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Development and Validation of HPLC Method for the Estimation of Levofloxacin in Bulk and Marketed Dosage form

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ABSTRACT:

Development and Validation of a High-Performance Liquid Chromatographic Analytical Procedure for Determining Levofloxacin in a Bulk and Marketed Dosage Formis described in this paper. The Separation was made with a C18 Symmetry (4.6 X 150mm,5µm) Column at ambient temperature, with isocratic mode and mobile phasePhosphate Buffer pH 2.8 : Acetonitrile 35:65 v/v. Eluent was monitored at 284nm and the flow rate was 1.0ml/min. LevofloxacinwaseffectivelySeparatedwithretentiontime(RT)of3.661minand5.116min respectively, within the
selected chromatographic conditions. The Method was validated for AnalyticalParameters: Specificity, Linearity, Precision, Accuracyand Limits ofDetection and Quantitation. The Calibration curves were linear in the concentration range of 10 µg/ml for Levofloxacin and the Regression was found to be 0.995 respectively (NMT 0.999) for Levofloxacin. The % recovery for 50%, 100% and 150% accuracy level of Levofloxacin was found to be within the range of 99.3-100.3% respectively for Levofloxacin. This Analytical Procedure is applicable for the Quality Control of Drug Formulations.

Keywords: Levofloxacin, Stationary Phase, Mobile Phase, HPLC, Validation, Phosphate Buffer, Acetonitrile.

INTRODUCTION:

Antibiotics:

Antibiotics are the most frequently falsified and adulterated pharmaceutical products, most likely due to their widespread usage.Antibiotic misuse promotes the development and dissemination of antibiotic resistance and can result in super infections. Drug-resistant strains of microorganisms arise due in part to the fact that many antibiotics are bacteriostatic in nature rather than bactericidal. Accurate assessment of antibiotic potency and bioactivity is crucial for addressing the resistance issue and ensuring safe antibiotic use. The determination of the true concentration of active ingredients in antibiotic preparation is crucial due to the growing issue of resistance.The actual efficacy of antibiotic preparations may be affected by slight variations in the concentration of the active ingredient. Since antibiotics are often the medications that stand between life and death, it is imperative to quantify the active pharmaceutical ingredient (API) in their preparation. Microorganisms have been shown to be completely destroyed or partially inhibited by these substances at

very low concentrations.[1] Both chemical and biological techniques can be used to assess an antibiotic's potency. Levofloxacin has been quantitatively determined in formulations as well as in human urine and serum using chemical techniques like capillary electrophoresis, ultraviolet (UV) spectrophotometry, high performance liquid chromatography (HPLC), and high performance thin layer chromatography (HPTLC). Nevertheless, no pharmacopoeia has yet to publish the microbiological test for levofloxacin potency determination. The most practical approach for figuring out an antibiotic's potency is the biological method.[2]Biological activity, active component estimation, and antibiotic stability monitoring are all supported by microbiological assay. The change in antimicrobial activity will indicate any minor modification to the antibiotic molecule that might go undetected using chemical techniques. Thus, microbiological assay is very helpful in clearing up any confusion regarding potential changes in the potency of antibiotics and the preparations that contain them. Effective and thoroughly characterized microbial strains are necessary for a microbial bioassay. Both culturable and non-culturable methods are used to identify and characterize microbial strains.

By evaluating the extent to which an antibiotic inhibits the growth of test microorganisms, a method known as microbial bioassay can be used to determine how potent an antibiotic is. Toxic solvents or specialized equipment are not needed for bioassays. The zone of inhibition's size and the antibiotic's dose are related in the widely used agar diffusion method of antibiotic assay. It has been theoretically examined how the diameter of inhibitory zones relates to the antibiotic concentration in a solution used in cups. An antibiotic can either stop or eradicate the growth of living microorganisms.Antibiotics' ability to inhibit microbial growth under controlled conditions can be used to illustrate their therapeutic value.

