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A SELECTIVE AND SENSITIVE UPLC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF FOUR GTIs IN LEVOFLOXACIN

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ABSTRACT

A selective and sensitive reversed phase UPLC-MS/MS method was developed and validated for the simultaneous determination of four GTIs (viz., LAL, LAC, LTA, LHP) in levofloxacin drug substance. The chromatographic separation was performed on Acquity UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm) using gradient elution of acetonitrile in methanol (75:25, v/v) and 5.0 mM ammonium acetate buffer at flow rate of 0.3 mL/min. Positive electrospray ionization (ESI) mode was operated for quantification of all four impurities. The total run time was 20 min, within which levofloxacin and its four impurities were well separated. The developed method was validated as per ICH guidelines in terms of specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness. The calibration curve shows good linearity over the concentration range of 0.15-2.0 ppm for all four impurities. The correlation coefficient obtained was >0.9998 in each case. Method has very low limit of detection (LOD) and limit of quantification (LOQ). The method provided the accuracy between 92.66-102.70% for all the four impurities. The developed method was successfully applied for five formulation batches of levofloxacin to determine the above mentioned impurities.

Keywords: Levofloxacin, UPLC-MS/MS, Quantification, Genotoxic Impurities, Positive mode, Electrospray Ionization.

INTRODUCTION

During the synthesis of active pharmaceutical ingredients (APIs), some starting materials, intermediates, reagents, and reaction by-products inevitably end up in the final products as impurities in new commercial drugs.[1] Among all, the genotoxic impurities (GTIs) that have the potential to induce genetic mutations, chromosomal breaks, and/or chromosomal, which may cause cancer in humans.[2] To overcome this, scientists have to identify GTIs early in process development, develop analytical methods and demonstrate the synthetic process controls. Hence, the impurity profiling of active pharmaceutical ingredients (APIs) is one of the most challenging tasks of analytical chemists. For these reasons, the European Medicine Agency (EMEA) and US FDA have published separate guidelines with respect to the limit of genotoxic impurities in new commercial drugs. Both agencies have set threshold of toxicological concern (TTC) of 1.5 µg/day for genotoxic impurities.[3,4] Levofloxacin belongs to the class of fluoroquinolone (or quinolone) antiinfectives. It is a synthetic chemotherapeutic agent used to treat severe or life-threatening bacterial infections. Levofloxacin functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV[5]. Chemically is known as (-)-(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-carboxylicacid hemihydrates. The compounds L-alaninol (LAL), levo acrylate (LAC),levotetrafluoro acrylate (LTA), levohydroxyl propane (LHP) are the key intermediates used in the synthesis of levofloxacin, which are already identified as GTIs[6].The chemical structure of GTIs and levofloxacin are demonstrated in Fig.- 1. Based on the TTC limit of 1.5 µg/person/day and on the maximum adult daily dose for levofloxacin of 750 mg/person, its GTIs are required to be controlled at a concentration limit of 2.0 µg/g (ppm) in the drug

substance. Therefore suitable analytical methods should be developed to meet the required limit of 1.5 µg/day intake of individual impurity.[7]

Ideally, conventional analytical instrumentations in pharmaceutical analysis such as HPLC with UV detection (for typical non-volatile analytes) or GC with FID detection (for volatile small molecules), should be employed as the standard first attempt for GTI analysis, but are often inadequate for accurate determination of analytes at low ppm levels, depending on properties of the analytes and sample matrices. Recently, hyphenated mass spectrometry has gained popularity in the field due to superior sensitivity and specificity.[8-9] Because of the advantage of mass selective detection, MS methods are generally less prone to interferences compared to the non-specific detectors such as UV. Therefore, efforts needed for method development for GTIs analyses could be greatly reduced.[10-12] There are several methods reported for the determination of levofloxacin and its impurities.[13-18] Based on literature survey,[19-23] no analytical method has been developed for the simultaneous determination of four mentioned GTIs in levofloxacin. Hence, we have developed a simple UPLC-MS/MS method that can quantify at permitted levels of all impurities in levofloxacin**.**This method is validated as per ICH guidelines in terms of limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, specificity and robustness.

