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ANALYSIS

DEVELOPMENT AND VALIDATION OF SPECTROFLUOROMETRIC AND SPECTROPHOTOMETRIC BIVARIATE METHODS FOR SIMULTANEOUS DETERMINATION OF PROPRANOLOL AND HYDROCHLOROTHIAZIDE IN DRUG TABLETS

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Abstract: Spectrofluorometric and spectrophotometric bivariate methods were developed for quantitative of Propranolol and Hydrochlorothiazide in drug tablets in an aqueous medium. The methods were tested according to ICH guidelines for linearity, recovery, and specificity and were found accurate, precise, sensitive, and free from interferences. The linearity was established over the ranges 0.025-0.30 and 0.010-0.20 mg/L in the first method, and 1.0-20.0 and 5.0-25.0 mg/L in the second method, for PRO and HCT, respectively. The excitation and emission wavelengths were 260, 345, and 270, 415 nm, for PRO and HCT in the first method, respectively. The recoveries were higher than 95% for both methods. Additionally, the results were statistically compared to that of standard chromatographic methods and showed that no significant difference among the methods regarding both accuracy and precision. Thus, the methods were successfully applied to the quantitation of PRO and HCT in combined formulations. The proposed methods are reliable, simple, sensitive, and adaptable and could effectively be an alternative method to HPLC.

Keywords: spectrofluorometry, bivariate method, hydrochlorothiazide, Propranolol, Inderide

Inderide tablets are types of fixed-dose combinations (FDCs); they are manufactured to contain Propranolol (PRO) as an active component in addition to hydrochlorothiazide (HCT), Figure 1. They are used to treat high blood pressure (hypertension) (1, 2). Propranolol (PRO) is a synthetic beta-adrenergic receptor-blocking agent chemically described as 2-Propanol, 1-[(1-methylethyl)amino]-3-(1-naphthalenyloxy)-, hydrochloride, (±)-. It affects some nerve impulses in certain parts of the body, like the heart. As a result, the heart beats slower and decreases blood pressure (3, 4). Hydrochlorothiazide is a diuretic. Its chemical name is: 6-Chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide. It reduces the amount of water in the body by increasing the flow of urine, which helps lower the blood pressure (5, 6).

Several analytical methods were used for simultaneous determination of HCT and PRO either alone or in combination with other drugs in pharmaceutical formulations such as spectrophotometric methods (7-11), HPTLC (12, 13), LC-MS (14), RP-

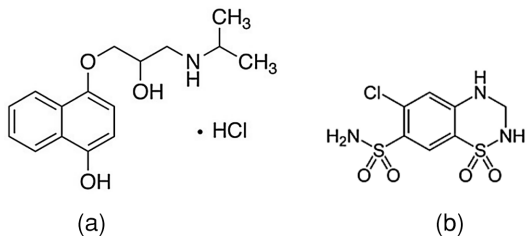

Figure 1. Schematic chemical structures of propranolol hydrochloride (PRO) (a) and hydrochlorothiazide (HCT) (b).

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HPLC (15-20), and micellar liquid Chromatography (21). In recent years, more attention has been given to spectroscopic techniques. Spectroscopic techniques are considered an alternative technique for routine analysis of drugs; they have been applied in a wide range of pharmaceutical applications because of the accessibility of its instrumentations, lower cost, and applicability in almost all laboratories (7-10, 22). However, the spectroscopic approach has the chief disadvantage of the difficulty to identify and quantify individual analyte in sample mixtures showing a complete overlap (22).

To solve this problem, several techniques have been investigated including bivariate method (22), spectroscopic derivates (23-25), multivariate calibration method (26), and spectrofluorometric method (27). This work aims to develop and validate simple, rapid, and sensitive spectrofluorometric and spectrophotometric bivariate methods for the simultaneous determination of propranolol and hydrochlorothiazide in pharmaceutical formulations in an aqueous medium.

MATERIALS AND METHODS

Materials and reagents

The HCT is purchased from Unichem Laboratories Limited, India (purity 99.2%, Batch No. ROHC190414). The PRO is supplied from A Johnson Mattey Company, UK (purity 99%, Batch No H26645). Magnesium stearate (Sigma-Aldrich, USA), croscarmellose sodium (Shaanxi Owens Industry Co., China), lactose (VWR), corn starch (Avonchem Limited, UK), colloidal silicon dioxide (Sigma-Aldrich), cellulose (Fisher Scientific SAS, France), sodium lauryl sulfate (Chemmax Chemical CO. China).

Commercial Inderide tablets

Inderide 40/25 tablets (Mylan pharmaceutical company, USA) contain 40 mg propranolol hydrochloride (Inderal®) and 25 mg hydrochlorothiazide; Inderide 80/25 tablets contain 80 mg propranolol hydrochloride (Inderal®) and 25 mg hydrochlorothiazide.

