Liquid Chromatographic Determination of Norfloxacin in Extended-Release Tablets

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Abstract

A stability indicating reversed-phase liquid chromatography method is developed and validated for the determination of norfloxacin in a new formulation of extended-release tablets. The LC method is carried out on a Luna C_{18} column (150 × 4.6 mm) maintained at 40°C. The mobile phase is composed of phosphate buffer (0.04 M, pH 3.0)–acetonitrile (84:16, v/v) run at a flow rate of 1.0 mL/min and detection at 272 nm. The chromatographic separation was obtained within 10 min, and it is linear in the concentration range of 0.05–5 µg/mL. Validation parameters, such as the specificity, linearity, precision, accuracy, and robustness, were evaluated, and results were within the acceptable range. Moreover, the proposed method was successfully applied for the assay of norfloxacin in the developed formulations.

Introduction

Norfloxacin, chemically known as 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1-ethyl-fluoro-1.4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid (NFX) (Figure 1) (1), is currently used as a broad spectrum antibacterial drug, the first choice drug for the treatment of diseases caused by *Campylobacter, E. coli, Salmonella, Shigella and V. cholera* (2,3). The drug is also used for the treatment of gonorrhea as well as infection of eyes and urinary tract (2). Resistance in Gram-negative bacteria has become common, making the therapeutic decisions more difficult. Increasing bacterial resistance to currently available quinolones has reduced their effectiveness and may compromise future use of this class of drugs (4,5).

The development of controlled-release formulations is a successful area in the pharmaceutical industry because expenses of new drug development are very high, and true innovation is at an all-time low. Hydrophilic matrices are one of the most used controlled delivery systems in the world due to the simple technology and low cost. Among the various hydrophilic polymers employed, hydroxypropyl methylcellulose (HPMC) is the most commonly used due to its versatility, compatibility with many drugs, and safety (6). Nevertheless, high molecular weight polyethylene oxides (PEOs) have been proposed as an alternative to HPMC (7). The correct choice of the hydrophilic polymer, molecular weigh, and quantity in the matrix formulation can provide an appropriate combination of swelling, dissolution, or erosion mechanisms to control drug-release kinetics (8,9).

Moreover, drug release at a constant rate is often desirable to maintain the plasmatic levels of the drug in the therapeutic range. This prevents peak and valley profile characteristics of conventional dosage forms in a multidose regime (10), which for antibiotics is very important, mainly in combination with a better patient compliance to the therapy, to avoid the discontinuation of the treatment and the development of resistant microorganisms.

Analytical methods have been published for the determination of NFX in pharmaceuticals by means of spectrophotometry, liquid chromatography (LC), and capillary electrophoresis (11–16).

As the excipients (including polymeric ones) and the technological process can affect the stability of the active pharmaceutical ingredient and the evaluation of the stability-indicating capability of LC methods becomes mandatory by the surveillance agencies (17,18), the aim of the present work was to develop and validate a stability-indicating method for the quality assessment of the new formulation of NFX extended-release tablets.

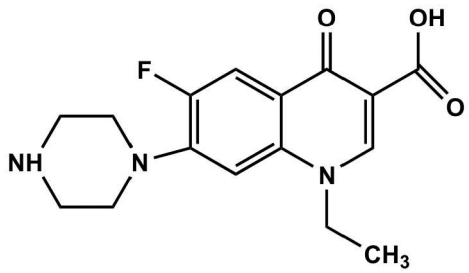

Figure 1. Chemical structure of NFX.

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Experimental

Chemical and reagents

NFX reference standard was kindly provided by Brazilian Pharmacopeia, and NFX raw material was from União Química Farmacêutica Nacional (Embu-Guaçu, SP, Brazil). The polymers used in this study, Polyox WSR N80, Polyox WSR 301, Polyox WSR 303, Polyox N60K, Methocel K100 LV, Methocel K100M, and Methocel K4M were kindly provided by Colorcon do Brazil Ltda (São Paulo, SP, Brazil). Others excipients used were: microcrystalline cellulose (Microcel 102, Blanver, Itapevi, SP, Brazil), magnesium stearate (M. Cassab, São Paulo, SP, Brazil), and colloidal silicon dioxide (Aerosil, Labsynth, Diadema, SP, Brazil). HPLC-grade acetonitrile was purchased from Tedia (Fairfield, OH). For all the analyses, ultrapure water was purified using a Milli-Q Gradient System (Millipore, Bedford, MA).

