RN Salgado 2012; Ich 2005).

2.2.2.3.3 Precision

Repeatability

Repeatability expresses the precision under the same operating condition over a short interval of time. It is also termed intra-assay precision. A minimum of six replicate sample preparation of the same sample or homogenous sample prepared at the 100% test concentration. Repeatability was done by analyzing 6µg/ml concentrations of berberine HCl in methanol six times on a single day.

Intermediate precision

Intermediate precision reflects within laboratory variations on different days. Intermediate precision was done by analyzing the same three concentrations on six different days. Reproducibility was determined by analyzing three different concentrations of berberine HCl (4 µg/ml, 6µg/ml, and 8 µg/ml in six different days (AM Fiorentino and RN Salgado 2012; Bhavyasri, Surekha, and Rambabu 2019; Ich 2005).

2.2.2.3.4 Limit of quantification (LOQ) and Limit of Detection (LOD)

The LOQ is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. There are different approaches to the determination of LOQ. LOQ is a parameter to the quantitation of the sample at a low level in the compound and is used particularly for the impurities, degradation products and/or residual solvents. LOD and LOQ can be established from signal to noise ratio method and linearity curve method (Ich 2005). The limit of detection (LOD) and the limit of quantification (LOQ) of the method were obtained from equations (2) and (3),

$$LOD = \frac{3.3 \times SD}{S} \text{.....(2)}$$

$$LOQ = \frac{10 \times SD}{S} \text{.....(3)}$$

Where S.D. is the intersection standard deviation and S is the average slope, obtained from the analytical curves of the linearity study.

2.2.2.3.5 Ruggedness

The ruggedness of the method was determined by carrying out the analysis by the different analysts in laboratories using a UV spectrophotometer and the respective absorbance of 6 µg/ml was noted. % RSD was calculated (AM Fiorentino and RN Salgado 2012; Ich 2005).

2.2.2.3.6 Robustness

The robustness of an analytical procedure measures its capacity to remain unaffected by small but deliberate variations in method parameters and indicates its reliability during normal usage. The robustness of the proposed method was also determined at different wavelengths i.e. 344nm, 345nm, and 346nm. Percentage Mean recovery was reported. Analytical methods are generally known as robust if the percent recovery is within 98-102% (Bhavyasri, Surekha, and Rambabu 2019; Ich 2005).

2.2.3 Preparation of Berberine HCl Phospholipid complex

Berberine HCl Phospholipid complex (BHCl-PC) was prepared by solvent evaporation method with slight modifications (Peng et al. 2012). A measured amount of berberine HCl and phospholipid in a suitable molar ratio of 1:1 was transferred to a round bottom flask containing 10 ml of ethanol. The dispersion containing a round bottom flask was attached to the reflux condenser and the solution was refluxed at 60°C for 12 h. The dispersion was subjected to a rota evaporator to evaporate the organic solvent at 60°C, 100rpm, and reduced pressure, resulting thin film of the BHCl-PC complex obtained around the surface of the flask. The prepared complex was vacuum-dried overnight and stored in an airtight container at below 20°C (Dora et al. 2017).

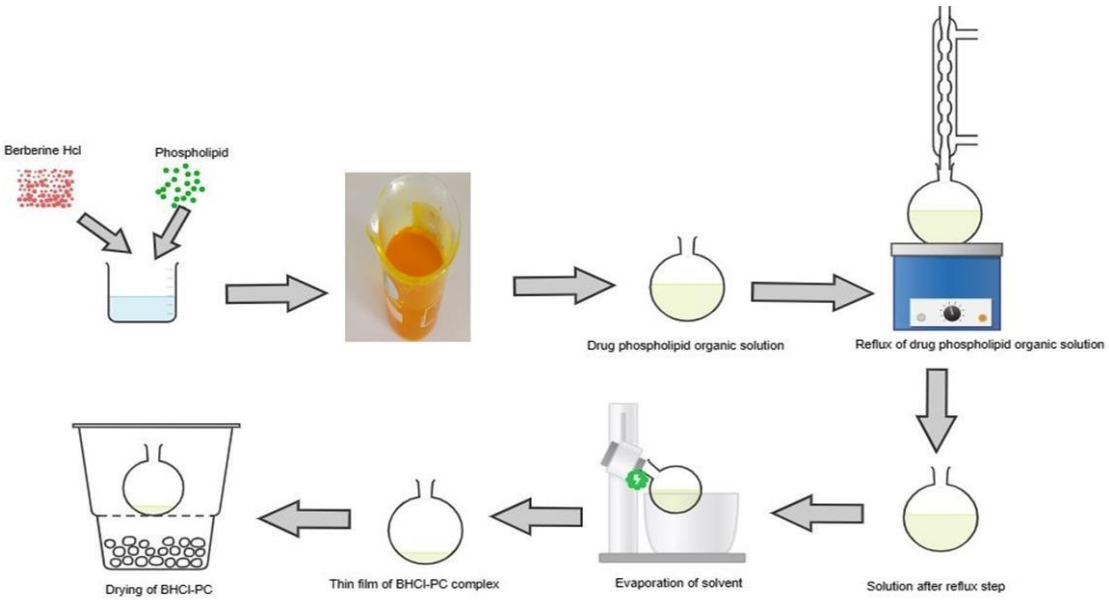

Figure 2: Method of preparation of BHCl-PC complex.

2.2.4 Screening of Berberine HCl Phospholipid complex

2.2.4.1 Effect of type of soya lecithin

The current screening parameters involve the investigation of the influence of the various phospholipids over the physicochemical properties of the drug phospholipid complex. Phospholipids like soya lecithin, Phospholipon 90G, and Lipoid S 100, were employed to prepare the complex. The

other parameters like a drug: phospholipid ratio 1:1, complexing solvent ethanol, the volume of complexing solvent 10ml, reflux temperature 60°C, and total period for the refluxing 12hr remained the same throughout the screening parameter. The prepared drug phospholipid complex was further characterized by its physical appearance, percentage drug content, complexation efficiency, and solubility in water and n-octanol (**Table 1**).

Table 1: BHCL-PC complex under the presence of the various phospholipid.

S. No.	Formulation code	Drug: Phospholipid molar ratio	Drug BHCl amount (molar) (molar)	Phospholipid amount			Solvent	Temperature (°C)	Reflux time (Hr)
				Soya lecithin	Phospholipid 90G	Lipoid S 100			
1	BPC1	1:1	0.02	0.02	-	-	Ethanol	60	12
2	BPC2	1:1	0.02	-	0.02	-	Ethanol	60	12
3	BPC3	1:1	0.02	-	-	0.02	Ethanol	60	12

2.2.4.2 Effect of type of complexing solvent

The current screening parameters involve the investigation of the influence of the various complexing solvents over the physicochemical properties of the drug phospholipid complex. The complexing solvent like ethanol, methanol, dichloromethane, and tetrahydrofuran was employed to prepare the complex. The other parameters like drug: phospholipid ratio 1:1, the volume of complexing solvent 10ml, reflux temperature 60°C, and total period for the refluxing 12hr remained the same throughout the current screening parameter. The prepared drug phospholipid complex was further characterized by the physical appearance, percentage drug content, complexation efficiency, and solubility in water and n-octanol (**Table 2**).

Table 2: BHCL-PC complex under the presence of the various complexing solvents.

S. No.	Formulation code	Drug: Phospholipid molar ratio	Drug BHCl amount (molar)	Phospholipid Lipoid S 100 amount (molar)	Solvent	Temperature (°C)	Reflux time (Hr)
1	BPC3	1:1	0.02	0.02	Ethanol	60	12
2	BPC4	1:1	0.02	0.02	Methanol	60	12
3	BPC5	1:1	0.02	0.02	Dichloromethane	60	12
4	BPC6	1:1	0.02	0.02	Tetrahydrofuran	60	12

2.2.4.3 Effect of molar ratio of phospholipid

The current screening parameters involve the investigation of the influence of the various molar ratios of the drug: phospholipid ratio 1:1, 1:1.5, 1:2, and 1:2.5 over the physicochemical properties of the drug phospholipid complex. The other parameters like complexing solvent methanol, the volume of complexing solvent 10ml, reflux temperature 60°C, and total period for the refluxing 12hr remained the same throughout the current screening parameter. The prepared drug phospholipid complex was further characterized by the physical appearance, percentage drug content, complexation efficiency, and solubility in water and n-octanol (**Table 3**).

Table 3: BHCL-PC complex under the presence of the various molar ratios of phospholipid.

S. No.	Formulation code	Drug: Phospholipid molar ratio	Drug BHCl amount (molar)	Phospholipid Lipoid S 100 amount (molar)	Solvent	Temperature (°C)	Reflux time (Hr)
1	BPC4	1:1	0.02	0.02	Methanol	60	12
2	BPC7	1:1.5	0.02	0.03	Methanol	60	12
3	BPC8	1:2	0.02	0.04	Methanol	60	12
4	BPC9	1:2.5	0.02	0.05	Methanol	60	12

2.2.4.4 Effect of reflux temperature

The current screening parameters involve the investigation of the influence of the reflux temperature 50°C, 60°C, and 70° over the physicochemical properties of the drug phospholipid complex. The drug: phospholipid molar ratio 1:2, complexing solvent methanol, volume of complexing solvent 10ml, and total period for the reflux 12hr remained the same throughout the process. The prepared drug phospholipid complex was further characterized by the physical appearance, percentage drug content, complexation efficiency, and solubility in water and n-octanol (**Table 4**).

Table 4: BHCL-PC complex under the presence of the various reflux temperature.

S. No.	Formulation code	Drug: Phospholipid molar ratio	Drug BHCl amount (molar)	Phospholipid Lipoid S 100 amount (molar)	Solvent	Temperature (°C)	Reflux time (Hr)
1	BPC10	1:2	0.02	0.04	Methanol	50	12
2	BPC8	1:2	0.02	0.04	Methanol	60	12
3	BPC11	1:2	0.02	0.04	Methanol	70	12

2.2.4.5 Effect of Reflux Timing

The current screening parameters involve the investigation of the influence of the reflux period 4hr, 8hr, and 12hr over the physicochemical properties of the drug phospholipid complex. The drug: phospholipid 90G 1:2 molar ratio, complexing solvent like methanol was employed to prepare the complex. The other parameters like the volume of complexing solvent 10ml, reflux temperature 60°C, and total period for the reflux was 12hr. The prepared drug phospholipid complex was further characterized by the physical appearance, percentage drug content, complexation efficiency, and solubility in water and n-octanol (**Table 5**).

Table 5: BHCL-PC complex under the presence of the various reflux timing.

S. No.	Formulation code	Drug: Phospholipid molar ratio	Drug BHCl amount (molar)	Phospholipid Lipoid S 100 amount (molar)	Solvent	Temperature (°C)	Reflux time (Hr)
1	BPC12	1:2	0.02	0.04	Methanol	60	4
2	BPC13	1:2	0.02	0.04	Methanol	60	8
3	BPC8	1:2	0.02	0.04	Methanol	60	12

2.2.5 Optimization of Berberine HCl phospholipid complex using the central composite design

After selecting the most important factors influencing BHCl-PC complexation efficiency and solubility in -octanol, the most popular response surface methodology (i.e., CCD) was used to determine the optimum levels of these variables. The optimization objective of this research was to obtain the drug-phospholipid complex with the higher drug complexation efficiency and high solubility of the complex in n-octanol solvent within the study range. For this purpose, a Central Composite Design (CCD) of RSM is carried out to investigate the interactions of two independent variables (amount of phospholipid and Reflux temperature (Reaction temperature) and their influence on the drug complexation efficiency and solubility of the complex in n-octanol solvent.

The CCD was chosen among other experimental designs because of its ability to generate response surfaces with fewer runs required. The CCD is a 2-level factorial design, augmented with center and axial points to fit quadratic models. The two independent input factors were varied over five levels: the high level (1), center points (0), lower level (-1), and two outer (axial) points (α and −α). The value for alpha (–1.414) is calculated to fulfill both rotatability in the design. The outer points represent the extreme values for each factor; i.e., maximum and minimum respectively. For a rotatable design having m factors, $\alpha = 2^{m/4}$. In our design $m = 2$ and $\alpha = 2^{2/4} = 1.414$. **Table 6** shown composition of

13 formulations as per central composite design and **Table 7** shown summary of inputs of the central composite design.
The DoE software was used to give information not only on the critical values required to achieve the desired response but also on the possible interactions of the selected independent variables on the dependent variables.
The total number of the required runs is calculated by Equation (4);

$N = 2n + 2n + nc$**……. (4)**

where N is the total number of experimental runs and n is the number of variables. The values $2n$, $2n$, and nc represent factorial runs, axial and center runs respectively. Therefore, a total number of 13 runs is performed according to Equation (4). They are listed with their coded values in Table 2. The runs were operated in a randomized arrangement to avoid systematic bias. According to the CCD matrix generated by Design-Expert software (A total of 13 experiments, including four factorial points, four axial points, and five replicated center points for statistical assessment of the pure error sum of squares, were constructed (Gonzalez-Mira et al. 2010).

Table 6: Composition of 13 formulations as per central composite design.

Formulation code	**Factor 1 A: Amount of phospholipid (Molar)**	**Factor 2 B: Reflux temperature (degree centigrade)**
DBPC1	0.03	60
DBPC2	0.04	70
DBPC3	0.03	74.142
DBPC4	0.03	45.858
DBPC5	0.03	60
DBPC6	0.03	60
DBPC7	0.0159	60
DBPC8	0.03	60
DBPC9	0.03	60
DBPC10	0.02	70
DBPC11	0.0441	60
DBPC12	0.02	50
DBPC13	0.04	50

Table 7: Summary of inputs of the central composite design.

Factor	**Name**	**Units**	**Low Actual**	**High Actual**	**Low Coded**	**High Coded**	**Mean**
A	Amount of phospholipid	Molar	0.02	0.04	-1	1	0.03
B	Reflux temperature	degree centigrade	50	70	-1	1	60
Response							
Y1: Percentage drug encapsulation (%)							
Y2: Amount of BHCl in n-octanol (mg)							

2.2.6 Preparation of mixture of the berberine HCl and permeation enhancer

Accurately measured amount of the drug berberine HCl and chemical permeation enhance was transferred to glass culture tube. The mixture containing glass culture tube was sonicated for 5min. to obtain the clear solution. The clear solution was filtered and stored at room temperature till further use (Fein et al. 2022; Stuettgen and Brayden 2020). **Table 8** showed the composition of different mixture of drug and chemical permeation enhancer.

Table 8: Composition of different mixture of drug and chemical permeation enhancer.

S. No.	Formulation code	Amount of drug (%w/v)	Amount of permeation enhancer (%w/v)	
			Phenyl piperazine	Methyl piperazine
1	BPE1	2	5	-
2	BPE2	4	5	-
3	BPE3	6	5	-
4	BPE4	8	5	-
5	BPE5	10	5	-
6	BPE6	2	-	5
7	BPE7	4	-	5
8	BPE8	6	-	5
9	BPE9	8	-	5
10	BPE10	10	-	5

2.2.7 Statistical analysis

Using this design, we were able to choose the best model among the linear, two-factor interaction model and quadratic model due to the analysis of variance (ANOVA) F-value. Predicting the response through the full second-order polynomial equation is as shown in Equation 5:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 AB + \beta_4 A^2 + \beta_5 B^2 \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \textbf{(5)}$$

where Y is predicted response(s), β_0 is intercept, β_1, and β_2, are linear coefficients, β_4, and β_5, are squared coefficients, β_5 are interaction coefficient. By using this equation, it is possible to evaluate the linear, quadratic, and interactive effects of the independent variables on the responses appropriately (Varshosaz et al. 2010).

2.2.8 Data Analyses

The design of statistical experiments, analysis of variance (ANOVA), analysis of the results, and prediction of the optimum responses were carried out using the Design Expert (version 07) statistical software. The analysis of variance (ANOVA) was used to determine the significance of the differences between the independent variables. All significant independent variable effects ($p < 0.05$) were included in the reduced model. To visualize the interaction effect of the variables on the responses, three-dimensional response surface plots were composed. The quality of fitting the polynomial model was expressed by the coefficient of determination R^2. A high R^2 value (close to 1) confirms the accuracy of the applied model. The R^2 should be at least 0.8 for models with a good fit. Finally, a confirmatory run was performed using the suggested optimum values to validate the model (Amiri et al. 2018; Laid et al. 2021). The relationship between the dependent and independent variables was further elucidated using response surface plots. These plots are useful to study the effects of various factors on the response at a given time and to predict the responses of dependent variables at intermediate levels of independent variables. Subsequently, a numerical optimization technique using the desirability approach and a graphical optimization technique using overlay plots were used to generate new formulations with the desired responses

2.2.9 Verification of the Models

To validate the chosen experimental design, the experimental values of the responses were quantitatively compared with predicted values, and the relative error (%) was calculated using the following equation (6):

Relative error (%) = Predicted Value-Experiment value/Predicted value× 100%…… (6)

One optimum formulation was chosen and estimated by generating a model that covered the complete experimental domain to assess the validity of the generated mathematical model. To determine the effectiveness of reduced model equations previously created utilizing the central composite design,

one optimized batch was developed. The optimal preparation conditions were A (0.04 molar) and B (62.03°C), which were further validated through three parallel experiments under the optimized preparation conditions (**Table 9**) (Srikanth et al. 2012).

Table 9: Composition of the optimized formulation as per design.

Formulation code	**Factor A: Amount of phospholipid (Molar)**	**Factor B: Reflux temperature (°C)**
DBPC14	0.04	62.03

2.2.10 Characterization of berberine HCl-Phospholipid complex

2.2.10.1 Physical appearance

The prepared drug phospholipid complex was visually observed for the presence of drug particles, and agglomerations.

