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Article

Stability-indicating HPLC-DAD method for the simultaneous determination of fluoroquinolone in combination with a non-steroidal anti-inflammatory drug in pharmaceutical formulation

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We developed and validated a stability-indicating assay method for the simultaneous determination of enrofloxacin and piroxicam in combination and in the presence of degradation products. Reverse-phase high-performance liquid chromatography analyses were carried out on a Vertisep C18 column and acetonitrile-water (48:52 v/v, pH 3.0) mobile phase with a 1.00 mL min^{-1} flow rate. The efficient chromatographic separation of these drugs and their forced degradation products was achieved in less than 5 min with a peak purity match factor higher than 950. The method used showed linearity in the concentration ranges of 0.25 to 16.0 μg mL^{-1} for enrofloxacin (r = 0.9997) and 0.125 to 8.0 μg mL^{-1} for piroxicam (r = 0.9999) as well as precision (relative standard deviation lower than 2%), accuracy (mean recovery 100 ± 2%), and robustness, according to ICH (International Conference on Harmonization) and AOAC (Association of Official Analytical Chemists) guidelines. This method can simultaneously determine the combination of these drugs in a veterinary formulation and separate the drug peaks from their forced degradation products. Additionally, its optimized chromatographic conditions can contribute to the quality control of this formulation in pharmaceutical manufacturing plants and minimize waste from the organic solvent.

Keywords: Enrofloxacin. HPLC. Piroxicam. Simultaneous determination. Stability-indicating method.

INTRODUCTION

Fluoroquinolones are a group of synthetic antibacterial agents that are widely used for the treatment of multiple bacterial infections in humans and in veterinary medicine because of their safety, broad antibacterial spectrum, and good tolerance (Chamseddin & Jira, 2011). These agents act by inhibiting DNA gyrase and topoisomerase IV, enzymes involved in bacterial DNA replication, transcription, and recombination (Garmyn *et al*., 2009).

Enrofloxacin (ENRO; Figure 1A) was the first of the fluoroquinolones developed exclusively for use in veterinary medicine and was approved in the late 1980s (Bimazutube *et al*., 2009). This drug is chemically known as 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, with the molecular formula $C_{19}H_{22}FN_3O_3$ and a molecular weight of 359.36 g mol^{-1} (British Pharmacopoeia, 2012). ENRO has good activity against a wide variety of Gram-positive and Gram-negative bacteria, including some anaerobes, and is

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also effective against *Mycoplasma* spp. (Bimazutube *et al.*, 2009).

ENRO has found veterinary applications in the treatment of skin and soft-tissue infections in dogs and cats, uncomplicated and complicated genitourinary tract infections, respiratory (pneumonia) and gastrointestinal diseases, footpad lesions, and mastitis in cattle, sheep, goats, pigs, and poultry. As regular part of animals' diets, the ENRO also reduces mortality and morbidity, improves animal welfare, and promotes growth (Sarmah *et al.*, 2006; Golovnev *et al*., 2012; Regitano & Leal, 2010). The extensive use of fluoroquinolones as prophylactics or additives in human and veterinary medicine has led to a significant increase in antimicrobial resistance and poses risks to the environment and public health from antimicrobial residues in foods (Sun *et al*., 2013; Regitano & Leal, 2010).

Piroxicam (PIRO; Figure 1B) is a non-steroidal anti-inflammatory drug (NSAID) used in humans for the treatment of gout, rheumatoid arthritis and osteoarthritis, gynecological diseases, and musculoskeletal disorders. It has anti-inflammatory, analgesic, and antipyretic activity and nonselectively inhibits the activity of the enzyme cyclo-oxygenase, COX-1, and COX-2 (Santos *et al*., 2011). PIRO is chemically known as 4-hydroxy-2-methyl-N-2-pyridyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide, with the molecular formula $C_{15}H_{13}N_3O_4S$ and a molecular weight of 331.35 g mol^{-1} (USP, 2008).

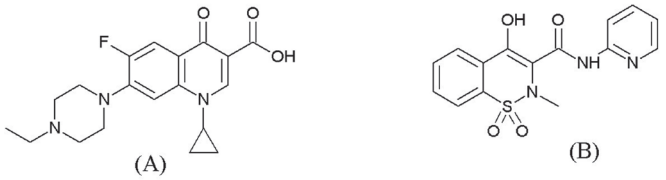

FIGURE 1 - Chemical structures of enrofloxacin (A) and piroxicam (B).