Samples

The composition of each NFX tablet formulation was: NFX (700 mg), polymer (20 or 30%), magnesium stearate (1%), colloidal silicon dioxide (0.5%), and microcrystalline cellulose (qs 1.07 g). For the production of the extended-release tablets, NFX and excipients were mixed for 10 min and then compressed by direct compression (Fellc compressing model F-10/8, São Paulo, SP, Brazil).

Methods

LC

A Shimadzu LC system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A_{VP} system controller, LC-10 AD pump, DGU-14A degasser, CTO-10AS_{VP} column oven, SPD-10A_{VP} UV detector, and a SPD-M10A_{VP} photodiode array detector was used. The detector was set at 272 nm, and peak areas were integrated automatically by a computer using a Shimadzu Class VP V 6.12 software program. The experiments were carried out on a reverse-phase Phenomenex (Torrance, CA) Luna C_{18} column (150 mm × 4.6 mm i.d., 5 mm, and pore size 100 Å). A security guard holder (4.0 mm × 3.0 mm i.d.) was used to protect the analytical column. The LC system was operated isocratically at 40ºC using a mobile phase of phosphoric acid (0.04 M, pH 3.0)–acetonitrile (84:16, v/v). This was filtered through a 0.45-µm membrane filter and run at a flow rate of 1.0 mL/min. The injection volume was 20 µL for both standard and samples.

Diffuse reflectance infrared fourier transform spectroscopy

The diffuse reflectance infrared fourier transform spectra (DRIFT) were measured in a Prestige spectrophotometer (Shimadzu) in a scan range of 400–4000 cm^{-1} with an average of over 32 scans at a spectral resolution of 4 cm^{-1} in KBr. A background spectrum was obtained for each experimental condition.

Mass spectrometry

The mass spectrometry (MS) experiments were performed on a triple quadrupole mass spectrometer (Micromass, Manchester, UK), model Quattro LC, equipped with an electrospray ionization (ESI) source in positive mode, set up in scan mode, using a Masslynx (v 3.5) software program. The samples were introduced into the mass spectrometer by direct infusion at 10 µL/min diluted in mobile phase. The best response for NFX was obtained with electrospray capillary potential of 3 kV, cone voltage of 30 V, RF lens voltage of 0.3 V, source temperature of 120°C, and ESI probe temperature of 400°C. The mass spectrometry data were acquired in the *m/z* range between 100–550 amu.

Procedure

Preparation of reference solutions

The stock solutions of NFX were prepared by weighing out 50 mg, transferring to individual 50-mL volumetric flasks, dissolving with 0.2 mL of acetic acid glacial, and diluting to volume with mobile phase, ultimately obtaining a concentration of 1 mg/mL. The stock solutions were stored at 2–8ºC protected from light. Working standard solutions were prepared daily by diluting the stock solutions to an appropriate concentration in mobile phase.

Preparation of decarboxylated norfloxacin

The hydrolysis of NFX during prolonged heating of its acid solution yields a decarboxylated degradant (DCN), which was prepared based on a described procedure (11). Two hundred fifty milligrams of NFX was refluxed with 70 mL of hydrochloric acid 2 M at 150°C for 48 h and protected from light. Then, the solution was cooled and adjusted to pH 7.5 with sodium hydroxide 2 M. After that, the solution was evaporated under vacuum to dryness. The residue was extracted with ethanol and filtered. To verify the identity of the obtained product, a sample was analyzed by means of DRIFT, LC–MS, and the proposed LC method.

