The objective of this study was to formulate and evaluate enrofloxacin solid lipid nanoparticles (SLNs) using a hot homogenization coupled with ultrasonication method for sustained oral delivery. The SLNs were prepared using tripalmitin as lipid carrier, tween 80 and span 80 as surfactants and polyvinyl alcohol (PVA) as a stabilizer.

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The results indicated that SLNs might be a promising delivery system to prolong and enhance the pharmacological activity of enrofloxacin.

The preliminary studies were carried out by preparing various blank SLNs formulations (Table 1) with different variables in order to optimize the particle size fit into nanoscale.

• Hot homogenization, followed by ultrasonication technique was selected to prepare blank SLNs
• Since the optimal type and amount of stabilizer, and the

INTRODUCTION

Enrofloxacin is a fluoroquinolone antimicrobial agent developed solely for use in animals. It has potent bactericidal activity against a range of clinically relevant Gram-negative and Gram-positive pathogens as well as Mycoplasma and Chlamydiae. Enrofloxacin and its active metabolite ciprofloxacin possess high bactericidal activity, killing the bacteria in a concentration dependent manner. The relative safety of enrofloxacin, its low minimum inhibitory concentrations, broad spectrum of activity, long post-antibiotic effect and good tolerance has encouraged their use in veterinary medicine [1].

Despite the therapeutic potential of enrofloxacin, the very poor aqueous solubility of enrofloxacin leads to difficulty in designs of pharmaceutical formulation and variations in bioavailability [2]. In addition, all the oral enrofloxacin formulations are available as conventional, immediate-release form that necessitates administration twice daily for several days or weeks [3]. Numerous efforts have been made to develop alternative formulations of enrofloxacin to reduce frequency of administration.

Nanoparticle-based drug delivery systems have considerable potential in improving the bioavailability of the drug and as well reducing the dosing frequency. The solid lipid nanoparticles (SLNs) introduced in 1991, are the forefront of the rapidly developing field of nanotechnology, which is the most effective lipid based colloidal carriers system. They are submicron-sized (50-1000 nm) carriers composed of a lipid matrix stabilized by a surfactant. SLNs possess good tolerability, stability, scaling up feasibility and the ability to incorporate hydrophilic/hydrophobic drugs [4]. The incorporation of poorly soluble drugs into SLNs can enhance gastrointestinal solubilization, absorption, and bioavailability of drugs [5]. Further, SLNs formulation has the ability to prolong, extend or sustain the release profile of the loaded molecules and hence reduce need for the repeated administration and increase the therapeutic value of the treatment [6].

Hence, the objective of this study is to formulate enrofloxacin SLNs with high loading capacity (LC) and sustained release profile using a hot homogenization and ultrasonication method.

METHODS

Drugs and chemicals

Enrofloxacin purchased from Himedia Laboratories Pvt. Ltd., India was used in this study. Tripalmitin (glyceryl palmitate), span 80 (polysorbate), tween 80 (sorbitate monoleate) and polyvinyl alcohol (PVA) procured from Sigma Aldrich Chemicals Pvt. Ltd., USA were utilized for the study. Dialysis membrane procured from Himedia Laboratories Pvt. Ltd., India was used. All other chemicals and solvents were analytical reagent grade and were used without further purification.
Tween 80 release of enrofloxacin SLNs and native enrofloxacin was quantified at 25°C. The samples were diluted appropriately with the de-ionized water for the measurements of particle size. Each value was the average of three measurements.

Surface morphology

Transmission electron microscopy (TEM)

The morphology of enrofloxacin SLNs was also analyzed using an AFM (PARK XE-100). Briefly, 1 mL of enrofloxacin SLNs and acetone (1 mL) were mixed. From the mixture, 10 µL was dispensed on a freshly cleaved mica substrate. After drying at room temperature, imaging of the samples was performed in non-contact mode with pyramidal silicon nitride tips.

