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Der Pharma Chemica, 2014, 6(2):111-121  
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ISSN 0975-413X  
CODEN (USA): PCHHAX

## Spectrophotometric and chromatographic methods for the simultaneous determination of rutin and ascorbic acid in their pharmaceutical formulation

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### ABSTRACT

Four different methods namely, first-derivative of ratio spectra, bivariate, thin layer chromatography and high performance liquid chromatography were used to determine rutin and ascorbic acid simultaneously in their pharmaceutical dosage form. The derivative ratio spectra method was based on measuring the peak amplitudes for ascorbic acid at 275 and 286 nm using  $20.0 \mu\text{g}\cdot\text{mL}^{-1}$  rutin as a divisor over a concentration range of 1.0 – 10.0  $\mu\text{g}\cdot\text{mL}^{-1}$  for ascorbic acid, while rutin can be measured directly at 363 nm without interference from ascorbic acid over a concentration range of 4.0-20.0  $\mu\text{g}\cdot\text{mL}^{-1}$  for rutin. Bivariate method is used for simultaneous determination of both drugs over a concentration range of 2.0 – 12.0 and 2.0 – 16.0  $\mu\text{g}\cdot\text{mL}^{-1}$  for ascorbic acid and rutin, respectively. The method was based on measuring the absorbance at the selected wavelengths. A TLC separation with densitometric detection of both drugs was achieved using ethyl acetate: methyl isobutyl ketone: formic acid: glacial acetic acid: water [20:12:2.8:1.2:20, v/v/v/v/v] as developing solvent. The method allowed determination of rutin and ascorbic acid in concentration ranges of 2.0-6.0 and 4.0-8.0  $\mu\text{g}\cdot\text{spot}^{-1}$ , respectively. Furthermore, a high performance liquid chromatographic procedure with ultraviolet detection at 270 nm was developed for the separation and determination of the studied drugs using a C8 column over a concentration range of 5.0-40.0 and 0.5 – 10.0  $\mu\text{g}\cdot\text{mL}^{-1}$  for rutin and ascorbic acid, respectively. The mobile phase is composed of ammonium acetate buffer: acetonitrile [80: 20, v/v], pH was adjusted to 6.3 by orthophosphoric acid. The proposed methods were successfully applied for the determination of the studied drugs in their mixtures and in pharmaceutical formulations containing them.

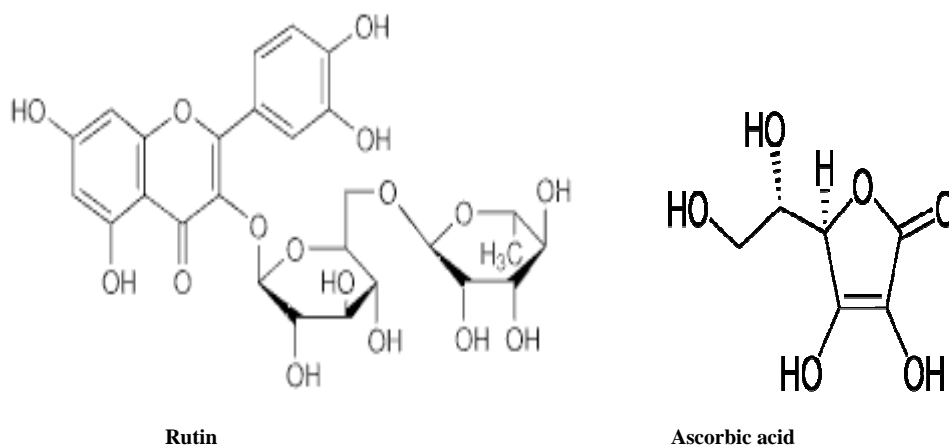
**Keywords:** Ratio spectra, Bivariate, TLC, HPLC, Rutin, Ascorbic acid

### INTRODUCTION

Rutin (RU, Fig.1) is chemically known as (quercetin-3-O-(6-O-rhamnosid) glucoside) [1]. It is a well known and widely occurring flavonoid. It is present in many foods, including buckwheat, onion, apple, tea, and red wine. It is highly consumed not only in food, but also due to its pharmacological properties. Studies have shown that RU scavenges free radicals, suppresses cellular immunity, has an antioxidant and anti-inflammatory effects, as well as anti-carcinogenic and antimicrobial potential, and even antihypertensive and as an adjuvant for type 2 diabetes treatment [2-7]. RU has been used in the treatment of peripheral vascular diseases, because of its vascular-protective property e.g. acute attack of piles, metrorrhagias, circulatory disturbances and capillary fragility disorders [3].

Vitamin C (ascorbic acid) (AA, Fig. 1) is chemically known as (5-methyl-2-oxo-1, 3-dioxolen-4-yl) methoxy-4-(1-hydroxy-1-methylethyl)-2-propyl-1-{4-[2-(tetrazol-5-yl) phenyl] phenyl} methyl-imidazol-5-carboxylate [1]. It is an essential vitamin for humans. Animals can make their own AA, but people must get this vitamin from food and other sources. Good sources of AA are fresh fruits and vegetables, especially citrus fruits. Its role as an endogenous antioxidant is well recognized. Historically, AA was used for preventing and treating scurvy. These days, AA is used most often for preventing and treating the common cold [8]. Moreover, supplementation of AA has been verified as an effective

therapy for the treatment of certain respiratory diseases, including allergic rhinitis [9] and chronic rhinosinusitis [10]. RU has been marketed in combination with AA in tablet dosage form (RUTA C 60® tablets). The combination of RU and AA is intended for oral administration for altering the increased fragility and permeability of capillaries.



