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Quantitative Estimation and Validation of Rafoxanide in Presence of its Alkali-Induced Degradation Product by Different Spectrophotometric Methods

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ABSTRACT

Objective: Development and validation of four simple, accurate, selective and sensitive UV spectrophotometric methods for the determination of rafoxanide in the presence of its alkali-induced degradation product without preliminary separation. **Methods**: **(A)** Dual wavelength method, where the difference in absorbance were calculated at 242 and 281 nm. **(B)** Area under the curve method, where area under the peak were measured at wavelength ranges (225-235 nm) and (275-295 nm). **(C)** Bivariate method, where the absorbance values were measured at selected wavelengths 250 and 280 nm. **(D)** First derivative method, where The amplitudes were measured at 253 nm. **Results**: Good linearity was found in the concentration range of 2.5–25 µg/mL for all the methods. The specificity of the methods were assessed by analysing synthetic mixtures containing the drug and its degradation product. Methods are validated as per ICH guidelines and accuracy, precision, repeatability and robustness are found to be within the acceptable limit. **Conclusion:** The proposed methods have been found to be accurate, precise and can be used for determination of the drug in pure form and pharmaceutical formulations as well as in the presence of its degradation product without any preliminary separation steps.

Keywords: *Area under the curve; Bivariate; Dual wavelength; First derivative; Rafoxanide*

INTRODUCTION

Rafoxanide is 3′-chloro-4′-(4-chlorophenoxy)-3,5-di-iodosalicylanilide (**Figure 1**). It is an anthelmintic used in veterinary medicine for the treatment of fascioliasis in cattle and sheep.[1] Literature survey reveals that rafoxanide was determined by several techniques including colorimetry [2], UV-Spectrophotometry [3], GC [4], UPLC [5], TLC [5,6], and HPLC [6-9].

The aim of this work is to develop and validate simple, sensitive, selective and cost effective spectrophotometric methods for the determination of rafoxanide in the presence of its alkali-induced degradation product without preliminary separation these methods namely dual wavelength [10,11], area under the curve [12,13], bivariate [14-16] and first derivative [17,18].

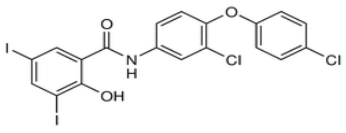

Figure 1. Structural formula of rafoxanide

MATERIALS AND METHODS

Instruments

Shimadzu dual beam UV-Visible 1800 Spectrophotometer, (Tokyo, Japan), equipped with 10

mm matched quartz cells.The bundled software, UV-Probe personal spectroscopy software version 2.21 (SHIMADZU).

Samples

Both pure rafoxanide (99.8%) (B. NO.RF/110315) and Flukanil® injection (B. NO. 1509104) were kindly supplied by Pharma-Swede, Egypt. 10th of Ramadan city, Egypt.

Chemicals and solvents

Hydrochloric acid, sodium hydroxide and methanol (El-Nasr Co., Egypt). Solvent used was methanol.

Standard solution

A stock solution of rafoxanide (100 µg/mL) was prepared by dissolving 10 mg of rafoxanide in 50 mL of methanol and complete to 100 mL with the same solvent.

Degraded sample

Accelerated alkali-induced degradation was performed by refluxing 100 mg of pure rafoxanide with 50 mL of 1 N sodium hydroxide solution for 7 hours. The solution was cooled to room temperature then neutralized to pH 7 by addition of 1 N hydrochloric acid solution, and then evaporated to dryness under vacuum. The obtained residue was extracted with methanol (3 x 25 mL), filtered into a 100-mL volumetric flask and diluted to volume with methanol to obtain a stock solution labeled to contain degradate derived from 1 mg/mL of rafoxanide. Working solution of degradate (100 µg/mL) was obtained by further dilution of the stock solution with the methanol.

Methods

Construction of calibration curves

Different aliquots equivalent to (25 – 250 µg) of both rafoxanide and its alkali-induced degradation product were accurately transferred from their standard solutions (100 µg/mL) into two separate series of 10-mL volumetric flasks and completed to volume with methanol. The absorption spectra (from 200 to 400 nm) of these solutions were recorded using methanol as a blank and stored in computer.

Dual wavelength method

The calibration curve was constructed by plotting the absorbance difference of the ratio spectra at 242 and 281 nm versus the corresponding concentrations in µg/mL and the regression equation was derived.

