Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 1, January – March 2014, p. 205 - 212

NOVEL HIGH-THROUGHPUT MICROWELL SPECTROPHOTOMETRIC ASSAY FOR DETERMINATION OF NON-FLUORINATED QUINOLONE ANTIBIOTIC ROSOXACIN IN ITS BULK AND CAPSULES

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This study describes, for the first, the development and validation of a novel high-throughput micorwell spectrophotometric assay for determination of the quinolone antibiotic rosoxacin (ROS) in its bulk and capsules. The assay involved the reaction between ROS and sodium nitroprusside (SNP) forming a red-colored product exhibiting maximum absorption peak (λ_{max}) at 455 nm. The reaction was carried out in 96-microwell plate and the absorbance of the colored-product was measured by microwell plate absorbance reader at 455 nm. The variables affecting the reaction were carefully investigated using the microwell format and the optimum conditions under were established. Under the optimized conditions, a linear relationship with good correlation coefficient (0.9992) was found between the absorbance of the ROS-SNP chromogen and ROS concentration in the range of 15 - 150 µg/mL. The limits of detection and quantification were 3.5 and 11.6 µg/mL, respectively. The assay showed high precision as the values of relative standard deviations (RSD) did not exceed 2%. The proposed assay was successfully applied to the determination of ROS in its capsules; the label claim percentages were 102.81 ± 1.43%. The results were compared favorably with those of a reference pre-validated method. The proposed assay is practical and valuable in terms of its routine application in determination of ROS in in its bulk and capsules in pharmaceutical quality control laboratories.

(Received December 6, 2014; Accepted February 3, 2014)

Keywords: Quinolone antibiotics; Rosoxacin; Sodium nitroprusside; Spectrophotometry; Microwell assay; High-throughput; Pharmaceutical analysis

1. Introduction

Rosoxacin (ROS; Fig. 1) is a relatively new oral non-fluorinated quinolone antibiotic used for treatment of bacterial infection of respiratory tract, urinary tract, gastrointestinal tract, central nervous system, immune-compromised patients [1]. ROS is known to be effective against penicillin resistant strains and is a single dose orally administered drug, which avoids all complications of parenteral administration seen with penicillin, especially anaphylactic shock [2]. The mode of ROS action depends on blocking of bacterial DNA replication by binding itself to an enzyme called DNA gyrase, which allows the untwisting required replicating one DNA double helix into two. ROS binds to and inhibits the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination. Accordingly, ROS has a broad-spectrum activity against both Gram-positive and Gram-negative bacteria [1-3].

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Spectrophotometry, because of its inherent simplicity, low cost, and wide availability is the most widely used analytical technique in the most pharmaceutical quality control laboratories [4]. However, only two spectrophotometric methods were described for determination of ROS [5,6]. These methods involved the conventional spectrophotometric technique; therefore their analytical throughputs were very low and limited their wide applications in determination of ROS. Accordingly, these methods could not fulfill the demand for the fast analytical procedures with high throughput that can provide same-day turnaround of results from large numbers of samples in pharmaceutical quality control laboratories. For these reasons, an alternative spectrophotometric method with high throughput was required for determination of ROS.

The present study described the development of microwell spectrophotometric assay with high throughput for determination of ROS. The proposed assay was based on the formation of red-colored chromogen with SNP reagent. The reaction was carried out in 96-microwell assay plate and the absorbance was measured by microwell absorbance plate reader. The proposed method, after its full validation, was adopted for the determination of ROS in its bulk and capsules.

2. Experimental

2.1. Apparatus

Microplate/cuvette reader (ELx 808, Bio-Tek Instruments Inc. Winooski, USA) was used for all the measurements in 96-microwell plates. UV-1601 PC (Shimadzu, Kyoto, Japan) ultraviolet-visible spectrophotometer with matched 1 cm quartz cells was used for recording the absorption spectra. 96-Microwell plates were a product of Corning/Costar Inc. (Cambridge, USA). Adjustable 8-channel-pipette was obtained from Sigma Chemical Co. (St. Louis, MO, USA). WSC-85 water purification system (Hamilton Laboratory Glass Ltd., Kent, USA) was used throughout the work for water purification.

