Spectroscopic studies on the interaction ofLevofloxacin with Picric acid as an anionic reagent - A Green Approach

A Thesis Submitted

In partial fulfillment for the award of

Degree of

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Submitted by
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Under the Supervision of
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JULY, 2016

DEDICATED TO MY PARENTS…..

Certificate

This is to certify that the thesis entitled **"Spectroscopic studies on the interaction of Levofloxacin with Picric acid as an anionic reagent – A Green Approach"** being submitted by PALAK NARANG, Registration No. 301402012 in partial fulfillment of the requirements for the award of degree of Master of Science in School of Chemistry and Biochemistry, Thapar University, Patiala, is a bonafide work carried under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. The report has not been submitted for the award of any degree in this or any other university.

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Candidate's Declaration

I hereby declare that the work being presented in this dissertation entitled **"Spectroscopic studies on the interaction of Levofloxacin with Picric acid as an anionic reagent - A Green Approach"** in partial fulfillment of the requirements for the award of degree of Master of Science in School of Chemistry and Biochemistry, Thapar University, Patiala, is an authentic record of my own work during the period of January 2016 to July 2016, under the supervision of **Prof. Susheel Mittal**, Senior Professor, School of Chemistry and Biochemistry, Thapar University, Patiala. The report has not been submitted for the award of any other degree in this or any other university.

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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S.No.	Contents	Page No.
1.	Introduction	1-6
2.	Literature Review	7 -13
3.	Gap in Studies	14
4.	Experimental Section	15-16
5.	Results and Discussion	17-26
6.	Conclusion	27
7.	References	28-30

ABSTRACT

Levofloxacin, an antimicrobial agent has been determined by using picric acid as an anionic reagent in aqueous medium. On mixing the reactants in distilled water a yellow colored product is separated within two minutes which was found to be an ion-association complex (LFX-PA) of both the reactants. Spectroscopic studies were carried out on the ion-pair complex in different solvent media to explain the interaction between levofloxacin and picric acid. The spectroscopic studies confirmed its formation as determined from substantial shift in respective λ_{max} values of the ion-pair. A study on the solvent effect by spectrophotometric method for LFX suggested that water is the best medium for the study. A Calibration curves were drawn in acetonitrile, water-acetonitrile and acetonitrile media using the absorbance values of the LFX. Based on better performance calibration curve was drawn in aqueous medium. A linear increase in absorbance was observed with increasing LFX concentration with a lower detection limit of 6×10^{-5} M in aqueous medium. Further, analysis of LFX in commercially available drug tablets proved that the proposed method is valid in real life sample analysis using the green approach.

INTRODUCTION

Quinolones are a class of synthetic broad spectrum antibiotic drugs used widely by human beings. Quinolones and their derivatives have been isolated from natural resources like plants, animals, bacteria etc. Quinolones apply their antibacterial effect by preventing bacterial DNA from duplicating and unwinding. Quinolones which have a fluorine atom attached to the central ring system typically at the C-6 position or C-7 position are called Fluoroquinolones. They are named with –oxacin suffix. They play important role in treatment of serious bacterial infections, especially hospital-acquired infections and in which resistance to older antibacterial classes is detected [1]. The parent compound of all fluoroquinolones is nalidixic acid. On the basis of structure-activity relationships modifications of nalidixic acid led to the synthesis of a large of derivatives.

Fluoroquinolones are extensively used in human and veterinary medicines. In modern times there has been remarkable development in the branch of fluoroquinolones. They can easily enter cells and therefore are frequently used to investigate intracellular infections in body. They are often useful for the treatment of large number of infections, including soft tissue infections, urinary tract infections, respiratory infections, typhoid fever, bone-joint infections, sexually transmitted diseases, prostatitis, community acquired pneumonia, prostatitis, acute bronchitis and sinusitis prostatitis. They are specifically used in bacterial urinary infections and even for infections whose antimicrobial agent has a great resistance [2].

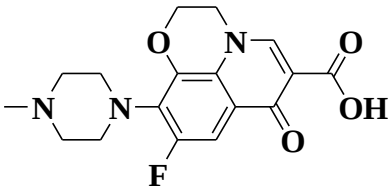
Fluoroquinolones have some severe effects, headache, notably nausea, confusion and dizziness. The fluoroquinolones should be utilized wisely. Inadequate use of fluoroquinolone antibiotic agents will probable worsens current problems with antibiotic resistance [3]. Mechanism of fluoroquinolones- the mechanism of activity of fluoroquinolones antimicrobials engage inhibition of DNA gyrase and bacterial topoisomerase IV (each of type II topoisomerses), enzymes required for DNA transcription, repair, replication and recombination, fever, prostatitis, uretheral infections, gonorrhea, blood poisoning, gonorrhea and sinusitis. The fluoroquinolones are very minute size molecules with weights between 300 and 500 Da. Many of these fluoroquinolones are Zwitter ions and consists different solubility characteristics with

changes in pH. These drugs are quite stable in both oral and intravenous dosage forms at or below 30°C [4].

TYPES OF FLUOROQUINOLONES

1. Ciprofloxacin (Cipro)
2. Levofloxacin (Levaquin/Quixin)
3. Gatifloxacin (Tequin)
4. Moxifloxacin (Avelox)
5. Ofloxacin (Ocuflox/Floxin/Floxacin)
6. Norfloxacin (Noroxin)

Newer generations of fluoroquinolones are being synthesized to increase the pharmacological properties and the antimicrobial spectrum of these antimicrobials. Various analytical methods including voltametric, chromatographic, potentiometric, titrimetric and spectrophotometric and so far have been reported for analysis of these drugs.



Structure of Levofloxacin

LEVOFLOXACIN

Levofloxacin is a widespread antibacterial agent with activity against scope in Gram-positive and Gram-negative bacteria and atypical organisms [5]. The chemical name is (-)-(S)-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 hemihydrate. It contributes in clinical and bacteriological adequacy in a region of infections, in addition to those introduced by both penicillin-susceptible and resistant strains of *S. pneumoniae [6]*. Levofloxacin is well bearable and is affilated with

few of the phototoxic, hepatic or cardiac adverse events seen with some other quinolones. Levofloxacin is quickly absorbed after oral administration. The main route of excretion of levofloxacin is through the kidneys, with 64–102% of a dose being recovered unvaried in the urine.

Development and acceptance of analytical methods is of primary importance to optimize the estimation of drugs in the pharmaceutical industry. The techniques used for the qualitative and quantitative determination of Levofloxacin are Spectophotometry, Voltammetry, Fluorescence, Flow Injection Technique, High Performance Liquid Chromatography (HPLC), Chemiluminescence, Nuclear Magnetic Resonance Spectroscopy, Capillary Zone Electrophoresis etc. In most of the analytical methods formation of ion-pair association complexes takes place. However, the challenges faced in this method generally require expensive instrument, labor-intensive sample preparation procedures, HPLC methods require complex and expensive equipment and disposal of solvents.

Chromatographic methods

Liquid chromatography, combined with tandem mass spectrometry (LC/MS) has also been rapidly embraced by the pharmaceutical industry as the definitive method for the determination of drug in various pharmaceuticals and biological fluids. In chromatographic methods, an automated solid phase extraction procedure, using either CN Isolute or C-18 Bakerbond cartridges are used for analysis of the drugs. Isocratic chromatographic conditions need to be established to keep analytical run-times to less than 10 min. MS/MS conditions also need to be adjusted to obtain adequate signal-to-noise response for all substances during each analysis [7]. Further, there has been a development and validation of a new multi-residue method for the determination of 28 basic/neutral pharmaceuticals (antiepileptics, antibacterial drugs, β-blockers, analgesics, lipid-regulating agents, bronchodilators, histamine-2-blockers, anti-inflammatory agents, calcium channel blockers, angiotensin-II antagonists and antidepressants) and illicit drugs in surface water with the usage of a new technique i.e. ultra performance liquid chromatography–positive electrospray tandem mass spectrometry (UPLC–MS/MS). The usage of the novel UPLC system with 1.7 μm particle size and 1 mm internal diameter column allowed for low mobile phase flow rates (0.07 mL min^{-1}) and short retention times (from 1.3 to 15.5 min) for all compounds analyzed [8].

