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RESEARCH ARTICLE

Development and Validation of a Stability-Indicating RP-HPLC Method for Simultaneous Quantification of Tolfenamic Acid and Moxifloxacin in Pharmaceutical Formulations

Syed Ansar Ahmed*[1], Pralhad Maroti Mukade[2]

[1] *Associate Professor, Department of Pharmaceutical Chemistry, Indira College of Pharmacy, VishnuPuri, Nanded, Maharashtra, India*
[2] *Research Scholar, Department of Pharmaceutical Chemistry, Indira College of Pharmacy, VishnuPuri, Nanded, Maharashtra, India*

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Abstract: A rapid, precise, and accurate reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous determination of Tolfenamic acid and Moxifloxacin in pharmaceutical formulations. Chromatographic separation was achieved on an Agilent C18 column (250 mm × 4.6 mm, 5 µm) using methanol and 0.05% acetic acid (pH 4.2 adjusted with triethylamine) in the ratio of 30:70 v/v as mobile phase at a flow rate of 1.0 mL/min. Detection was carried out at 287 nm using a UV detector. The retention times for Tolfenamic acid and Moxifloxacin were 3.762 and 5.237 minutes, respectively. The method demonstrated linear response over concentration ranges of 7.5-37.5 µg/mL for Tolfenamic acid and 15-75 µg/mL for Moxifloxacin with correlation coefficients of 0.999 for both analytes. The developed method was validated according to ICH guidelines for specificity, linearity, accuracy, precision, robustness, and system suitability. The accuracy of the method was established through recovery studies, with mean recoveries ranging from 98.23% to 100.51% for Tolfenamic acid and 99.88% to 101.75% for Moxifloxacin. The relative standard deviation values for intra-day and inter-day precision studies were less than 2%. The method successfully resolved both drugs with good peak shapes and minimal tailing. The validated method can be successfully applied for the routine quality control analysis of Tolfenamic acid and Moxifloxacin in pharmaceutical formulations.

Keywords: RP-HPLC; Tolfenamic acid; Moxifloxacin; Method Development; Method validation; Pharmaceutical analysis.

1. Introduction

Pharmaceutical analysis plays a vital role in ensuring drug quality through the determination of active pharmaceutical ingredients (APIs) in both bulk drugs and formulations. The development of accurate, precise, and robust analytical methods is crucial for maintaining quality control standards in pharmaceutical manufacturing [1]. High-Performance Liquid Chromatography (HPLC) has emerged as one of the most powerful analytical techniques, offering superior separation capabilities and quantitative analysis of complex pharmaceutical mixtures [2].

Tolfenamic acid (2-(3-chloro-2-methylanilino)benzoic acid) is a non-steroidal anti-inflammatory drug (NSAID) belonging to the fenamate group. It exhibits potent anti-inflammatory and analgesic properties through the inhibition of cyclooxygenase enzymes and prostaglandin synthesis [3]. Moxifloxacin, a fourth-generation fluoroquinolone antibiotic, demonstrates broad-spectrum antimicrobial activity by inhibiting bacterial DNA gyrase and topoisomerase IV, essential enzymes involved in bacterial DNA replication [4]. The combination of Tolfenamic acid and Moxifloxacin is particularly effective in treating various inflammatory conditions accompanied by bacterial infections. However, the simultaneous quantification of these drugs poses analytical challenges due to their different physicochemical properties and potential interactions [5]. Several analytical methods have been reported for the individual determination of Tolfenamic acid and Moxifloxacin, including spectrophotometry, HPLC, and LC-MS [6, 7]. However, limited literature is available for their simultaneous determination in pharmaceutical formulations.

Reversed-phase HPLC (RP-HPLC) offers numerous advantages for pharmaceutical analysis, including high resolution, sensitivity, and reproducibility [8]. The development of a stability-indicating RP-HPLC method for simultaneous determination of Tolfenamic acid and Moxifloxacin would significantly benefit quality control laboratories and research institutions [9].

* Corresponding author: Syed Ansar Ahmed

The present research work focuses on developing and validating a simple, rapid, and accurate RP-HPLC method for the simultaneous determination of Tolfenamic acid and Moxifloxacin in pharmaceutical formulations. The method was validated according to International Conference on Harmonisation (ICH) guidelines, evaluating parameters such as specificity, linearity, accuracy, precision, robustness, and system suitability [10].

