

Formulation of enrofloxacin SLNs and its pharmacokinetics in emu (*Dromaius novaehollandiae*) birds

P. Senthil Kumar · A. Arivuchelvan · A. Jagadeeswaran ·
N. Punniamurthy · P. Selvaraj · P. N. Richard Jagatheesan ·
P. Mekala

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Abstract The study was conducted to formulate the enrofloxacin solid lipid nanoparticles (SLNs) with sustained release profile and improved pharmacological activity and evaluate the pharmacokinetic behaviour of enrofloxacin SLNs after oral routes of administration in emus. The SLNs were prepared using tripalmitin as lipid carrier, Tween 80 and Span 80 as surfactants and polyvinyl alcohol (PVA) as a stabilizer by a hot homogenization coupled with ultrasonication method. The prepared enrofloxacin SLNs formulations were characterized for further investigation in emu birds. The pharmacokinetics of native enrofloxacin was studied after i.v. and oral bolus administration at 10 mg/kg in emu birds and compared with the disposition kinetics of enrofloxacin SLNs. Enrofloxacin and its metabolite ciprofloxacin in plasma were estimated using HPLC and the pharmacokinetic parameters were calculated by a non-compartmental analysis. The results demonstrated that the particle size, polydispersity index, zeta potential, encapsulation efficiency and loading capacity of the SLNs

were 154.72 ± 6.11 nm, 0.42 ± 0.11 , -28.83 ± 0.60 mV, 59.66 ± 3.22 and 6.13 ± 0.32 %, respectively. AFM and TEM images showed spherical to circular particles with well-defined periphery. In vitro drug release exhibited biphasic pattern with an initial burst release of 18 % within 2 h followed by sustained release over 96 h. Pharmacokinetic results showed that the $t_{1/2\beta}$, $AUC_{0-\infty}$, V_{darea}/F , MRT and bioavailability were 3.107, 1.894, 1.594, 2.993 and 1.895 times enhanced ($p < 0.01$), while CL_B and β were significantly ($p < 0.01$) decreased by 1.958 and 3.056 times compared to the values of native enrofloxacin administered orally. The ratio of AUC_{0-t} cipro/ AUC_{0-t} enro after administration of native enrofloxacin and enrofloxacin SLNs was less than 10 %. The $t_{1/2\beta}$ and MRT of the metabolite were longer than those of the parent substance. The PK/PD results confirmed that the SLNs extended the enrofloxacin concentration upto 48 h against pathogens susceptible to 0.125 μ g/mL in emus. The results indicated that SLNs might be a promising delivery system to prolong and enhance the pharmacological activity of enrofloxacin.

P. Senthil Kumar (✉)
Veterinary College and Research Institute, Orathanadu 614625,
Tamil Nadu, India
e-mail: senvetpharm@yahoo.com;
p.senthilkumar@tanuvas.org.in

A. Arivuchelvan · A. Jagadeeswaran · P. Selvaraj · P. Mekala
Veterinary College and Research Institute, Namakkal,
Tamil Nadu, India

N. Punniamurthy
Ethno Veterinary Herbal Training and Research Centre,
Thanjavur 613 403, India

P. N. Richard Jagatheesan
TANUVAS-Regional Research Centre,
Pudukkottai 622 304, India

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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 have encouraged their use in veterinary medicine (Scheer 1987). Despite the therapeutic potential of enrofloxacin, its very poor aqueous solubility leads to difficulty in designs of pharmaceutical formulation and variations in bioavailability (Martinez et al. 2006).

Emu (*Dromaius novaehollandiae*) belongs to ratite group. Bacterial infections are important causes of morbidity and mortality in domestic emus (Sales 2007). Restraining is not easy and causes stress. Hence, enrofloxacin with a sustained release profile is highly convenient for use in emus. However, all the oral enrofloxacin formulations are available in conventional, immediate-release forms that necessitate administration twice daily or daily for several days or weeks (Martinez et al. 2006). Numerous efforts have been made to develop alternative formulations of enrofloxacin to reduce the frequency of administration.

Solid lipid nanoparticles (SLNs), introduced in 1991, are at the forefront of the rapidly developing field of nanotechnology and are the most effective lipid-based colloidal carrier systems. They are submicron-sized (50–1,000 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 (Muller et al. 2000). The incorporation of poorly soluble drugs into SLNs can enhance gastrointestinal solubilization, absorption and bioavailability of drugs (Muller et al. 2002). Further, SLNs formulation has the ability to prolong or sustain the release profile of the loaded molecules, and hence reduce the need for repeated administration and increase the therapeutic value of the treatment (Xie et al. 2011a).

Hence, the present research was premeditated with the objectives of preparation and characterization enrofloxacin SLNs; determination of pharmacokinetics of enrofloxacin SLNs following oral bolus administration in domestic emu birds. This comprehensive study not only provides the pharmacokinetic data of enrofloxacin nanoparticles in domestic emus for the first time, but also lays down the pharmacokinetic comparison between native enrofloxacin and enrofloxacin nanoparticles. The recommendations on the dosage of enrofloxacin SLNs are made based on these findings.

Materials and methods

Drugs and chemicals

Enrofloxacin, ciprofloxacin hydrochloride (Himedia Laboratories Pvt. Ltd., India), tripalmitin, Span 80, Tween 80 and polyvinyl alcohol (Sigma Aldrich Chemicals Pvt. Ltd., USA) were used for the study. Dialysis membrane procured from Himedia Laboratories Pvt. Ltd., India was used.

For HPLC analysis, HPLC-grade acetonitrile, methanol, triethyl amine and phosphoric acid were purchased from Merck Specialities Ltd., India. Water for HPLC obtained from Millipore water purification system was utilized. All solvents and solutions for HPLC analysis were filtered through 0.2 μ HNN nylon membrane filter (Nupore) and degassed using a sonicator. All other chemicals and solvents were of analytical reagent grade and were used without further purification.

