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ORIGINAL PAPER

Spectrofluorimetric Study and Detection of Ketoconazole in the Presence of β-cyclodextrin

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Abstract The formation of a complex between ketoconazole and β-cyclodextrin was followed by spectrofluorimety. The inclusion of ketoconazole in β-cyclodextrin cavity enhanced the native fluorescence of the drug. The stoichiometry of the complex was 1:1 β-cyclodextrin to ketoconazole and the stability constant of the complex (log K_f) was determined to be 4.3±0.01 at pH=7.9 and 3.7±0.04 at pH=2.6. A sensitive spectrofluorimetric method for the detection of ketoconazole is presented. At optimized experimental conditions, a linear relationship between the fluorescence intensity of the solution and concentration of ketoconazole is observed in the range of 0.01–10 μg ml^{-1} (5×10^{-8} M–1.88×10^{-5} M). The method was applied to the detection of ketoconazole in pharmaceutical products and the results were satisfactory in comparison to the official method (relative error=2.8% and standard deviation=0.06 for tablets of ketoconazole). The recovery of ketoconazole from a blood serum sample, determined by the proposed method, was 97.1±2.4%.

Keywords KC · β-cyclodextrin · Spectrofluorimetry · Pharmaceuticals · Blood serum

Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides, which have the ability to form inclusion complexes with numerous guest molecules. The inclusion complex formation modifies the physicochemical characteristics of guest molecules [1, 2]. As a consequence of this effect and biocompatibility of CDs, they have been increasingly utilized as additives in pharmaceuticals, foods, cosmetics, pesticides, etc [3–5].

Upon complexation with CDs, many analyte molecules exhibit enhanced luminescence efficiencies compared to that observed in bulk water [6–8]. The included solute molecule experiences a less polar and more rigid local microenvironment [2, 9]. The polarity of the CD cavity has been estimated to be similar to that of oxygenated solvents such as dioxane, ethanol, or isopropyl ether [9–13], thus less polar solutes have a more tendency to enter the cavity of cyclodextrin from water. Nonradiative deactivations such as collisional relaxations are reduced in local microenvironment of CD cavity. On the other hand, the analyte molecules are protected from water-soluble quenchers like molecular oxygen. This fact can be used as a resource for improving the performance of analytical methods.

The presence of CDs can dramatically enhance the fluorescence signal of complexed solutes. The first application of CDs in this regard appears to have been reported by Kinoshita and co-workers for dansylated amino acids in a series of papers in the mid 1970s [14–16]. Fluorescence enhancements have since been observed for a wide variety of other analyte molecules. On the other hand, the inclusion into the CDs depends on both the size and polarity of the guest; hence the specificity of the method can be extended in addition to sensitivity. Analysts have used this property of CDs previously, and different techniques based on the fluorescence of inclusion complexes with CDs have been proposed for the detection of several pharmaceutical drugs and pesticides [17–21].

ketoconazole (KC), *cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1-*H*-imidazole-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy]-phenyl]piperazine (I), is a highly effective broad-

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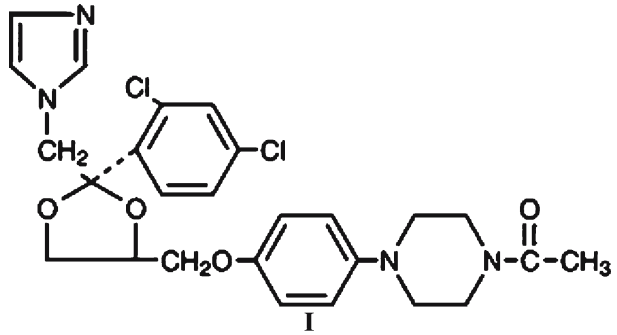
band spectrum antifungal agent used against several superficial and systemic mycoses [22, 23].



I

In a previous work [24], the enhancement of fluorescence of KC was observed in micellar medium and this property was used for the sensitive detection of KC in different samples successfully. In the present study, it was observed that β-cyclodextrin (β-CD) has a similar effect on the fluorescence intensity of KC; i.e. in β-CD solution, the fluorescence of KC is largely increased. This behavior was used for the sensitive detection of KC in different pharmaceuticals as well as blood serum samples.

Experimental

Apparatus

A Jasco FP-6200 spectrofluorimeter equipped with a Jasco ECT-272T temperature controller was used for the fluorescence measurements. A JENWAY 3345 pH/mV meter using a combined glass electrode was used for pH measurements of the experimental solutions.