Area under the curve method

The area under the curve of rafoxanide and its degradation product were measured at both wavelength ranges of (225-235 nm) and (275-295 nm). The calibration curves were obtained by plotting the area under curve values against the corresponding concentrations in µg/mL and the regression equations were derived.

Bivariate method

The absorbance was measured at two selected wavelength 250 and 280 nm. The calibration curves were obtained by plotting the absorbance values at the selected wavelengths for pure rafoxanide against its corresponding concentrations in µg/ml and the regression equations were derived.

First derivative method

The first derivative of zero-order spectra of rafoxanide and its degradation product were obtained. The amplitudes of first derivative for pure rafoxanide were measured at 253 nm, (zero crossing point for degradate). The calibration curve was constructed by plotting the amplitudes of first derivative for pure rafoxanide against its corresponding concentrations in µg/ml and the regression equation was derived.

Application to laboratory prepared mixtures

Different aliquots equivalent to (200 – 50 µg) of rafoxanide and (50 – 200 µg) of rafoxanide degradate were accurately transferred from their standard solutions (100 µg/mL) into a series of 10-mL volumetric flasks to prepare mixtures containing different ratios of both. The volumes were completed with methanol and the absorption spectra (from 200 to 400 nm) of these prepared mixtures were recorded using methanol as a blank. The concentrations of rafoxanide were calculated as described under linearity from the corresponding regression equation for each proposed method.

Application to pharmaceutical preparation

Appropriate volume of Flukanil® injection (75 mg/mL Rafoxanide) equivalent to 10 mg of rafoxanide was accurately taken and transferred into 100-mL volumetric flask and the volume was made up to 75 mL with methanol. The solution was shaken well then the volume was completed to 100-mL with the same solvent to obtain solution claimed to contain 100 µg/mL. The procedures stated under linearity were repeated using aliquots covering the working concentration range. The concentrations of rafoxanide in injection were calculated from the corresponding regression equations.

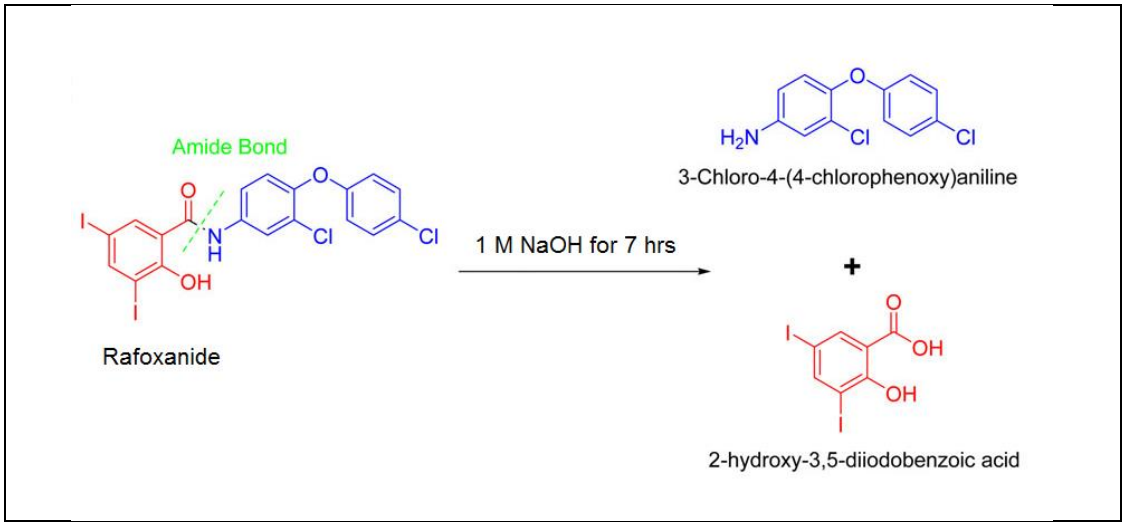

Figure 2. Suggested degradation pathway of rafoxanide

RESULTS AND DISCUSSION

Degradation product

Complete degradation was achieved, as investigated by thin layer chromatography using ethyl acetate : chloroform : hexane : acetonitrile (45:20:30:5, by volume) as a developing solvent, where two spots of the degradation product obtained with significant separation from that of intact one. Alkaline hydrolyses is expected to occur through the cleavage of amide bond as illustrated in the suggested degradation pathway (**Figure 2**).