Levofloxacin:

The synthetic broad-spectrum antibacterial agent levofloxacin is administered orally and intravenously. Its chemical formula is levofloxacin, a chiral fluorinated carboxyquinolone, which is the pure S-enantiomer of the racemic drug substance ofloxacin.[3]

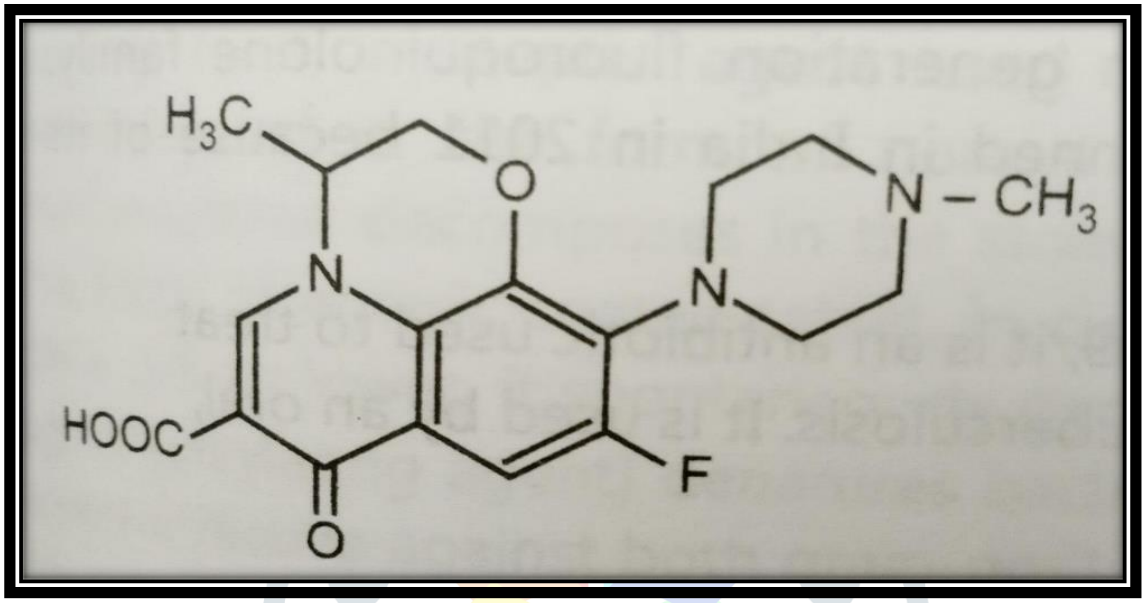
Levofloxacin, full name (s)-(-)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7- oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid.[4]It having molecular formula $C_{18}H_{20}FN_3O_4.½\ H_2O$ and molecular weight 370.38 g/mol. It is a synthetic broad spectrum antibacterial agent active against Gram-positiveand Gram-negative bacteriaincluding Staphylococcus species;Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus hemolyticus, Salmonella, Klebsiella, Serratia, Enterococcus, Proteus species, Enterobacter species, and other non fermentative rods of glucose. Levofloxacin additionally showed antibacterial activity against Chlaymydia trachomatis. Levofloxacin's primary mode of action involves inhibiting DNA gyrase. Its potency is twice that of its l-isomer ofloxacin.[5] Levofloxacin is a synthetic chemotherapeutic antibiotic that belongs to the fluoroquinolone drug class. It is used to treat bacterial infections that are either extremely serious or potentially fatal, or that do not improve with other antibiotic classes. Levofloxacin, an isomer ofOfloxacinthat ischirallyfluorinatedcarboxyquinolon, hasessentiallytakenitsplace inclinical practice. An oral antibacterial drug belonging to the third generation of flouroquinolones is levofloxacin. Levofloxacin inhibits DNA gyrase, topoisomerase IV, and type IItopoisomerases in Bacteria[6]. As with various other fluoroquinolones, the effectiveness of LEVO's activity is highly dependent on its blood concentration. A consistent dosage given within a predetermined window of time yields the bactericidal effect. It stops the bacteria from becoming resistant to the medication. The stability of the material in a pharmaceutical formulation is another factor that couldaffect howeffectivethetreatment is.[7]Levofloxacinis widelyusedtotreatavariety of bacterial infections and is considered to be a safe antibiotic.[8]

Levofloxacin isbactericidaland inhibitsbacterialDNAreplicationto produce its antimicrobial properties. Incomparisontoother antibiotics, it hasacomparativelylengthydurationofaction,.Levofloxacinislinkedto QTc-intervallengthening and should be administered cautiously in those who have other risk factors for lengthening(e.g. hypokalemia, concomitant medications). Levofloxacinhasshowninvitroefficacyagainst a

variety of gram-positive and gram-negative aerobic bacteria, and it may also have some activityagainst some anaerobic bacteria species and other pathogens including Chlamydia and Legionella. Levofloxacin resistance is possible and typically results from changes to DNA gyrase, topoisomerase IV.

