

## Development and Validation of Method for Bio-Quantification of Marbofloxacin in Sheep Plasma by Liquid Chromatography Tandem Mass Spectrometry

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Received date: July 17, 2018; Accepted date: August 22, 2018; Published date: August 27, 2018

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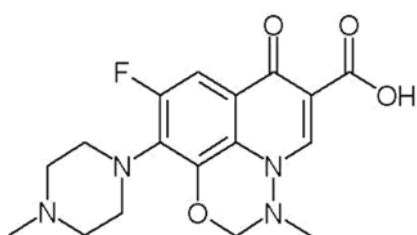
### Abstract

A simple, time saving, and sensitive liquid chromatography tandem mass spectrometric method has been developed and validated for determination of marbofloxacin from sheep plasma. Marbofloxacin was extracted from sheep plasma by liquid-liquid extraction. Detection was performed as transition of marbofloxacin into two product ions i.e., 363.00→320.00 and 363.00→245.00 using mass spectrometry. Chromatographic separation was performed using reverse phase C18 column at flow rate of 0.2 mL/min. The assay of marbofloxacin was linear over the range of 0.001 µg/mL to 10 µg/mL with regression co-efficient ( $r^2$ )>0.9994. The observed value of intra-day and inter-day precision of assay was found between 5.196 to 10.686% and 2.343 to 13.768%, respectively. The mean percent recovery of marbofloxacin from sheep plasma varied from 74.91 to 78.90%. The assay was used successfully for pharmacokinetics and bioavailability studies of marbofloxacin in sheep.

**Keywords:** Marbofloxacin; Sheep plasma; LC MS/MS; Validation

### Introduction

Marbofloxacin is a third-generation veterinary exclusive fluoroquinolone having broad spectrum antimicrobial activities against gram +ve and gram -ve bacteria. It has an empirical formula of  $C_{17}H_{19}FN_4O_4$  with a molecular weight of 362.36 g/mol. Chemically marbofloxacin is 9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-piperazinyl)-7-oxo-7H-pyridol(3,2,1-ij) (4,2,1) benzoxadiazin-6 carboxylic acid (Figure 1).



**Figure 1:** Chemical structure of marbofloxacin: 9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-piperazinyl)-7-oxo-7H-pyridol(3,2,1-ij)(4,2,1) benzoxadiazin-6 carboxylic acid.

It is a faint to light yellow crystalline powder, practically slightly soluble in water, sparingly soluble in methylene chloride, very slightly soluble in ethanol and least soluble in alkaline solution. It is having melting point of 268-269°C, and -20°C is an ideal temperature for storage under inert atmosphere [1]. Its density is 1.55 g/cm<sup>3</sup> [2].

Marbofloxacin like other fluoroquinolones inhibits the replication of bacterial DNA gyrase during bacterial reproduction. Marbofloxacin is a fluorinated quinolone introduced for exclusive use in veterinary medicine [3,4]. It has found a great application in treating many bacterial infections in veterinary medicine because of its lower MIC value, higher volume of distribution and expanded spectrum of antimicrobial activity. The drug having bright prospectus needs to be evaluated for pharmacokinetics profile in target species. This in turn needs sensitive and specific analytical assay for quantification of drug from plasma of target species. Many authors have reported HPLC method for quantification of fluoroquinolones including marbofloxacin from meat, milk, serum or plasma of veterinary species [5-7]. HPLC method has its own limitations due to co-elution of many metabolites and xenobiotics at a time due the junk or crude matrix like plasma of animal, particularly at very low-level present in plasma matrix and also due to detector being employed for the purpose is non-specific such as UV spectrophotometric in chromatography. Adoption of more specific techniques with molecular conformation methods becomes more appropriate for quantification at very low concentration from crude matrix. But there was no report on LC MS/MS method for quantification of marbofloxacin in plasma or serum of any veterinary species having good sensitivity. Hence, the present study was undertaken to establish and validate sensitive, rapid and specific LC MS/MS method for quantification of marbofloxacin from sheep plasma. Also, the investigation was extended to implement the new method for pharmacokinetic studies in target species.

