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DEVELOPMENT OF A HPLC METHOD FOR THE DETERMINATION OF ENROFLOXACIN AND CIPROFLOXACIN IN REPTILE PLASMA AFTER TRANSDERMAL DELIVERY

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ABSTRACT

A rapid and simple high-performance liquid chromatography method using diode array detection for determination of enrofloxacin and ciprofloxacin in snake's plasma was developed. Several snakes were treated transdermally with a single dose of enrofloxacin emulsions corresponding to an antibiotic dose of 5 mg/kg. Enrofloxacin and ciprofloxacin were analyzed using a core-shell silica particle stationary phase (Kinetex® RP–C18, 150 × 4.6 mm, particle size 5 μm) and 0.002 M phosphoric acid/acetonitrile (83:17, v/v) as mobile phase. Calibration curves were linear over the concentration range of 2–100 μM and 3–100 μM. The intra-day and inter-day coefficient of variations were below or equal to 10% for both compounds. Limits of quantification for enrofloxacin and ciprofloxacin were 2 μM and 3 μM respectively. Sample treatment procedure consisted of deproteinization with perchloric acid. The described HPLC method using core-shell silica particles results in better resolution, higher sensitivity and low back pressure.

Keywords: enrofloxacin, ciprofloxacin, HPLC, reptiles, plasma, transdermal application

INTRODUCTION

Enrofloxacin is often used in veterinary medicine to treat several bacterial diseases, as abscess, renal failure, carapace injury, oral cavity inflammation, cerebral meninges inflammation, gastrointestinal tract inflammation, lungs inflammation, wounds, abrasions, skin and mucous membrane infection [1]. Enrofloxacin belongs to fluoroquinolones (FLQ), which are a broad and potent group of active agents against both gram-positive and gram-negative bacteria. Ciprofloxacin is an active metabolite of enrofloxacin. Biotransformation may occur in some species such as poultry, cattle and reptiles. Metabolism of enrofloxacin to ciprofloxacin occurs through hepatic N-dealkylation [2].

Enrofloxacin is active against most of the gram-negative and gram–positive bacteria pathogens isolated from reptile species, such as *Aeromonas hydrophila, Klebsiella oxytoca, Morganella morganii, Providencia rettgeri, Pseudomonas aeruginosa* and *Salmonella arizonae* [3]. Few data are reported on pharmacokinetics of ciprofloxacin in reptiles. Previous publications only described pharmacokinetic data after intramuscular, intravenous or oral administration of enrofloxacin [4, 5, 6, 7, 8]. Besides, a wide variability in elimination half-life time ($t_{\frac{1}{2}\beta}$) of enrofloxacin was reported. For mammals, $t_{\frac{1}{2}\beta}$ is in the range of 1.2 - 3.3 h and usually higher for ectotherms (6.4 h in Burmese python, 23.1 h in Gopher tortoise and 27.9 h in Urutu pit viper) [5, 6]. For Indian star tortoise and Herman tortoise, $t_{\frac{1}{2}\beta}$ values were 5.1 h and 7.3 h respectively [7]. This variability should be taken into account to determine the appropriate dosage of enrofloxacin to use in the various species of reptiles. Oral, intramuscular and intravenous administrations require professional, trained personnel and specific equipment, including stomach tubes facilitating drug intake in case of oral administration, which additionally significantly raises costs of the therapy and stress level of animals. Intramuscular administration is characterized by relatively slow absorption, and intravenous injection can be also problematic because of the inaccessibility of the most peripheral vessels. Thus, transdermal application which is a non-invasive therapy could be an interesting alternative route to treat reptiles. Moreover, the use of transdermal application avoided first pass effect, drug-drug and drug-food interactions. The aim of the present work is to develop a performant HPLC method to determine enrofloxacin and ciprofloxacin concentrations in plasma from reptiles treated by transdermal drug delivery.

MATERIALS AND METHODS

Chemicals: Enrofloxacin and ciprofloxacin hydrochloride (figure 1) were provided by Fagron (Rotterdam, Netherlands). Methanol, gradient grade, and phosphoric acid 85% were purchased from Merck (Rahway, NJ, USA). Acetonitrile, gradient grade and hydrochloric acid were purchased from VWR® (Fontenay Sous Bois, France) and perchloric acid 70% from Sigma Aldrich (St. Louis, MO, USA). Deionized water was obtained by purification through water purification system Purelab Option (ELGA LabWater, High Wycombe, UK).

Instrumentation: The liquid chromatographic system consisted of a Waters Alliance 2795 pump, an auto injector and a Waters 2996 UV DAD detector (Waters Corporation, Milford, MA, USA). Integration of the detector output was performed using the Waters Empower® 2 software (Waters Corporation, Milford, MA, USA).