Reagents and solutions

Reagent-grade KC and its tablets (200 mg/tablet) and creams (2% w/w) were obtained from Behvazan pharmaceutical company, Rasht, Iran. KC shampoo (2% w/v) was from Shahre Daru pharmaceutical company, Tehran, Iran. Analytical-grade α, β, and γ-cyclodextrins were from Sigma and were used without further purification, except for vacuum drying. All of the other chemicals were from Merck (Darmstadt, Germany) and used as received. Doubly distilled deionized water was used throughout.

A working standard solution of 0.001 M KC was prepared daily by dissolving 5.31 mg of pure drug in doubly distilled water, containing a few drops of hydrochloric acid solution (0.01 M), and then diluting to the mark in a 10 ml calibrated volumetric flask using distilled water.

A 0.03 M of β-CD solution was prepared by dissolving 0.3405 g of the pure solid in water in a 10 ml volumetric flask. The solution was diluted to the mark using distilled water. Phosphate buffer (pH=7.9) was prepared by adjusting the pH of a sodium dihydrogen phosphate (0.01 M) solution with sodium hydroxide solution.

General procedure

An accurate volume of standard or sample solution containing an appropriate amount of KC was pipetted into a sample cell, 0.6 ml of β-CD solution (0.03 M), and 1 ml of the buffer solution were added and the mixture was diluted to 2.0 ml using distilled water. The fluorescence of the solution was measured against a reagent blank at 370 nm (λ_{ex}=288 nm).

Preparation of sample solutions

In order to prepare sample solution of tablets, five finely powdered tablets of KC were mixed thoroughly; an accurately weighed (17.25 mg) amount of it was dissolved in water containing 2 ml of hydrochloric acid (0.01 M). The mixture was stirred for 15 min, and then the resulted suspension was filtered. The clear solution was collected and diluted in a 25 ml volumetric flask to the mark using doubly distilled water. The same procedure was followed for the preparation of cream and shampoo samples. The method of standard addition [25] was used for the detection of KC in sample solutions using proposed procedure.

In order to precipitate proteins, which interfere in the proposed procedure, a saturated ammonium sulfate solution was added to a 50 ml blood serum sample. Then, the mixture was centrifuged and clear supernatant solution was separated and filtered [26]. In the measuring step, the following solutions were transferred to the quartz cell of the spectrofluorimeter: 0.60 ml of a β-CD solution (0.030 M), 0.40 ml of the buffer (0.05 M), 0.10 ml of a KC solution, and 0.90 ml of the blood serum sample. The fluorescence of the mixture was measured against a reagent blank at 370 nm (λ_{ex}=288 nm).

Results and discussion

Study of complex formation between KC and β-cyclodextrin

KC molecule has some native fluorescence, which has been used in the detection of the drug [27, 28]. In a previous paper, we reported the enhancement effect of micellar medium on the fluorescence of KC, which was employed in the sensitive detection of KC [24].

In this work, the effect of the presence of cyclodextrins, as inclusion complexing agents, is explored on the fluorescence intensity of KC.

In preliminary studies, the fluorescence intensities of aqueous solutions of KC were measured in the presence of α, β, and γ-cyclodextrins. As is shown in Fig. 1, β-CD had the most enhancement effect on the native fluorescence of KC. Absorbance as well as the excitation and emission spectra of KC were obtained in the presence of different concentrations of β-CD. The absorbance of KC increased in the presence of β-CD (not shown) and a large increase in fluorescence intensity of KC in the presence of β-CD was observed (Fig. 2). This observation indicates that KC molecule enters the cavity of β-CD.

In order to study the complexation reaction, mole-ratio plots were constructed in acidic and basic solutions. In practice, selected volumes of a β-CD solution were added to a solution of KC (1.0×10^{-5} M). After each addition, the fluorescence of the solution was measured. The resulting fluorescence intensity vs. β-CD/KC mole ratio plots are shown in Fig. 3. As is shown, at about 1:1 mole ratio a change in fluorescence intensity is observed, which is relatively sharp at pH=7.9. This is indicative of formation of a 1:1 inclusion complex of β-CD and KC. The 1:1 binding of KC with β-CD can be expressed by equilibrium:

$$KC + CD = KC - CD$$

in which CD represents cyclodextrin. The complex formation constant, K_f, was obtained using a nonlinear least-squares program KINFIT [29].