Certain pharmaceutical combinations of fluoroquinolones and NSAIDs, such as ENRO and PIRO, have been proposed in recent years for use in veterinary medicine. Clinical studies have demonstrated that the concomitant use of NSAIDs with antimicrobial decreases the severity of clinical symptoms in animals, increases appetite, and hastens the return to productivity, such as in weight gain or milk production, with higher production performance (Bednarek *et al*., 2003).

Several analytical methods have been described for the estimation of ENRO or PIRO alone or combined with other drugs in pharmaceutical formulations. Studies have employed high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), UV spectrophotometry, spectrofluorimetry, infrared spectroscopy, potentiometry, voltammetry, phosphorimetry, or chemiluminescence (Batrawi, Wahdan, Al-Rimawi, 2017; Starek *et al*., 2009; Kormosh, Hunka, Bazel, 2011; Ulu, 2009; Golovnev, Vasiliev, Kiriki, 2012; Khaled *et al*., 2012; Ensaifi, Khayamian, Taei, 2009; Souza *et al*., 2013; Pulgarin, Molina, Munoz, 2011).

However, we are unaware of a method to simultaneously quantify ENRO and PIRO in combination. No analytical method is reported in the official compendiums for the simultaneous determination of ENRO and PIRO in veterinary formulations. In addition, no stability-indicating HPLC method for the simultaneous determination of pharmaceutical combinations is found in the literature.

Therefore, the aim of this study was to develop and validate an isocratic reverse-phase stability-indicating HPLC-DAD method for the simultaneous determination of ENRO and PIRO and their respective degradation products in veterinary formulations.

MATERIAL AND METHODS

Chemicals and reagents

The ENRO reference standard (assigned purity of 99.50 %) was kindly donated by Biovet (Vargem Grande Paulista, Brazil). The PIRO reference standard (assigned purity of 100 %) was obtained from a local pharmacy. The injectable solution Zelotril Plus (sample), containing 100 mg mL^{-1} of ENRO and 12 mg mL^{-1} of PIRO (declared content), was also purchased locally. All reference substances, as well as the injectable solution, were protected from light throughout the study. HPLC-grade acetonitrile (Vetec, Brazil), analytical-grade phosphoric acid (Synth, Brazil), and fresh ultrapure water from a Milli-Q® Plus system were used in all analyses.

Analytical procedure

The HPLC analyses were performed in a system consisting of a Dionex® Ultimate 3000 (Thermo Fisher Scientific, USA), equipped with an Ultimate 3000 RS Variable Wavelength photodiode array detector and Ultimate 3000 pump. The system was connected to a microcomputer running the Chromeleon® 7.1 Chromatography Data System software.

The experimental conditions were optimized at room temperature (24 ± 2 °C) on a Vertisep C18 column (150×4.6 mm, particle size 5 µm) manufactured by Vertical, Inc., USA. All separations were obtained in

an isocratic mode using a mobile phase consisting of acetonitrile and water (pH 3.0) in the ratio of 48:52 v/v, respectively. The pH of the water was adjusted to 3.0 using phosphoric acid. The flow rate of the mobile phase was 1.0 mL min^{-1} and the sample injection volume was 20 µL. The photodiode array detector was set at 278 nm (ENRO) and 360 nm (PIRO).

Preparation of standard stock solutions

The stock solutions of reference standards were prepared in order to optimize the experimental procedure and decrease the chance of errors. Ten mg of each reference standard (ENRO and PIRO) was transferred to 100-mL volumetric flasks. The volumes were completed with acetonitrile and water in the ratio of 50:50 v/v to reach 100 µg mL^{-1} of each reference standard. These solutions were diluted and used to determine the linearity and accuracy of the method.

Preparation of sample stock solutions

The sample solutions were prepared in the same ratio as the labeled amounts in the veterinary formulation. An aliquot of 5 mL of sample (containing 100 mg mL^{-1} of ENRO and 12 mg mL^{-1} of PIRO) was transferred to a 500-mL volumetric flask. The content of the sample was extracted with 500 mL of acetonitrile and water in the ratio of 50:50 v/v under sonication for 10 min, obtaining a stock sample solution containing 1 mg mL^{-1} of ENRO and 0.120 mg mL^{-1} of PIRO. This solution was diluted and used to determine the precision and accuracy of the method. For the forced degradation study, 4 mL of the sample stock solution was transferred to a 100-mL volumetric flask and the volume was completed with acetonitrile and water in the ratio of 50:50 v/v (sample stock solution A).

Validation of the HPLC-DAD method

The method was validated based on the ICH and AOAC guidelines, following the validation parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness (AOAC, 2005; ICH, 2005).

