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Formulation and characterization of curcumin loaded liposome and its bio-enhancement

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Abstract
Background: Many decades of research has lead lot of innovative improvements in drug delivery by various drug delivery systems such as nanoparticles, micelles one of them is liposomal delivery. Recently liposomal or phospholipids complexes have received much attention by the researchers, owing to its biodegradability, biocompatibility and the ability to deliver a wide range of drugs.
Objective: The aim our study was to develop and evaluate liposome containing natural polyphenol. In the present study we select curcumin as a natural polyphenol for formulation of liposome because of the variety of biological activity it possesses. But it shows poor bioavailability and solubility that's why we chose piperine as bio enhancer with it.
Method: The liposome formulation containing the drug curcumin was prepared by ethanol injection method. It was freeze dried under vacuum pressure to obtained crude dried form of formulation. The prepared liposome formulations were evaluated for particle size, zeta potential, encapsulation efficiency and drug release.
Results: The drug encapsulation efficiency of the formulation were F1 59% and F2 61%. The mean particle size of selected batch was () and surface charge was (26.30 ±0.243). The study on *in vitro* release of the formulation in 7.4 phosphate buffer, the maximum % CDR observed at the period of 12 hrs. with an initial burst effect followed by sustained release they are thus may reduce frequency of dosing, thereby minimizing the occurrence of side effects, improve bioavailability and increase the effectiveness of the drug.

Keywords: liposome, curcumin, ethanol injection, piperine, bioavailability

Introduction

Medicinal plants have shown great promise in the treatment of many diseases and been a source of inspiration for novel drug compounds. There has been considerable public and scientific interest in the use of phytochemical derived from dietary components to fight against the human diseases. Phytochemicals are synthesized as secondary metabolites in plants, and stem from two major synthetic pathways shikimate and the acetate pathway. These phytochemicals are mainly phenolic compounds. The active components of dietary phytochemicals that are most often reported to be protective against various pathological conditions are curcumin, genistein, resveratrol, diallyl sulfide, Sallyl cysteine, allicin, lycopene, capsaicin, diosgenin, ursolic acid, silymarin, anethol, catechins, eugenol, saponins, phytosterols, inositol hexaphosphate, vitamin C, Dlimonene, lutein, folic acid, beta carotene, selenium, vitamin E, flavonoids, and dietary fiber etc (Aggarwal and Shishodia 2006) [1].
Polyphenols play an important role in the maintenance of health and prevention of diseases. Polyphenols in the human diet are derived mainly from vegetables, fruits and spices. Drinks and beverages such as coffee, green and black tea, as well as chocolate, red wine, olive oil, nuts, etc., are also good sources of polyphenols. Many of these polyphenol-rich natural resources have been traditionally used as medicines for the prevention of diseases, as well as maintenance of youth and longevity. The recent line of studies has confirmed that these traditionally used natural remedies are strong anti-oxidant and anti-inflammatory agents. In addition, many of them play important roles in regulating the immune system and are now being investigated as chemopreventive, neuroprotective, cardioprotective and hepatoprotective agents, either acting alone or in combinations (Conney, 2003; Aggarwal *et al*., 2007) [2, 3]. In particular, turmeric, a typical example of polyphenol-rich natural remedies, has been used for centuries in Indian traditional medicine (Ayurveda) and Traditional Chinese Medicine (TCM). Moreover turmeric, a popular curry spice, is also used as a food additive and preservative agent worldwide.
The reasons for low bioavailability of any agent inside the body are low intrinsic activity, poor absorption, and high rate of metabolism, inactivation of metabolic products and/or rapid elimination and clearance from the body. There are many researchers studied which is related to absorption, distribution, metabolism and excretion of curcumin, exposed poor absorption and rapid metabolism that strictly curtails its bioavailability (Wahlstrom and Blennow 1987; Anand *et al*., 2007) [4]. Resulting low serum levels, limited tissue distribution and short half-life. Regarding the distribution of curcumin, it showed its accumulation in the intestine, colon and liver, so this is one of the major reasons why it is showing most promising *in vivo* effects in gastrointestinal diseases when compared with other organ systems. The liver, and to a lesser extent the intestinal mucosa are the major organs

responsible for metabolism of curcumin. On absorption, curcumin gets converted into metabolite form i.e., glucuronides and sulfates or it is reduced to hexahydrocurcumin. To overcome these problems, there is no clear understanding whether these curcumin metabolites are pharmacologically as active as curcumin or whether these conjugates have effects that differ from those of curcumin (Rajasekaran 2011) [7]

