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ORIGINAL PAPER

Micelle-Enhanced Spectrofluorimetric Method for Determination of Cyproheptadine Hydrochloride in Tablets: Application to *In-Vitro* Drug Release and Content Uniformity Test

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Abstract A highly sensitive and simple spectrofluorimetric method was developed for the determination of cyproheptadine hydrochloride (CYP) in its pharmaceutical formulations. The proposed method is based on the investigation of the fluorescence spectral behaviour of CYP in a sodium dodecyl sulphate (SDS) micellar system. In aqueous solution, the fluorescence intensity of CYP was greatly enhanced (150 %) in the presence of SDS. The fluorescence intensity was measured at 410 nm after excitation at 280 nm. The fluorescence–concentration plot was rectilinear over the range 0.2–2.0 μg/mL, with lower detection limit of 0.06 μg/mL. The proposed method was successfully applied to the assay of commercial tablets as well as content uniformity testing. The application of the proposed method was extended to test the in-vitro drug release of CYP tablets, according to USP guidelines. The results were statistically compared with those obtained by official USP method and were found to be in good agreement.

Keywords Spectrofluorimetric · Cyproheptadine hydrochloride · Tablets · Content uniformity · In-vitro release

Introduction

Cyproheptadine hydrochloride (CYP), chemically known as 4-(5H-Dibenzo [a,d]cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride (Fig. 1), is a sedating antihistaminic drug with antimuscarinic, serotonin-antagonist, and calcium channel blocking action in pancreatic islet cells and smooth muscles [1]. It is used to treat some hormonal disorders and may also be used for treating side effects of taking antidepressants [2]. The United States Pharmacopeia [3] describes non-aqueous titration with perchloric acid as a titrant where the end point is located visually using crystal violet as indicator. Literature survey revealed the availability of few methods for the assay of CYP in pharmaceutical formulations. Liquid chromatography-mass spectrometry (LC-MS) [4], gas liquid chromatography [5, 6], and high performance liquid chromatography (HPLC) [7–13] have been used to assay CYP. Recently, HPLC has been used for the assay of CYP in foods [14]. Application of visible spectrophotometric methods [15–22], and derivative UV-spectrophotometry for the assay of CYP in two-component system [23] have also been reported. To the best of our knowledge, no spectrofluorimetric methods have been yet described for the determination of CYP in pharmaceutical preparations. This paper presents a new sensitive spectrofluorimetric method for the determination of CYP in tablets. 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.

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Experimental

Apparatus

- All fluorescence measurements were made using a RF-1501 Shimadzu spectrofluorometer, equipped with a 150 Watt Xenon arc lamp. The excitation and emission wavelengths were 280 nm and 410 nm, respectively, the slit widths were 5 nm for both excitation and emission, and the photomultiplier voltage was set to auto. Quartz 1 cm cuvette was used.

– A Hanna pH meter (Romania) was used for pH adjustments.

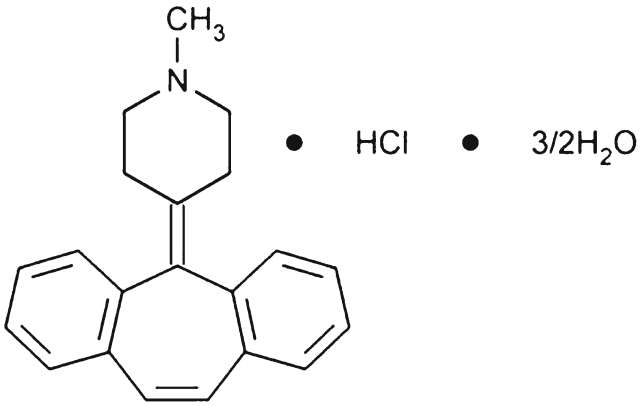

Fig. 1 Chemical structure of CYP

– Automatic tablet dissolution tester (8 cup system), Abbota Corporation, 178 Franklin Road, New Jersey 07869, United States.

Reagents and Materials

All the chemicals used were of Analytical Reagents grade, and the solvents were of HPLC grade.

