ISSN: 0975-8232

IJPSR (2010), Vol. 1, Issue 7 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received 26 March, 2010; received in revised form 02 June, 2010; accepted 13 June, 2010

NOVEL METHOD FOR SIMULTANEOUS ESTIMATION OF CIPROFLOXACIN HYDROCHLORIDE AND OFLOXACIN BY REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (RP-HPLC)

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Keywords:

Ciprofloxacin hydrochloride,

Ofloxacin,

RP-HPLC,

Dosage forms

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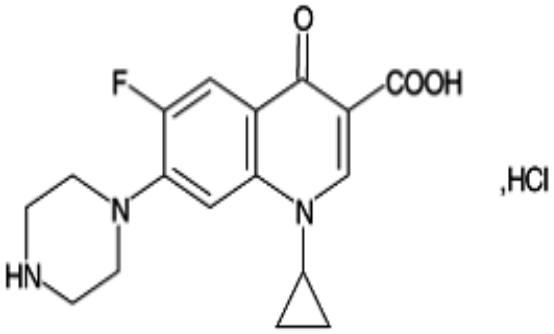
ABSTRACT

A novel method was developed for the determination of fluoroquinolones such as ciprofloxacin hydrochloride (CPL) and ofloxacin (OFX) by isocratic reverse phase-high performance liquid chromatography (RP-HPLC) coupled with UV detection. The developed method was rapid, accurate, reproducible, economical and sensitive for the simultaneous estimation of ciprofloxacin hydrochloride and ofloxacin. Fluoroquinolone antibiotics were separated on an analytical column (SIL 100Å, 125×4.6 mm ID) C_{18}-RP, 5 µm, at ambient temperature. The mobile phase was consisted of Phosphate Buffer (15 mM): Methanol: Acetonitrile: TEA (66:24:10:1% v/v), pH was 6.5 (adjusted with 80% HCl), at a flow rate 1ml/min and sample injection volume was 20-µL. UV detection at 289 nm and retention time (RT) was 3.01 and 6.03min for ciprofloxacin hydrochloride and ofloxacin respectively. The method was validated in terms of stabilizing accuracy, precision (Intra/Inter-day), linearity, specificity, stability and sensitivity. The proposed method has been successfully applied for the analysis of marketed tablets and can be used for the routine analysis of formulations containing any one of the above drugs without any alteration in the assay. The main advantage of this method is the common chromatographic conditions adopted for both formulations and less time consuming.

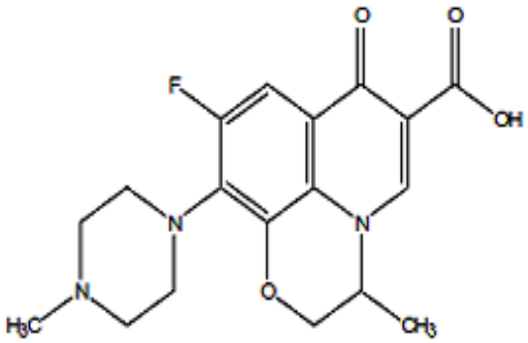
INTRODUCTION: In present days, the analytical chemistry has been practiced largely in laboratories in many diverse ways in both theoretical as well as practical form of science. Different methods have been developed, improved and validated and applied practically for analysis of various compounds. Development and validation of a new method of analysis is very tedious work. However, it is prerequisite to have accurate and reliable results. In spite of variations in individual approach, the method development often follows the well-established steps.

In most cases, the desired separation can be achieved with only few experiments, while in other cases considerable amount of experimentation may be required. However, the developed method should be as simple as possible, the best strategy being theoretical and empirical approach. Before proceeding with development for a particular sample it is absolutely essential to have detailed information about the sample and separation goal and instrument should be clearly defined.

