Development of Two Innovative 96-Microwell-Based Spectrophotometric Assays with High Throughput for Determination of Fluoroquinolone Antibiotics in their Pharmaceutical Formulations

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Abstract: ***Background:*** Fluoroquinolone antibiotics (FQAs) have broad-spectrum antibacterial activity, high potency, variable indications, and excellent pharmacokinetic profiles. Many spectrophotometric assays have been described for determination of FQAs in their pharmaceutical formulations; however, most of these assays have limited throughputs to be applied in quality control laboratories.

Objectives: This study was devoted to the development and validation of two innovative 96-micorwell-based spectrophotometric assays with high throughput for pharmaceuticals quality control of seven fluoroquinolone antibiotics (FQAs).

Methods: These FQAs were levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP), gemifloxacin (GEM), danofloxacin (DAN), enrofloxacin (ENR) and marbofloxacin (MAR). The first assay (assay I) was developed for LEV, NOR, CIP, and GEM via formation of red-colored metal complexes (MC) with $FeCl_3$. The second assay (assay II) was developed for LEV, DAN, ENR, and MAR via formation of red-colored charge transfer complexes (CTC) with 2,3-dich1oro-5,6-dicyano-1,4-benzoquinone (DDQ). The reactions of FQAs with both $FeCl_3$ and DDQ reagents were carried out in transparent 96-microwell plates and the absorbances of the colored-complexes were measured by absorbance microwell plate reader at 460 nm.

Results: The optimum reaction conditions were established for both assays; under which, Beer's law correlating the absorbances with the concentrations of FQAs were obeyed in the range of 10 – 100 µg/well with good correlation coefficients (0.9943 – 0.9982). The limits of detection were in the range of 4.5 – 7.5 µg/well, and the limits of quantification were in the range of 14.9 – 25.0 µg/well. Both assays showed high precisions as the values of the relative standard deviations (RSD) did not exceed 3.4%. The accuracy of both assays was proved by recovery studies as the recovery values were in the range of 98.1 – 102.6% (± 0.9 – 2.7%). The proposed assays were applied successfully for the determination of all FQAs in their tablets with good accuracy and precisions.

Conclusion: The proposed assays are simple, economic, and more importantly have high throughput; therefore, they are convenient and valuable for routine use for in pharmaceutical quality control laboratories for determination of FQAs in pharmaceutical formulations. An additional advantage of the proposed assays is that all the FQAs could be determined on a single system without modifications in the detection wavelength.

Keywords: Fluoroquinolone antibiotics, metal complexes, charge transfer complexes, spectrophotometry, 96-microwell-based assays, high analysis throughput.

1. INTRODUCTION

Fluoroquinolone antibiotics (FQAs) are synthetic agents with broad-spectrum anti-bacterial activity, high potency, variable indications, and excellent pharmacokinetic profiles. They act by selective inhibition of topoisomerase II ligase, leading to DNA fragmentation [1-4]. The effective and safe therapy with FQAs is basically depending on the quality of their pharmaceutical preparations. For quality control (QC) of FQAs, proper assay with high throughput is required. Spectrophotometric assays [5] are considered the most convenient methods because of their inherent simplicity, high sensitivity, low cost, and wide availability in most QC laboratories. As revealed from the survey of literatures, many spectrophotometric assays have been reported for determination of FQAs in their pharmaceutical formulations [6-13]. However, most of these assays were associated with some major drawbacks such as decreased selectivity due to measurement in ultraviolet region [6-8] and/or decreased simplicity of the assay procedure such as tedious liquid-liquid extraction steps in the assays based on formation of ion-pair associates [9-11], and consume large amounts of expensive and toxic organic solvents [12-15] in the assays based on charge transfer reactions [16,17]. As well, these assays were developed individually because of the difference in chemical structures of the FQAs. However, the availability of a spectrophotometric assay that allows the determination of many FQAs in pharmaceutical products would be useful for economic and convenience reasons. Moreover, the reported assays adapted the conventional spectrophotometric technical procedures which has limited analytical throughputs.

To improve the QC analysis and enhance its productivity, the technology scientists have been developing analytical instrument with high throughput capabilities [18]. High throughput assays enable the researchers to quickly process large numbers of samples in a reasonably time fashion. Through this process, rapidly identify active compounds, pharmaceutical formulation uniformity contents and other pharmaceutical industry activities could be achieved. In previous studies, Darwish *et al*. [19-25] has successfully apopted an absorbance microwell plate reader in the development of spectrophotometric assays high throughput for measuring many of the active drug contents in their pharmaceutical formulations. For these reasons, the present study was designed to employ the methodology for development of 96-microwell-based assay for FQAs. The formation of MC and CTC has a considerable importance and frequently applied for the analysis of FQAs; however, the analysis involved the conventional disadvantageous spectrophotometric techniques. Therefore, the present research proposal was devoted to employ formation of both MC and

CTC as basis in the development of the assays described herein for QC of the investigated FQAs by the proposed 96-microwell-based spectrophotometric assays.

