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Original Article

Simultaneous Estimation of Moxifloxacin and Tolfenamic Acid: Development and Validation of UV-visible, HPLC, and HPTLC Methods

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ABSTRACT

Objectives: This research emphasizes validating the HPLC, HPTLC, and UV-visible techniques for the simultaneous quantitative measurement of Moxifloxacin (MOX) and Tolfenamic Acid (TA) in veterinary formulations. **Materials and Methods:** The UV spectrophotometric analysis was performed at 291 nm for moxifloxacin and 272 nm for tolfenamic acid. For HPLC, chromatographic separation was achieved using a gradient technique on a reverse-phase C18 ODS column (250×4.6 mm, 5 µm particle size) with a mobile phase comprising 2% orthophosphoric acid and an acetonitrile with ratio of 80:20, with a 1 mL/min flow rate. Detection was accomplished at 254 nm. For HPTLC, separation was performed on pre-coated silica gel 60 F254 plates using a mobile phase of acetonitrile: water: formic acid: methanol:30% ammonia (8:1:1:1:2 v/v). Densitometric assessment for separate zones was performed at 230 nm. **Results:** The HPLC analysis of the current method exhibits the linearity range from 2 to 12 µg/mL for moxifloxacin and 1 to 6 µg/mL for tolfenamic acid in HPLC, 8 to 20 µg/spot for moxifloxacin and 4 to 9 µg/spot for tolfenamic acid in HPTLC, and 6 to 42 µg/mL for MOX and 3 to 21 µg/mL for TA in UV spectrophotometry. The methods were found to be accurate; recovery was achieved from 98 to 102%. The Relative Standard Deviation (RSD) for precision was found to be less than 2%. **Conclusion:** The proposed methods were successfully applied to the determination of MOX and TA in both bulk and veterinary tablet formulations. The methods were validated in accordance with ICH Q2-(R1) guidelines.

Keywords: HPLC, HPTLC, Moxifloxacin, Tolfenamic acid, Validation.

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INTRODUCTION

According to EU regulation 2017/625, the analytical techniques are used for official control of veterinary drugs in animals and animal products, primarily involving liquid chromatography, gas chromatography, ultraviolet, fluorescence, or Mass Spectrometry (MS) detection. The use of multiple liquid chromatography techniques for various chemicals has long been standard practice for detecting antibiotics and other veterinary medication residues (Khalifa and Shikoray, 2024).

Moxifloxacin, a fourth-generation fluoroquinolone antibiotic, is used to cure bacterial infections in veterinary medicine. It is particularly effective against gram-positive bacteria and targets topoisomerase II and topoisomerase IV enzymes (Meena *et al.*, 2019). It is a synthetic compound structurally characterized by a 4-oxo-1,4-dihydroquinoline-carboxylic acid moiety (National Center for Biotechnology Information [NCBI], 2025). Tolfenamic acid, a conventional anti-inflammatory agent, inhibits Cyclooxygenases (COX) enzymes, which play an important role in prostaglandin synthesis. In addition to its anti-inflammatory effects, it is an anticancer agent which acts through apoptosis (programmed cell death) (Feldman *et al.*, 2018). It also has potential effects on NF-κB, which is a protein complex that controls DNA transcription. Moreover, tolfenamic acid may lower the expression of Alzheimer's Disease (AD)-related genes and their products, potentially reduce the pathological burden, and improve cognitive function (Kankaanranta, 1993).

This article primarily highlights the method development for the simultaneous estimation of moxifloxacin and tolfenamic acid using UV, HPLC, and HPTLC methods. Few methods have been previously reported for the simultaneous estimation of this combination, including an RP-HPLC stability study

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(Ahmed *et al.*, 2024), a simple RP-HPLC method (Mali *et al.*, 2025), a UV-spectrophotometric method for MOX (Shende *et al.*, 2022; Gul *et al.*, 2015), and a UV method for TA assay (Tarkase *et al.*, 2012). However, no method has been reported for the combination of moxifloxacin and tolfenamic acid using UV, HPLC, and HPTLC techniques. Therefore, the current study focuses on the development and validation of methods for the concurrent estimation of this combination using UV, HPLC, and HPTLC.

