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Research Article

The Heat Stability of Enrofloxacin Antibiotic Residue in Milk Detected using HPLC

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Abstract | Enrofloxacin is one of the fluoroquinolone antibiotics used for therapy in animals. The presence of enrofloxacin antibiotic residue in milk could affect product quality and human health. This study aimed to determine the heating effect of enrofloxacin antibiotic in milk. The milk samples spiked with enrofloxacin antibiotic were submitted to thermal treatments. The milk samples were analyzed using high performance liquid chromatography (HPLC) method. This study showed that no heating (control), heating at 72 °C for 15 seconds (high temperature short time pasteurization), and 89 °C for 1 second (higher heat shorter time pasteurization) did not affect on enrofloxacin antibiotic residue concentration, however heating at 121 °C for 15 minutes (sterilization) affected the antibiotic enrofloxacin. The stability of enrofloxacin antibiotic could result problem in public health. Antibiotic residues could result microbial resistance, toxicity, carcinogenicity, allergy, and product rejection. Prevention and control of antibiotic residues should be carried out through socialization to the public, monitoring of antibiotic examinations on animal products and application of hazard analysis and a critical control point (HACCP) in the food industry.

Keywords | Antibiotic, Enrofloxacin, Heating, HPLC, Milk

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INTRODUCTION

Infectious diseases can cause problems in animal husbandry and lead to economic losses. Treatment of diseases caused by bacterial infections can be overcome with antibiotic therapy (Argudin et al., 2017; Priyanka et al., 2017; Mellado et al., 2018). Antibiotics can be applied for chemotherapeutic and prophylactic purposes. Safety and effective use of antibiotics must be regulated because improper antibiotics could cause residues in food products of animal origin. The animal products should be harvested after a sufficient withdrawal period from using the antibiotics to provide safe foods for public consumption from antibiotic residues. Control and detection of antibiotic residues in food of animal origin can protect humani-health as consumers (Jayalakshmi et al., 2017; Moghadam et al., 2016).

Many types of antibiotics have been used for diseases treatments in animals. One of antibiotics group can be given to animals are fluoroquinolone antibiotics (Trouchon and Lefebvre, 2016). Fluoroquinolone can be used for handling diseases in poultry, cattle, goats, sheep, pigs, horses, and fish. Fluoroquinolone antibiotics are important for the treatment of animal diseases, because they can be used for the treatment of quite serious diseases with specific infections. World Organization for Animal Health categorizes fluoroquinolone into the highest type of antibiotic (antimicrobial) criteria as critically important antimicrobials, so that their use and residue on food products of animal origin needs to receive attention (OIE, 2018).

Enrofloxacin is one of fluoroquinolone antibiotic used for therapy in animals. Enrofloxacin antibiotics are

broad-spectrum antibiotics, making them effective for the treatment of Gram positive and Gram negative bacterial infections. Enrofloxacin works by inhibiting deoxyribonucleic acid (DNA) gyrase (topoisomerase II) and topoisomerase IV that are needed by bacteria for DNA replication. This barrier produces a cytotoxic effect on the target cell. Some literature states that in addition to the benefits of treatment using the antibiotic enrofloxacin, it turns out this antibiotic residue can cause neurological disorders, cardiotoxic, blindness, embryonic death, and environmental toxicity (Trouchon and Lefebvre, 2016; Wei et al., 2018; Hruba et al., 2019). The use of enrofloxacin in animals can also cause residues and microbial resistance, so the use of enrofloxacin must be monitored and evaluated for food safety and human health (Moharana et al., 2015).

The European Commission (2010) states that the maximum residue limit (MRL) of the enrofloxacin in milk is 100 µg/kg (µg/L). The results of testing enrofloxacin residue in Czechoslovakia showed 115 out of 150 samples of cow's milk detected the presence of enrofloxacin residues in the range of 1.7-18.6 µg/L (Navratilova et al., 2011). Testing on 119 milk samples in Croatia showed enrofloxacin residues in the range 0.56-22.3 µg/L (Bilandzic et al., 2011). Some studies in India show that enrofloxacin were present in milk samples and some of them exceeded the MRL (Srinivasu et al., 2017; Nirala et al., 2017).

