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Research Article

DEVELOPMENT AND OPTIMIZATION OF EUGENOL LOADED NANOSTRUCTURED LIPID CARRIERS FOR PERIODONTAL DELIVERY

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ABSTRACT

In the present study, gel formulation of eugenol loaded nanostructured lipid carriers was developed for periodontal delivery such that it would effectively deliver drug in a sustained manner. A systematic study was performed for obtaining the nanostructured lipid carriers by using 3^2 factorial design approach with percentage of lipid and surfactant as independent variables at three different levels. Hot homogenization method was adopted for preparation of nanostructured lipid carrier dispersion. Nanostructured lipid carrier dispersion was characterized for particle size and entrapment efficiency. Results of regression analysis revealed that entrapment efficiency was dependent on lipid concentration. Optimized nanostructured lipid carrier dispersion was formulated into gel using Pluronic F127 and characterized for rheological behaviour and in vitro release. In vitro release from the gel showed its sustained release potential as compared to plain drug loaded gel. Stability study of nanostructured lipid carrier dispersion and its gel formulation as per ICH guidelines for six months revealed its stability over the entire period. The factorial design was found to be well suited to identify the key variables affecting the entrapment efficiency.

Keywords: Eugenol, Nanostructured lipid carrier (NLC), Factorial design, Entrapment efficiency, Rheology

INTRODUCTION

Nanoparticles made from solid lipids, the so-called solid lipid nanoparticles (SLN) were attractive as an interesting parenteral carrier system in the middle of 1990s[1,2]. SLN combines the advantages of polymeric nanoparticles such as controlled drug release and avoiding drug leakage with the advantages of emulsion and liposomes like low toxicity, good biocompatibility and higher bioavailability[2,3,4]. However, there were some problems associated with SLN such as limitation in drug loading capacity, drug expulsion during storage and high water content (70–95%) of aqueous SLN dispersions[5]. To overcome these problems, nanostructured lipid carriers (NLC) are developed in recent years. Conventionally, NLC are produced by controlled mixing of solid lipids with spatially incompatible liquid lipids. This leads to special nanostructures with improved properties for drug loading, modulation of the drug release profile and stability[5,6]. Depending on the productive method and the composition of lipid blend, different types of NLC can be obtained, i.e., imperfect, amorphous and multiple components[5,6,7]. If the content of liquid lipid is higher than the solubility of liquid lipid in the solid lipid, phase separation can occur to form nanocompartments. For number of drugs, the solubility in liquid lipid is higher than that of in solid lipid. Consequently, the highest drug loading can be achieved. Thus, a judicious selection of the type and amount of solid and liquid lipids is essential to obtain optimum loading.

Eugenol is a lipophillic drug used to cure 'dentinal hypersensitivity', a condition characterized by occurrence of pain or discomfort when mild stimulus is applied to exposed dentin. Various approaches have been tried till date for preparing compositions containing eugenol. Jadhav et al. have reported the development and evaluation of controlled-release mucoadhesive tablets containing eugenol for gingival application prepared by using carbopol 934P and hydroxypropylmethylcellulose K4M[8]. Markowitz et al. disclosed dental composition comprising of eugenol and an effective amount of anionic liposomes[9].

The diameter of the dentinal tubule in hypersensitive condition lies between 0.4 to 0.8 µ, thus presenting a physiological limitation for the drug delivery[10]. Hence, in order to penetrate and retain in dentinal tubules, the carrier particle size diameter should not be greater than 0.8 µ. Therefore, in the present study, an approach to use nanosized carriers such as NLC for the periodontal delivery of eugenol was attempted. The main factors influencing drug loading and subsequent release are the amount of lipids and surfactants used for preparing NLC. Hence, a systematic statistical, 3^2 factorial design was used to evaluate effects of different factors in the preparation of NLC dispersion. NLC were prepared by high pressure homogenization method. Drug loaded NLC dispersion was characterized for particle size and entrapment efficiency. Further NLC based gel containing optimized NLC dispersion was formulated using Pluronic F127 (PF 127). NLC based gels were characterized for rheological behaviour, gelation temperature (T_{gel}) and in vitro release. PF 127 represents both thermosensitive and mucoadhesive properties which makes it very interesting for formulation of gels[11,12]. Stability of NLC dispersion and NLC based gel was also studied at 40^0C and 75% relative humidity.

