A simple rapid liquid chromatography method for determination of enrofloxacin in chicken plasma was developed. Samples were prepared by protein precipitation with acetonitrile/ammonia mixture and dichloroethane extraction. Analytical separation was performed in a gradient grade on a Phenomenex Gemini C18 column and analytes were detected by fluorescence ($\lambda_{ex} = 300 \text{ nm}$ and $\lambda_{em} = 448 \text{ nm}$). The acidified mobile phase ($\text{pH} = 3.8$) was a mixture of acetonitrile and 0.05 M acetate buffer. The method was linear in the range 0.2 – 3.5 $\mu$g/mL, limit of detection was 40 ng/mL and limit of quantification was 120 ng/mL. The assay was accurate and precise and the mean recovery of enrofloxacin was 90.07 ± 0.89 %. The suitability of the assay for pharmacokinetic studies was determined by measuring enrofloxacin concentrations in chicken plasma after a single intravenous administration of the drug at a dose of 10 mg/kg. Enrofloxacin concentration decreased from 4.17 $\mu$g/mL in 5 min to 0.32 $\mu$g/mL 24 hours after drug administration. The obtained results confirmed the suitability of the method for enrofloxacin determination.

Key words: broiler chicken, enrofloxacin, HPLC, pharmacokinetics, validation

INTRODUCTION

Enrofloxacin is a derivative of quinolone carboxylic acid classified into the group of fluoroquinolones. It is widely used in veterinary medicine in cattle, pigs, poultry, fish, dogs and cats in the treatment of diseases caused by aerobic Gram-negative, Gram-positive bacteria and such pathogens as Mycoplasma, Chlamydia and Rickettsia (Brown, 1996). The mechanism of action of enrofloxacin is connected with its interfering with DNA synthesis by inhibiting DNA gyrase activity (Zechiedrich and Cozzarelli, 1995). Enrofloxacin possesses excellent pharmacokinetic properties such as high bioavailability and high volume of distribution (Martinez et al., 2006). Fluoroquinolones widespread application in
human and food-producing animals has increased the risk of inducing pathogen resistance which may lead to therapy failure (Brown, 1996).

Various techniques have been utilized for the determination of enrofloxacin e.g., microbiological assay (Sumano et al., 2001), spectrofluorimetry (Waggoner and Bowman, 1987), high pressure liquid chromatography (HPLC) with fluorescence (FL) (Knoll et al., 1999) and mass spectrometry (MS) detection (Kim et al., 2006). However, microbiological techniques are slow and suffer from poor precision and specificity and presently chromatographic methods, especially HPLC are the most popular (Carlucci, 1998; Anonymous, 2002). Most of the developed HPLC methods utilize fluorescence detection since it is more sensitive than ultraviolet (UV) detection. Besides, molecules of fluoroquinolones fluorize without previous derivatization which remarkably simplifies the analytical procedure (Lizondo et al., 1997).

The purpose of the present study was to develop a simple, rapid and reliable analytical method for the detection and quantification of enrofloxacin in chicken plasma samples using a HPLC assay with a reversed-phase C18 column and fluorescence detection. This method should enable enrofloxacin determination without expensive and time-consuming procedures like solid phase extraction (SPE) or nitrogen evaporation which were commonly used in other studies (Carlucci, 1998). The developed method should be suitable for pharmacokinetic studies in chicken.

MATERIAL AND METHODS

Reagents and materials
Enrofloxacin, anthranilic acid (the internal standard) and ammonium hydroxide were purchased from Fluka (Milwaukee, WI, USA). Ammonium acetate and HPLC-Grade methanol and acetonitrile were all obtained from J. T. Baker (Phillipsburg, NJ, USA). 1,2–dichloroethane and 85% phosphoric acid were bought from Polskie Odczynniki Chemiczne (Gliwice, Poland). Water used to prepare the mobile phase solutions was produced by Millipore (Billerica, MA, USA) water purification system. The ammonium acetate solution (0.05 M) was prepared by dissolving 3.854 g of ammonium acetate with double-deionized water, diluted to 1000 mL and filtered by glass vacuum solvent-filtration apparatus using 0.45 μm nylon membranes obtained from Supelco (Bellefonte, PA, USA). The samples were filtered through Titan Syringe Filter, Nylon, 0.45 μm, 17 mm obtained from Sun Sri (Rockwood, TN, USA).