2.2.10.2 Percentage of drug complexing efficiency

The complexing efficiency of berberine HCl with the phospholipid was determined by quantifying the amount of uncomplexed berberine HCl in the complex (Khatik et al. 2016; Yu et al. 2016). Accurately weighed 20 mg of BHCl-PC complex was transferred to a glass tube containing 5ml of distilled water and dispersed in deionized water under vigorous vortexing for 1min to form a uniform, homogenous dispersion. Solubility study confirmed that berberine HCl possesses higher solubility in water thus uncomplexed drug gets easily solubilized in water. The dispersion was transferred to a centrifugal tube and subjected to centrifugation at 1000 rpm for 15 min. The supernatant was diluted with methanol and the solution was scanned between a range of 200-400nm using the UV spectrophotometer. The absorbance of each sample was noted at the absorption maxima of the berberine hydrochloride and the amount of the berberine HCl drug was determined using the standard linearity curve. The drug complexation efficiency (%CE) of BHCl-PC complex was calculated by the following equation (7):

$$\textbf{\textit{Percentage Drug complexation efficency}} = \frac{\textbf{\textit{Total amount of drug in complex}} - \textbf{\textit{Amount of drug in supernatent}}}{\textbf{\textit{Total amount of durg in complex}}} \times \mathbf{100} \text{.....(7)}$$

2.2.10.3 Solubility of the complex in water and n-octanol solvent

The excess amount of drug, physical mixture of drug and phospholipid, and BHCl-PC complex was transferred to the glass tube containing the 5ml of n-octanol and 5ml of water separately. The glass tube was added to the water bath shaker. The agitation was executed at 100rpm and 28°C for 24 hours. The mixture of glass tubes was withdrawn and transferred to the centrifugal tube. The solution containing the centrifugal tube was centrifuged at 10000rpm for 15 minutes. The supernatant of each sample was diluted with methanol and the solution was scanned between a range of 200-400nm using the UV spectrophotometer. The absorbance of each sample was noted at the absorption maxima of the berberine hydrochloride and the amount of the berberine HCl drug was determined using the standard linearity curve (Dora et al. 2017; Freag, Saleh, and Abdallah 2018).

2.2.10.4 Total drug content and percentage drug loading

Accurately weighed 20 mg of BHCl-PC complex was transferred to a glass tube containing the 5ml of methanol under continuous stirring and sonicated the solution for 5min. The solution of the glass tube was withdrawn and transferred to the centrifugal tube and subjected to centrifugation at 10000rpm for 15min. The supernatant of each sample was diluted with methanol and the solution was scanned between a range of 200-400nm using the UV spectrophotometer. The absorbance of each sample was noted at the absorption maxima of the berberine hydrochloride and the amount of the berberine HCl drug was determined using the standard linearity curve (Dora et al. 2017).

$$\textbf{\textit{Total drug content}} = \frac{\textbf{\textit{Practical amount of drug in complex}}}{\textbf{\textit{Theoritical amount of durg in complex}}} \times \mathbf{100} \text{ .....(8)}$$

$$\textbf{Percentage drug loading} = \frac{\textbf{Practical amount of drug in complex}}{\textbf{Amount of BHCl–PC complex}} \times \mathbf{100}$$

…….(9)

2.2.10.5 *In-vitro* permeation study using franz diffusion apparatus

The permeation of the berberine HCl from the drug phospholipid complex was determined using the dialysis membrane through the Franz diffusion apparatus. The dialysis membrane was activated by soaking it in the 10% aqueous ethanol solution for overnight. The Franz diffusion cells have two compartment one is donor and other one is receptor. The receptor part was filled with the phosphate-buffered solution (PBS) pH 6.8. the activated dialysis membrane was sandwich between the donor part and receptor part of the Franz diffusion apparatus. Both part of the Franz diffusion apparatus was clamped and transferred to magnetic stirrer maintained at 100rpm and 37°C. The drug phospholipid complex equivalent to 500mg of berberine HCl was added to the donor part of the Franz diffusion apparatus. The sample was withdrawn at different time intervals like 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24hr. The withdrawn sample was replaced with the fresh drug release medium. Withdrawn sample was suitable diluted with the methanol solvent. The sample was scanned between 200-400nm using the UV-spectrophotometer. The UV spectrum was recorded and absorbance of each sample was noted at the absorption maxima of the berberine HCl.

2.2.11 Characterization of berberine HCl-permeation enhancer mixture

2.2.11.1 Physical appearance

The physical appearance investigation involves identification of colour, presence of drug particles and precipitation of drug.

2.2.11.2 Fourier Transform Infrared spectroscopy (FTIR) of optimized formulation

The FTIR spectra of BHCl-phenyl piperazine using the KBr pellet method in the region of 400-4000 cm^{-1} (Dora et al. 2017).

2.2.11.3 pH

PH of the prepared all berberine HCl-permeation enhancer mixture was investigated employing the pH meter. The pH meter was calibrated using the buffer solution. The pH electrode was transferred to the glass vials containing the formulation allow to keep in the solution for some time to stabilize the pH value. Note the pH value in triplicate manner.

2.2.11.4 Determination of drug content

The accurately measured volume of each mixture was transferred to the 10ml volumetric flask separately. Methanol solvent was added to each flask, sonicated for 1min to solubilized the content. The mixture was centrifuged at 15000rpm for 1min. The unsolubilized drug particles were settled down while the supernatant was suitable diluted with the methanol solvent. The sample was scanned between 200-400nm using the UV spectrophotometer. The UV spectrum was recorded and absorbance of each sample was noted at the absorption maxima of the berberine HCl.

2.2.11.5 Permeability calculation

The diffusion area (A) was calculated from the radius of the Franz cell and was 2.25 cm^2. Flux through membrane to receptor compartment (J; µg/cm^2/s) was calculated by dividing the amount of drug accumulated in the receptor compartment by A. The Fick's first law was derived to calculate flux (J) at steady state (Equation (1)):

$$J = \frac{dQ}{dt} \times A$$

where dQ is the amount of drug across the membrane (in moles), dt the permeation time (in seconds), and A the diffusion area (in cm^2). Note that J was obtained from the slope of the curve at steady state from typical mean cumulative concentration-time plots (minimum of triplicates), as further shown in

results section. Coefficient of variation (CV) of flux for each drug was also measured (ENV 2012; Kasim et al. 2004; Selzer et al. 2013). The apparent permeability (Papp) was calculated normalizing the flux (J) over the drug concentration in the donor compartment C_0, as described by the following formula:

$$Papp = \frac{J}{Co} \times A$$

2.2.11.6 *In-vitro* permeation study using Franz diffusion apparatus

The permeation of the berberine HCl from the mixture was determined using the dialysis membrane through the Franz diffusion apparatus. The dialysis membrane was activated by soaking it in the 10% aqueous ethanol solution for overnight. The Franz diffusion cells have two compartment one is donor and other one is receptor. The receptor part was filled with the phosphate-buffered solution (PBS) pH 6.8. the activated dialysis membrane was sandwich between the donor part and receptor part of the Franz diffusion apparatus. Both part of the Franz diffusion apparatus was clamped and transferred to magnetic stirrer maintained at 100rpm and 37°C. The drug permeation enhance mixture equivalent to 500mg of berberine HCl was added to the donor part of the Franz diffusion apparatus. The sample was withdrawn at different time intervals like 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24hr. The withdrawn sample was replaced with the fresh drug release medium. Withdrawn sample was suitable diluted with the methanol solvent. The sample was scanned between 200-400nm using the UV spectrophotometer. The UV spectrum was recorded and absorbance of each sample was noted at the absorption maxima of the berberine HCl (Shah et al. 2014).

2.2.12 Stability study of berberine HCl phospholipid complex and Berberine HCl-phenyl piperazine mixture

The stability study of the optimized berberine HCl phospholipid complex DBPC14 formulation and Berberine HCl-phenyl piperazine mixture BPE2 was assessed as per the International Council for Harmonization guidelines/ European Medicines Agency. The optimized berberine HCl phospholipid complex DBPC14 formulation and Berberine HCl-phenyl piperazine mixture BPE2 was transferred to the vails and stored at 4±2 °C (refrigerator), Room temperature (Desiccator), 25±2°C/60±5% RH (Room temperature) in the stability chamber. The samples were withdrawn at predetermined time intervals 0month, 1month, and 3months. The withdrawal samples were characterized for the physical appearance and percentage drug content (Pu et al. 2016; Yasir et al. 2021).

2.2.13 *In-vivo* pharmacokinetic study

2.2.13.1 Bioanalytical method development of Berberine HCl in plasma

2.2.13.1.1 Chromatographic conditions

The amount of berberine HCl was determined on an Agilent 1100 HPLC system. The analysis of BBR was performed employing an C18 (250mm×4.6mm, 5micron) and column temperature of 40°C. The mobile phase comprised of acetonitrile and 0.5 mol/L phosphate dibasic potassium (60:40 %v/v) with a flow rate of 1.0mL/min. The samples were charged 20µL to the HPLC and response of berberin HCl was observed at 345nm.

2.2.13.1.2 Preparation of the standard calibration curve of berberine HCl in plasma

An accurately measure 5mg of berberine HCl was transferred in the 1ml Eppendorf tube containing 0.5ml of plasma, vortexed to solubilize the drug in to the plasma. Accurately measured volume 0.5ml of the methanol, sonicated for 1minutes and centrifuge the solution at 10000rpm for the 10min. For the preparation of the standard linearity curve, a series of working solutions of between 0.01-0.06ml were withdrawn from the stock solution and transferred to the 10ml of volumetric flasks under gentle shaking separately. Methanol solvent was added and made the volume up to the mark result in a solution with concentration 1µg/ml, 2µg/ml, 3µg/ml, 4µg/ml, 5µg/ml, 6µg/ml, respectively. Each working solution was filtered using the 0.22µ membrane filter paper and charged in the HPLC. A linearity curve was prepared between the area and concentration (Zhang et al. 2014).

2.2.13.1.3 Extraction of berberine HCl from plasma samples

The protein from the plasma samples was separate employing the acetonitrile and methanol solvent. The accurately measured volume 100µl of acetonitrile and 100µl of methanol was added to the 200 µl plasma samples under continuous stirring. The mixture of sample was vortexed for 1min. and centrifuged at 5000 rpm for 15min. The supernatant was suitable diluted 10times with the mobile phase. The sample was filtered through the 0.22µ membrane filter and charged in the HPLC to determine the amount of berberine HCl (Zhang et al. 2014).

3.2 Result and Discussion

3.2.1 Preformulation study

The berberine HCl was subjected to investigate the preformulation studies like organoleptic property, melting point, partition coefficient, solubility, and determination of absorption maxima using the UV spectrophotometer.

3.2.1.1 Organoleptic property

The berberine HCl particles appeared a yellow color with bitter taste.

3.2.1.2 Melting Point determination

The melting point of berberine HCl was found to be 140.00±1°C to 145.33±0.58°C close to the value mentioned in the literature 145°C (Comincini et al. 2023).

3.2.1.3 Partition coefficient determination

The Partition coefficient of berberine HCl was found to be approximately -1.54±0.002 using a mixture solution of water and n-octanol by employing the shake flask method. The obtained value of partition coefficient was lies very close to the value mentioned in literature -1.50 (Battu et al. 2010; Kabary et al. 2018; Liu et al. 2016)

3.2.1.4 Solubility

Solubility of the berberine HCl was investigated in aqueous and non-aqueous solvents like water, ethanol, ethanol, and phosphate buffer pH 6.8. The solubility of the berberine HCl in solvents was shown in **Figure 3 and table 10.** Ther result of berberine HCl was sparingly soluble in methanol and slightly soluble in ethanol. In addition, the berberine HCl was slightly soluble in water and phosphate buffer pH 6.8. The solubility of berberine HCl was similar to the literatre (Zhu et al. 2013)(Ai et al. 2021).

Table 10: Solubility of berberine HCl in solvents.

Solvent	Solubility (mg/ml)	Outcome
Water	2.070±0.072	Slightly soluble
Methanol	12.159±0.026	Sparingly soluble
Ethanol	7.955±0.089	Slightly soluble
Phosphate buffer 6.8pH	1.288±0.005	Slightly soluble

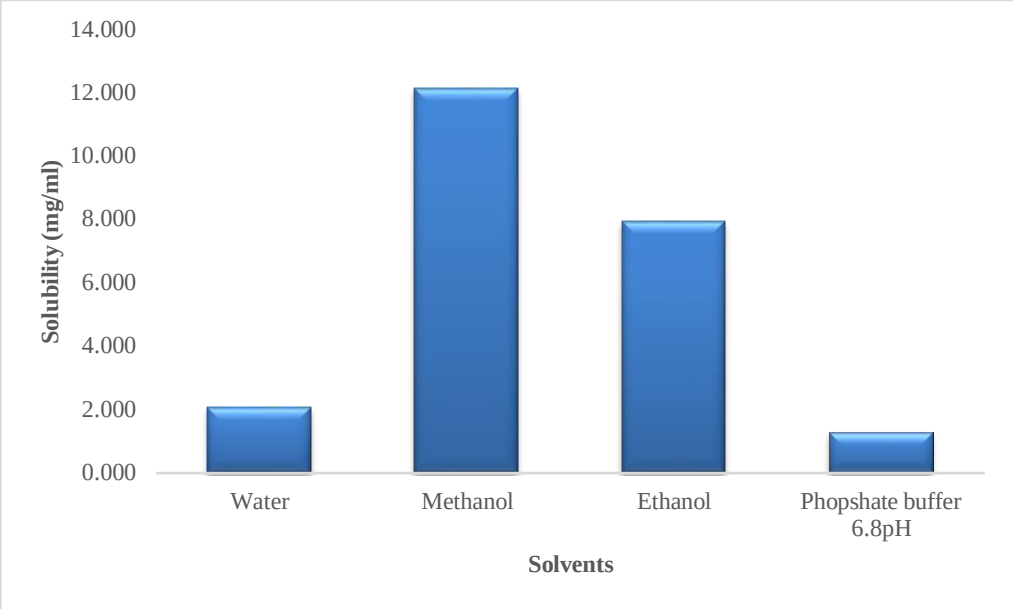

Figure 3: Solubility of berberine HCl in different solvents.

3.2.1.5 FTIR

The FTIR spectrum of berberine Hydrochloride revealed the existence of a methoxyl group (peak at 2981.24 cm^{-1}), and the peak at 1598.88 cm^{-1} is believed to correspond to the iminium ($C = N^{+}$) double bond present in the molecule. Moreover, the signals at 1,568.04and 1,505.49 cm−1 represent the aromatic C = C bending and the furyl group, respectively.

The FTIR spectrum of phospholipid lipoid S 100 [1742.68 cm^{-1} (C=O), 1163.57 cm^{-1} (P=O) and 1057.87 cm^{-1} (P-O)]. However, in the spectrum of complex, the characteristic absorption peak 1598.88 cm^{-1}, 1505.49 cm^{-1} and 1568.04 cm^{-1} were disappeared indicating some changes of berberine molecules. Moreover, the characteristic absorption peaks 1163.57 cm^{-1} (P=O) and 1057.87 cm^{-1} (P-O) has been shifted to lower wavenumber. These observations suggested that no new chemical bond was formed, but some physical interactions between the berberine benzene ring and polar head of phospholipid probably occured to form the complex.

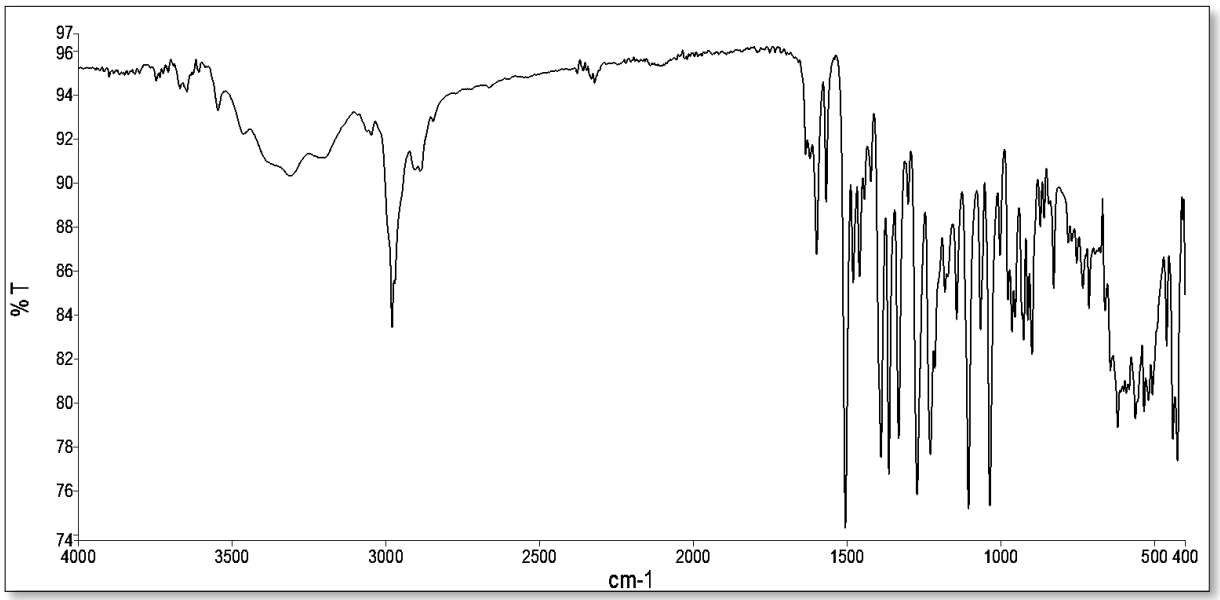

Figure 4: FTIR spectrum of berberine HCl.

