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ORIGINAL ARTICLE

A Synchronous Fluorescence Spectrofluorometric Method for the Simultaneous Determination of Clonazepam and Paroxetine Hydrochloride in Combined Pharmaceutical Dose Form

Kombine Farmasötik İlaç Şekillerinde Klonozepam ve Paroksetin Hidroklorür'ün Aynı Anda Belirlenmeleri İçin Senkronize Spektroflorimetrik Yöntem

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ABSTRACT

Objectives: First derivative synchronous spectrofluorimetry has been found to be superior because of its highly specific spectral discrimination and readily available solvent, it is economical, eco-friendly, and lacks an extraction procedure.

Materials and Methods: In the present study, a new simple, sensitive, and time-saving first derivative synchronous spectrofluorimetry method has been developed for simultaneous estimation of clonazepam (CLO) and paroxetine hydrochloride (PH) in pharmaceutical dose forms.

Results: CLO was determined at the emission wavelength of 512.79 nm (zero-crossing wavelength point of PH). Similarly, PH was measured at 336.00 nm (zero-crossing wavelength point of CLO). The first derivative amplitude-concentration plots were rectilinear over the range of 1-5 µg/mL for CLO and 5-25 µg/mL for PH. The method was validated statistically as per the ICH guidelines. The limits of detection were 0.055 and 0.033 µg/mL and quantification limits were 0.169 and 0.102 µg/mL for CLO and PH, respectively. The percentage recovery in commercial formulation was found to be in the range 100.45% and 99.38% for CLO and PH, respectively, by the proposed method, and percent relative standard deviation values for precision and accuracy studies were found to be less than 2.

Conclusion: This spectrofluorimetry method has been found to have several advantages such as simple spectra, high selectivity, and low interference. By virtue of its high sensitivity, this method could be applied to the analysis of both CLO and PH in their co-formulated dose forms.

Key words: First-derivative synchronous spectrofluorimetry, clonazepam, paroxetine hydrochloride

ÖZ

Amaç: Yüksek spesifiklikte spektral ayırıcılığı, kolay bulunan çözücülerin kullanımı, ekonomik ve çevre dostu oluşu ve ekstraksiyona gerek duymaması sebebiyle birinci türev senkron spektroflorimetri yönteminin üstün olduğu bulundu.

Gereç ve Yöntemler: Bu çalışmada farmasötik preparatlardan klonazepam (CLO) ve paroksetin hidroklorürün (PH) eş zamanlı tayini için basit, hassas ve zamandan tasarruf sağlayan birinci türev senkronize spektroflorimetri yöntemi geliştirilmiştir.

Bulgular: CLO, 512.79 nm'deki (PH'nin sıfır olduğu dalga boyu noktasında) emisyon dalga boyunda tayin edilmiştir. Benzer şekilde PH ise 336.00 nm'deki (CLO'nun sıfır olduğu dalga boyu noktasında) emisyon dalga boyunda ölçüm yapılarak tayin edilmiştir. Birinci türev için pik yüksekliğine karşı çizilen konsantrasyon grafiği CLO için 1-5 µg/mL, PH için 5-25 µg/mL aralığında doğrusal olarak bulunmuştur. Yöntemin istatistiksel validasyonu ICH kılavuzlarına uygun olarak istatistiksel gerçekleştirilmiştir. Teşhis sınırı CLO ve PH için sırasıyla 0.055 ve 0.033 µg/mL, tayin sınırı ise 0.169 ve 0.102 µg/mL'dir. Önerilen yöntem kullanılarak piyasa preparatlarındaki yüzde geri kazanım sonuçları CLO ve PH için sırasıyla %100.45 ile %99.38 arasında ve yüzde bağıl standart sapma değerleri de kesinlik ve doğruluk çalışmalarında 2'den daha düşük bulunmuştur.

Sonuç: Bu spektroflorimetri yönteminin, basit spektrumlar, yüksek seçicilik ve düşük girişim gibi birçok avantajı olduğu bulunmuştur. Yüksek duyarlılığından dolayı, bu yöntem CLO ve PH'nin birlikte formüle edilmiş dozaj formlarından analizi için uygundur.