- Cyproheptadine hydrochloride, was kindly provided by (Novartis Pharma Schweiz AG, Bern, Switzerland) and was used as received without further purification. The purity of the sample was found to be 99.69±0.51 according to the official USP non aqueous titration method [3].
- Sodium dodecyl sulphate (SDS; 95 %) and cetyl trimethyl ammonium bromide (CTAB; 99 %) were purchased from Winlab (UK). 0.5 % aqueous solution of each surfactant was prepared.
- β–cyclodextrin (β–CD) and hydroxy propyl–β–cyclodextrin (HP–β–CD) were obtained from Merck (Germany).
- Carboxymethylcellulose (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt), used as 0.5 % w/v aqueous solution.
- Tween–80 (El-Nasr Pharmaceutical Chemicals Company (ADWIC), Egypt), used as 0.5 % w/v aqueous solution.
- Methanol (Prolabo, France), n-propanol and acetonitrile (Sigma-Aldrich Chemie GmbH, Germany).
- Dimethyl sulphoxide was purchased from Riedel–deHäen (Germany).
- Glacial acetic acid, sodium acetate tri-hydrate, boric acid, sodium hydroxide, hydrochloric acid, and dimethyl formamide were all obtained from El–Nasr Pharmaceutical Chemical Co. (ADWIC; Egypt).
- Acetate buffer (0.2 M, pH 3.7–5.7) and borate buffer (0.2 M, pH 6.5–9.5) solutions were freshly prepared.
- 0.1 N hydrochloric acid for the dissolution test
- Triactin® tablets labeled to contain 4 mg CYP/tablet (batch #1210726), Product of Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt.

Standard Solutions

Stock solution of CYP was prepared by dissolving 10.0 mg of the drug in 100 mL of methanol. This solution was further diluted with the same solvent as appropriate. The standard solutions were stable for 10 days when kept in the refrigerator.

Construction of the Calibration Graphs

Aliquots of methanolic CYP standard solution was transferred into a series of 10 mLvolumetric flasks to give final concentrations of 0.2–2.0 μg/mL. 0.5 mL of 0.5 % w/v SDS solution was added to each flask. The volume was completed with distilled water, the contents of the flasks were mixed well and the relative fluorescence intensity (RFI) was measured at 410 nm after excitation at 280 nm. The corrected fluorescence intensity was plotted vs. the final drug concentration (μg/mL) to obtain the calibration graphs. Alternatively, the corresponding regression equations were derived.

Procedure for Tablets

Ten Triactin® tablets were weighed and well pulverized. A weighed quantity of the powdered tablets equivalent to 10.0 mg CYP was transferred into 100 mL volumetric flasks, about 80 mL of methanol was added and the flasks were sonicated for 30 min. The solutions were then diluted to volume with methanol, mixed and filtered. Aliquots covering the working concentration range of 0.2–2.0 μg/mL were transferred into 10 mL volumetric flasks. The procedure described under 'construction of the calibration graphs' was performed. The nominal contents of the tablets were calculated using the calibration graphs or the corresponding regression equations.

Procedure for Content Uniformity Testing for CYP

Ten different tablets were analyzed using the same procedure applied for the analysis of the studied compound in tablets. The uniformity of their contents was tested by applying the official USP guidelines [3] (*Chapter 905: Uniformity of Dosage Units*).

Procedure for In-Vitro Drug Release Test (Dissolution Test) for CYP

Dissolution test was performed on three tablets from the formulation present in the Egyptian market (Triactin® tablets). The dissolution USP apparatus II [3] using 900 mL of 0.1 N HCL maintained at 37±0.5^{0}C stirred at 50 rpm for 30 min. A 5 mL sample was withdrawn through a 0.45 μm syringe filter and replaced with another 5 mL of a suitable fresh dissolution medium at pre-selected intervals up to

30 min. The procedure described under 'construction of the calibration graphs' was applied on the filtered samples. The release studies were performed in three replicates and mean values were taken by applying the official USP method [3].

Results and Discussion

Cyproheptadine hydrochloride is formulated in a very minute amount in tablets (4 mg/tablet), therefore; there is an urgent need to develop a sufficiently sensitive and specific method for its determination in pharmaceutical preparations. Fluorimetric analysis, by virtue of its high inherent sensitivity, could successfully overcome this problem. CYP was found to exhibit an emission band of moderate strength at 410 nm in aqueous solution, after excitation at 280 nm. As a consequence, we attempted to enhance this emission band, in order to explore a new sensitive methodology for the analysis of CYP in its tablets. It is well known that the addition of a surfactant at a concentration above its critical micellar concentration to a given fluorophore solution increases its molar absorbtivity and/or the fluorescence quantum yield in many cases [24, 25]. This fact has been used to improve the performance of spectrofluorimetric methods of various analytes. The fluorescence properties of CYP in various micellar media were studied; there was an enhancement (about 150 %) of the fluorescence intensity in the presence of SDS compared with aqueous solution, therefore SDS was used as a fluorescence enhancer in order to develop a new sensitive spectrofluorimetric method for the determination of CYP.

