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Development and validation of a method for characterization and quantification of marbofloxacin in pharmaceutical formulation for animal use

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Marbofloxacin (MFN) is a fluoroquinolone drug used in veterinary medicine to treat dogs and cats with skin and appendage infections, urinary tract infections, gastrointestinal infections, and respiratory infections. The objective of this study was to develop and validate an analytical method for determining MFN in palatable tablets for animal use. The MFN raw material was characterized using thin-layer chromatography, infrared (IR) absorption spectroscopy, and melting range spectroscopy to ensure its quality for use in validating the spectrophotometric analytical method. Method validation was performed based on the parameters of specificity, linearity, precision, accuracy, limits of detection and quantification, and robustness. In the calibration curve from 5 to 15 µg mL^{-1}, the correlation value was 0.9990, in repeatability and intermediate precision the relative standard deviation was less than 1%, and the mean recoveries were 101.53 ± 0.4, the limits of detection and quantification were 0.033 and 0.625 µg mL^{-1}, respectively. The method developed and validated for the quality control of MFN was sensitive, linear, precise, accurate and robust for its determination in pharmaceutical formulations for veterinary use.

Keywords: Fluoroquinolone; Marbofloxacin; Spectrophotometry; Method Validation; Validation

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Introduction

Fluoroquinolones are the antibiotics of choice for treating genitourinary tract infections. The prototype of this pharmacological class is nalidixic acid, which was synthesized in 1962 and demonstrated activity against Gram-negative strains. Its use was initially for the treatment of genitourinary tract infections, but on the downside, this antibiotic quickly developed tolerance to microorganisms, generating resistance (1).

The mechanism of action of fluoroquinolones is to inhibit DNA gyrase and topoisomerase IV, both enzymes involved in the processes of replication, transcription, recombination, and repair of bacterial DNA. This effect, possibly associated with the binding of fluoroquinolones to DNA gyrase, is rapid bactericidal action (2). Bacterial resistance to fluoroquinolones is due to the emergence of enzymatic mutations in DNA gyrase and topoisomerase IV, or also mutations in genes that regulate different efflux systems (3).

In the 1980s, the first fluorinated quinolone for human use, norfloxacin, emerged. Since then, numerous fluoroquinolones have been synthesized and evaluated through changes in the substituents of the central nucleus, which ensured structural and, consequently, pharmacokinetic differences in each synthesized product (4-6).

To catalog fluoroquinolones and thus facilitate their use from a clinical point of view (7), they proposed a classification criterion based on potency and spectrum of antimicrobial action, thus emerging four generations of fluoroquinolones. The first generation includes compounds such as nalidixic acid, oxolinic acid, pipemidic acid, and cinoxacin. The second generation includes drugs such as norfloxacin, ciprofloxacin, enrofloxacin, harmofloxacin, and difloxacin. In turn, the third and fourth generations, introduced from the 1990s onwards, comprise broad-spectrum fluoroquinolones, with emphasis, in the third generation, on orbifloxacin, levofloxacin, marbofloxacin, sparfloxacin, and grepafloxacin, and, in the fourth generation, on trovafloxacin, gatifloxacin, moxifloxacin, gemifloxacin, and sarafloxacin (6).

A drug from the fluoroquinolone class for animal use that has been little studied but is available on the market is marbofloxacin (MFN). MFN is a third-generation fluoroquinolone currently used only in veterinary medicine. To date, no incidence of resistant pathogens has been reported, even after long-term treatment in cattle or domestic animals (8). This drug is produced by only one pharmaceutical laboratory in Brazil, in the form of palatable Marbopet® tablets, which is used in the treatment of infections of the skin and appendages, urinary tract, gastrointestinal tract, and respiratory tract, mainly in the treatment of dogs and cats (9).

The chemical structure of MFN (Figure 1) is composed of a benzoic nucleus condensed to a piperazine nucleus, a structure characteristic of a quinolone and with its own substituents. MFN is chemically named as 6-carboxylic acid-9-Fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7H-pyrido[3,2,1-

yl][4,1,2]benzoxadiazine, and has the molecular formula $C_{19}H_{22}FN_4O_4$ and a molecular weight of 362.4 g/mol. MFN is very slightly soluble in water and very slightly soluble in ethanol, very slightly soluble in methanol, and partially soluble in dichloromethane and occurs as pale yellow crystals (10).

