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Development and validation of an HPLC-DAD analytical method for quantitative and simultaneous determination of cefoperazone and prednisolone in intramammary ointment

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Cefoperazone (CPZ) is a third-generation cephalosporin used to treat bovine mastitis. The association with steroidal anti-inflammatory drugs, such as prednisolone (PRED), provides an improvement in the animal's clinical response, which justifies its use. An analytical method capable of simultaneously determining the association between CPZ and PRED by High Performance Liquid Chromatography coupled with a Diode Array Detector (HPLC-DAD) was developed. The method was applied in intramammary ointments, showing linearity, for CPZ, from 0.25 to 25 µg mL^{-1} (r=0.9999); and, for PRED, from 0.75 to 30 µg mL^{-1} (r=0.9997), with low limits of detection and quantification, precision with coefficients of variation less than 2%, in accuracy, presented recovery close to 100% for both drugs, in addition to demonstrating their robustness by the Youden test, which evaluated the effects and concluded that the altered parameters in the test were not sufficient to change the values obtained in the determinations. The test was also applied to the commercial sample for quality control purposes, with extraction efficiency being 69.66% for CPZ and 72.36% for PRED. Thus, the developed and validated method can be safely applied in the analysis of the studied drugs.

Keywords: Cefoperazone; prednisolone; validation, HPLC; quality control

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Introduction

Quality control of pharmaceutical products is essential to ensure that the desired therapeutic effect is achieved, reducing the risk of injury and promoting the health and well-being of users. Drugs belonging to the same therapeutic classification, with increasingly similar physicochemical properties, require more sensitive and selective methods. Chromatographic separation techniques stand out for their wide and efficient applicability in several areas (1,2).

Medicinal products for animal use must be strictly certified by quality control, coupled with good manufacturing practices, in the same way as medicinal products for human use. Irrational use, that is to say, when they are not in agreement with what is recommended in the package inserts and in pharmacological compendia, failing to meet the recommended criteria for clinical indication, such as the species, dosage, route of administration and withdrawal time, can lead to therapeutic ineffectiveness, thus, contributing to the spread of diseases among animals (whether the same species or not). It may also result in the presence of residues in food for human consumption above the maximum permitted limits (3,4).

Quality deviations from the standards required in the productive chain of livestock products for export may substantially affect the country's economy, as occurred in the case of the Brazilian beef imports by the United States in 2014 (3).

Among the different classes of drugs, antimicrobials deserve special emphasis since they are involved in an important issue of resistance to the treated pathogens – bacterial resistance – which contributes to increased human and animal morbidity and mortality (5). This fact becomes even more evident when observing that the development of new antimicrobials occurs at a slower pace compared to the advancement of resistant bacteria, resulting in a deficit in therapeutic advancement. (6,7).

An increase in antimicrobial resistance is detected, especially in the case of gram-negative bacteria, that have been shown to be more resistant to broad-spectrum beta-lactams (8). The indiscriminate use of antimicrobials has significantly contributed to increased microbial resistance, leading to a rise in usual dosage and intensification of residues in meat and milk. When drugs of higher generations are more frequently used, bacterial resistance occurs and, consequently, the number of deaths escalates (6). Antimicrobial therapy usually occurs associated with potent corticosteroids that can act as adjuvants in the inflammatory conditions that accompany the infectious process, considerably improving the therapeutic response (9).

In veterinary medicine, bovine mastitis is a disease that directly interferes with the national economy. It is a

bacterial infection that affects the teats of the lactating cows, making the milk unfit for consumption [10]. It is caused by *Pseudomonas sp., Staphylococcus sp., Streptococcus sp.* and *Escherichia coli*. Mastitis treatment usually involves the association of an anti-inflammatory drug with an effective antimicrobial (11).

Cefoperazone (CPZ), chemically illustrated in Figure 1, is a third generation cephalosporin employed in the allopathic treatment of mastitis in animals. It has an antimicrobial effect against the majority of mastitis-causing pathogens in dairy cattle, being one of the antimicrobial agents most employed in this therapy (Febler et al., 2012) (12). CPZ is a broad-spectrum antimicrobial, especially targeting the gram-negative bacteria by inhibiting the biosynthesis of antimicrobial cell wall (13-15).