Antibiotic residues in milk can affect product quality and human health. Milk processing techniques such as heating can decrease the concentration of antibiotic residues. A study conducted in Spain revealed that quinolones were stable in the heating milk process based on kinetic models (Roca et al., 2010). Results of research in Santiago regarding the cooking processes (microwaving, roasting, grilling, boiling and frying) did not affect the enrofloxacin residues in chicken muscle (Lolo et al., 2006). Furthermore, research in Thailand showed antimicrobial activities of enrofloxacin were quite stable in the heating process (Thamthaweechok et al., 2018). This study aimed to determine the effect of heating at various temperatures and length of time (72 °C for 15 seconds, 89 °C for 1 second, 121 °C for 15 minutes) on the concentration of enrofloxacin residue in milk.

MATERIALS AND METHODS

The research was conducted in Toxicology Laboratory of the Indonesian Research Center for Veterinary Science (IRCVS).

Chemicals and reagents

The chemicals and reagents in this study used were HPLC or analytical grade include enrofloxacin standard for HPLC (Sigma Aldrich, St. Louis, MO, USA), methanol (Merck, Darmstadt, Germany), trichloroacetic acid (TCA) and acetonitrile (Merck, Darmstadt, Germany), ultra pure water (UPW) (Merck, Darmstadt, Germany), potassium dihydrogen phosphate (Merck, Darmstadt, Germany), and sodium hydroxide (Merck, Darmstadt, Germany).

Milk samples

Milk samples used in this study were raw milk, originating from the Animal Husbandry Faculty of IPB University. Blank samples were confirmed of enrofloxacin residue free.

Research design

This research is an experimental study using a completely randomized design (CRD) regarding the effect of heating on the residual antibiotic enrofloxacin in milk. Milk samples (blank samples) were added with a standard antibiotic of enrofloxacin (Sigma-Aldrich) at a concentration of 200 µg/L and stirred until homogeneous. The treated milk was divided into 4 treatments, namely no heating/control (P1), with heating at 72°C for 15 seconds/ high temperature short time (HTST) pasteurization (P2), with heating at 89°C for 1 second/ higher heat shorter time (HHST) pasteurization (P3) (FDA, 2017) and with heating at 121°C for 15 minutes/ autoclaving sterilization temperature (P4) (WHO, 2019). Each treatment was conducted on 6 replications, and all milk samples were prepared and extracted, then analyzed using the HPLC method.

Sample preparation

Analysis of the antibiotic residue of enrofloxacin in milk using HPLC refers to the method developed by Cinquina et al. (2003) with modifications. In this procedure, 5 mL of spiked milk samples was added with 2.5 mL of 20% trichloroacetic acid (TCA) in methanol. The prepared solutions were homogenized for 15 seconds in a shaker and centrifuged at 1500 rpm for 10 minutes, then added with 12.5 mL of phosphate buffer (pH 7.4), and centrifuged 1500 rpm for 15 minutes. The supernatant was inserted into the solid phase extraction (SPE) column of OASIS HLB (500 mg, 6 cc) cartridges from Waters (Milford, MA, USA) which has been activated/conditioned using 6 mL of methanol, 6 mL of UPW, and 6 mL of phosphate buffer. After that, the SPE column is washed with 2 mL of UPW, and dried for 3 minutes. Then SPE was eluted with 2 mL of 1% TCA in acetonitrile. The samples were dried using flow nitrogen, then a 200 µL mobile phase solution is added, then the samples were ready to be analyzed using HPLC.

Chromatographic condition

Enrofloxacin antibiotic residue was analyzed using Shimadzu LC-20AD HPLC (Japan) with a photodiode array (PDA) detector and C_{18} Sunfire column (5 µm; 4.6 x 250 mm) (Waters, Ireland) for separation. The mobile phase used was a mixture of 0.02 M trichloroacetic acid

(TCA), methanol, and acetonitrile in a ratio of 74: 4: 22 (v/v), filtered using 13 mm PVDF 0.45 µm Acrodisc (Waters, USA) and sonicated for 10 minutes. The mobile phase flow rate in the HPLC was 1.4 mL/min, with an injected sample volume of 20 µL and detected at a wavelength of 277 nm.

