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Spectrofluorimetric Methods for the Determination of Gemifloxacin in Tablets and Spiked Plasma Samples

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Abstract Two new, sensitive and selective spectrofluorimetric methods have been developed for the determination of gemifloxacin (GFX) in tablets and spiked plasma samples. Gemifloxacin, as a primary amine compound, reacts with 7-chloro-4-nitrobenzofurazon (NBD-Cl) (for method A) and fluorescamine (for method B) which are a highly sensitive fluorogenic reagents used in many investigations. For method A, the reaction product was measured spectrofluorimetrically at 516 nm with excitation at 451 nm. The reaction proceeded quantitatively at pH 8.5, 80 °C in 7 min. For method B, the method was based on the reaction between GFX and fluorescamine in borate buffer solution of pH 8.5 to give highly fluorescent derivatives that were measured at 481 nm using an excitation wavelength of 351 nm. The fluorescence intensity was directly proportional to the concentration over the range 40–200 ng mL^{-1} and 100–1,200 ng mL^{-1} for method A and B, respectively. Successful applications of the developed methods, for the drug determination in pharmaceutical preparations and spiked plasma samples, were performed.

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Introduction

Gemifloxacin mesylate (GFX), 7-[(4Z)-3- (aminomethyl)-4-methoxyimino-pyrrolidin- 1-yl]-1-cyclopropyl-6-fluoro-4-oxo- 1,8-naphthyridine-3-carboxylic acid methanesulfonate (Fig. 1), is used for the treatment of pneumonia or bronchitis [1]. Gemifloxacin is recently being approved by the U.S. Food and Drug Administration for the treatment of the upper respiratory tract infections [2].

The literature survey revealed that a few analytical methods reported for the determination of gemifloxacin in pharmaceutical preparations or human plasma by visible spectrophotometry [3, 4], capillary electrophoresis [5] high performance liquid chromatography- tandem mass spectrometry [6, 7] microchip electrophoresis [8]. These methods were related with some major drawbacks such as having inadequate sensitivity, being time-consuming tedious, and dedicated to sophisticated and requiring expensive instruments. Spectrofluorimetry offers easy, less time consuming, sensitive analysis, by using simple and available reagents, which are able to be used for routine determinations of drug substances. Therefore spectrofluorimetric analysis are one of the major interests of analytical pharmacy.

No method has been reported for the determination of GFX in tablets and human plasma by spectrofluorimetry. Therefore, the two new spectrofluorimetric methods developed and proposed for determination of GFX in tablets and spiked plasma samples. Several derivatising agents for primary amines were introduced. Such as 7-Chloro-4-nitrobenzofurazan (NBD-Cl) and fluorescamine, which