2. EXPERIMENTAL

2.1. Apparatus and Tools

Absorbance microwell plate reader (ELx 808, Bio-Tek Instruments Inc., Winooski, USA) was used for all the spectrophotometric measurements. The reader is controlled by Gen5 software (Bio-Tek Instruments Inc., Winooski, USA) provided with the instrument. Double beam ultraviolet-visible spectrophotometer (UV-1800, Shimadzu Co. Ltd., Kyoto, Japan) with matched 1-cm quartz cells was used for scanning the UV-visible spectra. Transparent 96-microwell plates were a product of Corning/Costar Inc. (Cambridge, USA). Adjustable 8-channel-pipettes was obtained from Sigma-Aldrich Chemicals Co. (St. Louis, Missouri, USA). BRAND® PP reagent reservoirs with lids for multichannel pipettes were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Standards, Pharmaceutical Formulations and Reagents

The standards of FQAs were levofloxacin (LEV), norfloxacin (NOR), ciprofloxacin (CIP), danofloxacin (DAN), gemifloxacin (GEM), enrofloxacin (ENR) and marbofloxacin (MAR). All these standard materials were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, Missouri, USA). The purities of these standard materials were ≥ 99.5%.

The pharmaceutical formulations were tavanic (Sanofi-Aventis Pharma, France) labeled to contain 500 mg of LEV, noroxin (Algorithm, Lebanon) labeled to contain 400 mg NOR per tablet, cipropharm (Pharma international, Jordan) labeled to contain 500 mg of CIP, factive (Tabuk pharmaceuticals, Saudi Arabia) labeled to contain 320 mg of GEM and Laboratory-made tablets prepared to contain 25 mg per tablet for each of ENR, DAN and MAR.

The reagents were $FeCl_3$ (BDH Chemicals Co., Germany) and 2,3-dich1oro-5,6-dicyano-1,4-benzoquinone (DDQ; Sigma-Aldrich Chemicals Co. (St. Louis, Missouri, USA). $FeCl_3$ solution (0.5%, w/v) was prepared fresh daily by dissolving 50 mg of $FeCl_3$ in 10 ml of 0.05 M hydrochloric acid. DDQ solution (0.5%, w/v) was prepared fresh daily by dissolving 50 mg of DDQ in 10 ml acetonitrile. All other reagents and solvents were of analytical reagent-grade.

2.3. Preparation of Standard and Sample Solutions

2.3.1. Standard Solutions

For assay I, stock standard solutions (1 mg/ml) of LEV, NOR, CIP and GEM were prepared by dissolving an accurately weighed amounts (25 mg) of the standard material in 25 ml of 0.05 M HCl. For assay II, stock standard solutions (1 mg/ml) of LEV, DAN, ENR and MAR were prepared by dissolving an accurately weighed amounts (25 mg) of the standard material in 25 ml acetonitrile. These stock solutions were found to be stable for at least two weeks when kept in a refrigerator.

2.3.2. Pharmaceutical Formulation Sample Solutions

2.3.2.1. For Assay I

Twenty tablets of LEV, NOR, CIP and GEM were weighed, and finely powdered. An accurately weighed quantity of the powder equivalent to 25 mg of the active ingredient was transferred into a 25-ml calibrated flask and dissolved in about 15 ml of 0.05 M HCl. The contents of the flask were swirled, sonicated for 5 min, and then completed to volume with 0.5 M HCl. The contents were mixed well, filtered and the first portions of the filtrates were discarded. The filtrate solutions were diluted quantitatively with the same solvent to obtain solutions in the range of 100 – 1000 µg/ml. These solutions were analyzed by the proposed assay I, based on formation of colored MC upon their reactions with $FeCl_3$.