2.2. Chemicals and materials

Rosoxacin (ROS) was obtained from Sterling Winthrop Inc. (West Virginia, USA). ROS stock solution was prepared as 1 mg/mL in 1 M sodium hydroxide, kept in dark container because of its photosensitivity. Sodium nitroprusside (SNP; Sigma Chemical Co., St. Louis, USA), was 2% w/v aqueous solution, prepared fresh daily. All other materials used throughout this study were of analytical grade.

2.3. Capsules and preparation of sample solution

Eradicil® capsules labeled to contain 300 mg ROS per capsules was a product of Sanofi-Aventis (Paris, France). Ten Eradicil® capsules were evacuated and the contents were weighed. An accurately weighed quantity of the capsule contents equivalent to 50 mg of ROS was transferred into a 25-mL calibrated flask, and dissolved in about 15 mL of 1 M NaOH. The contents of the flask were swirled, sonicated for 20 min, and then completed to volume with 1 M NaOH. The contents were mixed well and centrifuged at 6000 rpm for 10 min; the supernatant was separated and filtered, the first portion of the filtrate was rejected. The filtered solution was diluted quantitatively to obtain working solutions in the range of 30-300 µg/mL.

2.4. General assay procedure

Accurately measured aliquots (100 µL of ROS solution (30–300 µg/mL) were transferred into separate well of 96-microwell assay plate. SNP aqueous solution (100 µL of 2%, w/v) was added and the reaction was allowed to stand for 10 min at room temperature (25 ± 2 °C). The absorbance of the solution in each well was measured by the microplate reader at 455 nm against reagent blank-well treated similarly.

3. Results and discussion

3.1. Strategy for assay development and its design

ROS was selected in this study because of its therapeutic importance and the need for an analytical method for its determination in its bulk and capsules as the reported methods for this purpose are very limited. Spectrophotometry based attempted based on its inherent simplicity and ease of its adaptation to automation by interfacing to different instruments (e.g. flow-injection analysis, microwell plate readers, etc.). SNP was considered in this study based on the facts that its reactivity with ROS and the reaction mechanism were proved in our previous study [5]. The proposed assay was designed to employ 96-microwell assay plate as a vessel for the reaction between ROS and SNP. In this design, the solutions were dispensed by 8-channel pipette, and the absorbance of the colored-reaction product was measured by 96-microwell-plate absorbance reader. This 96-microwell design of the proposed assay was considered based on the previous success of Darwish *et al.* [7,8] in the employment of this methodology for the high-throughput analysis of some other pharmaceuticals.

3.2. Absorption spectra and optimization of assay conditions

The reaction between ROS and SNP was performed, and the absorption spectra of ROS and its reaction product with SNP were recorded against. The product was red-colored exhibiting λ_{max} at 455 nm (Fig. 1). The optimization of experimental assay conditions affecting the reaction in the 96-microwell format was investigated by altering each reaction variable in a turn while keeping the others constant. All the measurements were carried out by the plate reader at 455 nm.

3.2.1. Effect of SNP concentration

The effect of SNP concentration on the reaction was checked. As shown in Fig. 2, the reaction of ROS was dependent on the concentration of SNP solution as the absorbance of the colored chromogen increased as the SNP concentration increased. The maximum readings were obtained when the SNP concentrations were in the range of 1.5-2.5% (w/v). Beyond these SNP concentrations, the reading significantly decreased. A concentration of 2% (w/v) was selected for subsequent investigations.

