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Piperine-phospholipid complexes – Development of novel Bioactive formulations for better Healthcare solutions

Nandkishor S. Talware[1,2*], Remeth J. Dias[3], V. Rama Mohan Gupta[4]

1.Research Scholar, Department of Pharmaceutical Sciences, Jawaharlal Nehru Technical University, (JNTU) Hyderabad, Telangana, India -500 085.

2.Shri Vile Parle Kelawani Mandal's Institute of Pharmacy,Dhule, Maharashtra, India -424 001.

3.Department of Pharmacy, Government Polytechnic, Vidyanager, Karad-Masur Road, Karad, Dist. Satara, Maharashtra, India-415 124

4.Pulla Reddy Institute of Pharmacy, Annaram, Medhak (Dt), Telangana, India -502 313.

ABSTRACT

Black pepper, also designated as 'King of spices' a characteristic familiar global spice related to the Piperaceae family and generally used in culinary and medicinal preparations. Its pungency is due to piperine, volatile elements and essential oil. Piperine is an amide alkaloid, effective bioactive present in piper species of black and long peppers; and reveals several potential therapeutic actions to intervene different disease conditions whereas functional group responses at active site liable to act as a xenobiotic bio-enhancer and an effective CNS stimulant. However, piperine is slightly soluble in water, limiting its pharmacological activities and biomedical services. It is solid crystalline nature, mild basic, initially tasteless while, a burning after taste. Therefore, this bioactive natural substance should be considered in the arenas of rational drug design and development of formulations. Recent developments in drug delivery system have to overcome its limitations, including poor bioavailability and blood–brain barrier permeability. Chaperons like phytosomes are encouraging tools to alter oral absorption of piperine. The study highlights the prepared and correctly recognized piperine-phospholipid complex (PPC) in terms of FT-IR (Fourier Transform Infra-red spectroscopy), DSC (differential scanning calorimetry), XRPD (X-ray powder diffractometry), and SEM (scanning electron microscopy). The PPC was found to be fine and loose, airy, light, rough surface with improved water solubility and bioavailability.

Keywords: Piperine, Bioavailability, Characterization, Herbosomes.

*Corresponding Author Email: nandkishorwani@rediffmail.com

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INTRODUCTION

Black pepper, 'King of spices', a characteristic familiar global spice related to the Piperaceae family and contains a most abundant pungent principle, piperine. Number of studies reported a variation in piperine content, viz- 9%, 4-5% and 2-7.4% in black pepper, long pepper (piper longum), black and white pepper (piper nigrum) respectively [1]. The cultivation aspects like weather, humidity and the location impacts the content of piperine [2]. Piperine is a creamy coarse extracted substance with molecular formula $C_{17}H_{19}NO_3$ having a melting point range of 128-130°C. Piperine is mild basic, which on hydrolysis (acid/alkali), can be transformed to piperidine and piperic acid[3]. Besides piper species, pipereine also obtains from seeds of Anethumsowa (Apiaceae), Fructus piperis Longi, Vicoaindica (Asteraceae),[4] in the leaves of Rhdodendron faurie (Ericaceae)[5], and bark of Careya arborea (Lecythidaceae)[6]. Black pepper has geometrical isomers of piperine, viz. piperine, isopiperine, chavicine and isochavicine, which do not possess pungency compared to piperine. [7]. While other alkaloids are viz. piperanine, piperettine, piperylin A, piperolein B, and pipericine, [8]. Piperine is practically exploited for its pungency [9] which is due to vanilloid elements; i.e., capsaicin, the pungent principle observed usually in hot chili peppers. Nonetheless, herbal plants having the alkaloid, piperine, are usually used in medicinal preparations for curing an array of disorders and as culinary spices globally [9,10].

Piperine is used in traditional remedies of Indian as well as in Chinese treatment. Piperine possesses various pharmacological activities modulating transporter and metabolic enzyme activities. Moreover, it is suggested that piperine can be used as an alternative medicine [11]. Piperine is reported to be used for the improvement of blood circulation, salivation, and stimulation of appetite [12]. Piperine can be used widely in pain management, chills, rheumatism arthritis, influenza, hypotension, vascular cell modulation and fever [13-15]. Piperine enhances the absorption and bioavailability of various drug molecules [16–18]. Some molecular mechanisms underlying piperine activities include a change in the membrane dynamics accompanied by the initiation of protein synthesis linked to the cytoskeleton functioning. This stimulates the passive absorption in the small intestine, thus, supporting the effective drug permeation through the epithelial barriers [19].

It also acts on many enzyme systems (including p-glycoproteins) [20,21]. Piperine has shown various biological activities such as anti-infective, antimicrobial, insecticidal, anti-inflammatory, antiamoebic, antiulcer, and antidepressant [22–31]. Piperine inhibits the expression of PPAR-γ on 3T3-L1 cell lines, thus used in the treatment of diseases related to obesity [32]. Piperine might be viewed as a potent immunomodulator, inhibiting airway inflammation a murine model of asthma

by the enhanced expression of TGF-beta gene in the lungs [33]. To inhibit antigen-induced allergic reactions that control degranulation, piperine can interfere with the IgE-mediated degranulation and cytokine production by RBL-2H3 cells [34]. Ahmad et al. discussed the biological values and diverse biological activities of piper nigrum, effect of piperine in the process of digestion, antioxidant properties, and the role in the management of various disorders [35–37]. Bioenhancers are agents capable of increasing the bioavailability when combined with a particular therapeutic agent without exerting any of its biological activity at the used dose. Bioenhancers are capable of increasing the absorption from the gastrointestinal tract or inhibiting enzymes involved in the biotransformation of the drug by preventing the drug transformation to metabolites and by decreasing the rate of elimination [38].

Despite the various therapeutic properties of piperine, its biomedical applications are still limited due to its poor bioavailability and low aqueous solubility, which can be enhanced by in situ intestinal absorption models via formulating it with polymers to form a novel drug delivery system [39]. Thus intent to design and develop phospholipid based novel drug delivery system- Herbosomes.

MATERIALS AND METHOD

Preparation of standard solution for calibration curve of piperine

Stock solution was prepared by dissolving 10 mg of piperine in 100 ml of methanol. Standard solutions were prepared from stock solution of piperine in the concentration range of 2-20µg/ml using methanol as solvent. The absorbance of piperine solutions were measured at λ_{max} 342 nm against methanol as blank and calibration curve was plotted between absorbance and concentration.

Method development and validation

Preparation of sample solution Three marketed formulations from different manufacturer (PP-1, PP-2 and PP-3) were taken in this study. Of each sample 5 gm were taken and converted into powdered form. Samples were transferred separately in 100 ml round bottom flask (RBF) and refluxed with 100 ml of ethanol for 1 hour. The extract was filtered and re-refluxes the marc left with 50 ml of ethanol for additional 1 hour. Filtrates were combined and subjected to concentration in rotary evaporator. Residue obtained was dissolved in methanol and volume was made upto 1000 ml with methanol. The absorbance of sample solutions were measured at λ_{max} 342 nm against methanol as blank. Same procedure was repeated for two different days.

Preparation of piperine- loaded phytosomes-

Different quantities of pure piperine (PIP) and Hydrogenated Soy Phosphatidyl Choline (HSPC) in molar ratio of HSPC: PIP in range of 0.5:1 to 1.5:1 were refluxed in 20 ml of dichloromethane.

After the volume of resulting solution was concentrated to 2-3 ml, then 10 ml of n-hexane was added with continuous stirring leading to the precipitation of the complex. The complex was then filtered, vacuum-dried and used for analysis.

