Original Research Article

Enhanced spectrofluorimetric determination of the multitargeted tyrosine kinase inhibitor, crizotinib, in human plasma via micelle-mediated approach

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Abstract

Purpose: To quantify the multi-targeted tyrosine kinase inhibitor, crizotinib, in human plasma and bulk powder by highly sensitive micellar enhanced spectrofluorimetric procedure.

Method: The developed procedure was based on measuring the fluorescence intensity of crizotinib (CRZ) in sodium dodecyl sulphate (SDS) micellar system at 404 nm after excitation at 271 nm. Validation of the developed procedure was carried out following ICH (International Council for Harmonization) guidelines.

Results: Maximum fluorescence intensity (FI) was attained by addition of 0.2 mL SDS and 0.2 mL HCl (1N) to CRZ aliquots and then dilution with distilled water. There was a linear relationship between the FI of CRZ and its concentration over the range, 5 – 400 ng/mL, with limit of detection and of quantification of 1.857 and 5.628 ng/mL respectively. The developed procedure was successfully applied to assay CRZ in pure powder form and spiked human plasma with mean recovery of 100.68 ± 0.37 and 99.98 ± 0.20 %, respectively.

Conclusion: The developed procedure is simple and sensitive, and can be applied to routine analysis of CRZ in pure powder form as well as in clinical laboratories for the determination of CRZ in plasma.

Keywords: Crizotinib, Spectrofluorimetry, Micelle, Human plasma, Sodium dodecyl sulphate

INTRODUCTION

Crizotinib (CRZ, Figure 1) is one of the new tyrosine kinase inhibitors (TKIs) which is chemically known as 3-[(1R)-1-(2, 6-dichloro-3-fluorophenyl) ethoxy]-5-(1-piperidin-4-ylpyrazol-4-yl) pyridine-2-amine. CRZ binds to the ATP-binding pocket of the correspondent kinase in a competitive manner [1-4]. Its targeting kinases include anaplastic lymphoma kinase (ALK), mesenchymal-epithelial transition factor (MET), and other tyrosine kinases.

It is used effectively in patients suffering from non-small cell lung cancer (NSCLC). Food and Drug Administration (FDA) has approved CRZ in 2011 for the management of NSCLC due to its success in two clinical trials [5]. CRZ is available in the market under the name of Xalkori® capsule and is manufactured by Pfizer, Inc. The absorption of CRZ is not influenced by foodstuff and its maximum plasma concentration attained after 4 h following administration of one dose. Currently, a binary combination of CRZ and

dasatinib is under evaluation for treating high-grade glioma (HGG) or diffuse intrinsic pontine glioma (DIPG). The positive results of using this binary combination may involve pharmacokinetic interaction between the two drugs as CRZ is considered an inhibitor of CYP3A. Unfortunately, CRZ pharmacokinetic disposition, in infants suffering from malignant brain tumors, is not known till now. Accordingly, reliable and accurate analytical methodology for CRZ determination is crucial for further pharmacokinetic studies. Literature review revealed that CRZ was assayed in plasma by liquid chromatography coupled with either tandem mass spectrometry [6-9] or fluorescence detection [10]. However, these chromatographic methods suffered from complexity and being expensive. Few spectrophotometric methods have been reported for determination of CRZ in dosage form [11-13]. Thus development of simple and sensitive analytical methodology for CRZ determination in plasma is demanded. Accordingly, in the current study a rapid, sensitive and reliable spectrofluorimetric method was conducted for the assay of CRZ in plasma. The present method involved micellar enhancement of FI of CRZ utilizing SDS as a sensing agent. Application of micellar enhancement approach in spectrofluorimetry was applied to assay small molecules by virtue of its high sensitivity [14-16] due to the ability of the formed micelle to decrease the free rotational motions and thus protect the analyte molecules from the radiationless deactivation process [16]. Additionally, this approach represents effective and echo-friendly methodology because there is no application of organic solvents throughout the whole study.

