ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

Development of protocol for screening of formulation attributes and the assessment of common quality problems in oleuropein loaded nanostructured lipid carriers

Sucharitha P[1,2], Satyanarayana SV[3], Bhaskar Reddy K*[4]

[1]Department of Pharmaceutics, Jawaharlal Nehru Technological University Anantapur, Ananthapuramu-515001, Andhra Pradesh, India
[2]Department of Pharmaceutics, Sri Venkateswara College of Pharmacy, RVS Nagar, Chittoor-517127, Andhra Pradesh, India
[3]Department of Chemical Engineering, Jawaharlal Nehru Technological University Anantapur, Ananthapuramu-515001, Andhra Pradesh, India
[4]Department of Pharmaceutics, Centre for Nanotechnology, Sri Venkateswara College of Pharmacy, Chittoor-517127, Andhra Pradesh, India

Article History:

Received on: 15.11.2018
Revised on: 14.02.2019
Accepted on: 17.02.2019

Keywords:

Gelation,
Oleuropein,
Particulate Characters,
Thermal Studies

ABSTRACT

The current study aims to explore and identify the screening of formulation components and evaluates the quality issues of nanostructured lipid carriers (NLCs) for the phenolic secoirodoid Oleuropein. The stepwise screening of the components for preparation of NLCs includes the selection of solid lipid, liquid lipid based on the relative solubility of Oleuropein in different lipids. Polaxomer 188 was selected as the main surfactant for the preparation of NLCs because of its good emulsification efficacy for the solid lipid liquid mix. Lecithin was used to enhance the stability of NLC. The optimized formulation was also evaluated for different quality issues. Thermal analysis by DSC revealed that the lipid particles maintained sufficiently good melting point even after nanonization. Absence of gelation and resilience for the stress provided by autoclaving further established the quality of the developed NLCs. In a nutshell, a systematic protocol was developed for designing of Oleuropein NLC with good particulate parameters of minimum particle size and maximum zeta potential with narrow polydispersity index.

* Corresponding Author

Name: Bhaskar Reddy K
Phone: +91-8328695204
Email: sucharitha89pharma@gmail.com

ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v10i2.545

Production and Hosted by

IJRPS | https://ijrps.com

INTRODUCTION

In pharmaceutical research, various strategies for developing a superior drug delivery system were emerging rapidly to cater to the therapeutic needs of the modern era. During the formulation of any carrier system, precise control over the material and process attributes was mandatory to get a quality product. Practically it was a cumbersome process to precisely tailor the properties of this carrier system. Lipid Nanocarriers holds an apex position in the current drug delivery technologies (Ana Beloqui *et al.,* 2015) Hence in this research, Nanostructured lipid carriers were chosen to load the drug Oleuropein which is a phenolic secoirodoid extracted from the Olive Leaf. The quality & efficacy of Nanostructured Lipid Carriers depends on the type of lipids, surfactants and methods of the formulation. In addition to the availability of wide range of lipids and surfactants, there are some critical quality issues associated with NLC, i.e. Polymorphic changes in lipids, presence of supercooled melts, gelation etc. (Anna Fabregas *et al.,* 2014) Hence, a structured protocol was need to be articulated to design a

physicochemically stable and pharmacologically effective NLC. In advancement to the existing research, Polyhydroxy surfactants were chosen in this study because of its advantage of projecting stronger lipophilicity and hydrophilicity compared to ethoxylated surfactant, minor susceptibility to pH change makes them favourable for designing of NLC when compared to other surfactants (Beloqui, MA *et al.,* 2013*).*

In the present study, we have tried to draft a comprehensive protocol for the selection of suitable material and process attributes for the design of NLC using Oleuropein as a model drug. We designed simple and effective experiments to choose a suitable solid lipid, liquid lipid, surfactant as well as the ratio between solid and liquid lipid. Furthermore, we evaluated the effect of various formulation methods on the particulate characters of NLC. Hence, in this work, we provide a stepwise protocol for the designing of NLC and evaluation of their quality characteristics.

MATERIALS

Olive Leaves was purchased from Rajasthan Olive Cultivation Limited, Rajasthan. Solid lipids like Compritol 888ATO, Precirol ATO5 and Tefose was gifted by Gattefose (Mumbai, India). Stearic acid, Palmitic acid was purchased from Merck, Germany. Dynasan 114 was gifted by Sasol, Germany. Liquid lipids like Oleic Acid, Labrafac and Gelucire 53/13 were received as a gift sample from Gattefose (Mumbai, India). Capmul, Capryol and Miglyol were received as a gift sample from Abitec Corporation Ltd. (Mumbai, India). Surfactants like Tween 80, Poloxamer 188, and Span 80 was purchased from Merck, Germany. Cremophore RH60, Lutrol F 68, Nurol, Plurol stearic (WL1009) and plantacare®810 were kindly gifted from Gattefose (Mumbai, India). Isotonisation agent Glycerol 85% from Fischer scientific, Dialysis tubing (Mol. Weight cut off: 6000-8000 Da and a horizontal width of 23mm) purchased from Sigma Aldrich (Bangalore, India). Plurol stearic (WL1009) and plantacare®810 was purchased from Gattefose (Mumbai, India). All other chemicals and reagents were of analytical grade and were used without further purification.

METHODS

Selection of Solid lipid and Liquid lipid by Equilibrium Solubility method

Screening of lipids was performed to predict the most suitable lipid matrix for Oleuropein which offers high solubility and high drug loading. Solubility studies were executed by taking 1000mg of each lipid in the screw-capped bottles followed by melting at a temperature of about >5°C of transition temperature for each lipid. To this melted lipid, an increasing amount of drug was added, and the mixture was stirred for about 60min at 500rpm using vortex mixer, and Saturation solubility was observed by the formation of a clear, pale yellow solution of molten lipid. Stabilize the sample for 24hrs, and the lipid mixtures were smeared on filter paper, which is a first visible sign to predict immiscibility between lipids. After the saturation solubility of the drug in lipid, the effect of different types of solid lipids and liquid lipids on particulate parameters was identified by Dynamic Light Scattering technique. In the end, subject the samples for centrifugation using ultracentrifuge. The volume of 0.5 to the 1ml supernatant layer was collected, diluted with ethanol and the drug concentration was analysed by using HPLC. Solubility studies were performed in triplicate, and the results were presented as mean $\pm$ SD (Ram R Patlolla *et al.,* 2010).

