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Novel spectrophotometric method for the analysis of ternary mixtures

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ABSTRACT

Though simple, rugged and reliable classical spectrophotometric methods have not proven adequate selectivity in the analysis of ternary mixtures with overlapped spectral data. Recently emerging methods incorporates several steps of mathematical manipulations to resolve the spectral overlap. The work introduces the novel H-point derivative ratio method (HDR) and compares its performance with the recently developed dual wavelength in ratio spectrum (DWRS). The resolution power of the H-point standard additions and the derivative ratio methods-resolve binary mixtures-merge to engender the HDR for the analysis of ternary mixtures. HDR and DWRS methods were applied to resolve the severely overlapped spectral data of the ternary mixture: caffeine, propyphenazone and paracetamol. The latter is the first line antipyretic in COVID19 symptomatic relief. One-way ANOVA statistical analysis proved the absence of significant differences between the analytical results of HDR, DWRS and the reported method. Validation parameters were assessed according to the ICH guidelines. The developed HDR method expressed sensitivity and selectivity potentials for routine analysis of the analytes in their pure powder form, ternary mixture and pharmaceutical formulation without prior extraction or interference from additives.

Keywords: H-point derivative ratio method; Dual wavelength in ratio spectrum method; caffeine; propyphenazone; paracetamol.

1. Introduction

Spectrophotometric analysis is a simple analytical technique that fits the purpose of most analytical laboratories in pharmaceutical analysis, without the need for sophisticated instrumentation, professional labor, costly supplies or highly pure solvents.

Spectrophotometry may be helpful in the analysis of simple mixtures by direct determination at the wavelength of maximum absorption (λ max). Some methods employed spectral manipulation for the determination of overlapped spectral data in binary mixtures such as derivative [1], [2], derivative ratio [3–6], dual-wavelength [7–9], H-point standard additions [10–12] and ratio difference [13–15] methods. However, only a few conventional methods based on simple mathematical manipulation were able to introduce solutions for the analysis of complex ternary mixtures. Conventional methods appear to be simpler than the complex mathematical algorithms employed in chemometric methods that may require additional knowledge in programming languages or expensive software for the data analysis.

The aim of the current work is to develop and validate sensitive and selective spectrophotometric methods for the determination of compounds in a ternary mixture. The proposed methods were applied to a ternary mixture of caffeine, propyphenazone and paracetamol widely formulated for the treatment of fever and pain.

The literature revealed that spectrophotometric determination was only feasible via chemometrics [16] and zero-crossing techniques [17–19] to resolve the overlapped spectra of the three components. The corresponding study adds a new effective manner to resolve spectral overlapping in the ternary mixtures.

Caffeine (CF) is 3,7-Dihydro-1,3,7-trimethyl-1H-purine-2,6-dione, can inhibit phosphodiesterase enzyme effect, CNS stimulant, and has an antagonistic effect at central adenosine receptors. Paracetamol (PC) is N-(4-Hydroxyphenyl) acetamide, described as an antipyretic and analgesic in COVID19. Propyphenazone (PZ) is 1,2-Dihydro-1,5-dimethyl-4-(1-methylethyl)-2-phenyl-3H-pyrazol-3-one. PC and PZ are used for the treatment of fever and pain as they have analgesic and antipyretic properties. Combination of the three drugs is used for the treatment of severe pain and mild fever.

The current work discusses the development of H-point derivative ratio and dual wavelength in ratio spectrum methods for the determination of CF, PZ and PC in the ternary mixture without prior chemical separation.

2. Experimental

Apparatus

SHIMADZU (Kyoto/ Japan) dual beam UV-visible spectrophotometer model UV-1650 PC connected to IBM compatible. The bundled software, UV-Probe software version 2.21 (SHIMADZU) was used to process absorption, derivative and ratio spectra. Scanning speed reaches 2800 nm/min.

Materials and reagents

Methanol (Piochem, Cairo, Egypt) spectroscopic grade was used.

CF, PZ and PC working standards were obtained from Eva Pharma, Egypt. Purity was reported to be 99.12 ± 1.25 %, 99.63 ± 0.93 % and 99.44 ± 1.92 %, respectively. Stopain® tablet, labeled to contain 50 mg CF, 150 mg PZ and 300 mg PC per tablet is manufactured by Eva Pharma, Egypt.

Solvent

Solvent consisted of 10 % methanol in water.

Standard stock solutions

Stock standard solution 100 µg/mL CF.

Stock standard solution 100 µg/mL PZ.

Stock standard solution 100 µg/mL PC.

Spectral characteristics of CF, PZ and PC

Into three separate 10 mL volumetric flasks, aliquots containing 30 µg of CF, 90 µg of PZ and 180 µg of PC were separately transferred from their respective stock standard solutions (100 µg/mL), and the volume was completed to mark the previously prepared solvent. The zero-order

(D^0) absorption spectrum of each solution was recorded against the solvent as a blank.