Preparation of sample solutions

To prepare the sample stock solution, the obtained extended-release tablets were crushed to a fine powder. An appropriated amount was transferred into individual 50-mL volumetric flasks, dissolved with 0.2 mL of acetic acid glacial, and diluted to volume with mobile phase, obtaining a concentration of 1 mg/mL of the active pharmaceutical ingredient. This solution was stored at 2–8°C protected from light. Working sample solutions were prepared daily by diluting the sample stock solutions to an appropriate concentration in mobile phase.

Validation of the Method

Analytical method development and validation play a major role in the discovery, development, and manufacture of pharmaceuticals (19). The International Conference on Harmonization (ICH) (20) requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. A stability-indicating method is the one that quantifies the drug and also resolves its degradation products (17,18,21). The method was validated to quantify NFX in a new formulation of extended-release tablets by the determination of the following parameters: specificity, linearity, accuracy, precision, robustness, and quantitation and detection limits.

Specificity

In order to determine the specificity of the method, a placebo solution was analyzed to evaluate the absence of interference from the formulation excipients (including the polymers) on the NFX peak. Moreover, the specificity was determined by subjecting a sample solution (1 mg/mL) to accelerated degradation by acidic, basic, neutral, oxidative, and photolytic conditions. After the procedures, the samples were diluted in mobile phase to a final concentration of 1 µg/mL. A sample solution in 5 M hydrochloric acid and 5 M sodium hydroxide, both refluxed at 100°C for 24 h, were used for the acidic and basic hydrolysis, respectively. The oxidative degradation was induced by storing the sample solution in 30% hydrogen peroxide at ambient temperature for 24 h and protected from light. Photodegradation was induced by exposing the samples to 200 watt h/m^2 of near UV light. Then, the specificity of the method was established by determining the peak purity of NFX in degradated samples using a PDA detector.

Linearity and range

Linearity was determined by constructing three independent calibration curves. For the construction of each calibration curve, seven standard concentrations of NFX in the range of 0.05–5 µg/mL were prepared in mobile phase. Three replicates of 20-µL injections were made for the standard solution to verify the repeatability of the detector response at each concentration. The peak areas of the chromatograms were plotted against the concentrations of NFX to obtain the calibration curve. The seven concentrations of the standard solutions were subjected to regression analysis by the least squares method to calculate calibration equation and correlation coefficient.

Precision and accuracy

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by six evaluations of the same concentration sample, on the same day, under the same experimental conditions. The intermediate precision was assessed by carrying out the analysis on three different days (inter-days) and also by other analysts performing the analysis in the same laboratory (between-analysts). The accuracy was evaluated by the recovery of known amounts (0.3, 0.5, and 0.7 µg/mL) of the reference substance added to a sample solution (containing 0.50 µg/mL of NFX and tablet excipients). This was done to obtain solutions with final concentrations of 0.80, 1.0, and 1.2 µg/mL, corresponding to 80, 100, and 120% of the nominal analytical concentration, respectively. Accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

Limits of quantitation and detection

The limits of quantitation (LQ) was taken as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, and the limits of detection (LD) was taken as the lowest absolute concentration of analyte in a sample that can be detected but not necessarily quantified. The LD and LQ were calculated from the slope and the standard deviation of the intercept of the mean of three calibration curves, which is determined by a linear regression model as defined by ICH.

Robustness

Two approaches are possible to evaluate robustness, either an one-variable-at-a-time (OVAT) procedure or an experimental design procedure. The OVAT procedure varies the levels of one factor while keeping the other factors at nominal levels to evaluate the effect of this former factor on the method response(s). When applying an experimental design, the effect of a given factor is calculated at several level combinations of the other factors. Thus, in an experimental design, a reported factor effect is an average value for the whole domain, and it represents more globally what is happening around the nominal situation (22–24).

The robustness was determined by analyzing the same samples under a variety of conditions of the method parameters such as: flow rate, column temperature, changing the mobile phase composition, and pH. The response surface method (RSM) design was applied to evaluate the relationships between one or more measured responses. Moreover, the D-optimal criteria was used to select design points to minimize the variance associated to the estimates of specified model coefficients with a low number of experiments.