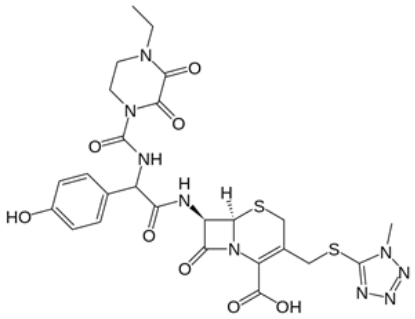

Figure l. Chemical structure of cefoperazone.

Prednisolone (PRED), a synthetic glucocorticoid that has intermediate-acting anti-inflammatory activity, is associated with CPZ to increase therapeutic efficacy, thus contributing to minimize bacterial resistance. Figure 2 shows the chemical structure of PRED (16).

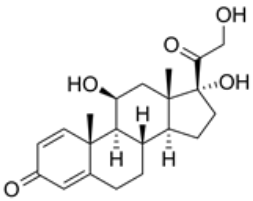

Figure 2. Chemical structure of prednisolone.

Several analytical methods have been used to determine CPZ in different matrices. High-performance liquid chromatography (HPLC) techniques are often used in biological matrices, such as plasma, due to their high sensitivity and accuracy (17).

For environmental samples, such as water, spectroscopy is an effective alternative to monitor pharmaceutical residues in the environment (18). Electrochemical methods, which have been gaining attention due to their high sensitivity and low reagent consumption, have proven to be effective in determining CPZ in pharmaceutical formulations, even in the presence of other drugs (19).

The determination of prednisolone in different matrices can also be performed by several analytical techniques. Silva et al. (2018) developed a high-performance liquid chromatography (HPLC) method with UV detection to quantify PRED in human plasma (20). Lima and Pereira (2021) developed an HPLC method with fluorescence detection for the analysis of PRED in pharmaceutical formulations (21). Pereira et al. (2019) applied mass spectrometry coupled to liquid chromatography to analyze the presence of PRED in water samples, contributing to the environmental monitoring of the compound (22). Souza et al. (2019) used UV-Vis spectrophotometry to determine the concentration of PRED in serum samples (23).

These studies demonstrate the diversity of analytical approaches available for the determination of CPZ and PRED in different matrices, contributing to quality control, clinical and environmental studies. Despite their clinical importance, analytical methodologies for the simultaneous determination of this association in veterinary drugs were not found in the literature. Therefore, given the relevance and need for analytical methods capable of determining CPZ and PRED simultaneously, the objective of this work was to develop and validate an analytical method for the simultaneous determination of these drugs in a formulation for animal use by High Performance Liquid Chromatography (HPLC), following the guidelines of RDC 166 of July 2017 of the National Health Surveillance Agency (24).

Experimental section

Equipment

To perform the chromatographic analysis, the Dionex® Ultimate 3000 (Thermo Fisher Scientific, USA) High Efficiency Liquid Chromatography (HPLC) was coupled to the Ultimate 3000 RS Variable Wavelength Diode Array Detector (DAD) and Ultimate 3000 Pump, analyzed by the Chromeleon 7.1 Software and 20 µL fixed loop. For constructing the calibration curves, software Microsoft Excel (2016) was used. Chromatographic separation was performed using reverse phase C18 columns: Acclaim® C18 (Thermo Scientific), Kinetex® C18 (Phenomenex), VertiSep C18 (Vertical®); in isocratic mode composed of solvents acetonitrile and water. MS TECNOPON mPA210 benchmark pH meter, Shimadzu AUY220 analytical scale and UNIQUE USC 1400A ultrasound bath were also used.

Reference standards and reagents

The reference standards were kindly donated by CEVA's research and development laboratory, with the analysis report issued by Sigma-Aldrich®: cefoperazone (batch SZBF103XV, valid until 04/13/2019, purity 99.8%) and prednisolone (batch SZBD009XV, valid until 09/01/2018, purity 98.2%). Commercial sample CEFAVET® was kindly provided by the manufacturer (CEVA Saúde Animal Ltda.) registered as batch 031/15, valid until 08/2018, containing 250 mg of CPZ and 4 mg of PRED.