Specificity

The specificity was evaluated through analysis of a placebo solution, as well as by analysis of the drug solutions after forced degradation studies.

Forced degradation studies were performed according to ICH guidelines (ICH, 2000). Aliquots of the sample stock solution were submitted to different stress conditions, and the resulting solutions were analyzed based on the mean of peak areas (n = 3) for the stressed sample solution. The residual amounts of active pharmaceutical ingredients (APIs) and the peak purity data were evaluated.

Acidic and basic degradations were performed by transferring 15 mL of sample stock solution A to 100-mL Erlenmeyer flasks and adding 15 mL of either 0.5 N hydrochloric acid or 0.5 N sodium hydroxide. The solutions were kept under vigorous mechanical stirring for 8 h at room temperature (24 ± 2 °C). After the stress period, the solutions were neutralized**.**

To promote oxidation, a mixture of 15 mL of sample stock solution A and 15 mL of 3% hydrogen peroxide solution was placed in a 100-mL Erlenmeyer flask and kept at room temperature (24 ± 2 °C) for 8 h.

To evaluate the thermal degradation, a mixture of 15 mL of sample stock solution A and 15 mL of the mobile phase was transferred to an Erlenmeyer flask for 8 h at 60 °C in the dark.

To study the radiation effect, a mixture of 15 mL of sample stock solution A and 15 mL of the mobile phase was transferred to 100-mL Erlenmeyer flasks. The flasks were exposed to two different stress conditions: i) direct sunlight and ii) cool-white fluorescent light (ISO 18909-2006) for up to 62 h and 92 h, respectively. The photo-degradation steps were evaluated at room temperature (24 ± 2 °C).

After the stress conditions, aliquots of 5 mL were removed and transferred to 10-mL volumetric flasks, the volumes were completed with the mobile phase, and the solutions were filtered before injection in a Millex 0.45 µm filter (Millipore, Bradford, USA).

Linearity

The linearity was determined via calibration curves (peak area versus concentration). For the curve of ENRO, ten concentration levels were used, and 11 for the PIRO curve, to establish the linear-regression lines by the least-squares method. All chromatographic determinations were performed in triplicate and at room temperature (24 ± 2 °C). The statistical evaluation was done by analysis of variance (ANOVA).

Precision

The intra-day precision (repeatability) was evaluated by analyzing the sample solution at a single concentration within the linearity range of the method. An aliquot of 1.0 mL of sample stock solution was transferred to a 100-mL volumetric flask. The final volumes were completed with the mobile phase to obtain final

concentrations of 10.0 and 1.2 μg mL^{-1} of ENRO and PIRO, respectively. The analyses were performed in six replicates on the same day. To estimate the inter-day precision, the sample solution was prepared fresh at the same concentration level for each drug, and the response was determined in six replicates. The procedure was repeated on three consecutive days. The intra- and inter-day precisions are expressed in terms of Relative Standard Deviation (%RSD).

Accuracy

The accuracy was determined by recovery studies, using the standard addition method as recommended by AOAC (AOAC, 2005). Recovery was analyzed by adding known amounts of standard solutions to the sample, followed by analysis using the proposed method. Aliquots of standard and sample stock solutions were transferred to 25-mL volumetric flasks and the final volumes were completed with the mobile phase.

The percentage of recovery (R) was calculated by comparing the theoretical and found concentrations, using the following equation: $R = [(C_F - C_U) / C_A] \times 100$; where C_F represents the concentration of the analyte measured in the fortified test sample; C_U, the concentration of the analyte measured in the unfortified test sample; and C_A, the concentration of the analyte added to the fortified test sample.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD was calculated based on the standard deviation of the response and the slope of the calibration curves. It was mathematically evaluated by the equation 3.3 σ/s, where σ represents the standard deviation of the analytical signal and s is the slope of the corresponding calibration curve. The LOQ was determined experimentally by injecting dilute solutions from the standard stock solution.

Robustness

The robustness was evaluated by slightly changing the chromatographic conditions and observing the effect of these changes on the peak area, retention time (t_R), and tailing factor (T_f) of the drugs analyzed. The factors (chromatographic conditions) selected to examine the robustness were the flow rate, percentage of acetonitrile, and pH of the water in the mobile phase. Each factor was investigated at three levels (– 1, 0, and + 1) (Sversut *et al*., 2014). Level 0 refers to the normal chromatographic conditions, i.e., the conditions employed in the proposed method. From this level, the chromatographic conditions were modified to a higher level (+1) or to a lower level (–1). When one condition was changed, the others remained at level 0. Replicate injections (n = 3) of the sample solution were performed under these small changes in the chromatographic conditions. Student's t-test was used for the statistical evaluation.