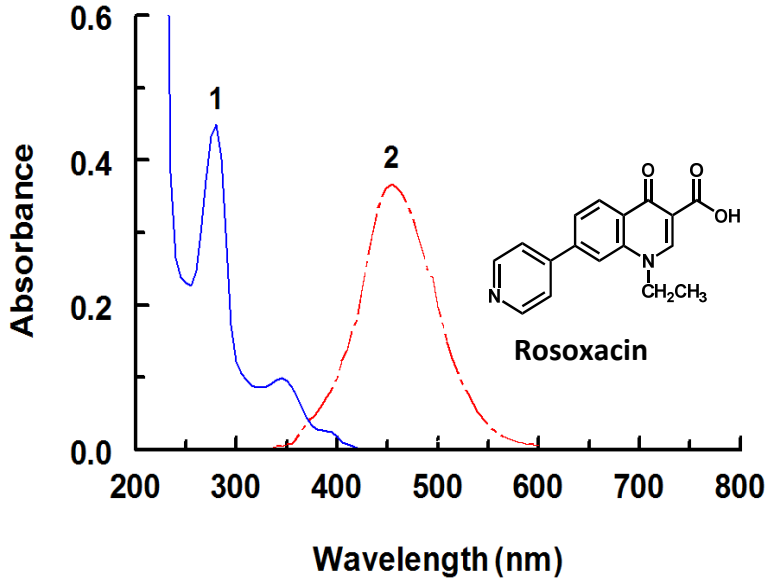

Fig. 1. Chemical structure of ROS and absorption spectra of its solution (20 µg/mL) a nd reaction solution of ROS (60 µg/mL) with SNP (2%, w/v).

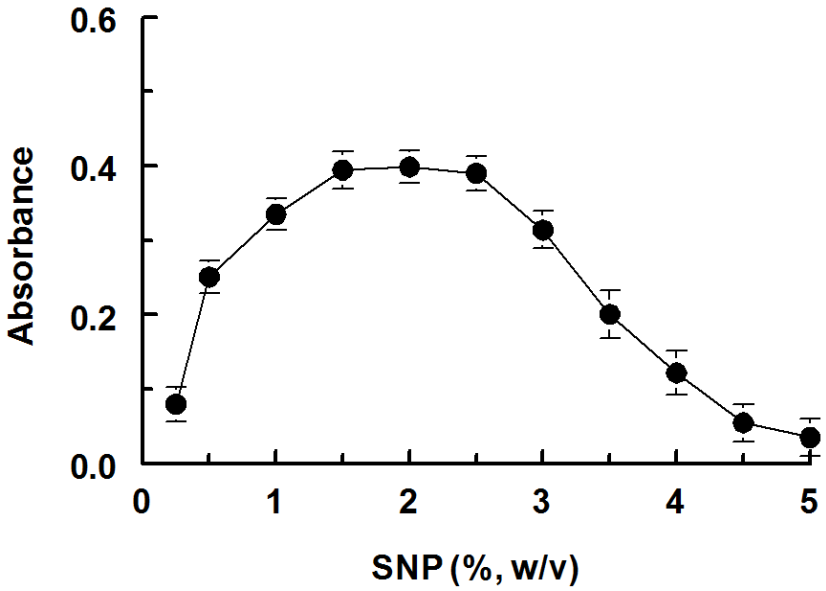

Fig. 2. Effect of SNP concentration on its reaction with ROS (60 µg/mL).

3.2.2. Effect of sodium hydroxide concentration

In our previous study (5), we demonstrated that the reaction between ROS and SNP proceeds in alkaline conditions and sodium hydroxide was the best among all the bases those were investigated (sodium hydroxide, borax, potassium hydroxide, disodium hydrogen phosphate, sodium carbonate and sodium bicarbonate). Studying the effect of sodium hydroxide concentration on the reaction revealed that 1 M was optimum; therefore this concentration was used for the subsequent investigations.

3.2.3. Effect of time and temperature

In order to study the effect of time of reaction between ROS and SNP and consequently the color development in the micowells, the reaction was allowed to proceed for varying times (2-25 min). It was found that reaction went to completion with 5 min (Fig. 3); however for higher precise reading, the measurements in the subsequent experiments were carried out after 10 min.

The reaction was found to be unaffected by higher temperature; therefore, further investigations were carried out at room temperature.

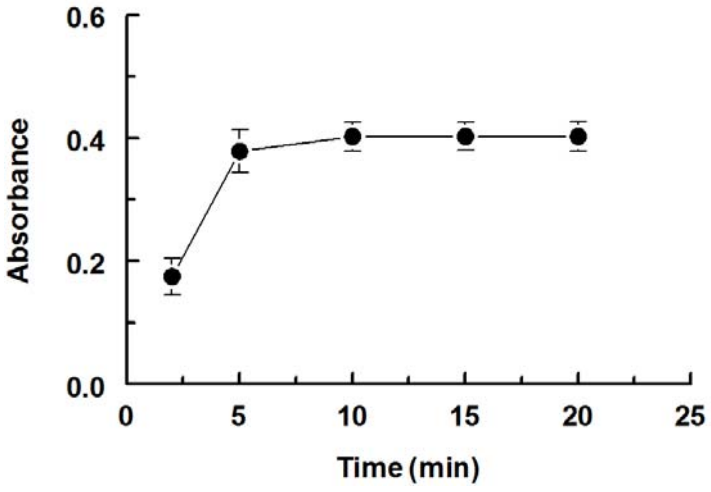

Fig. 3. Effect of time on the reaction of ROS (60 µg/mL) with SNP (2%, w/v).