Chromatography
Analyses were performed using the Agilent 1100 HPLC system consisting of a quaternary pump G1311A, degasser G1379A, autosampler G1313A, column thermostat G1316A and fluorescence detector G1321A. The analytical column was a reverse phase Gemini C18, 3 μm (150 x 3mm) obtained from Phenomenex (Torrance, CA, USA). The mobile phase consisted of a mixture of acetonitrile–acetate buffer (phase A: 0.05 M ammonium acetate/acetonitrile 2:8 (v/v); phase B: 0.05 M ammonium acetate/acetonitrile 8:2 (v/v). The pH of the
mobile phase was adjusted to 3.8 with 85% phosphoric acid. The analysis was performed at a controlled temperature of 35°C under gradient conditions and enrofloxacin was detected at λ\text{ex} = 300 nm and λ\text{em} = 448 nm. At t = 0 the mixture consisted of phase B exclusively, which changed linearly from t = 4 min to t = 6 min to reach composition of 80% A and 20% B which lasted until t = 10 min and then changed linearly within 2 min to the initial conditions. Total run time was 20 min. The injection volume (sample injected automatically by means of the autosampler) was 2 μL, the flow rate was 0.5 mL/min and enrofloxacin was quantified by measuring the peak area normalized by internal standard peak area and concentration. During routine analyses quality control points containing enrofloxacin at a fixed concentration: quality control low (QCL) = 1 μg/mL, quality control medium (QCM) = 2 μg/mL and quality control high (QCH) = 3 μg/mL were used to assess system stability (Anonymous, 2001).

**Drug standards**

The primary reference stock solution of enrofloxacin was prepared in methanol at 400 μg/mL concentration. Stock solution was subsequently diluted with methanol to yield solutions at concentrations 2, 5, 10, 15, 20, 25, 30 and 35 μg/mL. Anthranilic acid stock solution was prepared in methanol at a concentration of 1340 μg/mL and was diluted to 60 μg/mL working solution. Both enrofloxacin and anthranilic acid working solutions were prepared daily, while stock solutions were prepared every two weeks.

**Sample preparation**

The samples preparation was conducted according to the following procedure: chicken plasma samples were thawed out at room temperature and 250 μL of it was transferred to polypropylene tubes. Thereafter, 50 μL of anthranilic acid working solution (60 μg/mL) was added and after mixing, 600 μL of acetonitrile/ammonia solution (96:4, v/v) was added to precipitate the proteins. Thereon samples were vortexed for 10 s at the maximum speed. Samples were centrifuged (2200 x g, 20 min, 4°C) and the supernatant was transferred into tubes where 750 μL 1,2-dichloroethane was added. Tubes were vortexed, centrifuged (200 x g, 5 min, 4°C) and from the upper aqueous layer 100 μL was transferred to a new tube and mixed with 200 μL methanol. Finally samples were filtered through syringe filters to HPLC vials. Samples in which enrofloxacin concentration was over the upper limit of quantification were 2-times diluted with distilled water and reanalyzed.

Calibration samples were prepared in a similar manner; only in the first stage 225 μL of plasma was transferred to a tube and spiked with 25 μL enrofloxacin working solutions (yielding final concentrations 0.2 – 3.5 μg/mL).

**Method validation**

The method was validated according to directives of European Medicines Agency and Food and Drug Administration (Anonymous 1995, 2001). The linearity of the method was checked in the range 0.2 – 3.5 μg/mL. The range of the
Method was determined by means of 9 calibration curves (consisting of 8 previously prepared samples each) prepared during 3 subsequent days. Each sample was injected in five times replicate. The obtained data were also used to calculate accuracy and precision (repeatability and intermediate precision). Method accuracy and precision were determined for the whole linear range of the method. The extraction efficiency (recovery) was determined for enrofloxacin and anthranilic acid by comparing peak areas from drug-free samples spiked with known amounts of drugs (concentrations of quality control points for enrofloxacin and 10 μg/mL for internal standard) and standard solutions injected directly into the analytical column. Each sample was determined in quintuple. Limit of detection (LOD) and limit of quantification (LOQ) were estimated by means of additional analyses.

**Experimental design**

Clinically healthy broiler chickens (Ross, 28-31 days old, n = 24) were purchased at a poultry farm and placed in the animal house 1 week before administration of enrofloxacin. The animal house was maintained at room temperature (25 ± 2°C) and 45-65% relative humidity. Commercial fodder and water were provided *ad libitum*. The animals were housed and treated in accordance with the rules approved by the Local Ethics Commission (Ethic Commission Opinion No. 14/2006). Enrofloxacin was administered intravenously at a dose of 10 mg/kg b.w. into the left brachial vein. Blood samples (1.0 mL) were collected into heparinized tubes by means of insulin syringe at 0, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12 and 24 h after drug administration. The blood was withdrawn from every bird 4 to 5 times. For every time point 6 samples were obtained. The samples were centrifuged at 2000 x g for 10 min at 4°C and separated plasma was stored at -30°C until assay. All chickens were euthanatized by intraperitoneal injection of sodium pentobarbital (Vetbutal; Biowet Pulawy, Poland) 24 hours after the last sampling.

