Study on the Supramolecular Interaction of Cucurbit [7]uril with Ephedrine Hydrochloric Acid by Fluorescent Probe and its Analytical Application

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(Received on 9th June 2012, accepted in revised form 21st January 2013)

Summary: The inclusion interaction of cucurbit[7]uril (CB[7]) with ephedrine hydrochloric acid (EP) was studied using fluorescent probe method. Significant quenching of the fluorescence intensity of the CB[7]-palmatine (PAL) fluorescent probe was observed when EP was added, showing that EP was accommodated into the hydrophobic cavities of CB[7] and an endo-inclusion complex was formed. The hydrophobic interaction between CB[7] and EP mainly contributed to the formation of 1:1 type CB[7]-EP. Its complex constant was determined as $K = (2.82 \pm 0.52) \times 10^{11}$ M^{-1}. Based on the significant quenching of the supramolecular complex fluorescence intensity, a new spectrofluorimetric method with high sensitivity and selectivity was developed for the determination of EP in aqueous solution. The fluorescence quenching values (ΔF) showed good linear relationship with EP concentrations from 0.011 μg mL^{-1} to 2.8 μg mL^{-1} with a detection limit 0.0036 μg mL^{-1}. The proposed method had been successfully applied to the determination of EP in its pharmaceutical dosage forms and in urine samples with good precision and accuracy. In addition, molecular modeling calculations results indicated the partial inclusion of EP in the hydrophobic cavity of CB[7]. The interaction mechanism between the CB[7] and EP was confirmed by ^{1}H-NMR spectrum.

Keywords: Ephedrine hydrochloric acid, Cucurbit[7]uril, Fluorescent probe, Palmatine.

Introduction

Cucurbit[*n*]Urils (CB[*n*], *n* = 5–8, 10, Fig. 1) is a family of relatively new, pumpkin-shaped cavitands composed of *n* glycoluril units linked by a pair of methylene groups. The symmetrical CB[*n*] hosts resemble a hollow barrel with hydrophobic cavities and restrictive polar portals lined with ureido carbonyl groups location [1-3]. These characteristics enable CB[*n*] to form significant stable complexes with a variety of guest molecules in aqueous solution. Various organic drugs and biologically relevant molecules have been encapsulated by CB[*n*] [4, 5]. The formation of inclusion complexes often enhances or disturbs the photophysical and photochemical properties of the included guest molecules [6]. Several papers describing the recognition properties and applications of CB[7] have been published in the past few years [7-9]. However, little attention has been devoted to their fluorescent properties and potential analytical applications. Megyesi *et al.* [10] discovered that the formation of an inclusion complex between CB[7] and berberine leads to improved fluorescence intensity. Li *et al*. [11] studied the supramolecular interactions of CB[7]-isoquinoline alkaloid complex and its applications to the molecular recognition and determination of isoquinoline alkaloids and other drugs, owing to its excellent optical properties. The interaction between the non-fluorescent drug and CB[*n*] has only just begun to be studied using fluorescent probes titration [12]. The fluorescent probe system consisting of CB[7] and palmatine (PAL, Fig. 1) fluorescent probe has been successfully used for a variety of non-fluorescent drug determination because of its high sensitivity and selectivity [13].

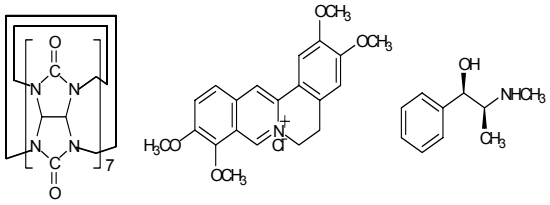

Fig. 1: The structures of CB[7], PAL, EP.