Determination of LC and encapsulation efficiency

To determine the entrapment of enrofloxacin in the SLNs, 0.1 mL of freshly prepared nanoemulsion was taken and diluted with 9.9 mL chloroform. The obtained suspension was centrifuged for 45 minutes at 6000 rpm. The supernatant was separated and filtered through 0.2 µm filter. The filtrate was diluted using chloroform and analyzed at 273.8 nm using a UV spectrophotometer (Systronics 2203 Smart, India). The control samples were performed in non-contact mode with pyramidal silicon nitride tips.

In vitro release studies

In vitro release of enrofloxacin SLNs and native enrofloxacin was performed by dialysis bag diffusion technique over a period of 120 hrs. Enrofloxacin nano suspension equivalent to 5 mg of enrofloxacin was filled in dialysis bag (Himedia Laboratory Pvt. Ltd, India). The receiver solution containing 100 mL of phosphate buffer with pH 6.7 was prepared and heated to 37°C under magnetic stirring at a speed of 100 rpm. The drug containing dialysis bag (molecular weight 12-14 kDa, pore size 2.4 nm) was dialyzed against receiver compartment. To determine the enrofloxacin diffused through the dialysis bag, 2 mL samples were withdrawn at regular intervals (0, 5, 10, 20, 30, 45, 60, 90 minutes, and 2, 4, 8, 12, 18, 24, 36, 72, 96 and 120 hrs) from the receiver solution and same amount of fresh receiver solution was added to maintain the volume constant. Enrofloxacin in the samples was measured spectrophotometrically at 273.8 nm using a UV spectrophotometer (Systronics 2203 Smart, India). The control nanoparticles without enrofloxacin were treated similarly and used as blanks for the measurements.

Compatibility studies using Fourier transform infrared (FT-IR) spectroscopic analysis

FT-IR spectral measurement for pure enrofloxacin, tripalmitin, span 80, tween 80, PVA and formulation were analyzed separately and then correlated for compatibility. In the present study, potassium bromide

Table 1: Formulation design of blank SLNs by hot homogenization and ultrasonication method

<table>
<thead>
<tr>
<th>Formulation (%)</th>
<th>Lipid (%)</th>
<th>Tween 80</th>
<th>Span 80</th>
<th>PVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.5</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F2</td>
<td>1.0</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F3</td>
<td>1.5</td>
<td>-</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F4</td>
<td>0.5</td>
<td>-</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>F5</td>
<td>1.0</td>
<td>-</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>F6</td>
<td>1.5</td>
<td>-</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>F7</td>
<td>0.5</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F8</td>
<td>1.0</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F9</td>
<td>1.5</td>
<td>2</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F10</td>
<td>0.5</td>
<td>4</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F11</td>
<td>1.0</td>
<td>4</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F12</td>
<td>1.5</td>
<td>4</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>F13</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F14</td>
<td>1.0</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>F15</td>
<td>1.5</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

SLNs: Solid lipid nanoparticles, PVA: Polyvinyl alcohol
(KBr) pellet method was employed. A small drop of sample was placed on one of the KBr plates. The second KBr plate was placed on the top and made a quarter turn to obtain an even film. Then, the plates were kept on the sample holder to run a spectrum.

Statistical analysis
The data obtained on particle size, PDI, zeta potential, LC and encapsulation efficiency were analyzed using a Statistical Package for Social Sciences (SPSS 11.00) [7]. All values are expressed as their mean±standard deviation (SD).

RESULTS
Formulation optimization
Various blank SLNs formulations were prepared on the basis of individual factors and the results are presented in Table 2. In all formulations, the mean±SD particle size was found within a range of 150.67±40.07-997.67±76.05 nm. Based on the comparison of visual clarity and particle size, the formulation F4, F10 and F13 having the mean±SD particle size from 150.67±40.07 to 229.67±57.57 nm were considered for enrofloxacin incorporation.