System suitability

The system suitability was carried out to evaluate the resolution and reproducibility of the system for the analysis to be performed using six replicate analyses of the drug at a concentration of 1 µg/mL. The parameters evaluated were peak area, retention time, theoretical plates, and asymmetry.

Analysis of the extended release tablets

For the quantitation of NFX in the extended-release tablets, 20 tablets of each batch were separated, accurately weighed, and crushed to a fine powder. An appropriate amount of each tablet was transferred into an individual 50-mL volumetric flask, dissolved with 0.2 mL of acetic acid glacial, sonicated for 15 min, diluted with mobile phase (sonicated again for 15 min), and diluted to volume. Ultimately, the NFX final concentration obtained was 1 mg/mL (stock solutions). For the analysis, the stock solutions were diluted to appropriate concentrations with mobile phase. An aliquot of 20 µL was injected for the analysis, and the amount of each drug per tablet calculated against the respective reference standard.

Results and Discussion

To obtain the best chromatographic conditions, the mobile phase was optimized to provide adequate peak symmetry and sensitivity. Potassium phosphate, sodium phosphate, sodium acetate, formic acid, and phosphoric acid buffers were tested. Methanol was tested as the organic solvent; however, a broad and non-symmetric peak was obtained. The use of phosphoric acid 0.04 M (pH 3.0) in combination with acetonitrile, which was optimized to 16% at 40°C, resulted in a relatively short retention time of 6.6 min, better peak symmetry (1.11), and a simple mobile phase (without salt buffer addition). For the selection of the best wavelength detection, a PDA detector was used. The optimized conditions of the LC method were validated for the analysis of NFX in the devel-

oped tablets and a typical chromatogram obtained by the proposed LC method is shown in Figure 2A.

A stability-indicating method is defined as an analytical method that accurately quantitates the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities. Forced degradation studies should be the first step in method development. The presence of degradants and impurities in pharmaceutical formulations can result in changes in their chemical, pharmacological, and toxicological properties affecting their efficacy and safety. Therefore, the adoption of stability-indicating methods is always required to control the quality of pharmaceuticals during and after production. This greatly contributes to the possibility of improving drug safety (17,18,25,26). For NFX, its decarboxylated degradant has particular significance because the pharmacological activity of the drug depends on the carboxylic group (27). On the other hand, this degradant was recorded as impurity in the bulk form and precipitate in the injection formulation (11). The prepared degradant (DCN) and the intact drug were submitted for FTIR, MS, and LC analysis. The spectrum scan of NFX standard exhibited a strong stretching vibration band at 1715 cm^{-1} together with a broad band around 2500–3500 cm^{-1} characterizing carbonyl and hydroxyl moieties of the carboxylic group, respectively. These two bands, in the spectrum of DCN, corresponded in position to that obtained for NFX standard, but the relative intensity decreased, which indicates decarboxylation. The mass spectra obtained is shown in Figure 3A. Characteristic signals were *m/z* 320 amu for NFX[H^+] and *m/z* 361 amu for NFX[CH_3CN-H^+], which is an adduct produced with NFX and acetonitrile (41 Da) from the mobile phase. Adduct is defined as an ion formed through the interaction between two species, usually an ion and a molecule, containing all the atoms of one specie plus one or several atoms of the other (28). In Figure 3B the signal on *m/z* 276 amu for DCN[H^+] is shown, supporting the identity of the degradation product. Moreover, the degraded sample was analyzed by the proposed LC method (Figure 2B), and its retention time was used to assume the identity of this product in the samples subjected to stress studies.

