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# Validation Methods for Determination of Enrofloxacin Level in Animal Medicine Products using High-Performance Liquid Chromatography Instrument

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**Abstract**

Enrofloxacin is an antibiotic that is often used as a veterinary drug. Analysis of enrofloxacin in veterinary drugs can be carried out using the high-performance liquid chromatography (HPLC) method. The HPLC method was chosen because it is capable of separating the active substance to be measured from other components with high speed and efficiency. This study aims to validate the appropriate analytical method for measuring enrofloxacin levels in veterinary drug products using HPLC. The analytical method was validated using HPLC brand Shimadzu® 6.1, column C18 150 × 4.6 mm; temperature 30˚C; UV-Vis detector wavelength 278 nm; and a flow rate of 1 mL/min. The mobile phase used was a mixture of 0.05 M $NaH_2PO_4$ pH 2.5 and acetonitrile with a ratio of 65:35 (volume: volume). The sample was a standard enrofloxacin solution with a concentration of 0.1 µg/mL; 1µg/mL; and 10 µg/mL were analyzed with three replications per sample concentration. The injection volume is 20 µL per injection. The parameters measured were selectivity, linearity, accuracy, precision, limit of detection, and limit of quantification. The results showed good selectivity because the peak area of enrofloxacin was not disturbed by other components. The results of measuring the concentration of standard solutions produce a linear line equation y = 291727x + 37070 with a correlation coefficient value of 0.9999 (r = 0.9999). The accuracy value of each concentration meets the standards ranging from 99.916% to 100.762%. The results of precision calculations are relative standard deviation (RSD) ranging from 5.333% to 10.831%. The detection limit value is 0.003 µg/mL and the quantification limit value is 0.011 µg/mL. The validation test results conclude that the analytical method developed has good validity and can be used to determine enrofloxacin levels in veterinary drug products.

**Keywords**: enrofloxacin, high-performance liquid chromatography, validation of analytical methods

## Introduction

Antibiotics are substances produced by various species of microorganisms and are toxic to other species of microorganisms, having the ability to inhibit bacterial growth (bacteriostatic) or kill bacteria (bactericidal) (Sumardjo, 2009). One of the antibiotics that is often used as a veterinary drug is enrofloxacin (Arief et al., 2016). Enrofloxacin can be given by injection or orally to animals, is a fluoroquinolone class of antibiotics that is effective as bactericidal on Mycoplasma, Gram-positive, and Gram-negative bacteria, but is relatively ineffective against obligate anaerobic microorganisms (Ramsey, 2014). The empirical formula of enrofloxacin is $C_{19}H_{22}FN_3O_3$, with a molecular weight of 359.4 g/mol. Physically, enrofloxacin has the form of a white or slightly yellow needle-shaped crystalline powder, odorless and tasteless, and available as hydrochloride, lactate, and sodium salts. Enrofloxacin is insoluble in water, slightly soluble in chloroform, and methanol, soluble in dimethyl formamide, and very soluble in acidic or alkaline media (Zahid and Isnindar, 2013). The mechanism of action works by inhibiting bacterial DNA-gyrase (type II topoisomerase), so that preventing the supercoiling of DNA and DNA synthesis (Plumb, 2011). Examination of levels or analysis of enrofloxacin in veterinary drugs, one of which can use the high-performance liquid chromatography (HPLC) method (Rakhmawatie and Rohmani, 2014).

High-Performance Liquid Chromatography (HPLC) or often also called High-Performance Liquid Chromatography (HPLC) is a method that uses high-pressure pump system column technology and sensitive detectors so that it can separate chemical compounds with high speed and efficiency (Anastasia, 2011; Harmita, 2009). High-Performance Liquid Chromatography (HPLC) equipment can separate a number of organic compounds, inorganic compounds, biological compounds, analysis of impurities, and analysis of nonvolatile compounds. The advantages of HPLC are ease of implementation, good resolution ability, speed of analysis, high sensitivity, and not causing damage to the material being analyzed (Satria et al., 2014). Analysis methods can vary, so the validation of the enrofloxacin analysis method in veterinary drug products must be carried out to obtain the accuracy and accuracy of enrofloxacin levels in veterinary drugs, and the results obtained can be accounted for (Rakhmawatie and Rohmani, 2014; Sofyani et al., 2018). Method validation analysis is a procedure for evaluating certain parameters based on laboratory experiments to prove that these parameters meet the requirements for use in measuring a compound. The validation parameters measured in this study include accuracy, precision, selectivity, linearity, the limit of detection (LOD), and the limit of quantification (LOQ) (Harmita, 2009).

This study aims to validate the appropriate analytical method for determining enrofloxacin levels in veterinary drug products using high-performance liquid chromatography (HPLC).