In this study, the effect of combining piperine, a known inhibitor of hepatic and intestinal glucuronidation, was evaluated on the bioavailability of curcumin in rats and healthy human volunteers. It is considered that if curcumin was given alone, it takes longer time to achieve moderate serum concentrations. Relatively the administration of piperine increased the serum concentration of curcumin in less time. Time to maximum was significantly increased while elimination half life and clearance significantly decreased, and the bioavailability was increased, this experiment is performed on rats. In humans if curcumin is given alone, serum levels were either undetectable or very low. Additional administration of piperine produced much higher concentrations; the increase in bioavailability was more as compared to rats. The present study on formulation and characterization of curcumin loaded liposome and its bio-enhancement is the first study on the dissolution profile and *in vivo* bioavailability studies demonstrated the significant improvement of curcumin performance from liposome compared to pure curcumin.

Material and methods

Preparation of Piperine

Powdered drug was extracted with ethanol (95%) in a soxhlet apparatus for 3 hours. Solution was filtered and concentrated under vacuum on water bath at 60^0c.10% alcoholic potassium hydroxide was added with constant stirring and was filtered. The alcoholic solution was kept overnight, from that the needles of piperine was separated out. By this crude piperine was obtained (Kolhe *et al*., 2011) [9].

Preformulation Studies

Preformulation testing is the first step in the rationale development of dosage forms of a drug substance. It can be defined as an investigation of physicochemical properties of a new drug substance alone and when combined with the recipients, to generate data useful to the formulator in developing safe, stable, potent, bioavailable and officious dosage form, which can be mass produced. The sample of curcumin and piperine was analyzed for physical appearance and compare with the standard. Physical appearance of the received curcumin and piperine complies with Pharmacopoeia standards. The melting point of Curcumin and Piperine was determined by using melting point apparatus. The sample drug was taken in capillary tube which was closed by one-sided and was placed in the melting point determining apparatus. The temperature was gradually increased automatically. The temperature (by using a thermometer) at which sample melted was noted. The solubility of curcumin and piperine was tested in various common solvents. A semi quantitative determination of the solubility was made by adding solvent in small incremental amount to a test tube containing fixed quantity of solute or vice versa. After each addition, the system is vigorously shaken and examined visually for any un-dissolved solute particles. The solubility was observed only by the visual inspection (Cartensen *et al*., 2010) [10].

Compatibility test by FTIR spectral studies

The FT-IR study of drug polymer sample in the form of nanoparticles was taken and dried KBr were mixed by triturating in ratio 2:98. This mixture of the sample and KBr was putted into the disc and pressed it to form pallet and placed the disc in to sample holder inside the instrument. Scanned it at the scanning range between 4000 cm−1 and 450 cm−1 and the resolution was 1 cm-1. The spectra obtained were interpreted for the functional group. Any changes in parent peak of functional group as compared to pure drug peak will indicate drug polymer interaction.

Determination of λ max

By appropriate dilution of drug solutions with methanol, solutions containing10 µg/ ml of Curcumin were scanned separately in the range of 400-800 nm to determine the wavelength of maximum absorption for the drugs (Chiou and Riegelman 2012).

Development of calibration curve

Calibration curve for Curcumin and was developed by UV spectrophotometric method. 1mg/ml solution of curcumin standard solution was prepared in methanol. From this 0, 1, 2, 3, 4, 5 and 6 µg/ml working standards were prepared and measured at 420.0 nm and for piperine in ethanol at 343 nm (Ganpati 2011) [12].

Preparation of Liposomes

Liposomal containing curcumin were prepared according to the thin film hydration method of Storm and Crommelin 1998. In this method the curcumin (25mg), soya lecithin (90mg, 100 mg, 110mg, 120mg) and cholesterol (20mg) were dissolved in Methanol and chloroform (10ml) and the solution was dried in a rotary evaporator under reduced pressure at 600C to form a thin film of lipid. Then the dried lipid was hydrated with 10ml of normal saline solution and vortex for one hour (above the gel – liquid transition temperature of the soya lecithin). This dispersion was centrifuged for 15 minutes at 3000 rpm. The volumes of drugs curcumin and piperine used were chosen so as to give Curcumin: Piperine according to dose ratios (mg/kg) of 10:1 ratio

respectively and solvent (methanol: chloroform) 10 ml according to volume (v/v) i.e., (9:1). The drug-free liposomes were prepared in the same manner to that of drug containing liposome except for omitting the drug.

