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Xanthene based resonance Rayleigh scattering and spectrofluorimetric probes for the determination of cyclobenzaprine: Application to content uniformity test

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Abstract: In this work, a resonance Rayleigh scattering technique and a spectrofluorimetric technique were applied to the quantification of cyclobenzaprine through two validated methods. The suggested methods are based on a facile association complex formation between cyclobenzaprine and eosin Y reagent in the acidic medium. The resonance Rayleigh scattering method relied on the enhancement in the resonance Rayleigh scattering spectrum of eosin Y at 370 nm after the addition of cyclobenzaprine. On the other hand, the spectrofluorimetric quantification relied on the quenching effect of cyclobenzaprine on the fluorescence strength of the eosin Y reagent at 545 nm (excitation wavelength at 300 nm). The suggested methods were linear over the ranges of 0.07-1.75 μg/mL and 0.15-2.0 μg/mL with detection limit values of 0.023 μg/mL and 0.048 μg/mL for the resonance Rayleigh scattering method and the spectrofluorimetric method, respectively. All reaction conditions for cyclobenzaprine–eosin Y formation were experimentally evaluated and optimized. In addition, both methods were validated based on ICH rules. Furthermore, the developed methods were practically applied to the analysis of cyclobenzaprine in its commercial tablet dosage form with acceptable recoveries. Moreover, the content uniformity test of the commercial cyclobenzaprine tablets was successfully applied using the proposed spectroscopic methods based on USP rules.

Keywords: cyclobenzaprine, eosin Y, pharmaceutical analysis, resonance Rayleigh scattering, spectrofluorimetry

1 Introduction

Cyclobenzaprine hydrochloride (CZP) is structurally similar to the first generation of the tricyclic antidepressant drugs [1]. The chemical structure of CZP is shown in Figure 1. Medicinally, CZP primarily acts as a skeletal muscle relaxant drug and has been commonly prescribed for the treatment of neck pain, back pain, and myofascial spasm for more than thirty years [2,3]. The studied drug does not affect the function of the central nervous system when medically prescribed at the recommended therapeutic doses [4]. As a result, CZP diminishes the hyperactivity of motor-neurons without considerable ataxia [4]. From a survey of the literature we found that most of the reported methods for the determination of CZP were related to chromatographic techniques (HPTLC, TLC, HPLC, and GC methods) [1,5-13], while spectrophotometric [14-17] and electrochemical [18,19] techniques were reported less frequently. Chromatographic techniques can require large volumes of hazardous organic solvents, tedious analytical procedures, may be time consuming and some methods require expensive detectors. Only one spectrofluorimetric method was reported for the determination of the studied drug [16]. The reported spectrofluorimetric method was

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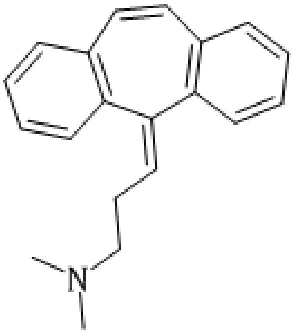

Figure 1: Chemical structure for cyclobenzaprine.