Using Bacillus subtilis, ATCC-6633, the antimicrobial activity of levofloxacin in ophthalmic solution was assessed. Levofloxacin's in vitro efficacy against 234 strains of Mycobacterium tuberculosis was assessed; the resulting MIC50 and MIC90 values were 0.25 mg/L and 0.5 mg/L, respectively.[9]

Structure:



FigNo.1:ChemicalStructureofLevofloxacin[10]

(s-enantiomerofofloxacin)

Mechanism Of Action :

Levofloxacin, like other fluoroquinolone antibiotics, exerts its antimicrobial activity via the inhibition of two key bacterial enzymes: DNA gyrase and topoisomerase IV. Both targets are type II topoisomerases, but have unique functions within the bacterial cell. DNA gyrase is an enzyme found only in bacteria that introducesnegative supercoils into DNAduring replication - this helps to relieve torsional strain caused by the introduction of positive supercoils during replication, and these negative supercoils are essential for chromosome condensation and the promotion oftranscription initiation. It is comprised of four subunits (two Asubunits and two B subunits) of which the A subunits appear to be the target of fluoroquinolone antibiotics.1 Bacterialtopoisomerase IV, in addition to contributing to the relaxation of positive supercoils, is essentialattheterminalstagesofDNAreplicationandfunctionsto“unlink”newlyreplicated chromosomestoallowforthecompletionofcelldivision.[11-15]

Pharmacokinetic:

Levofloxacinshowed potentialagainst severalaerobic gram-positive and gram-negative bacteria in vitro. It may also have some effect against some anaerobic bacterial species and other pathogens, including Legionella and Chlamydia. Levofloxacin resistance can arise from mutations in DNA gyrase or topoisomerase IV, or through changes in drug efflux. It's possible forlevofloxacin and other fluoroquinolones to become cross-resistant.[16]

Absorption:

Levofloxacin is rapidly and nearly entirely absorbed when taken orally, having the oral bioavailability of about 99%. Levofloxacin's intravenous and oral formulations may be interchangeable because of its almost total absorption.

Distribution:

The body has a large distribution of levofloxacin, with an average volume of distribution after oral administration of 1.09–1.26 L/kg (~89–112 L). Levofloxacin has a good penetration rate into a varietyof tissues, including skin, lung, prostatic, and fluids (such as blisters).

Metabolism:

Humans have only been shown to have two metabolites: desmethyl levofloxacin and Levofloxacin-N-Oxide, neither of which appears to have any visible pharmacological activity.Less than 5% of the oral dose was recovered in the urine as these metabolites after administration, suggesting that levofloxacin is metabolized very little in humans.[17]

Excretion:

Most levofloxacin that is administered is eliminated unaltered in the urine. After a single oral dose of levofloxacin, less than 4% has been eliminated in the feces within 72 hours and about 87% was eliminated unchanged in the urine in 48 hours.

MaterialAndMethods:

Instrumentation:

Agilent 1220 Infinity LC (G4288C) HPLC System used with a C_{18} Symmetry (4.6 x 150mm,5µm,Make:Xterra) Column. Final chromatographic mobile phase for finaloptimization was Phosphate Buffer PH 2.8 : Acetonitrile35:65 v/v. Detection carried out at 284nm.

DrugSample:

Levofloxacin gift samples were offered by "Indoco Remedies Ltd."A near by drug storeprovided the“Levofloxacin Syrup”. Syrup contains (Levofloxacin 200 mg). Manufactured by : LexicarePharma Pvt. Ltd. Analytical gradechemicals and reagents were utilized.

