Densitometric and UV-Spectrophotometric Methods for Simultaneous Determination of Spiramycin adipate in Binary Mixture with Oxytetracycline-HCl or Tetracycline-HCl

Asmaa O. El Demerdash[1], Sawsan A. Abdel Razeq[1*], Manal M. Fouad[1,2] and Hoda F. El Sanabary[3]

[1]*Department of Analytical Chemistry, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.*
[2]*Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), Cairo, Egypt.*
[3]*National Organization for Drug Control and Research (NODCAR), Giza, Egypt.*

Authors' contributions

This work was carried out in collaboration between all authors. Author AOED designed the study, performed the statistical analysis and wrote the first draft of the manuscript. Authors SAAR and MMF wrote the protocol and managed the analyses of the study. Author HFES managed the literature searches. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Five methods were developed for simultaneous determination of spiramycin adipate and oxytetracycline-HCl and/or tetracycline-HCl in their pharmaceutical formulations. The first one was a densitometric evaluation of thin-layer chromatograms using a mobile phase of methanol: Butanol: Chloroform: Ammonia 1% (5:1:1:1, by volume). The plates were visualized under UV lamp at 254 nm where spots appeared at R_f 0.76, 0.30 and 0.37 for spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl, respectively. The chromatograms of the drugs were measured densitometrically at 240 nm for spiramycin adipate and at 350 nm for both oxytetracycline-HCl and tetracycline-HCl in the range of 0.1-0.8 µg mL^{-1} and 0.1-1.0 µg mL^{-1}, respectively. Likewise, the simultaneous

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*Corresponding author: E-mail: razeqhegab@yahoo.com;

estimation of the cited drugs was performed by four spectrophotometric methods. Method A was a mean centering (MC) which provided spiramycin adipate determination at 232 nm together with oxytetracycline-HCl or tetracycline-HCl, both at 282, 286, 321, 324, 345 and 364 nm. Method B was a third derivative (^{3}D) through which spiramycin adipate could be estimated at 256 nm while the wavelength 281 nm was selected for oxytetracycline-HCl or tetracycline-HCl determination. Method C was a derivative ratio (^{1}DR) that was established to determine spiramycin adipate only in the presence of oxytetracycline-HCl or tetracycline-HCl at 225.6 and 240 nm. Method D was an induced dual wavelength (IDW) which allowed the determination of oxytetracycline-HCl in presence of spiramycin adipate and up to 50% of its impurity tetracycline. The calibration curves were linear over the concentration range of 5-70 µg mL^{-1} for spiramycin adipate and 5-60 µg mL^{-1} for both oxytetracycline-HCl and tetracycline-HCl. The obtained results were statistically analyzed and found to be in accordance with those given by reported methods. The validity of the methods was evaluated according to ICH guidelines.

1. INTRODUCTION

Spiramycin, is assigned as (4R,5S,6S,7R,9R,10R,16R) - (11E, 13E) -6- [O-2,6-dideoxy-3-C-methyl-a-L-ribo-hexopyranosyl -(1-4)-(3,6-dideoxy- 3-dimethylamino -b-D-glucopyranosyl) oxy]-7- formylmethyl -4-hydroxy-5-methoxy-9,16-dimethyl-10-[(2,3,4,6-tetradeoxy-4-dimethyl amino-D-erythro-hexopyra nosyl)oxy] oxacyclohexadeca -11,13-dien-2-one [1]. It is a product of fermentation by Streptomyces ambofaciens. It occurs naturally as a mixture of three components: spiramycin I, spiramycin II (its 4-O-acetyl derivative) and spiramycin III (its 4-O-propanoyl derivatives) [1,2]. It belongs to a group of macrolide antibiotics and almost exclusively used in veterinary medicine [2].
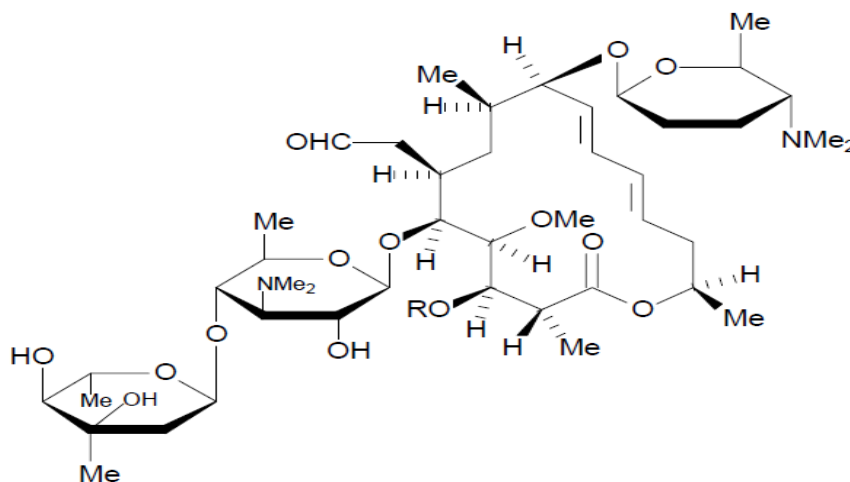
Spiramycin I   R = H
Spiramycin II   R = CO-CH$_3$
Spiramycin III R = CO-CH$_2$-CH$_3$

Tetracycline-HCl, is chemically designated as 2-Naphthacenecarboxamide,4-(dimethyl amino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo monohydrochloride,[4S-(4α,4aα,5aα,6β,12aα)]-4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydr-oxy-6-methyl-1,11- dioxo-2-naphthacenecarboxamide monhydrochloride while oxytetracycline-HCl is 5-hydroxytetracycline monhydrochloride [1,3]. Both acts as antibiotics by inhibiting subsequent binding of amino acyl transfer RNA to ribosomes resulting in termination of peptide chain growth [4].

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Tetracycline-HCl

Oxytetracycline-HCl
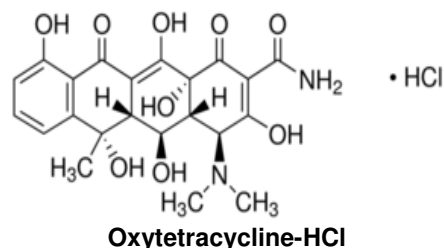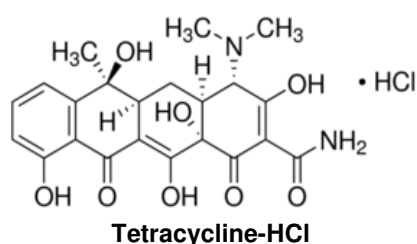
Several analytical methods have been reported for the determination of either spiramycin adipate alone or oxytetracycline-HCl and tetracycline-HCl in their pharmaceutical formulations, in biological fluids and/or in combination with other drugs using spectrophotometric (5-10), electrochemical (11-14), biochemical (15-18) and chromatographic techniques namely, densitometry (19-22), GC (23 and 24), HPLC (25-31) and capillary electrophoresis (32-35). A comprehensive literature search revealed the lack of any technique for the simultaneous determination of spiramycin adipate and oxytetracycline-HCl and/or tetracycline-HCl in their pharmaceutical formulations.

The aim of the present study is to develop simple, sensitive, selective and accurate densitometric and UV-spectrophotometric methods for the simultaneous determination of spiramycin adipate in pharmaceutical formulations together with oxytetracycline-HCl or tetracycline-HCl. Also, this study seeks to determine oxytetracycline-HCl in the presence of one of its impurities; tetracycline [3] in raw material.

2. EXPERIMENTAL

2.1 Instruments

- Densitometer model 3; equipped with WinCats software, Camag TLC scanner 3, Camag lincomat 5 autosampler and microsyring (100 µL).
- UV-Vis spectrophotometer (Shimadzu 1601, Japan).
- Thin layer chromatographic plates pre-coated with silica gel 60 F_{254}, 10x20 cm (Merck, Germany).

