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Pharmacokinetic Studies of an In Situ Forming Gel System for Controlled Delivery of Enrofloxacin In Pigs

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Abstract

The objective of this study was to evaluate the pharmacokinetic characteristics of enrofloxacin (ENR) *in situ* forming gel in pigs following a single intramuscular administration. Twelve healthy pigs were randomly divided into two groups (6 pigs per group), then administered 20 mg/kg body weight (b.w.) ENR *in situ* forming gel and 5 mg/kg b.w. ENR conventional injection respectively. High-performance liquid chromatography was used to determine ENR plasma concentrations. The important pharmacokinetic parameters of ENR *in situ* forming gel and conventional injection in pigs were as follows: MRT (mean residence time) 49.39 ± 8.97 h verse 11.75 ± 0.90 h, C_{max} (maximal concentration) 1.71 ± 0.24 μg/mL verse 1.76 ± 0.32 μg/mL, $t_{1/2\lambda z}$ (terminal elimination half-life) 42.92 ± 9.80 h verse 9.17 ± 0.61 h. Results suggest that the C_{max} of ENR *in situ* forming gel was in the range of ideal bactericidal concentration, and values of $t_{1/2\lambda z}$ and MRT were greatly extended, which could maintain the effective blood drug concentrations for a long time. Therefore, the pharmacokinetic characteristics of ENR *in situ* forming gel in pigs following a single i.m. administration were ideal. And the *in situ* forming gel system seems to be a feasible approach for ENR long-acting delivery.

Keywords: Enrofloxacin; Sustained delivery; *In situ* forming gel; Pharmacokinetic; Pigs

Introduction

Enrofloxacin (ENR), a synthetic agent of fluoroquinolone developed specifically for veterinary use, exhibits a broad spectrum of antibacterial activity [13]. It is active at low concentrations, compared with other classes of antimicrobial agents [14,22]. Similar to other fluoroquinolones, ENR produce their bactericidal properties by inhibiting the type IIA topoisomerase (DNA gyrase/topoisomerase II and topoisomerase IV), thereby preventing supercoiling and synthesis of bacterial DNA [9]. Due to the excellent antibacterial scope and activity, this kind of drugs has been widely applied in veterinary clinical since the date of listing.

Studies on the *in vivo* process of ENR conventional injection in animals showed that it had good absorption and high drug concentrations within the organism by injection, then it's suitable for treatment and prevention of bacterial infection in tissues [6, 10, 15, 36]. But the terminal elimination half-life ($t_{1/2\lambda z}$) in pigs was less than 9 ~ 12 h after i.m. (intramuscular) or s.c. (subcutaneous) injection [1, 2, 27, 31, 35]Thereby, the recommended dosing regimen of ENR conventional injection was administrated a single 2.5 ~ 5 mg/kg b.w. per day or twice a day, meaning that a course of treatment needs multiple dosing [40]. Obviously, in the clinical application the conventional injection caused a lot of inconvenience, for example, repeated stress response maybe worsen animals' illness or result in more disease, and the drugs' action was not sufficient when it was in peaks and valleys, what's more, frequency administration spent great human, financial and material resources.

On the other hand, studies revealed that the fluoroquinolones had biphasic concentration-dependent characteristics. When the blood drug concentrations were far more than the MIC (minimum inhibition concentration), they didn't have the killing effect, only had bacteriostatic action [4]. Then the drugs' action could not be exerted sufficiently. It also brings new challenges to the development of long-acting injection of this drug. The preparation methods of traditional long-acting injection, such as the process for making suspension or oil, were to increase blood drug concentration by adding the dose, which maybe also play against the drug's efficacy. For these reasons, there is a need for at least drug administration as possible, followed by longer duration of therapeutically effective drug concentration in the body.