The quinolones are synthetic antibiotics, chemically related to nalidixic acid. The general structure of quinolones antibacterial agents consist of a 1- substituted- 1, 4- dihydro- 4- oxopyridine- 3- carboxylic moiety combined with an aromatic or heteroaromatic ring. Almost all of the recent clinically useful quinolones bear a fluorine atom in the C-6 position of the quinolone, naphthyridine or benzoxazine ring system. Due to the presence of the fluorine atom in the molecules, these antibacterial agents are generally known as fluoroquinolones [1]. The quinolones and fluoroquinolones inhibit the replication and transcription of bacterial DNA by stabilizing the complex formed between DNA and topoisomerases. In Gram-positive bacteria, the stabilized complexes are between DNA and topoisomerase IV, with the drugs showing a 1000-fold selectivity for the bacterial enzyme over the corresponding enzyme in human cells. In Gram-negative bacteria, the main target for fluoroquinolones is the complex between DNA and topoisomerase II enzyme called DNA gyrase. It has the same role as topoisomerase IV in reverse and is required when the DNA double helix is being supercoiled after replication and transcription [2-3].



CIPROFLOXACIN HYDROCHLORIDE



OFLOXACIN

FIG. 1: CHEMICAL STRUCTURE OF FLUOROQUINOLONES

Ciprofloxacin hydrochloride is the most potent first generation fluoroquinolones (FQs) active against a broad range of bacteria, the most susceptible ones are the aerobic gram negative bacilli, especially the enterobacteriaceae and

Neisseria. The minimum inhibitory concentration (MIC) of ciprofloxacin against these is usually < 0.1 µg/ml, while gram positive bacteria are inhibited at relatively higher concentrations. A third generation ofloxacin has an asymmetric center and is sold as racemic mixture of both enantiomers one of which is active and one is not. Levofloxacin is the active enantiomer of ofloxacin and is twice as active as one would expect [4].

MATERIAL AND METHODS:

Regents and Materials: Ciprofloxacin hydrochloride (CPL) and ofloxacin (OFX) were received as a gift samples from Pegasus Farmaco (P) Ltd., Roorkee (India). HPLC Grade Acetonitrile, Water and di-potassium hydrogen orthophosphate as well as hydrochloric acid LR were supplied from s.d. Fine Chem Limited, Mumbai (India). Methanol was obtained from E. Merck, Mumbai (India) and triethylamine LR was obtained from Thomas Baker, Mumbai (India). Commercial pharmaceuticals containing ciprofloxacin hydrochloride and ofloxacin were provided from local drugstores.

Instrumentation: An LC-10AT VP pump from Shimadzu (Japan) was used to deliver the mobile phase to the analytical column. Sample injection was performed via a Hamilton Bonaduz (Switzerland) with a 20-µL loop. Detection was achieved by an SPD-10 A VP UV-Vis detector (Shimadzu, USA) and data acquisition software was Class-LC10/M10A. Degassing of solvents was achieved by Ultrasonic Bath (Toshcon Industries, Hardwar, Uttrakhand, India) before use. A Vortex Mixer (Khera Instrument Pvt. Ltd, Azadpur, Delhi, India) and centrifuge were employed for the sample pre-treatment. In order to choose the working wavelength for detection, UV spectra of fluoroquinolones were taken using a UV-Vis, Double Beam Spectrophotometer 2201, Systronics, Ambala, India.

Chromatographic Conditions [5-7]: The analytical column (SIL 100Å, 125×4.6 mm ID) C18-RP, 5 µm, from s.d. Fine Chem Limited (Mumbai, India) maintained at ambient temperature was used for the separation of the fluoroquinolones. Wavelength was selected by scanning standard drugs over the wide range of wavelength 200 nm to 400 nm. Both the components show reasonably good response at 289 nm.

The mobile phase consisted of Phosphate Buffer (15 mM): Methanol: Acetonitrile: TEA (66:24:10:1 % v/v), pH was 6.5 (adjusted with 80% HCl). The flow rate was 1 ml/min flow rate with the inlet pressure ranged from 135-140 Kgf and sample injection volume was 20-µL (table 2). High performance liquid chromatogram of fluoroquinolones is shown in Fig 2.