### Materials and Methods

Certified reference materials of marbofloxacin were procured from Nexia Enterprise, Mumbai, India. The marbofloxacin purity assay (%w/w on dried substance) was between 99.0 to 101.0%. The

marbofloxacin reference material procured passed all quality control tests as per standards described in European Pharmacopoeia 7.0. The drugs were stored in air tight glass bottle with stoppers at 4°C temperature. Water, acetonitrile and methanol of LC/MS grade were procured from J.T. Baker, Boston (USA). Formic acid (HPLC grade) was procured from S.D. Fine Chemical Limited, Mumbai. LC MS/MS apparatus used for marbofloxacin analysis comprised of dual pumps (Pro-Star 410 series), 212 LC solvent delivery system with auto sampler (Prostar-410). The ESI (Electron Spray Ionization) source was used for interphase with liquid chromatography and tandem mass spectrometric detection using Varian 310-MS TQ Mass spectrometer. The data were acquired and processed by 'MS Work Station' version 6.9.2 software from Varian Inc.

### Chromatographic conditions

Liquid chromatographic separation was performed on reverse phase high-performance chromatography system with isocratic elution mode using mobile phase of 10 mM formic acid in water: acetonitrile (90:10) on C18 Column (Polaris, 5  $\mu$ , 50  $\times$  2.0 mm, Varian) at 40°C with flow rate of 0.2 mL/min. The mobile phase was sonicated for degassing and filtered through 0.22  $\mu$ m membrane filter before use.

### MS-MS detection

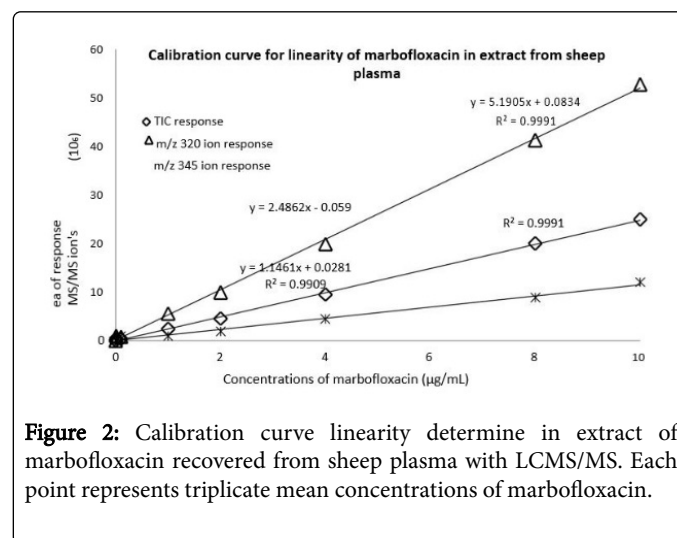
The LC MS/MS parameters were optimized by direct infusion of marbofloxacin at a concentration of 10  $\mu$ g/mL and flow rate of 0.02 mL/min while adjusting MS parameters for ionization to achieve maximum response intensity of precursor ion i.e., 363 in first mass analyzer (Q1). For further identification of product ions and their respective collision energy, MRM (Multiple Reaction Monitoring) were performed with scanning variable collision energy (from 5-80 eV) of selected precursor ions in collision cells (Q2) of triple quadrupole and analyzed with second mass analyzer (Q3). MRM in positive ionization mode were performed using dwell time of 0.5 second. The ions in MRM mode were produced by Collision Activated Dissociation (CAD) of selected precursor ions in collision cell of triple quadrupole and analyzed with the second mass analyzer of the system. MS/MS fragmentation of the precursor ion m/z 363 of marbofloxacin had typical mass spectra with two product ions i.e., Q2 Collision Energy of 15.0 eV for 320 and 30 eV for 345 product ions. The detector voltage was set at 1600 V for maximum gain.

