Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

# Determination of Median Lethal Dose of Enrofloxacin Microemulsion in Mice

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ARTICLE HISTORY (14-176)

Received: April 09, 2014
Revised: July 28, 2014
Accepted: December 23, 2014

**Key words:**
Acute toxicity test
Enrofloxacin
Median lethal dose
Mice
Microemulsion

**ABSTRACT**

As a novel formulation of enrofloxacin, the toxicity of enrofloxacin microemulsion was unknown. The present study was conducted to determine the median lethal dose ($LD_{50}$) of enrofloxacin microemulsion by using the acute toxicity test in mice. Based on the dose range of the pretest, mice in the group receiving the enrofloxacin microemulsion were intragastrically administered the dose levels of 1320.0, 1056.0, 844.8, 675.84, 540.67 and 432.6 mg/kg of body weight, respectively. $LD_{50}$ calculated by Bliss method was 740.08 mg/kg, and 95% confidence limit of $LD_{50}$ was 647.11~844.87 mg/kg, which indicated enrofloxacin microemulsion could be labeled as the hazard category 4 according to GHS and be considered as a low toxicity drug.



**To Cite This Article:** XF Yang, DY Liu, QG Han, YW Sun and GY Fan, 2015. Determination of median lethal dose of enrofloxacin microemulsion in mice. Pak Vet J, 35(2): 155-158.

## INTRODUCTION

Enrofloxacin is the first approved fluoroquinolone for special use in domestic animals (Ribeiro *et al*., 2011). The drug belongs to a broad-spectrum bactericidal antibacterial agent and has a high activity against *Escherichia Coli*, *salmonella*, *haemophilus*, *Pasteurella multocida*, *Staphylococcus aureus*, *Actinomyces pyogenes*, *Erysipelas bacteria*, *mycoplasma*, *Chlamydia*, etc. Furthermore, it has some merits including good absorption, high bioavailability, large distribution volume, and low plasma protein binding rate (Hwang *et al*., 2009). So enrofloxacin is widely used in pets and livestock for the treatment of gastrointestinal, urogenital, respiratory tract and skin infections caused by gram-negative and gram-positive bacteria (Hwang *et al*., 2009; Uivarosi *et al*., 2013; Hussain *et al*., 2014; Xiaolu *et al*., 2014).

Enrofloxacin is beset with the disadvantage of poor aqueous solubility (Seedher and Agarwal, 2009). The very poor aqueous solubility of the drug leads to the difficulties in clinic administration and the design of the novel preparation. It is well known that micro-emulsification is one of current common strategies for increasing the solubility of poorly water-soluble drugs and microemulsion has recently attracted much attention in pharmaceutical research areas. Microemulsion is a colloidal dispersion consisting of oil, surfactant, co-surfactant and aqueous phase at appropriate ratios with droplet diameter usually within the range of 10-100 nm (Nirmal *et al*., 2014). Some advantages of using microemulsion as an ideal drug delivery system mainly include high thermodynamic and kinetic stability, improvement the solubility, the dissolution and the oral absorption of poorly water-soluble drugs, enhancement of bioavailability, protection of the unstable drugs, easy preparation and a long shelf life (Moghimipour *et al*., 2013; Gundogdu *et al*., 2013; Singh *et al*., 2013).

Recently, a novel enrofloxacin microemulsion had been prepared for oral administration to improve the solubility of enrofloxacin in our laboratory (Yang *et al*., 2012). Enrofloxacin microemulsion characterized a transparent and uniform appearance, structural type of oil-in-water, droplet mean size of 22.45±2.92nm, high solubility, strong antibacterial activity and good stability. At present, the preparation and quality evaluation of enrofloxacin nanoemulsion and its content determination by UV spectrophotometry have been reported (Yang *et al*., 2012; Zhang *et al*., 2013). However, there was a lack of useful evaluation on the acute toxicity of enrofloxacin microemulsion till date. The commonly used term to describe the acute toxicity is the medial lethal dose ($LD_{50}$) which is the statistically derived single dose of a substance that produces death in 50% of a population of test animals administered by any of the methods like oral, dermal, inhalation, or intravenous (Anonymous, 2001). The more toxic the substance, the lower the $LD_{50}$ and smaller the dose needed to cause death. In the present study, $LD_{50}$ of enrofloxacin microemulsion was

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determined by the acute oral toxicity test in mice. The results obtained from this present study would provide useful preliminary information on the toxic nature of enrofloxacin microemulsion which could be used to select doses for further short-term and subchronic toxicity tests.

## MATERIALS AND METHODS

**Drugs and solutions:** Enrofloxacin was purchased from Zhengzhou Fansheng Biotechnology Co. Ltd, the lot number was 20090930. Different concentration suspensions of enrofloxacin were made with the distilled water as the solvent.

