High Performance Liquid Chromatographic Method for the Determination of Norfloxacin in Pharmaceutical Preparation After Pre-column Derivatization

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A sensitive high performance liquid chromatography (HPLC) method for the determination of Norfloxacin in pharmaceutical preparation is described. The proposed method was based on the derivatization of Norfloxacin with 4-chloro-7-nitrobenzofurazan in borate buffer at pH 9.0 to yield a fluorescent product. The chromatographic separation was achieved on a C_{18} column (150 mm×4.6 mm) using a mobile phase of methanol–water (75:25, v/v) solvent system at 1.1 mL/min flow-rate. The assay was linear over the concentration range of 10 to 500 ng/mL.

Keywords: NBD-Cl, Validation, HPLC, Derivatization

Norfloxacin (NFX) is a fluoroquinoline carboxylic acid [1] (fig. 1). It is effective against gram-positive and gram-negative bacteria through inhibition of their DNA gyrase, a critical enzyme to bacterial chromosome replication [2-4].

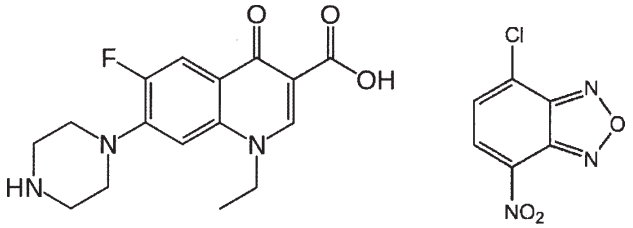

Fig.1. Chemical structures of Norfloxacin and 4-chloro-7-nitrobenzofurazan

The several analytical methods have been developed for the determination in pharmaceutical preparations and biological fluids.

A number of kinetic spectrophotometry [5,6] and, spectrophotometry [7] methods have been described for the determination of NFX pharmaceutical dosage forms, respectively. Determination of NFX pharmaceutical preparations by spectrophotometry and spectrofluorimetry method has also been described [8-10]. Several HPLC methods are available for the determination of NFX in biological fluids. A HPLC method has been reported for the assay of NFX urine and serum [11]and other researchers studied HPLC method for the determination of NFX in human plasma and urine [12]. A HPLC method of NFX in human serum and urine was developed [13].

Capillary electrophoresis method have been used for the determination of NFX in pharmaceuticals and real complex [16,17]. Determination of NFX in capsule, human serum and urine by chemiluminescence is presented in [18,19].

This report describes a sensitive, reproducible and accurate HPLC procedure with fluorescence detection for the determination NFX in tablets by means of the derivative formed with 4-chloro-7-nitrobenzofurazan. 4-chloro-7-nitrobenzofurazan usually reacts with primer and secondary amines.

The proposed method was fully validated for its linearity, limit of detection, limit of quantification, accuracy, precision, specificity and robustness. It was essential to establish an assay with an LOD in the low ng/ml range. Short separation times and high sensitivity are the main advantages of such a technique.

Experimental part

Materials and methods

Pure powder of NFX was obtained from Fluka its pharmaceutical preparation Noroxin® tablets (400 mg) was purchased from a local pharmacy. Mexiletine (IS), 4-chloro-7-nitrobenzofurazan (NBD-Cl) and other chemicals were purchased from Sigma and Merck . All solvents were of analytical grade.

The liquid chromatographic system was (Shimadzu Liquid Chromatography and consisted of a Model LC 20 AT solvent delivery system with an SIL-20AHT autosampler with a 5 µL loop, a RF-10AXL fluorescence detector. The analytical column was a Inertsil C_{18} column (150 mm×4.6 mm i.d., 5µm) with a guard column (4 mm×3 mm i.d., Inertsil) packed with the same material. The mobile phase was composed of methanol/water (75:25 v/v). Analyses were run at a flow rate of 1.1 mL/min at 40°C. The fluorimetric detector was set at 464 and 535 nm for the excitation and emission wavelengths, respectively.

