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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR DETERMINATION OF LEVOFLOXACIN IN TABLET USING UV AND FLUORESCENCE DETECTORS SIMELTANIOUSLY

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levofloxacin, RP-HPLC, Validation, UV, Fluorescence

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ABSTRACT: A Simple, sensitive, precise, accurate, and specific reversed –phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the determination of levofloxacinin pharmaceutical tablets. Levofloxacin is a third generation fluoroquinolones with a wide spectrum of action against gram-positive and gram-negative bacteria, anaerobic microorganisms and atypical pathogens. It is pure (-)-(S)-enantiomer of the racemic drug substance ofloxacin, which was introduced in 1997. Isocratic chromatography was performed on a C18 column with acetonitrile-methanol-phosphate buffer 0.1M15:25:60 (v/v/v) as mobile phase at a flow rate of 1 ml/min. UV detection was set at 287 nm. The fluorescence detector was set at excitation/emission wavelengths of 300/500 nm. The method was validated with respect to accuracy, linearity, precision, and selectivity. All the parameters examined met the current recommendations of U.S.P (30) for analytical method validation. The method can be reliably used for routine quality control analysis and to determine the levofloxacin content of marketed tablets.

INTRODUCTION: Levofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class and is used to treat severe or life-threatening bacterial infections or bacterial infections that have failed to respond to other antibiotic classes Levofloxacin is a chiral fluorinated carboxyquinolon, it is an isomer of Ofloxacin and has largely replaced it clinically. Levofloxacin is an oral anti-bacterial agent from the third generation of flouroquinolones [1]. Levofloxacin inhibits bacterial type II topoisomerases, topoisomerase IV and DNA gyrase. Levofloxacin, like other fluoroquinolones, inhibits the A subunits of DNA gyrase, two subunits encoded by the gyrA gene.

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This results in strand breakage on a bacterial chromosome, super coiling, and resealing; DNA replication and transcription is inhibited. Levofloxacin, approved by the FDA in the 1996. (-) -(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

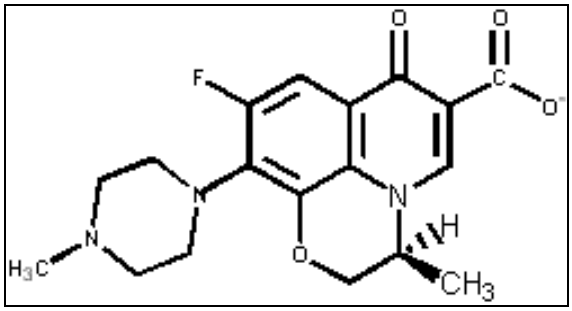

FIGURE 1: STRUCTURE OF LEVOFLOXACIN

Only few studies on determining levofloxacin content in pharmaceutical formulations have been published, involving nuclear magnetic resonance spectrometry Spectrophotometric and spectro

fluorimetric, ligand-exchange chromatography, as well as, HPLC methods [2-12]

Therefore, in the present investigation an attempt has been made to determine levofloxacin in dosages formusing RP-HPLC without internal standard. Method validation procedure (linearity, precision, accuracy, and selectivity) was based on the recommendations of U.S.P 30 for analytical methodvalidation[13, 14].

MATERIALS AND METHODS:

Levofloxacin working standard was obtained from the ministry of health- Syria and levofloxacin tablets were obtained from different local and regional manufactures. Acetonitrile, methanol, potassium dihydrogen phosphate, phosphoric acid reagent grade, acetic acid, sodium hydroxide, glacial acetic acid, and water of HPLC grade were purchased from PANREAC. A Jasco HPLC system equipped with an OPU-980 Intelligent HPLC gradient Pump, UV -970 Intelligent UV/VIS detector, manual injector and Nucleodurs C18 column (250 x 4.6mm, 5µm) was used. The mobile phase consisted of a mixture of acetonitrile - methanol - phosphate buffer (80:10: 10, v/v/v). The flow rate was set to 0.9 ml/min. and the detection wavelength was set at 287 nm.

Preparation of standard and stock solutions:

For detection using UV detector, Stock solution of levofloxacin 1 mg/ml was prepared in distilled water and diluted further to obtain standard solution of 40µg/ml. For detection using fluorescence detector, Stock solution of levofloxacin 1 mg/ml was prepared in distilled water and diluted further to obtain standard solution of 5µg/ml.