Selection of Solid and Liquid Lipid Ratio

Miscibility study was done to predict the concrete ratio of solid lipid to a liquid lipid with higher solubilizing capacity for drug oleuropein. The ratio of solid: liquid lipid chosen was 90:10, 80:20, 70:30, 60:40 and 50:50 (%w/w). Lipids were subjected to melt and blend them for about 30min at 500rpm using vortex mixer. Add 0.5% v/v concentration of surfactant solution to the lipid blend and homogenize the samples using High-Speed Homogenizer at 10000rpm for 10min. The samples were resided at RT for 24 hrs and observe particulate characters by Dynamic Light Scattering technique (Blanka Soto *et al.,* 2015).

Influence of Phospholipid (PL) /Lipid matrix (w/w) ratio on NLC

Various Proportions of PL and lipid blend i.e., 0.5:1,1:1, 1.5:1, 2:1, 2.5:1, 3:1, 4:1 (%w/w) were mixed using vortex mixer at an rpm of 500 and a duration of about 30min. To the homogenous blend, 0.5% v/v concentration of surfactant solution was added and homogenized the sample using High-Speed Homogenizer at 10000 rpm for about 10 min. keep the solutions aside for 24hrs and measure PS, PI and %EE (A.Kovacevic *et al.,*2011 and Yasmine M *et al.,* 2014).

Selection of Drug to Lipid Ratio

The influence of drug concentration on the loading and entrapment of NLC was investigated by taking different ratios of the drug: lipid blend. Melt and mix the blend for about 30min at 500rpm using vortex mixer. Add 0.5% v/v concentration of surfactant solution to the lipid blend and homogenize the samples using High-Speed Homogenizer at 10000rpm for 10min. The samples were resided at RT for 24 hrs and observe particulate characters by Dynamic Light Scattering

technique. The resultant mixtures were analysed for EE (%) and DL (%) (Fanfei Meng *et al.,* 2016, Fei Han *et al.,* 2008).

Preliminary Screening of Surfactant

The preliminary screening is a crucial step for the selection of surfactant to formulate NLC. Hence, Surfactants belongs to different categories such as Tween 80, Poloxamer 188, Span 80, Cremophore RA 60, Plutrol F68, Nurol and Planticare were screened and the effect of surfactant on PS, PDI and EE% was estimated by keeping the total surfactant concentration at 1% w/v constantly throughout the study. (Wang, T *et al.,* 2018).

Pre-Formulation Studies

FT-IR spectroscopy: Vibrational frequencies of NLC with and without drug as well as a physical mixture were recorded by using an FT-IR spectrophotometer (Eq. name) which is equipped with ATR sampling accessory 4000 to 400cm^{-1} at an optical resolution of 4cm^{-1}. Drug unloaded NLC was taken as a negative control and Oleuropein drug loaded NLC as a positive control to minimize the difference between spectra due to baseline shifts. IR spectrums were compared to investigate the interaction between drug and NLC (Wei Huang *et al.,* 2017).

Thermal studies: The thermal properties and phase transitions behaviour of the drug, lipid blend, placebo, NLC, Physical mix were investigated by using DSC (DSC 60; Schimadzu, Tokyo, Japan). DSC measurement was performed in a range of 10-190°C at a heating rate at 10°C/min to a temperature of 30°C throughout the analysis using Indium as the standard reference. Sample weight of about 2mg was used in this study. Melting Point values were determined from the endothermic peak and Crystallinity index was estimated using enthalpy of bulk material and enthalpy of NLC. The recrystallisation index (RI) was computed by the following equation (Hassan Hajj Ali *et al.,* 2018).

$$\%RI = \frac{\Delta HNLC}{\Delta HBL\ X\ Fraction\ of\ Lipid}\ X\ 100 \quad \text{Eq. (A.1),}$$

Where, $= Enthalpy\ of\ NLC;\ \Delta HBL = Enthalpy$

Optimization of formulation techniques to design NLC

Melt-Emulsification and Ultrasonication: Oleuropein loaded NLC were prepared by melt-emulsification and ultrasonication method. Briefly, a mixture of Solid lipid (Tefose) and Liquid lipid (Capmul) was taken in a ratio of 60:40, melted 5°C above the melting point of solid lipid. The drug: lipid ratio was maintained at 1.4%w/w, and the specified quantity of the drug was dissolved in melted lipid with the aid of vortex until a clear solution was obtained. Dissolve surfactant into an aqueous solution and heat the solution upto the temperature of lipid blend. At this juncture, hot lipid phase was added to hot surfactant solution and subject this mixture to ultrasonication for 5min (30:5 on: off lych) to obtain NLC. The obtained NLC was allowed to cool at RT and characterized further. The % of the aqueous phase was (95% by weight), and lipid phase was (5% by weight), Sodium azide (0.02%) was added to prevent microbial growth in NLC (Yasmine M *et al.,* 2014).

Ultrasonication: Oleuropein loaded NLC were formulated by using ultrasonication method. A specified quantity of lipid blend (Solid: Liquid lipid) and surfactant solution was heated to a temperature of 5°C excess to solid lipid in a vortex mixture separately. The aqueous solution was dropped into the lipid phase and homogenize the mixture using probe Sonicator (Q-Sonica, Germany) with an amplitude frequency of 80%, pulse of 30 secs for about 10min. Blank NLC's were prepared by using the same procedure without the drug. Cool the formulations to room temperature and characterized them further. The % of the aqueous phase was (95% by weight), and lipid phase was (5% by weight), Sodium azide (0.02%) was added to prevent microbial growth in NLC (Varsha B. Pokharkar *et al.,* 2015).