EXPERIMENTAL

Chemicals and Reagents

HPLC grade acetonitrile and ammonium acetate were purchased from Merck (Mumbai, India). Formic acid, trifloroacetic acid and methanol were obtained in their highest grade from SD fine chemicals limited (Mumbai, India). Reference substances of levofloxacin and all four GTIs were obtained from Sigma-Aldrich (St.Louis, MA, USA). Water was purified with a Milli-Q plus system from Millipore (Bedford, MA, USA).

Instrumentation

The instrumentation used was UPLC coupled with Mass spectrometer (XEVO TQ-S, Waters, USA), for method development and partial validation consisting of mass detector and auto sampler used in this experiment. Data acquisition and processing were conducted using the MassLynx version 1.4 software on a dell computer (Digital equipment Co).

Preparation of Stock and Standard Solutions

A stock solution of levofloxacin (5.0 mg/mL) was prepared by dissolving appropriate amount in the mobile phase B. Stock solutions of mixture of PGIs (LAL, LAC, LTA and LHP) at 0.5 mg/mL were also prepared in the above diluent. The diluted stock solution (0.01mg/mL) was prepared by diluting 1.0 mL of the 0.5 mg/mL solution to 50 mL with the same diluent. Then 0.5 µg/mL diluted stock solution was prepared by diluting 5.0 mL of 0.01 mg/mL stock solution to 100 mL. The working standard solution was prepared by accurately weighing about 50 mg of levofloxacin into 10 mL volumetric flask and made up to the mark after adding 10µL of 0.5µg/mL diluted stock solution to give 5.0ng/mL and 5.0mg/mL of PGIs with respect to levofloxacin which corresponds to 1.0 ppm of PGIs contamination relative to the drug substance. The PGIs samples for validation at 0.15, 0.5, 0.75, 1.0, 1.5 and 2.0 ppm concentrations relative to the drug substance were prepared in the same manner using 0.5 µg/mL of diluted stock solution. The concentration of the standard solutions and samples were optimized to achieve a desired signal–to–noise ratio (S/N) and good peak shape. All the standards were sonicated well and the filtered through 0.22 µm membrane filters before the analysis.

Chromatographic Conditions

All chromatographic experiments were carried out on an Acquity UPLC system coupled with MS/MS (Waters, USA). The analytical column used was Acquity UPLC BEH C18 (100 x 2.1 mm, 1.7µm). The gradient elution employed with solutions A and B as mobile phase components. The solution a contained 5.0 mM ammonium acetate buffer and solution B having a mixture of acetonitrile- methanol (75:25, v/v). The flow rate of the mobile phase was set at 0.3 mL/min and column temperature was maintained at 40^0C.

The gradient program was set as follows: time/% solution A: 0/65, 4.0/65, 6.5/45, 13/45, 15/65, 20/65, with equilibrium time of 2 min. The injection volume was 10 µL. All the solutions were filtered through 0.22 µm nylon filter before the analysis.

Mass Spectrometer

The MS/MS system used was Waters Xevo TQ–S triple quadruple mass spectrometer (USA) with electrospray ionization (ESI) probe operated in positive polarity. The control of the system and data collection was done by MassLynx version 4.1 software. Typical operating conditions were as follows: Capillary 3.3 kV, cone 25 V, Desolvation temperature 550^0C and source temperature 150^0C. Desolvation gas 750 L/hr, cone gas at 150 L/hr, nebulizer gas flow 6 bars and dwell time was 150 ms. Electrospray ionization in positive SIR mode was used for quantification of LAL at m/z 76.14, and MRM mode was used for the quantification of LAC, LTA and LHA at m/z 144.19/98.13, 320.31/274.22, and 350.32/86.09 respectively.

