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Development of liposome encapsulated curcumin for treatment of arthritis

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ABSTRACT

Liposomesof curcumin was prepared by Thin Film Hydration method (TFH) and Reverse Phase Evaporation Method (REV) methods and Freeze-thaw (F-T) cycles were carried out for liposomes prepared by both the methods, then optimized with regard to percentage drug entrapment by changing various process and formulation parameters, various compositions of optimized liposomal batches along with their PDE and mean vesicle size values are recorded. Prepared liposomes prior to size reduction were suitable for nasal delivery as then vesicle size distribution was in range of 10-20 pm that is required for nasal drug delivery. and finally optimized final batch parameter were evaluated and conclude ration of lipid and drug were-Drug: PC :CHOL (1:4:1), REV method followed by F-T cycles and Diethyl ether :water (5:1) solvent systems were used and PDE/D[4,3] before size reduction was 97.89±0 31/ 12.8±0.2 and PDE/D[4,3] after size reduction 98.3 ± 0.21/2.5 ± 0.01 were obtained. However, further processing for size reduction of liposomes was required for lung deposition in case of pulmonary drug deliver) and hence liposomes after size reduction to 3-5 pm were used for preparation of DPI formulations.

Keywords: Liposomes, Thin Film Hydration, Reverse Phase Evaporation and PDE

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1. INTRODUCTION

Liposomes were first reported as a particulate dispersion of solid spherical particles between 0.2-100 µm in diameter consisting of solid hydrophobic fat core such as triglycerides or fatty acids derivatives, stabilized by monolayer of phospholipids (**Figure. 1**). The internal core contains the drug dissolved or dispersed in solid fat matrix. Liposomes represent a new type of fat based encapsulation system developed for parenteral and topical delivery of bioactive compounds.[1-4] Inconsistent nomenclature is found in relation to Liposomes as nano-scale particles termed solid lipid nano particles (SLN**).**

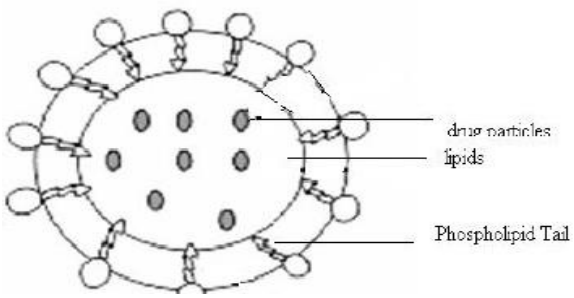


Figure 1: Structure of Liposomes

Agents for agricultural applications such as herbicides, fungicides and fertilizers can also be incorporated into lipospheres.[5-8] Lipospheres are distinct inner cores at room temperature. The lipospheres are distinct from microspheres of uniformly dispersed material in homogenous polymer since they consist of at least two layers, the inner solid particle and the outer layer of phospholipids.[9]

2. DRUG PROFILE[10-14]:

Curcumin is a phytopolylphenol pigment isolated from the plant Curcuma longa, commonly known as turmeric, with a variety of pharmacologic properties. Curcumin blocks the formation of reactive-oxygenspecies, possesses anti-inflammatory properties as a result of inhibition of cyclooxygenases (COX) and other enzymes involved in inflammation; and disrupts cell signal transduction by various mechanisms including inhibition of protein kinase C. These effects may play a role in the agent's observed antineoplastic properties, which include inhibition of tumor cell proliferation and suppression of chemically induced carcinogenesis and tumor growth in animal models of cancer.

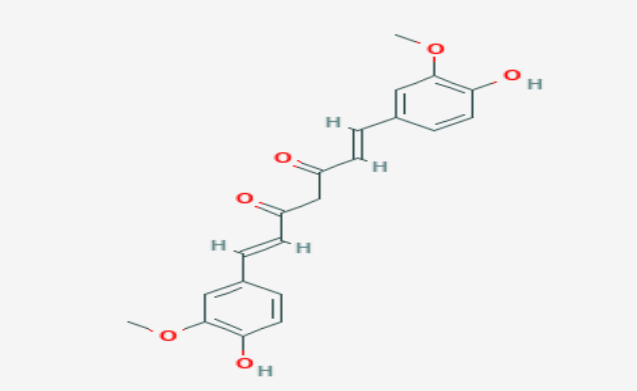


Figure 1: Chemical structure of Curcumin

Table 1: Physiochemical Properties of Curcumin

Properties	Specifications
IUPAC Name	(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione
Molecular formula	$C_{21}H_{20}O_6$
Mol. Wt.	368.385 g/mol
Solubility	Insoluble in water
Color	yellow
Nature of particle	A fine, white crystalline powder