Anahtar kelimeler: Birinci türev senkronize spektroflorimetri, klonazepam, paroksetin hidroklorür

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INTRODUCTION

The frequent association between panic disorders and depression is extensively documented in both clinical and epidemiologic studies, and it is considered to be more of a common phenomenon than an exception. Current treatment recommendations for comorbid depression and anxiety are based on clinical experience with the treatment of anxiety and depressive disorders when they occur independently. There are studies that have tried combinations of selective serotonin reuptake inhibitors (SSRI) (paroxetine) and benzodiazepine (clonazepam) in patients of depression and anxiety/panic disorders. Hence, the availability of a fixed dose combination of an anti-depressant such as paroxetine hydrochloride (PH) and an anti-anxiety drug such as clonazepam (CLO) would be a useful treatment option for the management of co-morbid depression and anxiety.[1-4]

CLO [5-(2-chlorophenyl)-7-nitro-2, 3-dihydro-1H-1,4-benzodiazepin-2-one], Figure 1 is a benzodiazepine drug that has anxiolytic, anticonvulsant, muscle relaxant, sedative, and hypnotic properties. CLO exerts its action by binding to the benzodiazepine site of GABA receptors, which causes an enhancement of the electrical effect of GABA binding on neurons, resulting in an increased influx of chloride ions into the neurons. These result in an inhibition of synaptic transmission across the central nervous system.[3-5]

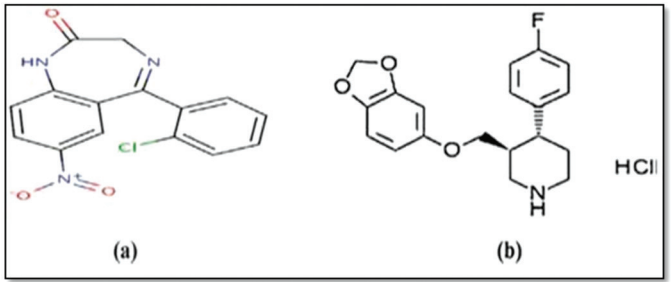

Figure 1. Chemical structure of (a) CLO and (b) PH

PH [(-)-Trans-4R-(4'-fluorophenyl)-3S-[(3', 4'-methylenedi-oxyphenoxy) methyl] piperidine hydrochloride], Figure 1b is an SSRI and potentiates 5-HT in the CNS. PH is indicated for the treatment of major depressive disorder, social anxiety disorder, obsessive-compulsive disorder, panic disorder, generalized anxiety disorder, and post-traumatic stress disorder. It exerts its antidepressant effect through selective inhibition for the reuptake of the neurotransmitter serotonin by presynaptic receptors.[3-5]

CLO and PH both are official drugs in the Indian Pharmacopoeia, United States Pharmacopeia, and British Pharmacopoeia when used individually, but the combination of CLO and PH is not official in any pharmacopoeia.[5-7] CLO and PH have been formulated in a fixed-dose combination and used in the treatment of depression, anxiety, and comorbidities.

Few analytical methods including spectrophotometry[8], colorimetry[9], stability-indicating high-performance liquid chromatography[10,11] high-performance thin layer chromatography[12] and ultra-performance liquid chromatography[13] have been reported for the simultaneous estimation of CLO and PH in combined pharmaceutical formulations. Chromatographic methods offer a high degree of specificity, yet they require large amounts of high purity organic solvents and generate a large amount of waste. Therefore, there is a need for an alternative to these techniques for the routine quality control analysis of the concerned drug.

To the best of our knowledge, no spectrofluorimetric method has yet been reported for the quantification of CLO and PH in combined formulations. Spectrofluorimetric methods have been found more selective than normal UV-spectroscopy due to the quantification of substance at excitation and emission wavelengths. Derivative spectrofluorimetry provides greater selectivity and spectral discrimination than common spectrofluorimetry. It is a powerful approach for the resolution of one analyte whose peak is hidden by a large overlapping peak of another analyte in multi-component analysis. Synchronous fluorescence spectroscopy has been found to have several advantages such as simple spectra, high selectivity, and low interference. The combination of synchronous scanning spectrofluorimetry with derivative techniques is advantageous in terms of sensitivity, spectral discrimination, and more reliable identification of chemical species in multi component analysis.[14,15]

The goal of the present work was to develop a simple, cost effective, sensitive, and rapid method for the simultaneous determination of CLO and PH in tablet form through first-derivative synchronous fluorimetry (FDSF) based on their native fluorescence. The emission spectra of CLO and PH overlap, which makes it difficult to analyze and determine their contents by conventional fluorimetry. These problems were minimized by using FDSF.