RP-HPLC determination of piperine in piperine-phospholipid complex

The content of piperine in the complex was determined by RP-HPLC method. Waters RP-HPLC system (Milford, USA) supported by quaternary pump was used for the analysis. A reverse phase C18 column (250 x 4.6 mm, 5 µm) was used as stationary phase. The assay was performed using isocratic conditions. Methanol and water (pH 2.3 by adding 0.1M ortho-phosphoric acid) in a ratio of 50:50 v/v was used as the mobile phase. All the samples were filtered through a 0.22 µm syringe filter previous to injection. For RP-HPLC analysis, 20 µl aliquot of the standard and sample solution were injected into the system. The flow rate was maintained constant at 1 ml min-1. Peaks were monitored at 342 nm using a UV-Vis detector. Analysis of each sample was done in triplicate (n=3). The method was validated using ICH guidelines (ICH 2005a).

Complexation efficiency

The complexation efficiency (CE) of piperine-phospholipid complex (PPC) was calculated based on the following formula from RP-HPLC:

% Complexation efficiency (CE) = (Amount of piperine in complex/Amount of piperine used) × 100

Optimization of PPC

Piperine-phospholipid complex (PPC) was optimized by taking three input variables namely, Ratio of phospholipid to drug (mole/mole) (A), Reaction time (hr) (B), and Reaction temperature (°C) (C). Response surface methodology (RSM) with a three factor-three coded level Box–Behnken design (BBD) was used for optimizing the preparation conditions. Several verification experiments were also conducted for establishing the accuracy of the results.

Table 1: Independent factors and their corresponding values for optimization of piperine phytosomes

Factors	Code	Range and levels		
		-1 (Low)	0 (Medium)	1 (High)
Ratio of phospholipid to drug (mole/mole)	A	0.5:1	1.2:1	1.5:1
Reaction time (hr)	B	1	2	3
Reaction temperature (°C)	C	40	50	60

Characterization of Phytosome

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of piperine, HSPC, physical mixture containing piperine and HSPC, and Phytosome were recorded by Fourier Transform Infrared Spectroscopy (Perkin Elmer Fourier Transform Infrared Spectrophotometer, Perkin Elmer, UK) using the potassium bromide (KBr). 5-10 mg of dried samples were grounded and mixed with spectra grade KBr and then pressed into a disk in hydraulic press. Samples were analyzed in the 4000 to 400 cm^{-1} spectrum.

Differential thermal analysis (DTA)

DTA curves using a Differential Scanning Calorimeter (Pyris Diamond TG/DTA, Perkin Elmer, Singapore) were recorded. 3.0 ± 0.2 mg of each individual sample were heated in an inert atmosphere having continuous flow of nitrogen gas at 150 ml min^{-1}. The studies were performed over the temperature range of 30-225°C at a heating rate of 12 °C min^{-1} using Alpha alumina powder as standard.

Particle size analysis

The mean particle diameter, polydispersity index and zeta potential of piperine phytosome was determined by using Zeta sizer Nano ZS90, Malvern Instruments Ltd, USA. 5 mg of piperine phytosome was dispersed in 10 ml of deionized water and readings were recorded.

Scanning Electron Microscopy (SEM)

The surface morphology of piperine phytosome was investigated by SEM. The sample was coated with a thin layer of about 30 µm of palladium in auto fine coater (Jeol JFC1600, Tokyo, Japan) and placed in the sample chamber of a scanning electron microscope (Jeol JSM 5200). Photomicrographs at different magnifications were recorded.

Powder X-ray diffraction (PXRD)

The intensity at different diffraction angles from 2°-50° of the samples were analyzed by a powder X-ray diffractometer (Rigaku Ultima III, Japan) using Cu K alpha radiation source at a voltage of 40 kV and a current of 30 Ma (1.2 kW). All the readings were recorded at room temperature.

***In-vitro* release studies**

In-vitro piperine release from piperine-phospholipid complex was performed using the dialysis bag method. Artificial gastric environment using 0.1 M HCl with pH 1.2 and intestinal environment using phosphate buffer solution (PBS) with pH 6.8 and 7.4 without enzymes were used as dissolution medium. The dialysis membranes (Himedia© LA 387) with a molecular weight cut-off of 12-14 kilo Dalton were used to hold piperine-phospholipid complex. The dialysis membranes were soaked in double distilled water for 12 hr prior to use. Piperine-phospholipid complex was redispersed in double distilled water, and 1 ml of suspension was added to the membranes, which were tightly bundled at the two ends. The bags were placed in 100 ml of the dissolution media in a

beaker. The beaker was shaken in a magnetic stirrer (Remi, India) at speed of 60 rpm and temperature 37±1°C. Aliquots of sample were collected periodically and refilled with a fresh dissolution medium for maintaining the sink condition. The amount of drug released from piperine-phospholipid complex was analyzed using RP-HPLC at 342 nm. All the operations were carried out in triplicate.

Dissolution efficiency (DE)

The %Dissolution efficiency of PIP, PPC and PIP+HSPC was determined in different medias at different time intervals according to the following equation:

$$\frac{{}_0\int^t y.dt.100}{y_{100}.t}$$

Where y is the percentage of drug dissolved at time (t). Dissolution efficiencies were determined in HCl at pH 1.2 and phosphate buffer at pH 6.8 and pH 7.4. The area under the curve (AUC) was calculated at each time point by the trapezoidal rule.

Solubility and oil–water partition coefficient

Solubility of PIP, PPC and PIP+HSPC was evaluated by adding them in excess to 5 ml of water or n-octanol at room temperature. The mixture was subjected to shaking for 24 hr followed by centrifugation at speed of 5000xg for 10 min. The Supernatant was collected, filtered and analyzed by RP-HPLC at 342 nm. For oil-water partition coefficient study, 2 mg of the samples (PIP, PPC and PIP+HSPC) were added to 1 ml deionized water previously saturated with n-octanol. The mixture was shaken for 30 min and the layers were allowed to separate. Amount of Piperine in each layer was analyzed by RP-HPLC at 342 nm. Each experiment was performed in triplicate.

Stability studies

PPC were stored in air tight glass vials and subjected to stability studies for long term conditions and accelerated conditions at temperature 40 ± 2 °C/75 ± 5% RH in stability test chamber as per the ICH guidelines (ICH, 2005b).

Formulation and evaluation of capsules containing Piperine Phytosomes

Piperine phytosomes were formulated into hard gelatin capsules containing Microcrystalline cellulose, corn starch, magnesium stearate, and talc in specified quantity (table 2) and mixed uniformly. Gelatin capsules were used to encapsulate the powder mixture using a hand-held capsule filling system. Further, the capsules were evaluated for the following physicochemical characteristics.

Table 2: Formulation of powder mixture containing Piperine phytosome

Sr. No.	Ingredients	Qty. (mg/capsule)

1	Piperine phytosome	185
2	Microcrystalline cellulose	80
3	Corn starch	20
4	Magnesium stearate	2
5	Talc	2

PREFORMULATION PARAMETERS

Powder mixture containing Piperine phytosome was evaluated for following Preformulation parameters

Angle of Repose

Angle of repose (α) was calculated using funnel method. The mixture was poured through a funnel that can be raised vertically until a maximum cone height (h) was obtained. The radius of the heap (r) was measured and angle of repose was determined: $\alpha = \tan^{-1} (h/r)$

Bulk Density

Apparent bulk density (ρb) was determined by placing pre sieved drug excipients mixture into a graduated cylinder and measuring the volume (V) and weight (M)

$$\rho b = M/V$$

Tapped Density

A measuring cylinder containing a known mass of mixture was tapped for a fixed number of taps. The minimum volume (V) occupied in the cylinder and the weight (M) of the mixture was measured. The tapped density (ρt) was calculated by following formula:

$$\rho t = M/ V$$

Hausner's Ratio

Hausner's ratio is an index of ease of flow of powder mixture; it can be calculated by using following formula:

$$\text{Hausner's ratio} = \rho t \backslash \rho b$$

Where, ρt = Tapped density ρb = Untapped bulk density

Carr's Index

The simplest way of measurement of free flow property of powder mixture is compressibility, an indication of the ease with which a material can be induced to flow is given by % compressibility (C) which can be calculated as follows:

$$C = (\rho t - \rho b) / \rho t * 100$$

Where, ρt = Tapped density ρb = Untapped bulk density

EVALUATION OF HARD GELATIN CAPSULE

Disintegration Time -

In vitro disintegration time of the capsules was determined by the disintegration tester. One capsule was put into each of six tubes of assembly and assembly was suspended in distilled water. Discs were added to each tube, temperature was maintained at 37±2°C and assembly was operated for 60 min.