Validation Study

The developed method was validated in terms of specificity, linearity, limit of quantification (LOQ), limit of detection (LOD), accuracy, precision, robustness, ruggedness and solution stability by following ICH guidelines.

The linearity of the method was evaluated by preparing and analyzing six point calibrators of 0.15-2.0 ppm for all the four impurities. The slope, intercept and regression coefficient values were determined by the least squares linear regression analysis. System precision of the mass spectrometric response was established by making six injections of the standard solution. The method precision was evaluated by spiking each analyte and determining the %RSD. Limit of detection (LOD) and limit of quantification (LOQ) were evaluated by considering the impurities concentration that would yield a signal–to–noise (S/N) ratios of 3:1 and 10:1 respectively. The LOD and LOQ values were experimentally verified by six injections of standard solutions of the compounds at the determined concentrations. Recovery of the method was performed by standard addition method to evaluate accuracy and specificity. Accordingly, the accuracy of the method was determined by spiking at LOQ, 0.5ppm and 1.0ppm of LAL, LAC, LTA and LHP separately to three batches of pure levofloxacin (5.0 mg/mL). Stability of the impurities in sample solution was done by analyzing spiked sample solution at different time intervals at room temperature.

RESULTS AND DISCUSSION

Method Development

The main objective of the present study was to achieve efficient separation between levofloxacin and four PGIs, and quantification of four PGIs in levofloxacin drug substance using UPLC-MS/MS. The signal intensity obtained in positive mode was much higher than that in negative mode for all the four PGIs. Then, the possibility of using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources under positive ion detection mode was evaluated during the early stage of method development. Positive ESI spectra revealed higher signals for the analytes compared to APCI source. Further the method development is therefore limited to ESI source.

Column Selection and Separation

The main objective of the present study was to achieve better separation among the closely eluting four GTIs in levofloxacin with symmetrical peak shapes, and the method should be able to determine all the impurities in a single run. Moreover, the developed method should be linear, accurate, reproducible, robust, stability indicating and enough for routine use in quality control laboratory.

Several attempts were made with different C18 UPLC columns (Inertsil ODS-3, 50 mm x 2.1 mm, 2.0 µm and Zorbax XDB C18, 50 mm x 4.6 mm, 1.8 µm), using gradient elution. Using the above columns the separation of the impurities was not satisfactory. On Acquity UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 µm) separation and response for all the four impurities were found to be good. On this column the impurities were well retained and separated from the levofloxacin drug substance peak. The sample

containing 5.0 mg/mL of levofloxacin and 1.0 ppm each of four impurities was prepared in the mobile phase B. The four impurities of levofloxacin were subjected to separation by reversed-phase LC on Waters Acquity BEH C18 (100 mm x 2.1 mm, 1.7 µm) column with 5.0 mM ammonium acetate buffer as solvent A and acetonitrile in methanol (60:40, v/v) as solvent B, in this case impurities viz., LAL and LPA were separated and the remaining two are merged together and levofloxacin was co-eluted with impurities. By changing the solvent B composition to 75:25 ratio all the impurities were eluted with desired peak shape as well as from the drug substance. Both isocratic and gradient elution modes were evaluated during the optimization, among which gradient elution was observed to be more efficient in achieving optimum separation of all the four impurities from the drug substance.

Optimization of Mass Spectrometric Parameters

Choosing the detection method is the most important task in pharmaceutical impurity analysis, from the instrument availability LC-MS/MS was first evaluated for the method development. However with LC-MS/MS the separation and peak shape was not good for all four closely related impurities. Hence LC-MS/MS is unsuitable for simultaneous determination of four closely related impurities in levofloxacin. Therefore we aimed to develop a UPLC-MS/MS method for determination of all four impurities in levofloxacin simultaneously. Due to the combination of a unique product ion and elimination of background noise, MRM results showed consistently low limits of detection even for complex matrices comparatively with SIM.