based on the ability of CZP to quench the fluorescence strength of a uranyl acetate reagent in the linear range of 1.0-10.0 μg/mL with a LOQ value of 0.850 μg/mL [16]. Eosin Y (a fluorescent reagent) is a member of the xanthene group of dyes and is a synthetic derivative of fluorescein, bearing four bromine atoms at positions 2', 4', 5', and 7' [20]. Recently, eosine Y has been utilized in the quantitative analysis of several pharmaceutical compounds such as asenapine, nicardipine, daclatasvir dihydrochloride, verapamil, albendazole, and β-blockers through spectrofluorometric or resonance Rayleigh scattering techniques [21-27]. Fluorescence spectrometry is a spectroscopic technique which can provide good selectivity, inherent simplicity and sensitivity without losing precision [28]. Therefore, in the presented work we aim to apply the resonance Rayleigh scattering technique for the first time for the determination of CZP either in pure form or in pharmaceutical tablets. In addition, we also aim to develop and validate a more sensitive spectrofluorimetric method for the determination of CZP compared to the reported spectrofluorimetric method [16]. The suggested resonance Rayleigh scattering method relies on measuring the enhancement in the resonance Rayleigh scattering of the eosin Y reagent after its reaction with CZP. By a reverse mechanism, the presented spectrofluorimetric method relies on measuring the quenching effect of CZP on the fluorescence strength of the eosin Y reagent. These methodologies have the merits of simplicity, low cost, selectivity, sensitivity, accuracy in their analytical procedure and require no extraction step. In addition, use of hazardous organic solvents was minimized in the analytical procedures of both methods. Both methods have been validated in compliance with ICH guidelines. The content uniformity test is a vastly important assessment of unit dosage form performance in pharmaceutical tablets [29]. No analytical method has been previously reported for testing the content uniformity (CU) of CZP in commercial tablets. Therefore, this is the first attempt to study the content uniformity in CZP pharmaceutical tablets according to USP guidelines.

2 Experimental

2.1 Apparatus

A Scinco (Serial No: FS-1304002) fluorescence spectrometer was applied throughout this study. All resonance Rayleigh scattering and spectrofluorimetric measurements were performed using a 5 nm slit width for the monochromators with a scanning speed of 1845 nm/min and a PMT voltage of 400 V.

2.2 Material, reagents, and standard solutions

Eosin Y (Merck, Darmstadt, Germany) was prepared in distilled water with a concentration of 3.7×10^{-5} M (0.024%g w/v) for both the spectrofluorimetric and the resonance Rayleigh scattering methods. A Teorell and Stenhagen buffer [30] was prepared and adjusted using a pH-meter to the required pH value. Cyclobenzaprine was kindly supplied from Global Napi Co. (Six-October City, Egypt). Multi-relax® 5 mg tablets and Multi-relax® 10 mg tablets produced by Multi-Apex Pharma Co. (Badr City, Egypt) were purchased from a local market with batch numbers of MT03370420 and MT04360620, respectively. Cyclobenzaprine standard solution was prepared via sonication of 10 mg of CZP powder with about 20 mL methanol solution in a 100-mL calibrated flask, which was then made up to the mark with distilled water.

2.3 Analytical procedures and the construction of calibration curves

Proper volumes (from 0.2 mL to 2 mL) of CZP solution, in the concentration ranges of 1.5-20.0 and 0.7-17.5 μg/mL were quantitatively added into 10.0-mL calibrated flasks for spectrofluorimetric methodology and resonance Rayleigh scattering methodology, respectively. One milliliter of Teorell and Stenhagen buffer (pH = 3.5) was added to all flasks followed by 1.0 mL and 0.8 mL of eosin Y solution (3.7×10^{-5} M) for spectrofluorimetric methodology and resonance Rayleigh scattering methodology, respectively. After five minutes, distilled water was added to the 10.0 mL mark and the flasks were shaken thoroughly. A reagent blank was prepared simultaneously at the same conditions for both methods. The resonance Rayleigh scattering method was synchronously scanned at $\lambda_{ex} = \lambda_{em}$ and calibrated by calculating the enhancement effect on resonance Rayleigh scattering spectrum (Δ IRRS) of the blank eosin Y solution at 370 nm (after subtracting the blank value) against the CZP concentration in µg/mL. The spectrofluorimetric method was calibrated by calculating the quenching effect on the fluorescence intensity of the eosin Y blank (Δ FI) at λ_{ex} = 545 nm (excitation wavelength = 300 nm) against the CZP concentration in µg/mL.

2.4 Procedures for the estimation of CZP in tablets

Twenty Multi-relax® 5 mg tablets were accurately weighed and finely powdered. A quantity tantamount to 20.0 mg of CZP from the powdered sample was weighed and quantitatively added into a 100-mL calibrated flask. The sample was dispersed in about 30 mL of methanol and sonicated at room temperature for 15 min. The flask was then filled up to the mark with methanol. The solution was filtered and diluted to the required concentrations with distilled water. In another preparation, ten Multi-relax® 10 mg pharmaceutical tablets were treated in the same manner. Procedures for the resonance Rayleigh scattering and the spectrofluorimetric methodologies were followed using five replicates.