**Pharmacokinetic and statistical analysis**

The concentration – time curve and the pharmacokinetic parameters were calculated using the mean plasma drug concentrations according to limited-sampling strategy model (Suarez-Kurtz et al., 1999). Pharmacokinetic variables were analyzed using a Biokinetica 3.1 computer program (Grabowski, 2009). Plasma concentration-time data were calculated using a one-compartment model and selected statistical moments parameters.

**RESULTS**

The linearity of the method was confirmed in the range 0.2 – 3.5 μg/mL. The coefficient of determination (R²) was 0.996 and the curve equation was y = 35.18x – 1.1196.

The accuracy of enrofloxacin determination is shown in Table 1. The obtained data suggest that the here presented method was accurate.
Table 1. The mean values of recovery for the consecutive concentrations of enrofloxacin during 3 subsequent days

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Day 1 Mean recovery (%)</th>
<th>SD</th>
<th>Day 2 Mean recovery (%)</th>
<th>SD</th>
<th>Day 3 Mean recovery (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>115.85</td>
<td>9.31</td>
<td>100.50</td>
<td>1.95</td>
<td>105.18</td>
<td>3.95</td>
</tr>
<tr>
<td>0.5</td>
<td>97.36</td>
<td>3.70</td>
<td>100.43</td>
<td>3.00</td>
<td>95.01</td>
<td>7.91</td>
</tr>
<tr>
<td>1</td>
<td>100.53</td>
<td>2.42</td>
<td>98.12</td>
<td>6.47</td>
<td>104.34</td>
<td>2.96</td>
</tr>
<tr>
<td>1.5</td>
<td>97.76</td>
<td>9.87</td>
<td>100.42</td>
<td>2.84</td>
<td>104.76</td>
<td>2.96</td>
</tr>
<tr>
<td>2</td>
<td>100.60</td>
<td>3.04</td>
<td>96.94</td>
<td>6.86</td>
<td>102.50</td>
<td>3.31</td>
</tr>
<tr>
<td>2.5</td>
<td>99.09</td>
<td>3.49</td>
<td>103.29</td>
<td>2.28</td>
<td>95.89</td>
<td>5.12</td>
</tr>
<tr>
<td>3</td>
<td>99.56</td>
<td>4.24</td>
<td>100.73</td>
<td>2.91</td>
<td>99.03</td>
<td>3.76</td>
</tr>
<tr>
<td>3.5</td>
<td>100.96</td>
<td>1.81</td>
<td>98.71</td>
<td>5.86</td>
<td>101.53</td>
<td>3.87</td>
</tr>
</tbody>
</table>

Repeatability of the method did not exceed 6.38% and intermediate precision did not exceed 8.44%. Results presented in Table 2 indicate that method of enrofloxacin determination was precise.

Table 2. Repeatability and intermediate precision of enrofloxacin determination in chicken plasma

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Repeatability SD (%)</th>
<th>Acceptance criterion (%)</th>
<th>Intermediate precision SD (%)</th>
<th>Acceptance criterion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>6.14</td>
<td>≤ 15%</td>
<td>8.44</td>
<td>≤ 20%</td>
</tr>
<tr>
<td>0.5</td>
<td>4.59</td>
<td>≤ 10%</td>
<td>4.60</td>
<td>≤ 15%</td>
</tr>
<tr>
<td>1</td>
<td>5.15</td>
<td>≤ 10%</td>
<td>6.37</td>
<td>≤ 15%</td>
</tr>
<tr>
<td>1.5</td>
<td>6.38</td>
<td>≤ 10%</td>
<td>6.38</td>
<td>≤ 15%</td>
</tr>
<tr>
<td>2</td>
<td>4.71</td>
<td>≤ 10%</td>
<td>6.27</td>
<td>≤ 15%</td>
</tr>
<tr>
<td>2.5</td>
<td>3.85</td>
<td>≤ 10%</td>
<td>5.56</td>
<td>≤ 15%</td>
</tr>
<tr>
<td>3</td>
<td>3.74</td>
<td>≤ 10%</td>
<td>4.52</td>
<td>≤ 15%</td>
</tr>
<tr>
<td>3.5</td>
<td>4.11</td>
<td>≤ 10%</td>
<td>5.49</td>
<td>≤ 15%</td>
</tr>
</tbody>
</table>

The mean recovery for enrofloxacin was 90.07 ± 0.89% and for the internal standard 95.48 ± 0.45%.
LOD was determined to be 40 ng/mL whereas the demonstrated LOQ was 120 ng/mL.
Representative chromatograms obtained after injection of enrofloxacin-free and enrofloxacin-fortified samples are shown in Figure 1. The drug-free sample
did not contain substances that would interfere at retention time of enrofloxacin, which makes this method suitable for pharmacokinetic analyses.

