

## **IN VITRO RELEASE AND PHARMACOKINETICS OF ENROFLOXACIN PHBV MICROSPHERE IN RATS**

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**Abstract:** Enrofloxacin loaded Poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) microspheres were synthesized by oil/water single emulsion technique. Encapsulation efficiency, *in vitro* release and pharmacokinetics of enrofloxacin loaded PHBV microspheres were evaluated in rats (5 mg/kg body weight). Optical microscopy demonstrated that the enrofloxacin loaded PHBV microspheres were regular and spherical in shape. The mean drug encapsulation efficiency of enrofloxacin loaded PHBV microspheres was  $43.03 \pm 2.36\%$  and about  $97.19 \pm 0.35\%$  enrofloxacin was released *in vitro* during first 24 hours due to burst release. While remaining amount of enrofloxacin was slowly release up to 13 days. After intramuscular administration of enrofloxacin and enrofloxacin loaded PHBV microsphere in rats (5 mg/kg body weight) the drug concentration of  $0.02 \pm 0.001$  and  $0.03 \pm 0.001$   $\mu\text{g/ml}$  in plasma was detected up to 6 and 72 h respectively and beyond then the drug was not detected in plasma. Significant increase values of elimination half-life, apparent volume of distribution, area under curve, area under first moment curve, mean residence time and significant decrease in total body clearance were observed in rats given enrofloxacin loaded PHBV microspheres compare to conventional enrofloxacin. It is concluded that it is possible to prolong the release of enrofloxacin through its incorporation into PHBV microspheres and maintain the therapeutic concentration over extended time periods.

**Keywords:** Enrofloxacin, Poly 3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV), microspheres, Encapsulation efficiency.

### **Introduction**

Antibiotics are an important class of therapeutic agents, used for the treatment of various infectious diseases in a variety of domestic and wild animals. There are many issues and problems associated with the administration of antibiotics to animals still exist. It includes the requirement of repeated drug administration to maintain plasma concentrations at an effective level and in some cases, the use of high doses to reach therapeutic concentrations at the site of action, can lead to toxic drug levels in the plasma as well as to adverse reactions (Ahmed

and Kasraian, 2002; Sun *et al.*, 2004). This compels us to develop drug delivery system, which can maintain the plasma concentrations of antibiotics within a safe therapeutic-window for extended-time periods and reduce number of doses and improve the therapeutic effectiveness in veterinary medicine (Vilos and Velasquez, 2012).

Polymeric microparticles with biodegradability and biocompatibility such as PHBV [Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)] provide platform for development of sustain release product based on microencapsulation technique. (Edlund and Albertsson, 2002). Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is a natural biocompatible polymer obtained from large-scale bacterial systems, and had shown promising results for biomedical engineering and drug delivery (Mitomo *et al.*, 2001; Yang *et al.*, 2015). Enrofloxacin, second generation bactericidal fluoroquinolone, widely used in the treatment of diseases caused by aerobic gram negative, gram positive bacteria and other pathogens like *Mycoplasma*, *Chlamydia* and *Rickettsia* (Brown, 1996; Martinez *et al.*, 2006). Low commercial cost, excellent pharmacokinetic properties such as high bioavailability and high volume of distribution (Modi *et al.*, 2012), bactericidal activity, lower minimum inhibitory concentration and its physiochemical properties favors enrofloxacin as good model to explore possibility of development of long or sustained release formulation for maximizing therapeutic effect using polymer. Therefore, in an effort to evaluate sustained release properties of enrofloxacin microsphere, the present study was undertaken to prepare enrofloxacin loaded PHBV microspheres and evaluate *in vitro* drug release and pharmacokinetics of enrofloxacin loaded PHBV microspheres in rats.

## **Materials and Methods**

### **Chemicals and reagents**

Enrofloxacin, Poly (3-hydroxybutyric acid-co-3- hydroxyvaleric acid) and Poly (vinyl alcohol) were obtained from Sigma-Aldrich St. Louis, USA. Dichloromethane, acetonitrile, triethylamine and ortho-phosphoric acid were purchased from Merck Specialities Private Limited, Mumbai. Phosphate buffered saline (PBS) was procured form Himedia Private Limited, Mumbai, India. Enrofloxacin (100mg/ml, Enrocin, Zoetis India Limited, Haridwar, India) injectable drug was procured form local market.