2.3.2.2. For Assay II

Marketed tavanic tablets (for LEV) and laboratory-made tablets (for DAN, ENR and MAR) were used. Tablets for ENR, DAN and MAR were prepared by mixing accurately weighed amounts of each active ingredient with starch (25 mg), hydroxypropyl cellulose (25 mg), microcrystalline cellulose (25 mg) and lactose monohydrate (25 mg). The amounts of active ingredients were 320, 25 and 25 mg for ENR, DAN and MAR, respectively. An accurately weighed quantity of the mixed powder equivalent to 25 mg of the active ingredient was transferred into a 25-ml calibrated flask and dissolved in about 15 ml of acetonitrile. The contents of the flask were swirled, sonicated for 5 min, and then completed to volume with acetonitrile. The contents were mixed well, filtered and the first portions of the filtrates were discarded. The filtrate solutions were diluted quantitatively with the same solvent to obtain solutions in the range of 100 – 1000 µg/ml. These solutions were analyzed by the proposed assay II, based on formation of colored CTC upon their reactions with DDQ.

2.4. General Procedures

2.4.1. For Assay I

Aliquots (100 µl) of the standard or sample solution containing 10 – 100 µg of LEV, NOR, CIP and GEM were separately transferred into each well of the 96-microwell assay plate. To each well, 100 µl of $FeCl_3$ solution (0.5%, w/v) was added, and the reactions were allowed to proceed at room temperature (25 ± 2 ºC) for 5 min. The absorbencies of the resulting solutions were measured at 460 nm by the absorbance microwell plate reader. Blank wells were treated similarly except 100 µl of 0.05 M HCl was used instead of the sample solution, and the absorbencies of the blank wells were subtracted from those of the sample wells.

2.4.2. For Assay II

Aliquots (100 µl) of the standard or sample solution containing 10 – 100 µg of LEV, DAN, ENR and MAR were separately transferred into each well of the 96-microwell assay plate. To each well, 100 µl of DDQ solution (0.5%, w/v) was added, and the reactions were allowed to proceed at room temperature (25 ± 2 ºC) for 5 min. The absorbencies of the resulting solutions were measured at 460 nm by the absorbance microwell plate reader. Blank wells were treated similarly except 100 µl of acetonitrile was used instead of sample solution, and the absorbencies of the blank wells were subtracted from those of the sample wells.

2.5. Determination of Molar Ratio

2.5.1. For Reaction with $FeCl_3$

The Job's method of continuous variation [26] was employed. Master equimolar molar solutions (2×10^{-3} M) of FQAs (LEV, NOR, CIP and GEM) and $FeCl_3$ were prepared in 0.05 M HCl. In each well of the 96-microwell assay plate, series of 200 µl/well of the master solutions of FQAs and $FeCl_3$ were made up comprising different complementary ratios (0:200, 25:175, 50:150, 100:100, 150:50, 175:25 and 200:0). The reactions were allowed to proceed for 5 min at room temperature and the absorbencies of the resulting solutions were measured at 460 nm. The measured absorbances were plotted see as a function of the mole fractions of FQAs in the reaction mixtures (FQAs + $FeCl_3$) The generated plots were used for determination the molar ratio of the reaction between FQAs and $FeCl_3$.

2.5.2. For Reaction with DDQ

The Job's method of continuous variation [26] was employed. Master equimolar molar solutions (2×10^{-3} M) of FQAs (LEV, DAN, ENR and MAR) and DDQ were prepared in acetonitrile. In each well of the 96-microwell assay plate, series of 200 µl/well of the master solutions of FQAs and DDQ were made up comprising different complementary ratios (0:200, 25:175, 50:150, 100:100, 150:50, 175:25 and 200:0). The reactions were allowed to proceed for 5 min at room temperature and the absorbencies of the resulting solutions were measured at 460 nm. The measured absorbances were plotted see as a function of the mole fractions of FQAs in the reaction mixtures (FQAs + DDQ) The generated plots were used for determination the molar ratio of the reaction between FQAs and DDQ.

3. RESULTS AND DISCUSSION

3.1. Reactions Involved and Assays Design

The formation of colored MC and CTC has been used as a basis of several spectrophotometric assays. These assays, in most cases, are instantaneous or at least very fast at room temperature and very mild and safe reaction conditions. Besides, these assays enabled more selective and sensitive determinations of certain substances, which found wide applications in pharmaceutical analysis. Examples for the assays based on formation of MC are the determination of mercury-containing drugs via formation of yellow color mercury complex with 1-salicyidene-5-(2-pyridylmethylene)-isothiocarbonohydrazide, and the determination of many drugs that are convertible into hydroxamic acid derivatives (e.g. esters, carboxylic amides, carbamoyl derivatives etc.) via formation of colored Fe(III)-hydroxamic acid complex [5]. Examples for the assays based on formation of CTC are the determination of sartans, statins, macrolides, antidepressants and tyrosine kinase inhibitors [20-25]. The formation of stable MC and CTC with organic pharmaceutical molecules necessitates the presence of at least two electron-donating groups in a steric arrangement permitting the formation of the chelate complexes with the metal ion and the CTC with electron acceptors. This criterion is fulfilled with the FQAs as their chemical structures contain carboxylic acid and hydroxyl groups that can form MC with metal ions [27-33], and other electron-donating terminal nitrogen atoms that enable FQAs to form CTC with electron acceptors [31-35]. Therefore, FQAs were selected as target analytes in the assays described in the present study. The documented advantages of the 96-microwell-based assays developed

in our laboratory [20-26] were the promoting factor to direct the present study towards the development of similar methodology for FQAs.

