Drug Design, Development and Therapy

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ORIGINAL RESEARCH

High Stability and Low Irritation of Enrofloxacin–Colistin Combination Injection Through a Tripartite Strategy

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Introduction: The rapid progression of bacterial resistance and the dearth of novel antimicrobial drug development impose a significant public health burden on the treatment of bacterial infections. Drug combination therapy has become an attractive strategy for combating multidrug-resistant bacterial infections. More importantly, matching the physicochemical properties of multiple components in formulations is essential for clinical application.

Methods: First, an enrofloxacin–colistin combination injection was developed using a tripartite strategy, defined as a three-step process involving the conversion of enrofloxacin to its salt form, the addition of 1,2-propanediol, and pH adjustment. Second, independent gradient model based on Hirshfeld surface (IGMH), nuclear magnetic resonance (NMR), and ultraviolet-visible spectroscopy (UV-vis) analysis were used to investigate the molecular mechanism of this process. Finally, the irritancy, toxicity, and efficacy of the combination injection were evaluated in vivo and in vitro.

Results: The tripartite strategy increased the solubility of enrofloxacin by 1500-fold from 0.18 mg/mL to 272.76 mg/mL, thereby preventing enrofloxacin precipitation during 6 months at both 30°C and 4°C, maintaining colistin stability, and reducing injection-site irritation. 1,2-Propanediol enhanced hydrogen bonding with enrofloxacin and inhibited its self-aggregation. Importantly, the combination injection exhibited no significant liver and kidney toxicity while demonstrating outstanding therapeutic efficacy against *Pasteurella multocida* pneumonia with 62.5% survival rate.

Discussion: The limited solubility of enrofloxacin has long hindered its co-formulation with pH-sensitive drugs. The tripartite strategy establishes a paradigm for overcoming challenges related to the crystal precipitation of insoluble drugs and the pH limitations in complex formulations. Our findings demonstrate that the tripartite strategy effectively enhances the solubility, stability, and therapeutic efficacy of enrofloxacin-colistin combinations, offering a novel solution to overcome challenges in developing complex antibacterial formulations for veterinary use.

Plain Language Summary:

(1) A highly stable and weakly acidic enrofloxacin–colistin combination injection was produced.
(2) 1,2-Propanediol enhanced enrofloxacin solubility and inhibited recrystallization.
(3) 1,2-Propanediol increased hydrogen bonding with enrofloxacin and inhibited molecular self-aggregation.
(4) The tripartite strategy resolved the insoluble drug-pH conflict, enabling multi-drug formulations.
(5) The enrofloxacin–colistin combination injection demonstrated excellent therapeutic efficacy with negligible toxicity or irritation.

Keywords: enrofloxacin, 1,2-propanediol, molecular dynamics simulation, solubility, recrystallization, compound injection

Introduction

The rapid rise in multidrug-resistant pathogens poses a major threat to public health.[1] However, the antibiotic development pipeline remains sparse, especially for broad-spectrum antibacterial molecules.[2] In this context, combining classical

Received: 6 June 2025
Accepted: 19 August 2025
Published: 6 September 2025

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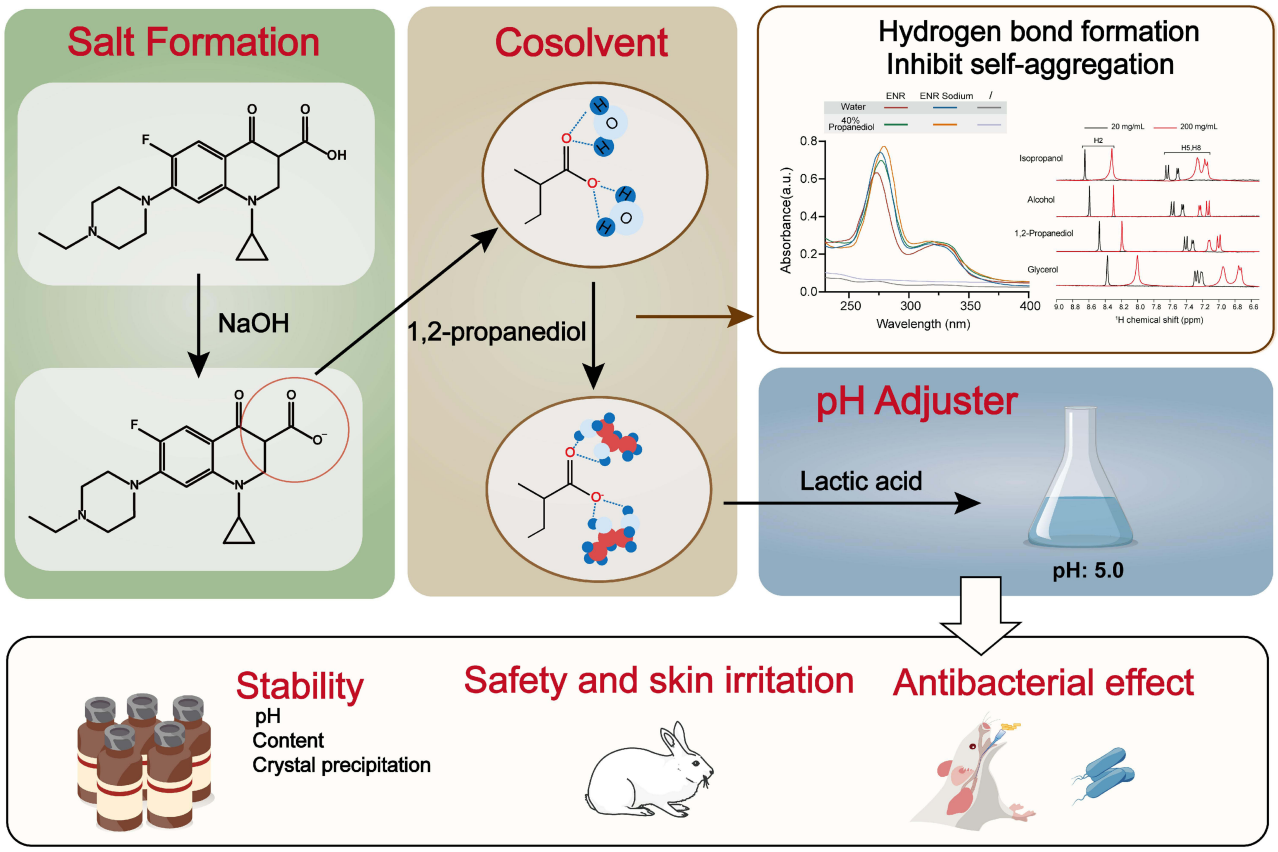
Graphical Abstract



antimicrobials has emerged as a key strategy to combat mixed bacterial infections and slow the progression of resistance. For example, the combination of β-lactam antibiotics and β-lactamase inhibitors, such as ceftazidime-avibactam, represents a successful clinical approach for dealing with drug-resistant bacterial infections.[3] This not only requires synergistic drug interactions but also poses challenges in formulation development.

