Utility of a green fluorone-based turn-off fluorescence probe for submicromolar determination and stability testing of two macrolides. Insights into reaction thermodynamics, quenching mechanism, and identification of the oxidative degradation products by ESI^+-MS

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Abstract

A smart, sensitive, and green turn-off fluorescence probe was developed and validated for the determination of two macrolides; spiramycin (SPM) and josamycin (JSM) at submicromolar concentration levels. The probe is based on the fluorone-based dye, eosin Y that efficiently interacts with the two compounds through ground-state ion-pair complex formation with quenching of its native fluorescence ($\lambda_{ex}/\lambda_{em}$ of 524/548 nm). The Stern-Volmer relationship confirmed the static fluorescence quenching mechanism. Furthermore, the thermodynamic parameters of the reaction were explored *via* the van't Hoff plot. The values of the fluorescence quenching were linearly correlated to the drug concentration over the ranges of 0.12-5.93 and 0.30-9.66 µM (0.1-5.0 and 0.25-8.0 µg/mL) for SPM and JSM, respectively. Therefore, the probe was utilized for quality control of the two compounds in their tablets with mean %recoveries of 98.12±1.72 and 97.22±1.51% for SPM and JSM, respectively. Statistical analysis of the results by *t*- and *F*-tests showed excellent agreement with the results of the comparison methods. Moreover, the developed probe was applied for stability testing of the two compounds under oxidative condition along with ESI^+-MS identification of the potential degradants. Besides, the greenness of the developed probe was ensured by different assessment metrics. Hence, the developed method is the first stability-indicating fluorimetric assay for the two compounds, and its chief merits include effortlessness, rapidness, sensitivity, cheapness and harmony with the green chemistry rules.

Keywords: Spiramycin; josamycin; eosin; turn-off fluorescence probe; stability-indication; thermodynamics.

1. Introduction

Macrolides (MCs) are a group of antibacterials originates chiefly from *Streptomyces spp.* and characterized by a macrocyclic lactone ring attached to one or more sugar residues. They have common physicochemical properties and similar antibacterial spectrum. MCs mechanism of action depends on reversible binding to the ribosomal 50S subunit leading to prevention of the transpeptidation or translocation reactions, inhibition of protein synthesis, and stopping of cell growth. They possess a broad antibacterial spectrum against many Gram positive and some Gram negative bacteria. As well, they are effective against *Mycoplasma spp.*, *Chlamydiaceae*, *Rickettsia spp*., and *spirochaetes*. Thus, they are used for the treatment of a wide variety of infections such as campylobacter enteritis, chancroid, diphtheria, legionnaires'disease, respiratory-tract, bone, and genital infections [1].

Spiramycin (SPM) and josamycin (JSM) are two quite recent MCs that are extensively used in many countries for the treatment of several bacterial infections in human [1] and also as antimicrobial veterinary medicines [2]. As well, SPM is also used for the treatment of the protozoal infections cryptosporidiosis and toxoplasmosis [1]. SPM chemical name is 4R,5S,6S,7R,9R,10R,11E,13E,16R)-6-[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-a-L-ribo-hexopyranosyl)-3-(dimethylamino)-b-D-glucopyranosyl]oxy]-4- hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-[[2,3,4,6-tetradeoxy-4- (dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one), while JSM chemical name is 4R,5S,6S,7R,9R,10R,11E,13E,16R)-4-(acetyloxy)-6-[[3,6-dideoxy-4-O-[2,6-dideoxy-3-C methyl-4-O-(3-methylbutanoyl)-a-L-ribo-hexopyranosyl]-3- (dimethylamino)-b-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2- oxoethyl)oxacyclohexadeca-11,13-dien-2-one [3]. The two MCs have a common 16-member macrolide nucleus (Fig 1A) and they are weak bases [1]. The British Pharmacopoeia (BP) [3] and the Japanese Pharmacopoeia (JP) [4] recommended the determination of the two MCs *via* a microbial assay for antibiotics. Though, this assay is not specific and lacks the necessary precision. There are also different analytical methods for the determination of these antimicrobials in different matrices. For instance, SPM has been determined by thin layer chromatography (TLC) [5-7], liquid chromatography (LC) [8-14], spectrophotometry [7, 15], and voltammetry [16]. As well, LC [11-13, 17,18], spectrophotometry [19], voltammetry [20], and fluorimetry [21] techniques have been

applied for the determination of JSM. Yet, many drawbacks are associated with these methods such as the weak sensitivity of the spectrophotometric [7,15,19], fluorimetric [21], LC [9,10,17], and voltammetric [16] methods, the costs and the experience needed for operating mass spectrometry [8,12-14], and the need for lengthy derivatization procedure prior some LC methods [11]. In addition, the TLC techniques reported for SPM [5,6] involved bioautography detection which is a microbial detection mode that needs more than one whole day to be performed. Moreover, most of the reported methods includes the use of plenty amount of hazardous and costly organic solvents [7-15] or the exposure to toxic mercury [16,20].

It is worthy to note that, very limited fluorescence-based methods have been reported for the two MCs although this technique has several merits such as sensitivity, specificity, and simplicity. In addition, fluorescence spectroscopy is more convenient than many other complicated techniques by avoiding multi-steps, time and organic solvents consumption, and by using easily operated, widely available, and cheaper instrument. Yet, the analytical literature includes only one HPLC method coupled with fluorescence detection for SPM and JSM, which depends on pre-column derivatization with cyclohexa-1,3-dione for 60 min at 90 °C [11]. JSM has been determined also by monitoring its ability to quench the fluorescence of 3-mercaptopropionic acid-capped CdTe quantum dots [21], yet the sensitivity of this method is extremely poor (12.0-120.0 µg/mL). It is also obvious that these methods needs many steps either for derivatization [11] or for the synthesis of the quantum dots [21]. Thus these methods are not suited for quality control analysis. The scarce number of fluorimetry methods for the two compounds is mainly attributed to their aliphatic nature and the deprivation of fluorophore groups or commonly-derivatized functional groups such as primary and secondary amines [22]. Thus, it is a challenge to develop a fluorescence-based method for the determination of these compounds.

Building on these considerations, exploration of the chemical structure of the two MCs (Fig 1A) showed that they possess tertiary amino groups. Accordingly, the idea of this study was originated for sensitive and selective determination of the two MCs *via* using the xanthene dye eosin Y (EY, 2',4',5',7'-tetrabromofluorescein) as a turn-off fluorescence probe. EY is an acidic fluorone dye (Fig 1A) that shows a strong fluorescence. It is widely used in histo-pathological laboratories as a diagnostic tool for staining cytoplasm. EY is also known to interact with cationic molecules in acidic medium leading to quenching of its native fluorescence [23]. Since SPM and

JSM have tertiary amino groups with pK_a values of 8.0 [24] and 6.8 [25], respectively, they are protonated in acidic medium and interacts with EY causing quenching of its native fluorescence. The value of fluorescence quenching (ΔF) is proportional to the drugs' concentrations. Hence, a new turn-off fluorescence probe for the determination of the two candidates has been successfully established and validated. The mechanism of fluorescence quenching and the thermodynamics of the reaction were also elucidated. The application of the new probe for the analysis of real pharmaceutical samples in addition to testing the stability of the SPM and JSM were also effectively investigated. The oxidative degradation products of the two compounds were identified for the first time using positive electrospray ionization-mass spectrometry (ESI^+-MS).

2. Experimental

2.1. Equipment and software

Fluorescence scanning and measurements were performed at room temperature (20 °C) utilizing a 1-cm quartz cuvette and a spectrofluorometer from Jasco, Tokyo, Japan (model FP-6500) built-in with 150W Xenon lamp. The excitation and emission monochromators band widths were fixed at 5 and 6 nm, respectively and the sensitivity of the instrument was adjusted to 650 V with a scan rate of 500 nm/min. Instrument control and data acquisition were done *via* Spectra Manager™ software (Jasco). Statistical computations were performed with Microsoft Excel 2013. A Quattro micro™ triple-quadrupole mass spectrometer from Waters Co. (Milford, MA, USA) equipped with an electrospray ionization source was used for MS analysis. The ESI^+-MS spectra were obtained at cone voltage of 20 V for SPM and 30 V for JSM, capillary voltage of 4 Kv, source temperature of 120 °C, desolvation gas temperature of 350 °C, flow rate of 500 L/h, and cone gas flow rate of 40 L/h.