Enrofloxacin microemulsion and blank microemulsion were all prepared according to the published method by Yang *et al*. (2012), but enrofloxacin was not added in blank microemulsion. In brief, 15.0% enrofloxacin was dissolved in the mixture of 3.0% isopropyl myristate, 28.8% polyoxyethylated castor oil-40 and 19.2% acetic acid, then mixed and drop-wise added 34% distilled water while continually stirring up to forming a clear transparent liquid which was enrofloxacin microemulsion prepared.

**Mice:** A total of 230 healthy Kunming mice of clean grade with the body weight of (20±2) g, half male and half female without multi-parity and pregnancy, were provided by Centre for Laboratory Animal of Xinxiang Medical University of China. Five mice which were raised in a plastic cage under controlled condition of temperature at 22±2°C, relative humidity of 50%~60% and artificial lighting set to12 h:12 h dark/light cycle, were allowed free access to water and standard commercial rodent diet. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Henan Province of China.

**Pretest:** Pretests were conducted to estimate the dose range from 100 to 0% mortality caused by the drug so that the proper dose levels could be established for $LD_{50}$ determination. Mice were acclimatized to the laboratory conditions for a week prior to the test. All 20 mice were randomly divided into 5 groups, 4 mice per group. Mice of each group were given by intragastric administration according to geometric concentration. After intragastric administration, the clinical symptoms and death number of mice were recorded, and the dose of 100% mortality caused by the maximum drug dose (a) and 0% mortality caused by the minimum drug dose (b) were determined by pretest respectively. Then under assumed condition of the group number (n) designed as 6, the dose geometric ratio (r) of official test was calculated according to the formula: $r = \sqrt[n-1]{b/a}$. And so did the dose range estimated from 100 to 0% mortality caused by enrofloxacin microemulsion respectively.

**Drug doses of acute oral toxicity test:** The doses of 100 and 0% mortality caused by enrofloxacin were 6870.0 mg/kg of body weight and 2250.0 mg/kg in the enrofloxacin pretest, respectively. In other words, a=6870.0 mg/kg and b=2250.0 mg/kg, then r=0.8 when n=6 groups. Based on the result of pretest, six dose levels were ultimately required for establishing $LD_{50}$ and the doses used in enrofloxacin groups with each group comprised of ten mice were 6870.0, 5496.0, 4396.8, 3517.4, 2813.9 and 2251.1 mg/kg, respectively.

In enrofloxacin microemulsion pretest, the computing methods of the drug doses were also the same as that of enrofloxacin. The doses of 100 and 0% mortality were all 1320.0 and 430.0 mg/kg for enrofloxacin microemulsion, respectively. Six dose levels used in enrofloxacin microemulsion groups were all 1320.0, 1056.0, 844.8, 675.84, 540.67 and 432.6 mg/kg, respectively. At the same time, a corresponding blank microemulsion group was set as the control of enrofloxacin microemulsion at each dosage.

**Formal test of acute oral toxicity test:** 190 healthy Kunming mice were randomly divided into nineteen groups (10 mice in each group, half male and half female). Among the groups, one group was the mutual control group, and the other 18 groups were enrofloxacin, enrofloxacin microemulsion and blank microemulsion. Three dose groups which were 60 individuals in each dose group were further divided into 6 subgroups, respectively. The ratio of doses between adjacent groups was 0.8. Prior to dosing, the mice were fasted for 12 h to ensure that the dose was administered on an empty stomach, during which they were permitted to drink water freely. 10 mice in each group were administrated twice by gastric perfusion, all of which were treated at the administrated drug volume of 0.02 mL/g of body weight. Mice in the control group was administered the same amount of distilled water.

Toxic signs and symptoms of mice were observed for 24 h after dosing. Meanwhile, the numbers of deceased mice in each group were counted.

**Calculation of $LD_{50}$:** $LD_{50}$ with 95% confidence limits was calculated by Bliss method using New Drug Statistic Treatment (NDST) software version 8.0 (Sun, 1998).

## RESULTS

**Toxic symptoms recorded during the test and $LD_{50}$ of enrofloxacin:** Most of mice were observed such symptoms as repose, decreasing activities and tiredness after administration. Mice began to die at about 2 h and their death time focused on 2~5 h after dosing. The impending death mice appeared labored breathing, shiver, convulsion and death finally. There were no obviously visible abnormalities of important organs such as liver, kidney, heart, lungs, brain, spleen, thymus in death mice by necropsy, but the stomach of most mice contained drug and appeared tympany. Seven days later, the living mice were killed by cervical dislocation, and it could be found that all organs were normal. Death did not appear in control group which behaved normal appetite and activities during the test.

