Synthesis, Characterization, and Pharmacodynamics Study of Enrofloxacin Mesylate

**Introduction:** Enrofloxacin is used in the treatment of a wide variety of bacterial infections in mammals. However, its poor solubility limits the clinical use.

**Methods:** In order to improve the solubility of enrofloxacin, the enrofloxacin mesylate (EM) were obtained by a chemical synthesis method. The characterization of EM was carried out using ultraviolet scan (UV), synchronous thermal analysis (SDT), fourier transform infrared spectrometer (FTIR) and mass spectrometry (MS), nuclear magnetic resonance (NMR) and X-ray powder diffraction analysis (XRPD). Acute toxicity of EM in Kunming mice was studied. Besides, pharmacokinetic studies were performed in New Zealand rabbits at a single oral dose of 10 mg/kg, and the antibacterial activity of EM was also evaluated.

**Results:** EM was successfully synthesized and purified. The stoichiometric ratio of mesylate to enrofloxacin was 1:1 and the aqueous solubility of EM was 483.01±4.06 mg/mL, the solubility of EM was about 2000 times higher than enrofloxacin. The oral lethal dose (LD$_{50}$) of EM was 1168.364 mg/kg, and the pharmacokinetics indicated that the oral relative bioavailability of EM was about 1.79 times and 1.48 times higher than that of enrofloxacin and enrofloxacin hydrochloride, respectively. In addition, the in vitro antibacterial activity of EM was not significantly changed compared with enrofloxacin and enrofloxacin hydrochloride.

**Conclusion:** EM has higher solubility, low toxicity for oral use, and increases the oral bioavailability in rabbit. This study may be of benefit for the development of new enrofloxacin drugs.

**Keywords:** enrofloxacin mesylate, characterization, antibacterial effect, acute toxicity, pharmacokinetics

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**Introduction**

Enrofloxacin, also known as ethyl ciprofloxacin, is a chemically synthesized third-generation fluoroquinolone.$^1$ The United States Food and Drug Administration (FDA) also approved enrofloxacin as a quinolone antibiotic for livestock and aquatic products in October 1996. Enrofloxacin exhibits good antibacterial activity against a variety of Gram-positive (G$^+$) bacteria and has special effects on mycoplasma. The advantage of enrofloxacin is that it has a broad spectrum of antibacterial, strong bactericidal power, rapid action, wide distribution in the body, what is more, enrofloxacin, as a fluoroquinolone drug for animals, kills bacteria by replicating DNA of tissue bacteria.$^{2,3}$ Enrofloxacin can be used in combination with other antimicrobial agents to kill pathogenic microorganisms, and no cross-resistance between other antibiotics. In previous reports, fluoroquinolone antibiotics have also been used in

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**Correspondence:** Hua-lin Fu
Department of Pharmacy, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, People’s Republic of China
Tel +86 028-86291162
Email fuhl2005@sohu.com

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Lin-lin Pei$^{1,*}$
Wen-zhu Yang$^{1,*}$
Jing-yuan Fu$^{1,*}$
Meng-xi Liu$^1$
Ting-ting Zhang$^1$
Dong-bo Li$^1$
Ruo-yue Huang$^1$
Li Zhang$^1$
Guang-neng Peng$^1$
Gang Shu$^1$
Zhi-xiang Yuan$^2$
Ju-chun Lin$^1$
Wei Zhang$^1$
Zhi-jun Zhong$^1$
Ling Zhao$^1$
Hua-lin Fu$^1$

$^1$Department of Pharmacy, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, People’s Republic of China; $^2$College of Pharmacy, Southwest Minzu University, Chengdu, Sichuan, People’s Republic of China

$^*$These authors contributed equally to this work

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combination with macrolides for treatment of Legionella pneumophila and in combination with beta-lactam antibiotics for treatment of bacteraemia caused by Gram-negative bacilli.

The chemical structure of enrofloxacin is shown in Figure 1. The quinoline ring is an essential structure for the antibacterial action of fluoroquinolones including enrofloxacin. Fluorine atom is a characteristic substituent of fluoroquinolones, which is on the C6 position of the drug, that can enhance DNA gyrase affinity, increase the permeability of the bacterial cell wall, and enhance the antibacterial effect against of G+ bacteria such as Staphylococcus. The introduction of piperazinyl in the C7 position of the drug, on the one hand, improved its antibacterial activity (such as anti-Pseudomonas aeruginosa activity) and its antibacterial spectrum, on the other hand, induced the production of side effects. The ethyl on the piperazine ring of the drugs, enhanced its lipophilicity and penetration ability. At the same time, it also reduces its toxic effects on the central nervous system. And the introduction of cyclopropyl at position N1 enhances the antibacterial effect of enrofloxacin. Because of its acidic carboxyl and alkaline nitrogen atoms, enrofloxacin is both acidic and alkaline, so its solubility greatly depending on the solvent and the pH value. The acid-base dissociation constants of enrofloxacin were pKa1=6.06 and pKa2=7.70, and the isoelectric point was 6.85. There is a study shown that enrofloxacin has the best solubility when the pH of the solution is 5.02. And the pH value of the solution does not have a significant effect on the hydrolysis rate.