**Fig. 1: Chemical structures of Ascorbic acid (AA) and Rutin (RU)**

Various methods for the individual determination of each drug were described like [11-14] for AA and [15-17] for RU. Only few methods for determining the active compounds in mixtures were reported. The simultaneous determination of RU and AA in their combined dosage forms has been achieved by UV-spectrophotometry [18-19] electrochemical method [20], voltammetry [21], chemiluminescence [22], capillary electrophoresis [23-26] and HPLC [27-29] and NIR FTIR [30].

## MATERIALS AND METHODS

### Instruments

A dual-beam UV-visible spectrophotometer [Shimadzu, Japan] model UV-1601 PC, with 1cm quartz cells, connected to an IBM compatible computer was used. Bundled, UV-PC personal spectroscopy software version 2.21 was used to process the absorption and the derivative spectra. The spectral bandwidth was 2nm with wavelength-scanning speed of 2800 nm min<sup>-1</sup>.

TLC plates [20 cm x 10 cm, 0.25 mm] coated with silica gel 60 F254 [Merck, Germany] were used.

Shimadzu TLC scanning densitometer model CS 9301PC [Kyoto, Japan] with Hamilton® micro syringe [100 µL] were used.

The chromatographic apparatus, a Shimadzu instrument, Model LC-10 AD VP, equipped with a variable wavelength UV-visible detector, Model SPD-10 AD VP, Degasser Model DGU-12 A and a 20-µl volume Rheodyne injector. The separation was performed on Zorbax C-8 (5µm, 150mm x 4.6 mm I.D) column. The samples were injected by the aid of a 100 µL Hamilton® analytical syringe.

### Materials

#### Samples

Reference RU and AA standards were kindly supplied by Kahira Pharmaceutical & Chemical Industries Company, Cairo, Egypt. The purity of RU was found to be 99.40 ± 0.76 (n=6), while that of AA was found to be 99.75 ± 0.63 (n=6) according to their reference methods [27, 31], respectively.

Pharmaceutical dosage form Ruta C® 60 tablets, batch No. 01159 labeled to contain 60 mg RU and 160 mg AA expressed as base per tablet, respectively are produced by Kahira Pharmaceutical & Chemical Industries Company, Cairo, Egypt.

### Reagents

All chemicals and reagents were of pure analytical grade.

-Methanol for spectroscopy work was obtained from El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt.

- For TLC work, glacial acetic acid and ethyl acetate were obtained from SDFCL, India. Methanol (HPLC grade), formic acid (HPLC grade) and methyl isobutyl ketone were purchased from E-Merck, Darmstadt, Germany.

-For HPLC work, de-ionized water, acetonitrile and o-phosphoric acid (E-Merck, Darmstadt, Germany) were of HPLC grade. Ammonium acetate was obtained from El-Nasr Pharmaceutical Chemicals Co., Cairo, Egypt.

Ammonium acetate buffer 0.065 M was freshly prepared by dissolving 5.0 g ammonium acetate in 1 L de-ionized water and adjusting pH to 6.3 by o-phosphoric acid.

### Standard solutions

Stock standard solutions of RU and AA [ $0.2 \text{ mg.mL}^{-1}$ ] in methanol were prepared for the spectroscopic methods and TLC chromatographic method. Stock standard solutions of [ $0.1 \text{ mg.mL}^{-1}$ ] of RU and AA were prepared in the mobile phase for HPLC method. All solutions were freshly prepared on the day of analysis.

### Procedures

#### Spectroscopic Methods

##### Derivative ratio spectrophotometric method

Aliquots from standard stock solutions of RU and AA were transferred into a series of 10-mL volumetric flasks. The volume was completed with methanol to prepare solutions in concentration ranges of  $1.0$ – $10.0 \text{ }\mu\text{g.mL}^{-1}$  for AA and  $4.0$ – $20.0 \text{ }\mu\text{g.mL}^{-1}$  for RU. The spectra of AA prepared solutions were scanned from 200 nm to 400 nm and stored in the computer. The stored spectra of AA were divided (amplitude at each wavelength) by the spectrum of  $20.0 \mu\text{g.mL}^{-1}$  of RU. The first derivative of the ratio spectra ( ${}^1\text{DD}$ ) with  $\Delta\lambda = 4 \text{ nm}$  and a scaling factor = 10 was obtained. The amplitudes of the first derivative peaks of AA were measured at 275 nm and 286 nm. Calibration graphs were constructed relating the peak amplitudes of ( ${}^1\text{DD}$ ) to the corresponding concentrations of AA. The spectra of RU were scanned from 200 nm to 400 nm and stored. The absorbance values ( ${}^0\text{D}$ ) of RU were measured at 363 nm without any interference from AA. Calibration graphs were constructed relating the absorbance values ( ${}^0\text{D}$ ) to the corresponding concentrations of RU. The regression equations were then computed at the specified wavelengths and used for determination of unknown samples of AA and RU.

