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IN-USE STABILITY OF ENROFLOXACIN SOLUTION FOR INJECTION IN MULTI-DOSE CONTAINERS

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The in-use stability study in this paper was designed as far as possible to simulate the practical usage of multi-dose containers products in veterinary practice and to establish the influence of storage conditions on drug's quality. According to literature data, shelf-live of enrofloxacin solutions for injection tested in this study is 28 days after opening. In-use (open container) stability testing of enrofloxacin injection solutions was studied during a period of 112 days, and the physical-chemical parameters and microbiological contamination were assessed. A spectrophotometric method was validated for the quantification of enrofloxacin. The validation method yielded good results and included the selectivity, linearity, intra-assay precision (1.26% RSD), inter-assay precision (1.52% RSD), limit of detection (0.18 μg/mL), limit of quantification (0.54 μg/mL) and accuracy. The results of spectrophotometric analyses were presented as the mean drug concentration of enrofloxacin vs. time of sampling. The findings of physical, chemical and microbiological parameters were in accordance with the producers' specifications and no extreme changes during prescribed storage occurred. The study was extended from the drug's proposed shelf-life after opening for the next 84 days and in that period no significant changes were recorded.

Key words: enrofloxacin, injectable solution, multi-dose container, quality

INTRODUCTION

Enrofloxacin is a synthetic 6-fluoroquinolone antibacterial agent (Figure 1) and the first fluoroquinolone developed for veterinary application. Essential for the broad spectrum of activity and for its excellent antimicrobial efficacy is the fluorine substituent at position C6 and the piperazine ring at position C7 (Brown, 1996). Several oral and parenteral formulations of enrofloxacin are available in veterinary medicine for the treatment of respiratory and alimentary bacterial infections in pets and livestock. The pharmacological behaviour of enrofloxacin has been established in several species including cattle (Kaartinen *et al.*1995; Kaartinen *et al.*, 1997), dogs (Kung *et al.*, 1993), fish (Intorre *et al.*, 2000; Gore *et al.*, 2005), pigs

(Anadon *et al.*, 1999; Manceau *et al.*, 1999), sheep (Mengozzi *et al.*, 1996) and poultry (Berg *et al.*, 1988; Anadon *et al.*, 1995).

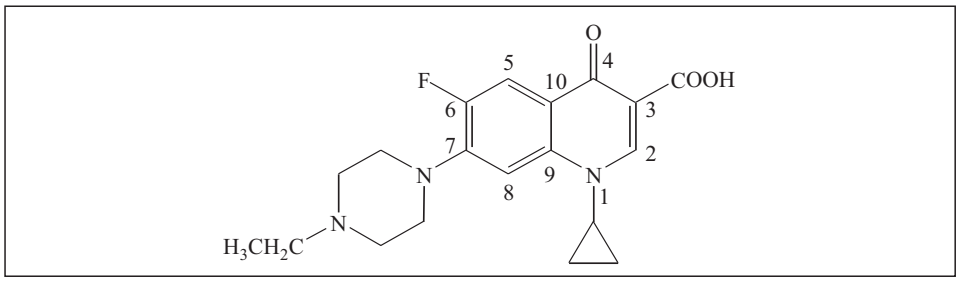

Figure 1. Chemical structure of enrofloxacin

Multi-dose containers of Veterinary Medicinal Products (VMP) for parenteral application are widely used in veterinary medicine, because of their convenience and low cost. Usually, they are glass containers (vials) for pharmaceutical use and intended to come into direct contact with the pharmaceutical preparation, which must remain sterile trough its storage and usage. The manufacturer of a pharmaceutical product is responsible for ensuring suitability of the chosen container in accordance to European Pharmacopoiea (2011). The first opening of multi-dose container and continuous sampling of its content can pose a risk to drugs potency regarding to microbiological contamination and/or physical-chemical degradation. Sabino and Weese (2006) reported a study of bacterial contamination in the multi-dose saline bottles and medication vials used in veterinary medicine. However, there is limited information about the quality issue of multi-dose injectable solution of VMP's during the period of their practical application.