Ephedrine (EP, Fig. 1) is a sympathy-omimetic agonist at both α- and β-adrenergic receptors, which determine an increase of cardiac rate and contractility, peripheral vasoconstriction, bronchodilatation and central nervous system (CNS) stimulation [14]. EP was widely used in cough-cold syrup, but it would cause drug addiction, and make mental damage to patient if abused [15]. In addition, EP as an illicit drug, has been promoted for use as a stimulant in athletes [16]. EP is on the list of substances prohibited by the Medical Commission of the International Olympic Committee (MCIOC) [17]. Therefore, the rapid and sensitive analysis of EP is of continual interest in the fields of foods, pharmaceuticals and forensic application [18]. A number of assays have been reported for the determination of EP in biological and pharmaceutical samples, including spectrophotometry [19-22], chemiluminescence [23], atomic emission spectrometric [24], GC [25], HPLC [26], GC-MS [27], HPLC-MS [28], capillary electrophoresis [29],

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and ^{1}H-NMR [30]. Considering that aqueous solutions of EP have no native fluorescence, it cannot be directly determined through the normal fluorimetric method. To our knowledge, however, usage of the fluorescent probe for the determination of EP has not been reported. We aim to find a simple, rapid, accurate, and sensitive method using the proposed procedures to determine the drug in their pharmaceutical dosage forms. Satisfactory assay results were obtained. The detection limit is 0.0036 μg mL^{-1}, which is lower than that of any other spectral method reported in the literature [20, 21, 23].

Results and Discussion

The Fluorescence Quenching of CB[7]-PAL Probe by EP

A strong fluorescence probe system containing optically inert CB[7] was developed for the determination of a non-fluorescent drug in the supramolecular complex formation with PAL that exhibits weak fluorescence [13]. CB[7]-PAL probe can emit strong fluorescence. Significant quenching of fluorescence intensity of the CB[7]-PAL complex with the addition of EP was observed. The fluorescence spectra of the CB[7]-PAL complex, in the presence of different concentrations of EP, are shown in Fig. 2. Fluorescence intensity decreased with increased EP concentration, which is likely due to the competition between EP and PAL molecules for occupancy of the CB[7] cavity. Parts of the PAL molecule can be expelled from the cavity of CB[7] by the introduction of the EP, thereby reducing the fluorescence intensity of CB[7]-PAL because of the formation of a new inclusion complex between EP and CB[7].

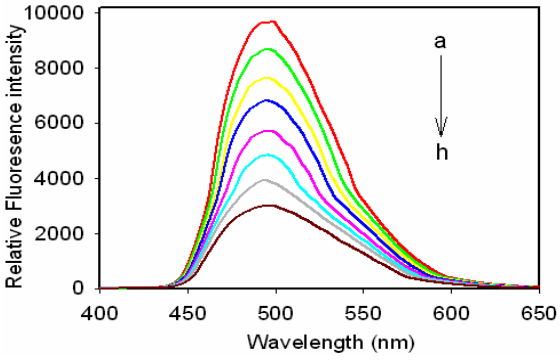

Fig. 2: Fluorescence spectra of CB[7]-PAL in the presence of EP in 1.0 mM HCl aqueous solution with λ_{ex} = 343 nm. The concentrations of EP (μg mL^{-1}): (a) 0; (b) 0.4; (c) 0.8; (d) 1.2; (e) 1.6; (f) 2.0; (g) 2.4; (h) 2.8. $C_{CB[7]}$ = 8.0 μM, C_{PAL} = 9.0 Mμ.

Apparent Association Constant and Stoichiometry

The addition of a nonfluorescent host (H) results in the formation of 1:1 host-guest inclusion complexes (H-G) which, in turn, enhances the fluorescence of the guest (G). The value of the enhanced fluorescence is dependent on the concentration of the added H [30, 31], as shown in the following equation:

$$F / F_0 = 1 + (F_\infty / F_0 - 1)\frac{[H]K}{(1+[H]K)} \quad (1)$$

where F_0 is the fluorescence intensity of G in the absence of H, F is the observed fluorescence intensity at each H concentration tested, F_∞ is the enhancement when 100% of G is complexed, and K is the equilibrium association constant for the 1:1 complexation.

$$H + G \Leftrightarrow H\text{-}G \quad (2)$$

$$K = \frac{[H\text{-}G]}{[H][G]} \quad (3)$$

The 1:1 complexation (and, hence, the applicability of Eq. 1) can be confirmed from the double reciprocal plot of 1/ (F – F_0) versus 1/[H]. The plot will be linear if only 1:1 complexation occurs and will be nonlinear if higher-order complexes also form.