2.2. Chemicals and reagents

Gift samples of SPM (Batch No. 20170508A) and JSM pure powders were got from Delta Pharma S.A.E (10^{th} of Ramadan City, Egypt) and SAJA Pharmaceutical Company (Jeddah, Saudi Arabia), respectively. Rovamycin® (1 g SPM/tablet) and Josaxin® (500 mg JSM/tablet) film coated tablets were products of Sanofi-Aventis (France) and SAJA Pharmaceutical Company and they were obtained from pharmacies. Eosin Y (Merck, Darmstadt, Germany), citric acid anhydrous, boric acid, acetic acid, sodium sulfite, hydrogen peroxide, and $Na_2HPO_4.12H_2O$

(Sigma Aldrich, St. Louis, USA) were used. *o*-Phosphoric acid (85%) and sodium hydroxide pellets were purchased from Winlab Co. (UK).

Aqueous solutions of EY (0.5 mM), H_2O_2 (0.9 and 0.45% w/v), and sodium sulfite (1.0 M) were prepared. Britton-Robinson buffer (BRB) with pH values in the range of 2.0 to 5.5 was prepared by adjusting the pH of a solution containing 0.04 M boric acid, 0.04 M acetic acid, and 0.04 M *o*-phosphoric acid with 0.2 M NaOH.

2.3. Standard solution

Standard solution of SPM (100 µg/mL) was prepared by weighing and dissolving 10.0 mg of authentic powder in 100 mL water with the aid of sonication for 10 min. Due to the very slight solubility of JSM in water, its standard solution was prepared by weighing 10.0 mg authentic powder and dissolving in 1 mL methanol then diluting to 100 mL with distilled water and sonication. The solutions were stored at 4 °C in the refrigerator and they were stable for at least 7 days in this condition

2.4. Procedures

2.4.1. Construction of calibration curves

To a group of 10 mL volumetric flasks, 1.0 mL of BRB (pH 2.2) was added followed by aliquots of the standard solutions of the cited drugs that yield final concentration range of 0.1-5.0 and 0.25-8.0 µg/mL for SPM and JSM, respectively. Approximately 5 mL of distilled water was added to each flask followed by 1.0 mL of EY solution (0.5 mM), then the final volumes were adjusted with water. The solutions were well-mixed and the fluorescence intensity of each solution (F) was measured at 548 nm after excitation at 524 nm. The initial fluorescence intensity of EY (F_o) was measured by repeating these steps omitting the drug. The quenched fluorescence values were calculated ($\Delta F = F_o – F$) and plotted versus drug concentration (µg/mL) to get the calibration curves and the regression equations were derived.

2.4.2. Job's method for determination of the reaction stoichiometry

Solutions of EY and the two MCs with the same molar concentration ($6.0x10^{-5}$ M) were individually prepared. Two sets of 10 mL volumetric flasks were prepared for the determination of the reaction stoichiometry for the two drugs. The procedure described for preparation of the

calibration graphs was followed using steadily varied mole fractions of MC: EY (0.1 MC: 0.9 EY to 0.9 MC: 0.1 EY) and fixing the total molar concentration. The fluorescence was directly measured after mixing of the solutions. The value of ΔF was calculated and plotted against the volume fraction of the drug [MC]/[MC] + [EY] to construct Job's plots. The maxima of the plots correspond to the stoichiometry of the reactants.

2.4.3. Study of the oxidative degradation

Aliquots of 0.5 and 0.8 mL of SPM and JSM standard solutions, respectively, were accurately added to 10 mL volumetric flasks followed by 1.0 mL of H_2O_2 solution (0.45 or 0.9%w/v). The solutions were heated in a thermostatically-controlled water bath at 40 °C, in addition, the experiment was carried out at room temperature (20 °C). After 10 min, samples were taken and 1.0 mL of 1.0 M sodium sulfite solution was added to each solution and the mixtures were allowed to react for 10 min at room temperature to terminate the action of H_2O_2. Next, the recommended volumes of BRB and EY were added and the steps of the general procedure for preparation of the calibration curves were completed. The concentrations of the two drugs were determined from the regression equation and the %degradation was calculated.

For ESI^+-MS study, aliquots containing 200 µg of the two drugs were mixed with 1 mL of 0.9% w/v H_2O_2 and incubated at 40 °C for 30 min to allow degradation of appreciable concentration of the drug. Then, the solutions were diluted 1:1 with methanol to facilitate vaporization in the MS and filtered with 0.45 µm cellulose acetate membrane filter. Twenty µL of each solution were injected and the positive ESI^+-MS spectra were recorded for the reaction mixture of each drug.

2.4.4. Analysis of tablets

Ten tablets were weighed, triturated to a fine powder, and well-mixed. An amount of the powder containing 1g of SPM or JSM was weighed and transferred into 100 mL volumetric flask. Methanol was added to the two flasks to adjust the final volumes followed by sonication for 30 min. Filtration was done to obtain clear solutions (10 mg/mL). These solutions were 100-times diluted with distilled water to prepare 100 µg/mL solutions. Suitable volumes of these solutions were withdrawn into 10 mL volumetric flasks and the procedure for construction of the calibration

graphs was followed. The concentrations were calculated from the regression equation and the %found±SD was computed.

3. Results and discussion

3.1. Fluorescence aspects and method optimization

The aqueous solution of EY displayed a strong fluorescence at 548 nm when excited at 524 nm in BRB of pH 2.2. After addition of the MCs, the fluorescence intensity of EY substantially decreased (Fig. 1B) which indicates the interaction of the drugs with EY. This reaction can be easily visualized by the naked-eye since the formed complexes are pink-colored and certainly distinctive from the blank color (Supplementary material, Fig S1). It was observed that some precipitation of the SPM-EY reaction product took place upon mixing of the buffered drug directly with EY followed by dilution with water. This was observable only with drug concentrations at the upper calibration limit. To overcome this problem, the buffered SPM was diluted with few mLs of water (about 5 mL) to keep it at a minimum concentration before mixing with EY, where no precipitation took place in such case [26]. This phenomenon was not detected in case of JSM, perhaps due to the slighter bulkiness of JSM-EY reaction product as will be explained later. For simplicity, a dilution step preceding the addition of EY was included in the general procedure for both drugs. The factors controlling the interaction of the two MCs with EY and the subsequent fluorescence quenching were inspected to achieve the best experimental settings for quantitative determination of SPM and JSM with the highest sensitivity.

3.1.1. Effect of pH, type, and volume of buffer

To assess the role of pH in the interaction of the tested MCs with EY, it was varied over the range of 2.0-5.5 using BRBs. The maximum fluorescence quenching (ΔF) was observed over pH range of 2.0-2.5 for SPM and 2.0-2.2 for JSM, further increase of the pH hinders the interaction between EY and the MCs, where a complete loss of fluorescence quenching was observed above pH 3.0 (Fig. 2A). pH 2.2 was selected as the optimum for the two compounds. Different volumes of BRB (pH 2.2) were utilized to investigate the best volume for the maximum ΔF and 1.0 mL of the buffer was eventually selected as the optimum. In addition, McIlvaine buffer with the same pH was tried, but better fluorescence quenching was attained using BRB.

3.1.2. Effect of EY volume

The influence of the variation in EY concentration on the ΔF was explored using different volumes of 0.5 mM aqueous solution (Fig. 2B). A maximum and steady ΔF was obtained using 0.5-1.2 mL for both compounds, while reduced values of ΔF were attained by using smaller or greater volumes. Consequently, 1.0 mL was chosen as the optimum volume for SPM and JSM to obtain the maximum ΔF value.

3.1.3. Effect of diluting solvent

Dilution of the reaction mixture was done using water, methanol, or ethanol to select the most appropriate diluent. The best response was obtained using water for dilution which added surplus advantages to the method regarding simplicity, costs, and greenness.

3.1.4. Effect of time

Experiments showed that the interaction of MCs and EY takes place and reaches the equilibrium immediately. The values of fluorescence quenching (ΔF) were measured immediately after mixing the reaction mixture and at increasing time intervals up to 60 min. ΔF values remained stable without significant variation for 60 min (Supplementary material, Fig S2). The rapidness and stability of the product make the developed method very convenient for application in quality control laboratories.

3.2. Quenching mechanism, reaction thermodynamics, and stoichiometry

3.2.1. Quenching mechanism

Fluorescence quenching can be a result of a diversity of molecular interactions such as collisional (dynamic) quenching, static quenching, excited state reactions, molecular rearrangement, or energy transfer [27]. Thus, some experiments were conducted herein to determine the type of quenching involved in the developed turn-off fluorescence probe based on EY-MCs interaction.

First of all, it is obvious that the absorption spectra of SPM and JSM (λ_{max} 231 nm for both [3,4]) and the fluorescence spectrum of EY ($\lambda_{emission}$ 548 nm) are free from any overlap which completely excludes the energy loss *via* fluorescence resonance energy transfer (FRET) [27].

