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DEVELOPMENT AND VALIDATION OF A HPLC-UV METHOD FOR SIMULTANEOUS DETERMINATION OF MOXIFLOXACIN HYDROCHLORIDE AND KETOROLAC TROMETHAMINE IN OCULAR FORMULATION

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High performance liquid chromatography, UV Spectrophotometry, Moxifloxacin Hydrochloride & Ketorolac Tromethamine.

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ABSTRACT: A simple, rapid, and sensitive high-performance liquid chromatographic method with UV detection has been developed and validated according to the ICH guidelines for the quantitation of Moxifloxacin Hydrochloride (MOX), Ketorolac Tromethamine (KET) in pharmaceutical dosage form. Chromatographic separation was carried out in a Zorbax eclipse plus, C₁₈ column (250 mm × 4.6 mm; 5 μm particle size) with simple mobile phase composition of 10 mM Potassium dihydrogen phosphate buffer with Triethylamine (pH 3.14) and acetonitrile (40:60, v/v) at a flow rate of 0.5 mL min⁻¹ where detector was set at 302 nm with a total run time of 8 mins. The method was linear over the concentration range of 40-100, μg mL⁻¹ with a correlation coefficient of 0.9891 and 0.994. Limit of quantifications (LOQ) of 13.3, 26.3 and limit of detections (LOD) 4.4, 8.7 μg mL⁻¹ for MOX, and KET respectively. Accuracy and precision values of both within-run and between-run obtained from six different sets of three quality control (QC) samples analyzed in separate occasions for both the analytes ranged from 98.13% to 99.75% and 0.95% to 2.15%, respectively. Extraction recovery of analytes in pharmaceutical formulation from 97.82% to 98.68%. The developed and validated method was successfully applied to quantitative determination of MOX and KET in pharmaceutical formulation.

INTRODUCTION: Moxifloxacin hydrochloride (MOX), 1-Cyclopropyl-6-fluoro-1, 4-dihydro-8-methoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo [3, 4-*b*]pyridin-6-yl]-4-oxo-3 quinolinecarboxylic acid hydrochloride (**Figure 1**), is a synthetic fourth-generation broad-spectrum fluoroquinolone antibiotic. It acts by inhibiting DNA gyrase, a type II topoisomerase and topoisomerase IV, which are involved in DNA replication and metabolism¹.

Ketorolac tromethamine (KET) (±)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid, a compound with 2-amino-2-(hydroxymethyl)-1,3-propanediol, a pyrrolizine carboxylic acid derivative (**Figure 2**), a nonsteroidal anti-inflammatory drug, is indicated for short-term management of moderate to severe pain and shows a high incidence of side effects like gastric bleeding¹. The primary mechanism of action responsible for ketorolac's anti-inflammatory, antipyretic and analgesic effects is the inhibition of prostaglandin synthesis by competitive blocking of the enzyme cyclooxygenase (COX). The combination of Ketorolac Tromethamine with Moxifloxacin Hydrochloride is extensively used for the treatment of postoperative inflammation and infection following cataract surgery¹.

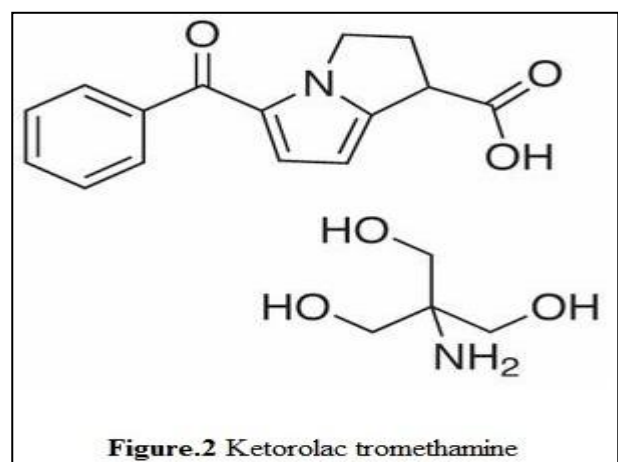
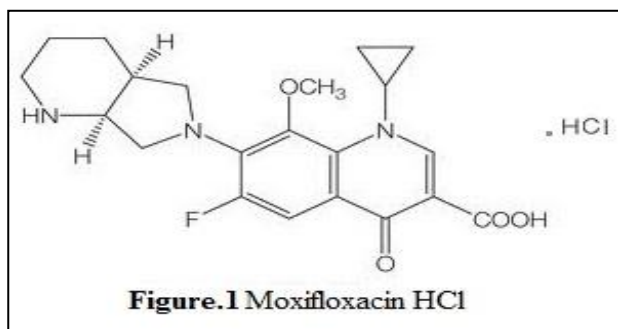
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Various methods like UV spectrophotometry^{2, 3, 4} estimation in biological fluids by HPLC^{5, 6} HPTLC⁸ UV spectrophotometry⁹ methods for determination of MOX with other drug are reported in literature.