Enrofloxacin, a fluoroquinolone antibiotic, targets DNA gyrase and topoisomerase IV, exhibiting potent antibacterial activity against bacterial pathogens like *Klebsiella pneumoniae (K. pneumoniae), Escherichia coli (E. coli)*, and *Haemophilus parasuis*. Enrofloxacin is widely used to treat respiratory, gastrointestinal, and urinary tract infections.[4,5] Nevertheless, the rapid emergence of resistant pathogens threatens its therapeutic value. Intervention strategies are urgently needed due to the unsatisfactory clinical efficacy of the single-component formulation. A primary resistance mechanism to enrofloxacin involves limited intracellular accumulation due to efflux pumps and outer membrane barriers of Gram-negative bacteria.[5] Colistin disrupts the bacterial outer membrane, counteracting efflux-mediated drug resistance and enhancing enrofloxacin accumulation.[6] Moreover, colistin neutralizes lipopolysaccharides, the endotoxins of Gram-negative bacteria, reducing tissue damage caused by bacteremia or bacterial lysis. Notably, colistin was once withdrawn from clinical use due to its nephrotoxicity and neurotoxicity. This combination could not only enhance the efficacy of enrofloxacin but also mitigate the safety risks associated with colistin. Consequently, co-administration of the two agents is considered an effective[7] strategy to combat bacterial resistance and systemic infections.

Despite their synergy, enrofloxacin–colistin combination formulations remain absent in the clinic, primarily due to their physicochemical incompatibilities. Enrofloxacin, as a Biopharmaceutics Classification System (BCS) class II drug, exhibits low solubility and strong penetration. Nevertheless, colistin shows high solubility and poor penetration.[8]

Enrofloxacin and colistin represent a typical synergistic pair with opposing biopharmaceutical properties. Enrofloxacin's bitterness and use in deep-seated infections make injectable formulations clinically preferred in veterinary practice.[9] However, its poor solubility compromises solution stability and risks of recrystallization via nucleation-mediated phase transitions during storage,[10–12] making injection development more demanding than tablets, enteric granules, suspensions, soluble powder, and nanoformulations.[13–15] Moreover, pH incompatibility further complicates co-formulation: commercial enrofloxacin injections (eg, Baytril®) are typically alkaline (pH 10–11) for stability, while colistin, despite being water-soluble, exhibits pH-dependent degradation (T1/2 < 2 h at pH > 7.0).[16,17] Therefore, it is necessary to develop an enrofloxacin-colistin injection with enhanced solubility and stability across compatible pH ranges.

In this study, we developed an enrofloxacin-colistin compound injection using the tripartite strategy comprising salt conversion, cosolvent addition and pH adjustment to balance solubility,[18] stability, and pH. IGMH, NMR, and UV-vis revealed that salt formation and 1,2-propanediol addition significantly improved enrofloxacin solubility and inhibited recrystallization. Hydrogen bonding between enrofloxacin and 1,2-propanediol increased the solvation free energy, restricted molecular mobility, and reduced self-aggregation. The final formulation demonstrated great safety, low irritation, and favorable efficacy in vitro and in vivo. In summary, we demonstrated that combining enrofloxacin salts, cosolvents and pH adjuster offers a simple and cost-effective approach for developing injectable formulations, which holds significant promise for clinical translation.

Materials and Methods

Materials

Enrofloxacin and colistin were obtained from Shanghai Yuanye Bio-Technology Co., Ltd (China). Pharmaceutical excipients sodium hydroxide and lactic acid were obtained from Sichuan jin shan zhiyao (China). Disodium edetate were purchased from Chengdu Huayi Pharmaceutical Excipients Manufacturing Co., Ltd (China). 1,2-propanediol were purchased from Jiangxi Yipsheng Pharmaceutical Co., Ltd (China).

Solubility Measurements

The solubility of enrofloxacin and enrofloxacin sodium salt was evaluated in water, different cosolvents (40%) and proportions of 1,2-propanediol solutions (20%, 60% and 80%). Excess powder was added to achieve saturation, and mixtures were stirred at 25°C for 48 h. Samples were held at room temperature for 6 and 14 days, and the supernatant was analyzed by high-performance liquid chromatography (HPLC) after centrifugation. Five independent experimental replicates were performed for each treatment group according to a previous study.[19]

HPLC Conditions

Chromatographic analysis for enrofloxacin utilized an Agilent SB-Aq C18 column (28°C) with isocratic elution (1 mL/min). The mobile phase (80:20, v/v) contained triethylamine-phosphate buffer (Phase A, pH 2.4) and acetonitrile (Phase B). Enrofloxacin was quantified by UV detection at 278 nm with 20 μL injections, demonstrating linearity over 10–300 ppm. Samples were diluted in acetonitrile prior to analysis. The method for colistin is described in Figure S3.

Ultraviolet-Visible Spectrum Detection

Enrofloxacin and its sodium salt (10 μg/mL) were dissolved in water and 40% 1,2-propanediol. A 200 μL sample was added to each well of a 96-well quartz plate, and UV–vis spectra were recorded from 230 nm to 800 nm using a microplate reader (Table S1).

NMR Measurements

A powdered mixture of enrofloxacin and NaOH was dissolved in D_2O solutions containing 40% (v/v) of each tested cosolvent, including 1,2-propanediol, glycerol, isopropanol, and ethanol. The mixture was then transferred to NMR tubes for immediate detection using a JNM-ECZ400S system (JEOL, Tokyo, Japan). ^{1}H NMR spectra were acquired at 20°C with standard parameters (Table S2).

https://doi.org/10.2147/DDDT.S536132

Binding Energy Calculations

Molecular conformations were explored using Molclus and MOPAC (PM6-DH+).[20,21] Structural optimization, vibration analysis, and energy calculations were performed in Gaussian 09[22] with B3LYP-D3BJ/6-31G, and binding energies were calculated at the M06-2X/ma-def2-TZVP level. Weak interactions were analyzed with IGMH[23] in Multiwfn 3.8[24] and visualized in VMD[25] (Table S3).

Preparation and Evaluation of Enrofloxacin–Colistin Compound Injection Solution

Synergistic Activity of Enrofloxacin and Colistin

Synergistic activity of enrofloxacin and colistin was evaluated by checkerboard assays with two-fold serial dilution of drugs.[26] The tested bacteria include clinically isolated strains of *K. pneumoniae, E. coli, Pasteurella multocida (P. multocida)*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Salmonella spp* (n = 10 in total). After incubating 20 h, the $OD_{600\ nm}$ value of each well was measured using a microplate reader.

Time-Dependent Killing Curve Measurement

Bacteria (1.5×10^6 CFUs/mL) were treated by 10 × or 1 × MIC of enrofloxacin or colistin alone, or 0.5 × MIC of the combination drug group. Incubated the mixture at 37°C in a shaker for 12 hours. Viable bacteria were counted at 2 hours, 4 hours, 6 hours, and 12 hours after drug addition, using the plate count method. As delineated in our previous publication,[27] each treatment group included five biologically independent replicates, with all experiments conducted separately.

Determination of pH Adjuster of Compound Injection

Enrofloxacin (2.5 g) was dissolved in saturated sodium hydroxide and divided into five portions. Different pH adjusters (n-butyric, lactic, malic, tartaric, or citric acid) were titrated dropwise and the volume of pH adjusters used was recorded when the solution was clarified. The final solutions were kept at room temperature to observe precipitation.

The Preparation Methods of Compound Injections

As shown in Figure 1, enrofloxacin (25 g) was dissolved in a solution containing 6 g sodium hydroxide, EDTA 2Na and water (450 mL) followed by stirring completely. Colistin (2.5×10^8 IU) was added to a solution containing 1,2-propanediol and water (450 mL total), and stirred until completely dissolved. The enrofloxacin solution is introduced into the colistin solution under continuous stirring, adjusted to the target pH with pH adjuster, and filtered to obtain a pale yellow, clear compound injection.