Preparation of enrofloxacin SLNs

Enrofloxacin SLNs were prepared by hot homogenization followed by the ultrasonication method. Enrofloxacin, tripalmitin and Span 80 were added at the ratio of 1:5:20 to get the organic phase of preparation. The lipid content in the organic phase was melted by heating at 70 °C using a magnetic stirrer with hot plate. The contents in the organic phase were mixed well by placing in the shaker (Spinix). An aqueous phase was prepared by dissolving Tween 80 and polyvinyl alcohol at the ratio of 20:20 and heating to the same temperature as the organic phase. The hot aqueous phase was added to the organic phase under magnetic stirring (Remi, Mumbai, India) at 1,000 rpm to form a pre-emulsion. The hot pre-emulsion was then homogenized at 10,000 psi for 3 min using a high-pressure homogenizer (Heidolph Electro, Germany) kept in a water bath maintained at 70 °C.

The hot emulsion so obtained was ultrasonicated (Sonics Vibra Cell, USA) using high-intensity (5/64" 2 mm tip diameter) microprobe with amplitude 20 % for 15 min to form a nanoemulsion. Then, the nanoemulsion was run under magnetic stirring at 1,000 rpm for 4 h to obtain enrofloxacin-loaded tripalmitin SLNs.

All the batches were prepared in triplicate and the average size was measured.

Characterization of enrofloxacin SLNs

Determination of particle size, polydispersity index and zeta potential

Particle size and polydispersity index (PDI) of enrofloxacin SLNs were measured by photon correlation spectroscopy (PCS) using Zetasizer NanoZS with the Malvern PCS software version 6.20. The aqueous SLNs dispersions were diluted with distilled water appropriately before analysis. Each value was the average of three measurements.

The zeta potential was measured by electrophoretic light scattering (ELS) mode using Zetasizer NanoZS. The particle charge of enrofloxacin SLNs was quantified at 25 °C. The samples were diluted appropriately with de-ionized water for the measurements of particle charge. Each value was the average of three measurements.

Surface morphology

Surface morphology and shape of the enrofloxacin SLNs were examined using transmission electron microscope (Philips, Tecrai10, Dutch). The nanosuspension was stained with 2 % (w/v) phosphotungstic acid and placed on copper grids for TEM investigation.

The morphology of enrofloxacin SLNs was also analysed using atomic force microscopy (PARK XE-100). Briefly, 1 mL of enrofloxacin SLNs and acetone (1 mL) were mixed. From the mixture, 10 μ L was dispersed in a freshly cleaved mica substrate. After drying at room temperature, imaging of the samples were performed in non-contact mode with pyramidal silicon nitride tips.

Determination of loading capacity 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 min at 6,000 rpm. The supernatant was separated and filtered through 0.2 μ m filter. The filtrate was diluted using chloroform and analysed at 273.8 nm using UV spectrophotometer. The SLNs formulated without enrofloxacin were treated similarly and used as control for the measurements. The assay was repeated three times using different preparations. The loading capacity and encapsulation efficiency were calculated as shown:

$$\text{Loading capacity} = \frac{\text{Weight of enrofloxacin in SLNs}}{\text{Weight of SLNs}} \times 100\%$$

$$\text{Encapsulation efficiency} = \frac{\text{Weight of enrofloxacin in SLNs}}{\text{Weight of enrofloxacin added}} \times 100\%$$

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 h. Enrofloxacin nanosuspension equivalent to 5 mg of enrofloxacin was filled in the 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 dialysed against the receiver compartment. To determine the diffusion of enrofloxacin through the dialysis bag, 2 mL samples were withdrawn at regular intervals (0, 5, 10, 20, 30, 45, 60, 90 min, and 2, 4, 8, 12, 18, 24, 36, 72, 96 and 120 h) from the receiver solution and the 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.

Pharmacokinetic study

Experimental design

The study was conducted in eight apparently healthy emus (4 male + 4 female) aged 18–24 months with a mean (\pm SE) body weight of 38.20 \pm 1.03 kg. The birds were under uniform conditions of housing (semi-intensive system) and feeding, according to the birds' requirements. Birds were offered feed and water ad libitum. Before the start of the experiment, the birds were examined clinically to rule out the possibility of any disease. Antibiotics and anthelmintics were not administered 2 months prior to the start of the experiment. The use of the birds and experimental design were approved by the Institutional Animal Ethics Committee (IAEC), TANUVAS, Chennai.

Administration of drugs and collection of blood samples

Trial I: Native enrofloxacin (10 mg/kg) was administered i.v. (bolus dose) to the emu through the jugular vein. Two millilitre blood samples were drawn by jugular venipuncture into heparinized tubes immediately before and at 0.083, 0.167, 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 8, 12, 18, 24 and 36 h after dosing.

Trial II: After 2 weeks of washout period, the same birds were orally administered the same dose of native enrofloxacin directly using a thin plastic tube attached to a syringe. Then, 2 ml of blood samples were drawn by the same method at 0.25, 0.50, 0.75, 1.5, 2, 3, 4, 6, 8, 12, 18, 24, 36, 48 and 60 h after dosing.

Trail III: With 2 weeks washout period, the same birds were orally administered enrofloxacin SLNs (10 mg/kg) directly using a thin plastic tube attached to a syringe. Blood samples (2 mL) were drawn by jugular venipuncture into heparinized tubes at 0.25, 0.50, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, 24, 36, 48, 60, 72, 84 and 96 h after dosing. The birds were checked for observable signs of toxicity for up to 7 days after administration of enrofloxacin SLNs.