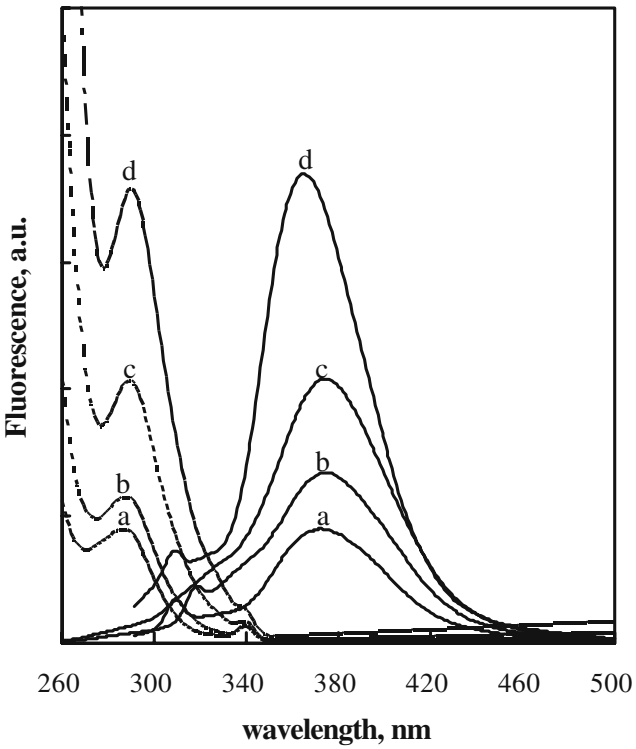


Fig. 2 Excitation and emission spectra of KC in the presence of different amounts of β-cyclodextrin. [KC]=1.0×10^{-6} M; [β-CD]: **a** 0.001 M; **b** 0.004 M; **c** 0.007 M; **d** 0.010 M

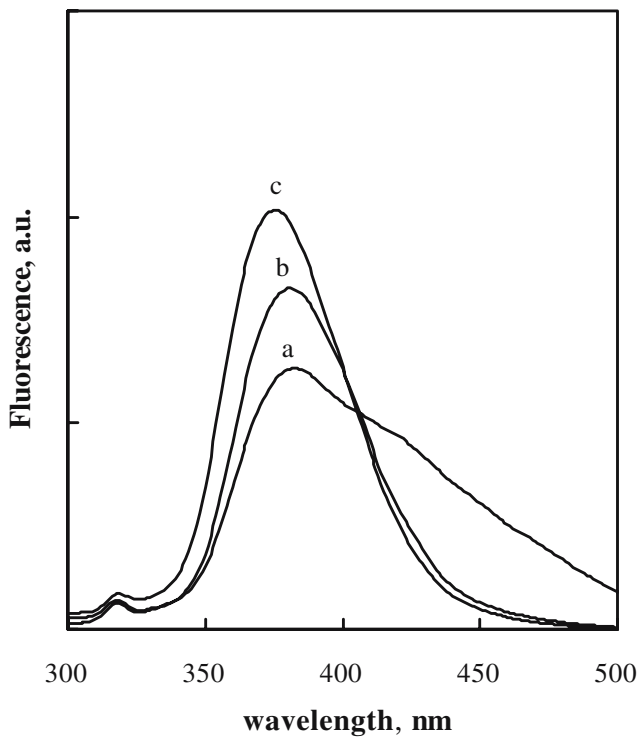


Fig. 1 Fluorescence spectra of KC in the presence of: **a** α-CD; **b** γ-CD; **c** β-CD. [KC]=1.0×10^{-5} M; [α-CD]=[β-CD]=[γ-CD]=0.01 M