In the interaction between CB[7] and PAL, the equilibrium reaction is as follows:

$$CB[7] + PAL \Leftrightarrow CB[7]\text{-}PAL \quad (4)$$

The equilibrium association constant is defined as follows:

$$K_{CB[7]\text{-}PAL} = \frac{[CB[7]\text{-}PAL]}{[CB[7]][PAL]} \quad (5)$$

Fig. 3 show the enhancement of PAL fluorescence as a function of added CB[7]. The inset shows the linear double reciprocal plot (r = 0.998), confirming the 1:1 stoichiometry of the complex. The solid line shows the best fit of the data to Eq. 1 using the nonlinear least squares method. Four such trials were performed, yielding average values of $K_{CB[7]\text{-}PAL}$ = (8.02 ± 0.76)×10^5 M^{-1}.

In the interaction between CB[7] and EP, the equilibrium equation is as follows:

$$CB[7] + EP \Leftrightarrow CB[7]\text{-}EP \quad (6)$$

The equilibrium association constant is defined as follows:

$$K_{\text{CB[7]-EP}} = \frac{[\text{CB[7]-EP}]}{[\text{CB[7]}][\text{EP}]} \quad (7)$$

When EP was added to the aqueous solution of the CB[7]-PAL complex, equilibriums (4) and (6) coexist in the solution. Thus, combining (4) and (6) gives the following equilibrium equation:

$$\text{CB[7]-PAL} + \text{EP} \Leftrightarrow \text{CB[7]-EP+PAL} \quad (8)$$

The equilibrium association constant is defined as follows:

$$K = \frac{K_{\text{CB[7]-EP}}}{K_{\text{CB[7]-PAL}}} = \frac{[\text{CB[7]-EP}][\text{PAL}]}{[\text{CB[7]-PAL}][\text{EP}]} \quad (9)$$

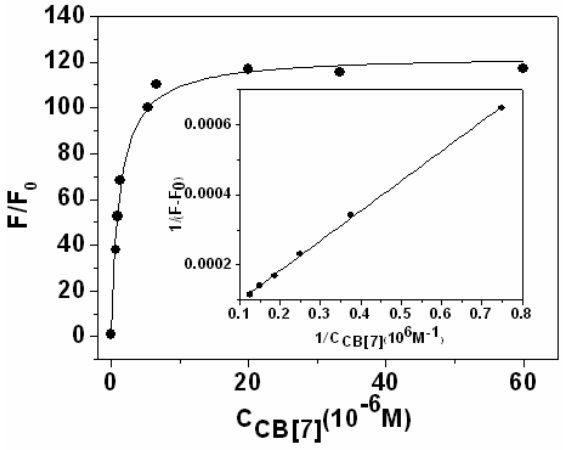

Fig. 3: Fluorescence enhancement of the fluorescence of a 2.0 μM solution of PAL as a function of added CB[7] in 1.0 mM HCl aqueous solution with $\lambda_{ex}/\lambda_{em}$ = 343/495 nm; the solid line shows the best fit of the data to Eq. 1. The inset shows the linear double reciprocal plot indicating 1:1 complexation

Wagner *et al*. [32] studied Eq. 1 and reported that it applies whether the fluorescence being measured is that of the guest or the host (i.e., they are interchangeable in Eqs. 2 and 3 because of the 1:1 complexation). In addition, Eq. 1 equally applies to the measurement of fluorescence quenching, as opposed to fluorescence enhancement [33]. The only difference is that the F/F_0 values are less than 1.0 in fluorescence quenching. In the present study, the CB[7]-PAL complex was considered the host molecule. Thus, Eq. 1 can also be applied to the quenching of CB[7]-PAL complexes (host) fluorescence with the addition of EP (guest). Eq. 1 can be used to determine the equilibrium association constant K (Eq. 9) from the fluorescence titration data of F/F_0 at a fixed CB[7]-PAL concentration as a function of EP concentration.