The optimized MS/MS parameters were as follow: Needle voltage: 5000 V, ESI Mode: Positive (MRM), Capillary voltage: 80 eV, Q1: -0.7 eV, Q2: -0.8 eV and Q3: -1.5 eV, Collision Energy (CE): 15.0 eV for 320 and 30.0 eV for 345, Nebulizing gas pressure: 50.0 psi, Drying gas temperature: 350°C and pressure: 20.0 psi. The peak areas were integrated using MS work station version 6.9.2 software.

### Preparation of calibration curve of marbofloxacin in plasma

For standardization and calibration, initially stock solution of marbofloxacin was prepared by dissolving 100 mg pure powder of marbofloxacin in small quantity of acetonitrile and final make was done up to 100 mL. For linearity, calibration stock solution was diluted using water: acetonitrile (90:10) to prepare different level of spiking standard solution in the range of 0.05 to 500  $\mu$ g/mL. Exactly 20  $\mu$ L volume of spiking standards were added to 980  $\mu$ L of blank sheep plasma to make final plasma concentration of marbofloxacin as 0.001, 0.01, 0.1, 1, 2, 4, 8 and 10  $\mu$ g/mL before extraction. Further spiked plasma samples processed, and recovered extracts were analyzed for

MS/MS response linearity. The linearity response and calibration curve thus constructed is depicted in Figure 2.



**Figure 2:** Calibration curve linearity determine in extract of marbofloxacin recovered from sheep plasma with LCMS/MS. Each point represents triplicate mean concentrations of marbofloxacin.

### Extraction from samples

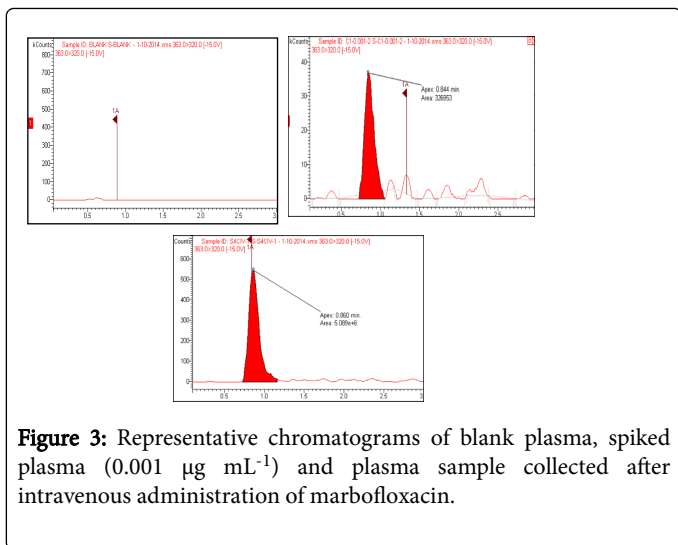
Accurately 0.2 mL aliquots of plasma samples were added to 1.8 mL of acetonitrile with 1% acetic acid in 5.0 ml capacity Eppendorf tube. This was followed by mixing and vortexing for 30 seconds. Mixture was dehydrated by adding dried 200 mg of anhydrous sodium sulphate and again mixed by vortexing, followed by centrifugation at 10000 RPM at 4°C. The supernatant was transferred to another Eppendorf tube of 1.5 mL capacity, further centrifuged at 10000 RPM at 4°C to settle down any particular matter. Finally, clean 1 mL supernatant was transferred to LC vial for injection of 5  $\mu$ L to LC MS/MS analysis.

### Method validation

Blank sheep plasma was used for method development. The blank plasma was processed with and without analyte (marbofloxacin) for development and validation of methods as described under.

