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"Revolutionizing Ophthalmic Drug Analysis: Simultaneous Quantification of Ofloxacin and Ketorolac Tromethamine using Reverse Phase High Performance Liquid Chromatography"

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Abstract:

A novel reverse phase high performance liquid chromatographic (HPLC) method was developed to simultaneously quantify Ofloxacin and Ketorolac Tromethamine in both bulk and ophthalmic dosage forms, providing a simple, accurate, rapid, and precise analytical approach. Employing a Eurosphere-100 C18 column (250 mm × 4.6 mm, 5 µm particle size) in isocratic mode with a mobile phase consisting of methanol and 0.005 M potassium dihydrogen phosphate buffer (50:50 v/v), adjusted to pH 3.5 ± 0.1 with ortho-phosphoric acid, facilitated efficient separation. The flow rate was maintained at 1.0 mL/min, and individual component absorbance was measured at 298 nm. Ketorolac Tromethamine and Ofloxacin exhibited retention times of 5.20, and 10.20 minutes, respectively.
The method demonstrated excellent linearity over the concentration ranges of 3-15 µg/mL for Ofloxacin and 5-15 µg/mL for Ketorolac Tromethamine, with correlation coefficient values of 0.9998 for both analytes. Percentage recovery for Ofloxacin and Ketorolac Tromethamine was determined to be 100.28% and 99.70%, respectively, indicative of the method's accuracy and reliability. This innovative HPLC technique offers a robust solution for the simultaneous analysis of these important pharmaceutical compounds, facilitating quality control and ensuring the efficacy of ophthalmic formulations.

Keywords: HPLC, Ofloxacin and Ketorolac Tromethamine etc.

Introduction:

The co-administration of multiple drugs can lead to clinically significant interactions, particularly with narrow therapeutic index medications, affecting their efficacy either before or after absorption. This study aimed to develop a precise analytical method for simultaneous quantification of Ofloxacin (OFLOX) and Ketorolac Tromethamine (KETO) to address potential therapeutic limitations. Ofloxacin, an antimicrobial agent, is chemically known as 9-fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1, 2, 3-de]-1, 4-benzoxazine-6-carboxylic acid. Various analytical techniques have been reported in the literature for OFLOX quantification, including spectrophotometry, potentiometry, conductometry, HPLC, electrophoresis, and LC/MS/MS, either alone or in combination. This research aimed to streamline the process by developing

a rapid, simple, and accurate method for concurrently assessing OFLOX and KETO levels, essential for optimizing therapeutic outcomes and minimizing adverse interactions.
Ketorolac Tromethamine, possessing anti-inflammatory and analgesic properties, is chemically identified as 5-benzoyl-2, 3-dihydro-1H-pyrrolizine-1-carboxylic acid, 2-(hydroxymethyl)-1, 3-propanediol. It is exclusively official in the USP. Literature reveals limited analytical methods for Ketorolac Tromethamine estimation, including spectrophotometry, HPLC, and HPTLC. The OFLOX and KETO fixed-dose combination is solely available in ophthalmic dosage form, with no reported method for simultaneous estimation. The current study aims to develop a precise, selective RP-HPLC method for swift assessment of OFLOX and KETO from the ophthalmic dosage form, filling the existing analytical gap effectively.

Materials and methods:

An Agilent 1100 series high-performance liquid chromatography (HPLC) system featuring an automatic injector with a 20 µL injection volume and an Ultra-Visible (UV-Vis) detector was utilized. The system includes a G1316A Thermostated Column Compartment and a stationary phase column (250 mm × 4.6 mm) with 5 µm particle size. Ofloxacin (OFLOX) was sourced from Crystal Pharma, while Ketorolac Tromethamine (KETO) was obtained from Global Pharma, Mumbai. Eye drops under the brand KETOFLOX (Allergan) containing 3 mg of Ofloxacin and 5 mg of Ketorolac Tromethamine per mL were acquired from a local pharmacy. Analytical grade potassium dihydrogen phosphate and ortho-phosphoric acid were used; along with HPLC grade methanol and water obtained from Qualigens. These materials and equipment were employed for the development of a precise HPLC method to simultaneously quantify OFLOX and KETO from the ophthalmic dosage form.