This mode provides significant enhancement of selectivity and sensitivity for screening and quantification. For MRM quantitation, specific mass transitions (daughter ions) were selected for LAC, LTA and LHP preparing standard solution of the analytes in the diluent and directly infusing into the electrospray ionization probe. For LAC with positive MRM, the transition selected was 144.19 (parent mass) →98.13 (fragment mass), as this is the most intense transition. In case of LTA the MRM transition of 320.31 (parent mass) →274.22 (fragment mass) was selected on the basis of response and for LHP the MRM transition of 350.3 (parent mass) → 86.09 (fragment mass) was selected. But for LAL there was no intense fragment peak was observed to maintain the MRM mode, hence in this case SIM mode was operated with positive ionization at m/z 76.14.

Method Validation

The developed method was fully validated by standard procedure to ensure adequate selectivity, sensitivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, solution stability and robustness[19-23]. The system suitability was checked by injecting 5.0 mg/mL of levofloxacin solution containing 5.0 ng/mL of all impurities monitored throughout the validation.

Specificity

The ability of an analytical method to unequivocally assess the impurities in presence of the other components (levofloxacin and excepients) can be demonstrated by evaluating the specificity of the method. The specificity of the developed UPLC-MS/MS method was verified by injecting a blank sample and individual impurity samples with standard concentration. No interfering peak was observed for any of the impurity with levofloxacin.The specificity chromatogram was shown in Fig.-1.

Linearity

The linearity of all the impurities was satisfactorily demonstrated with a six point calibration graph between LOQ - 200% (i.e0.15-2.0 ppm) of specification limit with respect to the sample concentration of 5.0 mg/mL. The average peak areas were plotted against the concentration of the analyte, resulting in a linear plot. The regression line was obtained by the least squares method, and expressed by the correlation coefficient (r) shown in Table-1.The result showed an excellent correlation between the peak and concentration of all impurities.

Limit of Quantification (LOQ) and Limit of Detection (LOD)

LOD and LOQ of all four impurities were evaluated based on their signal-to-noise ratios of 3:1 and 10:1 respectively, by injecting a series of dilute solutions of known concentration. The LOD of impurities was found to be 0.05 ppm and LOQ was found to be 0.15 ppm. Precision was also determined at LOD and LOQ levels by analyzing six individual preparations of all the four impurities and calculating their %RSD of the peak area for each impurity (Table- 1). Corresponding chromatograms were shown in Fig. - 2.