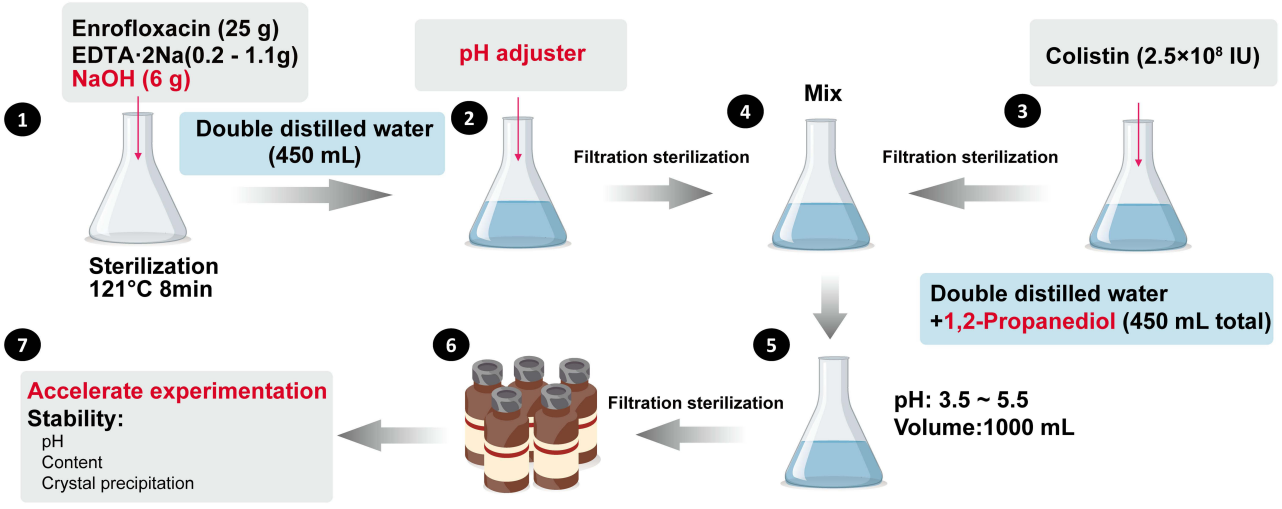


Figure 1 Preparation process of enrofloxacin-colistin sulphate compounded injection.

Orthogonal Test Optimization of Formula

To obtain the most stable compound injection solution, orthogonal tests with three factors at three levels were designed to optimize the dosage of 1,2-propanediol, pH, and EDTA 2Na (Table 1). Crystallization of the prepared compound solution was observed at 30°C for 90 days. Three preparation independent replicates were performed for each treatment group in separately conducted experimental trials.

Acceleration Stability and Low Temperature Stability

The compound solution was stored at 30°C, and samples were collected at 1, 2, 3, and 6 months to assess pH, active ingredient content, and related substances (ciprofloxacin) via HPLC. Appearance, colour, visible particles, and crystal formation were also examined to evaluate stability. For low-temperature stability, samples were stored at 4°C and crystal precipitation was observed at the same time points. All treatments were tested in triplicate (n = 3), with each replicate conducted independently.

Skin Irritation and Safety of Compound Injection

Adult male rabbits (n = 6) were given the compound solution intramuscularly at twice the recommended dose for 7 days. The right hind leg received the compound, while the left received saline as a control. Blood samples were collected via the marginal ear vein on days 0, 7, and 14 for hematological and biochemical analysis, including liver function (serum glutamic-pyruvic transaminase (ALT), serum glutamic-oxaloacetic transaminase (AST), total protein (TP), and albumin (ALB)), renal function (urea (UREA) and creatinine (CREA)), and other relevant indices. Skin irritation and local reactions were assessed on day 7. On day 14, liver, kidney, and muscle tissues at injection sites were collected, fixed in 4% paraformaldehyde, and subjected to hematoxylin and eosin (HE) staining for histopathology. Each replicate was conducted as an independent experiment.

Experimental Treatment of Pneumonic Infection Caused by Multidrug-Resistant P. multocida

Female ICR mice (20–25 g, n = 8) were anesthetized with 100 μL of 1% pentobarbital sodium via intraperitoneal injection. 75 μL of *P. multocida* P32 D7 bacterial solution (6×10^{10} CFUs/mL) was instilled into the mouse trachea. The mice were held in vertical position for 2–3 min to ensure instillation, then returned to their cages.

One hour post-infection, the compound was administered intramuscularly at the recommended dose, with subsequent doses at 24 and 48 h. Mice were monitored every 6 h. Upon death, lungs were harvested, homogenized, and diluted 10-fold with phosphate buffer saline (PBS). The diluted homogenate was plated onto sheep blood agar with 1/4 × MIC enrofloxacin, incubated at 37°C for 24 hours, and colony counts were counted. At 120 h post-infection, surviving mice were euthanized for the same procedure. Each treatment was performed as an independent experiment.

Statistical Analysis

Statistical significance was determined using the software package GraphPad Prism 9.0. The data are presented as the mean ± SD. Differences between groups were analyzed using independent-samples t-tests or analysis of variance and other special analyses. A P value of 0.05 or less was considered statistically significant. Differences were denoted as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; and ns, not significant.

Table 1 Orthogonal Design of Three Factors and Three Levels

Levels	1,2-Propanediol Content (%)	pH	EDTA 2Na Content (%)
1	30 ~ 35	3.5 ~ 4.0	0.025
2	35 ~ 40	4.5 ~ 5.0	0.05
3	40 ~ 45	5.0 ~ 5.5	0.075

https://doi.org/10.2147/DDDT.S536132

Results and Discussion

Salt Formation Increased the Solubility of Enrofloxacin

Enrofloxacin has a low solubility of 0.18 mg/mL in aqueous solution (pH 7.0, 25°C). It is more soluble in acidic and alkaline environments due to its amphiphilic nature.[28] At present, the production of alkaline injection is more mature, and the process of acidic injection makes it challenging. To increase the solubility of enrofloxacin by the available solubilization process, we used enrofloxacin sodium salt. Consistently, the addition of sodium hydroxide led to salt formation, significantly increasing the solubility of enrofloxacin. The results showed that the concentration of enrofloxacin in saturated solution increased by 299-fold with solubility changed from 0.18 mg/mL to 53.96 mg/mL (Figure 2A).[29] Nonetheless, enrofloxacin sodium salt injection carries the risk of crystal precipitation with prolonged or low-temperature storage. Therefore, the solubility of enrofloxacin needs to be further improved.

Enrofloxacin Exhibits Increased Solubility in 1,2-Propanediol

Considering both economic efficiency and practical formulation, cosolvent addition is a key strategy to improve enrofloxacin solubility.[30] First, four common cosolvents were used to enhance the solubility. We found that all four tested cosolvents, including 1,2-propanediol, glycerol, isopropanol, and ethanol enhanced the solubility of enrofloxacin (Figure 2B and C). Notably, enrofloxacin exhibited highest solubility (272.76 mg/mL) with 1,2-propanediol. The concentration of enrofloxacin increased gradually with increasing ratio of 1,2-propanediol, peaking at 40% (Figure 2D). These results indicate that 1,2-propanediol is a potent cosolvent for enrofloxacin. This aligns with the cosolvency principle, where solubility peaks at an optimal solvent ratio due to improved polarity matching and intermolecular interactions.[18,31] Solubility is a key factor affecting the precipitation of crystals from solution, which is crucial to the stability of the drug formulation. To assess long-term stability, drug concentrations in saturated solutions were monitored over 2, 6, and 14 days (Figure 2E). A continuous increase was observed in both 1,2-propanediol and