Several methods including spectrophotometry (Sastry *et al.*, 1995; Mostafa *et al.*, 2002), chromatography (Souza *et al.*, 2002; Cinquina *et al.*, 2003; Van Hoof *et al.*, 2005) and immunoassay (Zhang *et al.*, 2008) have been published for the determination of enrofloxacin in pharmaceutical preparations and biological fluids. The present study describes the spectrophotometric method for the determination of enrofloxacin in injectable solutions of VMP's. The proposed method is simple, economic and sensitive for routine determination in pharmaceutical preparations. Also, this method does not require complicated sample preparation and is less time consuming.

The aim of in-use (open container) stability testing in this paper was to investigate the quality of enrofloxacin solutions for injection in multi-dose containers during their storage and usage in environmental conditions. Therefore, in-use stability testing of enrofloxacin solutions for injection was studied for a period of 112 days. The purpose of this study was to detect the changes of physical, chemical and microbiological parameters and to compare the results of VMP's analyses during and after proposed shelf-life (after first opening).

MATERIALS AND METHODS

Pharmaceutical formulation

Three different batches within proposed shelf-life of Baytril® 10%, injectable solution (B1, B2, B3) were obtained from Bayer (Kiel, Germany), and three different batches within proposed shelf-life of Enroxil® 10%, injectable solution (E1, E2, E3) were obtained from Krka (Novo Mesto, Slovenia), as well. The VMP's were from Croatian market and they were packed in brown glass vials stoped with brominated butyl rubber stopper and sealed with an aluminium cap. According to product literature (Summary of Product Characteristics, SPC), recommended storage for these injectable solutions is at room temperature (25 °C). Shelf-life of these products stored in the original container is 36 months and shelf-life after opening is 28 days.

Study design

Samplings were performed over a period of 112 days using a 10 mL sterile syringe (Chirana, Stara Tura, Slovak Republic) and a sterile needle with 0.45 mm outer diameter (Tik, Kobarid, Slovenia). Aliquots of 2.5 mL were withdrawn for all batches on days: 1, 8, 15, 22, 28, 39, 43, 62 and 112. The products were stored at room temperature (25±3°C). The methods for the determination of physical-chemical parameters and microbiological contamination were defined in the producers' guidelines. Throughout the period of in-use (open container) stability testing, the physical properties (colour, clarity, presence of particulate matter and pH value) and chemical properties (identification and determination of the active substance) were monitored at each sampling. The pH values were measured with a pH-meter, while other physical properties were determined visually. Spectrophotometric analyses of enrofloxacin assays were performed by a validated method. The sterility test was performed according to European Pharmacopoeia (2011).

Chemicals

The enrofloxacin reference substance (assigned purity 99.6%) was purchased from Sigma-Aldrich (Seelze, Germany). Analytical grade sodium hydroxide was obtained from Kemika (Zagreb, Croatia). Glassware grade A was used. Deionised water for the preparation of solutions and samples was obtained from a NIRO-W system from Heal Force (Shangai, China).

Equipment and conditions

All spectral and absorbance measurements were carried out using Hach UV-visible spectrophotometer, model DR/4000U (Loveland, USA) equipped with quartz cells of 1 cm path length and controlled by software HachLink 2000, version 2.9. The absorbance was measured at 272 nm. The absorption spectra of the enrofloxacin reference standard and samples of Baytril® and Enroxil® injectable solutions were scanned under optimum conditions against a solvent blank over the range 190-600 nm, and recorded according to general procedures.

The pH values of injectable solutions were measured by Schott pH-meter (Mainz, Germany). These experiments were performed at room temperature (25± 3°C).

Sterility test was carried out by direct inoculation of the injectable solution in the culture media.

Standard and sample preparation

Standard stock solutions of enrofloxacin were prepared in 0.1 M sodium hydroxide in a concentration of 1 mg/mL, taking into account the purity of the standard. Working solutions for five-point calibration curve were established by dilution of stock standard solution using 0.1 M sodium hydroxide as diluent. Suitable amounts of injectable solutions were taken and prepared in 0.1 M sodium hydroxide in a concentration of 5 μg/mL. The samples were analyzed in sixplicate.

