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FORMULATION, CHARACTERIZATION AND *IN VITRO* EVALUATION OF NOVEL THIENOPYRIMIDINES AND TRIAZOLOTHIENOPYRIMIDINES LOADED SOLID LIPID NANOPARTICLES

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ABSTRACT
Thienopyrimidines and triazolothienopyrimidines loaded solid lipid nanoparticles (SLNs) were produced by microemulsion method. All the formulations were subjected to particle size analysis, zeta potential, compound entrapment efficiency and *in vitro* release studies. The SLNs formed were in nano-size range with maximum entrapment efficiency. Formulation with 195 nm in particle size and 84.20% of compound entrapment was subjected to scanning electron microscopy (SEM) for surface morphology, differential scanning calorimetry (DSC) for thermal analysis and short term stability studies. SEM confirms that the SLNs are circular in shape. The compound release behavior from SLN suspension exhibited biphasic pattern with an initial burst and prolonged release over 24 h.

KEY WORDS: Thienopyrimidines; Triazolothienopyrimidines; Solid lipid nanoparticles (SLNs); Particle size analysis; Entrapment efficiency; *In vitro* release study

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INTRODUCTION

Solid lipid nanoparticles (SLNs), introduced in 1991, to combine advantages and to avoid disadvantages of other colloidal carriers has attracted increased attention in recent years, and is regarded as an alternative carrier system to traditional colloidal systems, such as emulsions, liposomes and polymeric microparticles and nanoparticles[1-3]. Proposed advantages include, possibility of controlled compound release and compound targeting, increased compound stability, high compound payload, incorporation of lipophilic and hydrophilic compounds feasible, no biotoxicity of the carrier, avoidance of organic solvents, no problems with respect to large scale production and sterilization[4].

Many of pharmaceutical researchers have prepared SLNs as an alternative colloidal therapeutic systems, utilizing different approaches like modified high shear homogenization and ultrasound techniques[1], emulsification-diffusion method[5], solvent injection method[6], solvent diffusion method[7], microemulsion method[8] and hot homogenization technique[9].

SLN were prepared by the dispersion of warm oil-in-water (o/w) microemulsions in cold water; solid lipids with low melting points were used as internal phase of the microemulsions. Considering the droplet structure as the structural organization of o/w microemulsions, liquid oil nanodroplets are present in the warm o/w microemulsion; a rapid quenching of the warm o/w microemulsions in cold water permits the crystallisation of oil nanodroplets present in the microemulsion forming the solid nanospheres[10].

Thienopyrimidines and thiadiazolothienopyrimidines have been found to exhibit a variety of biological activities viz. anti inflammatory, antimicrobial, analgesic activities, inhibition of cancer cell proliferation, antagonism of α1 adrenoceptors and prevention of cartilage destruction in articular diseases. Consequently, thienopyrimidines have become a well sought-privileged class of compounds in drug discovery programs. We have reported the synthesis of some novel thienopyrimidines and thienotriazolopyrimidines for the evaluation of their antimicrobial properties[11]. In view of these reports and in continuation of our work on biologically active nitrogen and sulfur heterocycles, we now report herein the preparation, characterization and evaluation of thienopyrimidines and thienotriazolopyrimidines loaded solid lipid nanoparticles.

MATERIAL AND METHODS

Materials

Thienopyrimidines and thienotriazolopyrimidines derivative (Fig. 1) form our lab (Dr.IMK Group, Karnatak University, India), glyceryl monostearate (GMS) from Loba Chemie Pvt Ltd (Mumbai, India), tween 80 by Merck Ltd (Mumbai, India), egg lecithin from Himedia (Mumbai, India) and Millipore water by Millipore (India) Pvt. Ltd (Bangalore, India). Other chemicals are of analytical grades.

Preparation of SLN

Thienopyrimidines and thienotriazolopyrimidines derivative (compound) loaded SLNs were prepared from a warm o/w microemulsion technique[10]. Compound and GMS were melted at 70 ^{0}C. Warm acidic aqueous solution of egg lecithin was added to melted lipid-compound mixture (at 70 ^{0}C) in presence of co-surfactant, tween 80 under stirring. The warm microemulsion was then added carefully drop wise into ice cold water (2-3 ^{0}C) with continuous stirring (T25 basic Ultra Turrax, IKA, USA). The ratio between the microemulsion and the dispersion medium was about 1:10. The dispersion was subjected to ultra sonication for a period of 10 min to form nanosuspension (Table 1).