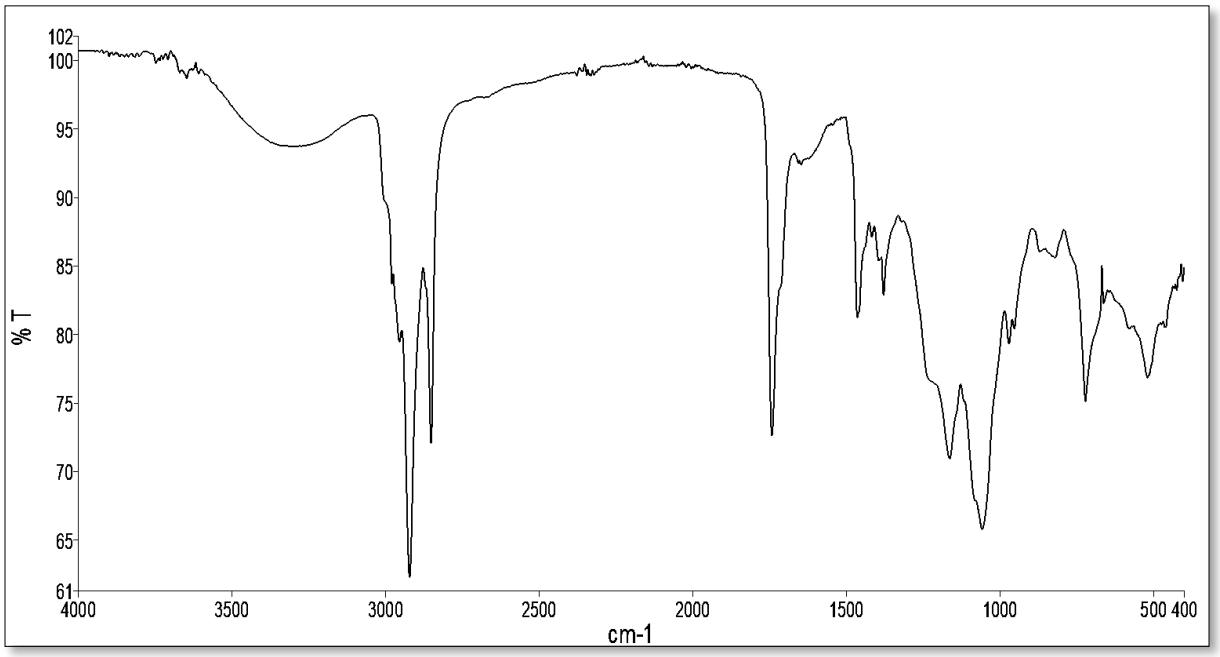

Figure 5: FTIR spectrum of lipoid S 100.

3.2.2 Determination of absorption maxima of berberine HCl

The absorption maxima of the berberine HCl was determined by scanning the solution of a certain concentration of 5µg/ml solution of berberine HCl in methanol between 200-400nm using the UV spectrophotometer. The UV spectrum revealed the absorption maxim at 345nm as shown in **Figure 6.** The similar wavelength 345nm matched with the previous literature (Koide et al. 2001)**.**

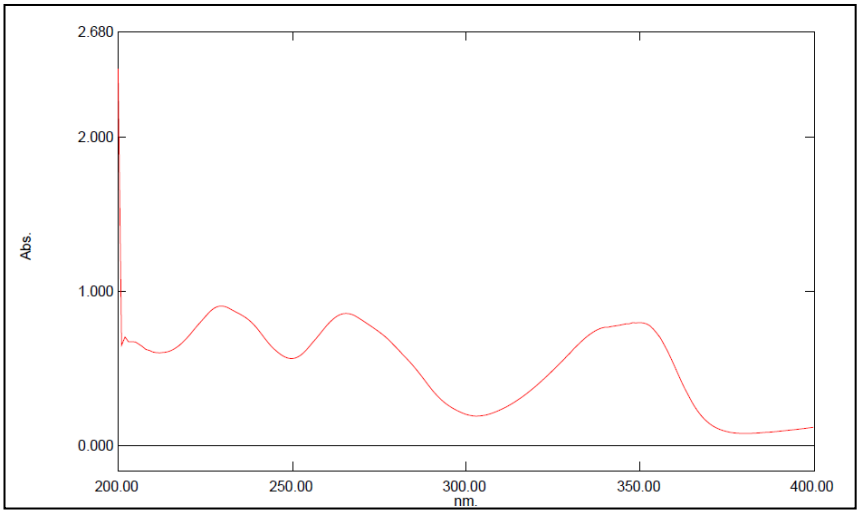

Figure 6: UV spectrum of berberine HCl in methanol (5µg/ml).

3.2.3 Method Validation

3.2.3.1 Linearity

The linearity of the berberine HCl in methanol was determined between a range of 1-11µg/ml in methanol solvent at absorption maxima 345nm. A linear graph was drawn between the concentration and absorbance at 345nm as shown in **Figure 7, 8 and 9.** The graphs are described by the regression equation: Y = a + bX (where Y = absorbance of drug solution; a = intercept; b = slope and X = concentration of the drug. **Table 11 and Figure 10** shown a linear response between concentration and mean absorbance of berberine HCl in methanol at 345nm.

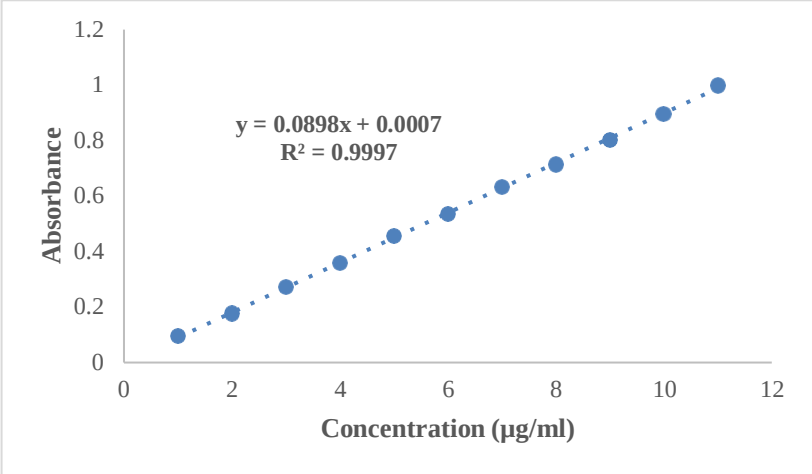

Figure 7: Standard calibration curve of berberine HCl in methanol at 345nm**.**

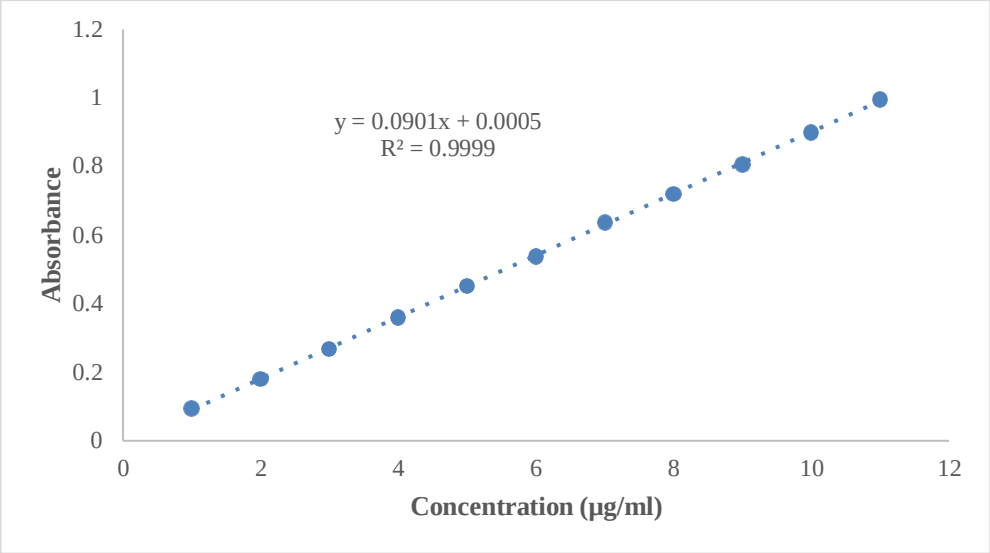

Figure 8: Standard calibration curve of berberine HCl in methanol at 345nm.

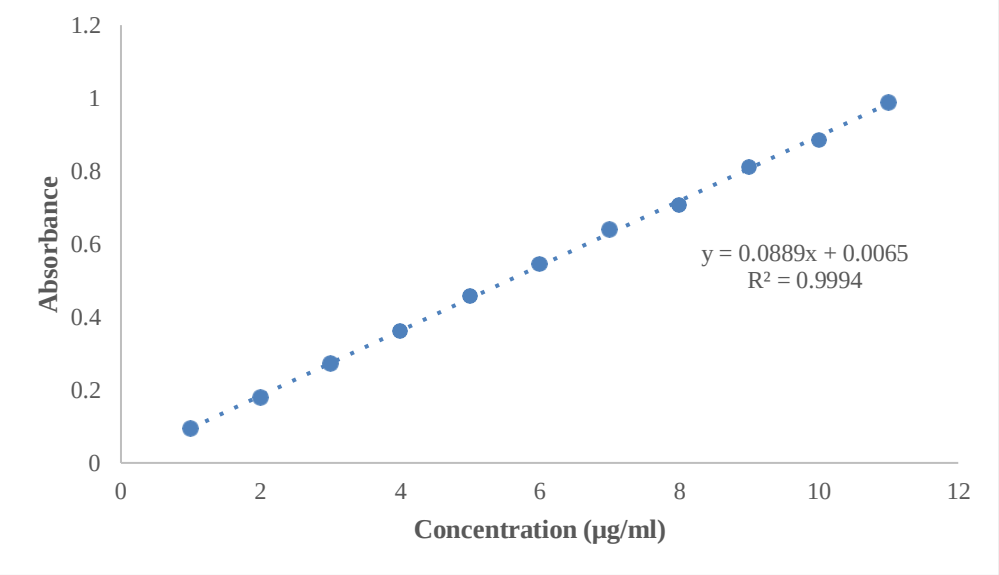

Figure 9: Standard calibration curve of berberine HCl in methanol at 345nm.

Table 11: A linear response between concentration and mean absorbance of berberine HCl in methanol at 345nm.

Concentration (µg/ml)	Absorbance at 345nm	Absorbance at 345nm	Absorbance at 345nm	Average Absorbance at 345nm	STD
1	0.095	0.094	0.094	0.094	0.001
2	0.177	0.18	0.179	0.179	0.002
3	0.271	0.268	0.273	0.271	0.003
4	0.358	0.359	0.362	0.360	0.002
5	0.455	0.452	0.457	0.455	0.003
6	0.535	0.538	0.545	0.539	0.005
7	0.633	0.637	0.64	0.637	0.004
8	0.714	0.72	0.707	0.714	0.007
9	0.802	0.806	0.812	0.807	0.005
10	0.896	0.9	0.885	0.894	0.008
11	0.999	0.995	0.988	0.994	0.006

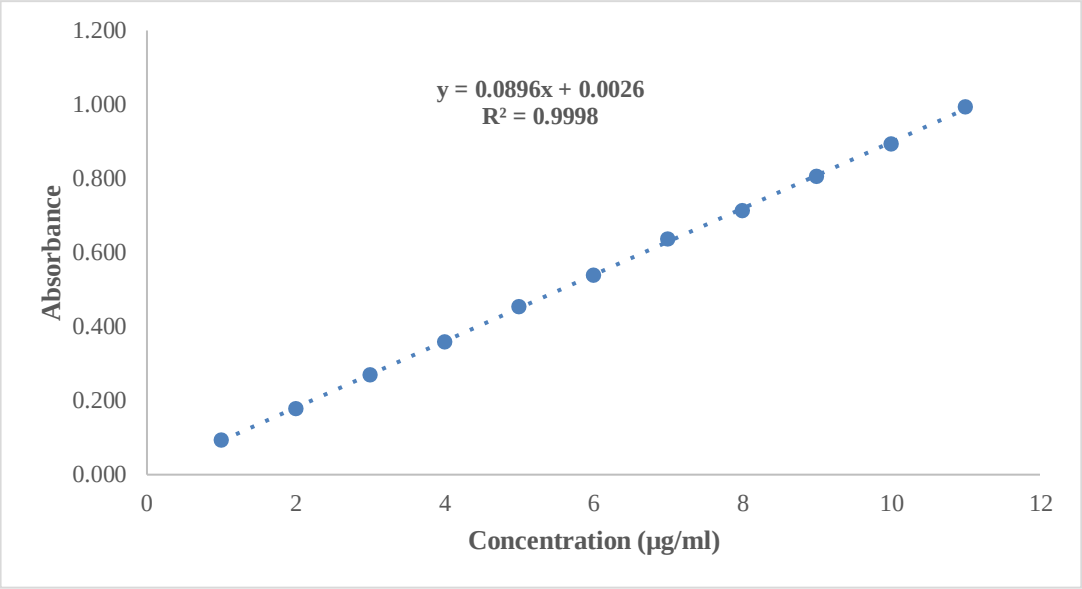

Figure 10: A linear response graph between concentration and mean absorbance of berberine HCl in methanol at 345nm.

Figure 10 shown the linear regression equations for berberine HCl in methanol of calibration curves y = 0.0896x +0.0026 with regression coefficient R^2 = 0.999 at 345nm. The concentration range selected 1-11µg/ml follows the lambert bear law.

3.2.3.2 Accuracy

The percentage recovery for berberine HCl at each level of 80%, 100%, and 120% was observed to be all the three concentration levels 99.256±0.426, 99.095±0.387 to 99.526±0.483. In addition, the %RSD at each level was found to be 0.429, 0.391, and 0.486 respectively (**Table 12**). The measured % RSD values for each level less than 1 indicated good accuracy of the developed method.

Table 12: Accuracy results of berberine HCl.

Level	Conc.(µg/ml)	% Recovery	Mean % Recovery	STD	%RSD
80%	4	98.884	99.256	0.426	0.429
	4	99.721			
	4	99.163			
100%	6	99.405	99.095	0.387	0.391
	6	99.219			
	6	98.661			
120%	8	99.247	99.526	0.483	0.486
	8	100.084			
	8	99.247			

3.2.3.3 Precision

Repeatability

The repeatability of the developed method was determined by determining the percentage recovery of the sample concentration solution 6µg/ml solution of berberine HCl in methanol running on the same day. The mean percentage recovery of berberine HCl was found to be 99.312±0.452 µg/ml with a % RSD value of 0.455 as given in **Table 13.** Inter-day precision was conducted by determining the percentage recovery of the berberine HCl on six different days (**Table 14**). In the current study, the percentage recovery of the berberine HCl in methanol was found to be 98.698±0.42 % to 99.805±0.140%. The percentage RSD on each day was found to be less than 2% indicating good precision of the developed method.

Table 13: Repeatability data of berberine HCl in methanol.

Concentration (µg/ml)	% Recovery	% Mean Recovery	STD	%RSD
6	99.777	99.312	0.452	0.455
	98.475			
	99.405			
	99.219			
	99.591			
	99.405			

Table 14: Interday Precision data of berberine HCl in methanol at six consecutive days.

Day 1				
Conc.(µg/ml)	**% Recovery**	**Mean % Recovery**	**SD**	**%RSD**
4	99.163	99.526	0.161	0.162
4	99.442			
4	99.163			
6	98.847	99.343	0.468	0.471
6	99.405			
6	99.777			
8	99.805	99.805	0.140	0.140
8	99.665			
8	99.944			
Day 2				
Conc.(µg/ml)	**% Recovery**	**Mean % Recovery**	**SD**	**%RSD**
4	98.605	99.070	0.426	0.430
4	99.442			
4	99.163			

6	99.591	99.591	0.186	**0.187**
6	99.405			
6	99.777			
8	99.247	99.479	0.213	0.214
8	99.526			
8	99.665			
Day 3				
Conc.(µg/ml)	**% Recovery**	**Mean % Recovery**	**SD**	**%RSD**
4	98.326	98.698	0.426	0.432
4	99.163			
4	98.605			
6	99.405	99.405	0.372	0.374
6	99.033			
6	99.777			
8	99.386	99.572	0.448	0.450
8	100.084			
8	99.247			
Day 4				
Conc.(µg/ml)	**% Recovery**	**Mean % Recovery**	**SD**	**%RSD**
4	99.442	98.791	0.702	0.711
4	98.047			
4	98.884			
6	99.219	99.095	0.215	0.217
6	98.847			
6	99.219			
8	99.805	99.758	0.351	0.352
8	100.084			
8	99.386			
Day 5				
Conc.(µg/ml)	**% Recovery**	**Mean % Recovery**	**SD**	**%RSD**
4	98.326	98.698	0.322	0.326
4	98.884			
4	98.884			
6	99.591	99.343	0.430	0.432
6	98.847			
6	99.591			
8	99.944	99.712	0.290	0.291
8	99.805			
8	99.386			
Day 6				
Conc.(µg/ml)	**% Recovery**	**Mean % Recovery**	**SD**	**%RSD**
4	99.163	98.977	0.322	0.326
4	98.605			
4	99.163			
6	99.777	99.343	0.598	0.602
6	98.661			
6	99.591			
8	100.084	99.805	0.279	0.280
8	99.805			
8	99.526			

3.2.3.4 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOD and LOQ were found to be 0.126 µg/ml and 0.380 µg/ml for berberine HCl respectively.

3.2.3.5 Ruggedness

The method of ruggedness was performed by two different analysts shown in **Table 15**. The results did not show any major statistical difference between operators. The % RSD values were in the range of was found to be within the limits (< 2 %) indicating acceptable ruggedness of the developed method.

Table 15: Ruggedness data of berberine HCl in methanol.