Standard solution and calibration curves

The HPLC method was validated using FDA (2019) guidelines. Standard enrofloxacin was prepared by dissolving 10 mg of enrofloxacin in 10 mL in methanol grade, then stored at about -20 °C. Working standard solution of enrofloxacin with concentration of 500, 250, 125, 62.5, 31.25, and 15.625 µg/L were prepared daily in mobile phase for calibration standards. The results fitted to linear regression analysis.

Statistical analysis

Data on heating treatment were analyzed using one-way analysis of variance (ANOVA) to see the effect of heating on the antibiotic enrofloxacin in milk. Furthermore, if it had a significant effect on the observed variables, it was continued by the Duncan's Multiple Range Test (DMRT) at a 95% confidence level.

RESULTS AND DISCUSSION

Analysis of enrofloxacin antibiotic residue in milk was carried out using an HPLC instrumentation with a PDA detector, with optimal results at a wavelength of 277 nm (Figure 1). Quantitative detection of antibiotic residues using an HPLC has advantages over microbial assays, because it can provide quantitative and confirmative information even to low concentrations below MRL (Jevinova et al., 2003). Some research on enrofloxacin antibiotic residues in milk has been carried out using HPLC devices (Cinquina et al., 2003; Moharana et al., 2015; Mohammed et al., 2016).

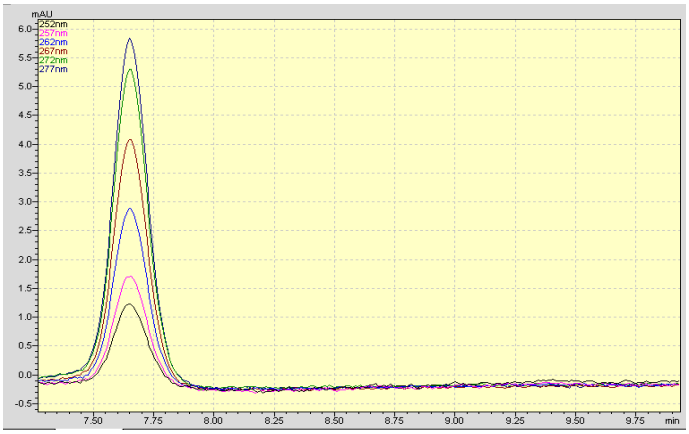

Figure 1: Profile of the standard wavelength peak of the antibiotic enrofloxacin.

The linearity of the calibration curve was carried out by making a line equations at 6 stratified concentrations of the antibiotic enrofloxacin 15.62 to 500 µg/L. The results of the linear regression analysis showed the coefficient of determination (R^2)= 0.999 (Figure 2). This showed a strong linearity relationship because there was a linear relationship between concentration and the peak area of the antibiotic enrofloxacin. The results of this study had a better coefficient of determination compared to research conducted by Navratilova et al. (2011) with a value of R^2 = 0.98.

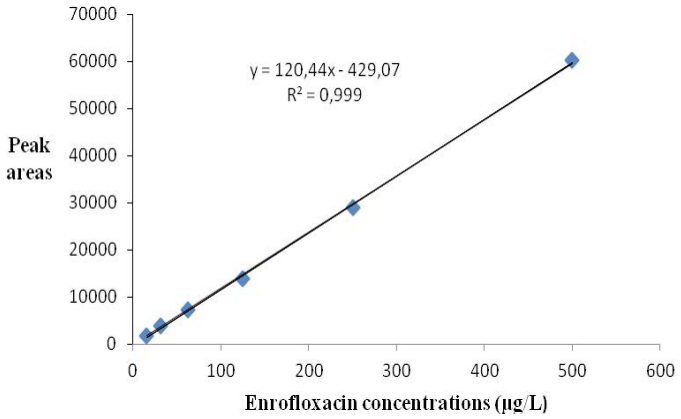

Figure 2: Enrofloxacin calibration curve using HPLC.