Figure 1. Chemical structure of marbofloxacin.

Regarding marbofloxacin, limited physicochemical information and only a few analytical methods for its determination are available in the literature. The British Pharmacopoeia (2013) (10) describes a high-performance liquid chromatography (HPLC) method for the identification and determination of MFN and its related impurities in the raw material. In addition, HPLC methods have been reported for the determination of MFN and other fluoroquinolones in raw materials (11, 12), biological samples (8, 13, 14), and food products (13, 15). However, simple and cost-effective analytical methods for the routine quality control of pharmaceutical formulations are still limited. Furthermore, an immunoenzymatic method for the determination of MFN and other fluoroquinolones in beef and pork was also reported (15). Hernández-Arteseros et al. (2000) (16) developed and validated an HPLC method for the simultaneous determination of MFN and other fluoroquinolones in animal products. However, to date, no analytical methodology for the quantitative determination of MFN in pharmaceutical formulations for animal use has been found in the literature.

Therefore, we emphasize the importance of this study with the development and validation of a sensitive and reliable analytical method, using Absorption Spectrophotometry in the Ultraviolet-Visible (UV/Vis) region for quality control of the commercial MFN sample available on the national market.

The study involves the process of obtaining the secondary working standard from an industrialized sample for use in the study of development and validation of the analytical method.

Experimental section

Commercial sample and Characterized Chemical Substance (CCS)

The sample used in this study was purchased locally (Marbopet® 82.5 mg), and the raw material used as CCS in this work was donated by the pharmaceutical company CEVA Saúde Animal Ltda., with a declared content of 100.2%, according to the report provided by the company, and was used without further purification.

For characterization and confirmation of its identity, analyses were performed using thin layer chromatography (TLC), infrared absorption spectroscopy (IR), and melting range.

Thin Layer Chromatography (TLC)

Methanolic solutions of the reference chemical were prepared at concentrations of 1 mg mL-1and 500 µg mL^{-1}, and of the commercial sample at concentrations of 1 mg mL^{-1}. These were applied to 6 x 8 cm Macherey-Nagel® 60 silica gel chromatographic plates with UV_{254} fluorescence indicator. Various eluent ratios were tested, as shown in Table 1, until the optimal separation conditions for each substance were established. The eluent ratios were evaluated using the ciprofloxacin monograph of the Indian Pharmacopoeia (2007) (17) as a reference, due to the lack of TLC methodology for MFN in the literature.

Table 1. Analysis of marbofloxacin (MFN) by thin layer chromatography (TLC).

Eluents	Proportion
Dichloromethane:Acetonitrile:Methanol:Ammonium hydroxide	(4:1:4:1)
Methanol:water:ethyl ether:dichloromethane	(8:1.2:1.5:7.7)
Dichloromethane:Methanol:Ammonium hydroxide	(8:1.5:0.5)
Acetonitrile:Ethyl ether	(0.5:9.5)
Acetonitrile:Dichloromethane	(0.5:9.5)
Chloroform:Methanol	(6:4)
Dichloromethane:Methanol:Ammonium hydroxide	(7.2:2.5:0.5)

The plates were developed with UV light and a 20% sulfuric acid ethanol solution, and Rfs were calculated to assess whether the secondary standard eluted in agreement with the commercial sample. This study was conducted in comparison with the commercial sample due to the impossibility of acquiring the primary standard due to its unavailability in the domestic market and the difficulties of exporting it to foreign markets.

The Rf values were calculated using the formula:

$$Rf = \frac{Dx}{De}$$

Where: Dx = distance traveled by the compound (raw material and/or industrialized sample) and De= distance traveled by the eluent (7 cm).

Infrared Absorption Spectroscopy (IR)

The secondary standard obtained was scanned in the infrared (IR) region, using Fourier transform (FTIR) in a Perkin Elmer® Spectrum 1000 spectrophotometer. The spectra were obtained using the Attenuated Total Reflection (ATR) technique, in the range of 4000 to 450 cm^{-1}.

Melting Range

A Gehaka® PF 1500 semi-automatic melting point meter was used, which has capillaries inserted into heating cells. The procedure was performed in triplicate.