Analyst 1					
S. No.	**Concentration (µg/ml)**	**% Recovery**	**% Mean Recovery**	**STD**	**%RSD**
1	6	99.405	99.405	0.424	0.427
2	6	99.033			
3	6	98.847			
4	6	99.963			
5	6	99.777			
6	6	99.405			
Analyst 2					
S. No.	**Concentration (µg/ml)**	**% Recovery**	**% Mean Recovery**	**SD**	**%RSD**
1	6	99.405	99.343	0.480	0.483
2	6	99.777			
3	6	99.963			
4	6	98.661			
5	6	99.033			
6	6	99.219			

3.2.3.6 Robustness

The robustness of the developed method was determined by evaluating the percentage recovery of the berberine HCl at three different absorption maxima. In the current investigation absorption maxima 344nm, 345nm, and 346nm were used to evaluated the robustness parameter. It was found that no significant variations were observed during the study thus indicating the robustness of the method. The % RSD values were found to be within the limits (<2 %) as given in **Table 16** and the method was found to be robust.

Table 16: Robustness parameter including percentage recovery of berberine HCl in methanol at a different wavelength.

Wavelength 344nm					
S. No.	**Concentration (µg/ml)**	**% Recovery**	**% Mean Recovery**	**STD**	**%RSD**
1	6	99.033	99.467	0.435	0.437
2	6	99.591			
3	6	98.847			
4	6	99.777			
5	6	99.963			
6	6	99.591			

Wavelength 345nm					
S. No.	**Concentration (µg/ml)**	**% Recovery**	**% Mean Recovery**	**STD**	**%RSD**
1	6	98.847	99.064	0.638	0.644
2	6	98.661			
3	6	98.103			
4	6	99.777			
5	6	99.591			
6	6	99.405			
Wavelength 346nm					
S. No.	**Concentration (µg/ml)**	**% Recovery**	**% Mean Recovery**	**STD**	**%RSD**
1	6	99.591	99.498	0.195	0.196
2	6	99.777			
3	6	99.591			
4	6	99.405			
5	6	99.405			
6	6	99.219			

3.2.4 Screening of phospholipid complex

3.2.4.1 Effect of type of phospholipid

In current activity, the effect of the type of phospholipids like soya lecithin, Phospholipon 90 G, and lipoid S100 having different amounts of phosphatidylcholine was investigated over the physicochemical properties like physical appearance, solubility in n-octanol and water solvent, percentage encapsulation efficiency and drug content of the BHCl-PC. By varying the type of phospholipid, the characterization parameters like percentage complexation efficiency, amount of solubilized BHCl in n-octanol and water solvent, drug content, and percentage loading of the prepared BHCl-PC were found to be in arrange of 32.858±0.321 to 49.812±0.436%, 0.966±0.012 mg to 1.688±0.022 mg, 2.126±0.009mg to 2.986±0.025mg, 32.272±0.401% to 47.188±0.298%, 10.605±0.132% to 15.506±0.098% (**Table 17).**

The BHCl-PC prepared from the phospholipid LIPOID S100 possesses higher complexation efficiency, percentage drug loading, and solubility in n-octanol solvent than the other two phospholipids because the lipoid S 100 comprises of higher amount of the phosphatidylcholine than the other two phospholipids. Previous literature confirmed that phospholipids possess a higher PC yielded a more stable phospholipid complex with the drug (Guihua et al. 2005; Lu et al. 2009). Thus, the phospholipid lipoid S 100 was selected for the preparation of BHCl-PC.

Table 17: Characterization parameter of BHCl-PC prepared under different phospholipid.

Formulation code	**Physical appearance**	**Percentage encapsulation efficiency (%)**	**Amount of BHCl solubilized in n-octanol (mg)**	**Amount of BHCl solubilized in water (mg)**	**Total drug content (%)**	**Percentage drug loading (%)**
Pure drug			0.180±0.02	2.685±0.026		
BPC1	Slightly yellow color sticky residue	32.858±0.321	0.966±0.012	2.126±0.09	32.272±0.401	10.605±0.132

BPC2	Slightly yellow color dried residue	40.330±0.343	1.399±0.023	3.190±0.028	41.895±0.147	13.767±0.048
BPC3	Slightly yellow color dried residue	49.812±0.436	1.688±0.022	2.986±0.025	47.188±0.298	15.506±0.098

3.2.4.2 Effect of complexing solvent

In current activity, the effect of complexing solvents like ethanol, methanol, dichloromethane, and tetrahydrofuran was investigated over the physicochemical properties like physical appearance, solubility in n-octanol and water solvent, percentage encapsulation efficiency, and drug content of the BHCl-PC. By varying the complexing solvent, the characterization parameters like percentage complexation efficiency, amount of solubilized BHCl in n-octanol and water solvent, drug content, and percentage loading of the prepared BHCl-PC were found to be in arrange of 11.919±1.297% to 58.982±0.259%, 0.566±0.024mg to 1.721±0.030mg, 2.554±0.026mg to 3.250±0.020mg, 10.280±0.196% to 56.472±0.614%, 3.378±0.064% to 18.557±0.202%. However, in our work, we found a maximum complexation efficiency of 58.982±0.259% with methanol and thus selected it as a final solvent for the preparation of BHCl-PC (**Table 18**).

Table 18: Characterization parameter of BHCl-PC prepared using a different complexing solvent.

Formulation code	**Physical appearance**	**Percentage encapsulation efficiency (%)**	**Amount of BHCl solubilized in n-octanol (mg)**	**Amount of BHCl solubilized in water (mg)**	**Total drug content (%)**	**Percentage drug loading (%)**
Pure drug			0.180±0.002	2.685±0.026		
BPC3	Slightly yellow color dried residue	49.812±0.436	1.688±0.022	2.986±0.025	47.188±0.298	15.506±0.098
BPC4	Slightly yellow color dried residue	58.982±0.259	1.721±0.030	3.250±0.020	56.472±0.614	18.557±0.202
BPC5	Yellow color sticky residue	33.764±0.321	1.063±0.026	2.554±0.026	29.866±0.427	9.814±0.140
BPC6	Yellow color sticky residue	11.919±1.297	0.566±0.024	2.817±0.032	10.280±0.196	3.378±0.064

3.2.4.3 Effect of molar ratio of drug-phospholipid

In current activity, the effect of the molar ratio of drug-phospholipid like 1:1, 1:1.5, 1:2, and 1:1.25 was investigated over the physicochemical properties like physical appearance, solubility in n-octanol and water solvent, percentage encapsulation efficiency and drug content of the BHCl-PC. By varying the drug-phospholipid molar ratio, the characterization parameters like percentage complexation efficiency, amount of solubilized BHCl in n-octanol and water solvent, drug content and percentage loading of the prepared BHCl-PC was found to be in arrange of 58.982±0.259% to 80.547±0.714%,

1.721±0.030 mg to 3.754±0.026mg, 3.109±0.018mg to 3.226±0.016mg, 56.472±0.614% to 77.215±0.700 %, 12.344±0.112% to 18.557±0.202% (**Table 19**).
Previous literature confirmed that the formation of drug–phospholipid complexes is based on the interaction such as hydrogen bonds and van der Waals forces between drugs and phospholipids (Maiti et al. 2007; Yanyu et al. 2006).

Table 19: Characterization parameter of BHCl-PC prepared using different molar ratios of drug-phospholipid.

Formulation code	**Physical appearance**	**Percentage encapsulation efficiency (%)**	**Amount of BHCl solubilized in n-octanol (mg)**	**Amount of BHCl solubilized in water (mg)**	**Total drug content (%)**	**Percentage drug loading (%)**
Pure drug			0.180±0.02	2.685±0.026		
BPC4	Slightly yellow color dried residue	58.982±0.259	1.721±0.030	3.250±0.020	56.472±0.614	18.557±0.202
BPC7	Slightly yellow color dried residue	72.355±0.429	2.601±0.040	3.109±0.018	71.085±0.458	17.487±0.113
BPC8	Slightly yellow color dried residue	80.547±0.714	3.754±0.026	3.226±0.016	77.215±0.700	15.180±0.138
BPC9	Slightly yellow color dried residue	76.013±0.355	3.635±0.039	3.120±0.021	75.405±0.682	12.344±0.112

3.2.4.3 Effect of reflux (reaction) temperature

In current activity, the effect of reflux temperatures 50^{o}C, 60^{o}c and 70^{o}C were investigated over the physicochemical properties like physical appearance, solubility in n-octanol and water solvent, percentage encapsulation efficiency, and drug content of the BHCl-PC. By varying the reflux temperature, the characterization parameters like percentage complexation efficiency, amount of solubilized BHCl in n-octanol and water solvent, drug content, and percentage loading of the prepared BHCl-PC were found to be in arrange of 70.282±0.375% to 80.547±0.714%, 2.802±0.036mg to 3.754±0.026mg, 2.872±0.012mg to 3.226±0.016mg, 68.558±0.591% to 77.215±0.700 %, 13.478±0.116% to 15.180±0.138% (**Tabel 20)**.

Table 20: Characterization parameter of BHCl-PC prepared using different reflux temperatures.

Formulation code	**Physical appearance**	**Percentage encapsulation efficiency (%)**	**Amount of BHCl solubilized in n-octanol (mg)**	**Amount of BHCl solubilized in water (mg)**	**Total drug content (%)**	**Percentage drug loading (%)**
Pure drug			0.180±0.020	2.685±0.026		
BPC10	Slightly yellow color dried residue	70.282±0.375	2.802±0.036	2.872±0.012	68.558±0.591	13.478±0.116

BPC8	Slightly yellow color dried residue	80.547±0.714	3.754±0.026	3.226±0.016	77.215±0.700	15.180±0.138
BPC11	Slightly yellow color dried residue	75.060±0.997	3.339±0.017	3.064±0.041	71.396±0.782	14.036±0.154

3.2.4.4 Effect of reflux (reaction) time duration

In the current activity, the effect of reflux (reaction) time duration 4hr, 8hr, and 12hr were investigated over the physicochemical properties like physical appearance, solubility in n-octanol and water solvent, percentage encapsulation efficiency, and drug content of the BHCl-PC. By varying the reflux time duration, the characterization parameters like percentage complexation efficiency, amount of solubilized BHCl in n-octanol and water solvent, drug content and percentage loading of the prepared BHCl-PC was found to be in arrange of 65.646±0.468% to 80.547±0.714%; 2.128±0.031mg to 3.754±0.026 mg; 2.904±0.063 mg to 3.226±0.016 mg; 59.427±0.456% to 77.215±0.700 %; 11.683±0.090% to 15.180±0.138% (**Table 21**).

Table 21: Characterization parameter of BHCl-PC prepared using different reflux (reaction) time duration

Formulation code	**Physical appearance**	**Percentage encapsulation efficiency (%)**	**Amount of BHCl solubilized in n-octanol (mg)**	**Amount of BHCl solubilized in water (mg)**	**Total drug content (%)**	**Percentage drug loading (%)**
Pure drug			0.180±0.02	2.685±0.026		
BPC12	Slightly yellow color dried residue	65.646±0.468	2.128±0.031	2.904±0.063	59.427±0.456	11.683±0.090
BPC13	Slightly yellow color dried residue	75.722±0.537	2.915±0.059	3.001±0.042	67.990±0.945	13.367±0.186
BPC8	Slightly yellow color dried residue	80.547±0.714	3.754±0.026	3.226±0.016	77.215±0.700	15.180±0.138

3.2.5 Optimization of BHCl-PC complex using the central composite design

Central composite design is one of the response surface models used in the design of experiments to hit the target reduce the variability and maximize the responses. The two significant variables selected, based on the screening of formulation and process parameters including the amount of phospholipid (0.02M to 0.04M) and reflux temperature (50^{o}C to 70^{o}C) were used to maximize the percentage drug complexation efficiency and solubility in n-octanol solvent, using central composite response surface methodology (**Table 22**).

Results that were obtained from all the formulations were assessed to get an appropriate study design. Contour plots, three-dimensional plots, and desirability plots were generated using Design Expert software. A quadratic model was suggested for percentage drug complexation efficiency and solubility in n-octanol solvent. The factors that had a significant effect on the responses were identified using ANOVA (Analysis of variance). The polynomial equations could be used to conclude after considering the magnitude of the coefficient and the mathematical sign it carries, i.e., positive or negative.

Table 22: Percentage of drug complexation efficiency and solubility in n-octanol solvent of formulation prepared as per the central composite design.

Formulation code	Response Y1 Percentage drug encapsulation (%)	Response Y2 Amount of BHCl solubilized in n-octanol (mg)
DBPC1	72.885	2.657
DBPC2	74.587	3.218
DBPC3	62.223	2.102
DBPC4	56.098	1.645
DBPC5	73.3	2.631
DBPC6	73.376	2.647
DBPC7	58.353	1.228
DBPC8	72.771	2.619
DBPC9	73.036	2.627
DBPC10	58.077	1.438
DBPC11	80.466	3.719
DBPC12	55.076	1.165
DBPC13	69.477	2.876

3.2.5.1 Percentage drug complexation

The results of the design of experiments indicated that this system was highly influenced by the amount of phospholipid and reflux temperature, which resulted in high drug encapsulation and high solubility in n-octanol for the preparation of BHCl-PC. From the response surface model, the regression equations (10) for the dependent variables were obtained using Design Expert software over the range of independent variables from the random order and are shown in **Table 23**.

$Y1 = 73.074 + 7.773A + 2.097B + 0.527AB - 1.827A^2 - 6.952B^2$ ····· **(10)**

where Y1 is the Percentage drug complexation and A, and B are the amount of phospholipid, and reflux temperature, respectively.

The sign and value of the quantitative effect represent the tendency and magnitude of the term's influence on the response, respectively. In the regression equation, a positive value indicates an effect that favors the optimization due to a synergistic effect, while a negative value indicates an inverse relationship or antagonistic effect between the factor and the response. Regression values represent the quantitative effect of process variables A, and B and their influence on the dependent responses Y1. These response data are shown in **table 22**. Further, close observation of data revealed the suitability of the response surface quadratic model when compared to the linear model, the two-factor (2FI) model, and cubic model.

Table 23: Regression values of the selected responses during optimization.

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	5.862	0.601	0.521	0.296	607.352	
2FI	6.169	0.603	0.470	0.120	758.227	
Quadratic	0.216	0.999	0.999	0.999	0.815	Suggested
Cubic	0.233	0.999	0.999	0.999	0.471	

ANOVA for the responses indicated that the quadratic regression model was significant and valid for each of the responses A ($p<0.0001$), and B ($p<0.0001$) and hence was appropriate to represent the observed data, respectively (**Table 24**). The adjusted R^2 values for the dependent responses are 0.999 (**Table 24**).

Table 24: ANOVA results for percentage drug complexation as the response (Y1).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	861.809	5	172.362	3697.753	< 0.0001	Significant
A-Amount of phospholipid	483.349	1	483.349	10369.484	< 0.0001	
B-Reflux temperature	35.167	1	35.167	754.451	< 0.0001	
AB	1.112	1	1.112	23.856	0.0018	
A^2	23.226	1	23.226	498.286	< 0.0001	
B^2	336.185	1	336.185	7212.331	< 0.0001	
Residual	0.326	7	0.047			
Lack of Fit	0.055	3	0.018	0.271	0.8443	Not significant
Pure Error	0.271	4	0.068			
Cor Total	862.135	12				
R-Squared	0.999					
Adj R-Squared	0.999					
Pred R-Squared	0.999					
Adeq Precision	173.577					

3.2.5.1.1 Three-dimensional (3D) plots

The independent variables, i.e. the amount of phospholipid (A) and the reflux temperature (B) and their interaction on the percentage drug complexation (Y1) as dependent responses were graphically represented by 3D surface plots by using RSM. When percentage drug complexation (Y1) was indicated as the response, good correlation was shown between observed and predicted values (**Table 25**). This Y1 was significantly influenced by the amount of phospholipid (A) and the reflux temperature (B) and their interactive term (AB) and polynomial model terms A^2 and B^2. The magnitude of the positive coefficient (73.074) of the term suggests that elevated levels of amount of phospholipid and the reflux temperature in the formulation could increase the percentage of drug complexation drastically. **Figure 11** shown Contour plots showing the interactive effects of (i) amount of phospholipid (A) and reflux temperature (B) on percentage drug complexation (Y1) and **Figure 12** shown 3-dimensional plot showing the interactive effects of amount of phospholipid (A) and reflux temperature (B) on percentage drug complexation (Y1).

The amount of phospholipid and the reflux temperature levels had a positive influence on the percentage of drug complexation and the results are inconsistent with the conclusion of other investigators (Varshosaz et al. 2010).

Table 25: Predicated and observed value.