Inderide excipients provided by the manufacturer were: colloidal silicon dioxide, croscarmellose sodium, magnesium stearate, microcrystalline cellulose, pregelatinized corn starch, and sodium lauryl sulfate.

Instruments and conditions

Spectrophotometric bivariate method

A Thermo Scientific (Erolution Array UV-VIS Spectrophotometer, USA) was used for UV-vis measurements using a 1-cm quartz cuvette, part number B0631107 from Perkin Elmer. The data analyses were carried out using a VISION collect (Version 1.5 Build 09) software.

Fluorometric method

Fluorescence spectra (in the range of 300-600 nm) and measurements were recorded using a Perkin Elmer (Fluorescence Spectrometer LS45, USA), model B0631107. Slit widths for grating excitation and emission monochromators were set at 10 nm. A 1 cm quartz cell was used. The data analyses were carried out using a Flwinlab software, version 4.00.03.

Sample preparations and procedures

Standard and working solutions for spectrophotometric bivariate method

Standard solutions of PRO and HCT were prepared by dissolving accurately weighed 125 mg of each in 250 mL of water (3 mL of methanol added in case of HCT to enhance solubility) and sonicated to ensure complete solubility to give 500 mg/L stock solutions.

Sufficient amount of PRO and HCT stock solutions were transferred series of small volumetric flasks to prepare working concentration in range of 1.0 to 25 mg/L of PRO (1.0, 5.0, 10.0, 15.0 and 20.0 mg/L), and HCT (5.0, 10.0, 15.0, 20.0 and 25.0 mg/L). The scanning of solutions was carried out in the range of 190-450 nm against water as blank.

Standard and working solutions for fluorometric method

Stock solutions of HCT and PRO were prepared by dissolving 100 mg of each pure powder (10.0 mL methanol added during HCT preparation and sonicated to dissolve completely) into 100 mL water and further diluted to obtain standard solutions containing 200 mg/L of each drug. The HCT and PRO solutions were stored and protected from light in the refrigerator.

The working concentrations of PRO and HCT were in range of 0.010 to 0.30 mg/L of PRO (0.025, 0.050, 0.10, 0.20 and 0.30 mg/L), and HCT (0.010, 0.025, 0.050, 0.10, and 0.20 mg/L). The excitation wavelengths were at 260 and 270 nm for PRO and HCT, respectively. Synchronous fluorescence spectra of the solutions were recorded by scanning both monochromators at a constant wavelength for each drug individually. The relative fluorescence intensity of the synchronous spectra was plotted versus the final concentration of the drugs (mg/L) to get the calibration curves.

Procedure for synthetic mixtures

Laboratory prepared mixtures of PRO/HCT were prepared in water using variable proportion of each compound. The ratios of the laboratory prepared mixtures are selected randomly with two mixtures (1.6 : 1, w/w PRO/HCT and 3.2 : 1 w/w PRO /HCT) reproducing the composition of the commercial Inderide® tablets.

Analysis by HPLC as a reference method

The high-performance liquid chromatography (HPLC) analysis, was performed for reference, was described previously by Shanmugasundaram et al. (20). In brief, separately, 100 mg of PRO and HCT were accurately weighed and dissolved in 10 mL water/methanol (10 : 1, V/V) in a 100 mL volumetric flask. The solution samples were vibrated in an ultrasonic bath until complete dissolution. Further dilution was performed to get 200 mg/L standard solutions of PRO and HCT for calibration curves.

The analysis was performed using an integrated U-HPLC with UV detection. The U-HPLC system was an Ultimate 3000 Series (Dionex/Thermo Scientific, USA) consisted of (SD, RS, BM, and BX) pumps, an auto-sampler, a column thermostat, and a UV detector. The separation was carried out on an RP-LiChrosorb C18 (250 mm, 5 µm × 4.6 mm) column, with a mobile phase consisting of acetonitrile : potassium dihydrogen phosphate solution (0.01 M, pH 3.0 adjusting with Orthophosphoric acid) in a ratio of (50 : 50, v/v) at isocratic mode, and a flow rate of 1.5 mL/min. The detection was performed at 270 nm.

The mixture of PRO/HCT was analyzed by applying the chromatographic conditions (20). The linearity of the method was evaluated with calibration curves made in the mobile phase ranging from 25–100 mg/L and 10–100 mg/L for PRO and HCT, respectively. For both compounds, a good linear relationship between peak area and standard concentration was found, as described by the linear regression equations: Y = 3710[PRO] + 629 (r^2 = 0.998) and Y = 45200[HCT] + 19680 (r^2 = 0.999).