##### Bivariate method

Several dilutions of the two drugs were made from the stock solutions and were used for the bivariate calibration. Spectra of the obtained solutions were recorded and stored into the computer. The regression equations were computed at 260 nm and 278 nm. The concentrations of RU and AA were calculated using the parameters of the linear regression functions evaluated individually for each component at the same wavelength and substituting in the following equations:

$$C_{\text{RU}} = m_{\text{A}2}(A_{\text{AB}1} - e_{\text{A}B1}) + m_{\text{A}1}(e_{\text{A}B2} - A_{\text{AB}2}) / m_{\text{A}2} m_{\text{B}1} - m_{\text{A}1} m_{\text{B}2}$$

$$C_{\text{AA}} = A_{\text{AB}1} - e_{\text{A}B1} - m_{\text{B}1} C_{\text{RU}} / m_{\text{A}1}$$

Where,  $A_{\text{AB}1}$  and  $A_{\text{AB}2}$  are the absorbance of A and B at 260 nm and 278 nm, respectively,  $e_{\text{A}B1}$  and  $e_{\text{A}B2}$  the sum of the intercepts of the linear calibration at the two wavelengths ( $e_{\text{A}B1} = e_{\text{A}1} + e_{\text{B}1}$ ),  $m_{\text{A}}$  and  $m_{\text{B}}$  are the slopes of the linear regressions and C is the concentrations [ $\mu\text{g.mL}^{-1}$ ]. The accuracy of the results was checked by applying the proposed bivariate method for determination of different samples of pure RU and AA. The concentrations were obtained from the corresponding regression equations from which percentage recoveries were calculated.

### Chromatographic Methods

#### TLC-Densitometric method

Aliquots equivalent to  $2.0$ – $6.0 \mu\text{g.spot}^{-1}$  of RU standard solution and  $4.0$ – $8.0 \mu\text{g.spot}^{-1}$  of AA standard solution [each,  $0.2 \text{ mg.mL}^{-1}$ ] were applied in the form of bands on TLC plates. The band length was 4 mm and dosage speed was  $150 \text{ nL S}^{-1}$ , the bands were applied 12.8 mm apart from each other and 15 mm from the bottom edge of the plate. Linear ascending development was performed in a chromatographic tank previously saturated with ethyl acetate: methyl isobutyl ketone: formic acid: glacial acetic acid; water [20:12:2.8:1.2:20, v/v/v/v/v] for 30 minutes at room temperature. The developed plates were air-

dried and scanned at 276 and 370 nm for AA and RU, respectively, using deuterium lamp, absorbance mode at 3 mm x 0.45 mm slit dimension and scanning speed of 20 mm S<sup>-1</sup>. Calibration curves relating the optical density of each spot to the corresponding concentration of RU and AA were constructed. The regression equations were then computed for the studied drugs and used for determination of unknown samples.

#### HPLC method

Aliquots from stock standard solutions [0.1mg.mL<sup>-1</sup>] of RU and AA were transferred into a series of 10-mL volumetric flasks. The contents of each flask were completed with the mobile phase to volume to get a concentration range of 5.0-40.0 µg.mL<sup>-1</sup> for RU and 0.5-10.0 µg.mL<sup>-1</sup> for AA. The samples were then chromatographed using the following chromatographic conditions: stationary phase: a 150 mm x 4.6 mm i.d. C8 Zorbax 5µm analytical column. The mobile phase consisted of ammonium acetate buffer: acetonitrile (80: 20, v/v), pH adjusted to 6.3 by orthophosphoric acid. The mobile phase was prepared daily, filtered & sonicated before use and delivered at a flow rate of 0.8 mL.min<sup>-1</sup>. [isocratically at ambient temperature (~25°C)] with UV detection at 270 nm. The injection volume was 50µL. The regression equations were computed and calculations were performed following the external standard technique. Different concentrations of unknown samples of AA and RU were determined using the obtained regression equations.

#### Assay of laboratory-prepared mixtures

Laboratory prepared mixtures containing different ratios of RU and AA were analyzed using the suggested methods, aliquots of RU and AA were mixed to prepare different mixtures and proceed as mentioned under each method, then concentrations were calculated from the corresponding regression equations.

#### Assay of pharmaceutical formulation (Ruta-C 60<sup>®</sup> tablets)

Ten tablets were weighed from the dosage form and the average weight was calculated, tablets were crushed to furnish a homogenous powder and certain amount of powdered tablets were dissolved by the aid of an ultrasonic bath for 2 hours and filtered. The solutions were diluted to the same concentration of the appropriate working solutions and proceed as described under each method.

## RESULTS AND DISCUSSION

### Spectroscopic Methods

#### Derivative Ratio Spectra Method

The derivative-ratio spectroscopy is a useful tool in quantification of drugs. It could be applied for the determination of AA in presence of RU. The zero – order absorption spectra of AA and RU are overlapped (Fig.2), where AA can't be assayed, while RU can be determined by direct measurement of absorbance at 363 nm without any interference from AA. The linearity was confirmed by plotting the measured absorbance value at 363 nm versus the corresponding concentrations of RU over a concentration range of 4.0-20.0µg.mL<sup>-1</sup> for RU. AA can be assayed in presence of RU by dividing the absorption spectra of different concentrations of AA by the absorption spectrum of (20.0µg.mL<sup>-1</sup>) RU and then the first derivative of ratio spectra (<sup>1</sup>DD) were recorded (Fig. 3).