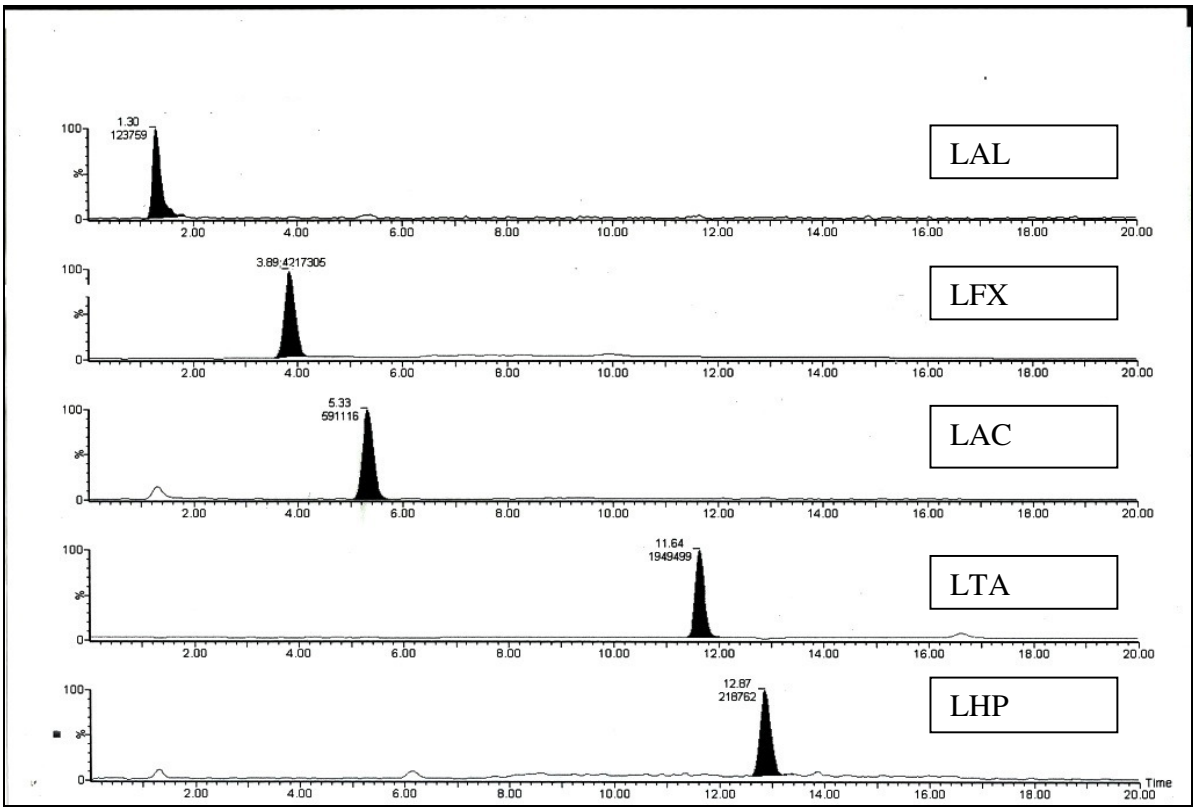

Fig.-1: Typical Chromatogram showing separation of all the impurities from Levofloxacin.

Precision

The precision of the method was verified by repeatability and intermediate precision. Repeatability was checked by injecting six individual preparations of levofloxacin sample (5.0 mg/mL) spiked with 1.0 ppm of its four impurities. The intermediate precision of the method was determined by performing the analysis on different days. %RSD of area for each impurity was calculated for both repeatability as well as intermediate precision. The %RSD was found to be less than 2.0% in both the cases, these results confirmed the overall precision of the method (Table- 1).

Accuracy

The accuracy of the method was demonstrated by evaluating the recovery of the impurities spiked into levofloxacin sample. Recoveries were assessed at LOQ, 1.0 ppm, and 1.5 ppm concentrations for all the four impurities with respect to 5.0 mg/mL nominal drug sample concentration. The recovery at all the three concentrations was excellent. Therefore, the accuracy of the method in the range of LOQ-150% was confirmed by the recovery data which was shown in Table-2 and the corresponding accuracy chromatogram was shown in Fig.-3. The developed method was successfully applied for the determination of the impurities in five different batches of levofloxacin formulation samples. No

impurities were found in three batches of levofloxacin samples. Moreover, only LAL was detected in two formulation batches of levofloxacin, but the concentration of LAL was found to be less than the detection level.