3. MATERIALS AND METHOD:

Table 2: Chemicals used during the Experimental work

Sr. No.	Chemical/Material	Source/Manufacturer
1	Curcumin	AK Scientific, San jose
2	Ethanol Absolute	ChangshuYanguan Chemicals, China
3	α-Tocopherol	Gateffose, India
4	Egg yolk phaspholipid	Thomas Baker Pvt. Ltd.
5	Cholesterol	Thomas Baker Pvt. Ltd
6	Methanol	SD Fine-chem Ltd, Mumbai
7	HCl	SD Fine-chem. Ltd, Mumbai
8	n-octanol	SD Fine-chem. Ltd, Mumbai

3.1 Preformulation Studies:

3.1.1 Melting point determination:

Capillary fusion method was used to determine the melting range of the drug.[15]

3.1.2 Solubility study

a) Lipid solubility: 100 mg of lipids were taken in culture tube and placed them in water bath shaker at 80°C and kept shaking until they melt. An excess amount of drug was added while shaking meanwhile 4 ml of distilled water was added and allowed their shaking for 72 h at 80°C. After 72 h centrifuge the sample at 15000 rpm followed by taking the supernatant and diluted with methanol if required. Scan the sample in UV spectroscopy between 200-400nm.[16]

b) Solvent solubility: Took 2 ml of each solvent in culture tube. Add excess amount of drug in 2 ml of solution using vortex shaker. Put the solution into water bath shaker for 48 h at 37°C. After 48 h. centrifuge the sample at 15000 rpm followed by taking the supernatant and diluted with suitable solvent if required. Scan the sample in UV spectroscopy UV spectroscopy (Shimadzu UV spectrophotometer, Japan) between 200-400 nm.[17-18]

3.1.3 Partition coefficient

A saturated solution of Curcuminwas prepared. And the above formed mixture was left undisturbed for about 24 h. Two layers were separate through separating funnel and the amount of solubilized Curcuminwas determined by measuring the absorbance at 320 nm against methanol blank through double beam UV/Vis spectrophotometer (Shimadzu) in both the solution. Partition coefficient was determined as ratio of concentration of drug in n-octanol to the concentration of drug in water and the value were reported as log P.[19]

$$P\ o/w = Cn\ (octanol)/Co\ (water)$$

3.1.4 Drug excipients compatibility study by FTIR

The compatibility of drug with excipients was ascertained by FT-IR. FTIR was used as tool to detect any physical and chemical interaction between drug and excipients.[20]

3.1.5 UV spectrum of Curcumin in Methanol

Double beam UV-visible spectrophotometer (Shimadzu Corporation, UV–1800, Kyoto, Japan) was used to determine the λ_{max} of drug. A stock solution of the drug (100 µg/ml) in methanol was prepared. The sample was then diluted with methanol (25 µg/ml). The resulting solution was scanned in the range 200-400 nm to determine the λ_{max} of the drug Curcuminexhibited characteristic λ_{max} at 320 nm.[21]

4. PREPARATION OF *CURCUMIN* LIPOSOMES:

The liposomes of curcumin were prepared by two methods viz,

(i) Thin Film Hydration method (TFH)[22-24] and

(ii) Reverse Phase Evaporation Method (REV)[25] methods.

4.1 Thin Film Hydration method (TFH):

EggPC/CHOL, curcumin & α-tocopherol (equivalent to 1% w/w of PC) in 5 ml chloroform/methanol (2:1) in 250 ml round bottom flask (Quick 111 neck B-24) Smooth, dry lipid film formation using rotary Hash evaporator at 120 rpm, vacuum of 500 mini Ig at 30°C The nitrogen atmosphere was maintained. Hydration of film carried out with the 0.5 mL double distilled water. Annealing of liposomes at room temperature at 2 hrs, separation of un-intrapped drug using centrifugation and Liposomes loaded with drug subjected for characterization.

