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First Derivative Synchronous Spectrofluorimetric Determination of Cyproheptadine Hydrochloride in Presence of its Oxidative Degradation Product at Critical Micelle Concentration

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ABSTRACT

Objectives: Simple and sensitive first derivative synchronous spectrofluorimetric method was developed for the determination of cyproheptadine hydrochloride in presence of its oxidative degradation product. **Method:** the method was based on measuring the synchronous fluorescence of the drug in water at $\Delta\lambda$ of 120 nm in the presence of a sodium dodecyl sulphate as a micellar system. The peak amplitude of the first derivative spectra was measured at 418 nm for the drug. The different experimental parameters affecting the synchronous fluorescence intensity of CYP were studied and optimized. **Results:** The peak amplitude–concentration plot was rectilinear over the range of 0.1–1.6 $\mu\text{g mL}^{-1}$. The limit of detection (LOD) was 0.02 and quantification limit (LOQ) was 0.06 $\mu\text{g mL}^{-1}$. **Conclusion:** The proposed method was successfully applied to commercial tablets. Statistical comparison of the results with those of the official method revealed good agreement and proved that there was no significant difference in the accuracy and precision of the two methods.

Keywords: Cyproheptadine Hydrochloride; First derivative synchronous spectrofluorimetry; Micellar medium

INTRODUCTION

Cyproheptadine hydrochloride (CYP), chemically known as 4-(5H-dibenzo [a,d]cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride (**Figure 1**), is a first generation antihistamine used for treating allergic reactions by blocking the effects of histamine and act as appetite stimulant effect and reduce or prevent experimental CNS ischemic damage due to its calcium channel blocking effect^{1,2}. It is used in treatment of nightmares associated with posttraumatic stress disorder^{3,4}. The United States Pharmacopeia⁵ describes non-aqueous titration using crystal violet as an indicator. The analytical methods that are reported for the determination of CYP in pharmaceutical formulations and biological samples include Liquid chromatography-mass spectrometry (LC-MS)^{6,7}, gas-liquid chromatography^{8,9}, and high-performance liquid

chromatography (HPLC)¹⁰⁻¹⁸. Also, several spectrophotometric methods were developed for its determination¹⁹⁻²⁸. And finally, a spectrofluorimetric method has been reported²⁹.

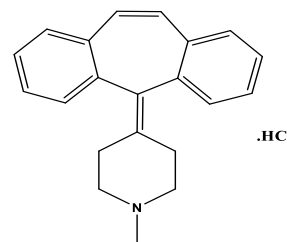


Figure 1. Structure formula of cyproheptadine.HCl.

The current paper implies the use of derivative synchronous processing to overcome overlapping

between CYP and its oxidative degradation product. The method allows a quick determination of CYP in bulk drug and in tablets without pretreatment of the sample with high accuracy and precision and without interference from excipients. The proposed method can be considered as a simple and fast alternative to the already existing HPLC procedures.

MATERIALS AND METHODS

Apparatus

- Jasco FP-6200 Spectrofluorometer (Japan), equipped with 150 Watt Xenon lamp, holographic grating monochromators of excitation and emission were used for all measurements. A 1 cm quartz cell was used and Slit widths for both monochromators were set at 10 nm.
- Jenway, 3510 pH meter (Jenway, U.S.A.).
- Hotplate (Torrey Pines Scientific, USA).
- Rotary evaporator (Scilogex-RE 100-pro, USA).
- Precoated TLC plates silica gel 60G_{F254} (Fluka, Chemie, Switzerland).

Materials and Reagents

All chemicals and reagents used throughout the work were of analytical grade.

- Cyproheptadine hydrochloride was kindly provided by Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt. The purity of the sample was found to be 99.69 ± 0.51 according to the official USP method [5].
- Triactin[®] tablets labeled to contain 4 mg CYP/tablet (batch No. #D215520), the product of Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt.
- Sodium dodecyl sulphate (SDS; 95 %) and cetyltrimethylammonium bromide (CTAB) was purchased from (Winlab, UK), prepared as 0.5 % aqueous solutions.