Development and Validation of the Analytical Method

Optimization of Conditions for Spectrophotometric Testing

Aliquots of 10.0 mg of secondary standard, obtained and qualified by the analytical techniques mentioned above, were weighed and diluted to 100.0 µg mL⁻¹. Aliquots of 5.0 mL of this solution were removed to perform new dilutions with solvents: distilled water, methanol, acetonitrile, 0.1 M HCl, 0.01 M HCl, 0.1 M NaOH, 0.01 M NaOH, acetonitrile:water (55:45) and acetonitrile:water (70:30), in order to obtain final solutions of 10.0 µg mL^{-1}, in 50.0 mL flasks. The solutions were read using a spectrophotometer, in scan mode from 200 to 400 nm, using the solvents mentioned above as blanks to determine the maximum absorbance of MFN in each solvent.
The procedure was performed on a Thermo Scientific® UV/Vis spectrophotometer – Model Evolution 60. The absorption spectra in the ultraviolet (UV) region of both the commercial sample and the obtained reference standard were performed in 10 mm quartz cuvettes. The maximum wavelength was defined during the experiment by scanning analysis as in the method established by Sahu, Afzal Azam, Banarjee (2011) (18).

Preparation of Characterized Chemical Substance (CCS)

The standard solution was prepared with 10.0 mg of MFN in a 100.0 mL volumetric flask. The volume was completed with the solvent solution defined in the preparative scanning test. This flask was subjected to ultrasound for 15 minutes. A 15.0 mL aliquot of the above solution was removed and diluted in a 100.0 mL volumetric flask with acetonitrile:water (70:30) to obtain a working standard solution containing 15.0 µg mL⁻¹ of MFN. This working standard solution was used to prepare the calibration standards over the validated concentration range (5.0–15.0 µg mL⁻¹).

Sample Preparation

To analyze the drug content in the tablets, the average weight of twenty units containing 27.5 mg MFN was first determined, weighed individually, and the average weight calculated. Subsequently, they were ground until a homogeneous powder was obtained. A quantity of powder containing 10.0 mg of MFN was duly weighed and diluted with acetonitrile:water (70:30) to a 100.0 mL volumetric flask, and a 5.0 mL aliquot was transferred to a 50.0 mL flask, where the final volume was completed with acetonitrile: water (70:30) to obtain a final concentration of 10.0 µg mL⁻¹. The assay was performed by interpolation using the calibration curve.

Validation of the Analytical Method

Specificity

The specificity of the proposed UV/Vis spectrophotometric method was assessed by analyzing a placebo solution prepared with the main excipients of the pharmaceutical formulation at their usual concentrations. The placebo solution was prepared following the same procedure used for sample preparation and analyzed at the selected analytical wavelength to verify the absence of interference from the excipients in the determination of MFN.

Linearity

Linearity was evaluated by constructing a calibration curve using standard solutions of MFN prepared in acetonitrile:water (70:30, v/v) at concentrations of 5.0, 7.5, 10.0, 12.5, and 15.0 µg mL⁻¹. Each concentration level was analyzed in triplicate, and the calibration curve was constructed using the mean absorbance values obtained from the three independent determinations.

Precision

Intermediate precision (inter-day) and repeatability (intra-day) were determined by analyzing the commercial MFN sample at a concentration of 10.0 µg mL^{-1}. Repeatability was performed with six replicate readings and intermediate precision was obtained by averaging the repeatability of 3 consecutive days and determining the relative standard deviation (RSD) between measurements.

Accuracy

The accuracy of the proposed method by UV/Vis spectrophotometry was evaluated through the recovery test, which was performed by adding known quantities of standard solution to the sample, as shown in Table 2.

Table 2. Added quantities of sample and standard solutions to perform the marbofloxacin recovery test using the UV/Vis spectrophotometric method.