Secondly, the Stern-Volmer plots were constructed by plotting F_o/F versus [MC] according to Stern-Volmer equation [27]: $F_o/F = 1+K_{SV}$ [MC] (Eq. 1)

{K_{SV} is the Stern-Volmer quenching constant and [MC] is the molar concentration of the macrolide}

The Stern-Volmer plots were found linear (r = 0.99-0.96) without upward curvature (Fig 3A) which is suggestive of a single type of fluorescence quenching, either dynamic or static. To distinguish the type of fluorescence quenching, the quenching dependence on temperature was examined by construction of Stern-Volmer plots at elevated temperatures (Fig 3A). It was evidenced that the elevated temperatures cause decrease of K_{SV} (Table 1) which is a typical static quenching behavior where non-fluorescent complexes are formed between EY and MCs which are less stable at higher temperatures. Another method to determine the quenching type that confirms this result is the examination of the absorption spectra of EY before and after addition of the MCs. As obvious in Fig 1C, a considerable change of the absorption spectrum of EY occurred by existence of the MCs, which indicates a ground-state complex formation and static quenching [27].

Further, the bimolecular quenching constants (K_q), which is an indicator for the fluorescence efficiency and approachability of the fluorophore (EY) to the quenchers (MCs), were calculated utilizing the data obtained from the Stern-Volmer plots according to Eq. 2 [27]:

$K_q = K_{SV}/\tau_0$ Eq. 2

{τ_0 is the fluorescence lifetime of EY = 1.1 ns in aqueous medium [28]}.

The obtained values of K_q for SPM and JSM ranged from $1.09x10^{14}$ to $0.3x10^{14}$ L/mol.S at different temperatures (Table 1). These values are greater by far than $1x10^{10}$ L/mol.S which results from the dynamic quenching. These values confirms the static quenching through molecular binding and complex formation [27].

3.2.2. Reaction thermodynamics

The thermodynamics of the reaction of EY and MCs was also studied by plotting Van't Hoff graph of Ln K versus 1/T according to Eq. 3 [29]:

$$\text{Ln K} = \left(-\frac{\Delta \boldsymbol{H}^{\circ}}{\boldsymbol{RT}}\right) + \frac{\Delta S^{\circ}}{R} \qquad \text{Eq. 3}$$

{K is the equilibrium constant equivalent to K_{SV} at the same temperature [30], ΔH° is the change in enthalpy of the reaction, ΔS° is the change in entropy of the reaction, R is the gas constant = 8.314 J K^{-1} mol^{-1}, T is the absolute temperature (°K)}.

The obtained plots are shown in Fig 3B. The positive slopes of the obtained straight lines indicated exothermic reactions. Meanwhile, the change in enthalpy ΔH° can be obtained from the slope (ΔH° = -slope x R) and the change in entropy can be calculated also from the intercept (ΔS° = intercept x R). Furthermore, the Gibb's free energy (ΔG°) associated with the MCs-EY reaction was calculated based on the data obtained from the van't Hoff graphs according to Eq. 4 [29]:

$$\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} = -RT\,(\text{Ln K}) \qquad \text{Eq. 4}$$

The obtained results are summarized in Table 1. The negative values of Gibb's free energy confirmed that the process is exergonic and the reaction is spontaneous. In the meantime, the negative values of both ΔH° and ΔS° suggested that the reaction spontaneity is temperature-dependent and it proceeds spontaneously if the temperature is low enough. This result is in accordance with the results of Stern-Volmer plots where the quenching reaction and the K_{SV} decrease by increasing the temperature [29].

Moreover, the negative ΔH° and ΔS° values are indicative of enthalpy-driven molecular binding of EY and MCs. This means weak entropy involvement in the complex formation and excludes any contribution of hydrophobic interactions. Instead, the forces predominantly accountable for the formation of the stable EY-MC complex are non-covalent electrostatic and/or van der Waals forces [31].

3.2.3. Stoichiometry and constitution of the complexes

As a consequence of the aforementioned results, the reaction stoichiometry and the composition of the formed complexes were studied adopting Job's method of continuous variation [32]. As illustrated in Fig 3C, the maximum ΔF values in the Job's plots (X_{max}) were achieved at mole fractions of 0.3 and 0.5 of SPM and JSM, respectively. This result indicated 1:2 (SPM: EY) and 1:1(JSM: EY) molar reactivity. This was rational given that, SPM possesses two tertiary amino groups per molecule while JSM has one tertiary amino group (Fig 1A). These basic

tertiary amines are protonated at the pH of the reaction (pH 2.2), thus having the ability to interact with EY. As a result, each SPM molecule reacts with two EY molecules while one JSM molecule reacts with one EY molecule. Since EY pK_a 2.02 and 3.80 are related to the phenolic OH and the carboxylic groups, respectively, it is expected that EY exists as a mono-ionic form at the pH of the reaction by dissociation of the phenolic hydroxyl group. The dissociation of the hydroxyl group predominates due to the strong electron-withdrawing effect of the two bromine atoms on the xanthine ring near it, thus decreasing the electron density on the oxygen atom of the -OH group and facilitating its dissociation [33]. Hence, the ion-pair complexes are formed *via* molecular bonding by electrostatic attraction of the positively charged MCs and the negatively charged EY (Fig 1A). A general equation for the complexation reaction can be written as follows:

$$n\,HEY + MC\text{–}(R_2N)_n \xrightarrow[pH\ 2.2]{} [EY]^-_n\,[MC\text{–}(R_2NH)_n]^{+n} \qquad \text{Eq. 5}$$

ion-pair complex

Where n is the number of tertiary nitrogen groups per MC molecule and HEY is the neutral form of EY.

3.3. Validation study of the analytical method

The guidelines of the ICH Q2(R1) [34] were followed in order to validate the developed method. Regression analysis of the data by the method of least squares shows excellent linear correlation of ΔF and drug concentration over the ranges of 0.1-5.0 and 0.25-8.0 µg/mL (0.12-5.93 and 0.30-9.66 µM) for SPM and JSM, respectively with correlation coefficient of 0.9999. The limits of detection (LOD) and quantification (LOQ) were also calculated based on the standard deviation of y-intercepts of regression lines (S_a) (LOD=3.3 S_a/b and LOQ=10 S_a/b, where b is the slope of the regression line) [34]. The obtained results for these validation criteria are collected in Table 2.

The developed method was found highly specific for the two analytes in presence of matrix components (such as formulation excipients and additives) and oxidative degradants. This specificity is attributed to the dependence of the method on the interaction of the two drugs with EY via a definite function group (tertiary amine), thus any component deprived of this group would not interfere with the method.

The accuracy of the method was also explored by comparing the results of the proposed method with the comparison method [9,18] by Student *t*-test and Variance ratio *F*-test. The calculated values of *t* and *F* were less than the theoretical values which shows no significant difference between the two methods in the accuracy and precision [35] (Table 3).

The repeatability of the method (intra-day precision) was also assessed by triplicate determination of 3 concentrations of each drug covering the linearity range of the developed method in a single day. Further, the intermediate precision (inter-day precision) of the method was also estimated by determination of 3 concentrations of each drug covering the linearity range of the method on 3 different days [34]. As the data in Table 2 demonstrates, the values of SD and relative standard deviation (%RSD) at the two precision levels are small confirming the high precision of the developed method.

Consideration of the method robustness [34] revealed constant ΔF values with minor intentional changes in EY volume (1.0±0.2 mL), buffer volume (1.0±0.1 mL), and pH of the buffer (2.2±0.1). These small deviation that may take place during experimental work has no significant effect on the values of ΔF. The stability of MCs standard solutions was also inspected by storing the solutions at room temperature in glass containers either protected from or exposed to artificial day light. The standard solutions were analyzed over 7 days using a freshly prepared standard solution every time for comparison. The results confirmed the stability of the solutions over the specified time period whether they were exposed or covered from light. This step was also applied to explore the stability of EY reagent solution and its stability was confirmed for about 3 months at room temperature.

These results confirmed the method validity and reliability [34] to be applied for quality control of SPM and JSM.

3.4. Method applications

3.4.1. Stability testing and identification of the oxidative degradation products

A glance to the chemical structures of the two studied MCs showed that they are rich in easily oxidized functional groups such as tertiary amine and aldehyde (Fig. 1A). As a consequence, it is expected that the two studied compounds are likely susceptible to oxidation. Keeping in mind that, the functional group responsible for the interaction of the two MCs with

EY is the tertiary amino group which is well-known by its propensity to oxidation to *N*-oxide on long term storage [36], the author was motivated to experimentally test the ability of the proposed method for indication of the oxidative degradation of SPM and JSM. Thus, the two MCs were exposed to oxidation with H_2O_2 at 20 and 40°C to mimic different climate conditions. It was found that oxidation of the drugs was associated with loss of their ability to interact with EY and subsequent loss of the quenching effect. Such result confirms that the tertiary amine was oxidized. Thus, the developed method is a specific stability-indicating assay method for SPM and JSM in presence of their oxidative degradation products.

