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Research Article

DEVELOPMENT AND VALIDATION OF HPLC-UV METHOD FOR THE ESTIMATION OF LEVOFLOXACIN IN HUMAN PLASMA

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ABSTRACT

A Simple, rapid, selective and sensitive HPLC method was developed and validated for the determination of levofloxacin from human plasma. The drug was extracted with ethyl acetate. levofloxacin was measured in plasma using a validated HPLC method with UV detector at 235nm chromatographic peaks were separated on 5µm intensil, C18 column (4.6x250mmx5µm) using 80:20 v/v Phosphate buffer pH 2.5, Acetonitrile as mobile phase at a flow rate of 1 ml/min. The chromatograms showed good resolution and no interference from plasma. The retention time of levofloxacin and internal standard were approximately 5.9±0.05 min and 10.1± 0.03 min respectively. The mean recovery from human plasma was found to be above 85%. The method was linear over the concentration range of 0.1 to 10µg/ml with coefficient of correlation (r^2) 0.9994. Both intraday and interday accuracy and precision data showed good reproducibility. This method was successfully applied to pharmacokinetics studies.

Keywords: Levofloxacin, Human plasama, UV

INTRODUCTION

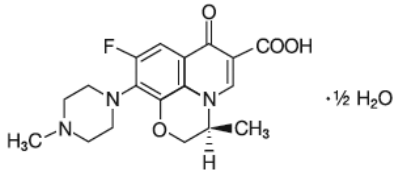

Fig. 1: The Chemical structure of Levofloxacin

Levofloxacin (fig-1) is a synthetic broad-spectrum antibacterial agent for oral and intravenous administration. Chemically, levofloxacin, a chiral fluorinated carboxyquinolone, is the pure (-)-(S)-enantiomer of the racemic drug substance ofloxacin. 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[1]. A simple reversed-phase HPLC method employing a single-step liquid-liquid extraction and UV detection for the rapid stereospecific determination of levoftoxacin in both human plasma and urine is reported[2]. S. Djabarouti et al. Reported HPLC with UV detection using an extraction method with a polymeric cartridge[3]. S. Watabe et al. Developed a HPLC method for simultaneous measurement of pazufloxacin, ciprofloxacin, and levofloxacin concentrations in spiked humanserum[4]. PF. Fang et al. Developed a HPLC–MS/MS method for high-throughput and simultaneous determination of isoniazid, levofloxacin and rifampicin in mouse plasma and different tissues including brain, lung, liver, kidney and small intestine[5]. H.A. Nguyen et al. described a method in order to determine simultaneously three FQs: levofloxacin, gatifloxacin and moxifloxacin by direct human serum injection without classical sample pre-treatment steps[6]. S. Siewert reported a HPLC method with fluorescence detection suitable for routine determination of levofloxacin in plasma and dialysate[7]. The aim of this study was to develop and to validate a rapid, selective and low-cost HPLC–UV method for determination of levofloxacin concentrations in spiked human plasma. The proposed method was carried out without changing the analytical conditions and utilized a simple sample preparation method. In future, this method will help in clinical study and routine analysis.

MATERIALS AND METHODS

Levofloxacin and gatifloxacin were purchased from Pei Li Pharmaceutical Ind. co. Ltd, Taiwan., HPLC grade acetonitrile, ethylacetate, methanol were purchased from SD fine chemicals, Mumbai, India. Analytical Grade Potassium Dihydrogen Phosphate and Sodium Hydroxide were purchased from SD fine chemicals, Mumbai, india. Pooled drug free expired human plasma was purchased from Red Cross Society, Warangal.

Chromatographic conditions

The HPLC system consisted of Alliance waters 2695 with dual λ Absorbance UV detector. The wavelength of detection as set at 235nm. Separation was carried out on inertsil C18 column (4.6x250mmx5µm) using 80:20v/v 20mM KH_2PO_4 buffer pH2.5, Acetonitrile as mobile phase at a flow rate of 1 ml/min. The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultrasonicator prior to use. Chromatography was carried out at room temperature 25^0c and maintains the column temperature at 32^0c.

Preparation of standard solutions

Stock solutions of levofloxacin (0.5mg/ml) and gatifloxacin (1mg/ml) internal standard were prepared in methanol. Further dilutions were carried out in 60% acetonitrile. Calibration standards were prepared freshly by spiking drug free plasma with levofloxacin stock solution to give the concentrations of 0.1, 0.2, 0.5, 1, and 2.5, 5, 8, 10 µg/ml.