3.2. Absorption Spectral Characteristics

The UV-visible absorption spectra of FQAs solutions were recorded in the range of 200 – 800 nm. The spectra had different shapes, absorption maxima and molar absorpitivities. These differences were attributed to the differences in the substituent groups attached to the main fluoroquinolone skeleton of the investigated FQAs (Fig. 1). In spite of these differences, all the investigated FQAs had UV-absorption cut-off at ≤ 425 nm, beyond which they did not show any light absorption. The spectrum of LEV, as a representative example, was given in Fig. 2. When FQAs solutions were separately mixed with each of $FeCl_3$ and DDQ solutions and the reactions were allowed to proceed at room temperature (25 ± 2 °C), the solutions turned red-color and their absorption spectra showed new absorption bands at much longer wavelengths than those of FQAs and reagents ($FeCl_3$ and DDQ); the absorption spectra of the reaction mixtures of LEV with each of $FeCl_3$ and DDQ were given as a representative example (Fig. 2). The absorption intensities of the new absorption bands increased with the reaction time and FQAs concentrations in the reaction solutions confirming the formation of MC and CTC with $FeCl_3$ and DDQ, respectively. Furthermore, the shape and pattern of the resulting absorption bands of FQAs with each of $FeCl_3$ and DDQ were coincide with those reported in literature [28-36]. Although the absorption bands of the MC and CTC of investigated FQAs with $FeCl_3$ and DDQ showed varying maximum absorption wavelengths; we decided to perform the spectrophotometric measurements at 460 nm. This decision was taken because all FQAs do not absorb lights at this wavelength (Fig. 2) and enable the analysis of all the FQAs on a single system without changing in the measurement wavelength.

3.3. Optimization of Reaction Conditions

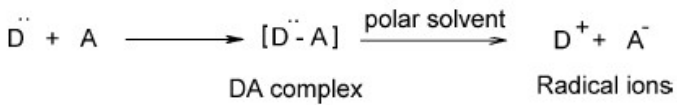
The optimization of experimental conditions affecting the reactions in the 96-microwell format was investigated by altering each reaction variable in a turn while keeping the others constant. LEV, NOR, CIP and GEM were used for optimization of reactions with $FeCl_3$; whereas, LEV, DAN, ENR and MAR were used for optimization of reactions with DDQ.

3.3.1. Reaction with $FeCl_3$

$FeCl_3$ dissolves freely in water and aqueous acid solutions. These solutions are faintly colored, and their colorations are reduced in 0.05 M HCl; therefore, the working solution of $FeCl_3$ was prepared in HCl. Variations in the concentrations of $FeCl_3$ indicated that a concentration of 0.5% (w/v) was optimum (Fig. 3). The effect of pH on the complex formation showed that the complexes are favorably formed and stable in acetate buffer solutions at pH 3.5 – 5.0, and comparable results were obtained when 0.05 M HCl. To simplify the procedures, 0.05 M HCl was used in the subsequent investigations. The complex between $FeCl_3$ and the FQAs formed instantaneously upon mixing of their solutions at room temperature and the reactions completed within 5 min (Fig. 4). Higher temperatures up to 70 °C had no enhancement or inhibitory effect on the formation of the complexes. Under these conditions, the complexes formed in a ratio of 1:1 ($FeCl_3$:FQAs) and they were found to be stable for at least 24 h. A summary for the optimization of the reaction conditions was given in Table 1.

3.3.2. Reaction with DDQ

Variations in DDQ concentrations indicated that the optimum DDQ concentration was 0.5%, (w/v) (Fig. 3), and 5 min was adeqaute for completing the reaction (Fig. 4). The reactions were carried out in different solvents of varying polarities (acetonitrile, methanol, n-propanol, n-butanol, acetone, tetrahydrofuran, 1,2-dichloromethane, dimethyl formamide, chloroform, 1,4-dioxan and carbon tetrachloride). The results indicated that acetonitrile was the best solvent for the reaction. This was attributed to its high dielectric constant that can promote the dissociation of the CTC to the colored radical ion of the DDQ molecule [36-38] as a predominent chromogen as given by the following equation:



Studying the effect of temperature in the range of 25 – 70 °C on the reactions indicated that maximum colour development was attained at room temperature (25 ± 2 °C) and higher temperature had negative effect on the reaction. Under these conditions, the complexes formed in a ratio of 1:1 (DDQ:FQAs). A summary for the optimization of the reaction conditions was given in Table 1.