MATERIALS AND METHODS

Materials (Chemicals and Reagents)

Moxifloxacin (MOX) was obtained from Cure worth Drugs and Intermediates Pvt. Ltd., and Tolfenamic Acid (TA) was obtained from Mehta Pharmaceuticals Industries, Mumbai. The fixed-dose combination of MOX (1500 mg) and TA (750 mg), marketed as Duromoxi Bolus, was sourced from Macquor Pharma, Panipat. Analytical-grade sodium hydroxide, formic acid, toluene, and ammonia were purchased from Modern Industries, Nashik, India. Acetonitrile and Methanol (HPLC grade) were obtained from Finar Limited, Gujarat, India, and ethyl acetate was purchased from Fine Chemicals, Mumbai, India.

Instrumentation

The UV-visible method was developed using a Shimadzu UV-visible double-beam spectrophotometer (UV-1800), with UV Probe Software for data analysis, and a pair of quartz cells (1 cm). The RP-HPLC method was developed using a Shimadzu Prominence-i LC-203ºC 3D HPLC system with PDA detector having 20 µL injection volume. The C18 ODS column, dimension (250 × 4.6 mm, 5 µm) and Lab Solutions software were used for computational analysis.

The HPTLC instrumentation consisted of a sample applicator with a 100 µL syringe volume. TLC scanning was performed using TLC software, while spot detection was done using a Crystal-3 UV cabinet. A Canon IXUS digital camera was used for capturing images of the TLC plates. For image documentation, the Easy Doc II Mini Photo Documentation System (Model 2019) was employed. The weighing of samples was performed using analytical balance (Shimadzu AY120), and sonication was accomplished using SIDILU Ultrasonic Bath.

Methods

Preparation of standard stock solution

For UV analysis, 10 mg each of MOX and TA standards were weighed correctly and transferred 100 mL volumetric flasks. Then it was dissolved in 0.1 N NaOH. The volumes were then adjusted to the mark with 0.1 N NaOH to get 100 µg/mL of MOX and TA each.

For HPLC and HPTLC, 10 mg of MOX and 5 mg of TA were weighed accurately individually and transferred to separate 10 mL volumetric flasks. 5 mL of solvent was added to each flask to make up the volume, to prepare 1000 ppm solution. It was sonicated to aid dissolution.

Preparation of sample solution

Ten tablets were accurately weighed, and the average weight was calculated. An amount equivalent to 100 mg of MOX and 50 mg of TA was transferred to a 100 mL of volumetric flask. The solution was diluted to 50 mL with a suitable solvent and sonicated for 15 min.

Development of spectrometric and chromatographic methods

For UV method

To perform UV analysis, concentrations were prepared ranging from 2 to 14 µg/mL using 0.1 N NaOH for both MOX and TA. The absorbance of these solutions was measured at 272 nm (iso-absorptive point) and 291 nm (λ_{max} of MOX). Absorptive values were then calculated using the calibration curve for MOX.

The concentrations of the two drugs in the mixture were calculated in 0.1 N NaOH at 272 nm and 291 nm using the equations below.

$$C_x = \frac{Q_M - Q_y}{Q_x - Q_y} \times \frac{A_1}{a_{x_1}} \quad C_y = \frac{Q_M - Q_x}{Q_y - Q_x} \times \frac{A_1}{a_{y_1}} \quad (10)$$

Where,

A1, A2 are absorbance of mixture at 272 nm and 291 nm.

ax1 and ay1 are absorptivity of MOX and TA at 272 nm and ax2 and ay2 at 291 nm respectively.

$$QM = A2/\ A1,\ QX = ax2/\ ax1 \text{ and } QY = ay2/\ ay1$$

For HPLC Method

A C18 ODS column was used as the stationary phase. The mobile phase consisted of Methanol: Acetonitrile (80:20 v/v). The pH of the mobile phase was adjusted to 4.0 using orthophosphoric acid. The flow rate was 1 mL/min. The mobile phase was filtered using Whatman filter paper and degassed prior to use. Wavelength detection was accomplished at 254 nm.