MATERIALS AND METHODS

Materials

Eugenol (99% extra pure) and stearic acid were purchased from Loba Chemei Pvt. Ltd. (Mumbai). Oleic acid was purchased from Merck Chemicals, India Pvt. Ltd. (Mumbai). Pluronic F68 (8400 g/mol) and Pluronic F127 (12600 g/mol) were a gift from BASF Corporation (Florham Park, New Jersey). Cremophore RH 40, tween 20 were purchased from Himedia Pvt. Ltd. (Mumbai). Sephadex® G25M was purchased from Amersham Biosciences (Uppsala, Sweden). All other chemicals used were of HPLC and analytical grade.

Preparation of NLC

NLC was prepared by hot homogenization method. Lipid phase consisted of a mixture of stearic acid (SA) (solid lipid) and oleic acid (OA) (liquid lipid) in the ratio of 7:3. The surfactant phase consisted of mixture of cremophore RH 40 (RH 40), Pluronic F68 (PF 68) and tween 20 (T 20) in the ratio of 5:3:2 respectively. Lipid phase containing solid lipid and liquid lipid were melted at 70°C, to which eugenol was added. Melted lipid phase was dispersed in hot surfactant mixture under mechanical stirring at 600 rpm for 30 min to obtain the pre-emulsion. This hot pre-emulsion was further processed using high pressure homogenizer (Niro Soavi, Italy) by applying 5 homogenization cycles at 500 bar pressure. The obtained lipid dispersion was cooled at ambient conditions of room temperature and solidified to obtain the aqueous NLC dispersion.

Effect of variables

To study the effect of variables on NLC characteristics, different batches were prepared using 3^2 factorial design (Table 1). Percentage of lipid and surfactant were selected as two independent variables whereas particle size and encapsulation efficiency were the dependent variables. Interindividual ratio of SA to OA and RH 40, F 68 and T 20 was kept constant.

Table 1: Experimental design with coded levels of variables and actual values

Batch Code	Variable X1 Percent of lipid	Variable X2 Percent of Surfactant
NLC-1	3 (-1)	1.5 (-1)
NLC-2	3 (-1)	2 (0)
NLC-3	3 (-1)	2.5 (+1)
NLC-4	4 (0)	1.5 (-1)
NLC-5	4 (0)	2 (0)
NLC-6	4 (0)	2.5 (+1)
NLC-7	5 (+1)	1.5 (-1)
NLC-8	5 (+1)	2 (0)
NLC-9	5 (+1)	2.5 (+1)

Values in parenthesis indicates coded levels

Preparation of NLC based gel

On the basis of factorial design approach, the optimized NLC dispersion was selected for formulating gel. The thermo reversibility of different concentrations (15%, 16% and 17%) of blank PF 127 gel and NLC based gel was characterized. Based on sol-gel phase transition measurements, 16% concentration was selected for further studies. PF 127 was used for gelling NLC dispersion on weight basis using cold method[13,14]. Selected amount of PF 127 (16%) was slowly added to cold NLC dispersion (5˚C) under constant stirring. The prepared dispersion was then refrigerated for five hours to obtain a homogenous dispersion.

Measurement of Gelation temperature

The prepared gels were evaluated for gelation temperature (T_{gel}) as described by Gilbert et al.[11]. T_{gel} was measured by heating the formulation (1-2°C/min) in a 15 ml borosilicate glass test tube. In each test tube 2 ml of formulation solution was placed and heated with gentle stirring until formulation solution gets gelled. Gel formation was considered as the point where there was no flow when the test tubes were gently inverted. All the measurements were performed in triplicate.