EXPERIMENTAL

Reagents and materials

Solvents utilized in this study were of HPLC grade (Merck, Darmstadt, Germany) while chemicals were of analytical grade. Reference powder of CRZ (purity ~ 99.6 %) was procured from Weihua Pharma Co. Ltd (Zhejiang, China). SDS (Sodium dodecyl sulphate; 95 %) was purchased from Winlab (Pontefract, London, UK) and prepared as 1 % aqueous w/v. Cremophor RH 40 and Cremophor EL were procured from BASF (Ludwigshafen, Germany) and prepared as 1 % v/v aqueous solutions. Both β–CD (β–cyclodextrin) and CMC (carboxymethylcellulose) were procured from Merck (Darmstadt, Germany) and prepared as 1 % w/v aqueous solutions. Tween 20, tween 80 and tween 85

were procured from Techno Pharmchem Haryana Company (New Delhi, India) and prepared as 1 % v/v aqueous solution. Ethanol and methanol were purchased from VWR Prolabo (Fontenay Sous Bois, France) and acetonitrile was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). 0.1 M Phosphate buffer and 0.1 M borate buffer, covering the pH ranges 2–12 were freshly prepared and ultrapure water was acquired through a Millipore Milli-Q® UF Plus water purifier (MA, USA). Plasma samples were obtained from King Khaled University Hospital (KSU, Riyadh, Saudi Arabia). All patients provided written informed consent, then fasting blood specimens were collected followed by plasma separation and storage at -70°C.

Instrumentation

Normal fluorescence measurements were recorded utilizing Jasco FP-8200 Fluorescence Spectrometer (Jasco Corporation, Japan) fitted with a xenon lamp (150 W) and standard 10 mm pathlength quartz cell with bandwidth of 5 nm for both excitation and emission monochromators. Quinine sulphate solution (0.01 μgmL^{-1}) was frequently utilized for calibration of Fluorescence Spectrometer. Data acquisition was executed by SpectraManager® software. pH measurements were done utilizing Hanna pH-Meter (Romania).

Preparation of standard stock and working solutions

Accurately quantity of CRZ (25 mg) was weighed and transferred into a 25 mL measuring flask, dissolved in methanol and made up t to mark with same solvent to prepare a stock solution of CRZ (1 mg/mL). CRZ stock solution was diluted afterwards with methanol to prepare working standard solution of 1 µg/mL. For fourteen days, these standard solutions were stable when stored in the refrigerator.

Sample preparation and generation of calibration curve

Calibration samples were prepared by accurately transferring aliquots of CRZ standard solutions to five mL calibrated flasks followed by addition of 0.2 mL of SDS and 0.2 mL of HCl (1 N) and completing volume by ultrapure distilled water to yield final concentrations of 5 - 400 ng/mL. Mixing of the flasks' contents took place followed by recording FI at 404 nm after excitation at 271 nm. Calibration curve was generated by plotting FI of calibration samples versus CRZ concentrations in ng/mL. Finally, the regression equation was computed and utilized to calculate

CRZ concentrations in different samples depending on their FI.

Analysis of human plasma samples

Accurate volume (20 µL) of standard solutions of CRZ (correspond to different concentrations of CRZ)) were spiked individually to a one mL portion of free drug human plasma and mixed well for 1 min to yield CRZ final concentrations of 150, 200 and 250 ng/mL. Thereafter, addition of 1 mL of NaOH (100 mM) /glycine buffer pH 12 and the Eppendorf tube was vortexed for 10 s. Afterwards, 5 mL of diethyl ether was added Tube was then vortexed once again for 30 s and then centrifuged at 10,000 rpm for 15 min for complete phase separation. Accurate volume of the upper organic layer was transferred to glass vials and dried using gentle stream of nitrogen. Ultimately, reconstitution of the residue took place in methanol and general steps of calibration curve generation were followed. Blank plasma samples were treated and prepared in a similar way. FI of CRZ was measured at the emission wavelength (404 nm) after excitation at 271 nm, then concentrations of CRZ were calculated from calibration graph of the drug constructed in plasma matrix.