Solvent diffusion method: Oleuropein loaded NLC was prepared by solvent diffusion method. A measured quantity of solid lipid, liquid lipid and drug was dissolved in 5 ml of mixed organic solvent of ethanol: acetone (1:1 v/v) in a water bath at 50°C. The resultant organic solution was quickly dispersed into a 20ml aqueous solution of surfactant at room temperature under mechanical agitation with 3000rpm for 30min until NLC suspension was obtained. Blank NLC were prepared with the same procedure except for drug. Prepared NLC's were placed in a vacuum desiccator for 24 hrs at Room temperature to evaporate the residual solvent. After 24hrs, centrifugation was done, and the NLC precipitate was collected and redispersed into 10ml of 0.3% w/v of sodium dodecyl sulphate aqueous solution to remove free drug adsorbed on the surface of NLC, then the precipitate was collected and characterized. The drug-free NLC suspensions were prepared with exactly the same procedure, and the formulated NLC were placed in a Vacuum desiccator for 24hr at RT to vaporize the residual organic solvent (Pimchanok Witayaudom *et al.,* 2017 and Harshad Vaghasiya *et al.,* 2013).

Characterization of NLC

Particle size Measurement (PS): In order to predict the suitability of formulated NLC, particle size analysis was performed on the day of their formulation. Dynamic Light scattering is the most widely used non-invasive technique to measure particle size and PDI based on electrophoretic mobility. The particle size of the formulated NLC's was measured at 25°C by photon correlation spectroscopy (Dynamic light scattering) using Zetasizer (Horiba). The measurement was conducted after dilution of samples with distilled water upto a concentration of 0.1% w/w, to get suitable scattering intensity and to weaken the opalescence. The mean diameter and PDI values were estimated at an angle of 90° in 10mm diameter cells at 25°C (Wei Huang *et al.,* 2017).

Zeta Potential (ZP): It reflects the electric charge on the particle surface, indicates physical stability of NLC. ZP was measured by using Zetasizer (Horiba). The samples were diluted with purified water and adjusting conductivity (50 µs/cm) with potassium chloride solution (0.1% w/v). The pH was in the range of 5.5-7.5. ZP was estimated from the electrophoretic mobility using Helmholtz Equation. Each sample was estimated 3 times and meant values and SD are represented (Jafar Ezzati Nazhad Dolatabadi *et al.,* 2018).

$$\frac{s\boldsymbol{p} = s\boldsymbol{m}\ \boldsymbol{X}}{\boldsymbol{4\pi}/s} \quad \text{Eq. (A.2),}$$

Poly Dispersity Index (PDI)

PI is a measure of particle width, and the values of PI are ranging from 0 to 1. In general, very narrow distributed particle possesses PI values of about 0.2 -0.3 which is ideal for stability and uniformity of dispersion (Hassan Hajj Ali *et al.* 2018).

Determination of %EE and % Drug loading

EE is referring to the amount of drug encapsulated compared with the amount of drug used. Whereas, DL refers to the drug encapsulated compared with the amount of lipid. The %EE of Oleuropein within NLC was measured by estimating the concentration of the drug (free drug) in aqueous of NLC dispersion. A measured volume of prepared NLC dispersion was diluted with distilled water and centrifuged at 25,000 rpm for 30 min at 25°C to settle the colloidal nanoparticles using ultracentrifuge. The amount of free drug in the aqueous phase was estimated spectrophotometrically at 275nm using UV-visible spectrophotometer (Wei Huang *et al*., 2017). Values of % EE and % DL was calculated by the following equation.

$$\%EE = \frac{\text{Amount of Oleuropein added} - \text{Amount of Oleuropein in NLC}}{\text{Amount of Oleuropein added}}$$

Eq. (A.3),

$$\%\ DL = \frac{\text{Amount of Oleuropein added - Amount of Oleuropein in NLC}}{\text{Weight of NLC}}$$

Eq. (A.4),

Creaming Stability Evaluation

A measured volume (10ml) of NLC dispersion was chosen and evaluated at 25°C by observing the height of top layer (cream) and height of the serum phase (HS) for 28days (Jakarwan Yostawonkul *et al., 2017* and Somasree Ray *et al.,* 2018).

$$\text{The Creaming Stability Index } (CSI) = 100X\frac{[(HT-(HC+HS)]}{HT} \quad \text{Eq. (A.5),}$$

HC= Height of Creamy layer; HT=Total height; HS=Height of serum layer

Gelation phenomena

Gelation phenomena in the colloidal suspension of lipid nanoparticles can transform the low viscosity suspension to the high viscosity gel. Such changes could be attributed to the aggregation of nanoparticles in the response of external stress such as syringing etc. The developed nanosuspension of the NLC was taken into a syringe with needle and pumped into a beaker. The process was repeated 20 times, and the particle size of the NLCs was determined before and after this process (soma Sree *et al.,* 2018).

Effect of sterilization

The effect of sterilization on the particle size of colloidal NLC was observed using an autoclave. The colloidal suspension of NLC was filled in screw-capped 50 mL reagent bottles. Autoclaving was conducted for 15 min at 15 psi or 121°C (Schwarz et al., 1994). Samples were allowed to cool to room temperature and are studied for the change in particle size using the DLS method (Wang *et al*. 2018).

RESULTS AND DISCUSSION

Pre-selection of Lipids

Oleuropein, a moderately soluble drug in water showed maximum saturation solubility at room temperature in Tefose and Capmul among the solid lipid, Liquid lipid chosen for the study. Recrystallisation behaviour and solubility of the drug in various solid and liquid lipids are mentioned in Table 1. Solid lipid, Compritol 888ATO, Stearic acid and Palmitic acid show recrystallization after 24hrs, which is not ideal for high drug entrapment. Hence those lipids are not recommended for study. But to identify their particulate character, the lipids are evaluated by DLS technique and the PS, PI and EE were reported.