It was found that upon dividing by 20.0 µg.mL<sup>-1</sup> of RU, best results were obtained in terms of sensitivity, repeatability and signal to noise ratio. Linear calibration graph was obtained for AA in concentration range of 1.0-10.0 µg.mL<sup>-1</sup> by recording the peak amplitudes at 275 and 286 nm using 20.0µg.mL<sup>-1</sup> of RU as a divisor. The regression equations were computed and found to be:

$${}^1\text{DD}_{275} = 0.531 C + 0.130 \quad (r^2 = 0.9997) \text{ for AA}$$

$${}^1\text{DD}_{286} = 0.547 C + 0.112 \quad (r^2 = 0.9996) \text{ for AA}$$

$${}^0\text{D}_{363} = 0.0284 C + 0.0109 \quad (r^2 = 0.9992) \text{ for RU}$$

Where, <sup>1</sup>DD is the peak amplitude of the first derivative ratio curve for (AA/RU), <sup>0</sup>D is the absorbance value of RU at 363 nm, C the concentration of AA (µg.mL<sup>-1</sup>) and r<sup>2</sup> is the correlation coefficient. The precision of the proposed method was checked by the analysis of different concentrations of authentic samples in triplicates. The mean percentage recoveries of AA were found to be 99.44 ± 0.85 at 275 nm and 100.76 ± 1.68 at 286 nm. The linearity ranges and analytical data for the calibration graphs are listed

in table 2. Results for analysis of laboratory-prepared mixtures with different proportions of the two drugs are given in table 3.

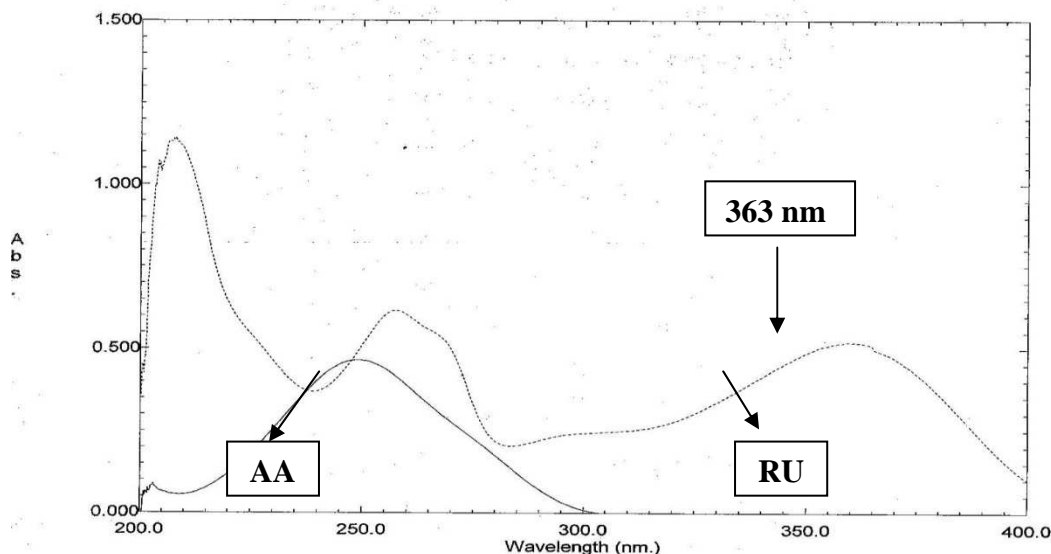


Fig. 2: zero-order spectra of  $10.0\mu\text{g.mL}^{-1}$  RU (.....) and  $10.0\mu\text{g.mL}^{-1}$  AA (—)

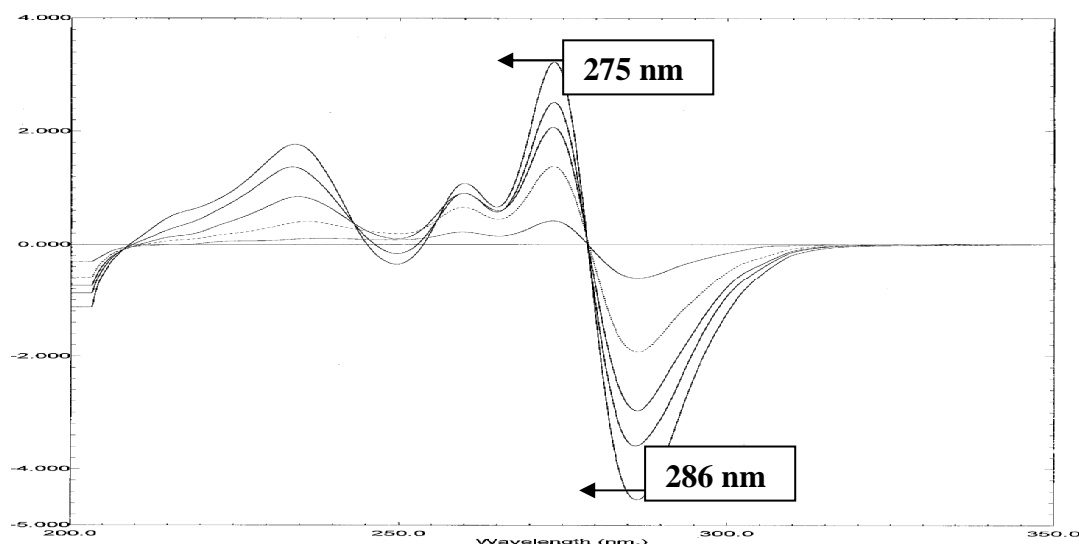


Fig. 3: First-order of ratio spectra of different concentrations of AA (using  $20.0\mu\text{g.mL}^{-1}$  RU as divisor)