Enrofloxacin is easily dissolved in methylene chloride and sodium hydroxide solution, dissolved in acetonitrile, slightly soluble in methanol and very slightly soluble in water. The lower water solubility is one of the major drawbacks of enrofloxacin. The literature shows that its solubility in water is only 0.23 g/L. The low solubility results in the low bioavailability and limits the study of enrofloxacin in vitro and in vivo. According to most of the literature on improving the solubility of drugs, when the solubility of the drug is less than 100 μg/mL usually shows dissolution-limited absorption. In this case, it is necessary to increase the dose of the drug to maintain the blood concentration, but it also leads to some side effects. So it is necessary to increase the solubility of enrofloxacin to meet clinical needs and research. To facilitate the clinical use of enrofloxacin, there are generally two major methods to increase the solubility of enrofloxacin: physical solubilization techniques and chemical solubilization techniques. Physical solubilization method is mainly used to achieve the solubilization effect by forming complex or a cyclodextrin inclusion compound between the active pharmaceutical ingredients (APIs) and excipients, while chemical method is through the APIs and acid or alkali form salt to increase solubility. Such as the synthesis of enrofloxacin citrate and enrofloxacin hydrochloride, etc. Salt formation can not only increase the dissolution rate and solubility of the drug, but also improve the bioavailability of the drug. In the pharmaceutical field, methanesulfonic acid is used to form salt with a chosen drug matter, salts of methanesulfonic acid are highly water-soluble and have no tendency to form hydrates. More than 30 registered drugs based on mesylate are known. This study uses the chemical solubilization technology to synthesize EM, and the synthesized EM was characterized by H-NMR, C-NMR, MS, FTIR, XRPD, and DSC-TGA analyses to determine its chemical structure. Then the toxicity, in vitro antibacterial activity and pharmacokinetics of EM were studied.

Materials and Methods

Materials

Enrofloxacin (≥98% by enrofloxacin), enrofloxacin sodium (content 83.88% by enrofloxacin) and enrofloxacin hydrochloride (content was 90.67%) was purchased from Zhejiang Guobang Pharmaceutical Co., Ltd. methanesulfonic acid (content 98%) was purchased from Chengdu Huaxia Chemical Reagent Co., Ltd. enrofloxacin mesylate (self-made, content is 74.49% based on enrofloxacin). Mueller-Hinton Agar (MHA) and Mueller-Hinton Broth
(MHB) medium were purchased from Hangzhou Microbial Reagent Co., Ltd. All strains were provided by Sichuan Provincial Key Laboratory of Animal Diseases and Human Health in Sichuan Agricultural University. Spectrum Two infrared spectrometer, PerkinElmer, USA; 6120B mass spectrometer, Agilent Technologies, Inc.; AVANCE III500M nuclear magnetic resonance spectrometer, Bruker Technology Co., Ltd. Vario EL cube elemental analyzer, Elementar, Germany; Q600 synchronous thermal analyzer Simultaneous DSC/TGA (SDT), American TA Instruments; D8 ADVANCE X-ray diffractometer, Brooke Technology Co., Ltd.; UV-2000 ultraviolet spectrophotometer, Shanghai Unico Instrument Co., Ltd.; SPX biochemical incubator, Ningbo Donghai Instrument Co., Ltd.; portable pressure steam sterilization pot, Shanghai Huaxian Medical Nuclear Instrument Co., Ltd; LC2010 high-performance liquid chromatography, Shimazu international trade (Shanghai) co., Ltd.

Kunming mice (20±2g) were used for the acute toxicity study. New Zealand white rabbits (2.5±0.3kg) were used for the pharmacokinetic study. All the experimental animals were provided by the Experimental Animal Center of Sichuan Agricultural University (Chengdu, China). Before the experiment, the animals were acclimatized at 25°C ±2°C under natural light/dark conditions for 1 week with free access to food and water. Twelve hours before dosing, the animals were made to fast but were allowed free access to water. All animal studies were approved by the Animal Ethical Experimentation Committee of Sichuan Agricultural University (SYXK [Chuan] 2019–187), and were performed according to the requirements of the People’s Republic of China National Act on the use of experimental animals.

**Synthesis of EM**

EM was prepared by improved modified solvent method. At 25°C, 1 g of enrofloxacin was dispersed with 2 mL of water, and 6.2 mL of mesylate with a concentration of 0.45 mol/L was added (the molar ratio of mesylate to enrofloxacin was 1:1). The solution was stirred at the speed of 20–30 r/min until the salt solution was clarified, and the solution was filtered, the obtained filtrate slowly evaporates solvent under 45°C water bath to obtain kosher salt solid. Then, 0.5 g of EM kosher salt was dissolved in 0.5 mL of water, slowly add the dissolved EM kosher salt solution into 30mL isopropanol, stir for 1.5 h, filter the mixed solution, dry the filtrate in a 45°C oven to obtain the refined EM salt.