The experiments showed that, about 60 and 80% of SPM and JSM, respectively, were degraded after incubation with 0.9% w/v H_2O_2 at room temperature (20 °C) for 10 min only, while a complete degradation of the two compounds took place by incubation with 0.45% H_2O_2 at 40°C for 10 min. The ESI^+-MS spectra of the oxidation reaction mixtures of the two drugs illustrated in Fig 4 revealed the structures of the potential oxidative degradants. As for SPM, the oxidation products include: the *N*-oxide carboxylic acid derivative (SI, *m/z* of 876.2 equivalent to $[M+H]^+$) and the di-*N*-oxide carboxylic acid derivative (SII, *m/z* of 892.4 equivalent to $[M+H]^+$), while for JSM, the sole oxidation product is the *N*-oxide carboxylic acid derivative (J1, *m/z* of 861.8 equivalent to $[M+H]^+$). These structures are very sensible since the tertiary amines and the aldehydes are easily oxidized to amine oxide and carboxylic acid, respectively, by oxidants like H_2O_2 and even by atmospheric oxygen [37]. The obtained results are rationalized and well-matched with the %degradation observed under room temperature where the higher degradation% of JSM (80%) is attributed to oxidation of the single tertiary nitrogen that is capable of reaction with EY while for SPM, one of its two main degradants, S1 contains an intact tertiary nitrogen atoms which is still reactive for EY. On the other hand, at mildly higher temperature (40 °C), complete oxidation was notable for the two drugs within 10 min.

These results reflect the high tendency of the two compounds to oxidation that could necessitate special precautions during manufacturing and storage to guarantee the oxidative stability. This is of a special importance given that traces of reactive peroxides may exist as impurities in the excipients, especially polymeric excipients such as microcrystalline cellulose, povidone, polyethylene glycol, and polysorbate, since peroxide is used during their synthesis to start the polymerization reaction. These peroxides are playing a chief role in drug oxidative

degradation [38]. Since some commercial formulations of the candidate MCs contain one or more of these excipients (e.g. polyethylene glycol 6000 and microcrystalline cellulose) [39], oxidation of the two compounds on storage is very likely. As well, the oxidation by atmospheric oxygen should be carefully considered for some protections.

This is the first report to study and identify the oxidative degradation products of the two MCs which imparts novelty and high importance to the study.

3.4.2. Quality control of pharmaceutical products

The simplicity, rapidness, minimum steps, and high sensitivity of the proposed method qualified it to analyze the two MCs in their formulations in quality control laboratories. Thus to appraise the developed method, it was used for the determination of SPM and JSM in their formulation (Rovamycin® film coated tablets and Josacine tablets, respectively). Percentages recoveries using the proposed method were 98.12±1.72 and 97.22±1.51% (n=3) for SPM and JSM. Statistical comparison of the results of the developed method and the comparison method [9,18] was performed using the Student t-test and the Variance ratio F-test. This shows that the calculated values of *t*- and *F*- were < the tabulated values (Table 3) indicating no significant difference between the two methods regarding accuracy and precision [35].

3.5. Is this a green analytical method?

As a part of the chemistry framework, analytical laboratories has a chief role for protection of the environment and human from hazards and bad consequences of chemical activities. For this purpose, it was important to continuously watch the analytical methods developed for application in analytical and quality control laboratories. Despite the ideal state for a green analytical method is to completely avoid the use of organic solvents, time-consuming derivatization procedures, energy consumption, and waste formation, this cannot be realized in most instances. The green property of the developed method is obvious at first glance by virtue of using water as the ultimate green solvent for dilution, no need to any energy intake, and minimal need of an inherently safe reagent (EY). Yet, in this connection, some metrics are currently available for true evaluation of the greenness of analytical methods instead of dependence on impressions or assumptions, such as the National Environmental Methods Index (NEMI) labeling, analytical eco-scale score [40], and recently the Green Analytical Procedure

Index (GAPI) [41]. Thus, herein an evaluation of the greenness of the developed method is conducted by applying these three tools (Table 4).

The NEMI method is dependent on a circular pictogram divided into four parts each of them is corresponding to a criteria associated with the reagents and wastes properties. The part is colored green up on satisfaction of the corresponding criterion. These criteria include: pH of the method should be ranged from 2 to 12, reagents are not in the persistent, bioaccumulative, toxic (PBT) list [42], reagents are not in the hazardous waste listings [43], and the volume of the waste is not greater that 50 g or mL. The NEMI pictogram for the proposed method, shown in Table 4, revealed that it meets all the requirements to be assigned as "green method" according to this metric. Yet, this evaluation tool is qualitative only since the quantity of reagents or the type of hazards are not included [40].

The analytical eco-scale score approach was then applied to evaluate the proposed method's greenness. This tool is semi-quantitative and depends on calculation of penalty points depending on the volume and type of hazard of the reagents according to the Globally Harmonized System (GHS) for labeling of chemicals, energy intake, occupational hazard, and waste volume and treatment method. The total penalty points are subtracted from a reference value 100 (for the ideal green analytical method) to give the score of the method. For the proposed method, the score is 94 (Table 4) which indicates "excellent greenness" according to Gałuszka et al [40].

Recently, Płotka-Wasylka [41] proposed GAPI as a more quantitative tool to evaluate the greenness of analytical procedures since it combines an eco-scale and a pictogram illustration. GAPI consists of 5 pentagrams which are divided into fields representing different aspects of the analytical method including instrumentation, reagents used, sample preparation, sample collection and preservation (Supplementary material, Table S1). Each field is colored red, yellow, or green referring to low, medium, and high environmental influences, respectively. GAPI has the advantages of giving plentiful information about the evaluated method, also it permits quick judgment of the method greenness by one look at the pictogram. As Table 4 and Table S1(Supporting information) illustrate, the proposed method satisfied most of the criteria of GAPI except fields No 1 and 15 (colored red) which are referring to off-line sampling and waste without treatment, respectively. Field No 10 is colored yellow since the reagents used has moderate health effects according to National Fire Protection Association (NFPA) [41], while field No 14 is

yellow since the waste generated is 10 mL/sample. This outcome is indicating the greenness of the method.

So, the results of the three metric tools are well-matched and approving the greenness of the developed method and its minimal impacts on the environment and human health and safety which adds a surplus merit to the developed method beside its simplicity, rapidness, and sensitivity.

3.6. Comparison of the proposed method and the published literature for SPM and JSM

Evaluation of the proposed method and comparing it with the published methods for analysis of the two cited MCs, with a main focus on the fluorescence-based techniques, showed its strengths and superiority in different aspects (Table 5). In this connection, the proposed method was found 39 times more sensitive than the reported fluorimetric method for JSM [21]. In addition, such method [21] needs a very long time for preparation of quantum dots (about 12 hr). On the other hand, the analytical literature lacks any fluorimetric method for SPM, though there is a HPLC-fluorescence detection method [11] for determination of SPM and JSM depends on a lengthy derivatization reaction with cyclohexa-1,3-dione at 90 °C followed by HPLC separation. Despite the sensitivity of this method is better to some degree than the proposed method (2-5 times), it necessitates about 65 and 70 min for the analysis of SPM and JSM, respectively. In contrary, the proposed method is very time-saving and conducted at room temperature which facilitate its application in quality control and stability testing.

Furthermore, other analytical methods based on detection modes other than fluorescence were also reviewed and compared (Table 5). The proposed method is 10-50 and 3-10 times as sensitive as the reported spectrophotometric [7, 15] and HPLC-UV [9, 10] methods for SPM, respectively. As well, it is 24-447 times more sensitive than the reported electrochemical methods [16] for SPM. Literature survey showed also TLC-bioautography detection methods [5,6] for determination of SPM. Yet, this detection technique is complicated and requires overnight incubation. While, the other TLC method for SPM suffers from narrow linearity range [7]. The sophistication, high costs, limited availability, and experience needed for mass spectrometry methods [8, 12-14] are still main difficulties facing its wide-spread applications despite its sensitivity.

Similarly, the sensitivity of the proposed method for JSM is greater by far than most of the reported methods (Table 5). The proposed method is 20-80 and 40 folds more sensitive than the reported HPLC-UV [17, 18] and spectrophotometry [19] methods for JSM, respectively. The published adsorptive stripping voltammetry method [20] for JSM has a comparable sensitivity to the developed method but many hazards are associated with the use of mercury in this technique.

It is also worthy to mention that, these published methods lack the stability-indicating character while the proposed method is stability-indicating and it is the first method to identify the oxidative degradants of SPM and JSM. To sum up, the advantages and strength points of the proposed method are obvious from the aforementioned discussion. These merits give the proposed method applicability and preference in quality control and stability testing of SPM and JSM.