Table 1: Screening of Formulation Variables based on Particulate Properties of Oleuropein NLC (Particle Size, Zeta Potential, Entrapment Efficiency and Poly Dispersity Index)

Solid Lipid	Melting range (°C)	Particle Size (nm)	Polydispersity index	Entrapment efficiency (%)
Compritol 888 ATO	65-77	155.4 ± 2.3	0.28 ± 0.012	73.2 ± 3.5
Precirol ATO 5	50-60	148.8 ± 3.0	0.22 ± 0.02	64.0 ± 3.4
Tefose	46-53	124.3 ± 4.2	0.14 ± 0.015	72.5 ± 2.6
Stearic acid	67-72	138.6 ± 2.4	0.16 ± 0.012	74.8 ± 2.4
Palmitic acid	62.9	146.6 ± 4.6	0.24 ± 0.020	78.6 ± 2.8
Liquid Lipid				
Oleic Acid	13 -14	120.4 ± 0.3	0.16 ± 0.026	75.3 ± 3.6
Labrafac	25 - 33	111.8 ± 0.5	0.18 ± 0.012	62.9 ± 2.4
Gelucire 53/13	42.5 - 47.5	128.3 ± 0.9	0.13 ± 0.009	82.8 ± 1.8
Capmul	---	95.3 ± 1.2	0.12 ± 0.007	85.7 ± 1.5
Capryol	---	101 ± 1.6	0.12 ± 0.013	87.9 ± 2.0
Miglyol	---	132.3 ± 1.5	0.26 ± 0.013	54.1 ± 2.8
The ratio of Solid Lipid Liquid Lipid				
90:10	---	120.3±2.4	0.345 ± 0.014	73.8 ± 1.4
80:20	---	118.6±2.5	0.260 ± 0.012	75.0 ± 1.6
70:30	---	115.7±2.4	0.240 ± 0.014	75.8 ± 1.4
60:40	---	109.6±2.6	0.146 ± 0.010	78.5 ± 1.2
50:50	---	111.5±3.6	0.140 ± 0.012	76.4 ± 1.2
Surfactant (w/w)				
Tween 80	---	243.64 ± 1.58	0.491 ± 0.021	48.11 ± 2.07
Poloxamer 188	---	190.630 ± 2.52	0.310 ± 0.035	54.65 ± 1.87
Span 80	---	223.29 ± 6.41	0.458 ± 0.074	56.47 ± 1.12
Cremophore RH60	---	218.52 ± 3.73	0.469 ± 0.059	46.07 ± 1.06
Plutrol F 68	---	268.05 ± 4.65	0.620 ± 0.067	35.89 ± 1.45
Nurol	---	198.47 ± 5.19	0.464 ± 0.027	59.07 ± 1.06
Planticare	---	221.00 ± 1.36	0.438 ± 0.013	62.83 ± 2.43

Selection of Solid Lipids

The results of solid lipid screening were depicted in table 1. For the selection of various excipients in the preliminary studies, the criteria of selection are PS<200nm, ZP>30mV and EE%

should be >50%. NLC prepared with solid lipid Tefose shows less particle size of about 124.3 ± 4.2nm compared to the other chosen solid lipids. Hence, Tefose is selected as a solid lipid for the further formulation and optimization of NLC.

Screening of Liquid Lipid

The results of solid lipid screening were depicted in table 1. Among the Liquid lipids chosen, Oleic acid and Capryol show recrystallization after 24hrs. The particulate parameters of the drug-loaded NLC project that the liquid lipid Capmul produces NLC of least particle size 195.3 ± 1.2 nm, PDI of 0.12 ± 0.007 and entrapment of about 85.7 ±1.5%. Hence, Liquid lipid Capmul were selected for the preparation of NLC, because of their better particulate properties, good physical compatibility with no phase separation.

Selection of Solid and Liquid Lipid Ratio

Different ratios of solid lipids and liquid lipids were examined to check for stability, physical compatibility and leaching out of the drug. The results are shown in Table 1, which shows close proximity in PS, EE but differs widely in PDI for all the formulations. Hence, the ratio of least particle size, high EE and best PDI was chosen to formulate NLC. The ratio 60:40 of Tefose: Capmul were found to be optimum with respect to the particulate properties and entrapment efficiency.

Effect of Drug: Lipid Ratio on Loading and Entrapment of drug

To predict the impact of drug concentration on lipid matrix solubility, various concentration of drug, lipid was taken (0.5:1 to 2:4) and analyzed for EE% and DL%. From the data, it was reflected that, the drug: lipid ratio of 1: 4 gives a maximum EE and DL. Hence the same ratio is selected for further studies. The changes in the PS and EE with different ratios of solid lipid and liquid lipid were shown in table 2. The particle size of NLC decreases with increase in the concentration of liquid lipid while the inverse is true with EE.