Injectable *in situ* forming systems have received considerable interest over the past few years, which had many distinct advantages including simple operation, low cost, and easy industrialized production [8, 20, 21, 24, 30]. The reversal thermal gelation (RTG) phenomenon constitutes one of the most promising strategies for the development of injectable drug delivery systems. The poloxamer 407 (P407) thermosensible *in situ* gels display low viscosity at ambient temperature, allowing the gel to flow freely into the body through a syringe during injection, and exhibit a sharp viscosity increase following a certain temperature rise, producing a semi-solid gel as the drug ' deposit ' at body temperature [25, 33]. Drugs release slow along with the gel to achieve the purpose of sustained-release. The *in vitro* process of thermo sensible *in situ* gel form of many drugs indicated that the release of this drug could sustain 4 ~ 7 days [23,28,29], which highly accorded with the medication cycle (3 ~ 5 days) in veterinary clinical application.

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To avoid the disadvantages of the ENR conventional injection, we developed a thermo sensible *in situ* forming gel with moderate poloxamers (P407 and P188) for ENR, which have the advantage of being injected as a liquid that changes into a solid gel immediately after injection. Through repeated experiments, we achieved that the gelation temperatures of the form we developed were 34 ± 0.5°C, and the gelation times were 7.5 ± 0.5 s in 37°C, *in vitro* release was about 40.7% in 24 h, and followed by sustained release was up to 100% within 144 h, which confirmed that the *in vitro* release of this form was good. Therefore, the objectives of this study were to evaluate pharmacokinetic characteristics of this form in pigs following a single IM administration, and then promote the use of it in veterinary clinical treatment and prevention.

Materials and methods

Drugs and reagents

98.5% (w/v) ENR was purchased from Guobang Pharmaceutical Corporation (Zhejiang, China). 99.9% (w/v) ENR analytical standards (Batch number, H0081206) were purchased from the China Institute of Veterinary Drug Control (Beijing, China). 5% (w/v) ENR injection was purchased from Hengtong Animal Pharmacy (Sichuan, China). 20% (w/v) ENR injectable *in situ* forming gel (Gelation temperatures, 33.5 ~ 34.5°C) was home-made with moderate poloxamers (P407 and P188). Acetonitrile was of high-performance liquid chromatography grade and purchased from Merck Corporation (Darmstadt, Germany). All other reagents used for extraction and analysis were analytical reagent grade or better and were commercially available.

Animals

Pharmacokinetics evaluation was performed on twelve clinically-healthy crossbred pigs (Duroc × Landrace × Yorkshire) weighing 10 ± 1.6 kg. In this trial, they were housed in semi-contained pens with access to water ad libitum and a commercial non-medicated chow at scheduled times. Pigs enrolled in the studies had no previous administration to any antibiotic and no drugs were given to the animals during the acclimation or experimental periods. All experimental procedures involving animals were conducted following the guidelines of Nanjing Agricultural University (Nanjing, China) Animal Ethics Committee.

Experimental design

When pigs were housed in experiment condition for a week of environmental adaptation, they were randomly divided into two groups (6 pigs per group, half mare and half female). Prior to ENR administration, all pigs were weighed and blood samples were taken from the precaval vein for control. For group one, each pig was administered a single i.m. injection in gluteal of 20 mg/kg b.w. ENR *in situ* forming gel and blood samples were collected from the precaval vein of each pig and collected in tubes containing heparin as anticoagulant at 0.25, 0.5, 2, 5, 9, 12, 24, 36, 48, 60, 72, 96, 120, 144, 168 and 180 h after dosing. For the other group, each pig was administered a single i.m. injection in gluteal of 5 mg/kg b.w. ENR conventional injection and blood samples were taken from the precaval vein of each pig and collected in tubes with anticoagulant (heparin) at 0.25, 0.5, 2, 5, 9, 12, 24, 36, 48, 60, 72, 84 and 96 h after dosing. Samples were centrifuged within 1 h after collection and plasma samples were stored frozen (-20°C) until analysis. All the samples were analyzed within one week after the experiments.