### Bivariate method

The bivariate calibration method may be competitive and in some cases even superior to commonly use derivative spectrophotometric methods as applied for the resolution of binary mixtures. The advantage of bivariate calibration method is its simplicity and the fact that derivatization procedures are not necessary. Unlike other chemometric techniques, there is no need for full spectrum information and no data processing is required. Calibration function was calculated ( $r > 0.9990$ ), *mi*- and *ei*-values were taken for the bivariate algorithm. In order to apply the bivariate method to the resolution of binary mixture of RU and AA, we first select the signals of the two components located at six wavelengths; 242, 248, 254, 260, 272, and 278 nm. The calibration curve equations and their respective linear regression coefficients are obtained with the aim of ensuring that there is a linear relationship between the absorbance values and the concentrations. All the calibration curves at the selected wavelengths showed satisfactory linear regression coefficients ( $r > 0.9990$ ). The slope values of the linear regression were estimated for both components at the selected wavelengths and used for determination of the sensitivity matrices *K*, proposed by Kaiser's method [32]. The determinants of these matrices were calculated and the wavelength set was selected for which the highest matrix determinant value was obtained, table 1. For the bivariate method determination of RU and AA was done using 260 nm and 278 nm. The linearity

ranges are listed in table 2. Results of analysis of laboratory-prepared mixtures with different proportions of the two drugs are given in table 3.

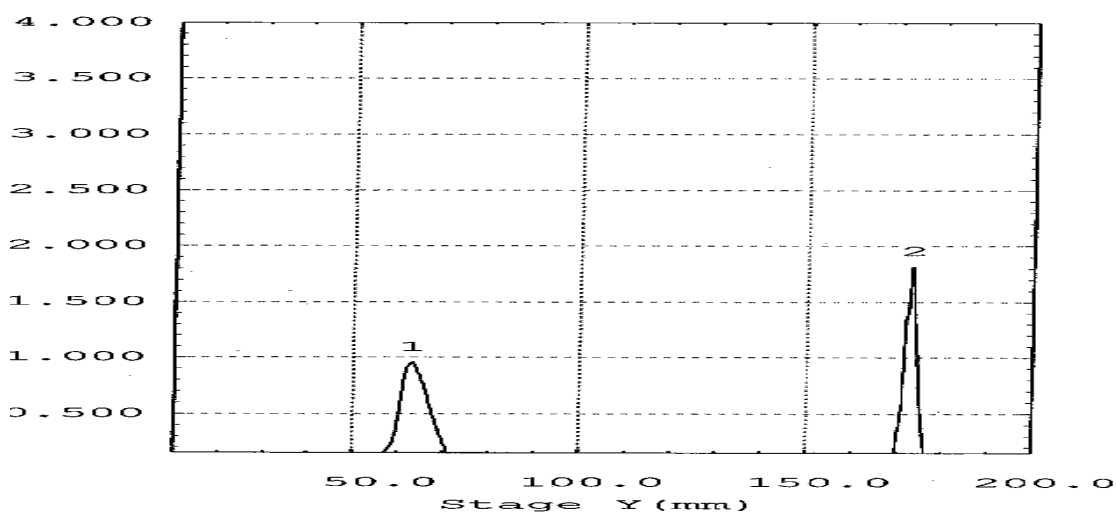
**Table 1: Application of Kaiser method for the selection of the wave length set for Rutin (RU) – Ascorbic acid (AA) mixture**

$\lambda / \lambda$	242	248	254	260	272	278
242	0	2.9	8.62	11.59	0	0
248		0	6.429	9.96	7.175	0.7182
254			0	4.154	2.979	2.374
260				0	0.0292	84.37*
266					0.456	3.9818
272					0	2.962
278						0

### Chromatographic Methods

#### TLC-Densitometric method

TLC densitometry overcomes the problem of overlapping absorption spectra of mixture of drugs by separating these components on TLC plates and determining each ingredient by scanning the corresponding chromatogram. The TLC densitometric method has the advantage of simultaneously determining the active ingredients in multi-component dosage forms [33]. A TLC-densitometric method could be used for the simultaneous determination of RU and AA without prior separation. Different solvent systems were tried for the separation of both drugs. Satisfactory results were obtained by using a mobile phase composed of ethyl acetate: methyl isobutyl ketone: formic acid: glacial acetic acid; water [20:12:2.8:1.2:20, v/v/v/v/v] where  $R_f = 0.2$  and  $0.4$  for AA and RU, respectively, (Fig.4). The separation allowed the determination of AA and RU with no interference, (Figs.5-6).



**Fig. 4: TLC chromatogram of mixture of (1) AA and (2) RU**

The linearity was confirmed by plotting the measured peak area versus the corresponding concentrations at 276 nm over a range of  $4.0-8.0 \mu\text{g}\cdot\text{spot}^{-1}$  for AA and at 370 nm over a range of  $2.0-6.0 \mu\text{g}\cdot\text{spot}^{-1}$  for RU, where a linear response was obtained, table 2, regression equations were found to be:

$$A = 2.58 C - 0.043 \quad r = 0.9998 \text{ (for RU).}$$

$$A = 0.88 C - 2.13 \quad r = 0.9998 \text{ (for AA).}$$

Where A is the area integrated under the peak  $\times 10^{-3}$  for RU and AA, C is the concentration in  $\mu\text{g}\cdot\text{spot}^{-1}$  and r is the correlation coefficient.