Quality control standards

Lowest quality control standards, Median quality control standards and highest quality control standards were prepared by spiking drug free plasma with levofloxacin to give solution containing 0.3, 4.5 and 9.5µg/ml respectively. They were stored at -20^0c till the time analyzed.

Sample preparation method

To 500µl of spiked plasma, 50µl gatifloxacin (100µg/ml) was added and vortexed. The drug was extracted with 3 ml of ethyl acetate followed by centrifugation at 4000 rpm/min on a cooling centrifuge for 15min at 4^0c. The organic phase was withdrawn and dried using lyophiliser. To the residue 400µl of mobile phase was added and respective samples were injected into column.

Validation

Specificity

A solution containing 0.1µg/ml was injected on to the column under optimized chromatographic conditions to show the separation of levofloxacin from the impurities from the plasma. The specificity of the method was checked for the interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of levofloxacin to internal standards and the best fit line was calculated .Wide range calibration was determined by solutions containing 0.1µg/ml to 10µg/ml

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against unextracted Quality control samples at HQC, MQC and LQC.

Limit of quantification

To estimate the LOQ, a drug free blank plasma sample was extracted and injected ten times and analyzed as described under optimized chromatographic conditions .The noise level was then determined, the limit of quantification for levofloxacin was determined.(signal to noise ratio=10).

Precision and accuracy

Intraday precision and accuracy was determined by analyzing quality control standards (0.3 µg/ml, 4.5µg/ml 9.5µg/ml) and LLOQ Quality control standards (0.1µg/ml) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control standards (0.3 µg/ml, 4.5µg/ml 9.5µg/ml) and LLOQC standards (0.1µg/ml) once on each of five different days.

Stability studies

The stability of levofloxacin was determined by measuring concentration change in control sample overtime. The plasma control samples were stored in eppendrorff tubes at -20^{0}c. Stability was tested by subjecting the plasma controls to three freeze thaw cycles and stored for 24hrs at room temperature.

RESULTS AND DISCUSSION

Under the chromatographic conditions employed, the sample showed sharp peaks of drug & internal standard with good resolution. The retention time of the drug was found to be 5.9 +0.05 min and the retention time of internal standard was 10.1±0.03min (figure-2). The method developed was validated for specificity, accuracy & precision, linearity and stability as per USFDA guidance[8]. The results of validating parameters are given below.

Specificity of the method was proven by the absence of the peaks near the reaction time of drug as well as the internal standard (figure-3).

The calibration function (peak area ratioVs Concentration) was linear over working range of 0.1 to 10µg/ml. with eight point calibration used for quantification by linear regression. The regression equation for the analysis was Y=2.06e-0.01x-3.79e-0.003 with coefficient of correction (r^2) = 0.9994. (figure-4).

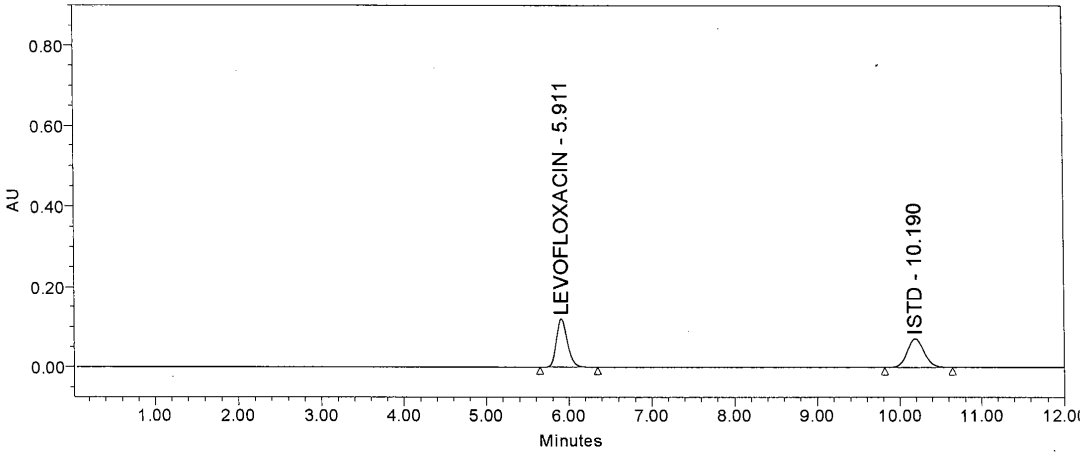

Fig. 2: Retention times of Aqueous Mixture consists of levofloxacin (5µg/ml) and gatifloxacin (5µg/ml)