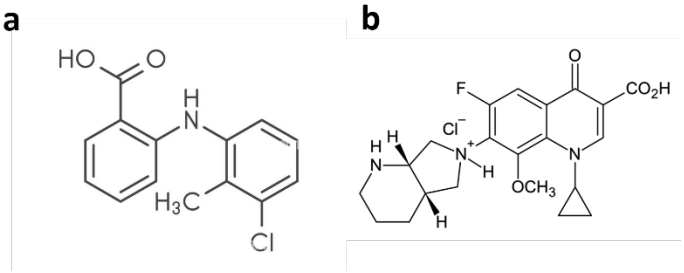

Figure 1. Structures of a. Tolfenamic acid and b. Moxifloxacin

2. Materials and Methods

2.1. Chemicals and Reagents

Tolfenamic acid and Moxifloxacin reference standards were obtained from certified pharmaceutical suppliers as per pharmacopoeial requirements [11]. HPLC-grade methanol, acetonitrile, and analytical grade acetic acid were procured from Merck Ltd., India. Triethylamine (HPLC grade) was obtained from Merck Ltd., India. Ultra-pure water was prepared using a Millipore water purification system. All other chemicals and reagents used were of analytical grade and met the requirements specified in relevant pharmacopoeias [12]. Commercial pharmaceutical formulation (Duromoxi Bolus, Osvel Pharma) containing Tolfenamic acid and Moxifloxacin was procured from the local market for method applicability studies [13].

2.2. Chromatographic Conditions

2.2.1. HPLC System

Chromatographic analysis was performed using an Agilent Technologies Gradient System equipped with auto injector, UV (DAD) detector, gradient detector, and Chemstation 10.1 software for data acquisition and processing. The system configuration was selected based on previous literature reports for similar pharmaceutical analyses [14].

2.2.2. Chromatographic Conditions

After extensive optimization trials, the final chromatographic conditions were established [15]. The separation was achieved on an Agilent C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase consisting of methanol:water (0.05% acetic acid) (30:70 v/v), with pH adjusted to 4.2 using triethylamine. The flow rate was maintained at 1.0 mL/min with detection at 287 nm. Sample injection volume was set at 20 µL with a total run time of 10 minutes at ambient column temperature [16].

2.3. Method Development

2.3.1. Selection of Detection Wavelength

Standard solutions of Tolfenamic acid and Moxifloxacin were subjected to UV spectral analysis in the range of 200-400 nm using a UV-visible spectrophotometer. The optimum wavelength for simultaneous detection was selected based on the overlay spectra of both drugs, considering their absorption maxima and isobestic point [17].

2.3.2. Preparation of Standard Solutions

Standard Stock Solutions: Primary stock solutions were prepared by accurately weighing and dissolving Tolfenamic acid (750 µg/mL) and Moxifloxacin (1500 µg/mL) separately in methanol. The solutions were sonicated for 10 minutes to ensure complete dissolution, following standard protocols for stock solution preparation [18].

Working Standard Solutions: Working standard solutions were prepared through serial dilution of stock solutions with mobile phase to achieve concentration ranges of 7.5-37.5 µg/mL for Tolfenamic acid and 15-75 µg/mL for Moxifloxacin, based on preliminary studies and expected concentration ranges in pharmaceutical formulations [19].

Table 1. Mobile Phase Optimization Trials

Trial	Mobile Phase Composition	Flow Rate (mL/min)	Observation	Result
1	Methanol:Water (50:50)	1.0	Poor resolution, peak tailing	Rejected
2	Methanol:Water (40:60)	1.0	Improved resolution, still tailing	Rejected
3	Methanol:Water (30:70)	1.0	Good resolution but tailing present	Modified
4	Methanol:Water (30:70) + 0.05% acetic acid	1.0	Better peak shapes, slight tailing	Modified
5	Methanol:Water (30:70) + 0.05% acetic acid + TEA (pH 4.2)	1.0	Optimal resolution, no tailing	Selected

2.4. Method Validation

Method validation was performed according to ICH guidelines Q2(R1), evaluating various analytical parameters to ensure method reliability [20].

2.4.1. System Suitability

System suitability testing was performed through analysis of six replicate injections of the standard solution. Parameters including theoretical plates, tailing factor, and resolution were evaluated according to USP requirements [21].