Construction of calibration curves

H-point derivative ratio method

Aliquots equivalent to 30–270 μg of CF from stock standard solution (100 μg/mL) were transferred into a series of 10 mL volumetric flasks containing fixed amount of PZ and fixed amount of PC, then the volume was completed with the previously prepared solvent. The zero-order absorption spectra of the prepared solutions were divided by the spectrum of 30 μg/mL PZ then the first derivate of the ratio spectra using delta lambda=4 and scaling factor=10 were obtained. The amplitude values of the obtained derivative data at 255.2 nm and 262.9 nm at which PC exhibits the same values were recorded and the two calibration curves were constructed. For PZ, aliquots equivalent to 30–300 μg of PZ from stock standard solution (100 μg/mL) were added to flasks containing fixed amounts of both CF and PC, and the volume was completed to the mark (10 mL) with the previously prepared solvent. Spectra of the prepared solutions were divided by the spectrum of 30 μg/mL PC. The first derivate of the ratio spectra was obtained on the bases of delta lambda = 4 and scaling factor =10, then the data at 216.9 nm and 219.1 nm at which CF exhibits the same values were utilized in calibration curves. For PC, aliquots equivalent to 30–360 μg of PC from stock standard solution (100 μg/mL) were used for the construction of calibration curves in the same manner. The zero-order absorption spectra of the prepared solutions were divided by the spectrum of 30 μg/mL PZ. Also, the first derivate of the ratio spectra was obtained considering delta lambda of 4 and a scaling factor of 10. Wavelengths of 253.0 nm and 259.2 nm at which CF has the same values were used in the construction of calibration curves.

Dual wavelength in ratio spectrum method

Aliquots equivalent to 15–240 μg of CF from stock standard solution (100 μg/mL) were transferred into a series of 10 mL volumetric flasks, and the volume was completed to the mark with the previously prepared solvent. The zero-order absorption spectra of the prepared solutions were divided by the spectrum of 30 μg/mL PZ. The peak amplitudes of the ratio spectra were measured at 213.8 and 218.3 nm (wavelengths at which PC exhibits the same amplitude). Calibration graphs relating the differences in the amplitudes at the chosen wavelength using PZ 30

μg/mL as a divisor ($\Delta P^{PZ\ 30\mu g/mL}_{213.8-218.3nm}$) to the corresponding concentrations of CF were constructed, and the regression equation was computed. For PZ, aliquots equivalent to 30–300 μg of PZ from stock standard solution (100 μg/mL) were used by the same manner. Spectra of the prepared solutions were divided by the spectrum of 30 μg/mL PC. The peak amplitudes of the ratio spectra were measured at 248 and 240.3 nm (wavelengths at which CF exhibits the same amplitude). Calibration graphs were constructed between the differences in the amplitudes at the chosen wavelength using PC 30 μg/mL as a divisor ($\Delta P^{PC\ 30\mu g/mL}_{248-240.3\ nm}$) and the corresponding concentrations of PZ. While for PC, aliquots equivalent to 60–330 μg of PC from stock standard solution (100 μg/mL) were used in development of calibration curve. The measured spectra were divided by the spectrum of 30 μg/mL PZ. The peak amplitudes of the ratio spectra were measured at 242.4 and 247.5 nm (wavelengths at which CF exhibits the same amplitude). The differences in the peak amplitudes at the chosen wavelength using PZ 30 μg/mL as a divisor ($\Delta P^{PZ\ 30\mu g/mL}_{242.4-247.5\ nm}$) were calculated at each corresponding concentration of PC for construction of calibration curve.

Laboratory-prepared mixtures

H-point derivative ratio method

For CF determination, laboratory prepared mixtures were prepared where 5 aliquots containing 30, 50, 70, 90 and 110 μg of CF were accurately added to 10 mL volumetric flasks containing different ratios of CF, PZ and PC and the final volume was achieved. Curves of standard additions were developed where the amplitudes of first derivative (delta lambda = 4 and scaling factor =10) of ratio spectra (using 30 μg/mL PZ divisor) after CF standard additions were represented on Y-axis and the concentrations of the added CF standards were represented on X-axis at the two selected wavelengths. For each mixture, two curves (at 255.2 nm and 262.9 nm) were obtained where the point of intersection upon extrapolation represents the H-point, whose abscissa represents CF concentration in the determined mixture. For PZ, laboratory prepared mixtures were prepared using aliquots containing 30, 50, 70, 90 and 110 μg PZ added to 10 mL volumetric flasks containing different ratios of CF, PZ and PC. The amplitudes of first derivative (delta lambda = 4 and scaling factor =10) of ratio spectra (using 30 μg/mL PC divisor) after PZ standard additions were represented on Y-axis and the concentrations of the added PZ standards were represented on X-axis for the two selected wavelengths. For each mixture, the two curves

were constructed at 216.9 nm and 219.1 nm. Upon extrapolation, the intersection point represents the H-point, whose abscissa represents PZ concentration in the determined mixture. Finally, for determination of PC, aliquots containing 30, 50, 70, 90 and 110 µg PC were accurately added to 10 mL volumetric flasks containing different ratios of CF, PZ and PC. The amplitudes of first derivative (delta lambda = 4 and scaling factor =10) of ratio spectra (using 30 µg/mL PZ divisor) after PC standard additions were represented on Y-axis and the concentrations of the added PC standards were represented on X-axis at the two selected wavelengths. The two curves (at 253.0 nm and 259.2 nm) were constructed. The H-point abscissa represents PC concentration in the determined mixture.

Dual wavelength in ratio spectrum method

Different aliquots from stock standard solution of CF (100 µg/mL), PZ (100 µg/mL) and PC (100 µg/mL) were transferred into a series of 10 mL volumetric flasks, and the volume was completed to mark with previously prepared solvent. Spectra of laboratory prepared mixtures having different ratios of CF, PZ and PC were firstly divided by the spectrum of 30 µg/mL PZ, then, the amplitude difference between 213.8 and 218.3 nm ($\Delta P^{PZ\ 30\mu g/mL}_{213.8-218.3nm}$) was obtained for determination of the concentration of CF, after substitution in the corresponding regression equation. PZ was determined in the same manner where the spectra of mixtures were divided by the spectrum of 30 µg/mL PC, then, the difference in amplitude between 248 nm and 240.3 nm ($\Delta P^{PC\ 30\mu g/mL}_{248-240.3\ nm}$) was calculated to determine the concentration of PZ, using the corresponding regression equation. Finally, the zero-order spectra of prepared mixtures were divided by the spectrum of 30 µg/mL PZ, then, the difference in amplitude between 242.4 nm and 247.5 nm ($\Delta P^{PZ\ 30\mu g/mL}_{242.4-247.5\ nm}$) was calculated and the concentration of PC was determined.