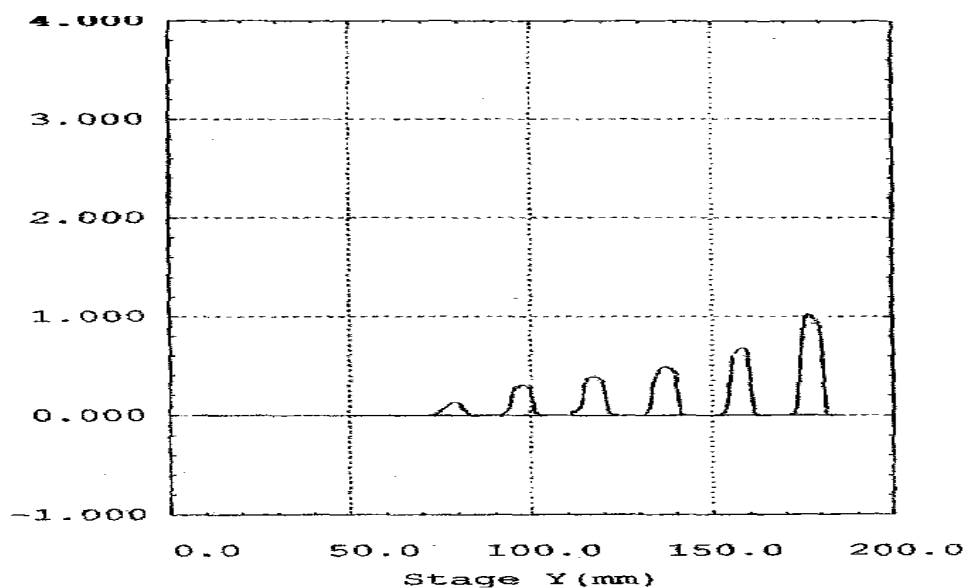


Fig. 5: Linearity graph of AA ( $R_f = 0.20$ ) over a concentration range ( $4.0-8.0 \mu\text{g.spot}^{-1}$ )

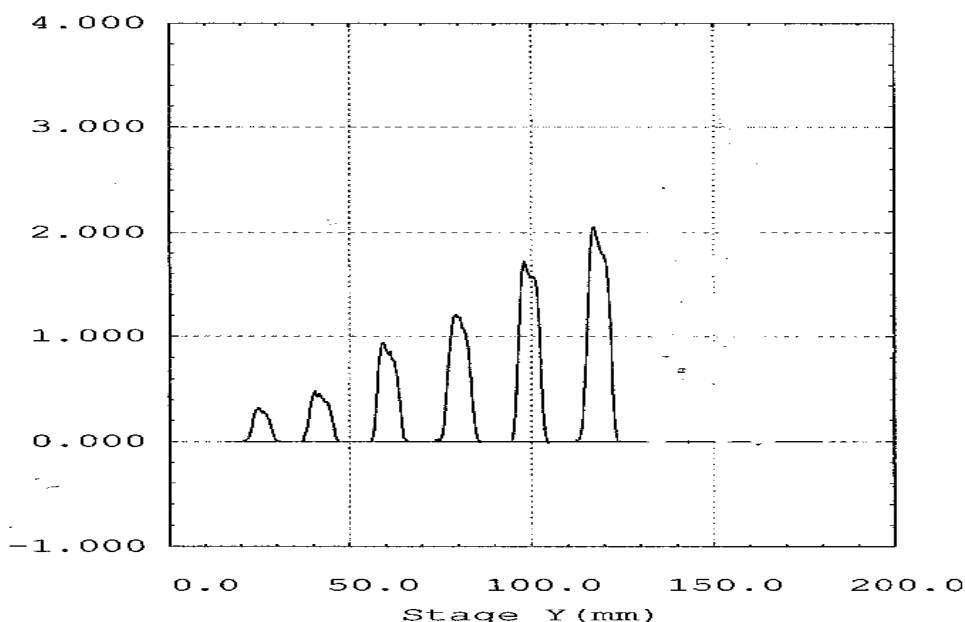
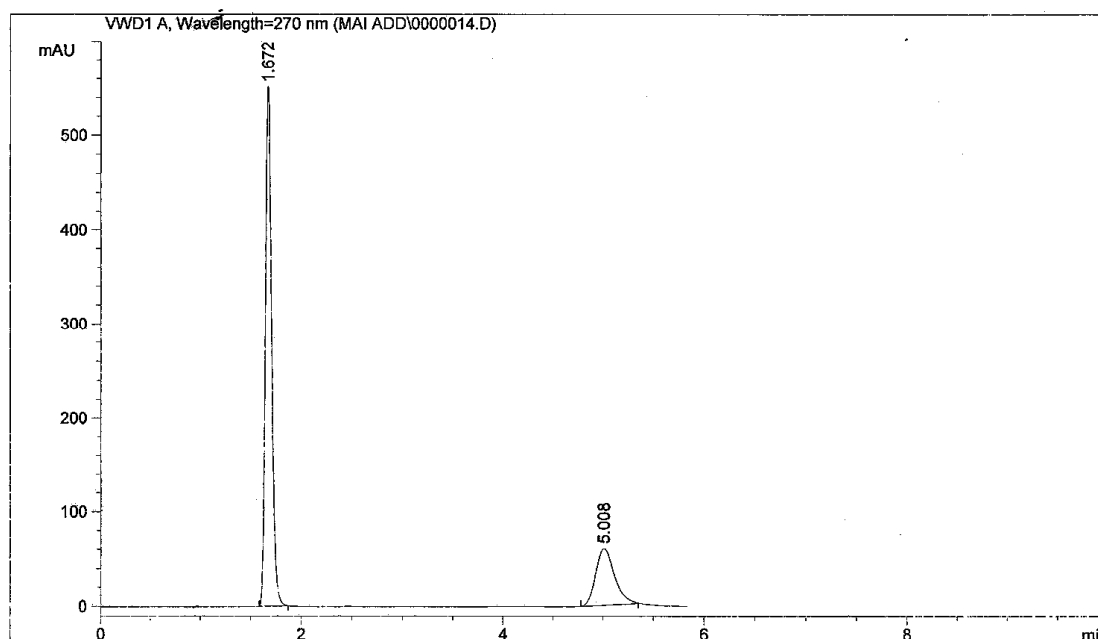