The mortalities of mice within 24 h were shown in Table 1. $LD_{50}$ of enrofloxacin was calculated by Bliss software, its regression equation between the probits of mortalities (Y) and the log of doses (D) was derived as y (Probit) = -24.83 + 8.1899 Log (D), $LD_{50}$ = 4387.6 mg/kg, 95% confidence limit of $LD_{50}$ was 3869.1~5015.9 mg/kg,

$LD_5$ = 2763.0 mg/kg, $LD_{95}$ = 6967.4 mg/kg, indicating that enrofloxacin was a low toxic substance.

**Toxic symptoms recorded during the test and $LD_{50}$ of blank microemulsion:** After intragastric administration, the mice exhibited decreased activities, prone immovability, lassitude, dispiritment, shiver, tired crouch and insensitivity to outside stimulation. Their feed and water intake declined and prior to the death, mice were observed restlessness, convulsion or asthenia and tachypnea. Mice started to die at about 1 h after administration. The dead mice anatomized were found a tympany stomach with the drug, and other abnormalities were not discovered. Seven days later, the survival mice were killed by cervical dislocation and their all organs such as hearts, livers, spleens, lungs, kidneys, stomachs were not found abnormalities.

The death numbers in 24 h were shown in Table 2. Based on Bliss method, the regression equation of $LD_{50}$ of blank microemulsion was y (Probit) = -18.833+8.2503 Log (D), $LD_{50}$ = 773.89 mg/kg. The 95% confidence limits of $LD_{50}$, $LD_5$ and $LD_{95}$ was 681.65~879.95, 488.99 and1224.8 mg/kg, respectively. It could be concluded that blank microemulsion had a low toxicity.

**Toxic symptoms recorded during the test and $LD_{50}$ of enrofloxacin microemulsion:** The toxic symptoms and anatomy results of the mice administrated by enrofloxacin microemulsion were corresponding to those of mice treated with blank microemulsion.

The death numbers in 24 h were shown in Table 3. The regression equation of $LD_{50}$ in enrofloxacin microemulsion group was y (Probit) = -17.015+7.6725 Log (D), $LD_{50}$ = 740.08 mg/kg, 95% confidence limit of $LD_{50}$ was 647.11~844.87 mg/kg, $LD_5$ = 451.74 mg/kg, $LD_{95}$ = 1212.50 mg/kg, indicating that enrofloxacin microemulsion were higher toxic than enrofloxacin, belonged to a low toxicity substance and the toxicity was mainly caused by blank microemulsion.

## DISCUSSION

The acute toxicity test is generally the first step of safety evaluation of preclinical new drugs. It provides data on the relative toxicity likely to arise from a single or brief exposure. More toxicology characteristics of new drugs can be obtained from the test as many as possible when new drugs are researched, and toxicity intensity data can also be provided to judge whether candidate new drugs has values of research and development in early stage. $LD_{50}$ is the most important parameter index to evaluate the acute toxicity and the basic standard of the acute toxicity classification or hazard category of different drugs. It not only provides the information on health hazards likely to arise from short-term exposure, but also contributes to establish a dose regimen in subsequent sub-chronic and chronic toxicity test studies when no other toxicology information is available. There are many methods used in calculating $LD_{50}$ such as the graphical method of Miller and Tainter, arithmetical method of Karber and statistical approach which include up-and-down procedure, fixed dose procedure, acute toxic class method, Bliss method, and sequential grouping method (Igbinosa *et al*., 2013). Among the different methods, the most classic, precise and sensitive method is Bliss method which is still the preferred statistical method (Li *et al*., 1995; Igbinosa *et al*., 2013). Moreover, the method was also recommended for the calculation of $LD_{50}$ by guiding principles of preclinical toxicology research of new drugs in China. In the present study, Bliss method was used for determination of $LD_{50}$ of enrofloxacin microemulsion in mice by intragastric administration.

As an antibiotic that is effective in treating a wide range of infections, enrofloxacin has been used specially for many years in clinical animals. In order to compare with $LD_{50}$ of enrofloxacin microemulsion under the same conditions controlled, $LD_{50}$ of enrofloxacin was firstly determined by the acute toxicity test. Its $LD_{50}$ for the laboratory mice was 4387.6 mg/kg in the present study, which was agreed with 4336 mg/kg reported (Enrofloxacin, CAS 93106-60-6). According to Globally Harmonized System (GHS) of Classification and Labeling of Chemicals (Anonymous, 2001), it should be classified as the hazard category 5 which meant it had a relatively low acute toxicity hazard.