2.5 Steps for content uniformity test

In this work, the content uniformity test was performed based on USP rules (Chapter 905) [31]. The test was achieved through the individual analysis of ten Multi-relax® 5 mg pharmaceutical tablets and ten Multi-relax® 10 mg pharmaceutical tablets by the current spectroscopic methodologies.

3 Results and discussion

As is known from reported studies, the resonance Rayleigh scattering spectrum of xanthene dyes such as fluorescein, erythrosin B, and eosin Y can be greatly enhanced via the formation of an association complex with some basic compounds [25-27,32,33]. In this study, the proposed methods were based on the association complex formation between CZP (containing one basic center, Figure 1) and the eosin Y reagent, giving a colored complex (Figure 2). The resonance Rayleigh scattering spectra of eosin Y, CZP and their formation product at pH 3.5 are shown in Figure 3. Figure 3 shows that the intensity for both the eosin Y and CZP spectra is very weak. However, their reaction with each other via the virtue of electrostatic and hydrophobic forces leads to a great improvement in the resonance Rayleigh scattering intensity and the appearance of a peak at 370 nm. Consequently, CZP can be quantitatively determined using the resonance Rayleigh scattering technique (Figure 3). By an opposite mechanism, the eosin Y reagent has also been applied to the quantitative estimation of some basic lipophilic compounds via quenching of its fluorescence strength [21-26,34]. Consequently, CZP (log P = 5.2, [35]) has the ability to quench the fluorescence strength of the eosin Y reagent at 545 nm (excitation wavelength = 300 nm) and can be estimated via the spectrofluorometric technique (Figure 4).

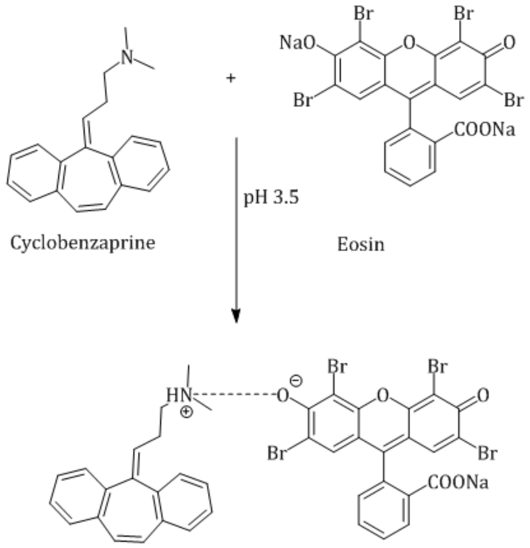

Figure 2: Association complex formation between cyclobenzaprine and eosin Y in the acidic medium.

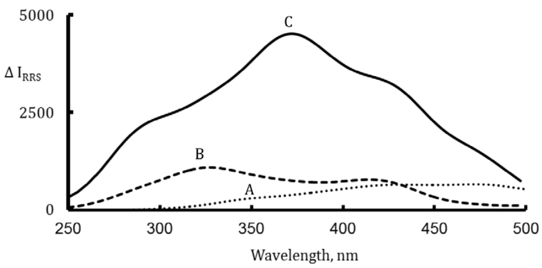

Figure 3: RRS spectra at pH 3.5: (A) for 3.0 µg/mL cyclobenzaprine, (B) for eosin Y (2.96×10^{-6} M), (C) for the reaction product between eosin Y and cyclobenzaprine (1.5 µg/mL).