Validation of proposed methods

Validation of the proposed methods was performed with respect to specificity, accuracy, precision, LOD and LOQ, linearity and range, and robustness according to the ICH Q2 (R1) guidelines (28).

Specificity

The specificity of the methods was achieved with analyses of different laboratory prepared mixtures of PRO/HCT.

Accuracy

Accuracy was confirmed by applying the proposed methods for determination of three concentrations (8.0, 12.0, and 16.0 mg/L) of PRO and HCT standards (n = 3), in the UV method; and (0.040, 0.080 and 0.15 mg/L) of standard PRO and HCT solutions (n = 3), in Fluorometric method. The recovery percentages were employed to evaluate the accuracy of the methods.

Precision

Precision inter-day (Repeatability) and intra-day (intermediate) were assessed in different periods of time using three concentrations (8.0, 12.0, and 16.0 mg/L) of PRO and HCT standards (n = 3), in the UV method; and (0.040, 0.080 and 0.15 mg/L) of standard PRO and HCT solutions (n = 3), in Fluorometric method. The percentage relative standard deviation (%RSD) of the predicted concentrations from the regression equation was taken as the measure of precision.

Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ) were estimated according to the ICH guidelines. The LOD was calculated using: $3.3 \times \sigma/s$ and the LOQ as $10 \times \sigma/s$, where σ is the standard deviation of the response of the blank, and s is the slope of the analytical curve.

Linearity and range

The linearity and range of the proposed methods were verified by analyzing five concentrations of PRO and HCT (n = 3). The assays were performed according to the experimental conditions previously mentioned. A linear relationship was established by plotting the absorption, and intensity against the concentrations mentioned in analytical methods.

Robustness

The robustness of the proposed methods was evaluated by the reliability of the analysis with respect to small variations in the experimental conditions. The spectrophotometric method includes changing in stock solution temperature by ± 5°C, changing optimum wavelength by ± 2 nm, and small changes in concentrations. Fluorometric method: changing excitation and emission wavelengths by ± 2 nm, and small changes in concentrations.

Analysis of commercial drugs

Ten tablets of both Inderide® 40/25 mg and Inderide® 80/25 mg were accurately weighed and finely powdered. An amount (0.4121 g) of Inderide®

40/25 powder was transferred into a 250 mL volumetric flask containing a solution of water and methanol (10 : 1, V/V). The solution was then sonicated for a few minutes and filtered using a 0.20-µm Whatman filter paper to remove insoluble components to get 146.6 mg/L PRO and 91.8 mg/L HCT (as stock). An appropriate aliquot (0.75 mL) from the stock was transferred to a 25 mL volumetric flask to prepare 4.39 mg/L PRO and 2.73 mg/L HCT. For Inderide® 80/25, the stock solutions were prepared as previously described for Inderide® 40/25.

RESULTS AND DISCUSSION

Methods development and optimization

The spectrophotometric bivariate analysis (UV-biv method)

The UV absorption spectra of PRO and HCT were recorded in a range of 190 – 350 nm. Figure 2 shows the absorption spectra of 20 mg/L of PRO in water and 20 mg/L of HCT in water/methanol (10 : 1, V/V) against distilled water as blank. PRO has two maximum absorption bands at wavelengths of 212 and 285 nm, while HCT has three maximum absorption bands at wavelengths of 222, 270, and 320 nm. It is clear that the two spectra are highly overlapped in a wide range starting from 200 to nearly 315 nm.

The fluorometric analysis

The excitation and emission wavelengths are key factors for the optimization of quantitation of PRO and HCT in binary mixtures. The quantitation is based on the direct measurements of their fluorescence. The fluorescence spectrum of PRO was evaluated at three excitation wavelengths at 260, 270, and 285 nm; the best sensitivity for the purpose of mixture analysis was achieved at 260 nm, Figure 3.

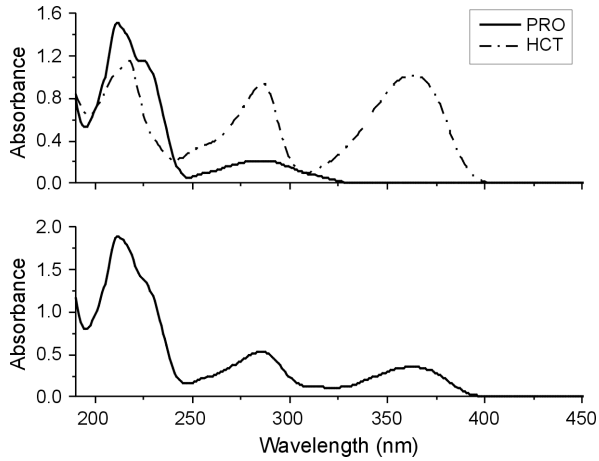

Figure 2. UV absorption spectra for an aqueous solution of (upper) 20 mg/L PRO and 20 mg/L of HCT (lower) a mixture of1.6 mg/L PRO and 1.0 mg/L of HCT.