EXPERIMENTAL

Materials

All chemicals and reagents were of analytical grade. The CLO and PH were obtained as gift samples from Vital Formulation, Anand and Torrent Pharmaceutical, and Ahmedabad, respectively. Pari CR Plus formulation (CLO 0.5 mg and PH 12.5 mg) were purchased from local pharmacies. Analytical grade methanol purchased from Merck, Mumbai, was used throughout these experiments.

Apparatus

Fluorescence spectra and measurements were recorded using a spectroflorophotometer (RF-5301PC series, Shimadzu Corporation, Japan), which allowed high sensitivity analysis based on a unique optical system that involves highly efficient Blazed Holographic Grating as well as low-noise circuitry that includes a digital filter. Spectroflorophotometer equipped with a 150 W xenon arc lamp and slit widths for both monochromators were set at 10 nm. A 1 cm^2 quartz cell was used for all measurements. Spectra and intensities were automatically

obtained using Shimadzu fluorescence spectroscopy software, RFPC version 2.04.

Preparation of standard solutions

Standard stock solutions each containing 1000 µg/mL of CLO and PH were prepared separately in the methanol. Working standard solutions (100 µg/mL) of the mentioned drugs were obtained by dilution of the respective stock solution in methanol. Working solutions were prepared separately by making serial dilutions from the standard solution to obtain concentrations between 1-5 and 5-25 µg/mL for CLO and PH, respectively, and fluorescence intensity was quantified using a spectroflorophotometer.

Spectrofluorimetric methods

For spectrofluorimetry, the solutions were scanned between 220 nm to 800 nm and emission wavelength was selected based on the maximum fluorescence intensity. The excitation and emission wavelength of both the drugs were found. The synchronous spectra of CLO and PH were obtained by keeping a constant interval between emission and excitation wavelength (i.e $\Delta\lambda$= 20). The obtained synchronous spectra of both drugs were converted to the 1st derivative spectra by optimizing $\Delta\lambda$= 20. The first derivative synchronized spectrum of CLO has zero intensity at 336.00 nm, whereas PH gives significant derivative response. The derivative spectrum of PH has zero intensity at 512.79 nm, and CLO gives significant derivative response by maintaining a constant interval $\Delta\lambda$= 20. Therefore, 512.79 nm and 336.00 nm were chosen for the estimation of CLO and PH, respectively. Different aliquots were transferred for both drugs to 10 nm volumetric flasks and the volumes were adjusted to the mark with methanol to obtain concentrations of 1-5 µg/mL of CLO and 5-25 µg/mL PH, respectively. The calibration curves between concentration and fluorescence intensity were plotted and corresponding regression equations were derived.

Method validation

The proposed methods were validated in accordance with International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines Q2 (R1) for the evaluation of various parameters: linearity, precision, accuracy, limit of detection, limit of quantification, specificity, and robustness.[16]

Linearity and range

The linear relationship between concentration and amplitude of both drugs were evaluated over the concentration range expressed in the concentrations range of 1-5 µg/mL for CLO, 5-25 µg/mL was selected for PH. The linearity ranges for the determination of CLO and PH by the proposed methods were repeated five times. Calibration curves were constructed by plotting the analyte intensity against the respective concentration.

LOD and LOQ

As per the ICH guidelines, the limit of detection and quantification of the developed method were calculated from the standard deviation of the response and slope of the calibration curve of each drug using the following formulas:

Limit of detection= $3.3x\sigma/S$

Limit of quantification= $10x\sigma/S$

Where, "σ" is standard deviation of response, "S" is Slope of calibration curve.

Precision

The precision of the developed methods were evaluated by performing intraday precision on the same day and interday precision studies on different days in three replicates. Repeatability and intermediate precision was performed for CLO at 1, 3, 5 µg/mL, and 5, 15, 25 µg/mL for PH. The percent relative standard deviation (% RSD) was calculated.

Accuracy

Accuracy of method was ascertained by performing a recovery study using the standard addition method; a known amount of standard drug was added to preanalyzed samples of CLO and PH at three concentration levels (50%, 100%, and 150%) in triplicate. The percentage recovery and RSD were calculated for each concentration.

Robustness

Robustness was performed by making deliberate changes in wavelength and different model of UV visible spectrophotometer. % RSD was calculated.