## Materials and Methods

The study was conducted at the Laboratory of the Department of Pharmacology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta Special Region. The material used as a standard solution was enrofloxacin produced by PT. Mandiri Citra Bandung determination was then made into a graded concentration to a concentration of 0.1 µg/mL; 1µg/mL; and 10 µg/mL. The mobile phase used was a mixture of 0.05 M $NaH_2PO_4$ pH 2.5 and acetonitrile with a ratio of 65:35. The ingredients for making the mobile phase were acetonitrile (J. T. Baker®), H3PO4 (J. T. Baker®), $NaH_2PO_4$ (EMSURE®), Aqua Pro Injection (PT. Ikapharmindo Putramas). The mobile phase was prepared by mixing 0.05 M $NaH_2PO_4$ pH 2.5 and acetonitrile (65:35). Preparation of $NaH_2PO_4$ 0.05 M pH 2.5 was carried out by weighing 2.9995 grams of $NaH_2PO_4$ and then dissolving it in aquabidest until the volume reached 500 mL, then homogenizing the mixture. After that, $H_3PO_4$ was added little by little, stirred, and measured with a pH meter until the pH of the solution reached 2.5. The mobile phase made has a volume of 500 mL, according to the comparison, it takes 325 mL of 0.05 M $NaH_2PO_4$ pH 2.5 and 175 mL of acetonitrile.

The analytical method for determining enrofloxacin levels in veterinary drug products is based on a modification of the veterinary drug residue analysis method by Patriana et al. (1997) used a Shimadzu 6.1 HPLC tool with a C18 column 150 × 4.6 mm; temperature 30˚C; UV-Vis detector wavelength 278 nm; and a flow rate of 1 mL/min. The prepared standard solution was then injected into the HPLC column using an HPLC syringe in the amount of 20 µL, three times for each solution concentration.

The HPLC results are in the form of a chromatogram which can be known for the retention time and the peak area of the compound components in solution. The determination of validation parameters can be measured based on retention time and peak area data. The validation parameters measured to analyze the research results include accuracy, precision, selectivity, linearity, the limit of detection (LOD), and the limit of quantification (LOQ).

## Result and Discussion

The validation parameters used in this study are selectivity, linearity, accuracy, precision, the limit of

detection (LOD), and the limit of quantification (LOQ). Selectivity is a parameter used to see the ability of a method to separate the active substance from other components in the solution. The selectivity of the method was determined by comparing the results of the enrofloxacin analysis with the blank (mobile phase). Analysis was performe on enrofloxacin and blank samples (a mixture of 0.05 M NaH2PO4 pH 2.5 and acetonitrile with a ratio of 65:35).

Comparison between the results of the enrofloxacin analysis and the blank was carried out by looking at the peak area formed and the retention time of each chromatogram. The selectivity value is determined by the resolution power (Rs) (Harmita, 2009). The resolution power is calculated to determine the quality of the separation of two adjacent peaks. Resolution power is said to be good if the value is ≥ 1.5 (Nurhidayati et al., 2015).

In the blank chromatogram, there is a peak area that appears at 1.517 minutes with a certain area, whereas, in the enrofloxacin sample, there is no peak at the same retention time. On the enrofloxacin chromatogram, the peak area appeared at 1,892 minutes (Figure 1). The peak area and resolution power (Rs) are presented in table 1. Based on the results in table 1, it appears that the resolution power values range from 2.602 to 3.396, so this HPLC method meets the selectivity parameter criteria.

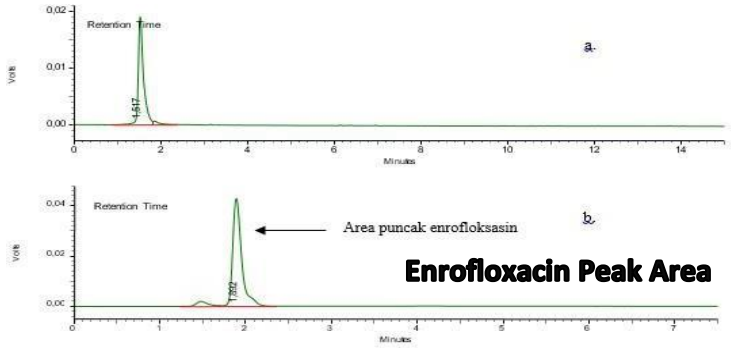


Figure 1. a. Blank chromatogram, b. Chromatogram of enrofloxacin concentration of 1 µg/mL.

Table 1. Peak area and resolution power data

| Concentration (µg/mL) | repetition | Peak area | X̄ peak area | Resolution power | X̄ resolution power |
|---|---|---|---|---|---|
| 0,1 | 1 | 60246 | | 2,428 | |
| | 2 | 64865 | 66465 | 2,321 | 2,602 |
| | 3 | 74284 | | 3,058 | |
| 1 | 1 | 308443 | | 3,336 | |
| | 2 | 340522 | 328553 | 2,664 | 2,926 |
| | 3 | 336694 | | 2,777 | |
| 10 | 1 | 2636607 | | 2,777 | |
| | 2 | 2949946 | 2954360 | 4,077 | 3,396 |
| | 3 | 3276526 | | 3,336 | |