Chromatographic methods rely on solid-phase extraction procedure as a sample preparation method and almost exclusively liquid chromatography coupled with electrospray ionization tandem mass spectrometry for separation and quantification of up to 30 compounds on C18 column with up to 50 min elution gradient time. All these chromatogprahic techniques require high cost instruments and are not cost-effective.

Chemiluminescence Methods

Chemiluminescence (CL) analysis of pharmaceuticals by the two most relevant automated controlled-flow methodologies-flow-injection analysis (FIA) and sequential-injection analysis (SIA) is also used widely because of the the absence of strong background light levels in CL methods which reduces noise signals and leads to improved detection limits (better sensitivity) and wide linear dynamic ranges. For drug determination by chemiluminescence, CL reagent is used mostly which a strong or moderate oxidant such as potassium permanganate, tris(2,2′-bipyridine)ruthenium(III) complex, potassium hexacyanoferrate(III), Ce(IV), hydrogen peroxide, oxygen or *N*-bromosuccinimide. Other less frequently used CL reagents are bromine or sodium hypochlorite. Further, the CL emission for studied drug can be enhanced by using a catalyst, an organized medium or a sensitizer. For example, Cephalosporines [9] were determined by permanganate-induced CL enhanced by glyoxal as sensitizer. A CL method for the assay of fluoroquinolones was devised by Song and coauthors [10], that enhanced weak CL from peroxynitrous acid (nitrite + hydrogen peroxide in acidic medium). In this method, fluoroquinolones served as energy-transfer species responsible for CL emission.

However, most of the reported CL drug assays are performed by means of lab-made flow luminometers which makes difficult comparison of the analytical numbers of merit of methods devised in different laboratories. Moreover, a CL reaction which could be used as a CL standard still remains a challenge opened to CL researchers due to which this technique is not appreciated much in drug analysis.

Voltammetric methods

The application of electrochemical techniques in the analysis of drugs and pharmaceuticals has increased greatly over the last few years. A large number of electroanalytical methods are available for quantification of pharmaceuticals. Their electrochemical behavior can be investigated using cyclic voltammetry and adsorptive stripping differential pulse voltammetry. For the determination of dopamine, levodopa, epinephrine and norepinephrine, copper (II) complex and silver nanoparticles modified glassy carbon paste electrode was constructed and used. The method for drug determination is based on the development of a new dinuclear copper (II)complex which contains an alcoholic OH group coordinated axially to each copper centers forming Cu_2O_2 non- planar ring, which forms the intermediate in the catecholamine reduction through the enzymatic activity i.e. tyrosinase. Whole of the mechanism take place in slightly acidic pH range [11].

Spectroscopic methods

Spectrophotometry enables to detect and quantify many drugs by using simple or even rather complicated derivatisation routines leading to the formation of coloured reaction products. UV–Vis spectrophotometry technique especially applied in the analysis of pharmaceutical dosage form which has increased rapidly over the last few years. The methods used for drug analysis so far are based on complex-formation reaction, oxidation-reduction process and catalytic effect. For example; determination of albendazole (anthelmintic) drug in bulk, tablet and suspension dosage forms was carried out in methanolic glacial acetic acid solution using spectrophotometric technique [12]. The determination of carbinoxamine maleate in pharmaceutical formulations was also carried out using spectrophotometric technique, in which formation of a ternary complex between copper (II), eosin, and carbinoxamine maleate take place in acidic pH conditions. Chelate formation takes place between carboxylic group and the nearest nitrogen atom in the drugs with copper and which further forms a ternary complex with eosin (sodium 2,4,5,7-tetrabromofluorescein) [13]. Development of an optimized spectrofluorimetric method for the determination of doxepin hydrochloride in pharmaceutical formulations is also based on dichloromethane extractable ion pair complex formation of the doxepin hydrochloride with eosin Y at pH 4.52 (sodium acetate–acetic acid buffer solution) [14]. Likewise, there are reports in which basic mechanism taking place for complex formation between drug and reagent relies on the maintenance of acidic pH.

Spectrophotometric method has various advantages such as good analytical selectivity, less time consuming, easy and less expensive and low interference level as compared with most of the other techniques. This method is very easy, simple and rapid so can be successfully used for the qualitative and quantitative determination of the drugs in biological fluids and for pharmaceutical analysis. Analysis is mostly based upon formation of colored ion-pair association complexes between the drug and the reagent which can be easily determined spectrophotometrically. The mostly complexes are formed by charge-transfer or ion-pair complex formation which require optimization of reaction conditions to generate ion-pair between drug and the reagent which becomes tedious in any type of analysis. In the proposed method, no optimization is required to generate ion-pair between drug and picric acid. The approach used so far is green due to use of water for the generation of ion-pair. Another uniqueness of the proposed method lies in the use of picric acid as a reagent which has a very low pK_a value i.e. 0.38 at 25°C and is highly acidic. Choice of anionic reagent plays an important role in ion-pair generation; picric acid with three electron-withdrawing groups and having very low pK_a was found to be the best anionic reagent to be used so far for ion-pair generation, in the proposed method.

LITERATURE REVIEW

As per the literature review, solution conditions play an important role in the determination of levofloxacin. Maintenance of acidic pH is the pre-requisite for the development of ion-pair. Generation of H^+ ion would take place only in the acidic pH which would ultimately lead to the formation of the ion-association between drug and reagent. Based upon this phenomenon, determination of levofloxacin in pharmaceutical formulations has been carried out using various techniques including Spectophotometry, Voltammetry, Fluorescence, Flow Injection Technique, High Performance Liquid Chromatography (HPLC), Chemiluminescence, Nuclear Magnetic Resonance Spectroscopy, Capillary Zone Electrophoresis etc. Formation of ion-pair association complexes or charge transfer complexes under optimum conditions is the basis of the determination of levofloxacin. All the reported methods so far work only under acidic pH as reported in below literature review.

Altiokka *et al*. (2002) [15] suggested a method for levofloxacin by flow injection analysis using techniques like potentiometry, UV detection and conductometry in pharmaceutical preparations under controlled conditios of pH. The limit of quantification (LOQ) and limit of detection (LOD) for FIA was found to be 1×10^{-7} M (S/N=10) and 3×10^{-7} M (S/N=3), respectively. Ocana *et al* (2004) [16] suggested a flow injection method by chemiluminescence detection in determination of levofloxacin, moxifloxacin and trovafloxacin in medicines. This method was based on luminescent properties of Ce(IV)–sulphite–fluoroquinolone and by addition of trivalent lanthanide as an emission sensitizer. Their concentration range was found to be of 0.5–3.5 μgmL^{-1}, 0.2–3.0 μgmL^{-1} and 0.008–0.400 μgmL^{-1}. The corresponding detection limits are found to be 0.100, 0.035 and 0.008 μgmL^{-1}, respectively.

Sun *et al*. (2006) [17] proposed a new analytical method for determination of ofloxacin and levofloxacin by chemiluminescence (CL) with flow-injection sampling which is very fast and sensitive method. It is based upon the the CL reaction of Ce(IV)–$Na_2S_2O_4$–ofloxacin/levofloxacin–H_2SO_4 system, in acidic medium. In the range of $1.0 \times 10^{-8} – 1.0 \times 10^{-7}$ g ml^{-1} and $1.0 \times 10^{-7} – 6.0 \times 10^{-6}$ g ml^{-1}, the concentration of levofloxacin or ofloxacin is linearly correlated (r^2 = 0.9988). The detection limit (S/N = 3) is 7×10^{-9} g ml^{-1}.