![Figure 1. Representative chromatograms showing A) drug-free plasma fortified with 10 μg/mL of anthranilic acid (AA) and B) plasma fortified with 1.5 μg/mL enrofloxacin (ENR) and 10 μg/mL of AA.](image)

The validated method was applied to the determination of enrofloxacin in plasma samples obtained from chickens participating in the pharmacokinetic studies. Enrofloxacin concentration decreased from 4.62 ± 1.02 μg/mL in 5 min to 0.32 ± 0.14 μg/mL in 24 hours after drug administration. The mean (± SD) plasma concentration-time profile are presented in Figure 2 and the pharmacokinetic parameters in Table 3.

![Figure 2. Plasma concentration-time profile of enrofloxacin in chickens (n = 6 for each sampling point, ± SD) following intravenous drug administration at a dose of 10 mg/kg b.w.](image)
### Table 3. Pharmacokinetic parameters describing the disposition of enrofloxacin (n = 6 for each sampling time) in chickens after its intravenous administration at a dose of 10 mg/kg b.w.

<table>
<thead>
<tr>
<th>Pharmacokinetic parameters</th>
<th>Value (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ ($\mu$g/mL)</td>
<td>4.62</td>
</tr>
<tr>
<td>$C_{\text{last}}$ ($\mu$g/mL)</td>
<td>0.32</td>
</tr>
<tr>
<td>$t_{\text{last}}$ (h)</td>
<td>24</td>
</tr>
<tr>
<td>$\beta$ (h$^{-1}$)</td>
<td>0.08</td>
</tr>
<tr>
<td>AUC (mg/L x h)</td>
<td>25.09</td>
</tr>
<tr>
<td>AUMC (mg/L x h$^2$)</td>
<td>175.38</td>
</tr>
<tr>
<td>MRT(o-t) (h)</td>
<td>6.99</td>
</tr>
<tr>
<td>$t_{1/2 \text{ beta}}$ (h)</td>
<td>9.14</td>
</tr>
<tr>
<td>Vd$_{ss}$ (l/kg)</td>
<td>2.79</td>
</tr>
<tr>
<td>Cl$_{(b)}$ (l/h x kg)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

$C_{\text{max}}$ – maximum plasma concentration; $C_{\text{last}}$ – last observed plasma concentration; $t_{\text{last}}$ – time of last observed plasma concentration; $\beta$ – elimination rate constant; AUC – area under the curve; AUMC – area under the first moment of curve; MRT – mean residence time; $t_{1/2 \text{ beta}}$ – elimination half-life; Vd$_{ss}$ – volume of distribution at the steady state; Cl$_{(b)}$ – total body clearance

**DISCUSSION**

Enrofloxacin is widely used in veterinary medicine and thus there are many developed methods of its determination both in plasma and tissues of many animal species. However, most of the described methods are quite complicated, expensive and time-consuming as they use procedures like solid phase extraction, evaporation under nitrogen stream (Gonzalez et al., 2006) or molecularly imprinted polymers (Sun et al., 2008). Our method was much simpler and consisted only of protein precipitation and dichloroethane extraction (sample evaporation was omitted). Due to it sample preparation was much faster and cheaper. To precipitate the proteins acetonitrile/ammonia mixture was applied which enabled us to dispose of small interfering peaks which were also observed by some authors (Vybiralova et al., 2006). The method was used in pharmacokinetic studies – the advantage was the small volume (0.25 mL) of the sample in comparison with other methods utilizing usually 1 mL of plasma (Gonzalez et al., 2006). By many methods ciprofloxacin (enrofloxacin metabolite) was also determined (Manceau et al., 1999; Gonzalez et al., 2006). However, our preliminary research (data not shown) indicated that the rate of enrofloxacin to ciprofloxacin conversion is not significant (maximum plasma concentration of ciprofloxacin was not more than 7% of maximum plasma concentration of enrofloxacin). This observation stays in accordance with results described by Ovando et al. (1999). That was the reason why the concentration of ciprofloxacin
in samples was not taken into account. Nevertheless, the obtained results indicate that our method enables ciprofloxacin determination in plasma samples.

