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Bioequivalence study and bioanalytical method development of Levofloxacin tablets in albino rat plasma using RP-HPLC method

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ABSTRACT

A rapid, sensitive and selective HPLC method for the determination of levofloxacin in rat plasma is developed and validated. Separation is done on a Phenomenex C18 RP column with a mobile phase of acetonitrile – 0.4 % triethylamine (pH 3.1) in the ratio of 18:82 % v/v and detection at 295 nm. The standard curve is linear (r > 0.998) over the concentration range of 20.0–5000 ng/ml. The lower limit of quantification (LOD) for levofloxacin is 10.0 ng/ml. The maximum concentration (C_{max}) obtained for two brands Levoflox and Generic formulation are 132.72 and 113.97 ng/ml respectively. The half life ($t_{1/2}$) of levofloxacin for Levoflox and Generic are calculated as 1.763 and 1.628 h. Area under the curve AUC_0^t of Levoflox and Generic is calculated as 271.18 and 279.84 ng h/ml and AUC_t^∞ is calculated to be 4.31 and 10.97 ng h/ml respectively and AUC_0^∞ is calculated to be 275.48 and 290.81 ng h/ml. Elimination rate constant (k_{el}) is calculated for Levoflox and Generic from the slope of log concentration versus time curve with regression analysis. Elimination rate constant is found to be 0.393 and 0.4272 h^{-1}. This study shows that there is no significant difference in kinetic parameters between two products. So the two formulations are considered to be bioequivalent.

Key words: RP-HPLC, Levofloxacin, bioequivalence.

INTRODUCTION

Levofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class [1] and is used to treat severe or life-threatening bacterial infections or bacterial infections that have failed to respond to other antibiotic classes [2] Levofloxacin is a chiral fluorinated carboxyquinolone. Levofloxacin interacts with a number of other drugs, as well as a number of herbal and natural supplements. Levofloxacin is associated with a number of serious and life-threatening adverse reactions as well as spontaneous tendon ruptures and irreversible peripheral neuropathy. Levofloxacin is rapidly absorbed after oral administration with maximum plasma concentrations being reached approx 1 h after a dose. It is widely distributed throughout the body, crosses the placenta and has been detected in breast milk. Levofloxacin undergoes limited metabolism and is excreted mainly as the unchanged drug in urine (80 to 85%) and faeces (2%) within 24 h.

In order to assure the therapeutic equivalence of these generic products, the bioequivalence study needs to be investigated and hence the present study was undertaken to compare the pharmacokinetic profiles and to evaluate the bioequivalence of the generic and branded Levofloxacin formulations in albino rats. Most of the drug in biological sample can be analyzed by HPLC method because of several advantages like rapidity, specificity, accuracy, and precision, ease of automation and elimination tedious extraction and isolation procedure.

MATERIALS AND METHODS

2.1. Materials

Levofloxacin and hydrochlorthiazide pure samples were obtained as a gift sample from Micro labs, Bangalore and Levoflox (500 mg) tablet (Cipla Ltd.,), Generic (Levoquin-500 mg) tablet were procured from Cheminova pharmaceuticals, baddi, HP, Analytical grade Orthophosphoric acid, Triethylamine and Disodium EDTA were obtained from SD fine chem. Limited, Mumbai

2.2. Instruments used

Electronic balance AY 220 (Shimadzu), pH meter (Eutech), Ultra sonicator (Soltech Pvt Ltd.,), Solvent filtration unit (Millipore), Ultra cooling centrifuge rpm-5000/sec (REMI), HPLC system (Shimadzu).

2.3. Configuration of the HPLC system

LC – 20AT prominence solvent delivery system (Pump), Rheodyne 7725i injector with 20 µl loop, SPD-M20A Prominence Diode array detector, LC-solution data station, Analytical column: Phenomenex – Luna, C18 (250 x 4.6 mm i.d.,5µ).

2.4. Study Design **[3-6]**

The study adheres to “Principles of Laboratory Animal Care” and is approved by the animal care committee IAEC/CPSEA-Institutional animal ethics /Committee for the purpose of control and supervision of experiments on animals.

Healthy Albino rats (both sex) of weight 150-250 g were taken and grouped. The rats were taken from KMCH college of pharmacy animal house, which were quarantined a week before. Rats are

divided in to two groups (12 rats/group), each group subdivided in to three subgroups A, B, C (4 rats/subgroup). Each group was given oral administration of Levofloxacin drug solution (100 mg/kg) as one group receives Levoflox (Cipla) and another group receives Generic.