The collected blood samples were centrifuged at 950 \times g for 20 min to separate the plasma. The plasma samples were stored at -4 °C until assay.

Drug assay

Determination of enrofloxacin and its active metabolite ciprofloxacin was performed by high-performance liquid

chromatography (HPLC). The method developed by Kung et al. (1993) was followed.

The HPLC system comprised an LC-20 AD double plunger pump, Rheodyne manual loop injector with a 20 μ L loop, column oven CTO-10 AS vp, SPD-M20A diode array detector and a software LC Solution for data analysis. The compound separation was achieved using a reverse-phase C18 column (Hibar 250-4, 6 RP-18 endcapped, particle size 5 μ m, 4.6 \times 250 mm, Merck, Darmstadt, Germany) as a stationary phase. The column was protected with 2–8 mm Phenomenax guard column (KJO-4282). The mobile phase consisted of a mixture of acetonitrile, methanol and water (containing 0.4 % phosphoric acid and adjusted to pH 3.0 using triethylamine) in the ratio of 17:3:80 (v/v/v). The flow rate of the mobile phase was 1 mL/min and the samples were analysed for 10 min at 40 °C. The scan range of PDA was 220–400 nm, and the detection wavelength was 278 nm. The mean (\pm SE) retention times for ciprofloxacin and enrofloxacin were 5.65 \pm 0.003 and 7.16 \pm 0.006 min, respectively.

Enrofloxacin and ciprofloxacin from the plasma were subjected to liquid–liquid extraction according to the method of Nielsen and Hansen (1997). To 0.5 mL of plasma, 0.75 mL of acetonitrile was added in the ratio of 1:1.5. The mixture was vortex mixed for 15 s and centrifuged for 15 min at 4 °C at a speed of 900 \times g. The clear supernatant thus obtained (0.5 mL) and twice the volume of HPLC-grade water (1 mL) were added in the ratio of 1:2. The aliquot was then filtered through 0.2 μ HNN nylon membrane filter and 20 μ L of filtrate was injected into the HPLC system.

Working standards of enrofloxacin (0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μ g/mL) and ciprofloxacin (0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μ g/mL) were prepared from respective stock solutions after diluting with plasma collected from emus. Standard calibration curves were prepared from plasma samples containing known concentrations of enrofloxacin and ciprofloxacin separately.

The standard curves of enrofloxacin and ciprofloxacin were linear in the range of 0.01–10.0 μ g/mL. The calibration curve for enrofloxacin was characterized by its regression coefficient ($r^2 = 0.999$), slope (19,070) and intercept (13,182), and was used to determine the analyte concentrations in the sample. The calibration curve for ciprofloxacin was characterized by its regression coefficient ($r^2 = 0.998$), slope (14,777) and intercept (6,507.4), and was used to determine the analyte concentrations in the sample.

The concentrations of enrofloxacin and ciprofloxacin in the plasma samples were determined by substituting the respective peak areas/peak heights in the linear regression formula after calibration of standard curves.

Absence of change in the retention time was considered the method found specific and selective. The mean absolute recovery was within the range of 97.778–107.45 % for plasma and the coefficient of variation (CV) was 2.129–7.676 %, suggesting the suitability of the method for analysis of enrofloxacin and ciprofloxacin in emu plasma. The intra-day and inter-day CVs were within the limits (<10 %) specified (enrofloxacin: 5.307–8.827 %, ciprofloxacin; 4.757–8.632 %) and hence the method was suitable for assay of both enrofloxacin and ciprofloxacin in emu plasma. The limits of detection and quantification were 0.01 and 0.025 μ g/mL for enrofloxacin and 0.025 and 0.05 μ g/mL for ciprofloxacin, respectively.

Pharmacokinetic analysis

The pharmacokinetic parameters were derived from concentration vs time curves obtained for each bird after administration of native enrofloxacin and enrofloxacin SLNs. Non-compartmental pharmacokinetic analysis was used to describe the pharmacokinetics of enrofloxacin and ciprofloxacin based on statistical moment theory using the pharmacokinetic software *PK function* (Usansky et al. 2011).

The elimination rate constant (β) was calculated from the log-linear portion of the elimination curve using linear regression analysis. The elimination half-life ($t_{1/2\beta}$) was calculated according to $t_{1/2\beta} = \ln 2/\beta$, where $\ln 2 = 0.693$. The area under the plasma concentration–time curve (AUC) and the area under the first moment curve (AUMC) were calculated using the trapezoidal rule and extrapolated to infinity by means of the elimination rate constant. The mean residence time (MRT = AUMC/AUC), total body clearance ($CL_B = \text{Dose}/\text{AUC}$), volume of distribution to steady state ($V_{dss} = CL_B \times \text{MRT}$) and apparent volume of distribution ($V_{darea} = \text{Dose}/\beta \times \text{AUC}_{0-\infty}$) were calculated after i.v. administration.