RESULTS

In the current study, micellar enhanced spectrofluorimetric method was developed for assaying CRZ (Figure 1) in plasma. This approach is characterized by the simplicity and sensitivity of spectroscopic technique that enables one to quantify CRZ in plasma. Hence, this approach is considered a typical alternative to the reported chromatographic methods for quantitation of CRZ in plasma. Different parameters affecting FI of CRZ were studied and optimized using one factor at a time in the optimization studies. Fluorescence spectra of CRZ in water and SDS micellar medium are displayed in Figure 2.
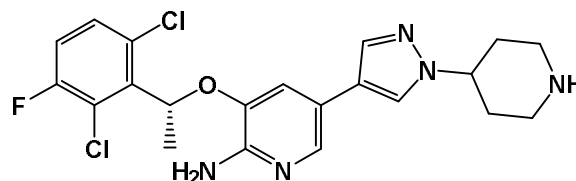
Figure 1: Chemical structure of Crizotinib (CRZ)

Experimental conditions optimization

Several experiments were performed to optimize the different parameters that affect the FI of CRZ. First of all, different types of organized media were added to aliquots of CRZ solution to inspect their effect on CRZ response. These organized media included SDS as an example for anionic surface active agent,
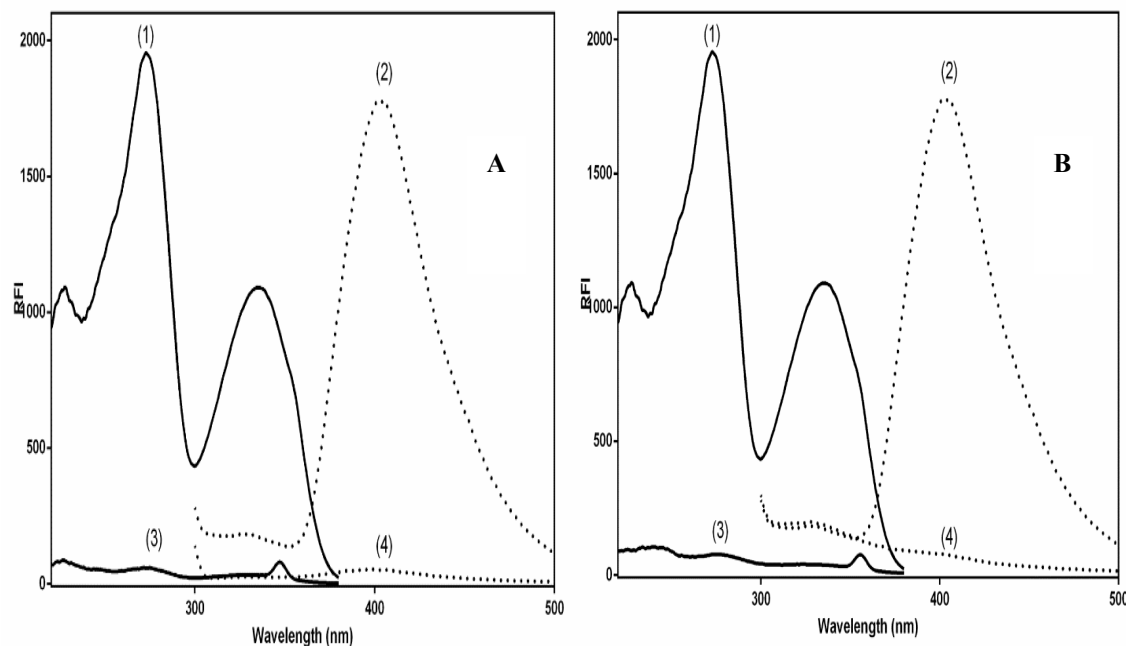
Figure 2: Excitation (1) and emission (2) spectra of CRZ (100 ng/mL) in SDS (1 %, w/v); (A) excitation (3) and emission (4) spectra of CRZ (100 ng/mL) in water; (B) excitation (3) and emission (4) spectra of SDS (1 %, w/v) in water

tween 20, 80 and 85, Cremophor El, Cremophor RH 40 as examples for nonionic surface active agents in addition to carboxymethyl cellulose (CMC), and macromolecules such as β-cyclodextrin. Accordingly, 0.5 mL of various organized media was added to CRZ standard solution. The best result was acquired by addition of SDS as seen in Figure 3.

Different volumes of 1 % w/v aqueous solution of SDS were added to CRZ standard solution. Figure. 4 anticipated that maximum FI of CRZ was attained by addition of 0.2 mL SDS. Any further increase in volume of SDS did not affect the FI of the cited drug and as a result, 0.2 mL 1 % w/v SDS solution was chosen throughout the study.

The effect of pH was also studied by adding different buffers to CRZ solution. The whole pH range was covered utilizing HCl, 0.1 M borate buffer and 0.1 M borate buffer individually. The data showed that maximum FI was recorded utilizing 1 N HCl (pH < 1) and on increasing pH, the FI decreased markedly as anticipated in Figure 5.