Measurement of size and zeta potential

Size and zeta potential of SLN were measured by Photon Correlation Spectroscopy (PCS) using zetasizer 3000 HSA (Malvern, U.K.). Samples were diluted appropriately with the aqueous phase of the formulation for the measurements and the pH of diluted samples ranged from 6.8 to 7.0.

Determination of entrapment efficiency (EE %)

The entrapment efficiency of the compound was determined by measuring the concentration of free compound in the dispersion medium[9]. The samples were centrifuged at 4000 rpm for 30 minutes. The amount of free compound was determined in the clear supernatant by UV spectrophotometer using supernatant of non-loaded nanoparticles as basic correction. The amount of incorporated compound was determined as a result of the initial compound minus the free compound. The entrapment efficiency could be calculated by the following equation[12].

$$EE\,(\%) = \left(\frac{W_{initial\ compound} - W_{free\ compound}}{W_{initial\ compound}} \right) \times 100$$

Scanning electron microscopy (SEM)

The morphological examination of SLN was performed by Scanning electron microscopy (Joel JSM 840A, Japan). Cleaned brass specimen studs were used for mounting the samples. Wet solvent paint was applied on these studs and while the paint was wet, the pellets were placed on each stud and allowed to dry. The sample was observed by SEM.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) analysis of the compound, bulk lipid and nanoparticles was conducted using a differential scanning calorimeter (DSC Q20 V24.4 Build 116, TA Instruments, USA) set at a heating rate of 10^0C/min

Short-term stability study

The selected SLN formulation was stored at 40ºC / 75% RH in stability chamber (Thermo Lab., Mumbai) for a period of one month and average particle size and entrapment efficiency was determined.

Effect of sterilization

To observe the effect of sterilization on particle size and entrapment efficiency, selected SLN formulation was autoclaved at 121 ^{0}C for 20 min.

In vitro release study

In vitro release studies were performed using modified Franz diffusion cells[9] having a surface area of 2.545 cm^2 and 75 ml of capacity. Dialysis membrane (LA 401) having pore size 2.4 nm, molecular weight cutoff 12000 – 14000 (HIMEDIA), was used. Membrane was soaked in distilled water for 12 hours before mounting in cell. Compound loaded SLN formulation equivalent to 5 mg of compound was placed in the donor compartment and the receptor compartment was filled with dialysis medium (phosphate buffer of pH 7.4, 75ml). The content of the cell was stirred with the help of magnetic stirrer at 37 ^{0}C. At fixed time intervals; 1ml of the sample was withdrawn from the receiver compartment through side tube. Fresh phosphate buffer of pH 7.4 was placed to maintain constant volume. Samples were analyzed for amount of compound released by UV spectrophotometer.

RESULTS AND DISCUSSION

The mean particle size, polydispersity index and zeta potential of colloidal carriers are important characteristics of SLNs from which the stability of compound-loaded SLNs can be predicted. Average particles size, polydispersity and zeta potential are shown in Table 2. All the formulations had shown particle in nanosize range (153-195 nm) with narrow size distribution (polydispersity index = 0.105-0.115). Besides production parameters, lipid matrix, surfactant blend and viscosity of lipid and aqueous phase influence the outcome of the procedure. Leaving all other parameters constant, in this study the only variable was composition of lipid matrix varying from 2.5% to 7.5%. Effect of lipid concentration on particle size and entrapment efficiency is recorded in Table 2. The results revealed that increasing the lipid content over 5–