### **Experimental animals**

Adult albino rats (n=18) weighing between 350 to 500 grams obtained from Jai Research foundation, Vapi, Gujarat and maintained at the laboratory animal house, College of Veterinary Science and Animal Husbandry, Navsari Agricultural University, Navsari. They

were kept under constant observation for two weeks prior to commencement of the experiment. Standard ration and water was provided *ad libitum*. All necessary management procedures were adopted to keep the animals free from stress. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC).

### **Synthesis of Enrofloxacin Microsphere**

Enrofloxacin loaded PHBV microspheres were synthesized by oil/water single emulsion technique. Briefly, 50 mg enrofloxacin was dissolved in 2.5 ml dichloromethane containing 100 mg PHBV. This resulting organic phase was then injected into aqueous phase containing polyvinyl alcohol (0.5%) to form oil in water emulsion. The high speed homogenizer was used for the emulsification operated at 25000 rpm for 60 seconds. The final emulsion was subsequently stirred at 800 rpm for 3 h at room temperature to evaporate dichloromethane. The microspheres were washed twice with deionised water and collected by centrifugation. Then microspheres were lyophilized and stored for further use (Mao *et al.*, 2008). Morphology of the microsphere was observed under optical microscope.

### **Drug encapsulation efficiency**

Experimentally, 2 mg of enrofloxacin loaded PHBV microsphere was dissolved in 200  $\mu$ l dichloromethane, followed by the addition of 800  $\mu$ l mobile phase (1% triethylamine in HPLC water and acetonitrile (82:18 v/v) adjusted to pH 3.0 with ortho-phosphoric acid) to precipitate the polymer. The resulting suspension was centrifuged (13000 rpm for 5 min) and the supernatant was subjected to high performance liquid chromatography (HPLC) (Yang *et al.*, 2015).

The experimental and Theoretical drug loading was calculated using the formula as follow:

Experimental drug loading = (Weight of detected drug) / (Weight of drug loaded microparticles)

Theoretical drug loading = (Weight of added drug) / (Weight of added drug + Weight of added polymer)

The encapsulation efficiency (EE%) was calculated as the ratio between the experimental drug loading and the theoretical drug loading.

### **In vitro release assay**

A portion of 5 mg of lyophilized microsphere was transferred into Eppendorf tube and suspended in 1.5 ml phosphate buffer solution. The samples were placed in water bath shaker at 37°C with continuous shaking. At predetermined time points (0.083, 0.25, 0.50, 0.75, 1, 1.5, 2, 4, 6, 8, 24, 48, 72, 96, 144, 168, 192, 216, 240, 264, 288, 312, 336 hours), the samples

(750 $\mu$ l) was collected and centrifuged (13000 rpm for 5 min). 500 $\mu$ l of supernatant was taken and subjected to analysis by high performance liquid chromatography (HPLC). Remaining 250 $\mu$ l and 500 $\mu$ l of fresh phosphate buffer solution was added to make 1.5 ml volume in Eppendorf tube (Bazzo *et al.*, 2012).

### **Pharmacokinetics of enrofloxacin microsphere**

Group I (n=6) rats were treated with conventional enrofloxacin (5 mg/kg body weight, intramuscular) and group II (n=12) animals were treated with enrofloxacin loaded PHBV microsphere suspended in sterile saline solution (5 mg/kg body weight, intramuscular). Blood samples were collected in K<sub>3</sub>EDTA vials, at different time interval (0.17, 0.50, 0.75, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96 hours) after the administration of enrofloxacin or enrofloxacin microsphere from retro orbital plexus. Plasma was separated soon after collection by centrifugation at 3000 revolution per min (rpm) for 10 minutes at 4°C. Separated plasma samples were transferred in labeled cryovials and stored at -20°C for further use. Samples were subjected to high performance liquid chromatography within 24 to 36 hours. (Bazzo *et al.*, 2012; Patel *et al.*, 2012).

