Research Article

Development and Validation of HPLC Assay Method for Determination of Marbofloxacin in Veterinary Formulation Injectables

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ABSTRACT

A novel gradient reverse-phase chromatographic analytical method was developed and validated for the quantitative determination of Marbofloxacin in veterinary formulation injectables using HPLC-DAD. Marbofloxacin was eluted in gradient mode using the mobile phase of 0.1% formic acid in 10mM ammonium formate in water (mobile phase-A) and 0.1% formic acid in methanol (Mobile Phase-B) at a flow-rate of 1.5mL/min on Inertsil ODS-3, C18×4.6mm, 5µm, particle size column. The present method was found to be sensitive and selective at very low levels of linearity range 1.986 to 14.892 µg/mL with a correlation coefficient of ≥ 0.999. The mean accuracy expressed as recovery of the method was 101.4%. The precision (%RSD) for intra & inter day was < 2%. The method has adequate sensitivity with detection and quantitation of 1.0 µg/mL. The method was strictly validated according to the ICH and AOAC guidelines. Based on the presented results, the proposed method was considered more precise, accurate with adequate sensitivity and robust. Acquired results demonstrated that proposed method can be applied for quantitative analysis of Marbofloxacin in veterinary formulation injections.

Keywords: Marbofloxacin; Quality control; HPLC-DAD; Assay method.

INTRODUCTION

Marbofloxacin is a broad spectrum, second-generation fluoroquinolone drug, frequently prescribed in veterinary practices. [1] The bactericidal activity of Marbofloxacin is concentration dependent mechanism [2], with susceptible bacteria cell death occurring within 20–30 minutes of exposure. It inhibits bacterial DNA gyrase enzyme which is responsible for supercoiling of DNA within the cells.[3] In fact, Marbofloxacin was shown to be the most effective drug against bacterial strains isolated from rabbits infected with upper respiratory tract diseases compared to Doxycycline, Enrofloxacin, Danofloxacin and tetracycline. [4]

Like other fluoroquinolones, Marbofloxacin has demonstrated a significant post-antibiotic effect for both gram – and + bacteria and is active in both stationary and growth phases of bacterial replication. [5]

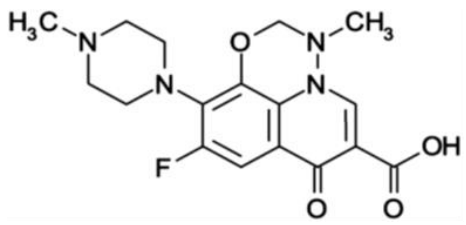

Figure 1: Chemical structure of Marbofloxacin

Marbofloxacin is a carboxylic acid derivative fluoroquinolone and chemically known as 9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3ij] [1,6,8] benzoxadiazin- 6-carboxylic acid. It has a molecular weight equivalent to 362.36 g/mol. Marbofloxacin is pale yellow crystalline powder with pKa of 5.38 and 6.16, [8] soluble in water, slightly soluble in ethanol, and very soluble in methanol. [7] The structural formulae of Marbofloxacin is shown in Figure 1.

Several analytical methods were found in the literature, which were successfully applied in the analysis of Marbofloxacin, in different matrixes. Marbofloxacin and other fluoroquinolones were determined in biological samples by high-performance liquid chromatography with UV detector (HPLC–UV) [9-11] and with fluorescence detector (HPLC–FL) [12-15] and by capillary electrophoresis with UV detection (CE–UV) [16] and electrospray mass spectrometry (CE–MS). [17]

In food of animal origin, various fluoroquinolones were detected including Marbofloxacin by HPLC–UV, [18,19] liquid chromatography– mass spectrometry (LC–MS) or liquid chromatography– tandem mass spectrometry LC–MS/MS, [20-25] by ultra-performance liquid chromatography (UPLC) coupled to MS (UPLC–MS), [26, 27] and high-performance thin-layer chromatography. [28] Few methods were applied in the determination of Marbofloxacin in infant foods, [29] and in environment samples. [30, 31]

The British Pharmacopoeia describes a HPLC method with detection in the infrared region for identification and determination of Marbofloxacin and its related impurities in raw material. [32, 33] However, currently no established highly sensitive analytical method was found for the determination of Marbofloxacin in veterinary formulation injectables. Hence, the objective of this paper was to

develop and validate an efficient, precise, accurate, and robust HPLC–UV method for quality control of Marbofloxacin in veterinary formulation injections.

MATERIALS AND METHODS

Chemicals and reagents

Methanol grade of HPLC, Ammonium formate extra pure grade and Formic acid analytical reagent grade were purchased from Scharlab S.L., Finar limited and Fisher chemicals respectively. The reference standard Marbofloxacin was purchased from Dr. Ehrenstorfer –LGC. Marbofloxacin injection samples (Marboflo-10%, WOOGENE B&G CO., LTD., Korea.) were acquired from the local market.

Preparation of Marbofloxacin standard stock solutions and calibration standards.