For HPTLC Method

The solutions were spotted using sample applicator syringe on an aluminum percolated silica F254 plate in the form of bands with an aetron 100 µL. The plate spotting was developed in an Aetron glass chamber of 10 × 10 cm previously saturated for 20 min with the mobile phase acetonitrile: water: formic acid: methanol: ammonia. 30% (8:1:1: 1:2) (v/v). The developed spots were scanned at 254 nm at a scanning speed of 10 mm/sec.

RESULTS AND DISCUSSION

Validation of the proposed method

The current method was validated following the (ICH Q2R1) guidelines, (Ahmed *et al.*, 2015, International Conference on harmonization [ICH], 2005) the detailed summary of regression and validation parameters for UV, HPLC and HPTLC for MOX and TA is provided in Table 1.

System suitability parameter

The performance of the proposed method was evaluated by assessing the system's overall suitability. Key parameters were considered such as theoretical plates, tailing factor, and percentage Relative Standard Deviation (%RSD).

Linearity (Preparation of calibration curves)

For an UV-visible spectrophotometry, the linearity was performed using 6-42 µg/mL concentrations for MOX and 3-21 µg/mL for TA. The calibration curve was constructed by plotting absorbance versus concentration as shown in Table 2 and Figure 1.

The linearity for HPLC was performed by using 2-12 µg/mL of MOX and 1-6 µg/mL of TA. These solutions were prepared using standard stock solutions. The calibration curve was constructed by plotting peak areas versus concentrations. The optimized chromatogram is shown in Figure 2.

HPTLC calibration curve was prepared using 8 to 20 (µg/uL) spots for MOX and 4 to 9 (µg/uL) for TA. The plates were dried and ran through the mobile phase chamber (the mobile phase chamber was saturated for 25 min). Finally, the spots were scanned through a UV cabinet, and volumes were noted from TLC software. The calibration curve was plotted by taking conc. vs. volume. Figure 3 shows the optimized TLC plate for MOX and TA and Figure 4 represents the lane profile graph of MOX and TA. The detailed linearity for all method data is given in Table 2.

Method precision

The precision is degree of agreement between a set of measurements taken from several homogenous samples under specified conditions is known as precision. Intraday and interday precision served as evidence of the analytical method's accuracy.

Intra-day precision

Three sets of different concentrations as low, middle, and high were prepared for intraday precision. After an hour, each set of samples was injected into the system on the same day, and the chromatogram was recorded to determine the reaction peak. The percentage RSD was calculated for every concentration.

Inter-day precision

Inter-day precision study, was accomplished using three sets of concentrations (low, middle, and high). Each set was analyzed on different days. The chromatograms were recorded for each injection, and the peak responses for each concentration set were determined on each day. The % RSD (Relative Standard Deviation) was calculated for each concentration level to assess the precision across the different days. Table 3 represents the

Table 1: Summary of regression and validation parameters for UV, HPLC & HPTLC.

Samples	UV				HPLC				HPTLC			
	Range	R^2	LOD	LOQ	Range	R^2	LOD	LOQ	Range	R^2	LOD	LOQ
MOX	6-42 µg/mL	0.999	3.08	6.38	2-12 µg/mL	0.998	0.6	2.1	32-112 µg/µL	0.992	0.2	2.4
TA	3-21 µg/mL	0.999	9.38	19.72	1-6 µg/mL	0.999	0.5	0.7	1.92-6.72 µg/µL	0.993	0.6	0.8

Table 2: Results of linearity data of MOX & TA in UV Spectroscopy.