Table 2: Influence of variables on the Particulate parameters of prepared Oleuropein Loaded NLC

Parameters	PS (nm)*	PDI *	EE (%)*	ZP (mv)*	DL%*
Effect of Lipid Concentration on Loading and Entrapment Efficiency of NLC					
1:2	---	---	68.34 ±3.2	---	70.42 ±2.6
1:4	---	---	76.52 ±2.6	---	77.62 ±2.4
1:6	---	---	64.42 ±2.4	---	60.32 ±2.2
1:8	---	---	61.54 ±3.2	---	54.50 ±2.8
Effect of Dru : Lipid Ratio on Loading and E trapment Efficiency of NLC					
0.5:4	---	---	64.80 ±3.6	---	64.34 ±2.6
1:4	---	---	75.60 ±3.4	---	68.42 ±3.0
1.5:4	---	---	79.26 ±2.8	---	74.34 ±2.4
2:4	---	---	60.42 ±2.6	---	64.34 ±2.6
Influence of Surfactant Combinations on the Particulate parameters of prepared Oleuropein Loaded NLC					
Tween 80:Span 80	181.00 ±2.50	0.291 ±0.02	56.70± 3.42	-12.1 ± 1.42	---
Tween 80: Poloxamer 188	175.30 ±1.09	0.260 ±0.01	65.20 ±1.42	-23.4 ± 1.64	---
Span80: Poloxamer 188	199.42 ±1.48	0.304 ±0.04	69.54 ±1.30	+18.4 ±1.54	---
Lecithin: Poloxamer 188	113.64 ±1.72	0.310 ±0.02	66.42 ±1.38	-33.6 ± 1.80	---
Plurol Stearique: Caprolyl	211.52 ±1.64	0.432 ±0.02	58.06 ±2.80	-39.8 ± 1.26	---
Lecithin: Caprolyl	103.47± 2.32	0.274± 0.04	71.10 ±1.54	-41.2 ± 2.10	---
Influence of Phospholipid/Lipid matrix (w/w) ratio on Particle Size and Entrapment Efficiency of Oleuropein Loaded NLC (n=3)					
0.5:1	113.4 ±3.6	---	65.8 ± 2.6	---	---
1:1	105.22 ±3.4	---	69.2 ± 3.0	---	---
1.5:1	98.0 ± 2.8	---	70.3 ± 2.4	---	---
2:1	90.0 ± 2.6	---	72.4 ± 2.8	---	---
2.5:1	91.0 ± 2.8	---	75.8 ± 2.6	---	---
3:1	87.0 ± 2.6	---	79.2 ± 2.0	---	---
4:1	89.0 ± 3.6	---	81.8 ± 2. 4	---	---

* Values are expressed as Mean ± SD (n=3)

Table 3: Effect of Various Formulation Techniques on the Particulate Characters of Oleuropein loaded NLC

Formulation Technique	PS (nm)		PDI	
	Blank NLC	OLE NLC	Blank NLC	OLE NLC
ME-US	144.20±1.90	153.42±1.52	0.192±0.004	0.199±0.002
US	162.80±0.80	184.52±1.52	0.214±0.002	0.221±0.004
SD	180.20±1.80	192.52±1.98	0.235±0.004	0.239±0.006

ME-US: Micro emulsification – Ultrasonication; US: Ultrasonication; SD: Solvent Diffusion; OLE: Oleuropein, Values are expressed as Mean ± SD (n=3)

Table 3: Effect of Various Formulation Techniques on the Particulate Characters of Oleuropein loaded NLC (Contd….)

Formulation Technique	EE (%)	ZP (mv)	
	OLE NLC	Blank NLC	OLE NLC
ME-US	73.4 ± 2.96	-40.0 ± 2.0	-42.0 ± 3.0
US	69.8 ± 3.12	-35.0 ± 3.0	-39.0 ± 2.0
SD	67.2 ± 2.56	-39.0 ± 2.0	-42.0 ± 2.0

ME-US: Micro emulsification – Ultrasonication; US: Ultrasonication; SD: Solvent Diffusion; OLE: Oleuropein, Values are expressed as Mean ± SD (n=3)

Effect of drug concentration on DL and EE%

The impact of drug concentration of DL and EE% was investigated and summarized in table 2. It was evident that drug concentration shows linear response on the EE%. Increase in the ratio of the drug from 0.5:4 to 1.5:4, the EE% increases to 79.26±2.8%. Further increase in the concentration of the drug, delivers EE which is attribute due to limited space for incorporation of the drug. Drug loading shows an increment with an increase in the concentration of drug from 0.5:4 to 1.5: 4 and a further increase in concentration to 2:4 ratios will revert the DL%. This correlation is sustained due to the saturation solubility of the drug in the lipid matrix and less concentration of the free drug.

Selection of Surfactant for the formulation of NLC

Selection of appropriate surfactant was significant to achieve desired particle size and stability. In general, a high concentration of surfactant favoured low PS, Narrow PDI and better stability and ended up with high toxicological potential. Hence an optimal concentration is needed to design NLC. Particulate characters were depicted in the mentioned Table 1&2. Lipid Nanoparticle, stabilized with Poloxamer 188 had a mean particulate diameter of about 190nm, Whereas NLC stabilized with Plantacare 810, exhibits a particle size of around 221nm, Cremophore RH60 shows PS of about 218nm. PI values less than 0.3 indicates narrow PSD. Hence, NLC stabilized by Poloxamer 188, and Plantacare shows highest drug encapsulation efficiency. The particle size of NLC may depend on various factors such as chemical structural, surfactants and chemical interaction. Hence, a combination of surfactant was ascertained in the formulation of NLC to prevent particle aggregation and to reduce or stops polymorphic transaction; thereby it improves stability of NLC. Because of its less toxicity and high capacity of penetration across BBB, Poloxamer 188 and Tween 80 combination were chosen for the study. Table 1&2 represent that all the system shows a particle size distribution in nm. The mean particle size for the formulation prepared by the surfactant alone was higher, and the mean particle size for the various surfactant combinations exhibit smaller particle size. The PDI also exhibit the same effect, i.e., surfactant alone shows higher PDI values whereas the combination shows narrow size distribution. Taking the particulate character represent in table 1&2 into consideration, a different combination of surfactant shows different emulsification efficiency, which is related to the particle size of the system. The combination of lecithin with polyhydroxy surfactant results in the formation of closely packed mixed films; thereby it reduces the particle size and PDI of the NLC. Hence, the combination of surfactant with stabilizer is a preferable choice for the NLC to obtain better stability. The mean diameter of all anionic NLC prepared with various combination of surfactants are in the range of 103.47 ± 2.32 to 211.52 ± 1.64 nm and PDI in varied from 0.26 to 0.432 (Table 2). ANOVA showed that combination off surfactant and type of surfactant had a significant effect on the particle size ($p \leq 0.0001$); lecithin: Poloxamer 188, Lecithin: Capryol Showed significant decrease in PS compared to others. These observations are due to the fact that lecithin favours a high oil/water interface, leads to the formation of smaller PS of NLC. The negative charged phospholipids molecule offers a –ve charge on the surface of the NLC, thereby it offers –ve zeta potential (-41.2 ± 2.10 mV) which indicate high stability of NLC. Increase in the drug concentration, increases in the particle size, which is due to agglomeration of particles and also high entrapment efficiency had a direct influence on the increase in PS.