7.5% results in larger particle size[13]. The choice of the emulsifiers and their concentration is of great impact on the quality of the SLN dispersion[14]. Investigating the influence of the emulsifier concentration on the particle size of SLN dispersions, we obtained best results with 2.5% egg lecithin. High concentrations of the emulsifier reduce the surface tension and facilitate the particle partition. The decrease in particle size is connected with a tremendous increase in surface area. The SLNs are stabilized with surfactant mixtures (egg lecithin/tween 80) to have lower particle sizes and higher storage stability. Use of surfactant mixtures with the aim to have lower particle size are also reported by Siekmann B and Westesen K[13] and Olbrich C and Muller R.H[15]. The measurement of the zeta potential allows predictions about the stability of colloidal aqueous dispersions[16]. Usually, particle aggregation is less likely to occur for charged particles with high zeta potential due to electric repulsion[17]. In general, lipid nanoparticles are negatively charged on the surface[18]. The determination of zeta potential was performed in aqueous SLN stored at room temperature. The values are shown in Table 2.

According to Professor Muller the prerequisite to obtain a sufficient loading capacity was a sufficiently high solubility of the compound in the lipid melt. Relative higher compound EE% was one of the major advantages of SLNs[2]. Figure 3 shows the compound EE% of Comp-GMS. The loading capacity of SLN was found to be satisfactorily high. The data showed EE% as high as 84.20% for some formulation. For SLN formulations, the entrapment efficiency is lower for the sample with lower lipid concentration (Table. 2). It has to be noticed that during the cooling process, the lipid solidifies and the compound is distributed into the shell of the particles, if the concentration of the compound in the melted lipid is well below its saturation solubility[12].

SEM image of the Comp-SLN derived from Joel JSM 840A has been presented in Fig. 2. SEM confirms that the Comp-SLNs are circular in shape. They are smooth and well separated on the surface.
The thermal curves of GMS bulk lipid and compound showed endothermic peaks at 59.32 ^{0}C and 182.02 ^{0}C respectively (Fig. 3). The melting endothermic peaks of the nanoparticles appeared at slightly lower temperature (59.22 ^{0}C). The decrease in melting temperature of nanoparticle formulated GMS lipid compared with the bulk has been attributed to their small size and presence of surfactants.

After one month storage at 40ºC / 75% RH, it has been found that particle size of Comp-GMS increases by 6nm and entrapment efficiency was lowered by 4.77 % (Table 3). Transitions of dispersed lipid from metastable forms to stable form might occur slowly on storage due to small particle size and the presence of emulsifier that may lead to compound expulsion from solid lipid nanoparticles. Therefore lowered entrapment efficiency observed on storage may be due to expulsion during lipid modification[9].

Effect of sterilization on particle size and entrapment efficiency was shown in Table 4. In selected Comp-GMS formulation, size of particles increases almost two times after sterilization, but still they are in nanosize. It was found that sterilization by autoclaving has least effect on entrapment efficiency. Therefore, sterilization by autoclaving can performed for SLNs of GMS stabilized with lecithin and tween 80.

Many research groups used vertical or flow-through Franz diffusion cells and dialysis bag/tubes for the study of compound release from solid lipid and polymeric nanoparticles and niosomes[19-26]. In order to evaluate the controlled release potential of the investigated formulations, the diffusion of Comp from the lipid particles was investigated over 24 h. Each sample was analyzed in triplicate. The results are shown in Fig. 4. The release rate of Comp depends on the total concentration of Comp in the formulation. Comp is released more quickly when using lower concentration because of the compound-enriched shell model proposed for these particles (Comp-SLN-1). Due to the large compound loading in Comp-SLN-3, the degree of diffusion can be decreased[12].

Percentage of compound released from SLNs up to 24 h were in the following order; Comp-SLN-1 (52.2%), Comp-SLN-2 (47.5%) and Comp-SLN-3 (42.8%). The release pattern revealed that there was an initially burst effect followed by a prolonged release of compound. This is because the compound may be located primarily in the shell of the particles. Other factors contributing to a fast release are large surface

area, high diffusion coefficient (small molecular size), low matrix viscosity and short diffusion distance of the compound. The compound enriched core is surrounded by a compound free lipid shell. Due to the increased diffusional distance and hindering effects by surrounding solid lipid shell, the compound has a sustained release profile.

The formulations were further subjected to release kinetic studies. The release data was fitted into first order and Higuchi equations. Release of compound from almost all the SLNs followed Higuchi equation better than the first order equation.