Chromatographic conditions: Analyses were performed on a Kinetex® column (150 × 4.6 mm, 5 µm particle size, RP–C18) maintained at 25 °C. Kinetex® is an analytical column with core-shell silica microparticles which results in faster elution of a given solute, high sensitivity and low back pressure. The mobile phase consisted of a mixture of 0.002 M phosphoric acid and acetonitrile (83:17, v/v). Separation was performed in an isocratic elution mode with 0.5 mL/min flow rate and detection wavelength was set at 280 nm. The volume of injection was 40 µL. The chromatographic run time was set up at 12 min.

Preparation of stock solutions, working standard solutions and quality control samples: Stock solutions of 1 mM enrofloxacin or ciprofloxacin were prepared in 0.1N HCl and stored at 4 °C. Working standard solutions at 0.01 and 0.1 mM for enrofloxacin and ciprofloxacin were prepared by dilution of the stock solution in deionized water. Working solutions of enrofloxacin and ciprofloxacin were used to spike plasma to obtain calibration standards at concentrations of 2, 5, 10, 50 and 100 µM. For the validation of the method, drug free plasma of reptiles prepared from blood samples collected on EDTA was used. Quality control samples consist of drug-free plasma spiked with known amount of enrofloxacin or ciprofloxacin at concentrations of 5, 25 and 100 µM. Stock solutions were stable at 3–4 °C for 14 days. Enrofloxacin and

ciprofloxacin did not reveal any degradation in plasma stored in acidic conditions.

Plasma sample preparation: 50 µL of perchloric acid 70% were added to aliquots of plasma (300 µL) in glass tubes. After agitation for 2 min the tubes were centrifuged at 3000 rpm for 20 min at 15 °C. Then supernatant was placed into the HPLC vials and 40 µL was injected into the column.

METHOD VALIDATION

The method was validated according the ICH guidelines [9].

(a). Linearity, LLOQ and LOD: Calibration curve was plotted using area under the peak versus the nominal concentration of enrofloxacin or ciprofloxacin. The Lower Limit of Quantification (LLOQ) was defined as the lowest drug concentration which can be determined with an accuracy of 80–120% and a precision below 20%. The Limit of detection (LOD) was determined by using a method signal-to-baseline noise (S/N > 3).

(b). Precision and accuracy: Precision was expressed as the percentage coefficient of variation (CV) and should not exceed 15% (< 20% in case of LLOQ) of nominal concentration. Accuracy was expressed as the percentage of error (PE) and should fulfil the same conditions. To determine intra-day and inter-day precision and accuracy, samples were analyzed 5 fold on the same day and once a day during 5 days respectively.

(c). Recovery: Recovery was determined at quality control concentration levels. The peak areas obtained after deproteinization were compared with peak areas resulting from standard solutions at the same concentrations.

(d). Experimental design*:* An investigation of the pharmacokinetics of both FLQs was carried out in 9 snakes from different species- Python regius, Boa constrictor, Acrantophis dumerili (3 snakes per species). Snakes were fed normally and fresh water was available ad libitum. Only adult or young adult animals were used in the experiment.

Enrofloxacin was used in a form of emulsion at two different concentrations: 0.5% and 5.0%. Emulsions were prepared using Pentravan® (Fagron, Rotterdam, Netherland), which is an oil-in-water emulsion. An emulsion was prepared by dispersing 250 mg of enrofloxacin in 50 g of Pentravan®, according to guidelines on good manufacturing practice for veterinary preparations.

Snakes received enrofloxacin at a dose of 5 mg/kg of body weight. Adequate amounts of emulsion were weighted and applied to the snakes' skin in an area behind the head of the animal corresponding to an area exhibiting better permeability. Blood samples were collected on EDTA tubes at 0, 1, 4, 24, 48, 72 and 96 h after administration. Each sample should not be higher than 1 mL to avoid potential anemia in animals. Blood Samples were centrifuged at 5000 rpm for 5 min at 20 °C**.**

RESULTS AND DISCUSSION

Chromatographic separation: Enrofloxacin and ciprofloxacin were eluted in less than 10 min with retention times of 9.1 and 7.25 min respectively (figure 2). No interference with the compounds of interest was found in plasma sample of different species of reptiles (figure 3). Chromatogram of plasma spiked with enrofloxacin was shown in figure 4.

Validation of the method: Calibration curves of ciprofloxacin were linear over the concentration range of 3–100 µM, whereas calibration curves of enrofloxacin were linear in the range of 2–100 µM, with high correlation coefficients ($R \geq 0.998$). The lowest limit of quantification for enrofloxacin and ciprofloxacin were 2 µM and 3 µM, respectively. The limit of detection was found to be 0.5 µM for both FLQs. Analytical recovery was found to be 91.7 ± 2.5% (n=3) for enrofloxacin. Between-batch precision and accuracy data for enrofloxacin and ciprofloxacin were summarized in Tables1 and 2.