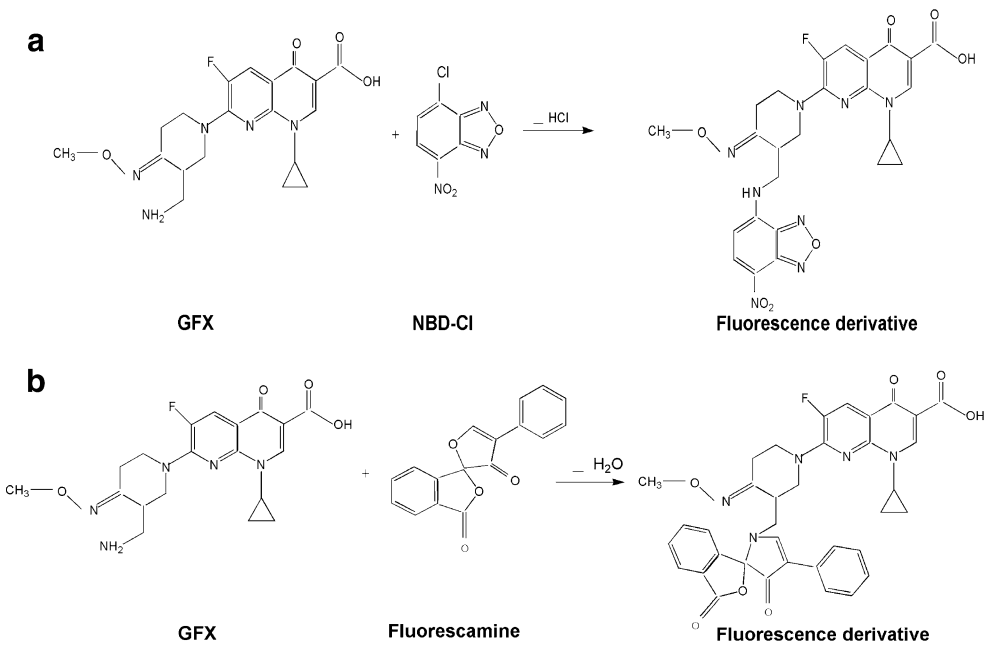

Fig. 1 Proposed reaction pathways between **a** NBD-Cl and **b** fluorescamine with GFX at pH 8.5 using borate buffer

was proposed by Ghosh et al. [9] and Undefriend et al. [10], respectively, and which have proved usefulness in numerous analytical applications for more than 30 years [11–22]. In this study spectrofluorimetric methods for the determination of gemifloxacin in tablets and spiked plasma samples using NBD-Cl and fluorescamine reagents were developed for the first time. The developed methods were also validated by specificity, linearity, accuracy, precision and robustness. Moreover the methods successfuly applied for the determination of GFX in tablets and spiked plasma without any interference from the matrix.

Experimental

Apparatus

Spectrophotometric measurements were carried out using a Shimadzu UV-160 A spectrophotometer with 1-cm glass cells.

Fluorescence spectra and measurements were taken on a Shimadzu spectrofluorimeter Model RF-1501 equipped with xenon lamp and 1-cm quartz cells. Excitation and emission wavelengths were set at 451 and 516 nm for method A and 351 nm and 481 nm for method B, respectively. pH measurements were made with WTW pH 526 digital pH Meter.

Reagents and Solutions

Gemifloxacin mesylate (GFX) was kindly supplied by Abdi Ibrahim Pharmaceuticals (Istanbul, Turkey) and its pharmaceutical preparation (Factive Tablet®, Abdi Ibrahim Pharmaceuticals, Istanbul, Turkey), containing 320 mg of gemifloxacin per tablets was purchased. NBD-Cl and other chemicals were purchased from Merck (Darmstadt, Germany). Fluorescamine, fluorescein and quinine sulfate was obtained from Sigma (MO, USA). All chemicals and reagents were of analytical-reagent grade.

A stock solution of gemifloxacin containing 1 mg mL^{-1} (calculated as free base) was prepared in water and diluted further with the water to obtain standard solution of 100, 10 and 1.0 μg mL^{-1}.

NBD-Cl solution was freshly prepared in methanol at 5 mg mL^{-1} concentration. The fluorescamine solution was freshly prepared at 1.5 mg mL^{-1} in acetone. Aqueous borate buffer (0.1 M, pH=8.5) solution was prepared by using boric acid, potassium chloride. 0.1 N HCl solution was used for the acidification.

General Procedure

For method A, an aliquot of 0.020–0.100 mL of standard solution of 100 μg mL^{-1} was mixed with 0.2 mL of buffer solution in a glass stoppered tube and then the volume was adjusted to 0.3 mL with water. After addition of 1.0 mL of NBD-Cl solution, the mixture was heated at 80 °C for 7 min in a thermostatted water bath. Then the mixture was cooled in an ice batch and acidified with 0.2 mL of 0.1 N HCl solutions. The derivative was extracted into 5 mL of chloroform on a vortex mixer. The phases were separated by centrifugation. 1 mL of the solution after chloroform extraction was transferred to 10 mL volumetric flask and the volume was brought to 10 mL with chloroform and mixed. The fluorescence intensities of the derivatives were measured at 516 nm with excitation at 451 nm against a blank prepared similarly.

For method B, 0.10–1.20 mL aliquots of standard solution of 10 μg mL^{-1} were transferred into a series of 10.0 mL volumetric flasks. To each flask, 0.2 mL of borate buffer solution of pH 8.5 and 0.05 mL fluorescamine solution was added and mixed well and then completed to the mark with distilled water. After 5 min, the fluorescence of the resulting solutions was measured at 481 nm with excitation at 351 nm.