Fig. 4 show the fluorescence quenching of CB[7]-PAL complexes with the addition of EP. The inset shows the linear double reciprocal plot (r = 0.999), indicating the formation of a 1:1 host-guest inclusion complex between EP and CB[7]. The solid line shows the best fit of the data to Eq. 1. Four such trials were performed, yielding average values of K = (3.52 ± 0.29)×10^5 M^{-1} for the CB[7]-PAL + EP equilibrium system. The association constant value of $K_{\text{CB[7]-EP}}$ (Eq. 7) can be calculated according to Eq. 9: $K_{\text{CB[7]-EP}}$ = (2.82 ± 0.52)×10^{11} M^{-1}. The value is extremely large, indicating very strong host-guest interactions with excellent size and shape matches. A comparison of $K_{\text{CB[7]-PAL}}$ with $K_{\text{CB[7]-EP}}$ obviously results in $K_{\text{CB[7]-EP}} >> K_{\text{CB[7]-PAL}}$. Thus, EP shows extremely stronger binding with CB[7] than PAL. Accordingly, considering the thermodynamic factor only, the PAL molecule can be expelled from the CB[7] cavity by the EP molecules.

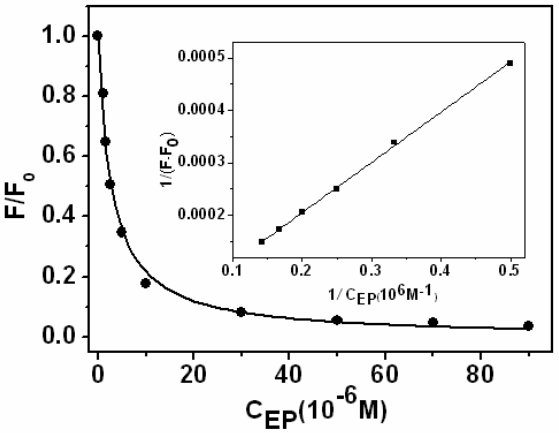

Fig. 4: Fluorescence suppression of the fluorescence of CB[7]-PAL as a function of added EP in 1.0 mM HCl aqueous solution with $\lambda_{ex}/\lambda_{em}$ = 343/495 nm; the solid line shows the best fit of the data to Eq. 1. The inset shows the linear double reciprocal plot indicating 1:1 complexation. $C_{\text{CB[7]}}$ = 2.0 μM, C_{PAL} = 2.25 Mμ.

Effect of PAL Concentration on the Fluorescence Intensity of the CB[7]-PAL Complex

The effect of varying PAL concentrations on the fluorescence intensity of the CB[7]-PAL complex was studied. The concentration of PAL was varied from 1.0 μM to 12.0 μM. The fluorescence intensity

of the CB[7]-PAL complex was gradually enhanced as PAL concentration increased until it reached the maximum inclusion equilibrium at CB[7] saturation. In the present paper, PAL served as a fluorescent probe; thus, determining the proper concentration was crucial. If the PAL concentration is too low, the sensitivity of the probe will also be low. Conversely, a very high concentration may not help determine the optimum detection limit of the analyte [23]. Taking everything into consideration, the optimal PAL concentration was 9.0 μM.