Solutions

Stock standard solutions of NFX and IS were prepared in methanol at a concentration of 100 µg/mL and stored at +4°C. These were diluted by using methanol to give appropriate solutions (10 µg/mL for NFX and IS). NBD-Cl solution was freshly prepared at 3 mg/mL in methanol. A borate buffer (0.1 M) was prepared by dissolving 0.620 g of boric acid and 0.750 g of potassium chloride in 100 mL of water. The *p*H was adjusted to 9.0 with 0.1 M sodium hydroxide solution and the volume was made up to 200 mL with water.

General Procedure

To a set of 12 mL volumetric flasks, increasing volumes from the standard solution of the NFX were quantitatively

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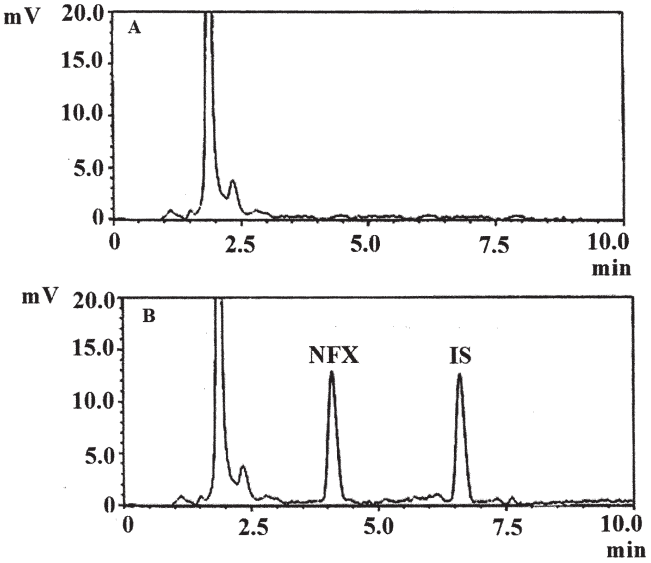

Fig. 2. Typical chromatograms obtained from separation of blank (A), Norfloxacin-NBD derivative (B) (4.24 min) and internal standard –NBD derivative (6.63 min) on a C_{18} column with mobile phase of methanol–water (75:25, v/v) at a flow rate of 1.1 mL/ min.

transferred so that to contain the drug within the concentration range 10-500 ng/mL. Next, 50 µL IS, 100 µL borate buffer *p*H 9.0 and 100 µL NBD-Cl solutions were added, and the reaction mixture was kept at 70 °C for 10 min in a water bath. It was cooled and acidified with 100 µL of 0.1 N HCl. The derivative was extracted two times with 2.5 mL ethyl acetate, and the organic layer was transferred to a tube. The organic phase was dried on anhydrous sodium sulfate. A 4.5 mL aliquot of the extract was evaporated under nitrogen at 45°C. The residue was then dissolved in 1 mL of the mobile phase. Then, the sample was injected into the HPLC system.

Procedure for the Assay of the Tablets

Contents of twenty tablets were weighed and net content of a tablet was calculated. Tablet powder equivalent to 100 mg of NFX was weighed and transferred to 100 mL calibrated flask, added 50 mL of methanol each and sonicated for 30 min. Then, it was made up to volume with methanol, mixed well and filtered. An aliquot of 1 mL of the filtrate was diluted to 100 mL to prepare the sample solutions (10 µg/mL).

Results and discussions

Optimization of the derivatization reaction conditions

The reactions of NFX and IS with NBD-Cl in borate buffer at *p*H 9.0 produce a yellowish fluorescence colour. Besides, different experimental parameters affecting the intensity of NFX-NBD derivative were investigated to find out the optimum parameters.

The effect of *p*H in the range of 8-10 was examined on the intensity of the derivative compounds. The maximum intensity of the associated compound was observed at those of *p*H 9. It is known that the maximum association between NBD-Cl and aimed compound is realized at the basic medium. But, NBD-Cl is also hydrolyzed in alkaline solution. Thus, the system stabilized by acidifying the reaction mixture to *p*H 2 (by adding 100µL 1N HCl) before measurement [20]. The influence of temperature and its duration on the intensity of the NFX-NBD derivative were also examined. Four different temperatures in the range of 50-80°C were investigated for NBD derivation. The best results were obtained at 70°C. The reaction time was tested between 10-60 min at 70°C and best reaction time was found to be 10 min. The amount of NBD-Cl solution is tested for 25-200 µL standard solution of drug. Experiment indicated that 100 µL NBD-Cl solution is enough for each drug, the final concentration of NBD-Cl is $1.5x10^{-3}$M.