3.4. Validation of the Proposed Assays

3.4.1. Linearity and Sensitivity

Under the above-mentioned optimum reaction conditions, assay procedures were carried out in 96-microwell assay plates and the color signals was generated (Fig. 5). The calibration graphs were constructed for the determination of LEV, NOR, CIP and GEM by assay I (via reaction with $FeCl_3$) and LEV, DAN, ENR and MAR by assay II (via reaction with DDQ). These graphs were generated by plotting the absorbencies as a function of the corresponding concentrations of FQAs. Regression equation for each one of the investigated FQAs was derived using the least-squares method; the results are given in Table 2. The correlation coefficients of the data obtained by the two assays were in range of 0.9943 – 0.9982. Beer's law was obeyed over the concentration range of 10 – 100 µg/well for all the FQAs. The limits of detection (LOD) and limits of quantitation (LOQ) were determined. The LOD values were found to be in the range of 4.5 – 7.5 and 6.1 – 7.2 µg/well using assays I and II, respectively. The LOQ values were found to be in the range of 14.9 – 25.0 and 20.3 – 24.0 µg/well using assays I and II, respectively. The small values of LOD and LOQ indicated the high sensitivity of the two proposed assays in the determination of the FQAs.

3.4.2. Precision and Accuracy

The precisions of both assays were accessed by replicates analysis of the FQAs solutions at three concentration levels for each (20, 50 and 80 µg/well). The intra-assay precision was assessed by analysis of 6 replicates of each sample as a batch in a single assay run, and the inter-assay precision was assessed by analysis of 3 replicates of each sample in two consecutive assay runs. The relative standard deviation (RSD) values did not exceed 3.40 and 2.91% for assay I and assay II, respectively (Tables 3 and 4). The accuracy of both assays was evaluated by the recovery studies for the investigated FQAs at three concentration levels of each drug (20, 50 and 80 µg/well). The recovery values were in the ranges of 98.1 – 102.6 ± 0.9 – 2.7% and 98.2 – 103.4 ± 1.2 – 3.1% for assay I and assay II, respectively (Tables 3 and 4). The small values of RSD and high recovery percentages (~100%) with low SD values indicated the high precision and accuracy of the two proposed assays. The achieved high level of precisions was attributed to the accuracy of the volumes that have been concomitantly dispensed in the microwells, and completeness of the reactions in small volumes of the reaction mixtures (200 µl) in each well of the 96-well assay plates.

3.5. Determination of FQAs in Pharmaceutical Formulations

The proposed assays were applied to the determination of FQAs in different pharmaceutical formulations and the results obtained are given in Table 5. In order to check the confidence and correlation between the results obtained by the two proposed assays using formation of MC and CTC with $FeCl_3$ and DDQ reagents, and the reference methods [28,34] for determination of FQAs, the results were statistically compared using t- and F-tests (for comparing the accuracy and precision, respectively). All the calculated *t*- and *F*-values at the 95% confidence level were lower than those of the tabulated values (Table 5). This proved the similarity in precision and accuracy of the proposed assays with the reference methods in the determination of FQAs in their pharmaceutical formulations.

CONCLUSIONS

Two novel microwell-based spectrophotometric assays (I and II) have been successfully developed and validated for the determination of 7 FQAs in their pharmaceutical formulations. Assays I and II were based on the formation of colored MC and CTC with $FeCl_3$ and DDQ reagents, respectively. The proposed assays are superior to the existing spectrophotometric methods for FQAs in terms of the analytical procedure simplicity, use of inexpensive analytical reagents (economic), consume minimum volumes of reagent (environmentally friendly "Green" approach) and have high throughputs. An additional advantage of the proposed assays is that all the FQAs could be determined on a single system without modifications in detection wavelength. These advantages encourage the application of the proposed assays in routine analysis of FQAs in quality control laboratories, as an alternative for the existing methods.