Influence of pH

The effect of pH on Δ*F* was studied over the pH range of 1.0 to 12.0. The results indicate that Δ*F* is maximum and almost constant in the pH range of 1.0 to 7.0. However, Δ*F* significantly decreased with further increases in pH. The reason is that alkali cations are readily coordinated to the carbonyl-fringed portals of CB[7] in alkaline medium. Binding of the alkali cation lowers the rate constant of the ingress of organic guests [10]. Results show that the presence of sodium salts in alkaline medium lowers not only the equilibrium constant of PAL binding with CB[7] but also the fluorescence quantum yield. Hence, using hydrochloric acid, the pH was adjusted to 3.0, which was the desired pH for all subsequent experiments.

Influence of Temperature and Reaction Time

The effect of temperature on Δ*F* was examined within 10 °C to 80 °C. All formed complexes were stable up to 35 °C. Above 35 °C, the fluorescence intensity greatly decreased due to the dissociation of the complexes at high temperatures. Hence, all subsequent measurements were performed at room temperature.

In addition, Δ*F* reached a maximum within 10 min after the EP was added and remained constant for at least 5 hours. Hence, the standard reaction condition was set to room temperature for 10 min.

The Response Mechanism of the Fluorescent Probe

PAL exhibits weak fluorescence emission in aqueous solution because the isoquinoline and the substituted benzene rings in PAL are not on the same plane. This configuration prevents a conjugated system from being formed. The isoquinoline and the substituted benzene rings in PAL were nearly on the same plane when CB[7] was added into the aqueous solution of PAL, thereby enhancing the fluorescence of PAL molecule. The electrostatic attraction between the positive charge of the heterocyclic nitrogen of the PAL and the high electron density of the carbonyl oxygens of the macrocycle was induced. The apolar part of PAL can penetrate into the host cavity. Inside the cavity, the degree of freedom of motion of the PAL molecule is reduced, thus also reducing the probability of radiationless transition. At the same time, the cavity can shield the excited single state of PAL from probable quenching processes that usually occur in aqueous solutions.

Molecular modeling calculations were optimized at the B3LYP/6-31G(d) [34] level of density functional theory [35] using the Gaussian 03 program [36]. Results confirmed the partial inclusion of PAL in the hydrophobic cavity of CB[7]. In the energy-minimized structure, the dihedral angle between the isoquinoline and the substituted benzene ring varies from 23.37° to –8.09°. The heterocyclic nitrogen is located in the vicinity of a carbonyl-laced portal. The partial immersion of PAL in the hydrophobic cavity of CB[7] reduces interaction with water. This state results in a less polar microenvironment which, in turn, leads to fluorescence enhancement.

When EP was added to the host-guest system of CB[7]-PAL, the PAL and EP competed to bind with CB[7]. Some parts of the PAL molecule were expelled from the CB[7] cavity with the introduction of the EP. The energy-minimized structure of CB[7]-EP complex are shown in Fig. 5, the benzene ring is embedded in the CB[7] cavity, and the nitrogen atom is located in the vicinity of a carbonyl-laced portal. The photochemical property of PAL is strongly dependent on its local microenvironment. The addition of the EP caused PAL to lose its protection in the CB[7] hydrophobic cavity, thus resulting in reduced PAL fluorescence intensity.

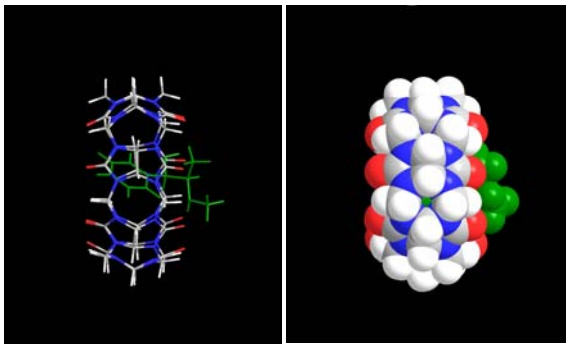

Fig. 5: Energy-minimized structure of CB[7]-EP complexes in the ground state using balls and tubes for the rendering of atoms. Color codes: EP, green; CB[7], oxygen, red; nitrogen, blue; carbon, gray; hydrogen, white.