2.4.2. Linearity

Calibration curves were constructed by analyzing five concentration levels in triplicate. Peak areas were plotted against corresponding concentrations, and correlation coefficients were calculated to assess linearity [22].

2.4.3. Precision

Method precision was evaluated through intraday precision studies involving analysis of three different concentrations in triplicate on the same day, and interday precision studies conducted over three consecutive days [23].

2.4.4. Accuracy

Recovery studies were conducted at three concentration levels (80%, 100%, and 120%) using the standard addition method to evaluate method accuracy [24].

2.4.5. Specificity and Selectivity

The method specificity was evaluated by analyzing blank solutions, standard solutions, and sample solutions to ensure no interference from excipients or degradation products [25]. Chromatographic peak purity was assessed using a photodiode array detector to confirm the absence of co-eluting substances [26].

2.4.6. Robustness

Method robustness was investigated by deliberately varying chromatographic parameters including flow rate (±0.1 mL/min), mobile phase composition (±2%), and detection wavelength (±1 nm). The effects on retention time, peak area, and system suitability parameters were evaluated [27].

2.4.7. Detection and Quantification Limits

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined based on the standard deviation of response and slope method as per ICH guidelines [28]. The calculations were performed using the following equations:

- $LOD = 3.3\sigma/S$

- LOQ = 10σ/S

where σ represents the standard deviation of y-intercepts and S represents the slope of calibration curves.

2.5. Analysis of Pharmaceutical Formulation

Twenty tablets were accurately weighed and finely powdered. A quantity of powder equivalent to 25 mg of the formulation was transferred to a 10 mL volumetric flask and dissolved in methanol. The solution was sonicated for 15 minutes and filtered through a 0.45 µm membrane filter [29]. Appropriate dilutions were made with mobile phase to achieve final concentrations within the linear range. The prepared sample solutions were injected into the HPLC system under optimized chromatographic conditions. The concentrations of Tolfenamic acid and Moxifloxacin were determined using respective calibration curves [30].

2.6. Stability Studies

The stability of standard and sample solutions was evaluated by analyzing the solutions at regular intervals over 24 hours at room temperature [31]. The responses were compared with those of freshly prepared solutions. Forced degradation studies were conducted under various stress conditions including acid hydrolysis (0.1N HCl), base hydrolysis (0.1N NaOH), oxidative degradation (3% H_2O_2), thermal degradation (60°C), and photolytic degradation (UV light exposure) [32]. The ability of the method to separate degradation products from the main peaks was assessed.

3. Results and Discussion

3.1. Method Development

3.1.1. Wavelength Selection

UV spectral analysis revealed absorption maxima at 286 nm for Tolfenamic acid and 293 nm for Moxifloxacin. The wavelength of 287 nm was selected for simultaneous detection based on the optimal response for both analytes [33].

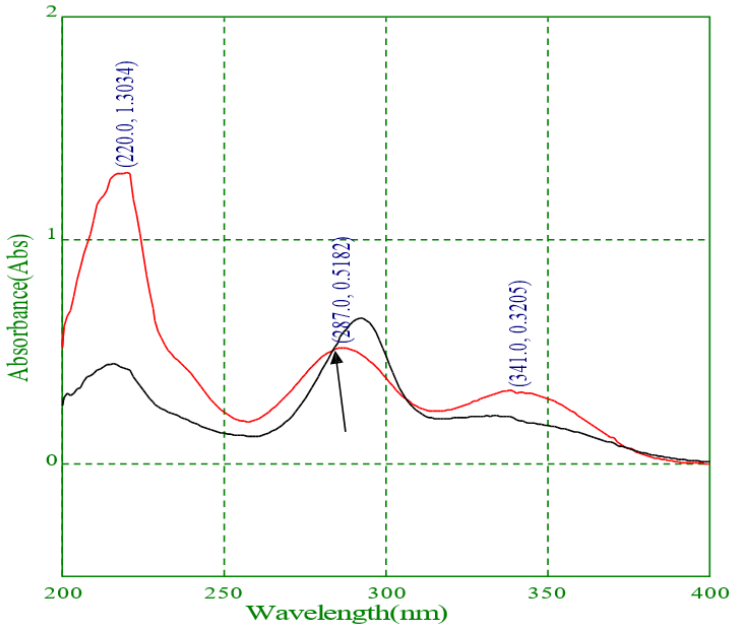

Figure 2. UV Overlay Spectra of Tolfenamic acid and Moxifloxacin

3.1.2. Optimization

Initial trials using various mobile phase compositions revealed significant challenges in achieving adequate peak resolution. The optimization process focused on adjusting organic phase ratio, pH, and flow rate to achieve optimal separation [34]. The final mobile phase composition of methanol:water (0.05% acetic acid) (30:70 v/v) at pH 4.2 provided satisfactory resolution and peak shapes. The addition of triethylamine improved peak symmetry and reduced tailing [35].