**Determination of Solubility**

Solubility of EM in water, hydrochloric acid solution (PH 1), and phosphate buffer (PH 7.6) solvents was

| Drugs                        | Water  | Hydrochloric Acid Solution (pH 1.0) | Phosphate Buffer (pH 7.6) |
|------------------------------|--------|------------------------------------|---------------------------|
| Enrofloxacin                 | 3.32±0.22 | 0.26±0.02a | 0.28±0.01                 |
| Enrofloxacin mesylate        | 0.019±0.002 | 483.01±4.06b | 486.88±8.26               |
| Enrofloxacin sodium          | 0.047±0.01 | 168.37±6.90c | 155.5±±2.00               |
| Enrofloxacin hydrochloride   | 0.031±0.007 | 11.90±0.22d | 6.77±0.49                 |

Notes: a, b, c, d represent highly significant difference. Only the differences of solubility in water were compared.
determined by the equilibrium method and compared with enrofloxacin, enrofloxacin sodium, and enrofloxacin hydrochloride. Excess enrofloxacin, enrofloxacin sodium, enrofloxacin hydrochloride, and EM were added in conical flask containing 2 mL water, hydrochloric acid solution (PH 1), and phosphate buffer (PH 7.6), respectively. In triplicate, after vortexed for 5 min, the conical flask was put in a water bath at 25 ± 2°C and shaken at 100 r/min for 24 h, until the solution was equilibrated, then the suspension was filtered with
0.22 μm nylon filter. The filtrate was diluted and determined by ultraviolet spectrophotometry (UV) quantitative analysis.

Characterization

Ultraviolet Scan (UV)
Enrofloxacin and EM in aqueous solution were prepared using water as a solvent to ensure the concentrations of enrofloxacin was 2 μg/mL – 9 μg/mL and scan at 200 nm–400 nm. Check whether the chromophoric group changes after enrofloxacin and methanesulfonic acid were salted.

Fourier Transform Infrared Spectrometer (FTIR)
To obtain the infrared scanning pattern of enrofloxacin, enrofloxacin hydrochloride and EM, FTIR were carried out in air under normal atmospheric conditions, using a spectrum two infrared spectrometer (PerkinElmer, USA). Enrofloxacin, enrofloxacin hydrochloride, and EM were pressed into KBr tablets, and infrared scanning was performed within the range of 400 cm⁻¹ to 4000 cm⁻¹ to ensure that the resolution of the instrument was no less than 2 cm⁻¹, in order to examine the differences of the chemical bond or functional group information in each drug molecule.²²

Mass Spectrometry (MS)
The Mass Spectrometry was performed using a 6120B mass spectrometry (Agilent Technologies). The condition is EI source. Nitrogen was used as the collision gas, and the pseudomolecular ions of the analytic were decomposed by using the optimal collision activation dissociation (CAD) condition and the corresponding stable isotope marker internal standard. The instrument parameters were set according to the reported method²³–²⁵ to ensure that the information of the molecular structure of EM could be obtained.

Nuclear Magnetic Resonance (NMR)
The nuclear magnetic resonance was performed using an AVANCE III500M nuclear magnetic resonance spectrometer (Bruker Technology), and uniform experimental parameters were used in all sample tests. The main parameters in hydrogen spectrum test are as follows: spectrum

Figure 5 The MS spectrum of enrofloxacin.
width (SWH) 8012.820 HZ, sampling data point (TD) 65,536, scanning times (NS) 16, delay time (D1) 1.0s, and receiving gain (RG) 12. The main parameters in the carbon spectrum test: spectrum width (SWH) 24,038.461 HZ, sampling data point (TD) 65,536, scanning times (NS) 512, delay time (D1) 2.0s, and receiving gain (RG) 194.26. In triplicate, enrofloxacin was dissolved in CDCl$_3$, TMS was used as internal standard and EM was dissolved in D$_2$O. Hydrogen and carbon spectra of enrofloxacin and EM were recorded at 400M HZ and 100M HZ, respectively.

**Elemental Analysis**

The C, H, O and N elements of EM were analyzed, in order to detect the proportion of four elements and infer their molecular composition.

**Synchronous Thermal Analysis (SDT)**

SDT thermal analysis has the characteristics of differential thermal analysis (DTA) and thermogravimetric analysis (TGA). It can provide the signal of melting point, melting heat, crystallization temperature and thermal stability of the test sample. The DSC-TGA analyses were performed using TA instruments equipment, aluminum oxide was used as reference. The heating rate was set at 10°C/min and the temperature range was 25–550°C, in an inert atmosphere of N$_2$.

**X-Ray Powder Diffraction Analysis (XRPD)**

The enrofloxacin and EM were scanned using an X-Ray powder diffractometer. The light source was Cu K$_\alpha$ radiation with a wavelength of 1.542 Å, and the DS (divergent slit) and SS (scattering slit) were 1°. The RS (receiving slit) was 0.2 mm. An operating voltage of 40 kV and current of 25 mA, the scanning angle was 5–45° with a speed of 0.06°/s.