Momani (2006) [18] developed a spectrophotometric technique for the determination of levofloxacin in human urine and tablets in acidic medium. He proposed that with oxidation of N-bromosuccinimide (NBS), a colored product is formed in acidic medium. Under the conditions, the sampling rate was approx 90 h^{-1}, the calibration curve obtained was linear over the range 10–300 µg ml^{-1} and the detection limit was found 3 µg ml^{-1}.

Fluorescence Technique

Wong *et al*. (1997) [19] developed a method for the determination of levofloxacin in urine and human plasma. Levofloxacin was isolated from the biological matrix in a single step by liquid liquid extraction process. The method was linear from 23 µg ml^{-1} to 1464 µg ml^{-1} in urine and from 0.08 to 5.18 µg ml^{-1} of levofloxacin. Gonzalez *et al* (2000) [20] proposed a spectrofluorimetric method for determination of levofloxacin in tablets, serum and spiked human urine. The method permits the determination of Levofloxacin of 20-3000 mg ml^{-1} in aqueous solution of acetic acid–sodium acetate buffer. This method allows direct measurement of Levofloxacin in urine 420 µgml^{-1} and spiked human serum 5 µg ml^{-1} in 8 mM solution of sodium dodecyl sulphate at pH 5.

Neckel *et al.* (2002) [21] reported simultaneously determination of levofloxacin and ciprofloxacin in microdialysis and plasma samples by reversed-phase high-performance liquid chromatography (HPLC) technique and fluorescence detection. The sample was prepared and separated in the isocratic mode within 10-12 min. The calibration curve for levofloxacin was linear from 0.0156 to 5 µgml^{-1} and 0.02–12.5 µgml^{-1}. The limits of quantification for ciprofloxacin and levofloxacin were 0.1 µgml^{-1} and 0.015 in microdialysis samples 0.1µgml^{-1} and 0.02 in plasma samples, respectively. This can be used as a routine method to investigate the microdialysis and plasma samples.

Djabarouti *et al.* (2003) [22] described a method for the determination of levofloxacin in bone tissues, plasma and bronchoalveolar lavage. The method used was based upon ultraviolet radiation at 299nm and separation with Supelcosil ABZ^{+} column. The sample was extracted on completely automated liquid-solid extraction with the help of OASIS cartridge. It has found to be linear in the concentration from 0.25–25 µg/ml for levofloxacin in plasma, 1–6 µg/ml in

bronchoalveolar lavage and 0.5–10 µg/g for bone tissues. This method was also applied to pharmokinectic analysis of levofloxacin in human infectious such as bone and tissues.

Santoro *et al.* (2006) [23] developed a method for the estimation of fluoroquinolones of third generation i.e lomefloxacin (LOM), gatifloxacin (GAT), levofloxacin (LEV) and pefloxacin (PEF) in medicines and in preparations of injections. The mobile phase consists of H_2O:CH_3CN (80:20, v/v) with 0.3% of triethylamine and at pH 3 with phosphoric acid. The flow rate of 1.0 mL/min was pumped and detection under UV was done with wavelength in the range of 279 to 295 nm. The calibration curves were found to be linear over a concentration range from 4.0 to 24.0 µg/mL.

Ulu (2008) [24] proposed a method for the determination of three antibacterials fluoroquinolones (FQ) i.e. Enrofloxacin (ENR), Ofloxacin (OFL) and Levofloxacin (LEV) with 2,3,5,6-tetrachloro*p*-benzoquinone (chloranil,CLA) through charge transfer (CT) complex formation. At the optimized conditions, the complexes of the FQ–CLA showed the maxima excitation from 359-363nm and maxima emission 442-488nm. The detection limit is 16 μgmL^{-1} for ENR, 17 μgmL^{-1} for LEV, 8 μgmL^{-1} for OFL, respectively. Xia *et al*. (2012) [25] developed a method for the estimation of fluoroquinolones i.e Norfloxacin, Gatifloxacin, Ofloxacin and Ciprofloxacin based upon the mechanism of quenching of fluorescence. Under the optimized conditions, the calibration curves were found to be linear in the concentration range of 0.01 to 4.0 μgml^{-1} for all the drugs. The limit of detection was found out to be 0.009 $\mu g\ mL^{-1}$ (Ofloxacin), 0.010 $\mu g\ mL^{-1}$ (Ciprofloxacin), 0.012 $\mu g\ mL^{-1}$ (Norfloxacin) and and 0.016 $\mu g\ mL^{-1}$ (Gatifloxacin).

Locatelli *et al.* (2015) [26] reported an MEPS–HPLC–PDA method for the estimation of levofloxacin and ciprofloxacin in human sputum sample taken from cystic fibrosis (CF) patients. The drugs were resolved using an isocratic solution with a period of time of 15 min. The concentration range is from 0.05 to 2 µg/ml for both the reagents in human sputum. The limit of quantification was found to be 0.05 µg/mL for both reagents. The standard curves matched with weighed matrix showed a good linearity range upto 2 µg/mL. Ren and Zhu (2015) [27] developed a new fluorimetric method for the determination of Levofloxacin(LFX) , which is based on the fact that fluorescence intensity of Levofloxacin could be enhanced dramatically by methyl-β-cyclodextrin, (methyl-β-CD) and cetyltrimethyl ammonium bromide

(CTAB). The results showed that under certain conditions, methyl-β-CD and LFX can form a stable inclusion complex, which make LFX better adapt to the less polar and more rigid micro environment, so the fluorescence intensity of LFX increases; CTAB can change the micro environment, improve the fluorescence quantum yield, and fluorescence intensity increased further.

Spectrophotometric Technique

Mostafa *et al*. (2001) [28] suggested a method for the determination of an antibacterial drugs i.e Enrofloxacin, Ciprofloxacin and Perfloxacin by charge transfer complex formation with three different types of acceptors. This method was developed for the estimation of Enroxil oral solution, Peflacin tablets, Ciprocin tablets and Peflacin ampules. Tetracyanoethylene (TCNE) was also used in the determination forming the charge transfer complexes with the aborbance at 335 nm for ciprofloxacin and at λ_{max} 290 nm for both enrofloxacin and pefloxacin. The reagent 2,3 -dichloro-5,6-dicyano-p-benzoquinone (DDQ) is used for the estimation of Perfloxacin which leads to the formation of charge transfer complexes with max absorbance at 460nm. The mean percentage accuracy for the estimation of peflacine tablets and peflacine ampules are 100.40 ±0.76 and 99.91±0.623, respectively.

Lakshmi *et al.* (2004) [29] developed three rapid methods for the determination of levofloxacin in pharmaceutical formulations. The two methods are based upon the formation of ion-association complexes of fluoroquinolones with bromocresol green and Eriochrome black T. After the extraction with chloroform, the absorbance was measured at 420nm and 490nm. The third method is based upon the formation of blue coloured chromogen with reagent Folin-Clocalteau which showed absorbance at 720nm.

Brashy *et al.* (2004) [30] proposed two methods for the determination of fluoroquinolones levofloxacin, norfloxacin and ciprofloxacin. In the first method, norfloxacin and levofloxacin is treated with dye bromocresol green (BCG) in solvent dichloromethane while ciprofloxacin is treated with the same dye bromocresol green (BCG) but in aqueous acidic buffer. In case of levofloxacin and *norfloxacin*, instantaneously yellow colored compounds are formed and in case of ciprofloxacin the extraction with dichloromethane is done. The species formed was detected spectrophotometrically at their absorption maxima at 411 nm for levofloxacin and 412

nm for norfloxacin and ciprofloxacin. The second method included the reaction of p- chloranilic acid with levofloxacin and norfloxacin with tetracyanoethylene (TCNE) in solvent acetonitrile to provide the complexes with maximum absorbance at 521 and 333 nm for both the two drugs, respectively. In the first method, the calibration graphs were linear from the range 1- 20 μg ml $^{-1}$ in the second method, the concentration range was over 15-250 μg ml $^{-1}$ for levofloxacin and 0.8-16 μg ml $^{-1}$ for Norfloxacin.