Blood samples were withdrawn at specified pre-determined time intervals (0.33 to 12 h) using tail vein puncture. The collection tube containing (Disodium EDTA), blood samples were immediately transferred to the tube and shaken well and centrifuged using ultra cooling micro centrifuge at 5000 rpm/sec to separate plasma. The separated plasma samples were transferred to a labeled air tight sample tubes and kept in deep freezer for further analysis.

2.5. Estimation of levofloxacin in plasma [7-14]

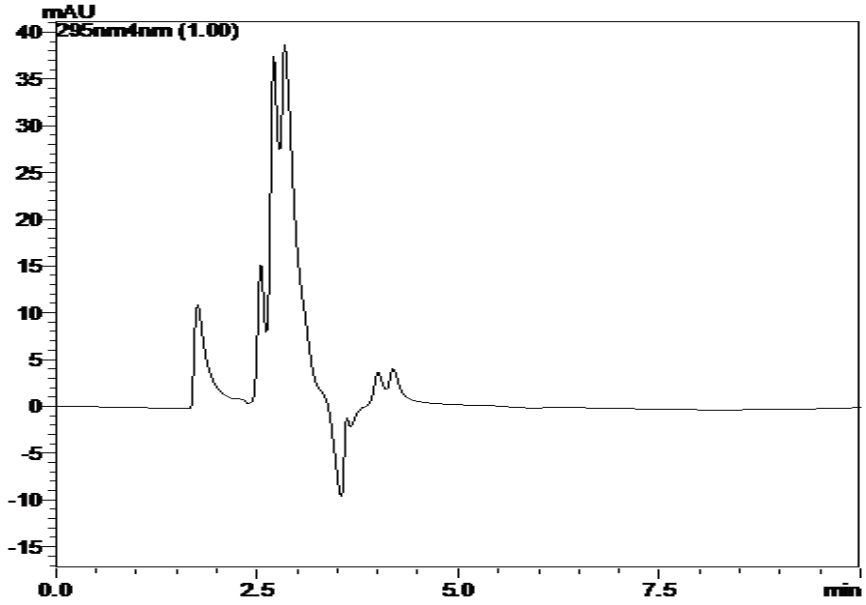
2.5.1. Preparation of standard solution

Standard graph of Levofloxacin was prepared by taking 0.2 ml of the drug free plasma and 0.05 ml of working standard solution of Levofloxacin was added to yield a final respective concentration as 20, 40, 60, 80, 100 ng/ml of Levofloxacin in Plasma. To each calibration standard 0.05 ml of Hydrochlorthiazide (internal standard) solution was added and vortexed. To this calibration standards 0.1 ml of extraction solvent (Methanol) was added, vortexed and centrifuged for 15 min at 5000 rpm. 20 µL of this supernatant was injected to HPLC and chromatogram was recorded. A standard graph was plotted using concentration and peak area ratio of Levofloxacin.

2.6.2. Preparation of sample solution

Sample Solution was prepared by taking 0.2 ml of sample plasma, 0.1 ml of internal standard (15 µg/ml) and 0.1 ml of precipitating agent (methanol) were added and mixed. The resulting solution was vortexed and centrifuged at 5000 rpm for 15 min. The supernatant layer was separated and analyzed.

Figure 2: Blank Plasma Chromatogram



The standard solution and sample solution were injected with the above chromatographic condition and the chromatograms were recorded. The retention time of Levofloxacin and internal standard was 6.84 and 8.67 min, respectively. The response factor (peak area ratio of standard peak area and the internal standard peak area) of the standard solution and the sample were calculated and the concentration of the Levofloxacin present in the plasma samples was calculated. The HPLC chromatograms for the estimations were included in the figures 2-5.