### Matrix factor

The blank plasma was processed without analyte (marbofloxacin) to evaluate the presence of any interference at retention time as well as MS detection and quantification of analyte. The plasma matrix effects were evaluated at lower as well as higher concentrations to avoid any error in precision, selectivity and sensitivity. The representative chromatograms of blank sheep plasma calibration standards (0.001  $\mu$ g/mL) and unknown plasma sample are depicted in Figure 3.



**Figure 3:** Representative chromatograms of blank plasma, spiked plasma ( $0.001 \mu\text{g mL}^{-1}$ ) and plasma sample collected after intravenous administration of marbofloxacin.

### Calibration curve and linearity from sheep plasma extract

Eight-point calibration curves (five different runs) were produced (in triplicates) using MS/MS response peak area against concentration of marbofloxacin. A linear regression was calculated using least square method (Table 1).

Numbers of Runs	Slope	Intercept	$r^2$
1	5.46237	0.08904	0.99958
2	5.76462	0.0459	0.99856
3	4.90429	0.2909	0.99985
4	5.32706	0.55093	0.99973
5	5.73298	-0.0304	0.99953
Mean	5.43826	0.18926	0.99945
SD	0.35051	0.23452	0.00051
%RSD	6.44534	123.911	0.0512

**Table 1:** Statistical values of linearity of calibration curve of marbofloxacin validated from sheep plasma. Where,  $r^2$  stands for regression co-efficient, SD for standard deviation of mean, %RSD for relative standard deviation. The data represent for five different run of calibration curve at concentration ranged from 0.001-10.0  $\mu\text{g/mL}$  extracted from sheep plasma.

### Intraday and interday accuracy and precision

Intra-day precision was evaluated by comparing the results of five replicates prepared on same day at three different concentrations (0.1, 1.0 and 10.0  $\mu\text{g/mL}$ ). The Interdays precision was estimated by comparing results of five replicates prepared over period of five days. The %RSD (Relative standard deviation) was calculated (Table 2).

Precision Repeatability	Spiked Concentration ( $\mu\text{g/mL}$ )	Obtained concentration ( $\mu\text{g/mL}$ )	%RSD	% Bias

Intraday	0.1	$0.119 \pm 0.013$	10.686	19.064
	1.0	$0.951 \pm 0.064$	6.702	-4.868
	10	$9.748 \pm 0.504$	5.196	-2.516
Interday	0.1	$0.11 \pm 0.01$	13.768	0.089
	1.0	$0.96 \pm 0.03$	3.192	-0.043
	10	$10.10 \pm 0.24$	2.343	0.010

**Table 2:** Intraday and Interday variability of the Marbofloxacin LC MS/MS assay at three levels of concentrations.

### Recovery percent

Recovery was carried out to quantify the loss of analyte drug during process of extraction. The recovery was calculated by comparing the results for extracted standard samples of plasma with standard prepared in extracted blank matrix at the same concentration representing 100% recovery. Three concentrations were employed viz., 0.1, 1.0 and 10  $\mu\text{g/mL}$  for recovery analysis (Table 3).

Spiked Concentration ( $\mu\text{g/mL}$ )	Recovered concentration ( $\mu\text{g/mL}$ )	% Recovery	%RSD
0.100	$0.075 \pm 0.005$	74.906	6.423
1.000	$0.752 \pm 0.046$	75.198	6.086
10.000	$7.890 \pm 0.667$	78.903	8.453

**Table 3:** Recovery percent of Marbofloxacin from plasma of sheep through validated method. % RSD=percent relative standard deviation.