***In vitro* characterization**

Mean particle size and Vesicles Morphology analysis

Mean vesicle size morphology of Drug loaded liposomes was determined using Scanning electronic microscope at room temperature by keeping angle of detection at 90 degree (Kumar *et al*., 2010) [13].

Zeta potential measurement

Zeta Potential of optimized liposomes obtained from ethanol evaporation and rotary evaporator method was determined using Zeta sizer 300HSA (Malvern instrument, Malvern, UK).

Drug-compatibility study by FTIR

Drug – Polymer interaction was investigated by FTIR analysis of drug, lipid, cholesterol, placebo liposomes and drug loaded liposomes.

Drug Entrapment Studies [72]

The percentage drug entrapped (PDE) was determined by Ultra-centrifugation. The liposomal formulations were subjected for ultracentrifugation (Ultra Centrifuge. Remi laboratories, Mumbai, India) at 11000 rpm for 30 min in an ultracentrifuge in order to separate the entrapped drug from the free drug. Then the clear supernatant was separated and analyzed for drug content after appropriate dilution by UV-Visible Spectrophotometer. This indicates amount of free drug. The liposomal pellet was redispersed in methanol and analyzed for drug content after appropriate dilution by UV-Visible Spectrophotometer. This indicates amount of drug entrapped. The entrapment capacity of liposomes was calculated as follows (Srilatha *et al*., 2013).

$$PDE = [(T-C)/T] \times 100$$

Where T is the total amount of drug that is detected both in the supernatant and sediment, and
C is the amount of drug detected only in the supernatant.

***In vitro* drug release studies**

In vitro-release studies were performed using dialysis membrane method. It was soaked in warm water at 45^0 C for 30 minutes before using it for release study. This membrane was then carefully clamped to one end of the hollow glass tube and considered as the donor compartment. The dissolution medium i.e., PBS (PH 7.4) (200ml) was taken into the receiver compartment. The donor compartment was immersed into the receiver compartment so that the edge just touched the receiver compartment. Before the release test, 0.5 ml of curcumin formulation was diluted to 3 ml in release medium and placed into the hollow glass tube as the donor compartment at 37^0c at 100 rpm by using magnetic stirrer and bead. Samples (5 ml) were removed from the receptor compartment at predetermined intervals and replaced with fresh medium immediately. The samples were analyzed using UV-visible spectrophotometer at 420 nm. Drug release was monitored for 12 hrs.

Kinetic Treatment of Dissolution Data

In order to describe the kinetics of the release process of drug in the different formulations, models were fitted to the dissolution data of optimized formulations using linear regression analysis. In order to study the exact mechanism of drug release, drug release data was analyzed according to Zero Order Kinetics; first order kinetics, Higuchi square root equation, Hixon -Crowell equation. The criterion for selecting the most appropriate model was chosen on the basis of goodness of fit test (Dinesh *et al*., 2009).

Zero Order Kinetics

Drug dissolution from pharmaceutical dosage forms that do not disaggregate and release the drug slowly assuming that area does not change and no equilibrium conditions can be represented by the following equation. Qt = Q0 + K0t, Qt is the amount of drug dissolved in time t Q0 is the initial amount of drug in the solution K is the zero-order release constant.

First Order Kinetics

The application of this model to drug dissolution studies used to describe absorption and/or elimination of drugs. To study the first order release rate kinetics the release rate data were fitted to the following equation. Log Qt = log Q0 + K1t / 2.303 Qt is the amount of drug released in time t Q0 is the initial amount of drug in the solution K1 is the first order release constant

Higuchi Model

Higuchi developed several theoretical models to study the release of water-soluble and low soluble drugs incorporated in semi-solid and/or solid matrixes. Mathematical expressions were obtained for drug particles dispersed in a uniform matrix behaving as the diffusion media, the equation is Qt = KH.t1/2. Qt is the amount of

drug released in time t KH is higuchi dissolution constant. Higuchi describes drug release as a diffusion process based in the Fick's law, square root time dependent.