CONCLUSION

Solid lipid nanoparticles represent a particulate system, which can be produced with an established technique, microemulsion process allowing production on industrial scale. It can be achieved after the selection of optimal formulation and process parameters. Thienopyrimidines and triazolothienopyrimidines loaded SLN showed narrow particle size distribution with high entrapment efficiency. *In vitro* release study of compound loaded SLNs in phosphate buffer of pH 7.4, exhibited a biphasic pattern with an initial burst and prolonged release over 24 h.

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Table 1: Compositions of SLN formulations

Formulation code	Concentration of compound (mg)	Concentration of lipid (%)	Concentration of surfactants, 1:1 (%)
Comp-SLN-1	10	2.5	2.5
Comp-SLN-2	10	5.0	2.5
Comp-SLN-3	10	7.5	2.5

Table 2: Particle size, Polydispersity index, Zeta potential and Entrapment efficiency

Formulation code	Average diameter (nm)*	Polydispersity index*	Zeta potential (mV)*	Entrapment efficiency (EE%)*
Comp-SLN-1	153.0±1.00	0.105±0.001	-20.40±0.95	62.20±1.92
Comp-SLN-2	170.0±1.00	0.111±0.001	-22.67±0.80	68.87±1.51
Comp-SLN-3	195.0±1.00	0.115±0.001	-24.37±0.92	84.20±1.63

* Mean ± SD., *n* = 3

Table 3: Effect of time of storage (at 45^{0}C / 75%RH) on particle size and entrapment efficiency

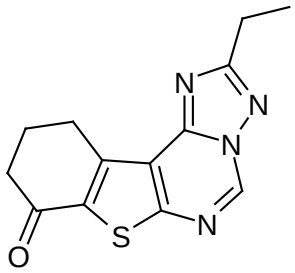
Formulation code	Particle size (nm)*		Entrapment efficiency (%)*	
	Zero day	One month	Zero day	One month
Comp-SLN-3	195.0±1.00	201.4±1.00	84.20±1.63	79.43±0.97

* mean ± SD., *n* = 3

Table 4: Effect of Sterilization on Particle size and Entrapment efficiency

Formulation code	Particle size (nm)*		Entrapment efficiency (%)*	
	Before	After	Before	After
Comp-SLN-3	195.0±1.00	383.0±2.64	84.20±1.63	80.50±0.88

* mean ± SD., *n* = 3



2-Ethyl-8,9,10,11-Tetrahydro[1]benzothieno[3,2-*e*][1,2,4]triazolo[1,5-*c*]pyrimidine-8-one

Figure 1: Thienopyrimidines and thienotriazolopyrimidines derivative

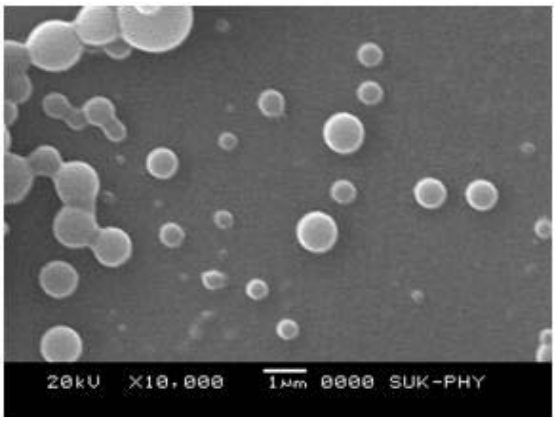
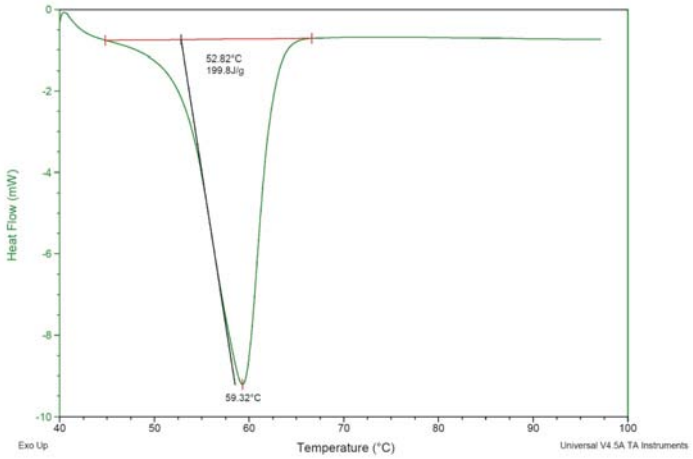
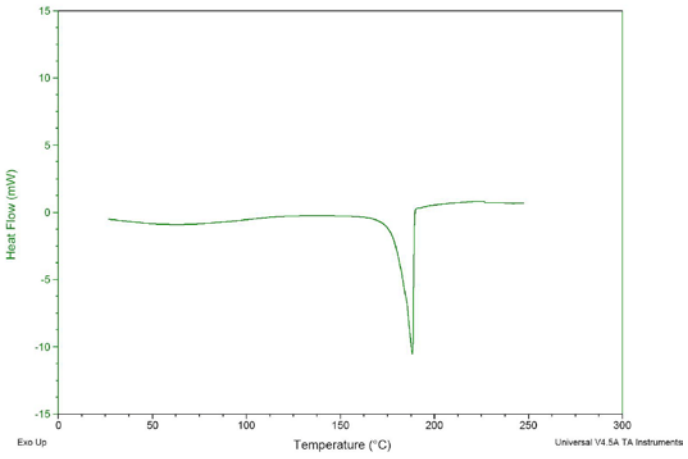