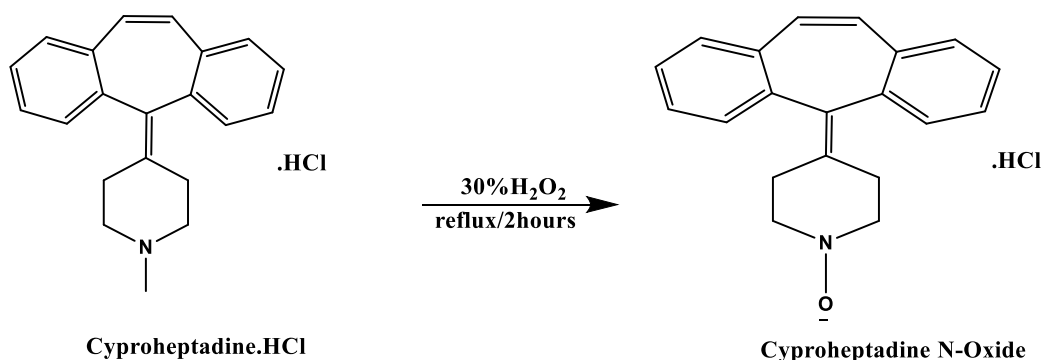
- Acetonitrile, n-propanol, ethanol and methanol were obtained from Tedia (USA).
- Tetrahydrofuran (THF) 99% (Lab scan, USA).
- β -Cyclodextrin (β -CD) (Fluka Chemie, Germany).
- Glacial acetic acid, boric acid, sodium hydroxide, methylcellulose (MC), n-hexane, triethylamine and hydrochloric acid were obtained from El-Nasr Pharmaceutical Chemical Co. (ADWIC; Egypt) & phosphoric acid (Riedel-deHäen, Germany).
- Britton-Robinson (BR) buffer solutions (pH 2-9) were prepared by mixing a solution of (0.04 molL⁻¹ phosphoric acid, 0.04 molL⁻¹ acetic acid and 0.04 molL⁻¹ boric acid). Buffer solutions were adjusted by adding the necessary amount of 2.0 molL⁻¹ NaOH solution in order to obtain the appropriate pH.

Standard solution of intact CYP

A standard solution of CYP (100 μ g mL⁻¹) was prepared by dissolving 10 mg of the drug powder in 50 mL methanol and the volume was completed to 100 mL with the same solvent. Working solution of CYP (10 μ g mL⁻¹) was obtained by further dilution of the stock solution with bidistilled water.

Standard solution of degraded sample

Ten mg of pure CYP powder was dissolved in 45 mL methanol and transferred to a 100-ml round bottomed flask to which 5 ml of 30 % H₂O₂ were added. The solution was heated under reflux for 2 hours and evaporated to dryness under vacuum. The obtained residue was extracted with methanol (2x10 mL), filtered into a 100-ml volumetric flask and diluted to volume with methanol to obtain a stock solution labeled to contain degradation product derived from 100 μ g mL⁻¹ of CYP. Working solution of cyproheptadine hydrochloride oxidative degradation product DCYP (10 μ g mL⁻¹) was obtained by further dilution of the stock solution with bidistilled water.



Scheme 1. Proposed degradation pathway of cyproheptadine hydrochloride

Construction of calibration graph

Aliquots of CYP standard solutions covering the working concentration ranges of 1–16 µg were transferred into a series of 10mL volumetric flasks followed by 0.5 mL of 0.5 % SDS solution and the solution was completed to the volume with bidistilled water. At a constant wavelength difference, $\Delta\lambda$ of 120nm and scan rate of 400nmmin⁻¹ using 10nm excitation and emission windows both monochromators was scanning for the recording of the synchronous fluorescence spectra of the solutions. The first derivative spectra were derived from the normal synchronous fluorescence spectra. The amplitudes of the first derivative spectra were measured at 418nm. A blank experiment was carried out simultaneously. The peak amplitude of the first derivative was plotted versus final concentration (µgmL⁻¹) to get the calibration graph.