The purity of reagents used in the development, optimization and validation of the method was above 99.5%: acetonitrile, o-phosphoric acid, and ultrapure water obtained by the Direct-Q® 3UV MilliPak® Express 20 system (Merck, USA).

Chromatographic conditions (Development)

In the development of the HPLC method, three C18 reverse phase chromatographic columns were tested, also used in the studies on cephalosporin separation by Li et al. (2016) (25), Wang et al. (2015) (26) and Zhou et al. (2010) (27): 1) Kinetex C18 - Phenomenex®; 2) VertiSep C18 - Vertical®; and 3) Acclaim® C18 - Thermo Scientific. In all assays the acetonitrile:water pH 3.0 (60:40 v/v) phase was used, with flow rate of 0.3 mL min^{-1} (column 1); 0.5 mL min^{-1} (column 2); and 1.0 mL min^{-1} (column 3), operating in isocratic mode. Different eluting systems were applied by diversifying the acetonitrile/water pH 3.0 ratio (28) acidified with o-phosphoric acid. The room temperature was maintained at 25°C. Chromatographic peak areas were detected at 240 nm for PRED and 260 nm for CPZ.
The parameters analyzed for inclusion of the trial procedure as part of the analytical method met the eligibility criteria: number of plates (more than 2000), asymmetry factor (close to 1.0) and resolution (more than 2.0) (29).

Preparation of solutions

Standard and work solutions

Standard solutions were prepared by quantitatively weighing 10.0 mg of each CPZ and PRED reference standard and transferred to individual 100 mL volumetric flasks. The volume was completed with acetonitrile, resulting in the final concentration of 100 µg mL^{-1}. These solutions made up the standard solution, stored in a refrigerator (2-4°C) for 30 days. Work solutions were prepared by diluting the appropriate quantity of standard solution in acetonitrile and water pH 3.0 in a 70:30 v/v ratio. All development and validation were performed after the association of drugs in the same volumetric flask with known concentrations of each drug.

Preparation of intramammary ointment

By means of liquid-liquid extraction, 100 µL of ointment (Cefavet® lot 031/15) were transferred to a 50 mL volumetric flask and the volume was completed with acetonitrile, resulting in work solutions with 50 µg mL^{-1} of CPZ. It was then subjected to ultrasonic bath for five minutes and then the analytical solutions were filtered with 0.22 µm membrane separating the ointment-formed button in the polar solvent before injecting it into the chromatograph. Because of the large difference in the labeled content of the drugs present in the commercial sample, a volume of 200 µL ointment was used for PRED analysis, resulting in a 1.6 µg mL^{-1} concentration. The assay followed the steps described in the previous paragraph.

Preparation of an ointment placebo

At a 1:1 g/g lanolin and petroleum jelly ratio, an ointment was prepared for testing sensitivity and interference of excipients.

Calculation of the actual content (%) of CPZ and PRED present in the sample

The CPZ and PRED content in the ointment sample was calculated by Equation (1), and the percentages, by Equation (2).

$$CO = \frac{PA \, x \, CRC}{ARC} \qquad (1)$$

$$PC\ (\%) = \frac{CO \, x \, 100}{TC} \qquad (2)$$

Where: CO = Concentration of CPZ/PRED in ointment (µg mL^{-1}); PC (%) = Percentage concentration of CPZ / PRED in ointment (%); PA = CPZ/PRED peak area in ointment; CRC = Concentration of reference chemical; ARC = Area of reference chemical; TC = Theoretical concentration of drugs (CPZ and/or PRED) in the final product (µg mL^{-1}).

Method Validation

The main analytical parameters were determined to validate the method: linearity, precision, accuracy, selectivity, ruggedness and limits of detection and quantification, according to the literature recommendations (24, 30, 31).

Selectivity

For conducting this test, the method was validated in the ointment placebo sample composed of lanolin and petroleum jelly in the 1:1 g/g ratio, simulating the basic components of the commercial sample. The manufacturer reserves the right not to share such classified information in a legal way.