4.2 Reverse Phase Evaporation Method (REV Method):

EggPC/CHOL, Curcumin & α-tocopherol (equivalent to 1% w/w of PC) in 2.5 ml of Diethyl ether in a glass boiling lube (Quick Fit neck B-24). 0.5 mL of double distilled water injected rapidly with force using 23-gauge hypodermic needle attached to 5 ml syringe. Boiling tube closed with glass stopper; sonicated for 5 minutes in bath sonicator, attach this tube to rotary' flash evaporator to dry' the contents at 37 °C under vacuum of 500 mmHg until gel form. Vacuum released, tube removed frorn evaporator and subjected to vigorous mechanical agitation on vortex mixer for 5 minutes. When gel collapsed to fluid, boiling tube fitted again to rotary' flash / evaporator for removal of organic solvent. A cycle of 10 minutes drying and 5 minutes vortexing repeated twice. Final liposomal suspension subjected for removal of last traces of organic solvent under high vacuum (500 mmHg) for 15 minutes then separation of un-entrapped drug using centrifugation and Liposomes loaded with drug subjected further for characterization.

5. EVALUATION PARAMETERS:

5.1 Liposome Morphology:

Transmission Electron Microscopy (TEM) was used to study the vesicles' morphology. According to morphological evaluation analysis, all vesicle types seemed to have a similar spherical or oval shape. These oval-shaped vesicles may have resulted from the liposomes' deformation, which might occur during the sample preparation. In addition, TEM failed to reveal any structural differences among these vesicle types, indicating that phospholipid type did not have a significant impact on vesicle structure.[26]

5.2 Differential Scanning Calorimetry:

Characterization Differential Scanning Calorimetry (DSC) is used to characterize the melting and crystallization behavior of crystalline materials. The DSC curves of curcumin, the physical mixture with or without curcumin, and three kinds of curcumin-loaded liposomes were recorded.[27]

5.3 Determination of vesicle Size:

The vesicle size determination of the prepared liposomes carried out by light scattering was based on laser diffraction using Malvern. MasterSizer SM 2000k (Malvern Instillments Inc, UK).[28]

5.4 Determination of vesicular shape & lamellarity:

All the batches of the liposomes alter sufficient dilution were viewed under Olympus (BX 40F4, Japan) with the provision of dark background and attachment of polarizing lens, to study their shape and lamellarity.[29]

5.5 Determination of Percentage of entrapment efficiency (PDE)[30-31]:

The mini-column centrifugation method was used to assay entrapment efficiency. Sephadex G50 was chosen, swollen in distilled water for at least 12 h and stored at 4 °C. A small piece of cotton was inserted in the bottom of the barrels of a 2 cm^3 injection syringe, which was then filled with sephadex G50. Excess water was centrifuged off at 1,500 rpm for 3 min, and 0.5 mL PBS (pH6.5) was added, then centrifugation repeated twice. An equal volume of curcumin-loaded liposome suspension was also determined and the calculated drug amount was designated as WTotal:

$$\% \text{ drug entrapeed (PDE)} = W\text{Entrapped} / W_{Total} X100$$

5.6 *In vitro* drug release study from liposomes:

Concentrated liposomal suspension, 0.5 ml was taken in a test tube of opening diameter of 20 mm. The open end was covered with a semi-permeable dialysis membrane (Himedia Laboratories Pvt. Ltd.) and tied with a thread. The test tube was inverted and placed over the surface of 100 ml water present in a 250 ml beaker in such a way that the membrane just touched the water surface. The test tube was secured by a clamp fixed with a stand. The water in the beaker was stirred with a magnetic stirrer so that no vortex could form in the beaker. The temperature was maintained at 37 °C. The drug released from the liposomes permeates across the membrane and enters into the receptor chamber medium. Samples of 2 ml were taken out from the receptor chamber medium, suitably diluted, and the absorbances were taken by UV-spectrophotometer at 320 nm against a blank of fresh medium.[32-33]

6. RESULTS & DISCUSSION

6.1 Organoleptic properties:

Organoleptic properties of drug Curcumin found to be as per I.P. monograph.