**The Antibacterial Activity in vitro**

Microbroth dilution method$^{28,29}$ was used to study in vitro antimicrobial activity of EM. Individual colonies grown on MHA medium for 18–24 h were placed...
in 5mL MHB medium, shaken for 16 h at 37°C and 100 r/min, and then diluted to $1.0 \times 10^8$ CFU/mL with MHB broth for later use. The minimum inhibitory concentration (MIC) test systems were created by diluting different drug solutions in 96-well plates to a volume of 100 μL, and the enrofloxacín sodium, enrofloxacín hydrochloride and EM were double diluted\textsuperscript{28} to final concentrations ranging from 25 to 0.0488 μg/mL, and then 100 μL bacterial suspensions was added to each drug-containing well. The MIC was determined visually as the first dilution step with a complete growth inhibition\textsuperscript{30–32} and the minimum bactericidal concentration (MBC) was determined after the MIC. 10 μL mixtures were extracted from the wells in the 96-well plate that were visually free of bacterial growth, and then uniformly coated on the MHA medium after serial dilution, and incubated at 37°C for 18–24 h. The number of colonies on the plate was calculated, and the minimum concentration killing 99.9% of the bacteria was recorded as MBC.\textsuperscript{30–32} The MIC and MBC of standard strains and clinical strains of Escherichia coli, Salmonella and Staphylococcus aureus were determined.

### Acute Toxicity Study

In accordance with the requirements of veterinary drug research technical guidelines, 5 dose groups were set up

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**Figure 7** The $^1$H-NMR spectrum of enrofloxacín.

**Table 2** The $^1$H-NMR (400 M) Data of Enrofloxacín (CDCl$_3$)

| Atomic Number | δ (ppm) |
|---------------|---------|
| 2             | 8.7 (1H, S) |
| 5             | 7.9 (1H, d, J=12.8 HZ) |
| 8             | 7.35 (1H, d, J=7.2 HZ) |
| 1a            | 3.57 (1H, m) |
| 2' 6'         | 3.38 (4H, t, J=0.8 HZ, J=9.2 HZ) |
| 3' 5'         | 2.69 (4H, d, J=4.8 HZ, J=2.0 HZ) |
| 7'            | 2.54 (2H, dd, J=7.2 HZ, J=7.2 HZ) |
| 1b            | 1.4 (2H, dd, J=6.8 HZ, J=6.8 HZ) |
| 1c            | 1.21 (2H, m) |
| 8'            | 1.15 (3H, m) |
in this experiment, namely, 625 mg/kg, 884 mg/kg, 1250 mg/kg, 1767 mg/kg and 2500 mg/kg, respectively. The state and behavior of the mice were observed after administration, and the mortality was recorded. The improved Karber method was used to calculate the \( \text{LD}_{50} \) of EM.

**Pharmacokinetic Study**

The pharmacokinetic of enrofloxacin, enrofloxacin hydrochloride and EM was determined by high-performance liquid chromatography (HPLC). After oral administration with a single oral dose of 10 mg/kg, the rabbit ear vein blood samples were collected at 0 h, 0.083 h, 0.167 h, 0.333 h, 0.667 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 4 h, 6 h, 9 h, 12 h, 24 h, 36 h and 48 h, respectively. The peak concentration (\( C_{\text{max}} \)) and the time at which \( C_{\text{max}} \) was observed (\( T_{\text{max}} \)) were calculated. Additionally, DAS software was used to process the data to obtain the pharmacokinetic parameters which were analyzed by SPSS19.0.

**Results and Discussion**

**Determination of Solubility**

The bioavailability of drugs is a vital concern in the pharmaceutical field, the low aqueous solubility of candidate drugs will lead to the increase of drug concentration, which will lead to some adverse reactions. EM

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**Figure 8** The \(^1\text{H-NMR} \) spectrum of enrofloxacin mesylate.