Method validation

The spectrophotometric method was in-house validated according to the ICH guidelines (Anonymous, 2005). The following validation criteria were defined according to the characteristics of the sample: selectivity, linearity, linear range, intra-assay and inter-assay precision, limit of detection, limit of quantification, accuracy and robustness.

Linearity of the system was determined by analysis of three replicates in five concentrations of standard solutions. The limit of detection and limit of quantification were obtained from the calibration curve for enrofloxacin. These calculations were based on the standard deviation of the response and slope of the calibration curve. The intra-assay precision of the method was determined by performing six replicated samples, using solutions of enrofloxacin at 5 μg/mL over one day under the same conditions. The inter-assay precision was assessed by performing six replicated samples at the same concentration level over three different days. Results for each type of precision were expressed by the relative standard deviation (% RSD). The accuracy of the method was evaluated by the recovery studies which were carried out by adding known amounts of the enrofloxacin reference substance to the injectable solution at one concentration level (5 μg/mL). The evaluation of robustness was considered during the validation process.

Statistics

The data collected in the study was analyzed statistically using ANOVA. Differences were considered significant at $p<0.05$.

RESULTS

The physical parameters (colour, clarity, presence of particulate matter) that were monitored visually, as well as pH value measurements showed no significant changes (Table 1).

Table 1. Physical and microbiological properties of three different batches (B1, B2, B3) of Baytril® 10%, injectable solution and three different batches (E1, E2, E3) of Enroxil® 10%, injectable solution

Parameter	Specification	Results*					
		B1	B2	B3	E1	E2	E3
Colour	Slightly yellow solution	Complies	Complies	Complies	Complies	Complies	Complies
Clarity	Clear solution	Complies	Complies	Complies	Complies	Complies	Complies
Presence of particulate matter	Practically without particles	Complies	Complies	Complies	Complies	Complies	Complies
pH	10.5 – 12.0	10.6	10.6	10.5	10.6	10.8	10.6
Sterility	Sterile	Complies	Complies	Complies	Complies	Complies	Complies

*Mean of eighteen analyses

The spectrophotometric method for identification and determination of enrofloxacin in injectable solution of VMP's was validated. The validation method yielded good results (Table 2) and included selectivity, linearity, intra-assay precision, inter-assay precision, limit of detection, limit of quantification and accuracy. Characteristic maximal wavelength value was obtained at 272 nm and no endogenous interfering peaks were detected.

Table 2. Validation parameters for spectrophotometric method

Parameter	Result		
Linear range (μg/mL)	0.6 - 8.0		
Limit of detection (μg/mL)	0.18		
Limit of quantification (μg/mL)	0.54		
Linearity*			
Intercept	0.0119	0.0027	0.0136
Slope	0.0954	0.1067	0.0933
Correlation coefficient	0.9999	0.9991	0.9997
Precision (% RSD)			
Intra-assay precision (n=6)	1.26		
Inter-assay precision (n=6)	1.52		
Recovery (mean** ± SD %)	100.1 ± 0.06		
**n=12	100.2 ± 0.12		
	99.9 ± 0.03		

*Three calibration curves

The robustness was established by variations in method parameters (stability of analytical solutions, influence of reagents, influence of solvent and influence of temperature) using the same equipment and the same conditions by two analysts on different days (1.52% RSD). The measurement uncertainty of the method (Anonymous, 2000) was calculated on the basis of recognized sources of measurement uncertainty (Figure 2). The measurement uncertainty of the method was 1.3 mg/mL.

The results of spectrophotometric analyses are presented as the mean drug concentration of enrofloxacin vs. time of sampling (Table 3 and Table 4). The obtained enrofloxacin assays of injectable solutions are within the producers' specifications.