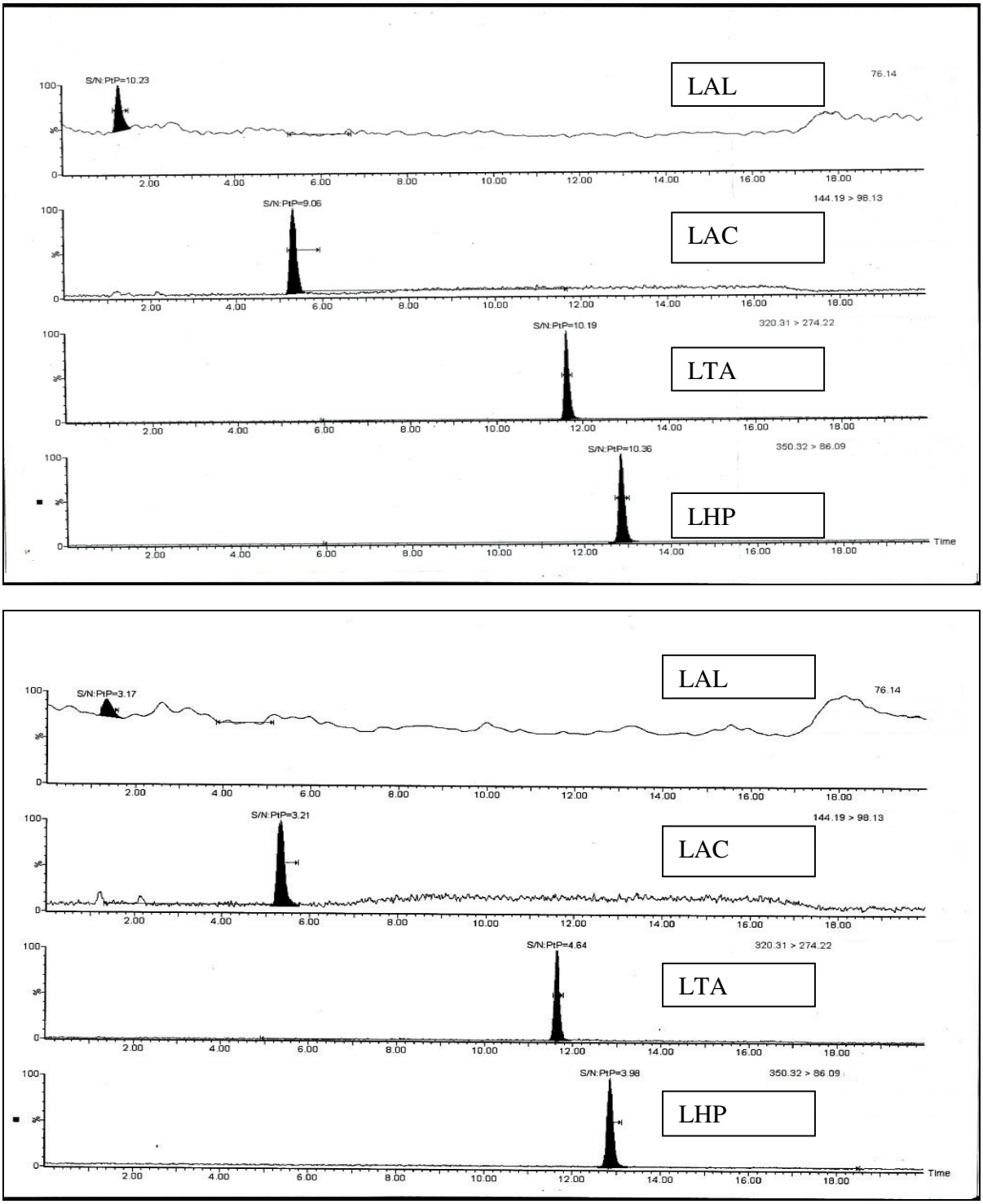

Fig. -2: Typical chromatograms showing LOQ (top) and LOD (bottom) of all the impurities

Table- 1: Linearity, LOD, LOQ and Precision data of LAL, LAC, LTA, LHP impurities with respect to 5 mg/mL of levofloxacin.

Parameter	LAL	LAC	LTA	LHP
Linearity range (ppm)	0.15-2.0	0.15-2.0	0.15-2.0	0.15-2.0
slope	435,211	628,443	216,429	433,284
r	0.9998	0.9999	0.9998	0.9997
LOD	0.05	0.05	0.05	0.05
Precision at LOD				
% RSD	1.24	1.52	0.72	0.84
LOQ				
Precision at LOQ	0.15	0.15	0.15	0.15
% RSD				
	0.93	0.49	2.42	0.96
Precision				
Intraday (n=6)				
% RSD		1.66		
Interday (n=6)	2.14		0.94	1.43
% RSD		0.98		
	1.45		1.22	1.21

Where, n is number of determinations.