A general problem in HPLC analysis of fluoroquinolones is the severe peak broadening and tailing on reversed-phase columns, due to specific interactions with the column support. Residual sylanol groups and metal impurities in the traditional phase columns are known to be the cause of tailing in reversed-phase liquid chromatography, which leads to incorrect peak integration and inaccurate data (Ramos et al., 2003). To avoid it, choosing the both mobile phase and the column is crucial. Most of the separations are nowadays managed on C18 columns, although also C8 (Samadinou et al., 2005) and PLRP-S columns are used (Yorke and Froc, 2000). At first we used Zorbax XDB Eclipse column, but tailing was significant. Finally, the column of choice was Phenomenex Gemini C18 – a column with mixed silica polymeric support which prevented peak tailing.

In comparison to other methods, due to omitting sample evaporation, LOQ of our method was quite high (0.12 μg/mL in our study versus e.g. 0.04 μg/mL obtained by Manceau et al. (1999), 0.06 μg/mL reported by Knoll et al. (1999) and 0.05 μg/mL acquired by Gonzalez et al. (2006)). Nevertheless, the method was still sensitive enough for pharmacokinetic studies in plasma (the method described by da Silva et al. (2006) was less sensitive and was successfully used in pharmacokinetic studies). The linearity of our method was similar to that obtained by da Silva et al. (2006) and was narrower in comparison with methods described by Gonzalez et al. (2006) and Manceau et al. (1999), but wider in comparison with the method described by Knoll et al. (1999). The values of recovery of enrofloxacin, accuracy and precision obtained in our study were similar to those observed by other authors and stayed in accordance with criteria of international agencies (Anonymous, 1995; 2001).

The curve obtained in the pharmacokinetic research had a characteristic shape for this route of administration which is in accordance with other authors (Anadon et al., 1995). The value of the area under the curve obtained in our study was lower in comparison with the results acquired by Anadon et al. (1995), but it was higher than the value obtained by Knoll et al. (1999). Elimination half–life was very similar to the one obtained by Anadon et al. (1995), but it was longer than that reported by Knoll et al. (1999). The value of the volume of distribution obtained in our study was significant indicating that enrofloxacin distributes easily into the tissues. These observations stay in accordance with other results (Anadon et al., 1995; Knoll et al., 1999).

Summarising, our liquid chromatography method can be useful for enrofloxacin determination in broiler chicken plasma. It represents a simple and specific assay and was validated in accordance with guidelines for validation of analytical methods. This method was employed in pharmacokinetic studies, proved to be accurate and reliable and gave satisfactory results during analysis of about 1000 samples.
ACKNOWLEDGMENTS:
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ODREĐIVANJE KONCENTRACIJE ENROFLOKSACINA U KRVNOJ PLAZMI PILIĆA
HPLC METODOM RADI PROUČAVANJA FARMAKOKINETIKE

JAKUBOWSKI P, JAROSZEWSKI JJ, GRABOWSKI T, MARKIEWICZ W i MASLANKA T

SADRŽAJ

U ovom radu je opisan brz i jednostavan HPLC metod za određivanje koncentracije enrofloskacina u krvnoj plazmi pilića. Uzorci su bili pripremljeni precipitacijom belančevina pomoću mešavine acetonitril/amonijak i ekstrakcijom diholoretanom. Analitička separacija je bila izvedena na koncentracionom gradijentu kolone Phenomenex Gemini C18, a vrednosti su određivane fluorescencom λex = 300 nm i λem = 448 nm. Kisela mobilna faza (pH = 3,8) je predstavljala mešavinu acetonitrila i 0,05 M acetatnog pufera. Ovaj metod je bio linearan u opsegu od 0,2 do 3,5 μg/mL. Granica detekcije je bila 40 ng/mL, a granica kvantifikacije 120 ng/mL. Metod je bio siguran i precizan i njime je detektovano 90,07 ± 0,89% enrofloskacina. Pogodnost metoda za farmakokinetika pročuvavanja je utvrđivana određivanjem koncentracije enrofloskacina u krvnoj plazmi pilića posle jednokratne itavenske aplikacije leka u dozi od 10 mg/kg. Koncentracija enrofloskacina se smanjivala od 4,17 μg/mL u petom minutu do 0,32 μg/mL 24 časa posle aplikacije leka. Postignuti rezultati potvrđuju prikladnost ove metode za određivanje koncentracije enrofloskacina.