**Table 3** The \(^1\text{H-NMR} \) (400M HZ) Data of Enrofloxacin Mesylate (\( \text{D}_2\text{O} \))

| Atomic Number | \( \delta \) (ppm) |
|---------------|-----------------|
| 2             | 8.4 (1H, s)     |
| 5             | 7.33 (1H, d, \( J=7.6 \text{ HZ} \)) |
| 8             | 7.18 (1H, d, \( J=12.8 \text{ HZ} \)) |
| 3'            | 3.86 (2H, d, \( J=8.4 \text{ HZ} \)) |
| 5'            | 3.7 (2H, d, \( J=8.0 \text{ HZ} \)) |
| 1a            | 3.57 (1H, m)    |
| 2' 6' 7'      | 3.3 (6H, m)     |
| 9'            | 2.73 (3H, s)    |
| 8' 1b         | 1.36 (5H, m)    |
| 1c            | 1.09 (2H, m)    |
has been synthesized successfully in this study, a white, slightly bitter, loose solid powder that can dissolve in water easily, and the EM reduces the bitterness of enrofloxac in. The yield of EM was 94.41% and the content of enrofloxac in EM was 74.49%. The solubility results for enrofloxac, EM, enrofloxac sodium, and enrofloxac hydrochloride are shown in Table 1. It can be seen that the solubility of EM is much higher than enrofloxac hydrochloride and enrofloxac sodium, so it has a good dissolution advantage. The higher solubility in water may have been associated with the smaller lipo-hydro partition coefficient of the drug, the larger the distribution coefficient of lipid-water, the drugs more soluble in fat, and vice versa. Except for, the lipo-hydro partition coefficient of EM is 0.019±0.002, which is smaller than enrofloxac (3.32±0.22) and other enrofloxac salts (enrofloxac sodium, 0.047±0.01; enrofloxac hydrochloride, 0.031±0.007). In addition, this may be related to the polar surface area of the drug molecule. With the increase of the polar surface area, the solubility becomes higher. Due to the introduction of multiple oxygen atoms, the polar surface area of the EM molecule may be the largest among all prepared enrofloxac salts. EM is a new crystal that is different from enrofloxac, it is possible that the crystal structure of EM is more conducive to its dissolution, but the crystal structure of EM needs further study. Thus, the solubility is greatly improved.

Characterization

Ultraviolet Scan (UV)

The UV scanning results of the enrofloxac in and EM aqueous solutions are shown in Figure 2. The UV absorption curves of the enrofloxac in and the EM are generally the same. This shows that the UV-chromophoric groups were not destroyed after enrofloxac was formed into salt with methanesulfonic acid. However, the peaks of enrofloxac are shifted: $\lambda_{\text{max}}$=271 nm to $\lambda_{\text{max}}$=276 nm, that indicate that salt formation infects chromophoric groups.
Fourier Transform Infrared Spectrometer (FTIR)
The infrared scanning results of the main bands of enrofloxacin and EM are shown in Figure 3. The infrared spectrum of EM is basically the same as the enrofloxacin, such as at 3446 cm\(^{-1}\), O-H stretching vibration of the carboxyl; stretching vibration peak of carbonyl C=O at 1729 cm\(^{-1}\); stretching vibration peak of C=C bond at 1629 cm\(^{-1}\) and 1506 cm\(^{-1}\); bending vibration of C-H bond at 1475 cm\(^{-1}\); the characteristic absorption peak of the C-F bond at 1289 cm\(^{-1}\). These prove that EM and enrofloxacin both contain the same structural groups such as F-Ph, -CH2, -COOH, C=O and so on. In addition, there are many new characteristic peaks on the infrared spectrum of EM, such as 3492 cm\(^{-1}\), methanesulfonic acid O-H absorption peak; 2457 cm\(^{-1}\)-2655 cm\(^{-1}\) amine salt absorption peak; 1058 cm\(^{-1}\) and 1195 cm\(^{-1}\) methanesulfonic acid S=O absorption peak; 638 cm\(^{-1}\) is the methanesulfonic acid S-O absorption peak. This shows that methanesulfonic acid may bind with the basic nitrogen atom of enrofloxacin and form an amine salt. The above hypothesis was further confirmed by comparing the infrared spectra of enrofloxacin hydrochloride with EM (Figure 4). It can be seen that both of them have similar amine salt peaks, and that EM has more absorption peaks of the methanesulfonic acid characteristic group than enrofloxacin hydrochloride.

Mass Spectrometry (MS)
Figures 5 and 6 show the mass spectra of enrofloxacin and EM, respectively. The molecular ion peak ([M+H]+) in the mass spectrum of Figure 6 is 360.1728, which is in agreement with the molecular weight of enrofloxacin C\(_{19}\)H\(_{22}\)FN\(_{2}\)O\(_{3}\), indicating that enrofloxacin is the base portion of EM.

Nuclear Magnetic Resonance (NMR)
The \(^1\)H-NMR spectrum of enrofloxacin is shown in Figure 7. The spectrum shows 21 hydrogens (excluding -COOH active hydrogen) except the solvent, which is consistent with the molecular structure of enrofloxacin. According to the enrofloxacin hydrogen spectrum data reference\(^3^5\), enrofloxacin is now assigned to Table 2. The \(^1\)H-NMR spectrum of EM is shown in Figure 8. The spectrum shows 24 hydrogens (one -SO\(_2\)H and one -COOH may exchange with D\(_2\)O). According to the attribution of enrofloxacin and the chemical environment of hydrogen atoms, the hydrogen atom of EM will be assigned to Table 3. Among them, \(\delta\) 2.73 (S, 3H, CH\(_3\)) is exactly one molecule of three hydrogens on the methanesulfonic acid methyl group. The rest is hydrogen on the base of enrofloxacin. Except for the piperazine ring and ethyl, the chemical shift values of other hydrogen atoms are not changed much. Two hydrogens at the 3' position on the piperazine ring (\(\delta\) 3.86, d, J = 8.4 HZ, 2H, CH\(_2\)) and two hydrogens at the 5' position (\(\delta\) 3.7, d, J = 8.0 HZ, 2H, CH\(_2\)) shift to low field; two hydrogens at the 7' position on the ethyl (\(\delta\) 3.3, m) and three hydrogens at the 8' position (\(\delta\) 1.36, m) all move to the lower field. This may be due to the fact that the formation of a quaternary ammonium salt at the N4' position introduces a positive charge, giving the surrounding H a different degree of shielding effect.\(^3^6\)