2.2 Materials and Reagents

- Pure spiramycin adipate, B. N. 20160308, was kindly supplied by Wux Fortune Pharmaceutical company; Ltd. with purity of 99.6 % as referred by the supplier.

- Pure oxytetracycline-HCl, B.N. YT140402132, was kindly supplied by Pioneer International Trading; Ltd.; 100.1 % purity as referred by the supplier.
- Pure tetracycline-HCl, B.N. DOX-098, was kindly supplied by Pharmachem S.A.; 99.9 % purity as referred by the supplier.
- Birdamycin water soluble powder (W.S.P.); B.N. DBM-001, labeled to contain 65 mg of spiramycin adipate and 100 mg of oxytetracycline-HCl per g, the product of Minnesota Mining and Manufacturing Company, Egypt; was kindly supplied by the company.
- Capimycin water soluble powder (W.S.P.); B.N. 60450, labeled to contain 118 mg of spiramycin adipate and 200 mg of tetracycline-HCl per g, the product of Capital Company, Egypt; was kindly supplied by the company.
- Methanol, HPLC grade (Scharlau chemie, Spain).
- Butan-2-ol, (BDH Chemicals ltd, England).
- Chloroform, HPLC grade (Honil Limited, London, UK).
- Ammonia Solution 33%, (Adwic. Cairo, Egypt).
- HCl, (Sigma Aldrich, Germany); 0.1 N solutions prepared in distilled water.

2.3 Standard Solutions

Standard methanolic solutions (1 mg mL^{-1}) of spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl were prepared to be used in the densitometric method. For the UV-spectrophotometric methods, standard solutions of the three drugs (0.1 mg mL^{-1}) were prepared separately in 0.1 N aqueous HCl.

2.4 Procedures

2.4.1 Linearity

2.4.1.1 Densitometric method

Accurately measured aliquots containing 0.1-0.8 mg spiramycin adipate were transferred from its

standard solution (1 mg mL^{-1}) into a series of 10-mL volumetric flasks; using methanol as a diluent. Into another set of the flasks, aliquots of standard methanolic solutions of oxytetracycline-HCl and tetracycline-HCl (1 mg mL^{-1}) equivalent to 0.1-1.0 mg were carefully transferred then the volume was completed with methanol. Ten µL of each solution were applied to TLC plate pre-coated with silica gel 60 F$_{254}$ (10 × 20 cm) previously impregnated with 5% EDTA solution pH 9.0 and dried in a horizontal position for at least two hours at room temperature. The plates were spotted 2 cm apart from each other and 2 cm apart from the bottom edge. The chromatographic chamber was pre-saturated for 30 minutes with the mobile phase, and then developed by ascending chromatography with methanol: butanol: chloroform: ammonia 1% (5:1:1:1, by volume) through a distance of 7 cm at room temperature. The plates were allowed to dry in air and spots were scanned densitometrically at 240 nm for spiramycin adipate and at 350 nm for oxytetracycline-HCl and tetracycline-HCl. The calibration curve representing the recorded area under the peak against the corresponding drug concentration was constructed and the regression equations were computed.

2.4.1.2 Spectrophotometric methods

Aliquots of the standard solutions (0.1 mg mL^{-1}) in 0.1 N HCl equivalent to 0.05-0.70 mg spiramycin adipate or 0.05-0.60 mg oxytetracycline-HCl or tetracycline-HCl, respectively; were introduced into three separate sets of 10-ml volumetric flasks and the volume was completed to the mark with 0.1 N HCl. The spectra of the prepared standard solutions were scanned over the range of 200 - 400 nm then stored in the computer.

2.4.1.3 Mean centering (MC) method

From the stored data, the spectra of spiramycin adipate were divided by 30 µg mL^{-1} oxytetracycline-HCl or tetracycline-HCl spectrum and the obtained spectra were then mean centered. The amplitude of the obtained mean centered peaks was measured at 232 nm and plotted against the corresponding spiramycin adipate concentrations. The regression equation was deduced. The same procedure was followed for oxytetracycline-HCl or tetracycline-HCl to be quantified using spectrum of 5 µg mL^{-1} spiramycin adipate as a divisor. The calibration curves relating the peak amplitudes at 282, 286,

321, 324, 345 and 364 nm were to oxytetracycline-HCl or tetracycline-HCl corresponding concentrations were plotted and the regression equations were calculated.

2.4.1.4 Third derivative (^{3}D) method

^{3}D spectra of each drug were recorded using Δλ = 8 nm and scaling factor = 10. Calibration curves were deduced by plotting the peak amplitude at 256 nm for spiramycin adipate and at 281 nm for oxytetracycline-HCl or tetracycline-HCl spectra versus their corresponding concentrations and the regression equations were computed.

2.4.1.5 Derivative ratio (^{1}DR) method

Ratio spectra were obtained using a divisor of 30 µg mL^{-1} oxytetracycline-HCl or tetracycline-HCl. Then the first derivative of the ratio spectra (^{1}DR) is calculated using Δλ = 8 nm and scaling factor = one. The first derivative signals at 225.6 and 240 nm was measured and then plotted against its corresponding concentration from which the regression equation was deduced.

2.4.1.6 Induced dual wavelength method (IDW)

Firstly, an equality factor for pure tetracycline-HCl at two selected wavelengths was precisely calculated (F = [A270/A300] = 2.31). In the second step, the zero-order absorbance at 300 nm of the stored oxytetracycline-HCl spectra was multiplied by the studied equality factor (F) of pure tetracycline-HCl. Finally, the absorbance difference (ΔA) of the zero order spectra at 270 nm and 300 nm, after multiplying the later by F, was recorded. A calibration curve was constructed between the obtained absorbance difference (ΔA) versus the corresponding concentration and the regression equation was computed.

2.4.2 <u>Assay of laboratory prepared mixtures of the two drugs</u>

2.4.2.1 Densitometric method

Aliquots from standard spiramycin adipate methanolic solution (1 mg mL^{-1}) equivalent to 0.1-0.7 mg drug were mixed with different volumes of standard oxytetracycline-HCl and tetracycline-HCl (1 mg mL^{-1}) equivalent to 0.1-0.9 mg pure drugs. Each flask was completed to the mark with methanol and the prepared mixtures were analyzed by densitometry as described under "2.4.1. Linearity".

2.4.2.2 Spectrophotometric methods

Different aliquots of standard drugs solutions (0.1 mg mL^{-1}) 0.1 N HCl equivalent to 0.05-0.65 mg spiramycin adipate and 0.05-0.55 mg oxytetracycline-HCl or tetracycline-HCl were introduced into two separate series of 10-mL volumetric flasks. Into a third set containing 0.03 mg spiramycin adipate, aliquots equivalent to 0.01-0.59 mg of oxytetracycline-HCl from its solution were mixed with diverse portions from tetracycline solution equivalent to 0.59-0.01 mg of it. The volumes were completed with 0.1 N HCl and the zero-order spectra of the mixtures were recorded at 200–400 nm first. Then the corresponding manipulating steps for each method were applied as detailed under "2.4.1. Linearity" and the concentration of each drug was calculated from the corresponding regression equation.

2.4.3 <u>Application to pharmaceutical formulations</u>

The contents of five Birdamycin W.S.P. and Capimycin W.S.P. were thoroughly mixed separately. A weight of a fine powder of Birdamycin equivalent to 65 mg spiramycin adipate and 100 mg oxytetracycline-HCl was dissolved in 80 mL methanol. The contents of the flask were sonicated for 10 min to dissolve the active ingredient completely then filtered and the volume was completed with methanol to prepare a clear solution labeled to contain 0.65 mg mL^{-1} spiramycin adipate and 1 mg mL^{-1} oxytetracycline-HCl. Similarly, a weight of a fine powder of Capimycin equivalent to 59 mg spiramycin adipate and 100 mg tetracycline-HCl was treated as previously mentioned to obtain a solution claimed to contain 0.59 mg mL^{-1} spiramycin adipate and 1 mg mL^{-1} tetracycline-HCl. These two sample solutions were analyzed for assay determination by the proposed densitometric method.