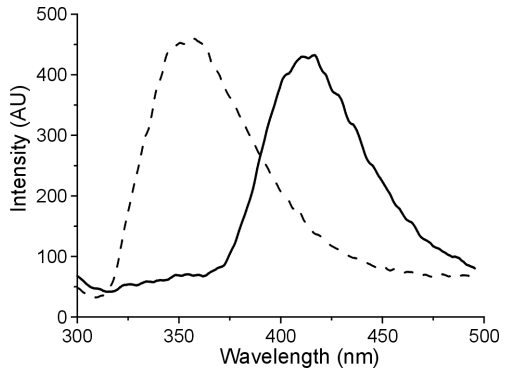

Figure 3. Fluorescence spectra of 0.10 mg/L PRO in water using λ_{exc} = 260 nm (dash line) and 0.10 mg/L HCT in MEOH/water using λ_{exc} = 270 nm (solid line).

Table 1. Regression equations and validation parameters result for PRO and HCT.

Parameter	UV-biv method		Fluorometric	
	PRO	HCT	PRO	HCT
Linearity range (mg/L)	1.0-20.0	5.0-25.0	0.025-0.30	0.010-0.20
$\lambda_{excitation}$ (nm)			260	270
$\lambda_{emission}$ (nm)			345	415
$\lambda_{optimum}$ (nm)	235, 270	235, 270		
Regression equation (Y^a)				
Slope (a) ± SD - at 235 nm - at 270 nm	 0.098 ± 0.002 0.016 ± 0.001	 0.022 ± 0.0008 0.064 ± 0.011	3584.8 ± 89.6	3468.6 ± 77.6
Intercept (b) ± SD - at 235 nm - at 270 nm	 0.015 ± 0.003 0.091 ± 0.002	 0.013 ± 0.0009 0.014 ± 0.002	65.9 ± 1.4	70.5 ± 2.0
Correlation coefficient (*R*) - at 235 nm - at 270 nm	 0.995 0.996	 0.998 0.999	0.991	0.996
LOD (mg/L)[b]	0.24	0.53	0.0031	0.0011
LOQ (mg/L)[b]	0.72	1.6	0.0093	0.0032
Accuracy (recovery, %)[c]	98.7	99.4	97.7	97.7
Specificity (recovery± SD,%)[d]	99.5 ± 0.4	98.9 ± 0.6	97.7 ± 1.1	96.9 ± 1.1
Precision, RSD (%) - Repeatability[e] - Intermediate precision[f]	 1.46 1.22	 1.05 0.97	 1.26 1.69	 1.48 1.63

[a]Y = a*C* + b, *C* is concentration in mg/L and *Y* is absorbance or fluorescence (arbitrary unit). [b]*LOD* and *LOQ* were calculated as following: *LOD* = 3.3σ/s and *LOQ* =10σ/s where σ is the standard deviation of replicate of determinations of blank, s is the slope of regression equations. [c]Average (*n* = 3) ,of three concentrations (8.0, 12.0, and 16.0 mg/L) for PRO and HCT in the UV-biv method; and (0.040, 0.080 and 0.15 mg/L) PRO and HCT, in Fluorometric method. [d]Average± SD (*n* = 3), of synthetic mixtures for PRO and HCT. [e]Repeatability (*n* = 3), of three concentrations (8.0, 12.0, and 16.0 mg/L) for PRO and HCT in the UV-biv method; and (0.040, 0.080 and 0.15 mg/L) PRO and HCT in Fluorometric method, repeated three times within the day. [f]Intermediate precision (*n* = 3), of three concentrations (8.0, 12.0, and 16.0 mg/L) for PRO and HCT in the UV-biv method; and (0.040, 0.080 and 0.15 mg/L) PRO and HCT in Fluorometric method, repeated three times in three days.

Table 2. Slopes values of calibration curves for PRO and HCT at 7 selected wavelengths.

Slope (HCT)	Slope (PRO)	λ (nm)
0.067	0.095	210
0.033	0.098	235
0.011	0.008	244
0.064	0.016	270
0.017	0.013	286
0.014	0.012	290
0.015	0.005	310

Figure 3 shows that PRO has a maximum emission at 345 nm. The fluorescence spectra of HCT were tested at one excitation wavelength at 270 nm. The HCT emission spectrum showed a maximum emission at 415 nm.

Method validation

The results of method validation are summarized in Table 1.