Table 1. Analytical performance data for the proposed method

Parameters	Proposed method
Wavelength (nm)	418
Linearity range (µgmL ⁻¹)	0.1-1.6
LOD (µgmL ⁻¹)	0.02
LOQ (µgmL ⁻¹)	0.06
Regression equation* $y = bx + a$	
Slope (b)	16.536
Intercept (a)	0.7562
Correlation coefficient (r)	0.9997
Accuracy (% mean recovery)	99.22
Precision(%RSD)	
-Repeatability	0.834
-Intermediate precision	0.907

* $y = bx + a$ where y is the amplitude of the first derivative synchronous fluorescence and x is the concentration.

Analysis of laboratory prepared mixtures

The general procedure of the method was applied using aliquots of CYP solution (10 µgmL⁻¹) containing (11–2 µg) with aliquots of its degradation product solution (10 µgmL⁻¹) containing (5–14 µg). The concentrations of CYP were calculated from the corresponding regression equation.

Analysis of pharmaceutical preparation

Ten Triactin® tablets were weighed and well pulverized. A weighed quantity of the powdered tablets equivalent to 10mg CYP was transferred into 100- mL volumetric flask and about 70 mL of methanol was

added and the flasks were sonicated for 30 min. The solutions were then filtered and diluted to volume with methanol. Aliquots covering the working concentration range were transferred into 10-mL volumetric flasks. The procedure described under ‘construction of the calibration graph’ was performed. The nominal contents of the tablets were calculated using the corresponding regression equation.

Analysis of pharmaceutical preparation

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RESULTS AND DISCUSSION

No degradation of CYP was observed by heating the drug under reflux using aqueous, acidic (1M HCl) or basic (1M NaOH) conditions, whereas complete degradation was attained when the drug was heated under reflux with 30% H₂O₂ for 2 hours

Scheme 1). The obtained solution was tested by TLC on silica gel 60 GF₂₅₄ plates. Separation of the intact drug and its corresponding degradation product was achieved by using mobile phase consisting of methanol: n-hexane: triethylamine: acetonitrile (10: 10:10: 0.1, by volumes) and UV detection at 286 nm.

Confirmation of degradation product using IR technique

There is no characteristic peak in IR to differentiate between CYP and its degradation product **Figure 2 (a,b).**

Confirmation of degradation product using 1H NMR technique

Reveal disappearance of 2.68 (S, 3H, CH₃) methyl group attached to N atom of piperidine ring As shown in **Figure 3 (a,b)** which confirmed that its conversion to N-Oxide

Confirmation of degradation product using mass spectrometry

Mass spectrometry was performed for the oxidative degradation product and molecular ion peak was obtained at m/z= 324.8 indicating that its molecular weight is 324.8 as shown in **Figure 4.**

Table 2. Determination of cyproheptadine hydrochloride in presence of its oxidative degradation product in their laboratory mixtures by the proposed method

Intact ($\mu\text{g ml}^{-1}$)	Degradate ($\mu\text{g ml}^{-1}$)	Percent of degradate	Intact found ($\mu\text{g ml}^{-1}$)	% Recovery of intact
1.1	0.5	31.25 %	1.083	98.42
0.7	0.9	56.25 %	0.696	99.38
0.5	1.1	68.75 %	0.491	98.13
0.3	1.3	81.25 %	0.295	98.44
0.2	1.4	87.5 %	0.201	100.50
Mean \pm % RSD				98.98 \pm 0.982

Table 3. Determination of CYP in Triactin® tablets by the proposed method and application of standard addition technique

Proposed method	Triactine® tablets	Standard addition technique			
	% Recovery* \pm % RSD	Taken ($\mu\text{g/mL}$)	Pure added ($\mu\text{g/mL}$)	Pure found* ($\mu\text{g/mL}$)	% Recovery
First derivative synchronous	101.33 \pm 1.233	0.3	0.3	0.302	98.91
			0.6	0.606	100.86
			0.9	0.89	98.82
	Mean \pm % RSD				