The \(^13\)C-NMR spectrum of enrofloxacin is shown in Figure 9, which shows the resonance of 19 carbon atoms, corresponding to 19 carbon atoms in the enrofloxacin molecule. Its attribution is shown in Table 4. The \(^13\)C-NMR spectrum of EM is shown in Figure 10, which shows the resonance of 20 carbon atoms, corresponding to 20 carbon atoms in the EM molecule. Its attribution is shown in Table 5. Of these, \(\delta\) 38.46 (Q, CH\(_3\)) is just methyl sulfonate on methanesulfonic acid. The rest is carbon on the base of the enrofloxacin. Except for the piperazine ring and the ethyl group, the

Table 4 The \(^{13}\)C-NMR (100 HZ) Data of Enrofloxacin (CDCl\(_3\))

| Atomic Number | \(\delta\) (ppm) | Remark |
|---------------|-----------------|--------|
| 3             | 167.02, S       |        |
| 3a            | 167.02, S       |        |
| 6             | 152.43–154.94, S|        |
| 2             | 147.34, D       |        |
| 7             | 145.91, S       |        |
| 9             | 139.1, S        |        |
| 10            | 119.57, S       |        |
| 5             | 112.14, D       |        |
| 10            | 108.02, S       |        |
| 8             | 104.75, D       |        |
| 2' 6'         | 52.44, T        |        |
| 7             | 52.26, T        |        |
| 3' 5'         | 49.79, D        |        |
| 1a            | 35.3, D         |        |
| 8'            | 11.97, Q        |        |
| 1b 1c         | 8.2, T          |        |
chemical shift values of other carbon atoms are not much changed. Both C3′ and C5′ carbons ($\delta$46.41, T, CH$_2$) on the piperazine ring move towards the high field; C7′ ($\delta$50.86, T, CH$_2$) and C8′ ($\delta$ 7.46, Q on the ethyl group, CH$_3$) all move to the high field to varying degrees. This may be due to the fact that after N4′ forms a quaternary ammonium salt, the positive charge is concentrated on the hydrogen ion connected to the nitrogen atom. The C atom around the N4′ atom is not directly connected with the positive charge, the induction effect is weakened, and the electric field effect makes each C shifts to the high field.\textsuperscript{36}

A comprehensive analysis of the hydrogen spectrum indicated that methanesulfonic acid combined with enrofloxacin N4′ to form a salt. The theoretical formula of the formed EM salt may be C$_{19}$H$_{22}$FN$_3$O$_3$⋅CH$_4$O$_3$S. Combined with other characterization methods, the molecular formula of EM is C$_{19}$H$_{22}$FN$_3$O$_3$⋅CH$_4$O$_3$S.

**Synchronous Thermal Analysis (SDT)**

The DSC-TGA curve of enrofloxacin and EM is shown in Figure 11. The melting point of enrofloxacin and EM is 225.5°C and 298.5°C, respectively. The heat absorption peak on the DSC curve and the significant mass loss on the TGA curve after 298.5°C indicate that EM dissolves during melting and it has better thermal stability. Beyond that, the mass loss of EM between 30°C and 75°C is due to the volatilization of adsorbed solvent. After 75°C and before decomposition temperature, there was no significant mass loss in the TGA curve, indicating that EM molecules did not contain crystal water.

**Elemental Analysis**

The elemental analysis result of EM is Table 6. The proportion of N, C, H and S elements of EM is similar to the theoretical formula C$_{19}$H$_{22}$FN$_3$O$_3$⋅CH$_4$O$_3$S. Combined with other characterization methods, the molecular formula of EM is C$_{19}$H$_{22}$FN$_3$O$_3$⋅CH$_4$O$_3$S.

**X-Ray Powder Diffraction (XRPD)**

The powder diffraction pattern of enrofloxacin and EM is shown in Figure 12. X-ray powder diffraction
**Antibacterial Activity in vitro**

The MIC and MBC determination of enrofloxacin and each enrofloxacin salt are based on enrofloxacin. The inhibitory effects of enrofloxacin and its salts on six strains of bacteria are shown in Table 7. The MIC values of EM which prepared in this experiment, enrofloxacin, enrofloxacin hydrochloride in Chinese Veterinary Pharmacopoeia, and enrofloxacin sodium commonly used in clinical trials were both in the range of 0.0727–1.5625 μg/mL. The experimental results show that the bacteriostatic effect of EM synthesized by ourselves is basically the same as that of enrofloxacin, which has not changed its bacteriostatic effect, but has greatly increased the solubility of enrofloxacin, solved the problem that enrofloxacin is insoluble in water, and enriched the use of enrofloxacin. Enrofloxacin exhibits antibacterial activity mainly through the binding of antibacterial active sites with bacterial DNA gyrase to prevent the replication of bacterial DNA. The salt formation may not change its antibacterial active groups, so the bactericidal effect does not change significantly.