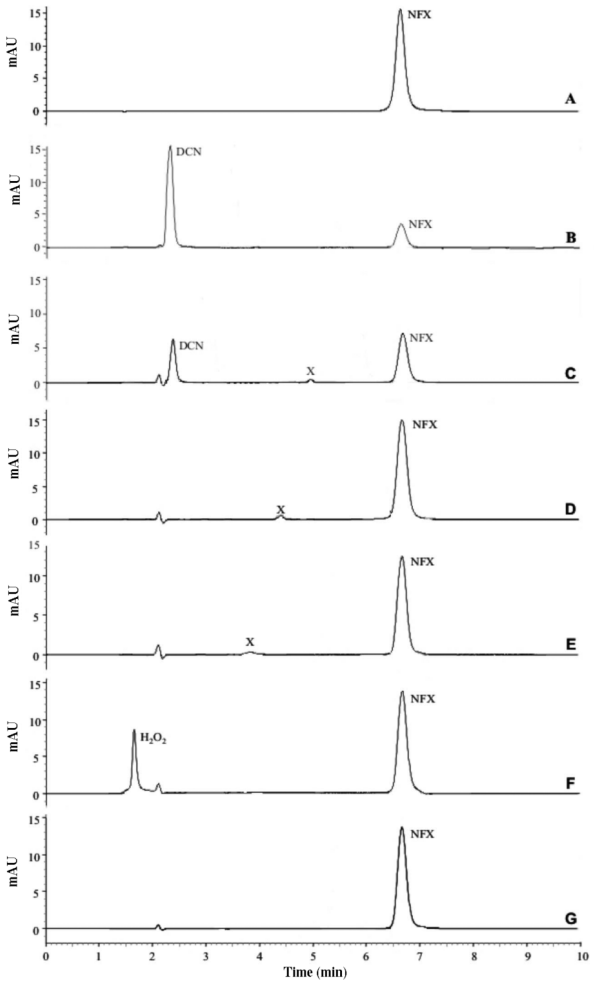

Figure 2. LC chromatograms of NFX developed formulation (1 µg/mL), A; decarboxylated degradant, B; after acidic condition, C; after basic condition, D; after photolytic condition, E; after oxidative condition, F; after neutral condition, G. Chromatographic conditions: Luna C_{18} column (150 × 4.6 mm, 5 mm), 40ºC; mobile phase: phosphate buffer (0.04 M, pH 3.0)–acetonitrile (84:16, v/v); flow rate: 1.0 mL/min; detection: 272 nm.

The specificity of the analytical method for NFX is indicated in Figure 2, where the excipients did not interfere on NFX peak. Under acidic condition, one additional peak was observed with the same retention time of DCN, thus confirming its identity. The basic and photolytic conditions generated one additional peak. Under oxidative and neutral conditions, there was no change in the area, and no additional peak was detected. The studies with the PDA detector showed that the NFX peak was free from any coeluting peak, with values of peak purity index higher than 0.9999. This demonstrates that the proposed method is specific.

The calibration curves constructed for NFX were found to be linear in the 0.05–5 µg/mL range. The value of the determination coefficient calculated (r^2 = 0.9999, y = 186846 ± 1960x – 951.8 ±

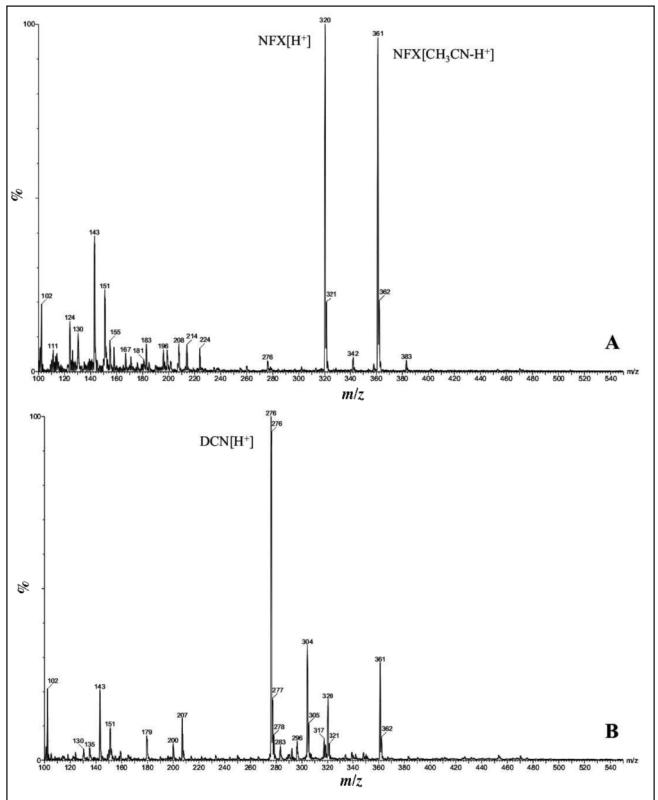

Figure 3. Mass spectra for NFX, A; and decarboxylated degradant, B.