There were no significant differences among results of the Baytril® 10% solution batches (p_{B1} = 0.28; p_{B2} = 0.49; p_{B3} = 0.06) during proposed shelf-life after first opening and out of the proposed shelf-life after opening (next 84 days). Also, there were no statistically significant differences among results for Enroxil® 10% solution batches (p_{E1}=0.21; p_{E2}=0.42; p_{E3}=0.57) within and out of proposed shelf-life after first opening.

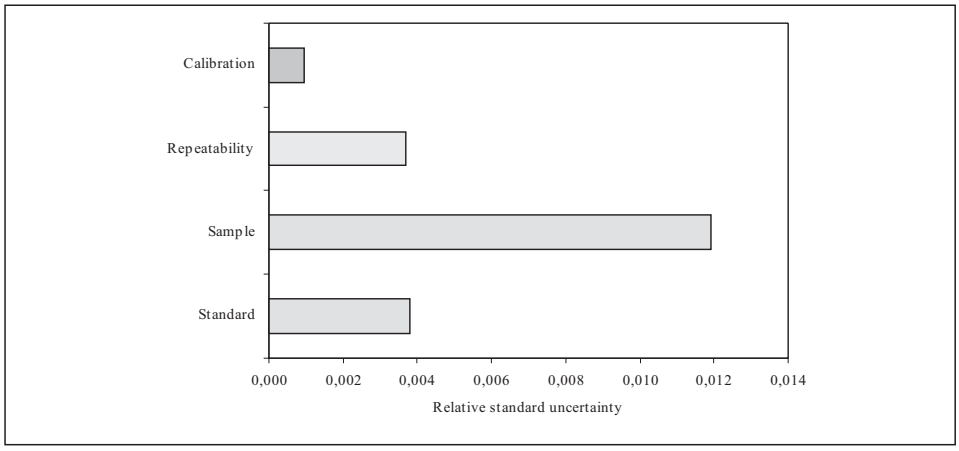

Figure 2. The sources of measurement uncertainty

Table 3. The enrofloxacin assays of the Baytril® 10% batches during in-use (open container) testing

		Baytril batch no. 1	Baytril batch no. 2	Baytril batch no. 3
Day 1	M	101.11	99.12	99.43
	SD	0.42	1.08	0.26
Day 8	M	98.84	99.83	99.62
	SD	1.89	0.72	0.94
Day 15	M	98.30	98.13	99.28
	SD	0.31	0.08	0.92
Day 22	M	100.91	100.72	99.28
	SD	0.74	2.38	0.92
Day 28	M	98.47	99.47	100.96
	SD	0.66	0.22	0.60
Day 39	M	99.99	100.09	100.10
	SD	0.01	0.77	0.27
Day 43	M	99.77	99.46	100.53
	SD	0.57	0.66	0.23
Day 62	M	99.33	99.52	101.22
	SD	1.72	1.12	0.19
Day 112	M	100.41	100.25	100.58
	SD	0.44	0.45	0.29

M (%) = mean of six analyses; SD = standard deviation

Table 4. The enrofloxacin assays of the Enroxil® 10% batches during in-use (open container) testing

		Enroxil batch no. 1	Enroxil batch no. 2	Enroxil batch no. 3
Day 1	M	100.55	99.89	99.42
	SD	1.74	1.41	1.74
Day 8	M	98.28	97.72	97.53
	SD	1.47	1.74	1.54
Day 15	M	98.47	99.42	99.04
	SD	0.13	0.13	0.40
Day 22	M	98.19	99.99	99.23
	SD	1.87	0.67	0.13
Day 28	M	98.09	98.57	97.62
	SD	0.67	0.53	0.54
Day 39	M	99.89	99.70	98.09
	SD	0.80	0.27	0.14
Day 43	M	97.91	98.85	98.28
	SD	1.20	0.94	0.67
Day 62	M	100.36	99.89	99.13
	SD	0.13	0.54	0.80
Day 112	M	99.51	100.27	99.13
	SD	0.27	0.27	0.27

M (%) = mean of six analyses; SD = standard deviation

The sterility test has shown no growth of micro-organisms in the studied batches.