Drug Content -

Drug Content was determined by measuring absorbance of prepared solution of content inside capsule in ethanol, at 342nm using UV-visible spectrophotometer.

In-vitro Drug Release Study -

The release rate of Piperine was determined by using IP Dissolution Test Apparatus Type II (basket type). The capsules were placed in a dry basket at the beginning of each test. Lower the basket in the dissolution medium and apparatus was run at a speed of 50 rpm, The dissolution test was performed using 900 ml of phosphate buffer pH 6.8, at temperature of 37±0.5°C and speed at 50 rpm. 5 ml of sample were withdrawn at time intervals of 5 minute for 60 minutes. This was maintained at constant temperature. The samples were filtered through Whatman filter paper no. 41. Absorbance of these samples was measured at 342 nm using UV-Visible spectrophotometer.

Release Kinetics

In vitro drug release study were fitted with various kinetic equations like zero order (cumulative percent drug released vs. Time), first order (Log cumulative percent drug retained vs. Time), Higuchi (cumulative percent released vs. √T), and Peppas (log of cumulative percent drug released vs. log Time). The kinetic model that best fits the dissolution data was evaluated by comparing the regression coefficient (r) values obtained in various models.

Stability Study

Short term stability study was performed for period of six months under the conditions of 25°C and 60% RH. Where as, Accelerated stability studies were performed for period of six months by keeping the sample in stability chamber under the conditions of 45°C and 75% RH. The capsules were evaluated at intervals of 1, 2, 3, and 6 month for Organoleptic properties, disintegration time, drug content and in-vitro drug release.

RESULTS AND DISCUSSION

Standard solution for calibration curve of Piperine

Absorption maxima of piperine was found to be 342nm (figure 1). The absorbance characteristics showed that piperine obeys Beer Lambert's law within the concentration range 2-20 µg/ml at the λ-max of 342nm with the regression value (R2 value)of 0.9956 (figure 2).

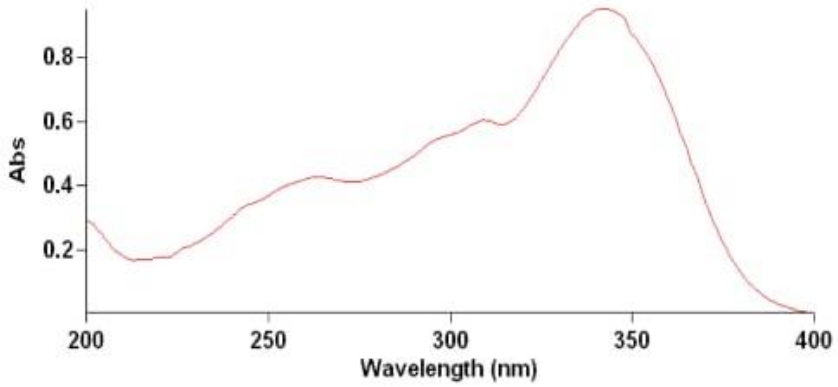


Figure 1: UV absorption spectra of piperine

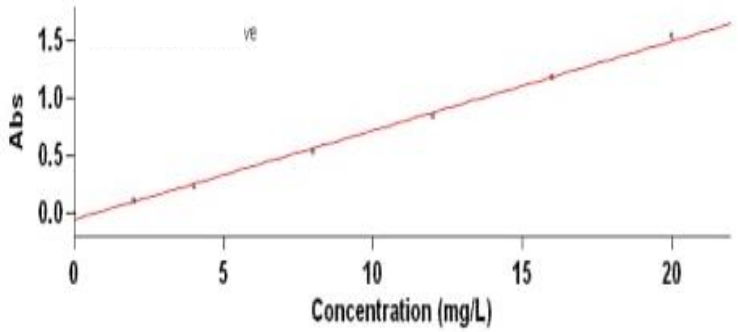


Figure 2: Calibration curve of Piperine

Method development and validation

Almost parallel results were obtained for two days of all the three samples with %RSD value less than 0.12, indicating the reproducibility of the method. Recovery studies were performed by standard addition method and the average percentage recovery of the three samples PP-1, PP-2 and PP-3 were found to be 98.51%, 99.12% and 98.92% respectively. Results obtained from the recovery study indicates the accuracy and precision of the method.

Table 3: Table for Piperine content in the samples

Sample		Conc. (µg/ml)	Mean[a]	SD	%RSD
PP-1	1st day	5.16	0.3495	0.0002	0.05
	2nd day	5.16	0.3493	0.0001	0.03
PP-2	1st day	4.26	0.2798	0.0004	0.12
	2nd day	4.26	0.2800	0.0002	0.08
PP-3	1st day	4.49	0.2982	0.0003	0.06
	2nd day	4.49	0.2979	0.0001	0.08

[a]Values expressed as mean of three readings

Optimization by Response Surface Methodology

Response surface methodology (RSM) is a rapid and efficient statistical tool to elucidate functional relationship between experimental responses and input variables. The effect of process variables on experimental response through the use of three dimensional (3D) graphs is effectively depicted by RSM. It consists of a series of mathematical and statistical processes to predict optimum conditions of input variables required in order to achieve maximum output results. This reduces the number of experimental trials leading to a reduction in time and labour. This output is used in the form of complexation efficiency for Piperine-phospholipid complex (PPC).

Out of various designs such as central composite, Doehlert matrix and three-level full factorial design employed for response surface analysis. Box-Behnken is the preferred one due a variety of advantages such as it possesses more accuracy than other designs, enables formation of sequential designs, provides parameter estimation for quadratic model, identifying lack of fit detectivity of the model, etc. Input variables and their respective optimization values have been listed in Table 3.2. The seventeen preparation conditions obtained were analyzed by Design-Expert 10.0 software (Trial version). The regression equation was as follows:

$$\%CE = +80.70 - 1.52 \times A + 12.10 \times B + 5.05 \times C - 0.78 \times AB + 5.72 \times AC - 1.68 \times BC + 8.91 \times A2 - 8.09 \times B2 - 7.79 \times C2$$

The variance analysis of regression model for predicting % complexation efficiency was confirmed from the F-test and P-value. P-value of lack of fit higher than 0.05 indicates the reliability of the model in predicting experimental result. Three-dimensional (3-D) response surface plot and contour plot between the independent variables and their corresponding response (% CE) are presented in Figure 3. The model predicted maximum complexation efficiency of 91.74% for Piperine-Phospholipid Complex.