*Average of three determinations

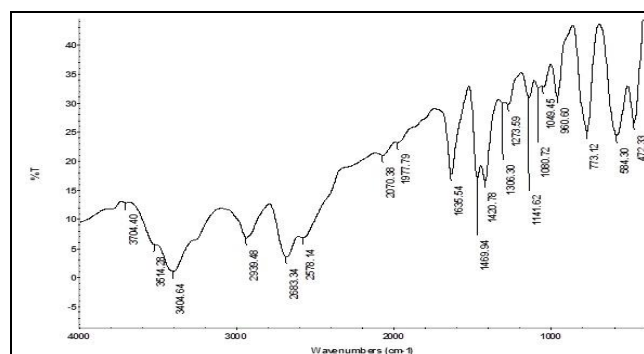


Figure 2(a). IR spectrum of CYP on KBr disc

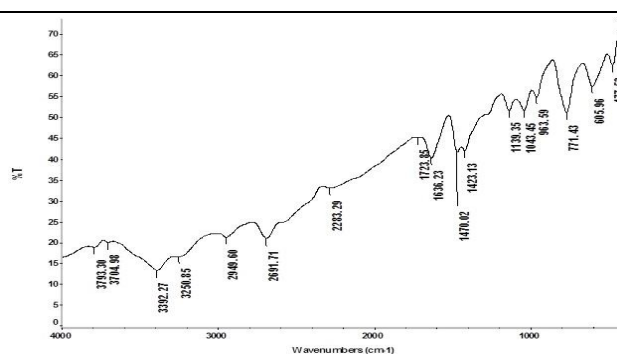


Figure 2(b). IR spectrum of DCYP on KBr disc

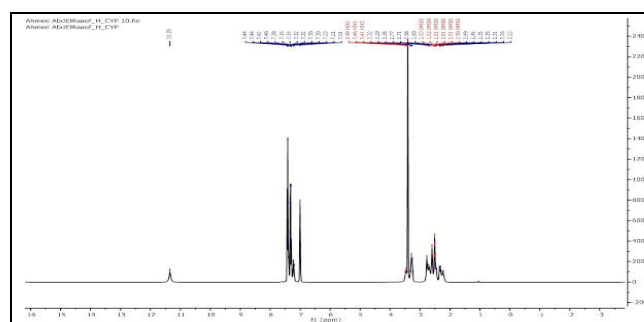


Figure 3(a). ¹H NMR spectrum of CYP in DMSO

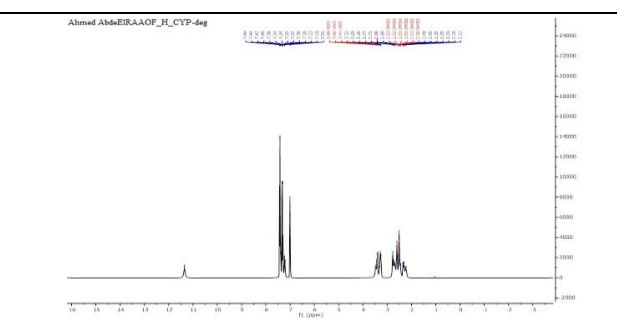


Figure 3(b). ¹H NMR spectrum of DCYP in DMSO

Spectral characteristic

Both CYP and its oxidative degradation product exhibit native fluorescence at 400 and 352 nm, after excitation at 281nm, respectively, the emission spectra of CYP and DCYP are overlapped as shown in Figure. 5. Which hindered the use of normal fluorescence for determination of CYP in presence of its DCYP. Moreover, the derivatization of normal fluorescence spectra does not solve the problem. Such overlapping can be resolved by measuring the synchronous fluorescence at $\Delta\lambda=120$ nm, then deriving the first derivative