Conc. of solution (µg/mL)	MOX at 291 nm		MOX at 272 nm		Conc. of solution (µg/mL)	TA at 291 nm		TA at 272 nm	
	Mean Abs±SD (*n*=3)	% RSD	Mean Abs±SD (*n*=3)	% RSD		Mean Abs±SD (*n*=3)	% RSD	Mean Abs ±SD (*n*=3)	% RSD
6	0.560±0.0005	0.103	0.211±0.001	0.473	3	0.164±0.002	1.266734	0.193±0.001	0.897
12	1.023±0.001	0.097	0.418±0.001	0.239	6	0.396±0.003	0.757576	0.293±0.003	1.095
18	1.562±0.001	0.073	0.632±0.002	0.329	9	0.580±0.011	2.054596	0.386±0.003	0.934
24	2.083±0.001	0.048	0.830±0.001	0.183	12	0.796±0.002	0.315893	0.496±0.002	0.533
30	2.574±0.003	0.124	1.098±0.001	0.091	15	0.984±0.002	0.211408	0.595±9.003	0.635
36	3.0323±0.0005	0.019	1.303±0.002	0.153	18	1.196±0.002	0.221217	0.684±0/007	1.096
42	3.575±0.003	0.105	1.514±0.002	0.185	21	1.393±0.004	0.328972	0.784±0.002	0.265

Table 3: **Interday and Intraday precision in UV, HPLC & HPTLC (*n**=6 determinations).**

	Interday precision						Intraday precision					
	MOX			TA			MOX			TA		
	Conc.	Mean conc. ±SD	% RSD	Conc.	Mean conc. ±SD	% RSD	Conc.	Mean conc. ±SD	% RSD	Conc.	Mean conc. ±SD	% RSD
UV [Abs]	6	3.1±0.01	0.48	3	0.1±0.001	0.90	6	0.5±0.002	0.48	3	0.16±0.002	1.53
	24	3±0.48	1.06	12	0.5±0.3	0.51	24	3.1±0.033	1.06	12	0.585±0.05	0.85
	42	3.1±0.01	0.54	21	1±0.005	0.51	42	3.2±0.01	0.54	21	1.0±0.018	1.79
HPLC [Area]	2	35283±4.3	0.01	1	26514±3.05	0.01	2	35573±3.51	0.03	1	26509±2	0.007
	6	92611±2.08	0.02	3	79246±2.51	0.03	6	92606±2.51	0.03	3	79241±2.64	0.003
	12	183942±2.3	0.01	6	155324±5.29	0.05	12	183944±4	0.05	6	155320±2.08	0.001
HPTLC [Vol]	8	94.8±0.03	0.03	4	487.1±5.52	1.13	8	95.6±0.54	0.57	4	487.4±1.26	0.25
	12	107±0.06	0.05	6	559±0.80	0.14	12	108.6±1.16	1.07	6	558±0.56	0.10
	20	132±0.03	0.06	9	684±1.23	0.18	20	132±0.86	0.86	9	683±0.04	0.23

Table 4: **Recovery study (Average of three determinations).**

Method	Conc. of added drug	% Recovery		% RSD	
		MOX	TA	MOX	TA
UV	80%	98.23%	98.6%	0.4	1.1
	100%	98.53%	101.6%	0.2	1.1
	120%	101.3%	100.6%	1.5	0.5
HPLC	80%	100%	100%	0.06	0.2
	100%	99.56%	99.45%	0.05	0.4
	120%	99.99%	98.99%	0.09	0.6
HPTLC	80%	101%	99.99%	0.5	0.5
	100%	100%	101.4%	0.8	0.3
	120%	100.5%	100%	0.6	0.4

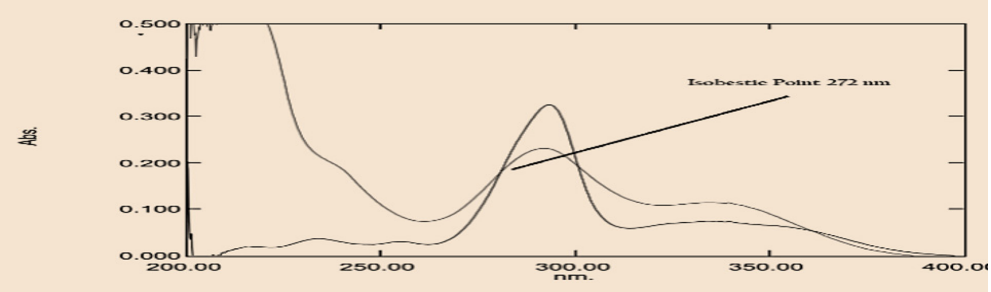

Figure 1: UV scan of MOX and TA showing absorptive points.

interday and intraday precision data of MOX and TA conducted for UV, HPLC and HPTLC.