Brashy *et al.* (2004) [31] proposed two methods for the determination of norfloxacin, levofloxacin and ciprofloxacin in tablets and spiked human urine. In both the methods formation of binary complex takes place between the fluoroquinolone and the two xanthenes dyes i.e merbromin in aqueous buffered medium and eosin Y. Under the optimized experimental conditions, the max absorption of complexes were showed at 547 nm for eosin Y and 545 nm for dye merbromin. The calibration graphs were found to be linear in the concentration range of 2–8 μg ml^{-1} for the all three fluoroquinolones. In case of dye merbromin, the concentration range was found in the 2–15 μg ml^{-1}.

Sebaiy (2011) [32] proposed a method for the estimation of levofloxacin HCl, gatifloxacin and lomefloxacin HCl in pharmaceutical formulations. The method involves the formation of yellow and blue coloured ion-pair complexes between the provided three drugs and dyes, bromophenol red and fast green FCF with their absorption maxima at 426-430 nm and 631 nm, respectively. The stoichiometry of the complexes was found to be 1:1. In the fast green FCF method, Beer's law was observed in range of 0.2-1.8 μg/ml^{-1} for all given three drugs and in case of bromophenol red dye, it was observed in the range of 10-70 μg/ml^{-1} for lomefloxacin HCl and levofloxacin HCl and 10-55 μg/ml^{-1} for gatifloxacin. The amounts acquired by the suggested methods are between 99.80% and 100.37%. Shoaibi and Gouda (2012) [33] proposed a method for the determination of tropicamide (TPC). In this method formation of ion pair complexes take place between the basic nitrogen of the moiety drug with the methyl orange (MO) and bromocresol purple (BCP) in the acidic medium. The complexes were extracted in chloroform and maximum absorbance was found at 408 nm and 427 nm for BCP and MO, respectively. Beer's law was observed in the range 1.0–16 μg ml^{-1} with value of correlation coefficient (n=6) ≥0.9991.

Prashanth *et al*. (2013) [34] suggested the two spectrophotometric methods for the estimation of ofloxacin (OFX). Formation of ion-pair association complexes take place by reaction between drug OFX and acidic sulphonphthalein dyes i.e bromocresol green and bromocresol purple in solvent dichloromethane. The conditions were optimized such as reaction time, reaction medium and reagent concentration to attain the maximum sensitivity. Both the dyes are treated with drug OFX and formation of yellow coloured ion- pair complexes take place. The Beer's law is showed in the concentration range of 1.0- 16 μgml^{-1} of OFX. The molar absorptivity values obtained are be 2.40×10^4 and 1.97×10^4 l mol^{-1} cm^{-1} for both the procedures. A Job's plot showed (1:1) ratio of absorbance versus molar ratio of drug.

Mahmound (2014) [35] proposed three spectrophotometric methods for the determination of lomefloxacin HCL, levofloxacin HCL and sparfloxacin in pure and pharmeceutical dosage by using an oxidimetric reagent i.e bromate-bromide. These drugs were treated with insitu generated bromine in excess and further they are reacted with either of methylene blue, thymol blue or methyl orange. Their absorbance was detected at 678nm, 545nm and 510nm, respectively. In case of methylene blue, Beer's law was obeyed in the range of 0.05-1.0 µg/ml for lomefloxacin HCL and levofloxacin HCL and 0.1- 1.4 µg/ml for sparfloxacin, 0.25-2.75 µg/ml for lomefloxacin HCL and levofloxacin HCL and 1.0 – 5.5 µg/ml for sparfloxacin in case of thymol blue and 0.1-1 µg/ml^{-1} for lomefloxacin HCL and levofloxacin HCL ,0.1-1.8 µg/ml^{-1} for sparfloxacin in case of methyl orange.

Qassim (2015) [36] proposed a reproducible spectrophotometric method for the determination of Ciprofloxacin in pure and pharmaceutical formulation. The described method was based upon the formation of ion-pair association complexes between drug Ciprofloxacin Hydrochloride and dyes Bromothymol Blue and Bromocresol Purple which were extracted by chloroform. Formation of orange-yellow coloured compound took place and its absorbance was detected at 440 nm and 450 nm, respectively. The calibration curves were found out to be linear in the concentration range of 5-55 μgml^{-1} for Bromocresol Purple and 9-72 μg ml^{-1} for Bromothymol Blue complexes, respectively.

Voltammetric Technique

Radi and Sherif (2002) [37] proposed a method for the estimation of levofloxacin by square wave anodic stripping voltammetry on glassy carban in human urine. The drug was assembled on a glassy carban electrode and oxidation peak was found at +0.4 V versus Ag/AgCl for 300s in acetate buffer at pH 5.0. The linear calibration graph was found in the concentration range of 6.0×10^{-9} to 5.0×10^{-7} M. The detection limit was obtained to be 5.0×10^{-9} M. Radi *et al*. (2003) [38] developed a method in which interaction of drug levofloxacin with double stranded calf thymus DNA was investigated at glassy carban electrode by cyclic voltammetry. The linear dependence graphs of the peak currents was found in the concentration range from 5.0×10^{-7} to 5.0×10^{-6} M. the detection limit of procedure was found to be 25 $\mu g/ml^{-1.}$ This method was tested in human urine samples for estimating the levofloxacin. Humans were given a oral dosage of 500 mg of drug for urinary infections. Samples of urine were collected per dose at 0-12, 12-24 and post dose at 24-36 h.

Sun *et al*. (2007) [39] described the interaction between the heparin and crystal violet by using technique voltammetry and absorbtion spectrophotometry at acidic pH 3.0 Britton-Robinson (B-R) buffer solution. Formation of supramolecular ion association complex takes place as heparin molecule is negatively charged easily bind to the positively charged crystal violet which leads to the change in the max absorption of crystal violet at 592 nm decreased and after the addition of molecule heparin, two new absorption peaks were found at wavelength 510 nm and 363 nm. The change in oxidation peak current was also observed in crystal violet on glassy carbon electrode (GCE) at +0.84V simultaneously with no change in peak potential which indicated the formation of supramolecular ion association complex. This method also developed the new spectrophotometric method for the molecule heparin in the linear range between 0.10–4.0 mg L^{-1}. The value of relative standard deviation for eleven parallel estimations of 0.40 mgL^{-1} in heparin was found to be 1.69% and the limit of detection was 0.09 L^{-1}. .

GAP IN STUDIES

A lot of work has been done in the field of determination fluoroquinolones using various techniques such as Voltammetry, Spectophotometry, Fluorescence, Flow Injection Technique, High Performance Liquid Chromatography (HPLC), Chemiluminescence, Nuclear Magnetic Resonance Spectroscopy, Capillary Zone Electrophoresis etc under the optimized conditions in acidic medium. The methods reported in literature included DMF, DCM for the extractive determination of ion-pair. There is no report on the ion-pair complex based determination of levofloxacin in which various conditions like solvent medium, pH, temperature and reaction time need not to be optimized. The proposed method is the first report in which green solvent i.e water has been used as a medium for the formation of ion-pair complex without any optimization of various reaction conditions. This method has a significance of easy formation of ion-pair, less reaction time and use of green solvent (Water). The proposed method is suitable for the determination of the drug in a wide concentration range 6×10^{-5} M to 24×10^{-5} M, with a low limit of detection (LOD) as 6×10^{-5} M.

EXPERIMENTAL

4.1 Instrumentation

Fourier transform infrared (FTIR) analysis of samples was carried out in KBr pellets using Agilent Resolutions Pro (Cary 660) Spectrometer in the range of 400-4000 cm^{-1}. Analytic Jena Spectrophotometer using slit width of 1.0 cm and matched quartz cells was used for spectrophotometric measurements

4.2 Materials and Reagents

All chemicals and reagents were of analytical reagent grade and used without further purification. The solvents used in spectrophotometric were of HPLC grade (Sd Fine, India). Distilled acetonitrile was stored on molecular sieves before use. Distilled water was used throughout the experiment. Pure levofloxacin as a hemihydrate was provided by Saurav Chemicals, Derabasi (Patiala). All the spectrophotometric studies were performed at room temperature.