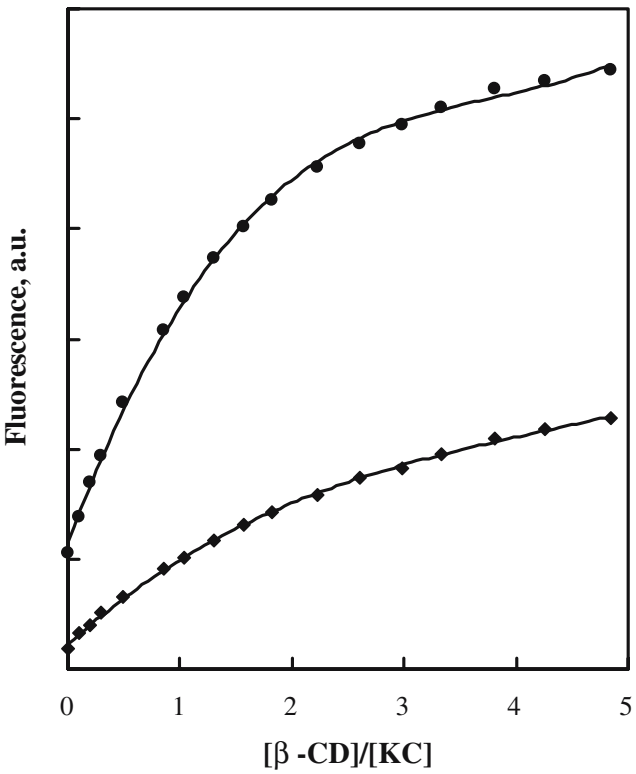


Fig. 3 Fluorescence intensity against β-CD/KC mole-ratio at (*filled diamond*) pH 2.6; (*filled circle*) pH 7.9. λ_{ex}=288 nm, λ_{em}=370 nm

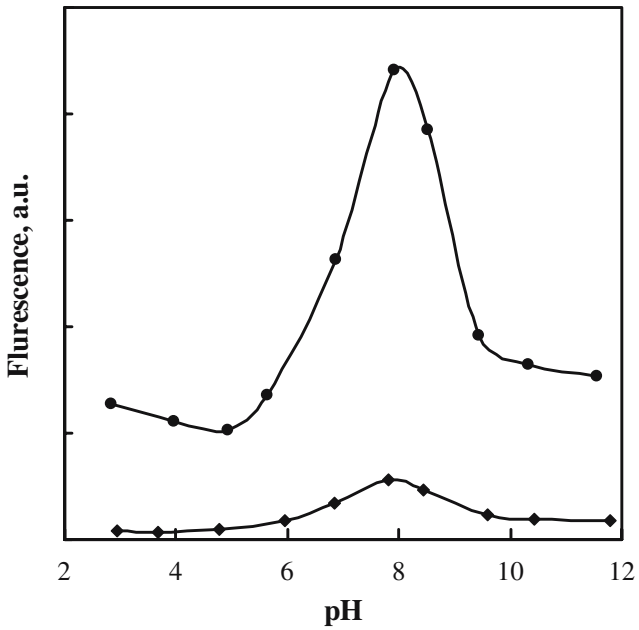


Fig. 4 Effect of pH on the fluorescence intensity of KC: (*filled diamond*) in the absence of β-CD; (*filled circle*) in the presence of β-CD. λ_{ex}=288 nm, λ_{em}=370 nm, [KC]=1.0×10^{-6} M, [β-CD]= 0.010 M

The magnitude of the stability constant of complex formation (log K_f) was obtained to be 4.3±0.01 (pH 7.9), and 3.7±0.04 (pH 2.6). The obtained stability constant in acidic solutions is comparable to the value of this quantity obtained previously by potentiometric method (log K_f=3.5) [30].

Inclusion of KC in CD cavity retards the rate of non-radiative relaxations such as collisional relaxations and quencher effects, thus the quantum efficiency of the fluorescence of KC increases and enhancement in fluorescence intensity is observed.

The value of K_f at pH 7.9 is larger than that at pH 2.6. This observation may be explained on the basis of protonation of KC. In acidic solutions, KC can accept a proton via its piperazine moiety [31], and some hydroxyl groups of cyclodextrin molecules are also protonated. At higher pH values, KC is deprotonated and has no charge. The larger stability constant of the complex KC–CD at pH 7.9 may be indicative of the better interaction of neutral form of the drug with the cavity of β-CD.

Detection of KC concentration

Effect of pH

Based on the enhancement effect of β-CD on the fluorescence of KC, a sensitive method was proposed for the detection of low concentrations of the drug. For this purpose, several experimental conditions were optimized.

The influence of the pH of the test solution on the fluorescence intensity of KC/β-CD complex was studied in the range of pH 2.5–12 (adjusted with either HCl or NaOH solutions). As is shown in Fig. 4, at pH value of 7.9, KC shows the largest fluorescence. In this pH value KC is considered to be neutral [31], so its neutral form is the main species that is included in cyclodextrin cavity and the enhanced fluorescence observed may be from this form of KC. The pH was maintained at 7.9 using a phosphate buffer solution. A same experiment was done for a KC solution in the absence of β-CD. As is shown in Fig. 4, the fluorescence intensity is much lower than that in the presence of β-CD, although the maximum fluorescence is obtained in the same range of pH.