Standard Order	Actual Value	Predicted Value
DBPC1	55.076	54.952
DBPC2	69.477	69.444
DBPC3	58.077	58.091
DBPC4	74.587	74.691
DBPC5	58.353	58.427
DBPC6	80.466	80.412
DBPC7	56.098	56.205
DBPC8	62.223	62.135
DBPC9	72.885	73.074
DBPC10	73.376	73.074

DBPC11	72.771	73.074
DBPC12	73.036	73.074
DBPC13	73.3	73.074

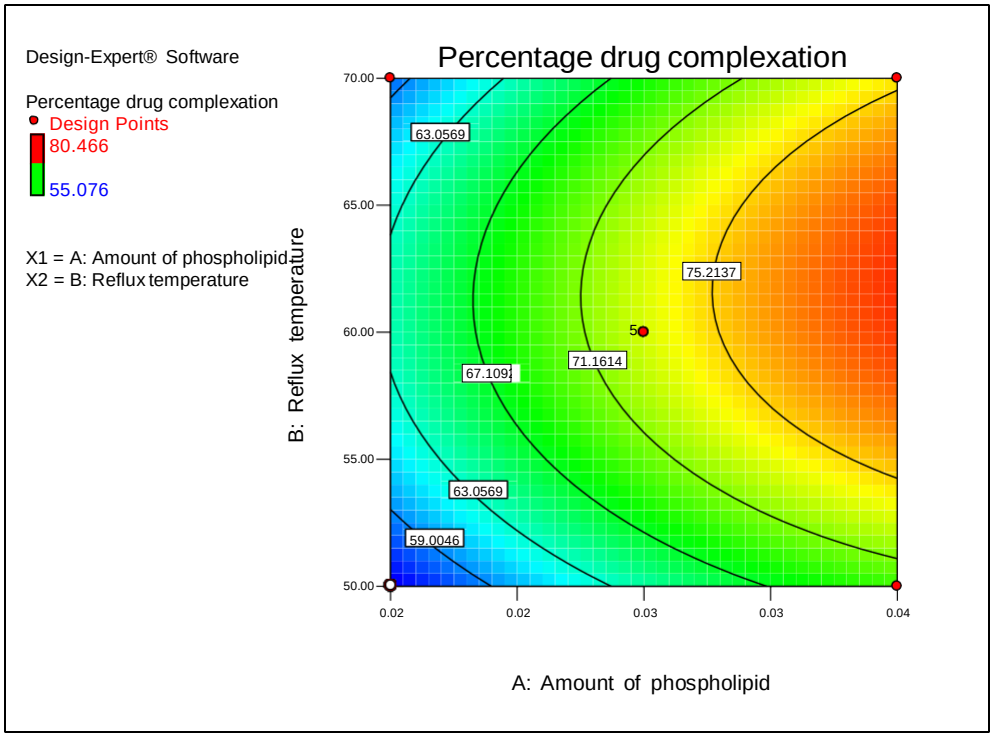

Figure 11: Contour plots showing the interactive effects of (i) amount of phospholipid (A) and reflux temperature (B) on percentage drug complexation (Y1).

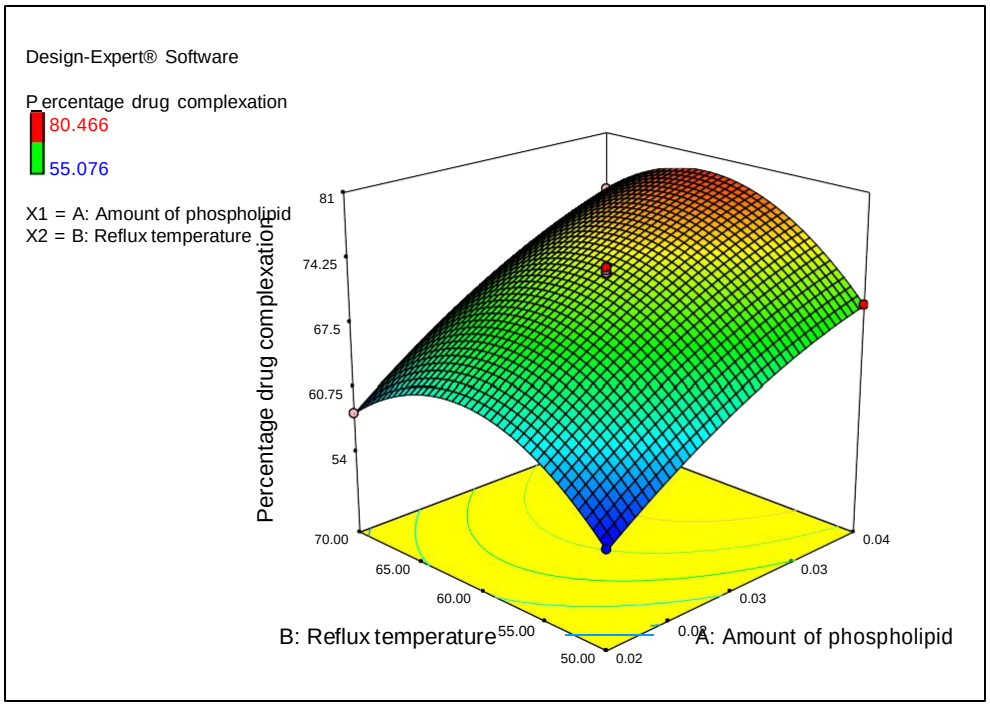

Figure 12: 3-dimensional plot showing the interactive effects of amount of phospholipid (A) and reflux temperature (B) on percentage drug complexation (Y1).

The above result might be explained based on the hypothesis that berberine HCl is adequately solubilized in a drug phospholipid complex. Previous literature confirmed that the formation of drug–phospholipid complexes is based on the interaction such as hydrogen bonds and van der Waals forces

between drug and phospholipids (Maiti et al. 2007; Yanyu et al. 2006). On increasing the molar ratio of phospholipid more sites of phospholipid will be available for the interaction with the sites of the berberine HCl. At low phospholipid molar ratio, phospholipids cannot occupy each possible position of berberine HCl, which might be involved in the interactions with phospholipids by the formation of hydrogen bonds and intermolecular forces, and until the amount of phospholipid raised to 0.04M, it might allow Berberine HCl to be surrounded by enough phospholipids and the apolar parts of the phospholipids are located around the spatial structure of berberine HCl. Such a berberine HCl–phospholipid complex structure effectively improves the liposolubility of berberine HCl (Lu et al. 2009).

3.2.5.2 Solubility in n-octanol solvent

The results of the design of experiments indicated that this system was highly influenced by the amount of phospholipid and reflux temperature, which resulted in high drug encapsulation and high solubility in n-octanol for the preparation of BHCl-PC.

From the response surface model, the regression equations 11, for the dependent variables were obtained using Design Expert software over the range of independent variables from the random order and are shown in **Table 26.**

$Y2 = 2.636 + 0.877A + 0.158B + 0.017AB - 0.081A^2 - 0.0381B^2$ **… (11)**

where Y2 is the Solubility in n-octanol solvent and A, and B are the amount of phospholipid, and reflux temperature, respectively.

The sign and value of the quantitative effect represent the tendency and magnitude of the term's influence on the response, respectively27. In the regression equation, a positive value
indicates an effect that favors the optimization due to a synergistic effect, while a negative value indicates an inverse relationship or antagonistic effect between the factor and the response. Regression values represent the quantitative effect of process variables A, and B and their influence on the dependent responses Y2. These response data are shown in **Table 26**. Further, close observation of data revealed the suitability of the response surface quadratic model when compared to the linear model, the two-factor (2FI) model, and the cubic model.

Table 26: Regression values of the selected responses during optimization.

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	0.319	0.862	0.834	0.751	1.832	
2FI	0.336	0.862	0.816	0.694	2.256	
Quadratic	0.013	1.000	1.000	1.000	0.003	Suggested
Cubic	0.014	1.000	1.000	1.000	0.002	

ANOVA for the responses indicated that the quadratic regression model was significant and valid for each of the responses A ($p<0.0001$), and B ($p<0.0001$) and hence was appropriate to represent the observed data, respectively (**Table 27**). The adjusted R^2 values for the dependent responses are 0.9997 (**Table 27**).

Table 27: ANOVA results for solubility in n-octanol solvent as the response (Y2).

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	7.367	5	1.473	8546.266	< 0.0001	Significant
A-Amount of phospholipid	6.149	1	6.149	35667.993	< 0.0001	
B-Reflux temperature	0.199	1	0.199	1153.466	< 0.0001	
AB	0.001	1	0.001	6.904	0.0340	
A^2	0.046	1	0.046	265.806	< 0.0001	
B^2	1.011	1	1.011	5862.376	< 0.0001	

Residual	0.001	7	0.000			
Lack of Fit	0.000	3	0.000	0.348	0.7936	Not significant
Pure Error	0.001	4	0.000			
Cor Total	7.368	12				
R-Squared	0.9998					
Adj R-Squared	0.9997					
Pred R-Squared	0.9996					
Adeq Precision	286.6553					

3.2.5.2.1 Three-dimensional (3D) plots

The independent variables, i.e. the amount of phospholipid (A) and the reflux temperature (B) and their interaction on the solubility in n-octanol solvent (Y2) as dependent responses were graphically represented by 3D surface plots by using RSM. When solubility in n-octanol solvent (Y2) was indicated as the response, good correlation was shown between observed and predicted values. This Y1 was significantly influenced by the amount of phospholipid (A) and the reflux temperature (B) and their interactive term (AB) and polynomial model terms A^2 and B^2. The magnitude of the positive coefficient of the term suggests that elevated levels of the amount of phospholipid and the reflux temperature in the formulation could increase the berberine HCl solubility in n-octanol solvent. **Figure 13** shown the response surface model for berberine HCl solubility in n-octanol solvent in response to the investigated factors.

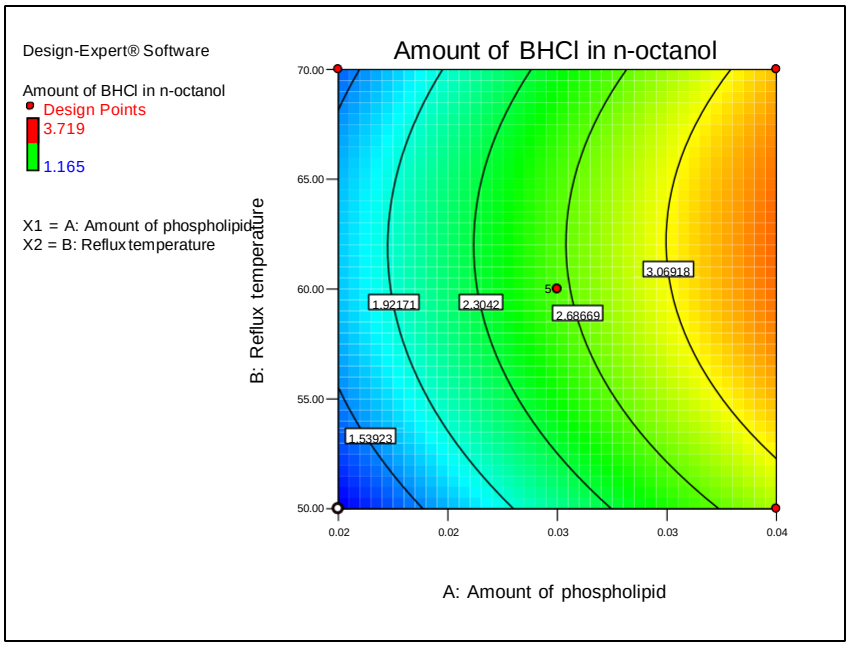

Figure 13: Contour plots showing the interactive effects of (i) amount of phospholipid (A) and reflux temperature (B) on berberine HCl solubility in n-octanol solvent (Y2)

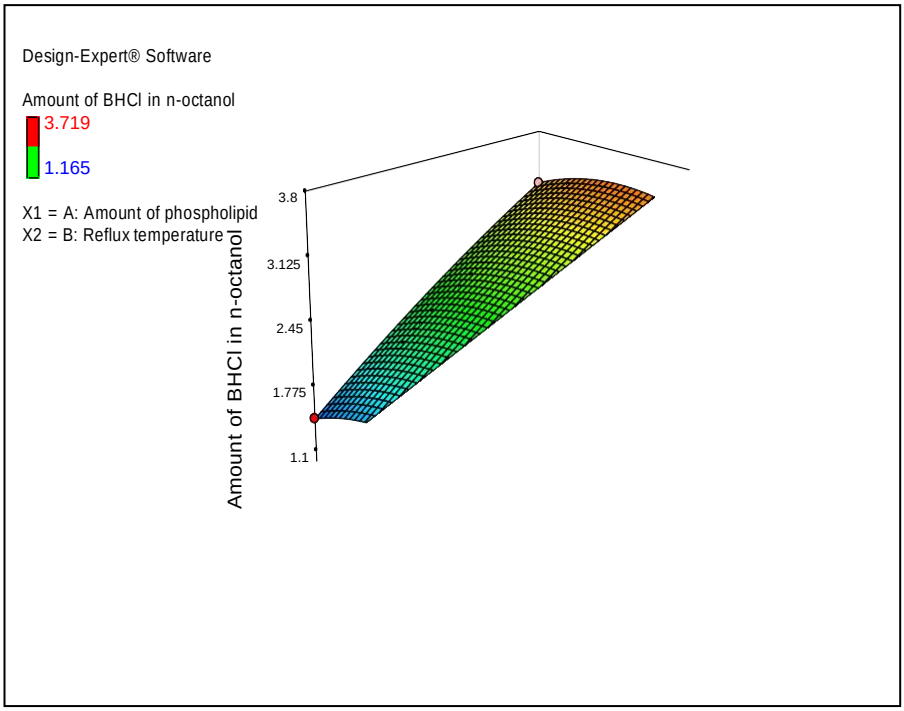

Figure 14: Three-dimensional plot showing the interactive effects of (i) amount of phospholipid (A) and reflux temperature (B) on berberine HCl solubility in n-octanol solvent (Y2).

3.2.6 Optimization of independent variable and validation

After analyzing the polynomial equations, and depicting the dependent and independent variables, a further optimization and validation process using the Design Expert software was undertaken with desirable characteristics to probe the optimal formula solution of BHCl-PC. This depended on the prescriptive criteria of maximum percentage drug complexation, and maximum n-octanol solubility. The composition of optimum formulation was shown in **Table 28**, which fulfilled the requirements of optimization. At these levels, the predicted values of Y1 (Percentage drug complexation), and Y2 (Amount of BHCl in n-octanol) were 79.265%, and 3.451565mg respectively. Therefore, to confirm the predicted model, a new batch of BHCl-PC according to the optimal formulation factor levels was prepared. **Table 29** shown Observed value of both responses for optimized formulation.

Therefore, to confirm the predicted model, a new batch of BHCl-PC according to the optimal formulation factor levels was prepared. A comparison between these observed results and theoretical predictions indicated the reliability of CCD in predicting a desirable SLN formulation (Dudhipala and Veerabrahma 2015).

Table 28: Composition and predicted value of both responses for optimized formulation.

Number	Amount of phospholipid (Molar)	Reflux temperature (^oC)	Percentage drug complexation (%)	Amount of BHCl solubilized in n-octanol (mg)	Desirability	
DBPC14	0.04	62.03	79.26545	3.451565	0.923555	Selected

Table 29: Observed value of both responses for optimized formulation.

Number	Percentage drug complexation (%)	Amount of BHCl solubilized in n-octanol (mg)
DBPC14	79.790	3.473

3.2.7 Desirability

The solutions generated by Design Expert Software were sorted in descending order of desirability values and the formulation having the highest desirability was selected for optimization. The desirability value ranges from zero to 1 for any given response. The value of 1 represents the ideal case and zero indicates one or more responses are falling outside the desirable limits. In this case as depicted in **Figure 15** desirability of the optimized formulation is 0.9234.

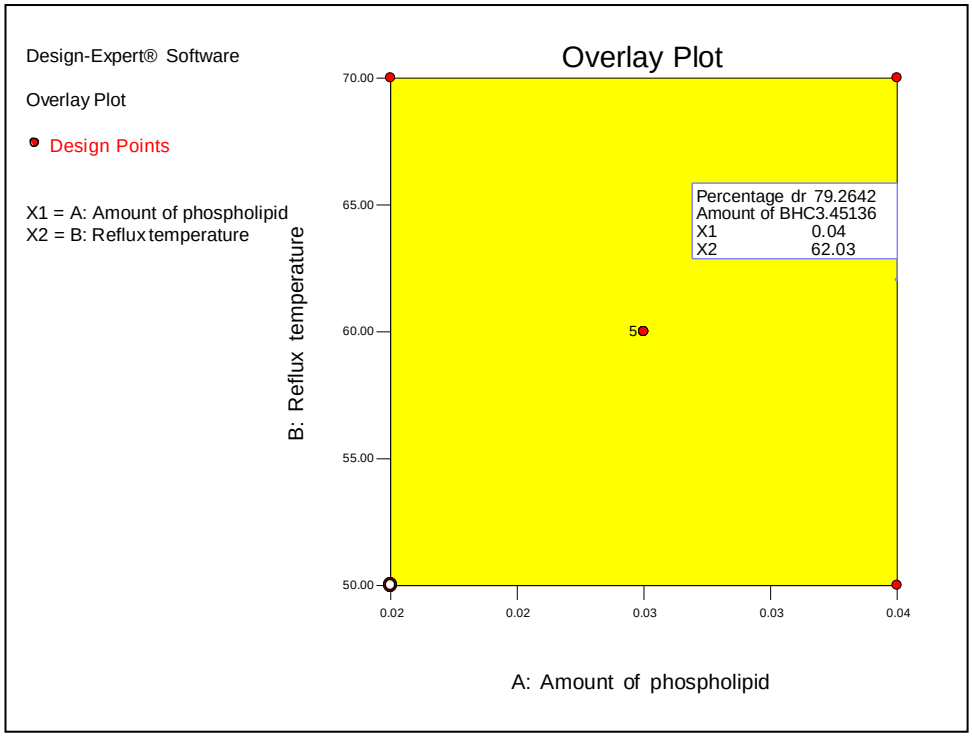

Figure 15: Over counterplot of optimized formulation.

3.2.8 Characterization parameters of berberine HCl phospholipid complex

3.2.8.1 Physical appearance

All prepared berberine HCl-phospholipid complexed formulation were characterized for their color and physical appearance shown in **Table 30**. The prepared berberine HCl complex berberine HCl-phospholipid complexed were yellow in colour residue.