### **Measurement of enrofloxacin**

Plasma proteins were precipitated by mixing 100  $\mu$ l of plasma sample and 100  $\mu$ l of acetonitrile in a clean microcentrifuge tube. The mixture was vortexed for 1 minute and centrifuged at 3000 rpm for 10 minutes at 4°C. The 20  $\mu$ l supernatant was injected into HPLC system (Shimadzu, LC-20AP) through auto sampler. Enrofloxacin was assayed from plasma by adopting procedure as described by Patel *et al.* (2012) with minor modifications. Mobile phase consisted of a mixture of 1% triethylamine in HPLC water and acetonitrile (82:18 v/v) adjusted to pH 3.0 with ortho-phosphoric acid. The eluent was monitored at 278 nm with a flow rate of 1.5 ml/min. To fulfill the requirement of partial validation of modified method, intraday and interday absolute recovery, precision and accuracy were evaluated including mean and coefficient of variance (C.V.) for five standard concentrations (100, 25, 3.125, 0.195 and 0.024  $\mu$ g/ml). At all concentrations, C.V. was less than 3.82% and mean correlation coefficient ( $R^2$ ) was 1. The limit of detection and limit of quantification were observed as 0.006 and 0.0024  $\mu$ g/ml, respectively. Various pharmacokinetic parameters calculated from plasma concentration time profile after single dose intramuscular administration of enrofloxacin and enrofloxacin loaded PHBV microsphere (5 mg/kg body weight) in rats using software PK solution (version 2.0).

## Results and Discussion

Optical microscopy demonstrated regular and spherical shape of enrofloxacin loaded PHBV microspheres (Figure.1). In present study, the mean encapsulation efficiency of  $43.03 \pm 2.36$  % was observed for enrofloxacin loaded PHBV microspheres (Table 1) synthesized by oil/water single emulsion technique. Whereas Yang *et al.* (2015) reported lower encapsulation efficiency value ( $27.57\% \pm 2.87\%$ ) following spray drying technique for preparation of enrofloxacin loaded PLGA microspheres. However, Bazzo *et al.* (2012), Mao *et al.* (2008) and Sindhuri and Purushotamann, (2011) prepared ibuprofen loaded PHBV and PLA polymeric microspheres, ABT 627 (hydrophobic model drug) loaded PLGA microspheres, and norfloxacin loaded carbopol and sodium carboxyl methyl cellulose microspheres respectively and reported higher encapsulation efficiency value compared to present study. This variation in encapsulation efficiency may be due to difference in physiochemical properties of drug (lipid solubility) and polymer (Lizondo *et al.*, 1997).

The *in vitro* release results of present study showed about  $97.19 \pm 0.35\%$  of enrofloxacin released in first 24 hr while remaining amount released upto 13 day (Figure 2). Whereas, Yang *et al.* (2015), Bazzo *et al.* (2012) and Mao *et al.* (2008) reported release upto 12 h, 19 h and 30 days with enrofloxacin loaded PLGA microsphere, ibuprofen loaded PHBV microsphere and ABT 627 (hydrophobic model drug) loaded PLGA microspheres respectively. Reason of high initial burst in present study may be due to non-homogeneous dispersion in the polymeric matrix and surface adsorption of drug on microsphere (Mao *et al.*, 2008; Vilos and Velasquez, 2012).

Following intramuscular administration enrofloxacin loaded PHBV microsphere in rats (5 mg/kg body weight), drug concentration ( $0.03 \pm 0.001$  µg/ml) was detected upto 72 h (Figure 3). In accordance to present study, Vilos *et al.* (2012) and Kumar *et al.* (2015) reported drug plasma concentration up to 72 h (intraperitoneal) and 60 h following administration of ceftiofur PHBV microsphere (intramuscular) in rats and enrofloxacin solid lipid nanoparticles (PO) in emu birds respectively. However Bazzo *et al.* (2012) reported drug concentration upto 12 h following ibuprofen loaded PHBV and PLA in wistar rats. In addition to this Niteshkumar (2011) reported plasma concentration up to 72 h (intraperitoneal) with long acting commercial available enrofloxacin in rats. It indicates that microsphere preparation in the *in vivo* condition can be tool for preparation of sustained release formulation for veterinary application.

Pharmacokinetic parameters of enrofloxacin and enrofloxacin loaded PHBV microsphere are shown in table 2. Following intramuscular administration enrofloxacin loaded PHBV microsphere in rats (5 mg/ kg body weight), significant increase in elimination half-life ( $t_{1/2\beta}$ ), apparent volume of distribution ( $V_{d_{area}}$ ), area under curve (AUC), area under first moment curve (AUMC) and mean residence time (MRT) and significant decrease in total body clearance ( $Cl_B$ ) were observed in rats in comparison to plain enrofloxacin administered rats. This indicate potential of enrofloxacin loaded PHBV microsphere for preparation of sustain release formulation. In accordance to present study lower value of total body clearance ( $0.3330 \pm 0.07$  L/h/kg) and higher AUC ( $30.420 \pm 0.760$   $\mu\text{g}\cdot\text{h}/\text{ml}$ ) were reported in emu birds following oral administration of enrofloxacin solid lipid nanoparticles (Kumar *et al.*, 2015), However lower elimination half-life ( $t_{1/2\beta}$ : 0.99 h) and AUC ( $2.11$   $\mu\text{g}\cdot\text{h}/\text{ml}$ ) was observed following intravenous administration of enrofloxacin loaded PLGA microsphere (Yang *et al.*, 2015). In addition to this Niteshkumar, (2011) reported similar values for elimination half life ( $42.84 \pm 2.43$  h), apparent volume of distribution ( $6.76 \pm 0.30$  L/kg), AUC ( $46.44 \pm 4.39$   $\mu\text{g}\cdot\text{h}/\text{ml}$ ) and mean residence time ( $66.87 \pm 2.48$  h) following intraperitoneal administration of commercial available long acting preparation of enrofloxacin in albino rats.