Robustness

To determine the robustness of the developed method the experimental conditions were deliberately changed and the impact on chromatographic performance was observed. To study the effect of flow rate it was changed to 0.27 and 0.33 mL/min (altered by 10% of flow). The effect of column temperature was studied at 38^0C and 42^0C. However, in all these experiments the mobile phase components were not changed. In all the deliberately varied chromatographic conditions the selectivity as well as the performance of the method was unchanged, which proves the robustness of the method.

Table- 2: Accuracy data of levofloxacin spiked with its four impurities at LOQ, 1.0 ppm and 1.5 ppm concentrations.

Theoretical conc. (ppm)	LAL	LAC	LTA	LHP
Accuracy at LOQ level (n = 3)				
Amount added	0.15	0.15	0.15	0.15
Amount recovered	0.154	0.148	0.146	0.139
% recovery	102.66	98.66	97.33	92.66
% RSD	0.83	0.92	1.16	1.54
Accuracy at 1.0 ppm conc. (n=3)				
Amount added	1.0	1.0	1.0	1.0
Amount recovered	0.946	1.027	0.937	0.931
% recovery	94.60	102.70	93.70	93.10
% RSD	1.34	0.82	1.84	2.28
Accuracy at 1.5 ppm conc. (n=3)				
Amount added	1.5	1.5	1.5	1.5
Amount recovered	1.482	1.514	1.394	1.518
% recovery	98.80	100.93	92.93	101.20
% RSD	1.42	0.86	2.94	1.53

Where, n is number of determinations.

Stability in Solution and in the Mobile Phase

No significant changes in the concentrations of all four impurities were observed during solution stability experiments. The percentage recoveries of standard solutions at different time intervals were within the range of 96.5-102.8% of their nominal values. The results from the solution stability experiment confirmed that standard solutions were stable up to 36hr during the analysis. The corresponding solution stability data was presented in Table- 3.