Ketorolac Tromethamine was determined using HPTLC^{7, 8} HPLC^{10, 14} methods. However, a Few analytical methods were reported for the simultaneous determination of Moxifloxacin Hydrochloride and Ketorolac Tromethamine in a mixture, namely, rapid liquid chromatography–electrospray ionization mass spectrometry (LC-MS)¹ HPLC using a diode array detector¹³, RP-HPLC¹⁴ and HPTLC¹⁵.

An extensive review of the literature did not revealed any HPLC method for simultaneous determination of both drugs. Therefore, attempts were made to develop and validate simple, precise, and sensitive, isocratic reverse phase stability indicating high performance liquid chromatographic method for simultaneous determination of both drugs in pharmaceutical formulations.

The proposed methods were validated in compliance with the ICH guidelines and were successfully applied for determination of Moxifloxacin Hydrochloride and Ketorolac Tromethamine in their pharmaceutical formulations.



MATERIALS AND METHODS:

Chemicals and reagents:

MOX, KET were procured from pharmaceuticals industry. Acetonitrile HPLC Grade, Phosphoric acid analytical grade from Merck (Mumbai, India), Potassium dihydrogen phosphate analytical grade from Merck (Mumbai, India), Triethylamine analytical grade from Merck (Mumbai, India), HPLC-grade water (resistivity 18.2 MΩ) cm was generated from a Milli-Q water purification system, was used throughout the analysis.

Samples are procured from pharmaceutical industry and they are considered as **Sample I** and **Sample II** respectively and both the samples are ocular formulations.

Instrumentation and chromatographic conditions:

HPLC apparatus consisted of a Agilent Technology (USA) Model, G1311A Quaternary pump, G1365D variable wave length UV detector, Auto-sampler (G1329A), Column oven (G13368) and EZ CHROM ELITE Version 331SOP software. Chromatographic separation was performed isocratically at room temperature using a Agilent Zorbax eclipse Plus, C₁₈ (250 mm x 4.6 mm, 5 μm particle size) Mobile phase consisted of a mixture of Potassium dihydrogen phosphate buffer (2.7363 gm to 1000ml+ 1 ml Triethylamine, pH 3.14 by dil H₃PO₄) and Acetonitrile (40:60, v/v) at a flow rate of 0.5 ml min⁻¹ and sample injection of 20 μL was injected at 25°C. Eluent was monitored with a UV detector set at 302 nm.

Preparation of stock and working solutions:

24.5 gm of MOX and 25.5 gm of KET taken in a 25 ml volumetric flask and dissolving in mobile phase to obtain concentration of 1000 μg/mL. The stock solution stored in amber colored labeled volumetric flask at 8 °C.

Preparation of calibration standards and quality control (QC) samples:

Five calibration standards (CC) of both MOX and KET at concentration of: 20, 40, 60, 80, and 100 μg mL⁻¹ were prepared by spiking 0.2, 0.4, 0.6, 0.8, 1.0 ml respectively to 10 ml by Mobile phase. Three QC sample 40, 60, 80 μg mL⁻¹ were used. All standards stored in amber colored labeled volumetric flask at 8 °C.

Sample preparation:

2ml of sample diluted with mobile phase mixed properly. Samples were further diluted by mobile

phase which have final concentration $20\mu\text{g mL}^{-1}$ of both MOX and KET and then injected into the HPLC system.