### Results and Discussion

Statistics values of linearity of calibration curve of marbofloxacin validated from sheep plasma were presented (Table 1) where, the five replicates of calibration curve were prepared from extracts with a range of concentration from 0.001 to 10  $\mu\text{g/mL}$  standard concentrations in sheep plasma. The mean values of slope, intercept and regression co-efficient ( $r^2$ ) are given in Table 1. The relative standard deviation of slope of calibration curve was calculated to be 6.4453%. The calibration curve has average values of regression co-efficient  $r^2 > 0.9994$ . The evaluation of matrix factors revealed no interference with retention period of analyte. So, the present assay is highly specific and selective. The method was found to be highly accurate and precise. The intraday and interday precision (%RSD) was in the range of 5.196 to 10.686 and 2.343 to 13.768%, respectively at three different concentration i.e., 0.1, 1 and 10  $\mu\text{g/mL}$  (Table 2). The average recovery for marbofloxacin at 0.1, 1.0 and 10  $\mu\text{g/mL}$  in plasma samples was found to be 74.906, 75.198 and 78.903%, respectively (Table 3). The LOQ (Limit of Quantification) and LOD (Limit of Detection) of method developed were observed to be 0.333  $\text{ng/mL}$  and 1.000  $\text{ng/mL}$ , respectively.

The method development needs intensive optimization at every step involving selection of detector, mobile phase, column and other chromatographic condition. The optimization and standardization of detector parameters are one of the most important factors in development of novel and rapid method for quantification of any compound. Thus, compound under investigation was required to be

tuned to achieve maximum intensity for identification of parent mass and daughter mass.

While ionization of marbofloxacin having mass of 363 in MS with ESI source, the product ions of 320 and 345 were observed. Based on literature review and previous experience of HPLC of other fluoroquinolones, the C18 column was adopted as a stationary phase for separation. The selection and subsequent optimization of mobile phase was also undertaken. The mobile phase with different solvent including methanol could not give optimum height of peak. The shifting of methanol with acetonitrile was found to be optimum. The different proportions of mobile phase were also tried with variable results. After trying different composition, the acetonitrile acidified with using formic acid produced desired intensity and results. The small concentration of formic acid (10 mM) in aqueous phase was tried and found to give best peak shape and maximum intensity.

The formic acid acted as a mobile phase modifier as well as source of  $H^+$  ions for ionization. Thus, mobile phase was optimized and selected, but the experiment with column of conventional length (250 mm) resulted in distortion of peak shape and longer retention time of marbofloxacin. This seemed to be time consuming and costlier approach. So, column having shorter length of 50 mm was tried and this resulted in short retention time as well as improvement in symmetry of peak shape to satisfactory level. The variable injection volume ranging from 2 to 50  $\mu$ L were injected into the LC MS/MS system. There were no observable effects of increasing injection volume beyond 5  $\mu$ L on peak heights and intensity counts. So finally, 5  $\mu$ L was selected as injection volume.

For extraction, protein precipitation was optimized using different proportion of acetonitrile and sample volume. The trials with dichloromethane, ethyl acetate and ether were resulted without improvement in recovery. However, acidification of acetonitrile with 1% acetic acid gave best recovery of marbofloxacin from sheep plasma. The addition of drying agent magnesium sulphate resulted in poor recovery while replaced with anhydrous sodium sulphate improved recovery to the extent of 20 to 25%. The solid phase extraction was tried out, but it didn't give any significant improvement considering the cost and time involved, therefore it was decided not to use solid phase extraction. The final liquid-liquid extraction protocol involving use of acidified acetonitrile with 1% acetic acid and drying agent sodium sulphate as described here was adopted for extraction of analyte.

Generally, in veterinary drug kinetic research, the basic approaches to quantify the concentrations of antimicrobials include microbial assay, spectrophotometry, liquid chromatography and mass spectrometry. The use of HPLC is a usual approach for quantification of antimicrobial drugs. Since most fluoroquinolones have fluorescence, liquid chromatography with fluorescent detector is mainly used as method of choice for quantification. The fluorescence of any compound depends on pH of medium. The highest fluorescence is obtained at pH value from 2.5 to 4.5 whereas anionic species generally don't show fluorescence. The marbofloxacin has poor native fluorescence and therefore UV detection is preferred by most workers. Alternatively, a newer approach of LC MS/MS has been attempted in present study. The mass spectrometry is considered as a more sensitive and specific method for quantification of antimicrobial drugs in biological matrix with high accuracy and precision. LC MS/MS instrumentation has provided the capability to monitor more drugs and metabolites with conformation, at very low concentrations, in a shorter span of time and with reduced sample preparation [8,9]. This