4.3 Standard stock solutions

Stock solutions of levofloxacin (LFX) [10^{-3} M] and picric acid (PA) [10^{-3} M] were prepared in distilled water and acetonitrile, respectively in two separate 50 mL volumetric flasks.

4.4 Procedure for drugs in pure form

In a 25 mL separatory funnel, 3mL each of LFX [10^{-3} M] and PA [10^{-3} M] were added in 1:1 stoichiometric ratio. Shook the mixture well for 5-10 minutes. On keeping the reaction medium for few minutes in separatory funnel a bright yellow colored solid separated out from the reaction mixture as a ion-pair complex of LFX with PA. Bright yellow colored solid was filtered out through Whatmann filter paper, washed several times with distilled water and dried overnight in oven at 60-70°C.

4.5 Procedure for drugs in dosage form

Three series of single tablets in triplicate of levofloxacin were powdered and dissolved in distilled water to give a final concentration of 10^{-3} M levofloxacin. Solutions were prepared taking into consideration mass of levofloxacin in each tablet as provided by the manufacturer. Same procedure was applied for ion-pair formation as described in section 4.4.

4.6 UV-Vis Technique

All the spectrophotometric studies were carried out using 20 µM solutions prepared from stock solutions of 10^{-3} M concentrations. All the measurements were done at room temperature from freshly prepared solutions. Initial runs were carried in the range 200-1100 nm to know the maximum wavelength values.

4.6.1 Preparation of solutions

Stock solutions of LFX and PA were prepared in CH_3CN, CH_3CN:H_2O (9:1 v/v) and H_2O solvent media. For UV- vis spectra, 20 µM solutions of LFX, PA and LFX-PA were prepared by taking 60 µL of 10^{-3} M stock solution and then making the total volume to 3 mL by CH_3CN, CH_3CN:H_2O (9:1 v/v) or H_2O depending upon the solvent medium under study. Initial readings were taken in CH_3CN as solvent medium and then effect of solvent was studied by changing the solvent from pure CH_3CN to CH_3CN:H_2O (9:1 v/v) and then to 100% H_2O.

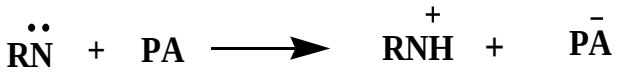
4.6.2 Calibration curve analysis

For quantitative analysis, ion-pair was generated first by changing the volume of [10^{-3} M] LFX and [10^{-3} M] PA. Volume taken for ion-pair generation was studied in the range 3 mL-12 mL and their corresponding solution of concentration 20 µM were prepared separately in three different solvent media (CH_3CN, CH_3CN:H_2O (9:1 v/v) and H_2O). UV-vis spectra of these freshly prepared solutions were recorded to determine the change in λ_{max} with change in concentration of LFX and calibration curve was plotted between the absorbance values and LFX concentration in three solvent media to analyze the better solvent for drug (LFX) determination.

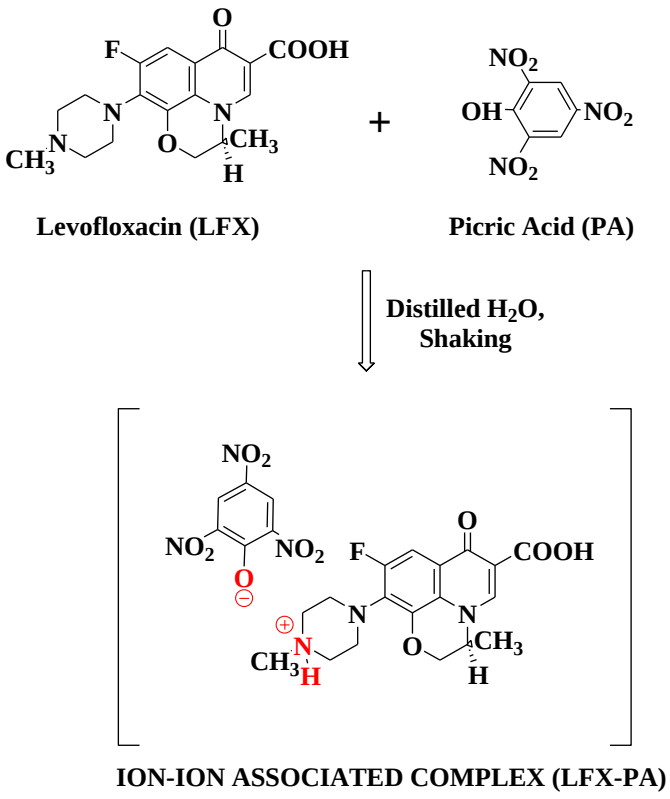
RESULTS AND DISCUSSION

The fluoroquinolone contains a terminal nitrogen atom in its piperazine moiety which gets protonated in acidic medium to form positively charged quaternary ammonium group which in turn forms ion pair complex with anionic dyes. This work was undertaken with picric acid (PA)

as the anionic dye which contains three nitro groups and can easily generate negative charge on the hydroxyl group. The reaction scheme can be given as follows:



Ion pair (LFX-PA) is formed between the levofloxacin (LFX) and picric acid (PA) in pure aqueous medium. Spectrophotometric studies were performed to elucidate the formation of ion pair. The proposed mechanism for the ion pair formation by reaction of picric acid and levofloxacin is shown in Scheme 1.



Scheme 1: Proposed mechanism of ion-pair (LFX-PA) formation

5.1 Characterization of the Ion-Association complex by FTIR spectroscopy

FTIR spectra of ion-association complex shows the presence of characteristics absorption bands due to varied force constants in the acceptor and donor species due to ion-association mechanism taking place. In case of LFX, characteristic peak at 3264 cm^{-1} was due to carboxylic group, bands between 2800-3000 cm^{-1} appear due to alkane group stretching, 1724

cm^{-1} due to carbonyl group stretching, 1291 cm^{-1} and 1089 cm^{-1} due to stretching vibrations of amines and halogen group, respectively. PA contains three nitro groups for which stretching frequencies cannot be equivalent. Nitro groups ortho to phenolic hydroxyl group get hydrogen bonded intramolecularly with hydroxyl group. As a result of these hydrogen bonded nitro groups, the asymmetrical stretching frequency of nitro group gets rotated out of plane to the ring, while the para nitro group is coplanar to the ring [40]. Due to different types of interaction of nitro groups, asymmetrical stretching frequencies of three nitro groups are not resolved and appear at one broad band at 1528 cm^{-1}.

In case of ion- ion association complex (LFX-PA), characteristic absorption bands of LFX i.e, the alkanes stretching frequencies (2800-3000 cm^{-1}), carbonyl group stretching (1724 cm^{-1}) and amine stretching frequency (1291 cm^{-1}) get shifted to their respective lower frequencies. While the asymmetric stretching of nitro groups of PA gets shifted to higher frequency and appears at 1552 cm^{-1} as shown in Figure 1. Major characteristic band of LFX-PA appears at 3450cm^{-1} due to $N^{+}H$ formed after an ion association between LFX and PA. Characteristic peaks of LFX, PA and LFX-PA are tabulated in Table 1.