Figure 6 shows the synchronous fluorescence spectra of CYP at 401 and DCYP at 363nm. It is clear that there is strong overlapping between the two spectra. This led us to adopt first derivative synchronous fluorescence spectroscopy (FDSFS) method for the determination of CYP. The fluorescence spectra of CYP and DCYP were well separated with a zero-crossing point where the resulted first derivative synchronous fluorescence spectra of CYP and its degradation product allow the determination of CYP in the presence of its degradation product at 418 nm (**Figures 7,8**). Different experimental parameters affecting the fluorescence of CYP were carefully studied and optimized. Such factors were changed individually while others were kept constant. These factors included $\Delta\lambda$ selection, pH, type of diluting solvent and surfactant.

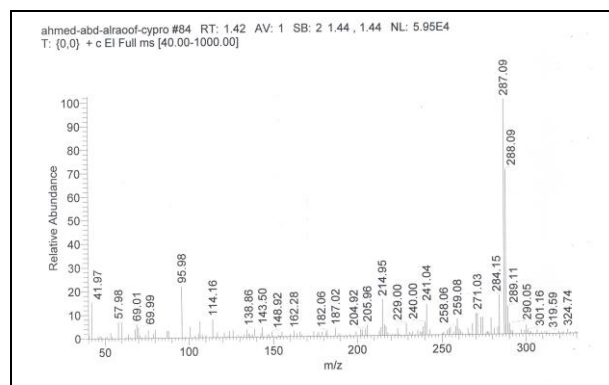


Figure 4. Mass spectrum of DCYP degradate

Effect of constant wavelength difference ($\Delta\lambda$ selection)

Since the adjustment of instrumental parameters is very important to obtain a good spectrum shape with a narrow bandwidth and higher sensitivity, so different $\Delta\lambda$ have been tested at 10 nm intervals and each time the synchronous spectrum has been recorded. The change in $\Delta\lambda$ leads to a considerable change in the spectrum shape and the best synchronous spectrum was

obtained for $\Delta\lambda = 120$ nm which enhance the resolution and sensitivity of the analytical method (**Figure.9**).

Effect of surfactant type

Study of the impact of the surfactant was accomplished using 0.5 % aqueous solutions of anionic surfactant (SDS), a cationic surfactant (CTAB) and different macromolecules such as β -CD, and methylcellulose. The greatest synchronous fluorescence intensity was acquired utilizing SDS as displayed in (**Figure.10**). Although the exact origin of this enhancement remains uncertain, it is possibly related to the interaction of CYP with the hydrophobic aggregates formed by SDS.

Effect of the Volume of SDS

The influence of SDS on the relative fluorescence synchronous intensity (RSFI) was studied using increasing volumes of 0.5 % w/v SDS. It was found that increasing volumes of SDS solution resulted in a corresponding increase in RSFI up to 0.4 mL, after which no more increase in RSFI was attained. Therefore, 0.5 mL 0.5 % w/v SDS solution was chosen as the optimum volume for CYP (**Figure 11**).

Effect of diluting solvent

Dilution with different solvents such as water, methanol, ethanol, 1-propanol, and tetrahydrofuran (THF) was attempted. THF decreased the synchronous fluorescence intensities of CYP, since they initiated intersystem crossing process (similar to heavy atom effect) ^{30, 31}. On the other hand, a distinct and sharp decrease in the relative synchronous fluorescence intensities was observed in the SDS system using methanol, ethanol, acetonitrile or 1-propanol. For example in propanol, this effect is attributed to their penetration of alcohol into micelles to decrease the surfactant monomer concentration (CMC) and increase the counterion dissociation of micelles resulting in a minimum of conductance, where short-chain alcohols (methanol, ethanol and propanol) were found to decrease the conductance of micellar solutions due to the lowering of dielectric constant of solvent ^{30, 31}. The synchronous fluorescence intensities of CYP and DCYP were higher in water compared to other solvents. Moreover, its use adds another advantage of the lowest blank reading. The results are summarized in **Table 1** and **Figure 12**.