Linearity

Linearity was determined by obtaining analytical calibration curves in triplicates, carried out on three different days, from the dilution of the standard solutions prepared simultaneously. For CPZ, concentrations of 0.25; 0.75; 1.25; 2.50; 5.0; 15.0 and 25.0 µg mL^{-1} were used; for PRED, 0.75; 1.25; 5.0; 10.0; 15.0; 20.0 and 30.0 µg mL^{-1}. All determinations were performed in triplicates and at room temperature (25 ± 2°C). CPZ solutions were analyzed by HPLC-DAD at 260 nm, and PRED at 240 nm. The results were evaluated by linear regression analysis and linearity, by linear correlation coefficient.

Precision

Precision was determined by the intra-day (repeatability) and inter-day (intermediate precision) assays. Both parameters were evaluated at three levels of concentration, as recommended by RDC ANVISA n.166/2017 (Determination of the Collegiate Board of Directors) (24), with concentrations of 1.0, 5.0 and 20.0 µg mL^{-1} for CPZ+PRED, that is, within the linearity range with a low, medium and high level of the calibration curve. For repeatability, triplicates of the solutions were prepared and analyzed on the same day and by the same analyst. For intermediate precision, triplicates of solutions were prepared by different analysts and examined on different days. This assay was performed for three consecutive days and the results were expressed in terms of the relative standard deviation (RSD) calculated by Equation (3):

$$RSD = (\frac{SD}{\bar{X}})\, x\, 100 \quad (3)$$

Where: SD is the standard deviation of the intercept and $\bar{X}$ is the mean of the determinations.

Accuracy

Accuracy was determined by the recovery test where known quantities of reference standard CPZ and PRED were added to solutions of samples whose concentrations were fixed. The drugs were evaluated at three levels of recovery, taking into consideration the method linearity, 10, 15 and 20 µg mL^{-1} as R1, R2 and R3, respectively, for CPZ. For PRED, the accuracy was also evaluated at three concentration levels, 5.6; 7.6 and 9.6 as R1, R2 and R3, respectively. Table 1 illustrates the preparation of the solutions used in the accuracy test for CPZ and PRED. The percentage of recovered CPZ and PRED was calculated using Equation (4) below (30):

$$\%\, R = \frac{(Ca - Cna)}{Ctp} x\, 100 \quad (4)$$

Where: R: recovery, Ca: drug concentration found in the standard added sample (µg mL^{-1}), Cna: drug concentration found in the standard non-added sample (µg mL^{-1}) and Ctp: theoretical standard concentration added to the sample (µg mL^{-1}).

Table 1. Preparation of cefoperazone (CPZ) and prednisolone (PRED) solutions for the recovery test by HPLC.

Volumetric flasks (10mL)	**Rates of CPZ and PRED standard solution 50 µg mL^{-1}**	**Rates of sample solution of ointment (CPZ 50 µg mL^{-1} and PRED 1.60 µg mL^{-1})**	**CPZ and PRED final concentration (µg mL^{-1})**
Sample	---	1.0 / 10	5.0 / 1.6
R1	1.0 / 0.400	1.0 / 9.6	10 / 5.6
R2	2.0 / 0.600	1.0 / 9.4	15 / 7.6
R3	3.0 / 0.800	1.0 / 9.2	20 / 9.6
Standard	1.0 / 0.160	---	5.0 / 1.6

R1, R2 and R3: Recovery 1, Recovery 2 and Recovery 3.

Robustness

The Youden´s test (32) was used to analyze the ruggedness of the method. The test was made by means of dosage and by using CPZ and PRED standard solutions in the dilutions of mobile phase (acetonitrile and water pH 3.0, 70:30 v/v adjusted with o-phosphoric acid) at a concentration of 10 µg mL^{-1} for standard CPZ and 15 µg mL^{-1} for standard PRED. Seven variables were evaluated, combined in eight different experiments, where the (normal) nominal conditions are represented by the upper case letters of the Latin alphabet, and the altered conditions, by the lower case letters of the same alphabet, as shown in Table 2. The altered conditions are presented in Table 3.

Table 2. Factorial combination of the analytical parameters for the ruggedness evaluation by the Youden´s test.