Characterization

Particle size distribution of NLC

NLC dispersions were characterized for average particle size (z-average size) and polydispersity index using laser diffractometer (Malvern Mastersizer 2000SM version 5.22, Malvern Instruments Corp., U.K). The particle size diameters were determined (in triplicate) at 90th, 50th and 10th percentile of particles undersized.

Entrapment efficiency (EE) of drug loaded NLC

Entrapment efficiency was determined by using minicolumn centrifugation method on a Sephadex® G25M column[15]. 100 µL of NLC dispersion was loaded onto the gel column and eluted continuously with double distilled water. The eluants were collected in numbered tubes to separate NLC entrapped drug. These collected NLC were ruptured using sufficient volume of methanol and percent encapsulation was calculated from total amount of eugenol present in 100µL of NLC by HPTLC analysis using following equation 1.

$$EE = (Q_e / Q_t) \times 100 \quad \text{----------1}$$

where Q_e -Amount of encapsulated drug present in elute and Q_t - Total amount of drug added.

This method was validated by applying free drug solution instead of NLC.

High performance thin layer chromatography (HPTLC) analysis of eugenol

Estimation of eugenol concentration was carried out using HPTLC method. In a typical HPTLC analysis, the samples were spotted in the form of bands with Camag 100 µl sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium plate 60 F-254 (20 cm×10 cm) (E. Merck, Darmstadt, Germany) using Camag Linomat IV Automatic Sample Spotter (Switzerland). Linear ascending development was performed in a 20 cm x 10 cm twin trough glass chamber (Dimensions: length × width × height = 12 cm×4.7 cm×12.5 cm; Camag, Switzerland). Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 280 nm for all measurements and operated by CATS software (Camag V 3.15). The mobile phase consisted of petroleum ether: dichloromethane: formic acid (4:3:0.1v/v/v). Concentrations of the compound subjected to chromatography were determined from the intensity of diffusely reflected light. Evaluation was carried out using peak areas with linear regression. Method was validated for a range of 2-14 µl eugenol in pH 6.8 phosphate buffer (r^2 = 0.9994).

Particle size distribution of NLC based gel

Particle size analysis of NLC based gel was carried out by laser diffraction (Malvern Mastersizer 2000SM ver. 5.22, Malvern Instruments Corp., U.K). Prior to analysis, about 1 g of the semisolid NLC based gel was dispersed in 100ml of double distilled water by shaking to obtain uniform dispersion. All measurements were performed in triplicate.

Drug content of NLC based gel

For determination of drug content, about 1 g of NLC based gel was weighed in 10 ml volumetric flask, dissolved in 5 ml of methanol and volume was made up to 10 ml with methanol. The samples were mixed thoroughly, filtered using 0.45 µ membrane filter and analyzed using HPTLC at 280 nm.

Rheological Characterization of NLC based gel

Rheological analysis of NLC based gel was performed using a stress control Viscotech rheometer (Rheologica Instruments AB, Lund, Sweden), equipped with Stress Rheologic Basic Software, version 5, using cone-plate geometry with the diameter of the cone being 25 mm and a cone angle of 1°, operating in the oscillation and static mode. The gap was maintained at 0.5 mm. All measurements were carried out at a temperature of 37 ± 0.1°C. Oscillation stress sweep tests were carried out at a constant frequency of 1 Hz in a stress range of 1–500 Pa. Oscillation frequency sweep tests were performed over a frequency range from 1 to 100 Hz at a constant stress of 20 Pa.

In vitro drug release studies of NLC based gel

Dissolution study was performed by dialysis method. Typically, 2 g of plain drug loaded gel and NLC based gel were placed in two different dialysis tubes (Molecular weight cut off 12000 g/mol). These dialysis tubes were then placed in a vessel containing 100 ml of pH 6.8 phosphate buffer, maintained at a temperature of 37 ± 0.5°C and stirred at 100 rpm. Samples were collected periodically and replaced with fresh dissolution medium. After filtration through Whatmann filter paper, the concentration of eugenol was determined using HPTLC analysis (by spotting 10 µL/ spot). Kinetic analysis of the release data was done using PCP-Disso v2.08 software (Poona College of Pharmacy, Pune, India).