Volumetric flask	Sample Solution 20.0 µg mL^{-1} (mL)	Standard Solution 20.0 µg mL^{-1} (mL)	Theoretical Final Concentration* (µg mL^{-1})
1	0.0	3.0	6.0
2	3.0	2.0	10.0
3	3.0	3.0	12.0
4	3.0	4.0	14.0
5	3.0	0.0	6.0

*Final volume 10.0 mL

The recovery tests were performed with the above aliquots prepared in 10.0 mL flasks from the standard

solution and sample solution, previously prepared, both with a concentration of 20.0 µg mL^{-1} and completing their volume with acetonitrile: water (70:30). The spectrophotometric reading of the five flasks was performed in triplicate. The percentage recovery was calculated as indicated by the Association of Official Analytical Chemists (2006) (19), according to the following equation:

$$\%R = \frac{(\mathrm{Ca} - \mathrm{Cna})}{\mathrm{Ctp}} x100$$

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}).

Limit of detection and Limit of quantification

The limit of detection (LD) and limit of quantification (LQ) were calculated according to the International Conference on Harmonization (2005) (20):

$$\mathrm{LD} = 3.3 \times \left(\frac{SD}{a}\right)$$

$$\mathrm{LQ} = 10.0 \times \left(\frac{SD}{a}\right)$$

Where: SD represents the standard deviation of the y-intercept, and a is the slope of the calibration curve.
The LD and LQ values were determined from the lowest concentration (5.0 µg mL^{-1}) of the calibration curve, where successive dilutions were performed obtaining 4 reading concentrations: 5.00 µg mL^{-1}, 2.50 µg mL^{-1}, 1.25 µg mL^{-1}and 0.625 µg mL^{-1}. From the determination of the absorbance of these concentrations, the average of the values of the triplicate readings was calculated, and subsequently the value of the correlation coefficient.

Robustness

The robustness test was performed by varying the solvent ratio (± 5.0 mL) and wavelength variation (± 3 nm). Considering these conditions, new sample solutions were prepared using acetonitrile:water (65:35) and acetonitrile:water (75:25). Each solution was read in triplicate, and then, using the average result obtained, the MFN content in each solution was calculated using the straight-line equation obtained in the calibration curve.

Results and Discussion

Qualitative analysis of Characterized Chemical Substance (CCS) for chemical identification

Thin Layer Chromatography (TLC)

In the TLC assay, the mobile phase was first defined using tests based on the monograph of ciprofloxacin hydrochloride, a fluoroquinolone similar to MFN.
The best result was obtained with the mobile phase composed of dichloromethane:methanol:ammonium hydroxide in the proportions (7:2.5:0.5), which provided efficient migration of the drug.
At a concentration of 1.0 mg mL^{-1}, the Rf calculated for the commercial samples (A1 and A2) was 0.586 and for the reference chemical substance (P1) the Rf was 0.571. The Rf of the reference chemical substance (P2) at a concentration of 500 µg/mL was 0.557 (Figure 2).

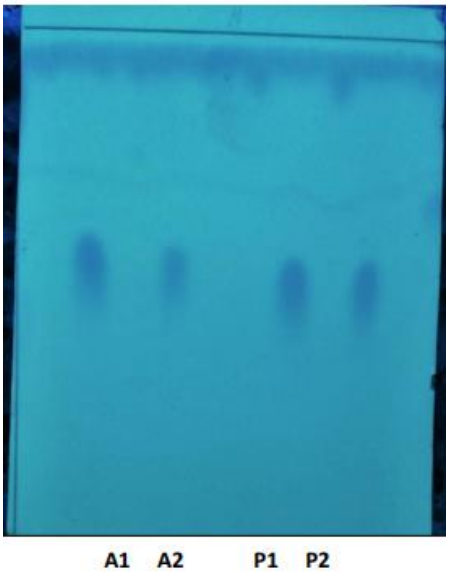

Figure 2. Chromatogram obtained from the commercial sample (A1 1 mg mL^{-1} and A2 1 mg mL^{-1}) and reference chemical substance (P1 1 mg mL^{-1} and P2 500 µg mL^{-1}) using dichloromethane:methanol:ammonium hydroxide (7.0;2.5;0.5) as eluent and 254 nm UV lamp as developer.