Variables	**Experiments**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
V1	A	A	A	A	A	a	a	a
V2	B	B	B	b	B	B	b	b
V3	C	c	C	c	C	c	C	c
V4	D	D	D	d	D	d	D	D
V5	E	e	E	e	E	E	e	E
V6	F	F	F	F	F	f	f	F
V7	G	G	G	G	G	G	G	g
Results	*s*	*t*	*U*	*v*	*W*	*x*	*y*	*z*

Source: Youden; Steriner, 1975.

Table 3. Analytical parameters and their levels of alteration used in the ruggedness test for cefoperazone (CPZ) and prednisolone (PRED) by HPLC.

	Variables	Level of alteration
V1	pH of mobile phase	A: 3.0 a: 2.8
V2	Mobile phase ratio (acetonitrile and water pH 3,0, v/v)	B: 70:30 (v/v) b: 72:28 (v/v)
V3	Wavelength (nm)	C: 240 (PRED) e 260 (CPZ) c: 242 (PRED) e 258 (CPZ)
V4	Flow (mL min^{-1})	D: 0.90 $mL.min^{-1}$ d: 0.92 $mL.min^{-1}$
V5	Room temperature (°C)	E: 25 e: 27
V6	Acetonitrile brand	F: Tedia f: Biosolvent
V7	Ultrasound bath time (min.)	G: 0 g: 5

Limits of detection (LOD) and quantification (LOQ)

The limits were analyzed experimentally; the lowest concentration analyzed with acceptable RSD comprises the LOD value, and the smallest quantified amount, with precision and accuracy, comprises the LOQ. Also theoretically, under the guidance of RDC - ANVISA 166/2017 (24), the limits were calculated by Equations (5) and (6).

$$LOD = \frac{3.3 \times SD}{\alpha} \quad (5)$$

$$LOQ = \frac{10 \times SD}{\alpha} \quad (6)$$

Where: LOD is the limit of detection, LOQ is the limit of quantification, SD is the standard deviation of the intercept and α is line slope of the calibration curve.

Results and Discussion

General aspects of method development

After testing various combinations between chromatographic columns and mobile phase, flow and proportions, a better response was obtained with an Acclaim® C18 column (250 mm x 4.6 mm, 5 µm – Thermo Scientific, USA) with mobile phase acetonitrile: water pH 3.0 adjusted with o-phosphoric acid (70:30 v/v), flow 0.9 mL min^{-1}, injection volume 20 µL. Figure 3 shows the chromatogram obtained with the developed method.

The chromatographic run has a complete elution of the drugs in less five minutes. It present good resolution, higher than 10.03, number of plates higher than 6600 for both drugs and an asymmetry factor between 0.97 and 0.99.

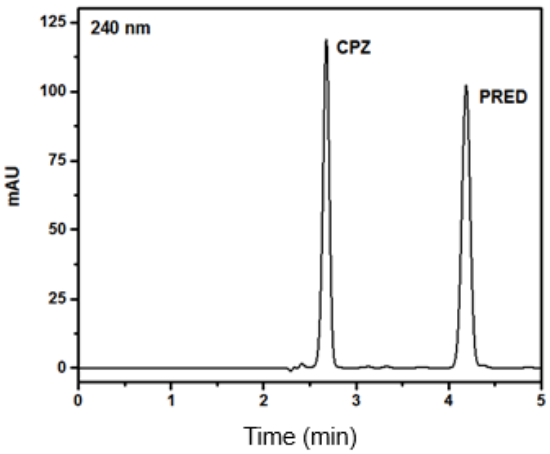

Figure 3. Chromatogram of reference standard cefoperazone (CPZ, 2.67 min) and prednisolone (PRED, 4.20 min) with 5 µg mL^{-1} for both drugs.

Selectivity

The selectivity of the method was confirmed, since in the retention times of both drugs there was no interference from any of the excipients that could interfere in the analysis of the drug, as demonstrated in Figure 4 at 260nm and in Figure 5 at 240nm.