For the spectrophotometric methods, an amount of the fine powder of Birdamycin equivalent to 13.0 mg spiramycin adipate and 20 mg oxytetracycline-HCl or of Capimycin equivalent to 11.8 mg spiramycin adipate and 20 mg tetracycline-HCl were dissolved in 100 mL 0.1 N HCl. These prepared solutions claimed to contain 0.130 mg mL^{-1} spiramycin adipate and 0.2 mg mL^{-1} oxytetracycline-HCl or 0.118 mg mL^{-1} spiramycin adipate and 0.2 mg mL^{-1} tetracycline-HCl, respectively.

For each proposed method, the details under "2.4.1. Linearity" were followed and the concentration of each drug was calculated from the corresponding regression equation.

3. RESULTS AND DISCUSSION

3.1 Densitometric Method

Premier studies on the mentioned drugs were performed to achieve satisfactory simultaneous separation. Due to the strong tendency of tetracyclines to form complexes with trace metals in the adsorbents used, the plates were impregnated with a 5% EDTA solution pH 9.0. This was effective to overcome lower separation performance because of spots tailing or being retained on the baseline [21]. Optimization of a mobile phase was carried out using different developing systems with different ratios and the best separation of spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl was achieved using a mobile phase composed of methanol: butanol: chloroform: ammonia 1% (5:1:1:1, by volume). The R$_f$ values were 0.76 for spiramycin adipate and 0.30 and 0.37 for oxytetracycline-HCl and tetracycline-HCl, respectively; when visualized under UV lamp at 254 nm. While the chromatogram of the drugs was measured densitometrically at 240 nm for spiramycin adipate and at 350 nm for both oxytetracycline-HCl and tetracycline-HCl; Figs. 1 and 2.

3.2 Spectrophotometric Methods

Four techniques of great utility for resolving overlapped spectra were established for the simultaneous separation of spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl, Figure 3. Mean centering (MC) and third derivative (^{3}D) methods were used for spiramycin adipate determination with oxytetracycline-HCl or tetracycline-HCl simultaneously in binary mixture. Derivative ratio (^{1}DR) method was performed to detect spiramycin adipate only in the presence of oxytetracycline-HCl or tetracycline-HCl. Induced dual wavelength (IDW) method revealed oxytetracycline-HCl in presence of its impurity tetracycline.

3.2.1 <u>Mean centering (MC) method</u>

It is a simple method based on mean centering of ratio spectra. For the determination of spiramycin adipate, 30 µg mL^{-1} oxytetracycline-HCl or tetracycline-HCl was selected as a divisor and the mean-centering of the ratio spectra was calculated in the wavelength range of 200-400

nm where good linearity was observed at 232 nm. For oxytetracycline-HCl or tetracycline-HCl estimation, the suitable divisor was 5 µg mL^{-1} spiramycin adipate over the wavelength range of 270-370 nm where determination was done at 282, 286, 321, 324, 345 and 364 nm; Figs. 4 and 5.

3.2.2 Third derivative (^{3}D) method

Derivative spectroscopy uses first or higher derivatives of absorbance with respect to wavelength for quantitative analysis. To optimize the derivative order, the first to the third derivative spectra of the solutions containing separately the respective drugs were recorded. Comparing these spectra, it was noticed that although oxytetracycline-HCl and tetracycline-HCl could be measured at 350 nm in the zero order; or at 280 nm or 285 nm in the first and the second derivative spectra without interference from spiramycin adipate as shown in Figs. 3, 6 and 7, respectively; neither the zero-order method nor the first and the second derivative methods present zero crossing points for spiramycin adipate. Consequently, the simultaneous determination is possible by the third derivative because there was a characteristic zone for each compound, so this order of derivative was selected. Spiramycin adipate was estimated at 256 nm at which a linear correlation was observed whereas oxytetracycline-HCl and tetracycline-HCl were detected at 281 nm; Fig. 8.

3.2.3 Derivative ratio (^{1}DR) method

The fundamental parameters affecting the resultant ratio spectrum were examined. Mainly, the effect of a divisor concentration should be tested. Multiple concentrations of oxytetracycline-HCl or tetracycline-HCl were tried where 30 µg mL^{-1} oxytetracycline-HCl or tetracycline-HCl was chosen as a divisor regarding the method selectivity and sensitivity. Effect of delta lambda was also studied; reliable results were obtained using Δλ = 8 nm. Finally, the peak amplitude at 225.6 and the trough amplitude at 240 nm using scaling factor 1 showed good linearity; Fig. 9.

3.2.4 Induced dual wavelength method (IDW)

This method is applied for a binary mixture of two substances with complete overlapped spectra. The basis of this method is the selection of two wavelengths at which the interfering substance exhibits unequal absorbance values. By employing the λ$_{max}$ of the targeted substance together with the equality factor of the interfering substance, the difference in the absorbance values of interfering substance is induced to be zero, whereas the targeted substance shows significant difference in absorbance values [36]. For oxytetracycline-HCl determination, tetracycline should be cancelled at the two selected wavelengths, 270 (λ$_{max}$ of oxytetracycline-HCl) and 300 nm; Figure 3. An equality factor of pure tetracycline at the two chosen wavelengths was calculated (F = [A270/A300] = 2.31). This factor equalizes the absorbance of tetracycline at the two selected wavelengths. The ΔA of the zero order spectra of the mixture at 270 nm and 300 nm, after multiplication of the later by F, was deduced. The calculated absorbance difference (ΔA) is only related to oxytetracycline-HCl, while tetracycline is eliminated. The concentration of oxytetracycline-HCl was derived by substituting the ΔA values in the regression equation.

3.3 Method Validation

The proposed methods were validated according to ICH guidelines [37].

3.3.1 Linearity

A linear correlation was found between the peak areas of the separated spots and the response and the corresponding drug concentration in the range of, 0.1-0.8 µg/spot and 0.1-1.0 µg/spot by densitometric method and 5-70 µg mL^{-1} and 5-60 µg mL^{-1} by spectrophotometric methods for spiramycin adipate and both oxytetracycline-HCl and tetracycline-HCl, respectively. The regression parameters were calculated and presented in Tables 1 and 2.

3.3.2 Accuracy and precision

The accuracy of the developed methods was tested using three concentrations of the cited drugs within the linearity range. The mean recoveries were 98.43-101.37%, 99.76-100.79% and 99.29-100.44% for spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl, respectively. Also, precision was evaluated within the same day and over a period of two months by calculating intraday and interday RSD %. Intraday RSD % was ranged between 0.04-1.33, 0.05-1.65 and 0.23-1.28, while interday RSD % was amounted to be 0.80-1.84, 0.13-1.84 and 0.91-1.68 for spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl, respectively. This

indicated the repeatability and reproducibility of the proposed methods, Tables 1 and 2.