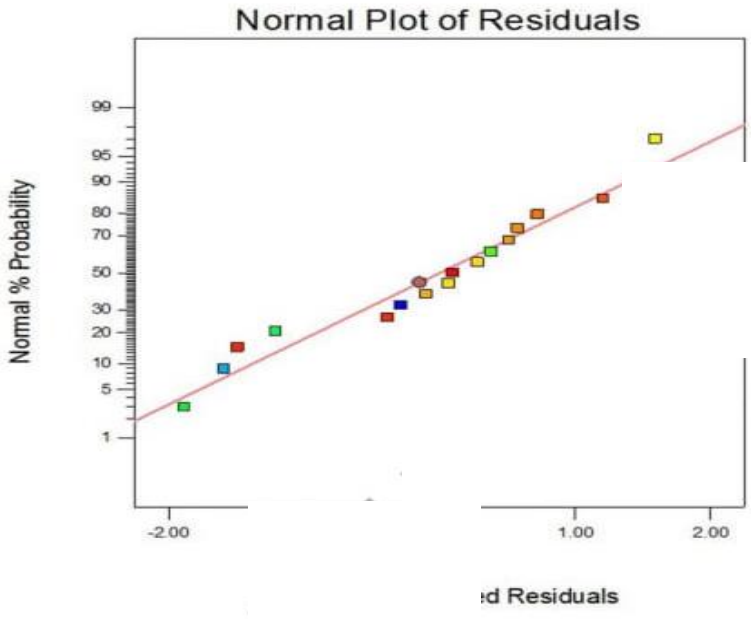


Figure 3: Three-dimensional (3-D) response surface plot between the independent variables and dependent variable (a) Ratio of phospholipid to drug and reaction time, (b) Reaction temperature and reaction time, (c) Ratio of phospholipid to drug and reaction temperature

The conditions required to achieve this involves maintaining ratio of HSPC: PIP at 1.2 2:1, reaction temperature to 55 °C and time to 1.35 hr. The normal probability plot as depicted in Figure 4 shows a straight line with little deviation. This indicated the proposed model to be adequate for the data set used. The model was validated by formulating Piperine-Phospholipid Complexes maintaining these preparation conditions in triplicate. The experiment yielded Piperine-Phospholipid Complex with complexation efficiency of 91.64 ± 0.25% as mean of three experiments indicating the model to be ideal for formulation. Therefore, this formulation of PPC was used for further studies.

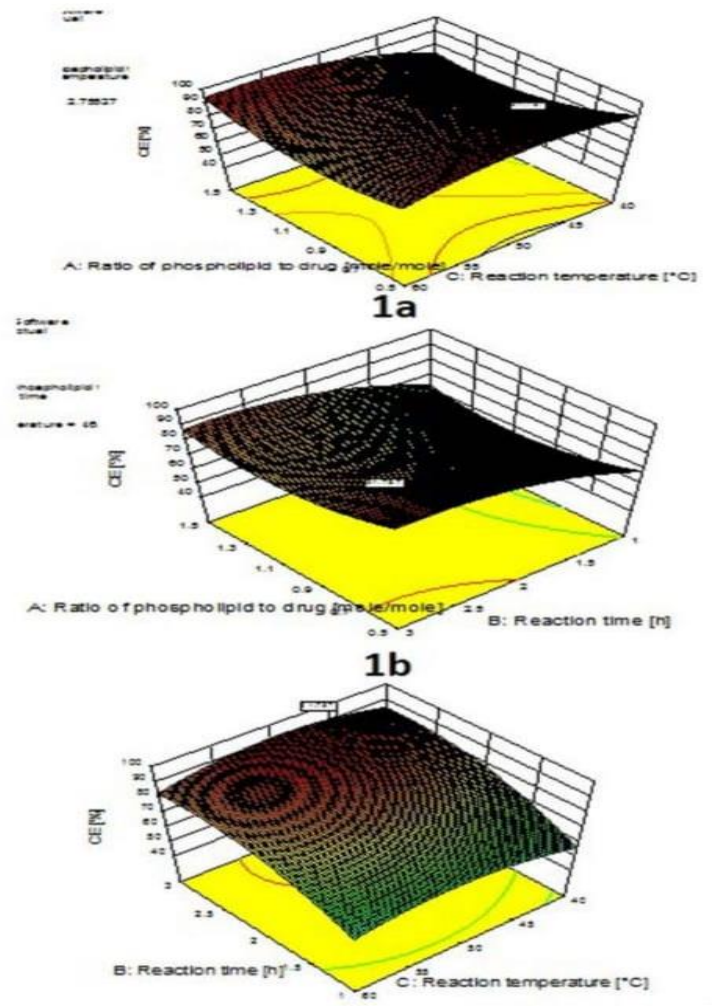


Figure 4: Normal probability plot of standardize residuals for the proposed mode

RP-HPLC method validation

The method was found to be selective to PIP and IS (biberine) with no internal interference observed in their retention times. The method showed linearity over the concentration range of 0.1-20 µg/ml as indicated by correlation coefficient (r^2) value of 0.9953. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 5.2 mg/ml and 18.6 mg/ml, respectively. The intra and interday accuracy was found to be less than 5% whereas the intra and interday precision was estimated to be less than 10% indicating the developed method to be accurate, precise and reliable.

Characterization of Phytosome

Fourier transform-Infrared spectroscopy (FT-IR)

Formation of piperine-phospholipid complex was confirmed by FT-IR spectroscopy, comparing the spectrum with that of pure piperine as shown in Figure 5. The FT-IR spectrum of phospholipid (HSPC) showed the characteristic signals at 2918 cm^{-1} and 2850 cm^{-1} related to the distinctive C-

H stretching band present in the long fatty acid chain. O-P-O antisymmetric double bond stretching bands at 1247.51 cm^{-1} and C=O stretching band at 1737.56 cm^{-1} were also seen. The C=O stretching bands are due to the presence of ester carbonyls and are due to free and hydrogen bonded carbonyls groups. Stretching band at 1091 cm^{-1} indicating P-O-C stretching and stretching band at 970cm^{-1} for the choline moiety [$-N^{+}(CH_2)_3$] present in polar head were also observed. The FT-IR spectra of piperine showed absorption band at 3000 cm^{-1} corresponding to aromatic C-H stretching, 1635 cm^{-1} for stretching of –CO-N=, 1608 cm^{-1} characteristic of asymmetric stretching of diene (C=C), 930 cm^{-1} most characteristic, probably related to C-O stretching, 1132 cm^{-1} characteristic of in-plane bending of phenyl CH, 995 cm^{-1} due to C-H bending for trans -CH=CH-. The physical mixture of PIP+HSPC gave characteristic peaks at 3000.12 cm^{-1} and 1634.76 cm^{-1} which indicated the presence of aromatic C-H bond and –CO-N= distinct for piperine . Whereas bands at 1187.89 cm^{-1} and 1031.25 cm^{-1} indicated the presence of asymmetric and symmetric stretching of =C-O-C respectively.

On the other hand 1735.87 cm^{-1} and 1231.98 cm^{-1} indicated the characteristic C=O stretching band and O-P-O antisymmetric double bond stretching bands of the phospholipid respectively. Thus the physical mixture gives a quite combined spectra of individual HSPC and PIP. This indicates that no interaction takes place between the two moieties in the physical mixture. In case of the FT-IR spectrum of PPC the characteristics peaks at 3000 cm^{-1}, 1250 cm^{-1} and 1030cm^{-1} were not seen. The C=O stretching band at 1737.56 cm^{-1}, O-P-O asymmetric double bond

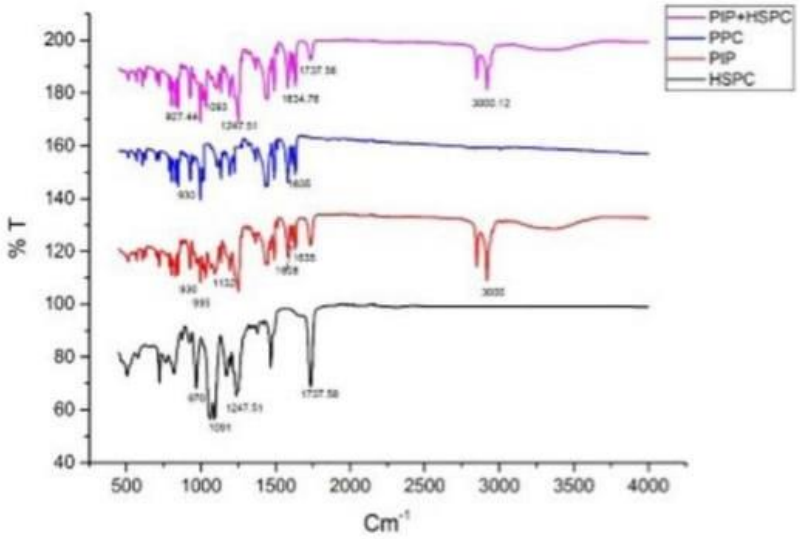
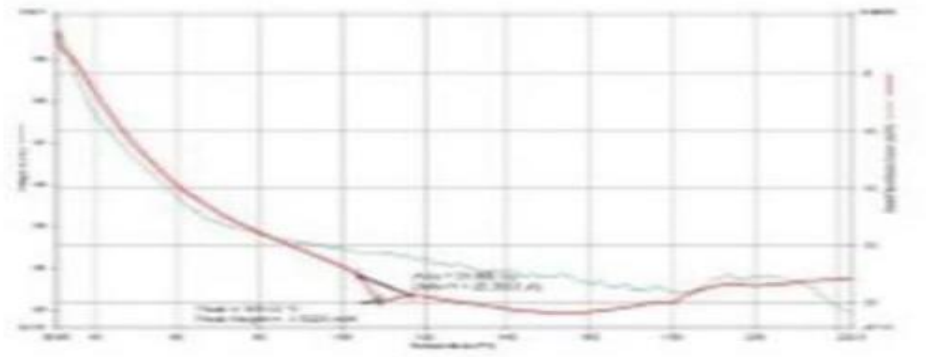