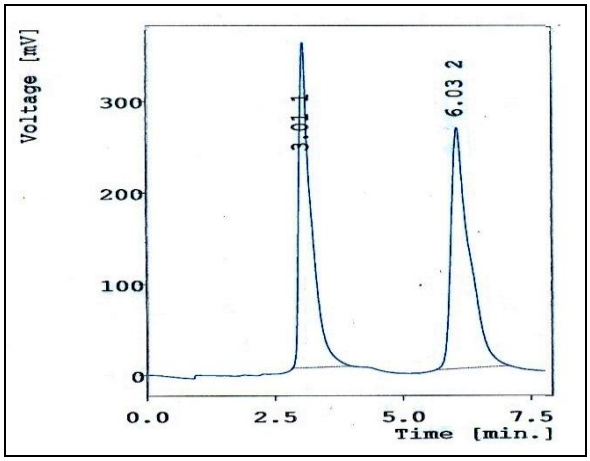


FIG. 2: HIGH PERFORMANCE LIQUID CHROMATOGRAM OF FLUOROQUINOLONES (CPL 3.01 MIN. AND OFX 6.03 MIN.)

TABLE 1: OPTIMIZED CHROMATOGRAPHIC CONDITIONS

PARAMETER	OPTIMIZED CONDITION
Mobile Phase	Phosphate Buffer: Methanol: Acetonitrile: TEA (66:24:10:1%v/v), pH- adjusted to 6.5 with 80% HCl
Column	C18-RP (SIL 100Å, 5 µm, 125×4.6 mm ID), S.D. Fine Chem. Limited
Flow Rate	1 ml/min
UV Detection	289 nm
Injection Volume	20 µl
Temperature	Ambient
Retention Time of CPL	3.01 min
Retention Time of OFX	6.03 min
Run Time	8.0 min

Standard and Working Solutions: Individual standard stock solutions of ciprofloxacin hydrochloride (400µg/ml) and ofloxacin (400µg/ml) were prepared by accurately weighing 4mg of each compound in volumetric flasks and volume was made up to 10 ml with mobile phase. Working solutions and mixed working solutions (MWS) prepared from standard stock solutions. MWS was used in the preparation of analytical and calibration standards. Analytical standards were prepared from MWS by diluting it with mobile phase to obtain a concentration range of 10-200 µg/ml for ciprofloxacin hydrochloride and ofloxacin. All standard stock solutions and mixed working solutions were stored refrigerated at 4 ^{0}C and found to be stable for two month.

Calibration and Quality Control Samples: Calibration standards were prepared from MWS over a range of 10, 20, 40, 80, 160 & 200 µg/ml for ciprofloxacin hydrochloride and ofloxacin. Quality control (QC) samples at three different levels [Lower (20µg/ml), Medium (80µg/ml) and High (200µg/ml)] were prepared in triplet once each day and used to assess accuracy and precision of the assay method. The calibration standards and quality control samples were prepared fresh on each day of validation.

Method Validation [8-11]: The proposed isocratic HPLC method was validated in terms of HPLC system reproducibility, sensitivity, linearity, specificity, recovery, limit of detection (LOD), limit of quantitation (LOQ), and intra/inters day accuracy and precision for ciprofloxacin hydrochloride and ofloxacin.

Accuracy and Precision: Accuracy and Precision were determined by injection of quality control standard (low, medium and high concentrations) in three replicates. Accuracy and precision values of method for ciprofloxacin hydrochloride and ofloxacin are summarized in Table 2. The results show that the method is accurate and precise with deviation less than 5%.

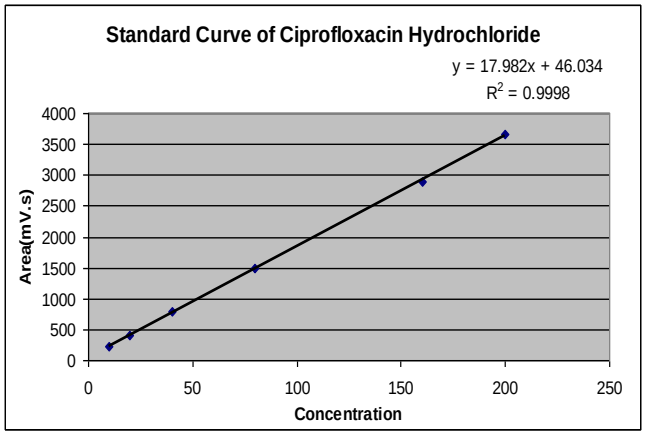
TABLE 2: ACCURACY (%BIAS) AND PRECISION (%RSD) OF CIPROFLOXACIN HYDROCHLORIDE AND OFLOXACIN