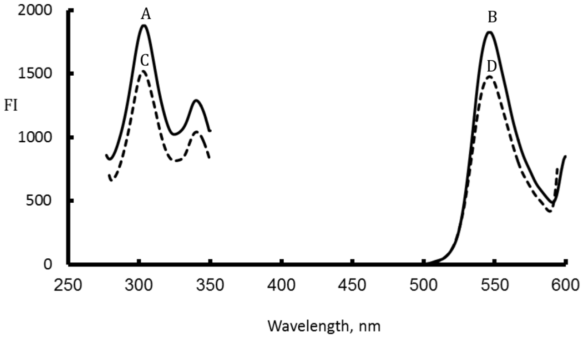

Figure 4: Fluorescence spectra for eosin Y (3.7×10^{-6} M, pH 3.5): (A,B) excitation and emission spectra for eosin Y, (C,D) excitation and emission spectra for eosin Y after its reaction with cyclobenzaprine (1.5 µg/mL).

3.1 Mechanisms for the resonance Raleigh scattering enhancement

3.1.1 The molecular volume enlargement

According to the Rayleigh scattering equation, the intensity of resonance Rayleigh scattering is definitely influenced by increasing the molecular volume. When the molecular volume, could not be easily calculated, the molecular weight can be used instead, by using Eq. 1 [33]:

$$I = KCMI_0 \tag{1}$$

where: I represents the intensity of resonance Rayleigh scattering, M represents the molecular weight, while I_0, K, and C represent the incident light intensity, a coefficient, and concentration, respectively. If all parameters in the previous formula are kept constant, the intensity of resonance Rayleigh scattering will be in a direct proportionality with the molecular weight (M). In the present work, it was observed that increasing the compound molecular weight from 275.39 (CZP+) to 923.28 (CZP-eosin Y) resulted in an augmentation of the intensity of resonance Rayleigh scattering of the solution which was obtained from the reaction of cyclobenzaprine with eosin Y in slightly acidic solution.

3.1.2 Hydrophobic interface formation

The signal of resonance Rayleigh scattering has a direct link to the hydrophobicity of the molecule [36]. Initially CZP is present as positively charged ions (CZP+) whereas eosin Y dye (acid dye) is negatively charged. Thus the ions of the drug and the dye could be easily hydrated with water molecules, therefore, both would have very low resonance Rayleigh scattering intensity. After the interaction of CZP with eosin Y with each other, an ion-pair associate will be formed and neutralization of both ions will occur. Consequently, a hydrophobic interface is produced between the formed complex and the aqueous solution.

3.1.3 Rigidity and molecular planarity effect

The interaction of the negatively charged dye and positively charged drug ions to form the binary complex, also resulted in the restriction of the movement of the aryl group because of the structure becomes more rigid, the molecule would be highly planar in addition to the increase in the molecular volume. All these phenomena induce a great augmentation in the intensity of scattering for the resultant solution [37].

3.2 Experimental conditions

Various reaction conditions that influence either the enhancement in the resonance Rayleigh scattering spectrum of the eosin Y reagent or the quenching effect on the fluorescence intensity of eosin Y were studied and optimized to achieve the best results for the proposed spectroscopic methodologies.

3.2.1 pH of the medium

The effect of pH on the binary complex formation between CZP and eosin Y was investigated for both methods in the pH range of 2.6-4.6. It was found that CZP-eosin Y complex formation was highly affected by the variation in pH value of the medium. The highest values for Δ IRRS and Δ FI were achieved in the pH range 3.2-3.8 for both the suggested spectroscopic methods. pH values above or below this range led to lower Δ IRRS and Δ FI values, thus the chosen pH value in this work was 3.5 for both spectroscopic methodologies (Figure 5).

3.2.2 The volume of eosin Y solution

Optimization of the eosin Y volume to achieve the highest values for the resonance Rayleigh scattering enhancement or the fluorescence quenching effect were evaluated. It was found that 0.8 and 1.0 mL of 3.7×10^{-5} eosin Y solution were the most suitable volumes to give the highest fluorescence quenching effect for the spectrofluorimetric method and highest resonance Rayleigh scattering enhancement values for the resonance Rayleigh scattering method, respectively (Figure 6). The reaction would be incomplete if the eosin Y concentration was lower. In agreement with the reported data [33], we found that concentrations of xanthene dye higher than the reported level leads to self-aggregation of the eosin Y self-aggregation, minimizing the studied intensities.