4. Conclusion

In this study, a newly developed green turn-off fluorescence probe based on EY was presented and validated for the determination of SPM and JSM antibiotics. The interaction of the two MCs with EY resulted in the formation of ground state ion-pair complexes associated with quenching of EY fluorescence via a static mechanism. The thermodynamic study of the reaction showed that it is a spontaneous enthalpy-driven exothermic reaction. The main advantages of the new probe are the simplicity, rapidness, and high sensitivity that allowed the determination of the two MCs at submicromolar concentrations with LODs of 22.5 and 77.3 nM. The developed method is a specific stability-indicating assay allowing the determination of SPM and JSM in presence of their oxidative degradation products. The oxidative degradation products were identified by ESI^+-MS. The developed methods was effectively applied for the determination of SPM and JSM in their tablets with %found of 98.12±1.72 and 97.22±1.51%, respectively. An added advantage of the designed probe is its greenness as confirmed by applying different green analytical chemistry metrics.

Conflict of interest

The author declared no conflict of interest.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] S.C. Sweetman, Martindale: The Complete Drug Reference, 36th ed., Pharmaceutical Press, London, UK, 2009.

[2] Committee for Veterinary Medicinal Products, The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Units, UK, Accessed 13 February 2019 at: https://www.ema.europa.eu/documents/mrl-report/spiramycin-summary-report-2-committee-veterinary-medicinal-products_en.pdf and https://www.ema.europa.eu/documents/mrl-report/josamycin-chicken-summary-report-committee-veterinary-medicinal-products_en.pdf.

[3] The British Pharmacopoeia, Her Majesty's Stationary Office, London, 2013, electronic version.

[4] The Japanese Pharmacopeia, The Ministry of Health, Labor, and Welfare, 16th Ed, pp. 995-998, 1424-1425, 2011.

[5] M.B.M. Ahmed, Y.H.A. Sree, S.M. Abdel-Fattah, N.S. Hassana, M.M. E.-D. Saad, Determination of tylosin, spiramycin, and erythromycin residues in Egyptian buffaloes' meat by thin-layer chromatography-bioautography, J. Planar Chromatogr.-Mod. TLC 26 (2013) 409-416.

[6] U. Vincent, G. Gizzi, C. von Holst, J. De Jong, J. Michard, Validation of an analytical method for the determination of spiramycin, virginiamycin and tylosin in feeding-stuffs by thin-layer chromatography and bio-autography, Food Addit. Contam. 24 (2007) 351-359.

[7] A.O. El Demerdash, S.A. Abdel Razeq, M. M. Fouad, H.F. El Sanabary, Densitometric and UV-spectrophotometric methods for simultaneous determination of spiramycin adipate in binary mixture with oxytetracycline-HCl or tetracycline-HCl, Int. Res. J. Pure Appl. Chem. 17 (2018) 1-21.

[8] M. Voigt, C. Savelsberg, M. Jaeger, Photodegradation of the antibiotic spiramycin studied by high-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry, J. Toxicol. Environm. Chem. 99 (2017) 624-640.

[9] M.M. Elkhoudary, R.A. Abdel Salam, G.M. Hadad, Development and optimization of HPLC analysis of metronidazole, diloxanide, spiramycin and cliquinol in pharmaceutical dosage forms using experimental design, J. Chromatogr. Sci. 54 (2016) 1701–1712.

[10] A. Mahmoudi, Efficient and simple HPLC method for spiramycin determination in urine samples and in pharmaceutical tablets, Sep. Sci. plus 1 (2018) 253–260.

[11] D.B. Gomis, A.I.A. Ferreras, M.D.G. Álvarez, E.A. García, Determination of Spiramycin and Josamycin in Milk By HPLC and Fluorescence Detection, J. Food Sci. 69 (2004) 415-418.

[12] M. Sismotto, J.A.R. Paschoal, J.A. Teles, R.A.E. de Rezende, F.G.R. Reyes, A simple liquid chromatography coupled to quadrupole time of flight mass spectrometry method for macrolide determination in tilapia fillets, J. Food Comp. Anal. 34 (2014) 153-162.

[13] X. Song, T. Zhou, J. Li, M. Zhang, J. Xie, L. He, Determination of Ten Macrolide Drugs in Environmental Water Using Molecularly Imprinted Solid-Phase Extraction Coupled with Liquid Chromatography-Tandem Mass Spectrometry, Molecules 23 (2018) 1172.

[14] X. Song, T. Zhou, J. Li, Y. Su, J. Xie, L. He, Determination of macrolide antibiotics residues in pork using molecularly imprinted dispersive solid-phase extraction coupled with LC-MS/MS, J. Sep. Sci. 41 (2018)1138-1148.

[15] R. El Sheikh, A.A. Gouda, K.M. Khalil, Sensitive and selective spectrophotometric determination of spiramycin in pure form and in pharmaceutical formulations, Int. J. Pharm. Sci. Res. 4 (2013) 2234-2243.

[16] R.M. Youssef, H.M. Maher, Electrochemical study of spiramycin and its determination in pharmaceutical preparation, Drug Test. Anal. 2 (2010) 392-396.

[17] J.J. Nasr, Sh. Shalan, F. Belal, Simultaneous determination of tylosin and josamycin residues in muscles, liver, eggs and milk by MLC with a monolithic column and time-programmed UV detection: application to baby food and formulae, Chem. Cent. J. 8 (2014) 37.

[18] N. Yining, Study on separation and determination of josamycin by HPLC, Chin. J. Pharm. Anal. 13 (1993) 386-388.

[19] N.A. Alarfaj, S.A.A. Razeq, H.M Alsehaly, Determination of josamycin and ciprofloxacin in their pharmaceutical dosage forms by spectrophotometry, Asian J. Chem. 23 (2011) 3362-3366.

[20] A.H. Alghamdi, M.A. Alshadokhy, A.A. Alwarthan, Development of adsorptive stripping voltammetric procedure for the determination of josamycin, a macrolide antibiotic, Jordan J. Chem. 1 (2006) 171-182.

[21] J. Peng, K. Nong, G. Mu, F. Huang, CdTe quantum dots as fluorescent probes for josamycin determination, Bull. Korean Chem. Soc. 32 (2011) 2727- 2731.

[22] N.D. Danielson, P.A. Gallagher, J.J. Bao, Chemical reagents and derivatization procedures in drug analysis, In: R.A. Meyers (Ed.), Encyclopedia of Analytical Chemistry, John Wiley & Sons Ltd, Chichester, 2000, pp.1-34.

[23] S.M. Derayea, D.M. Nagy, Application of a xanthene dye, eosin Y, as spectroscopic probe in chemical and pharmaceutical analysis; a review, Rev. Anal. Chem. 37 (2018) 1-14.

[24] A.C. Moffat, M.D. Osselton, B. Widdop, Clarke's Analysis of Drugs and Poisons, 4th ed., Pharmaceutical Press, UK, 2011, 2064, 2212- 2213.

[25] S. Omura, Macrolide Antibiotics: Chemistry, Biology, and Practice, Academic Press, UK, 2002.

[26] A. M. El-Brashy, M. E.-S. Metwally, F. A. El-Sepai, Il Farmaco 59 (2004) 809

[27] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Third Edition, Springer, NY, 2006.

[28] P. Fita, M. Fedoseeva, E. Vauthey, Ultrafast excited-state dynamics of eosin B: A potential probe of the hydrogen-bonding properties of the environment, J. Phys. Chem. A 115 (2011) 2465–2470.

[29] R. Chang, J.W. Thoman Jr., Physical Chemistry for the Chemical Science, University Science Books, Canada, 2014.

[30] M.A Rashid, S.N.I. Rabbi, T. Sultana, Md.Z. Sultan, Md.Z. Sultan, Pharm. Anal. Acta 2015, 6:8, DOI: 10.4172/2153-2435.1000408

[31] S. Miyamoto, P A Kollman, What determines the strength of noncovalent association of ligands to proteins in aqueous solution?, PNAS 90 (1993) 8402-8406.

[32] C. Tablet, I. Matei, M. Hillebrand, The Determination of the stoichiometry of cyclodextrin inclusion complexes by spectral methods: possibilities and limitations, In: A. Innocenti (Ed.), Stoichiometry and Research- The Importance of Quantity in Biomedicine, Intechopen, UK, 2012, pp. 50-51.

[33] V.R. Batistela, D.S. Pellosi, F.D. de Souza, W.F. da Costa, S.M. de Oliveira Santin, V.R. de Souza, W. Caetano, H.P. M. de Oliveira, I.S. Scarminio, N. Hioka, pK_a determinations of xanthene derivates in aqueous solutions by multivariate analysis applied to UV–Vis spectrophotometric data, Spect. Acta A 79 (2011) 889–897.

[34] ICH, Q2 (R1), Validation of Analytical Procedures: Text and Methodology, International Conference on Harmonization, Geneva https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (Accessed 18 February 2019)

[35] J.N. Miller, J.C. Miller, Statistics and chemometrics for analytical chemistry, 6th edn. Pearson Education Limited, Harlow, England, 2010.