Figure 5: FT-IR data of PPC, PIP+HSPC, PIP, HSPC

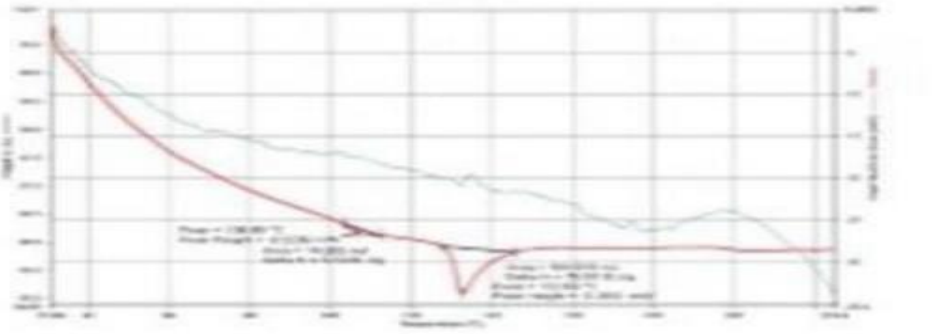
stretching bands at 1247.51 cm^{-1} and stretching band at 970cm^{-1} for the choline moiety were reduced in intensity and broadened in shape compared to HSPC indicating the involvement of

polar head group of phospholipid in the bond formation. In addition absorption band at 1635 cm^{-1} and 930 cm^{-1} and 996.32 cm^{-1} indicating -C=O bond were broadened and reduced in phospholipid complex compared to Piperine indicating the participation of -C=O group of Piperine interacting with the head group of phospholipid. It may be possible that PIP is bonded to HSPC by hydrogen bonding interactions.

Differential thermal analysis (DTA)



HSPC



PIP+HSPC

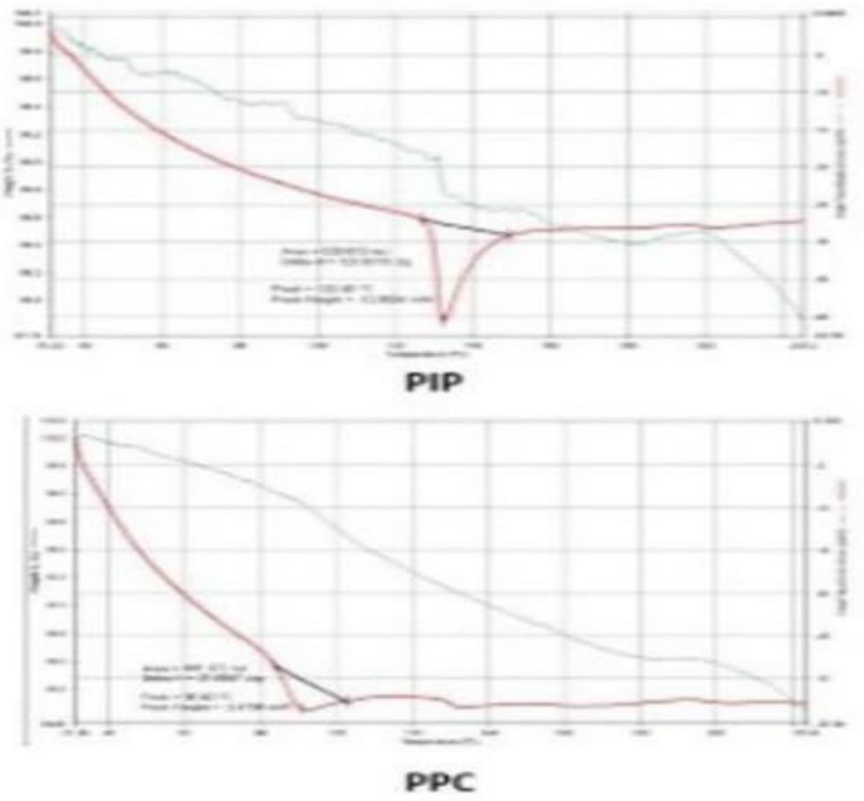


Figure 6: DTA curve of HSPC, PIP+HSPC, PIP, PPC

The drug excipient compatibility can be well determined through DTA, which provides a curve showing endothermic peak with respect to temperature. Disappearance or appearance of endothermic peaks, change in peak shape and its onset, melting point, peak temperature, and relative peak area or enthalpy gives an idea on the type of interaction taking place between drug and excipient. HSPC showed one major peak at 109.26°C, which was due to the melting phase transition of HSPC shown in Figure 6. The pure piperine showed a sharp endothermic peak at 132.40°C corresponding to its melting point. PIP + HSPC showed two major peaks at 106.89°C and 132.69°C suggesting the melting point of HSPC and PIP respectively, thus showing that the components did not interact with each other and retained their individual identity. DTA of piperine-phospholipid complex showed that the endothermal peaks of PIP and HSPC have disappeared and the phase transition temperature has become lower compared to the phase transition temperature of HSPC. This might be the result of complexation between PIP and the HSPC polar head molecule. This association makes the carbon-hydrogen chain in HSPC rotate freely hence engulfing the polar head in the process resulting in decrease in phase transition temperature.

Particle size analysis

The mean particle diameter of the complex was found to be 205.85±12 nm as shown in Figure 7 and the polydispersity index was found to be 0.32±0.06. The low polydispersity index (value less than 0.5) indicates a narrow range of particle size distribution. The smaller particle size enables the drug loaded phytosome to permeate through physiological barriers effectively. Another important parameter Zeta potential provides a measure of degree stability of colloidal dispersions of drug-phospholipid complex. Zeta potential values greater than ±30 mV partially indicates the physical stability of colloidal dispersion. Zeta potential value of -63±2.12 mV of the complex in solution form confirms its stability.

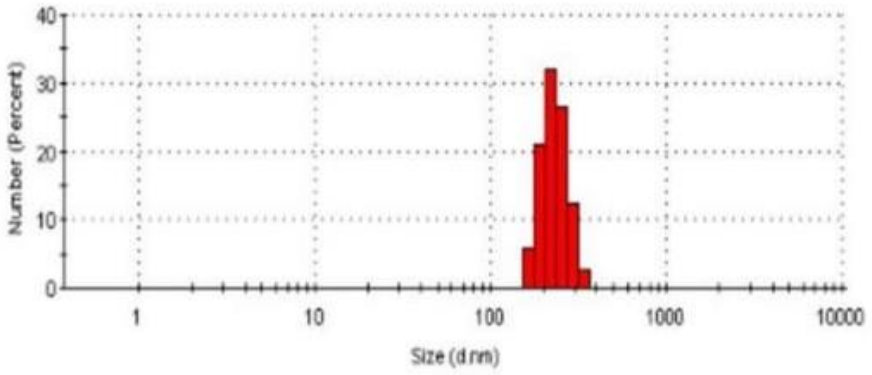


Figure 7: Particle size distribution of PPC

Scanning Electron Microscopy (SEM)

The photomicrographs from SEM shown in Figure 8 presented a roughly spherical nature of piperine phytosome. At 5000× magnification, it was observed that piperine was efficiently complexed with HSPC and formed a roughly spherical structure.