Therefore, it can be assumed that the reference chemical substance used has a similar chemical identity to the commercial sample, which allows its use in quantitative analyses for the determination of MFN.
MFN is a fluoroquinolone widely used in veterinary medicine. However, little information has been found in the literature regarding its physicochemical properties. To date, only the British Pharmacopoeia (2013) (10) provides information on MFN, but only for the raw material, making no mention of any pharmaceutical form.
Qualitative analyses such as TLC, IR, and MFN UV spectroscopy confirmed that the drug in question was indeed the quinolone targeted in this study and did not indicate the presence of other aggregated compounds, such as excipients.
In the TLC assays, the similarity of the secondary working standard to the commercial sample was evidenced by the Rf values, suggesting that it is the same substance, and that the proposed extraction method did not cause alterations in the chemical structure of the MFN

molecule. Although no TLC identification technique for MFN was found in the literature, the method used for the characterization of ciprofloxacin hydrochloride listed in the Indian pharmacopoeia was adapted and considered valid for the separation and identification of both the standard and the sample.

In the analysis of the IR spectral profile of MFN, the characteristics common to fluoroquinolones were all evidenced, as well as the vibrations corresponding to the particular constituents of MFN, which allows us to presume that the pattern obtained in this study has the chemical identity of the raw material MFN, even though the spectral profile of MFN was not found in the literature.

The melting point range of MFN showed the characteristic profile of the fluoroquinolone class, which is melting followed by decomposition. Therefore, it is presumed that the characteristic profile is that of a fluoroquinolone. However, the melting point range of MFN cannot be determined due to the unavailability of this physicochemical data in the literature.

Infrared Absorption Spectroscopy (IR)

Figure 3 shows the IR absorption spectrum of the secondary standard obtained from the commercial sample of MFN. The common vibrations between MFN and the other fluoroquinolones are a strong band at 1725.7 cm^{-1}, characteristic of the C=O stretching of the carboxylic acid, and a strong band at 1623 cm^{-1}, characteristic of the C=C stretching of the quinolone nucleus.

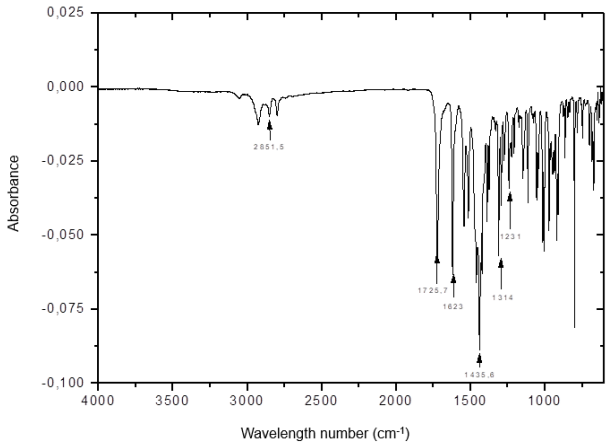

Figure 3. Infrared (IR) spectrum of the raw material marbofloxacin.

A characteristic absorption band of aryl fluoride, normally observed between 1300-1100 cm^{-1}, was evidenced in the test at 1231 cm^{-1}. The particular features found in the IR spectrum of MFN were a strong intensity band at 2851.5 cm^{-1} characteristic of the methyl stretching, a strong intensity band at 1435.6 cm^{-1} characteristic of the N-CH_3 group of aromatic compounds, and a strong intensity band at 1314 cm^{-1} characteristic of the C-O-C group of the methoxyl group.

The absorption bands found in the IR spectrum, as well as the spectral profile of MFN, considering the comparison with the literature for other fluoroquinolones such as ciprofloxacin, and that the other bands present indicate the groups that differentiate MFN from ciprofloxacin, it is then assumed that the spectral profile of chemical identity obtained corresponds to MFN (21-26).

Melting Range

The data obtained in this study indicate that MFN melting occurred in the range of 220 to 240 °C, followed by a carbonization process from the final melting temperature, characterizing a melting followed by thermal decomposition. This range is lower than the reported melting point of pure marbofloxacin, which is 268–269 °C (decomposition) (27), suggesting that the sample may contain impurities or exist in a different solid form.

Development and Validation of the Method

Table 3 presents the results of the optimization of the spectrophotometric conditions for MFN. It was observed that MFN presented the highest absorbance when dissolved in the solvent mixture acetonitrile:water in the ratio 70:30, using a standard solution with a concentration of 10 µg mL^{-1}.