Figure 3: HPLC Chromatogram of Plasma spiked Levofloxacin 100 ng/ml and internal standard Hydrochlorthiazide 500 ng/ml

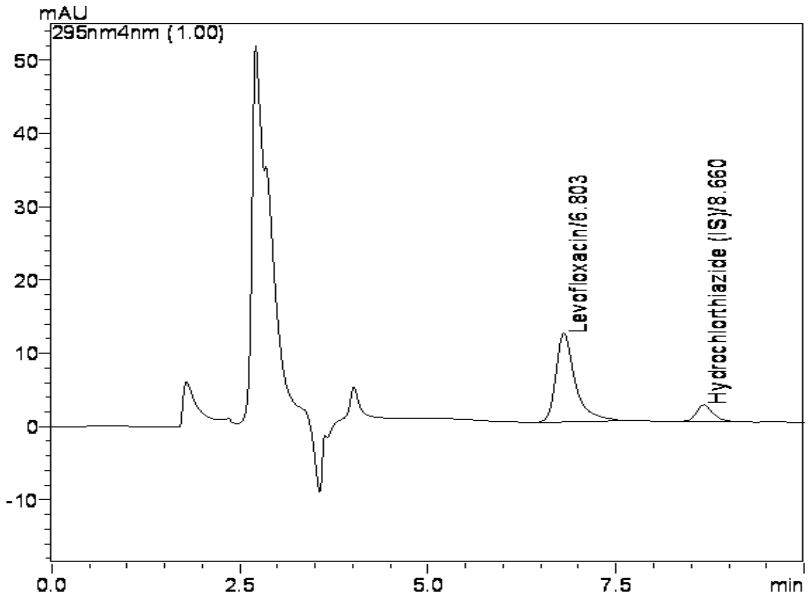


Figure 4: HPLC Chromatogram of Levofloxacin after 40 min (oral administration) of Levoflox and spiked internal standard Hydrochlorthiazide 500 ng/ml

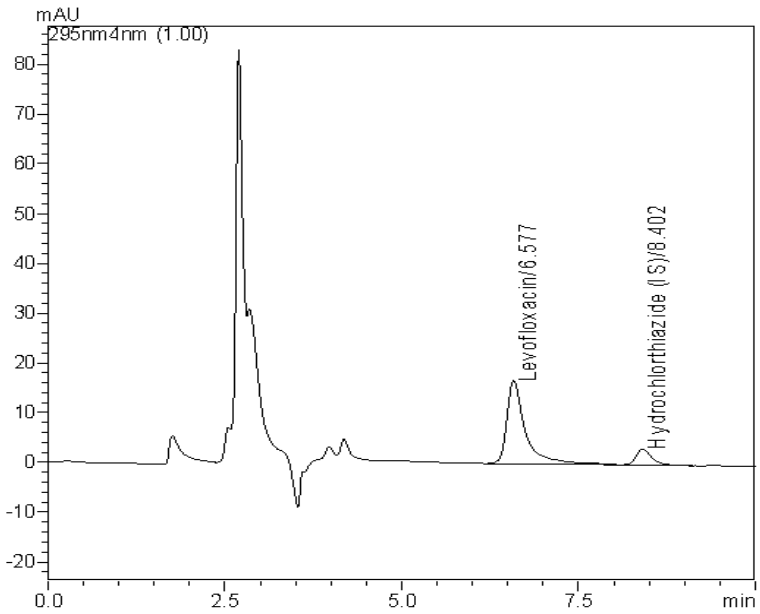
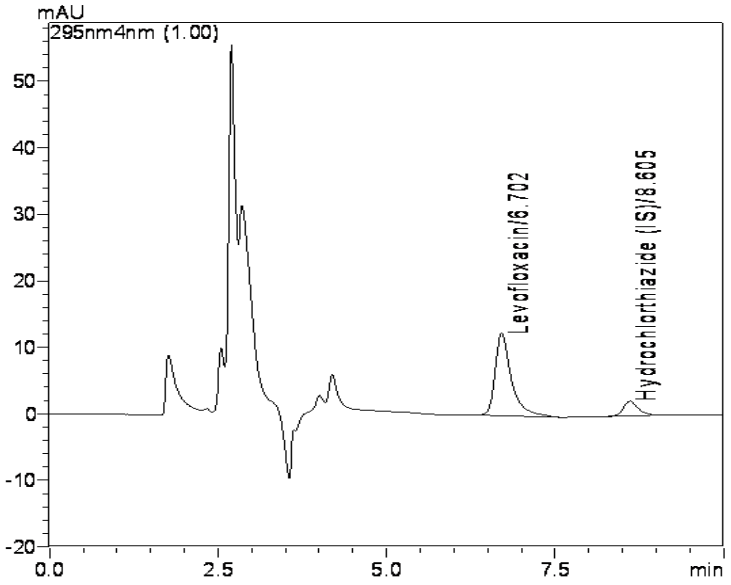