Table 30: Physical appearance

S. No.	Formulation code	Physical appearance
1.	DBPC1	Slightly yellow color dried residue
2.	DBPC2	Slightly yellow color dried residue
3.	DBPC3	Slightly yellow color dried residue
4.	DBPC4	Slightly yellow color dried residue
5.	DBPC5	Slightly yellow color dried residue
6.	DBPC6	Slightly yellow color dried residue
7.	DBPC7	Slightly yellow color dried residue
8.	DBPC8	Slightly yellow color dried residue
9.	DBPC9	Slightly yellow color dried residue
10.	DBPC10	Slightly yellow color dried residue
11.	DBPC11	Slightly yellow color dried residue
12.	DBPC12	Slightly yellow color dried residue
13.	DBPC13	Slightly yellow color dried residue

3.2.8.2 FTIR of optimized formulation

The phospholipid complex of berberine HCl and phospholipids (lipoid S 100) showed changing of peaks in comparison to drug and excipient **(Figure 16)**. The berberine HCl peaks at 1800 – 2800 cm^{-1} were not observed in a complex spectrum. The lipoid S 100 peaks at 1230 and 1760 cm-1 were observable in the complex spectrum. These changes indicated that berberine HCl and lipoid S 100 formed a complex by hydrogen bonding between the methoxy and the C=O group of the phospholipids.

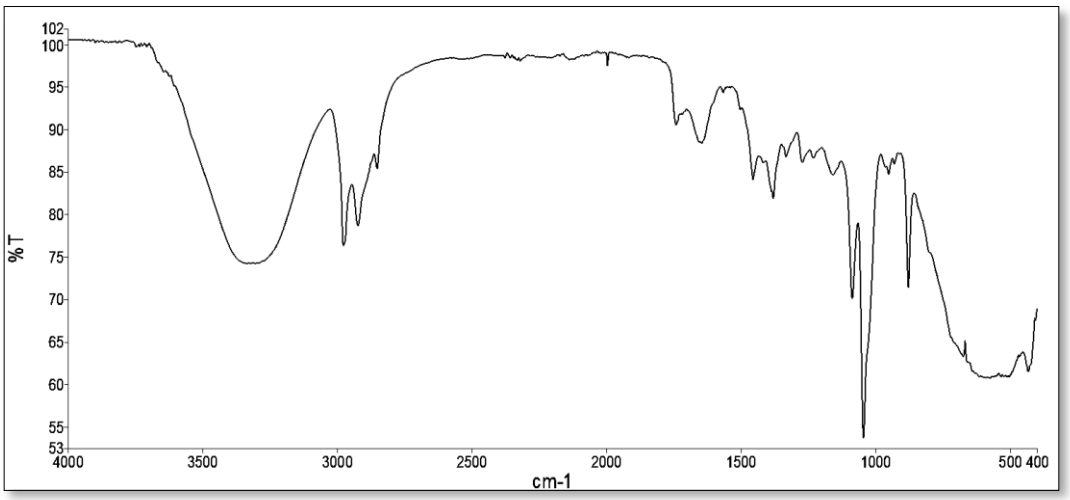

Figure 16: FTIR spectrum of DBPC14 complex formulation.

3.2.8.3 Percentage drug complexation

Percentage drug complexation of the berberine HCl with phospholipid in all berberine HCl-phospholipid complexed formulation were shown in **Figure 17.** Percentage drug complexation of the Percentage drug complexation of the berberine HCl with phospholipid in all berberine HCl-phospholipid complexed formulation were observed between 56.098±0.851% to 80.466±0.621%

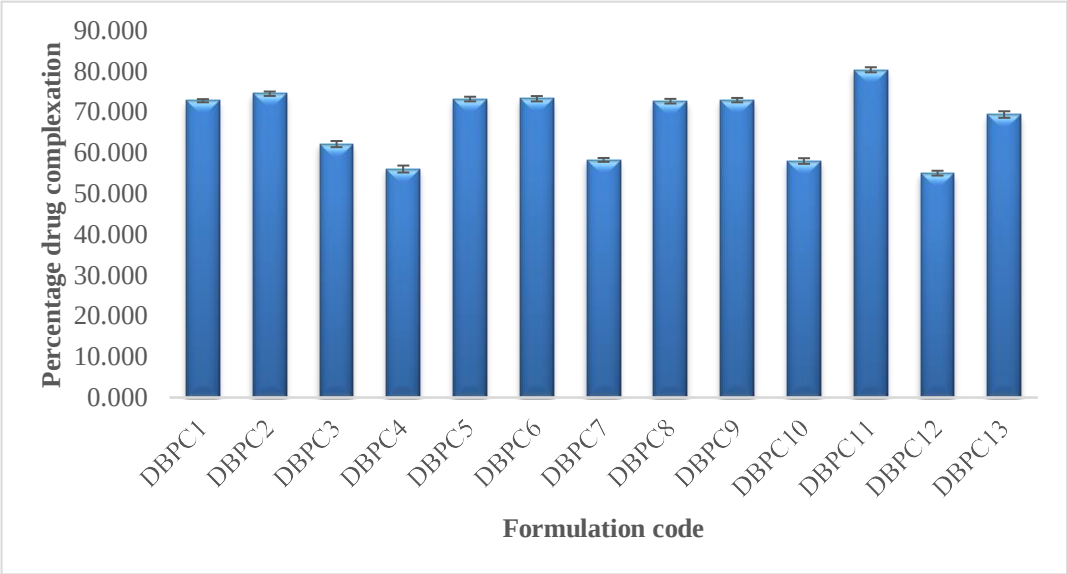

Figure 17: Percentage drug complexation of the berberine HCl with phospholipid in all berberine HCl-phospholipid complexed formulation.

3.2.8.4 Solubility of berberine HCl in n-octanol solvent

Solubility of the berberine HCl from the berberine HCl-phospholipid complexed formulation in n-octanol solvent was demonstrated in F**igure 18.** Solubility of the berberine HCl from the berberine

HCl-phospholipid complexed formulation in n-octanol solvent were observed between 1.165±0.050mg to 3.719±0.103mg.

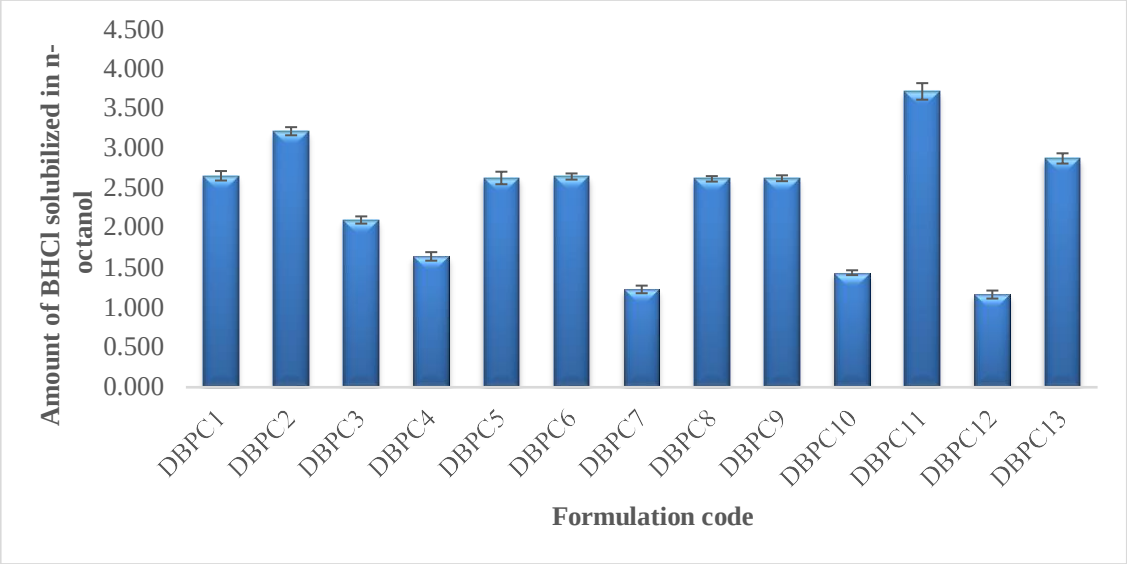

Figure 18: Solubility of the berberine HCl from the berberine HCl-phospholipid complexed formulation in n-octanol solvent.

3.2.8.5 Solubility of berberine HCl in water solvent

Solubility of the berberine HCl from the berberine HCl-phospholipid complexed formulation in water solvent was demonstrated in **Figure 19.** Solubility of the berberine HCl from the berberine HCl-phospholipid complexed formulation in n-octanol solvent were observed between 1.074±0.033mg to 2.225±0.012mg.

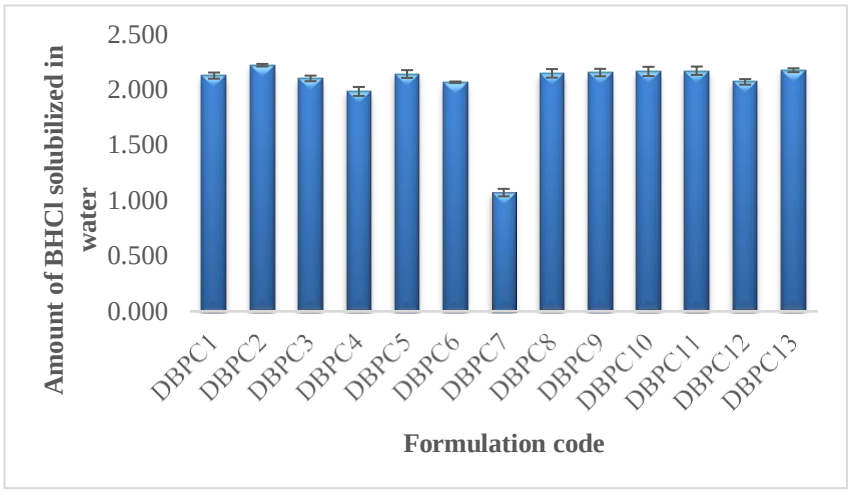

Figure 19: Solubility of the berberine HCl from the berberine HCl-phospholipid complexed formulation in n-octanol solvent

3.2.8.6 Total drug content

Total drug content of the berberine HCl in all berberine HCl-phospholipid complexed formulation were shown in **Figure 20.** Total drug content of the berberine HCl in all berberine HCl-phospholipid complexed formulation were observed between 57.777±0.856% to 82.511±0.693%.

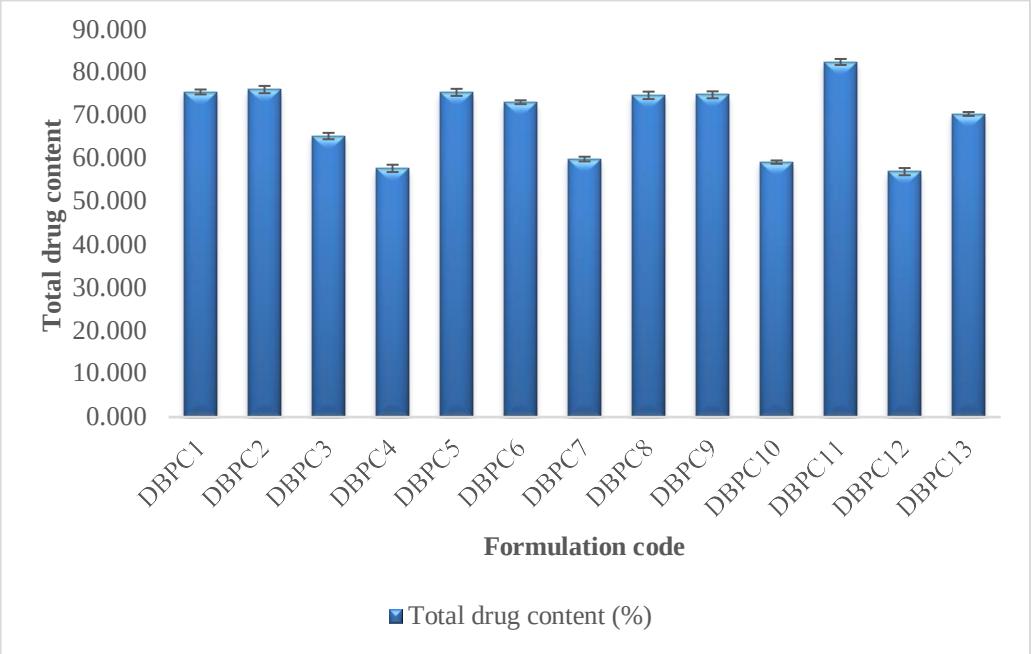

Figure 20: Total drug content of the berberine HCl in all berberine HCl-phospholipid complexed formulation.

3.2.8.7 Percentage drug loading

Percentage drug loading of the berberine HCl in all berberine HCl-phospholipid complexed formulation were shown in **Figure 21.** Percentage drug loading of the berberine HCl in all berberine HCl-phospholipid complexed formulation were observed between 14.213±0.211% to 22.881±0.209%.

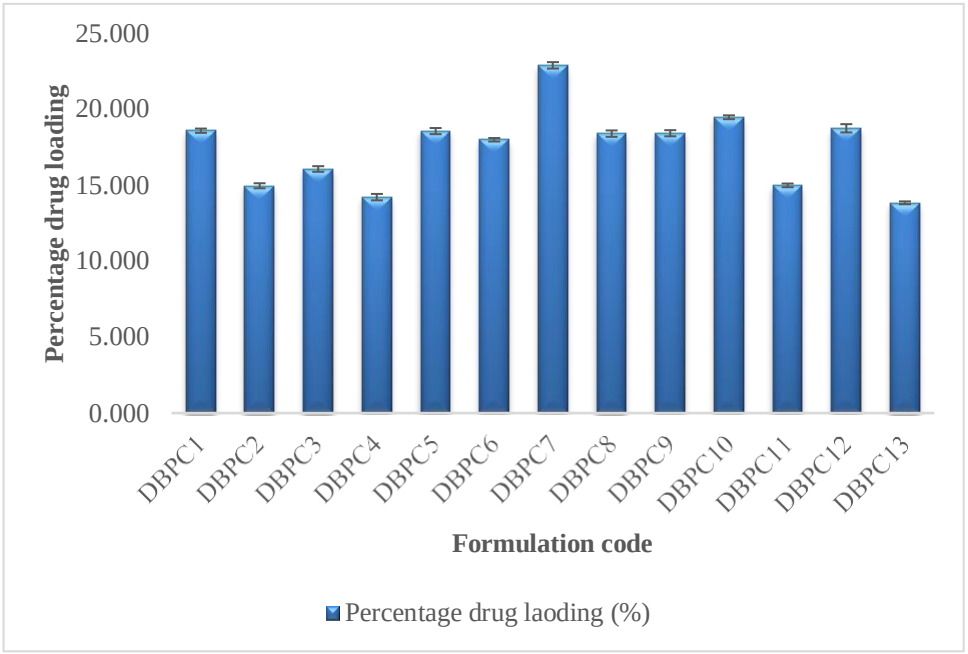

Figure 21: Percentage drug loading of the berberine HCl in all berberine HCl-phospholipid complexed formulation

3.2.8.8 *In-vitro* characterization parameter of the optimized formulation

In-vitro characterization parameter for optimized berberine HCl complexed formulation were shown in **Table 31.**

Table 31: *In-vitro* characterization parameter for optimized berberine HCl complexed formulation

Formulation code	Percentage encapsulation efficiency (%)	Solubility of BHCl in n-octanol (mg/ml)	Solubility of BHCl in water (mg/ml)	Total drug content (%)	Percentage drug loading (%)
DBPC14	79.790±0.426	3.473±0.022	2.186±0.025	82.466±0.498	16.213±0.098

3.2.8.9 *In-vitro* permeability study

The permeation of berberine HCl from selected berberine HCl-phospholipid complexed formulation DBPC14 was investigated through the dialysis membrane in phosphate buffer pH 6.8 tested from the employing Franz diffusion cells within 24 h and compare with the permeation of pure drug berberine HCl.

It should be noted that recently reported studies demonstrated that drug release medium does not interfere with membranes. Berberine drug possess the partition coefficient was used as a model penetrant/drug of medium polarity (log P = -1.54±0.002) (Elsheikh et al. 2018).

Dialysis membrane was selected for the current *in-vitro* permeation study. It is evident that the berberine HCl phospholipid complex enhances te permeation of the berberine HCl 3-fold higher than the permeation of pure drug. The values obtained from the permeation experiments were expressed as the permeated amount of the drug per unit of membrane surface area, see in **Figure 22.**

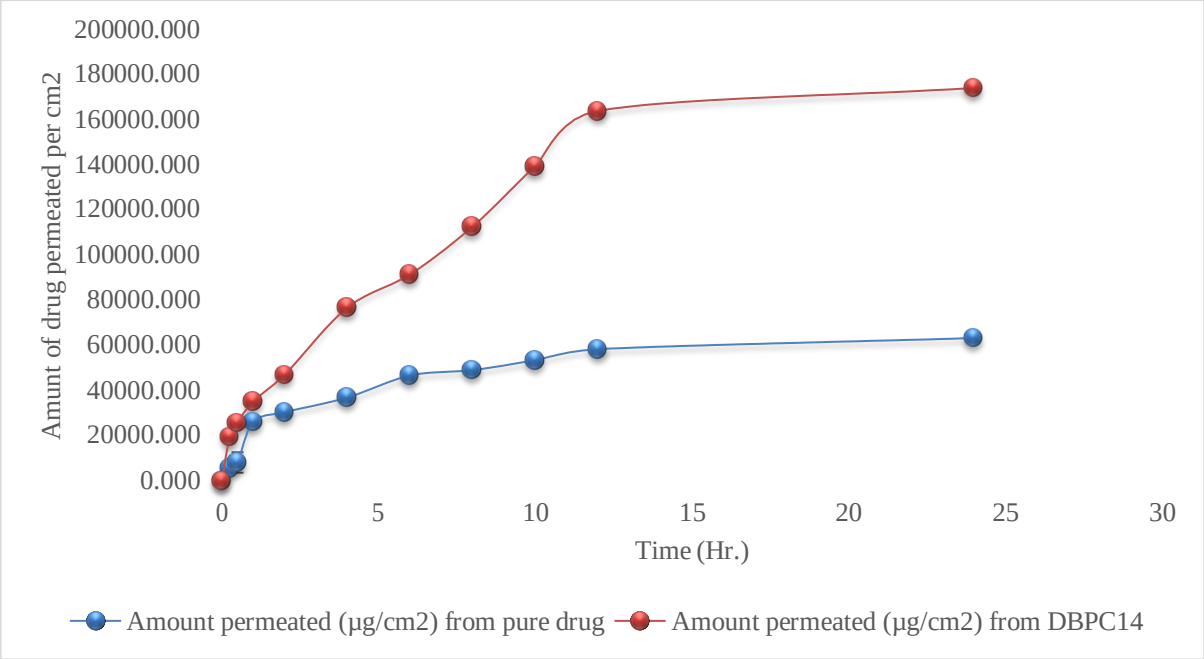

Figure 22: Comparison of permeated amounts of berberine HCl per unit area [µg/cm^2] from pure drug and berberine HCl permeation enhancer mixture.