Preparation of Sample solutions:

Twenty tablets (which were previously subjected to mass uniformity test) were weighed and finely powdered. Amass equivalent to 100 mg of levofloxacin was weighed and transferred in a 100 ml volumetric flask, mixed with distilled water, and sonicated for 30 minutes. The solution was filtered through 0.45µ filter paper. The filtrate was transferred to a 100 ml volumetric flask and diluted to the mark with buffer. For detection using UV detector, Stock solution of levofloxacin 1 mg/ml was prepared in buffer and diluted further to obtain standard solution of 40µg/ml.

For detection using fluorescence detector, Stock solution of levofloxacin 1 mg/ml was prepared in buffer and diluted further to obtain standard solution of 5µg/ml. The solution of the samples was injected in triplicate and chromatographed.

Accuracy:

The accuracy of the method was assessed by determination of the recovery of the method at three different concentrations (corresponding to 75,100, and125% of the standard solution concentration for detection using UV, and 60, 100 and 140% for detection using fluorescence detector) along with the excipients. Each concentration was injected in triplicate.

Linearity:

Five concentrations were prepared containing 10, 20, 30, 40, 50µg/ml for detection using UV, and 0.1,0.3,0.5,0.7,1µg/ml for detection using fluorescence detector. Each solution was injected in five. Linearity was evaluated by linear-regression analysis.

Precision:

Precision of the method was determined by performing repeatability test. Repeatability of the method was checked by carrying out six independent assays at the standard concentration levels. Precision was determined as the relative standard deviations (RSD) of the drug recoveries at 100 % concentration levels.

Selectivity:

Selectivity of the method was assessed by preparing tablet powder without levofloxacin with the same excipients as those of in the commercial formulations. For RP-HPLC the solutions were prepared using the same procedure as for the standard solution.

Detection limit and Quantification limit:

The detection limit DL and Quantification limit QL were measured from the signal-to-noise ratio**.** The DL was defined as the concentration level corresponding to peak area of three times the baseline noise. The QL was defined as the lowest

concentration level of a peak area with a signal-to-noise ratio higher than 10.

Optimization of the chromatographic conditions:

In order to develop a suitable and valid LC method for the determination of levofloxacin, various chromatographic conditions were employed using different mobile phases. The system containing acetonitrile-methanol-phosphate buffer 0.1M 15: 25:60 (v/v/v) as a mobile phase at a flow rate of 1ml/min was found to be satisfactory and gave well resolved peak for levofloxacin.

A UV scan was performed and 287 nm was selected as a detection wavelength for estimation of levofloxacin using HPLC. A fluorescence scan was performed and excitation/emission wavelengths of 300/500 nm was selected as a detection wavelength for estimation of levofloxacin using HPLC. Complete resolution of the peak with clear baseline separation was obtained. The retention time for levofloxacin was 4.31 min. (**Figure 2**).