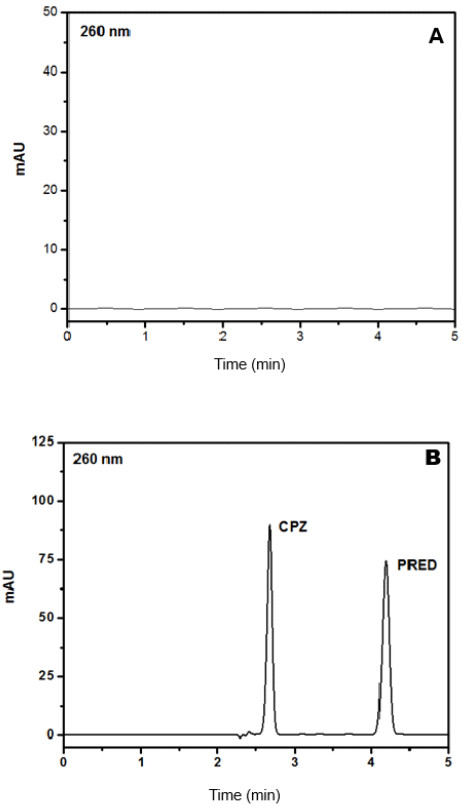

Figure 4. Chromatogram (A) of the ointment formulated with lanolin and petroleum jelly for the test of interference of excipient and impurity, and (B) the reference standards of cefoperazone and prednisolone, obtained with the Acclaim® C18 column and acetonitrile: water pH 3.0 (70:30 v/v) mobile phase analyzed at 260 nm by HPLC-DAD.

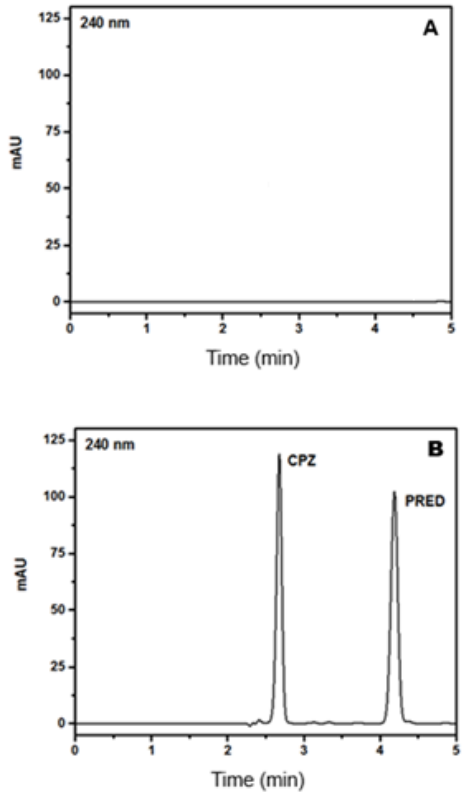

Figure 5. Chromatogram (A) of the ointment formulated with lanolin and petroleum jelly for the test of interference of excipient and impurity, and (B) the reference standards of cefoperazone and prednisolone, obtained with the Acclaim® C18 column and acetonitrile: water pH 3.0 (70:30 v/v) mobile phase analyzed at 240 nm by HPLC-DAD.

Linearity

The calibration curves were obtained simultaneously, where the concentrations studied for CPZ comprised linearity of 0.25 to 25 µg mL^{-1} (r= 0.9999 and y= 0.6776x + 0.0719) and, for PRED, from 0.75 to 30 µg mL^{-1} (r= 0.9997 and y= 1.0786x + 0.0209). The correlation coefficient (r) superior to 0.999 indicates that, under the concentration studied, both analytes demonstrate linear responses directly proportional to the concentration of the analytes.

Precision

The method was precise as it presented RSD values below 2% in the three studied levels (Table 4). Campos (2015) obtained RSD higher than that found in this method, where precision parameters were close to a 5% variation for PRED (33), a variation higher than the present study for both repeatability (RSD 1.84%) and intermediate precision (RSD 1.13%), demonstrating that the proposed method is exact in all levels analyzed.

Table 4. Result of the precision test of simultaneous analysis of cefoperazone (CPZ) associated with prednisolone (PRED) by HPLC-DAD.