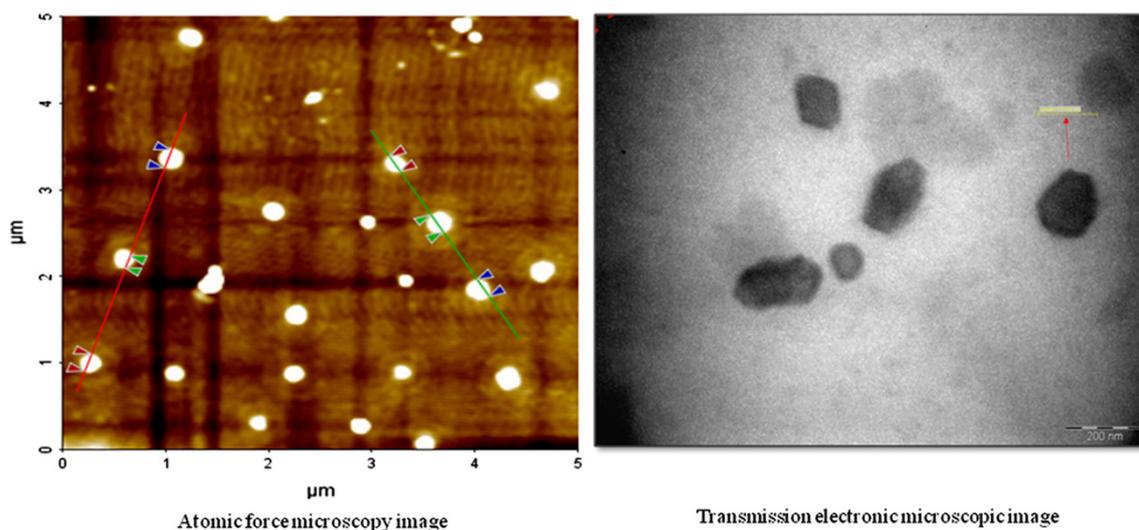
After oral administration, AUC, AUMC and MRT were calculated as above. Comparing the corresponding oral and i.v. route of administration, the bioavailability (F) after oral administration was calculated as $F = \text{AUC}_{0-\infty}(\text{oral})/\text{AUC}_{0-\infty}(\text{i.v.}) \times 100$; mean absorption time as $\text{MAT} = \text{MRT}_{\text{oral}} - \text{MRT}_{\text{i.v.}}$; total body clearance as $CL_B = \text{Dose} \times F/\text{AUC}_{0-\infty}$; apparent volume of distribution as $V_{darea} = \text{Dose} \times F/\beta \times \text{AUC}_{0-\infty}$.

Pharmacokinetic/pharmacodynamic (PK/PD) integration

The ratios $C_{\text{max}}/\text{MIC}$ and AUC/MIC were calculated for hypothetical MIC_{90} (0.05, 0.125, 0.25 and 0.5 μ g/mL)

Table 1 Mean(\pm SD) particle size, PDI, zeta potential, EE and LC of selected enrofloxacin SLNs formulations

Particle size (nm)	PDI	Zeta potential (mV)	EE (%)	LC (%)
154.717 \pm 6.149	0.422 \pm 0.109	-28.83 \pm 0.603	58.33 \pm 3.51	6.03 \pm 0.97

**Fig. 1** Atomic force microscopic and transmission electron microscopic image of enrofloxacin SLNs

values using the means of C_{\max} and AUC obtained in this study.

Statistical analysis

Statistical analysis of the data was performed by using SPSS 17.0 software. The results were expressed as mean \pm SE. Harmonic mean was used with data not distributed normally. Test of significance such as *t* test and analysis of variance (one way ANOVA) were applied to find out the difference between and among various groups, respectively (Snedecor and Cochran 1989). Comparison of the means of different subgroups was performed by Duncan's multiple range tests as described by Kramer (1957).

Results

The mean (\pm SD) particle size, PDI, zeta potential, encapsulation efficiency and loading capacity of the enrofloxacin SLNs are given in Table 1. AFM and TEM analysis showed that the enrofloxacin SLNs were spherical and circular in shape (Fig. 1). The particles were well dispersed with good particle size distribution. The surfaces of the nanoparticles were smooth.

In vitro release of enrofloxacin from SLNs formulation and native enrofloxacin is illustrated in Fig. 2. 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 h, followed by a slow and sustained release. The amount of cumulated drug release over 96 h was 51.1 %. In the native enrofloxacin, the release was 93.67 % within 2 h and reached 100 % by 24 h.

The mean (\pm SE) plasma concentrations of enrofloxacin and its metabolite ciprofloxacin after native enrofloxacin (i.v. and oral) and enrofloxacin SLNs (oral) administration are depicted graphically in Fig. 3. After i.v. administration of native enrofloxacin, enrofloxacin could be detected up to 18 h in one bird, while in seven birds the drug was detected up to 24 h. The highest mean concentration was 14.756 μ g/mL at 5 min and the lowest was 0.054 μ g/mL at 24 h. The mean (\pm SE) values of plasma concentration of enrofloxacin following oral administration of native enrofloxacin rapidly increased from 0.591 \pm 0.073 μ g/mL at 15 min to 2.207 \pm 0.098 μ g/mL within 1.5 h and then declined to 0.004 \pm 0.004 μ g/mL at 36 h. Detectable concentrations of enrofloxacin after oral administration of native enrofloxacin were found up to 24 h in seven birds, while in one bird the drug was detected up to 36 h. The plasma concentration of the active metabolite ciprofloxacin was observed from 15 min to 24 h for both the routes of i.v. and oral administration of native enrofloxacin.

The mean (\pm SE) plasma concentration of enrofloxacin after oral administration of enrofloxacin SLNs was 0.964 \pm 0.074 μ g/mL at 15 min, reached a significantly

Fig. 2 In vitro release of native enrofloxacin and enrofloxacin SLNs (mean \pm SD, $n = 3$)