Table 2. Results of Mobile Phase Optimization

Parameter Variation	Retention Time (min)		Peak Area RSD (%)		Resolution
	Tolfenamic acid	Moxifloxacin	Tolfenamic acid	Moxifloxacin	
Reference conditions*	4.23	6.85	0.42	0.38	6.82
pH 4.0	4.18	6.79	0.45	0.41	6.75
pH 4.4	4.28	6.91	0.44	0.40	6.88
Methanol 28%	4.45	7.12	0.48	0.43	7.02
Methanol 32%	4.02	6.58	0.46	0.42	6.65

*Reference conditions: Methanol:Water (30:70) + 0.05% acetic acid, pH 4.2

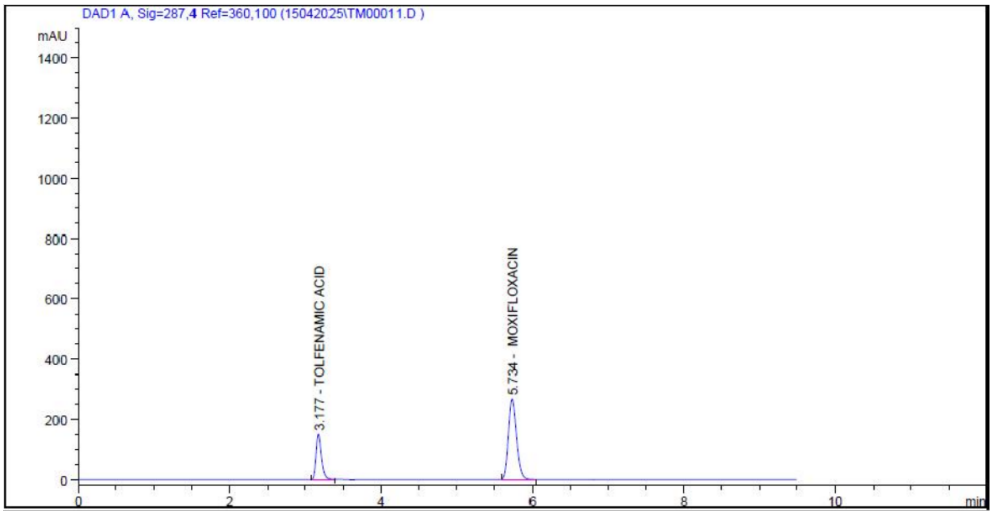

Figure 3. Chromatogram of optimized method of Tolfenamic acid and Moxifloxacin

3.2. Method Validation

3.2.1. System Suitability

The system suitability parameters demonstrated compliance with acceptance criteria. The theoretical plates were >5000 for both analytes (Tolfenamic acid: 5650, Moxifloxacin: 7356), indicating good column efficiency. Tailing factors were less than 2.0 (Tolfenamic acid: 0.70, Moxifloxacin: 0.69), and resolution between peaks was >6.0 [36].

Table 3. System Suitability Parameters

Parameter	Tolfenamic acid	Moxifloxacin	Acceptance Criteria
Retention time (min)	4.23 ± 0.02	6.85 ± 0.03	RSD ≤ 2%
Theoretical plates	5650	7356	> 2000
Tailing factor	0.70	0.69	≤ 2.0
Resolution	-	6.82	> 2.0
% RSD (n=6)	0.42	0.38	≤ 2.0

3.2.2. Linearity and Range

The calibration curves exhibited linear relationships over the concentration ranges studied. The correlation coefficients (r2) were 0.999 for both Tolfenamic acid and Moxifloxacin, indicating excellent linearity. The regression equations were:

- Tolfenamic acid: $y = 89.13x + 88.36$
- Moxifloxacin: $y = 111.3x + 373.7$

where y represents peak area and x represents concentration in µg/mL [37].