RESULTS AND DISCUSSION

Method optimization

Choice of stationary phase

Initially, different stationary phases were tested on chromatographic parameters. The chromatographic parameters obtained with the Kinetex C18 and VertiSep C18 were within acceptable limits according to ICH and AOAC (AOAC, 2005; ICH, 2005). However, the VertiSep C18 was chosen for the development and validation of the analytical method because it provided better values for the chromatographic parameters for both drugs.

Although the Kinetex C18 has lower organic-solvent waste, the Vertisep C18 was chosen. Its performance is better in the chromatographic analysis (resolution > 2.0, plates > 2000 and asymmetry of peaks < 2.0), increasing the reliability of the method.

Choice of mobile phase

Some mobile phases were tested in a Vertisep C18 column to provide the best chromatographic separation of drugs. The effects of the different compositions of the mobile phases on the chromatographic parameters are summarized in Table I**.**

The reported values of pK_a for ENRO were pK_{a1} = 6.0, corresponding to the carboxyl group, and pK_{a02} = 8.8, corresponding to the basic piperazinyl group and the isoelectric pH = 7.32 (Chakravarthy *et al*., 2015). The mobile phase pH affects the acid-base behavior of this drug and can reduce the retention time in the stationary phase. In acidic pH, the N-ethyl group (tertiary alkylamine) accepts a proton, and in basic pH the carboxyl group (carboxylic acid) donates a proton, showing the amphoteric character of the ENRO (Beale, Block, 2011). At pH 3.0 the tertiary alkylamine group remained ionized, leading to lower retention time in the reverse stationary phase. The use of a buffered mobile phase is dispensable because the proposed pH value is at least 2 units below the pK_a, providing a completely ionized tertiary alkylamine group (Beale, Block, 2011).

PIRO has reported pK_a values (USP, 2008) of 3.79 and 4.76 and is poorly soluble in polar solvents (Batlouni, 2010). However, the amine and hydroxyl groups in its structure allow the use of a mobile phase with a lower

proportion of organic solvent, which optimizes the method.

PIRO was more retained in the stationary phase when mobile phases composed of a larger amount of water (mobile phase F; Table I) were used, and when acetonitrile was substituted for methanol (mobile phase H; Table I). The pH adjustment of the mobile phases secured the ionization of the proton acceptor groups. However, pH adjustment alone was not sufficient; it was necessary to optimize the proportion of the organic modifier in the mobile phase.

Comparing mobile phases B and F (Table I), both are composed of acetonitrile and water at pH 3.0. It was clear that reducing the proportion of the organic modifier (acetonitrile) in mobile phase F significantly affected the retention time of PIRO, and therefore the chromatographic parameters could not be determined. When comparing the mobile phase B with D and E, in mobile phases B and E all the chromatographic parameters evaluated in Table I are acceptable, which is not confirmed with the value found in theoretical plates in mobile phase D. However, mobile phase B contains a lower proportion of organic solvent, which makes the method advantageous. In mobile phase C, with a higher pH than in mobile phases B and D, and only acetonitrile and water were used at the same proportions as in mobile phase B, there was a reversal of the elution, in which PIRO had a lower retention time than ENRO. This was due to a lower degree of ionization of the tertiary alkylamine group of ENRO in the less-acidic pH.

In these studies, suitable separation with high resolutions, satisfactory theoretical plates, and optimum peak symmetry were achieved with mobile phase B, acetonitrile:water (48:52 v/v, pH 3.0). Under the optimized chromatographic conditions, it was possible to obtain efficient separation of APIs in the veterinary formulations. Peak identity was confirmed by the retention time and by the reference-spectra match factor. Sharp, symmetrical peaks of ENRO and PIRO were obtained at retention times of 1.33 and 4.89 min, respectively (Figure 2). All values for the reference-spectra match factor were higher than 950, indicating the high similarity between the analytes and library reference spectra (Agilent Technologies, 2008).