Mobile Phase:

A 0.005 M potassium dihydrogen phosphate buffer solution was prepared by combining methanol with HPLC grade water in a 50:50 volume-to-volume ratio. The pH of the solution was adjusted to 3.5 ± 0.1 using ortho-phosphoric acid. Initially, potassium dihydrogen phosphate (5.8045 g) was dissolved in 500 mL of HPLC grade water. Subsequently, an additional 500 mL of HPLC grade water was added to achieve a final concentration of 0.05 M. The resulting solution was then filtered through a 0.22 µm membrane filter for further use.

Standard stock solution:

Standard stock solutions were prepared individually for OFLOX and KETO. For OFLOX, 25 mg of the standard was precisely weighed and transferred into a 25 mL volumetric flask. The compound was dissolved in the mobile phase, and the flask was shaken for 0.5 hours. The volume was then adjusted to the mark with the mobile phase to obtain a solution containing OFLOX at a concentration of 1000 µg/mL. Similarly, for KETO, 25 mg of the standard was accurately weighed and transferred into a 25 mL volumetric flask. The substance was dissolved in the mobile phase, followed by shaking for 0.5 hours. The volume was then adjusted to the mark with the mobile phase to achieve a solution containing KETO at a concentration of 1000 µg/mL.

Working standard solution:

A combined working standard solution was prepared by diluting the stock solutions of OFLOX and KETO in mobile phase to achieve concentrations of 3µg/mL for OFLOX and 5µg/mL for KETO.

Sample solution:

Eye drops equivalent to 3 mg of OFLOX and 5 mg of KETO were precisely measured and transferred to a 100 mL volumetric flask containing 50 mL of mobile phase. The flask was then sonicated for 0.5 hours, and the volume was adjusted to the mark with mobile phase. The resulting solution was filtered through a 0.45 µm membrane filter. Subsequently, 1 mL of this solution was diluted to 10 mL with mobile phase to obtain a solution theoretically containing OFLOX at a concentration of 3 µg/mL and KETO at a concentration of 5µg/mL.

Table No. 01: Chromatographic conditions:

Sr. No.	Parameter	Value
1.	Mobile phase composition	Methanol: 0.05 M potassium dihydrogen phosphate buffer (50:50 v/v)
2.	pH adjustment	3.5 ± 0.1 with ortho-phosphoric acid
3.	Flow rate	1 mL/min
4.	UV detection wavelength	298 nm
5.	Column temperature	Ambient

Assay:
Twenty microliters of the test and standard solutions were separately injected (n = 3) into the HPLC injector, and chromatograms were recorded. The amounts of both drugs were then calculated based on the respective peak areas.

Table No. 02: Linearity and calibration:

Sr. No.	Concentration (µg/mL)	OFLOX Volume (mL)	KETO Volume (mL)
1.	03	0.30	0.50
2.	06	0.60	1.00
3.	09	0.90	1.50
4.	12	1.20	2.00
5.	15	1.50	2.50

Each concentration was prepared in six 10 mL volumetric flasks, and the volume was made up to the mark with mobile phase for both OFLOX and KETO. The solution (20 µL) was then injected into the column using a Hamilton Syringe. Measurements were repeated three times for each concentration to generate calibration curves of the area under the curve versus concentration for both drugs.

Method validation:
The analytical technique was validated according to the requirements outlined by the United States Pharmacopeia (USP) and the International Council for Harmonisation (ICH) standards for factors such as recovery, accuracy, ruggedness, and repeatability.

Recovery study:

An analytical technique's accuracy refers to how closely the test findings generated by that method align with the real value. It is important to establish the correctness of an analytical technique across its whole range. A predetermined quantity of a standardized solution containing pure pharmaceuticals (OFLOX and KETO) was introduced into a pre-analyzed sample solution (OFLOX 3 µg/mL and KETO 5 µg/mL). These solutions underwent analysis.
A smaller relative standard deviation (RSD) number indicates higher accuracy of the approach. The average recoveries of OFLOX and KETO were 100.25% and 99.67% respectively, with RSD values being within the specified ranges.