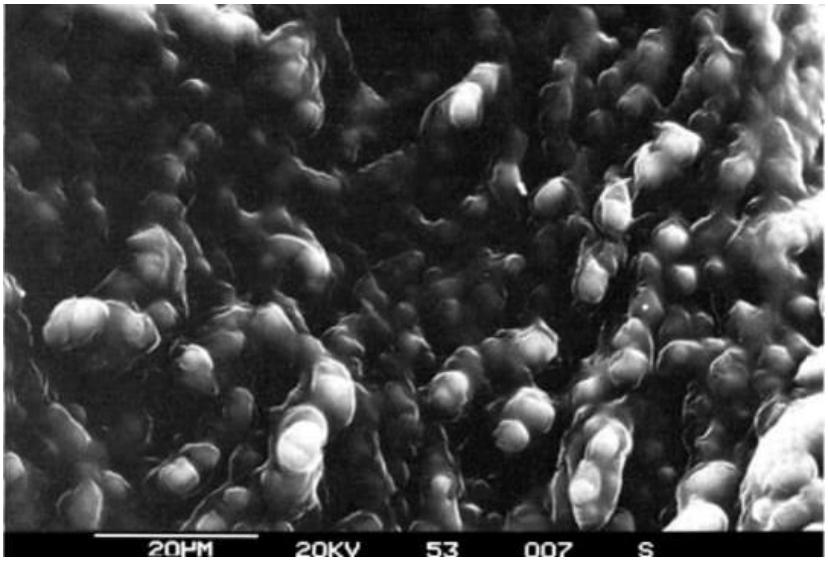


Figure 8: SEM of PPC

Powder X-ray diffraction (PXRD)

The crystalline nature of drug is measured by X-ray diffractogram through evaluating intensity of the sample at different diffraction angle in the form of a graph. The graph is characterized by complete absence or disappearance or reduction in the intensity of large diffraction peaks, which provides an idea about the crystallinity of the sample. Piperine showed intense diffraction peaks of crystallinity at a diffraction range of 2θ=2°C-50°C suggesting it's the crystalline nature. For the pure HSPC, one broad peak was depicted at a diffraction angle of 20.1°, indicating the presence of amorphous structure (Figure 9). PIP+HSPC showed sharp characteristic diffraction peaks of PIP along with a broad peak at 20.1° this indicates there was no interaction between PIP and HSPC. All these characteristic peaks of PIP were absent in diffractogram of PPC along with reduction in intensity. Thus it can be assumed that piperine in the phospholipids lipid matrix was in amorphous form due to its integration into the amorphous HSPC. These data validates the finding of DTA studies which indicated towards the reduced crystallinity of piperine in the prepared piperine-phospholipid complex by lowering of melting point.

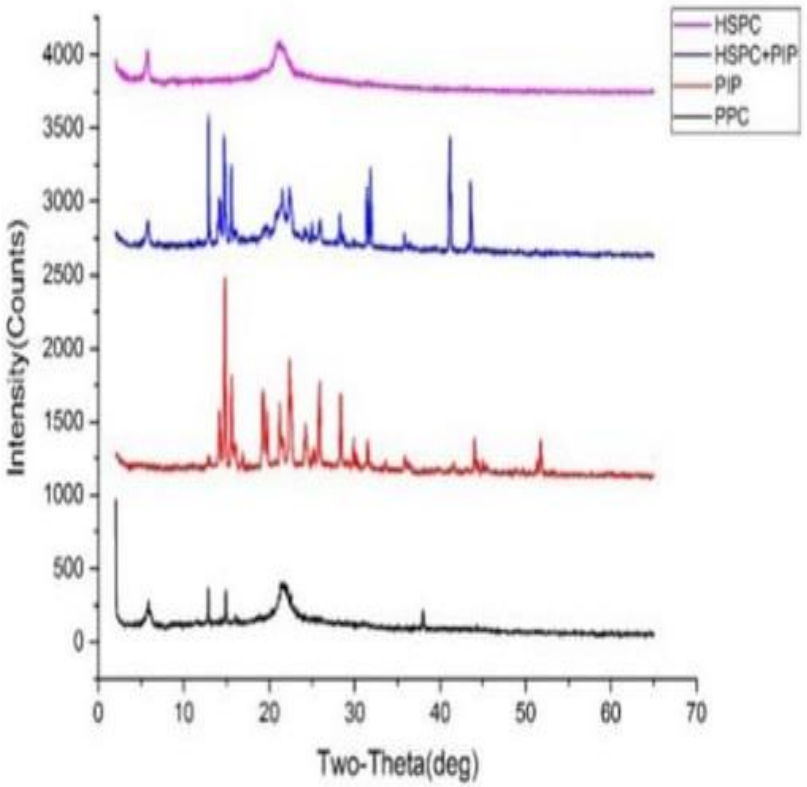


Figure 9: PXRD data of PPC, PIP+HSPC, PIP, HSPC

In vitro release studies

Cumulative percentage of piperine released from PPC in three different media has been shown in Figure 10. Primarily fast release of PIP was observed in all the three dissolution media followed by a slow and gradual release. Therefore, the release profile may be divided into two phases: the first initial burst release phase and the second sustained release phase.

Cumulative percentage of piperine released in artificial gastric juice was quick and reached a stable phase after 12 hours with piperine content of 92.56%. The piperine released in intestinal juice with pH of 6.8 and 7.4 was found to be in a sustained release manner with 82.32 and 85.63% piperine content obtained after 24 hours, respectively. The results indicated that significant increase in dissolution behavior of piperine compared to piperine which is essential for rapid absorption. The sustained release property of Phytosome in intestinal conditions may enable it to be present in the body for a longer period of time, consequently enhancing its bioavailability.

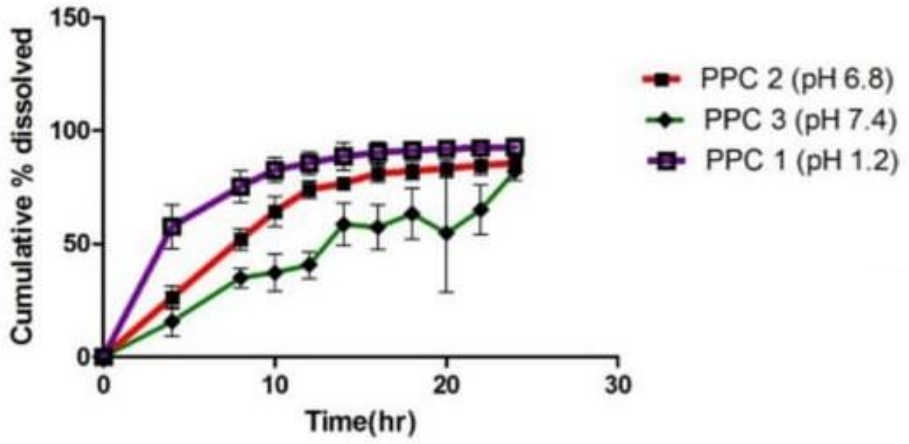


Figure 10: In vitro drug release profile from PPC in three different media

Dissolution efficiency

Dissolution efficiency (DE) affords valuable data regarding the dissolution profiles of individual batches.