3.3.3 **Selectivity**

It was ascertained by analyzing laboratory prepared mixtures of spiramycin adipate together with oxytetracycline-HCl or tetracycline-HCl in different ratios by the densitometric and the spectrophotometric methods. The developed methods were proved to be effective for the simultaneous separation of spiramycin adipate with oxytetracycline-HCl or tetracycline-HCl as indicated by percentage recoveries range ± SD of 99.35-100.37 ± 0.83-1.86 and 99.81-101.25 ± 0.57-1.67 for spiramycin adipate and oxytetracycline-HCl; or 99.62-101.15 ± 0.43-1.86 and 99.53-101.07 ± 1.03-1.30 for spiramycin adipate and tetracycline-HCl, respectively; Tables 3 and 4. It is noteworthy to mention that the densitometric and the induced dual wavelength methods were found to be valid for detecting oxytetracycline-HCl in the presence of up to 90% and 50% tetracycline, respectively; without interference from spiramycin adipate with mean recoveries of 100.10% ± 1.67 and 100.34% ± 0.95, respectively; Table 3.

The selectivity of the suggested methods was further evaluated by successful analysis of the cited drugs in their pharmaceutical formulations namely, Birdamycin and Capimycin W.S.P. The results presented in Tables 5 and 6; revealed no interference by excipients and additives proving selectivity of the method.

The accuracy of the proposed methods was assured by applying the standard addition technique. As shown in Tables 5 and 6, the mean recoveries justify the accuracy of the method. The obtained results were statistically [38] compared with those obtained from the reported methods which are a UV-spectrophotometric measurement for the first derivative spectra (^{1}D) at 218.3 nm for spiramycin adipate [6] and a vis-spectrophotometric determination of the formed charge transfer complex between p-chloranilic acid and oxytetracycline-HCl or tetracycline-HCl at 540 nm [10].

As shown in Table 3, calculated *t*- and *F*-values were less than theoretical ones, indicating that there was no significant difference between the proposed and the reported methods. However, the proposed methods were found to be more selective. Consequently, they could be successfully employed for the quantitative estimation of spiramycin adipate, oxytetracycline-HCl or tetracycline-HCl in their combined formulations without interference from excipients and additives. Moreover, the suggested densitometric and induced dual wavelength methods were valid for the assay of oxytetracycline-HCl in the presence of trace concentrations down to less than 0.1 ug/ spot and 2 µg mL^{-1} tetracycline, respectively; without interference from spiramycin adipate; proving high selectivity and sensitivity.
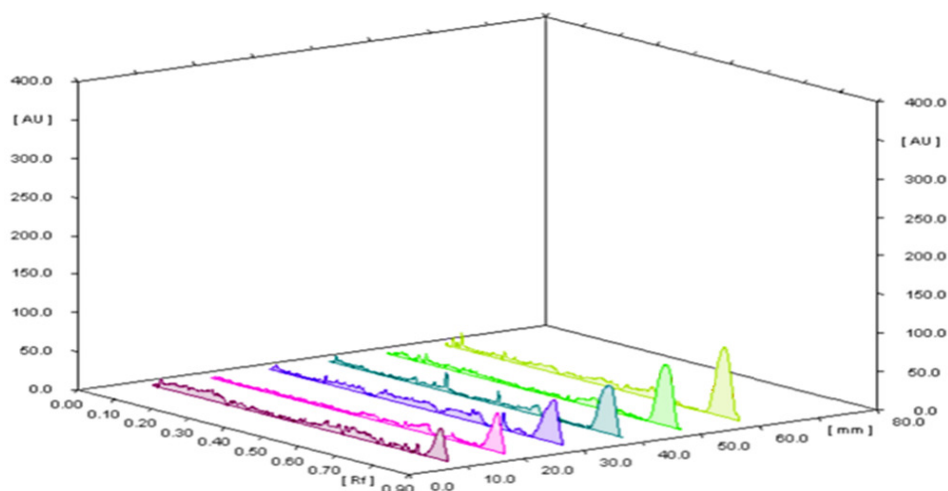

Fig. 1. Densitometric chromatogram of spiramycin adipate (0.1 – 0.8 µg / spot) at 240 nm

Table 1. Regression parameters and assay validation results for the determination of spiramycin adipate and oxytetracycline-HCl by the proposed methods

Parameters	Spiramycin adipate					Oxytetracycline-HCl								
	Densitometry	Spectrophotometric methods				Densitometry	Spectrophotometric methods						Third derivative	Induced dual wavelength
		Mean centering method	Third derivative	First derivative ratio			Mean centering method							
	at 240 nm	at 232 nm	at 256 nm	at 225.6 nm	at 240 nm	at 350	at 282 nm	at 286 nm	at 321 nm	at 324 nm	at 345 nm	at 364 nm	at 281 nm	
linearity range	0.1 – 0.8 µg/spot	5 – 70 µg mL^{-1}				0.1 – 1.0 µg/spot	5 – 60 µg mL^{-1}							
Regression Parameters														
Slope ± S.D.	3521.880 ± 35.2845	0.054 ± 0.0003	0.0002 ± 8.39E-07	0.003 ± 1.34E-05	0.0038 ± 1.75E-05	10589.150 ± 64.9795	196.655 ± 1.7023	157.567 ± 1.4111	141.147 ± 1.6229	145.695 ± 84.7297	185.788 ± 2.3882	178.745 ± 1.9828	0.0003 ± 1.65E-06	0.0028 ± 1.22E-05
Intercept ± S.D.	628.104 ± 18.1638	0.007 ± 0.0136	-0.0001 ± 3.59E-05	9.72E-05 ± 0.0006	-9.68E-05 ± 0.0007	410.042 ± 38.4424	-123.088 ± 60.3190	-98.028 ± 49.9756	-109.112 ± 57.4755	-120.076 ± 60.9001	-167.787 ± 84.5780	-142.365 ± 70.2210	-0.0003 ± 5.85E-05	-0.0015 ± 0.0004
S.D. of residual	21.6072	0.0182	0.0001	0.0008	0.001	50.3329	83.8028	69.4326	79.8523	84.6115	117.5066	97.5601	0.0001	0.0006
Correlation coefficient	0.9996	0.9999	0.9999	0.9999	0.9999	0.9998	0.9997	0.9997	0.9995	0.9994	0.9993	0.9995	0.9999	0.9999
Accuracy (R%± S.D.)	100.66 ± 0.40	100.40 ± 1.98	100.43 ± 0.58	101.37 ± 0.11	100.34 ± 0.53	99.83 ± 0.53	100.32 ± 1.03	100.21 ± 1.17	99.76 ± 1.18	99.78 ± 0.93	99.88 ± 1.48	100.78 ± 1.45	100.79 ± 0.63	100.38 ± 0.46
Precision (RSD%, n=9) *Intraday*	0.88-1.33	0.13 – 0.25	0.58 – 1.15	0.12 – 0.19	0.04 – 0.16	0.83 – 1.65	0.05 – 0.43	0.11 – 0.19	0.12 – 0.69	0.12 – 0.67	0.09 – 0.54	0.05 – 0.44	0.17 – 1.08	0.39 – 0.62
Interday	1.40-1.84	0.81 – 1.49	1.14 – 1.54	0.80 – 1.48	0.97 – 1.15	1.30 – 1.53	0.46 – 1.09	0.61 – 0.98	0.36 – 0.94	0.13 – 0.86	0.45 – 0.80	0.42 – 0.95	1.01 – 1.51	0.74 – 1.84

Table 2. Regression parameters and assay validation results for the determination of spiramycin adipate and tetracycline-HCl by the proposed methods