After encapsulation of enrofloxacin in the selected blank SLNs (F4, F10 and F13), mean±SD particle size, PDI, zeta potential, entrapment efficiency (EE) and LC were evaluated and are presented in Table 3. All the enrofloxacin SLNs formulations had shown nanosize range of 154.717-238.33 nm. From these studies, most efficient formulation of F13 with the particle size, PDI, zeta potential, EE and LC of 154.717±6.149 nm, 0.422±0.109-28.83±0.603 mV, 58.33±3.51 and 6.03±0.97, respectively were considered as final preparation.

Characterization of enrofloxacin SLNs
Particle size, PDI and zeta potential
The mean±SD particle size, PDI and zeta potential of the formulations (F13) are given in Table 3.

Surface morphology
Transmission electron microscopic studies revealed that the enrofloxacin SLNs were spherical in shape (Fig. 1). In general, the particle size was with a diameter of <200 nm. These observations are consistent with PCS data of enrofloxacin SLNs recorded in this study.

Drug LC and encapsulation efficiency
The mean±SD encapsulation efficiency and LC of enrofloxacin SLNs (F13) are presented in Table 3.

In vitro release studies
In vitro release of enrofloxacin from SLNs formulation and native enrofloxacin is illustrated in Fig. 3. The release curve of enrofloxacin SLNs exhibited a biphasic pattern. There was an initial burst release with about 39.23% drug released within the initial 24 hrs, followed by a slow and sustained release. The amount of cumulated drug release over 96 hrs was 51.1%. In the native enrofloxacin, the release was 93.67% within 2 hrs and reached 100% by 24 hrs.

Table 2: Mean±SD particle size of the blank SLNs formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Particle size (nm) (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>673.33±35.55</td>
</tr>
<tr>
<td>F2</td>
<td>852.67±39.52</td>
</tr>
<tr>
<td>F3</td>
<td>997.67±76.05</td>
</tr>
<tr>
<td>F4</td>
<td>229.67±57.57</td>
</tr>
<tr>
<td>F5</td>
<td>491.67±53.08</td>
</tr>
<tr>
<td>F6</td>
<td>571.33±60.33</td>
</tr>
<tr>
<td>F7</td>
<td>703.33±27.57</td>
</tr>
<tr>
<td>F8</td>
<td>879.67±28.91</td>
</tr>
<tr>
<td>F9</td>
<td>902.3390.16</td>
</tr>
<tr>
<td>F10</td>
<td>192.67±56.86</td>
</tr>
<tr>
<td>F11</td>
<td>416.67±54.22</td>
</tr>
<tr>
<td>F12</td>
<td>588.00±119.38</td>
</tr>
<tr>
<td>F13</td>
<td>150.67±40.07</td>
</tr>
<tr>
<td>F14</td>
<td>312.33±69.01</td>
</tr>
<tr>
<td>F15</td>
<td>416.67±23.03</td>
</tr>
</tbody>
</table>

SLNs: Solid lipid nanoparticles, SD: Standard deviation

Table 3: Mean±SD particle size, PDI, zeta potential, EE and LC of selected enrofloxacin SLNs formulations

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>EE (%)</th>
<th>LC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4</td>
<td>238.33±30.90</td>
<td>0.59±0.072</td>
<td>−29.33±1.42</td>
<td>49.33±3.79</td>
<td>4.60±1.47</td>
</tr>
<tr>
<td>F10</td>
<td>195.67±68.14</td>
<td>0.55±0.05</td>
<td>−30.67±3.05</td>
<td>50.67±7.64</td>
<td>5.03±0.95</td>
</tr>
<tr>
<td>F13</td>
<td>154.717±6.149</td>
<td>0.422±0.109</td>
<td>−28.83±0.603</td>
<td>58.33±3.51</td>
<td>6.03±0.97</td>
</tr>
</tbody>
</table>

SD: Standard deviation, SLNs: Solid lipid nanoparticles, PDI: Polydispersity index, EE: Entrapment efficiency, LC: Loading capacity
Compatibility studies using FT-IR spectroscopic analysis
The FT-IR spectra of drug, tripalmitin, span 80, tween 80, PVA and formulation were exhibited the peaks of specific functional groups at their respective frequencies as presented in Fig. 4.