Table 4: Dissolution efficiency of PIP, PIP+HSPC and PPC

Time (hr)	pH 1.2			pH 6.8			pH 7.4		
	PIP	PIP + HSPC	PPC	PIP	PIP + HSPC	PPC	PIP	PIP + HSPC	PPC
4	1.21 ± 0.235	1.78 ± 0.425	6.22 ± 0.221**	0.43 ± 0.035	0.68 ± 0.025	1.22 ± 0.121**	0.38 ± 0.022	0.61 ± 0.210	1.31 ± 0.126**
8	2.11 ± 0.231	3.54 ± 0.421	16.21 ± 0.203**	0.54 ± 0.134	0.76 ± 0.021	1.31 ± 0.003**	0.42 ± 0.014	0.75 ± 0.126	1.54 ± 0.543**
10	4.89 ± 0.119	6.21 ± 0.325	26 ± 0.198**	0.56 ± 0.019	0.81 ± 0.023	1.65 ± 0.098**	0.53 ± 0.176	0.80 ± 0.218	1.79 ± 0.165**
12	5.54 ± 0.215	7.25± 0.415	31 ± 0.176**	0.72 ± 0.015	0.85 ± 0.015	1.79 ± 0.076**	0.62 ± 0.021	0.87 ± 0.005	2.43 ±0 .004**

Values are expressed as Mean±SD, *p < 0.05; **p < 0.01 (significant with respect to PIP)

As observed in Table 4 the Dissolution efficiency of PPC increased significantly in all the buffers as compared to PIP. Though, no significant change was observed for PIP+HSPC and PIP in any of the pH indicating phospholipid complexation to be the key reason for increasing dissolution efficiency.

Solubility and oil water partition coefficient

In distilled water, solubility of PPC was found to increase significantly (**p<0.01) as compared to PIP (1.18±0.007 vs. 0.04±0.006 mg/ml). Solubility of PIP+HSPC was also found to increase in distilled water as compared to solubility of PIP (0.126±0.005 vs 0.04±0.006) but not significantly. The solubility might have increased because of partial interaction between HSPC and PIP. Solubility of PPC was found to increase significantly (**p<0.01) in n-octanol as compared to PIP (3.84±0.041 vs 1.2±0.005). The PIP+HSPC solubility in n-octanol also increased, although not to a significant extent. Thus, complexation with HSPC was found to enhance the water solubility of the hydrophobic phytomolecule piperine. Oil-water partition coefficient study showed a decrease in log p value of PPC when compared to PIP (1.82 vs 0.51) indicating a decrease in lipophilicity of PIP.

Stability studies

Phytosomes containing piperine were evaluated for the time period of 0, 1, 3 and 6 months for any change in appearance, zeta potential and drug content. No significant changes were observed in the appearance, complexation efficiency, and zeta potential at any of the month when compared to day 0.

Capsules containing piperine phytosomes

Preformulation parameters of Powder mixture containing piperine phytosomes

Angle of repose was found to be 26^0, Bulk density was determined to be 0.48 gm/cm^3 and tapped density as 0.59 gm/cm^3. From density data, % compressibility and Hausner's ratio was calculated and found to be 13% and 1.19 respectively. This indicates the good micromeritic properties of Powder mixture containing piperine phytosome.

Evaluation of Hard Gelatin Capsule

Disintegration Time

The average disintegration time for hard gelatin capsule was found to be 4.7±0.468 min.

Drug Content

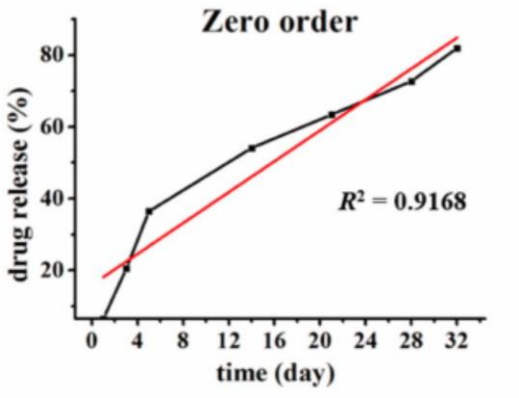
The percentage drug content in capsules were found to be between 97.19±0.544% to 98.79±0.612%, which was within the acceptable limits as per IP.

In- vitro Drug Release

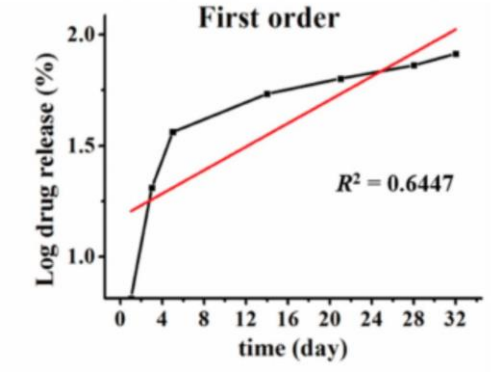
The % drug release using IP Dissolution Test Apparatus Type II was found as 99.09±0.411% at the end of 60 minutes.

Drug Release Kinetics Data Analysis

Kinetics and mechanism of drug release from Capsule was evaluated on the basis of Zero Order, First Order, Higuchi equation, Hixon-crowell model and Peppas model. The data was most suited to the Higuchi model with correlation coefficient of 0.9939.



(a)



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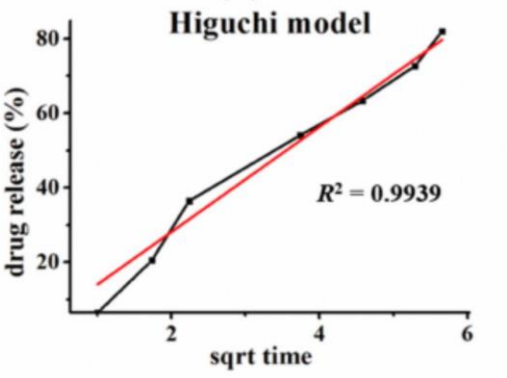

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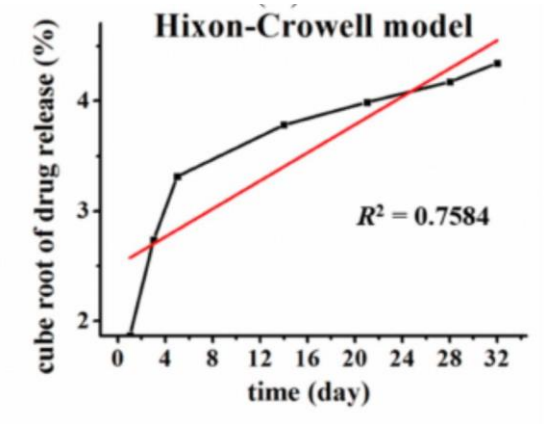

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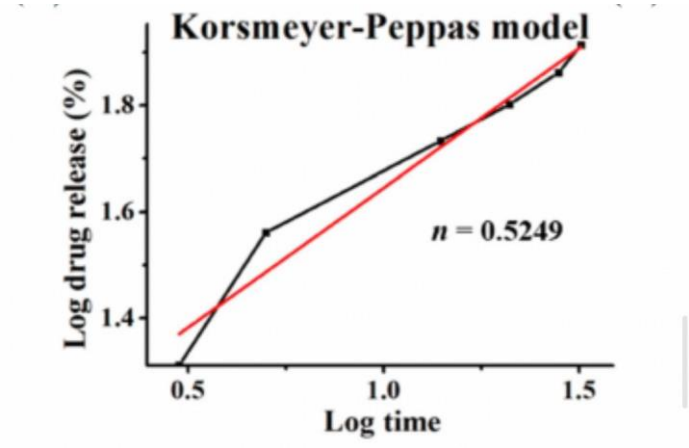

(e)

Figure 11: Drug Release Data fitted into various Kinetics models a) Zero Order, b) First Order, c) Higuchi model, d) Hixon-crowell model and e) Peppas model

Stability Study

Stability Study as per ICH Guideline Stability study of hard gelatin capsule containing Powder mixture of piperine phytosome was done to see the effect of temperature and humidity on capsules during the storage time. Capsules were evaluated periodically for Organoleptic properties, disintegration time, drug content and in-vitro drug release. Stability study results show that there was no significant change in Organoleptic properties like shape, colour, disintegration time, drug content and in-vitro drug release of the formulation for Short term Stability Study and Accelerated stability studies shown in Table 5 and Table 6 respectively.