Parameters	Spiramycin adipate					Tetracycline-HCl							
	Densitometry	Spectrophotometric methods				Densitometry	Spectrophotometric methods						Third derivative
		Mean centering method	Third derivative	First derivative ratio			Mean centering method						
	at 240 nm	at 232 nm	at 256 nm	at 225.6 nm	at 240 nm	at 350 nm	at 282 nm	at 286 nm	at 321 nm	at 324 nm	at 345 nm	at 364 nm	at 282 nm
linearity range	0.1 – 0.8 µg/spot	5 – 70 µg mL^{-1}				0.1 – 1.0 µg/spot	5 – 60 µg mL^{-1}						
Regression Parameters													
Slope ± S.D.	Previously mentioned in table 1	0.0559 ± 0.0003	Previously mentioned in table 1	0.0029 ± 1.37E-05	0.0039 ± 2.34E-05	8754.928 ± 55.6663	194.936 ± 0.7832	151.222 ± 0.5275	120.313 ± 0.6227	123.487 ± 0.5455	158.940 ± 0.8272	166.857 ± 1.1446	0.0003 ± 3.52E-06
Intercept ± S.D.		-0.0007 ± 0.0137		0.0008 ± 0.0006	-0.0004 ± 0.0010	249.940 ± 32.9327	26.709 ± 27.7355	36.709 ± 18.6820	43.215 ± 22.0512	47.312 ± 19.3185	28.276 ± 29.2937	20.685 ± 40.5386	-0.0001 ± 0.0001
S.D. of residual		0.0183		0.0008	0.0013	43.1190	38.5336	25.9554	30.6363	26.8397	40.6986	56.3186	0.0002
Correlation coefficient		0.9999		0.9999	0.9999	0.9998	0.9999	1.0000	0.9999	0.9999	0.9999	0.9998	0.9995
Accuracy (R%± S.D.)		100.00 ± 1.78		100.97 ± 0.88	98.43 ± 0.15	100.44 ± 0.79	100.21 ± 0.13	100.18 ± 0.15	99.29 ± 0.50	99.29 ± 0.57	99.64 ± 0.54	99.66 ± 0.29	100.28 ± 0.51
Precision (RSD%, n=9) **Intraday**		0.02 – 1.08		0. 0.12 – 0.21	0.09 – 0.54	0.61 – 1.28	0.58 – 0.99	0.68 – 1.11	0.56 – 0.87	0.24 – 0.88	0.50 – 0.84	0.69 – 0.96	0.23 – 0.67
Interday		0.75 – 1.48		0.65 – 1.20	0.46 – 0.99	1.20 – 1.55	1.19 – 1.54	0.97 – 1.50	1.02 – 1.68	0.92 – 1.07	0.92 – 1.22	1.11 – 1.47	0.91 – 1.38

Table 3. Determination of spiramycin adipate and oxytetracycline-HCl in mixtures using the proposed methods

Drugs ratio			Densitometry		Drugs ratio		Spectrophotometric methods											Drugs ratio			Induced dual wavelength
			at 240 nm	at 350 nm			Mean centering method							Third derivative		First derivative ratio					
							at 232 nm	at 282 nm	at 286 nm	at 321 nm	at 324 nm	at 345 nm	at 364 nm	at 256 nm	at 281 nm	at 225.6 nm	at 240 nm				
Spiramycin adipate	Tetracycline	Oxytetracycline-HCl	Recovery % of Spiramycin adipate	Recovery % of Oxytetracycline-HCl	Spiramycin adipate	Oxytetracycline-HCl	Recovery % of Spiramycin adipate	Recovery % of Oxytetracycline-HCl						Recovery % of Spiramycin adipate	Recovery % of Oxytetracycline-HCl	Recovery % of Spiramycin adipate		Spiramycin adipate	Tetracycline	Oxytetracycline-HCl	Recovery % of Oxytetracycline-HCl
0.4	0.4	0.4	101.57	100.57	-		-	-	-					-	-	-		30	1	59	100.82
0.4	0.8	0.8	100.91	98.85															3	57	100.93
0.8	0.4	0.4	98.08	100.01															6	54	100.73
0.1	0.1	0.9		100.31	10	10	99.10	101.72	101.85	101.69	101.29	101.36	101.19	98.50	99.33	98.19	99.16		9	51	99.342
0.4	0.2	0.8		99.97	10	20	100.37	100.17	100.40	100.76	100.36	100.43	99.80	99.00	101.50	98.61	99.79		12	48	98.579
0.3	0.3	0.7		100.80	20	10	100.12	101.71	101.20	101.67	101.27	101.88	101.73	100.00	99.67	100.97	101.39		15	45	100.53
0.5	0.5	0.5		101.65	30	30	98.21	101.69	101.62	100.92	100.51	100.12	100.83	98.67	99.00	101.78	101.00		18	42	100.16
0.6	0.7	0.3		100.32	30	60	98.30	100.29	100.66	100.08	99.57	99.40	99.76	99.33	99.89	100.77	100.14		24	36	100.97
0.2	0.9	0.1		98.32	60	30	100.03	101.94	101.50	101.03	100.84	100.91	101.10	100.83	101.67	101.34	100.74		30	30	101.75
Mean% ± SD			100.19 ± 1.86	100.10 ± 1.67	-		99.35 ± 0.95	101.25 ± 0.80	101.20 ± 0.57	101.02 ± 0.60	100.64 ± 0.65	100.68 ± 0.89	100.74 ± 0.80	99.39 ± 0.89	100.18 ± 1.13	100.28 ± 1.50	100.37 ± 0.83	-			100.43 ± 0.95

Table 4. Determination of spiramycin adipate and tetracycline-HCl in mixtures using the proposed methods

Drugs ratio		Densitometric method		Spectrophotometric methods											
				Mean centering method							Third derivative		First derivative ratio	
Spiramycin adipate	Tetracycline-HCl	at 240 nm	at 350 nm	at 232 nm	at 282 nm	at 286 nm	at 321 nm	at 324 nm	at 345 nm	at 364 nm	at 256 nm	at 281 nm	at 225.6 nm	at 240 nm
		Recovery % of Spiramycin adipate	Recovery % of Tetracycline-HCl	Recovery % of Spiramycin adipate	Recovery % of Tetracycline-HCl						Recovery % of Spiramycin adipate	Recovery % of Tetracycline-HCl	Recovery % of Spiramycin adipate	
0.4	0.4	101.57	98.73	-	-						-	-	-	
0.4	0.8	100.91	100.57											
0.8	0.4	98.08	99.50											
10	10	-		101.87	101.68	101.28	100.22	100.56	101.21	101.73	101.50	98.67	99.172	100.41
10	20			99.36	101.84	101.92	100.67	100.75	100.79	101.09	100.50	101.33	100.45	99.2564
20	10			98.83	100.02	99.79	98.10	98.09	98.23	98.59	101.25	99.00	100.12	99.7179
30	30			100.60	101.52	101.30	100.31	100.06	101.58	99.36	101.67	99.11	98.77	98.812
30	60			98.45	100.84	100.65	99.86	99.94	99.75	98.81	100.83	100.17	100.16	99.7009
60	30			100.26	99.18	98.94	98.46	98.25	101.27	100.72	101.17	98.89	100.63	99.8291
Mean% ± SD		100.19 ± 1.86	101.07 ± 1.13	99.89 ± 1.27	100.85 ± 1.06	100.65 ± 1.04	99.60 ± 1.06	99.61 ± 1.16	100.47 ± 1.27	100.05 ± 1.30	101.15 ± 0.43	99.53 ± 1.03	99.88 ± 0.74	99.62 ± 0.54