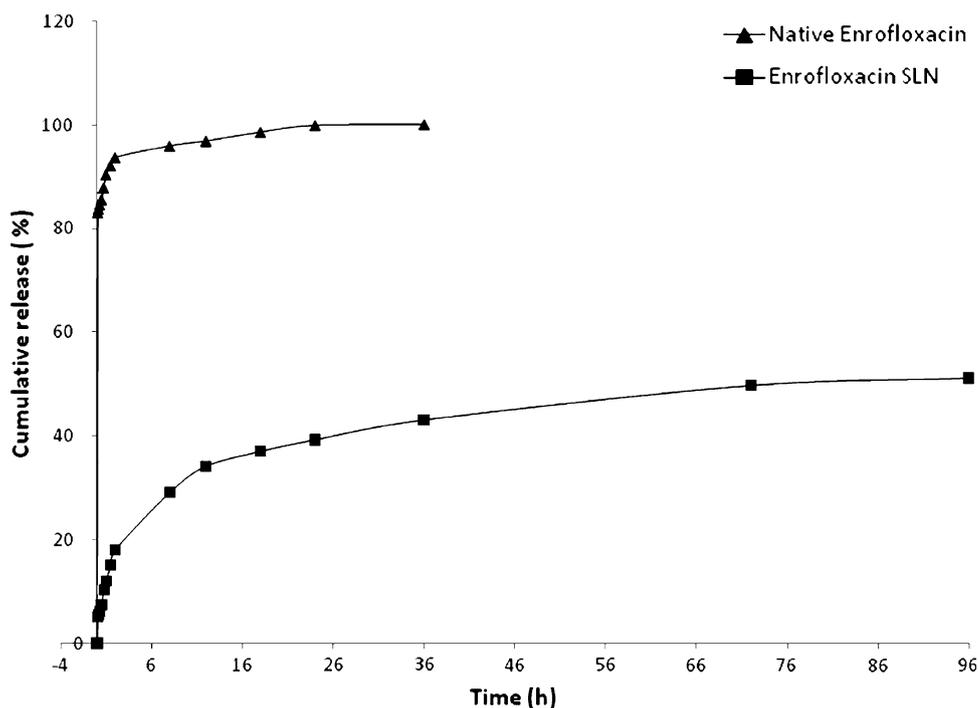
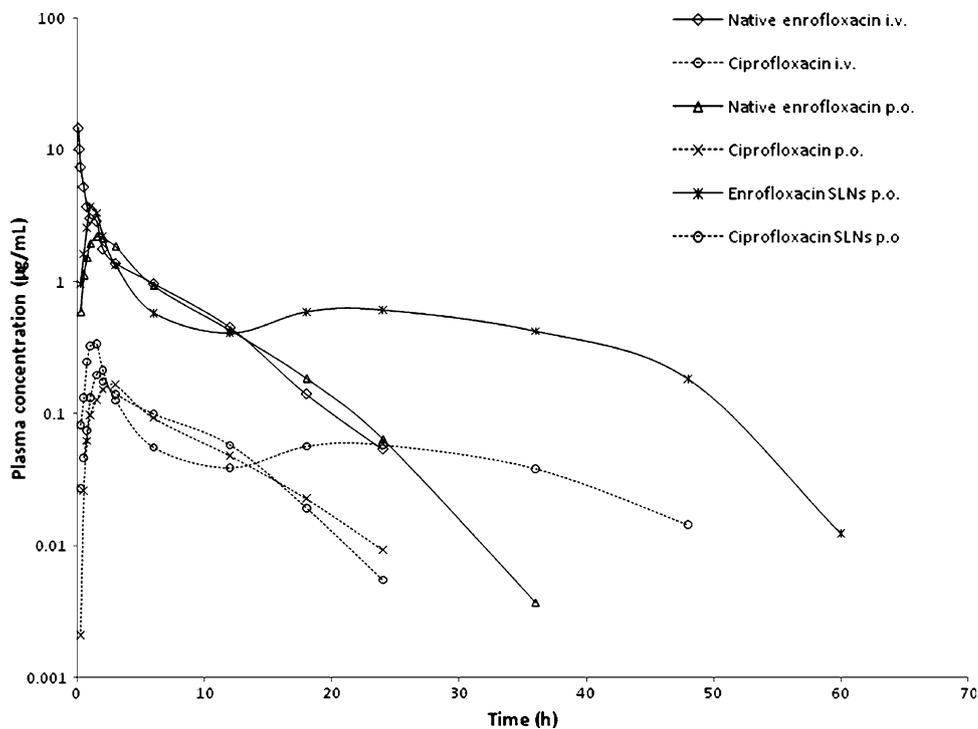


Fig. 3 Semilogarithmic plot of mean plasma enrofloxacin and its metabolite ciprofloxacin concentration ($\mu\text{g/mL}$) vs. time in emus ($n = 8$) following administration of native enrofloxacin and enrofloxacin SLNs (10 mg/kg)



higher peak concentration of $3.721 \pm 0.128 \mu\text{g/mL}$ at 1 h, then decreased sharply to the same levels of native drug 2 h post-administration. Although the plasma drug concentration decreased to $0.580 \pm 0.032 \mu\text{g/mL}$ at 6 h, the concentration was maintained over $0.012 \mu\text{g/mL}$ for up to 60 h. After enrofloxacin SLNs administration, enrofloxacin could be detected in plasma up to 60 h in four birds and up

to 48 h in four birds. The metabolite ciprofloxacin could be observed from 15 min to 48 h after the administration of enrofloxacin SLNs orally.

Enrofloxacin showed $\text{AUC}_{0-\infty}$ of $20.085 \pm 3.493 \mu\text{g h/mL}$ with large apparent volume of distribution ($3.921 \pm 1.005 \text{ L/kg}$) and slower elimination half-life ($4.364 \pm 0.179 \text{ h}$) following i.v. administration (Table 2).

