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ENANTIOMERIC SEPARATION OF LEVOFLOXACIN IN DRUG PRODUCT AND DRUG SUBSTANCE USING CHIRAL STATIONARY PHASE

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ABSTRACT

A chiral liquid chromatographic method was developed for the enantiomeric purity of Levofloxacin in drug substance as well as in drug product. The chromatographic separation was achieved on Chiralpak IC 150 X 2.1mm X 5µm, column using a mobile phase system consisting of n-hexane and isopropyl alcohol in the ratio of 95:5 (v/v). The mobile phase was pumped through column at the flow rate of 1 mL min-1. The resolution between the enantiomers was found to be more than three. The developed method was subsequently validated and proved to be accurate, specific and precise. The experimentally established limit of detection and quantification for (R)-enantiomer of Levofloxacin were found to be 0.509 µg mL^{-1} and 1.316 µg mL^{-1} respectively for 20 µl injection volumes. The percentage recovery of (R)-enantiomer was ranged between 95 to 105 % in drug product as well as in drug substance. The proposed method was found to be suitable and accurate for the quantitative determination of chiral purity of Levofloxacin in drugs substance as well as in drug product.

Keywords: Levofloxacin, Chiral Purity, Normal Phase, Development, Validation

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INTRODUCTION

Levofloxacin (LEVO), the levorotatory isomer of ofloxacin, exhibits activity against a broad spectrum of Gram-positive and Gramnegative bacteria. It is used to treat various infectious diseases such as community acquired and nosocomial pneumonia, skin structure infection, urinary tract infections or sepsis [1]. Several analytical techniques have been used for the determination of levofloxacin in different matrices, including adsorptive square wave anodic stripping voltammetry [2] flow injection analysis with absorption photometric, potentiometry and conductometry detection [3]. However, these methods do not present selective signals to discriminate a single analyte in mixtures so separation procedures or multivariate calibration algorithms are needed. A literature survey reveals various separation methods for the determination of levofloxacin such as reversed phase high performance liquid chromatography
(HPLC) with UV absorption spectrophotometric detection [4–12], HPLC with fluorescence detection [13–16], and capillary electrophoresis with chemiluminescence detection [17]. These methods are quite complex, mostly because of the sample preparation, which involves solid-liquid extraction [18], liquid-liquid extraction [19] and protein precipitation combined with centrifugation steps. Therefore, these methods are relatively expensive and time consuming for routine use in clinical pharmacology, and chemical and pharmaceutical laboratories. Recently, room-temperature phosphorimetry was used to selectively detect LEVO in samples containing ciprofloxacin and norfloxacin, but interferences were found when applied to urine samples [20]. Hence it is desirable to develop a liquid chromatographic method for the determination of chiral purity of Levofloxacin in final drug substance as well as in drug product which serves a reliable, accurate and sensitive.

EXPERIMENTAL SECTION

Chemicals

Levofloxacin and its respective (R)-enantiomers were kindly supplied by Research and Development department of Wockhardt Limited, Aurangabad, India, and the chemical structure is shown in figure-1. HPLC grade n-hexane, methanol, isopropyl alcohol (IPA), were purchased from Merck Ltd, India.

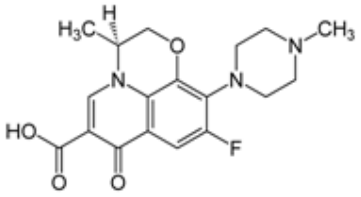

Figure 1
Levofloxacin

Equipment

The instrument used was a Waters 2695 separation module coupled to Photo Diode Array (PDA) detector equipped with an auto injector and utilized for method development and validation. Empower software was used for data acquisition and system suitability calculations.

Solution preparation

Preparation of Mobile Phase

Prepared a mixture of n-hexane, isopropyl alcohol in the ratio of 95:5 (v/v) mixed and degassed.

Preparation of diluents

Mobile phase was used as blank.

Preparation of system suitability solution

Accurately weighed and transferred about 1 mg each of Levofloxacin working standard and its (R) enantiomer in to a 50 mL volumetric flask. About 10 mL of isopropyl alcohol was added, sonicated to dissolve and diluted to volume with mobile phase. The solution was filtered through 0.45 µm nylon filter with discarding first few milliliter of filtrate.

Preparation of standard solution

Accurately weighed and transferred about 100 mg of Levofloxacin working standard in to a 100 mL volumetric flask. About 10 mL of isopropyl alcohol was added, sonicated for about 20 minutes with intermittent shaking and diluted to volume with mobile phase. The resulting solution was further diluted with mobile phase to get a solution containing 10 µg mL^{-1} concentrations. The solution was filtered through 0.45 µm nylon filter with discarding first few milliliter of filtrate.

Sample preparation (For Levofloxacin drug substance)

Accurately weighed and transferred about 100 mg of Levofloxacinin to a 100 mL volumetric flask. About 10 mL of isopropyl alcohol was added, sonicated for about 20 minutes with intermittent shaking and diluted to volume with mobile phase. The solution was filtered through 0.45 µm nylon filter with discarding first few milliliter of filtrate.