OptimizedChromatographicconditions:

The chromatographic conditions were optimized by using C_{18} column (150 x 4.6mm, particlesize 5µ). Final chromatographic mobile phase for final optimization was Phosphate Buffer pH2.8 : Acetonitrile 35:65v/v. Detection was Carried out at 284nm.

Preparationofstandardstocksolution:

10 mg of Levofloxacin working standards were accurately weighed and transferred into a 100 ml cleandryvolumetricflaskaddabout70mlofdiluentswasaddedandsonicatedtodissolve it completelyand the volume was made up to the mark with the same solvent.(Stock solution) Further 1.2ml of Levofloxacin was pipetted from the above stock solution into a 10 ml volumetric flask and diluted upto the mark with diluent.

Preparationofsamplesolution:

01 ml of Levofloxacin was weighedandtransferred intovolumetric flask. The 5ml syrup equivalentto the amount of active ingredient present in 1 ml (Levofloxacin 200 mg was transferred into a 100 ml clean dry volumetric flask, 70 ml of diluent was added to it and was shaken for 5 minutes. Then make up the final volume 100 ml with selected solvent (stock solution). 1ml of stock solution was transferred to a 10 ml (200µg/ml) volumetric flask and diluted with diluent upto the mark and the solution was filtered through 0.45 µmfilter before injecting into HPLC system.

VALIDATIONOFANALYTICALMETHOD:

Specificity:

The chromatograms of standard and sample are identical with nearly same retention time. No interferenceduetoplaceandsample attheretentiontimeofanalyte. The is no interferencedue to blank at the retention time of analyte. which shows that the method was specific. As shown in Fig. No 01 & 02