Applicability of the proposed method for analysis of formulation

For the analysis of the marketed formulation, 20 tablets were weighed and finely powdered. From the tablet sample, an amount equivalent to 0.5 mg of CLO and 12.5 mg PH were accurately weighed and taken into a 100 mL volumetric flask. About 30 mL of methanol was added and the mixture was sonicated for 15 min. The mixture was diluted to volume with methanol, mixed well and filtered to obtain the sample stock solution, 5 µg/mL of CLO and 125 µg/mL of PH. The resultant solution was used for the analysis of CLO and for analysis of PH; 1 mL from the above solution was withdrawn and the volume was made up to 10 mL to make 12.5 µg/mL for PH. The resultant solutions were then used to estimate both drugs at their particular λ_{max} for both methods. The analysis was repeated in triplicate.

RESULTS AND DISCUSSION

Synchronous fluorescence spectrofluorimetric method

Different solvent systems were tested in order to find the best conditions such as solubility, fluorescence activity, stability, and spectral discrimination (clear separation) of both drugs. From the results, it was found that CLO and PH gave comparatively high fluorescence intensity in methanol. CLO exhibited native fluorescence at emission wavelength of 460 nm after excitation at 258 nm and similarly PH exhibited fluorescence at emission wavelength of 545 nm after excitation at 288 nm in methanol. It was revealed that the fluorescence spectra of these drugs overlapped considerably. As a result, the conventional spectrofluorimetric method did not permit the simultaneous determination of both drugs.

The overlapped spectra were resolved by using a first-order derivative synchronous spectrofluorimetric method, which was used to choose the suitable wavelengths that make the estimation proportional to CLO and PH concentrations with the zero cross over point. It was necessary to record the synchronous spectra of CLO and PH shown in Figure 2 and 3, corrected for the blank signal. There was large overlap of the spectra; the quantification of CLO and PH through synchronous spectrofluorimetry was not possible. This overlap was highly discriminated by using a first-derivative synchronous spectrofluorimetric method (Figure 4).

The first-derivative synchronized spectrum of CLO has zero intensity at 336.00 nm, whereas PH gives significant derivative response. The derivative spectrum of PH has zero intensity at 512.79 nm, whereas CLO gives a significant derivative response by maintaining a constant interval $\Delta\lambda$= 20. Therefore, 512.79 nm and 336.00 nm were chosen for the estimation of CLO and PH, respectively, in tablet form (Figure 5, 6).

Method validation

The validation of the methods was performed according to ICH recommendations.

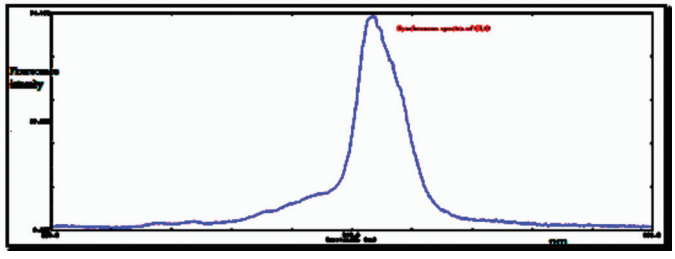

Figure 2. Synchronous spectrum of CLO

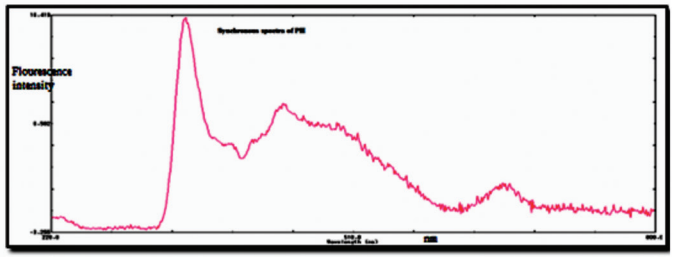

Figure 3. Synchronous spectrum of PH

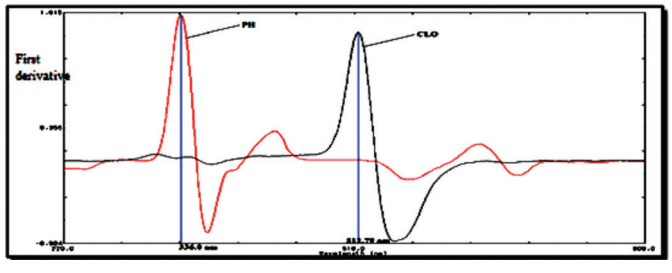

Figure 4. Overlay of 1st order derivative spectrum of CLO and PH

Linearity

The calibration ranges for CLO and PH were established through considerations of the practical range necessary according to Beer-Lambert's law. Linearity was evaluated using the least square regression method. The responses for CLO were found to be linear in the concentration range of 1-5 µg/mL with a correlation co-efficient (r^2) value of 0.9986 as depicted in Figure 7. Similarly, the responses for PH were linear in the concentration range of 5-25 µg/mL with a correlation coefficient (r^2) value of 0.9982, as shown in Figure 8. The values of correlation coefficients of CLO and PH were close to unity, indicating good linearity. The characteristic parameters for the constructed equations are summarized in Table 1.