Weighed 10.0 mg of Marbofloxacin reference standard and transferred into a 10 mL volumetric flask and added 2 mL of Diluent (used Mobile Phase-A), mixed well. Made up to the mark with diluent and mixed well to obtain a concentration of 992.8 µg/mL after correcting for purity. Sonicated it for 15 minutes.

The Marbofloxacin calibration curve was constructed from a standard stock solution of 992.8 µg/mL, from which successive dilutions were prepared to obtain concentrations in the range of 1.986 to 14.892 µg/mL (25% to 150%).

Sample solution preparation

Weighed 0.1 g of Marboflo-10% w/v sample into a 100 mL volumetric flask and added10 mL of diluent, mixed well, sonicated in ultrasonic bath until sample was completely dispersed and made the volume up to the mark with diluent. Mixed well. The final solution was diluted again with the diluent to obtain final concentration of 10.0 µg/mL., and filtered through a 0.22 µm membrane filter, filled the autosampler vial and injected into the HPLC system.

Instrumentation and Chromatographic conditions

The HPLC analysis was carried out with a Agilent 1260 Infinite II, equipped with an UV diode array detector (photodiode array). The data was recorded using Agilent ChemStation software for LC system. The chromatographic conditions were optimized, and adequate separations were obtained with a Inertsil ODS-3, C18 150 x 4.6 mm, 5 µm. The system was operated in the gradient mode with flow rate of 1.500 mL/min (mobile Phase-A was 0 -3 min 90%, → 3 – 12min 60%, → 12.5 -15 min 90%). Mobile phase consists Mobile Phase-A (0.1% Formic acid in 1000 ml of deionized water with 0.630 g of Ammonium formate) and Mobile Phase-B (0.1% formic acid in Methanol). The injection volume was set at 10 µL, and UV detection at 327 nm. All analyses were conducted at room temperature (25°C).

Method validation

The proposed method was validated in accordance with International Conference on Harmonization (ICH) and AOAC guidelines on validation of analytical methods. [30, 31] The parameters assessed were Specificity, Precision, Accuracy, linearity, Limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability.

Specificity: The specificity of the proposed method was demonstrated that there were no observed interfering peaks in blank sample at the retention time of analyte (Marbofloxacin). The blank sample which were used for the validation is equivalent to commercial formulation (excipients mixture).

Linearity: The linearity of the method was determined through the calibration curve, constructed using five different concentration levels (25%, 50%, 100%, 125% & 150%) range from 1.986 µg/mL to 14.892 µg/mL. The linearity parameters were calculated using the least squares method.

Precision: The precision of any analytical procedure express the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample analyzed on the same day (intra-day) or on subsequent days (inter-day) under the prescribed conditions.

The Precision of the proposed method was evaluated by analyzing replicates at concentration level of 10.0 µg/mL sample solution, and the result were expressed as relative standard deviation (RSD) between the values found.

Accuracy: The accuracy of the proposed method was determined by spiking the standard solution in the blank sample (placebo) to obtain the final concentrations of 4.964, 9.928, and 12.410 µg/mL. The percent mean recovery of the sample solution demonstrates the accuracy of the proposed method.

Detection and Quantitation Limits: The detection limit (LOD) and quantitation limit (LOQ) are defined as the lowest concentration that can be detected and quantified with acceptable accuracy and precision, respectively. The proposed LOD and LOQ concentrations were verified by proposed method.

Robustness: The evaluation of robustness of an analytical procedure has been defined by ICH as “a measure of its capacity to remain unaffected by small but deliberate variations in method parameters”. The robustness of the method was determined by intentionally altered the method experimental procedures and chromatographic characters are evaluated. Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1 ml/min), wavelength of detection (±2 nm) and acetonitrile content in mobile phase (±2%) were studied to determine the robustness of the method.

RESULTS AND DISCUSSION

A simple HPLC assay method was developed and applied successfully in quantification of Marbofloxacin in veterinary formulation injections. The method was fully validated, and the validation parameters were presented in the following section.

The specificity of the method results was indicating that the Retention time, theoretical plates, and peak asymmetry of the chromatographic peak obtained during this study found to be satisfactory. No interfering peaks in blank sample at the retention time of analytic (Marbofloxacin) were observed. The representative chromatograms shown in **Figure-2 and Figure-3.**