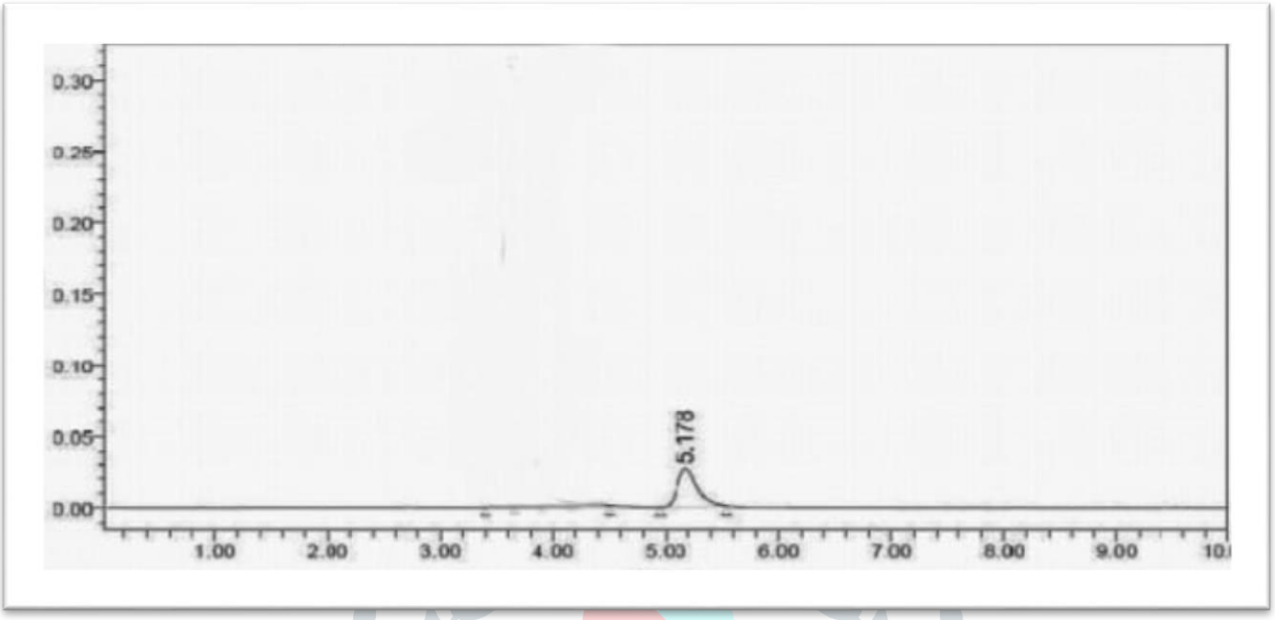


Fig.No.03.StandardchromatogramforLevofloxacin

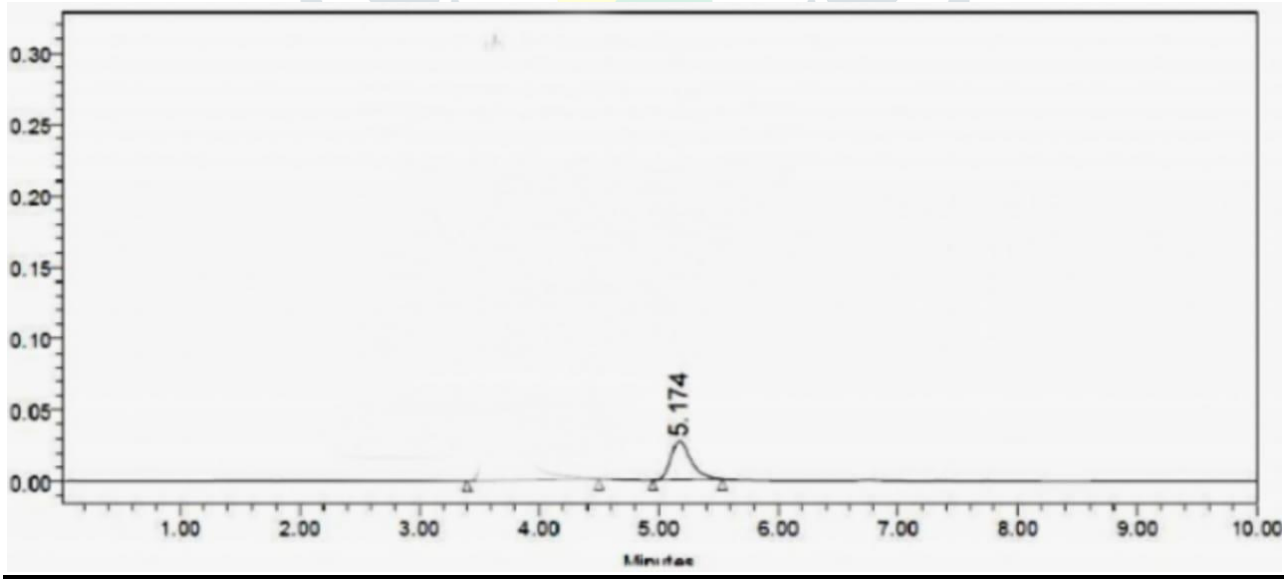


Fig.No.04.SamplechromatogramforLevofloxacin

There is no interferenceduetoblankattheretention timeof analyte.which shows that the method wasspecific.

Linearity:

Linearity studywasperformed Intheconcentrationrangeof10-50 µg/ ml. TheCalibration curve for the linearity are shown in Fig.No.05 for levofloxacin