[36] S.W. Baertschi, K.M. Alsante, R.A. Reed, Pharmaceutical Stress Testing: Predicting Drug Degradation, 2nd Ed., Informa Healthcare, UK, 2011.

[37] L. G. Wade, Organic Chemistry, 8th Ed., Pearson, USA, 2013.

[38] Y. Wu, J. Levons, A.S. Narang, K. Raghavan, V.M. Rao, Reactive impurities in excipients: profiling, identification and mitigation of drug–excipient incompatibility, AAPS PharmSciTech. 12 (2011) 1248–1263.

[39] Rovac film coated tablets (Spiramycin), Delta Pharma S.A.E., Egypt http://deltapharma.eg/wp-content/uploads/woocommerce_uploads/2016/07/rovacinsert.pdf (accessed 26 February 2019)

[40] M. Tobiszewski, M. Marć, A. Gałuszka, J. Namieśnik, Green chemistry metrics with special reference to green analytical chemistry, Molecules 20 (2015) 10928-10946.

[41] J. Płotka-Wasylka, A new tool for the evaluation of the analytical procedure: Green Analytical Procedure Index, Talanta 181 (2018) 204-209.

[42] Persistent bioaccumulative toxic (PBT) chemicals covered by the TRI program, United States Environmental Protection Agency https://www.epa.gov/toxics-release-inventory-tri-program/persistent-bioaccumulative-toxic-pbt-chemicals-covered-tri

(accessed 20 February 2019)

[43] Hazardous waste listings, Unites States Environmental Protection Agency, 2012 https://www.epa.gov/sites/production/files/2016-01/documents/hw_listref_sep2012.pdf (accessed 20 February 2019)

Figure legends

Figure 1. (A) Chemical structures of the two MCs and EY, and a schematic illustration of the reaction mechanism and the probe principle, (B) fluorescence spectra of (1,1') EY (0.05 mM) and its reaction products with (2, 2') JSM (6.0 µg/mL), and (3, 3') SPM (5.0 µg/mL) in BRB (pH 2.2), where (1,2,3) are excitation spectra and (1', 2', 3') are the emission spectra, and (C) absorption spectra of EY (0.05 mM) before (1) and after addition of SPM (10.0 µg/mL) (2) and JSM (10.0 µg/mL) (3).

Figure 2. Effects of (A) pH of BRB and (B) volume of EY on the fluorescence quenching by SPM (4.0 µg/mL) and JSM (6.0 µg/mL).

Figure 3. (A) Stern-Volmer plots (at different temperature settings), (B) van't Hoff plots, and (C) Job's plots for SPM and JSM.

Figure 4. The ESI^+-MS spectra and the structures of the oxidative degradation products with the affected functional groups highlighted red for (A) SPM and (B) JSM.

Table 1. A summary of the Stern-Volmer and the thermodynamic parameters for the reaction of the two macrolides with EY

Stern-Volmer parameters				
Drug	Temperature (°K)	Stern-Volmer quenching constant (K_{SV}) $x10^5$ (L/mol)	Correlation coefficient (r)	Bimolecular quenching constant (K_q) $x10^{14}$ (L/mol.S)
Spiramycin	293	1.201	0.99	1.09
	303	0.710	0.96	0.64
	313	0.553	0.96	0.50
Josamycin	293	0.750	0.99	0.68
	303	0.440	0.99	0.40
	313	0.330	0.99	0.30
Thermodynamic parameters				
Drug	The reaction enthalpy change (ΔH°) (kJ mol^{-1})	The reaction entropy change (ΔS°) (J mol-1 K)	Gibb's free energy ΔG° (kJ) (temperature °K)	
Spiramycin	-32.24	-12.76	-28.50 (293)	
			-28.37 (303)	
			-28.25 (313)	
Josamycin	-34.13	-23.05	-27.38 (293)	
			-27.15 (303)	
			-26.92 (303)	

Table 2. Linearity, accuracy, and precision data for the two studied MCs by the developed method

Linearity data		
Parameter	SPM	JSM
Concentration range (μg/mL)	0.1-5.0	0.25-8.0
Limit of detection (LOD) (μg/mL)	0.019	0.064
Limit of quantification (LOQ) (μg/mL)	0.06	0.194
Correlation coefficient (r)	0.9999	0.9999
Slope (b)	50.03	28.79
Intercept (a)	19.54	26.33
Standard deviation of the residual ($S_{y/x}$)	0.48	0.94
Standard deviation of the intercept (S_a)	0.30	0.56
Standard deviation of the slope (S_b)	0.11	0.14
Relative standard deviation (%RSD)	0.71	1.05
Percentage error (%Error)	0.32	0.43

Accuracy and precision data				
Conc. (μg/mL)	Intra-day		Inter-day	
SPM	Accuracy (%Found±SD)	Precision (%RSD)	Accuracy (%Found±SD)	Precision (%RSD)
0.5	99.29±1.63	1.64	100.61±1.39	1.38
3.0	99.84±0.62	0.62	100.16±0.36	0.36
5.0	99.67±0.16	0.16	99.90±0.24	0.24
JSM				
1.0	101.42±2.21	2.18	99.40±0.52	0.52
5.0	99.83±0.38	0.38	100.17±0.39	0.39
8.0	100.13±1.10	1.10	99.23±0.38	0.39

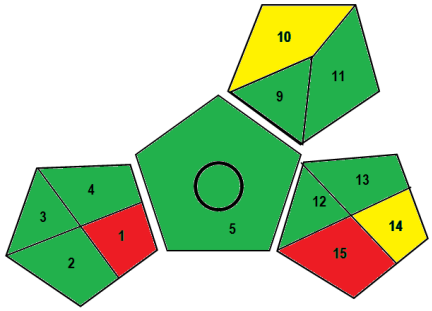
Table 3. Results for the determination of the two MCs in pure form and formulations

Matrix	%Found ± SD		t-test[c]	F-test[c]
	Proposed method[a]	Comparison method[b] [9, 18]		
SPM in pure form	99.85±0.71	100.32±1.02	0.82 (2.37)	2.06 (5.79)
Rovamycin® tablets (1 g SPM/tablet)	98.12±1.72	99.25±1.54	0.85 (2.78)	1.25 (19.00)
JSM in pure form	99.72±1.05	100.2±0.65	0.70 (2.57)	2.61 (19.25)
Josaxin® tablets (500 mg JSM/tablet)	97.22±1.51	98.5±1.65	0.99 (2.78)	1.19 (19.00)

[a]n=5 for SPM pure form, n=6 for JSM pure form, and n=3 for dosage forms. [b]n=3

[c]The values between parenthesis are the tabulated t and F values [35].

Table 4. Results for evaluation of the greenness of the developed method by different green analytical chemistry metric tools

1-NEMI pictogram	2-Green Analytical Procedure Index (GAPI)[a]
	

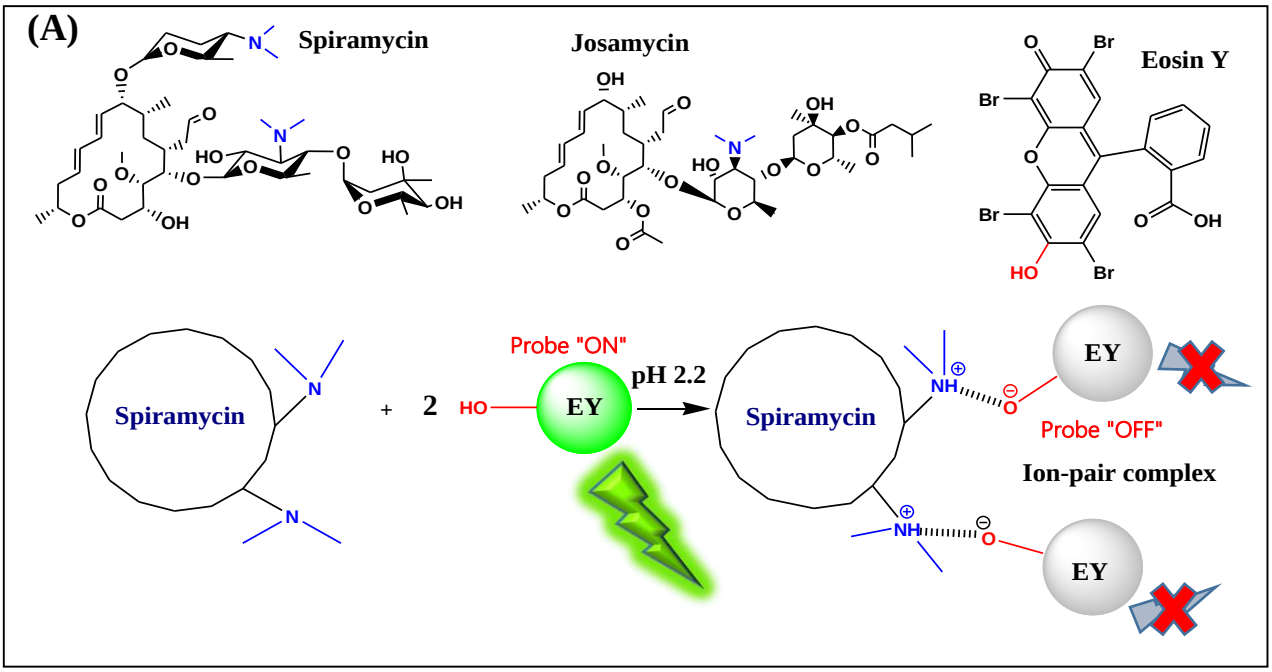
3-Analytical eco-scale score			
Reagents			
Reagent, volume (mL)	**Number of pictograms**	**Word sign**	**Penalty points**
Eosin Y, 1	1	Warning	1
BRB, 1	1	Danger	2
Item			**Penalty points**
Spectrofluorimeter			0
Waste			3
Occupational hazards			0
Total penalty points			Σ 6
Analytical eco-scale score			94