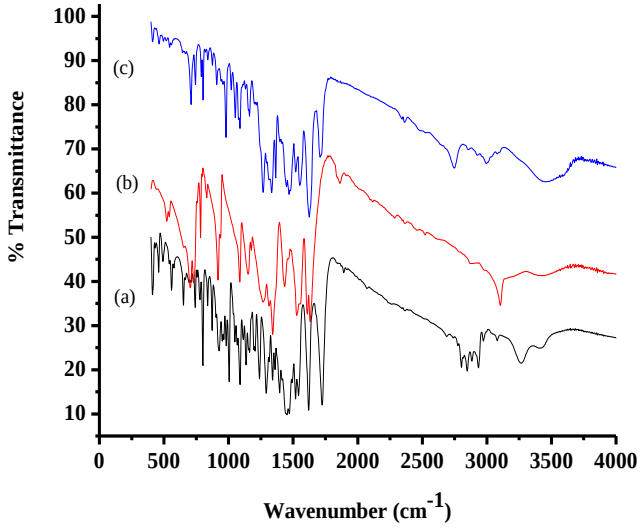


Figure 1: FTIR spectrum of (a) LFX (b) PA (c) LFX-PA Complex

Table 1: Characteristics absorption frequencies of LFX, PA and LFX-PA.

Absorption frequencies in cm^{-1}			Assignment of
LFX	**PA**	**LFX-PA**	**Bands**
3411	3432	--	υ_{OH} (free)
3264	3104	--	υ_{OH} (H-bonded)
		3450	υ_{NH+}
2935	--	2955	υ_{C-H}
2847	--	2748	
2802	--	--	
1724	--	1709	$\upsilon_{c=o}$ (stretching)
1291	--	1267	υ_{C-N} (stretching)
--	1528	1552	$\upsilon_{(NO2)\ asym}$
--	1342	1333	$\upsilon_{(NO2\ )\ sym}$
1089	--	--	Halogen (F group)
--	782	909	$\upsilon_{(NO2)\ stretching}$

5.2

Spectrophotometric characterization of the ion-association complex of LFX-PA

The UV-Vis spectrum studies on the drug LFX and PA at 20 µM each were investigated in CH_3CN as solvent medium. LFX showed absorption bands at 226 nm and 298 nm while PA showed bands at 235 nm and 330 nm due to л-л* and n-л* transitions, respectively. The ion pair (LFX-PA) shows the absorption bands at 220 nm, 290 nm, 335 nm and 375 nm, as shown in Figure 2. The UV-Vis. spectrum of the ion-pair complex shows a broad band having maximas (λ_{max}) at 335 nm and 375 nm. The band at 330 nm in the spectrum of LFX gets broadened in LFX-PA with two maximas at 335 nm and 375 nm. This is due to dispersal of electron cloud of lone pair on phenolate anion (of PA) under electrostatic attraction by quaternary amine of LFX.

Absorption bands of LFX-PA (226 nm and 298 nm) got blue shifted by few nanometers from those of LFX. The absorption ratio of band at 235 nm of PA decreases sharply from 1.28 a.u. to

0.316 a.u., indicating 4 fold sharp absorption changes due to interaction of LFX with PA. A new broad band appeared in the absorption spectrum of LFX-PA at 375 nm responsible for bright yellow color of the product formed.

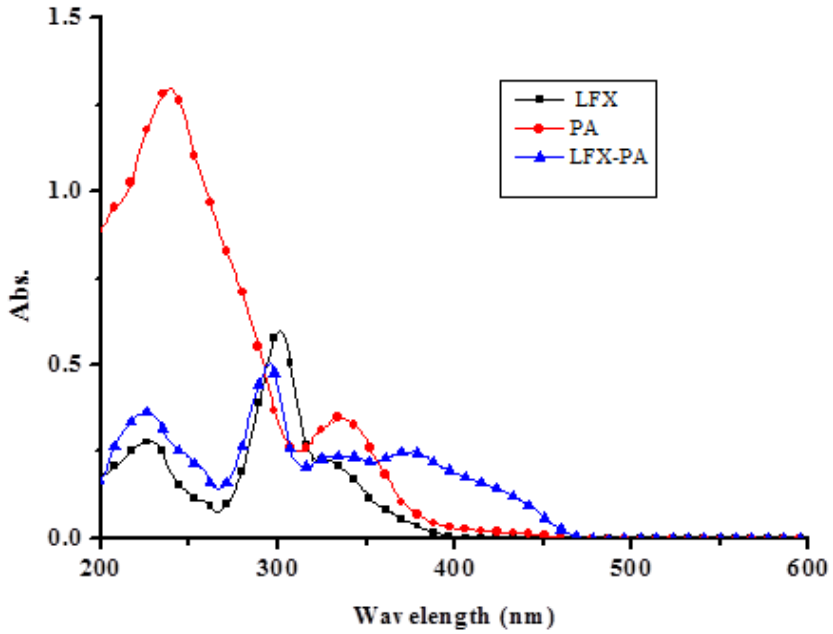


Figure 2: Absorption spectra of LFX, PA and LFX-PA in acetonitrile medium.

Time for formation of LFX-PA ion pair

The extent of time required for the complete formation of ion pair was studied carefully and shown in Figure 3. An absorption intensity of PA at 235 nm was monitored over the reaction time. A sharp decrease of band intensity at 235 nm indicated progress of the reaction in terms of ion-pair formation. It is clear from the figure that complete formation of ion pair needs 5 - 6 minutes before filtration through the Whatmann filter paper.

Stoichiometry

The nature of binding of reagent to the drug was determined by continuous variation method [41]. The results indicated that 1:1, [PA]: [drug] ion-pair is formed through electrostatic interactions between positively charged protonated LFX and negatively charged PA, as shown in the proposed mechanism (Scheme 1).

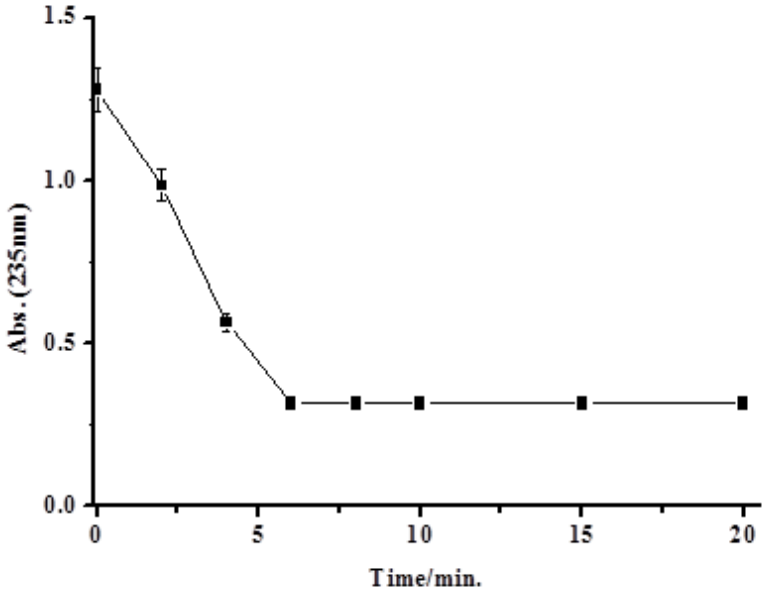


Figure 3: Effect of time on ion-pair formation.

It is proposed that in the aqueous medium the reactants LFX and PA are more stable in their respective ionic forms and the two species are held together through electrostatic forces, resulting in the formation of ion-pair (LFX-PA). We suggested that species formed between LFX and PA has an ion-association character. This interpretation is in fair agreement with the

results of the examination of fluoroquinolone compound and anionic forms of other organic substances [42-44].