ANALYTE	CONCENTRATION (µg/ml)	% BIAS		% R. S. D.	
		INTRA DAY	INTER DAY	INTRA DAY	INTER DAY
Ciprofloxacin hydrochloride	20	-3.1	-2.05	1.91	1.35
	80	2.73	1.51	0.56	0.69
	200	-0.54	0.03	3.53	2.2
Ofloxacin	20	0.45	0.93	1.61	1.24
	80	1.68	0.81	0.25	1.35
	200	-1.30	-0.43	0.87	0.69

Sensitivity: The method was sensitive with a limit of quantitation (LOQ) of 0.47µg/ml and 0.14µg/ml for ciprofloxacin hydrochloride and ofloxacin respectively. The limit of detection (LOD) for ciprofloxacin hydrochloride and ofloxacin was 1.59 µg/ml and 1.56 µg/ml respectively.

Specificity: Specificity of the method was tested by comparison of peaks of drug mixture with those of reference standards. There was no interference from drug mixture (should not be same RT), indicating the high specificity of the method.

Linearity: For linearity studies (calibration curve), six different concentrations in the range of 10-200 µg/ml of ciprofloxacin hydrochloride and ofloxacin were prepared from standard stock solutions using mobile phase. The linear regression equation of ciprofloxacin hydrochloride and ofloxacin was found to be y = 17.982x + 46.034 and y = 34.718x + 44.838, respectively, shown in Fig 3. The correlation coefficient values were found to be 0.9998 and 0.9999 for ciprofloxacin hydrochloride and ofloxacin, respectively.



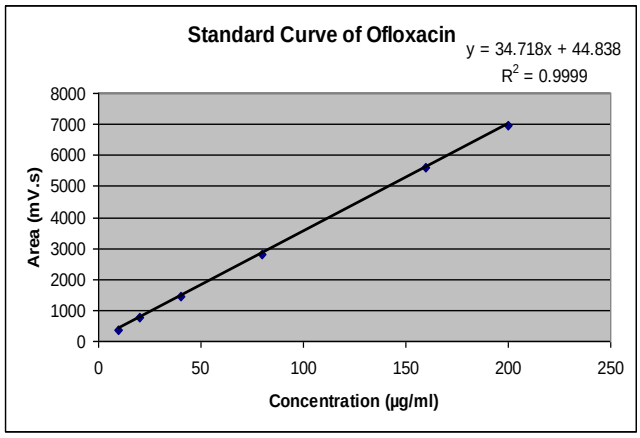


FIG. 3: CALIBRATION CURVE FOR CIPROFLOXACIN HYDROCHLORIDE AND OFLOXACIN RESPECTIVELY

Stability: The stability of drug solutions was verified by storing sample solutions in refrigerator for six weeks and analyzing them at intervals of 1st week, 4th week and 6th week. The stability was determined by comparing the results of 4th week and 6th week with that of the freshly prepared solution of 1st week. The RSD was found to be less than 11%, within the acceptable limits of less than 20%.

Pharmaceutical Sample Preparation [12-13]**:**

Film coated Tablets: Twenty commercial film coated tablets (Ciplox-500 and OFLOX-200) were finely powdered in a porcelain mortar. Individual 5mg of finely powdered ciprofloxacin hydrochloride and ofloxacin tablet were accurately weighed, transferred to a centrifuge tube and 10ml of mobile phase was added. The sample was centrifuged at 2000rpm for 30minutes. The supernatant was transferred to a 10ml of volumetric flask to have a concentration of 364.6µg/ml for ciprofloxacin hydrochloride and 330.5µg/ml for ofloxacin (stock solution). From the stock solution, different aliquots were taken and diluted in volumetric flask to have a concentration viz. 9.12, 36.46 and 145.84µg/ml for ciprofloxacin hydrochloride and 16.53, 66.1 and 165.25µg/ml for ofloxacin.