Effect of pH

The influence of pH on the synchronous fluorescence intensity of the studied drug was studied using Britton-Robinson (BR) buffer solutions (pH 2-9). The result was found that no subsequent increase in the fluorescence intensity upon using any of the buffers. Therefore, the buffer wasn't included throughout the parameters study.

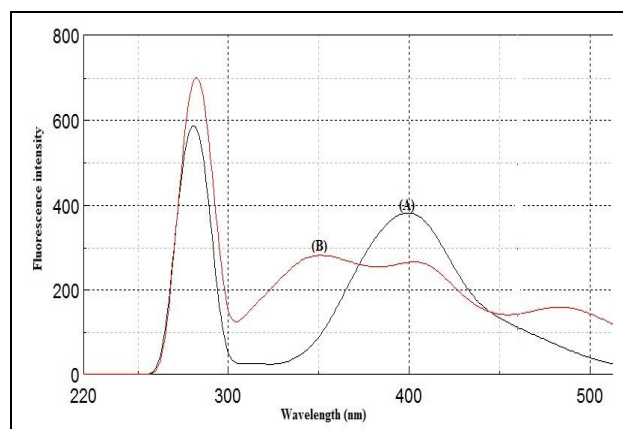


Figure 5. Emission spectra of (A) CYP (0.7 µg/ml) and (B) DCYP product (0.7 µg/ml), in water after excitation at 281nm using $\Delta\lambda=120$ nm.

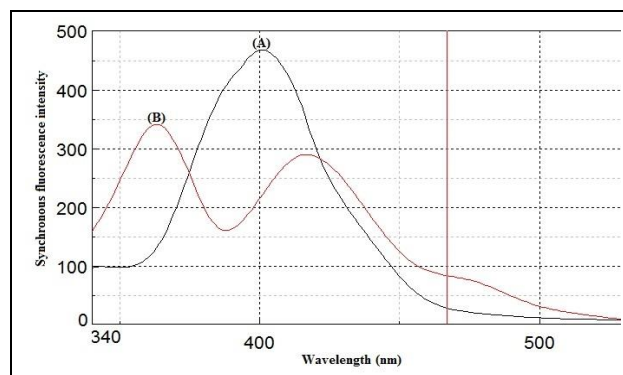


Figure 6. Synchronous fluorescence spectra of (A) CYP (0.7 µg/ml) and (B) DCYP (0.7 µg/ml), in water using $\Delta\lambda=120$ nm.

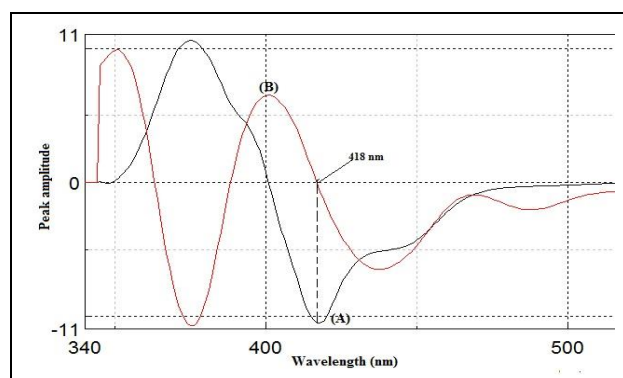


Figure 7. First derivative synchronous fluorescence spectra of (A) CYP (0.7 µg/ml) and (B) DCYP (0.7 µg/ml) in water using $\Delta\lambda=120$ nm.

Validation of the method

The proposed method was tested for linearity, range, limit of detection (LOD), limit of quantification

(LOQ), selectivity, accuracy and precision according to International Conference on Harmonization (ICH) guidelines³².

Linearity and range:

Under the described experimental conditions, the calibration graph for the method was constructed by plotting the amplitudes of the first derivative of the synchronous spectra versus drug concentrations in µg/mL. The calibration graphs were rectilinear over the concentration range of 0.1-1.6 µg/mL with a high value of the correlation coefficient. The regression parameters are supplied in **Table 1**.