Table 3: Organoleptic Properties of Curcumin

Sr. no.	Properties	Inferences
1	Colour	Yellow
2	Odour	Odourless
3	Form	Crystalline
4	Melting point	181-184° C
5	Log p Values	3.230± 0.061

Table 4: Solubility studies of Curcumin for different lipids

S. No	Lipid Name	Solubility (mg/ml) Mean±S.D
1	EPC	14.048±0.002
2	Cholesterol	13.723±0.058
3	A-tocopherol	14.645±0.004

Table 5: Solubility studies of Curcumin for different lipids

S. No	Solvent Name	Solubility (mg/ml) Mean±S.D
1	Methanol	59.238±0.872
2	Ethanol	38.857±0.571
3	0.1N HCl	0.198±0.001
4	PBS 6.8	0.041±0.0003
5	PBS 7.4	0.001±0.0003
6	Chloroform	44±0.571
7	Acetone	51.619±0.872

* Each value is average of three independent determinations

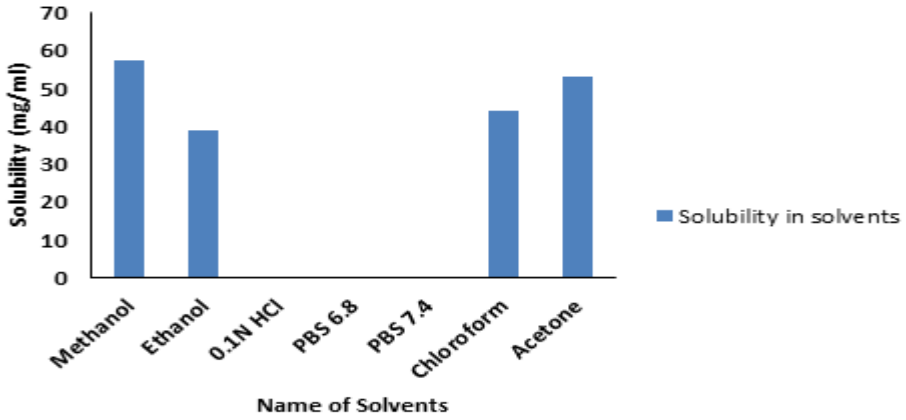


Figure 2: Solubility study of drug in different solvents

6.2 Drug Excipient compatibility study by FTIR

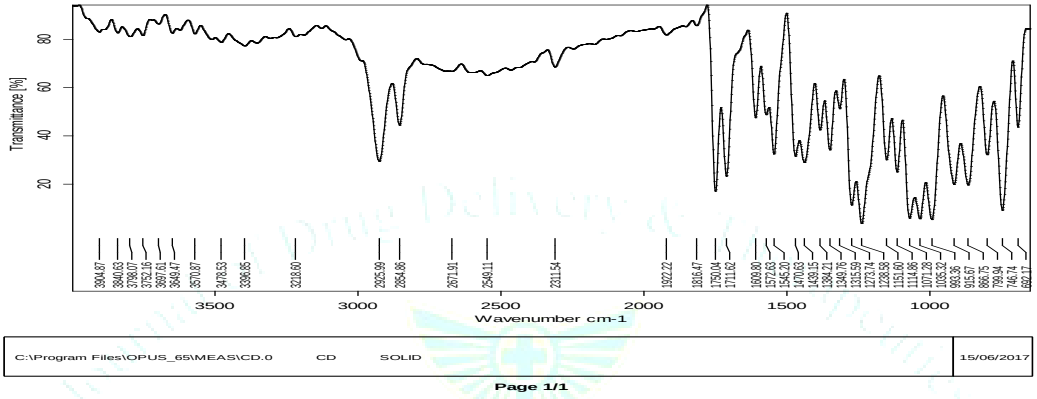


Figure 3: FTIR spectra of standard curcumin

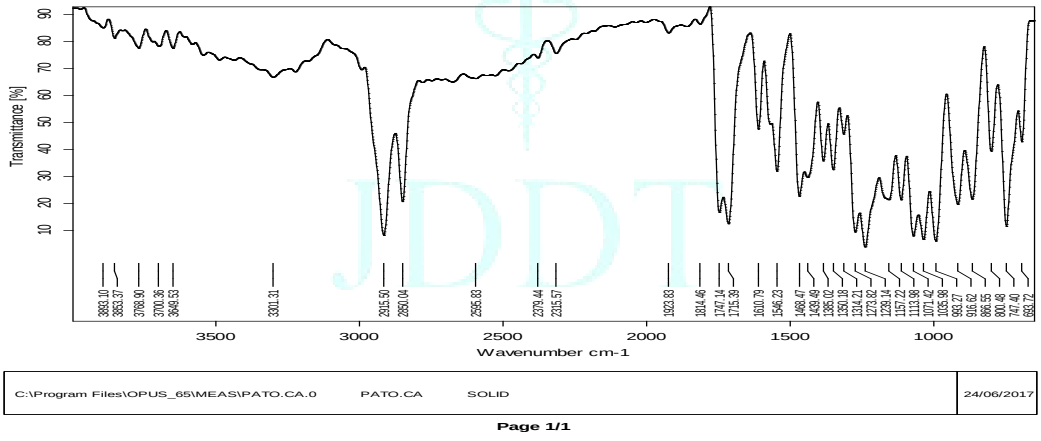


Figure 4: FTIR spectra of physical mixture containing drug and PC

6.3 Determination of absorption maxima in Methanol:

Table 6: Calibration curve of Curcumin in Methanol (λ_{max} = 320 nm)

S. No.	Concentration (µg/ml)	Absorbance (Mean ±SD)
1	1	0.052±0.003
2	5	0.195±0.002
3	10	0.412±0.003
4	15	0.551±0.001
5	20	0.767±0.002
6	25	0.899±0.005

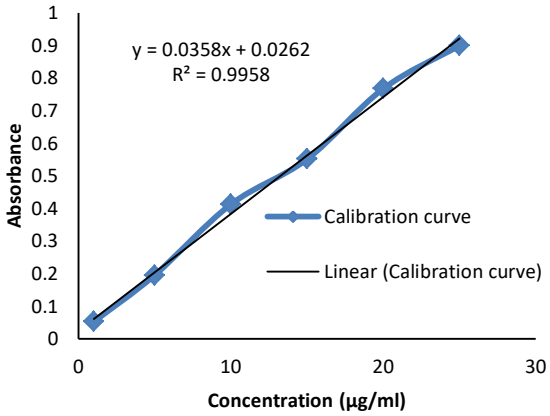


Figure 6: Graph of standard calibration curve of Curcumin in Methanol

7. EVALUATION PARAMETER:

7.1 Effect of Melting Point on Combination of Lipid And Drug:

The melting points for the three kinds of physical mixture I (PC:Curcumin) were found to be generally between 126.8°C and 143.6°C. Curcumin alone exhibited a melting peak of approximately 182.3° C. The physical mixture with curcumin showed a melting point of approximately 179.3° C; however, this phenomenon was not observed in liposomes. Moreover, the melting point of lipid material in the three kinds of liposomes dropped 6–17 °C compared with the corresponding physical mixtures I and II (CHOL:Curcumin).

7.2 Effect of Process Variables:

Table 7: Selection of Process Variables for Preparation of Curcumin Liposomes

Independent Variables	Level		
	1	2	3
Chloroform methanol ratio	2.1	1.1	1.2
Vacuum	400	500	600
Speed of rotation	80	120	160
Temperature	20	30	40

7.3 Influence of F-T cycles:

Influence of F-T cycles on PDF of liposomes were also evaluated and PDF before and after exposure of liposomes to FT cycles are recorded in table 8. Significant improvement in PDF was observed after exposure of liposomes to F-T cycles Significant variation ($p<0.05$) variation in vesicle size after F-T cycle was observed.

Table 8: Influence of Frecze-thaw cycles on Percent Drug Entrapment of curcumin liposomes

Batch	Molar ratio (Drug PC CHOL)		TFH Method PDF ± SEM	REV Method PDF ± SEM
1	1:2:0	B	5 765±2 25	8 254+2 00
	A1		11 54+1 21	15 67+0 74
	A2		15 13+0 58	15 85+0 41
	A3		15 25+0 42	15 25+0 42
2	1:3:0	B	16 25±2 35	2 i 08+2 52
	A1		35 43±2 13	43 87+0.11
	A2		42 69+0 97	44 38+0 79
	A3		43.847+0.37	44 254+0 35
3	1.4:0	B	71.23+2 45	78 650+2 69
	A1		88 32+1 18	96 98+1 09
	A2		96 10+0 65	97 32+0.45
	A3		96 02+0 31	97 56+0.29
4	1:5:0	B	74 36+2.69	83 32+2 87
	A1		91 31+201	95 61+0.98
	A2		95 86+1 08	96 56+0 25
	A3		95 98+0 28	96 69+0 26
5	1:4:1	B	76.275+3 15/15.0+0 2*	88 789+3 00/12 6+0.2*
	A1		89 54+2.54	97 98+1.36
	A2		96 87+1 17	99 01+0.44
	A3		97.98+0.29	98.99+0 21
6	1:3:2	B	35 014+2 11	40 519+2 31
	A1		49 23+1.16 '	55 36+1.21
	A2		57.01+0.74	56 45+0.47
	A3		58 321+0.21	56 551+0.26