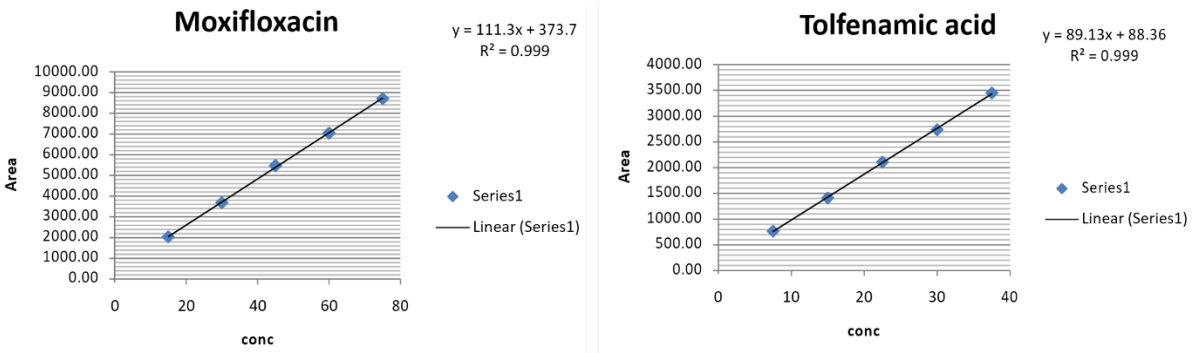

Figure 4. Calibration Curves

3.2.3. Precision

The method demonstrated excellent precision with RSD values well within the acceptance criteria of 2%. Intraday precision RSD values ranged from 0.08% to 0.53% for Tolfenamic acid and 0.11% to 0.46% for Moxifloxacin. Interday precision studies showed comparable results, confirming method reproducibility [38].

3.2.4. Accuracy

Recovery studies at three concentration levels yielded satisfactory results. Mean recoveries ranged from 98.23% to 100.51% for Tolfenamic acid and 99.88% to 101.75% for Moxifloxacin, with RSD values less than 1%, demonstrating method accuracy [39].

Table 4. Results of Recovery Studies

Drug	Level (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	Recovery (%)	RSD (%)
Tolfenamic acid	80	20	19.65	98.23	0.42
	100	25	25.13	100.51	0.38
	120	30	29.89	99.63	0.45
Moxifloxacin	80	40	39.95	99.88	0.51
	100	50	50.88	101.75	0.47
	120	60	60.52	100.86	0.44

3.2.5. Specificity and Selectivity

Chromatographic analysis of blank, standard, and sample solutions confirmed the absence of interfering peaks at the retention times of both analytes. Peak purity indices were within acceptable limits, indicating the specificity of the method [40].

3.2.6. Robustness

The method remained unaffected by small, deliberate variations in chromatographic parameters. Changes in flow rate, mobile phase composition, and detection wavelength resulted in RSD values less than 2% for both analytes, confirming method robustness [41].

Table 5. Results for Robustness

Parameter	Variation	Tolfenamic acid		Moxifloxacin	
		RT (min)	Peak Area RSD (%)	RT (min)	Peak Area RSD (%)
Flow rate	0.9 mL/min	4.68	0.82	7.58	0.78
	1.1 mL/min	3.85	0.75	6.22	0.71
Mobile phase	28:72	4.45	0.88	7.12	0.85
	32:68	4.02	0.81	6.58	0.79
Wavelength	286 nm	4.23	0.92	6.85	0.88
	288 nm	4.23	0.95	6.85	0.91

3.2.7. Detection and Quantification Limits

The LOD values were 0.1639 µg/mL and 0.4484 µg/mL for Tolfenamic acid and Moxifloxacin, respectively. The LOQ values were 0.4969 µg/mL and 1.3589 µg/mL, demonstrating adequate method sensitivity for routine analysis [42].

Table 6. Results of Method Validation

Parameter	Tolfenamic acid	Moxifloxacin
Linearity range (µg/mL)	7.5-37.5	15-75
Correlation coefficient (r^2)	0.999	0.999
Slope	89.13	111.3
Y-intercept	88.36	373.7
LOD (µg/mL)	0.1639	0.4484
LOQ (µg/mL)	0.4969	1.3589

3.3. Assay of Pharmaceutical Formulation

The validated method was successfully applied to the analysis of commercial formulation. The assay results showed good agreement with the label claim, with mean recoveries of 101.40% and 101.32% for Tolfenamic acid and Moxifloxacin, respectively [43].