Korsmeyer and Peppas Model

This model is generally used to analyze the release of pharmaceutical polymeric dosage forms, when the release mechanism is not well known or when more than one type of release phenomena could be involved. Mt / M = K.tn. Mt /M is the fraction of drug release K is the release constant t is the release time *n* is the diffusion exponent for the drug release that is dependent on the shape of the matrix dosage form.

***In vivo* study**

Pharmacokinetic Study for Determination of Curcumin

In vivo study was investigated in Sprague dawley rat. All the animal's experiments were conducted according to the rules and guidelines of committee for the purpose of control and supervisions of experiments on animals (CPCSEA). The study was approved by Institutional animal ethical committee (Aukunuru *et al*., 2009) [16].

Construction of curcumin standard graph

For the evaluation of pharmacokinetics of the curcumin formulations in rat a standard graph of curcumin was developed by following method.100 mg of pure curcumin was dissolved in 100 ml of methanol and sonicated for 10 mins to form stock solution. From that 10 ml was taken and diluted with same solvent methanol up to 100ml and again it was sonicated to form standard solution. From standard solution further dilution were performed i.e., 0.5ml was transferred from the standard solution into test tubes and diluted to 10 ml with methanol to form 5 micorgram/ml. The solutions so prepared (5 microgram/ml, 10 microgram/ml, 15 microgram/ml, 20 microgram/ml and 25 microgram/ml). The samples were analyzed in HPLC at 420 nm wavelength taking acetic acid: acetonitrile (90:10) ratio as a mobile phase at a flow rate of 0.5 ml /min using C18 column. The retention time and peak area are noted and data obtained through HPLC analysis was further used to interpolate the experimental peak area values to get the corresponding concentration of curcumin in plasma (Umasankar *et al*., 2013)

Animal experimentation

Animals were divided into 4 groups; Group 1 received: - liposomal curcumin; Group 2 received: - liposomal curcumin+ pure piperine; Group 3 received: - liposomal curcumin: piperine formulation; Group 4 (standard) received: - pure curcumin. Blood samples (0.5ml) from the experimental rats were collected by retriorbital plexus technique into a series of micro centrifuge tubes containing 0.3 ml of sodium citrate solution. Blood samples were collected at different time intervals like 30 min1 hr for, 2, 3, 6, 12, 18, and 24 hrs. The collected blood sample were centrifuged at a speed of 5000 rpm for 10 min and plasma was separated into another micro centrifuge tube by using micropipette and stored in deep freeze until analysis. The drug was extracted out from plasma by adding in a methanol solvent and it was centrifuged for 10 min from which the organic layer of drug comes out other than sediment. The organic layer of drug was then injected into the HPLC system for the further processing of determination of plasma drug concentration and other parameters i.e., AUC, Cmax etc to report the oral bioavailability enhancement of the drug.

Result and Discussion

Preformulation Studies

Physical appearance of the drug Curcumin and Piperine was complies with IP. Curcumin was found to be bright yellow orange amorphous powder and piperine found to be pale yellow crystals. The melting point of drug sample of Curcumin was found to be 179-187°C and piperine found to be 130^0c. After performing melting point test it was found that the melting point of Curcumin and piperine drug sample obtained in range as given in Indian pharmacopoeia.

Solubility analysis

Solubility of pure drug sample of Curcumin and Piperine was analyzed with water, ethanol, methanol, and chloroform and petroleum ether. It was observed the curcumin was soluble with ethanol, methanol and water and insoluble water and petroleum ether while piperine was soluble all the ethanol, methanol, chloroform and petroleum ether and insoluble with water.

Identification test of pure drug by FT-IR spectrophotometer

The spectra obtained from FT-IR Spectroscopy studies at wavelength from 4000 cm-1 to 450 cm-1 are shown and the Characteristic peaks obtained are shown table 1 and figure 1.

λ max spectrum

The absorption spectrum of pure drug was scanned 400-800 nm with10 µg/ ml prepared in methanol. The λ max of pure curcumin was to be found to be 420 nm which is nearest to the standard i.e., 421- 425nm and for piperine sample was scanned at 200-400 nm in ethanol the λ max of pure piperine was to be found to be 341 nm nearest to standard i.e., 343 nm.