***D[4,3]**, **B**=Before Freeze thaw Cycle, **Al**= After 1st Freeze thaw cycle **A2**= After 2nd Freeze thaw cycle, **A3**= After 3rd Freeze thaw cycle.

Table 9: Effect of Hydration time on PDE of liposomes prepared by TEH and REV method

TIme of Hydration	TFH method	REV method
2	68 14+3 17	88 7S9±3.00
4	76 27±3 15	88 71512 86
8	76 29±3 21	--
12	--	--

Table 10: Effect of time and temperature of freezing on PDE evaluated in Batch 05

Time/temperature	TFH method	REV method
Before	76 2717 73	88 79±7 35
30 min/-20°C	76 32±7 45	87 98±6 98
60 min/-20°C	78 65±2 13	88 67±0 98
120min/-20°C	79.54±1 21	89 98±0 32
180 min/-20°C	79 24±I 12	97 0110.29
30min/-40°C	77 96±6 83	89 1316 42
60min/-40°C	88 13± 1 05	96 6110.78
120min/-40°C	89.49+0.98/15.1+0.2*	97.89+0.31/ 12.8+0.2*

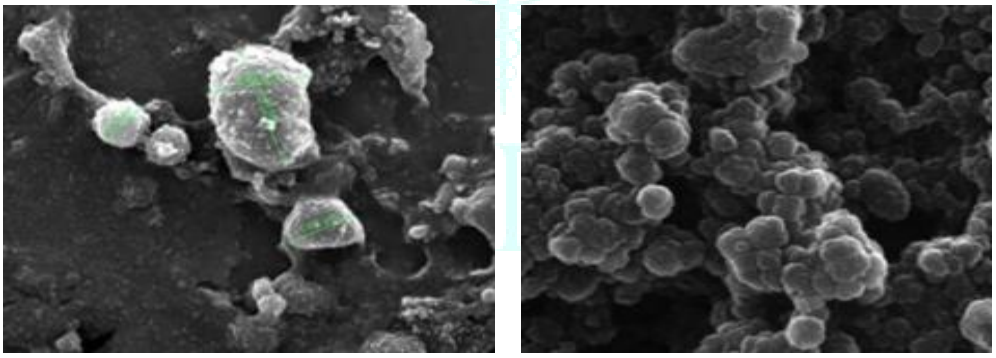
* D,[4,3]

Table 11: Effect of Post Sonication Freeze-thaw cycle on Batch 05

Sonication Time	PDE before Freeze-thaw cycle/ Liposome size (D[4,31)	PDF after Freeze-thaw cycle/Liposome size (D[4,3])
0 min	--	98 3±02ll/128±02
10 min	84 14± i 26/8 4 ±0 14 9	98 27 ± 0 20/8 5 ± 0.03
20 min	72 06± I 15/2 5±0 II	98 3 ± 0 21/2 5 ± 0 01
30 min	37.34± I 51/2 1 ±0 II	88 29 ± 0 35/2 2 ± 0 11

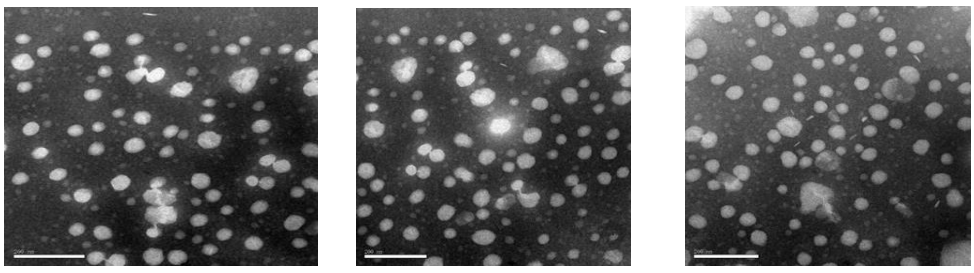
7.4 Size Reduction of Optimized Liposomal batch (Batch 05):

Size reduction of curcumin liposome was carried out using sonication in a 10 ml borosil beaker under ice bath, b) probe sonicator Twenty minutes sonication lime was found to be optimum with' regard to required size distribution (below 5 pm) Post sonication F-T cycles helps in regaining the equilibrium with the leaked drug.