Linearity and range

The linearity of the method was determined for both analytes by both methods by analyzing the different concentration solutions. PRO exhibited excellent linearity in the concentration of 1.0 to 20.0 mg/L for the UV-biv method at λ = 235 nm and 0.025 to 0.20 mg/L in the Fluorometric method at $\lambda_{excitation}$ = 260 nm with good correlation coefficient ($r^2 > 0.988$). HCT was linear in the range of 5.0 – 25.0 mg/L for the first method λ = 270 nm and 0.010 to 0.20 mg/L for the second method at $\lambda_{excitation}$ = 270 nm with an excellent correlation coefficient ($r^2 > 0.996$), Figure 4. The corresponding regression equations of PRO and HCT and their statistical parameters such as linear range, regression equa-

tions, and correlation coefficients were calculated (see Table 1).

Specificity

The specificity of the proposed methods was assessed for the determination of PRO and HCT in synthetic mixtures with excipients that were similar to those that existed in the drug tablets. Since the absorption and emission spectra of PRO and HCT were partially overlapped, it becomes difficult to estimate concentration one of them in the presence of the other. The applications of bivariate and fluorescence approaches were helpful for analyzing overlapping analyte.

The bivariate approach was applied to suggest a pair of wavelengths that could be used to quantify of PRO and HCT in binary combination mixtures. The absorbance was measured at seven randomly selected wavelengths: 210, 235, 244, 270, 286, 290, and 310 nm. The sensitivity values (slope) were determined from the linear curves at those wavelengths; after that, sensitivity determinant (Kaiser's determinant) was created, Tables 2 and 3. The results in Table 3 imply that the higher sensitivity was obtained using measurements at **235** and **270** nm. The concentration of HCT and PRO in laboratory prepared mixtures (example: mixture 2 from Table 4, Fig. 2) and commercial preparations (Table 7) were calculated using the parameters of the linear regression of PRO and HCT at 235 and 270 nm (Table 1).

Fluorescence spectra of PRO and HCT were partially overlapped in the region between 350 and 480 nm. In order to separate PRO spectra from the

Table 3. The Kaiser's determinant (K × 105) for PRO/HCT mixtures.

310	290	286	270	244	235	210	λ/λ
105	5.71	7.44	5.10	50.9	353	0	210
131	97.6	124	574	81.4	0		235
6.80	1.60	0.70	33.6	0			244
8.64	53.6	56.0	0				270
10.5	1.55	0					286
10.3	0						290
0							310

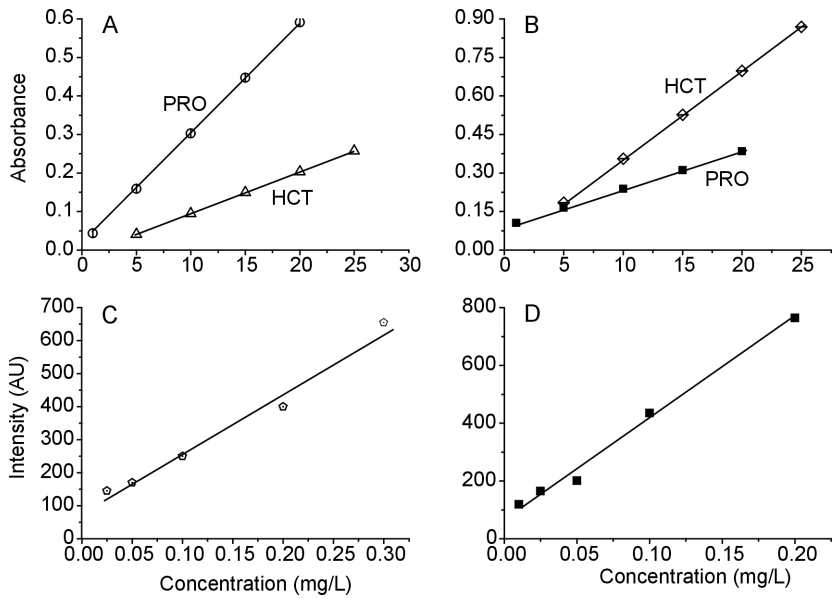

Figure 4. Calibration curves of (A) PRO 1.0 to 20.0 mg/L and HCT 5.0 to 25.0 mg/L, UV method at 235 nm (B) PRO 1.0 to 20.0 mg/L and HCT 5.0 to 25.0 mg/L, UV method at 270 nm (C) PRO 0.025 to 0.20 mg/L, Fluorometric method at $\lambda_{excitation}$ = 260 nm (D) HCT 0.010 to 0.20 mg/L, Fluorometric method at $\lambda_{excitation}$ = 270 nm.

Table 4. Recovery study results for PRO/HCT in synthetic mixtures by the proposed methods.