3.3 Characterization parameter of berberine HCl chemical permeation enhancer mixture

3.3.1 Physical appearance

The physical appearance of all prepared mixtures is shown in **Table 32**. The result indicated the berberine drug in phenyl piperazine chemical permeation enhancer was clear, transparent and dark brown colour at 20mg/ml and 40mg/ml. But increasing drug amount in constant amount of the phenyl piperazine did not enhance solubility of the berberine HCl and appeared as drug particles at bottom of the glass culture tube. The methyl piperazine did not solubilize drug thus in all mixture and drug particles were observed at the bottom.

Table 32: Physical appearance of berberine HCl chemical permeation enhancer mixture

Formulation code	Physical appearance
BPE1	Clear and Dark Brown colour solution
BPE2	Clear and Dark Brown colour solution
BPE3	Dark Brown colour solution and drug not solubilized
BPE4	Dark Brown colour solution and drug not solubilized
BPE5	Dark Brown colour solution and drug not solubilized
BPE6	Dark Brown colour solution and drug not solubilized
BPE7	Dark Brown colour solution and drug not solubilized
BPE8	Dark Brown colour solution and drug not solubilized
BPE9	Dark Brown colour solution and drug not solubilized
BPE10	Dark Brown colour solution and drug not solubilized

3.3.2 FTIR

FTIR spectrum of a mixture of berberine HCl-phenyl piperazine mixture confirmed the characteristic peaks of the drug appeared with less intensity or coincided with peaks of phenyl piperazine indicating solubilization of the drug in permeation enhancer **(Figure 23 and 24)**.

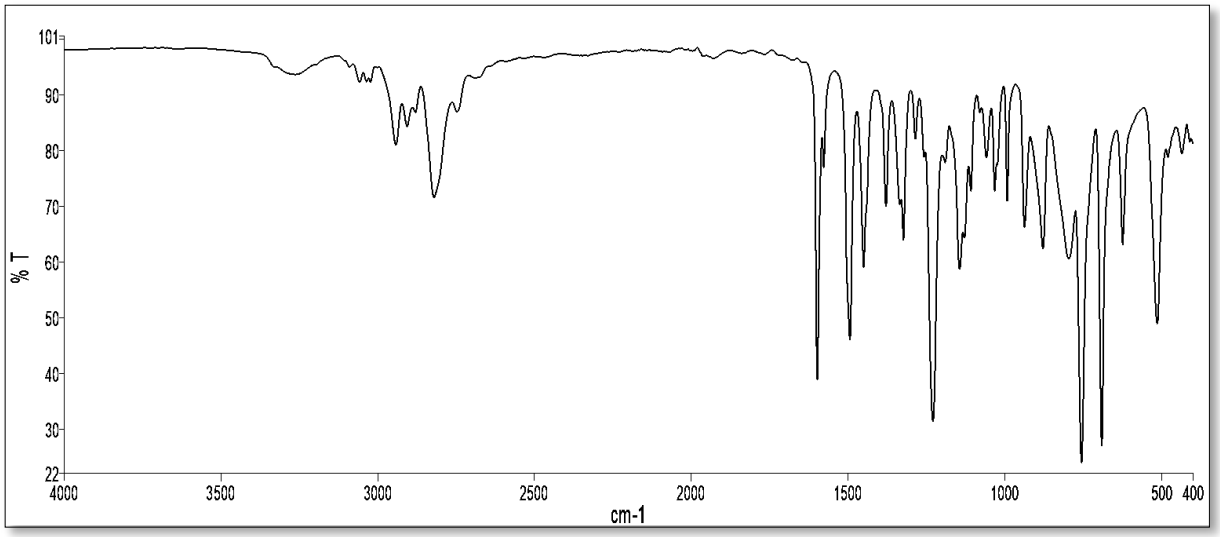

Figure 23: FTIR spectrum of phenyl piperazine

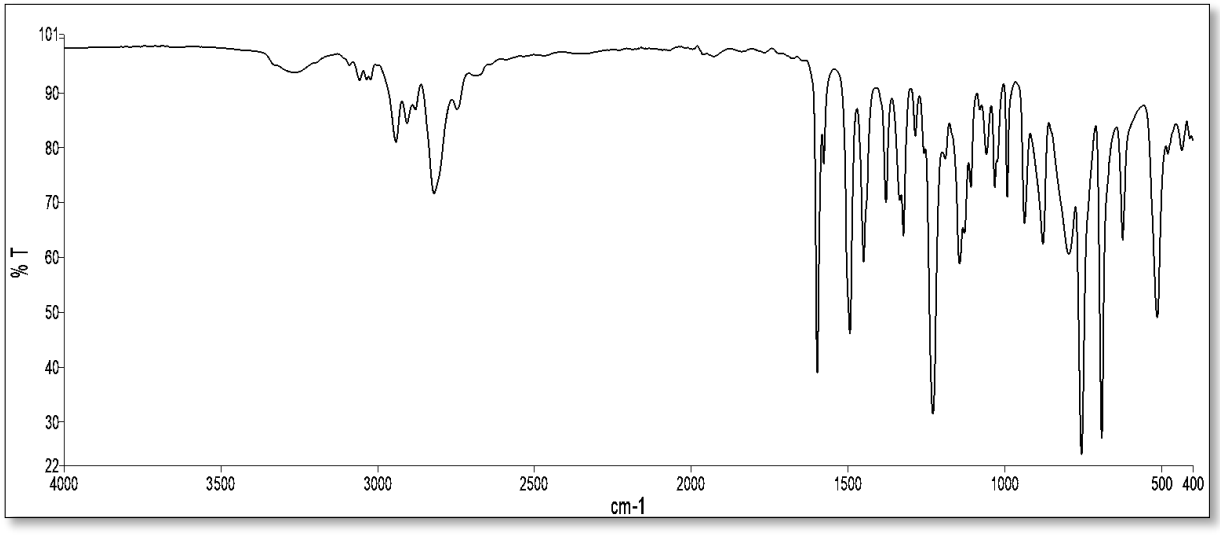

Figure 24: FTIR spectrum of a mixture of berberine HCl-phenyl piperazine mixture.

3.3.3 pH

pH of all prepared berberine HCl chemical permeation enhancer mixtures was observed to be in between a range of 4.373±0.032 to 5.153±0.047. pH of all prepared mixtures is shown in **Figure 25**.

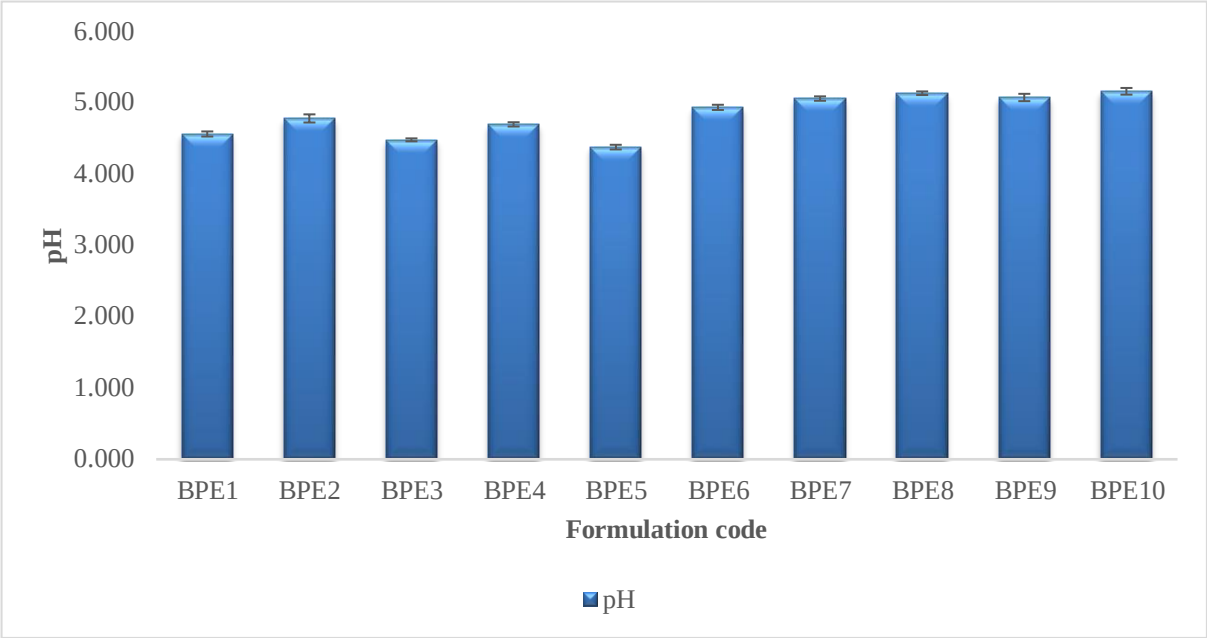

Figure 25: pH of berberine HCl chemical permeation enhancer mixture.

3.3.4 Percentage drug content

Percentage drug content of all prepared berberine HCl chemical permeation enhancer mixtures was observed to be in between a range of 20.281±0.178% to 99.814±0.426%. Mixture of the berberin HCl in phenyl piperazine at 40mg/ml concentration demonstrated higher percentage drug content 99.814±0.426% of berberin HCl. The percentage drug content of all prepared mixtures is shown **in Figure 26.**

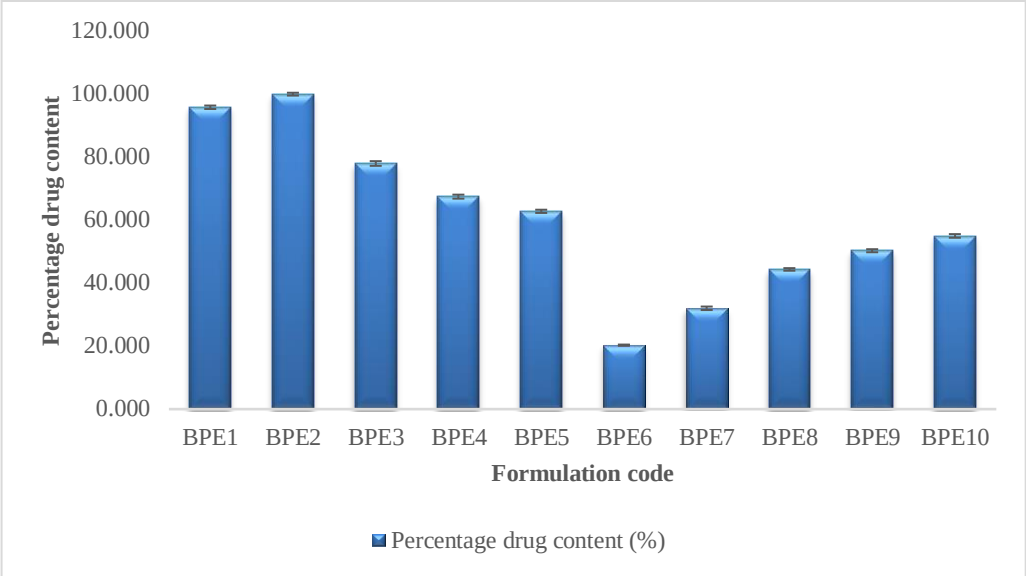

Figure 26: Percentage drug content of berberine HCl chemical permeation enhancer mixture

3.3.5 Percentage drug loading

Percentage drug loading of all prepared berberine HCl chemical permeation enhancer mixtures was observed to be in between a range of 1.277±0.022% to 6.266±0.051%. The percentage drug loading of all prepared mixtures is shown in **Figure 27**.

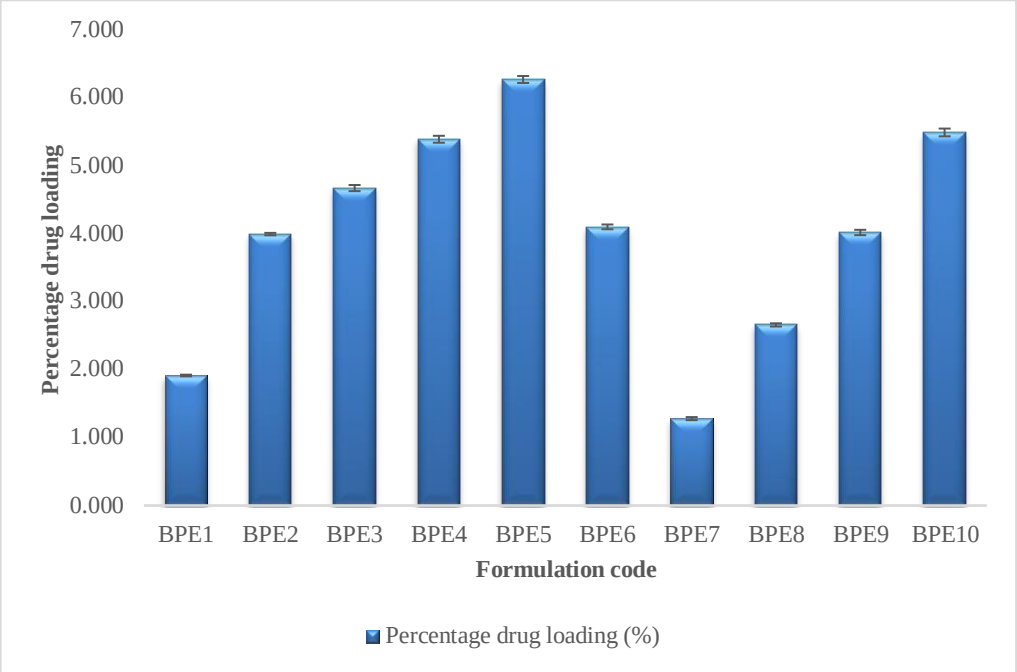

Figure 27: Percentage drug content of berberine HCl chemical permeation enhancer mixture.

3.3.6 Permeability flux

It is evident that the addition of chemical permeation phenyl piperazine caused the enhancement of berberine HCl permeation. Based on the above-mentioned results, the permeated amount of berberine HCl with the chemical permeation enhancer at minute 60 reached approx. 2-fold higher values than from the formulation without the chemical permeation enhancer, see **Figure 28**., and mixture BPE2 was the most effective.

At berberine HCl solubility 40mg/ml in the chemical permeation enhancer the Permeation flux (µg/cm^2/hr) and Apparent permeability (Papp) (cm/hr) was found to be higher 7325.667±18.938 µg/cm^2/hr and 14.651±0.398cm/hr, respectively.

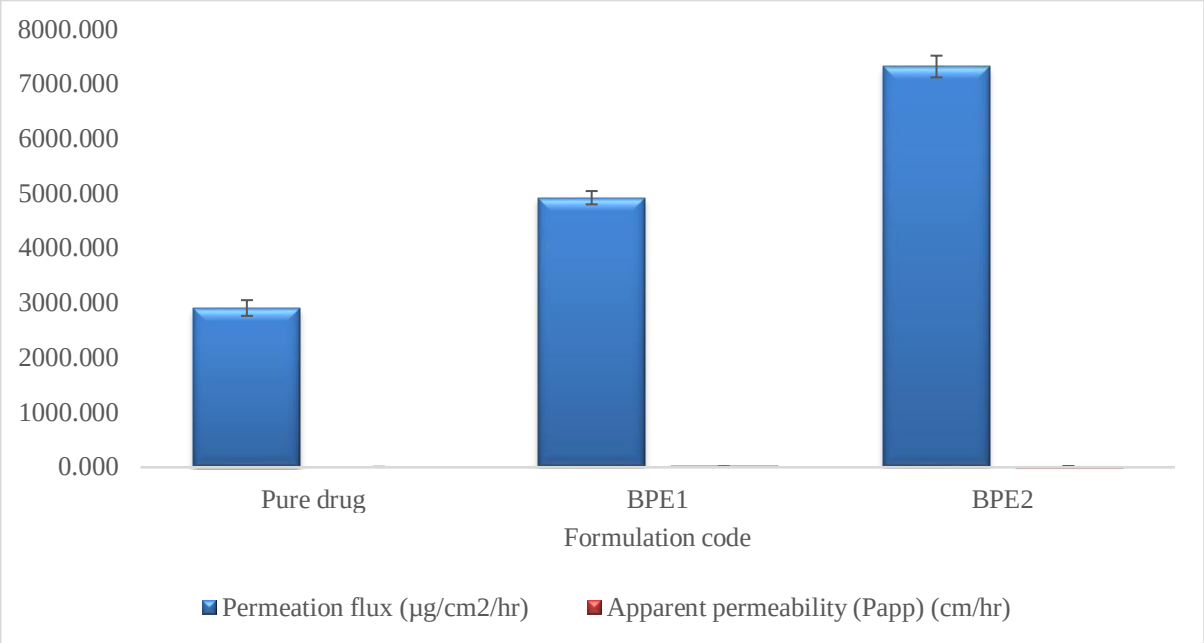

Figure 28: Permeation flux (µg/cm^2/hr) and Apparent permeability (Papp) (cm/hr) parameters for berberine HCl and berberine HCl with chemical permeation enhancer.