[a]A detailed interpretation of the references to the colors in each field is given in the supplementary material (Table S1).

Table 5. Comparison of the proposed method and the published literature for SPM and JSM

Compound	Method	Linearity range (µg/mL)	LOD (µg/mL)	Remarks	Ref
SPM	-TLC-densitometry	0.1-0.8 µg/spot	ND	-Densitometry detection at 240 nm using a mobile phase of methanol: butanol: chloroform: ammonia (1%) (5:1:1:1, by volume). Narrow linearity range.	7
	-Spectrophotometry	5.0-70.0	ND	-Mean centering, derivative, and ratio derivative methods. 0.1 N HCl was used as a solvent.	
SPM	HPLC-UV	1.0-20.0	0.11	Gradient elution using 0.05 m phosphate buffer and methanol as the mobile phase. UV detection at 242 nm. The run time is about 8 min.	9
SPM	HPLC-UV	0.3-25.0	0.03	ODS column and a mobile phase composed of acetonitrile:2-methyl-2-propanol:0.03 M potas-sium phosphate buffer (pH 6.5), with 1.5% triethylamine(33:7: up to 100, v/v/v) were used. The run time was about 7 min.	10
SPM and JSM	HPLC-Fluorescence detection	0.05-0.5	0.008 for SPM and 0.013 for JSM	Derivatization with cyclohexa-1,3-dione in ammonium acetate buffer (pH 7.0) for 60 min at 90 °C, separation on ODS column at 45 °C using acetonitrile-methanol-phosphate buffer (pH 6.0) (45:5:50, v/v/v) mixture as a mobile phase. Fluorescence detection at $\lambda_{ex}/\lambda_{em}$ 375/450 nm. Run times for SPM and JSM are 5 and 10 min, respectively.	11
SPM	Spectrophotometry	1.0-10.0 2.0-18.0 2-12.0, respectively	0.062 0.287 0.399, respectively	Methods based on derivatization with quinalizarin and alizarin red S (λ_{max} 568 and 527 nm, respectively), and reduction of Fe(III) by SPM in acid medium and subsequent interaction of Fe(II) with ferricyanide to form Prussian blue (λ_{max} 760 nm).	15
SPM	-Differential pulse polarography -Square wave polarography	-20.0-80.0 -0.8-80.0	-8.5 -0.46	The use of mercury (hanging mercury drop electrode) entails toxicity and hazardous effects.	16
JSM	HPLC-UV	5.0-500.0	ND	A micellar mobile phase of 0.17 M sodium dodecyl sulphate, 14% methanol and 0.3% triethylamine in 0.02 M phosphoric acid buffered at pH 4.0, UV detection at 232 nm. Run time is about 10 min.	17
JSM	HPLC-UV	20.0-1000.0	NA	ODS column was used with a mobile phase of methanol—0.02 M KH_2PO_4 (65:35, pH=3.3). The detection wavelength was 232 nm.	18
JSM	Spectrophotometry	10.0-160.0	2.4	Derivatization with 2,4-dinitrophenyl hydrazine and measuring the absorbance of product in HCl/methanol solvent at 411 nm.	19
JSM	Adsorptive stripping voltammetry	0.124-0.2898	0.0196	The use of mercury (hanging mercury drop electrode) entails toxicity and hazardous effects.	20
JSM	Fluorimetry	12.0-120.0	2.5	The Method is based on quenching of the fluorescence of 3-mercaptopropionic acid-capped CdTe quantum dots. Synthesis of quantum dots needs about 12 hr.	21
SPM and JSM	Fluorimetry	0.1-5.0 for SPM and 0.25-8.0 for JSM	0.019 for SPM and 0.064 for JSM	Quenching of EY fluorescence at 548 nm (λ_{ex} 524 nm) at room temperature.	This method

ND: not determined, NA: not available.


(A)
Spiramycin
Josamycin
Eosin Y
Spiramycin
+ 2
HO
EY
Probe "ON"
pH 2.2
Spiramycin
NH
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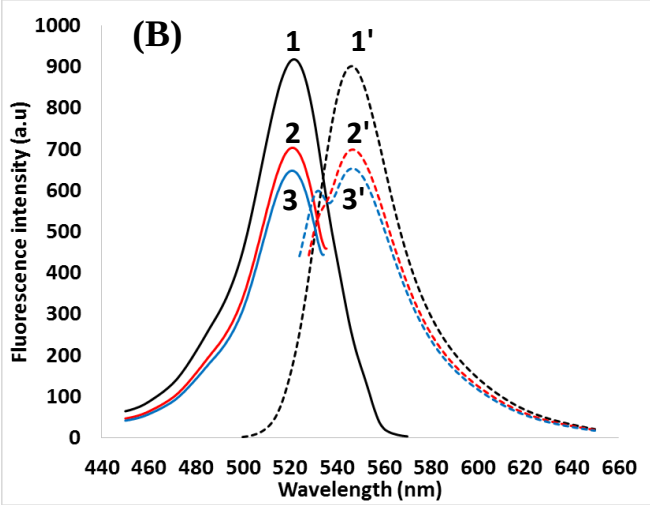
EY
Probe "OFF"
Ion-pair complex
NH
O
EY


(B)
1
1'
2
2'
3
3'
Fluorescence intensity (a.u)
1000
900
800
700
600
500
400
300
200
100
0
440 460 480 500 520 540 560 580 600 620 640 660
Wavelength (nm)

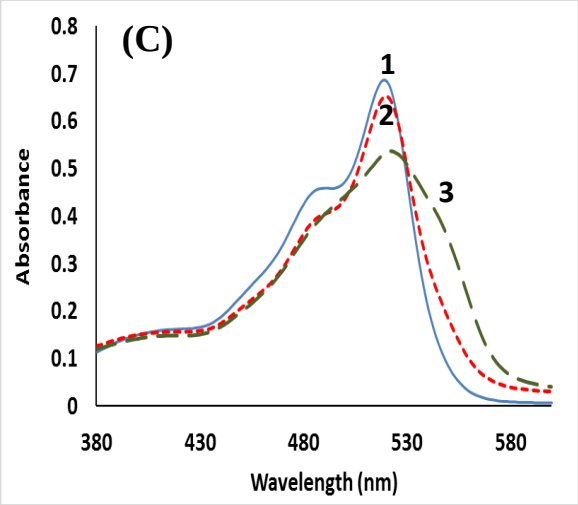

(C)
1
2
3
Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
380 430 480 530 580
Wavelength (nm)

Figure 1

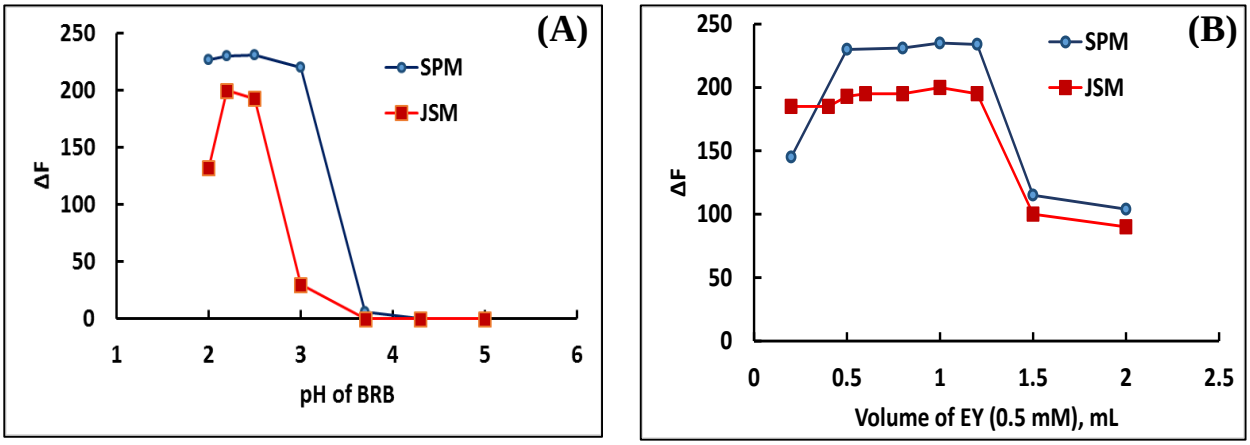

(A)
250
200
150
100
50
0
ΔF
1
2
3
4
5
6
pH of BRB
SPM
JSM
(B)
250
200
150
100
50
0
ΔF
0
0.5
1
1.5
2
2.5
Volume of EY (0.5 mM), mL
SPM
JSM