Mixture	PRO			HCT		
	Conc.(mg/L)	Found[b]	% Recovery	Con.(mg/L)		% Recovery
	UV-biv method					
1 (1.0 : 1.0)	10.0	9.98	99.8	10.0	9.89	98.9
2 (1.6 : 1.0)	16.0	15.8	98.9	10.0	9.95	99.5
3 (3.2 : 1.0)	20.0	19.9	99.7	6.25	6.20	98.9
4 (4.0 : 1.0)	20.0	19.9	99.4	5.00	5.91	98.1
Mean ± S.D [a]	99.5 ± 0.4					98.9 ± 0.6
	Fluorometric method					
5 (1.0 : 1.0)	0.050	0.0489	97.7	0.050	0.0484	96.8
6 (1.6 : 1.0)	0.080	0.0769	96.1	0.050	0.0477	95.4
7 (3.2 : 1.0)	0.160	0.1571	98.2	0.050	0.0487	97.5
8 (4.0 : 1.0)	0.200	0.1971	98.6	0.050	0.0489	97.9
Mean ± S.D[a]	97.7 ± 1.1					96.9 ± 1.1

[a]Standard deviation; [b]average of three measurements.

Table 5. Accuracy and precision data for PRO and HCT in the proposed methods.

	UV-biv				Fluorescence			
	Conc. (mg/L)	% Recovery [a]	Intra-day [b] %RSD	Inter-day [c] %RSD	Conc. (mg/L)	% Recovery [a]	Intra-day [b] %RSD	Inter-day [c] %RSD
PRO	8.0	98.2	1.30	1.14	0.040	98.4	1.72	1.61
	12.0	99.1	1.96	0.98	0.080	97.5	1.30	1.83
	16.0	98.8	1.12	1.53	0.150	97.3	0.76	1.01
HCT	8.0	99.6	0.48	0.63	0.040	96.1	1.72	1.23
	12.0	98.9	1.52	0.44	0.080	97.2	1.91	1.88
	16.0	100.1	1.16	1.84	0.150	98.7	1.44	1.76

[a] the mean of three determinations. [b] same analyte on the same day. [c] same analyte in several days apart.

fluorescent of HCT, a background subtraction approach was utilized. In this approach, fluorescence spectra for any two analytes can be approximated as the mathematical sum of the emission spectra of both analytes, one analyte as a signal, and the second as a background (27, 29, 30). Satisfactory results were obtained at a low concentration of analytes in combination mixtures which were needed to minimize the effect of internal absorption.

Quantification of PRO and HCT in mixture 1 (1 : 1, w/w PRO/HCT) (see Table 4) was done as follows: emission spectra of the mixture 1 and HCT with concentration exactly equal to that in the mixture 1 were recorded and HCT stored at 260 nm. The background subtraction was used to extract the fluorescence of PRO from the emission of the mixture using HCT as a background (Fig. 5A). A similar approach was used to quantify HCT in the same mixture, the emission spectra of mixture 1 and PRO at 270 nm were used, PRO was the background (Fig. 5, B). The same approach was applied to mixture 2 (1.6 : 1, *w/w* PRO/HCT), Figure 5C and D.

Satisfactory mean recoveries were obtained 99.5% ± 0.4 and 98.9% ± 0.6 for PRO and HCT, respectively, in the UV-biv method, and 97.7% ± 1.1 and 96.9% ± 1.1 for PRO and HCT, respectively, in the Fluorescence method. The specificity was also evaluated regarding possible spectral interferences from the excipients in commercial tablets. The UV and emission spectra of drug tablets solutions did not show any additional peaks when compared to the UV and emissions of synthetic mixtures (see Fig. 6).

The concentrations of PRO and HCT of mixtures were determined from the regression parameters of the calibration curves (see Table 1).