890.6, where x is concentration and y is the peak absolute area). This is the linearity of the calibration curve for the method. The validity of the assay was verified by means of ANOVA, which demonstrated significant linear regression and non-significant linearity deviation ($p < 0.01$).

Table I. Inter-day and Between-Analysts Precision Data of the Method

Sample	Inter-day			Between-analysts		
	Day	Rec.* (%)	RSD† (%)	Analysts	Rec. (%)	RSD (%)
1	1	99.99	0.59	A	100.00	0.05
	2	101.07	0.59	B	99.91	0.05
	3	100.96	0.59	C	100.00	0.05
2	1	99.06	0.39	A	101.72	0.28
	2	99.63	0.39	B	101.15	0.28
	3	98.88	0.39	C	101.46	0.28

* Rec = recovery: mean of three replicates; † RSD = relative standard deviation

Table II. Accuracy of the Method

Added conc. (µg/mL)	Mean conc. found* (µg/mL)	RSD† (%)	Accuracy (%)
0.30	0.30	1.09	100.48
0.50	0.50	1.16	100.23
0.70	0.69	0.40	98.70

* Mean of three replicates; † RSD = relative standard deviation.

Table III. Chromatographic Conditions and Range Investigated During Robustness Testing

Exper.	Factors				Responses*		
	ACN (%)	Flow (mL/min)	pH	Temp (°C)	RSD (%)	Assay (%)	Peak Symmetry
1	18.00	1.00	2.70	45.00	0.43	99.78	1.09
2	16.00	1.20	3.30	35.00	0.31	100.48	1.10
3	16.00	1.20	2.70	45.00	0.35	99.84	1.09
4	14.00	1.00	3.30	45.00	0.35	100.23	1.10
5	16.00	1.20	3.30	45.00	0.49	99.99	1.09
6	14.00	1.00	2.70	35.00	0.54	100.17	1.11
7	14.00	0.80	3.00	35.00	0.41	100.05	1.11
8	14.00	0.80	2.70	40.00	0.28	100.56	1.10
9	14.00	1.20	3.00	35.00	0.89	100.02	1.10
10	18.00	0.80	3.00	35.00	0.52	100.41	1.10
11	18.00	0.80	3.30	40.00	0.17	99.61	1.09
12	16.00	1.20	2.70	35.00	0.44	100.41	1.10
13	14.00	1.00	3.30	45.00	0.22	99.76	1.08
14	18.00	1.00	2.70	45.00	0.31	100.12	1.09
15	14.00	0.80	3.00	35.00	0.70	100.34	1.10
16	16.00	0.80	2.70	35.00	0.63	99.90	1.09
17	18.00	1.00	2.70	35.00	0.38	100.37	1.09

* Mean of three replicates.

The precision evaluated as the repeatability of the method was studied by calculating the relative standard deviation (RSD) for six determinations of 1 µg/mL concentration performed on the same day and under the same experimental conditions. The RSD value obtained was 0.98%.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (inter-day); the RSD values obtained were 0.59% and 0.39%. Between-analysts precision was determined by calculating the RSD for the analysis of two samples of the pharmaceutical formulation by three analysts; the values were found to be 0.05% and 0.28% (Table I).

The accuracy was assessed from three replicate determinations of three different added standard solutions containing 0.3, 0.5, and 0.7 µg/mL of NFX. The results are shown in Table II with a mean value of 99.90% and RSD of 0.97%. This demonstrates that the method is accurate within the desired range.