Integrating the pooled enrofloxacin loaded PHBV microsphere pharmacokinetics data and MIC<sub>90</sub> values available in literature (Asuquo and Piddock, 1993; McKellar *et al.*, 1999) for *Escherichia coli* and *Pasteurellamultocida*, intramuscular administration of enrofloxacin loaded PHBV microsphere (5 mg/kg) is sufficient to maintain plasma concentration of drug above the MIC<sub>90</sub> up to 72 h when it is administered intramuscularly. Whereas for the *Staphylococcus intermedius* (whose MIC<sub>90</sub> value is 0.125  $\mu\text{g}/\text{ml}$ ) enrofloxacin loaded PHBV microsphere (5 mg/kg) is sufficient to maintain plasma concentration of drug above the MIC<sub>90</sub> up to 36 h when it is administered intramuscularly.

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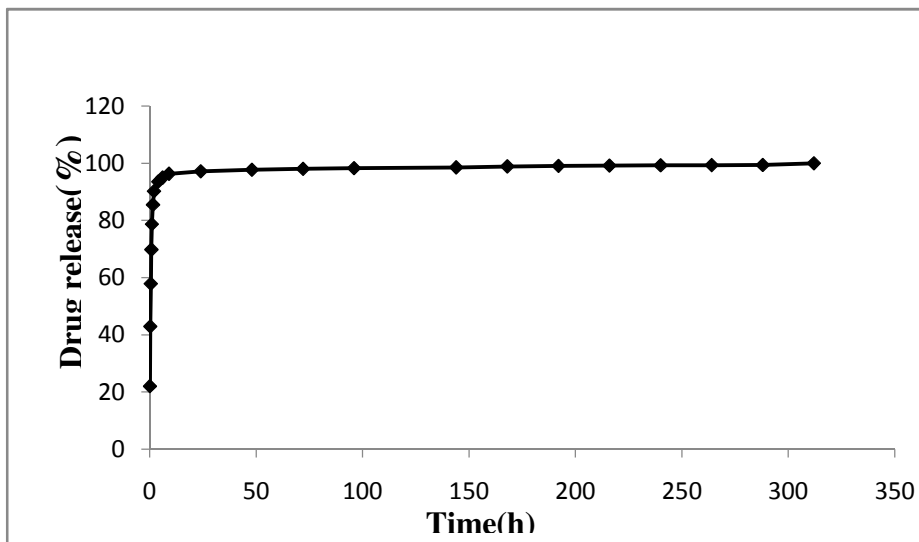
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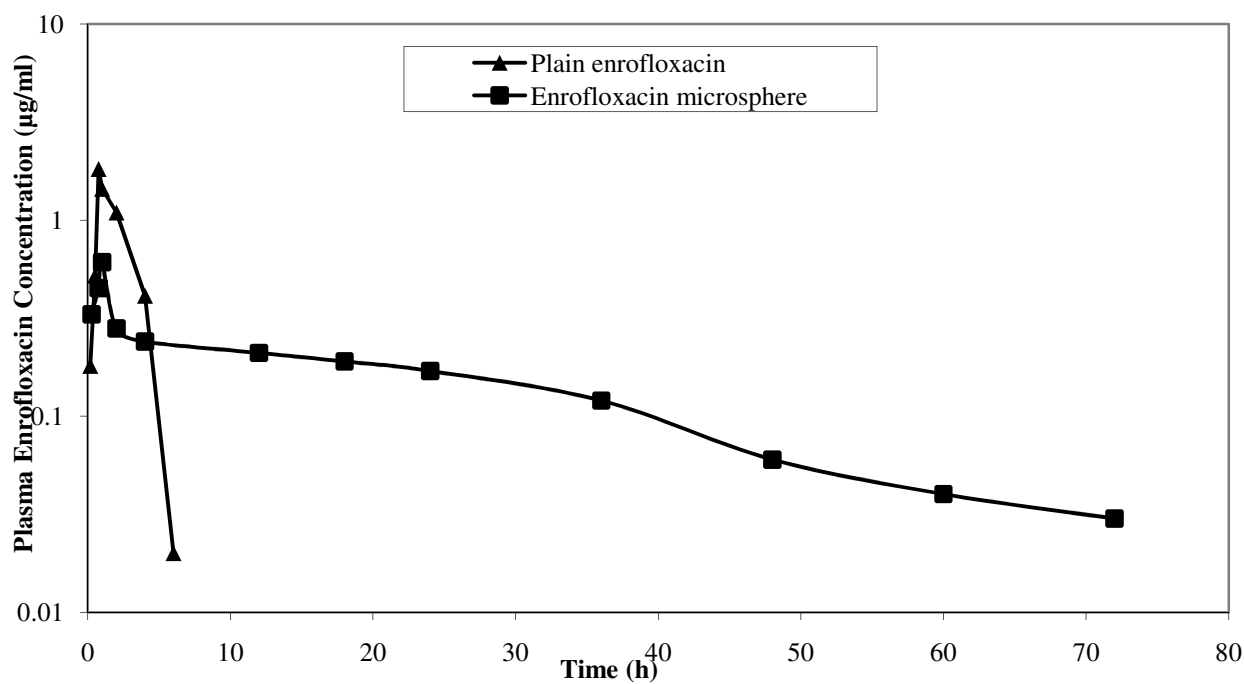