Stability studies of NLC based gel

Stability of optimized NLC dispersion and NLC based gel formulation was studied at 40^0C temperature and 75% relative humidity for six months. Effects of temperature and relative humidity on particle size, in vitro release performance and drug content were studied for NLC dispersion and for NLC based gel during the stability period.

RESULTS AND DISCUSSION

The selection of lipids and surfactants and their proportions was done on the basis of pseudoternary phase diagram (data not shown). After this initial screening of different solid lipids and liquid lipids, stearic acid (SA) and oleic acid (OA) were chosen for the preparation of NLC. Also, it was found that among the various surfactants screened, cremophore RH 40 (RH 40), Pluronic F68 (PF 68) and tween 20 (T 20) were effective in size reduction and stabilization.

Preparation of NLC dispersion

Lipids SA and OA in 7:3 ratio were found effective and hence, selected for the preparation of NLC. Use of any single surfactant could not produce stable nanosized NLC. A single surfactant would either produce a gel (RH 40) or larger particle size (T 20). Therefore, different surfactant combinations were screened. On cooling up to temperature below melting point of the lipid, nanoemulsion drops changed to solid particle following prodigious change in surface state. Thus, mixture containing RH 40 and T 20 was found coagulated under storage for short period of time and then resulted in gelation. Extra emulsifier was needed for the surface of newly formed particle hence could not cover the naked new surface immediately leading to gelation during cooling[16,17]. Therefore, surfactant mixture containing three different surfactants was tried. The amount of surfactants and lipids were found to be more prominent factor in the preparation of NLC. Of the various ratios screened, the system containing mixture of surfactants i.e. RH 40, PF 68 and T 20 in the ratio of 5:3:2 resulted in small uniform NLC. In order to predict the more clear effect of these two factors on entrapment efficiency, a 3^2 factorial design was adopted. Percent of lipid and surfactant were found to be critical parameters in the preparation and stabilization of NLC and hence selected as independent variables in the 3^2 factorial design.

Gelation temperature (T_{gel})

T_{gel} is the temperature at which the liquid phase makes a transition to gel phase. Suitable T_{gel} range for periodontal gel formulation would be 34 - 37^0C. If T_{gel} is lower than 34^0C, gelation occurs at room temperature leading to difficulty in manufacturing, handling and administering the product. If it is higher than 37^0C, the formulation will remain as a liquid at body temperature, resulting in drainage and washout from the periodontal cavity. Therefore, the NLC formulation must have a suitable T_{gel}, between 34-37^0C, so as to maintain sol phase at room temperature (25^0C) and transform into a gel phase instantly upon administration inside the periodontal pocket.

T_{gel} of NLC based gels containing 15%, 16% and 17% of PF 127 were found to be 38, 36 and 32°C respectively. It was observed that T_{gel} decreased as the concentration of PF 127 increases. This may be due to increased viscosity after dissolution of polymers in aqueous media. Water is a good solvent for PEO as well as PPO chains of pluronic. At lower temperatures, hydrophilic chains of co-polymer become desolvated due to breakage of hydrogen bonding between the solvent and these chains. This phenomenon favours hydrophobic interactions among the polyoxypropylene domains and thus leads to gel formation at lower temperature[18]. As the NLC based gel containing 16% PF 127 exhibited optimum T_{gel} close to body temperature, it was selected for further study.

Statistical Data

Different batches were prepared as per the factorial design and evaluated for particle size and EE (Table 2).