Table 5: Short term Stability Study

Time interval (months)	Shape	Colour	disintegration time (min)	% drug content	% drug release
0	Cylinder with hemispherical ends	Pale green	4.6 ± 0.279	97.96 ± 0.633	99.08 ± 0.318

1	Cylinder with hemispherical ends	Pale green	4.9 ± 0.322	97.15 ± 0.461	98.84 ± 0.530
2	Cylinder with hemispherical ends	Pale green	4.9 ± 0.471	96.97 ± 0.674	98.07 ± 0.581
3	Cylinder with hemispherical ends	Pale green	5 ± 0.523	96.91 ± 0.396	98.07 ± 0.424
6	Cylinder with hemispherical ends	Pale green	5.4 ± 0.583	96.04 ± 0.510	97.57 ± 0.267

Table 6: Accelerated stability studies

Time interval (months)	Shape	Colour	disintegration time (min)	% drug content	% drug release
0	Cylinder with hemispherical ends	Pale green	4.6 ± 0.214	97.18 ± 0.633	99.10 ± 0.318
1	Cylinder with hemispherical ends	Pale green	4.9 ± 0.396	96.25 ± 0.53	98.93 ± 0.489
2	Cylinder with hemispherical ends	Pale green	4.9 ± 0.510	96.97 ± 0.581	98.15 ± 0.612
3	Cylinder with hemispherical ends	Pale green	5.2 ± 0.691	96.91 ± 0.279	98.15 ± 0.424
6	Cylinder with hemispherical ends	Pale green	5.3 ± 0. 322	96.04 ± 0.523	97.96 ± 0.267

DISCUSSION

Piperine is found in black pepper (piper nigrum), white pepper, and long pepper (piper longum) belonging to the family Piperaceae. Among all spices, black pepper is well known as a distinctive spice worldwide. It is also known as the 'King of spices.' It has a distinctive pungent flavour due to the presence of an alkaloid piperine, along with volatile oils, and essential oils. The content of piperine varies from plant to plant belonging to the Piperaceae family and varies from 2% to 7.4% in vines of black and white pepper (piper nigrum). Piperine represents diverse biological activities, such as Antioxidant, Antitumor, Antiasthmatics, Antipyretic, Analgesic, Anti-inflammatory, Antidiarrheal, Anxiolytic, Antidepressant, Hepatoprotective, Antibacterial, Antifungal, Anti-metastatic, Anti-thyroid. Piperine also has biotransformative effects and can enhance the bioavailability of different drugs such as rifampicin, sulfadiazine, tetracyline, and phenytoin by increasing their absorption, by slowing down the metabolism of the drug, or by the combination of two.

Despite excellent therapeutic properties of piperine, it is slightly soluble in water (40 mg/L at 18 °C). The poor water solubility of such compounds prevents them from dissolving in aqueous gastrointestinal fluids. The low solubility of piperine in water and its poor dissolution is the rate-controlling step in the absorption process of piperine. The pharmaceutical activities of piperine are limited due to its low water solubility.

A strategy of using phyto-phospholipid complexes represents a promising approach to increase the bioavailability of such active constituents. Phyto-phospholipid complexes also known as Phytosomes or Herbosomes are prepared by complexing active constituents at defined molar ratios with phospholipids under certain conditions. Phyto-phospholipid complexes are more readily absorbed and generate higher bioavailability compared to free active constituents. Phytosomes as lipid-based nanocarriers play a crucial function in the enhancement of pharmacokinetic properties of herbal-originated phytocompounds. Furthermore, the production of complexes can protect phytoconstituent from destruction by external forces, such as hydrolysis, photolysis, and oxidation. Absorption maxima of piperine was found to be 342nm and it obeys Beer Lambert's law with the regression value (R2 value)of 0.9956. Further piperine was complexed with Hydrogenated Soy Phosphatidyl Choline (HSPC) to develop piperine loaded Phytosomes. IR spectroscopy was used in order to investigate the possible interaction between piperine and HSPC in the phytosome. The physical mixture have given a quite combined spectra of individual HSPC and piperine indicating that no interaction takes place between the two moieties in the physical mixture. -C=O bond were broadened and reduced in phospholipid complex compared to Piperine, indicating the participation of -C=O group of Piperine interacting with the head group of phospholipid.

The drug excipient compatibility was determined through DTA, Physical mixture of PIP + HSPC showed two major peaks indicating that the components did not interact with each other and retained their individual identity. Disappearance of endothermic peaks of piperine-phospholipid complex accounts for the interaction and formation of complex. The results from X-ray diffractogram, indicate that piperine is no longer present in a crystalline state and its phospholipid complex is in an amorphous form. PIP+HSPC showed sharp characteristic diffraction peaks indicating there was no interaction between PIP and HSPC, but all these characteristic peaks of PIP were absent in diffractogram of phospholipid complex along with reduction in intensity concluding that piperine is in the phospholipids lipid matrix.

The polydispersity index value was found to be less than 0.5 indicating a narrow range of particle size distribution and Zeta potential value was found to be greater than ±30 mV partially indicating the physical stability of colloidal dispersion. The morphological studies were performed by SEM, the phospholipid complex showed a drastic change in the morphology and shape of particles compared to piperine, exhibiting a fluffy, porous and rough surface, indicating an apparent interaction in the solid-state that may have resulted in improved solubility and enhanced dissolution rate when compared to pure drug. In vitro release studies indicated dissolution

efficiency of PPC increased significantly in all the buffers as compared to Piperine . Complexation with HSPC was found to enhance the water solubility of the hydrophobic phytomolecule piperine. Piperine phytosomes were formulated into dosage form as hard gelatin capsules. Initially, Powder mixture containing piperine phytosome was prepared and evaluated for Preformulation parameters like Angle of repose, Bulk density tapped density, % compressibility and Hausner's ratio. The results indicated good micromeritic properties of Powder mixture containing piperine phytosome and excipients. Then powder mixture containing phytosomes was filled into the empty hard gelatin capsule shells. The hard gelatin capsules were then evaluated for dissolution studies, disintegration study, drug content, and Short term Stability Study and Accelerated stability study. All the evaluation parameters were found to be within limit. Kinetics and mechanism of drug release from Capsule was evaluated on the basis of Zero Order, First Order, Higuchi equation, Hixon-crowell model and Peppas model. Higuchi model was found to be best fit with R^2 = 0.9939. Short term stability study was performed for period of six months under the conditions of 25°C and 60% RH. Where as, Accelerated stability studies were performed for period of six months at 45°C and 75% RH and found that there was no significant change in Organoleptic properties like shape, colour, disintegration time, drug content and in-vitro drug release of the formulation indicating that capsules are stable for longer period of time.

CONCLUSION

Based on the results of the study, it can be concluded that the phytosomes may be considered as promising drug delivery system for improving the bioavailability of the piperine molecule. . The physicochemical properties of piperine changed remarkably after it was complexed with the phospholipid and these characteristics especially, the improved solubility might contribute to improve oral absorption of the drug. Further, by physicochemical and analysis studies it was found, there is successful formation of Piperine phytosome through hydrogen bond. Also, the bioactivity was maintained after piperine was complexed with phospholipid and the production process of the complex did not change or destroy the molecular structure of active ingredient i.e. piperine in the complex. Piperine phytosomes formulated as hard gelatin capsules do not show significant change in Organoleptic properties, disintegration time, drug content and in-vitro drug release of the formulation even at Accelerated conditions. Thus, the dosage form was considered an optimized formulation. As these drug-lipid complexes have been reported to be stable and more bioavailable, the phospholipid complex of piperine may serve as a value added herbal drug delivery system.

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