**Figure 1: Optical photomicrograph of the enrofloxacin loaded PHBV microspheres (100X)**





**Figure 2:** *In vitro* drug release results for the enrofloxacin loaded PHBV microsphere. Each point represents mean  $\pm$  SE.



**Figure 3:** Comparative Semilogarithmic plot of enrofloxacin concentration in plasma versus time following single dose intramuscular administration of enrofloxacin and PHBV loaded enrofloxacin microsphere. Each point represents mean  $\pm$  S.E.

**Table 1: Drug encapsulation efficiency enrofloxacin loaded PHBV microspheres for different batches.**

Batch/ Calculation	Batch- A	Batch-B	Batch-C	Batch-D	Batch- E	Batch-F	Mean± SE%
Theoretical drug loading(%)	33.0	33.0	33.0	33.0	33.0	33.0	33.0 ± 0.00
Experimental drug loading(%)	16.5	12.4	13.5	12.1	14.3	16.4	14.2 ± 0.78
Drug encapsulation efficiency(%)	50.00	37.57	40.90	36.66	43.33	49.69	43.03 ± 2.36

**Table 2: Comparison of pharmacokinetics parameters (Mean ± S.E) of enrofloxacin and enrofloxacin microsphere after intramuscular administration (5mg/kg BW) in rats.**

Pharmacokinetic Parameter	Unit	Enrofloxacin (Mean ± S.E)	Enrofloxacin loaded PHBV microsphere (Mean ± S.E)
K <sub>a</sub>	h <sup>-1</sup>	1.51 ± 0.028	1.66 ± 0.0392
β	h <sup>-1</sup>	1.41 ± 0.042	0.04 ± 0.002**
t <sub>1/2K<sub>a</sub></sub>	h	0.46 ± 0.009	0.67 ± 0.239
t <sub>1/2β</sub>	h	0.49 ± 0.015	17.13 ± 0.659**
C <sub>max</sub>	μg /ml	2.62 ± 0.181	0.61 ± 0.031**
T <sub>max</sub>	h	0.96 ± 0.042	1.00 ± 0.000
AUC <sub>(0 - ∞)</sub>	μg.h/ml	4.04 ± 0.035	9.73 ± 0.239**
AUMC	μg.h <sup>2</sup> /ml	8.12 ± 0.126	264.72 ± 8.608**
Vd <sub>(area)</sub>	L /kg	0.77 ± 0.124	12.72 ± 0.460**
Cl <sub>(B)</sub>	L/h <sup>1</sup> /kg	1.07 ± 0.164	0.52 ± 0.012*
MRT	h	2.01 ± 0.014	27.18 ± 0.363**

\*Significant at p&lt;0.05, \*\*highly significant at p&lt;0.01