Figure 2

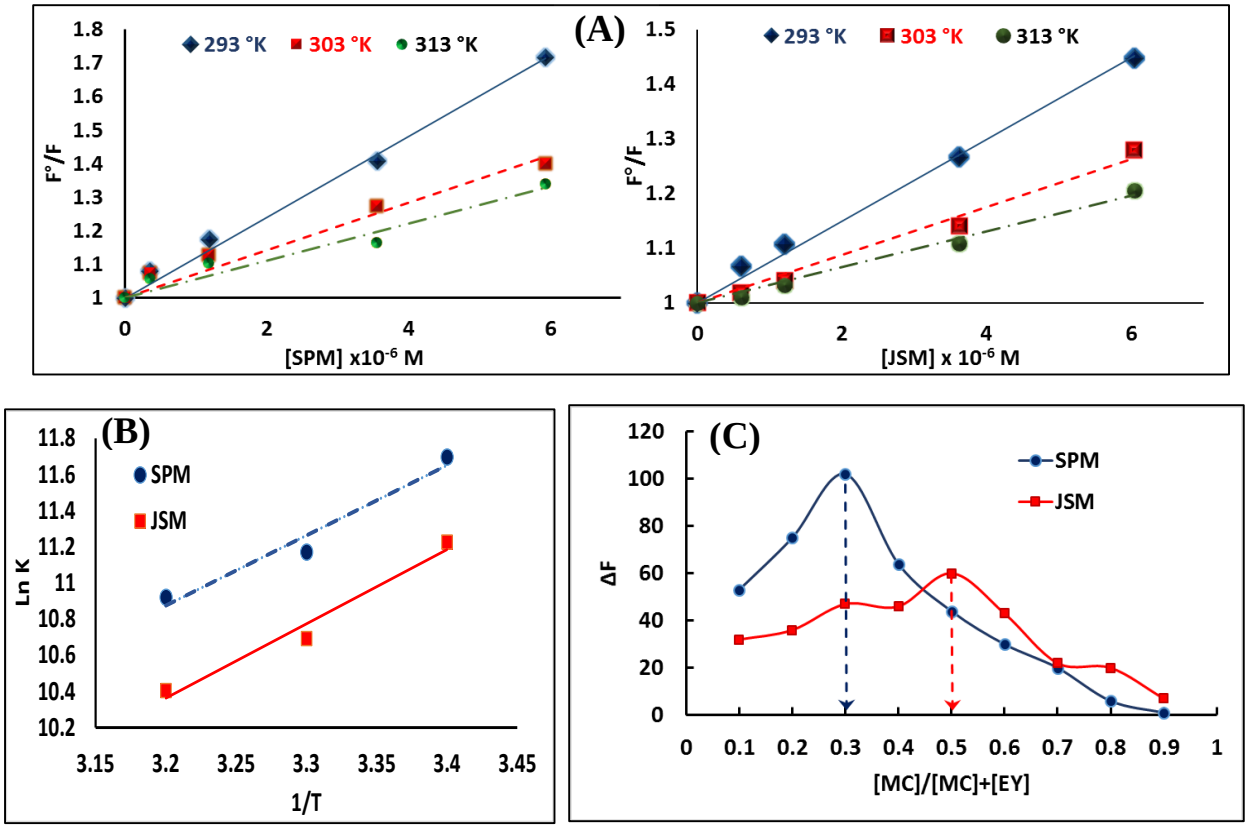

(A)
293 °K
303 °K
313 °K
F°/F
[SPM] x10-6 M
[JSM] x 10-6 M
(B)
SPM
JSM
Ln K
1/T
(C)
SPM
JSM
ΔF
[MC]/[MC]+[EY]

Figure 3

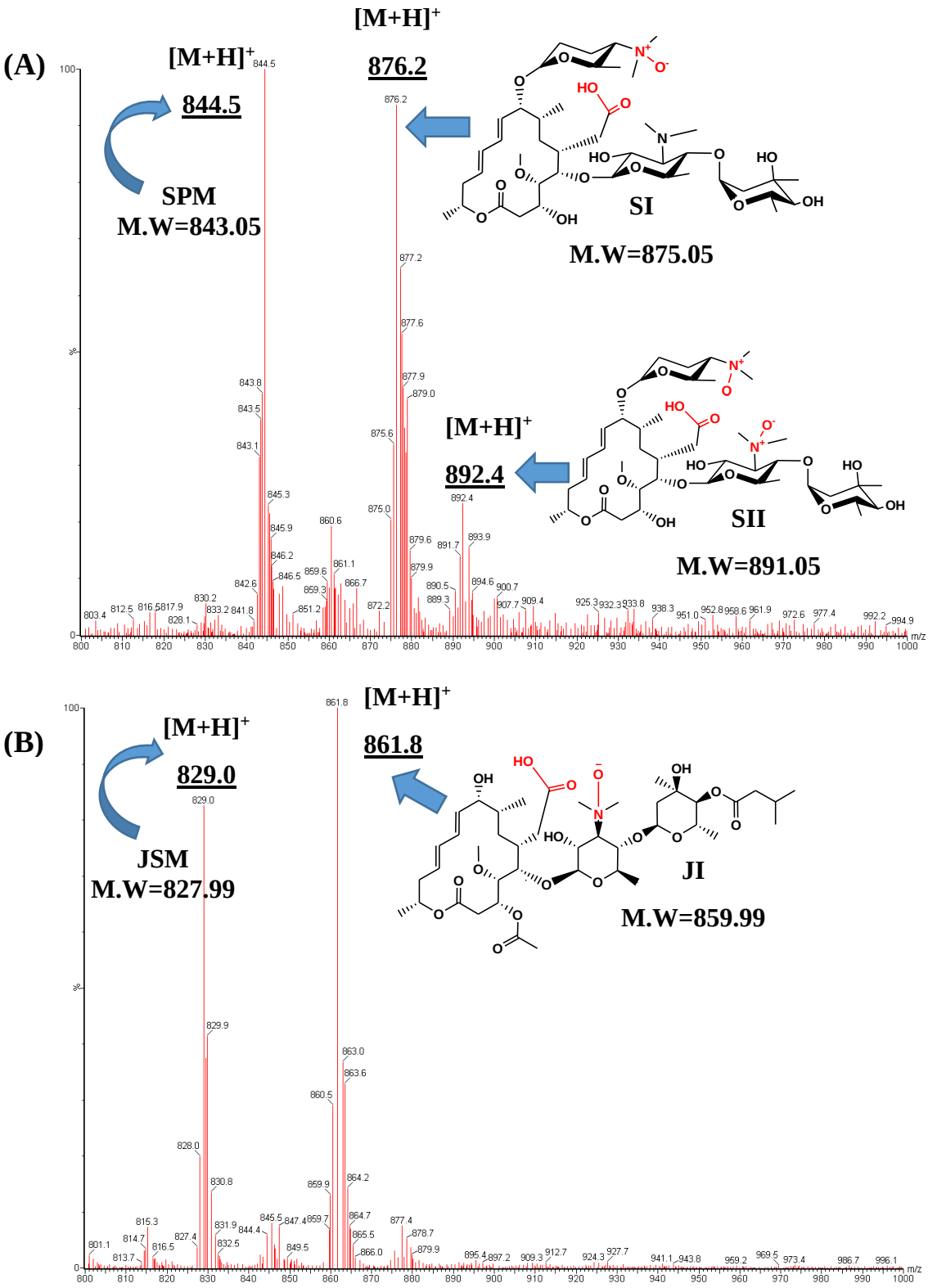

(A)
[M+H]+
844.5
SPM
M.W=843.05
[M+H]+
876.2
SI
M.W=875.05
[M+H]+
892.4
SII
M.W=891.05
(B)
[M+H]+
829.0
JSM
M.W=827.99
[M+H]+
861.8
JI
M.W=859.99

Figure 4