Spectral characteristics

The zero-order absorption spectra of rafoxanide and its dgradate shows severe overlapping, as shown in **Figure 3**. To overcome the interference from the degradate, we devolepe four spectrophotometric methods namely dual wavelengh, area under the curve, bivariate and first derivative method. These methods are found to be very easy to apply, rapid, simple, sensitive, accurate and precise.

Dual wavelength method

In this method, we select two wavelengths where the absorbance at these wavelengths have the same values for the degradate and different value for the drug. 242 and 281 nm were selected due to the difference in absorbance between these wavelengths is zero for degradate, while it is directly proportional to the concentration of the drug as shown in **Figure 3**.

Area under the curve method

In this method, the area under the curve for rafoxanide and its degradation product were recorded over wavelength ranges of (225–235 nm) and (275–290 nm) (**Figures 4** and **5**). The absorptivity 'a' values of rafoxanide and its degradation product were determined at each wavelength range. The concentrations of rafoxanide in presence of its degradation product can be obtained from the following equation:

$$C_{(x)} = (A_1 b_2 – A_2 b_1) / (a_1 b_2 – a_2 b_1)$$

Where $C_{(x)}$ is rafoxanide concentration, A_1 and A_2 are the area under the curve of the mixture at the wavelength range (225 - 235) nm and (275 – 290) nm, respectively, a_1 and a_2 are absorptivity coefficient of the drug at first and second wavelength range respectively and b_1 and b_2 are absorptivity coefficient of the degradate at first and second wavelength rang respectively.

Bivariate method

In order to apply the bivariate method for determination of rafoxanide in presence of its degradation product, the absorbance values of both drug and degradate individually at seven different wavelengths were recorded in the region of overlapping; 240, 250, 260, 270, 280, 290 and 300 nm. The calibration curve equations and their respective linear regression coefficients were obtained directly with the aim of ensuring that there is a linear relationship between the absorbance and the corresponding concentration. All of the calibration curves at the selected wavelengths showed a satisfactory linear regression coefficient ($r^2 > 0.9987$). According to Kaiser method [16], the slope values of the linear regression equations for both drug and degradate at the selected wavelengths were used to calculate the sensitivity matrices (K) to find out the optimum pair of wavelengths at which the binary mixture was recorded.

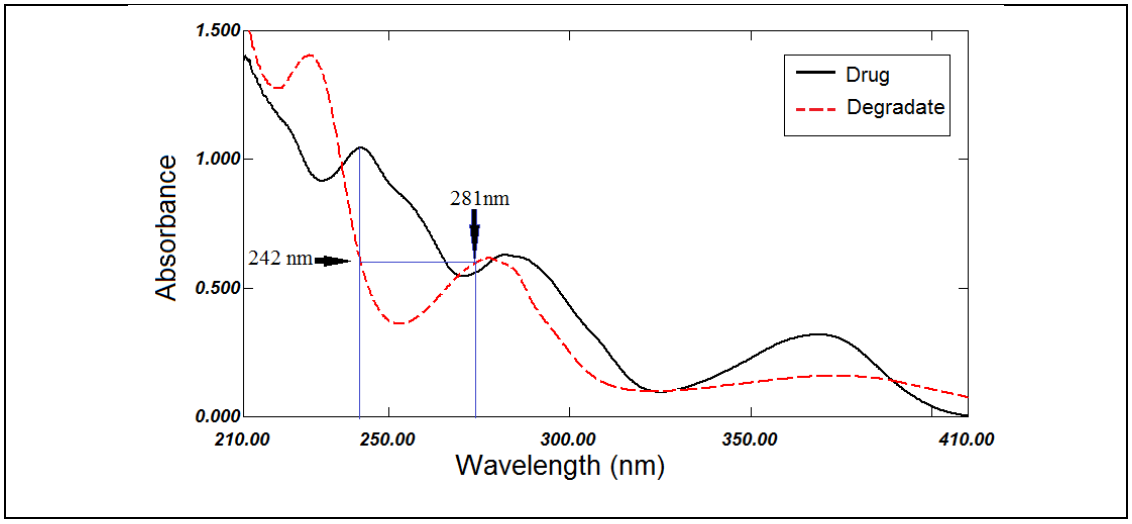

Figure 3. Zero-order absorption spectra of rafoxanide (15 µg/ml) and its alkaline degradate (15 µg/ ml) (----) showing selected wavelengths for dual wavelength method.