DISCUSSION
Formulation optimization
Hot homogenization followed by ultrasonication was reported to be an economic, simple, reproducible and most reliable method for the preparation of SLNs. In this method, the preparation of SLNs does not require any organic solvents, which could be difficult to remove after nanoparticle synthesis [8]. By this method, it is possible to scale up to industrial level. Hence, hot homogenization coupled with ultrasonication method was employed in the present study to formulate SLNs.

Lipids must be selected based on their ability to solubilize the drug [9]. The lipid tripalmitin had high dissolution of enrofloxacin. The solubilizing potential along with already reported biocompatibility and acceptability of lipid tripalmitin for oral route has favored its selection for the present study. In the present study, as the lipid concentration increased from 0.5 to 1.5%, the mean particle sizes and particle size distribution also increased, which is in agreement with Müller et al. [4] and Westesen et al. [9] higher concentration of lipid content increased the viscosity of the lipid dispersion which affected the homogenization efficiency and increased rate of particle agglomeration, hence, the lipid content of the SLNs dispersion should not exceed 5% [4]. The inclusive level of tripalmitin in this study was 0.5% which is well below the recommended rate.

According to Souto and Müller [10], the right selection of surfactant with proper concentration was required to prepare physicochemically stable lipid nanoparticles. In the current study, the SLNs dispersion stabilized by the combination of surfactant (2% span 80 and 2% tween 80) had smaller particle size when compared with formulation stabilized by single surfactant. These findings are in accordance with Mehner and Mäder [6] who had lower particle sizes and better stability when stabilized the formulation with surfactant mixtures compared with formulations with only one surfactant. Combination of surfactants prevented particle agglomeration more efficiently and also reduced the particle size of the SLNs [10]. In the present study, the mean particle size of SLNs tended to decrease with increase in the surfactant concentration. This observations are in agreement with the findings of Bunjes et al. [11] and Lippacher et al. [12].

In the current study, 2% PVA was used to stabilize the SLNs formulation. According to DeMerlis and Schoneker [13], PVA is the most commonly used emulsifier in the formulation of nanoparticles due to its excellent mechanical strength, biocompatibility and nontoxicity, and has been approved by the US FDA for medical and food applications.

From the optimization study, the suitable blank SLNs formulation containing 0.1% tripalmitin, 2% span 80, 2% tween 80 and 2% PVA were considered for further incorporation of drug.

Formulation of enrofloxacin SLNs
The blank SLNs selected in the optimization study were used to entrap 0.1% enrofloxacin using a hot homogenization coupled with ultrasonication method. According to Muller et al. [4], high temperature was performed in hot homogenization technique and thus, this method could not be used for temperature sensitive drugs. In this study, the
temperature for the preparation of SLNs did not exceed the melting point of enrofloxacin (219-233°C), hence the stability and antibacterial activity will be maintained.

Homogenization followed by ultrasonication technique applies high shear stress disrupting lipid particles down to the submicron range. According to Schwarz et al. [14], a sufficient high-energy input was necessary to break down the droplets into the nanometer range. A high energy such as high production temperature, high stirring rate, longer emulsification time and stronger ultrasound power were applied in this study to obtain a finer dispersion of formulation. In the present study, the homogenization pressure 10,000 psi was applied for 3 minutes and followed by ultrasonication resulted the mean±SD particle size of 154.7±6.149 nm with narrow size distribution. The result suggests that the hot homogenization and ultrasonication method was a feasible and compatible method for preparing enrofloxacin loaded tripalmitin SLNs.

Characterization of enrofloxacin SLNs

The loading of drug with the blank SLNs in the present study resulted in a slight increase in the mean±standard error (SE) particle sizes from 150.67±0.07 to 154.72±0.15 nm. These findings are in consistent with Jensen et al. [15] who explained that the increase in size of SLNs after incorporation of drug reflected the dissolution of the drug in the lipid phase.