Before F-T cycle **After F-T cycle**

Figure 7: Photomicrographs of the optimized liposomal batches



A **B** **C**

Figure 8: Morphology of curcumin-loaded liposomes.(A) PC:Drug, (B) CHOL:Drug (C) PC:CHOL:Drug

7.5 Differential scanning colorimeter (DSC) Analysis:

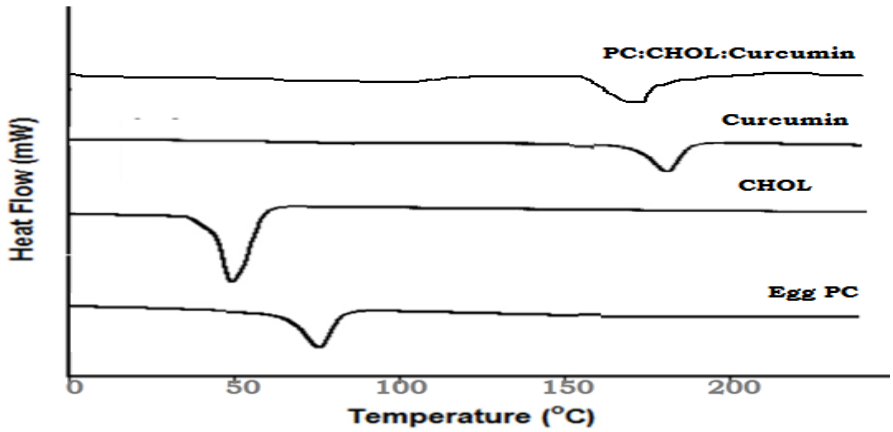


Figure 9: Differential Scanning Calorimetric Thermogram of PC, CHOL, Curcumin and physical mixture of PC:CHOL:Curcumin

7.6 Comparisons of all formulation:

The curcumin liposomes prepared by two different methods compared, REV method showed higher PDE at all the drug: PC: CHOL ratio compared to TFH method. The high entrapment found for MLV vesicles prepared from water/organic solvent emulsions (REV method) depend on maintaining a core dining the process of liposome formation (Prdgeon et al,, 1987) Even during the Freeze-thaw cycles, equilibrium with respect to PDE attained faster (after FT cycle) for REV method compared to that of TFH method (after 3rd cycle) The difference in number of freez thaw cycles required to establish equilibrium on prepared liposomes for both the methods, may be due to the structural differences observed for liposomes produced by both the methods as TFH method resulted into multillamellar and REV method resulted into oligolamellar liposomes.

Table 12: Analytical profile of Final Liposomal batches of curcumin

Batch	Curcumin Liposomes
Composition	Drug: PC :CHOL (1:4:1)
Method of Preparation	REV method followed by F-T cycles
Solvent system	Diethyl ether :water (5:1)
PDE/D[4,3] before size reduction	97.89±0 31/ 12.8±0.2
PDE/D[4,3] after size reduction	98.3 ± 0.21/2.5 ± 0.01

8. CONCLUSION:

On physicochemical evaluation melting point and partition coefficient was found to be 181-184°C and 3.230± 0.061 and it shows that the curcumin is lyophilic in nature. On FTIR spectroscopy and DSC analysis, there was no incompatibility between drug and lipid. On UV-visible spectrophotometer analysis absorption maxima of Curcumin was found to be 420 nm. Drug was freely soluble in methanol followed by chloroform and acetone. Liposomesof curcumin was prepared by Thin Film Hydration method (TFH) and Reverse Phase Evaporation Method (REV) methods and Freeze-thaw (F-T) cycles were carried out for liposomes prepared by both the methods, then optimized with regard to percentage drug entrapment by changing various process and formulation parameters, various compositions of optimized liposomal batches along with their PDE and mean vesicle size values are recorded. Prepared liposomes prior to size reduction were suitable for nasal delivery as then vesicle size distribution was in range of 10-20 pm that is required for nasal drug delivery.

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