Table 2: Summary of results obtained for all experimental batches

Batch code	Particle size- d 0.9 (nm) ±S.D	Polydispersity index ±S.D	Entrapment efficiency (%) ±S.D
NLC-1	206±2	0.620±0.002	71.53±0.403
NLC-2	201±2.52	0.611±0.001	72.65±0.414
NLC-3	197±2.51	0.607±0.002	73.81±0.668
NLC-4	208±2.08	0.614±0.001	89.21±0.450
NLC-5	206±2.08	0.619±0.002	91.710±0.410
NLC-6	202±2.5	0.611±0.003	93.43±510
NLC-7	228±2.51	0.621±0.001	94.38±0.645
NLC-8	210±2	0.617±0.002	97.430±0.564
NLC-9	209±2	0.620±0.002	96.39±0.456

Obtained data was subjected to multiple regression analysis using Unistat software (Megalon, USA) and fitted in following equation 2:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1 X_1 + \beta_{22} X_2 X_2 + \beta_{12} X_1 X_2 \quad (2)$$

Response surface plots was generated using Factorial-3^2 module of PCP Disso V 3 software (IIPC, PCP, India).

Effect on particle size of NLC

The Z-average size (nm) which corresponds to hydrodynamic radius was determined using Malvern Mastersizer (Table 2). The average particle size of all the batches was estimated between 200 and 228 nm. Polydispersity index (PI) was also determined (Table 2) which is measure of particle homogeneity and it varies between 0 to1. Closer the value of PI to zero, higher is the uniformity between the particle's size. Thus, the particle size distribution was found to be unimodal with PI ranging from 0.607 to 0.621 for NLC batches. The concentration of lipid showed significant effect on particle size as shown in the response surface plot (Fig. 1). Effect of process variables was found insignificant in case of particle size reduction.

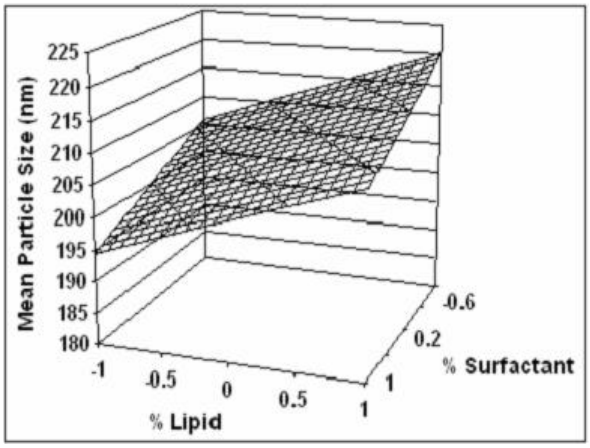

Fig. 1: Response surface plot for particle size

To understand the effect of lipid and surfactant concentration on particle size, coefficients observed were subjected to multiple regression analysis using Unistat software (Megalon, USA)

$$Y = 207.44 + 7.16X_1 - 5.66X_2 \quad (3)$$

The above equation 3 revealed that with increasing concentration of lipid, particle size also increased. This is in agreement with Shah et al. who reported that with increase in the lipid content, particle size increases, which could be attributed to the increased globule size of the dispersion[19]. Further, the particle size measurement also revealed that as the concentration of surfactant increased, particle size decreased. This may be due to better stabilization of internal structure of dispersion at higher surfactant concentration preventing coalescence. This was in agreement with the general literature.

Effect on entrapment efficiency (EE) of NLC

The most important parameter that needs to be monitored during preparation of NLC is its EE. It was determined by minicolumn centrifugation method. Concentration of lipids showed a significant effect on EE as observed from response surface plot (Fig. 2).

To understand the effect of lipid on EE, coefficients observed were subjected to multiple regression analysis using Unistat software (Megalon, USA)

$$Y = 91.45 + 11.83X_1 + 1.505X_2 - 7.213X_1X_2 \quad (4)$$

The effects were more clearly understood from the response surface plot, which showed that, EE was minimum at lower level of lipid. Maximum EE was seen at highest level of lipid and intermediate level of surfactant. This is in agreement with Qingzhi et al., who reported that the increase in lipid content can afford more space to encapsulate drug under the given surfactant concentration in outer phase[2].