3.2.4. Stability of the chromogen

The effect of time on the stability of the ROS-SNP chromogen was studied by following the absorption intensity of the reaction solution at different time intervals. It was found that the absorbance of the chromogen remains stable for at least 30 min. This allowed comfortable analytical processing of large batches of samples. This gives a high throughput property to the proposed assay when applied for analysis of large number of ROS samples in pharmaceutical quality control laboratories.

A summary for the optimum conditions for the proposed microwell spectrophotometric assay for ROS based on its reaction with SNP is given in Table 1.

Table 1. Summary for the optimization of experimental conditions for the proposed microwell spectrophotometric assay for ROS based on its reaction with SNP

Variable	Studied range	Optimum
SNP concentration (%, w/v)	0.25 – 5	2
Time (min)	2 – 20	10
Temperature (°C)	25 – 60	25
Chromogen stability time (min)	5 – 30	30
Measuring wavelength (nm)	340 – 600	455

3.3. Validation of the proposed assay

Assay validation was conducted according to The International Conference of Harmonization (ICH) guidelines for validation of analytical procedures [9].

3.3.1. Calibration and sensitivity

Under the optimum reaction conditions (Table 1), the calibration curve for the determination of ROS by proposed microwell spectrophotometric assay based on its reaction with SNP was constructed by plotting the absorbances as a function of the corresponding concentrations (Fig. 4). The regression equation for the results was A = 0.0144 + 0.0057 C (r = 0.9992), where A is the absorbance at 455 nm, C is the concentration of ROS in µg/mL in the range of 15 - 150 µg/mL, and r is the correlation coefficient. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the following formula was used: LOD or LOQ= ×SDa/b, where ×= 3.3 for LOD and 10 for LOQ, SDa is the standard deviation of the intercept, and b is the slope. The LOD and LOQ were found to be 3.5 and 11.6 µg/mL, respectively.

The parameters for the analytical performance of the proposed method are summarized in (Table 2).

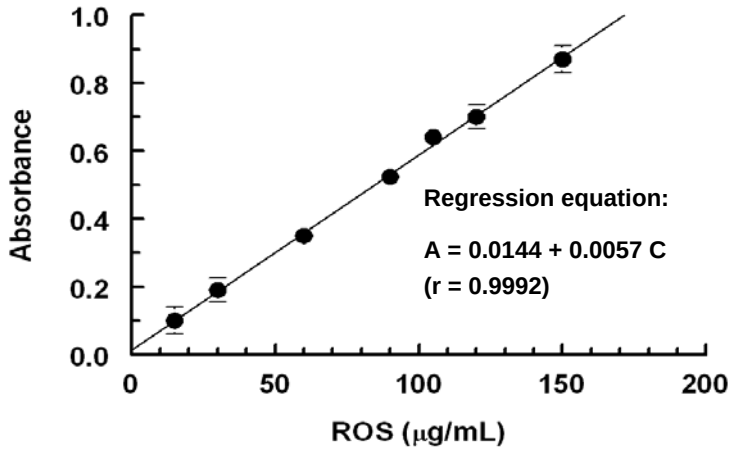

Fig. 4. Calibration curve for determination of ROS by the proposed microwell spectrophotometric assay based on its reaction with SNP. A, C and r are the absorbance, ROS concentration and correlation coefficient, respectively.

Table 2. Quantitative parameters for the analytical performance of the proposed microwell spectrophotometric assay for ROS

Parameter	Value
Linear range (μg/mL)	15 – 150
Intercept (a)	0.0144
Standard deviation of intercept	0.0061
Slope	0.0057
Standard deviation of slope	0.0012
Correlation coefficient	0.9992
Limit of detection, LOD (μg/mL)	3.5
Limit of quantification, LOQ (μg/mL)	11.6

3.3.2. Accuracy and precision

The accuracy of the proposed method was evaluated by the recovery studies. The recovery values were 98.54 ± 1.91 - 101.41 ± 1.47% (Table 3), indicating the accuracy of the proposed assay.