Precision:
The precision of an analytical technique refers to the level of concordance seen among the individual test outcomes when the method is regularly used on several samplings of a uniform sample.
An analysis was conducted on the fluctuation of findings within the same day (intra-day) and between different days (inter-day). The intra-day precision was assessed by assessing three different concentrations of OFLOX (3, 6, and 9 µg/mL) and three different concentrations of KETO (5, 10, and 15 µg/mL) on the same day, repeated three times. The inter-day accuracy was assessed by evaluating the same drug concentrations on a daily basis for a period of 3 days.

Ruggedness:
An analytical method's ruggedness refers to the level of consistency in test findings while analyzing the same sample under various settings, including multiple labs, analysts, equipment, and reagent batches.

Results and Discussion:

Ofloxacin (OFLOX) is a man-made antibacterial agent belonging to the fluoroquinolone class. It functions by suppressing the activity of the bacterial DNA gyrase enzyme, which is necessary for DNA replication. As a result, it leads to the destruction of bacteria. Ketorolac Tromethamine is a pharmacological compound that exhibits both anti-inflammatory and analgesic properties. It functions by suppressing the activity of the cyclooxygenase enzyme and the production of prostaglandins.
The market study indicated that the aforementioned combination has recently been released to the market. Additionally, the literature review found no known methodologies for the simultaneous estimate of OFLOX and KETO in their combined dose form.
Therefore, an effort has been made to create a chromatographic technique for the concurrent determination of Ofloxacin and Ketorolac Tromethamine in their pharmaceutical formulation.
A reverse phase high-performance liquid chromatography (HPLC) technique was devised to simultaneously estimate the quantities of Ofloxacin and Ketorolac Tromethamine in an ocular dose form. The separation was accomplished using a Eurosphere-100 C18 column and a mobile phase consisting of methanol and a 0.05 M potassium dihydrogen phosphate buffer in a 50:50 volume ratio. The pH of the mobile phase was adjusted to 3.5 ± 0.1 using orthophosphoric acid. The flow rate of the mobile phase was maintained at 1.0 mL/min. The detection of the compounds was performed at a wavelength of 298 nm.

Assay results:
The assay results indicate that, using the suggested approach, the content of OFLOX and KETO in the two medications was found to be 99.64% and 100.77% respectively, based on duplicate analysis (n = 3) (Table-1). The Ketorolac Tromethamine and Ofloxacin had retention durations of 5.20 and 10.2 minutes, respectively, as shown in Figure no. 01. The linearity of the data was evaluated by plotting concentration against area. The resulting graphs showed a linear relationship throughout the concentration range of 3-15 µg/mL for Ofloxacin and 5-25 µg/mL for Ketorolac Tromethamine. The correlation coefficient values for both medicines were determined to be 0.9998 (Table-04).

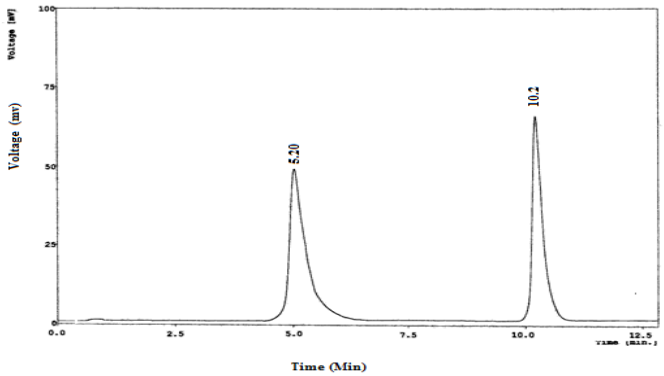

Figure no.01: Typical chromatogram of the sample solution containing Ofloxacin and Ketorolac Tromethamine at retention time of 5.20 and 10.2 min, respectively.

Table No. 03: Result of HPLC:

Formulation (eye drop)	Actual concentration (mg)		% OFLOX* ± SD	% KETO* ± SD
	OFLOX	KETO		
KETOFLOX (Allergan)	3.0	5.0	99.63 ± 0.60	100.72 ± 0.76

*Average of 3 determination; SD = Standard deviation.