Application to pharmaceutical preparations

Twenty tablets were weighed and the average tablet weight was obtained, then the tablets were ground and well mixed in a mortar to ensure a good homogeneity. Into a 250 mL beaker, one average tablet weight (equivalent to 300 mg PC, 150 mg PZ and 50 mg CF) was transferred, 200 mL of the solvent were added and sonicated for 15 minutes, then filtered into 250 mL measuring

flask. The residue was washed using about 10 mL of the solvent for three times, then, the volume was completed with the same solvent to the mark.

H-point derivative ratio method

For determination of CF, a volume of 0.15 mL of the previously extracted solution was accurately transferred into each 10 mL measuring flask then aliquots containing 30, 50, 70, 90 and 110 µg CF were accurately added, and the volume was completed to the mark. After application of the first derivative on ratio spectra, the curves of standard additions were constructed at 255.2 nm and 262.9 nm as mentioned in procedures of laboratory prepared mixtures. Upon extrapolation, the point of intersection represents the H-point, whose abscissa represents CF concentration in the determined dosage form. By the same manner, PZ and PC were determined. For PZ determination, a volume of 0.15 mL of the previously extracted solution was accurately transferred into each 10 mL volumetric flask, then aliquots containing 30, 50, 70, 90 and 110 µg PZ were accurately added. Also, aliquots containing 30, 50, 70, 90 and 110 µg PC were transferred into 10 mL volumetric flasks containing a 0.15 mL of the previously extracted solution for the determination of PC. The first derivative of ratio spectra was obtained as mentioned previously, then, the curves of standard additions were constructed at 216.9, 219.1 nm, and 253.0, 259.2 nm for the determination of PZ and PC, respectively, in the determined dosage form.

Dual wavelength in ratio spectrum method

A volume of 0.15 mL of the previously extracted solution was accurately transferred into a 10 mL measuring flask and the mark with reached by the solvent. The same procedure under laboratory prepares mixtures was followed to determine the concentration of each component in the dosage form.

3. Results and discussion

The absorption spectra of CF, PZ and PC show high degree of interference as shown in **Figure 1**. The application of the direct spectrophotometry fails to determine either of them in their mixture.

The ability of the newly introduced spectrophotometric methods to resolve overlapped spectral data of three components and to determine selectively each component without interference from

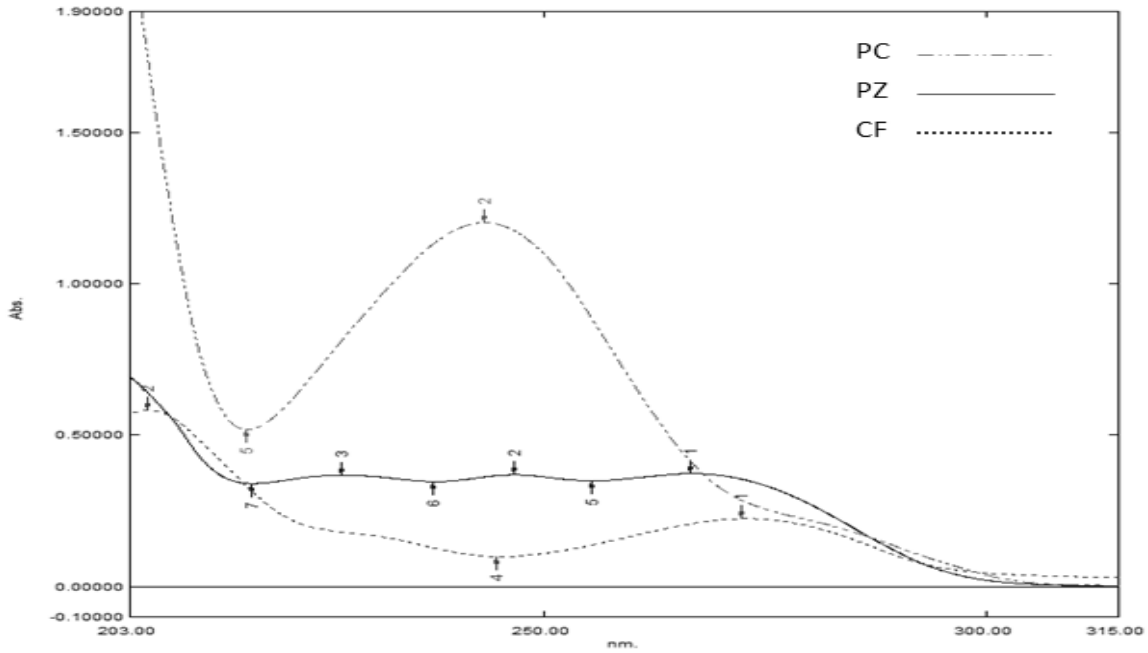

Figure 1. UV absorption spectra of CF 3 µg/mL (…), PZ 9 µg/mL (__) and PC 18 µg/mL (··—··).

the other components was proven experimentally via the practical application on ternary mixtures of CF, PZ and PC.

The traditional H-point standard additions method was applied for the analysis of binary mixtures [20], [21]. H point method has been applied on zero-order absorption spectra with simultaneous addition of both analytes [22], however, this method suffered from weak robustness. This problem was solved by the application of the H-point standard additions method principle on the ratio spectra using a normalized divisor of the interfering substance, where measurement can be done at any two points which increased the sensitivity and robustness of the method [23], but still the method is only dedicated to the assay of components in binary mixtures.