Calibration curve of Curcumin and Piperine

Standard calibration curve in the absorbance of curcumin were 0.376, 0.682, 0.999, 1.377 and 1.713; piperine were 0.275, 0.485, 0.696, 1.073 and 1.317 at different concentration 2, 4, 6, 8, 10 µg/ml. at 420 nm. The regression value were found to be (0.9983 & 0.9865)

Preparation of Liposome Formulation

In this present study, curcumin loaded liposome formulation were prepared by thin film hydration method using piperine as a bio-enhancer and lipid, cholesterol (excipients) and methanol and chloroform (solvent).This technique offers a simple and mild preparation because other method such as (1.Ethanol injection method, 2. Ether injection method) used during formulation needs continuous process, (time consuming)for lyophillization process but in thin film hydration method after drying of thin film of formulation only one day lyophillization takes place for obtaining the crude formulation. The procedure used for the preparation of liposome formulation produced good yield, which indicates minimum loss of formulation during the preparation and recovery.

Table 1: Characteristic peaks of curcumin, piperine and mixture of curcumin and piperine

Characteristic peaks of curcumin		
Functional group	**Peak (cm-1) theoretical**	**Peak (cm-1) Practical**
NH-CO-NH	3326	3323.35
CONH	3251	3250.05
C-H	3030	3030.17
CO- NH-CO-NH	1689	1689.64
Characteristic peaks of piperine		
Symmetric and asymmetric stretching of C=C (diene) 1	1635; 1608	1633.71;1605.33
Aromatic stretching of C=C (benzene ring) 1	1608; 1580	1586.55; 1612.49
Stretching of -CO-N	1635	1633.56
Asymmetric and symmetric CH2 stretching, aliphatic C-H stretching	2925; 2840	2929.52; 2848.30
CH2 bending	1450	1452.60
Asymmetrical stretching =C-O-C	1250; 1190	1253.71; 1195.87
C-O stretching (most characteristic)	930	929.69
Out-of-plane C-H bending 1,2,4-trisubstituted phenyl (two adjacent hydrogen atoms)	850; 830; 805	846.75; 831.32;804.32
Characteristic peaks of mixture of curcumin and piperine		
CONH	3251	3250.05
Symmetric and asymmetric stretching of C=C (diene) 1	1635; 1608	1633.71;1605.33
C-H	3030	3034
Aromatic stretching of C=C (benzene ring) 1	1608; 1580	1612.49;1582.49
CO- NH-CO-NH	1689	1689.16
Stretching of -CO-N	1635	1632.55
Asymmetric and symmetric CH2 stretching, aliphatic C-H stretching	2925; 2840	2930.38; 2848.30
Asymmetrical stretching =C-O-C	1250; 1190	1253.71; 1195.87
C-O stretching (most characteristic)	930	929.69
Out-of-plane C-H bending 1,2,4-trisubstituted phenyl (two adjacent hydrogen atoms)	850; 830; 805	846.75;831.32;806.25

Particle size Vesicle morphology

Particle size analysis of the formulation was done by Zeiss system by analytical services in NIT, Raipur. The particle sizes of formulation shown in figure 2. Particle size of formulation found to be 315nm, 360nmrespectively. The mean particle size of liposome formulation was found to be in the range of (315 nm-360nm). The ability of liposome to alter the bio-distribution and pharmacokinetics of drugs, have important *in vivo* therapeutic applications. In this respect, the size and surface characteristics of liposomes are of prime importance.