Heating the antibiotic residue of enrofloxacin was done with 4 treatments, namely no heating/control (P1), heating at 72 °C for 15 seconds/ HTST (P2), heating at 89 °C for 1 second/ HHST (P3) and heating at 121°C for 15 minutes/ sterilization (P4). The results of the four HPLC chromatograms of treatments are shown in Figure 3. Enrofloxacin was detected at the retention time of 7.4 minutes. The highest percentage of antibiotic residue degradation is heating at 121 °C for 15 minutes/ sterilization (Table 1).

Table 1: Results of the percentages of the enrofloxacin residue degradation on heating treatments.

Treatments	P2	P3	P4
The percentages of the antibiotic residue degradation (%)	0.045	2.25	6.34

Results of analysis of variance (ANOVA) heating treatment of enrofloxacin antibiotic residues in milk differed significantly, with a value of P= 0.013 (P <0.05), so there were a heating effect on the concentration of the antibiotic residue of enrofloxacin in cow's milks which were detected using HPLC.

Statistical analysis was confirmed with Duncan Multiple Range Test (DMRT) analysis, because the results of the ANOVA heating treatment had a significant effect. The results of the DMRT analysis showed no significant

difference between P1, P2, and P3, but there were differences with P4 (Table 2). The treatment no heating (P1), heating at 72°C for 15 seconds (P2), and heating at 89°C for 1 second (P3) did not affect the concentration of the enrofloxacin antibiotic. Heating temperature at 121°C for 15 minutes using an autoclave affected on the concentration of the antibiotic enrofloxacin. This may occur due to the influence of the use of autoclaves with high temperature heating treatment and a pressure of 1 atm. The sterilization process affected on the antibiotic enrofloxacin, but not all the antibiotic residues of enrofloxacin were degraded so that it could endanger human health.

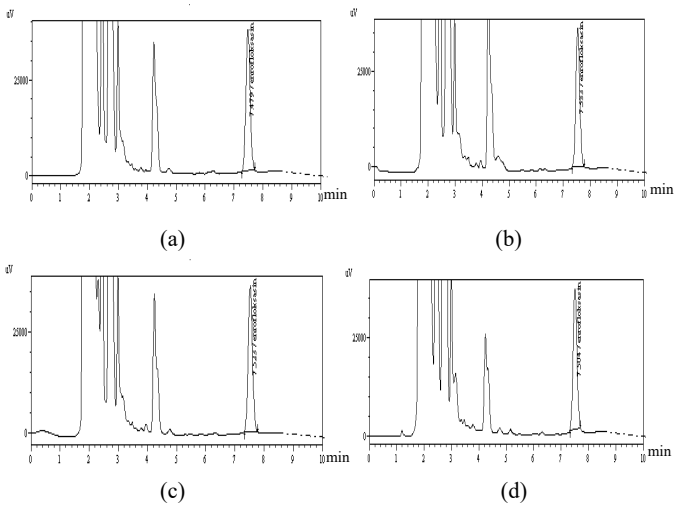

Figure 3: Chromatograms of treatments enrofloxacin in milk using HPLC: no heating (a), heating at 72 °C for 15 seconds (b), heating at 89 °C for 1 second (c), and heating at 121 °C for 15 minutes (d).

Table 2: Results of Duncan Multiple Range Test (DMRT) analysis of heating treatments of enrofloxacin residues in milk using HPLC.

Treatments	N	Residue concentration of enrofloxacin (Average ± SEM)
No heating/control (P1)	6	156.4±1.91^{a}
Heating at 72 °C for 15 seconds (P2)	6	156.3±1.66^{a}
Heating at 89 °C for 1 second (P3)	6	152.9±1.89^{a}
Heating at 121 °C for 15 minutes (P4)	6	146.5±2.93^{b}

Note: Different superscript show significantly different at P <0.05. SEM: standard error of the mean.