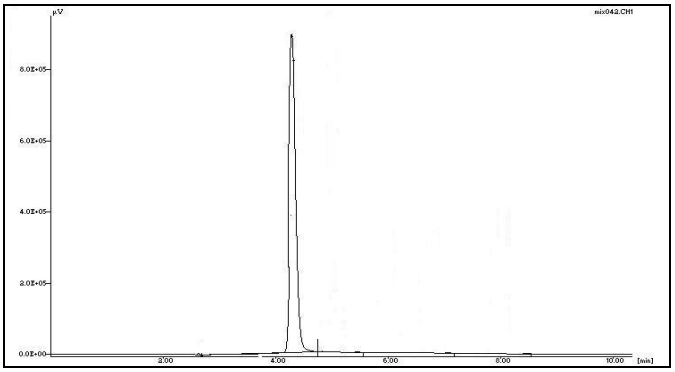

FIGURE 2: HPLC CHROMATOGRAM OF LEVOFLOXACIN USING UV DETECTOR

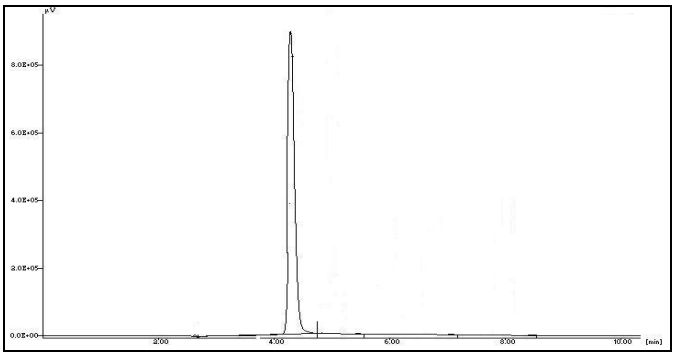

FIGURE 3: HPLC CHROMATOGRAM OF LEVOFLOXACIN USING FLUORESCENCE DETECTOR

System suitability:

System suitability was performed before each validation run, five replicate injections of system suitability were performed. Retention time, area, asymmetry, theoretical plates, and capacity, for the five suitability injections were determined.

TABLE 1: RESULT OF LINEARITY OF THE PROPOSED METHOD USING UV DETECTOR

Standard No	Concentration µg/ml	Concentration	Concentration %	RSD%	
1	10	9.85	98.5	0.52	
2	20	20.3	101.5	1.2	
3	30	29.0	96.66	0.76	
4	40	40.37	100.92	1.35	R^2=0.9996
5	50	49.74	99.48	0.82	Y=9.18X-1.46

TABLE 2: RESULT OF LINEARITY OF THE PROPOSED METHOD USING FLUORESCENCE DETECTOR

Standard No	Concentration µg/ml	Concentration	Concentration %	RSD%	
1	0.1	0.10	99.5	0.83	
2	0.3	0.29	99.98	0.61	
3	0.5	0.47	95.2	0.67	
4	0.7	0.69	96.6	0.12	R^2=0.9997
5	1.0	0.99	105	1.2	Y=9.2951x +0.3066

TABLE 3: ACCURACY OF THE PROPOSED METHOD USING UV DETECTOR

Amount added%	sets	Average amount recovered	Mean recovery%	RSD%
75	3	76.25	101.66	1.31
100	3	99.98	99.98	0.62
125	3	130.06	104.05	0.52

TABLE 4: ACCURACY OF THE PROPOSED METHOD USING FLUORESCENCE DETECTOR

Amount added%	sets	Average amount recovered	Mean recovery%	RSD%
60	3	59.07	98.46	0.64
100	3	101.3	101.3	0.42
140	3	114.94	103.35	0.51

TABLE 5: PRECISION OF THE PROPOSED METHOD USING UV DETECTOR

Standard No	Area	
1	3527145	
2	3527432	
3	3525456	
4	3524987	
5	3524157	Average=3526328
6	3528788	RSD=0.045

TABLE 6: PRECISION OF THE PROPOSED METHOD USING FLUORESCENCE DETECTOR

Standard No	Area	
1	4961615	
2	4942545	
3	4938784	
4	4966521	
5	4950326	Average=4951989
6	4952142	RSD=0.196

TABLE 7: ASSAY RESULTS FOR LEVOFLOXACIN IN MARKETED TABLET DOSAGE FORM BY PROPOSED METHOD USING UV DETECTOR

Tablet	Amount claimedmg	Amount recovered	Mean%oflable(claim)	RSD%	RSD%
1	500	494.8	98.9	0.148	0.896
2	500	489.3	97.8	1.27	
3	500	500.33	100	0.754	

TABLE 8: ASSAY RESULTS FOR LEVOFLOXACIN IN MARKETED TABLET DOSAGE FORM BY PROPOSED METHOD USING FLUORESCENCE DETECTOR

Tablet	Amount claimed mg	Amount recovered	Mean%oflable(claim)	RSD%	RSD%
1	500	510	102	0.32	
2	500	488	97.6	0.8	0.8
3	500	499	99.8	1.3	

Validation of the developed methods

Calibration data for levofloxacin using UV detector was shown in **Table 1**. The linearity plot of levofloxacin was found to be linear. The linear equation and correlation coefficient were: **Y=9.18x-1.46**, 0.996 respectively (**Figure 4**). This demonstrates the suitability of this method for the analysis of levofloxacin in tablets.