LIST OF ABBREVIATIONS

FQAs = Fluoroquinolone antibiotics

LEV = Levofloxacin

NOR = Norfloxacin

CIP = Ciprofloxacin

GEM = Gemifloxacin

DAN = Danofloxacin

ENR = Enrofloxacin

MAR = Marbofloxacin

DDQ = 2,3-Dich1oro-5,6-dicyano-1,4-benzoquinone

SD = Standard deviation

RSD = Relative standard deviation

QC = Quality control

MC = Metal complex

CTC = Charge transfer complex

UV = Ultraviolet

LOD = Limit of detection

LOQ = Limit of quantitation

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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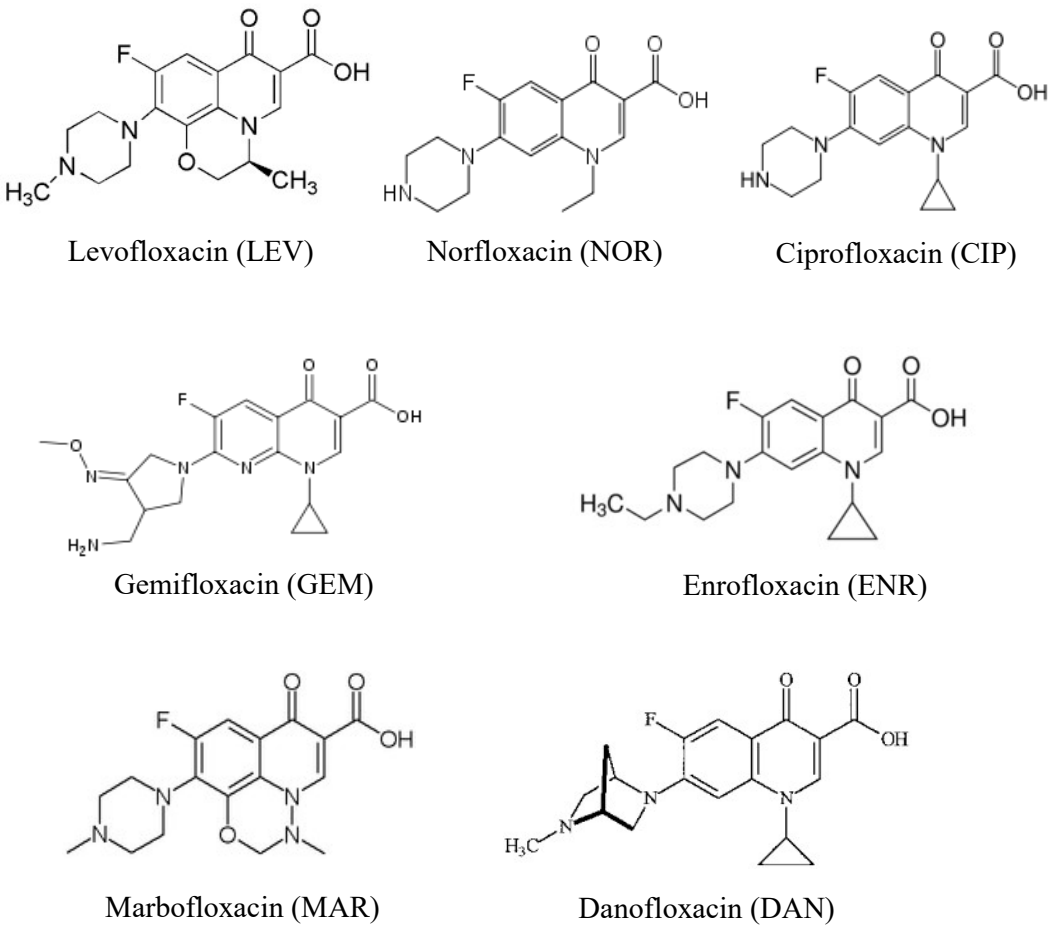

Fig.1. Chemical structures and abbreviations of the investigated FQAs.

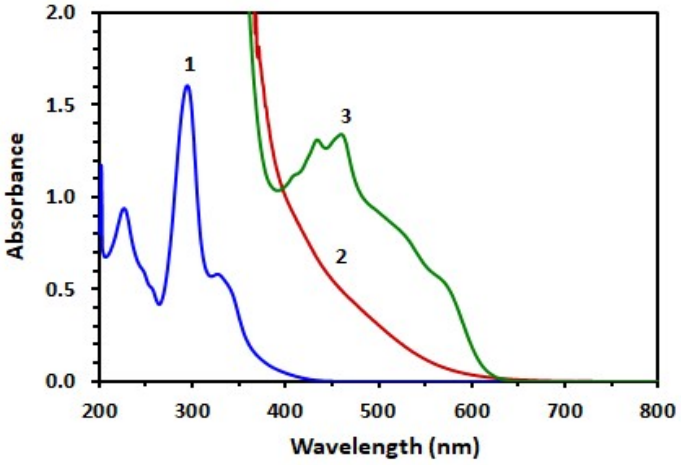

Fig. 2. Absorption spectra of (1) LEV (20 µg/ml), (2) reaction mixture of LEV (200 µg/ml) with $FeCl_3$ (2%, w/v, in 0.05 M HCl) and (3) reaction mixture of LEV (200 µg/ml) with DDQ (0.5%, w/v, in acetonitrile).