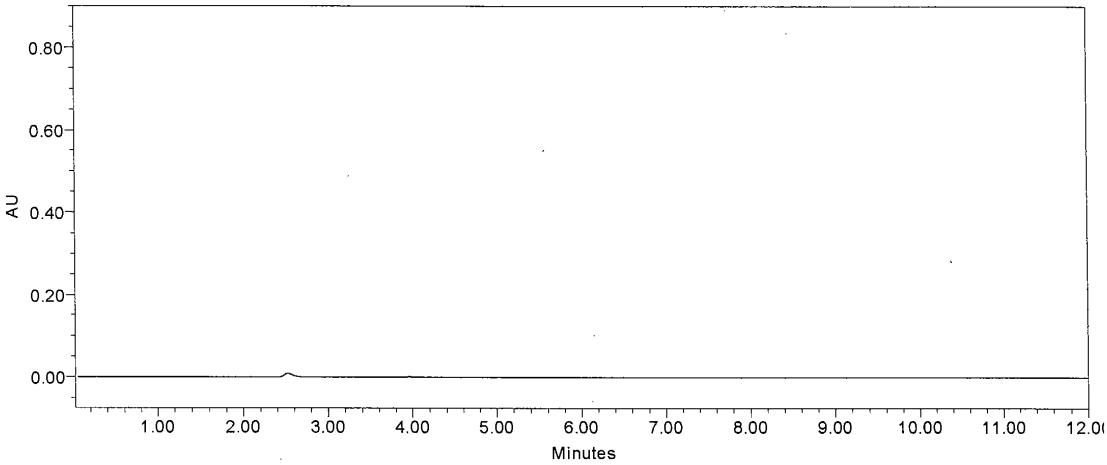

Fig. 3: Blank plasma sample showing no interference at the RT of levofloxacin and gatifloxacin.

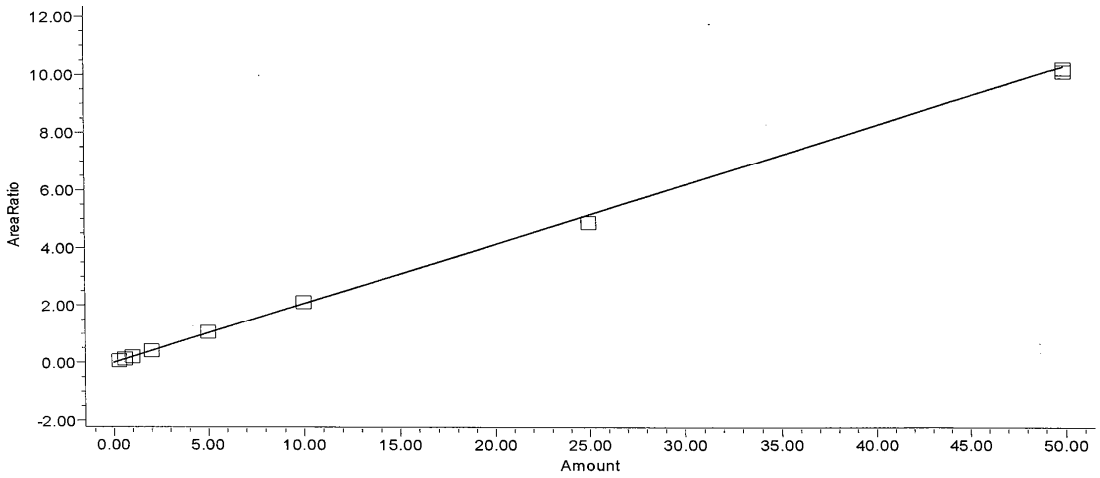

Fig. 4: Spiked concentrations (0.1µg/ml to 10µg/ml) were plotted against peak area ratio Vs concentration with eight point calibration used for quantification by linear regression