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

LOD and LOQ were calculated according to ICH guidelines³² from the following equations:

$$\text{LOD} = 3.3 \text{ Sa} / \text{slope}$$

$$\text{LOQ} = 10 \text{ Sa} / \text{slope}$$

Where Sa is the residual standard deviation of a regression line. LOD and LOQ values of CYP by the proposed method was listed in **Table 1**.

Selectivity

The specificity of the proposed procedure was assured by applying it to laboratory prepared mixtures of the intact drug with its degradation product. The proposed procedure was adopted for the selective determination of intact CYP in presence of up to 87.5% of its degradation product. The percentage recovery \pm RSD % was 98.97 ± 0.982 , as shown in **Table 2**, and the results of the standard addition technique (**Table 3**) prove that the proposed method can selectively analyze the drug without any interference from its excipients.

Accuracy

The accuracy of the proposed method, calculated as the mean percent recovery (%R), was assessed by applying the proposed procedures for the triplicate determination of three concentration levels covering the specified range for each drug (0.3, 0.9 and 1.2 µg/mL). The concentrations of unknown solutions were obtained from the corresponding regression equations and the mean percent recovery, shown in **Table 1**, performed that the accuracy of the proposed method. Accuracy of the method was further assured by the use of the standard addition technique. It was performed by addition of known amounts of pure CYP to known concentrations of the pharmaceutical preparation and the resulting mixtures were assayed, and the result obtained was compared with the expected result (**Table 3**). The good recovery of the pure added CYP suggested the good accuracy of the proposed method.

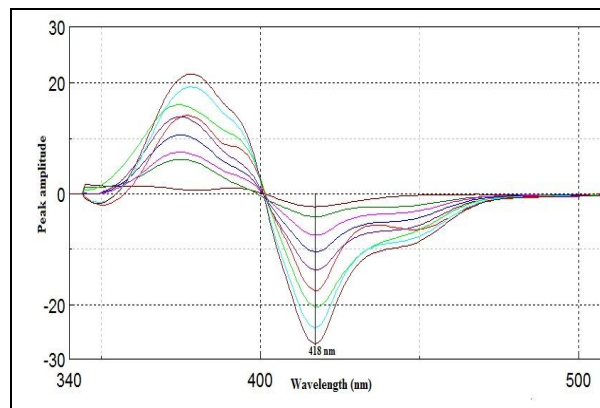


Figure 8. First derivative synchronous fluorescence spectra of CYP at different concentrations (0.1- 1.6 µg/ml) in water using $\Delta\lambda=120$ nm.

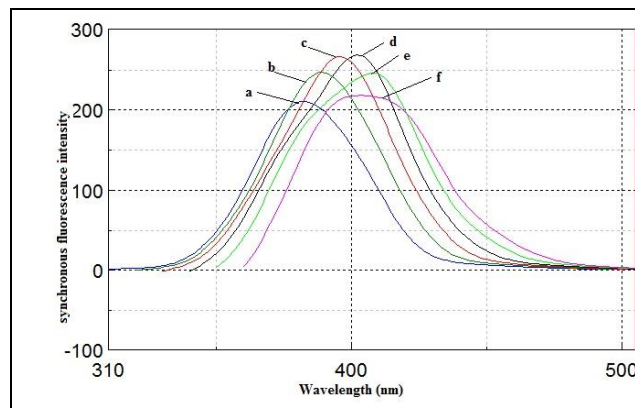


Figure 9. Synchronous fluorescence spectra showing the effect of the constant wavelength difference $\Delta\lambda$ on the Synchronous spectra of cyproheptadine hydrochloride in SDS micellar medium. (a) 90, (b) 100, (c) 110, (d) 120, (e) 130 and (f) 140 nm.