Figure 5: HPLC Chromatogram of Levofloxacin after 40 min (oral administration) of Generic and spiked internal standard Hydrochlorthiazide 500 ng/ml



RESULTS

3.1. Separation and Chromatography

Methanol was selected as a good precipitating agent for the drug, since it shows maximum recovery. The chromatogram was recorded for the standard calibration and plasma sample under the developed chromatographic conditions. The retention time of levofloxacin and hydrochlorthiazide are 6.86 and 8.68 min. The chromatogram is well resolved without any interference from one another. Moreover, peaks do not show any tailing or fronting and it shows straight baseline. The concentration of levofloxacin in rat plasma was determined from the calibration curve of the spiked plasma by regression analysis. It shows very good linearity in the range of 20-100 ng/ml and the r^2 value was found to be 0.9984.

3.2. Validation of the method [12]

3.2.1. Accuracy and precision

The accuracy and precision study was performed at two levels namely intra-day and inter-day. The developed method shows good accuracy and precision. The intra-day accuracy ranges between 97.02 and 99.04 with precision between 1.211 and 0.406. The intra-day accuracy ranges between 97.90 and 99.87 with precision between 0.905 and 0.061.

3.2.2. Linearity and range

The calibration curves were linear over the range of 20 to 100 ng/ml. The correlation coefficient (r) was > 0.9984. The slope of the calibration curves for Levofloxacin was 0.06125 ± 0.001422. The mean intercept of calibration curve for Levofloxacin was 0.01900 ± 0.09433.

3.2.3. Limit of Detection and Limit of Quantification

The LOD was 500 pg/ml. The LLOQ was 10 ng/ml with coefficient of variation of 0.61% and accuracy 99.81%. The ULOQ was 50 ng/ml with coefficient of variation of 0.045% and accuracy 100.11%.

3.2.4. Recovery from plasma

The extraction efficiency of Levofloxacin from rat plasma at the concentrations of 1, 10 and 100 ng/ml was found to be 91.61%, 92.55% and 93.57% with precision of 2.275, 1.117, and 0.621. The mean recovery for internal standard was 93.56%.

3.2.5. Specificity

No significant interfering peaks were observed at retention time of either analyte or internal standard in four different lots of drug spiked rat plasma samples used for analysis.

2.3.6. System suitability

System suitability parameters such as column efficiency (theoretical plates), resolution factor and HETP of the optimized methods were found satisfactory.

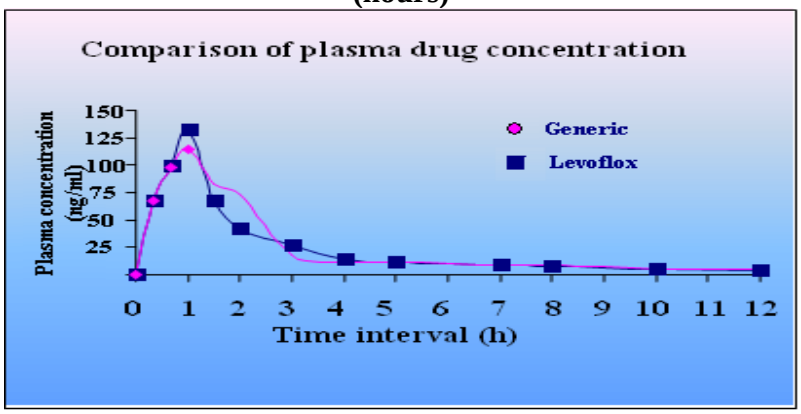
3.3. Pharmacokinetic study

After a single oral dose of 100 mg/kg of Levofloxacin the sample was in measurable amount in plasma up to 12 hours. The pharmacokinetic parameters of the samples were calculated manually and using prism, graph pad version 5, Microsoft excel software and the results are given in the table 1 and figure 1.