Figure 2: SEM of compoud loaded SLN



(a)



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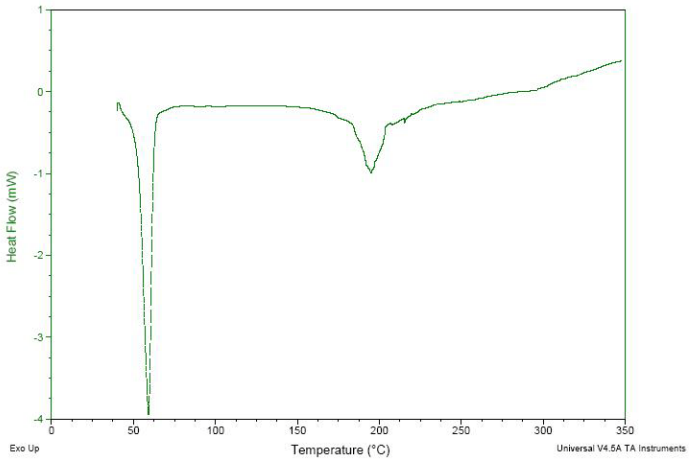

(c)

Figure 3: DSC thermograms of (a) bulk lipid (b) compound (c) compound loaded SLN

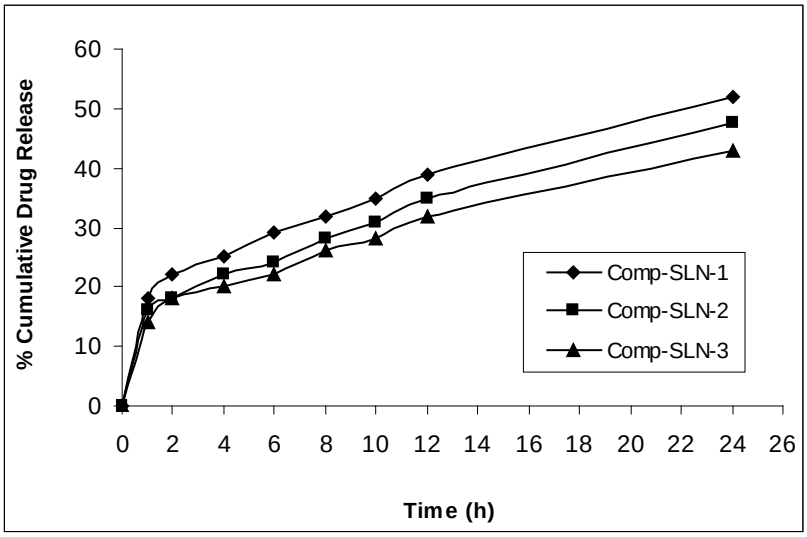

Figure 4: *In vitro* release study of compound loaded SLN

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