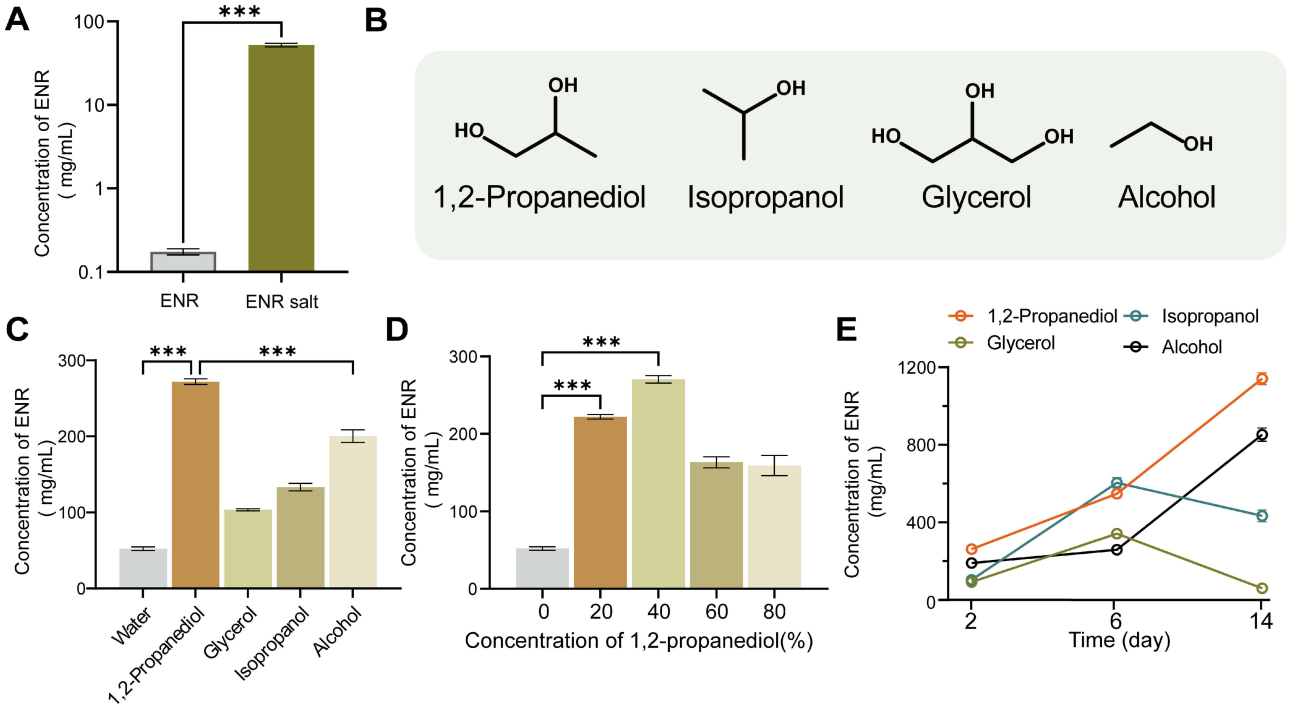


Figure 2 Enrofloxacin exhibits enhanced solubility in 1,2-propanediol. (**A**) Saturated solubility of enrofloxacin and enrofloxacin salt in water. The saturated solubility of enrofloxacin is increased 299-fold by its sodium salt. Enrofloxacin is abbreviated as ENR in the figures in this article. (**B**) Structures of four cosolvents. (**C**) Saturated solubility of enrofloxacin in different cosolvent solutions at 40% (v/v). (**D**) Saturated solubility of enrofloxacin in various concentrations of 1,2-propanediol. Enrofloxacin exhibits the highest saturated solubility in 40% 1,2-propanediol solution. (**E**) Enrofloxacin concentrations of saturated solutions containing different cosolvents (40%) stored for 2 d, 6 d and 14 d. Experiments in B, C, and D were performed as five independent experiments (n=5), and the mean ± SD is shown. Differences were denoted as follows: ** $p \leq 0.001$; and ns, not significant.

ethanol groups, with 1,2-propanediol exhibiting the most marked enhancement over time. In contrast, glycerol and isopropanol exhibited an initial rise followed by a decline. The sustained solubility in 1,2-propanediol and ethanol reflects optimized hydrogen-bond networks and solvent stability, whereas glycerol's excessive viscosity and isopropanol's volatility disrupt supersaturation. These findings highlight the dual benefit of 1,2-propanediol in enhancing solubility and inhibiting crystallization. In summary, salt formation and 1,2-propanediol addition together offer viable solutions to bridge the critical gap between solubility enhancement and physical stability maintenance in existing enrofloxacin formulations.

GMH Analysis of Enrofloxacin with 1,2-Propanediol

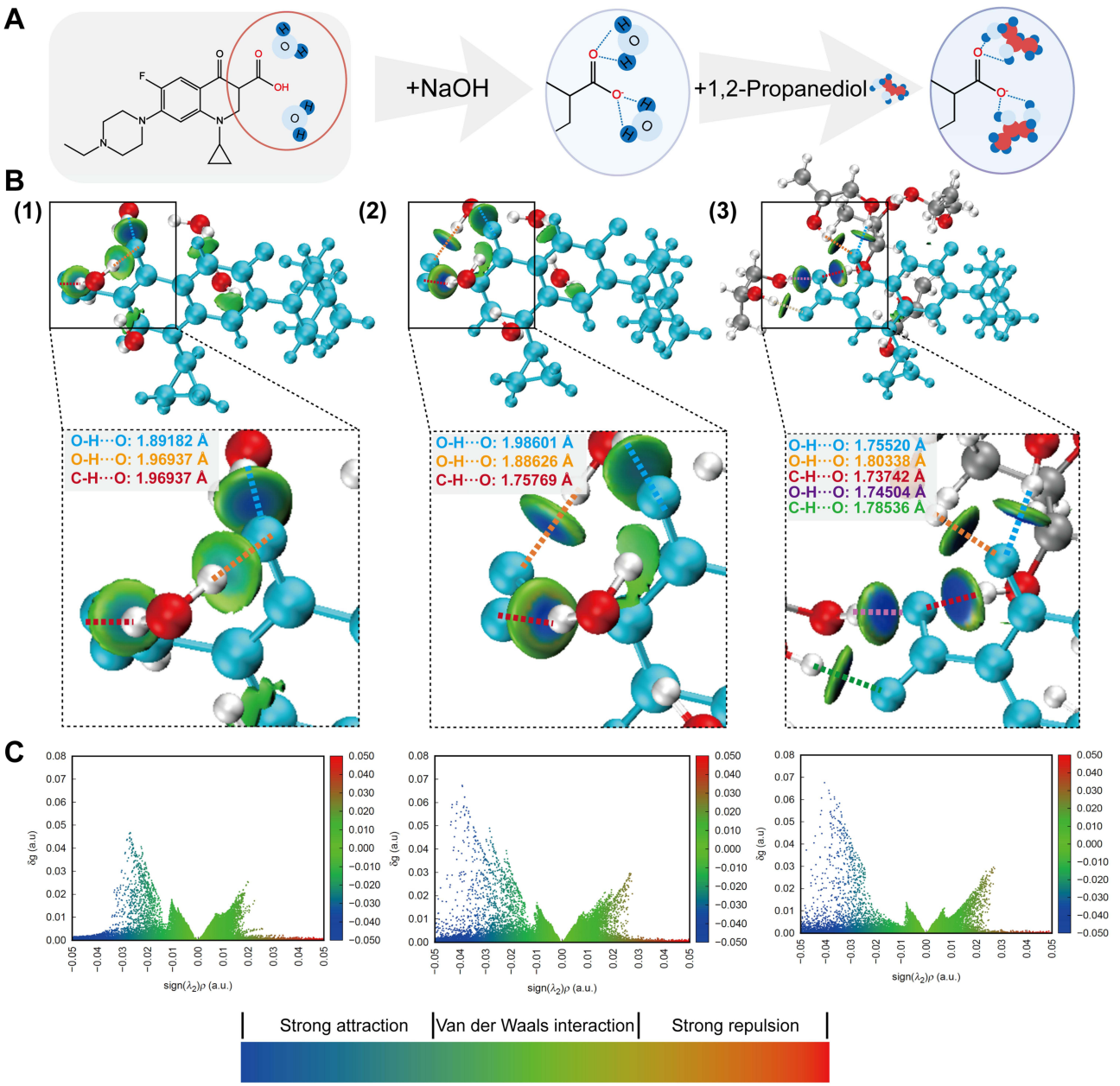
Given the enhanced solubility and stability of enrofloxacin with 1,2-propanediol, we elucidated the molecular mechanism of 1,2-propanediol.[32] We hypothesized that this effect is mediated by hydrogen bond formation between the two hydroxyl groups of 1,2-propanediol and the carboxyl and carbonyl groups of enrofloxacin (Figure 3A). We determined the hydrogen bond formation using IGMH, a new approach for probing strong and weak interactions in molecules.[33] It uses the product of electron density (ρ (r)) and the sign of the second lowest eigenvalue of the electron density Hessian matrix (sign (λ2)) to distinguish between interaction types. The δg values quantify the interaction strength. In IGMH diagram, blue regions indicate strong attractive forces (eg, hydrogen or halogen bonds), green represent weak interacting forces such as van der Waals forces and red shows prominent repulsive interaction.[34] We found only enrofloxacin sodium-water and enrofloxacin sodium-1,2-propanediol systems exhibited spikes near 0.04 a.u. in sign $(\delta_2)\rho$, indicating hydrogen bond formation (Figure 3B). Notably, enrofloxacin sodium forms more hydrogen bonds with 1,2-propanediol than with water or free enrofloxacin (Figure 3C). This aligns with binding energy results that enrofloxacin sodium–1,2-propanediol had the strongest interaction, followed by sodium–water, with the weakest for free enrofloxacin–water (Figure 3D). These results provide computational evidence of hydrogen bond-mediated cosolvent effects in fluoroquinolone systems. Although the long-range effects of the real solvent environment were not fully captured, this approach provides a novel molecular perspective for the crystallisation control, warranting further validation via more solvent molecules or molecular dynamics. Collectively, these results suggest that cosolvent 1,2-propanediol enhances solubility and inhibits crystallization by increasing hydrogen bonding, potentially disrupting drug molecules self-aggregation.