Method validation:

The proposed methods were validated in compliance with the ICH guidelines and were successfully applied for determination of Moxifloxacin Hydrochloride and Ketorolac Tromethamine in their pharmaceutical formulations.

This method was validated to meet the acceptance criteria with the ICH guidelines of method validation.¹⁶

Selectivity:

Selectivity of the method was determined by analyzing blank (mobile phase), to demonstrate the lack of chromatographic interference at the retention time of the analytes.

Limit of detection (LOD), Limit of quantitation (LOQ) and Linearity:

Limit of detection (LOD), Limit of quantitation (LOQ) was determined by the following equation $3.3\sigma/S$ and $10\sigma/S$, where as σ = standard deviation of the response and S = slope of the calibration curve. Calibration curves were acquired by plotting the peak-area of the analytes against the nominal concentration of calibration standards. Analyte concentration of different CC and QC samples were prepared as mentioned above.

Accuracy and precision:

Accuracy of an analytical procedure is the closeness of agreement between accepted conventional true values (reference values) and the values found. The accuracy of the proposed methods was tested by the determination of MOX and KET at different concentration levels within the linear range of each compound.

Precision was studied by determination of intra-day and inter-day precision. Intra-day precision was determined by injecting five standard solutions of three different concentrations on the same day and inter-day precision was determined by injecting the same solutions for three consecutive days. Relative standard deviation (RSD %) of the peak area was then calculated to represent precision.

Extraction recovery:

Recoveries of MOX and KET were determined in the addition standard ($40, 60, 80\mu\text{g mL}^{-1}$) by

comparing the experimental and true values ($40, 60, 80\mu\text{g mL}^{-1}$).

RESULTS AND DISCUSSION:

Optimization of chromatography:

In the work chromatographic conditions were optimized to obtain high sensitivity, reproducibility, and sample throughput for the determination of MOX and KET. Both analytes contain basic nitrogen atoms and therefore have the potential to cause the peak tailing due to interaction of this basic nitrogen atoms with the silanol group of the stationary phase during chromatographic separation. In order to obtain symmetrical peaks with better resolution, the chromatographic condition i.e. pH of the buffer, concentration of the organic modifier and silanol blocker were optimized.

Various chromatographic condition such as mobile phase composition, analytical column with different packing materials (C8, C18, Phenyl, Cyano) and configuration (10, 15, 25 cm) were used to obtain sharp peak with reduce tailing, and better resolution with no peak impurity. Finally Agilent Zorbax Eclipse Plus C18 column was selected which provided reduced peak tailing and acceptable peak purity index.

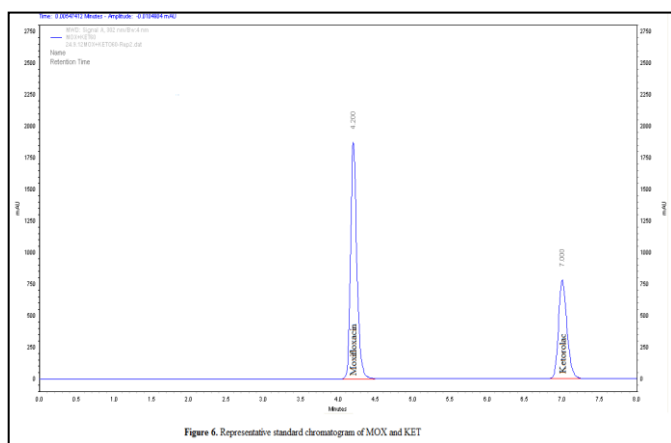
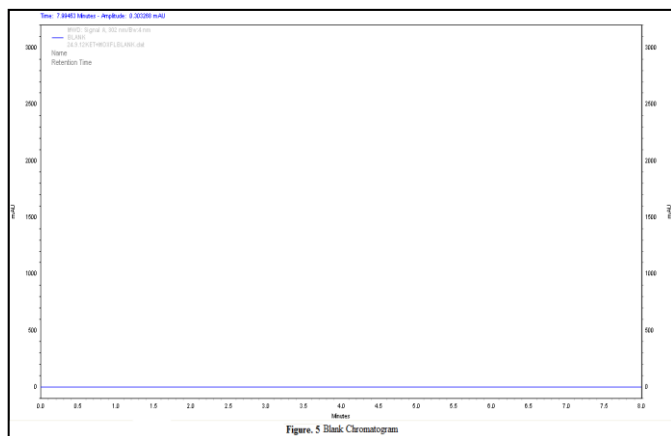
Eclipse plus C18 packing is made by first chemically bonding a dense monolayer of dimethyl-n-octadecylsilane stationary phase to a specially prepared, an improved ultra-high purity (>99.995% SiO₂), ZORBAX Rx-SIL porous silica support. The bonded-phase packing is doubly endcapped using proprietary reagents and procedures to obtain maximum deactivation of the silica surface. Mobile phase composition was selected base upon the peak parameter (symmetry, tailing, resolution and peak purity index etc.), run time, case of preparation and cost.