Fluorescence Spectra of CYP in Aqueous Solution and in the SDS System

The fluorescence spectra of CYP in both aqueous and SDS systems were studied. Figure 2 illustrates the fluorescence spectra of CYP in the two systems. The first system was aqueous solution, while the second was the same solution but in the presence of SDS as a fluorescence enhancer. The percentage of fluorescence enhancement in the presence of SDS was 150 % compared with the native fluorescence intensity of the drug in aqueous medium.

Optimization of the Experimental Conditions

Effect of Organized Media

The effect of different organized media on the fluorescence intensity of CYP was studied by adding 0.5 mL of an aqueous solution of each one of them to the drug solution. Different surfactants, like sodium dodecyl sulfate (SDS) [anionic surfactant], cetrimide [cationic surfactant], carboxymethylcellulose (CMC), tween 80 [non ionic] as well as β-cyclodextrin and hydroxy-β-cyclodextrin were tried.

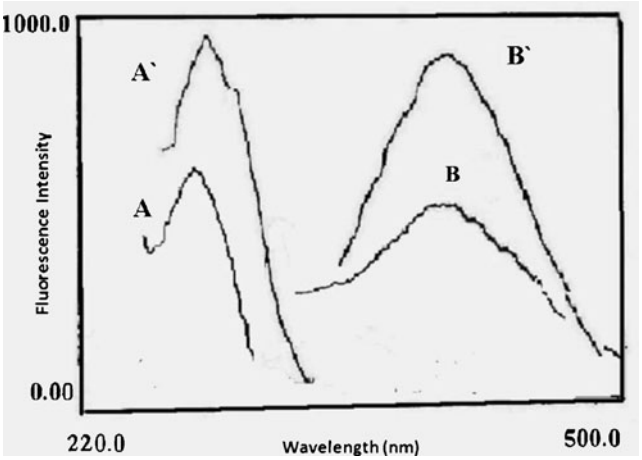

Fig. 2 Fluorescence spectra of: (A, B) CYP (2 μg/mL) in water, (A`, B`) CYP (2 μg/mL) in water (0.5 mL 0.5 % w/v SDS); where: (A, A`) Excitation spectra; (B, B`) Emission spectra

All the organized media studied caused slight decreases in the RFI of the drug as shown in Fig. 3. Only SDS gave a considerable increase in the RFI, so it was used through the present study.

Effect of the Volume of SDS

The influence of SDS on the RFI 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 RFI up to 0.3 mL, after which no more increase in RFI was attained. Therefore, 0.5 mL 0.5 % w/v SDS solution was chosen as the optimum volume for CYP (Fig. 4). Increasing SDS volume more than 1 mL not preferred due to high blank reading.

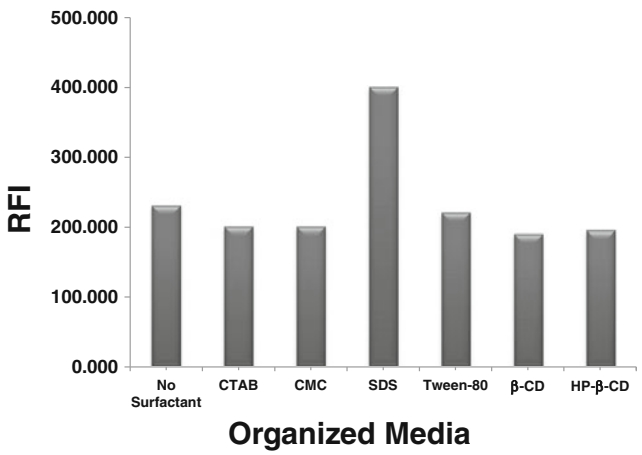

Fig. 3 Effect of the type of organized media (0.5 mL 0.5 % w/v solution of each) on RFI of CYP (1 μg/mL)

Effect of pH

The influence of pH on the micelle–enhanced fluorescence of CYP was studied using different types of buffers covering the whole pH range, such as 0.2 M acetate buffer over the pH range 3.7–5.7 and 0.2 M borate buffer over the pH range 6.5–9.5. It was found that no subsequent increase in the fluorescence intensity upon using any of the buffers (Table 1). Therefore, no buffer was incorporated throughout the study.