Enrofloxacin Salt and 1,2-Propanediol Interact via Hydrogen Bonding

To further assess the role of the hydrogen bonds in the solubility enhancement of enrofloxacin by 1,2-propanediol, the UV-visible spectrum of enrofloxacin and its sodium salt in water and in 40% 1,2-propanediol aqueous solution were recorded.[35] Both salt formation and 1,2-propanediol addition induced a redshift in the absorption peak (Figure 4A). The shift being most pronounced when both strategies were combined, resulting in a peak shift from 272 nm to 280 nm. This implies that the combined use of salt formation and cosolvent enhances hydrogen bonding interactions, thereby lowering the energy required for electronic transitions. Moreover, both salt and cosolvent increased the absorption peak intensity, possibly due to enhanced electron density in the conjugated system.[36] Although the contributions from solvent polarity changes cannot be ruled out, such spectral shifts are consistent with solvation effects enhanced by hydrogen bonding, as reported in fluoroquinolone–solvent interactions.[37,38] Collectively, these findings suggest that 1,2-propanediol enhances hydrogen bonding between enrofloxacin and the solvent, with this effect further amplified in the salt form.

1,2-Propanediol Inhibit Enrofloxacin Molecule to Form Self-Associates

Molecular self-aggregation in solution promotes crystallization.[39] To investigate the impact of 1,2-propanediol on this process, the molecular self-aggregation of enrofloxacin was determined using ^{1}H-NMR analysis. We found the chemical shifts of H2, H5, and H8 protons in enrofloxacin were monitored from 10 to 100 mg/mL in aqueous solution (Figure 4B). Increasing concentration caused downfield shifts of the proton signals in the conjugated system (Figure 4C). This reflects concentration-dependent self-association, where π–π interactions cause shielding and shift the conjugated proton peaks upfield.[19] Subsequently, the influence of four cosolvents on π–π stacking was compared. Chemical shifts of H2, H5, and H8 were recorded at 10 and 100 mg/mL enrofloxacin solutions containing 40% of each cosolvent (Figure 4D). In all tested cosolvent systems, increasing enrofloxacin concentration continued to induce upfield shifts, indicating persistent molecular self-



D

Solute	Solvent	binding energy	
		kcal/mol	eV
Enrofloxacin	Water	-36.4	-1.5763
Enrofloxacin salt	Water	-66.8	-2.8967
Enrofloxacin salt	1,2-Propanediol	-94.6	-4.1022

Figure 3 1, 2-propanediol contributes strong free energy of solvation by forming hydrogen bonds with enrofloxacin molecules. (**A**) Pattern diagram of 1, 2-propanediol increasing the solubility of enrofloxacin salt. (**B**) Molecular simulations and corresponding (**C**) IGMH analyses of enrofloxacin with water molecules and 1, 2-propanediol molecules. (1) Enrofloxacin-water, (2) enrofloxacin sodium-water, and (3) enrofloxacin sodium-1,2-propanediol systems. (**D**) Binding energy of enrofloxacin with water molecules and 1, 2-propanediol molecules.

association. At fixed concentrations, chemical shifts varied among different cosolvents, likely reflecting differences in solvent environments. However, the magnitude of concentration-dependent shifts varied by cosolvent. As shown in Figure 4E, 1,2-propanediol resulted in the lowest ΔH values among all solvents tested. In particular, ΔH8 decreased from 0.56 ppm in