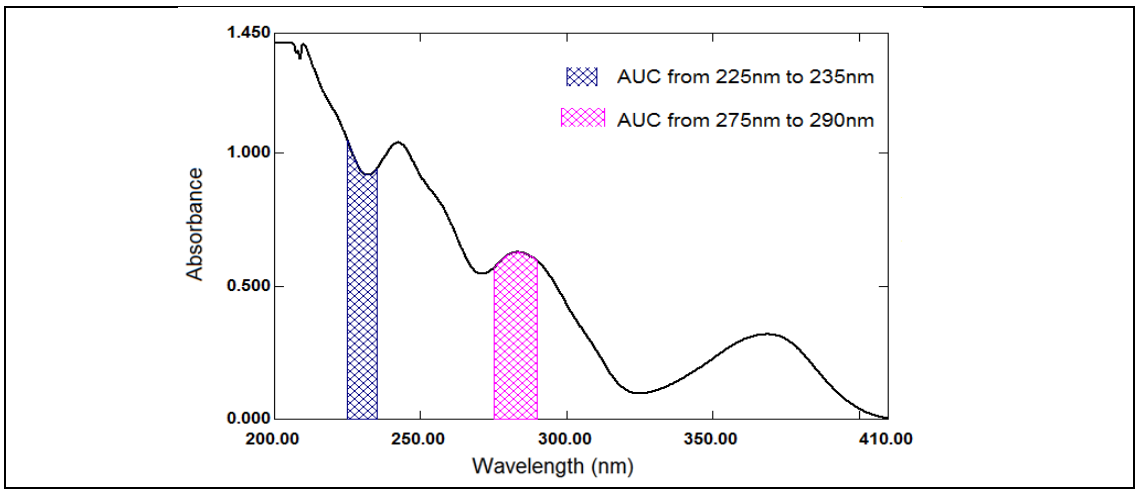

Figure 4. Area under the curve ranges of intact rafoxanide (15 µg/mL).

It was found that, the slopes at 250 and 280 nm gave the maximum value of K (**Table 1**) and thus chosen for the analysis using the following two equations:

$$A_{AB1} = m_{A1} C_A + m_{B1} C_B + e_{AB1}$$
$$A_{AB2} = m_{A2} C_A + m_{B2} C_B + e_{AB2}$$

The resolution of each equation set allows the evaluation of C_A and C_B values:

$$C_A = (A_{AB1} - e_{AB1} - m_{B1} C_B) / m_{A1}$$
$$C_B = [m_{A2} (A_{AB1} - e_{AB1}) + m_{A1} (e_{AB2} - A_{AB2})] / m_{A2} m_{B1} - m_{A1} m_{B2}$$

Where C_A and C_B are the concentration of drug and degradate respectively, m_{A1} and m_{A2} are the slope values of the drug at 250 and 280 nm, m_{B1} and m_{B2} are the slope values of the degradate at 250 and 280 nm, A_{AB1} and A_{AB2} are the absorbance of the mixture at 250 and 280 nm, e_{AB1} and e_{AB2} are the sum of the intercepts of drug and degradate at 250 and 280 nm respectively. This simple mathematic algorithm allows the resolution of the two components by measuring the absorbance of their mixture at the two selected wavelengths.

First derivative method

Derivative spectrophotometry offers a powerful tool for a better resolution of the interference between overlapped spectra. In this method, first derivative corresponding to each spectrum was

Table 1. Application of Kaiser method for the selection of the wavelength set for the mixture of rafoxanide and its degradate.

λ/λ	240	250	260	270	280	290	300
240	0	-1260.79	-593.42	663.96	726.38	45.19	-258.61
250		0	385.39	1251.24	1374.34	763.75	307.22
260			0	791.88	868.9	373.29	65.55
270				0	-2.88	-357.36	-418.44
280					0	-390.76	-458.9
290						0	-167.67
300							0

Table 2. Regression and analytical parameters of the proposed spectrophotometric methods for determination of rafoxanide.