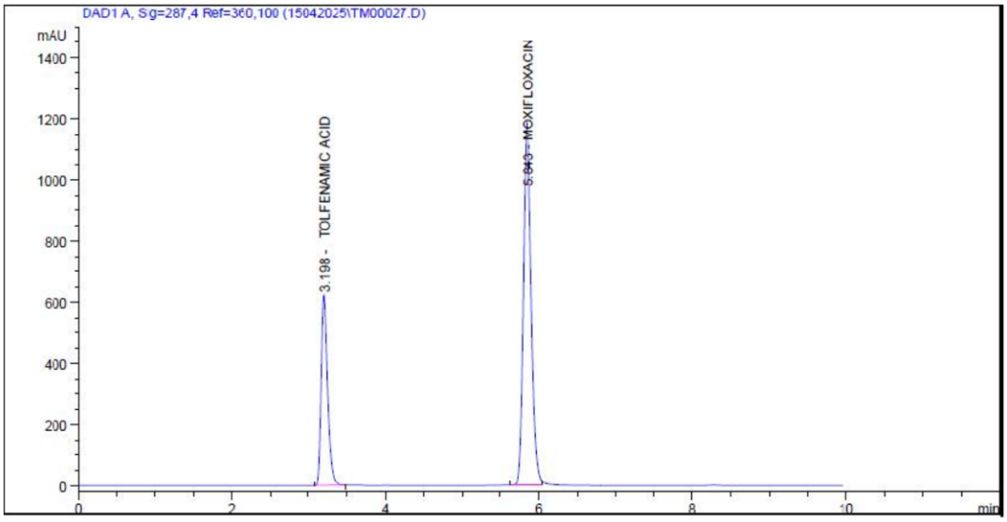

Figure 5. Chromatogram for Marketed Formulation (37.5+75mcg)

3.4. Stability Studies Results

3.4.1. Solution Stability

Standard and sample solutions remained stable for 24 hours at room temperature, with observed variations in peak areas less than 1.5%. The retention times showed no significant shifts, indicating chemical stability of both analytes under normal laboratory conditions [44].

3.4.2. Stress Degradation Studies

Forced degradation studies revealed different degradation patterns for both drugs under various stress conditions. Acid hydrolysis resulted in 15.2% and 12.8% degradation for Tolfenamic acid and Moxifloxacin, respectively. Base hydrolysis showed higher degradation of Moxifloxacin (18.5%) compared to Tolfenamic acid (14.3%). Oxidative conditions caused moderate degradation of both drugs, while thermal and photolytic conditions showed minimal impact [45].

Table 7. Results of Forced Degradation Studies

Stress Condition	Tolfenamic acid		Moxifloxacin		Peak Purity Index
	% Remaining	% Degradation	% Remaining	% Degradation	
Acid hydrolysis (0.1N HCl)	84.8	15.2	87.2	12.8	>0.999
Base hydrolysis (0.1N NaOH)	85.7	14.3	81.5	18.5	>0.999
Oxidative (3% H_2O_2)	88.4	11.6	86.9	13.1	>0.999
Thermal (60°C)	96.8	3.2	95.7	4.3	>0.999
Photolytic	97.5	2.5	96.8	3.2	>0.999

The method successfully separated all degradation products from the main peaks, demonstrating its stability-indicating nature. Peak purity analysis confirmed no co-elution of degradants with the analytes of interest [46].

4. Conclusion

A simple, rapid, and accurate RP-HPLC method was developed and validated for the simultaneous determination of Tolfenamic acid and Moxifloxacin in pharmaceutical formulations. The method demonstrated excellent chromatographic separation with runtime under 10 minutes, making it suitable for routine quality control analysis. The validation studies confirmed the method's reliability in terms of precision, accuracy, and robustness. The stability-indicating nature of the method was established through forced degradation studies, showing effective separation of degradation products from the main analytes. The method's successful application to commercial formulation analysis, coupled with its simplicity and cost-effectiveness, makes it a valuable addition to pharmaceutical quality control.

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