The current work introduces a modification on H-point standard additions method to enhance the method power in resolving spectral overlapping that the method be able to analyze the ternary mixtures. This modification relies on the fact that the application of derivative manipulation on the ratio spectra can eliminate the interference of one of the mixture components (used as a divisor). The principle of H-point standard additions method can be applied on the derivative ratio spectra for the determination of the other components.

The H-point standard additions method was applied on the first derivative of the ratio spectrum to resolve overlapped spectral data of ternary mixtures. The novel H-point derivative ratio method (HDR) merges H-point standard additions and derivative ratio principles for determination of the ternary mixtures. In determination of ternary mixtures, one component was cancelled by the derivative ratio theory and another component was cancelled by application of H-point standard additions, thus the third analyte can be accurately determined.

In the suggested method, the application of the derivative ratio principle removed the interference of one of three components, then, two wavelengths were selected at which a second component exhibits the same amplitude, while the third component exhibits different behavior at the selected wavelengths and can be determined.

For CF, the divisor involved in derivative ratio principle (PZ) was cancelled and the two wavelengths selected were 255.2 nm and 262.9 nm at which PC was cancelled (exhibited the same values), leaving the chance for CF only to be determined. On the other hand, PZ was determined using PC as a divisor in the derivative ratio application. The two selected wavelengths were 216.9 nm and 219.1 nm where CF exhibits the same values. Finally, determination of PC was achieved using PZ as a devisor and the two wavelengths were 253.0 nm and 259.2 nm.

Additional aliquots of one component were added to the ternary mixtures to determine the concentration of that component in the mixture by H-point standard additions theory. Aliquots containing 30, 50, 70, 90 and 110 µg of each component to be determined were accurately added to a separate 10 mL volumetric flask containing laboratory prepared mixture or pharmaceutical dosage form prepared solutions containing the three components.

Curves of standard additions - after the addition of increment amounts of the component to be determined - were constructed where the amplitudes belong to the first derivative of ratio spectra of each solution to be examined were represented on Y-axis while concentrations of the added standard were represented on X-axis at the two selected wavelengths.

By plotting the amplitudes versus the added standard concentrations, two straight lines of different slopes and intercepts were obtained. As the value of one component is constant at the two selected wavelengths, The straight lines obtained at the different wavelengths by applying the theory of H point standard additions will intersect at a common point [20], [21] which is the H

point, **Figure 2**, **Figure 3** and **Figure 4**. The abscissa refers to CF, PZ and PC concentrations respectively.

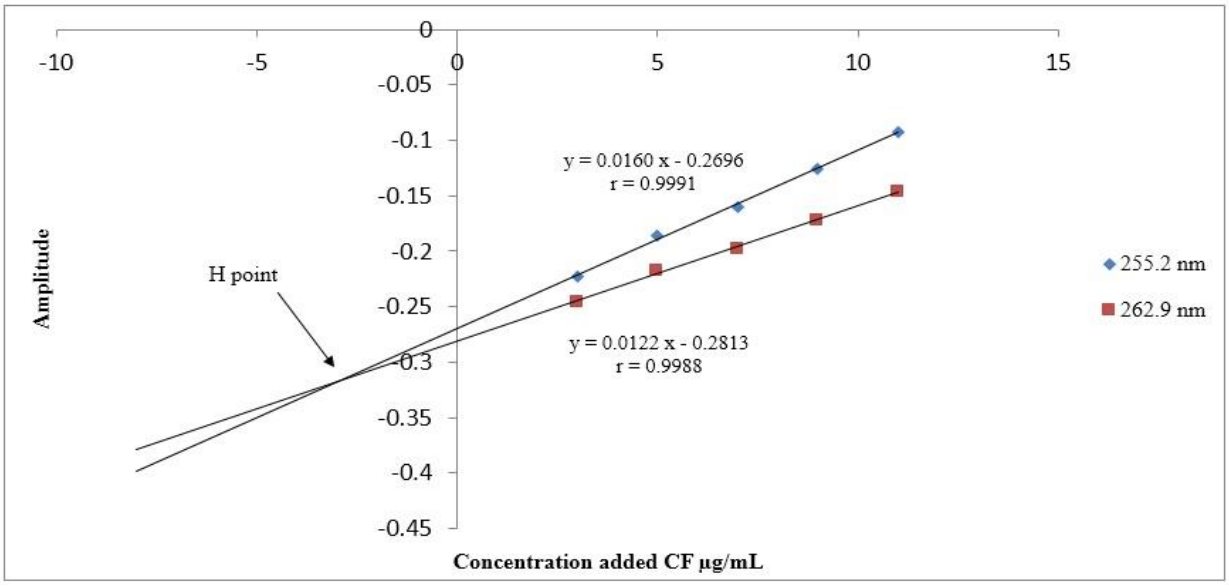

Figure 2. Plot of H-point derivative ratio method for a laboratory prepared mixture at 255.2 nm and 262.9 nm to the corresponding added concentrations of standard CF.