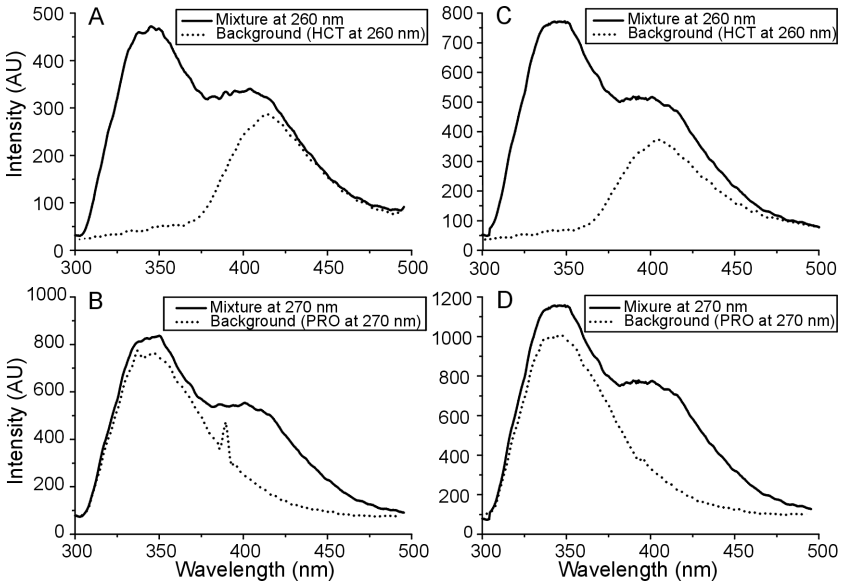

Figure 5. Emission spectra of (A) mixture (10:10, *w/w* PRO/HCT) and 10 mg/L HCT at $\lambda_{excitation}$ = 260 nm (B) mixture (10 : 10, *w/w* PRO/HCT) and 10 mg/L PRO at $\lambda_{excitation}$ = 270 nm (C) mixture (16:10, *w/w* PRO/HCT) and 10 mg/L HCT at $\lambda_{excitation}$ = 260 nm (D) mixture (16:10, *w/w* PRO/HCT) and 16 mg/L PRO at $\lambda_{excitation}$ = 270 nm.

Table 6. Results of robustness.

	PRO	HCT
Parameter	(Recovery ± RSD,%)	(Recovery ± RSD,%)
Temperature (±5°C)[a]		
16	98.7 ± 1.2	100.1 ± 1.22
26	97.8 ± 1.0	98.6 ± 1.3
Optimum Wavelength (± 2nm)[b]		
233	99.1 ± 0.89	
237	99.3 ± 0.54	
267		98.8 ± 0.66
272		98.1 ± 1.41
Excitation wavelength (± 2nm)[c]		
262	100.1 ± 1.11	
258	99.5 ± 1.44	
272		99.3 ± 1.21
268		98.4 ± 1.77
Emission wavelength (± 2nm)[c]		
343	98.5 ± 1.87	
347	99.0 ± 1.91	
413		100.0 ± 1.76
417		99.6 ± 0.99

[a]The ambient temperature was 21°C. The recoveries were at the optimum wavelengths 235 and 270 nm, resp. [b]PRO and HCT (n = 5), three concentrations (8, 12, 16 mg/L), absorption was at 235 and 270 nm, resp. [c]PRO and HCT (n = 5), three concentrations (0.040, 0.080, 0.15 mg/L), excitation and emission were 260 and 345 for PRO and 270 and 415 nm for HCT, resp.

Precision and accuracy

In UV-biv method, intra-day RSD (repeatability) was 1.46 and 1.05% for PRO and HCT. In the Fluorometric method, it was 1.26 and 1.48% for PRO and HCT, respectively. In UV-biv method, inter-day (intermediate precision) was 1.22 and 097% for PRO and HCT, respectively, and it was 1.69 and 1.63% for PRO and HCT, respectively, in the Fluorometric method (see Table 5).

Satisfactory recoveries were obtained, 98.7 and 99.4% for PRO and HCT, respectively, in the UV-biv method, and 97.7% for both in the Fluorometric

method (see Table 5). The results in Table 5 indicate good accuracy and precision (RSD% < 2.0) for both analytes.

LOD and LOQ

In the UV-biv method, the *LOD* for PRO and HCT was 0.0070 and 0.021 mg/L at 235 nm, and 0.013 and 0.0060 mg/L at 270 nm, respectively, in Fluorometric method, it was 0.0040 and 0.0369 mg/L for PRO and HCT, respectively. The *LOQ* in the UV-biv method was determined 0.021 and 0.012 mg/L at 235 nm and 0.040 and 0.0020 mg/L for PRO and HCT, respectively, and it was 1.12 and 0.0013 mg/L for PRO and HCT, respectively, in the fluorometric method.

Robustness

The effect of small changes in temperature, absorption wavelengths, and excitation and emission wavelengths were assessed. The solution acidity (pH) was not modified at any condition to ensure the integrity of the active ingredients of standard solutions (see Table 6).

It was observed that the small changes did not affect the recoveries of the drugs or the system suitability parameters of the proposed methods.

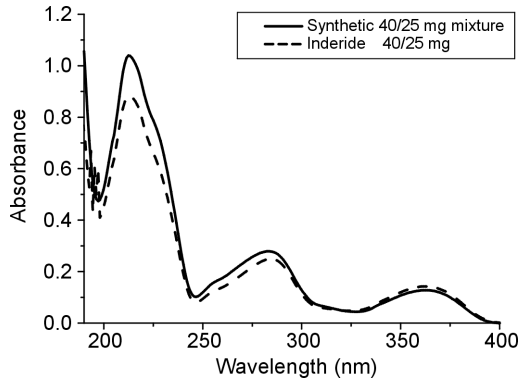

Figure 6. UV absorption spectra for a 14.8 mg/L PRO and 9.2 mg/L HCT mixture of Inderide® 40/25 mg compared to a synthetic mixture of 16 mg/L PRO and 10 mg/L HCT.