The formation of CB[7]-PAL complex had been confirmed by ^{1}H-NMR spectroscopy [12]. Fig. 6 shows the ^{1}H-NMR spectra of the CB[7]-EP complex. The results of the ^{1}H-NMR experiments are consistent with the theoretical structures of the complex. Compared with the proton resonances of the unbound EP molecules (Fig. 6), the signals of H_2, H_3, H_4, H_5, H_6, H_7, H_8, and H_{11} protons of the bound EP significantly shifted upfield. This behavior is characteristic of this part of the molecule encapsulated in the CB[7] cavity [37]. The chemical shift of the H_{10} protons is practically unchanged, which indicates negligible interaction with CB[7]. These findings indicate that the PAL molecules were expelled from the CB[7] cavity due to the addition of EP. These results are consistent with the foregoing discussion.

In summary, combination of hydrophobic interaction of the cavity of CB[7] as well as ion-dipole interaction between the carbonyl portal of CB[7] and N^+ ion (of PAL, and EP) leads to the formation of host-guest complex. Because of the excellent size and shape matches, EP bound with CB[7] more tightly than PAL.

Effect of Interfering Substances

Prior to the application of the proposed spectrofluorimetric method to real samples, the effect of commonly used tablet excipients on the determination of 0.2 μg mL^{-1} of EP was studied under optimum experimental conditions. A 3000-fold mass in excess of each substance over EP was first tested. When interference occurred, the ratio was progressively reduced until the interference ceased. The tolerance limit was defined as the ratio of the concentration of the foreign species to that of EP which resulted in an error of less than ± 5%. The results are shown in Table-1. The determination was obviously free of interference from the usual excipients. During the testing of real samples for the determination of pharmaceutical preparations, no other interfering substances having similar structure with EP in excipients were observed. Thus, no background interference was present, indicating good selectivity in the method used to test the drug in both raw material and dosage forms.

Calibration Graph and Sensitivity

Under the optimum experimental conditions described above, a linear relationship between ΔF intensity and the concentration of EP was obtained in the range of 0.011–2.8 μg mL^{-1}. A correlation coefficient of 0.9997 and a detection limit of 0.0036 μg mL^{-1} were also determined. The linear regression equation obtained was $\Delta F = 2392.2C + 15.653$. The proposed method proved to have higher sensitivity than any other spectral method for determining EP reported in the literature, as presented in Table-2.