TABLE 3: THE RESULTS OF SAMPLE SOLUTION ANALYSIS FOR CIPROFLOXACIN HYDROCHLORIDE TABLET

CONC. (µg/mL) (Claimed)	AREA (mV. s) (Mean ± SD)	CV (%)	CONC. (µg/mL) (Found)	% RSD
9.12	210.19 ± 16.88	8.02	9.13	-0.15
36.46	735.01 ± 3.84	0.52	38.32	-5.1
145.84	2667.46 ± 24.25	0.91	145.78	0.04

TABLE 4: THE RESULTS OF SAMPLE SOLUTION ANALYSIS FOR OFLOXACIN TABLET

CONC. (µg/mL) (Claimed)	AREA (mV. s) (Mean ± SD)	CV (%)	CONC. (µg/mL) (Found)	% RSD
16.53	601.82 ± 7.42	1.23	16.04	2.98
66.1	2218.07 ± 7.89	0.35	62.6	5.29
165.25	5479.96 ± 38.98	0.71	156.55	5.26

RESULT AND DISCUSSION:

Chromatography: A typical chromatogram for ciprofloxacin hydrochloride and ofloxacin using C_{18}, HPLC column with mobile phase composed of Phosphate Buffer (15 mM) : Methanol : Acetonitrile : TEA (66:24:10:1%v/v) and pH adjusted to 6.5 with 80% HCl at 1.0ml/min flow rate is shown in Fig 2. Under the assay conditions described above the examined fluoroquinolones were well resolved with retention times 3.01min for ciprofloxacin

hydrochloride and 6.03min for ofloxacin, indicating the perfect separation.

Validation Study: Method validation was performed in terms of linearity, accuracy and precision, sensitivity and specificity. The accuracy and precision of method based on inter-day and intra-day repeatability was performed by replicate injections (n = 3) of three standard solutions (20, 80 and 200 µg/ml). Results are shown in Table 2.

Analysis of Dosage Form: High performance liquid chromatogram of fluoroquinolones in pharmaceutical formulation is shown in Fig 4. The experimental results for these tablets are given in Table 3 and Table 4.

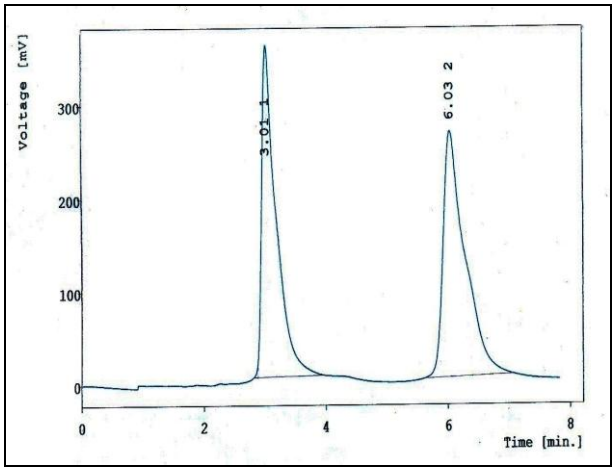


FIG 4 HIGH PERFORMANCE LIQUID CHROMATOGRAM OF FLUOROQUINOLONES (CPL TABLET 3.01 MIN. AND OFX TABLET 6.03 MIN.)

CONCLUSION [14-16]: The present study describes a highly sensitive, accurate and reproducible HPLC method for determination of ciprofloxacin hydrochloride and ofloxacin. This method has several advantages over the previously reported methods. Sample preparation is simpler, and the chromatographic column is available commercially. The procedure for sample preparation is rapid and inexpensive. Retention time for ciprofloxacin hydrochloride and ofloxacin is less as compared to previous developed methods. The very low quantification limit obtained with a UV detector helped to avoid use of fluorimetric detection, which demands more expensive equipments. UV detectors give more reproducible and stable responses than fluorimetric detectors.

Another advantage of this method is the use of an isocratic mobile phase of very simple composition, which gives the column a longer life time. From the results, it can be concluded that proposed method has been successfully applied for the analysis of marketed tablets and can be used for the routine analysis of formulations containing any one of the above drugs without any alteration in the assay. The main advantage of the method is the common chromatographic conditions adopted for both formulations.

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