Effect of β-CD concentration

In order to obtain the optimum concentration of β-CD, the fluorescence of KC solution (1.0×10^{-6} M and 1.0×10^{-5} M) was monitored while changing the amount of β-CD. The results are shown in Fig. 5. As is observed, the fluorescence of the two test solutions is maintained almost constant at β-CD concentrations larger than 0.01 M. A 0.01 M concentration of β-CD was quite appropriate for further studies.

Effect of temperature

The influence of the temperature of the test solution on the fluorescence intensity of KC in the β-CD solution was

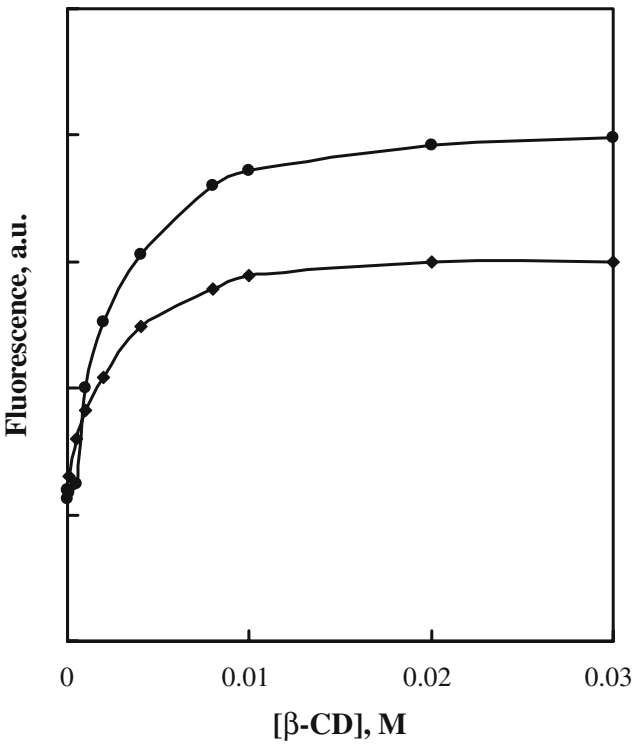


Fig. 5 Effect of β-CD concentration on fluorescence intensity of KC. (*filled diamond*) [KC]=1.0×10^{-6} M; (*filled circle*) [KC]=1.0×10^{-5} M. λ_{ex}=288 nm, λ_{em}=370 nm

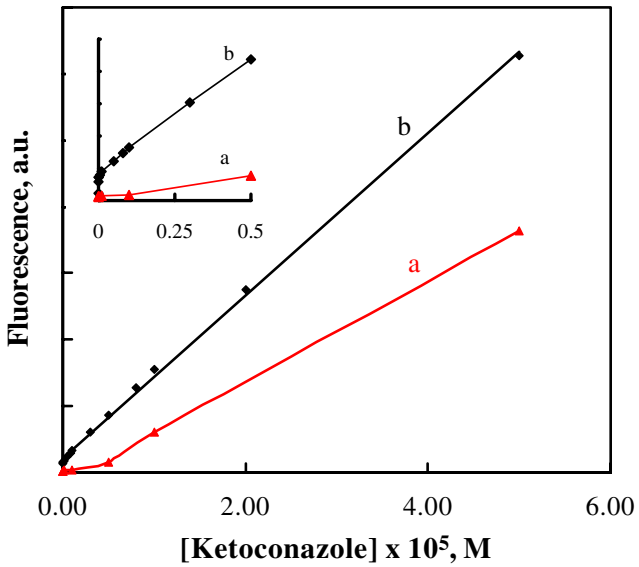


Fig. 6 Fluorescence intensity against KC concentration. **a** in the absence of β-CD; **b** in the presence of β-CD. [β-CD]=0.01 M; pH=7.9; t=25°C; λ_{ex}=288 nm, λ_{em}=370 nm. *Inset*: Fluorescence intensity against KC concentration in the concentration range: 0–5.0×10^{-6} M

studied in the range of 17–40 °C (not shown). A gradual decrease in fluorescence was observed by increasing temperature. The temperature value of 25 °C was selected as the optimum temperature.