Table 7. Statistical analysis results of the comparison between the two proposed methods and a literature method.

	UV-biv		Fluorometric		Literature method[a]	
Product	PRO	HCT	PRO	HCT	PRO	HCT
Inderide® (40 mg PRO+25 mg HCT)						
Average Found (mg)[b]	38.33	24.52	37.88	24.22	38.90	24.60
RSD%	0.57	0.60	0.90	0.81	0.81	0.76
n	6	6	6	6	5	5
t (2.26) [c]	1.37	1.27	1.96	0.80		
F (6.26) [c]	3.08	1.60	1.25	1.14		
Inderide®						
(80 mg PRO+25 mg HCT)						
Average Found (mg)[b]	77.52	23.44	77.52	23.74	77.71	23.36
RSD%	1.18	0.78	1.06	0.54	0.97	0.90
n	6	6	6	6	5	5
t (2.26) [c]	0.31	0.467	1.40	0.868		
F (6.26) [c]	1.48	1.24	1.19	3.16		

[a]Reported RP-HPLC method (ref. 20). [b]Mean, $n = 6$. [c]Tabulated *t* and *F* values at p = 0.05.

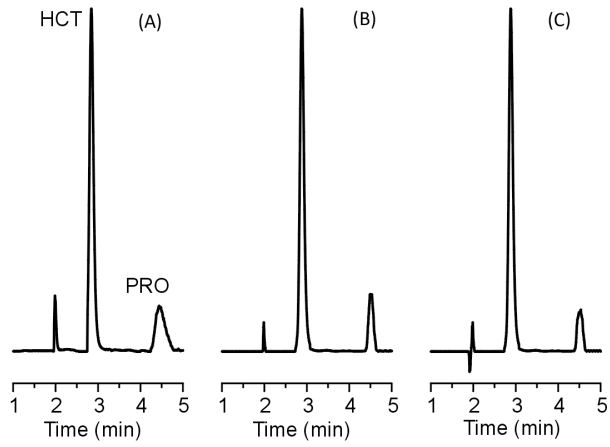

Figure 7. The chromatograms of a synthetic mixture 80 mg/mL PRO and 25 HCT mg/mL in the mobile phase (panel **A**); Inderide® 80/25 in the mobile phase (panel **B**), and Inderide® 40/25 in the mobile phase (panel **C**).

Recoveries ranged from 97.8 to 100.1% and RSD below 2% in all cases.

Analysis of tablets

The UV-biv and fluorometric procedures were applied for quantitation of PRO and HCT from Inderide® 40/25 mg and Inderide® 80/25 mg. Recovery studies results for Inderide® 40/25 mg were 95.8 and 98.1% for PRO and HCT, respectively, in the UV-biv method, and 94.7 and 96.9% for PRO and HCT, respectively, in the Fluorometric method; and for Inderide® 80/25 mg were 96.9 and 95.9% for PRO and HCT, respectively, in the UV-biv method, and 96.9 and 94.6% for PRO and HCT, respectively, in the Fluorometric method (see Table 7). The analysis results were in acceptable agreement with the amount of PRO and HCT in Inderide® tablets. Furthermore, under the previously described chromatographic conditions spiked PRO/HCT mixture and commercial Inderide® 40/25 mg and Inderide® 80/25 mg were analyzed, Figure 7. The PRO and HCT peaks are clearly resolved with reasonable retention times at 2.95 ± 0.1 and 4.45 ± 0.1 min, respectively.

Statistical analysis was carried out to compare the results of the proposed spectroscopic methods and an HPLC reference method. The determined *t* and *F* values were less than the critical *t* and *F* values, indicating that there is no significant difference in analysis results at $p = 0.05$ between the proposed methods and the reference method (see Table 7). This indicates that the proposed methods are applicable for routine analysis of drugs**.**

CONCLUSIONS

Two simple spectrophotometric and spectrofluorometric methods were successfully applied for the determination of PRO and HCT from synthetic mixtures and pharmaceutical formulations. The methods were simple, accurate, and precise. The methods have an advantage of the availability of chemicals and equipment in any analytical laboratory. The statistical comparison results confirm that there were no significant differences between the proposed methods and the reference method. Thus, the proposed methods were found reliable for routine applications on drug combinations.

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Conflict of interests

The authors declare no conflict of interest.

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