Parameters	Dual wavelength	Area under the curve		Bivariate		First derivative
Wavelength (nm)	242&281	(225-235)	(275-290)	250	280	253
Range (µg/mL)	2.5 - 25					
Slope (*b*)	0.0280	0.6688	0.6205	0.0609	0.0421	0.0092
Intercept (*a*)	0.0004	-0.3918	-0.1147	0.0004	-0.0084	-0.0026
Coefficient of determination (*r*2)	0.9998	0.9998	0.9999	0.9999	0.9999	0.9998
LOD	0.382	0.366	0.302	0.317	0.325	0.406
LOQ	1.156	1.110	0.914	0.962	0.984	1.229
Accuracy [a]	100.23	99.95		99.93		100.15
Repeatability (RSD)[b]	1.417	1.196		1.243		1.209
Intermediate precision (RSD)[c]	1.230	1.299		1.118		1.348

[a] Average of three determinations for three concentrations (10, 15 and 20 µg/mL) for rafoxanide repeated three times.
[b] The intraday (n = 3), average of three concentrations (10, 15 and 20 µg/mL) for rafoxanide repeated three times within the day.
[c] The interday (n = 3), average of three concentrations (10, 15 and 20 µg/mL) for rafoxanide repeated three times in three days.

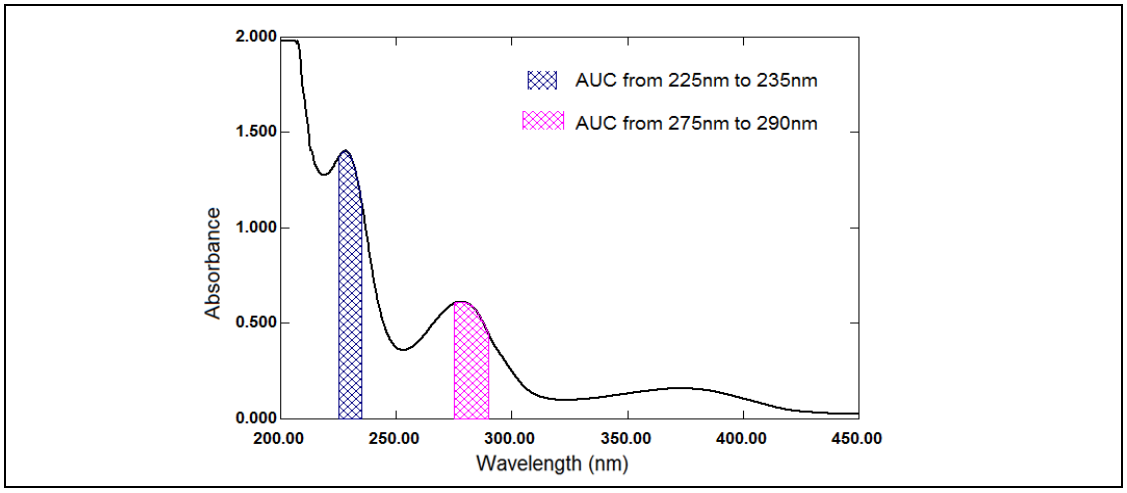

Figure 5. Area under the curve ranges of rafoxanide degradation product (15 µg/mL)

Table 4. Statistical comparison between the results obtained by applying the proposed spectrophotometric methods and reported method for determination of rafoxanide in Flukanil® injection.

Parameter	Dual wavelength	Area under the curve	Bivariate	First derivative	Reported [a] method[5]
Mean	99.66	99.44	100.13	99.94	100.37
SD	1.489	1.264	1.290	1.177	1.149
RSD%	1.494	1.271	1.288	1.178	1.145
N	5	5	5	5	5
Variance	2.216	1.598	1.664	1.386	1.320
***t*-test [b]**	0.844 (2.31)	1.219 (2.31)	0.310 (2.31)	0.583 (2.31)	____
***F*-value [b]**	1.679 (6.39)	1.211 (6.39)	1.261 (6.39)	1.050 (6.39)	____

[a] TLC densitometric method
[b]The values in the parenthesis are the corresponding theoretical values of *t* and *F* at (P = 0.05)

Table 5. Application of standard addition technique to the analysis of Flukanil® Injection by applying the proposed methods.