**Acute Toxicity Study**

After administration, the mice showed: dizziness, less movement, glassy eyes, depressed spirit, sluggish breathing and slow movement, some mice even showed severe ataxia; after a short period of depression, poisoned mice suddenly became excited and restless, jumping, some performance for the whole body tremor, rolling or running, eventually collapse and die, death began at 5 min, and the peak of death was 13–19 min in the high dose group, 9 min in the low and medium dose group, and 6–9 min in the high dose group. The death statistics of mice in each group are shown in Table 8. After SPSS 19.0 analysis, the oral LD50 of EM was 1168.364 mg/kg, indicating that the oral drug safety of EM was high.

**Pharmacokinetic Study**

After administration of enrofloxacin, EM and enrofloxacin hydrochloride, according to the blood concentration of each group, the pharmacokinetic parameters are shown in Table 9. The Cmax of EM was 1.391 ±0.158 mg/L, which was significantly higher than enrofloxacin hydrochloride (0.989±0.195 mg/L) (P < 0.01) and enrofloxacin hydrochloride (0.877±0.155 mg/L) (P < 0.01). Because of its good solubility, EM could form a higher concentration of the drug in the

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**Table 5** The $^{13}$C-NMR (100 M HZ) Data of Enrofloxacin Mesylate (D$_2$O)

| Atomic Number | δ (ppm) | Remark |
|---------------|---------|--------|
| 4             | 175.37, S |        |
| 3a            | 168.41, S |        |
| 6             | 151.83–154.33, S | Significant splitting of the carbon signal connected to F |
| 2             | 147.91, D |        |
| 7             | 144.01, S |        |
| 9             | 138.68, S |        |
| 10            | 118.28, S |        |
| 5             | 110.29, D |        |
| 3             | 106.35, S |        |
| 8             | 105.36, D |        |
| 2' a          | 62.27, T  |        |
| 2' b          | 50.86, T  |        |
| 3' 5'         | 46.41, T  |        |
| 9'            | 38.46, Q  |        |
| 1a            | 36.07, D  |        |
| 1b 1c         | 8.58, T   |        |
| 8             | 7.46, Q   |        |

**Table 6** The Elemental Analysis of Enrofloxacin Mesylate

| Elemental     | N (%) | C (%) | H (%) | S (%) |
|---------------|-------|-------|-------|-------|
| The measured values | 8.870 | 50.480 | 5.677 | 7.206 |
| The theoretical value | 9.227 | 52.736 | 5.754 | 7.040 |

characterization showed that EM has a distinct diffraction peak, compared with enrofloxacin, EM has the characteristics of diffraction peak in 20 were 7.93, 9.61, 11.89, 22.07, 22.95, 23.68, 24.36, 24.97, 35.29, 35.97, respectively, indicating that it is a crystal. In addition, the numbers of diffraction peaks of EM were greatly different from enrofloxacin, and the energetic peak of EM is less than that of enrofloxacin, indicating that EM crystals may have small particle size, which facilitates their rapid dissolution. The angular positions, and relative intensity, as well as the shape of the diffraction peaks of EM, are greatly different from enrofloxacin, indicating that mesylate and enrofloxacin are not a simple physical mixture, but mesylate reacts with enrofloxacin to form a crystal different from enrofloxacin. Under crystal state, the molecule maintains its stable arrangement in space with hydrogen bond, due to the introduction of salt bond, the interaction force of molecules inside the crystal cell is increased, which is conducive to the stability of the crystal.
gastrointestinal tract, to promote the drug absorption. There was no significant difference in the peak time (T_{max}) of enrofloxacin, EM and enrofloxacin hydrochloride. The area under the curve (AUC_{0-t}) of EM was significantly higher than that of enrofloxacin and enrofloxacin hydrochloride (P < 0.05). Using enrofloxacin as a reference drug, the relative bioavailability of EM and enrofloxacin hydrochloride can be calculated according to the following equation:

\[
F = \frac{AUC_{0-t}^S}{AUC_{0-t}^R} \times 100\%
\]

AUC_{0-t}^R is the area under the curve of the reference drug; AUC_{0-t}^S is the area under the curve of the drug under test.

![Figure 11](image1.png) The DSC-TGA analysis of enrofloxacin and enrofloxacin mesylate.