Sample preparation (For Levofloxacin drug product)

Accurately weighed and transferred tablet powder equivalent to 100 mg of Levofloxacinin to a 100 mL volumetric flask. About 10 mL of isopropyl alcohol was added, sonicated for about 20 minutes with intermittent shaking and diluted to volume with mobile phase. The solution was filtered through 0.45 µm nylon filter with discarding first few milliliter of filtrate.

Chromatographic conditions

The chromatographic conditions were optimized using chiral stationary phase (CSPs) Chiralpak IC-3, 250 mm X 4.6 mm, 3 µm, The mobile phase consisting of n-hexane and isopropyl alcohol in the ratio of 95:50 (v/v) was pumped through the column at the flow rate of 1.0 ml min^{-1}. The column oven compartment was maintained at 40°C and the detection was carried out at a wavelength of 235 nm. The injection volume was 20µl.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The aim of this work is to separate the enantiomers and for the accurate quantification of (R)-enantiomer. A solution containing 1 mg mL^{-1} of Levofloxacin and 0.01 mg mL^{-1} of R-isomer prepared in mobile phase was used in the method development. To select the best chiral stationary phase and mobile phases that would give optimum resolution and selectivity for the enantiomers. But satisfactory separation was not found in all above experiments. There is an indication of separation on Chiralpak IC-3 column using a mobile phase consisting of n-hexane and isopropyl alcohol in the ratio of 95:5 (v/v). The phase improved the chromatographic efficiency and resolution between the enantiomers. A satisfactory separation was achieved on Chiralpak IC-3 column (resolution between enantiomers was found greater than 3) using the mobile phase system n-hexane and isopropyl alcohol in the ratio of 95:5 (v/v) (Figure – 2). It is presumed that it could be due to high probability of interaction, better resolution was found on Chiralpak IC-3 column. Due to the better chromatographic results obtained on the Chiralpak IC-3 column, further method validation was carried out on the same column. In the optimized method, the typical retention times of Levofloxacin and its (R)-enantiomer were found about 10.3 min and 11.8 min, respectively. The typical chromatogram of resolution between Levofloxacin and its R-Isomers is shown in figure – 2.

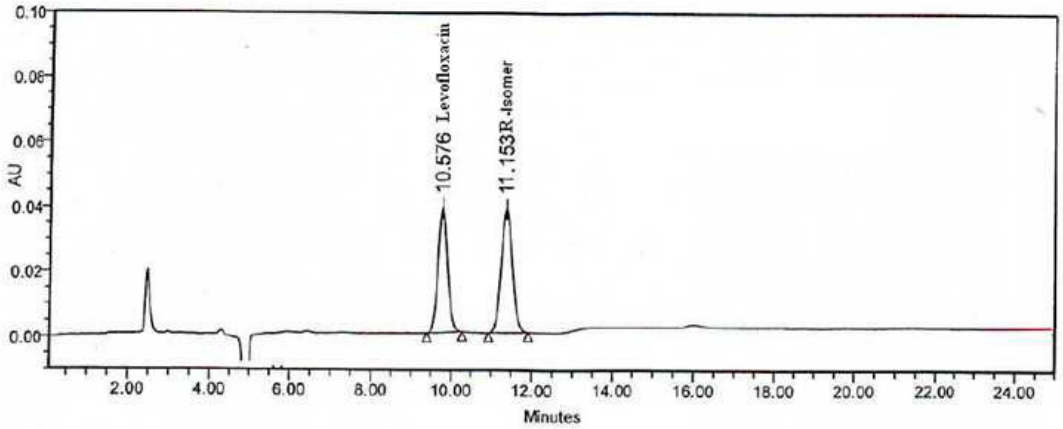

Figure 2
Typical HPLC chromatograms of resolution between Levofloxacin and R-Isomer

Method validation

No table of figures entries found.The optimized chiral purity method for Levofloxacin was validated according to ICH guidelines [15], with respect to specificity, accuracy, precision (repeatability and intermediate precision), linearity, range and robustness. System suitability parameters were also assessed.

System suitability test

The system suitability test was performed according to USP 30 and BP 2007 indications. The observed RSD values at 1% level of analyte concentration were well within the usually accepted values (<2%). Theoretical plates (N), USP tailing factor (T_f) and USP resolution (R_s) between Levofloxacin and R-isomer were also determined. The results obtained were all within acceptable limits (Table-1).

Table 1
System suitability parameters

Analyte (n=6)	Tailing factor (T_f)[a]	Efficiency (plates m-1)[a]	Resolution (R_s)[a]	RSD (%)
R-Isomer	1.07	8983	2.78[b]	0.86

Specificity

The specificity of the method was checked by injecting blank solution, excipient (for drug product) solution without drug substance, sample solution and sample solution spiked with all related known impurities at 1% level (for drug substance as well as drug product). There was no interference from blank, excipient and related known impurities at the retention time of analyte peak. Specificity was also checked by exposing the sample under stressed conditions like acid hydrolysis (1mL Trifluoro acetic acid), base hydrolysis (2mL, Diethyl amine), Oxidation (30% hydrogen peroxide 2mL), heat (80°C), light (1.2 million lux hours), humidity (40°C/75%RH). The results are tabulated in table 2.