Table 3. Study of solvent selection for the spectrophotometric assay from marbofloxacin solutions at a concentration of 10 µg/mL.

Solvents	Maximum wavelength (nm)	Absorbance
Distilled water	290	0.149
Methanol	300	0.212
Acetonitrile	303	0.434
0.1M HCl	298	0.193
0.01M HCl	298	0.216
0.1M NaOH	290	0.180
0.01M NaOH	290	0.256
Acetonitrile:water (55:45)	295	0.532
Acetonitrile:water (70:30)	298	0.863

Under these conditions, the maximum wavelength (λ max) was determined at 298 nm, with a maximum absorbance of 0.863. The spectrum corresponding to this analysis is illustrated in Figure 4.

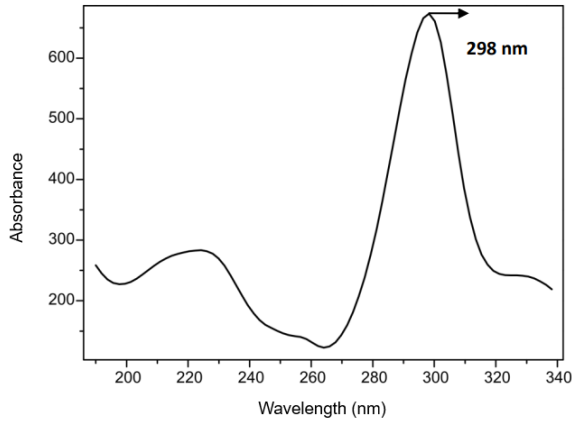

Figure 4. Absorption spectrum in the ultraviolet region of the marbofloxacin 10 µg mL^{-1} raw material used as a reference chemical substance, using water:acetronitrile (70:30) as a solvent.

MFN was characterized by UV spectrophotometry, since the characteristic profile of a fluoroquinolone was evidenced by the presence of an absorption band around 200-240 nm referring to amino chromophore groups, and another band of strong intensity between 260-320 nm referring to carboxylic acid chromophore groups.

The method was developed and validated using an acetonitrile:water (70:30, v/v) mixture, as this solvent system provided the highest absorbance intensity (0.863) for MFN at the selected wavelength of 298 nm, indicating greater sensitivity for the spectrophotometric determination. In addition, the proposed method is simple, inexpensive, and easy to perform, with straightforward solvent handling and disposal. Silva et al. (2022) (28) proposed an eco-friendly spectrophotometric method using a water:ethanol (80:20, v/v) mixture with a λmax of 296 nm. Although ethanol is preferable from a green chemistry perspective because of its lower environmental impact and reduced waste toxicity, the present method demonstrated superior analytical sensitivity, providing substantially lower limits of detection (LD, 0.033 µg mL^{-1}) than those reported for the hydroalcoholic method (0.39 µg mL^{-1}).

Specificity

The specificity of the proposed spectrophotometric method is shown in Figure 5. In the spectrum, it was observed that no absorbance or interference peaks of the placebo occurred in the presence of MFN, at a wavelength of 298 nm.

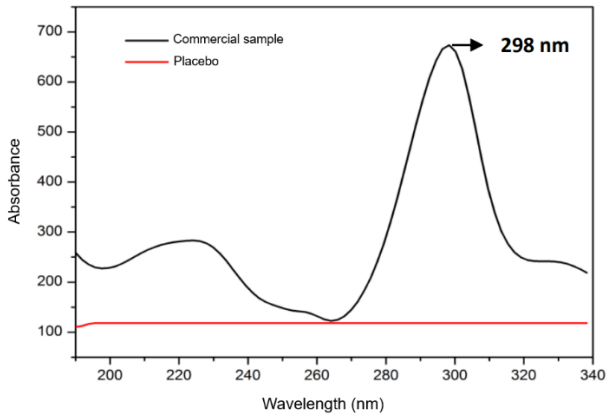

Figure 5. Spectrum of the commercial sample of marbofloxacin and placebo in UV/Visible Spectrophotometry to assess the specificity of the method.