From these data, it was believed that at low pH, CRZ is in the cationic form and thus interact favorably with the SDS negative sulphonyl (OSO_3^-) group. Calculations carried out by Chemicalize [17] supported this thought which pointed out that the CRZ acquired maximum positive charge at low pH which in turn reflects that the cationic form of CRZ is the dominant form. The suggested interaction between CRZ and SDS is displayed in Scheme 1.
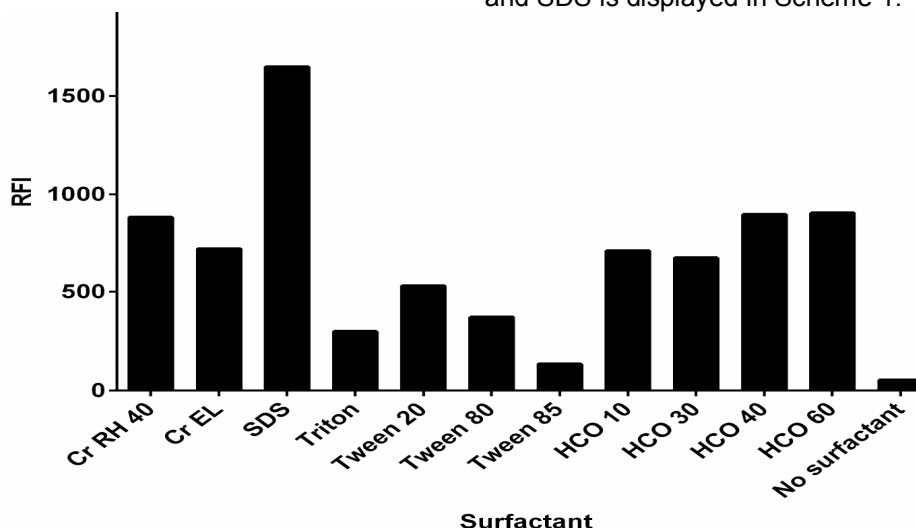

Figure 3: Effect of the type of organized media (0.2 mL, 1 % w/v solution of each) on fluorescence intensity of CRZ (100 ng/mL)
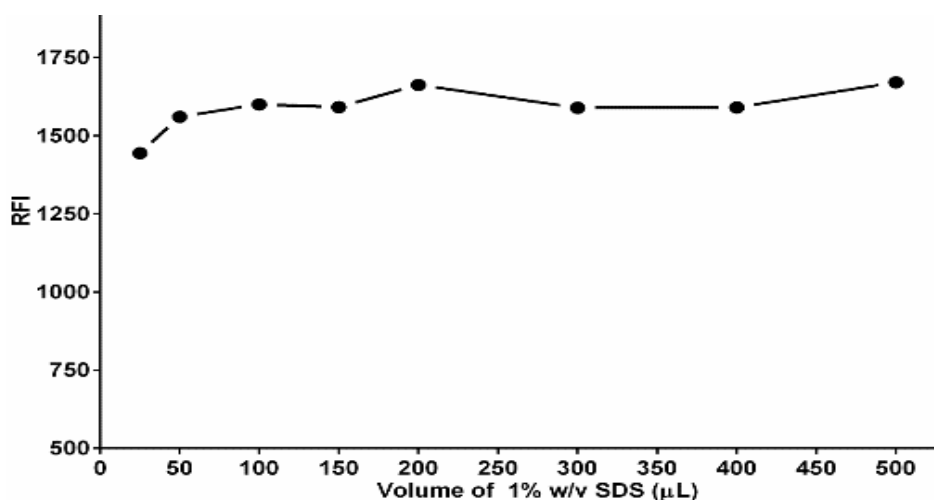