Table 5. Statistical analysis of determination of spiramycin adipate and oxytetracycline-HCl in their pharmaceutical formulations by the proposed methods in comparison with the reported methods [6 and 10]
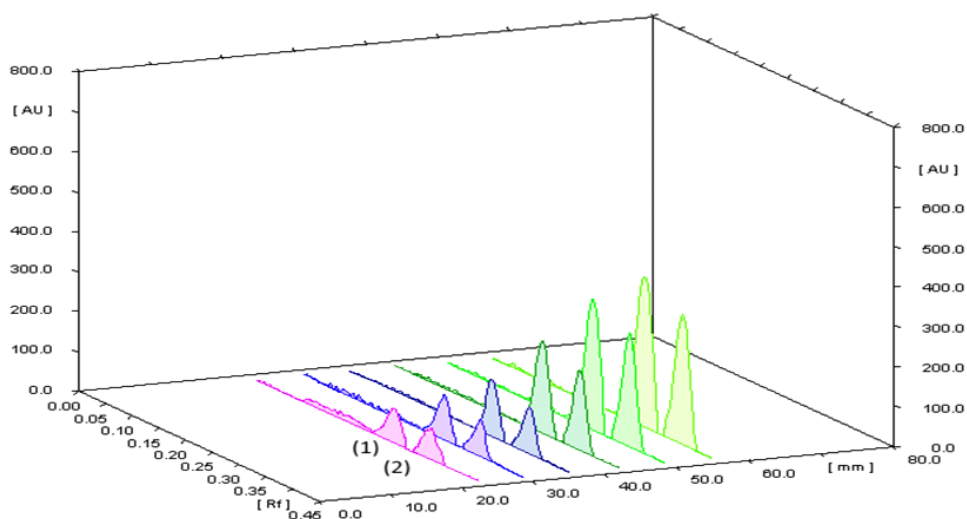
Parameters	Spiramycin adipate						Oxytetracycline-HCl									
	Densitometry	Spectrophotometric methods				Reported method [6]	Densitometry	Spectrophotometric methods								Reported method (10)
		Mean centering method	Third derivative	First derivative ratio		First derivative		Mean centering method						Third derivative	Induced dual wavelength	
	at 240 nm	at 232 nm	at 256 m	at 225.6 nm	at 240 nm	at 218.3 nm	at 350 nm	at 282 nm	at 286 nm	at 321 nm	at 324 nm	At 345 nm	at 364 nm	at 281 nm		at 540 nm
Mean %	100.72	99.54	100.48	100.25	100.96	100.70	100.95	103.93	103.80	103.53	103.17	103.60	103.73	103.90	102.54	102.77
S.D.	0.998	0.829	0.614	0.724	0.576	1.403	1.322	0.897	1.086	1.147	1.022	1.136	1.027	1.076	1.143	1.164
Variance	0.996	0.687	0.377	0.525	0.332	1.970	1.748	0.805	1.179	1.315	1.043	1.291	1.055	1.157	1.306	1.354
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
t – test (2.31)	0.025	1.590	0.316	0.631	0.392	-	2.304	1.776	1.451	1.052	0.586	1.152	1.386	1.602	0.310	-
F – test (6.39)	1.978	2.868	5.226	3.754	5.936	-	1.291	1.683	1.149	1.030	1.298	1.049	1.284	1.170	1.037	-
Standard addition Mean%± S.D.	100.17 ± 1.64	99.64 ± 1.08	100.11 ± 1.49	99.37 ± 0.98	100.29 ± 0.95	-	100.05 ± 1.47	100.88 ± 0.32	101.22 ± 0.35	100.86 ± 0.51	100.43 ± 1.04	100.25 ± 1.13	99.93 ± 1.32	99.94 ± 1.79	101.09 ± 0.76	-

➢ *Ref [6] UV- measurements for the first derivative spectra of spiramycin adipate.*
➢ *Ref [10] Vis-spectrophotometric determination of the formed charge transfer complex between p-chloranilic acid and oxytetracycline-HCl.*

Table 6. Statistical analysis of determination of spiramycin adipate and tetracycline-HCl in their pharmaceutical formulations by the proposed methods in comparison with the reported method [6 and 10]

Parameters	Spiramycin adipate						Tetracycline-HCl								
	Densitometry	Spectrophotometric methods				Reported method[6]	Densitometry	Spectrophotometric methods						Third derivative	Reported method (10)
		Mean centering method	Third derivative	First derivative ratio		First derivative		Mean centering method							
	at 240 nm	at 232 nm	at 256 nm	at 225.6 nm	at 240 nm	at 218.3 nm	at 350 nm	at 282 nm	at 286 nm	at 321 nm	at 324 nm	at 345 nm	at 364 nm	at 281 nm	at 540 nm
Mean %	100.75	102.42	102.43	102.26	101.72	100.70	100.89	103.10	103.21	102.54	103.75	103.47	103.32	102.52	101.97
S.D.	0.629	0.926	1.118	1.353	1.540	1.403	0.984	1.206	0.899	1.285	1.366	0.990	0.881	0.988	1.980
Variance	0.395	0.858	1.251	1.832	2.371	1.970	0.968	1.455	0.808	1.652	1.865	0.980	0.777	0.976	3.920
N	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
t – test (2.31)	0.073	2.291	2.159	1.795	1.096	-	1.100	1.089	1.272	0.540	1.650	1.507	1.390	0.552	-
F – test (6.29)	4.984	2.297	1.575	1.075	1.204	-	4.049	2.694	4.853	2.373	2.103	4.002	5.047	4.019	-
Standard addition Mean%± S.D.	100.54 ± 0.92	100.53 ± 1.70	99.75 ± 1.16	100.03 ± 1.08	98.99 ± 0.68	-	99.06 ± 1.00	100.08 ± 0.73	99.80 ± 1.53	100.29 ± 1.30	100.03 ± 1.21	100.17± 1.12	100.11 ± 0.84	99.29 ± 0.92	-

➢ *Ref [6] UV- measurements for the first derivative spectra of spiramycin adipate.*
➢ *Ref [10] Vis-spectrophotometric determination of the formed charge transfer complex between p-chloranilic acid and tetracycline-HCl.*

Fig. 2. Densitometric chromatogram of (1) oxytetracycline-HCl
(0.1 - 1 μg / spot) and (2) tetracycline-HCl (0.1 - 1 μg / spot) at 350 nm
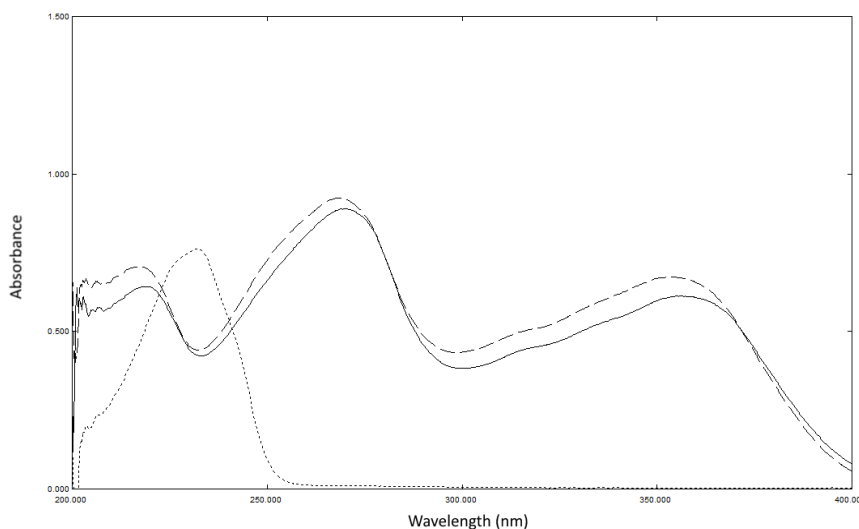
**Fig. 3. Absorption spectra of spiramycin adipate 30 μg mL^{-1} (…),**
**oxytetracycline-HCl 30 μg mL^{-1} (– – –) and tetracycline-HCl 30 μg mL^{-1} (—) in 0.1 N HCl**

3.3.4 <u>Stability of standard solutions</u>

The stability of the spiramycin adipate, oxytetracycline-HCl and tetracycline-HCl solutions (1 mg mL^{-1}) in methanol or (0.1 mg mL^{-1}) in 0.1 N HCl were evaluated by the densitometric and spectrophotometric methods, respectively. This was investigated through storing on laboratory bench and in the refrigerator at 4°C. The spiramycin adipate solutions were found to be stable for one week at room temperature and two weeks in refrigerator. While the methanolic solutions (1 mg mL^{-1}) of oxytetracycline-HCl and tetracycline-HCl were found to be stable for one day at room temperature or one week in refrigerator, whereas the stability of the standard solutions of both drugs in 0.1 N HCl was one week at room temperature or two weeks in refrigerator.