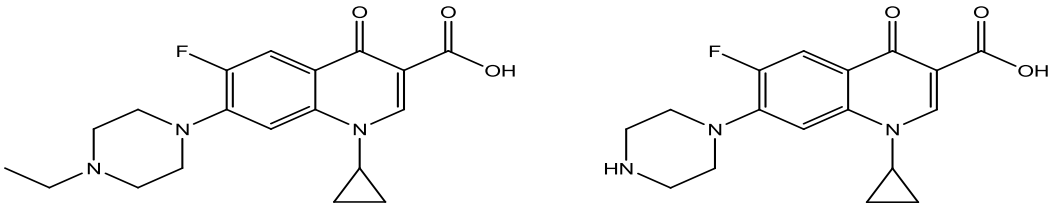
Application of the method: The reported HPLC assay was used to determine enrofloxacin and ciprofloxacin concentration in snake's plasma following transdermal application.

In the described assay, none of the samples achieved quantifiable amount of enrofloxacin or ciprofloxacin. The highest concentrations were detected in plasma sample collected 1 h after transdermal application and it seems to decrease immediately. The use of 0.5% and 5.0% enrofloxacin emulsion in reptile's treatment appears not sufficient to reach therapeutic concentration of enrofloxacin in plasma. Ciprofloxacin was detected only in one snake after topical application of 5% enrofloxacin emulsion.
About the application of the drug on the skin of reptiles, neither skin decoloration nor behavior

disorder was observed. Our results confirm the permeation of enrofloxacin across the skin of reptiles. FLQs demonstrate concentration−dependent bacteria eradication in *in vitro* environment. The AUC/MIC ratio can be used as a good predictor of efficacy *in vivo* (AUC- area under the plasma concentration-time curve in $\frac{\mu g*h}{mL}$). It should be taken into consideration that MIC_{90} for the most susceptible bacteria are below ≤ 0.125 µg/mL, except from *Pseudomonas aeruginosa*, which MIC_{90} can reach value of 0.5 µg/mL and higher. The ratio AUC/MIC should be higher than 125, whereas maximum concentration should be 8-10 times higher than MIC. Threshold values should be assumed to lower possibility of resistance development and to optimize a planned therapy [8]. *Escherichia coli, Klebsiella spp.*, and *Proteus spp*. are the most sensitive for enrofloxacin gram-negative pathogens, with MIC_{90} <0.1 µg/mL [5]. Studies reveal that the same dose (5 µg/kg) after intramuscular administration in estuarine crocodile reach the fixed threshold [4] whereas in juvenile Burmese python, the threshold level was not obtained using the same dose and route of administration [5]. The present study did not provide reliable pharmacokinetic data, that unable an assessment of the efficacy of the transdermal treatment using method described above.

CONCLUSIONS

A rapid and simple HPLC method for the determination of enrofloxacin and ciprofloxacin in reptile's plasma was developed using a core-shell silica particles stationary phase which exhibits less band broadening compared to fully porous particles and results in better resolution, higher sensitivity and low back pressure. Outcomes imply that enrofloxacin in concentration of 0.5% and 5.0% in emulsion formulation for transdermal administration in reptiles is not sufficient to ensure an effective treatment and avoid a spread of resistant strains. Further studies are required to assess higher dosage of enrofloxacin such as use of a prolonged treatment or change the formulation composition to reach therapeutic levels.



1a **1b**

Figure 1. Molecular structure of enrofloxacin (**1a**) and ciprofloxacin (**1b**).