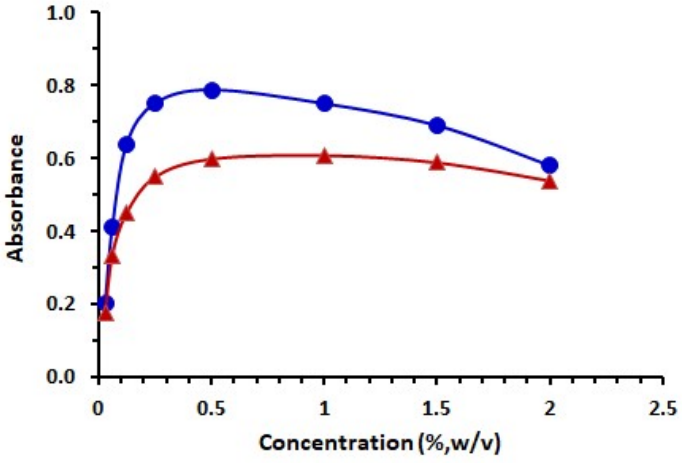

Fig. 3. Effect of $FeCl_3$ (▲) and DDQ (●) on their reaction with LEV. Concentrations of LEV were 40 µg/well in 0.05 M HCl and 100 µg/ml in acetonitrile for reaction with $FeCl_3$ and DDQ, respectively.

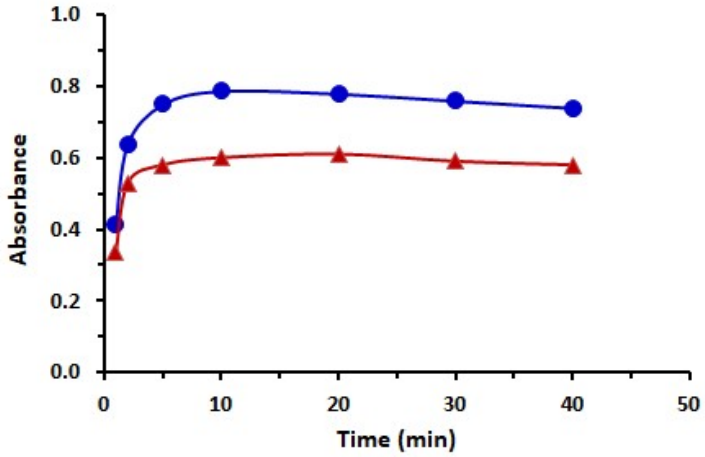

Fig. 4. Effect of time on the reaction of $FeCl_3$ (▲) and DDQ (●) on their reaction with LEV. Concentrations of LEV were 40 µg/well in 0.05 M HCl and 100 µg/ml in acetonitrile for reaction with $FeCl_3$ and DDQ, respectively.

Fig. 5. An image of the assay plate of the proposed 96-microwell-based spectrophotometric assay of FQAs involving formation of CTC with DDQ. The plate contains the calibration solutions of varying drug concentrations (upper wells) and test samples (lower wells).

Table 1. Summary for the optimization of the variables affecting the reaction of FQAs with $FeCl_3$ and DDQ employed as analytical reagents in the development of the two proposed 96-microwell-based spectrophotometric assays

Variable	Studied range	Optimum
Assay I, via formation of MC with $FeCl_3$		
Concentration of $FeCl_3$ (%, w/v)	0.04 – 2	0.5
pH of the reaction medium	3 – 9	3.5 – 5.0
Type of acid	Hydrochloric, sulphuric, nitric and acetic	Hydrochloric
Concentration of HCl (M)	0.1 – 1	0.5
Temperature (°C)	25 – 70	25
Time (min)	0 – 40	5
Assay II, via formation of CTC with DDQ		
Concentration of DDQ (%, w/v)	0.04 – 2	0.5
Solvent	Different [a]	Acetonitrile
Temperature (°C)	25 – 70	25
Time	0 – 40	5

[a] Solvents were: acetonitrile, methanol, n-propanol, n-butanol, acetone, tetrahydrofuran, 1,2-dichloromethane, dimethyl formamide, chloroform, 1,4-dioxan and carbon tetrachloride.