Drugs	Theoretical concentration (µg mL^{-1})	Concentration found (µg mL^{-1} ± RSD%)			
		Repeatability[a]			Intermediate precision[b]
		Day 1	Day 2	Day 3	
CPZ	1,0	0,. 6 ± 0.33	0.95 ± 1.48	0.95 ± 1.74	0.95 ± 1.33
	5,0	4.93 ± 0.15	5.05 ± 1.07	5.03 ± 0.15	5.01 ± 1.22
	20,0	18.91 ± 0.74	18.76 ± 0.49	18.92 ± 1.71	18.86 ± 1.04
PRED	1,0	0.99 ± 0.16	0.98 ± 0.65	0.98 ± 0.09	0.98 ± 0.50
	5,0	4.89 ± 0.12	4.89 ± 1.84	4.86 ± 1.13	4.88 ± 1.13
	20,0	19.69 ± 0.70	19.56 ± 0.25	19.62 ± 1.62	19.62 ± 0.93

a-Mean of three determinations carried out at three levels (triplicate of each level); b- Mean of nine determinations carried out at three levels of concentration on different days. Chromatographic conditions: Acclaim® C18 column, acetonitrile:water pH 3.0 (70:30 v/v) mobile phase, flow 0.9 mL min^{-1} and injection volume 20 µL; RSD: relative standard deviation.

Accuracy

The mean recovery in the accuracy test for CPZ and PRED was 100.6% and 100.3%, respectively; the results of each recovery level are shown in Table 5. The accuracy parameter was shown to be valid in view of the recovery interval required by the official compendiums, which require recovery not inferior to 98% and not superior to 102%.

Table 5. Accuracy of analytical method applied for recovery of cefoperazone (CPZ) and prednisolone (PRED).

	Final concentratione xpected ($\mu g\ mL^{-1}$)	Final concentration found ($\mu g\ mL^{-1}$)[1]	Recovery (%)	Mean recovery (%)	RSD[2] (%)
	10.0	9.9	99.7		
CPZ	15.0	15.3	101.6	100.6	1.0
	20.0	20.1	100.4		
	5.60	5.6	100.5		
PRED	7.60	7.5	98.8	100.3	1.4
	9.60	9.8	101.7		

1-Mean of three determinations. 2-RSD: relative standard deviation

Robustness

Table 6 shows the contents obtained in each of the experiments and Table 7, the effects of each alteration on the method. In the test presented, the proposed changes yielded no significant effect since all alterations for CPZ had effects smaller than 1.73 and, for PRED, less than 1.70. These values correspond to twice the standard deviation of all average of the respective experiments.

Table 6. Cefoperazone (CPZ) and prednisolone (PRED) contents determined in the ruggedness analysis of the method.

Experiment	Contents (%)	
	CPZ	PRED
1	100.87	99.40
2	100.90	100.72
3	100.12	99.00
4	100.34	100.18
5	100.32	97.55
6	99.45	100.40
7	101.47	100.35
8	97.50	101.44
Mean	100.12	99.88
Standard deviation	1.22	1.21

Table 7. Effects on the Youden´s test applied to the validation of the method for cefoperazone (CPZ) and prednisolone (PRED).

Variable	Effects	
	CPZ	PRED
pH	0.88	0.11
Mobile phase ratio	0.53	0.73
Wavelength	1.15	1.02
Flow	0.13	1.20
Room temperature	1,27	0.36
Acetonitrile brand	0.26	0.47
Ultrasound bath	0.82	0.41

Limits of detection (LOD) and quantification (LOQ)

From the lower concentration of the calibration curves, the experimental LOD was 0.10 $\mu g\ mL^{-1}$ ± 6.1% and the LOQ was 0.25 $\mu g\ mL^{-1}$ ± 0.76% for CPZ. For PRED, the experimental limits of 0.10 $\mu g\ mL^{-1}$ ± 8.5% and 0.25 $\mu g\ mL^{-1}$ ± 0.3% were obtained for detection and quantification, respectively. The theoretical values were calculated according to equations 5 and 6 previously mentioned. The LOD results for CPZ were 0.37 $\mu g\ mL^{-1}$ and for PRED 0.18 $\mu g\ mL^{-1}$, and the LOQ results were 1.11 $\mu g\ mL^{-1}$ and 0.54 $\mu g\ mL^{-1}$ for CPZ and PRED, respectively.