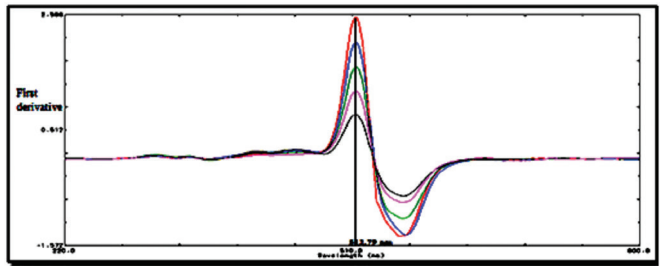

Figure 5. 1st order derivative spectrum of CLO at 512.79 nm (1-5 µg/mL)

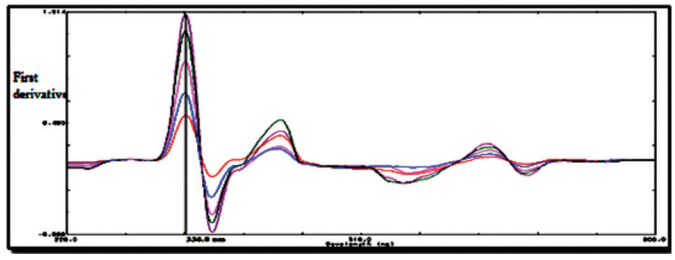

Figure 6. 1st order derivative spectrum of PH at 336.00 nm (1-5 µg/mL)

Table 1. Linear regression parameters for CLO and PH

Parameters	CLO	PH
Linearity range (µg/mL)	1-5	5-25
Correlation coefficient (r^2)	0.9986	0.9982
Slope ± SD[b] (S_b)	0.470±0.005	0.063±0.009
Confidence limit of slope	0.435 to 0.506	0.058 to 0.068
Intercept ± SD[b] (S_a)	0.274±0.008	0.087±0.012
Confidence limit of intercept	0.156 to 0.392	0.006 to 0.169
Limit of detection (µg/mL)	0.055	0.033
Limit of quantification (µg/mL)	0.169	0.102
Bartlett's test[b] (χ^2)	0.0110	0.0054

[b]: Mean of five determinations, SD: Standard deviation, RSD %: Relative standard deviation, b χ^2 critical value: 9.488 at α=0.05, [a]: Confidence interval at 95% confidence level and 4 degree of freedom (t=2.78)

Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection and limit of quantification were determined based on the standard deviation of response (y-intercept) and slope of the calibration curve according to ICH guidelines. LOD and LOQ for CLO were found as 0.055 µg/mL and 0.169 µg/mL, and 0.033 µg/mL and 0.102 µg/mL for PH, respectively, as tabulated in Table 1.

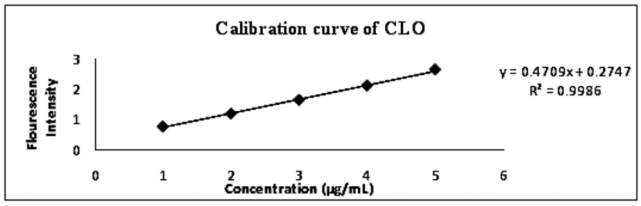

Figure 7. Calibration curve of CLO at 512.79 nm

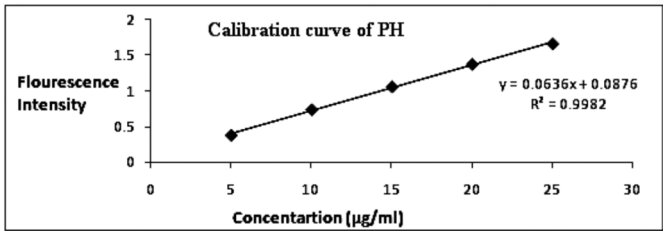

Figure 8. Calibration curve of PH at 336.00 nm

Table 2. Precision study

Amount of drug (µg/mL)	Intraday precision		Interday precision	
	Amount of drug found ± SD (µg/mL)	RSD %	Amount of drug found ± SD (µg/mL)	RSD %
CLO				
1	1.056±0.003	0.454	1.069±0.007	0.946
3	2.899±0.007	0.473	2.956±0.026	1.563
5	5.003±0.019	0.723	5.072±0.036	1.360
PH				
5	4.951±0.002	0.663	4.969±0.005	1.309
15	15.010±0.008	0.826	15.187±0.019	1.801
25	24.787±0.019	1.170	24.817±0.022	1.375

n=3 concentration/3 replicates, SD: Standard deviation, RSD %: Relative standard deviation