Table 1: Recovery - Levofloxacin

	LQC			MQC			HQC		
ID	**Unextracted (area ratio)**	**Extracted (area ratio)**	**%Recovery**	**Unextracted (area ratio)**	**Extracted (area ratio)**	**%Recovery**	**Unextracted (area ratio)**	**Extracted (area ratio)**	**%Recovery**
1	0.029	0.022	75.86	0.425	0.36	84.71	0.948	0.818	86.29
2	0.03	0.024	80.00	0.417	0.36	86.33	0.904	0.786	86.95
3	0.03	0.024	80.00	0.44	0.38	86.36	0.93	0.789	84.84
4	0.03	0.024	80.00	0.417	0.36	86.33	0.901	0.763	84.68
5	0.029	0.023	79.31	0.424	0.37	87.26	0.909	0.782	86.03
6	0.03	0.024	80.00	0.449	0.380	84.63	0.904	0.786	86.95
Mean	0.030	0.024	79.195	0.429	0.368	85.938	0.916	0.787	85.955
±SD	0.001	0.001		0.013	0.010		0.019	0.018	
%CV	1.74	3.56		3.04	2.67		2.06	2.25	

The % mean recovery for levofloxacin in LQC, MQC and HQC was 79.19%, 85.93% and 85.95% respectively.

The intraday and interday precision and Accuracy of the method was found to be 0.13 to 3.22% and 98.48 to 107.12% respectively for the quality control samples. This is within the acceptance limits of precision is 15% and accuracy is 85 to 115% (table-2). The limit of Quantification was found to be0.1µg/ml. at such concentration the inter day precision was 4.89 and the accuracy was 92.9%. Which is within the acceptance limits of precision is 20% and accuracy is 80 to 120%.

Table 2: Precision & accuracy of quality control standards

Batch ID	**QC ID**	**LQC**	**MQC**	**HQC**	**Batch ID**	**QC ID**	**LQC**	**MQC**	**HQC**
	Actual conc.(µg/mL)	0.3	4.5	9.5		Actual conc.(µg/mL)	0.3	4.5	9.5
	1	0.269	4.133	9.098		1	0.314	4.621	10.136
	2	0.268	4.078	9.093		2	0.313	4.6	10.172
	3	0.268	4.137	9.092		3	0.316	4.655	10.183
	4	0.267	4.136	9.08		4	0.32	4.618	10.179
	5	0.263	4.135	9.104		5	0.322	4.607	10.186
Intraday	6	0.265	4.138	9.114	Interday	6	0.312	4.666	10.203
	Mean	0.271	4.126	9.097		Mean	0.316	4.628	10.177
	± SD	0.003	0.024	0.012		± SD	0.004	0.027	0.022
	% CV	3.22	0.57	0.13		% CV	1.27	0.58	0.22
		90.48	91.69	95.76			105.39	102.84	107.12
	% Accuracy					% Accuracy			

Stability was assessed by comparing against the freshly thawed quality control samples. The %mean stability for HQC and LQC were 112.35 and 100.39 respectively, which is within the acceptance limits of 85 to 115%. Plasma Quality control samples of levofloxacin were found to be stable for at least one month (table-4).

levofloxacin is soluble in methanol. Hence standard solutions were prepared in methanol. The proportion of acetonitrile in the mobile phase was optimized to 80% and 20% mobile phase was made up of with phosphate buffer (pH 4). A slight increase and decrease in concentration of acetonitrile and pH by 2% does not affect the reaction times.

The extraction of levofloxacin was based on liquid-liquid extraction technique. Various solvent systems were tried for recovery studies. The maximum recovery was obtained with a mixture of phosphate buffer (pH 2.5) and ethyl acetate. Five drugs were attempted for selection as internal standard. The other drugs tried were found to be overlapping with reaction time of levofloxacin under the optimized chromatographic conditions.

Table 3: Precision & accuracy of LLOQC standard

	LLOQC
Actual conc.(µg/ml)	0.1
1	0.095
2	0.104
3	0.095
4	0.093
5	0.102
Mean	0.09666667
±SD	0.005
%CV	4.89
% Accuracy	92.9

Table 4: Freeze-thaw Stability of quality control standards

Freeze – thaw III Cycles		
QC ID	**LQC**	**HQC**
Actual conc.(µg/ml)	0.3	9.5
1	0.302	10.496
2	0.301	10.625
3	0.302	10.547
4	0.305	10.994
5	0.306	10.891
6	0.291	10.488
Mean	0.301	10.674
± SD	0.005	0.216
% CV	1.77	2.03
% Accuracy	100.39	112.35

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