3.2.3 Order of addition and time of the reaction

CZP should be protonated firstly in an acidic medium (pH = 3.5) to facilitate the binary complex formation with the xanthene dyes [22]. Therefore, the selected addition order for the current work was CZP-solution first, then buffer, then lastly the eosin Y solution. At room temperature (25 ± 3), the CZP-eosin Y complex was formed immediately on mixing the solutions.

3.2.4 Diluting solvent

Various diluting solvents such as acetonitrile, distilled water, DMF, ethyl alcohol and DMSO were tested. It was observed that the best resonance Rayleigh scattering enhancement and fluorescence quenching effect values were obtained with distilled water (also the most environmentally friendly solvent) for both methodologies.

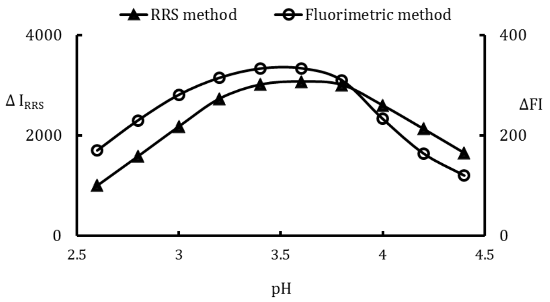

Figure 5: Effect of pH on the fluorescence quenching effect and RRS enhancement of the association complex formation between cyclobenzaprine (1.0 µg/mL) and eosin Y.

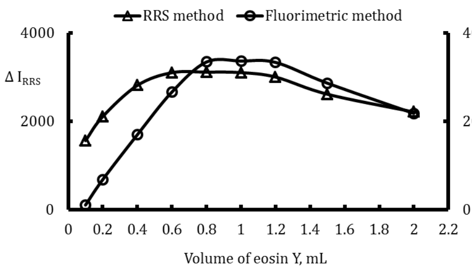

Figure 6: Effect of eosin Y volume (3.7×10^{-5} M) on the fluorescence quenching effect and RRS enhancement of the association complex formation with cyclobenzaprine (1.0 µg/mL).

3.3 Methods validation

The two developed analytical methodologies were assessed in line with ICH guiding principles [38]. The study included examining the linear range, accuracy, precision, method robustness, the detection limit (LOD), and the qauntitation limit (LOQ).

3.3.1 Linearity, ranges, and analytical parameters

A series of standard solution were assayed by the general procedure and the values of either Δ FI or Δ IRRS were plotted versus CZP concentration in µg/mL to build up the corresponding calibration curves for the spectrofruorimetric or resonance Raylieg scattering the methods, respectively. The linearity of the developed methods was in the range of 0.7-1.75 and 0.15-2.0 µg/mL for both methods, respectively. Furthermore, the data were analyzed by least square curves fitting and the different statistical data were computed and listed in Table 1. For estimating the values of LOD and LOQ, the next two formula were employed [38]:

$$LOD = 3.3\ \mathrm{Sa/b} \quad (2)$$

$$\text{and LOQ} = 10\ \mathrm{Sa/b} \quad (3)$$

Table 1: Analytical parameters for the determination of CZP by the proposed spectroscopic methods

Parameter	Fluorimetric method	RRS method
Linear range (µg/mL)	0.15-2.0	0.07-1.75
Intercept	16.57	20.71
Standard deviation of intercept (S_a)	4.69	20.81
Standard deviation of residuals ($S_{y/x}$)	6.72	30.33
Slope	318.32	3001
Standard deviation of slope (S_b)	4.33	19.8
Number of determinations	6	6
Correlation coefficient (r)	0.9996	0.9996
Determination coefficient (r^2)	0.9992	0.9993
Limit of quantitation (µg/mL)	0.147	0.069
Limit of detection (µg/mL)	0.048	0.023

where: *b* refers to the calibration curve slope and *Sa* refers to intercept standard deviation. The resultant detection limit values were 0.023 and 0.048 µg/mL for the resonance Rayleigh scattering and the spectrofluorimetric methods, respectively, while the resultant quantitation limit values were 0.069 and 0.147 µg/mL for the respective methods.