Quantum Yield Determination

Quantum yields of the derivatives were determined according to the Eq. 1 [23].

$$\Phi_x = \Phi_{st}\left(A_{st} \cdot I_x \cdot n_x^2 / A_x \cdot I_{st} \cdot n_{st}^2\right) \quad (1)$$

where Φ_{st} is the quantum yield of the respective standard; A_{st} and A_x are absorbances of the standard (st) and the derivative (x), respectively at the excitation wavelength of the derivative; I_{st} and I_x are the integrated area of the emission spectra of the standard (st) and the derivative (x), respectively; and n_{st} and n_x are the refractive indices of the solvent of the standard (st) and the derivative (x), respectively.

The quantum yield of the derivatives were determined by using fluorescein in 0.1 M NaOH and quinine sulfate in 0.5 M H_2SO_4 as standards for GFX-NBD and GFX-fluorescamine derivatives respectively. According to the equation, quantum yields of fluorescein [24] and quinine sulfate [25], in their solvents mentioned above, were used to find the quantum yields of the derivatives in addition to the measurements.

Assay Procedure for Tablets

Five tablets were separately weighed and powdered. Tablet powder equivalent to 50 mg of GFX base was accurately weighed and transferred into a 50 mL calibrated flask. About 25 mL of water was added and then extraction was performed mechanically for 20 min and sonicated for 20 more minutes. The volume was brought to 50 mL with water and final solution was filtered. Aliquots of filtrate were diluted further with water then proceeded as described under "General Procedure". The nominal contents of the tablets were calculated using either the calibration graph or the corresponding regression equation.

Assay Procedures for Spiked Plasma Samples

An aliquot of plasma (100 μl) in a centrifuge tube was spiked with different concentrations of GFX and extraction was carried out by using chloroform 5.4 mL and acetic acid 0.1 mL by vortex for 1 min and centrifuged at 10,000 rpm at 6 min [26]. The organic phases were evaporated to dryness in a water bath at 50 °C. The residual mass was reconstituted with 0.1 mL water. The analyses were performed as under *General Assay Procedure*. All the procedures for blank were performed in the same manner. The percentage recoveries were calculated by using the corresponding calibration graphs for plasma.

Results and Discussion

As the fluorogenic technique is among the most sensitive methods, it has been chosen for developing a method of analysis of GFX. This drug contains an amino group, which is a suitable candidate for derivatization by NBD-Cl (Fig. 1a) and fluorescamine (Fig. 1b).

NBD-Cl, as an electroactive halide reagent, was initially introduced as an analytical reagent for the determination of primary or secondary amines [27]. Several pharmaceutical compounds have been determined through this approach [11–16]. Under the described experimental conditions, the fluorophore exhibits its highest fluorescence intensities for GFX at excitation wavelenght of 451 nm and emission wavelenght of 516 nm. Figure 2a shows the excitation and emission spectra of the fluorescent product.

Fluorescamine is a fluorogenic agent which has been extensively used in the field of pharmaceutical analysis