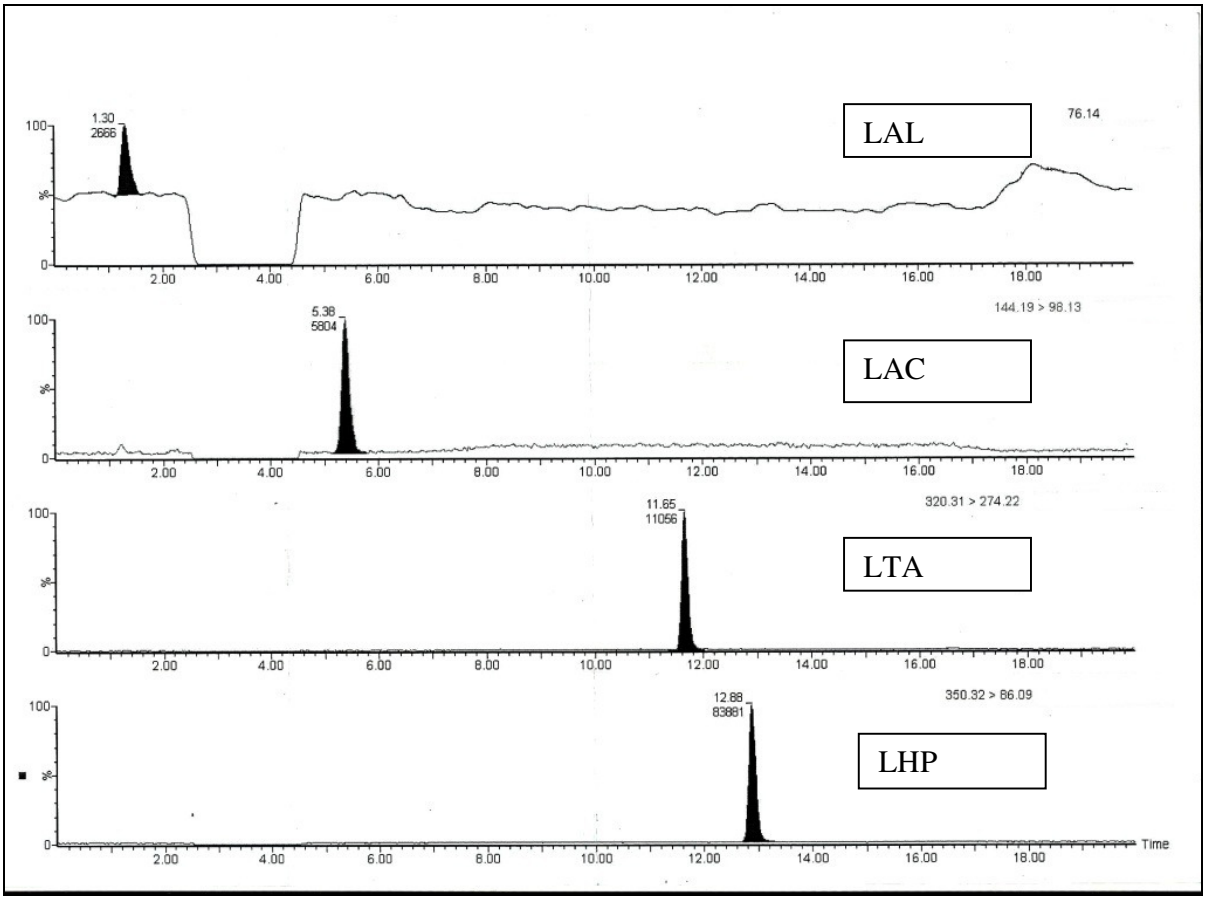

Fig.-3: Typical chromatogram representing impurities spiked in Levofloxacin sample.

Table- 3: Solution stability data of levofloxacin spiked with its impurities at LOQ concentration.

Parameter	Time (hr)			
	0	12	24	36
LAL				
Theoretical conc. (ppm)	0.15	0.15	0.15	0.15
Measured conc. (ppm)	0.147	0.152	0.134	0.139
% recovery (mean±%RSD)	98.65±0.83	101.80±0.22	89.4±1.22	92.8±2.16
LAC				
Theoretical conc. (ppm)	0.15	0.15	0.15	0.15
Measured conc. (ppm)	0.146	0.149	0.154	0.144
%recovery(mean± %RSD)	97.4±0.87	99.4±1.81	102.80±1.33	96.4±1.85
LTA				
Theoretical conc. (ppm)	0.15	0.15	0.15	0.15
Measured conc. (ppm)	0.142	0.147	0.145	0.145

%recovery(mean± %RSD)	94.8±2.11	98.5±1.92	96.9±1.28	97.2±0.89
LHP				
Theoretical conc.(ppm)	0.15	0.15	0.15	0.15
Measured conc. (ppm)	0.144	0.138	0.152	0.145
%recovery(mean± %RSD)	96.0±0.84	92.5±1.22	101.80±0.93	97.1±1.18

The %RSD value calculated from three determinations.

CONCLUSION

In this study a rapid and sensitive UPLC-MS/MS method was developed for quantitative determination of four GTIs in levofloxacin. The UPLC-MS/MS method was validated as per ICH guidelines. Newly developed method was found to be simple, sensitive, selective, cost effective and stability indicating. Detection limit for impurities was found to be as low as 0.05 ppm and was found to have excellent resolution for four impurities indicating high sensitivity and selectivity of the validated method. The method was also applied for the determination of mentioned impurities from the formulation batches of levofloxacin. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of levofloxacin dosage forms.

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