Figure 4: Effect of Sodium dodecyl sulfate (SDS) volume (1 % w/v) on fluorescence intensity of CRZB (100 ngmL^{-1})
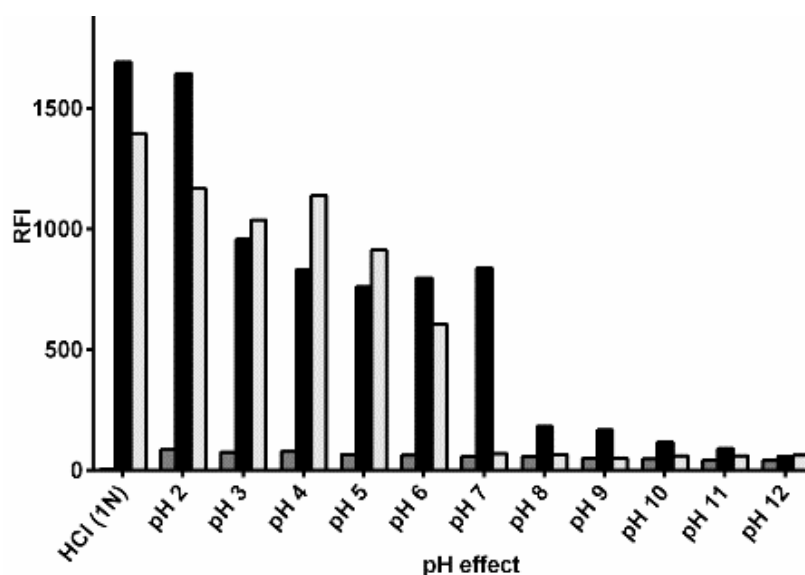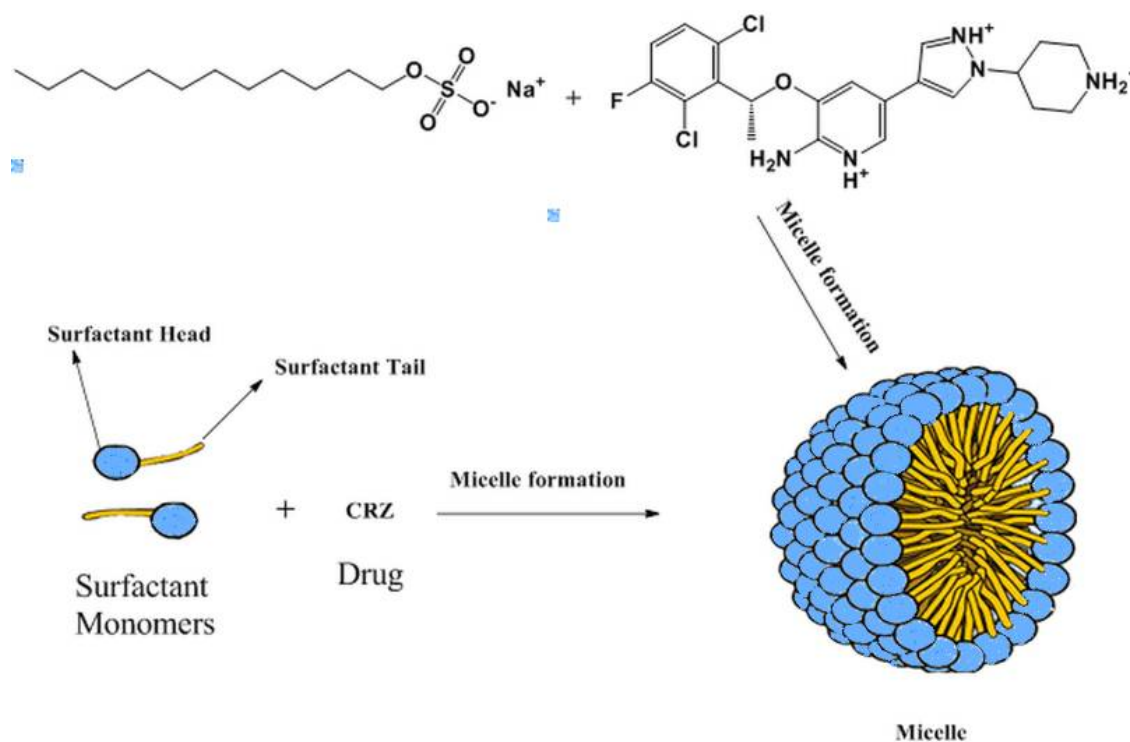
Figure 5: Effect of pH on the FI of 100 ng/mL CRZ in 0.2 mL 1 %, w/v Sodium dodecyl sulfate (SDS) solution in water (black column CRZ with surfactant, white column CRZ without surfactant and gray column surfactant only)

Scheme 1: Suggested mechanism for CRZ-SDS micelle formation

Ultimately, the factor of dilution was taken into account by adding various solvents such as; water, methanol, ethanol and acetonitrile. The response was almost equal utilizing different solvents as displayed in Figure 6. Accordingly, water was used for dilution to increase the greenness of the proposed method. The stability of FI of CRZ was also studied versus time and it was revealed that the fluorescence property of CRZ in SDS micellar medium was developed immediately and remained stable for at least 60 min.