The zeta potential for samples formulated with Lecithin: Capryol was higher than that of samples made with Tween 80, Poloxamer 188 and span 80 combinations. The Zp for plurol stearique: Capryol also exhibits a close resemblance with Lecithin: Capryol formulations. In non-ionic surfactant, the molecules are complexed around the interface of nanoparticles, which forms complex micelles, and the interaction effects are much smaller than the emulsifier molecule results in decreased ZP. Lecithin, a charged ionic surfactant forms a stable polymeric layer on the surface of the particle, which improves the electrostatic repulsion and promotes complete steric stabilization. Hence, it is very necessary to add an ionic surfactant (lecithin) in combination with a polyhydroxy surfactant to acquire the NLC of excellent stability

Influence of PL/Lipid matrix (w/w) ratio

Phospholipids forms shell around the nanostructures lipid carrier, which is a pre-requisite for the stability of NLC. From the data in table 2, it was evident that the increase in Phospholipid concentration increases the PS, which is due to surface active effect and reduction in surface tension. Increase in PL enhances the EE% of drug which is due to an uptrend in the miscibility of the drug in the lipid matrix. Taking account of stability, PS and EE% of NLC, the ratio of PL: Lipid – 3:1 was chosen for subsequent studies.

Influence of Formulation Techniques on NLC

Particulate characters were estimated for blank and drug-loaded NLC by using various methods, and the results are shown in Table 3. Form the

results it was observed that Melt Emulsification – Ultra-sonication (ME-US) method exhibits minimal particle size (144.2 nm for blank NLC and 153.42 nm for drug-loaded NLC) with narrow uniform size distribution with good zeta potential (-40.0 mV for blank NLC and -42.0 mV for drug-loaded NLC). From this data, ME-US method was chosen as the best method for formulation of NLC.

Pre-Formulation Studies

FT-IR: The IR spectrum for Oleuropein and Oleuropein NLC was shown in Fig. 1, which screens out the presence of aromatic OH group at 3208cm -1 and 2952cm -1 respectively in the spectrum, which confirm the presence of phenolic OH group in the extracts. The wavenumber at 1653.1cm -1 indicates the presence of alkenes and 1407.1cm -1 indicates the presence of aromatic compounds with conjugated diene. No shift in the wavenumber or there is no formation of new peaks or disappearance of new peaks was observed. Hence, the drug and additives were found to be compatible.

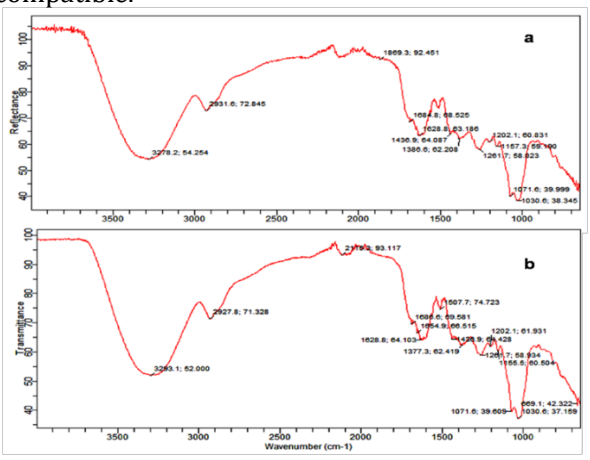

Figure 1: a) FT-IR Spectrum of Oleuropein Extract and (b) FT-IR spectrum of Oleuropein Loaded NLC Extract.

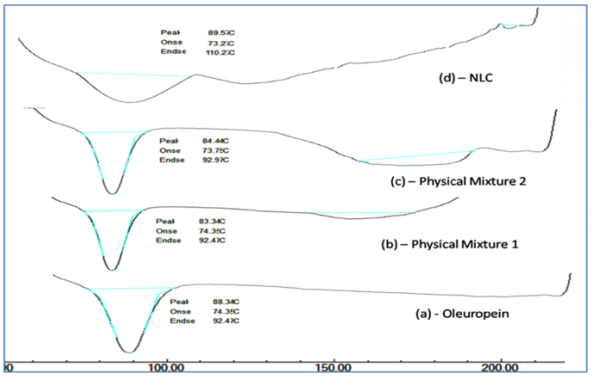

Figure 2: Comparative DSC Thermograms of a) Oleuropein Extract (b) Physical Mixture – 1(Oleuropein: Tefose, Capmul, in a ratio of 10:5:5) (c) Physical Mixture -2 (Oleuropein: Tefose, Capmul, Poloxamer 188, Tween 80 in a ratio of 10:5:5:5:5); (d) Thermal peak of Oleuropein loaded NLC

Thermal Analysis

DSC is a useful tool for the estimation of the degree of Crystallinity, Polymorphism and incompatibility between drug and lipid. DSC thermograms of drug, lipids, Physical mixture and drug-loaded NLC formulation are presented in fig 2. Oleuropein drug shows a sharp melting endothermic peak at 88.3°C. The lipids show a slight pre-shift in the peak with a melting temperature ranging from 83 to 84°C. The DSC parameters like Melting temperature, onset temperature and Crystallinity Index (CI %) are given in Fig. 2. Thermal analysis of Oleuropein loaded NLC was done after 7 days of production.