Linearity is a parameter to assess the ability of an analytical method to provide a good response and is proportional to the concentration of analyte in the sample. The linear relationship is shown by the correlation coefficient parameter (r) for linear regression analysis y = a + bx (Harmita, 2009). Enrofloxacin linearity analysis using HPLC can be obtained by making a curve of the relationship between enrofloxacin concentrations (x-axis) and the average area of the peaks formed (y-axis). Enrofloxacin linearity was determined using three concentration levels, namely 0.1 µg/mL; 1µg/mL; and 10 µg/mL. The average area value in table 1 is analyzed and produces a linear equation y = 291727x + 37070. Based on the curve it is known that the value of r = 0.9999. Harminta (2009) states that good linearity has a correlation coefficient of 1 ≤ r ≥ 0.9990. Thus, it can be concluded that the relationship between concentration and peak area is linear and meets the requirements for a

good r-value so that the HPLC method used meets the criteria for linearity parameters. Validation of the enrofloxacin analysis method using spectrophotometry by Ambarwati et al. (2013), HPLC by Wijayanti and Setiawan (2017), and microbiological assay by Souza et al. (2004) gave results that were not much different, namely r = 0.999. The linearity value of enrofloxacin can be seen in Figure 2 below.

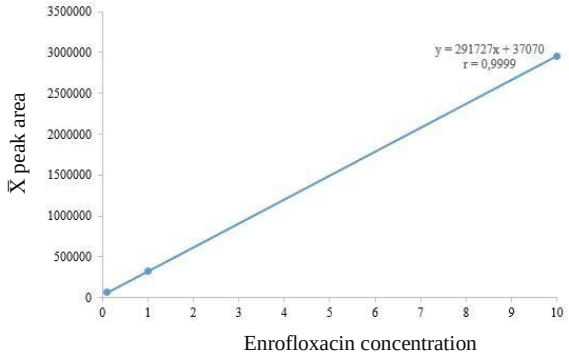


Figure 2. Enrofloxacin linearity curve

Accuracy is the closeness of the determination results obtained with the actual results. According to Harmita (2009), the recovery of analytes can be calculated using the formula:

$$\% \text{ recovery } = \frac{analytical\ concentration}{actual\ concentration} \times 100\%$$

The accurate calculation results are presented in Table 3. Based on the results in table 3, the recovery value ranges from 99.916% to 100.762%. The recovery value is not much different from the results of research by Ambarwati et al. (2013), who obtained an average recovery value of 99.44% using spectrophotometry, and the results of research by Souza et al. (2004), who obtained a recovery value of 98.7%-99.7% using a microbiological assay. So that the HPLC method meets the accuracy parameter criteria.

The precision parameter describes the closeness of the test results in several repetitions (Sugihartini, et al., 2014). Precision is measured as standard deviation (standard deviation) or relative standard deviation (coefficient of variation/relative standard deviation). The specified RSD (relative standard deviation) requirements vary depending on the level of the analyte being analyzed. The RSD value at 1 ppm is 16% (Harmita, 2009). Precision calculation results for a concentration of 0.1 µg/mL; 1µg/mL; and 10 µg/mL are presented in Table 3. The calculation results show that each concentration has an RSD value of ≤ 16%. Based on this, it can be concluded that the HPLC method used meets the precision parameter criteria.

Table 2. Recovery value and precision value

| Concentration (µg/mL) | Recovery value (%) | Deviation standard | RSD (%) |
|---|---|---|---|
| 0,1 | 100,762 | 7154,464 | 10,764 |
| 1 | 99,916 | 17529,630 | 5,333 |
| 10 | 100,001 | 319982,231 | 10,831 |

Harmita (2009) states that the limit of detection (LOD) is the smallest amount of analyte in a sample that can be detected and gives a significant response compared to a blank. Based on the calculations, the limit of detection was obtained at a concentration of 0.003 µg/mL. This means that 0.003 µg/mL is the minimum amount that can be detected by the HPLC method. The limit of detection (LOD) obtained in this study can detect lower levels of enrofloxacin than the spectrophotometric method by Ambarwati et al. (2013), and HPLC by Wijayanti and Setiawan (2017) with detection limit values of 0.757 µg/mL and 0.005 µg/mL respectively.

The limit of quantification (LOQ) is the smallest quantity of analyte in a sample that still meets the

criteria. Wijayanti and Setiawan (2017) stated that the quantification limit value for the enrofloxacin analysis method with HPLC was 0.010 µg/mL. Based on the research results, the quantification limit value (LOQ) was obtained at a concentration of 0.011 µg/mL. This means that 0.011 µg/mL is the minimum amount that can be detected by the HPLC method which can be accounted for quantitatively.

## Conclusion

Based on the results of the study, the use of a high-performance liquid chromatography (HPLC) brand Shimadzu 6.1, with a 150×4.6 mm C18 column, temperature 30˚C, UV-Vis detector wavelength 278 nm using a mobile phase mixture of 0.05 M NaH2PO4 solution pH 2.5 and acetonitrile with a ratio of 65:35 and a flow rate of 1 mL/minute have good validity in terms of accuracy, precision, selectivity, and linearity. The detection limit was obtained at a concentration of 0.003 µg/mL and the quantification limit at a concentration of 0.011 µg/mL. Thus, this method can be used to measure enrofloxacin levels in veterinary drug products.

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