Effect of Diluting Solvent

The effect of different diluting solvents on the RFI of CYP in the presence of SDS was investigated using water, methanol, acetonitrile, n–propanol, dimethyl sulphoxide and dimethyl formamide. It was found that water was the best solvent for dilution, as it gave the highest RFI, and the lowest blank reading (Table 1). Distinct and sharp decrease in the relative fluorescence intensities was observed in the SDS system using methanol, acetonitrile or n–propanol. This effect is attributed to their denaturating effect on the micelles, where short–chain alcohols (methanol and propanol) are solubilized mainly in the aqueous phase and affect the micellization process by modifying the solvent properties. Addition of these alcohols also results in a reduction of the size of the micelles, but with a progressive breakdown of the surfactant aggregate at very high concentration [26]. Both dimethyl sulphoxide and dimethyl formamide decreased the fluorescence intensities of CYP, since they initiated an intersystem crossing process (similar to the heavy atom effect) [27].

Effect of Time

The effect of time on the RFI of the drug was also studied. It was found that the fluorescence intensity was immediately developed and remained stable for more than 2 h.

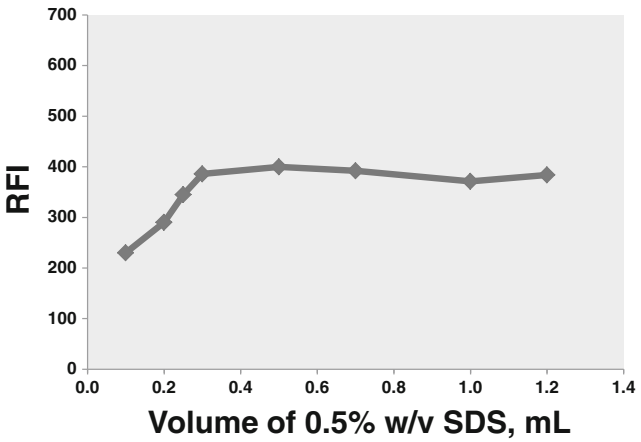

Fig. 4 Effect of the volume of 0.5 % w/v SDS on RFI of CYP (1 μg/mL)

Table 1 Effect of diluting solvents on the fluorescence intensity of CYP (1 μg/mL)

Solvent	CYP		
	$\lambda_{ex.}$ (nm)	$\lambda_{em.}$ (nm)	F.I.
Water	280	410	320
Acetonitrile	284	409	150
n-propanol	284	407	175
Methanol	280	410	166
Dimethyl sulfoxide	287	412	20
Dimethyl formamide	285	410	7
Acetate buffer (pH 4.5)	283	411	300
Borate buffer (pH 7)	282	418	291
Borate buffer (pH 9.4)	287	418	315

Effect of Temperature

Another factor that may affect the fluorescence intensity is temperature. The effect of temperature was studied in the range 40–100 °C in a thermostatically controlled water bath. It was found that increasing the temperature resulted in a decrease in the RFI. This effect can be explained by higher internal conversion as the temperature increases, facilitating non radiative deactivation of the excited singlet state [28]. Therefore, all the experiments were carried out at room temperature.

Validation of the Method

Linearity and Range

The calibration graph for the determination of CYP by the proposed method was constructed by plotting the relative fluorescence intensity (RFI) versus the concentration. The graph was found to be rectilinear over the concentration range cited in Table 2.

Statistical analysis [29] of the data gave high values of the correlation coefficients (r) of the regression equations, small values of the standard deviation of residuals ($S_{y/x}$), of intercept (S_a), and of slope (S_b), and small value of the percentage relative standard deviation and the percentage relative error (Table 2). These data proved the linearity of the calibration graph for the studied drug.