Table 2
Results of stress study

Condition	Conditions	Time	Temperature (°C)	%Degradation
Acid hydrolysis	1mL, TFA	1Hr	25°C± 2°C	1.31
Base hydrolysis	2mL, DEA	2Hr	60°C± 2°C	4.13
Oxidation	2mL, 30% H2O2	2Hr	60°C± 2°C	6.67
Thermal	-	24Hrs	80°C	1.12
Photolytic	250 watt h/m^2	22Hrs	-	0.87
Humidity	40°C/75% RH	8 days	-	0.45

The peak purity indices for the analytes in stressed solutions and spiked sample were determined with PDA detector under optimized chromatographic conditions found to be better (purity angle < purity threshold) indicating that no additional peaks were co-eluting with the analytes and evidencing the ability of the method to assess unequivocally the analyte of interest in the presence of potential interference.

Method precision and ruggedness

In order to determine the Method precision and ruggedness of the method, six independent preparation of sample solution containing 1.0 mg mL-1of Levofloxacin spiked with (R)-enantiomer (0.5%) was prepared and calculated the percentage (% w/w) of (R)-isomer and was determined of percentage (weight / weight) of each enantiomer. The results are tabulated in table-3.

Table 3
Validation results of precision, Regression statistics, LOD and LOQ

Validation parameters	Results
Repeatibility (n=6, %RSD)	
Retention time (R)-enantiomer	0.61
Retention time (S)-enantiomer	0.76
Peak area (R)-enantiomer	1.30
% w/w of (R)-enantiomer	3.53
Intermediate Precision (n=12, %RSD)	
Retention time (R)-enantiomer	0.54
Retention time (S)-enantiomer	0.62
Peak area (R)-enantiomer	0.91
% w/w of (R)-enantiomer	3.28
Regression statistics (S-enantiomer)	
Calibration range (µg/mL)	1.271 to 7.628 µg mL-1
t-stat	150.56
P-value	7.15E-24
95% confidence interval (Lower)	9.206
95% confidence interval (Upper)	9.472
Correlation coefficient	0.999078
LOD-LOQ (S-enantiomer)	
Limit of detection (µg/mL)	1.316
Limit of quantification (µg/mL)	0.509
Precision at LOQ (%RSD)	8.23

Linearity and range

The nominal concentration of Levofloxacinin test solution was 1 mg mL^{-1}. Taking into account that typical impurity tolerance levels is 0.5 % for R-isomer and response function was determined by preparing standard solution of R-isomer at different concentration levels ranging from lower limit of quantification to 150 % of impurity tolerance level. The regression statistics are shown in table 4.

Table 4
Recovery Data

Spike level (%)	Amount added (%w/w)	Amount recovered (%w/w)	Recovery (%)	% RSD
Drug Substance				
	0.203	0.208	102.46	
50%	0.197	0.204	103.55	3.47
	0.201	0.195	97.01	
	0.503	0.499	99.20	
100%	0.502	0.489	97.41	2.65
	0.497	0.510	102.62	
	0.754	0.749	99.34	
150%	0.745	0.754	101.21	1.59
	0.747	0.766	102.54	
Drug product				
	0.203	0.199	98.03	
50%	0.198	0.205	103.54	2.90
	0.196	0.201	102.55	
	0.504	0.492	97.62	
100%	0.498	0.507	101.81	3.30
	0.496	0.517	104.23	
	0.755	0.740	98.01	
150%	0.749	0.777	103.74	2.92
	0.752	0.768	102.13	

Determination of limit of quantification and detection (LOQ and LOD)

The linearity performed above, used for the determination of limit of quantification and detection. Residual standard deviation (σ) method was applied and were predicted the LOQ and LOD values using following formula (a), (b) and established the precision at these predicted levels. The results are tabulated in table-3.

$$LOQ = \frac{10 \times \sigma}{R} \qquad \text{---------- (a)}$$

$$LOD = \frac{3.3 \times \sigma}{R} \qquad \text{--------- (b)}$$

Where
σ = Residual standard deviation of response, R = Slope of the calibration curve

Accuracy

Accuracy was evaluated by the determination of R-isomer in solution prepared by standard addition method. The experiment was carried out by adding known amount of R-isomer corresponding to three concentration levels of 50 %, 100 % and 150 % of the impurity tolerance level in sample solution (Drug substance as well as in drug product). The samples were prepared in triplicate at each level. The quantification of added analyte (% weight/weight) was carried out by using an external standard of R-isomer prepared at the analytical concentration. The experimental results revealed that approximate 95 - 105 % recoveries were obtained for R-isomer in drug substance as well as in drug product. Therefore, based on the recovery data (Table - 4) the estimation of R-isomer that are prescribed in this report has been demonstrated to be accurate for intended purpose and is adequate for routine analysis.

CONCLUSION

A simple, rapid and accurate normal phase chiral LC method was described for the enantiomeric separation of Levofloxacinin drug substance as well as in drug product. The method was validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the quantitative determination of chiral impurity ((R)-enantiomer) in drug substances and drug product.

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