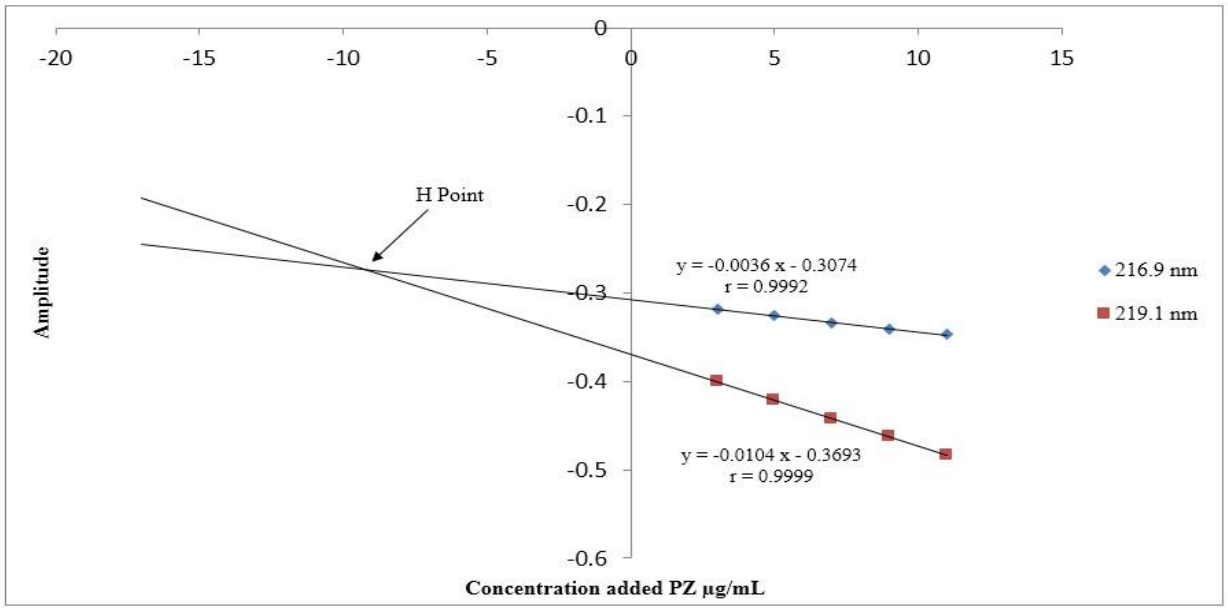

Figure 3. Plot of H-point derivative ratio method for a laboratory prepared mixture at 216.9 nm and 219.1 nm to the corresponding added concentrations of standard PZ.

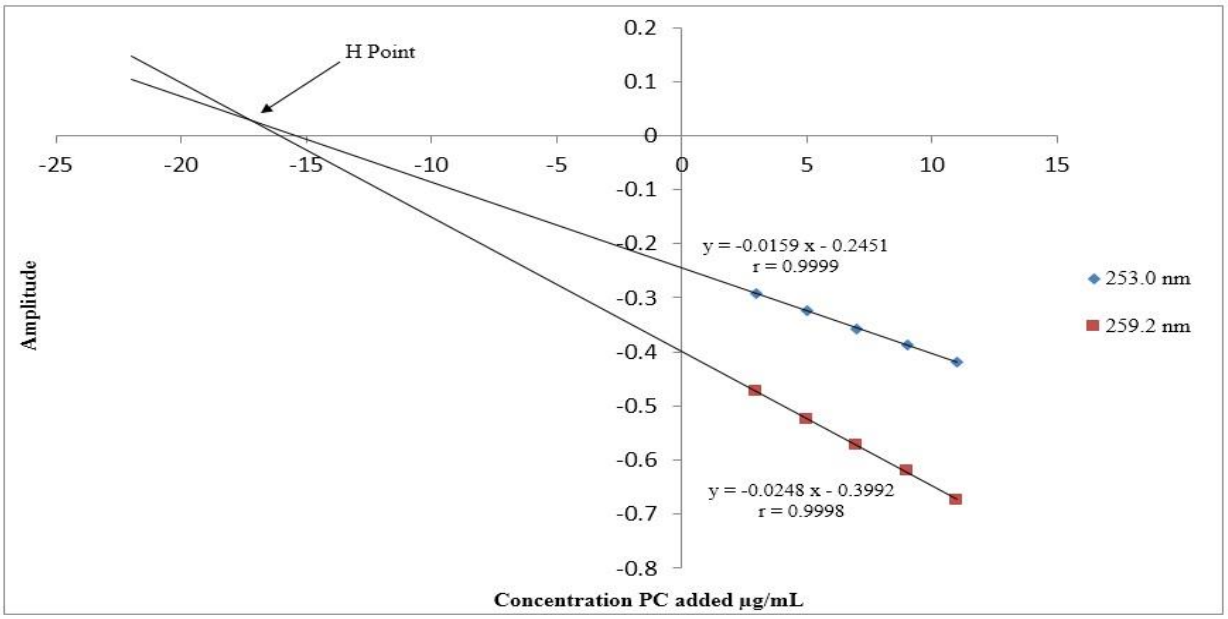

Figure 4. Plot of H-point derivative ratio method for a laboratory prepared mixture at 253.0 nm and 259.2 nm to the corresponding added concentrations of standard PC.

The method succeeded in determination of the laboratory-prepared mixtures containing different ratios of CF, PZ and PC and was validated as per the ICH guidelines [24]. The data showed that the method is accurate, precise and specific over the specified range, **Table 1**.

On the other hand, dual wavelength in ratio spectrum method merges dual wavelength and ratio difference methods for determination of the ternary mixtures. One interfering component is cancelled by the application of dual wavelength theory—but in the ratio spectra—at which the second interfering component—the divisor—is cancelled by the difference in the ratio spectra, **Figure 5**, **Figure 6** and **Figure 7**. The method differs from ratio difference method in that we do not have the opportunity to select any wavelength on the ratio spectrum, but the selected wavelength pair must exhibit exactly the same amplitude for the first interfering component in the ratio spectrum.