The most suitable mobile phase composition was found to be acetonitrile and phosphate buffer (pH 3.14) in the ratio of 60:40 (v/v), respectively. Under the chromatographic conditions outline, highly symmetrical, sharp peaks of MOX and KET were obtain at retention time about 4.2 and 7.0 min. respectively.

Selectivity:

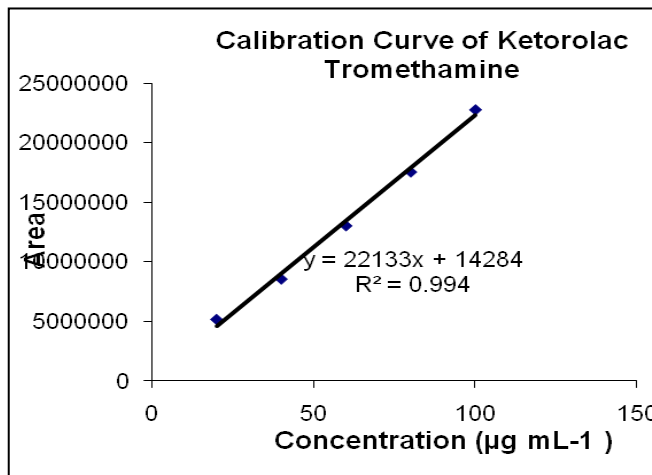
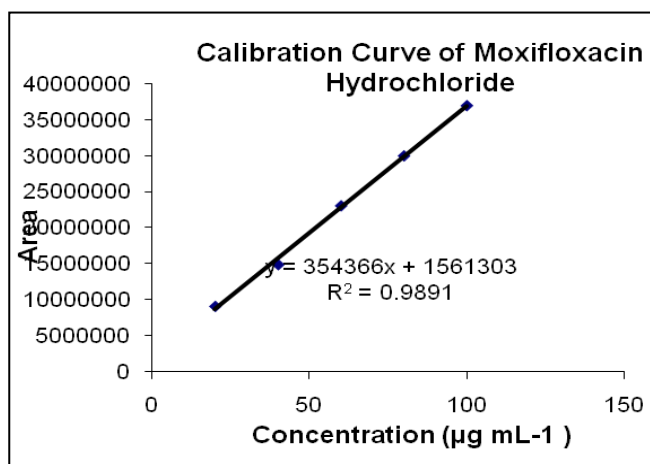
The method was found to selective as no significant interfering peak are observed at the retention times of MOX and KET which were 4.2, and 7.0 min

respectively. Total chromatographic run time was 10.0 min. (Figure 5 & 6) shows the representative chromatograms of blank spiked with analytes .



Limit of detection (LOD), Limit of quantitation (LOQ) and Linearity:

Limit of detection (LOD) was established 4.4 and 8.7 $\mu\text{g mL}^{-1}$ for MOX and KET respectively. Limit of quantification (LOQ) was established 13.3 and 26.3 $\mu\text{g mL}^{-1}$ for MOX and KET respectively. Calibration curves were linear over the concentration range 40–100 $\mu\text{g mL}^{-1}$ of both analytes. Regression coefficient 0.9891 and 0.994 for MOX and KET respectively. (Figure 3 & 4)



Standard curve had a reliable reproducibility over the standard concentrations across the calibration range. All back-calculated concentrations did not differ from the theoretical value as no single calibration standard point was dropped during the validation.