Limit of Quantitation (LOQ) and Limit of Detection (LOD)

Limit of quantitation (LOQ) and limit of detection (LOD) were calculated according to the ICH Q2 (R1) recommendation [30]. The limits of quantitation (LOQ) were determined by establishing the lowest concentrations that can be measured below which the calibration graph is non–linear. The

Table 2 Analytical performance data for the spectrofluorimetric determination of CYP

Parameter	CYP
Wavelength [$\lambda_{ex.}/\lambda_{em.}$] (nm)	280/410
Linearity range (μg/mL)	0.2 - 2.00
Intercept (*a*)	−13.61
Slope (*b*)	412.7
Correlation coefficient (*r*)	0.9997
S.D. of residuals ($S_{y/x}$)	8.74
S.D. of intercept (S_a)	7.35
S.D. of slope (S_b)	5.99
% RSD [a]	1.38
% Error [b]	0.62
LOD (μg/ml)[c]	0.06
LOQ (μg/ml)[d]	0.18

[a] Percentage relative standard deviation for three replicate samples

[b] Percentage relative error for three replicate samples

[c] Limit of detection

[d] Limit of quantitation

limits of detection (LOD) were determined by evaluating the lowest concentrations of the analytes that can be readily detected. The results are also summarized in Table 2. The values of LOQ and LOD were calculated according to the following equation [30]:

$$\mathrm{LOQ} = 10\,S_a/b$$

$$\mathrm{LOD} = 3.3\,S_a/b$$

Where S_a is the standard deviation of the intercept of the regression line and b is the slope of the calibration graph.

Accuracy

To prove the accuracy of the proposed method, the results of the assay of the drug were compared with those obtained by official USP method [3] both in pure form and in tablets. Statistical analysis [29] of the results obtained by the proposed and reference methods using Student's *t*-test and variance ratio *F*-test showed no significant difference between them regarding accuracy and precision, respectively (Tables 3 & 4).

Precision

The intra-day precision was evaluated by determination of three concentrations of each drug in pure form on three successive occasions. The inter-day precision was also evaluated through replicate analysis of three concentrations for a period of 3 successive days. The results of intra-day and inter-day precision are summarized in Table 5.

Table 3 Assay results for the determination of CYP in pure form by the proposed and reference methods

Parameter	Proposed method			Official USP method [3]		
	Amount taken (μg/mL)	Amount found (μg/mL)	% Found	Amount taken (μg/mL)	Amount found (μg/mL)	% Found
	0.20	0.203	101.50	0.5	0.4991	99.81
	0.50	0.505	101.00	1.0	1.0054	100.54
	1.0	1.002	100.20	2.0	2.0260	101.30
	1.5	1.470	98.00	3.0	3.0474	101.58
	2.0	2.020	101.00			
Mean			100.34			100.81
± S.D.			1.39			0.80
t			0.594			
F			3.03			

The values of tabulated values of *t* and *F* at *P*=0.05 [29] are (2.36) and (9.12) respectively

Robustness of the Method

The robustness of the adopted method was demonstrated by the constancy of the RFI with minor changes in the experimental conditions, such as the change in the pH, and the volume of SDS (0.5±0.2 mL). These minor changes that may take place during the experimental operation did not affect the RFI.

Table 4 Assay results for the determination of CYP in commercial tablets by the proposed and official USP methods

Dosage form	Proposed method			Official USP method [3]		
	Amount taken (μg/mL)	Amount found (μg/mL)	% Found[a]	Amount taken (μg/mL)	Amount found (μg/mL)	% Found
Triactin® tablets[b]	0.50	0.5545	99.52	0.5	0.4897	97.94
	1.0	1.0374	101.18	1.0	0.9950	99.50
	1.5	1.3078	98.71	2.0	1.9634	98.17
	1.8	1.6845	97.92	3.0	3.0036	100.12
	2.0	2.2157	100.91			
Mean			99.65			98.93
± S.D.			1.40			1.05
t			0.84			
F			1.77			

[a] The average of three separate determinations

[b] Labeled to contain 4 mg CYP/tablet (batch# 1210726), product of Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt

[c] The values of tabulated values of *t* and *F* at P=0.05 [29] are (2.36) and (9.12) respectively

Table 5 Accuracy and precision data for the determination of CYP by the proposed method

Amount taken (μg/mL)	% Found	% RSD	% Error
Intra-day			
0.5	99.12±0.63	0.63	0.36
1.0	100.88±0.47	0.47	0.27
1.5	100.48±0.85	0.84	0.49
Inter-day			
0.5	100.56±1.15	1.14	0.66
1.0	99.97±2.20	2.20	1.27
1.5	99.89±1.55	1.55	0.90

Specificity

The specificity of the method was investigated by observing any interference encountered from common tablet excipients. It was shown that these compounds did not interfere with the results of the proposed method (Table 4).