Fig. 6: Linearity graph of RU ( $R_f = 0.40$ ) over a concentration range ( $2.0-6.0 \mu\text{g.spot}^{-1}$ )

The precision of the proposed method was checked by the analysis of different concentrations of authentic samples in triplicates. The mean percentage recovery was found to be  $99.81 \pm 1.72$  for RU and  $99.56 \pm 1.28$  for AA. To assess the specificity, accuracy and selectivity of the TLC method for assay of both drugs without interference from one another, synthetic mixtures of RU and AA at various concentrations within the linearity range were prepared and analyzed, table 3.

#### HPLC method

A simple isocratic high-performance liquid chromatography method was developed for the determination of RU and AA in pure form and in pharmaceutical formulation using a 150 mm x 4.6 mm, i.d. C8 Zorbax 5 $\mu\text{m}$  analytical column. The mobile phase consisted of ammonium acetate buffer: acetonitrile (80: 20, v/v), pH adjusted to 6.3 by orthophosphoric acid. The mobile phase was prepared daily, filtered & sonicated before use and delivered at a flow rate of  $0.8 \text{ mL}\cdot\text{min}^{-1}$  [isocratically at ambient temperature ( $\sim 25 \text{ }^\circ\text{C}$ )] with UV detection at 270 nm. The injection volume was  $50 \mu\text{L}$ . RU and AA were well separated and the average retention time for RU was 5.008 min. while that of AA was 1.672 min. as shown in figure 7.



**Fig.7: Liquid chromatogram of AA (10.0 $\mu\text{g}\cdot\text{mL}^{-1}$ ) at  $R_t$  1.672 min and RU(20.0 $\mu\text{g}\cdot\text{mL}^{-1}$ ) at  $R_t$  5.008 min**

The linearity of the detector response for both drugs was determined by plotting peak area ratios to the internal standard versus concentration. The linearity ranges and analytical data for the calibration graphs are listed in table 2. Linearity ranges were found to be 5.0-40.0  $\mu\text{g}\cdot\text{mL}^{-1}$  for RU and 0.5-10.0 $\mu\text{g}\cdot\text{mL}^{-1}$  for AA using the following regression equations:

$$A = 0.47 C - 0.39 \quad r = 0.9993 \text{ (for RU).}$$

$$A = 2.05 C + 1.15 \quad r = 0.9994 \text{ (for AA).}$$

Where, A is the peak area ratio, C is the concentration of RU and AA [ $\mu\text{g}\cdot\text{mL}^{-1}$ ] and r is the correlation coefficient.

The precision of the method was evaluated by repeating three experiments on the same day (within-day precision) and over 3 days (day-to-day precision). The variability in the peak area ratios on the concentration of 20  $\mu\text{g}\cdot\text{mL}^{-1}$  of RU and 5.0 $\mu\text{g}\cdot\text{mL}^{-1}$  of AA was determined as the precision of the assay. The relative standard deviation values from intra-day and inter-day analysis were found to be 0.69 and 1.22 % for RU, and 0.34 and 0.64 % for AA, respectively. Results for HPLC analysis of laboratory-prepared mixtures with different proportions of the two drugs are given in table 3.

The robustness of the HPLC method was investigated by analysis of samples under a variety of experimental conditions such as small changes in the pH [5-7], small changes in ammonium acetate buffer / acetonitrile ratio in the mobile phase and small changes in mobile phase flow rate [0.7 – 1.0  $\text{mL}\cdot\text{min}^{-1}$ ]. It was found that the method was robust when the mobile phase ratio and flow rate were varied. During these investigations, the retention times were modified, however the areas and peak symmetry were conserved.