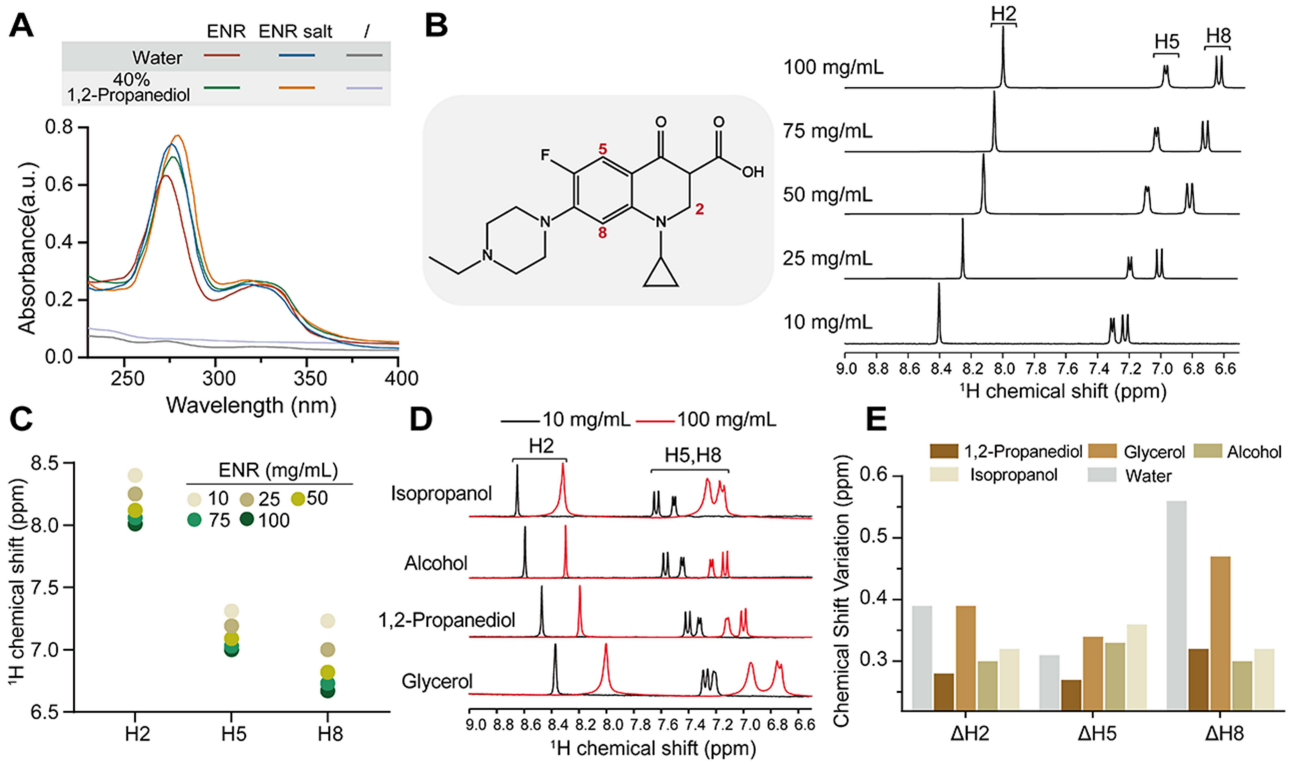


Figure 4 1,2-Propanediol increases solubility by forming hydrogen bonds and improves solution stability by inhibiting molecular self-aggregation. (**A**) UV-visible spectra of different enrofloxacin solutions (5 μg/mL). (**B**) ^{1}H NMR spectrum (H2, H5 and H8) for different concentrations of enrofloxacin solutions. (**C**) Change in chemical shift of characteristic hydrogen with increasing solution concentration. (**D**) ^{1}H NMR spectrum of the H2, H5 and H8 in enrofloxacin solution with different cosolvents at 40% (v/v). (**E**) ΔH2, ΔH5 and ΔH8 in enrofloxacin solution with different cosolvents (40%).

water to 0.32 ppm, highlighting a marked reduction in self-association. This attenuated shift suggests that 1,2-propanediol disrupts self-aggregation via steric hindrance and/or hydrogen bond competition.[40,41] Specifically, 1,2-propanediol may compete with enrofloxacin for intermolecular hydrogen bonding, thereby suppressing self-aggregation through both hydrogen-bond disruption and spatial hindrance. Taken together, the hydrogen bond enhancement and self-association suppression together explain how 1,2-propanediol sustains enrofloxacin supersaturation.

Combined Antimicrobial Potency of Enrofloxacin and Colistin

The combination of colistin and enrofloxacin is expected to be synergistic.[42] We assessed the synergy of enrofloxacin and colistin using the checkerboard assay. Enrofloxacin and colistin exhibited robust synergistic activity against *K. pneumoniae, E. coli, Salmonella*, and *P. multocida* with fractional inhibitory concentration (FIC) index less than 0.5 (Figure S1 and Table S4). To observe the combination effect of enrofloxacin and colistin on persistent bacterium, a time-dependent killing curve was conducted (Figure S2). As expected, the combination of 0.5 × MIC of enrofloxacin and colistin overcame bacterial persistence effectively, reducing viable bacteria below the detection at 12 hours. It is consistent with the group under the treatment with colistin at 10 × MIC alone. Notably, enrofloxacin failed to control the bacterial growth at 10 × MIC. Therefore, the combined use of enrofloxacin and colistin can effectively inhibit the production of persister bacteria and reduce the required antibiotic dose, which slows down the development of resistance.

Determination of pH Adjuster in the Formulation

Given the physicochemical incompatibility between enrofloxacin and colistin, suitable pH adjusters are needed to improve the stability and reduce injection-site irritation of the compound formulation. Compared to other pH adjusters, lactic acid and malic acid required smaller volumes for pH adjustment and showed no precipitation after 48 hours at room temperature (Table 2). Lactic acid, with one hydroxyl and one carboxyl group, forms soluble salts with enrofloxacin

https://doi.org/10.2147/DDDT.S536132

Table 2 Determination of pH Adjuster in the Formulation

pH adjuster	Dosage (mL)	Crystallization (Hours)
n-Butyric acid	2.2	No Cryst within 48
Lactic acid	1.4	No Cryst within 48
Malic acid	0.8	No Cryst within 48
Tartaric acid	0.7	48
Citric acid	35	12

and avoids high viscosity or complex ionization. Moreover, lactic acid adjustment yielded a favorable pH range (4–5), which contributes to reduced injection-site irritation. Based on its effectiveness in achieving stability and biocompatibility, along with cost-efficiency, lactic acid was selected as the optimal pH adjuster for the compound injection.

Formulation Optimization and Stability Assessment

An orthogonal test with three factors including 1,2-propanediol, pH, and EDTA 2Na at three levels identified two lead formulations (Groups 1 and 6) with no crystallization over 90 days (Table 3). Accelerated stability testing (30°C, 180 days) further confirmed Group 6 as the optimal formulation, maintaining a pH of 4.9±0.1 and drug content above 97% under both 30°C and 4°C storage (Table 4 and Table 5 and Table S5). After 10 months of stability assessment, both enrofloxacin and colistin retained over 90% of their original content using HPLC (Table S6). Therefore, Group 6 was ultimately selected as the final formulation. The results demonstrate that 1,2-propanediol as cosolvent and lactic acid as pH adjuster effectively enhance the drug solubility and stability. Due to the limited sample size (n = 3), the results suggest better performance for Group 6 but are insufficient to confirm statistical significance. This limitation underscores the need for further quantitative analysis of recrystallization to optimize the formulation. The preparation process of this injection demonstrated the clinical translational potential of the tripartite strategy, which could also serve as a basis for developing other types of formulations, such as soluble powders and nanoformulations.

Skin Irritation and Safety of Compound Injections

Irritation potential and safety profiles constitute critical evaluation metrics for compound formulations. First, an irritation test was conducted using adult male rabbits to evaluate the enrofloxacin-colistin compound injection (Figure 5A). Blood biochemical analyses revealed all hepatic markers (ALT: 28–80 U/L; AST: 25–130 U/L) and renal parameters (CREA: 0.5–2.5 mg/dL; UREA: 20–45 mg/dL) remained within normal physiological ranges (Figure 5B–D).[43] Complete blood counts showed no statistically significant deviations from the untreated group, collectively indicating negligible hepatorenal toxicity. Furthermore, a histological analysis of liver and kidneys was performed by H&E staining, displaying no appreciable

Table 3 Stability of Compound Solutions with Different 1,2-Propanediol Content, pH, and EDTA 2Na Content, Under the Three-Factor Three-Level Orthogonal Design

Group	1,2-Propanediol Content (%)	pH	EDTA 2Na Content (%)	Crystallization (Days)
1	35	5.0	0.05	No Cryst within 90
2	35	4.0	0.025	60
3	35	4.5	0.075	30
4	40	4.0	0.05	30
5	40	4.5	0.025	60
6	40	5.0	0.075	No Cryst within 90
7	45	4.5	0.05	60
8	45	5.0	0.025	60
9	45	4.0	0.075	30