Method validation

Specificity

The excipients present in the pharmaceutical dosage forms did not affect the analysis because no peak

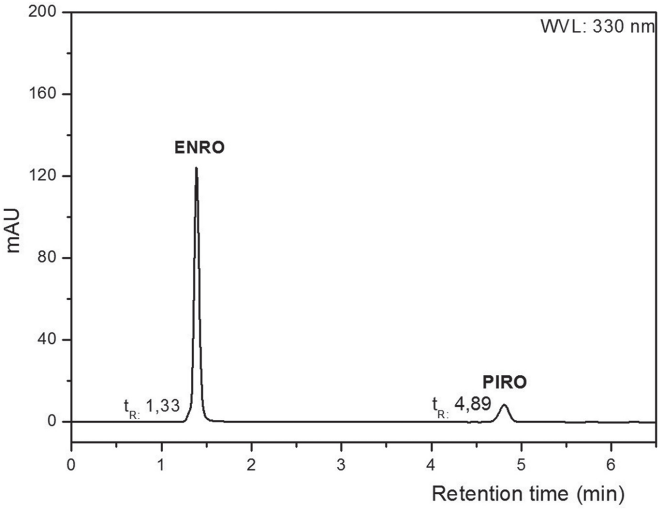

FIGURE 2 - Chromatogram of enrofloxacin and piroxicam in a pharmaceutical formulation. t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (48:52, v/v); pH 3.0; column Vertisep C18; flow rate 1.0 mL min^{-1}; injection volume 20 µL.

TABLE I - Effects of different compositions of mobile phases[a] on chromatographic parameters[b] using Vertisep C18 column

	Resolution		Theoretical plates		Tailing factor		Retention time	
	ENRO	PIRO	ENRO	PIRO	ENRO	PIRO	ENRO	PIRO
A	23.58		3625	8877	1.92	0.88	1.25	4.46
B	22.30		2910	8779	1.09	0.95	1.33	4.89
C		10.32	602	2567	1.47	1.01	9.09	1.03
D	17.01		1392	8358	0.86	0.93	1.36	4.14
E	21.27		3989	8926	1.47	0.95	1.26	3.81
F	*		2141	*	0.74	*	2.89	*
G	1.53		852	5878	1.78	0.99	1.36	2.40
H	*		2542	*	1.59	*	1.91	*

[a] Mobile phase compositions: **A-** acetonitrile:water (50:50, v/v), pH 3.0; **B-** acetonitrile:water (48:52, v/v), pH 3.0; **C -** acetonitrile:water (48:52, v/v); **D -** acetonitrile:water (52:48, v/v), pH 3.0; **E-** acetonitrile:water (55:45, v/v), pH 3.0; **F -** acetonitrile:water (25:75, v/v), pH 3.0; **G -** acetonitrile:water (75:25, v/v), pH 3.0; **H -** methanol:water (50:50, v/v), pH 3.0. Conditions: flow rate 1.0 mL min^{-1}; injection volume 20 µL. [b]The asterisk (*) indicates that the non-steroidal anti-inflammatory drug was retained in the column and the chromatographic parameters were not determined.

was observed at drug retention times, which proves the specificity of the method.

The specificity of the method was also evaluated by checking the peak purity of all analytes after the forced degradation studies (Figure 3). Chromatographic peak purity data were evaluated from the spectral analysis report supplied by the photodiode array detection. Peak purity match factor (PPM) values higher than 950 indicate a homogeneous peak (Maio *et al.*, 2006). Almost all PPM values for API peaks in chromatograms of stressed standard solutions were higher than 950. The peak purity value was lower than 950 in basic and sunlight degradation for ENRO after 8 and 62 hours, respectively. The peak purity value for PIRO was lower than 950 only in sunlight degradation after 8 hours.

Almost all stress conditions were sufficient to degrade the drugs. In studies of acidic, basic and oxidative degradation, PIRO was more stable than ENRO. However, in studies of degradation by sunlight and fluorescent light, ENRO was more stable than PIRO. In the study of thermal degradation, both drugs were equally stable.

Under acidic hydrolysis (Figure 3A), ENRO and PIRO were degraded up to 23.63% and 2.5%, respectively. Under basic hydrolysis (Figure 3B), PIRO suffered no degradation, while ENRO was totally degraded (PPM = 694), precluding determination of the content.

Starek *et al.* (2009) observed two degradation products of PIRO under acidic conditions (HCl 1 N; 60 and 120 °C for 1 h) and only one product under basic conditions (NaOH 1 N; 60 and 120 °C for 1 h). To identify these degradation products, ^{1}H NMR and LC-MS-MS were carried out, and the spectrum data of the first product showed the presence of signals corresponding to pyridine-2-amine. The second product was identified as 2-methyl-2H-benzo[e][1,2]thiazin-4(3H)-one 1,1-dioxide in acidic hydrolysis. In basic hydrolysis, only the product pyridine-2-amine was identified.

A similar result was seen in the study of Modhave *et al*. (2011), where in acidic and basic media (HCl 0.1 N and NaOH 0.5 N; 80 °C for 24 h), hydrolysis occurred in the amide group of PIRO, resulting in the formation of the same degradation products reported by Starek *et al.* (2009).