Repeatability

Repeatability is an exactness of measurements made under the same circumstances in a brief period of time usually over several injections on various days.

To assess repeatability, a standard solution containing a mixture of drugs was injected six times. The % RSD (Relative Standard Deviation) of the resulting peak areas was then calculated to evaluate the consistency of the results.

Accuracy

The accuracy of the method was evaluated by calculating the recovery of MOX and TA using the standard addition method at three different concentration levels (80%, 100%, and 120%) relative to a pre-analyzed marketed sample. For UV-visible spectrophotometry, known amounts of standard solutions were prepared with concentrations of 12 µg/mL for MOX and 6 µg/mL for TA. For RP-HPLC, standard solutions containing 8 µg/mL of

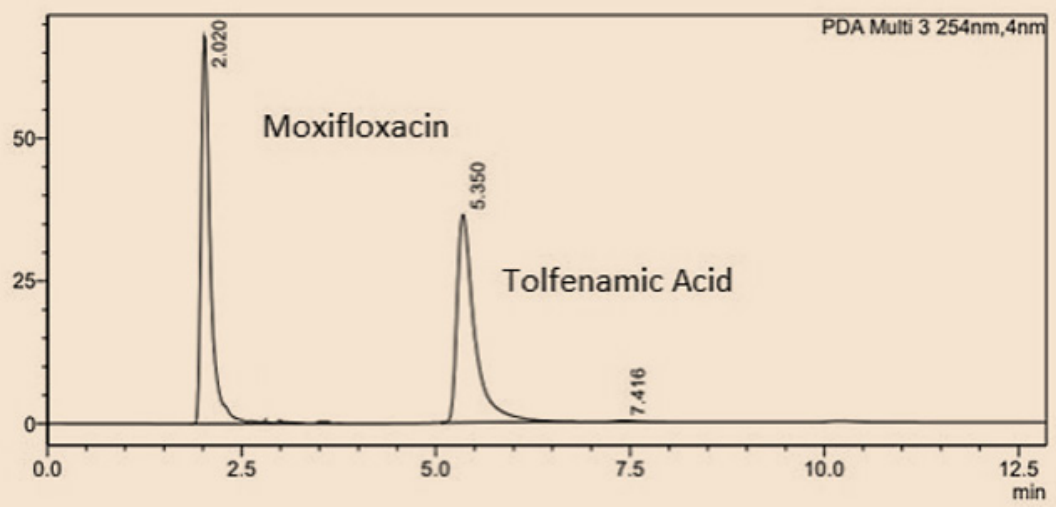

Figure 2: Optimized Chromatogram of MOX TA in HPLC.

Table 5: Robustness results for UV, RP-HPLC and HPTLC Method.

Drug	UV				RP-HPLC				HPTLC			
	Change of instrument (UV 1800)		Change of instrument (UV 1900)		Change Flow rate (0.9 mL/min)		Change in Flow rate (1.1 mL/min)		Change in Mobile Phase		Change in Mobile Phase	
MOX	SD	%RSD	SD	%RSD	SD	%RSD	SD	%RSD	SD	%RSD	SD	%RSD
	0.012	0.44	0.012	0.44	2.081	0.002	2.94	0.003	1.389	1.38	1.32	1.2
TA	0.04	0.86	0.08	1.63	2.516	0.003	3.29	0.004	4.23	0.80	1.20	0.2

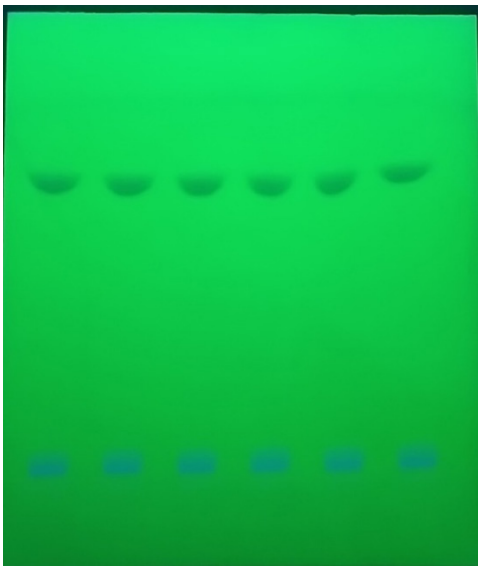

Figure 3: Optimized TLC plate MOX & TA.