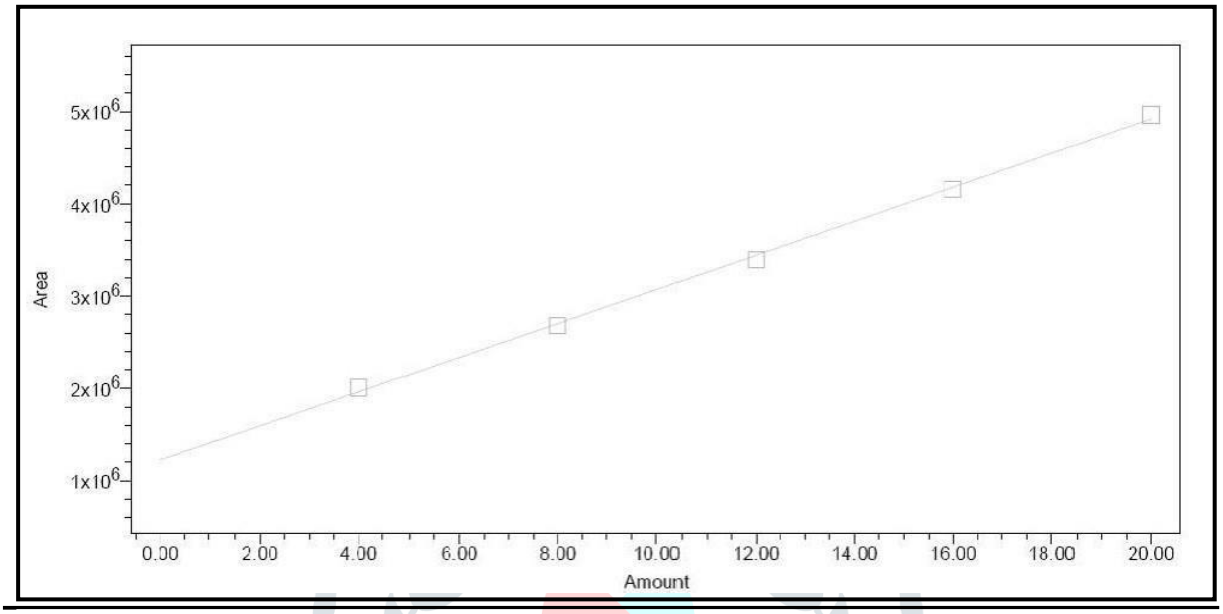


Fig.No.05.CalibrationcurveofLevofloxacin

Correlationco-efficientofLevofloxacinwasfoundtobe0.995respectively(NMT0.999).

Accuracy:

Thepercentagerecoveriesofpuredrugfromtheanalyzedsolutionofformulationare calculatedintherecoveryrangefrom50%to150%.Thesummaryofaccuracyresultsare tabulatedin

TableNo.01.%Recovery resultsforLevofloxacin

Sample No.	SpikeLevel	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	50%	5	4.96	99.2%	100.3%
		5	4.99	99.8%	
		5	5.1	102%	
2	100%	10	9.92	99.2%	99.4%
		10	9.94	99.4%	
		10	9.98	99.8%	
3	150%	15.3	15.1	98.6%	99.3%
		15.3	15.2	99.3%	
		15.3	15.3	100%	

The%recoveryfor50%,100%and150%accuracylevelofLevofloxacinwasfoundtobe within the range of 99.3-100.3% respectively (98.0 to102.0%)

Precision:

TheRSDof% Recoveryfor Levofloxacin chromatogramsorepeatabilityprecisionand intermediate precision is calculated.

Repeatability:

TableNo.03SamplevaluesforrepeatabilityofLevofloxacin

Levofloxacin		
Injection No	**Peak area**	**%Recovery**
1	3480636	99.4%
2	3463599	100%
3	3498779	99.0%
4	3497870	99.8%
5	3490276	99.2%
Mean	3486232	99.48%

SD	14601.3	0.415
%RSD	0.42	0.42

The%RSDforareaoffivestandard injectionsofrepeatabilityofLevofloxacinwas foundto be 0.42

1.**Intermediateprecision(analysttoanalyst variability)**

Comparison of both the results obtained for analysts shows that the assay method was rugged for analyst-analyst variability. The results of intermediate precision (Ruggedness) were found to be within the limits and are tabulated in Table given below