Precision

The intraday and interday precision were determined by the analysis of three different concentrations of CLO and PH, within the linearity range, through three replicate analyses of three pure samples of both drugs on a single day and on three consecutive days, respectively. As indicated in Table 2, data showed RSD % less than 2%. The precision of the method was considered acceptable based on its intended use.

Accuracy

Recovery study by spiking the standard at 3 concentration levels, 50, 100 and 150% showed RSD % of less than 2% with acceptable percent recovery, indicating that the proposed method was accurate and could be applicable for routine analysis of formulation (Table 3).

Robustness

The method remained unaffected by deliberate small changes in parameters such as wavelength (±1 nm) and model of UV spectrophotometer. The tabulated values indicate that the method was robust in terms of changed wavelength and model of UV spectrophotometer. The data are presented in Table 4.

Analysis of marketed dosage form

The proposed method was applied to the assay of commercially available tablets containing CLO (0.5 mg) and PH (12.5 mg). The percentage potency in the commercial formulations was found as 100.45% for CLO and 99.38% for PH using the proposed method. The RSD % for the formulations was less than 2 for both drugs, as shown in Table 5. The percentage recoveries of the amount of CLO and PH in tablet dose form, expressed as a percentage assay, were in good agreement with the label claims, thereby suggesting that there was no interference from any of the excipients that normally present in tablets.

Table 3. Recovery study for CLO and PH by proposed method

Drugs	Taken (µg/mL)	Level %	Amount of standard added (µg/mL)	Total amount of drug found (µg/mL)	Recovery ± SD %	RSD %
CLO	2	50%	1	3.024	101.21±0.015	0.015
		100%	2	4.007	100.90±0.026	0.025
		150%	3	5.029	100.84±0.034	0.034
PH	10	50%	5	15.118	101.20±0.071	0.070
		100%	10	20.267	100.15±0.211	0.211
		150%	15	25.244	100.78±0.232	0.232

n=3 concentration/3 replicates, SD: Standard deviation, RSD %: Relative standard deviation

Table 4. Robustness of proposed method

Sr. No	Variable parameters	Amount of drug found ± SD	RSD %
CLO			
1	Equipment 1 UV Spectrophotometer model-UV-1700	5.051±0.0358	1.354
	Equipment 2 UV Spectrophotometer model-UV-1800	5.029±0.0356	1.348
2	Wavelength 513.79 nm	4.906±0.0289	1.315
	Wavelength 511.79 nm	4.762±0.0280	1.276
PH			
1	Equipment 1 UV Spectrophotometer model-UV-1700	4.621±0.001	0.618
	Equipment 2 UV Spectrophotometer model-UV-1800	4.951±0.002	0.663
2	Wavelength 335.00 nm	4.321±0.004	1.138
	Wavelength 337.00 nm	4.969±0.005	1.309

SD: Standard deviation

Table 5. Analysis of marketed dose form

Formulation	Drug	Label claim	Assay ± SD %	RSD %
Pari CR plus	CLO	0.5 mg	100.451±0.926	1.295
	PH	12.5 mg	99.381±0.300	0.331

n=3 replicates, % RSD: Relative standard deviation; SD: Standard deviation

CONCLUSION

In the present study, a new simple, sensitive, and time-saving first-derivative synchronous spectrofluorimetry method was developed for simultaneous estimation of CLO and PH in pharmaceutical dose forms. First-derivative synchronous spectrofluorimetry has been found to be superior because of its highly specific spectral discrimination, readily available solvent, cost effectiveness, eco-friendly nature, and lack of extraction procedure. This spectrofluorimetry method has been found to have several advantages such as simple spectra, high selectivity, and low interference. By virtue of its high sensitivity, this method can be applied to the analysis of both CLO and PH in their co-formulated dose forms.

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Conflict of Interest: No conflict of interest was declared by the authors.

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