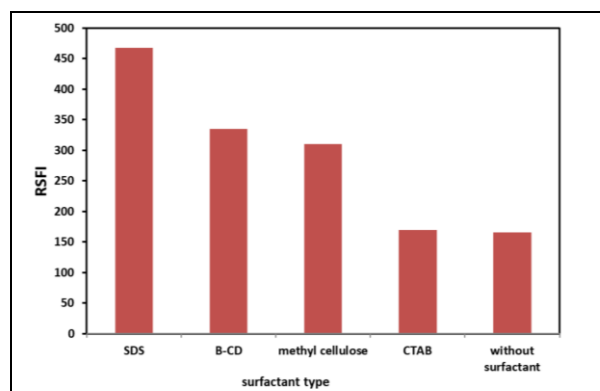


Figure 10. Effect of the surfactant type (0.5 mL 0.5 % w/v solution of each) on RSFI of CYP (0.7 µg/mL).

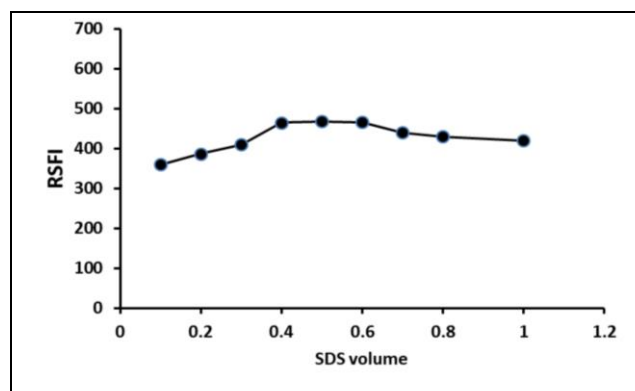


Figure 11. Effect of the volume of 0.5% w/v SDS on RSFI of CYP (0.7 µg/mL).

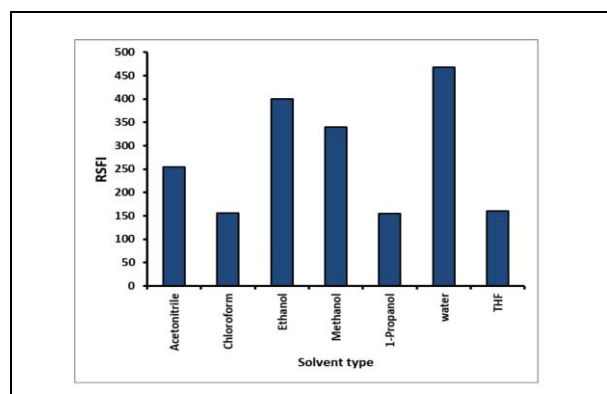


Figure 12. Effect of the solvent type on RSFI of CYP (0.7 µg/mL).

Precision

Precision of the proposed method, calculated as percent relative standard deviation (% RSD) of the

percent recoveries, was checked by applying the proposed procedures for triplicate determination of three concentration levels covering the specified range for each drug (0.3, 0.9 and 1.2 µg/mL) in the same day (intra-day analysis) for repeatability and on three different days (inter day analysis) for intermediate precision. The results in **Table 1** indicate the precision of the method.

Application to pharmaceutical formulation

The proposed method was applied for the determination of CYP in the pharmaceutical formulation, Triactin[®] tablets. Satisfactory results were obtained in good agreement with the label claim, and the results of the standard addition technique indicate no interference from excipients and additives (**Table 3**).

Statistical analysis

Table 4 showed a statistical comparison of the results obtained by the proposed method and the official

method in pure form. The calculated t and F values were less than the theoretical ones indicating that; there was no significant difference between the proposed and the official method with respect to accuracy and precision.

CONCLUSION

The present study described a fully validated and novel micellar enhanced first derivative synchronous spectrofluorimetric method for the determination of cyproheptadine hydrochloride in presence of its oxidative degradation product with enhanced sensitivity and specificity. The proposed method does not require elaborate treatment for the sample or tedious procedure for the extraction. As well as, the method is sensitive enough for the analysis of lower concentration of cyproheptadinehydrochloride.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

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