Table 4 Crystallization and pH Changes in Accelerated Stability Test at 30°C

Group	Case of Crystallization					pH				
	0 d	30 d	60 d	90 d	180 d	0 d	30 d	60 d	90 d	180 d
1	–	–	–	+	++	4.95	4.78	4.78	4.75	4.7
2	–	–	+	++	+++	4.02	3.96	4.03	3.99	3.95
3	–	+	+	+++	+++	4.42	4.34	4.36	4.33	4.33
4	–	+	++	+++	+++	4.03	3.98	4.03	3.98	3.95
5	–	–	+	++	++	4.52	4.43	4.46	4.44	4.43
6	–	–	–	–	–	4.98	4.82	4.82	4.82	4.80
7	–	–	+	++	+++	4.49	4.41	4.43	4.39	4.36
8	–	–	+	++	+++	4.97	4.82	4.83	4.80	4.82
9	–	+	++	+++	+++	4.08	4.03	4.1	4.05	4.03

Notes: – No crystal precipitation was observed; + A small amount of crystal precipitation; ++ moderate crystal precipitation; +++ A large number of crystals precipitation.

Table 5 Phase Pair Content of Enroxacin in Accelerated Stability Test at 30°C (%)

Group	0 d	30 d	60 d	90 d	180 d
1	105.14 ± 1.32	100.09 ± 2.93	102.32 ± 1.52	101.17 ± 2.75	100.23 ± 2.17
2	96.45 ± 1.50	96.10 ± 3.23	100.24 ± 2.68	98.97 ± 3.04	101.03 ± 3.26
3	102.21 ± 2.28	107.64 ± 3.05	99.71 ± 1.91	90.23 ± 2.34	91.86 ± 2.31
4	97.65 ± 1.80	107.25 ± 2.64	100.75 ± 2.42	105.26 ± 3.53	103.25 ± 2.13
5	99.74 ± 1.59	98.84 ± 2.84	95.22 ± 3.01	100.54 ± 3.56	97.49 ± 1.26
6	101.26 ± 1.71	103.72 ± 1.23	101.40 ± 1.83	99.84 ± 2.63	103.31 ± 3.26
7	97.10 ± 0.80	98.41 ± 2.48	102.67 ± 1.67	103.98 ± 2.27	98.60 ± 2.23
8	101.57 ± 1.87	106.38 ± 3.20	98.38 ± 3.11	105.26 ± 3.93	94.52 ± 2.47
9	94.43 ± 2.44	95.46 ± 3.16	94.72 ± 1.75	95.78 ± 2.53	97.25 ± 1.94

histological abnormalities or damages compared with the untreated rabbits (Figure 5E). The skin of rabbits treated with compound injection did not show redness, swelling, inflammation or histological lesions, and was not significantly different from that of rabbits injected with saline (Figure 5F). These results indicate that the compound injection exhibits no significant liver or kidney toxicity and minimal skin irritation. This safety profile is mainly attributed to the reduced toxicity from enrofloxacin-colistin co-administration and the physiologically compatible pH of the injection. Moreover, the pharmaceutical excipients we used, such as 1,2-propanediol, are widely used and applied within recommended concentration limits.[44] Since long-term injection is rare in livestock, chronic toxicity risks are minimal. In addition, the enhanced solubility achieved by our strategy allows for dilution to lower 1,2-propanediol levels when needed while maintaining sufficient drug concentration. Overall, our strategy effectively reduces the potential toxicity and irritation caused by both the excipients and the formulation.

Efficacy of Compounded Injections in vivo and in vitro

The therapeutic effect of compound injection against bacterial infection in vivo was further evaluated in a respiratory infection mouse model (Figure 6A). The compound injection treatment group showed a significantly higher survival rate (62.5%) compared to the PBS group (survival rate of 25.0%) at 120 hours post-infection (Figure 6B). Moreover, in surviving mice at the endpoint of observation, the bacterial load was significantly reduced in the lungs after triple administration of compound injection (Figure 6C). In order to confirm the in vitro activity of the compound injection, the antimicrobial activity against 31 isolates of porcine origin including *A. pleuropneumoniae, P. multocida, S. suis* and *H. parasuis* were tested. The antimicrobial activity of the compound injection was superior for the first three with minimum inhibitory concentration (MIC) less than