Effect of solvent

Levofloxacin is an amine containing three nitrogen atoms with a lone pair on each atom. The nitrogen atom of piperazine being attached to electron donating methyl group is most basic in nature and gets protonated easily on coming in the vicinity of hydroxyl group of picric acid. This protonation of LFX results in the formation of an ion-pair with PA in an instantaneous reaction. The proposed method of detection of the antibiotic is based on its characteristics to form ion-pair with strongly acidic species like picric acid. The hypothesis that levofloxacin forms stable ion pair has been verified by UV- Visible spectroscopy. UV spectrum of the substrate (LFX), its ion-pair and the reagent (PA) were recorded separately in acetonitrile medium. The spectra were taken again by changing the solvent medium from pure acetonitrile to its mixture with 10% water and in pure aqueous media. A description of absorbances recorded in all the three media are given in Table 2. Some interesting observations can be made from the absorbance values as summarized below:

1. UV-Vis spectrum of LFX-PA in acetonitrile shows four absorbance bands at 220 nm, 290 nm, 335 nm, and 375 nm. The bands at 220 nm and 290 nm can be assigned due to л- л* transition and 335 nm and 375 nm can be assigned to n- л* transition.
2. In partially aqueous medium the n- л* transitions are severely affected with almost quenching of absorbances, where water being polar in nature would tend to stabilize the ion-pair. In pure aqueous medium, the absorption band at 375 nm is lost completely proving thereby the existence of ion-pair of levofloxacin with picric acid.

In case of LFX, two bands observed at 226 nm and 298 nm are due to л- л* transition. On addition of water in acetonitrile medium, major λ_{max} gets red shifted and appears at 301 nm with a low absorption band at 334 nm; due to the stabilization of LFX transition and hence move to high λ value. In case of pure water medium, absorption band at 334 nm gets further intensified as compared to that in CH_3CN:H_2O (9:1; v/v) medium.

Absorption spectra of picric acid were also observed in different solvent media. In pure acetonitrile medium, the charge transfer band was observed at 330nm. This absorption band gets shifted to 366 nm in CH_3CN:H_2O (9:1; v/v) medium. In pure water medium, these bands are observed at 356 nm 404 nm respectively. Since the ion-pair complex shows sharp and clear bands in water as a medium, hence water can be used as a suitable medium for quantitative study of LFX after its conversion to LFX-PA complex. Further studies were carried out using water as a given solvent for the determination of the drug.

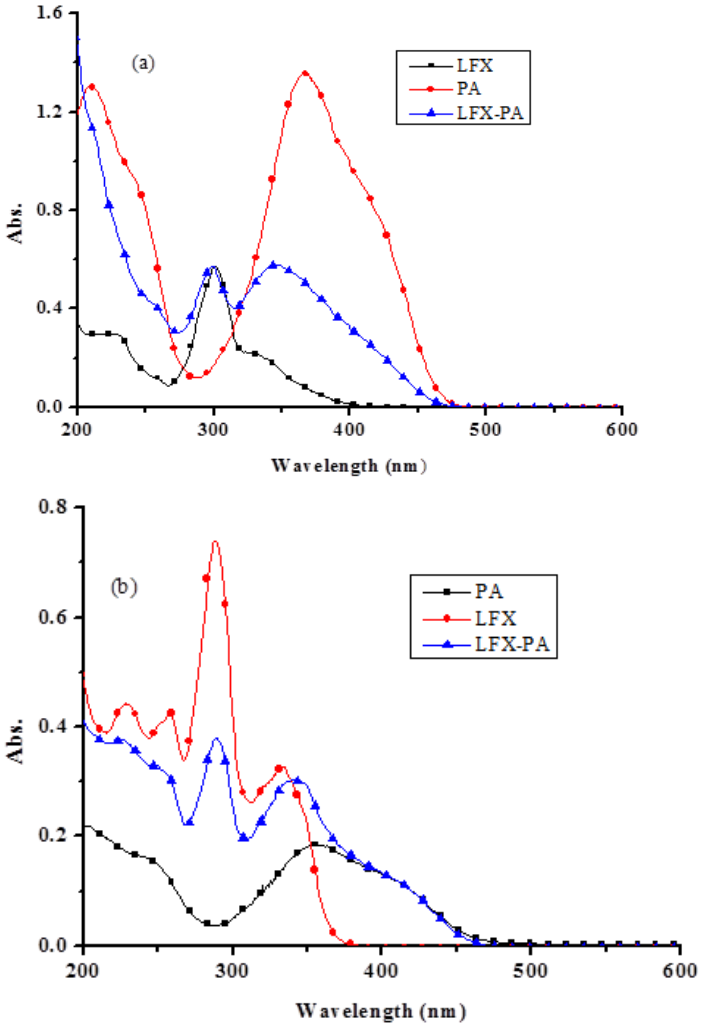
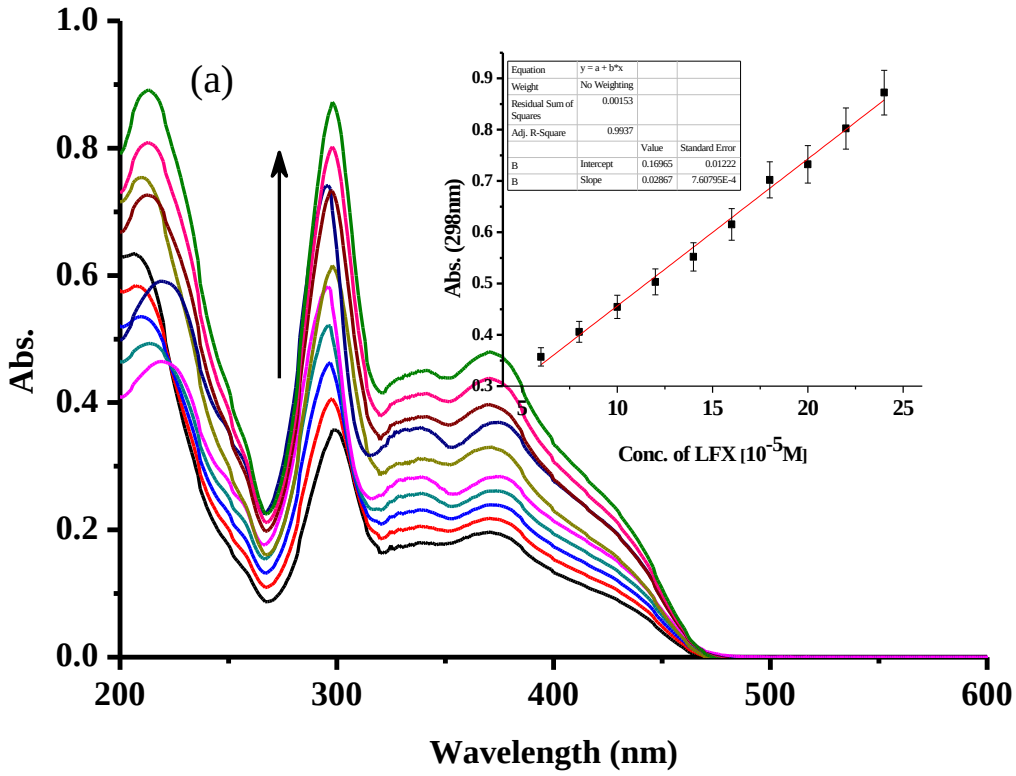


Figure 4: Absorption spectra of LFX [10^{-3}M], PA [10^{-3}M] and LFX-PA [10^{-3}M] in (a) CH_3CN: H_2O medium (b) H_2O medium.

Quantitative study

Picric acid has been used as a reagent for the extractive spectrophotometric determination of levofloxacin. Typical calibration graphs for the determination of the drug were obtained under the optimized conditions as shown in Figure 5 in different solvent systems. In case of acetonitrile, absorbance values for LFX-PA increased linearly with increasing concentration of LFX in the working range 6×10^{-5} - 24×10^{-5} M at 298 nm, as shown in Figure 5a with correlation coefficient value 0.99, intercept 0.17 and slope 0.028. Similarly, calibration graphs were plotted for LFX in CH_3CN:H_2O (9:1; v/v) and H_2O as solvent media in the concentration range (6×10^{-5} - 24×10^{-5} M and 6×10^{-5} - 24×10^{-5} M, respectively) which also showed linear responses with correlation coefficient values 0.966 and 0.967, respectively (Figure 5b and 5c).