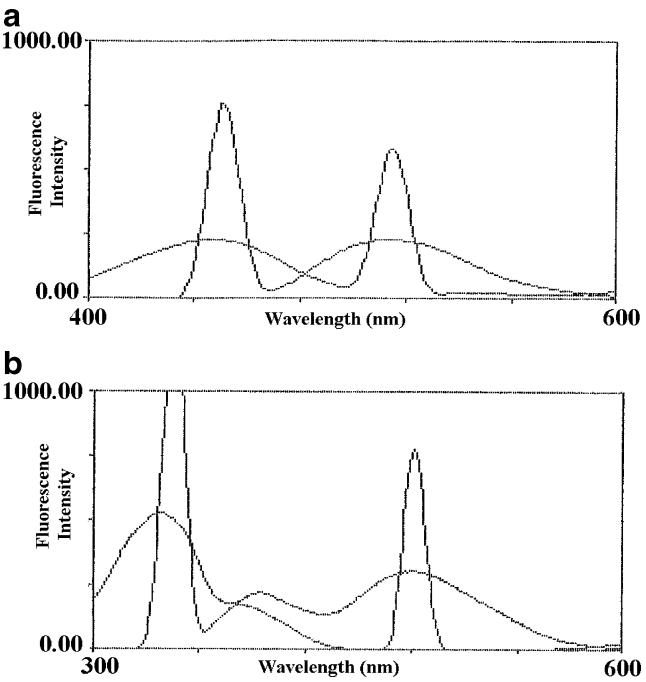

Fig. 2 **a** Excitation and emission spectra of the reaction products of GFX with NBD-Cl in chloroform. **b** Excitation and emission spectra of the reaction products of GFX with fluorescamine in water

[17–22]. It reacts instantaneously with primary amines in aqueous solutions to give highly fluorescent pyrrolinone derivatives [28]. The method is performed in borate buffer of pH 8.5 to yield a highly fluorescent derivative that is measured at 481 nm with excitation at 351 nm (Fig. 2b).

Study of Experimental Parameters

The different experimental parameters affecting the development of the reaction product were carefully studied and optimized. Such factors were changed individually while others were kept constant. These factors were: pH, volume of the reagent, temperature and heating period.

Effect of Alkalinity, pH and HCl Concentration

The effect of pH on fluorescent intensity was studied using different pH values. The pH was varied over the pH range of 8–10 using borate buffers where the maximum absorbance was obtained at pH 8.5 for both methods as shown in Fig. 3.

The hydrolysis product of NBD-Cl (4-hydroxy-7-nitrobenzo-2-oxa- 1,3-diazole) may cause problems by interfering with the fluorescence product. The fluorescence of the hydrolysis product is quenched by decreasing the pH of the reaction medium to less than 1 [29]. Therefore, the reaction mixture was acidified prior to the measurement of the fluorescence intensity. The required amount of HCl for acidification was found to be 0.2 M by adding 0.1 mL.

Effect of Temperature, Time

The effect of temperature on fluorescence intensity was studied. For method A, the derivatization reaction was carried out at 60 °C to 80 °C. Complete color development was attained after 7 min. in water bath at 80 °C. For method B, it was found out that the fluorophore was formed immediately, reached maximum intensity after 5 min.

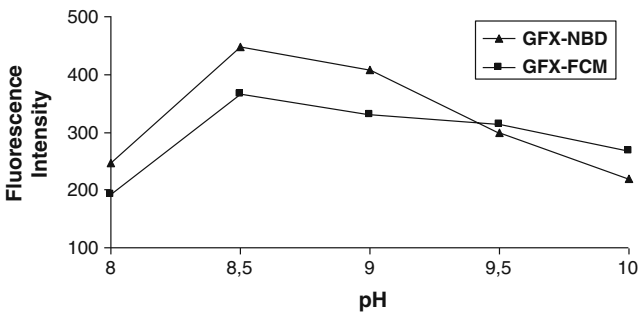

Fig. 3 Effect of pH on the development of the reaction products of GFX with NBD-Cl and fluorescamine

Effect of Reagents Concentration

The effect of the concentration of NBD-Cl on the color intensity was studied over the range 0.1–0.5%. It was found out that 1.0 mL of 0.5% NBD-Cl solution was sufficient for production of maximum and reproducible color intensity as shown in Fig. 4.

The effect of the concentration of fluorescamine on the fluorescence development was studied. It was found out that 0.05 mL of 0.15% fluorescamine solution was sufficient for the maximum fluorescence.

Effect of Organic Solvents and Stability of the Derivative

In order to select the most appropriate organic solvent, different solvents were tested for method A: methanol, ethyl acetate, dichloromethane, chloroform and acetonitrile. Chloroform was found as optimum solvent to give the highest absorbance. The effect of time on the stability of the GFX-NBD derivative in chloroform was studied at different time intervals. The color remains stable for 12 h.

For method B, the possibility of enhancing the sensitivity of the fluorophore by using different organic solvents, was also investigated. No significant changes were observed with methanol, ethanol and water so, consequently, the water was chosen for the experimental work. The samples prepared under these conditions, remained stable for at least 6 h.