Calibration data for levofloxacin using fluorescence detector was shown in **Table 2**. The linearity plot of levofloxacin was found to be linear. The linear equation and correlation coefficient were: Y= 9.295X + 0.3066, 0.9997 respectively (**Figure 5**). This demonstrates the suitability of this method for the analysis of levofloxacin in tablets.

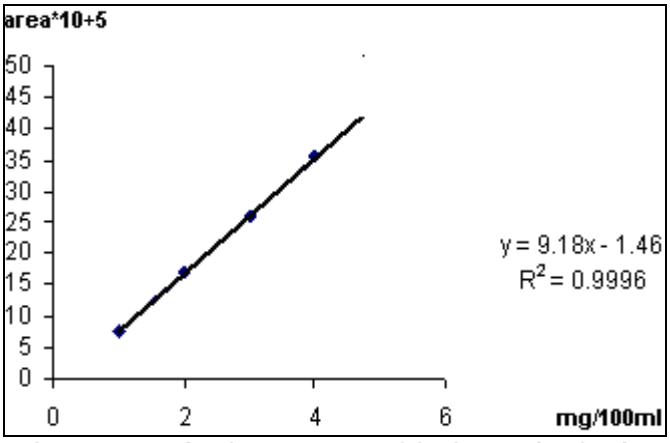

FIGURE 4: RELATION BETWEEN CONCENTRATION AND PEAK AREAOF LEVOFLOXACIN USING UV DETECTOR.

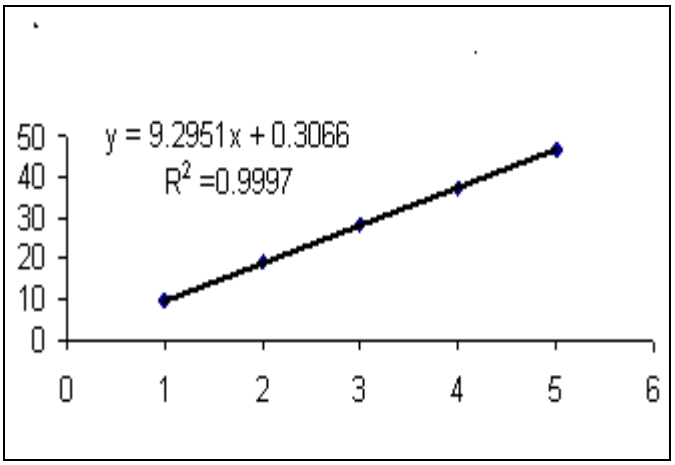

FIGURE 5: RELATION BETWEEN CONCENTRATION AND PEAK AREAOF LEVOFLOXACIN USING FLUORESCENCE DETECTOR

The results of accuracy (**Table 3,4**) showed that the method is accurate with percentage recovery of 99.98-104.05%,and 98.46-103.35% using UV detector and fluorescence detector respectively, and acceptable RSD less than 2% at each level, and. The percentage relative standard deviation of assay values for repeatability (n=6) was 0.045, 0.196 using UV detector and fluorescence detector respectively. (**Table 5, 6**)

The purity of analyte peak and the RSD value of < 2 indicate that the method is selective for analysis of levofloxacin in its dosage form (**Table 7, 8**).

The percentage relative standard deviation for the assay values (n=5) for levofloxacin peak were within the acceptance limit of 2%.The DL and QL were found to be 1.2µg/ml and5µg/ml respectively using UV detector, and 0.22µg/ml and 0.5 2µg/ml respectively using fluorescence detector.

Determination of levofloxacin in tablets:

The developed method was applied for the determination of levofloxacin in tablets. The results of these assays ranged from 97.8% to 100% and from 97.6% to 102% of the claim concentration (**Table7, 8)** using UV and fluorescence detector respectively. The results indicated that the method is selective for the assay of levofloxacin without the interference of the excipients used in the tablet.

RESULTS AND DISCUSSION:

An accurate, fast, sensitive, and precise isocratic reverse phase high performance liquid chromatographic method has been developed for the determination of levofloxacin in tablet dosage form. The developed method was found to be simple and have short run time which makes the method rapid. Several studies in the literature for the determination of the tested compound depends on UV, HPTLC or volumetric methods, few used HPLC upon then less used RP-HPLC. Nevertheless, the results of the study indicate that the developed HPLC method is simple, precise, accurate and less time consuming.

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