$LD_{50}$ of enrofloxacin microemulsion in mice by intragastric administration was 740.08 mg/kg, which was lower than that of enrofloxacin. It could be labeled as the hazard category 4 according to GHS and be considered as a low toxicity drug. Because $LD_{50}$ of blank microemulsion was 773.89 mg/kg, and the toxic symptoms and pathological observation of mice treated were similar

**Table 1:** The results of the acute toxicity test of enrofloxacin

| Dose (mg/kg) | Log of dose (D) | No. of mice | No. of survival | No. of death | Mortality (%) | Experimental probit unit (Y) | Regression probit unit (Y) |
|---|---|---|---|---|---|---|---|
| 6870.0 | 3.8370 | 10 | 0 | 10 | 100 | — | 6.5948 |
| 5496.0 | 3.7400 | 10 | 3 | 7 | 70 | 5.5240 | 5.8011 |
| 4396.8 | 3.6431 | 10 | 5 | 5 | 50 | 5.0000 | 5.0075 |
| 3517.4 | 3.5462 | 10 | 8 | 2 | 20 | 4.1585 | 4.2137 |
| 2813.9 | 3.4493 | 10 | 9 | 1 | 10 | 3.7183 | 3.4200 |
| 2251.1 | 3.3524 | 10 | 10 | 0 | 0 | — | 2.6263 |

**Table 2:** The results of the acute toxicity test of blank microemulsion

| Dose (mg/kg) | Log of dose (D) | No. of mice | No. of survival | No. of death | Mortality (%) | Experimental probit unit (Y) | Regression probit unit (Y) |
|---|---|---|---|---|---|---|---|
| 1320.0 | 3.1206 | 10 | 0 | 10 | 100 | — | 6.9132 |
| 1056.0 | 3.0237 | 10 | 2 | 8 | 80 | 5.8415 | 6.1137 |
| 844.8 | 2.9268 | 10 | 4 | 6 | 60 | 5.2529 | 5.3142 |
| 675.8 | 2.8298 | 10 | 6 | 4 | 40 | 4.7471 | 4.5144 |
| 540.6 | 2.7329 | 10 | 9 | 1 | 10 | 3.7183 | 3.7146 |
| 432.6 | 2.6360 | 10 | 10 | 0 | 100 | — | 2.9152 |

**Table 3:** The results of the acute toxicity test of enrofloxacin microemulsion

| Dose (mg/kg) | Log of dose (D) | No. of mice | No. of survival | No. of death | Mortality (%) | Experimental probit unit (Y) | Regression probit unit (Y) |
|---|---|---|---|---|---|---|---|
| 1320.0 | 3.1206 | 10 | 0 | 10 | 100 | — | 6.9281 |
| 1056.0 | 3.0237 | 10 | 2 | 8 | 80 | 5.8415 | 6.1845 |
| 844.8 | 2.9268 | 10 | 3 | 7 | 70 | 5.5240 | 5.4410 |
| 675.8 | 2.8298 | 10 | 6 | 4 | 40 | 4.7471 | 4.6973 |
| 540.6 | 2.7329 | 10 | 8 | 2 | 20 | 4.1585 | 3.9535 |
| 432.6 | 2.6360 | 10 | 10 | 0 | 100 | — | 3.2101 |

between blank microemulsion and enrofloxacin microemulsion was primarily caused by the drug carrier the blank microemulsion. That is to say, the blank microemulsion itself possessed certain low toxicity. This reason was in agreement with the hazard category 4 of blank microemulsion based on its $LD_{50}$ according to GHS. It had been reported that treatment with polyoxyethylated castor oil vehicle alone produces asthenia, tachypnea, convulsions, and ultimately death in dogs and mice (Torchilin, 2011; Labatec-Pharma, 2012; Li *et al*., 2012). And it became evident that the polyoxyethylated castor oil as a surfactant causes serious side effects such as hypersensitivity reactions in some patients (Gelderblom *et al*., 2001; Singla *et al*., 2002). Since polyoxyethylated castor oil acted as surfactant phase, a relative higher level (28.8%), was one of the important ingredients, and animal reactions exhibited were all similar in blank microemulsion and enrofloxacin microemulsion groups, it was thought to be responsible in the present study. Therefore, it may be concluded that the acute toxicity of enrofloxacin microemulsion was primarily due to the toxicity of polyoxyethylated castor oil. However, the cause of polyoxyethylated castor oil related acute mortality was not investigated in this study.

**Conclusion:** $LD_{50}$ of enrofloxacin microemulsion in mice was determined as 740.08 mg/kg by the acute toxicity test and Bliss method, and 95% confidence limit of $LD_{50}$ was 647.11~844.87 mg/kg. The result obtained from the present study indicated the toxicity of enrofloxacin micro-emulsion could be labeled as the hazard category 4 according to GHS and be considered as a low toxicity drug.

**Author contribution:** GY and XF conceived and designed the experimental protocols. XF, DY and YW executed the experiment. DY and QG analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

**Acknowledgement:** This work was supported by the grant of Science and Technology Research Important Project of Education Department Henan Province (Grant No. 13A230306).

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