Validation results

Linearity and sensitivity

Method linearity was confirmed by generation of the calibration curve through plotting the FI of CRZ, as y-axis, against the corresponding amounts of CRZ in ng/mL, as x-axis. Linearity of CRZ in organized medium (SDS) ranged between 5 and 400 ng/mL as depicted in Table 1. Statistical results [18] were also displayed in Table 1 showing minimal values of; standard

deviation of slope, standard deviation of intercept and standard deviation of residuals in addition to low values of % RSD and % error for triplicate samples. Additionally, determination coefficient value was almost equal unity. These above-mentioned values confirmed the linearity of the adopted method. Additionally, method linearity was checked by plotting ratio of fluorescence intensity- CRZ concentration (as y-axis) against log CRZ concentration (as x-axis) as shown in Figure 7 [19].

For assessment of method sensitivity, limits of quantification (LOQ) and detection (LOD) were found to be 5.628 and 1.857 respectively. LOQ and LOD were calculated utilizing the following equations:

$$LOQ = 10(\rho/n)\dots\dots\dots\dots\dots\dots\dots (1)$$

$$LOD = 3.3(\rho/n)\dots\dots\dots\dots\dots\dots\dots (2)$$

where, ρ and n are the standard deviation of intercept and slope of regression line slope, respectively. The results are displayed in Table 1.

Literature [20] revealed that peak plasma concentration of CRZ is 274 ng/mL, which is quite higher than the LOQ of our adopted method, and therefore, determination of CRZ in human plasma samples can be proceeded reliably.
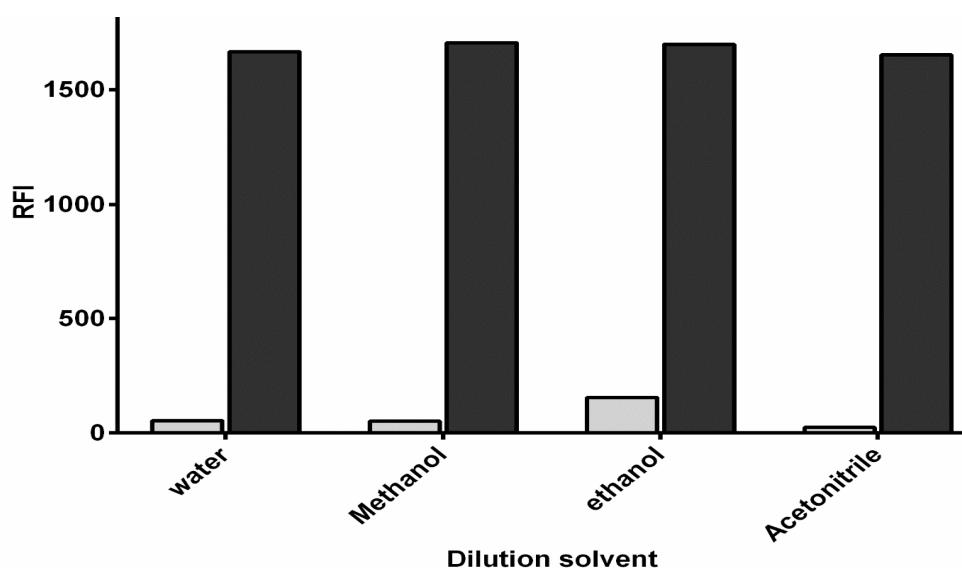