**Table 2: Assay parameters and validation of the proposed methods for determination of RU and AA**

parameters	The proposed methods								
	Bivariate method		Derivative ratio method			TLC method		HPLC method	
	RU	AA	RU		AA	RU	AA	RU	AA
			363 nm	275 nm	286 nm				
LOD ( $\mu\text{g.ml}^{-1}$ )	0.65	0.26	0.12	0.07	0.09	0.02 ( $\mu\text{g.spot}^{-1}$ )	0.05 ( $\mu\text{g.spot}^{-1}$ )	0.103	0.010
LOQ ( $\mu\text{g.ml}^{-1}$ )	1.95	0.77	0.39	0.23	0.297	0.066 ( $\mu\text{g.spot}^{-1}$ )	0.165 ( $\mu\text{g.spot}^{-1}$ )	0.342	0.035
Range ( $\mu\text{g.ml}^{-1}$ )	2.0-16.0	2.0-12.0	4.0-20.0	1.0 – 10.0	1.0 - 10.0	2.0-6.0 ( $\mu\text{g.spot}^{-1}$ )	4.0-8.0 ( $\mu\text{g.spot}^{-1}$ )	5.0-40.0	0.5-10.0
Slope	0.024	0.041	0.028	0.531	0.547	2.58	0.88	0.47	2.05
Intercept	0.03	-0.021	0.01	0.13	0.112	-0.043	-2.13	-0.39	1.15
Mean $\pm$ S.D.	100.06 $\pm$ 1.11	100.38 $\pm$ 1.28	99.79 $\pm$ 0.81	99.44 $\pm$ 0.85	100.76 $\pm$ 1.68	99.81 $\pm$ 1.72	99.56 $\pm$ 1.28	100.57 $\pm$ 1.60	99.68 $\pm$ 0.72
Correlat. Coeff. (r)	0.9995	0.9994	0.9992	0.9997	0.9996	0.9998	0.9998	0.9993	0.9994
RSD% <sup>a*</sup>	0.13-0.17	0.22-0.25	0.58 – 0.61	0.75-0.77	0.81-0.83	0.44-0.47	0.99-1.02	0.69-0.72	0.34-0.37
RSD% <sup>b*</sup>	0.6-0.7	0.47-0.51	0.75 – 0.78	0.83-0.86	1.04-1.06	1.78-1.80	1.04-1.07	1.22-1.26	0.64-0.67

<sup>a,b</sup> Intra-day and inter-day (n = 3) relative standard deviations of samples of RU and AA.

**Table 3: Determination of RU and AA in laboratory prepared mixtures by the proposed methods**

Drug	Derivative ratio method		Bivariate method		TLC method	HPLC method
	275 nm	286 nm	260 nm	278 nm		
AA	99.53 $\pm$ 0.69	101.08 $\pm$ 0.97	99.46 $\pm$ 0.46	99.13 $\pm$ 0.58	99.09 $\pm$ 1.40	99.96 $\pm$ 1.21
	at 363 nm		260 nm	278 nm		
RU	101.35 $\pm$ 1.01		100.99 $\pm$ 1.40	99.24 $\pm$ 0.67	101.72 $\pm$ 1.13	100.98 $\pm$ 1.60

A statistical comparison of the results obtained by the proposed methods and the reference methods for pure RU [27] and pure AA [31] is shown in table 4. The values of the calculated T and F are less than tabulated ones, which reveals that there is no significant difference with respect to accuracy and precision between the proposed methods and the reported and official ones.

**Table 4: Statistical analysis of results obtained by applying proposed methods and the reference ones for analysis of pure RU and AA**

Values	The proposed methods									Reference methods	
	Bivariate method		<sup>1</sup> DD method			TLC method		HPLC method		RU [27]	AA [31]
	RU	AA	AA		RU	RU	AA	RU	AA		
			275	286	363 nm						
Mean	100.06	100.38	99.44	100.76	99.79	99.81	99.56	100.57	99.68	100.03	99.18
S.D.	1.11	1.28	0.85	1.68	0.81	1.72	1.28	1.60	0.72	1.23	0.86
N	6	6	6	6	6	6	6	6	6	6	6
Variance	1.23	1.64	0.72	2.82	0.66	2.96	1.64	2.56	0.52	1.51	0.74
T[2.23]*	0.24	1.91	0.36	1.93	0.43	0.24	1.19	1.52	1.38	.....	.....
F[5.05]*	1.23	2.22	1.03	3.81	2.29	1.96	2.22	1.69	1.42	.....	.....

\*The figures in parenthesis are the corresponding tabulated values at P=0.05.  
Analysis of Laboratory-prepared mixtures

The validity of the proposed methods for the simultaneous determination of RU and AA was assessed by analysis of laboratory prepared mixtures containing different ratios of RU and AA and calculating the concentrations from the corresponding regression equations. The results are shown in table 3.

#### Analysis of Tablets

The validity of the proposed methods for the analysis of the pharmaceutical formulation and the effect of possible interferences from common excipients were studied by assaying Ruta-C 60<sup>®</sup> tablets (labeled to contain 60 mg RU and 160 mg AA expressed as base per tablet, respectively), the results are present in table 5.

Table 5: Determination of RU and AA in Ruta-C 60 tablets B.N: 01159 by the proposed methods

Drug	Derivative ratio method		Bivariate method		TLC method	HPLC method
	275 nm	286 nm	260 nm	278 nm		
AA	99.31 ± 0.59	100.37 ± 0.73	99.71 ± 0.51	101.14 ± 1.39	99.27 ± 1.23	100.78 ± 1.59
	363 nm		260 nm	278 nm		
RU	101.6 ± 0.77		99.38 ± 0.69	100.85 ± 1.16	99.04 ± 1.43	98.87 ± 1.45

### CONCLUSION

The proposed methods are accurate and precise and could be used for determination of RU & AA in their mixtures and in their pharmaceutical formulation without prior separation. The most striking feature of the spectrometric methods is their simplicity and rapidity. For spectroscopic methods there was no need for time-consuming sample preparation steps such as filtration, degassing that are needed for the HPLC procedure. The HPLC method is a versatile method and may offer advantages over the derivative method for the selective determination. The TLC-method has some advantages over HPLC such as a short run time, large sample capacity and minimal volume use of solvent. With these two methods, one can gain the advantages of speed, low-cost, and environmental protection without sacrificing accuracy.

### Acknowledgment

The author thanks Kahira Pharmaceutical & Chemical Industries Company, Cairo, Egypt, for providing Rutin and Ascorbic acid standards and their dosage form as gift samples for this work.

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