The specificity of the method was confirmed by the analysis of the placebo, which did not present any reading or interference in the wavelength of analytical interest, and the linearity was confirmed by the correlation coefficient (r), which satisfied the recommendations of RDC n. 166/2017 of Anvisa, whose method is considered linear when the correlation coefficient is above 0.990 (29), as well as the sensitivity, which was demonstrated through the determination of low drug limits with adequate precision and accuracy.

The specificity of the method was confirmed by the absence of interference from excipients. It is worth noting that forced degradation studies conducted by other authors indicate that MFN is highly photosensitive undergoing up to 30% degradation under UV light although it remains stable under acidic and basic conditions for short periods (28, 30).

Linearity, LD, LQ, Precision, Accuracy and Robustness

The linearity of the method was evaluated from the mean of three independent analyses using calibration standards at concentrations of 5.0, 7.5, 10.0, 12.5, and 15.0 µg mL^{-1}. The calibration curve was constructed by plotting absorbance as a function of MFN concentration. The straight-line regression equation obtained was $y = 0.1084x + 0.2268$, with a correlation coefficient of 0.9990 (Table 4). The experimental LD and LQ values found for the spectrophotometric method were 0.033 and 0.625 µg mL^{-1}, respectively.

The linearity obtained (r = 0.9990) in the 5 to 15 µg mL^{-1} range is consistent with the results of Oliveira et al. (2023) (30), who demonstrated robust linearity for MFN in both HPLC (1 to 10 µg mL^{-1}) and spectrophotometric methods. The high sensitivity of the UV/Vis method validated here allows for precise quantification even at low concentrations, which is essential for the rigorous quality control of veterinary formulations.

Table 4. Parameters obtained in the linearity test to calculate the marbofloxacin calibration curve.

Parameter	Value	Standard Error
Slope (m)	0.1084	0.0011
Intercept (b)	0.2268	0.0670
Coefficient of determination (r^2)	0.9999	-
Correlation coefficient (r)	0.9990	-

Given this, the method was considered linear, since the correlation coefficient of the calibration curve presented a value between 0.99 and 1.00, meeting the criterion established by RDC n. 166/2017 (29), which accepts values equal to or greater than 0.99 as adequate, being even more reliable when equal to or greater than 0.999. Therefore, the equation of the line was used to calculate the drug concentrations in all analyses performed during the validation process.

The relative standard deviation (RSD) found between the intra-day and inter-day analyses performed on the sample were: 0.09% for the repeatability analysis, 0.20% for the intermediate precision analysis, and the contents obtained were 101.6% (± 0.09) and 104.4% (± 0.20), respectively, as can be seen in Table 5. The experimental results obtained for the accuracy test are shown in Table 6.

Table 5. Results obtained in the repeatability and intermediate precision analysis using the proposed method.

Method	Declared theoretical concentration (µg/mL)	Experimental concentration found (µg/mL)			RSD (%)	Content (%)
Repeatability	10.0	Day 1 101.8 ± 0.17	Day 2 101.4± 0.16	Day 3 101.6 ± 0.02	0.09	101.6 ± 0.09
Intermediate precision	10.0		101.6 ± 0.20		0.20	104.4 ± 0.20

Table 6. Results obtained in the recovery analysis using the proposed method.

	Fortified theoretical concentration (µg/mL)[a]	Experimental concentration found (µg/mL)[ab]	Recovery (%)	
			Result[c]	Average[d]
Sample	10.0	9.78	98.00	101.53 ± 0.4
	12.0	12.27	106.83	
	14.0	13.84	99.75	

[a]Theoretical sample concentration: 20.0 µg/mL; [b]Average of 3 determinations; [c]Concentration of the analyte in the unadulterated sample (Cna): 6.0 µg/mL; [d]95% confidence interval (t-Distribution).

Regarding the repeatability and intermediate precision of the developed method, these were determined in percentages of RSD with results below 2.0%, indicating that it has precision for quantifying MFN in the pharmaceutical form studied.

The accuracy of the method, determined by recovery tests, yielded values that meet the recommendations of international method validation guidelines. Considering that the obtained value is between 98 and 102% and the RSDs are less than 2.0%, the developed method has an accuracy considered adequate for the quantification of MFN in the pharmaceutical form under study.

The preservation of the spectral profile and the accuracy observed in recovery tests (101.53%) in our study suggest that the method is capable of quantifying the active drug without interference from potential primary degradation products or the tablet matrix.