Analytical method

Apparatus conditions: The HPLC system (Agilent 1200 series; Agilent Technologies Co. Ltd., Palo Alto, Santa Clara, CA, USA) comprised a G1311A quaternary pump, a G1328B MAN injector, a G1314B UV detector, a G1316A column heater and online/offline analysis software. The UV detector was set to monitor 278 nm. Chromatographic separations were performed on Eclipse XDB-C18 column (5-μm, 250 × 4.6 mm). The column temperature was maintained at 35°C. The composition of the mobile phase was phosphoric acid (adjust pH to 3.5 with triethylamine; 0.1 M)-acetonitrile (84: 16, v/v). The flow rate of the mobile phase was set to 0.8 mL/min.

Sample preparation: The extraction method was performed as described by Guo M [18] with some modifications. Briefly, the frozen plasma samples (0.2 mL) were thawed at room temperature and added 2 mL of methylene chloride. The mixture was mechanically shaken for 3 min and centrifuged for 10 min at 8000 × *g*, and then the lower layer was manually transferred into a tube. Then the organic layer was evaporated at 50°C under nitrogen and the residue was reconstituted in 0.2 mL of mobile phase. At last, the samples were vortex mixed for 5 min, centrifuged at 12000 × *g* for 10 min and 20 μL volumes was injected into HPLC system.

Pharmacokinetic data analysis

ENR plasma concentration verse time date of each animal was subjected to the non-compartmental analysis (NCA) using the computer program WinNonlin version 5.2 (Pharsight, CA, USA). Lambda z is a first-order rate constant associated with the terminal (log linear) segment of the curve. It was estimated by the linear regression of the terminal data points. The terminal elimination half-life ($t_{1/2\lambda z}$) was calculated by $t_{1/2\lambda z} = 0.693/\lambda z$. Areas under the plasma concentration-time curves ($AUC_{0\to\infty}$ and $AUC_{0\to t}$) were calculated by the method of trapezoids. Peak plasma concentrations (C_{max}) of drug and times to reach peak concentration (t_{max}) were determined from the individual plasma concentration–time curves.

Statistical analysis

All data were presented as mean ± standard deviation (SD). The pharmacokinetic parameters determined were compared by one-way analysis of variance using statistical software (SPSS version 12; SPSS Inc., Chicago, IL, USA). In all cases, $p < 0.05$ was considered statistically significant.

Results

No adverse reaction or apparent sign of toxic effects related to ENR were observed in the days following injection. The serum concentration vs. time data of ENR injectable *in situ* forming

gel formulation and conventional injection following single i.m. administration and the pharmacokinetic parameters are presented in Figure and Table , respectively.

In this study, blood concentrations of ENR *in situ* forming gel were monitored over 180 h while the conventional injections were just 72 h. There were significant differences ($p < 0.01$) between ENR *in situ* forming gel and conventional injection in pharmacokinetic parameters $t_{1/2\lambda z}$. The terminal elimination half-life calculated for ENR *in situ* gel 42.92 ± 9.80 h was significantly higher than that of the conventional injection which was 9.17 ± 0.61 h. The area under the blood concentration vs. time curve (AUC) of ENR *in situ* gel was 48.00 ± 6.44 μg·h/mL, obtained for hours 0 to 180 by the trapezoid rule and normalized for injected dose, while that of conventional one was 19.96 ± 4.07 μg·h/mL. The mean residence time (MRT) of ENR *in situ* gel and conventional injection were 49.39 ± 8.97 h and 11.75 ± 0.90 h, respectively. The C_{max} value of ENR injectable *in situ* forming gel had a similar level with the conventional injection, which was 1.71 ± 0.24 μg/mL reached after 2.50 ± 1.22 h while the conventional injection was 1.76 ± 0.32 μg/mL reached after 2.00 ± 0.00 h.