Table 2: Validation parameters for the determination of FQAs by the two proposed 96-microwell-based spectrophotometric assays

Parameter	Assay I, via formation of MC with $FeCl_3$			
	LEV	NOR	CIP	GEM
Intercept	0.0257	0.0475	0.0660	0.0751
Slope	0.0147	0.0194	0.0203	0.0127
Correlation coefficient (r)	0.9976	0.9951	0.9943	0.9966
LOD (µg/well)	4.8	7.3	7.5	4.5
LOQ (µg/well)	15.9	24.2	25.0	14.9
	Assay II, via formation of CTC with DDQ			
	LEV	DAN	ENR	MAR
Intercept	0.0435	0.0517	0.1512	0.1115
Slope	0.0094	0.0115	0.0102	0.0096
Correlation coefficient (r)	0.9959	0.9960	0.9982	0.9945
LOD (µg/well)	6.2	6.1	6.6	7.2
LOQ (µg/well)	20.6	20.3	22.1	24.0

Table 3. Precision and accuracy of the proposed 96-microwell-based spectrophotometric assay for FQAs via formation of MC with $FeCl_3$

FQAs/ conc. (μg/well)	Relative standard deviation (%)[a]		Recovery (% ± SD) [a]
	Intra-assay	Inter-assay	
LEV			
20	0.32	0.09	98.1 ± 1.4
50	0.26	1.07	101.0 ± 1.9
80	0.96	1.45	102.3 ± 2.3
NOR			
20	0.63	2.10	102.6 ± 1.5
50	1.89	2.70	100.6 ± 1.9
80	1.80	2.26	99.5 ± 2.1
CIP			
20	0.36	0.06	101.4 ± 0.9
50	0.42	3.40	100.2 ± 1.8
80	0.53	1.67	99.8 ± 2.7
GEM			
20	0.53	2.53	99.8 ± 1.2
50	2.43	2.82	102.4 ± 1.6
80	0.92	1.15	101.2 ± 2.1

[a] Values are mean of 3 determinations ± SD.

Table 4. Precision and accuracy of the proposed 96-microwell-based spectrophotometric assay for FQAs via formation of CTC with DDQ

FQAs/ conc. (μg/well)	Relative standard deviation (%)[a]		Recovery (% ± SD) [a]
	Intra-assay	Inter-assay	
LEV			
20	0.18	1.08	100.3 ± 1.2
50	1.88	2.91	102.6 ± 2.3
80	2.31	2.38	103.4 ± 3.1
DAN			
20	0.21	2.14	100.1 ± 1.4
50	1.16	0.33	99.5 ± 1.8
80	0.03	1.23	101.2 ± 2.6
ENR			
20	0.90	1.22	98.6 ± 2.1
50	2.39	2.34	102.1 ± 2.4
80	0.48	0.48	101.4 ± 1.9
MAR			
20	0.32	1.13	99.2 ± 2.4
50	1.92	2.13	101.6 ± 1.9
80	1.66	2.52	98.2 ± 2.6

[a] Values are mean of 3 determinations ± SD.

Table 5. Analysis of tablets formulations for their contents of the FQAs by the proposed 96-microwell-based spectrophotometric assays (I and II) and reference methods

Tablets formulation	Label claim (% ± SD) [a]		t-value [b]	F-value [b]
	Proposed	Reference [c]		
Assay I (via formation of MC with $FeCl_3$				
Noroxin	100.7 ± 0.9	98.9 ± 0.7	2.08	1.55
Ciropharm	98.3 ± 1.0	97.2 ± 1.5	1.44	2.34
Tavanic	100.8 ± 1.0	99.4 ± 0.7	2.03	2.22
Factive	99.4 ± 0.8	98.6 ± 0.7	1.29	1.08
Assay II (via formation of CTC with DDQ				
Tavanic	99.6 ± 1.8	100.1 ± 1.1	2.10	2.55
Enrofloxacin[d]	99.1 ± 1.3	99.6 ± 1.1	2.55	1.39
Danofloxacin[d]	100.2 ± 1.6	99.6 ± 1.2	2.63	1.74
Marbofloxacin[d]	99.8 ± 1.2	100.2 ± 0.9	2.35	2.13

[a] Values are mean of 5 determinations ± SD.

[b] The tabulated values of t- and F- at 95% confidence limit are 2.78 and 6.39, respectively

[c] References 28,34.

[d] Laboratory-made tablets.