14
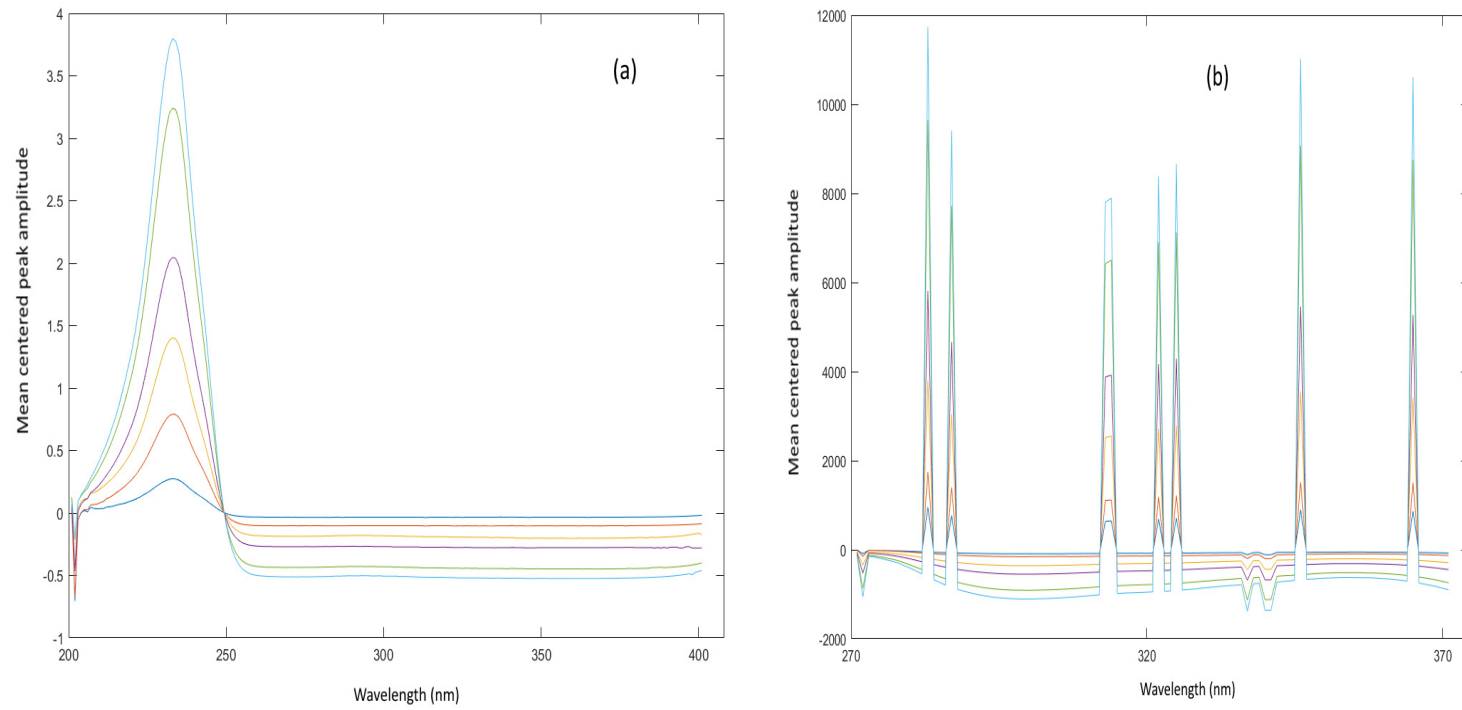
Fig. 4. Mean centered ratio spectra of (a) spiramycin adipate (5-70 µg mL^{-1})
using 30 µg mL^{-1} oxytetracycline-HCl as a devisor and (b) oxytetracycline-HCl (5-60 µg mL^{-1}) using 5 µg mL^{-1} spiramycin adipate as a devisor
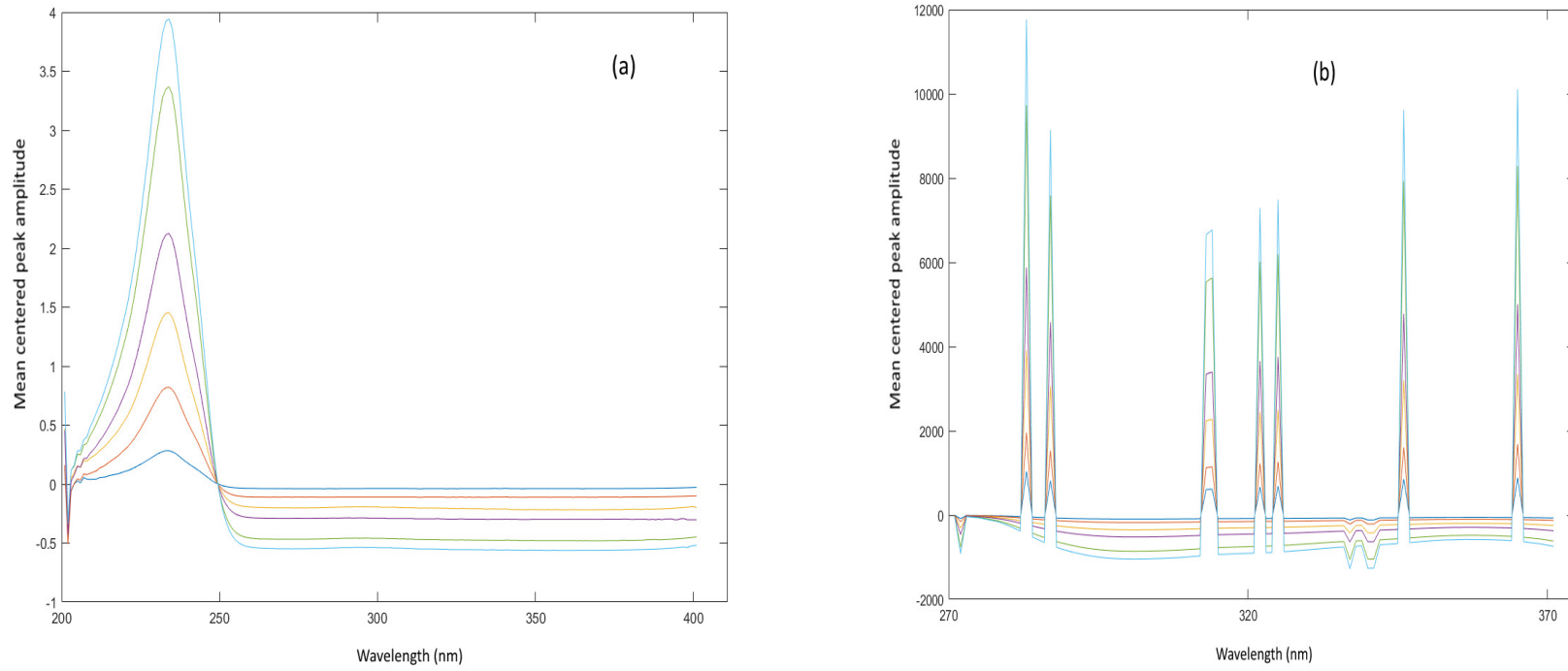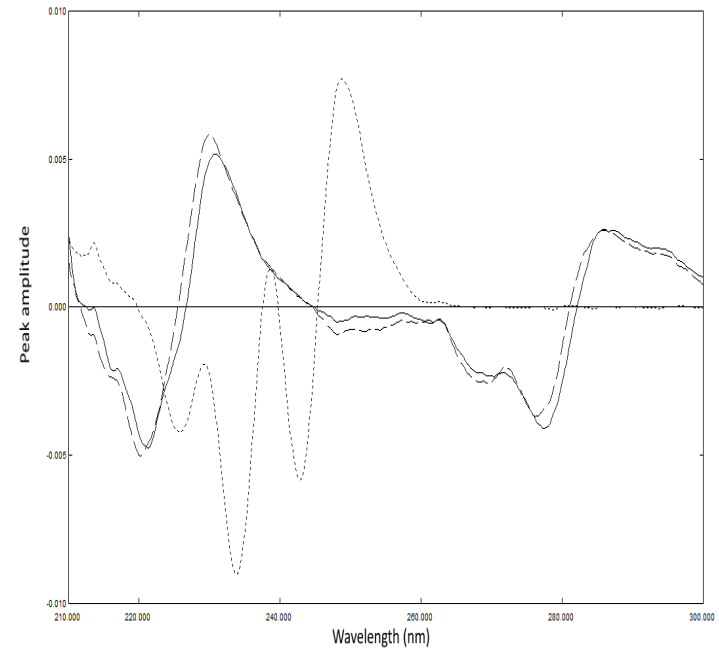
**Fig. 5. Mean centered ratio spectra of (a) spiramycin adipate (5-70 µg mL^{-1}) using 30 µg mL^{-1} tetracycline as a devisor and (b) tetracycline-HCl (5-60 µg mL^{-1}) using 5 µg mL^{-1} spiramycin adipate as a devisor**
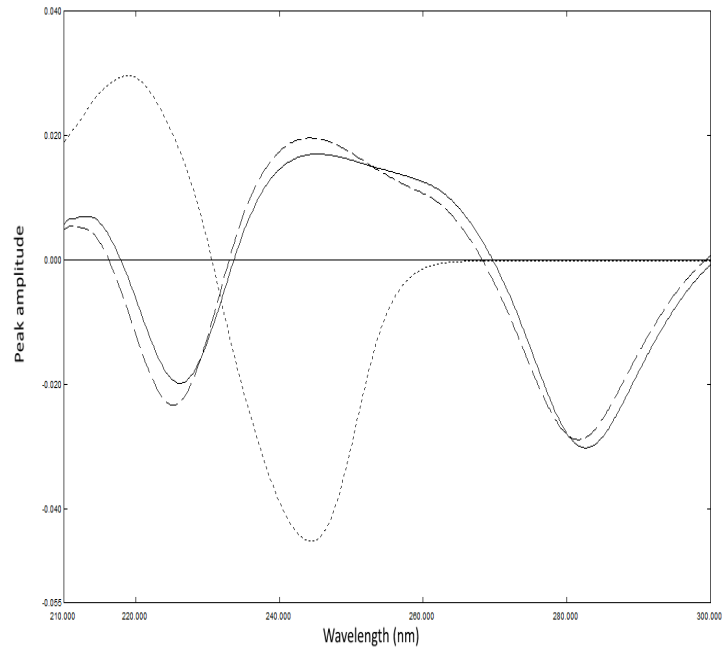
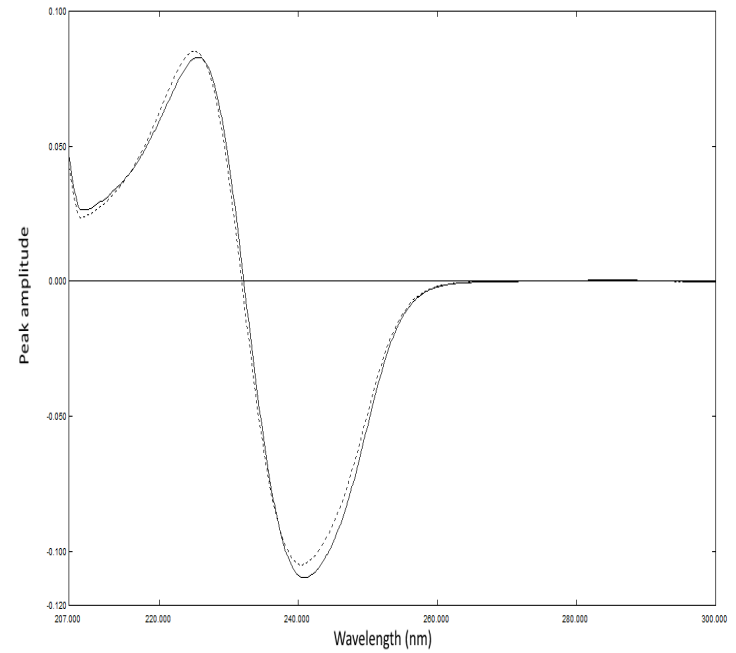**Fig. 6. First derivative spectra of spiramycin adipate 30 µg mL^{-1} (…), oxytetracycline-HCl 30 µg mL^{-1} (– – –) and tetracycline-HCl 30 µg mL^{-1} (—).**