Accuracy and precision:

The accuracy and precision of the proposed methods were tested by the determination of MOX and KET at different concentration levels within the linear range of each compound. The low SD (< 1) of six determinations indicated the high accuracy and precision of the proposed method. Collective results are shown in Tables 1 & 2.

The inter- and intra-day determination of MOX and KET over 3 consecutive days by the same analyst using the same instrument is shown in Tables 1 & 2. The low RSD (< 2%) reflects the ruggedness of the methods.

TABLE 1: ASSESSMENT OF ACCURACY AND PRECISION OF MOXIFLOXACIN HYDROCHLORIDE

	QC Sample ($\mu\text{g mL}^{-1}$)	Mean ($\mu\text{g mL}^{-1}$)	S.D.	R.S.D. (%)	Accuracy (%)
Intra Day (n=6)	40.00	39.71	0.70	1.76	99.28
	60.00	59.52	1.28	2.15	99.19
	80.00	79.38	1.25	1.57	99.22
Inter Day (n=18)	40.00	39.54	0.70	1.78	98.86
	60.00	59.43	1.16	1.95	99.05
	80.00	79.22	1.35	1.70	99.03

S.D. = Standard deviation; R.S.D. (%) (Relative standard deviation) = [(S.D./Mean) X 100]; Accuracy (%) = [(Mean / Conc. Added) X 100]; n = number of replicates.

TABLE 2: ASSESSMENT OF ACCURACY AND PRECISION OF KETOROLAC TROMETHAMINE

	QC Sample ($\mu\text{g mL}^{-1}$)	Mean ($\mu\text{g mL}^{-1}$)	S.D.	R.S.D. (%)	Accuracy (%)
Intra Day (n=6)	40.00	39.90	0.56	1.40	99.75
	60.00	59.26	0.78	1.31	98.76
	80.00	79.08	0.75	0.95	98.84
Inter Day (n=18)	40.00	39.43	0.70	1.78	98.58
	60.00	58.88	0.74	1.25	98.13
	80.00	78.78	1.00	1.28	98.47

S.D. = Standard deviation; R.S.D. (%) (Relative standard deviation) = [(S.D./Mean) X 100]; Accuracy (%) = [(Mean / Conc. Added) X 100]; n = number of replicates.

Extraction recovery:

Recovery results were found to be satisfactory as these were consistent, precise and reproducible are summarized in **Table 3**.

TABLE 3: EXTRACTION RECOVERY OF ANALYTES (n = 6)

Analyte	QC Sample ($\mu\text{g mL}^{-1}$)	Extraction recovery (%)	R.S.D. (%)
MOX	40.00	98.68	0.64
	60.00	98.55	1.06
	80.00	97.82	1.07
KET	40.00	98.07	0.88
	60.00	98.35	0.80
	80.00	98.14	0.68

R.S.D. (%) (Relative standard deviation) = [(Standard deviation / Mean) X 100]; n = number of replicates.

Implementation to Pharmaceutical formulation:

This newly developed method was applied to determine the MOX and KET in pharmaceutical formulation (eye drops). Result were summarized in **Table 4**.

TABLE 4: ESTIMATION MOXIFLOXACIN AND KETOROLAC IN DIFFERENT FORMULATION

Sample	Analyte	Concentration found % (w/v)	%
Sample I	MOX	0.511	102.18
	KET	0.519	103.98
Sample II	MOX	0.493	98.69
	KET	0.494	98.85

CONCLUSION: Here, we have developed and validated a HPLC-UV method that has significant advantages over the previously published method as it provides simple mobile phase composition for chromatographic separation, shorter run time for analysis, simple sample preparation as well as improved sensitivity. Therefore, this new method leads to a simple, feasible, cost effective, rapid method with high degree of accuracy and specificity to quantify simultaneously MOX and KET in pharmaceutical formulations with HPLC-UV. It will be extremely helpful for successfully analyzing the MOX and KET in ocular formulations.

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