DISCUSSION

The importance of in-use stability testing is to establish a period of time during which a parenteral VMP supplied in multi-dose containers may be used following the removal of the first dose without adversely affecting the integrity of the product (Anonymous, 2002). In-use stability testing, as well as stability testing in the development and manufacture of VMP is evidence on how the quality of an active substance or drug product varies with time under the influence of various environmental factors. The integrity of VMP in multi-dose containers after opening and/or consecutively sampling is an important quality issue. Chemical and physical degradations of drug content may change its pharmacological characteristics that can result in reduced therapeutic efficacy, as well as toxicological consequences. Physical stability of parenteral solutions is a relevant factor due to the solution's interaction with the container and/or changes in the chemical composition. Solutions, particularly parenteral solutions, may have a

tendency of slight discoloration without showing detectable changes in the content of active substances (Carstensen and Rhodes, 2000). Chemical stability of pharmaceutical products depends on physical changes like pH or presence of particulate matter, but also on environmental factors such as temperature, humidity and light that can cause oxidation, hydrolysis, photochemical reactions or complex interactions with excipients (Yashioka and Stella, 2000). For parenteral products like multi-dose injectable solutions, oxidation can become problematic in the process of withdrawing doses from a container as the amount of oxygen increases with each dose withdrawn (Sutton *et al.*, 1998).

There is limited information about the quality issue of VMP multi-dose container of injectable solutions during the period of practical usage in veterinary medicine. Most of the reported studies of the stability of enrofloxacin described the stability of standard and sample solutions (Souza *et al.*, 2002; Urbaniak and Kokot, 2009; Okerman *et al.*, 2007) and the stability of the standard in biological matrices (Gonzalez *et al.*, 2006).

Various methods for the determination of enrofloxacin in pharmaceutical preparations of VMP have been published (Sastry *et al.*, 1995; Mostafa *et al.*, 2002; Souza *et al.*, 2002; Cinquina *et al.*, 2003; Van Hoof *et al.*, 2005; Zhang *et al.*, 2008), but the proposed spectrophotometric method for the determination of enrofloxacin assay has the advantage of being simple, environmentally safe, low cost, sensitive and suitable for routine analysis in quality control laboratories. The method reported herein was validated by evaluation of the parameters (Table 2) as described in the ICH guideline (Anonymous, 2005). The calibration curves were prepared over the concentration range 0.6 to 8.0 μg/mL, and the linearity was good, as shown by the fact that the determination coefficients (r^2) are grater than 0.999 for all curves. No interference or interfering peaks were observed in the absorption spectra and good recoveries were obtained from fortified samples. The limit of detection, calculated as the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable certainty, is 0.18 μg/mL. The limit of quantification, calculated as the smallest measured content of the identified analyte in the sample that may be quantified with a specified degree of accuracy (1.26 % RSD), is 0.54 μg/mL. The method demonstrates good selectivity, acceptable intra-assay precision and inter-assay precision.

The measurement uncertainty of the method was calculated on the basis of recognized sources of the measurement uncertainty: mass, standard purity, linearity, sample preparation and repeatability. From the data obtained (Figure 2), it turns out that the greatest contribution to total measurement uncertainty comes from sample preparation. It was expected because the sample preparations include operations (like dilution etc.) that can affect the precision and accuracy of the method.

Contamination and degradation of enrofloxacin solution may be caused by many factors including: working techniques of the veterinarian, injection of environmental air into the container during its usage, number of withdrawals, duration of use, storage before and after usage, type of container and type of container's closure (Plott *et al.*, 1990). Microbiological changes in pharmaceutical

products like proliferation of micro-organisms and maintenance of sterility must be considered, as well. The proliferation of micro-organisms and/or their endotoxins may be responsible for some physical changes of the same product like discolouration, odours, gas formation, loss of viscosity etc. These changes may cause adverse reactions in treated animals.