Fig. 7. Second derivative spectra of spiramycin adipate30 µg mL^{-1} (…), oxytetracycline-HCl 30 µg mL^{-1} (– – –) and tetracycline-HCl 30 µg mL^{-1} (—).
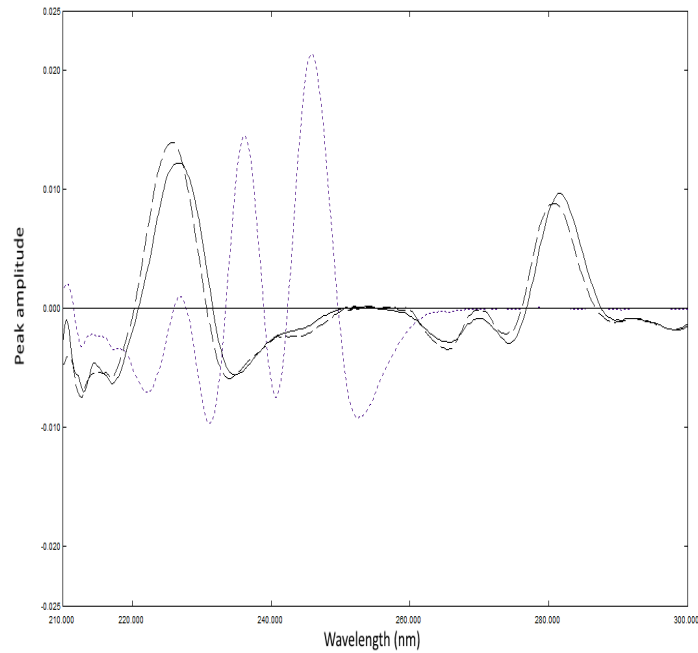
Fig. 8. Third derivative spectra of spiramycin adipate 30 µg mL^{-1} (…), oxytetracycline-HCl 30 µg mL^{-1} (– – –) and tetracycline-HCl 30 µg mL^{-1} (—).

Fig. 9. First derivative of the ratio spectra of spiramycin adipate 30 µg mL^{-1} using 30 µg mL^{-1} oxytetracycline-HCl (—) or tetracycline-HCl (---) as a devisor.

4. CONCLUSION

Simple, sensitive, accurate, precise and rapid assay methods were adopted for the simultaneous determination of spiramycin adipate and oxytetracycline-HCl or tetracycline-HCl either in their bulk powders or in pharmaceutical formulations. Both densitometry and induced dual wavelength methods were effective for assay and purity testing of oxytetracycline-HCl which represent an advantage over the previously published methods. The established methods could be successfully employed for the routine analysis of the studied drugs in quality control laboratories.

5. FUTURE RESEARCH RECOMMENDA-TIONS

New methods like HPLC could also applied.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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