In a study of fluoroquinolones in which ENRO was subjected to acid medium, it lost the carboxylic acid functional group, generating a degradation product which was subsequently identified by 1 H-NMR (Allah, 2004).

Our results showed that ENRO is more reactive under acid hydrolysis conditions because decarboxylation

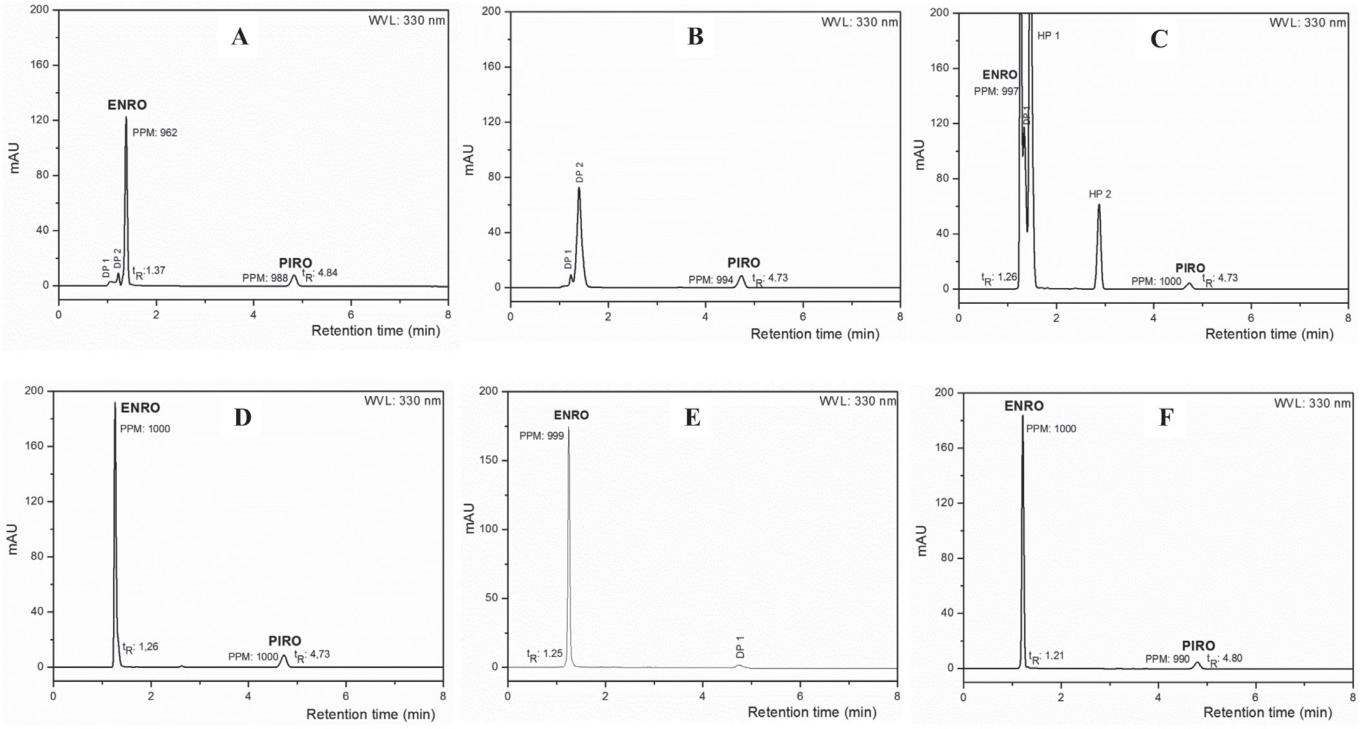

FIGURE 3 - Chromatogram of enrofloxacin and piroxicam under acidic hydrolysis (A), alkaline hydrolysis (B), oxidation (C), high temperature (D), 8 hours of sunlight (E) and 92 hours of fluorescent light exposure (F). DP, degradation product; PPM, peak purity match factor; t_R, retention time. *Conditions*: mobile phase composed of acetonitrile:water (48:52, v/v); pH 3.0; column Vertisep C18; flow rate 1.0 mL min^{-1}; injection volume 20 µL.

in ENRO is faster than the hydrolysis of the amide group in PIRO (competitive reactions). In basic conditions, this is more evident, and ENRO undergoes complete degradation.

Under oxidative conditions (Figure 3C), ENRO was degraded up to 29.3% and PIRO was degraded up to 9.5%. The HP1 and HP2 peaks are not degradation products, because injection of hydrogen peroxide alone resulted in the same retention time and a similar profile.