Table 2 Pharmacokinetic parameters of enrofloxacin after administration (10 mg/kg) of native enrofloxacin (i.v. and p.o.) and enrofloxacin SLNs (p.o.) in emus

Variable	Unit	Routes of administration					
		Native enrofloxacin				Enrofloxacin SLNs	
		Intravenous		Oral		Oral	
		Enrofloxacin	Ciprofloxacin	Enrofloxacin	Ciprofloxacin	Enrofloxacin	Ciprofloxacin
β	/h	0.159 ± 0.007	0.152 ± 0.006	0.162 ± 0.015	0.129 ± 0.004	0.054 ± 0.003	0.044 ± 0.005
AUC _{0–t}	µg h/mL	19.553 ± 3.518	1.518 ± 0.258	15.756 ± 1.416	1.423 ± 0.130	29.511 ± 0.880	2.675 ± 0.081
AUC _{0–∞}	µg h/mL	20.085 ± 3.493	1.561 ± 0.262	16.056 ± 1.436	1.496 ± 0.128	30.420 ± 0.760	2.970 ± 0.153
AUMC _{0–t}	µg h ² /mL	90.670 ± 19.068	10.591 ± 2.058	102.756 ± 16.766	10.575 ± 1.106	538.882 ± 36.290	46.035 ± 1.673
AUMC _{0–∞}	µg h ² /mL	104.619 ± 19.920	11.889 ± 2.058	109.083 ± 17.395	12.892 ± 1.063	603.401 ± 27.268	75.657 ± 10.032
MRT	h	5.105 ± 0.216	7.454 ± 0.223	6.616 ± 0.475	8.625 ± 0.173	19.807 ± 0.590	25.463 ± 3.288
MAT	h	–	–	1.511 ± 0.475	–	14.702 ± 0.590	–
V _{d area} /F	L/kg	–	–	3.881 ± 0.234	–	6.186 ± 0.357	–
V _{d area}	L/kg	3.921 ± 1.005	–	3.171 ± 0.269	–	15.291 ± 1.147	–
V _{dss} /F	L/kg	–	–	4.168 ± 0.191	–	6.520 ± 0.196	–
CL _B	L/h kg	0.629 ± 0.164	8.256 ± 2.385	0.507 ± 0.003	6.897 ± 0.509	0.3330 ± 0.007	3.421 ± 0.177
CL _B /F	L/h kg	–	–	0.646 ± 0.052	–	–	–
t _{1/2β}	h	4.364 ± 0.179	4.595 ± 0.163	4.125 ± 0.361	5.393 ± 0.186	13.012 ± 0.717	16.913 ± 2.061
C _{max}	µg/mL	–	0.197 ± 0.029	2.397 ± 0.052	0.169 ± 0.08	3.815 ± 0.059	0.358 ± 0.011
t _{max}	h	–	1.417 ± 0.834	2.167 ± 0.279	3.167 ± 0.167	1.167 ± 0.105	1.33 ± 0.105
AF	%	–	–	79.941 ± 7.147	–	151.462 ± 3.782	–
AUC _{0–t} cipro/ AUC _{0–t} enro		7.764		9.031		9.063	

After oral administration, enrofloxacin peak plasma concentration (C_{max}) of 2.397 ± 0.052 µg/mL was achieved at (t_{max}) 2.167 ± 0.279 h with bioavailability of 79.941 ± 7.147 %, whereas for enrofloxacin SLNs the mean (\pm SE) peak plasma concentration (C_{max}) was 3.815 ± 0.059 µg/mL at 1.167 ± 0.105 h. The $t_{1/2\beta}$, AUC_{0–∞}, AUMC_{0–∞}, MRT, MAT and V_{darea}/F were 3.107, 1.894, 5.531, 9.730, 2.993 and 1.594 times enhanced significantly ($p \leq 0.01$) than the values of native enrofloxacin. Total plasma body clearance (CL_B) and elimination rate constant (β) of drug was significantly ($p \leq 0.01$) decreased by 1.958 and 3.056 times, respectively, in enrofloxacin SLNs compared to native enrofloxacin. In SLNs groups, the bioavailability was 1.895 times higher than the bioavailability recorded for native enrofloxacin. The C_{max} of enrofloxacin SLNs was 1.499-fold higher than those obtained with the native enrofloxacin.

After i.v. and oral administration of native enrofloxacin, the ciprofloxacin AUC_{0–t} was 7.764 and 9.031 % of enrofloxacin AUC_{0–t}, respectively (Table 2). The ratio of AUC_{0–t}cipro/AUC_{0–t}enro after oral administration of enrofloxacin SLNs was 9.063 %. The elimination half-life ($t_{1/2}$) and MRT of the metabolite after administration of native enrofloxacin and enrofloxacin SLNs were longer than those of the parent substance. The clearance of the

active metabolite recorded in this study was faster compared to the enrofloxacin.

The PK/PD integration parameters of C_{max}/MIC and AUC_{0–24}/MIC were calculated from the obtained PK parameters as presented in Table 3.

Discussion

Hot homogenization followed by ultrasonication technique applies high shear stress disrupting lipid particles down to the submicron range. According to Schwarz et al. (1994), a sufficient high-energy input was necessary to break down the droplets into the nanometer range. 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 min, followed by ultrasonication and resulted in the mean (\pm SD) particle size of 154.717 ± 6.149 nm with narrow size distribution.

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 of enrofloxacin are not affected. According to Luo et al.