Chromatography

Several parameters were examined for the optimization of HPLC analysis of NFX-NBD derivative compound. When it was overlooked the NFX-NBD compound, it is observed that the molecule exhibits highly non-polar structure. Therefore, it was thought that mobile phases have to be consisted of solvent-double distilled water without any *p*H adjustment and methanol was preferred as a solvent in this study. The mobile phase consisted of methanol/water and acetonitrile/water at various ratios such as 90:10, 85:15, 80:20, 75:25, 70:30 and 60:40 were tested. The variation at the mobile phase leads to considerable changes in the chromatographic parameters. Optimum mobile phase was methanol/water (75:25) for this system. This system was adopted for the analysis because provided a better separation of the derivatives and the retention times were much more reproducible. It was found to suitable retain NFX-NBD derivative in the column. It is observed that it retains for short time and the column good resolution of the peak symmetry and a reduction in the tailing factor.

Different column temperatures (30, 35, 40 and 45°C) were investigated to optimize the chromatographic analysis. The peak width slowly decreased with increase of the temperature; however, the temperature of 40°C was chosen as optimal in order to prolong the column lifetime.Then, the influence of the flow-rate of the mobile phase on the peak normalization was studied. Flow-rate of 1.1 mL/min was the most appropriate for the analysis of NFX-NBD. A solution of NFX-NBD (50 ng/mL) was prepared and injected through the column. NFX-NBD and IS-NBD derivative appeared at 4.24 min and 6.63 min, respectively. The typical HPLC chromatograms of NFX-NBD and IS-NBD derivative are demonstrated in figure 2.

Method Validation

Linearity

Calibration plots were constructed by plotting concentration against NFX-NBD compound to IS peak area ratio showed good linearity in the range 10-500 ng/mL. As can be seen from the data, the method is much more sensitive than most of the reported methods [5-7,12-19].

Limit of detection and quantification

The limit of detection was calculated by LOD = 3.3r/S, where r is the standard deviation of the response of the blank and S is the slope of the calibration curve. The limit of quantification was calculated by LOQ = 10r/S under the ICH guidelines [21]. The LOD and LOQ values for NFX were found to be 0.2 and 0.6 ng/mL, respectively. The results indicate us the method is much more sensitive than most of the reported methods [6,7,12-19] (table 1).

Precision and Accuracy

Precision and accuracy were tested by spiking three different concentrations derivatives and they were injected consequently for three days (table 2).

Recovery

The % recovery of the added pure drug was calculated as, % recovery = $[(Ct - Cs)/Ca] \times 100$, where Ct is the total

Parameters	*Value*
Linear regression equation,	
A=aC+b	
C (concentration, ng/mL)	
Slope (a)	0.002
Intercept (b)	0.0095
Standard deviation of intercept	1.21×10^{-4}
Standard deviation of slope	1.00×10^{-3}
Limit of detection (ng/mL)	0.20
Limit of Quantification (ng/mL)	0.60
Correlation coefficient (r)	0.9997

Table 1
RESULTS OF LINEARITY DATA OF NFX (n=6)

Added concentration (ng/mL)	**Found concentration** (ng/mL) mean ± SD	**Precision (RSD%)**	**Accuracy (RME %)**
Intra-day			
10	$9.96 \pm 7.0 \times 10^{-3}$	0.07	-0.40
250	$250.2 \pm 1.41 \times 10^{-1}$	0.06	+0.08
500	$500.4 \pm 4.1 \times 10^{-1}$	0.08	+0.08
Inter-day			
10	$9.94 \pm 7.1 \times 10^{-3}$	0.07	-0.60
250	$250.5 \pm 1.1 \times 10^{-1}$	0.04	+0.20
500	$500.3 \pm 3.0 \times 10^{-1}$	0.06	+0.06