Dosage taken (µg/mL)	Pure add (µg/mL)	Dual wavelength	Area under the curve	Bivariate	First derivative
		Recovery % of pure found			
5	5	99.00	99.67	101.80	101.30
	10	98.43	98.73	101.75	101.74
	15	99.67	98.78	100.22	98.26
	20	101.54	101.52	101.73	100.87
Mean ± RSD		99.66±1.355	99.68±1.604	101.23±0.869	100.54±1.554

Table 6. One-way ANOVA testing for the different proposed methods used for the determination of rafoxanide in Flukanil® injection.

Source of variation	Degree of freedom	Sum of squares	Mean square	*F* value
Between exp.	4	2.728	0.682	0.417 (2.866)
Within exp.	20	32.741	1.637	

The values between parentheses are the theoretical *F* values. The population means are not significantly different.

recorded, using Δλ = 4 nm and scaling factor = 10. The amplitudes of the first derivative at 253 nm (zero crossing point for degradate) were proportional to the concentrations of the drug without interference from its degradate, as shown in **Figure 6**.

Methods validation

Methods validation was performed according to ICH guidelines [19] for all the proposed methods. Linearity, range, LOD, LOQ, accuracy and precision of the proposed methods were shown in **Table 2**. The specificity of the methods was checked by the analysis of laboratory prepared mixtures of the drug with its alkali-inducd degradation product as shown in **Table 3**. The developed methods have been also applied for determination of rafoxanide in Anthimizole® veterinary powder and the results obtained were acceptable with small RSD % values. Results obtained by the proposed methods were statistically compared to those obtained by the reported TLC densitometric method [5] and no significant difference was observed (**Table 4**). The validity of the proposed procedures is further assessed by applying the standard addition technique and the results obtained in **Table 5** showing no excipients

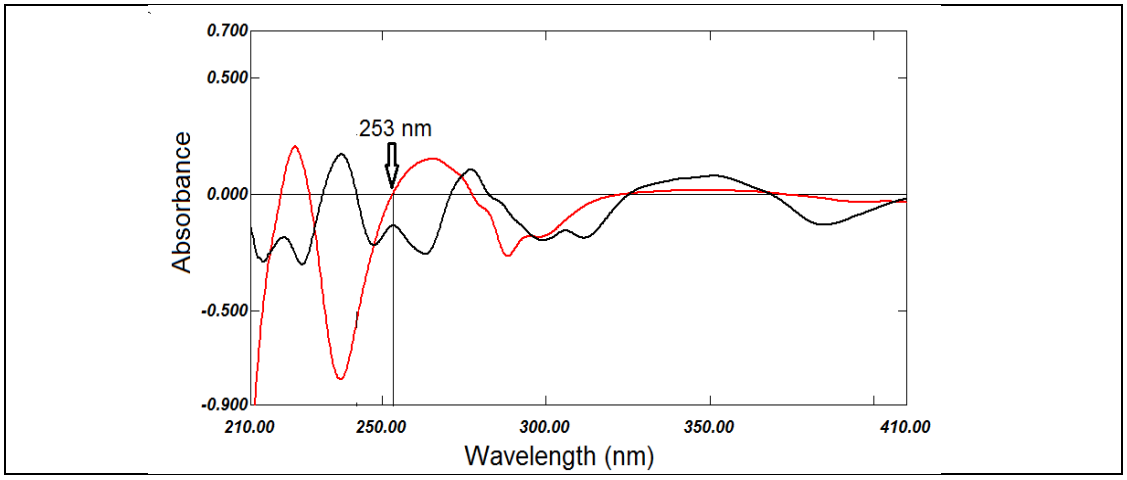

Figure 6. First derivative of the absorption spectra of intact rafoxanide (15 µg/mL) and of its degradation product (15 µg/mL)

interference. One-way ANOVA was applied for the purpose of comparison of developed methods, the results showed that there was no significant difference between the proposed methods for the determination of rafoxanide and the reported method (**Table 6**).

CONCLUSION

In conclusion, the described spectro-photometric methods have the advanteges of being simple, accurate and precise methods and can be applied for determination of rafoxanide in bulk, pharmaceutical formulation and in the presence of its degradation product without sample pretreatment and without interference from excipients or degradate. Moreover, the developed methods do not require sophisticated techniques or instruments and can be easily applied for routin analysis of the studied drug. Dual wavelength method was applied directly on zero order absorption spectra and it does not need any data processing or derivatization, so it considered as the simplest one, while area under the curve method seems to be the most sensitive, accurate and precise method and has the advantage of resolve the severe overlapping by simple mathematical processing.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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