A study with ENRO and its degradation products found that ENRO was stable in all the degradation conditions except in oxidation degradation, where slight degradation was observed (0.86%) when performed by adding 30% peroxide solution (H_2O_2) at 70 °C for 1 hour (Chakravarthy, Sailaja, Kumar, 2015).

Another stability-indicating study with ENRO in combination with amoxicillin found no degradation for ENRO. There was no evidence of degradation of the drug when exposed to acidic (HCl 0.1 N for 4 h; room temperature), alkaline (NaOH 0.1 N; 65 °C for 7 days), oxidative (H_2O_2 0.3% for 7 days; room temperature), thermal (70 °C for 3 weeks), and photo (UV light for 3 days) stress conditions (Batrawi, Wahdan, Al-Rimawi, 2017).

The oxidation reactions of drugs using H_2O_2 involves electron-transfer mechanisms. Groups such as amines, sulfides, phenols, double bonds, benzylic carbon, allylic carbon, and α-carbon attached to a heteroatom can undergo oxidation to N-oxides, hydroxylamine, sulfoxides, sulfones, quinones, hydroperoxides, hydroxides, and ketones ENRO and PIRO can undergo oxidation with H_2O_2, the former because of the amine group (piperazine), and the latter because of the enol group (Charde *et al*., 2013).

Under thermal degradation (Figure 3D), ENRO and PIRO were not degraded; they retained the content of 100% and PPM of 1000. Fluoroquinolones are chemically stable and resistant to increased temperatures because of the stability of the quinolone ring (Babić, Perisa, Skorić, 2013). A study with PIRO showed that 2 hours of heating at 100 °C produced a gradual loss of absorbance, indicating degradation of the molecule, possibly due to hydrolysis of PIRO by cleavage of the amide bond attached to the 2-pyridine ring (Ahmad, Aminuddim, Nazin, 2011).

Under photodegradation, when sunlight was the stressor agent (Figure 3E), ENRO was degraded up to 1.35% and PIRO was totally degraded (PPM = 700), precluding determination of the content after 8 hours of exposure. However, after 62 hours of exposure, ENRO was also totally degraded (PPM = 584).

When white fluorescent light was the stressor agent (Figure 3F), ENRO was degraded less than 1% and PIRO 63.58% after 92 hours of exposure. Light-stress conditions can induce photo-oxidation by free radical mechanisms (Charde *et al*., 2013).

Several types of stress-produced oxidative processes via free radicals have been reported. The hydroxyl radical, for example, typically reacts through two competitive mechanisms, hydrogen removal or hydroxylation. According to Santoke *et al*. (2009), the addition of the hydroxyl radical to the aromatic ring in fluoroquinolones occurs because the aromatic ring of quinolones is activated, due to the presence of donor groups such as amines bonded to the aromatic ring (e.g., Piperazine) leading to the formation of phenols. The cyclopropane functional group present in ENRO has been shown to have low reactivity with the hydroxyl radical. Defluorination can occur at the carbon-fluorine position, which undergoes rapid HF elimination to form phenoxyl radicals. The replacement of hydrogen and piperazine groups also occurs in ENRO, which can also be replaced by the hydroxyl radical.

Another study of photodegradation with fluoroquinolones, using an irradiation spectrum similar to sunlight, observed changes in the piperazine ring and the carboxylic acid ester group, and also that the ethyl group of ENRO was first removed (Burhenne *et al*., 1997). Another study found that photolysis of ENRO involved the fragmentation of C-F and C-COOH bonds, suggesting that photolysis of ENRO was accompanied by decarboxylation, defluorination and also N-dealkylation processes (Li, Niu, Wang, 2011).

Lin *et al.* (2010) observed that exposure of ENRO to fluorescent light caused the formation of ciprofloxacin as an intermediate, in pond water and sediment slurry; the study also showed that natural-light irradiation had a greater effect on the degradation of fluoroquinolones than fluorescent and ultraviolet light. Lin *et al.* (2010) also observed that the half-life of ENRO was 14.1 days (pond water) and 71 days (sediment slurry) upon exposure to fluorescent light.

The difference in stability between fluoroquinolone ENRO and oxicam PIRO during the photodegradation process can be explained by the presence of an enol group in PIRO. The enol group is very susceptible to reaction with singlet oxygen, forming dioxetane intermediates (Modhave *et al*., 2011). The subsequent ring cleavage and the transacylation process could explain the formation of N-methylsaccharine and N-(2-pyridyl) oxamic acid, the main products detected by HPLC-MS analysis in the studies of Lemp, Zanocco and Gunther (2001) and Miranda, Vargas and Serrano (1991).