For the calculation of the LD and LQ, the calibration equation for NFX was generated by using the mean values of the three independent calibration curves. The mean of the slope and the standard deviation of the intercept of the independent curves were 186,846 and 951.8, respectively. The values calculated for

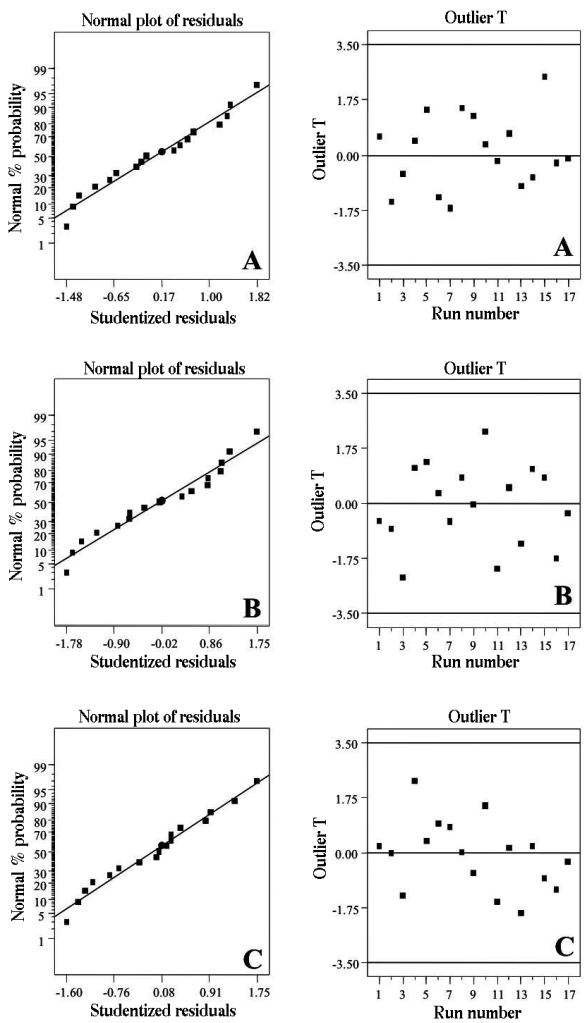

Figure 4. Normal plot of residuals and outlier T values for responses peak area RSD%, A; assay, B; and peak symmetry, C.

the LD and LQ were 0.01 and 0.05 µg/mL, respectively. The LQ evaluated experimentally for NFX was also 0.05 µg/mL and was included in the calibration curve of the method.

To evaluate the robustness of an analytical method usually the OVAT approach is applied; however, it is not recommended. The most important reason is that when the factors are examined in given intervals, the effects are estimated for a smaller domain around the nominal levels with the OVAT than with the experimental design approach. Moreover, the OVAT approach requires more experiments, especially when the number of examined factors becomes larger, and secondly, the importance of factor interactions cannot be taken into account (22,23). The experimental ranges of the selected variables evaluated are given in Table III. The analysis of variance ANOVA was performed, and the model terms (variables) were not significant ($p < 0.05$). The normal plot of residuals and outlier T for the responses evaluated are shown in Figure 4, and the results demonstrated that the method was robust. Moreover, the stability of the analytical solution was analyzed, and it was found to be stable up to 48 h (99.65%, assay).

The system suitability results showed that the parameters were within the suitable range. The RDS values calculated for peak area and retention time were 0.98 and 0.15%, respectively. The mean asymmetry and theoretical plates ± RSD were 1.11 ± 0.34% and 8226 ±1.3%, respectively.

The LC method validated in this paper was applied for the determination of NFX in the new extended-release formulations without prior separation of the excipients. The values obtained ranged from 99.43–102.35%.

Conclusion

The results of the validation studies showed that the LC method is specific, accurate, and possesses significant linearity and precision characteristics without any interference from the formulation excipients and degradation products. Moreover, the proposed method was successfully applied for the quantitative analysis of NFX in the developed extended-release dosage forms.

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