Application in commercial sample Cefavet®

Figure 6 shows the chromatograms obtained from the determination of the CPZ and PRED content. Figure 6-A shows the CPZ and PRED reference standards with a concentration of 5 $\mu g\ mL^{-1}$ at a wavelength of 260 nm, determined for the detection of CPZ. In 6-B, the chromatogram refers to the commercial sample at a concentration of 5 $\mu g\ mL^{-1}$, at a wavelength of 240 nm, used for the detection of PRED. Figure 6-C shows the superposition of the spectra of Figures A and B, in order to demonstrate that the chromatographic peaks obtained at the wavelengths of 240 and 260 nm correspond and that the elution occurred at the same time.

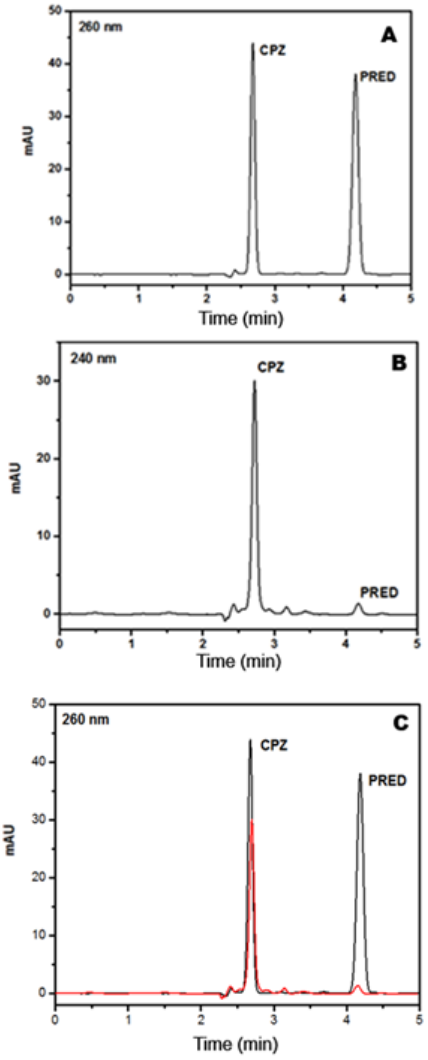

Figure 6. Chromatograms obtained from the determination of cefoperazone (CPZ) and prednisolone (PRED) content in an intramammary ointment sample by HPLC, where "A" illustrates the reference standards at a concentration of 5 µg mL^{-1} for CPZ and PRED, "B" refers to the chromatogram of the commercial sample; and "C" is the overlay of chromatograms A and B.

The content found by the liquid-liquid extraction efficiency for CPZ was 69.6 ± 1.1% and, for PRED, 72.4 ± 0.7%. However, the simultaneous analysis of drugs in complex matrices has demonstrated lower extraction efficiency, despite the use of sensitive techniques such as liquid chromatography. Tang et al. (2012) analyzed fresh milk samples and the result was 66.6 to 75.7% among the levels studied to analyze CPZ associated with other antimicrobials (34).

Conclusions

The analytical method was developed and validated by high-performance chromatography to determine, concurrently and quantitatively, cefoperazone associated with prednisolone in intramammary ointment. This method has advantages because the analysis time is relatively short, which saves organic solvents and instrument use. The method uses a small amount of sample and requires simple preparation, which makes it possible to be used both in routine laboratories and pharmaceutical industries.

The method was shown to be linear with a correlation coefficient superior to 0.999 for both drugs. It was also shown precise, by repeatability and intermediate accuracy, with coefficients of variation lower than 2.0%. In the test of accuracy the method demonstrated recovery values close to the true value, therefore, exact.

Through the study of robustness, we can observe that the small variations occurred in the routine of the method application, such as temperature difference, pH alterations, acetonitrile brand, mobile phase ratio, solvent flow and wavelength did not significantly altered the results obtained, thus demonstrating the ruggedness of the method.

In the analysis of the content of cefoperazone and prednisolone, it was concluded that complex samples pose an analytical challenge, since they require a more elaborate preparation, involving more steps, and generate a larger number of sample and solvent residues, facts avoided with the method developed and validated in this work.

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Conflict of interest

The authors report that there is no conflict of interest.

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