Dragomiroiu *et al.* (2015) observed that exposure of PIRO under different temperature and luminosity

conditions showed that direct light exposure leads to significant degradation of the drug (about 24.76%) when the stock solution was placed in direct sunlight for 4 hours.

The largest amounts of degradation products (5 degradation-product peaks) were generated under sunlight conditions after 62 hours of exposure for the ENRO and PIRO combination. Smaller amounts of degradation products were obtained under thermal and fluorescent light conditions, where no impurity peak was observed.

Linearity

Linear calibration curves were evaluated in the concentration ranges from 0.25 to 16.0 μg mL^{-1} for ENRO and 0.125 to 8.0 μg mL^{-1} for PIRO. The correlation coefficients were 0.9997 and 0.9999 for ENRO and PIRO, respectively. The statistical ANOVA evaluation indicated a significant linear regression. These results indicated a linear correlation between the peak areas and drug concentrations.

Precision

For ENRO and PIRO, the values of %RSD were lower than 0.91% (repeatability) and 0.82% (intermediate precision). Therefore, the proposed method has good precision for the simultaneous determination of these drugs.

Accuracy

The mean recovery values were 99.79 ± 1.11% and 99.40 ± 1.24% for ENRO and PIRO, respectively. These recovery values are within acceptable limits [50] (100 ± 2%) and suggest good accuracy of the proposed method.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD values were 0.096 and 0.017 μg mL^{-1} for ENRO and PIRO, respectively (signal to noise ratio of 3:1). The LOQ were 0.25 and 0.125 μg mL^{-1} for ENRO and PIRO, respectively. These results indicated the sensitivity of the method.

Robustness

The results for robustness indicated that small changes in the chromatographic conditions did not significantly modify the peak areas, retention time, and tailing factors of the drugs.

As seen in Table II, a 1% change in acetonitrile, 0.3% in the pH and 3% in the flow rate of the mobile phase only slightly affected these chromatographic parameters and did not compromise the analyses of the drugs.

TABLE II - Robustness evaluation of the proposed HPLC method

Chromatographic changes		Drugs					
		ENRO			PIRO		
Factor[a]	Level	Area	t_R[b]	T_f[c]	Area	t_R[b]	T_f[c]
A: % acetonitrile in mobile phase (v/v)							
47	-1	39.81	1.31	1.15	1.21	4.95	0.95
48	0	40.35	1.33	1.12	1.24	4.89	0.93
49	+1	40.55	1.29	1.13	1.20	4.82	0.96
Mean		40.24	1.31	1.13	1.22	4.89	0.95
± S.D. (n=3)		± 0.95	± 1.53	± 1.35	± 1.71	± 1.33	± 1.61
B: pH of mobile phase							
2.99	-1	40.42	1.32	1.11	1.22	4.81	0.96
3.00	0	40.35	1.33	1.12	1.24	4.89	0.93
3.01	+1	40.51	1.34	1.14	1.26	4.86	0.95
Mean		40.43	1.33	1.12	1.24	4.85	0.95
± S.D. (n=3)		± 0.20	± 0.75	± 0.02	± 1.61	± 0.83	± 1.61
C: Flow rate (mL min^{-1})							
0.97	-1	40.39	1.35	1.10	1.21	4.87	0.96
1.00	0	40.35	1.33	1.12	1.24	4.89	0.93
1.03	+1	40.28	1.31	1.13	1.23	4.82	0.94
Mean		40.34	1.33	1.12	1.23	4.86	0.94
± S.D. (n=3)		± 0.14	± 1.50	± 1.38	± 1.25	± 0.74	± 1.62

[a] Three factors (A, B and C) were slightly changed at three levels (+1, 0, −1); each time a factor was changed from level 0, with the other factors remaining at level 0. [b] Retention time. [c] Tailing factor.

Application of the method

The proposed HPLC-DAD method is suitable for simultaneous determination of ENRO and PIRO in veterinary dosage formulations in routine analysis. For the commercial formulation Zelotril Plus® (100 mg mL^{-1} ENRO and 12 mg mL^{-1} PIRO), the contents found were 99.91 ± 0.81% of ENRO and 96.62 ± 0.08% of piroxicam.

CONCLUSIONS

A stability-indicating HPLC-DAD method was developed and validated for simultaneous determination of enrofloxacin and piroxicam in veterinary formulations. This method showed specificity, precision, accuracy, sensitivity, and robustness. In addition, the method was successfully applied to separate the active pharmaceutical ingredients from their forced degradation products. The results suggest that the proposed method can be used in the routine quality-control analysis.

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