The robustness test demonstrated that small deliberate variations in the analytical conditions did not significantly affect the quantification of marbofloxacin by the proposed UV/Vis spectrophotometric method (Table 7).

Table 7. Results obtained in the robustness analysis using UV-Visible Spectrophotometry.

Spectrophotometric conditions	Content (%)
I – acetonitrile Vetec®:water (65:35) at 300 nm	97.17
II – acetonitrile Vetec®:water (75:25) at 306 nm	97.31
III - acetonitrile Química Moderna®:water (70:30) at 303 nm	99.26
IV - mobile phase pH 2.9[a]	100.19
V - mobile phase pH 3.1[a]	99.08
VI - solution temperature 25°C[a]	99.92
VII - solution temperature 27°C[a]	100.17

[a]acetonitrile:water (70:30) at 303 nm.

Variations in the acetonitrile:water ratio (65:35 and 75:25, v/v) combined with changes in the analytical wavelength (300 and 306 nm) resulted in assay values of 97.17% and 97.31%, respectively, whereas the nominal analytical condition (acetonitrile:water, 70:30, v/v, at 303 nm) provided an assay value of 99.26%. Likewise, the use of acetonitrile from a different manufacturer did not affect the assay results.

The influence of pH was evaluated by varying the solution to pH 2.9 and 3.1, resulting in assay values of 100.19% and 99.08%, respectively. Similarly, solution temperatures of 25 °C and 27 °C yielded assay values of 99.92% and 100.17%, respectively. All assay values remained within the acceptance range of 90–110% recommended by official compendia (10, 17, 27), demonstrating that the proposed method is robust against small deliberate variations in solvent composition, analytical wavelength, solvent manufacturer, pH, and solution temperature. Therefore, the method is suitable for the routine quantification of MFN in the evaluated pharmaceutical formulation.

The interchangeability between HPLC, UV spectrophotometric, and microbiological methods for the analysis of MFN tablets has already been statistically demonstrated in the literature through ANOVA tests, which showed no significant differences among the results obtained by these techniques. This reinforces the applicability of UV/Vis spectrophotometry as a fast, low-cost, and reliable alternative for routine laboratory analysis, providing results comparable to more complex and expensive techniques such as high-performance liquid chromatography. In addition, while chromatographic methods are primarily focused on the separation and quantification of impurities, the combined use of physicochemical and microbiological methods is recommended to ensure the actual antimicrobial potency of the pharmaceutical product (28, 30).

Therefore, it can be concluded that the developed UV spectrophotometric method for the analysis and quantification of MFN in veterinary pharmaceutical formulations is valid, as it demonstrated linearity, precision, accuracy, robustness, specificity, sensitivity, and simplicity, without requiring laborious analytical steps. Furthermore, the proposed extraction procedure was efficient in obtaining a representative solution of the active pharmaceutical ingredient from the dosage form, with no interference from excipients. Consequently, the method can be confidently applied for routine quality control in pharmaceutical laboratories. Importantly, the method also aligns with the principles of Green Analytical Chemistry, as it minimizes solvent consumption, reduces waste generation, and avoids the need for complex instrumentation, contributing to more sustainable and environmentally responsible pharmaceutical analysis.

Conclusions

The developed and validated method proved to be sensitive, linear, precise, accurate, and robust for the quantification of MFN in veterinary pharmaceutical formulations. Furthermore, the method demonstrated simplicity, speed, cost-effectiveness, and ease of reproducibility, making it suitable for routine analytical use by quality control laboratories in pharmaceutical industries.

Author contributions

K.R.W.O.: Conceptualization, Methodology, Formal Analysis, Investigation, Data Curation, Writing – Original Draft Preparation, Visualization. P.E.S.: Formal Analysis, Writing – Original Draft Preparation, Writing – Review and Editing, Visualization. M.S.A.: Formal Analysis, Writing – Original Draft Preparation, Visualization. T.F.M.C.: Formal Analysis, Writing – Original Draft Preparation, Visualization. N.M.K.: Conceptualization, Methodology, Formal Analysis, Data Curation, Resources, Visualization, Supervision, Project Administration.

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Conflict of interest

The authors declare no conflicts of interest.

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