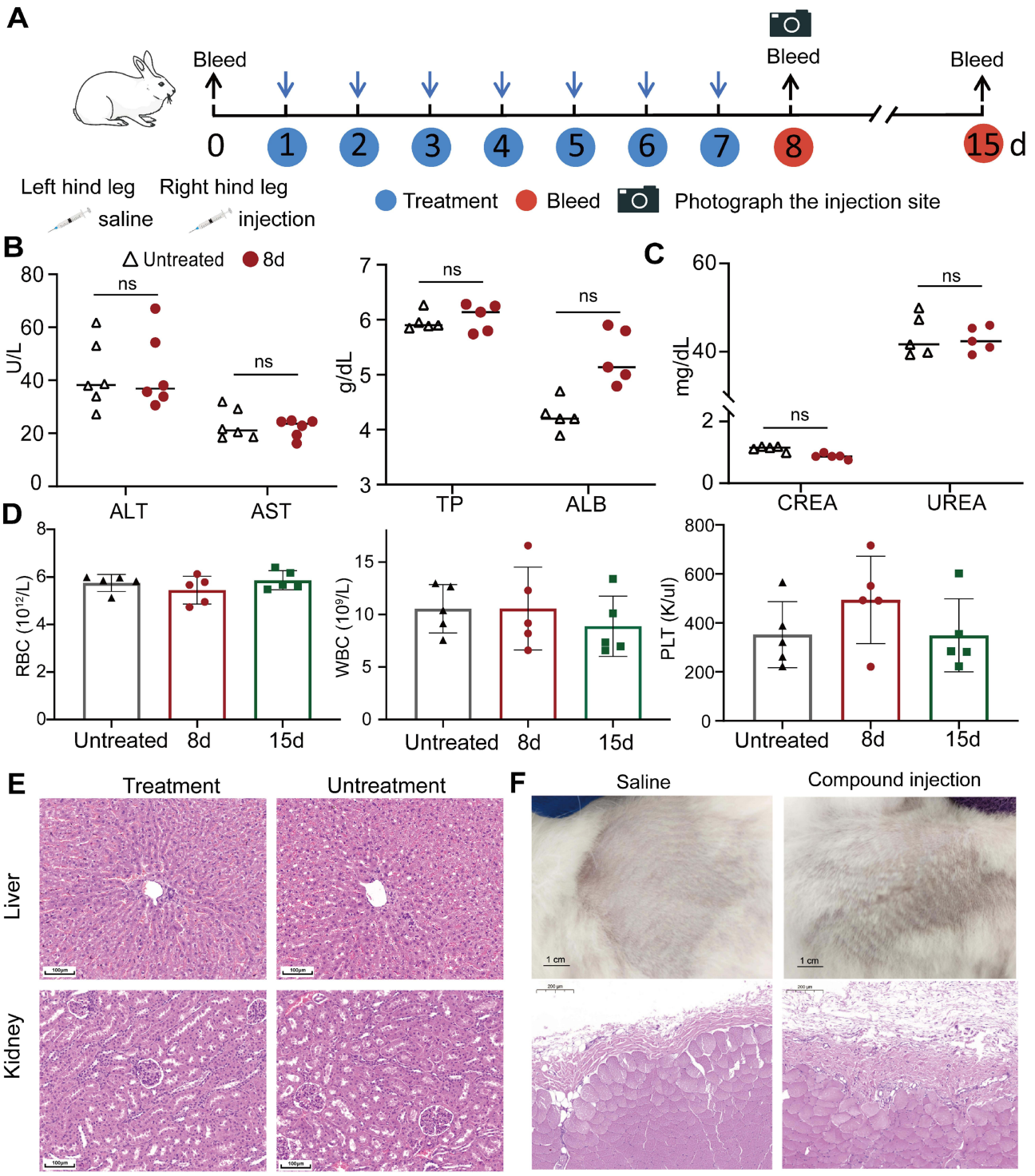


Figure 5 In vivo toxicity and irritation of enrofloxacin-colistin sulphate compounded injections. (**A**) Scheme of the experimental protocol of toxicity and skin irritation in rabbits. (**B**) Liver (ALT, AST, TP and ALB) and (**C**) kidney (CREA and UREA) blood biochemistry indices after enrofloxacin-colistin sulphate compounded injections (n = 6 for ALT and AST, n = 5 for others based on biological sample availability). (**D**) RBC, WBC, and PLT indices at 8d and 15d post-treatment (n = 5). (**E**) Histopathological changes of the liver and kidney after enrofloxacin-colistin sulphate compounded injections by H&E staining. (**F**) Comparison of skin surface and histology at the injection site of saline and compound injections. Differences were denoted as follows: ns, not significant.

10%. However, the effect was highly variable for *H. parasuis* with minimum inhibitory concentration from 0.24% to 62.4%, which is due to colistin resistance (Figure 6D). These results demonstrate the potential for clinical application of compounded injections.

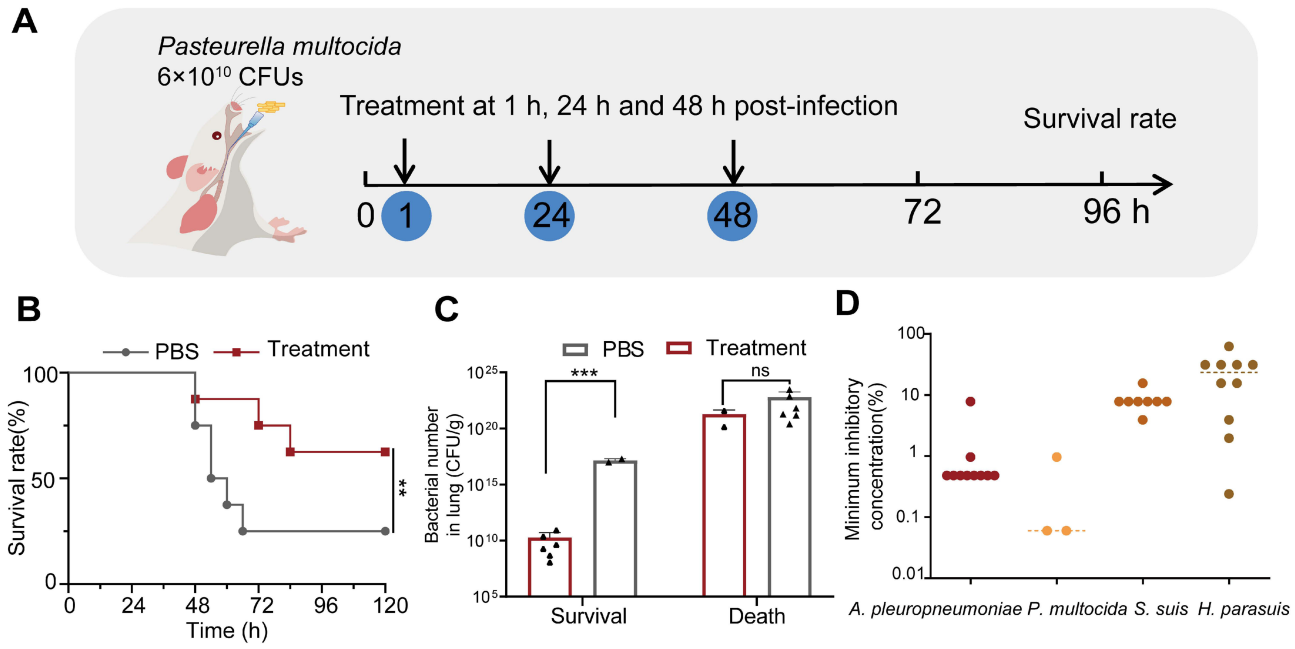


Figure 6 Efficacy of enrofloxacin-colistin sulphate compounded injections in vivo and in vitro. (**A**) Scheme of the experimental protocol of the mouse lung infection model. (**B**) Survival rates of mice infected with *P. multocida* (6×10^{10} CFUs) under treatments (n = 8). (**C**) Bacterial number in the lung. (**D**) Minimum inhibitory concentration of enrofloxacin-colistin sulphate compounded injections to clinical isolates of swine origin (n = 31). MIC values are expressed as the percentage dilution of the compounded injection in the final test well. Differences were denoted as follows: *** $p \leq 0.001$; and ns, not significant.

Conclusion and Perspectives

In this investigation, a tripartite strategy involving salt formation, cosolvent (40% 1,2-propanediol) addition, and pH adjustment was employed to develop a stable and minimally irritant enrofloxacin–colistin combination injection. This strategy enabled a 1500-fold increase in enrofloxacin solubility (from 0.18 to 272.76 mg/mL), with excellent solution stability over 6 months at both 30°C and 4°C. The addition of pH adjusters maintained the formulation within a biocompatible pH range (4–5), which not only stabilized colistin but also reduced injection-related irritation. Moreover, the compounded injection exhibited excellent efficiency in *P. multocida* infection model, achieving a 62.5% survival rate, and caused no significant toxicity or injection-site irritation. These results suggest the therapeutic potential of combining enrofloxacin and colistin, more importantly, addressing longstanding formulation barriers to their co-administration.

Compared to other solubility enhancement strategies such as nanocarriers or lipid-based systems, offering target delivery and extending circulation time but requiring more complex manufacturing and regulatory pathways, the tripartite strategy balances efficacy, simplicity, and scalability, making it more immediately applicable for clinical translation, especially in resource-limited settings. However, the injection used in this study is based on a solution formulation, which may result in suboptimal pharmacodynamics due to pharmacokinetic discrepancies between the two drugs. Although the favorable in vivo efficacy of enrofloxacin and colistin in combination, the potential risk of inconsistent pharmacokinetics remains a concern when applying this strategy to other drug pairs. Nevertheless, using this tripartite strategy as a formulation foundation, potentially in combination with delivery systems, may help overcome this limitation.

In summary, this tripartite strategy provides a practical platform for co-formulating multiple poorly soluble drugs that often present formulation challenges in conventional methods. Future investigations may further explore its applicability across other drug combinations and administration routes.

Ethics Statement

All animal experiments were performed in strict accordance with the Regulations on the Administration of Laboratory Animals (11-14-1988) issued by the State Council of the People's Republic of China. The experimental protocols complied with relevant ethical guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of China

https://doi.org/10.2147/DDDT.S536132

Agricultural University (Approval IDs: YXK-2016-0008 and AW11503202-2-1). The laboratory maintained a valid animal research license (SYXK-2021-0012), granted by the Beijing Association for Science and Technology.

Funding

This work was supported by the Ministry of Agriculture and Rural Affairs of China.

Disclosure

The authors report no conflicts of interest in this work.

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