The in-use stability study in this paper was designed as far as possible to simulate a practical usage of the multi-dose containers products in veterinary practice and an influence of storage conditions on drug's quality. At intervals comparable to those that occur in practice, appropriate quantities of solution were removed by the withdrawal method regularly used and described in the product literature (Summary of Product Characteristics, SPC). The subject of this study was quality control of VMP batches during proposed and out of proposed shelf life after opening. Therefore, the appropriate physical (colour, clarity, presence of particulate matter, pH value), chemical (identification and active substance assay) and microbiological (sterility) parameters susceptible to change during usage of these solutions for injection were monitored regarding to the producers' specifications.

The producers' recommendation of shelf-life after the first opening of the original packing was 28 days at storage up to 25^{o}C, accordingly the samples have been taken under normal environmental condition of use. The study was extended from the drug's proposed shelf-life for next 84 days in order to determine possible changes in the quality of multi-dose containers of enrofloxacin solutions for injection after the proposed period of validity of the open product. At the beginning of study, all batches had analyses results within the producer’s specification. Based on obtained physical and microbiological analyses and regarding to enrofloxacin stated amounts in multi-dose solutions for injection, there were no significant changes or extreme loss of assay during prescribed storage of 28 days after first opening, as well as next 84 days. The quality analyses of all VMP's batches showed no significant differences at 95% confidence level during our in-use stability testing, while the results of sterility test indicate that injection solution of enrofloxacin is safe for administration during 112 days after the first opening of multi-dose container.

In our study extraction of samples was under aseptic conditions and contamination by equipment was eliminated. However, multi-puncture and damage of the closure can be the potential source of parenteral VMP contamination in field conditions. Generally, we did not expect contamination of enrofloxacin solutions for injection during the proposed shelf-life after first opening, stored at recommended temperature, because of VMP's formulation and excipients.

CONCLUSIONS

The results of in-use stability testing quality control of enrofloxacin solution for injection can be useful for veterinarians considering to administration of drug, environmental conditions, and drug’s storage before and after first opening and therapeutic efficacy. This study proved that there were no changes in enrofloxacin

assay, physical and microbiological parameters of injectable solutions under the recommended conditions of storage including all factors that might affect the risk of contamination. The study was extended from the drug's proposed shelf-life for further 84 days and in that period no significant changes occurred. We hope that the results of this study provide some perspective of in-use stability testing in multi-dose injection containers solutions and their quality, this being the bases for further investigations.

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STABILNOST INJEKCIJSKE OTOPINE ENROFLOKSACINA U OTVORENIM VIŠEDOZNIM SPREMNICIMA

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SADRŽAJ

Istraživanja u ovom radu provedena su sa svrhom ispitivanja stabilnosti injekcijskih otopina enrofloksacina nakon otvaranja njihovih spremnika te utjecaja uvjeta skladištenja na kvalitetu testiranog lijeka. U provedenoj studiji simulirana je

praktična primjena injekcijskih otopina enrofloksacina u veterinarskoj medicini nakon prvog otvaranja višedoznih spremnika. Rok valjanosti svih testiranih injekcijskih otopina enrofloksacina bio je 28 dana od prvog uzorkovanja lijeka. Stabilnost injekcijskih otopina bila je testirana tijekom 112 dana provedbom fizikalno-kemijskih analiza i određivanja mikrobiološke čistoće. Sadržaj enrofloksacina u injekcijskim otopinama analiziran je validiranom spektrofotometrijskom metodom. Validacijom je potvrđena dobra selektivnost, linearnost, ponovljivost (1.26% RSD) i međupreciznost metode (1.52 % RSD) te je određena granica detekcije (0.18 μg/mL), granica određivanja (0.54 ìg/mL) i točnost metode. Rezultati spektrofotometrijske analize prikazani su kao srednja koncentracija enrofloksacina u odnosu na vrijeme uzorkovanja lijeka. Tijekom propisanog skladištenja otvorenih injekcijskih otopina enrofloksacina nije bilo značajnih promjena, a dobiveni rezultati fizikalnih, kemijskih i mikrobioloških analiza bili su u skladu sa specifikacijama proizvođača. Istraživanje je produženo za 84 dana od preporučenog roka valjanosti svake otvorene injekcijske otopine, no ni u tom periodu nisu zapažene značajne promjene u kvaliteti lijeka.