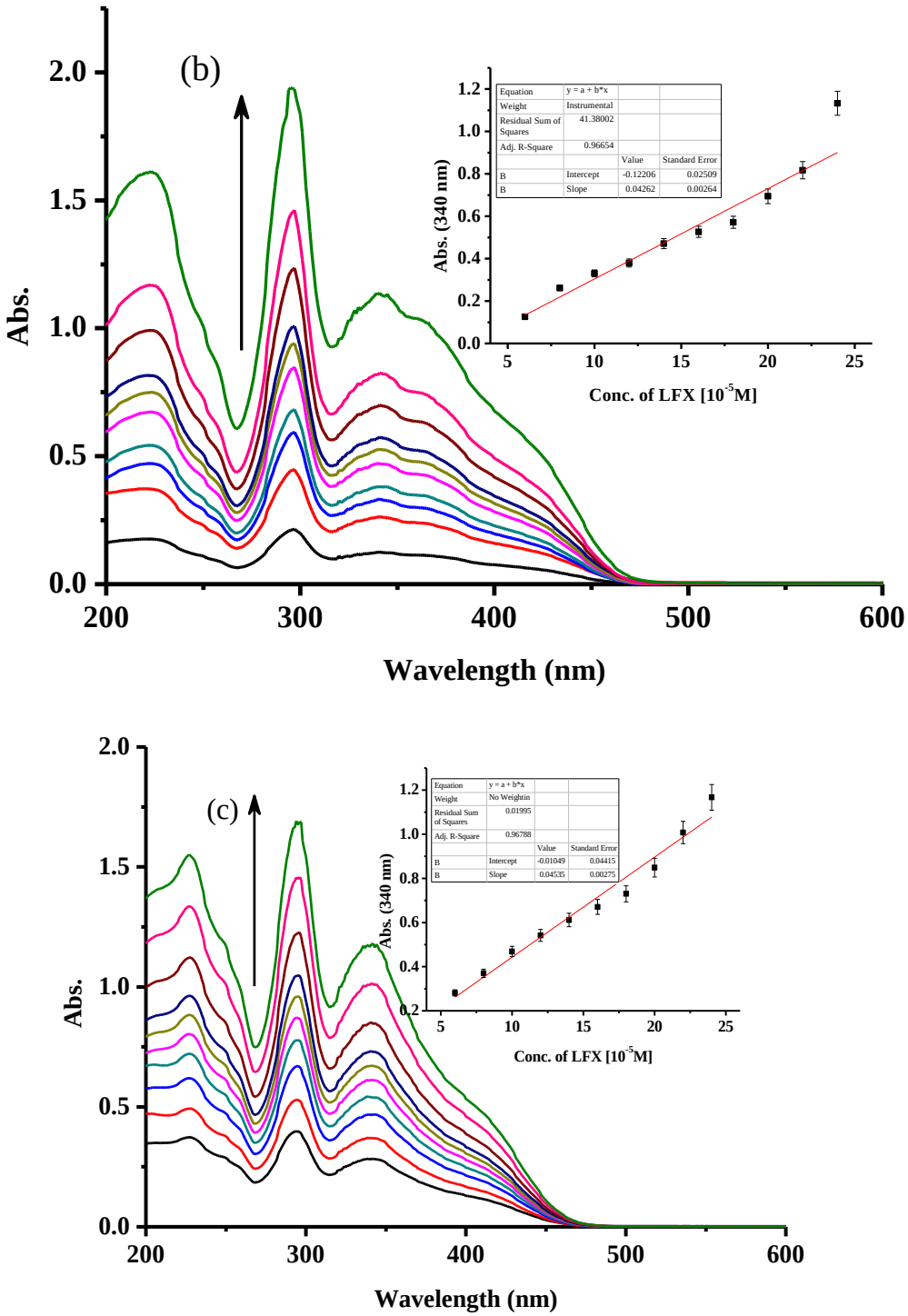


Figure 5: Absorption changes of LFX-PA with increasing concentration of PA (6×10^{-5} M - 24×10^{-5} M) in reaction mixture in (a) CH_3CN (b) CH_3CN: H_2O (c) H_2O as solvent medium. Insets showing their respective calibration plots.

Table 2: Absorption values of LFX, PA, LFX-PA in different solvent systems.

S. No.	SOLVENT	REAGENT	λ_{max} (nm)	Absorbance Values
1.	CH_3CN	LFX [10^{-3}M]	226	0.277
			298	0.577
		PA[10^{-3}M]	235	1.28
			330	0.338
			220	0.349
		LFX-PA[10^{-3}M]	290	0.456
			335	0.238
			375	0.262
2.	$CH_3CN:H_2O$ (9:1; v/v)	LFX[10^{-3}M]	301	0.568
			334	0.193
		PA[10^{-3}M]	209	1.305
			366	1.356
		LFX-PA[10^{-3}M]	295	0.547
			340	0.570
3.	H_2O	LFX[10^{-3}M]	228	0.439
			259	0.421
			289	0.738
			334	0.322
		PA[10^{-3}M]	356	0.180
			404	0.127
			229	0.375
		LFX-PA[10^{-3}M]	251	0.320
			289	0.373
			339	0.303

5.3 Real life sample analysis

The proposed method was successfully applied for the determination of levofloxacin content in commercial tablets. To establish validity of this proposed method, recovery test was performed by using standard addition technique. Recovery is calculated as:

% recovery = (measured levofloxacin × 100) / added levofloxacin

The calibration graph for LFX (as LFX-PA ion pair) was repeated at least 5 times and a slope of 0.03±0.001 was observed. Using this calibration curve levofloxacin was determined from the commercial samples purchased from the market. Following the recommended procedure for the extraction of the levofloxacin from the commercial samples into the aqueous medium, the concentrations of active component of the drug was determined and results are in given Table 3. Concentration values obtained by this method for different series of tablets are shown in the table. Results given in Table 3 reveal that recoveries obtained were in the range 94.6% to 99.9% proving the validity of the proposed method.

Table 3: Recovery studies using standard addition method.

Pharmaceutical Preparations	**Proposed method**		
	Spectrophotometric method		
	Taken (μg/mL)	**Found (μg/mL)**	**Recovery (%)***
Levomac tablets[a] (Levofloxacin, 500 mg/tablet)	20.0	18.9	94.6
	40.0	39.2	98.0
	60.0	59.4	99.0
	100.0	98.6	98.5
Levac tablets[b] (Levofloxacin, 750 mg/tablet)	20.0	19.9	99.9
	40.0	39.5	98.8
	80.0	79.4	99.3
	100.0	99.3	99.3
Levomac tablets[c] (Levofloxacin, 250 mg/tablet)	20.0	19.7	98.8
	40.0	39.5	98.9
	60.0	58.9	98.3
	100.0	99.6	99.6

* Average of at least 3 determinations.
[a] MACLOEDS PHARMACEUTICAL LTD., MUMBAI. (Batch No. KLB502A).
[b] Franklin Laboratories Pvt. Ltd. Roorkee. (Batch No. LYC75-14).
[c] MACLOEDS PHARMACEUTICAL LTD., MUMBAI. (Batch No. ALC401C).

CONCLUSIONS

A new spectrophotometric method was developed for the determination of levofloxacin at ppm level. The most important findings are that the method uses water - a green solvent as a medium of spectrophotometric determination. Picric acid having three nitro groups was used as an anionic reagent for the spectrophotometric determination of levofloxacin. Picric acid facilitates the removal of H^+ ion from phenolic hydroxyl group in aqueous medium, which eventually forms the ion association at the nitrogen center of piperazine moiety of levofloxacin. The method described herein has many advantages; easy synthesis of ion pair complex, green method of detection of levofloxacin as no organic solvent used for its synthesis, stability of the complex, less time consuming, sensitive, spectrophotometriclly active. This is the first report of its kind where maintenance of pH is not required while doing spectrophotometric determination by ion-association method. Reproducibility of the method was also checked by repeating the calibration curves, a number of times. The method has a low RSD of ±2% and is workable in the concentration range 6×10^{-5} M to 24×10^{-5} M, of LFX.

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