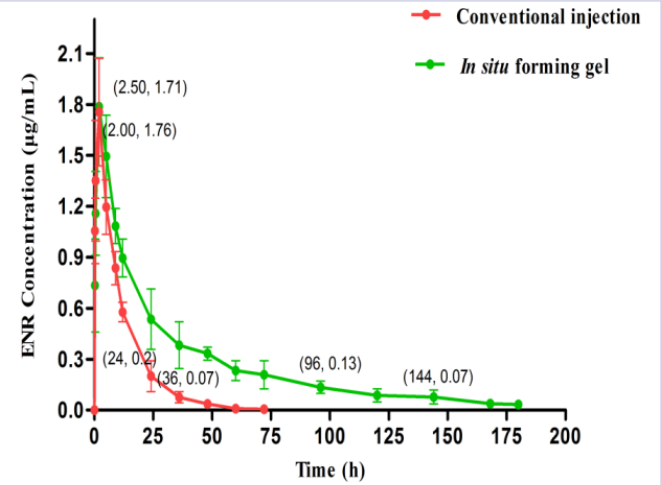

Figure 1: Plasma concentration–time profile of ENR *in situ* forming gel in pigs following i.m. administration of 20 mg/kg b.w. and conventional injection in pigs following i.m. administration of 5 mg/kg b.w. (mean ± SD, n = 6).

Table 1: Pharmacokinetic parameters of ENR *in situ* forming gel (20 mg/kg b.w.) and conventional injection (5 mg/kg b.w.) in pigs (n = 6) following i.m. administration

Value for indicated group		
Parameters	**Conventional injection**	***In situ* forming gel**
MRT (h)	11.75 ± 0.90	49.39 ± 8.97
C_{max} (μg /mL)	1.76 ± 0.32	1.71 ± 0.24
t_{max} (h)	2.00 ± 0.00	2.50 ± 1.22
$t_{1/2\lambda z}$ (h)	9.17 ± 0.61	42.92 ± 9.80 **
$AUC_{0\to t}$ (μg·h/mL)	19.96 ± 4.07	48.00 ± 6.44
$AUC_{0\to\infty}$ (μg·h/mL)	20.12 ± 3.97	49.86 ± 5.59

**P < 0.01. MRT: mean residence time, C_{max}: maximal concentration, t_{max}: time needed to reach C_{max}, $t_{1/2\lambda z}$: terminal elimination half-life, AUC: area under the blood concentration vs. time curve. Data are presented as mean ± SD.

There were significant differences ($p < 0.01$) between ENR *in situ* forming gel and conventional injection, in pharmacokinetic parameters $t_{1/2\lambda z}$. As the doses between ENR *in situ* forming gel and conventional injection are different, the significance analysis of the parameters MRT, C_{max}, t_{max} and AUC which are positive correlated with dose is meaningless.

Discussion

In our study, a novel approach based on thermoreversible *in situ* forming gel with poloxamers for prolonged release of ENR had been implemented. The *in vivo* pharmacokinetic results showed that the mean residence time (MRT) and the terminal elimination half-life ($t_{1/2\lambda z}$) of the drugs we developed were respectively 49.39 ± 8.97 h and 42.92 ± 9.80 h after a single 20 mg/kg b.w. i.m. injection. Significantly, the *in vivo* characteristics of ENR thermoreversible *in situ* forming gel we developed in pigs were superior to that of the ENR conventional injection [2, 27], and also more ideal than the reported results of ENR sustained-release injection [37, 39]. Studies have been conducted on the pharmacokinetics of different ENR forms after different dose under different injection route extensively. For example, Zeng et al. (2002) [39] found that the $t_{1/2\lambda z}$ value of ENR suspension in pigs was 18.95 h after a single 10 mg/kg b.w. i.m. injection; Messenger et al. (2011) [26] reported after a single 7.5 mg/kg b.w. s.c. injection , the $t_{1/2\lambda z}$ value of ENR (Baytril 100) in pigs was 26.6 h; Xiao et al. (2002) [37] found that at the dose of 18.75 mg/kg b.w. under i.m. injection, the $t_{1/2\lambda z}$ value of ENR long-acting injection solution was 19.47 h, and so on. Though all these dosing regimens ($t_{1/2\lambda z}$, 18.95 ~ 26.6 h) have improved compare to the recommended ($t_{1/2\lambda z}$, 9 ~ 12 h), the $t_{1/2\lambda z}$ value is still further lower than that of ENR long-acting forms in our study ($t_{1/2\lambda z}$ = 42.92 h).