No significant shift in the peak was observed in the physical mixture which indicates good compatibility of drug and lipids. From the thermograms, it was found that no melting peak of oleuropein was detected for drug-loaded NLC which indicates that drug was completely soluble in the lipid matrix of NLC. It was attributed due to the change in the crystal lattice of lipid during the formulation of NLC, and also small particle size and large surface area of NLC leads to a decrease in crystallization point. The CI % of drug and bulk lipid NLC was found to be 100% and 15% indicating the complete crystallization of lipid after production. According to Siekmann and Westesen (1994), the decrease in the melting point of NLC is due to their large surface to volume ratio and rapid crystallization of lipid matrices.

Creaming stability

Creaming stability of the formulation observed after 28 days of the storage at 25°C. All the samples are homogenous, and there are no signs of phase separation upto 21 days, and after that, NLCs solidified which results in the formation of a thin cream layer on the top. Formation of thin creamy layer is attributed due to the polymeric transition of solid lipid, the increased surface to total volume ratio, insufficient surfactant and hydrophobic interactions between lipid particles. The CSI was found to be 97.84 ± 0.21% after 28 days of storage at 25°C, whereas CSI was 99.89 ± 0.13% for the sample stirred at 5°C

Gelation phenomena

Stress-induced gelation will be a possible reason for concern in lipid Nanocarriers. Morphological changes can occur during formulation, storage of administration (parenteral administration) poses a formulation related to physical instability. However, gelation was fetal to the patient because it will block veins or may lead to other complications. After multiple syringing, the Particle size of the NLC was found to be 156.22 ± 3.1 nm. Hence, no significant change was observed

in NLC even when it was exposed to the stress through multiple syringing.

Effect of sterilization

The developed NLC suspension was subjected to the standard autoclave cycle. Physical observation after autoclaving showed slight changes in turbidity. However, there was a complete absence of lumps or aggregates even under a microscope. The particle size of the post-autoclaved NLC was found to be 165 ± 3.6 nm, which was slightly higher than the optimized size of pre-autoclaved NLC (156.22 ± 3.1 nm). Hence, no significant difference was observed, and NLC was suitably autoclaved for terminal sterilization without any loss of stability and integrity.

CONCLUSION

NLCs are promising nanoparticles with a variety of therapeutic applications. However, the quality of a good NLC formulation depends upon the précised selection of the material and process attributes. The present study defines a stepwise protocol for selection of excipients for NLCs by employing simple experiments. This protocol can be utilized in a logical selection of material and process attributes to achieve a good quality formulation with the defined objectives. Furthermore, the developed formulation was found to be invulnerable against the common quality problems of lipid formulations.

Acknowledgement

The Principal author acknowledges Department of Science and Technology, New Delhi for the financial support of this project under DST women's scientist scheme (DST Ref No: SR/WOS A/LS 1262/2015(G) Dated 30.05.2016). We are also thankful to Abitec Corp. (USA), BASF (India) and Gattefossé (Saint-Priest, France) for providing the generous gift samples. Authors are also highly thankful to Dr R. Venkataswamy, Chairman and Mr R. V. Srinivas, Vice Chairman, Sri Venkateswara College of Pharmacy, RVS Nagar, Chittoor, Andhra Pradesh for pursuing and successful completion of this research work.

REFERENCES

Ana Beloqui, Maria Angeles Solinis, Alicia Rodriguez, Antonio, Veronique 2016. Nanostructured Lipid Carriers, Promising drug delivery systems for future clinics, *Nanomedicine*, 12, 143-161.

Anna Fabregas, Noemi Sanchez-Hernandez, Josep Ramon Tico, Encarna garccia Montoya, pilar Perez Lozano, Josep M. Sune Negre., 2014. A new optimized formulation of cationic solid lipid nanoparticles intended for gene delivery: Development, Characterization and DNA binding efficiency of TCERG 1 Expression plasmid. *International Journal of Pharmaceutics*. 473, 270-279.

Beloqui, MA, Solinics, A., Delgado, C., Evora, Adel Pozo Roodruguez., 2013. Bio-distribution of Nanostructured Lipid Carriers after intravenous administration to rats: Influence of technological factors. *Eur J Pharm Biopharm*, 84, 309-314.

Blanka Soto, Sabrina Weber, Andreas Zimmer, Gabriella Farkas, Andras Kelemen, Maria Budai, Szilvia Berko, 2015. Optimization and design of ibuprofen-loaded Nanostructured Lipid Carrier with a 2^3 full factorial design. *Chemical Engineering Research and design,* 104, 488-496.

Fanfei Meng, Sajid Asghar, Yurui Wang, Xin Jin, Zhilin Wang, Jin Wang., 2016. Design and evaluation of lipoprotein resembling Curcumin encapsulated protein free nanostructured lipid carrier for brain targeting, *Int J Pharm*, 506, 46-56.

Fei Han, Sanming Li, Ran Yin, Hongzhuo Liu, Lu Xu., 2008. Effect of surfactants on the formulation and characterization of a new type of colloidal drug delivery system Nanostructured Lipid Carriers. *Colloids and Surfaces A: Physicochem. Eng. Aspects*, 315, 210-216.

Hailong Yu., Peipei Liu., Hui Tang., Jian Jing Xiang., Lanlan Chen., 2016. Oleuropein, a natural extract from plants, offers neuroprotection in focal cerebral ischemia/reperfusion injury in mice. *Eur J Pharmacol,* 775,113-119.