Pharmaceutical Applications

Application of Procedure to Analysis of CYP in Tablets

The proposed method was successfully applied to CYP assay in its tablets. The average percent recoveries of different concentrations were based on the average of three replicate determinations. The results obtained were in good agreement with those obtained with the official USP method [3].

Table 6 Results of content uniformity testing of CYP tablets using the proposed method

Parameter	Tablet[a] no.	Percentage of the label claim
Data	1	99.51
	2	101.49
	3	100.65
	4	101.35
	5	98.46
	6	97.95
	7	99.46
	8	97.34
	9	102.46
	10	102.94
Mean		100.16
S.D.		1.92
% RSD		1.914
% Error		0.61
Acceptance value (AV)[3]	4.608	
Max. allowed AV (L1)[3]	15.0	

[a] Triactin® tablets: Labeled to contain 4 mg CYP/tablet (batch # 1210726), product of Kahira Pharmaceutical and Chemical Industries, Cairo, Egypt

Table 7 Results of in-vitro dissolution test data for CYP tablets using the proposed method

Time (minutes)	% Drug release [3, 31] (Mean±SD)
5	13.50±0.82
10	29.95±1.25
15	44.68±0.79
20	60.18±0.27
25	75.97±0.64
30	81.92±1.04
35	88.76±1.04

Each result is the average of three separate determinations

Content Uniformity Test for CYP

Due to the high sensitivity of the proposed method and its ability to rapidly measure the fluorescence intensity of a single tablet extract with sufficient accuracy, the method is ideally suited for content uniformity testing which is a time-consuming process when using conventional assay techniques. The steps of the test were adopted according to the USP [3] procedure. The acceptance value (AV) was calculated and it was found to be smaller than the maximum allowed acceptance value (L1). The results demonstrated excellent drug uniformity as shown in Table 6.

In-Vitro Drug Release (Dissolution Test) for CYP

Dissolution test was performed on three tablets from the formulation present in the Egyptian market (Triactin® tablets). The amount of drug released was then determined with the help of the calibration curve and the percentage of drug released was calculated [31] (Table 7). According to the USP

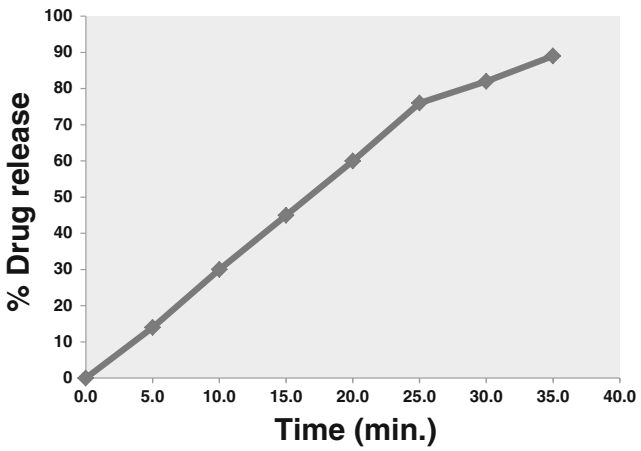

Fig. 5 Dissolution profile for CYP tablet according to USP guidelines

requirements [3]; not less than 80 % of the labeled amount of CYP is dissolved in 30 min (Fig. 5).

Conclusion

A simple and sensitive spectrofluorimetric method was developed for the determination of CYP. The proposed method is rapid, less time–consuming and does not require elaborate treatment compared to the reported chromatographic methods. By virtue of its simplicity and rapidity, the proposed method could be applied to the analysis of the studied drug in its tablets. The proposed method is very suitable to be applied in content uniformity testing. Additionally, it has been adapted for dissolution testing of CYP tablets as a rapid and simple alternative to the reported HPLC methods. The proposed method is a non–pollutant methodology and meets the requirements of green chemistry, since no organic solvents are used in the procedure.

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