The dual wavelength in ratio spectrum method determines an analyte in a ternary mixture and removes the interference imposed by the other two components. Component A is used as a divisor to obtain a horizontal straight-line ratio spectrum where the difference between any two wavelengths is zero. Interference of component B is removed by selecting two wavelengths at which that component has exactly the same amplitude in the same ratio spectrum so the amplitude

Table 1. Validation parameters of the proposed methods for the determination of CF, PZ and PC according to ICH guidelines.

Method	DWRS			HDR		
Parameter	CF	PZ	PC	CF	PZ	PC
Accuracy [a] (Mean ± SD)	100.69 ± 1.13	101.09 ± 0.76	99.81 ± 0.51	101.22 ± 0.39	100.45 ± 0.63	100.82 ± 0.77
Specificity [b] (Mean ± SD)	99.87 ± 1.58	100.44 ± 0.78	100.65 ± 1.29	100.29 ± 1.07	99.89 ± 1.32	100.35 ± 1.15
Repeatability [c]	0.721	0.510	0.334	± 1.032	± 1.325	± 1.029
Intermediate precision [d]	0.450	0.652	0.793	± 0.863	± 0.911	± 0.811
Range	1.5 – 24 µg/mL	3 – 30 µg/mL	6 – 33 µg/mL	3 – 27 µg/mL	3 – 30 µg/mL	3 – 36 µg/mL
Slope	0.0268	0.0017	0.0036	at 255.2 nm : 0.0161 at 262.9 nm : 0.0122	at 216.9 nm : - 0.0036 at 219.1 nm : - 0.0104	at 253.0 nm : - 0.0159 at 259.2 nm : - 0.0247
Intercept	0.0219	0.0001	0.0002	at 255.2 nm : - 0.0812 at 262.9 nm : - 0.0834	at 216.9 nm : - 0.2173 at 219.1 nm : - 0.2212	at 253.0 nm : - 0.1104 at 259.2 nm : - 0.1169
Correlation coefficient (r)	0.9996	0.9998	0.9997	at 255.2 nm : 0.9991 at 262.9 nm : 0.9988	at 216.9 nm : 0.9992 at 219.1 nm : 0.9999	at 253.0 nm : 0.9999 at 259.2 nm : 0.9998
LOD	0.41 µg/mL	0.71 µg/mL	1.35 µg/mL	at 255.2 nm : 0.55 µg/mL at 262.9 nm : 0.57 µg/mL	at 216.9 nm : 0.90 µg/mL at 219.1 nm : 0.88 µg/mL	at 253.0 nm : 0.63 µg/mL at 259.2 nm : 0.59 µg/mL
LOQ	1.23 µg/mL	2.16 µg/mL	4.08 µg/mL	at 255.2 nm : 1.67 µg/mL at 262.9 nm : 1.71 µg/mL	at 216.9 nm : 2.73 µg/mL at 219.1 nm : 2.68 µg/mL	at 253.0 nm : 1.89 µg/mL at 259.2 nm : 1.78 µg/mL

[a] Accuracy (mean of percentage recoveries ± SD) assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range.
[b] Specificity (mean of percentage recoveries ± SD) of recovery percentage data of the laboratory prepared mixtures.
[c] The intraday (n = 3), RSD of three concentrations repeated three times within the day.
[d] The interday (n = 3), RSD of three concentrations repeated three times in three successive days.

difference at these two wavelengths is zero. Thereby, the analyte C will only contribute to the difference between the selected pair of wavelengths and can be determined.

The proposed method begins with scanning zero-order spectra of the prepared standard solutions of CF, PZ and PC in the previously prepared solvent, then, different divisor concentrations were tried. The divisors should be selected carefully to compromise between maximum sensitivity and minimal noise. The divisor concentration 30 µg/mL gave the best results regarding average recovery percent when used for the prediction of CF, PZ and PC concentrations.

Different wavelengths were chosen on the ratio spectra of the drugs and the linearity at those wavelengths singly was assessed. A good linearity at 213.8 and 218.3 nm for CF, at 248 and 240.3 nm for PZ and at 242.4 and 247.5 nm for PC was obtained.

The amplitude difference at 213.8 and 218.3 nm in the ratio spectrum obtained using 30 µg/mL PZ as a divisor ($\Delta P^{PZ\ 30\mu g/mL}_{213.8-218.3nm}$) was plotted versus the corresponding concentration of CF, and the regression equation was computed as shown in **(Eq. 1)**:

$$\Delta P^{PZ\,30\mu g/mL}_{213.8-218.3nm} = 0.0268C_{CF} + 0.0219 \qquad [1]$$

Where ΔP is the amplitude difference and C is the concentration. Also the linear correlation was obtained between the difference in amplitude of the ratio spectra at 248 and 240.3 nm for PZ using 30 µg/mL PC as a divisor ($\Delta P^{PC\ 30\mu g/mL}_{248-240.3\ nm}$) and at 242.4 and 247.5 nm for PC using 30 µg/mL PZ as a divisor ($\Delta P^{PZ\ 30\mu g/mL}_{242.4-247.5\ nm}$) against the corresponding concentration of PZ and PC, respectively, as shown in **(Eq. 2)** and **(Eq. 3)**:

$$\Delta P^{PC\,30\mu g/mL}_{248-240.3nm} = 0.0017C_{PZ} + 0.0001 \qquad [2]$$

$$\Delta P^{PZ\,30\mu g/mL}_{242.4-247.5nm} = 0.0036C_{PC} + 0.0002 \qquad [3]$$

The proposed method was successfully applied for the simultaneous determination of CF, PZ and PC in laboratory prepared mixtures of different ratios of CF, PZ and PC.

The validation parameters according to ICH guidelines [24] are presented in **Table 1**, where the results obtained by the new method proved to be accurate, precise and specific over the specified range.