MOX and 4 µg/mL of TA were added to the pre-analyzed sample solution. In HPTLC, known quantities of standard solutions were applied to spots on the pre-analyzed sample solution, which contained 20 µg/µL of MOX and TA.

Detection Limit and Quantification Limit

The Detection Limit and the Quantitation Limit of drug were determined by calculating the Signal-to-Noise ratio (S/N), with a value of 3.3 for DL and 10 for QL, as per the guidelines set by the International Conference on Harmonization (ICH). DL and QL were calculated using below formula.

$$\mathbf{LOD} = 3.3 \times \sigma/S,$$

$$\mathbf{LOQ} = 10 \times \sigma/S$$

Where, σ = standard deviation of the response,

S = slope of the calibration curve.

Robustness

The robustness of the methods was evaluated with deliberate variations in the method parameters. The changes in the responses of MOX and TA were observed as follows: For UV-visible spectrophotometry, the instrument was altered; for RP-HPLC, the flow rate was varied by ±1 mL/min; and for HPTLC, the mobile phase composition was modified by ±1 mL. Table 5 shows the robustness results for UV, RP-HPLC and HPTLC study of MOX and TA.

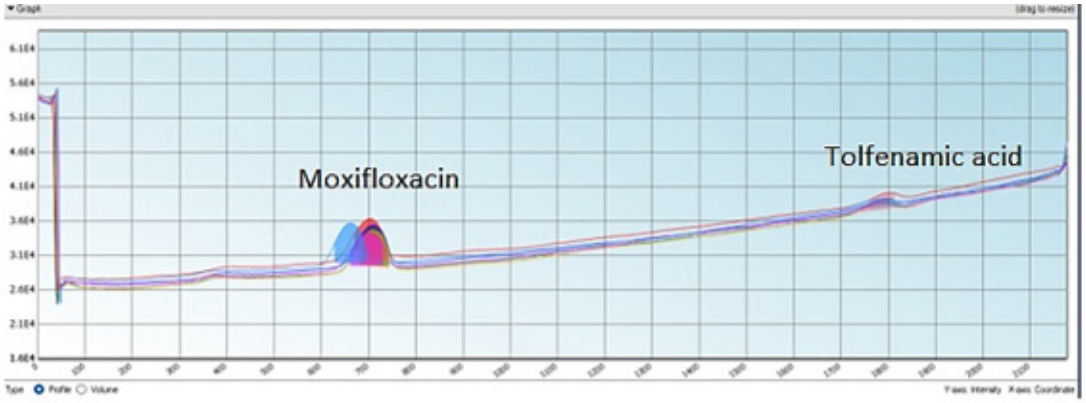

Figure 4: Lane profile graph of MOX & TA.

CONCLUSION

The validated methods demonstrated reliability and reproducibility for the simultaneous estimation of MOX and TA in veterinary formulations, making them suitable for routine quality control analysis.

To estimate moxifloxacin and tolfenamic acid simultaneously in bulk and veterinary-marketed tablet formulations, this study involved the development and validation of UV-Vis, HPLC, and HPTLC methods. In accordance with the ICH Q2(R1) guidelines, the method was validated for accuracy, linearity, repeatability, intraday precision, interday precision, LOD, and LOQ.. The above findings imply that the proposed UV, RP-HPLC, and HPTLC methods can be applied to routine analysis of tolfenamic acid and moxifloxacin tablets.

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ABBREVIATIONS

UV: Ultra-violet spectroscopy; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **HPTLC:** High Performance Thin Layer Chromatography; **MOX:** moxifloxacin; **TA:** Tolfenamic acid; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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