The results showed that enrofloxacin was relatively stable in the heating process. Enrofloxacin, which belongs to the fluoroquinolone antibiotic group, was more resistant to heat than the betalactam antibiotic group (ampicillin, amoxicillin). The heating process could kill bacteria, but it could not inactivate antibiotics (Thamthaweechok et al., 2018). Previous research conducted by Roca et al. (2010), stated that enrofloxacin was stable to the heating process with results based on kinetic models. Enrofloxacin, ciprofloxacin, and norfloxacin antibiotics are more stable than other fluoroquinolones (danofloxacin and gemifloxacin) because they have a piperazine ring bound to position 7 of the quinolone structures (De Bairros et al., 2018).

The stability of antibiotic residues in milk could be a public health problem. Antibiotic residues in foodstuffs can cause microbial resistance, toxicity, carcinogenicity, allergy, and product rejection (Asredie and Engdaw, 2015; Bacanli and Basaran, 2019). Some research showed that some types of bacteria were resistant to the antibiotic enrofloxacin, namely *Campylobacter* (Panzenhagen et al., 2016; Roasto et al., 2016; Mansouri-najand et al., 2012), *Actinonobacillus* (Wang et al., 2010), *Escherichia coli* (Pereira et al., 2020; Niasono et al., 2019; Li et al., 2017; Piras et al., 2015), *Enterococcus* (Kataoka et al., 2014; Liu et al., 2012), *Mycoplasma* (Lysnyansky et al., 2013), *Salmonella* (Li et al., 2017), *Streptococcus* (Yongkiettrakul et al., 2019), *Pasteurella* (Oh et al., 2018; Bourely et al., 2019; Cuevas et al., 2020).

Prevention and control of antibiotic residues should be carried out such as encouraging concern about antibiotic residues in individuals, groups or community organizations through socialization from veterinarians, institutions, local and central government; development of procedures for rapid detection of antibiotic residues in animal products; antibiotics are used only for treatment in sick animals according to their causes (Nisha, 2008).

The prevention of antibiotic residues in Indonesia is carried out by stipulating the Minister of Agriculture Regulation No. 14/2017 regarding the Classification of Veterinary Drugs, since 1st January 2018 the government has banned the use of antibiotic growth promoters (AGP) in the feed. This prohibition is also reinforced by MOA No. 22/2017 concerning Registration and Distribution of Feed, which requires a declaration of not using AGP in feed formulas produced for producers who wish to register feed for trade. Besides, it is hoped that the prohibition of using antibiotics as feed additives can prevent antibiotic residues from occurring in animal products. The use of antibiotics for therapy in animals should be carried out by a veterinarian or animal health personnel under the supervision of a veterinarian. Other regulations concerning the guarantee of safe, healthy, intact, and halal food are based on the Minister of Agriculture Regulation No. 95 of 2012 concerning veterinary public health and animal welfare. If these regulations are implemented properly, food products of animal origin can be created that are protected from antibiotic residues and guaranteed food safety for the public.

Furthermore, the prevention of the presence of antibiotic residue can be conducted by concerning the withdrawal time on diseases treatment and monitoring of antibiotic residue on animal products. The application of hazard analysis and critical control points (HACCP) in the food industry can also improve the quality of food products. The HACCP system applies good manufactory practices to ensure all food products are safe for public consumption (Mohammed et al., 2016; Jayalakshmi et al., 2017; Priyanka et al., 2017).

CONCLUSIONS AND RECOMMENDATION

The degradation of enrofloxacin antibiotic in milk was affected by heating treatment at 121 °C for 15 minutes, but it was persisted on heating treatments at 72 °C for 15 seconds and 89 °C for 1 second. The stability of the antibiotic residue of enrofloxacin in milk could be a public health problem.

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NOVELTY STATEMENT

Our research showed the degradation of enrofloxacin antibiotic in milk was affected by heating treatment at 121 °C for 15 minutes which is suitable, but the stability on common pasteurization processing in the dairy industry at 72 °C for 15 seconds and 89 °C for 1 second could be a public health problem.

AUTHOR'S CONTRIBUTION

This research was designed jointly by Prima Mei Widiyanti, Mirnawati B Sudarwanto, Etih Sudarnika, and Raphaella Widiastuti. The authors have read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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