Calibration curve

The fluorescence intensity of the KC solution under optimum experimental conditions showed a linear relationship with the concentration of KC in the range of 0.01–10 μg ml^{-1} (5×10^{-8} M–1.88×10^{-5} M), as is shown in Fig. 6. It is obvious from Fig. 6a that, in the absence of β-CD, the linear range of the calibration curve starts from higher concentrations of KC, and the slope of the curve is lower as compared to Fig. 6b, which was obtained in the presence of β-CD (the plot is magnified as an inset for very low concentrations of KC).

The limit of detection (LOD) of the method, which was calculated according to the equation LOD=$k_b s_b/m$ (k_b=3; s_b is the standard deviation of blank measurements; and m is the slope of the calibration curve) [32] was obtained to be 0.01 μg ml^{-1} (5×10^{-8} M).

Table 1 Effect of some interferents on the detection of KC by the proposed method

Interferent	Tolerance limit[a]
L-cystin	900
L-leucin	900
Kreatin	1,000
L-tyrosin	900
Miconazole	800
Fluconazole	800

[a] Interferent to KC mole ratio

Table 2 Detection results of KC in pharmaceutical formulations

Sample	Labeled	Found[a] (X±SD)	
		Proposed method	Official method
Tablet (mg/Tablet)	200	201.04±0.06	207±2
Cream (m/m)%	2	1.92±0.06	1.97±0.1
Shampoo (m/v)%	2	1.90±0.45	–

[a] Average of three replicate measurements

Effect of interferents

We have examined the effect of some compounds potentially interfering during detection of KC by the proposed method (Table 1). In such experiments, to an accurately prepared solution of KC ([KC]=1.0×10^{-6} M) an excess of the interferent was added and the concentration of KC was detected by the proposed method. The recovery was obtained, which is the percent ratio of KC concentration obtained by the proposed method to that had been prepared. The tolerance limit of the interferent is expressed as the interferent-to-KC concentration ratio assuring recovery of KC between 95–105%. According to the data in Table 1, all the interferents tested have no considerable effect on the detection of KC by the proposed method. The results of analysis of real samples by the proposed method were also free from the influence of interferents.

Applications

Detection of KC in pharmaceutical preparations The proposed method was successfully applied to the detection of KC in tablets, creams, and shampoo samples. As is shown in Table 2, there is a satisfactory agreement between the results of the proposed and official method [33] as well as the labeled amounts on the pharmaceutical products. The official method is a non-aqueous acid-base titration method. In comparison with the official method, the proposed procedure is simpler, performed in aqueous solution, more sensitive (especially needed for measurements in biological

Table 3 Results of recovery studies of KC from blood serum samples

[KC], M	Recovery[a] (X±RSD%)
7.0×10^{-8}	97.95±0.91
1.0×10^{-7}	98.92±0.16
1.0×10^{-6}	97.10±2.4

[a] Average from three replicate measurements

fluids), and more selective. Despite of these advantages, the proposed method needs more expensive equipment than the official method.

Detection of KC in blood serum samples The applicability of the new method to the detection of very low concentrations of KC in blood serum samples was investigated. Usually it is necessary to measure the concentration of drugs in biological fluids in order to optimize the therapeutic and limit the toxic effects of the drug. The method was applied to the recovery of different concentrations of KC from a blood serum sample. After removal of proteins from blood serum, the KC content of the solution was determined using the proposed method. The recovery of KC from blood serum samples is shown in Table 3. Therapeutic concentration of KC in blood serum is about 9.4×10^{-8} M (0.05 mg L^{-1}) [34], which is within the linear range of the proposed method.

Conclusions

The formation of an inclusion complex between KC and β-CD in aqueous solution reduces the non-radiative relaxation of KC when it is irradiated with a beam of radiation at $\lambda=$ 288 nm. Thus, an enhancement in fluorescence intensity of KC is observed.

The fluorescence against β-CD/KC mole-ratio plots was constructed and the stoichiometry and stability constants of the inclusion complexes formed were determined in acidic and basic solutions.

Based upon the enhancement effect observed, a sensitive spectrofluorimetric method for the detection of KC was proposed. Under optimized experimental conditions, the method was applied to the detection of KC in pharmaceutical formulations as well as the recovery from blood serum samples spiked with the drug. The results of the proposed method were satisfactory as compared to the results of the official method and also in comparison to labeled amounts of KC on pharmaceutical products.

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