Stoichiometry of the Reaction

The molar ratio of NBD-Cl or fluorescamine to GFX in the reaction mixture was studied according to Job's method of continuous variation [30]. Utilizing equimolar solution of GFX and NBD-Cl or fluorescamine, the stoichiometry of the reaction was found as 1:1 ratio (drug/reagent), confirming that one molecule of GFX reacts with one molecule of NBD-Cl or fluorescamine.

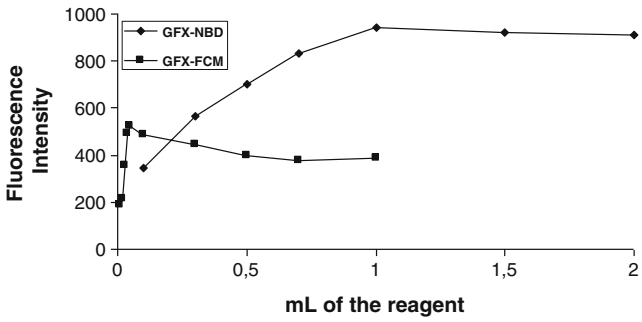

Fig. 4 Effect of volume of NBD-Cl (0.5%, *w/v*) and fluorescamine (0.15%, *w/v*) on the development of the reaction products of GFX with NBD-Cl or fluorescamine

Table 1 Analytical parameters of the proposed method

Parameters	Method A (using NBD-Cl)	Method B (using fluorescamine)
Wavelength (nm)	λex: 451, λem:516	λex: 351, λem: 481
Concentration range[a] (ng mL^{-1})	40–200	100–1200
Regression equation[b]		
Intercept ± SD	−32.064±0.412	57.152±0.678
Slope ± SD	5.1433±0,032	0.773±0,00212
Correlation coefficient (r^2)	0.9991	0.9996
Precision		
Intra-day[c], RSD%	0.697	0.567
Inter-day[d], RSD%	0.902	1.021
LOD (ng mL^{-1})	0.240	2.63
LOQ (ng mL^{-1})	0.801	8.77

[a] Average of five determinatons

[b] $I_f = m\,C + b$ where C is the concentration in ng mL^{-1} and I_f is the fluorescence intensities

[c] n=5 correspond to replicate analysis for each level

[d] Results of five different days

Quantum Yield Values of the Derivatives

The quantum yield of GFX-NBD and GFX-fluorescamine derivatives were found as 0.6174 and 0.5068 respectively.

Validation of the Method

Calibration and Sensitivity

Under the optimum reaction conditions relative fluorescence intensity was found to be linearly correlated to GFX concentration over the range of 40–200 and 100–1,200 ng mL^{-1} for method A and B, respectively. Linear regression analysis of the concentration-fluorescence intensity data gave the following equation: $I_f = 5.1433\,C - 32.064$ (for method A) and $I_f = 0.7728\,C + 57.152$ (for method B) where C is the concentration in ng mL^{-1} and I_f is the fluorescence intensities (n=5). The limits of detection (LOD) and limits of quantitation (LOQ) were determined using the formula: $\mathrm{LOD\,or\,LOQ} = \kappa \mathrm{SDa/b}$, where κ=3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The parameters for the analytical performance of the proposed methods are summarized in Table 1.

Accuracy, Specificity and Precision

To check accuracy of the proposed method, the standard addition technique was applied. A different amount of pure sample solution was added to three different concentrations of the standard drug solution and assayed. The percent recovery of the added standard to the assay samples was calculated from Eq. 2:

$$\text{Recovery } \% = [(C_t - C_u)/C_a] \times 100 \quad (2)$$

Where C_t is the total concentration of the analyte determined; C_u is the concentration of the analyte present in the formulation; and C_a is the concentration of the pure analyte added to the formulation. The results of analysis of the commercial dosage forms and the recovery study as shown in Table 2. The average percent recoveries obtained were quantitatively as 100.02% and 100.37% for method A and B respectively, indicating good accuracy of the method.