TableNo.04IntermediateprecisionresultsforLevofloxacin

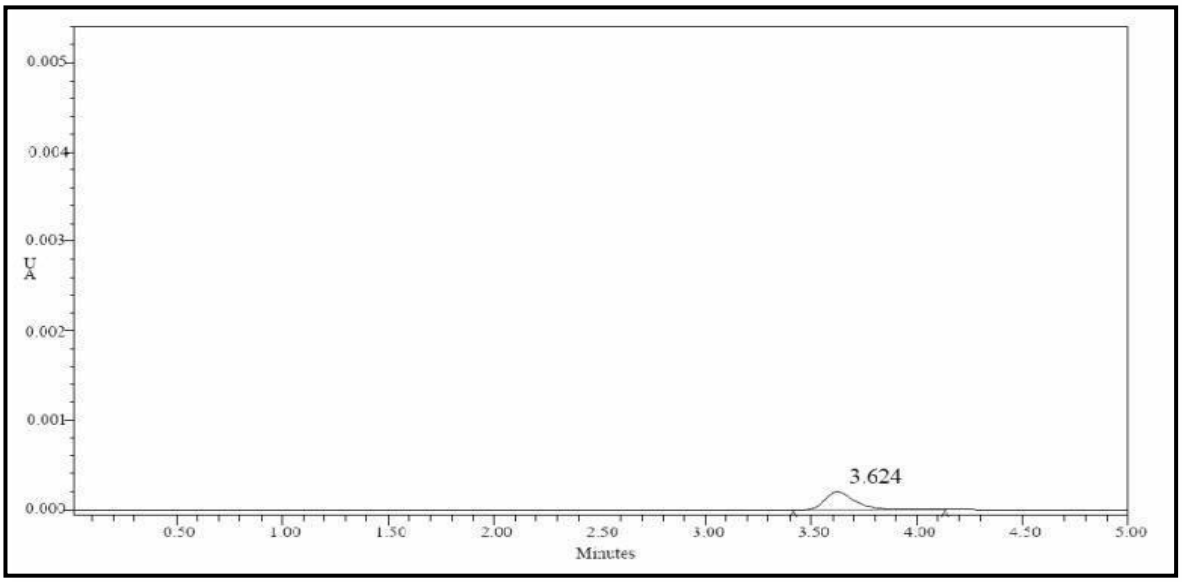
Parameter	**Peak Area**	**%Assay**
Avg	3486743	99.10%
%RSD*	0.41	0.38

The % RSD for the area of five standard injections for intermediate precision of Levofloxacin was found to be 0.42 for day-1, analyst- 1 and 0.43 for day-2, analyst -2 respectively.

LimitofDetection:(LOD)

limit of detection was calculated from the linearity curve method using slope, and standard deviation of intercepts.The of calibration curve

01.Levofloxacin



CalculationofS/Nratio-

a.Average baseline noiseobtainedfromblank-52µV

b.SignalobtainedfromLODsolution(0.25%oftarget assayconcentration)-154

S /N=154/52=2.96

CalculationofS/NRatio:

a.Average baseline noise obtained from blank -52µV

b.Signal obtained from LOD solution (0.25 % of target assay concentration) - 154µV

S/N=154/52=2.96

Limitofdetectionwasfound to be2.96 forLevofloxacin.

LimitofQuantification:(LOQ)

The limit ofquantificationwascalculatedfromthe linearitycurve methodusingslope,and standard deviation of intercepts of calibration curve.