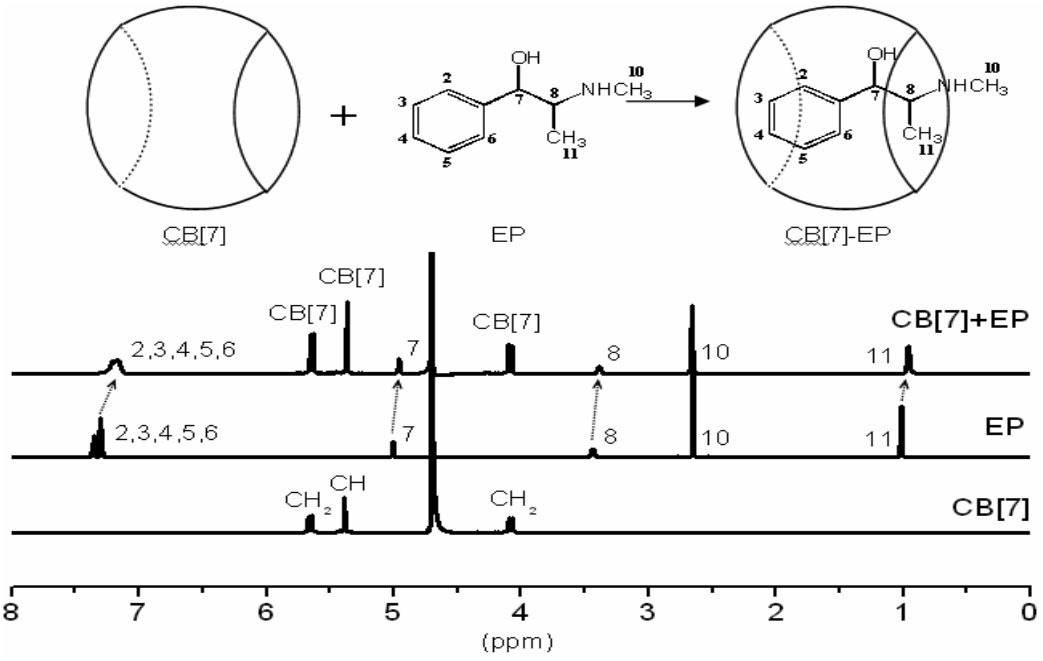

Fig. 6: ^{1}H-NMR spectra (600 MHz) of CB[7], EP, CB[7]-EP complex in D_2O.

Table-1: Effect of interference (tolerance error ± 5.0%).

Tolerance ratio in mass	Interference
3000	Starch, sucrose, lactose, glucose,mannitol, sorbitol, boracic acid, hexane diacid,
2000	Methyl cellulose,
1500	Gelatin, glycin,
1000	Sodium hydroxymethyl cellulose,tryptophan, gum acacia power,
500	Sodium carboxymethyl cellulose,
100	NH_4^+, K^+, Na^+,
50	Mg^{2+}, Ca^{2+}, Fe^{2+}, Zn^{2+},

Table-2: Comparison with other Spectral methods for the determination of EP.

Technique	Linear range (μg mL^{-1})	Detection limit (μg mL^{-1})	Application	Reference
Spectrophotometry	100.0–1000.0	1.92	Tabellae	[20]
	15–75	0.92	Tabellae	[21]
Chemiluminescence	0.2–20	0.03	Tabellae	[23]
The proposed method	0.011–2.8	0.0036	Tabellae, human urine	

Table-3: Analytical results and recovery tests of EP in pharmaceutical formulation.

Drugs	Label claim (mg/grain)	Fluorescent probe method			Reference method [26]	
		Found (mg/grain)	Equivalent nominal content (%) ± S.D.[a]	Recovery(%) ± S.D.[a]	Found (mg/grain)	Equivalent nominal content (%) ± S.D.[a]
EP[b]	10	9.93 (*t*,1.54; *F*, .92)	99.3 ± 0.54	99.65 ± 0.59	9.853	98.53 ± 0.68
EP[c]	5	4.88 (*t*,1.65; *F*, .18)	97.60 ± 0.72	97.82 ± 0.78	4.847	96.94 ± 0.82

[a]Average of five determination. (The tabulated values of *t* and *F* at the 95% confidence limit are *t* = 2.31 and *F* = 6.39.)
[b]EP Tablets (Tianjin Lisheng Pharmaceutical Co., Ltd, Tianjin, China)
[c]EP Tablets (Shanxi Tongda Pharmaceutical Co., Ltd, Shanxi, China)

Analysis of Pharmaceutical Formulations

The proposed fluorescent probe was applied in the determination of EP in pharmaceutical preparations. Ten tablets of EP were carefully pulverized. From this powder, an equivalent of 10 mg of EP was accurately weighed, placed inside a 100 mL calibrated flask, dissolved in 20 mL water, and swirled and sonicated for 3 min. The resulting solution was diluted to volume with water. The first 10 mL of the filtrate was discarded, after which 10 mL of the remaining filtered sample solution was diluted to 100 mL with double-distilled water. Further dilutions were performed to obtain the sample solutions using the same testing methods as described in experimental procedure. The results are presented in Table-3. The concentrations of EP determined using the proposed fluorescent probe are consistent with the obtained through methods found in the literature [26]. The relative standard deviations obtained from the proposed method were less than 1.00%. Moreover, the *t*- and *F*-tests showed that the proposed method had better precision and accuracy compared with the method. The recoveries were in the range of 97.82% to 99.65%.