Table 2 Results of recovery studies by standard addition method

Proposed methods	Amount taken (ng mL^{-1})[a]	Amount added (ng mL^{-1})	Total amount found[b] (ng mL^{-1}) (Mean ± S.D.[c])	Recovery (%)	RSD (%)
Method A (using NBD-Cl)	40	40	79.87±0.75	99.68	0.94
		100	140.45±1.23	100.45	0.88
		160	199.90±1.41	99.94	0.71
Method B (using fluorescamine)	200	100	299.89±1.76	99.89	0.59
		500	701.21±3.09	100.24	0.44
		1000	1209.78±7.87	100.98	0.65

[a] Factive Tablet ® (320 mg)

[b] Five independent analyses

[c] Standard deviation

Table 3 Determination of GFX in tablets by the proposed methods (n=5)

	Label claim[a] (mg/per tablet)	Mean[b] ± S.D	Recovery (%)	RSD (%)
Method A (using NBD-Cl)	320	321.73±1.88	100.54	0.59
Method B (using fluorescamine)		322.59±2.52	100.81	0.78

[a] Factive Tablet ® (320 mg)

[b] five independent analyses

The specificity of the method was investigated by considering the interference liabilities from the common tablet excipients, such as talc, lactose, starch, mannitol and magnesium stearate. No interference was observed from these excipients.

The inter- and intra-day precisions were examined by analysis of GFX with the three concentrations (n=5) for seven consecutive days. The RSD values for intra-day precisions were found 0.697% and 0.567, and inter-day precisions were found 0.902% and 1.021%, for method A and B, respectively, indicating good precision. The obtained results are summarized in Table 1.

Robustness

Robustness was assessed by testing the susceptibility of measurements to deliberate variation of the analytical conditions. It was found that variation in the NBD-Cl or fluorescamine concentrations (%, w/v±0.5), temperature (optimum ±2 °C), and time (optimum ±0.25 min) did not significantly affect the procedures; recovery and the RSD values did not exceed 2%. The most critical factor affecting the results was pH that should be adjusted to be in the range of 8.5±0.2.

Applications of the Methods

Determination of GFX in Tablets

The proposed methods was successfully applied to the analysis of marketed product (Factive Tablet ®). The obtained results are satisfactorily accurate and precise as indicated by the excellent% recovery and SD<2 (Table 3). Experiments showed that there was no interference from the additions and excipients, e.g. lactose, glucose, fructose, magnesium stearate and starch.

Determination of GFX in Plasma Samples

The proposed method could be successfuly applied for the determination of GFX in spiked plasma. Allen et all were investigated pharmacokinetic properties of GFX [31]. With reference to this study, GFX was rapidly absorbed, and maximum concentrations of the drug substance (*C*max) in plasma increased linearly with dose. *C*max was achieved approximately 1 h after dosing and the mean *C*max values was found as 1.48±0.39 μg mL^{-1} following a single oral dose of 320 mg GFX.

The extraction procedures and applying of the proposed methods to plasma samples were described at "Assay Procedures for Spiked Plasma Samples". Therefore, the proposed methods are able to be applied succesfuly to the analysis of GFX in human plasma. The obtained results shown in Table 4 are satisfactorily accurate and precise.

Conclusion

The proposed spectrofluorimetric methods are quite simple and do not require any pretreatment of the drug and tedious extraction procedure. Considering the limits of detection and/or concentrations ranges, the proposed methods are more sensitive than previously published methods. The method were successfuly applied for determining GFX in

Table 4 The recoveries of GFX from plasma (n=5)

Methods	Added (ng mL^{-1})	Determined (ng mL^{-1})	Recovery (%)	RSD (%)[a]
Method A (using NBD-Cl)	40	33.62	84.04	4.27
	100	80.03	80.03	3.49
	200	159.56	79.78	3.01
Method B (using fluorescamine)	100	82.02	82.02	4.56
	500	406.05	81.21	3.65
	1200	942.48	78.54	2.89

[a] five independent analyses

tablets and proved being highly sensitive, accurate, precise, simple and without interference from excipients. The methods were also applied to the spiked plasma samples without any interference from the matrix. Hence the proposed methods can be used for routine analysis of GFX in pharmaceutical industries, hospitals and research laboratories.

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