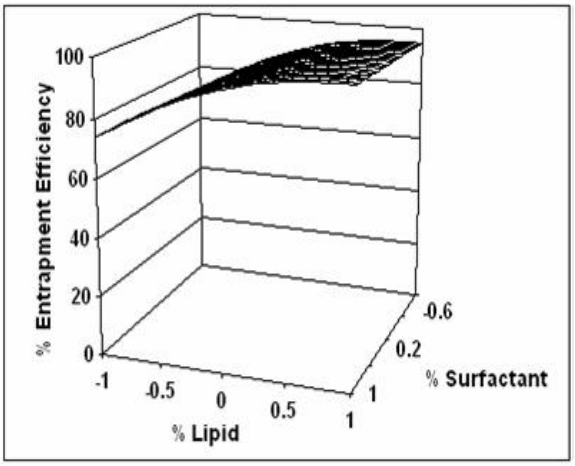

Fig. 2: Response surface plot for entrapment efficiency (EE)

Jenning et al.[21] and Souto et al.[22] reported that the incorporation of liquid lipids into solid lipids could lead to massive crystal order disturbance, and the resulting matrix of lipid particles would indicate great imperfections in the crystal lattice. Thus, enough space was left to accommodate drug molecules leading to improved drug loading capacity and entrapment efficiency. Also, eugenol being a lipophillic molecule, higher loading could be achieved at high lipid level. Further, a very small positive coefficient value for X_2 i.e. amount of surfactant indicated that as compared to amount of lipid, surfactant does not significantly alter the loading.

Particle size and drug content of NLC based gel

The NLC based gel exhibited particle size of 225 nm. The results obtained show that there was no significant change in particle size after gelling of NLC dispersion. Drug content of prepared NLC gel was found to be 99.54±0.283 % by HPTLC.

Rheological characterization of NLC based gel

In order to get comprehensive information on the rheological behaviour of the NLC based gel systems, oscillatory measurements yielding information about viscous and elastic properties of the investigated carrier were performed. An oscillation frequency sweep test is a dynamic test measuring the response of a system as a function of frequency at constant stress amplitude. It revealed the storage modulus G' (elastic response) which is a measure of energy stored and the loss modulus G'' (viscous response) which reflects the energy lost. If performed within the linear viscoelastic region (LVR), a frequency sweep provides a fingerprint of a viscoelastic system under non-destructive conditions. Thus the systems are examined in their rheological ground state without disrupting the structure like continuous shear techniques[23].

The response of the dynamic measurement of 16 % gel based NLC system is shown in Fig. 3. The 16 % gel based NLC system had shown weak dependency of both G' and G'' on the applied frequency which is a typical behaviour for a viscoelastic solid. The storage modulus is higher than the loss modulus over the whole frequency range, indicating the presence of a gel-like structure. The higher values of the storage modulus show that the investigated system is more elastic than viscous in the investigated frequency range. Thus, taking into consideration sol-gel transition temperature and rheological properties, 16% gel based NLC system was found to be stable and appropriate for periodontal application.

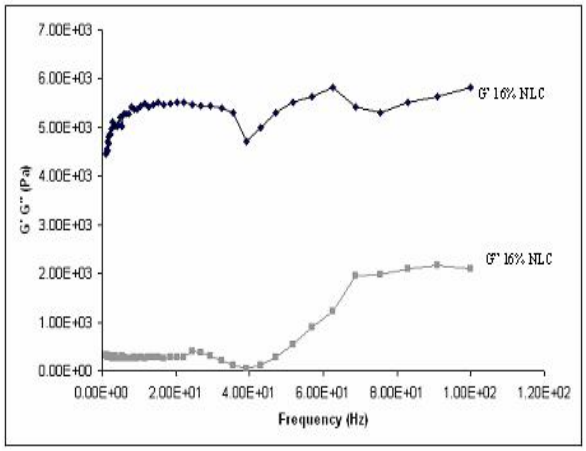

Fig. 3: Effect of Frequency (Hz) on elastic (G') and viscous (G") modulus

In vitro release of NLC based gel

Drug release studies is an important step during the development stages of new formulation and is a routine quality control test for assuring uniformity of finished product. The release profiles from the present study indicated that NLC based gel showed a biphasic pattern of drug release i.e. initial burst release (25%) in the first hour and thereafter a retarded release of the drug from the lipid matrix for more than 8 hours when compared with plain drug loaded gel (Fig. 4). Depending on the solubility of the respective drug, crystallization of the melt can result in monolithic solid solution or a solid dispersion containing the API in a homogenous distribution or forming clusters. Further, depending on the melting point of the agent and lipid, the drug could be associated with the nanoparticles in three different states: at the nanoparticles surface, in the core as a reversible complex, or in the core as irreversible complex[24].