A narrow particle size distribution was an indication of nanoparticles stability and homogeneous dispersion [16]. PDI values ranging from 0 to 0.5 were considered to be monodisperse and homogenous, but those of more than 0.5 indicated non-homogeneity and polydispersity [8,17]. In the present study, the particle size distribution was monodisperse and homogenous as formulation has less mean±SE PDI of 0.42±0.11.

According to Schwarz and Mehnert [18] and Zimmermann et al. [19], the negative charge of zeta potential was conferred by the lipids used in the SLNs. In agreement with this, the tripalmitin utilized in this study provided negative charge of zeta potential. Nanoparticle with zeta potential values >+25 mV or <-25 mV typically have high degrees of stability due to electric repulsion between particles. Dispersions with a low zeta potential value will aggregate due to Van Der Waal inter-particle attraction [4]. In this study, the mean±SD zeta potential of ~24.90±1.00 mV was recorded and it could provide proper stability to the enrofloxacin SLNs. According to Srivavas and Sagar [20], the zeta potential with negative charge might not interfere in the absorption of the formulation.

TEM and AFM images revealed spherical and circular in shape with the presence of some particle aggregates. The presence of aggregates might be due to redistribution of particles after preparation. The images represented that the particles were ranging from 100 to 200 nm and well dispersed with smooth surfaces.

The enrofloxacin SLNs obtained in the present study had relatively medium drug EE (59.67%). This could be attributed to the physicochemical properties of the drug, most importantly, its lipophilic nature [21]. To get sufficient LC, the drug should have sufficiently high solubility in the lipid melt. The crystallization habits of tripalmitin nanoparticles also varied with the quantity of drug incorporated [22]. High temperature in production and high surfactant concentration might influence the drug loading and the shape of the loading profile [4]. The percentage encapsulation efficiency data obtained in this study are consistent with the findings of Xie et al. [23].

In the present study, enrofloxacin was having a higher melting point (219-233°C) than the lipid base (67°C). Hence it was expected that lipid phase solidify first upon cooling during the high homogenization production process with the drug forming a core in the lipid phase [24]. Hence, the formulated SLNs in this study might be drug enriched core model. Sadiq and Rassol [25] (2014) was formulated stibinin enriched core model using tripalmitin lipid. In vitro release data obtained under sink conditions are consistent with drug release reported from different SLNs by Ji et al. [26] and Xie et al. [27]. The initial fast release (burst effect) could be attributed to the presence of a small fraction of unentrapped drug or drug embedded near the SLNs surface. Other factors contributing to a fast release were large surface area, high diffusion coefficient (small molecular size), low matrix viscosity and short diffusion distance of the drug. The slow release was mainly due to the low diffusion of drug molecules through the lipid matrix of the nanoparticles and hindering effects by surrounding solid lipid shell [4,11]. Slow drug release contributes to maintaining the effective therapeutic drug concentrations.

In the formulation spectrum, the peak at 1656.30/cm revealed the presence of C=O stretching of the carboxylic group of enrofloxacin, peak at 2345.21/cm referred to OH vibration bond, 2070/cm and 2954/cm were the stretching vibration of the secondary and tertiary amine moiety of the drug, 1254.70/cm represented the C-N stretching which indicated presence of enrofloxacin without any change in the formulation [28].

From the IR spectra, it was clear that functionalities of drug have remained unchanged, including intensities of peak. This suggested that during the process of formulations, surfactants, lipid and stabilizer have not reacted with the drug to give rise to reactant products. Hence, it was only physical mixture and there was no interaction between which is on favor to proceed for formulations.

CONCLUSION

Enrofloxacin was successfully incorporated into tripalmitin-SLNs by a hot homogenization coupled with ultrasonication method. The physicochemical study of enrofloxacin loaded tripalmitin SLNs showed desired particle size, PDI, zeta potential, LC and encapsulation efficiency. The enrofloxacin SLNs had a sustained release effect in the in vitro release study. FT-IR study concluded that no interaction occurred between the drug excipients and polymer used in this study.

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REFERENCES