Figure 6: Effect of the diluting solvent on fluorescence intensity of CRZ (100 ng/mL); white column without surfactant and black column with surfactant
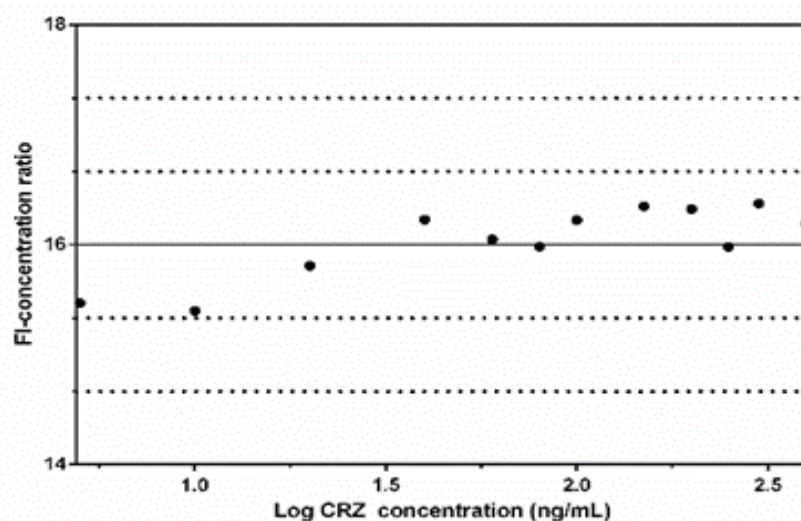

Figure 7: Validation of linearity of the analytical method of CRZ

Table 1: Analytical performance data for the spectrofluorimetric determination of CRZ

Parameter	CZB
Wavelength [λ_{ex}/ λ_{em}] (nm)	271/404
Linearity range (ng/mL)	5-400
Intercept *(a)*	- 5.810
Slope *(b)*	16.24
Correlation coefficient *(r)*	0.9999
S.D. of residuals ($S_{y/x}$)	36.90
S.D. of intercept (S_a)	9.140
S.D. of slope (S_b)	0.0502
% RSD[a]	0.355
% Error[b]	-0.699
LOD (ng/mL) [c]	1.857
LOQ (ng/mL) [d]	5.628

[a] % Relative standard deviation (n = 3); [b] % relative error for three replicate samples; [c] limit of detection; [d] limit of quantitation

Accuracy and precision

Accuracy, intra- and inter-day precisions were computed following the guidelines stated by International Conference on Harmonization (ICH) for validation of analytical procedures [21]. Results of accuracy and precisions were displayed in Tables 2 and 3. For calculation of intra- and inter-day precisions, triplicate samples corresponding to four different concentrations of CRZ were assayed in one day and in three different successive days respectively.

Precisions were labelled in terms of percentages recovery. Average percentage recovery was around 100 % and RSD values were low (Table 3). Accuracy was described in terms of % RSD and standard error of mean (SEM). The small values of both % RSD and SEM were indicative for the accuracy of the current spectrofluorimetric method.

Robustness

Table 4 summarized the results of the method robustness by evaluating the liability of determinations to deliberate changes of the experimental conditions. It was found that small changes that possibly occur during the experimental runs will have no significant effect on the fluorescence intensity of the cited drug. The temperature values were kept constant by the aid of thermostatic water bath.

Selectivity

Method selectivity was evaluated by assaying CRZ in plasma (as an example for biological matrix). Table 5 displayed the results for analysis of plasma samples, indicating the selectivity of the suggested procedure as there was no observed interference from endogenous plasma components.

Table 2: Accuracy of the suggested spectrofluorimetric method for assaying CRZ

Day	Actual Conc. (ng/mL)	Mean Conc. (ng/mL)	± SD	% Nominal	% RSD	SEM
1	10	10.07	0.316	100.72	3.136	0.182
2	10	10.12	0.154	101.24	1.523	0.089
3	10	10.07	0.275	100.66	2.735	0.159
1	100	99.24	1.475	99.24	1.487	0.852
2	100	100.86	1.326	100.86	1.315	0.766
3	100	99.52	0.869	99.52	0.873	0.501
1	200	200.7	0.945	100.35	0.471	0.545
2	200	200.71	0.741	100.36	0.369	0.428
3	200	203.27	0.71	101.64	0.349	0.41
1	300	301.06	1.071	100.35	0.356	0.618
2	300	307.48	3.16	102.49	1.028	1.824
3	300	304.11	2.103	101.37	0.692	1.214

SEM = standard error of the mean; 1, 2 and 3 represents measurements obtained on day 1, day 2 and day 3, respectively (n = 3 for each day)

Table 3: Intra- and inter-day precision for assay of CRZ by the proposed spectrofluorimetric method