Generally, drug release follows more than one type of mechanism. In case of release from the surface, drug adsorbed on the surface of nanoparticles dissolves instantaneously when it comes in contact with the release medium. The early phase of the release corresponds to the release of drugs physically bound to the surface of the nanoparticles and the delayed phase due to the release of entrapped drug due to diffusion of drug from the rigid matrix structure[25].

The prolonged release in second phase may also be attributed to higher viscosity of solid lipid thus slowing down the release. Another reason to this might be the higher concentration of liquid oil (OA and eugenol) in the system. After lipid crystallization, the solubility of oil in solid lipid exceeded. Hence, oil precipitates leading to formation of fine droplets of oil incorporated in solid lipid thereby providing prolonged release[4,26]. At the end of 8 hours about 65% release was obtained in case of gel based NLC, whereas the plain drug loaded gel had shown around 97% release in 2 hours. Burst release as well as sustained release, both is of interest for periodontal delivery. Hence from in vitro studies, we can predict that NLC based gel can retain in periodontal pocket for longer time providing initial fast release for quick action and then prolonged release of eugenol. These findings are in agreement with Joshi et al[14]. The drug release data from the NLC gel was fitted into different models. The value of r^2 was found to be highest for the Higuchi model (r^2 = 0.9819), indicating that the test product follows matrix diffusion based release kinetics.

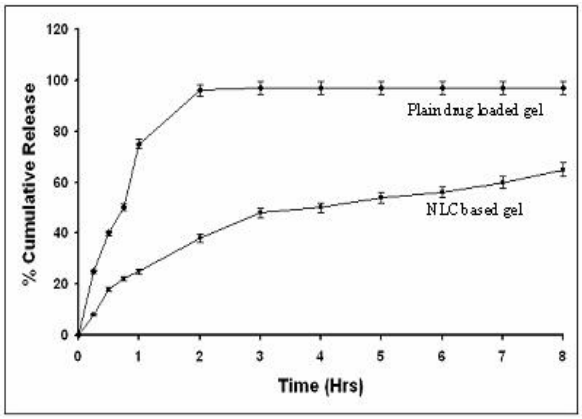

Fig. 4: In vitro release profile of NLC based gel and plain drug loaded gel

Stability study of NLC dispersion and NLC based gel

Stability of NLC dispersion as well as NLC based gel was carried out as per ICH guidelines for six months. Insignificant effect was observed on particle size, drug content and in vitro performance throughout the stability period for NLC dispersion and NLC based gel (data not shown).

CONCLUSION

Eugenol loaded NLC were prepared by hot homogenization method. Factorial design was found to be well-suited and sound approach to obtain a stable NLC formulation. As particle size of NLC was found to be less than 400 nm, it can retain in periodontal cavity for longer duration. Amount of lipid was found to have profound effect on entrapment efficiency. High entrapment efficiency was attributed to the imperfections in the crystal lattice, leading to increased drug loading. NLC based gel exhibited initial faster onset of release followed by sustained release as evident from comparative study with plain drug loaded gel. Thus the developed NLC based gel formulation can be effective for the periodontal delivery providing initial fast release for quick action with subsequent prolonged release of eugenol. NLC dispersion and NLC based gel were found to be stable for six months as per ICH guidelines.

The developed technology platform can be extended for many other potential actives required to elicit prolonged action. It is further suggested that by changing the ratio of solid lipid and liquid lipid, release profile can be modified.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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