The intra-assay precision of the proposed method was determined on samples of ROS solutions at varying concentration levels (Table 4) by analyzing 6 replicates of each concentration as a batch in a single assay run. The inter-assay precision was determined by analysis the same samples as duplicates in three consecutive days. The relative standard deviations (RSD) did not exceed 2 % (Table 4) proving the high precision of the proposed assay for the routine application in the analysis of ROS in quality control laboratories.

Table 3. Recovery studies for determination of ROS by the proposed microwell spectrophotometric assay

Sample number	ROS		Recovery (% ± SD)[a]
	Nominal (μg/mL)	Found (μg/mL) [a]	
1	20	19.71	98.54 ± 1.91
2	50	50.10	100.25 ± 1.04
3	120	121.69	101.41 ± 1.47

[a] Values are mean of three determinations.

Table 4. Precision of the proposed microwell spectrophotometric assay at different concentrations of ROS

ROS concentration	Relative standard deviation (%)	
(μg/mL)	Intra-assay, n = 6	Inter-assays, n = 6
20	1.82	1.96
50	0.94	1.08
120	1.42	1.84

3.3.3. Robustness

Robustness was examined by evaluating the influence of small variation in the assay variables on its analytical performance. In these experiments, one parameter was changed whereas the others were kept unchanged, and the recovery percentage was calculated each time. It was found that small variation in one of the parameters did not significantly affect the procedures; recovery values were 97.8 - 101.4% ± 0.52 - 1.84% (Table 5). This indicated the reliability of the proposed assay during its routine application for the analysis of ROS.

Table 5. Robustness and ruggedness of the proposed microwell spectrophotometric assay for determination of ROS

Parameters	Recovery (% ± SD) [a]
Recommended conditions [b]	100.1 ± 0.52
SNP concentration (%, w/v)	
1.8	100.4 ± 1.09
2.2	97.8 ± 0.98
NaOH concentration (M)	
0.9	101.2 ± 1.05
1.1	98.6 ± 1.84
Reaction time (min)	
8	101.4 ± 1.52
12	98.6 ± 0.98

[a] Values are mean of 6 determinations.
[b] The recommended conditions are given in the Experimental Section.

3.4. Application for analysis of ROS in capsules

It is evident from the above-mentioned results that the proposed method gave satisfactory results with ROS in bulk powder. Thus, its pharmaceutical dosage form (Eradicil® capsules) was subjected to the analysis of their ROS contents by the proposed method. The percentage found from the label claim was 102.81 ± 1.43% (Table 6). This result was compared with those obtained by a reported method (8); the label claim percentage was 101.43 ± 1.35%, with respect to the accuracy (by t-test), and precision (by F-test). It was found that the calculated t- and F-values (1.57 and 1.12 for t- and F-value, respectively) were lower than the tabulated ones (2.31 and 6.61 for t- and F-value, respectively). This indicated that there were no significant differences between the means and variance between the two methods in terms of the accuracy and precision.

Table 6: Analysis of ROS-containing-capsules by the proposed and the reported methods

Capsules	Recovery (% ± RSD) [a]		t-value [c]	F-value [c]
	Proposed	Reported [b]		
Eradicil® capsules	102.81 ± 1.43	101.43 ± 1.35	1.57	1.12

[a] Values are mean of 5 determinations.
[b] Reference 8.
[c] The t

abulated values of t- and F- at 95% confidence limit are 2.31 and 6.61, respectively

4. Conclusions

This study described the successful development of a new microwell spectrophotometric assay for the determination of ROS in its bulk and capsules based on its reaction with SNP as a chromogenic reagent. The assay described herein offered the following advantages:

Providing a high throughput analytical methodology that can facilitate the processing of large number of ROS samples in a relatively short time when applied in pharmaceutical quality control laboratories. This property was attributed to the use of multi-channel pipettes for efficient dispensing of the solutions, carrying out the analytical reaction in 96-well plates (as reaction vessels), and measuring the color signals in the 96 wells at ~ 30 seconds by the plate reader.

Use of an inexpensive, stable reagent with excellent shelf life, and available in any pharmaceutical QC laboratory.

Acknowledgment

The authors would like to extend their appreciation to the Deanship of Scientific Research at King Saud University for its funding of this research through the research group project No. RGP-VPP-225.

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