3.3.2 Accuracy and precision parameters

The accuracy for both methods was inspected at three concentration levels for each method using (recovery ± SD) to examine the intended parameter. Results shown in Table 2 confirmed the sufficient degree of accuracy of the developed methodologies. In a similar way, the inter-day precision and intra-day precision were examined at the same concentration levels for each method. The values of RSD were calculated and employed in the evaluation of the precision. The values of RSD shown in Table 3 indicated the appropriate precision as the values did not exceeded 2%, These results prove the reliability of the developed methods.

Table 2: valuation of accuracy for the suggested spectroscopic methods

Method	Conc. (µg/mL)	% Recovery ± SD[a]
Fluorimetric	0.5	101.01 ± 1.58
	1.0	98.99 ± 1.61
	1.5	100.90 ± 1.15
RRS	0.5	98.70 ± 1.50
	1.0	101.72 ± 0.84
	1.5	101.98 ± 1.18

[a]: Mean of three determinations, SD: Standard deviation

Table 3: Evaluation of inter-day precision and intra-day precision for the suggested spectroscopic methods

Method	Conc. (µg/mL)	Inter-day precision	IntraIntra -day precision
		% Recovery ± RSD[a]	% Recovery ± RSD[a]
Fluorimetric	0.5	101.01 ± 1.57	98.08 ± 1.96
	1.0	100.36 ± 1.13	101.4 ± 1.46
	1.5	101.88 ± 1.44	98.32 ± 1.3
RRS	0.25	98.81 ± 1.03	101.81 ± 1.07
	0.75	101.0 ± 1.37	100.84 ± 1.87
	1.25	99.87 ± 0.97	99.91 ± 1.74

[a]: Mean of three determinations, RSD: Relative standard deviation

3.3.3 Robustness

The robustness for the suggested spectroscopic methods was evidenced by investigation of the influence of minor changes such as pH change (3.5 ± 0.1) and changes in the volume of eosin Y solution for both approaches. It was found that these minor changes did not produce any significant effect on the resonance Rayleigh scattering enhancement or the fluorescence quenching effect values which prove the robustness for the suggested methodologies.

3.4 Application to pharmaceutical tablets

Commercial tablet formulations (Multi-relax® 5 and 10 mg tablets) containing CZP were assayed by the proposed approaches. The estimated recovery percentages for Multi-relax® 5 mg tablets were 101.83 and 101.37 using the resonance Rayleigh scattering and the spectrofluorimetric methods, respectively. While, the values in the case of Multi-relax® 10 mg tablets were 101.80 and 100.98 for both methods, respectively. These Multi-relax® 5 and 10 mg tablets were assayed also by another reported method [15], and the obtained recovery percentages were statistically compared with that of both proposed methods. The values of the student's t- and variance ratio F- tests were estimated and compared with the theoretical values at the 95% confidence interval. It was observed that, the estimated values of both parameters were lower than the tabulated one. This is considered as a sign of the absence of any variation in the accuracy and precision between the reported and developed methods in the sassy of CZP (Table 4). Moreover, the interval hypothesis was applied to compare the results of the developed and reference methods [39,40]. As the estimated values of t- (paired) and F- tests at the 95% confidence interval did not exceed the tabulated ones, it was proved that the tested methods are sufficiently accurate and precise. The true bias in the test of interval hypothesis was assessed through applying Eq. 4 [39]:

$$\theta^2\left[x_1^2-\frac{Sp^2t^2}{n_1}\right]-2\theta x_1x_2+\left[x_1^2-\frac{S_p{}^2t^2}{n_2}\right]=0 \tag{4}$$

The following two equations were applied to estimate the lower limit (θ_L) and upper limit (θ_U):

$$\theta_U=\frac{-b+\sqrt{b^2-4ac}}{2a} \tag{5}$$

$$\theta_L=\frac{-b-\sqrt{b^2-4ac}}{2a} \tag{6}$$

where:

$$a = (x_1^2 - S_p{}^2 t^2 / n_1) \tag{7}$$

$$b = 2x_1 x_2 \tag{8}$$

$$a = (x_2^2 - S_p{}^2 t^2 / n_2) \tag{9}$$

where: x_1 and n_1 are the mean and number of measurements obtained by the suggested method, respectively. The same values for the reported, method are denoted x_2 and n_2. S_p is the pooled standard deviation and t is one-sided t-test using confidence level of 95%. The values of the lower (θ_L) and upper (θ_U) limits for the determination of CZP in Multi-relax® 5 mg and 10 mg tablets are demonstrated in Table 4.