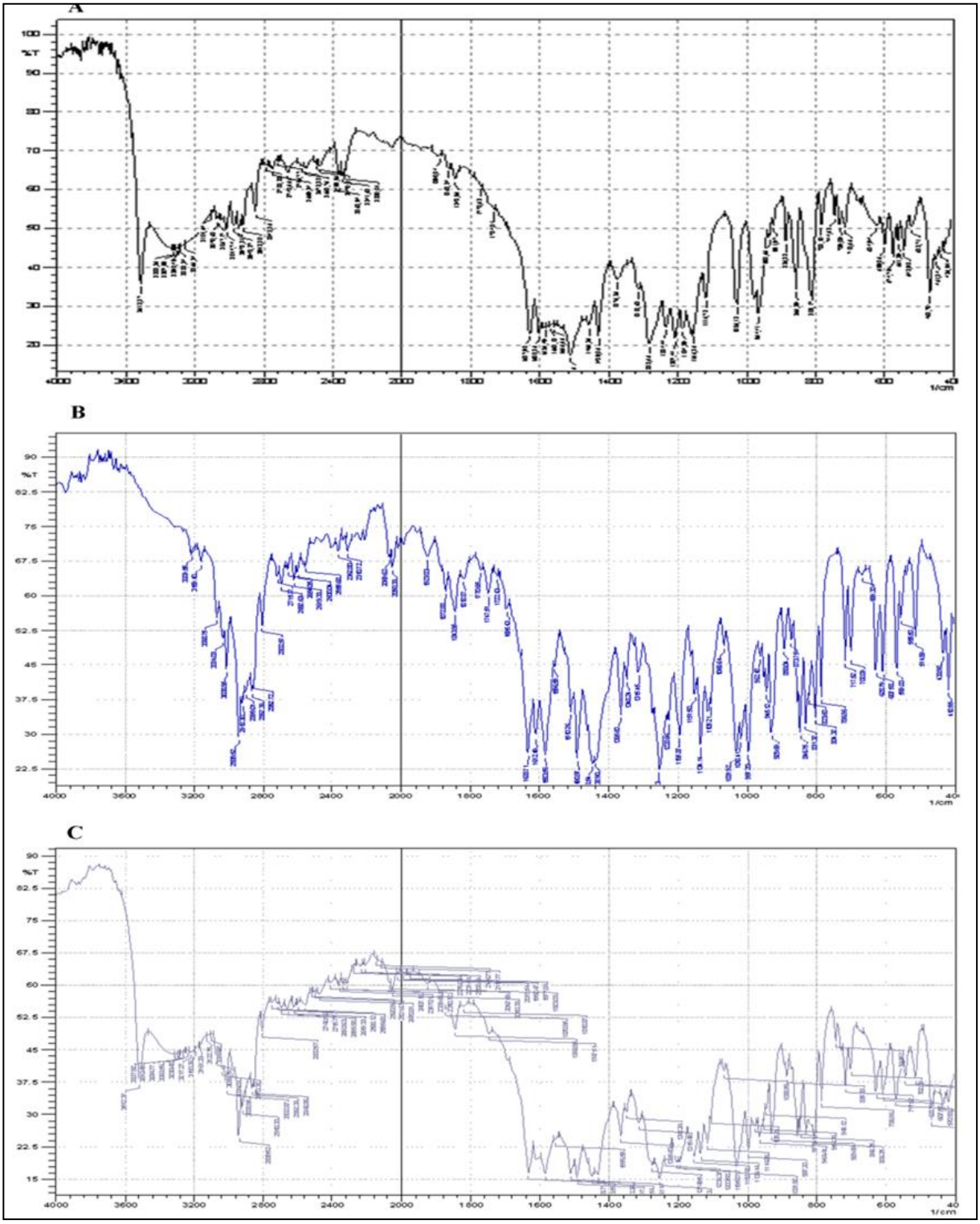

Fig 1: IR spectrum of curcumin [A], piperine [B] and mixture of curcumin and piperine [C]