Table 3 PK/PD parameters of native enrofloxacin and enrofloxacin SLNs considering MICs of 0.05, 0.125, 0.25 and 0.5 µg/mL

Ratio	MIC (µg/mL)	Intravenous (native enrofloxacin)	Oral (native enrofloxacin)	Oral (enrofloxacin SLNs)
C_{max}/MIC	0.05	295.11 ± 44.52*	47.94 ± 1.04	76.29 ± 1.18
	0.125	118.04 ± 17.81*	19.17 ± 0.42	30.52 ± 0.47
	0.25	59.02 ± 8.90*	9.59 ± 0.21	15.26 ± 0.24
	0.5	29.51 ± 4.45*	4.79 ± 0.10	7.63 ± 0.12
AUC_{0-24}/MIC	0.05	391.06 ± 70.35	315.11 ± 28.31	374.43 ± 10.97
	0.125	156.42 ± 28.14	126.05 ± 11.32	149.77 ± 4.39
	0.25	78.21 ± 14.07	63.02 ± 5.66	74.89 ± 2.19
	0.5	39.11 ± 7.03	31.51 ± 2.83	37.44 ± 1.10

* For C_{max} , a value of 14.755 µg/mL (mean peak plasma concentration at 5 min) was used for the calculation

(2006), the size of nanoparticles ranging from 100 to 200 nm was favourable for better per oral performance of incorporated drugs. The particle size of the enrofloxacin SLNs obtained in this study are within the accepted range for oral administration. The preparation of enrofloxacin SLNs did not require any organic solvents, which could be difficult to remove after nanoparticle synthesis. Dilbaghi et al. (2013) formulated enrofloxacin-loaded SLNs by solvent diffusion technique using an organic solvent. The simple manufacturing techniques such as hot homogenization and ultrasonication make it possible to produce SLNs in a large scale and reproducible manner. The result suggests that the hot homogenization and ultrasonication method is feasible and compatible for preparing enrofloxacin SLNs.

A narrow particle size distribution was an indication of nanoparticles stability and homogeneous dispersion (Olb-rich et al. 2002). PDI values ranging from 0 to 0.5 were considered to be monodisperse and homogenous, but those of more than 0.5 indicated nonhomogeneity and polydispersity (Zhang et al. 2009; Anton et al. 2008). In the present study, the particle size distribution was monodisperse and homogenous as the formulation has less mean (\pm SE) PDI of 0.42 ± 0.11 .

Nanoparticle with zeta potential values greater than +25 mV or less than -25 mV typically have high degrees of stability due to electric repulsion between particles. Dispersions with a low zeta potential value aggregate due to Van Der Waal inter-particle attraction (Muller et al. 2000). In this study, the mean (\pm SD) zeta potential of -24.90 ± 1.00 mV was recorded and could provide proper stability to the enrofloxacin SLNs. According to Schwarz and Mehnert (1997) and Zimmermann et al. (2000), the negative charge of zeta potential was conferred by the lipids used in the SLNs. In agreement with this report, the tripalmitin utilized in this study provided negative charge of zeta potential.

AFM images revealed spherical and circular shape with the presence of some particle aggregates. The presence of

aggregates might be due to redistribution of particles after preparation. The images of AFM and TEM represented that the particles ranged from 100 to 200 nm and well dispersed with smooth surfaces.

The enrofloxacin SLNs obtained in the present study had relatively medium drug entrapment efficiency (59.67 %). To get sufficient loading capacity, the drug should have sufficiently high solubility in the lipid melt (Bunjes et al. 2002). The percentage encapsulation efficiency data obtained in this study are consistent with the findings of Xie et al. (2011b).

In vitro release data obtained under sink conditions are consistent with drug release reported from different SLNs by Ji et al. (2011) and Xie et al. (2011b). The initial fast release (burst effect) could be attributed to the presence of a small fraction of untrapped 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 (Muller et al. 2000; Mehnert and Mader 2001).

No overt signs of toxicity or abnormal behaviour were observed when enrofloxacin SLNs were administered to emus through the oral route. Published data regarding the pharmacokinetics of drug-loaded SLNs in ratites and other domestic animals are limited. Hence, the results obtained in the present study are interpreted by comparing the pharmacokinetic results reported for laboratory animals administered with SLNs. The plasma concentration of enrofloxacin after enrofloxacin SLNs administration in emus showed biphasic release pattern. The initial fast (burst release) release of the drug could be due to desorption and diffusion of enrofloxacin accumulated at the oil–water interface and in the outer shell of nanoparticles (Xie et al. 2008; Muller et al. 2000; Han et al. 2009; Wang et al. 2011). The initial release should be sufficiently rapid to

ensure that therapeutic drug levels are achieved in a timely manner *in vivo*. The subsequent slow release was mainly due to the slow diffusion of drug molecules through the lipid matrix of the nanoparticles (Mehnert and Mader 2001; Muller et al. 2000) which maintains the effective therapeutic drug concentrations for a longer period. In the present study, the sustained release performance of enrofloxacin-loaded SLNs provided plasma concentrations of enrofloxacin exceeding 0.012 $\mu\text{g/mL}$ for 60 h, which was therapeutically effective for many common pathogens (Prescott and Yielding 1990). The bi-exponential release of enrofloxacin from SLNs observed in this study is in accordance with the reports of Xie et al. 2011b (ofloxacin-loaded palmitic acid SLNs in mice), Xie et al. 2011a (enrofloxacin-loaded palmitic acid SLNs in mice), Kurtz et al. 1994 (doxorubicin-loaded SLNs in rats) and Pandita et al. 2011 (paclitaxel-loaded in SLNs in mice).

The plasma concentration of enrofloxacin recorded in this study was not consistent with the *in vitro* release profile. The *in vivo* degradation of SLNs could be the main reason it is an important parameter in determining drug release *in vivo* (Olbrich et al. 2002).