3.3.7 *In-vitro* Permeability coefficient

The permeation of berberine HCl through the dialysis membrane with or without chemical permeation enhancer was investigated from the phosphate buffer pH 6.8 tested from the employing Franz diffusion cells within 24 h.

It should be noted that recently reported studies demonstrated that drug release medium does not interfere with membranes. Berberine drug possess the partition coefficient was used as a model penetrant/drug of medium polarity (log P = -1.54±0.002) (Elsheikh et al. 2018).

Dialysis membrane was selected for the current in vitro permeation study. The values obtained from the permeation experiments were expressed as the e permeated amount of the drug per unit of membrane surface area, see in **Figure 29.** The dependences of the permeated amount of the drug per unit of surface area in time for the pure drug berberine HCl and the most effective drug permeation enhancer mixture for better lucidity.

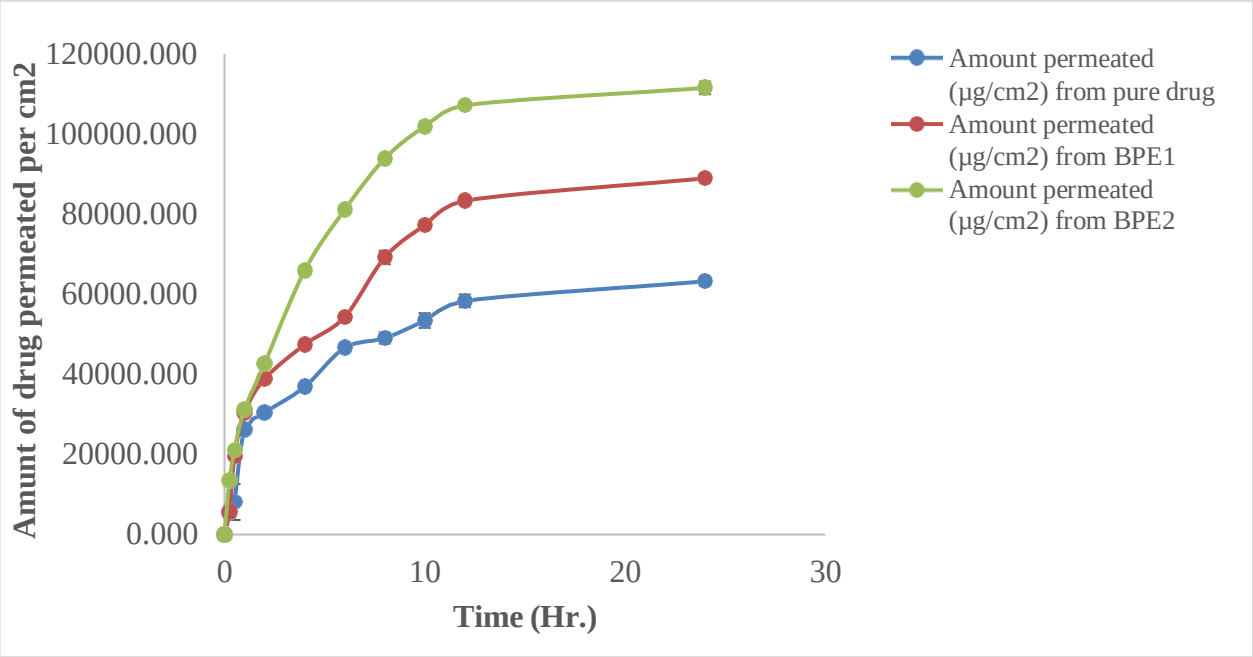

Figure 29: Comparison of permeated amounts of berberine HCl per unit area [µg/cm^2] from pure drug and berberine HCl permeation enhancer mixture.

3.3.8 Stability study

Stability study of optimized berberine HCl phospholipid complex DBPC14 formulation and Berberine HCl-phenyl piperazine mixture BPE2 was assessed at three different storage conditions 4±2 °C (Refrigerator), Room temperature (Desiccator), 25±2°C/60±5% RH. The formulations were characterized for the physical appearance, percentage drug content, and percentage drug loading of berberine HCl shown in **Table 33-34.**

Table 33: Physical appearance, Percentage drug content, and percentage drug loading of berberine HCl phospholipid complex DBPC14 formulation.

Time interval	**Storage condition: 4±2 °C**		
	Physical appearance	Percentage total drug content (%)	Percentage drug loading (%)
0th month	Slightly yellow color dried residue	82.797±0.284	16.278±0.056
1st month	Slightly yellow color dried residue	82.513±0.142	16.222±0.028
3rd month	Slightly yellow color dried residue	82.466±0.082	16.213±0.016

Time interval	**Storage condition: Room temperature (Desiccator)**		
	Physical appearance	Percentage total drug content (%)	Percentage drug loading (%)
0th month	Slightly yellow color dried residue	82.797±0.284	16.278±0.056
1st month	Slightly yellow color dried residue	82.561±0.217	16.231±0.043
3rd month	Slightly yellow color dried residue	82.182±0.357	16.157±0.070
Time interval	**Storage condition: 25±2°C/60±5% RH**		
	Physical appearance	Percentage total drug content (%)	Percentage drug loading (%)
0th month	Slightly yellow color dried residue	82.797±0.284	16.278±0.056
1st month	Slightly yellow color dried residue	82.324±0.217	16.185±0.043
3rd month	Slightly yellow color dried residue	82.135±0.082	16.148±0.016

Table 34: Physical appearance, Percentage total drug content, and percentage drug loading of Berberine HCl-phenyl piperazine mixture BPE2 formulation.

Time interval	**Storage condition: 4±2 °C**		
	Physical appearance	Percentage total drug content	Percentage drug loading
0th month	Clear and Dark Brown colour solution	82.797±0.284	16.278±0.056
1st month	Clear and Dark Brown colour solution	82.513±0.142	16.222±0.028
3rd month	Clear and Dark Brown colour solution	82.466±0.082	16.213±0.016
Time interval	**Storage condition: Room temperature (Desiccator)**		
	Physical appearance	Percentage total drug content	Percentage drug loading
0th month	Clear and Dark Brown colour solution	82.797±0.284	16.278±0.056
1st month	Clear and Dark Brown colour solution	82.561±0.217	16.231±0.043
3rd month	Clear and Dark Brown colour solution	82.182±0.357	16.157±0.070
Time interval	**Storage condition: 25±2°C/60±5% RH**		
	Physical appearance	Percentage total drug content	Percentage drug loading
0th month	Clear and Dark Brown colour solution	82.797±0.284	16.278±0.056
1st month	Clear and Dark Brown colour solution	82.324±0.217	16.185±0.043
3rd month	Clear and Dark Brown colour solution	82.135±0.082	16.148±0.016

Tables 33 and 34 indicated no significant changes were observed in the physical appearance, percentage drug content, and percentage drug loading of the berberine HCl in optimized berberine HCl phospholipid complex DBPC14 formulation and Berberine HCl-phenyl piperazine mixture BPE2 was assessed at three different storage different condition 4±2 ℃ (Refrigerator), Room temperature (Desiccator), 25±2°C/60±5% RH.

3.3.9 *In-vivo* pharmacokinetic

The determination of berberine concentration in plasma and the pharmacokinetics study of berberine HCl suspension, berberine HCl-phenyl piperazine mixture (BPE2), and berberine-HCl-phospholipid complex (DBPC14) accomplished using the HPLC method.

3.3.9.1 Preparation of linearity curve of the berberine HCl in plasma

The linearity curve of the berberine HCl in plasma was shown in **Table 35.**

Table 35 Linearity curve of berberine HCl in plasma.

Con.(µg/ml)	Area
1	78947±21.932
2	146617.3±34.429
3	214831±173.865
4	291410.3±164.148
5	359740±247.322
6	432350.3±263.306

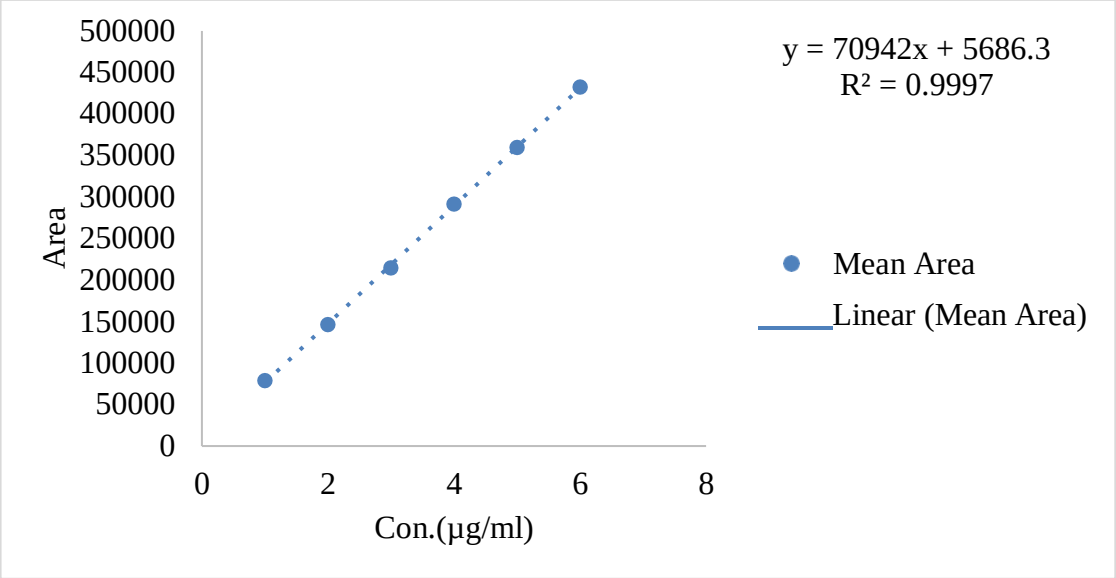

Figure 30: Linearity curve of the berberine HCl in plasma.

3.3.10 Pharmacokinetic Study

The plasma concentration-time curves after oral administration of berberine HCl suspension, berberine HCl-phenyl piperazine mixture (BPE2), and berberine-HCl-phospholipid complex (DBPC14) were shown in **Figure 6.51** and the pharmacokinetic parameters were summarized in Table 6.44. As seen in the berberine plasma concentration-time curves, a second peak appeared around 8 h after the oral administration of berberine HCl suspension, berberine HCl-phenyl piperazine mixture (BPE2) and berberine-HCl-phospholipid complex (DBPC14), which was resulted from the enterohepatic circulation in accordance with the previous research findings (Feng et al. 2010).

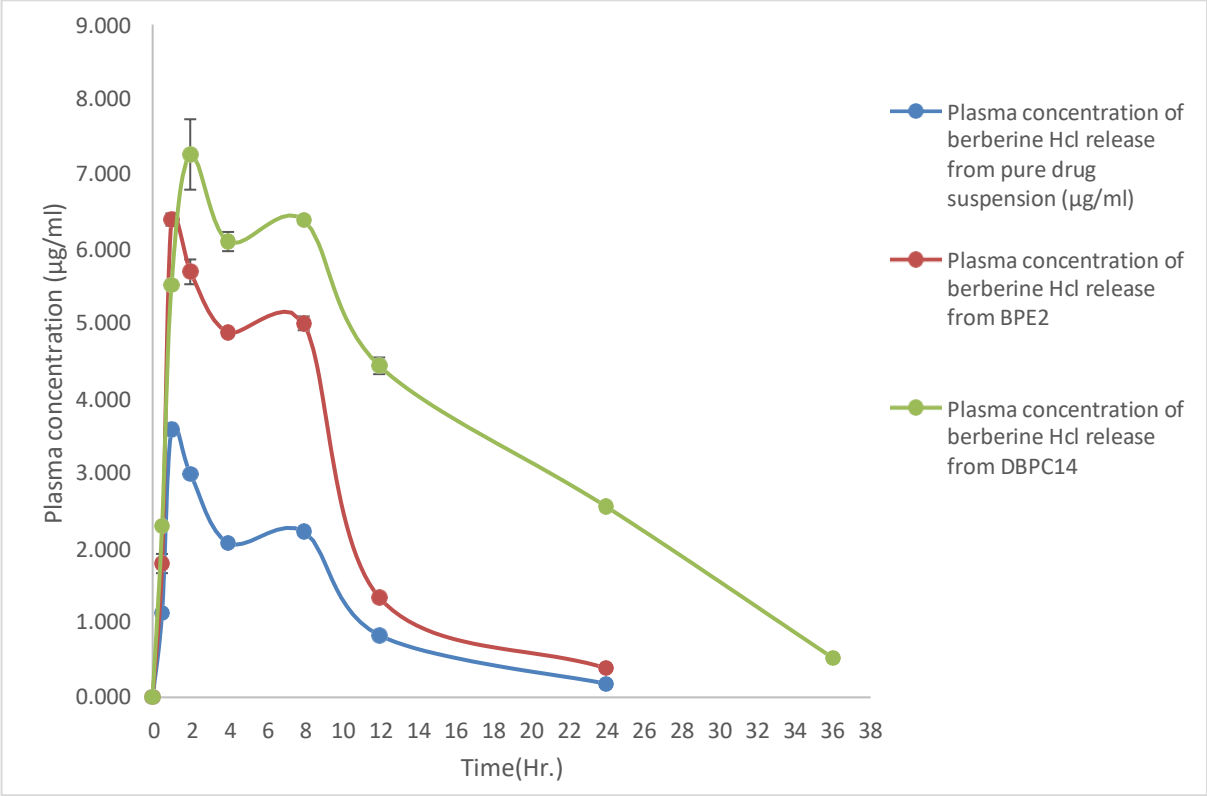

Figure 31: *In-vivo* pharmacokinetic profile of berberine HCl suspension, berberine HCl-phenyl piperazine mixture (BPE2) and berberine-HCl-phospholipid complex.

Table 36: Pharmacokinetic parameters after oral administration of berberine HCl suspension, berberine HCl-phenyl piperazine mixture (BPE2) and berberine-HCl-phospholipid complex (DBPC14).

Pharmacokinetic parameter	**Pure drug suspension**	**Berberine HCl-Phenyl piperazine mixture (BPE2)**	**Berberine HCl phospholipid complex DBPC14**
$t_{1/2}$ (h)	5.427±0.048	7.244±0.969	8.194±0.052
T_{max} (h)	1.000±0.001	1.000±0.002	2.000±0.001
C_{max} (µg/ml)	3.582±0.012	6.391±0.082	7.261±0.471
AUC_{0-t} (µg/ml*h)	30.513±0.318	65.245±0.688	129.286±1.261
AUC_{0-inf_obs} (µg/ml*h)	31.935±0.315	66.952±0.883	135.537±1.193
MRT_{0-inf_obs} (h)	8.099±0.064	8.912±0.293	13.857±0.046

The AUC_{0-t} and C_{max} of berberine HCl phospholipid complex DBPC14 formulation was higher than the pure drug berberine HCl suspension and berberine HCl-Phenyl piperazine mixture (BPE2). Significant increase in AUC_{0-t} (129.286±1.261 µg/ml×h) of berberine HCl from berberine HCl phospholipid complex DBPC14 was observed when it was compared to AUC_{0-t} of berberine HCl (30.513±0.318 µg/ml×h) and Berberine HCl-Phenyl piperazine mixture (BPE2) (65.245±0.688 µg/ml×h) (**Table 36**). Short half-life of berberine HCl (5.427±0.048 hr) was significantly increased in Berberine HCl phospholipid complex DBPC14 (8.194±0.052 hr). Permanency (Mean Residence Time; MRT) of drug with phospholipid complex was found ~1.5 fold higher than Pure drug berberine HCl suspension and berberine HCl-Phenyl piperazine mixture (BPE2) (Dora et al. 2017).

The higher C_{max} obtained by the DBPC14 group compared to the pure drug berberine HCl suspension group could be ascribed to the efficient absorption caused by the nanoscaled particle size and enhanced affinity related to the lipid rich cell membranes by the effective and attractive hydrophobic modification of the DBPC14. Moreover, DBPC14 systems entirely exhibited higher absorption and

relative bioavailability than berberine HCl under the action of amorphous form dispersion of drug in carriers, and the solubilization, wetting effect and P-gp inhibit effect. The combined carriers could enormously expand the contact area with the gastrointestinal tract, also resulting in a better absorption and improved bioavailability (Zhang et al. 2014). The results demonstrate that the absorption of the berberine HCl in the gastrointestinal tract was also markedly improved by the berberine HCl phospholipid complex DBPC14 than and berberine HCl suspension, therefore significantly increased the bioavailability of berberine HCl which is essential for further clinical application (Yu et al. 2017).

4. Conclusion

In conclusion, the optimization of berberine chloride formulation using permeation enhancers holds significant promise for the treatment of diabetes. Through a comprehensive preformulation study, various parameters were evaluated, including physical characteristics, solubility, and FTIR analysis, indicating the successful formation of berberine chloride-phospholipid complexes. These complexes demonstrated enhanced permeation and bioavailability, particularly the DBPC14 formulation. The stability study further confirmed the robustness of the optimized formulation over a three-month period. In the *in-vivo* pharmacokinetic analysis highlighted the superior performance of the DBPC14 complex compared to the pure drug and other formulations, suggesting its potential for improved therapeutic outcomes in diabetes treatment. Overall, the study highlights the importance of utilizing quality by design strategies in formulation development to ensure consistent quality and efficacy of phospholipid-based formulations for diabetes management.

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