superiority of LC MS/MS assay over traditionally used HPLC assay is gaining wide acceptance and approval from regulatory authorities too. The LC MS/MS method for quantification of marbofloxacin from plasma of sheep or any other veterinary species has not been reported. But LC MS/MS assay for quantification of marbofloxacin residues in food product of animal origins have been developed and published [9]. LC MS/MS method for multi-residue analysis of quinolones along with other antimicrobials from cow milk was developed and validated [10], where the values of LOD and LOQ were found to be 0.375  $\mu$ g/kg and 0.75  $\mu$ g/kg, respectively and value of regression co-efficient was calculated to be 0.990. Comparable values of LOD (0.3 ng/mL), LOQ (1.0 ng/mL) and regression co-efficient ( $r^2 > 0.9994$ ) were observed in present LC MS/MS assay. The Interday and intraday precision were reported in the range of 6-9% and 7-10%, respectively with recovery of 88.00% [10]. In present assay, the respective values of intraday precision and interday precision were observed to be 5.196 to 10.686 and 2.343 to 13.768 percent. These values are in close agreement with reported values. The mean recovery of marbofloxacin from sheep plasma was found to be 74 to 78 percent (Table 3). The lower values of recovery in present study may be due to difference in extraction procedure and species specific biomatrix. In another HPLC and LC MS/MS method developed and validated for simultaneous determination of fluoroquinolones in food of animal origins (bovine, porcine, poultry and fishes), the recoveries of marbofloxacin from bovine and porcine tissues were in the range of 70.6 to 96.4% and 87.1 to 103.3%, respectively. LOD and LOQ values observed to be as 0.003 and 0.001  $\mu$ g/kg, respectively [9]. The validation parameters of present report are in close agreement with present study. A validated liquid chromatography-tandem mass spectrometric method for the quantification of eight quinolones in bovine muscle, milk and aquaculture products were also reported [11]. The values of LOD and LOQ were found to be in close agreement with values obtained in present study. The average %RSD for milk, meat and aquaculture products was reported to be in the range of 13 to 15%. The similar range was also observed in the present assay of marbofloxacin.

The present analytical method was successfully applied for pharmacokinetic study of marbofloxacin in sheep plasma following single dose intravenous administration @2 mg/kg drug to sheep. The plasma concentrations (Mean  $\pm$  SE) of marbofloxacin in relation to time in healthy sheep were assayed adopting this method. The marbofloxacin concentration in sheep plasma samples were estimated to be  $7.31 \pm 0.64$  g/mL,  $1.37 \pm 0.30$  g/mL and  $0.04 \pm 0.01$  g/mL at 2 minutes, 1.0 hour 24 hours, respectively, thus validated method doing well for said purpose. This method also can be endowed with potential for application pharmacokinetics of marbofloxacin in other species of domestic animals.

## Conclusion

In conclusion, the developed method was easy, simple, rapid, cost effective and having great practical utility. This LC MS/MS assay was highly precise and accurate with acceptable range of recovery. The same can be applied for pharmacokinetic and bioavailability study also. Additionally, the method developed was advantageous over HPLC with respect to its simplicity and accuracy. Thus, present study fulfills the objective of development and validation of LC MS/MS method for bio-quantification of marbofloxacin in sheep plasma.



## Acknowledgement

Authors sincerely express thanks to Director of Research, SD Agricultural University, Sardarkrushinagar and Research Scientist, Spice Research Center, Jagudan, Gujarat, India for providing facilities for research work.

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