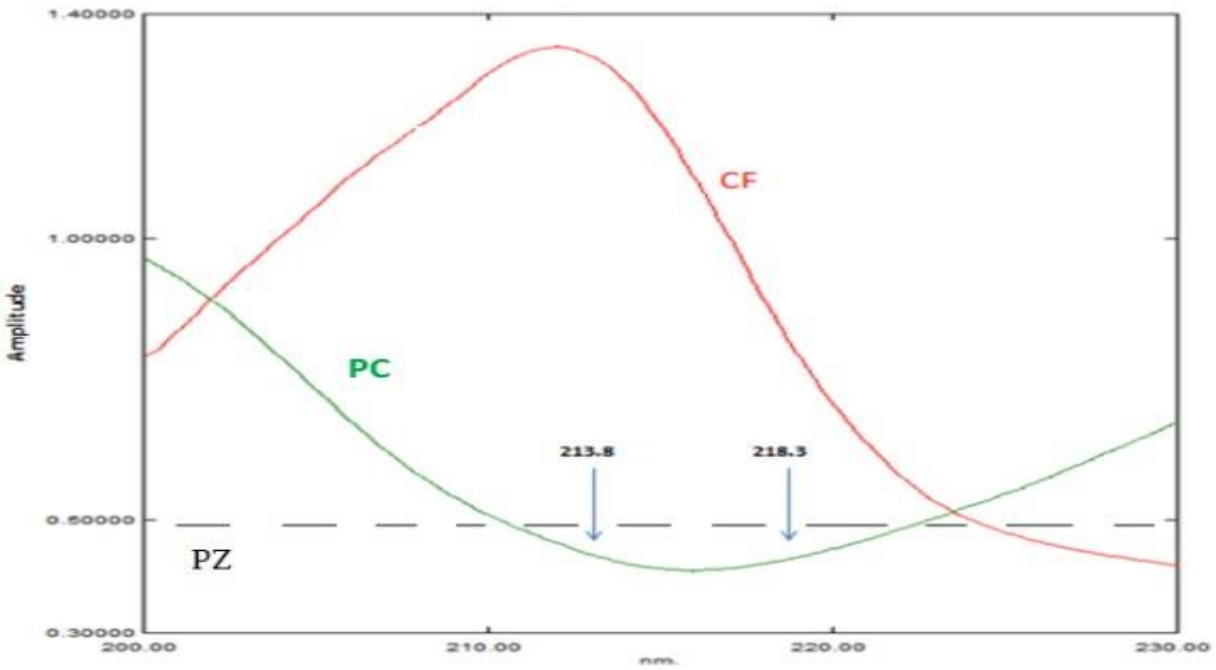

Figure 5. Ratio spectra of CF (15 µg/mL), PZ (15 µg/mL) and PC (15 µg/mL) divided by PZ (30 µg/mL) showing the wavelengths selected for determination of CF.

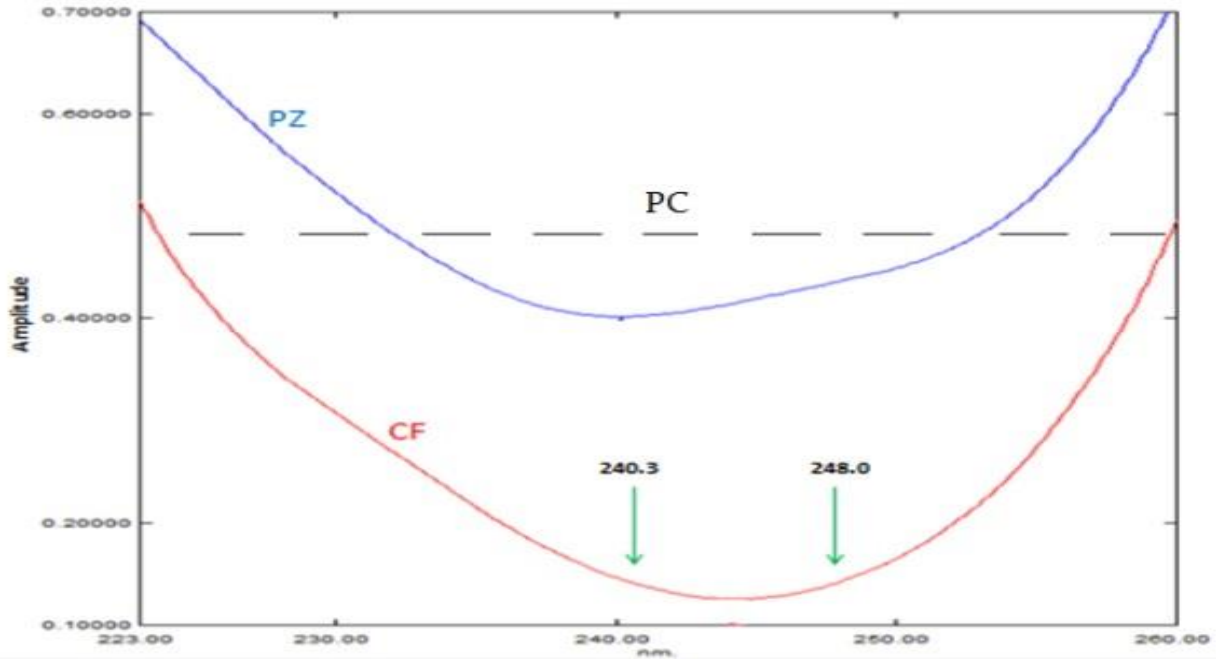

Figure 6. Ratio spectra of CF (15 µg/mL), PZ (15 µg/mL) and PC (15 µg/mL) divided by PC (30 µg/mL) showing the wavelengths selected for determination of PZ.