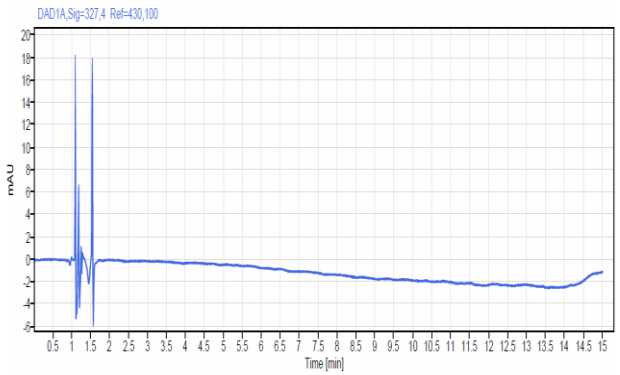

Figure 2: Chromatogram for the blank sample

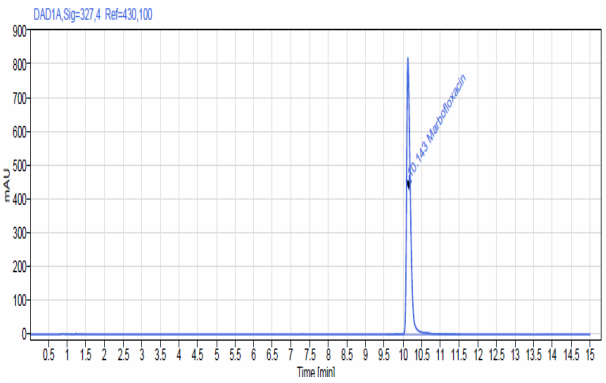

Figure 3: Chromatogram for the standard sample

The standard calibration curve showed good linearity in the range of 1.989 – 14.892 µg/ml, for Marbofloxacin with the correlation coefficient (r2) of 0.9993. A typical calibration curve has the regression equation of y = 42.221x + 40.923 for Marbofloxacin. The results were given **Figure 4 and Table 1.**

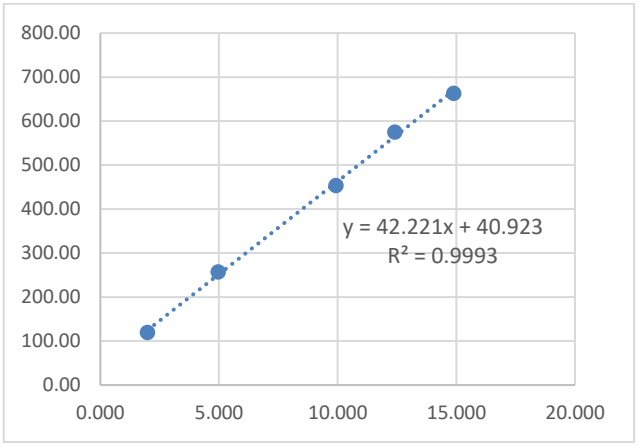

Figure 4: Calibration curve standard for Marbofloxacin

Table 1: Results of the linearity and range

S.No	Conc.	Area
1	1.989	119.84
2	4.964	257.55
3	9.928	453.9
4	12.41	575.42
5	14.892	663.35

Accuracy: recovery study**:** The recovery of the method was determined by additional spiking drug standard solution at three concentration levels with a previously analyzed test solution. The recovery results were found 101.5 – 101.7%. The overall mean recovery (%), and RSD (%) were given in Table 2. Based on the data given in the below table was indicating method is accurate.

Precision: Intra-assay & inter-assay: The intra & inter day variation of the method was carried out by analyzing six replicated of a 10.0 µg/mL sample solution and the results were presented as relative standard deviation (RSD) between the values found. The mean assay % RSD was found to be < 2% within a day and day to day variations for Marbofloxacin. Hence the data indicating that the proposed method was precise. The results were given **Table 3.**

Table 2: Results obtained in the recovery test

Final theoretical concentration (µg/mL)	Final experimental concentration (µg/mL)	Recovery Results (%)
4.964	5.038	101.5
9.928	10.097	101.7
12.41	12.547	101.1
Overall mean % recovery at three different concentration level		101.4
S.D		0.306
%RSD		0.301

Table 3: Results obtained in intra- and inter-day precision analysis

Theoretical concentration (µg/mL)	Experimental concentration ± RSD (%)			
	Repeatability			Intermediate precision
	Day1	Day2	Day 3	
9.928	102.77±1.785	104.266 ± 1.74	99.74±1.92	101.20 ±0.341
The above data for average of six determinations				

Robustness: The results obtained for the evaluation of robustness showed that a small change in the proportion of the mobile phase, its flow rate, and wavelength has no significant impact on the Marbofloxacin chromatograms. However, observed an insignificant variation in retention time and peak symmetry when used same equivalent HPLC column with different make. So such small deliberate changes in the mobile phase, column type, and wavelength do not have negative impact on the quantitative determination of drug in veterinary injectable samples. The results were given **Table 4.**

Table 4: Results obtained in the evaluation of robustness test

Change in parameter	% RSD (n = 6)
Flow (1.4 ml/min)	0.19
Flow (1.6 ml/min)	0.03
Wavelength of Detection (329 nm)	0.07
Wavelength of detection (225 nm)	0.51
0.1 formic acid in methanol: Phosphate buffer (10.2 : 89.8)	0.11
0.1 formic acid in methanol: Phosphate buffer (9.8 : 90.2)	0.12

CONCLUSIONS

A new RP-HPLC method for the quantification of Marbofloxacin was successfully developed, validated and applied for quantification of Marbofloxacin in veterinary formulation injections.

Based on the obtained data the method was found to be simple, reliable, linear, accurate, sensitive and reproducible as well as cost effective for the quantitative analysis of Marbofloxacin in bulk and in Veterinary formulation injections. All the validation parameters met the requirements of AOAC and ICH guidelines.

Therefore, the validity of proposed method supports its use for routine quality control analysis of Marbofloxacin in Veterinary formulation injections.

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