Nominal conc. (ng/mL)	Intra-day		Inter-day	
	Measured conc. (ng/mL)	*Recovery (% ± RSD)* [a]	*Measured conc. (ng/mL)*	*Recovery (%, ± RSD)* [a]
10	10.07	100.87±0.316	10.07	99.50±1.475
100	99.24	99.87±0.869	99.52	99.03±1.326
200	200.7	100.78±0.741	203.27	100.67±0.819
300	301.06	100.41±1.071	304.11	100.36±1.68

[a] Mean of three determinations

Table 4: Robustness of the proposed spectrofluorimetric method

Parameter variation	Recovery (%, ± SD [a])
No variation [b]	99.68 ± 0.88
SDS volume (µL)	
180	100.02±2.223
220	102.79±0.545
Acid concentration (N)	
0.8	101.31±1.306
1.2	104.25 ±0.631
Acid volume (µL)	
0.95	100.07± 0.545
1.05	104.25± 0.452
Temperature (C °)	
20	103.33 ± 0.770
30	098.25± 0.981

[a] Mean of three determinations; [b] following the general calibration procedures

Table 5: Assay results for CZB in pure form and human plasma samples

Pure form		Plasma	
Amount taken (ng/mL)	% Found	Amount taken (ng/mL)	% Found
40	100.83	150	99.9
100	100.26	200	99.98
300	100.95	250	0.2
Mean	100.68		99.98
± S.D.	0.367		0.199

Application to CRZ in human plasma

The suggested spectrofluorimetric procedure was applied for assaying CRZ in plasma samples. The maximum plasma concentration of CRZ is reached after oral administration of a single CRZ dose of 250 mg by 4 h [22]. The C_{max} of CRZ is reported to be 274 ng/mL [20]. Accordingly, CRZ could be assayed efficiently by the adopted method as the C_{max} lied in our dynamic range (Table 1). Mean of the relative recoveries and % RSD of CRZ in plasma samples are presented in Table 5 (99.98 and 0.199 %, respectively).

DISCUSSION

Fluorescence spectrum of any molecule depends on absorbing of electromagnetic radiation by the molecule. On examining the fluorescence behavior of CRZ, it was found that CRZ exhibited two peaks. The first one represented the excitation band with maximum intensity at 271 nm while the other one represented the emission band with maximum intensity at 404 nm.

This indicated the intrinsic fluorescence property of CRZ. As a trial to increase the FI of CRZ and hence the sensitivity of the proposed method, different surfactants were added to CRZ solution. It was found that addition of SDS, an anionic surfactant, led to increment of CRZ fluorescence intensity by enhancement of its absorption at 271 nm (excitation wavelength). This was confirmed by calculation of the molar absorptivity of CRZ in SDS ($\varepsilon_{micellar}$) and that of CRZ in HCl solution (ε_{HCl}) at 271 nm. The value of $\varepsilon_{micellar}$ was $5.085 \times 10\ 5$ while that of ε_{HCl} was 3.3×10^5. Upon calculation of quantum yield of CRZ in SDS and HCl respectively, it was noticed that there is no difference between the two values at 271 nm indicating that the enhanced effect of SDS was due to increasing CRZ molar absorptivity only.

The quantum yield of CRZ in SDS medium was calculated Eq 3 [15]:

$$\emptyset c = \emptyset q(Fc/Fq \times Aq/Ac) \dots\dots\dots\dots\dots (3)$$

where $\emptyset c$ and $\emptyset q$ are the quantum yields of CRZ in SDS medium and quinine, respectively. Fc and Fq are the integral fluorescence intensities of the CRZ and quinine, respectively; Ad and Aq are the absorbance values of CRZ and quinine at excitation wavelength, respectively. In order to reduce the error of the inner effect, we selected concentration of CRZ of absorbance value < 0.05 [23].

CONCLUSION

The current work is the first spectrofluorimetric approach, to the best of our knowledge, for the assay of CRZ in spiked human plasma samples as well as bulk powder form. The adopted method, based on measuring native fluorescence of the cited drug, is sensitive and simple and gave reproducible results for CRZ determination in very low concentration levels.

Another advantage is the green nature of the method because no organic solvent was used except during extraction of CRZ from plasma matrix. Thus, the developed procedure can be used in clinical and quality control laboratories.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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