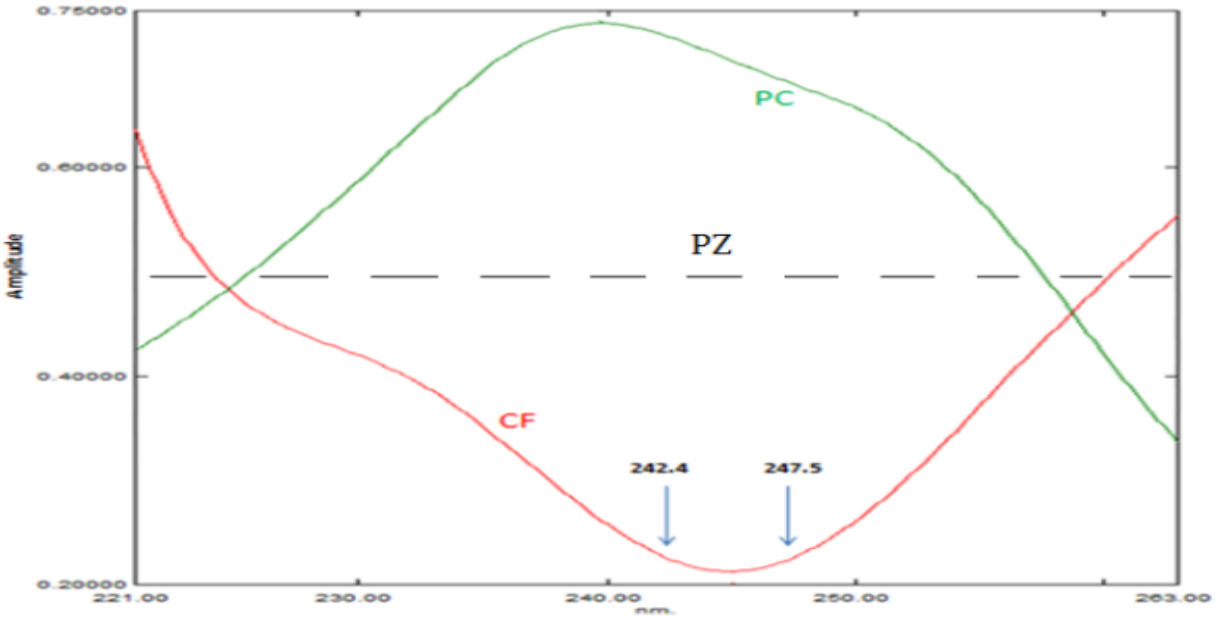

Figure 7. Ratio spectra of CF (15 µg/mL), PZ (15 µg/mL) and PC (15 µg/mL) divided by PZ (30 µg/mL) showing the wavelengths selected for determination of PC.

The two suggested methods were found to be applicable and valid for the analysis of Stopain® tablet with no interference from the excipients. The validity of the proposed procedure was tested by the application of standard addition technique, **Table 2**.

Table 2. Analysis of pharmaceutical preparation and application of standard addition technique.

Method	Component	Proposed Method R%[a] ± SD	Standard addition R%[b] ± SD
Dual wavelength in ratio spectrum (DWRS)	CF	101.89 ± 1.65	100.43 ± 1.07
	PZ	102.12 ± 1.43	100.36 ± 1.59
	PC	101.62 ± 2.05	101.60 ± 0.27
H-point derivative ratio (HDR)	CF	102.21 ± 0.98	99.88 ± 1.45
	PZ	99.13 ± 1.73	100.04 ± 1.19
	PC	101.84 ± 1.88	99.65 ± 1.28

[a] Average of three determinations (Stopain® tablet labeled to contain 300 mg PC, 150 mg PZ and 50 mg CF per tablet).
[b] Average of nine determinations over three concentration levels.

One-way ANOVA Statistical comparison at 5% significance level [25] was performed on the recovery percent data obtained from the application of the two proposed methods on the pharmaceutical dosage form as shown in **Table 3**. The test proved that there were no significant differences (P>0.05) between the proposed methods and reference spectrophotometric method [19]. The described methods can be used for accurate determination of CF, PZ and PC in their ternary mixtures and in pharmaceutical preparations.

Table 3. One-way ANOVA statistical analysis within 95% confidence interval on recovery percentage results obtained from the reported method and application of the two proposed methods on pharmaceutical preparation.

	One Way ANOVA Dependent variable: Recovery percentage data					
	Source	Sum of Squares	df [a]	Mean Square	F [b]	P-value
CF	Between Groups [c]	0.793	2	0.397	0.027 (5.14 [d])	0.973
	Within Groups	86.684	6	14.447		
	Total	87.477	8			
PZ	Between Groups [c]	6.607	2	3.303	0.325 (5.14 [d])	0.717
	Within Groups	56.246	6	9.374		
	Total	62.853	8			
PC	Between Groups [c]	42.311	2	21.155	3.725 (5.14 [d])	0.089
	Within Groups	34.074	6	5.679		
	Total	76.384	8			

[a] Degrees of freedom.
[b] F is the ratio of mean square to error mean square.
[c] Between reported method (Derivative ratio spectra-zero crossing spectrophotometric method) [19] and the two corresponding methods.
[d] The tabulated value of F.

4. Conclusion

H-point standard additions carried out in the derivative ratio spectrum engenders the novel hybrid HDR method. The latter resolves overlapped spectral data of the ternary mixtures. The method successfully compares to the recent DWRS method for the determination of a ternary mixture of CF, PZ and PC in presence of pharmaceutical excipients without prior separation. The

two methods were validated and show adequate sensitivity and selectivity for the analysis of the studied mixture.

- **Conflict of Interest**

The Authors declare no conflict of interest.

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