Due to the addition of poloxamer 407 (P407), thermosensible *in situ* gel system has the reversal thermal characteristics, and addition of proper poloxamer 188 (P188) could adjust its gelation temperatures [7, 12, 38]. The preparation of this kind of thermosensible *in situ* gel often adopts the " Cold method " [34], which is fully equipped with the feasibility of industrial production. In medicine, multiple related preparations are in the process of new drug application (NDA). Some other additives, such as carboxymethylcellulose sodium (CMC-Na), polyvinylpyrrolidone (PVP), carbomer, and even sodium chloride (NaCl), can affect the gelation strength and gelation time of thermosensible *in situ* gel. Because the patent of this drug is in the examination period, it is inconvenient to discuss its formula in this paper. But the results indicated that this prescription composition was ideal.

The characteristics of antibacterial activity of the fluoroquinolones and the relationship between the MIC and the blood drug concentration have been in-depth study [4]. As the ratio of fluoroquinolones concentration to MIC increases from 1:1 to the optimal bactericidal concentration (usually 10:1), the fluoroquinolones presents concentration-dependent killing effect. The concentration-dependent killing effect may plateau off when the ratio of fluoroquinolones concentration to MIC reaches

15:1 to 20:1 and at ratios greater than 20:1 the fluoroquinolones may become bacteriostatic. The MIC of ENR for the pathogens of common infectious diseases in clinical is 0.001 ~ 0.1 μg/mL [32]. One of the characteristics of ENR is that the MIC for sensitive bacteria is low. For example, the MIC values of ENR for swine pathogenic *escherichia coli* and *erysipelas coli* respectively are 0.05 and 0.1 μg/mL; for swine *actinobacillus pleuropneumoniae*, the MIC_{90} is no more than 0.06 μg/mL; for swine *actinobacillus*, *escherichia coli*, *salmonella krefeld*, *klebsiella pneumonia*, *pasteurella multocida* isolated in clinical, the MIC_{90} values are lower than 0.128 μg/mL [3, 5].

In our study, the mean plasma concentration following the injection of *in situ* gel in pigs was 0.13 μg/mL at 96 h, 0.07 μg/mL at 144 h, indicating that the drugs we developed had much longer acting time than common injection. In spite of the dosage of ENR *in situ* gel was four times as large as that of ENR conventional injection; the C_{max} of them had a similar level. And the C_{max}/MIC ratio of ENR *in situ* gel was less than 20, thus avoiding excessive concentration result in bacteriostatic. This can be explained with the higher distribution volume that leads to lower peak plasma levels within the pigs. Therefore, when compared with the control injection, the *in situ* gel can lead to a reduction of the severity of systemic side effects induced by ENR. Furthermore, to maximize clinical efficacy and reduce selection of resistant bacteria, C_{max}/MIC ratio ≥ 10 : 1 or AUC/MIC_{90} ratio ≥ 125 : 1 may be required [11, 16, 17, 19]. In our study, the AUC of ENR *in situ* gel in pigs which was 48.00 ± 6.44 μg·h/mL at a single 20 μg/mL b.w. dose. Obviously, the AUC/MIC_{90} ratio of ENR *in situ* gel was more than 125, suggesting that the ENR *in situ* gel had a better clinical efficacy.

Conclusion

The C_{max} of ENR *in situ* gel was in the range of ideal bactericidal concentration, and the values of $t_{1/2\lambda z}$ and MRT were greatly extended, which could maintain the effective blood drug concentrations for a long time. Therefore, following a single i.m. administration, the pharmacokinetic characteristics of ENR *in situ* gel in pigs were ideal. On the other hand, the ratios of C_{max}/MIC and AUC/MIC_{90} suggested that the ENR *in situ* gel had a better clinical efficacy. According to this reasonable formulation design, it is worth popularizing in clinical application.

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