3.5 Application to content uniformity test

It is recommended to investigate the uniformity of content for the tablet formulation units if the percentage of their active constituents does not exceed 25% of the total weight of the tablet or if the content of the active constituent is less than 25 mg [31,41,42]. The contents of CZP in Multi-relax® tablets are 5 and 10 mg. Consequently, the uniformity of Multi-relax® 5 mg tablets and Multi-relax® 10 mg tablets units can be evaluated by the content uniformity test as stated in the USP guidelines [31]. If a complicated analytical procedure is used, the steps for performing the test of content uniformity will be time consuming and laborious. In the two presented methods, the procedures are very simple and so both methods are appropriate for this purpose. To consider tablet formulations to be uniform according to USP guiding rules, the acceptance value (AV) of ten individually analyzed tablets should not exceed the maximum allowed acceptance value (L1). The AV values of the two studied tablet formulations could be estimated by employing the next equation:

$$AV = KS + |M - \bar{x}| \tag{10}$$

where: S, K, $\bar{x}$, and M are the standard deviation, acceptability constant, mean of individual tablet contents, and reference value, respectively. As shown in Table 5 the estimated values of AV for the examined tablet formulations did not exceed L1.

Table 4: Estimation of CZP in pharmaceutical tablets (Multi-relax® tablets) by the proposed spectroscopic methods

Method	Tablets	%Recovery[a] ± SD	t-value[b]	F-value[b]	θ_L	θ_U
Fluorimetric	5 mg	101.83 ± 1.53	1.2	4.59	1.004	1.025
RRS		101.37 ± 1.13	0.76	2.51	1.003	1.016
Reported		100.39 ± 2.42				
Fluorimetric	10 mg	101.80 ± 1.67	1.65	3.05	1.002	1.029
RRS		100.98 ± 1.06	2.14	1.22	1.004	1.044
Reported		99.41 ± 1.85				

[a]: Average of five determinations

[b]: Tabulated value at 95% confidence limit; F = 6.338 and t = 2.306.

Table 5: Content uniformity test for CZP in its commercial tablets by the proposed spectroscopic methods

Parameter	Multi-relax® 5 mg tablets		Multi-relax® 10 mg tablets	
	Fluorimetric method	RRS method	Fluorimetric method	RRS method
Recovery	97.28	103.94	101.17	104.28
	102.68	95.91	103.09	102.98
	100.24	102.28	106.29	104.44
	97.18	103.71	99.66	103.28
	102.10	103.11	104.50	102.58
	103.65	101.78	102.43	105.27
	104.63	104.74	101.42	95.18
	103.62	102.94	101.14	102.94
	104.16	101.94	102.15	101.94
	104.55	104.38	103.34	104.38
Mean ($\bar{X}$)	102.53	102.31	102.52	102.73
S.D	2.44	2.61	1.90	2.84
Acceptance value	6.05	5.07	4.64	8.35
Maximum allowed acceptance value		15		

4 Conclusion

In the acidic aqueous solution, cyclobenzaprine can be reacted with eosin Y to give an ion-pair associate throughout the hydrophobic and electrostatic forces. Depending on this interaction a new fluorimetric and resonance Rayleigh scattering protocols were built up and systematically evaluated for the assay of cyclobenzaprine The two methods are selective, sensitive, simple, and rapid. Additionally, the steps for sample cleanup in the two methods are very simple and did not involve any boring step. In addition, the measurements was carried out in aqueous solution which make the procedures are more environmentally safe. Finally, the developed methods were efficiently employed for the analysis of commercial tablets that contain cyclobenzaprine, and the content uniformity of the cited formulations were tested.

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