Analysis of Spiked Human Urine

The proposed method was applied in the determination of the EP in spiked samples of human urine. Accuracy was assessed by investigating the recovery of EP at three concentration levels covering the specified range (five replicates of each concentration). The results (Table-4) showed that percentage recoveries were 97.10–99.48% with an S.D. of less than 2.0 for spiked human urine, indicating both good accuracy and precision.

Table-4: Determination of EP in spiked urine (n = 5).

Samples	Amount added (μg mL^{-1})	Amount found (μg mL^{-1})	Recovery (%) ± S.D.[a]
Urine 1	0.1	0.0971	97.10 ± 1.4
Urine 2	0.5	0.4917	98.34 ± 1.06
Urine 3	1.0	0.9948	99.48 ± 0.93

[a]Average of five determination.

Experimental

Instruments

Fluorescence spectra and intensity measurements were obtained using a Hitachi F-4500 spectrofluorimeter equipped with a 150 W xenon lamp (Japan). The slit widths of both excitation and emission monochromators were set to 5 nm. The fluorescence spectra were recorded at a scan rate of 1200 nm min^{-1}. All measurements were performed using a standard 10 mm path-length quartz cell at 25.0 °C ± 0.5 °C. The pH values were measured using a pHS-3 TC digital precision pH meter (Shanghai, China). ^{1}H-NMR spectra were obtained using a Bruker DRX-600MHz spectrometer (Switzerland) in D_2O.

Reagent and Chemicals

PAL and EP were obtained from the Chinese National Institute for the Control of

Pharmaceutical and Biological Products (Beijing, China) without further treatment. EP was dissolved in double-distilled water to prepare stock standard solutions of 100 μg mL^{-1}. PAL was dissolved in double-distilled water to prepare stock solutions with final concentration of 1.0 mM. CB[7] was prepared and characterized according to reported procedure [3]. CB[7] stock solution of 1.0 mM was prepared by dissolving CB[7] in double-distilled water. Stock standard solutions were stable for several weeks at room temperature. Standard working solutions were prepared by diluting the stock standard solutions with double-distilled water before use. All other chemicals were of analytical reagent grade, and double-distilled water was used throughout the procedure.

Experimental Procedure

0.8 mL solution of 0.1 mM CB[7] was poured into a 10 mL colorimetric flask, to which 0.9 mL of the 0.1 mM PAL solution and 1.0 mL of 0.01 M hydrochloric acid were also added. Suitable amounts of EP solution were then sequentially added to the flask. The mixture was diluted to volume with double-distilled water and shaken for 15 min at room temperature. The fluorescence intensity values of the solution ($F_{PAL\text{-}CB[7]\text{-}EP}$) and the blank solution ($F_{PAL\text{-}CB[7]}$) were measured at 495 nm using an excitation wavelength of 343 nm.

Conclusion

The new fluorescent probe system for CB[7]-PAL complexes was devised for the determination of analytes. Considering the significant quenching observed in the fluorescence intensity of CB[7]-PAL in the presence of EP, the spectrofluorimetric method developed was of high sensitivity and selectivity for the determination of EP in aqueous solution. The proposed method is more sensitive than the other spectral methods reported in the literature. Moreover, the method had been successfully applied to the determination of EP in its pharmaceutical dosage forms and in urine samples. This method can also be used in a fluorescence sensor for the detection of non-fluorescent or weakly fluorescent substances. Related studies are in progress in our laboratory. At the same time, the interaction models of the supramolecular complexes were established through theoretical calculations. The interaction mechanism between the fluorescent probe and EP was confirmed via the ^{1}H-NMR spectrum. The association constants of the complexes formed between the host and the guest were calculated.

Acknowledgement

This work was supported by the Research Fund for the Doctoral Program of Higher Education of China (No.20091404110001). Helpful suggestions by anonymous referees are also gratefully acknowledged.

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