Table 2
INTRA- AND INTER-DAY PRECISION AND ACCURACY OF THE ASSAY NFX

Concentration of drug in formulations (ng/mL)	**Concentration of pure drug added (ng/mL)**	**Recovery mean ± SD**	**RSD (%)**
50	50	101.0 ± 0.21	0.19
50	200	99.10 ± 0.44	0.18
50	450	100.09 ± 0.07	0.01

Table 3
RECOVERY RESULTS FROM THE STANDARD ADDITION METHOD
(n= 3)

drug concentration measured after standard addition, *C*s the drug concentration in the formulation sample and *C*a is the drug concentration added to formulation. The results showed that the recoveries were in the range of 99.10-101.0%. These results are given in table 3.

The robustness of a method is its resilience to minor changes in the analytical conditions, for example, mobile phase composition, flow rate and column oven. The results were in the table 4. It can be concluded that this method is robust, because slight variation of these experimental conditions have little or no effect on the results.

Specificity

No interference was observed when tablet matrix of microcrystalline cellulose, sodium lauryl sulfate, calcium stearate, talc, titanium dioxide, yellow iron oxide was used. The HPLC method did not interfere by the formulation excipients, since there was not another peak in the retention time of NFX.

System suitability

System suitability tests were performed and chromatographic parameters calculated from experimental data, such as retention time (tr), capacity factor (k'), tailing factor (T), resolution (Rs), N (theoretical plate number) are given in table 5. All the values for the system suitability parameters are within the acceptable range.

Determination of NFX in pharmaceutical formulations

The applicability of the developed method was checked by analyzing commercially available pharmaceutical preparation. The formulations selected were Noroxin 400 mg tablets. Comparing the results obtained by the proposed method with the offical method [22] using the *t*-

Parameter	Modification	Mean±SD (ng/mL)	RSD(%)
Mobile phase ratio (v/v)	74 : 26	250.3 ± 1.42 x 10^{-1}	0.06
	76 : 24	250.2 ± 1.40 x 10^{-1}	0.06
Flow rate (mL/min)	1.0	250.4 ± 1.42 x 10^{-1}	0.06
	1.2	250.1 ± 1.35 x10^{-1}	0.05
Column oven (°C)	41	250.6 ± 1.43 x 10^{-1}	0.05
	42	250.7 ± 1.45 x 10^{-1}	0.06

Table 4
ROBUSTNESS DATA FOR THE PROPOSED METHOD (250 ng/mL)

Component	Capacity factor (k')	Tailing factor (T)	Theoretical plates (N) (meters)	Resolution (Rs)
NFX	8.42	0.9	7139	4.4

Table 5
SYSTEM SUITABILITY PARAMETERS (n=3)

	Proposed method	Official method [22]
Noroxin 400 mg Recovery (%) ± SD	99.51 ± 1.0 X 10^{-1}	99.46 ± 1.3 X 10^{-1}
t	1.42	
F	1.69	

Table 6
DETERMINATION OF NFX IN PHARMACEUTICAL PREPARATIONS BY THE PROPOSED AND OFFICIAL METHOD (n =6, t=2.23, F=5.05)

test for the accuracy and F-test for the precision assessment. According to the t- and F-tests, no significant difference was found between the calculated and theoretical values of both the proposed and the official method at 95% confidence level. This indicates good level of precision and accuracy (table 6).

Conclusions

A HPLC method has been developed and validated for the determination of NFX in tablet dosage form by isocratic mode elution.

In this study, the purpose of the derivatization reaction is the raise of sensitivity thus the possibility of working in low concentrations has been occurred. The method is conventional with easy mobile phase composition, easy to prepare with little or no variation. It has a rapid assay (4.24 min) and the proposed method does not need sophisticated and expensive instrumentation, and it can have practical importance for the quality control of drugs preparations.

In summary, this paper describes a sensitive and accurate proposed method for the quantification of NFX suitable to monitor plasma concentrations during clinical pharmacokinetic studies on humans.

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