Harshad Vaghasiya, Abhinesh Kumar, Krutika Sawant., 2013. Development of solid lipid nanoparticles based controlled release system for topical delivery of terbinafine hydrochloride. *European Journal of Pharmaceutical Sciences*. (In-Press)

Hassan Hajj Ali, Florentin Michaux, Aya n. Khanji, Jordane Jasniewski, Michel Linder., 2018. Chitosan Shea butter solid lipid nanoparticles assemblies for the preparation of novel nanoparticles in microparticles system containing Curcumin. *Colloids and Surfaces A: Physicochem Engineering Aspects*. (In-Press)

Intakhab Alam., Sunjula Baboota., Alka Ahuja., Mushir Ali., Javed Ali., 2012. Intranasal administration of nanostructured lipid carriers containing CNS acting drug: Pharmacodynamic studies and estimation in blood and brain, *Journal of Psychiatric Research*, 46, 1133-1138.

Jafar Ezzati Nazhad Dolatabadi, Aida Azami, Ali Mohammadi, Hamed Hamishehkar, Vahid Panahi-Azar, Yalda Rahbar Saadat, Amir Ata Saei., 2018. Formulation, Characterization and cytotoxicity evaluation of Ketotifen loaded Nanostructured

lipid carriers. *Journal of Drug Delivery Science and Technology*, 46, 268-273.

Jakarwan Yostawonkul, Suvimol Surassmo, Tawin lempridee, Wittaya Pimtongo, Kunat Suktham, Warayuth Sajomasang, Parrarapond Gonil., 2017. Surface modification of nanostructured lipid carriers by oleoyl quaternized chitosan as a mucoadhesive nanocarrier. *Colloids and Surfaces B: Biointerfaces*, 149, 301-311.

Kovacevic, A, Savic., S, Vuleta., G, Muller., RH, Keck., CM., 2011.Polyhydroxy surfactants for the formulation of lipid nanoparticles: Effect on Size, Physical stability and particle matrix structure. *Int J Pharm.* 406, 163-172.

Noha Nafee, Shaimaa Makled, Nabila Boraie., 2018. Nanostructured lipid carriers versus solid lipid nanoparticles for the potential treatment of pulmonary hypertension via Nebulization. *European Journal of Pharmaceutical Sciences*, 125, 151-162.

Oihane Gartziandia, Enara Herran, Jose Luis Pedraz, Eva Carro, Manoli Igartua, Rosa Maria Hernandez., 2015. Chitosan-coated nanostructured lipid carriers for brain delivery of proteins by intranasal administration. *Colloids and Surfaces B: Biointerfaces,* 134, 304-313.

Paolo Blasi, Aurellie Schoubben, Giovanna Valentina Romano, Stefano Giovagnoli, Alessandro Di Michele, 2013., Lipid Nanoparticles for brain targeting. Technological Characterization. *Colloids and Surfaces B: Biointerfaces*, 110, 130-137

Pardeike, J, Weber, S, Haber, T, Wagner, J, Zarf., HP, Plank., H, Zimmer., A., 2011. Development of Itraconazole Loaded Nanostructured Lipid Carrier formulation for pulmonary application. *Int J Pharm,* 419, 329-338.

Pimchanok Witayaudom, Utai Klinkesorn., 2017. Effect of surfactant concentration and solidification temperature on the characteristics and stability of nanostructured lipid carriers (NLC) prepared form rambutan kernel fat. *Journal of Colloid and Interface Science*, 505, 1082-1092.

Ram R Patlolla, Mahavir Chogule, Apurva R Patel, Tanise Jackson, Prasad N.V. Tata, Mandip singh, 2010. Formulation characterization and pulmonary deposition of nebulized Celecoxib encapsulated nanostructured lipid carriers. *J Control Release*, 144, 2333-241.

Slavomira Doktorovova, Eliana Souto, Amelia Silva, 2014. Nanotoxicology applied to solid lipid nanoparticles and nanostructured lipid carriers- A systematic review of *in-vitro* data. *Eur J Pharm Biopharm*. 87, 1-18.

Somasree Ray, Pratyusha Sinai, Bibek Laha, Sabyasachi Maiti, Uttam Kumar Bhattacharyya, Amit Kumar Nayak., 2018. Polysorbate 80 coated cross-linked chitosan nanoparticles of ropinirole hydrochloride for brain targeting. *Journal of Drug Delivery Science and Technology*. 48, 21-29.

Tejas B. Devakar, Avinash R. Tekade, Krishanchandra R. Khandelwal., 2014. Surface engineered nanostructured lipid carriers for the efficient nose to brain delivery of ondansetron HCl using Delonix regia gum as natural mucoadhesive polymer. *Colloids and surfaces B: Biointerfaces.* 122, 143-150.

Varsha B. Pokharkar, Mallika R Jolly, Dipak D. Kumbhar, 2015. Engineering of a hybrid polymer-lipid nanocarrier for the nasal delivery of Tenofovir disoproxil fumarate: Physicochemical, Molecular, Microstructural and stability evaluation. *European Journal of Pharmaceutical Sciences*, 71, 99-111.

Wang, T., Bae, M., Bae, M., Lee, Luo Y., 2018. Solid Lipid polymer hybrid nanoparticles prepared with natural biomaterials: A new platform for oral delivery of lipophilic bioactive, *Food Hydrocolloids*, 19(10):3112

Wei Huang, Huating Dou, Houjiu wu, Zhigao Sun, Hua Wang and Linhua Huang., 2017. Preparation and Characterization of Nobiletin loaded Nanostructured Lipid Carriers. *Journal of Nanomaterials*, 10: 1-10.

Xiaoyun Zhang, Jianping Liu, Hua Liu, Jingman Ni, Wenli Zhang, Yanbin Shi, 2010. Formulation optimization of dihydroartemisinin nanostructured lipid carrier using response surface methodology. *Powder Technology*, 197, 120-128.

Yasmine M Gaba., Amani O Kamel., Omaima A Sammour., Ahmed H Elshafeey., 2014. Effect of Surface charge on the brain delivery of nanostructured lipid carrier's in situ gels via nasal route. *Int J Pharm*. 473, 420-457.