Table 1. Pharmacokinetic parameters of two products

S.No.	Parameters	Levoflox (reference product)	Generic (test product)
1	AUC_{0-t} (ng. h/ml)	271.18	279.84
2	$AUC_{0-\infty}$ (ng. h/ml)	275.18	290.81
3	C_{max} (ng /ml)	132.72	113.97
4	t_{max} (h)	1.0	1.0
5	K_{eli} (h^{-1})	0.3930	0.4272
6	$t_{½}$ (h)	1.763	1.628

Figure 1: Comparison of plasma concentration of levofloxacin and generic drug. It shows the Plasma concentration of the drugs under analysis (ng/ml) at various time intervals (hours)



The maximum concentration (C_{max}) obtained in two brands Levoflox and generic were 132.72 and 113.97 ng/ml respectively. The half life ($t_{1/2}$) of levofloxacin for Levoflox and Generic were calculated and found to be 1.763 and 1.628 hours. Area under the curve AUC_0^t of Levoflox and Generic was calculated as 271.18 and 279.84 ng h/ml and AUC_t^∞ was calculated to be 4.31 and 10.97 ng h/ml respectively and AUC_0^∞ was calculated to be 275.48 and 290.81 ng h/ml. Elimination rate constant (k_{el}) was calculated for Levoflox and Generic from the slope of log concentration versus time curve with regression analysis. Elimination rate constant was found to be 0.3930 and 0.4272 h^{-1}.

The pharmacokinetic parameters such as $C_{max,}$ $T_{max,}$ AUC_0^t, AUC_0^∞, K_{el} and $t_{1/2}$ are compared for the bioequivalence study by statistical analysis. The p value obtained from one-way ANOVA and t-test were found to be 0.9914, which shows there is no significant difference between two products.

DISCUSSION

The bioanalytical method was developed and validated and the pharmacokinetic parameters were studied. Bioequivalence of two brands of Levofloxacin was studied from the acquired data. Levoflox 500 mg tablets were taken as a reference product to evaluate the pharmacokinetic profile of Generic (levaquin) 500 mg tablets which was taken as (Test Product). The pharmacokinetic parameters $C_{max,}$ $t_{max,}$ AUC $_{(0\text{-}t),}$ AUC $_{(0\text{-}\infty),}$ $t_{1/2,}$ K_{eli} of test Product (Generic 500mg) were found to be similar to those of reference product (Levoflox 500 mg).

CONCLUSION

A rapid, sensitive and selective HPLC method for the determination of levofloxacin in rat plasma was developed and validated. Sample preparation was assured by one-step protein precipitation method. Separation occurred on a Phenomenex C_{18} RP column (5 µm, 25 cm x 4.6 mm ID) with a mobile phase of acetonitrile-0.4 % triethylamine (pH 3.1), (18:82% v/v) and detection at 295 nm. The standard curve was linear (r > 0.998) over the concentration range of 20.0–5000 ng/ml. The lower limit of quantification for levofloxacin was 10.0 ng/ml using 20 µl plasma samples. This method was successfully applied to the bioequivalent study of levofloxacin in rats for two formulation, standard (levoflox) and test (generic-levaquin). For bioequivalence study, parameters like $C_{max,}$ $T_{max,}$ AUC_0^t, AUC_0^∞, K_{el} and $t_{1/2}$ are compared by statistical analysis. The maximum concentration (C_{max}) obtained in two brands Levoflox and generic were 132.72 and 113.97 ng/ml respectively. The half life ($t_{1/2}$) of levofloxacin for Levoflox and Generic were calculated and found to be 1.763 and 1.628 hours. Area under the curve AUC_0^t of Levoflox and Generic was calculated as 271.18 and 279.84 ng h/ml and AUC_t^∞ was calculated to be 4.31 and 10.97 ng h/ml respectively and AUC_0^∞ was calculated to be 275.48 and 290.81 ng h/ml. Elimination rate constant (k_{el}) was calculated for Levoflox and Generic from the slope of log concentration versus time curve with regression analysis. Elimination rate constant was found to be 0.3930 and 0.4272 h^{-1}. The p value obtained from one-way ANOVA and t-test were found to be 0.9914, which shows there is no significant difference between two products.

The bioanalytical method developed is simple and shows good accuracy, specificity and reproducibility. It can be used for the estimation of Levofloxacin in biofluids. The separation method developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of levofloxacin and internal standard (Hydrochlorthiazide) without any interference.

Pharmacokinetic parameters such as C_{max}, t_{max}, $AUC_{(0-t)}$, $AUC_{(0-\infty)}$, $t_{1/2}$, K_{eli} calculated for the two product, Levoflox (reference) and generic (test), shows no significant difference.

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