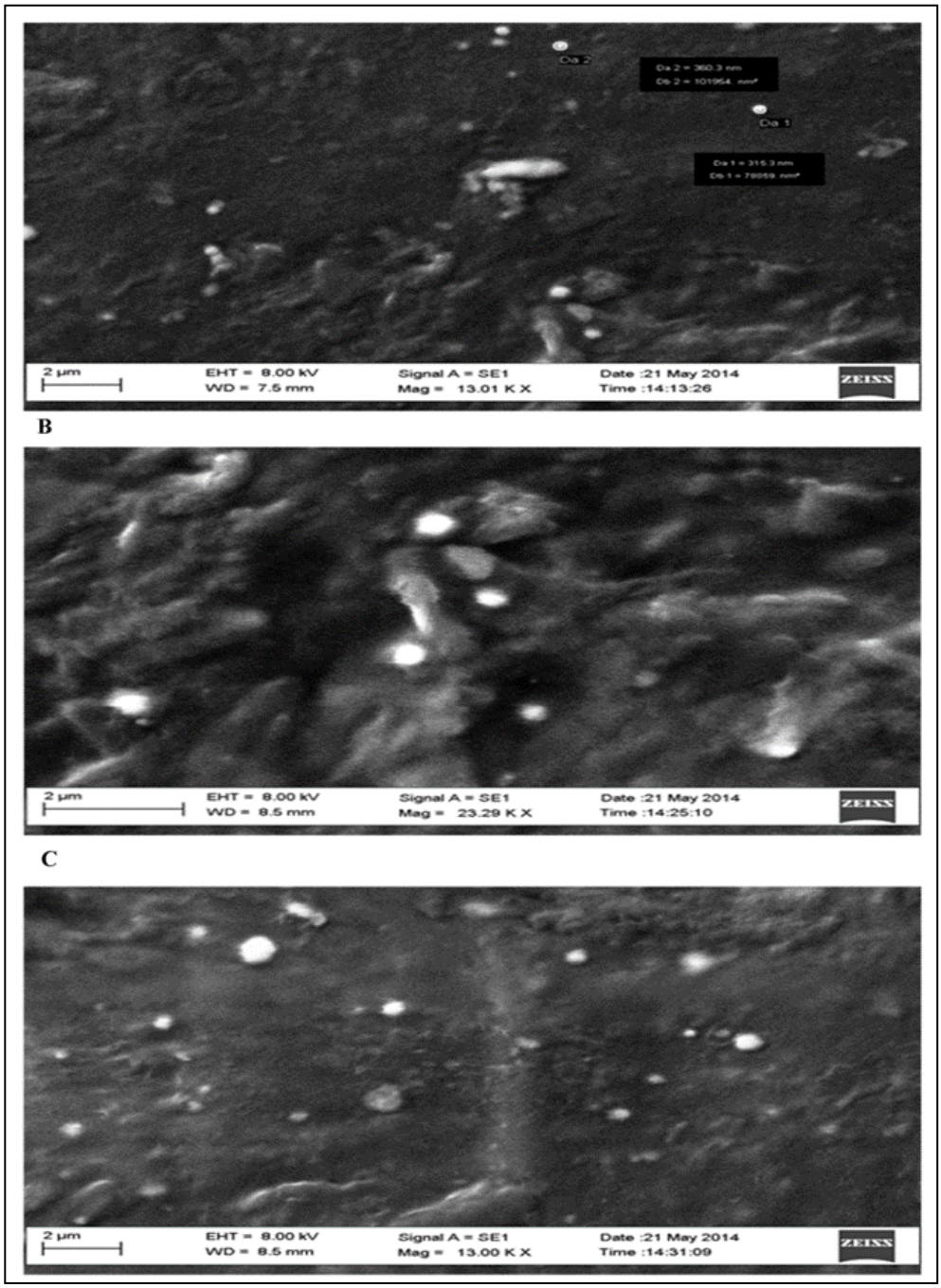

Fig 2: SEM photograph of CU-LPs Curcumin [A] piperine [B] Formulation [C]

Surface charge

Surface charge analysis of the CU-NPs was done by the Malvern system by services. Zeta potential of formulated Liposome was in the range of (26.30 ±0.243) which indicates that they are moderately stable. The zeta potential is a measure of the charge of the particles, as such the larger the absolute value of the zeta potential the larger the amount of charge of the surface. In a sense, the zeta potential represents an index for particle stability. For the case of charged particles, as the zeta potential increases, the repulsive interactions will be larger leading to the formation of more stable particles with a more uniform size distribution.

Drug encapsulation efficiency

The encapsulation efficiencies of the formulations were F1 59% and F2 61%. The results of encapsulation efficiency of the formulations was found to be almost similar without any significant differences, but it can be observed that, as a particle size was increased encapsulation efficiency was also increased.

***In vitro* drug release studies**

The *in vitro* drug release of drug curcumin from liposome formulations was carried out by using dialysis membrane in 7.4 pH phosphate buffer for 12 hrs. The *in vitro* release profile of obtained for formulation, are

shown in table 2. The comparisons of *in vitro* release profile of CU-LPs formulation. The cumulative percentage release of curcumin from the prepared Liposomes was varied from depends upon the drug ratio for 12 hrs.

Table 2: *In vitro* release profile of liposome formulation F1 and F2

Time (hr)	F1	F2
	%CDR	%CDR
0.5	0.061	0.052
1	0.364	0.254
1.5	0.425	0.606
2	0.986	0.858
3	1.147	1.108
4	1.708	1.262
6	1.869	1.516
8	2.130	1.670
10	2.391	1.932
12	2.752	2.086

Release kinetics

Drug release data obtained with all four formulations was analyzed according to % CDR Vs Time (Zero order rate kinetics), Log cumulative percent drug retained Vs Square root of Time (First order rate kinetics), %CDR Vs Square Root of Time (Higuchi model) and Log % CDR Vs Log Time (Kosmeyer-Peppas model). Calculated regression co-efficient for different formulations are shown in Table 3. The values were compared with each other for model fitting equation. The model giving a regression coefficient close to unity was taken as order of release. The best fit model was observed to (zero order release) for formulation.