Levofloxacin

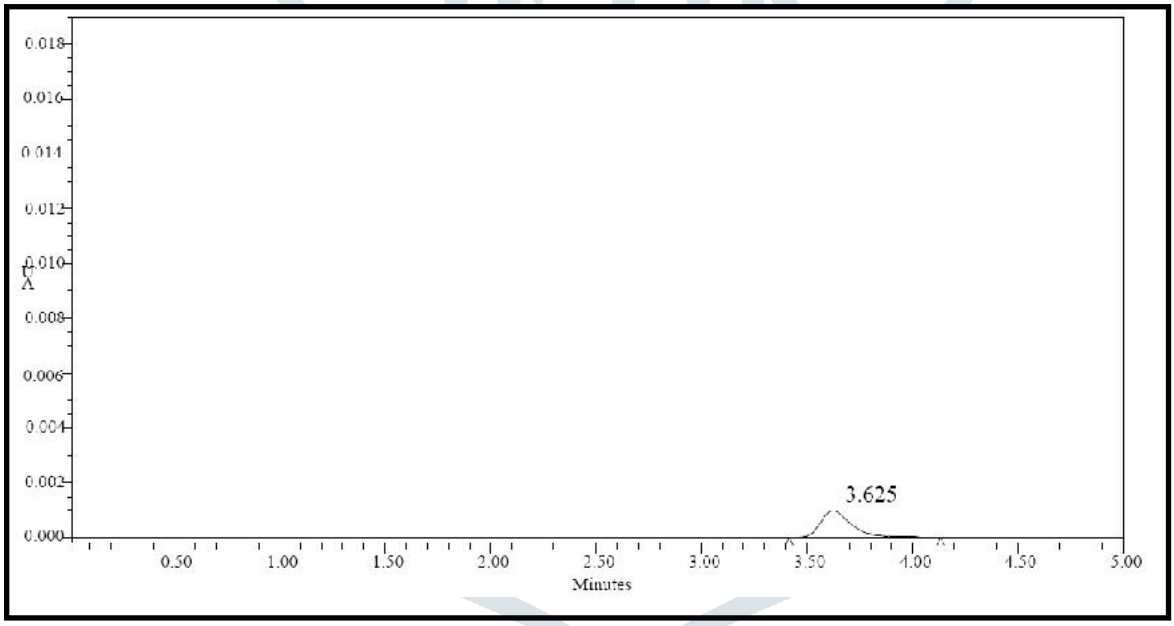


Fig.No.09.LOQChromatogramofLevofloxacin

CalculationofS/NRatio:

a. Average baselinenoiseobtainedfromblank-52µV
b. Signalobtained formLOQsolution(1%oftarget assay concentration) - 522µV
   S/N=522/52=10.

Limitofdetectionwasfoundtobe2.96forLevofloxacin.

Robustness:

Effect of variation in flow rate:

As the % RSD of retention time and asymmetrywere within limits for variation in flow rate (±0.1ml).

Hencetheallowable flowrateshould be within0.4 mlto 0.6ml.Thechromatograms arerecordedandshowninFig. Theresultsofrobustness for effectofvariationinflowrateare tabulatedin.Table given below

TableNo.06RobustnessresultsforLevofloxacin

DrugSample	Sr.No	Flow rate(ml/ min)	System suitability results	
			USPPlate count	USPTailing
LVF	1	0.4	4859	1.62
	2	0.5	4890	1.58
	3	0.6	4895	1.58

The%RSDofretentiontimeandasymmetrywerewithinlimits forvariationin flow rate (±0.1 ml).

1.Effect of variation in mobile phase composition:

The chromatogramsareshow.Theresultsofrobustnessfor effect of variationinmobilephasecompositionaretabulatedinTable given below

TableNo.07Resultsforvariationinmobilephasecomposition

Drug Sample	Sr. No	Change inorganic Composition in the mobilephase	Systemsuitabilityresults	
			USP Plate count	USPTailing
LVF	1	10%less	4899	1.52
	2	*Actual	4857	1.52
	3	10%more	4879	1.61

The % RSD of retention time and asymmetry were within limits for variation in composition of mobile phase. Hence the method was found to be robust.

SystemSuitability:

% RSD ofretention time was found to be 0.2, % RSD ofpeak area was found to be 0.2. Theoreticalplateswerefoundtobe morethan3500. USPtailing factorwasfoundtobe1.48for Levofloxacin. All the parameters were within the limit

TableNo.08Chromatogramvaluesforsystemsuitability ofLevofloxacin

Injection	Retentiontime	Peakarea	USPPlatecount	USPTailing
1	3.666	5305432	6859	1.62
2	3.654	5318619	6890	1.58
3	3.649	5319646	6998	1.58
Mean	3.656	5314566	6915.667	1.59333
SD	0.008	7926.638	72.96803	0.020394
%RSD	0.2389	0.1491	1.055	1.499

%RSDofretentiontimewasfoundtobe0.2,%RSDofpeakarea was foundtobe 0.2.Theoreticalplateswerefoundtobemorethan3500.USPtailingfactorwasfoundtobe1.48for Levofloxacin. All the parameters were withinthelimit.

SUMMARYOF RESULTS:

TableNo.09Summaryofresults

Sr.No	Parameter	Requirement	Result	Acceptance Criteria
			LVF	
1.	Specificity	No interference	Pass	No interference
2.	Linearity	Correlation coefficient	0.9998	NLT0.999
3.	Accuracy	50%recovery	100.3%	100±2.0%
		100%recovery	99.4%	
		150%recovery	99.3%	
4.	Precision (repeatability)	%RSD	0.42	NMT2%
5.	Intermediate Precision	%RSD	0.03	NMT1%
6.	Robustness	%RSD	0.43	NMT1%
7.	System Suitability	RT	3.654	-

	a.	Tailingfactor	1.6	NMT2
	b.	Plate count	4859	NLT3000
	c.	Assayvalue	98.7%	100±2.0%

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