![Figure 12](image2.png) The X-ray powder diffraction analysis of enrofloxacin and enrofloxacin mesylate.
The relative bioavailability of EM and enrofloxacin hydrochloride was F=179.78±28.91%, 121.02±18.85%, respectively. The data showed that the relative bioavailability of EM was the best. Indicating that salt formation using enrofloxacin and mesylate improved the bioavailability of enrofloxacin.

**Conclusion**

New salt formation of enrofloxacin was successfully synthesized in this study by the chemical methods. And its structure was analyzed by ultraviolet and infrared spectroscopy, mass spectrometry and nuclear magnetic resonance, etc. The molecular formula is determined as C_{19}H_{22}FN_{3}O_{3}·CH_{4}O_{3}S. The in vitro antibacterial test showed that the antibacterial effect of EM was not significantly different from that of enrofloxacin, enrofloxacin hydrochloride and enrofloxacin sodium. The acute toxicity test showed that EM was safe to take orally, and the pharmacokinetic results showed that EM significantly improved the bioavailability of enrofloxacin, which ensured the efficacy of enrofloxacin.

**Table 7** The MIC and MBC Determination Results of Enrofloxacin and Its Salts

| Drugs (by Enrofloxacin) | E. coli ATCC25922 | Clinical | Salmonella G9-23 | Clinical | Staphylococcus Aureus ATCC25923 | Clinical |
|-------------------------|-------------------|----------|-----------------|----------|-------------------------------|----------|
| Enrofloxacin            | 0.0977 (μg/mL)    | 1.5625   | 1.5625          | 0.3906   | 1.5625                        | 1.5625   |
| Enrofloxacin hydrochloride | 0.0855 (μg/mL) | 1.4167   | 1.4167          | 0.3542   | 1.4167                        | 1.4167   |
| Enrofloxacin sodium     | 0.0814 (μg/mL)   | 1.3028   | 1.3028          | 0.3257   | 1.3028                        | 1.3028   |
| Enrofloxacin mesylate   | 0.0772 (μg/mL)   | 1.1639   | 1.1639          | 0.2910   | 1.1639                        | 1.1639   |

**Table 8** The Death Number Statistical Results of Mice in Every Groups

| Groups | 1 | 2 | 3 | 4 | 5 |
|--------|---|---|---|---|---|
| Dose (mg/kg) | 625 | 884 | 1250 | 1767 | 2500 |
| The original number | 10 | 10 | 10 | 10 | 10 |
| The number of deaths | 0 | 4 | 6 | 7 | 10 |

The relative bioavailability of EM and enrofloxacin hydrochloride was F=179.78±28.91%, 121.02±18.85%, respectively. The data showed that the relative bioavailability of EM was the best. Indicating that salt formation using enrofloxacin and mesylate improved the bioavailability of enrofloxacin.

**Table 9** The Pharmacokinetic Parameters of Enrofloxacin, Enrofloxacin Mesylate and Enrofloxacin Hydrochloride (n=6)

| Pharmacokinetic Parameters | Unit | Enrofloxacin | Enrofloxacin Mesylate | Enrofloxacin Hydrochloride |
|----------------------------|------|--------------|-----------------------|---------------------------|
| t_{1/2α}                  | h    | 0.811±0.267a | 1.030±0.248a          | 1.473±0.329a               |
| t_{1/2β}                  | h    | 19.144±4.587 | 11.823±3.306          | 21.692±4.389               |
| V_{1/0}                   | L/kg | 6.583±1.148a | 4.265±1.015a          | 6.778±1.293a               |
| K_{10}                    | l/h  | 0.3±0.034    | 0.205±0.086           | 0.269±0.187                |
| K_{12}                    | l/h  | 0.608±0.181a | 0.46±0.036a           | 0.226±0.091a               |
| K_{21}                    | l/h  | 0.2±0.054a   | 0.242±0.052a          | 0.126±0.019b               |
| AUC_{(0-τ)}               | mg/L|h    | 6.993±1.104a | 12.572±2.022a          | 8.463±1.318b               |
| AUC_{(0-∞)}               | mg/L|h    | 9.323±1.526b | 12.726±2.243a          | 9.437±1.621b               |
| T_{max}                   | h    | 1.05±0.175   | 1.5±0.447             | 1.417±0.204                |
| C_{max}                   | mg/L| 0.877±0.155c | 1.391±0.158a          | 0.989±0.195b               |

Note: a, b represent highly significant difference and a, b, c represent significant difference.

Abbreviations: t_{1/2α}, plasma half-life for the distribution phase; t_{1/2β}, plasma half-life for the elimination phase; V_{1/0}, apparent volume of distribution; K_{10}, first-order elimination rate constant; K_{12}, transport rate constant from central compartment to periphery compartment; K_{21}, transport rate constant from periphery compartment to central compartment; T_{max}, peak time; C_{max}, maximum concentration; AUC, area under the curve.
in clinical use. Furthermore, the EM has higher solubility and thermal stability, and more conducive to clinical use. It is hope that the outcome of this study may help to the development of new enrofloxacin drugs.

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Disclosure

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