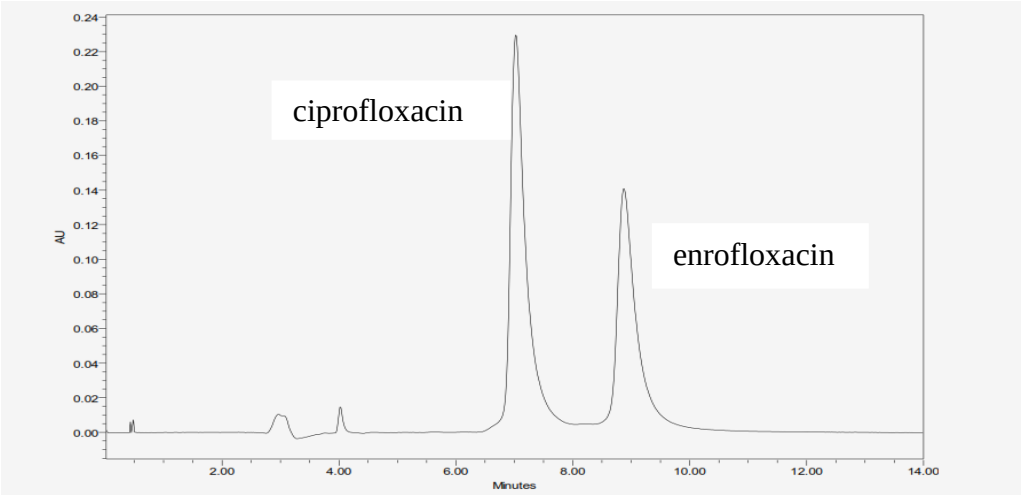


Figure 2. Chromatogram of standards solutions spiked with ciprofloxacin and enrofloxacin at a concentration of 50 µM.

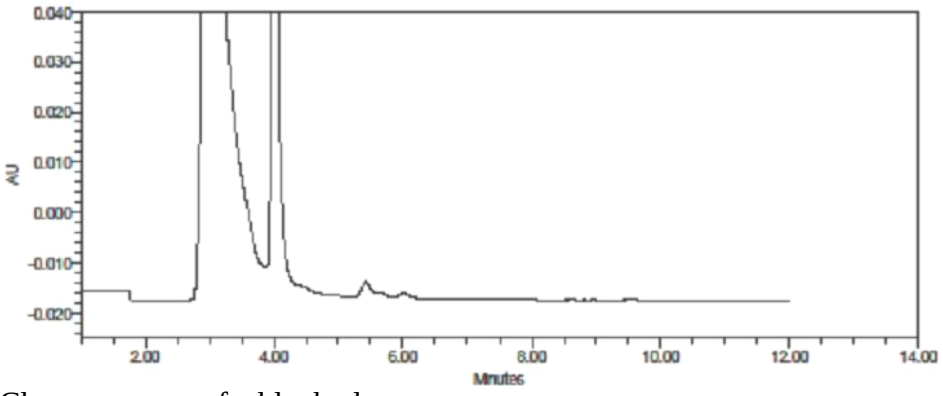


Figure 3. Chromatogram of a blank plasma.

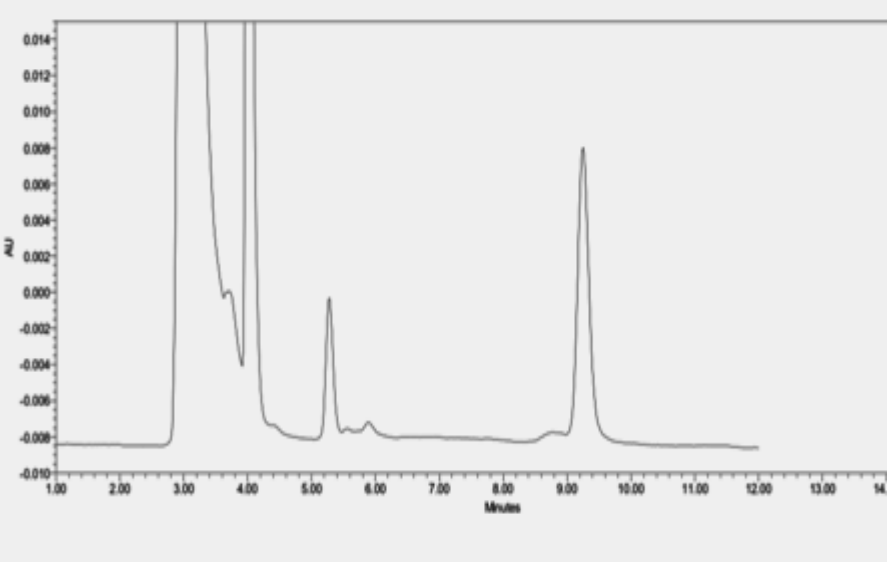


Figure 4. Chromatogram of plasma sample spiked with enrofloxacin at a concentration of 30 µM after deproteinisation with perchloric acid.

Table 1. Inter-day precision and accuracy assessment of enrofloxacin determination (n=5).

Nom. Conc. of enrofloxacin [µM]	**Exp. conc. of enrofloxacin [µM]**	**CV (%)**	**PE (%)**
100	101.12 ± 2.50	4	1
25	24.91 ± 0.37	5	0
5	5.12 ± 0.39	7	2
2 (LLOQ)	2.06 ± 0.6	10	3

Table 2. Intra-day precision and accuracy assessment of ciprofloxacin determination (n=5).

Nominal conc. of ciprofloxacin [µM]	Exp. Conc. of ciprofloxacin [µM]	CV (%)	PE (%)
100	99.58±1.62	9	0
25	25.04±0.58	7	0
5	4.95±0.37	7	1
3 (LLOQ)	3.23±0.22	8	8

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