Significantly higher ($p < 0.01$) $AUC_{0-\infty}$ and C_{\max} values with shorter ($p < 0.01$) t_{\max} was observed for enrofloxacin SLNs compared to native enrofloxacin after administration of the same dose in emus. The increased absorption of enrofloxacin-loaded SLNs might have been contributed via six possible mechanisms. First, the SLNs formulations entering into the GI tract stimulated secretions of bile salts (BS), phospholipids (PL) and cholesterol, due to the presence of lipids in the formulation (Fleisher et al. 1999; Dahan and Hoffman 2008). The SLNs products along with the gastric shear movement formed a crude emulsion. It promoted the solubilization of the co-administered lipophilic drug. Secondly, the SLNs are degraded by lipase/co-lipase complex anchored onto their surface. Triglycerides of SLNs are degraded into surface-active monoglycerides, forming micelles. Drugs present in the degrading lipids may be entrapped in the micelles. The micelles can interact with bile salts present in the gut, leading to the formation of mixed micelles. Then, the lipids are absorbed via chylomicron formation primarily into the lymphatic system and simultaneously the drug goes with the lipid, which is called ‘Trojan horse effect’ (Muller et al. 2000). Thirdly, the cellular lining of the gastrointestinal tract is composed of absorptive enterocytes interspersed with membranous epithelial (M) cells. M cells that cover lymphoid aggregates, such as Peyar’s patches, take up microparticles by a combination of endocytosis or transcytosis (Norris et al. 1998; Andrianov and Payne 1998). Fourth, the permeability of gut wall was enhanced by lipids present in the SLNs and thus increased the drug absorption (Constantinides and Wasan 2007). Fifth, the activity of

p-glycoprotein efflux transporters in the GI wall was suppressed by lipids and surfactants and, hence, increased the fraction of drug absorbed (Dintaman and Silverman 1999; Nerurkar et al. 1996). Sixth, the lipids in the GI tract provoked delay in gastric emptying which resulted in increased residence time of the co-administered lipophilic drug in the small intestine. This enabled better dissolution of the drug at the absorptive site and thereby improved the absorption (Citters and Lin 1999).

The MRT for enrofloxacin SLNs was significantly increased compared to native enrofloxacin in this study. According to Duchene and Ponchel (1997); Vasir et al. (2003), the adhesive properties of nanoparticles with gastrointestinal tract wall increase their residence time in the gastrointestinal tract. Moreover, Xie et al. (2011b) explained that nanoparticles could protect the drug from chemical and enzymatic degradation and gradually release drug from the lipid matrix into blood, resulting in a several-fold increase in MRT. Mehnert and Mader (2001) reported that the drug transported as lipid vesicles remained intact for extended periods and, thereby, resulted in prolonged release of the encapsulated drug.

The relative bioavailability obtained in this study (189.47 %) is comparable to that reported by Suresh et al. (2007) for lovastatin SLNs (173.0 %). In concurrence with Xie et al. (2011a), the triglycerides utilized in the SLNs formulation enhanced the lymph formation and simultaneously promoted the lymph flow rate. The possible mechanisms for increased absorption of enrofloxacin SLNs discussed under $AUC_{0-\infty}$ could be the reasons for higher bioavailability obtained in the present study. According to Suresh et al. (2007), the increased relative bioavailability was due to transport of SLNs by intestinal lymph which avoided first pass hepatic metabolism of drugs. Investigation of lymph at regular intervals for enrofloxacin could have provided valuable information in this study and support the suggestion of Suresh et al. (2007) regarding lymphatic transport of SLNs.

The result after administration of enrofloxacin SLNs indicated longer $t_{1/2}$ and prolonged clearance (CL_B) of enrofloxacin in emus. The increase in AUC and F reflects the higher availability of enrofloxacin in SLNs-treated groups, which is attributed to the low clearance of enrofloxacin in these birds.

The degree of metabolism varies considerably across species (Cox et al. 2004). In the present study, ciprofloxacin AUC_{0-t} was lower than 10 % of enrofloxacin AUC_{0-t} after administration of native enrofloxacin and enrofloxacin SLNs. Similar results were obtained by De Lucas et al. (2004) in ostrich for native enrofloxacin. Helmick et al. (1997) reported that the plasma concentration of metabolite ciprofloxacin was not consistent in emus. However, Anadon et al. (1995) observed a high hepatic conversion of

enrofloxacin to ciprofloxacin in chicken. The ratio of AUC_{0-t} cipro/ AUC_{0-t} enro recorded in this study indicated limited, but rapid conversion of ciprofloxacin in the liver of emu birds after native enrofloxacin and enrofloxacin SLNs administration.

The use of SLNs sustained the therapeutic concentrations up to 48 h, and enhanced the bioavailability and volume of distribution besides appreciably increasing the AUC/MIC ratio. However, these derived values do not take into account the contribution made by the active metabolite ciprofloxacin and therefore underestimate enrofloxacin efficacy. From these results, it is obvious that the use of enrofloxacin SLNs administration at 10 mg/kg every 48 h is able to produce an ideal clinical outcome against pathogens susceptible to 0.125 $\mu\text{g/mL}$.

From the results of the executed experiments, it can be concluded that hot homogenization coupled with ultrasonication method is suitable for producing SLNs with optimal particle size, shape, PDI, zeta potential, drug loading and encapsulation efficiency. In vitro release of enrofloxacin SLNs exhibited biphasic pattern with an initial burst release followed by sustained release over 96 h. Enrofloxacin SLNs were rapidly absorbed after oral bolus administration and therapeutic concentrations in plasma were achieved for an extended period of time. SLNs significantly improved the bioavailability, $t_{1/2\beta}$, $AUC_{0-\infty}$, V_{darea}/F and MRT while significantly decreasing the CL_B and elimination rate constant compared to native enrofloxacin. In the present study, the SLNs played an important role in the drug delivery system and significantly changed the in vivo pharmacokinetic behaviour of drug molecules. The results of pharmacokinetic parameters of enrofloxacin SLNs strongly support the potential application of SLNs in emus as sustained delivery system for enrofloxacin.

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Conflict of interest The authors declare no conflicts of interest.

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