Table No. 04: Statistical Data for Linearity and Calibration Range:

Sr. No.	Parameters	OFLOX	KETO
1.	Linear range (µg/mL)	3-15	5- 25
2.	Slope	18.143	6.83
3.	Coefficient of variation	0.9998	0.9998

The method of estimation was validated based on the fixed parameters for the following parameters:

Recovery studies: Recovery studies involved the addition of a known amount of standard solution containing pure drugs (OFLOX and KETO) to a preanalyzed sample solution (OFLOX 3 µg/mL and KETO 5 µg/mL). The resulting solutions underwent analysis, yielding results within the acceptable range of above 99% and below 101% (refer to Table-05).

Table No.05: Result for Recovery Studies:

Sr. No.	Sample solution (µg/mL)	Amount of standard drug added (µg/mL)	% Recovery* ± SD	% RSD
1.	OFLOX	3.00	100.28 ± 0.35	0.15
2.	KETO	5.00	99.70 ± 0.60	0.37

*Average of 5 determination; SD = Standard deviation; RSD = Relative standard deviation.

Precision:
Precision studies, including intra-day and inter-day analysis precision parameters, were conducted, demonstrating results within the acceptable limit of % RSD below 2.0. This indicates the reproducibility of the method, as shown in Table-06.

Ruggedness:
Ruggedness studies focused on a single parameter, specifically different analysts. Results indicated that the % RSD fell within the range of 0.1-1.4, which is less than 2, across different analysts. This study highlights the method's ruggedness under varying performance conditions, as illustrated in Table-07.

System suitability test:
According to USP-24 guidelines, a system suitability test was conducted on freshly prepared standard stock solutions of OFLOX and KETO. Twenty µL of each drug was injected under optimized chromatographic conditions, and various parameters were analyzed to assess the system's suitability. The values obtained from the system suitability test are presented in Table-08.

Table No. 06: Results of Precision Studies:

Sr. No.	Component	Intra-day precision		Inter-day precision	
		Area under curve	% RSD	Area under curve	% RSD
OFLOX (µg/mL)					
1.	3	0516.12	1.32	0515.84	2.32
2.	6	0938.05	1.36	0937.05	1.98
3.	9	1400.82	1.45	1401.27	1.78
KETO (µg/mL)					
1.	5	0331.26	1.76	0332.09	1.89
2.	10	0686.47	1.57	0686.08	1.77
3.	15	1013.32	1.55	1012.97	1.16

The precision study data for both inter-day and intra-day measurements were collected for the simultaneous estimation of Ofloxacin and Ketorolac Tromethamine in an ophthalmic dosage form.
*Average of three determination; RSD = Relative standard deviation.

Table No.07: Ruggedness Studies:

Sr. No.	Drug	Label claim (mg/mL)	Amount found (%)	
			Analyst I	Analyst II
1.	OFLOX	3.0	099.98	100.09
2.	KETO	5.0	100.24	100.10

The table demonstrates the reproducibility of the proposed method.'

Table No. 08: System Suitability Test Parameters:

Sr. No.	System suitability parameters	Proposed method	
		KETO	OFLOX
1.	Retention time (t_R)	5.96 min	11.5 min
2.	Capacity factor (k')	1.50	4.16
3.	Theoretical plate number (N)	10732	94044
4.	Tailing factor (T)	-	-
5.	Resolution factor (R)	-	2.73

The table delineates a range of validation parameters.

Ethical Approval:
There are no conflicts of interest to disclose. This study did not involve any research with animal or human subjects, nor was it conducted in private or protected areas.

Consent to Participate:
Participation in the research is entirely voluntary, and the outcomes may not directly benefit us.

Consent to Publish:
Written informed consent for the publication of their details was obtained from the study participants or their next of kin.

Authors Contributions:
All authors participated in the conceptualization and design of the study. Dr. Shailesh B. Patil and Prof. Jitendra D. More conducted material preparation, data collection, and analysis. The final manuscript was reviewed and approved by all authors.

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Competing interests:
The authors do not have any relevant financial or non-financial interests to declare.

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