Table 3: Best Fit Model for all formulations

Formulations	Zero order	First order	Higuchi matrix	Peppas plot	Best fit Model
	R^2	R^2	R^2	R^2	
F1	0.9465	0.8636	0.884	0.7044	Zero order
F2	0.9859	0.9749	0.9353	0.8322	Zero order

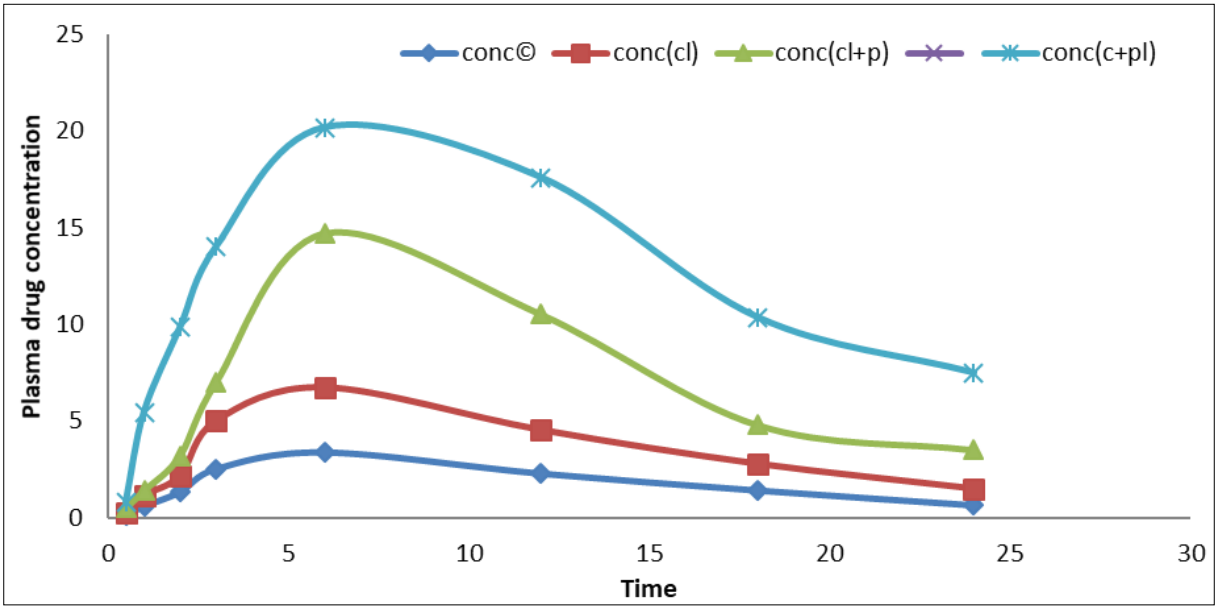

Fig 3: Plasma drug concentrations

Table 4: parameters of *in vivo* study

Parameters	Curcumin liposome formulation	Curcumin liposome+ pure piperine	Curcumin piperine liposome	Pure Curcumin
Cmax(µg/ml)	3.362	14.724	20.17	2.501
Tmax(h)	6	6	6.7	3
AUC0∞ (µg/ml/h)	51.3	223.373	419.409	47.724
MRT	13.813	15.22	20.52	13.261
Kel'	0.0723	0.0657	0.04873	0.0754

Conclusions

This study has shown that the thin film hydration method can be used as a feasible method to formulate curcumin loaded liposomes. Careful selections of various procedures are critical, firstly to achieve stabilization during formulation. These findings indicate the suitability of formulation procedure for preparation of formulation of poorly water-soluble drug. sFT-IR studies confirm the compatibility of curcumin with the various excipients used in our study. Preparation of curcumin liposome using thin film hydration technique by the use of soy lecithin, cholesterol and other excipients as a carrier is found to be feasible, economical and more productive. Curcumin to piperine 10:1 ratio found to be the optimum formulation with reference to various physicochemical characters such as drug content, dissolution profile etc. Dissolution profile and *in vivo* bioavailability studies demonstrated the significant improvement of curcumin performance from liposome compared to pure curcumin.

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