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Determination of urotropine using cucurbit[7]uril-palmatine complex as a highly sensitive fluorescent probe

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A new method for sensitive and selective determination of urotropine has been developed using the cucurbit[7]uril-palmatine complex as a fluorescent probe. The complex exhibits high fluorescence in aqueous solution, which is quenched in the presence of urotropine. The fluorescence quenching value, ΔF, is directly proportional to the concentration of urotropine in the range of 0.004–1.26 μg mL^{-1}, with detection limit as sensitive as 0.0013 μg mL^{-1}. The proposed method has been successfully applied to determine urotropine in food samples with good precision and accuracy. The stoichiometry and binding affinity as well as the nature of the binding behavior are determined using spectrofluorimetry, ^{1}H NMR and molecular modeling theoretical calculations.

Keywords: Analytical chemistry, Fluorescent probes, Urotropine, Cucurbit[7]uril, Palmatine, Competitive inclusion

Cucurbit[*n*]urils (CB[*n*]s, *n* = 5–8, 10, Fig. 1), a family of macrocyclic synthetic host molecules, are cyclic oligomers composed of a varying number of glycoluril units bridged by methylene groups, and posses unique guest binding properties in water[1-3]. Numerous organic drugs and biologically relevant molecules have been encapsulated by CB[*n*]s[4,5]. Among all the members in the cucurbituril family, CB[7] has elicited much attention. Unlike CB[6] and CB[8], which show poor solubility, CB[7] possesses a high level of solubility in neutral water. Also, it exhibits better ability to complex a variety of guest molecules than its water-soluble analogue CB[5] because of a more voluminous cavity. Accordingly, it has been used extensively in supramolecular chemistry.

Palmatine (PAL, Fig. 1) is a clinically important isoquinoline alkaloid[6]. Although the aqueous solution of PAL exhibits weak native fluorescence, significant fluorescence intensity enhancement was observed upon its inclusion in CB[7][7].

Urotropine (URO, Fig. 1), also known as 1,3,5,7-tetraazatricyclo[3.3.1.1^{3,7}]decane, methenamine, hexamine and hexamethylenetetramine, is widely used as a food additive as a preservative. It is approved for usage for this purpose in the EU (item "E239")[8]. However, URO has been forbidden as a food additive in many countries, such as China, USA and Australia, due to the undesirable release of the highly toxic formaldehyde[9,10]. A number of assays have been reported for the determination of URO, including spectrophotometry[11-13], spectrofluorimetry[13], ion-pair extraction[14], HPLC[15], GC[16], gas-liquid chromatography[17], proton NMR[18]. Although these methods provide a rapid and sensitive analysis, they require complicated sample preparation procedures, rigorous experimental conditions, expensive equipment, and well-trained operators. In contrast to these techniques, spectrofluorimetry can provide an operationally simple and cost-effective detection method together with high sensitivity and selectivity[19]. To the best of our knowledge, usage of the fluorescent probe method to determine URO has never been reported. Furthermore, the proposed method was successfully applied to determine URO with higher sensitivity than any other spectral method reported in the literature[11-13].

Experimental

The fluorescence measurements were performed with an Agilent Cary Eclipse fluorescence spectrofluorometer (Agilent, Australia) using a standard 10 mm path-length quartz cell at 25.0±0.5 °C. Excitation and emission band-widths were set to 5 nm. The fluorescence spectra were recorded at a scan

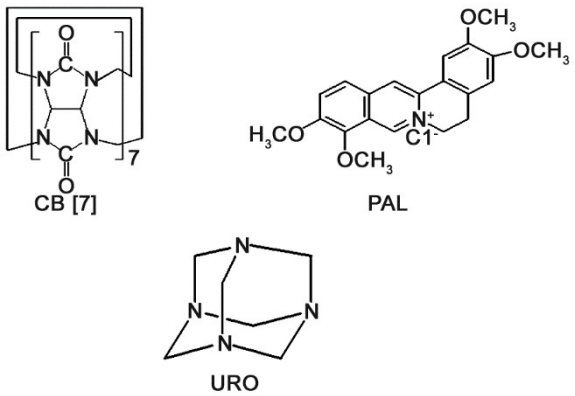

Fig. 1 — Structures of CB[7], PAL, and URO.

rate of 600 nm min^{-1}. ^1H NMR spectra were obtained using a Bruker DRX-600MHz spectrometer (Switzerland) in D_2O.

All reagents used were of analytical-reagent grade. Doubly distilled water was used throughout. Palmatine (PAL) and urotropine (URO) were obtained from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and used without further treatment. CB[7] was synthesized and characterized according to reported procedure[2]. URO was dissolved in doubly distilled water to prepare stock standard solutions of 100 μg mL^{-1}. Stock solutions of CB[7] and PAL were prepared as 1.0 m*M*. Standard working solutions were prepared by diluting the stock standard solutions with doubly distilled water before use.

For the estimation of URO, CB[7] solution (0.8 mL, 0.1 m*M*) was poured into a colorimetric flask (10 mL), to which PAL solution (0.8 mL, 0.1 m*M*) was also added. Suitable amounts of URO solution were added sequentially to the flask. The mixture was diluted to volume with doubly distilled water. The fluorescence intensity values of the solution ($F_{PAL\text{-}CB[7]\text{-}URO}$) and the blank solution ($F_{PAL\text{-}CB[7]}$) were determined after incubation for 15 min at room temperature.

For the analysis of soy food samples, the samples were ground before determination. From this powder, 2.00 g of each sample was placed in a centrifuge tube, to which a certain quantity of URO and 5 mL acetonitrile were added. The mixture was vortex-extracted for 15 min and centrifuged at 4000 rpm for 10 min, then 2 mL of the clear supernatant was removed and taken in a 10-mL colorimetric tube and evaporated to dryness under nitrogen flow. The residue was diluted to volume with doubly distilled water for subsequent analysis. Further dilutions were performed to obtain sample solutions using the same detection method as described above in the experimental procedure.

Results and discussion

PAL exhibits very weak fluorescence emissions in aqueous solution, with the maximum excitation and emission wavelengths at 235 nm and 370 nm. However, in acidic or neutral medium and at room temperature, PAL reacts with CB[7] to form stable complex, with greatly enhanced fluorescence intensity[7].

On addition of URO, dramatic quenching of the fluorescence intensity of the CB[7]-PAL complexes was observed . The fluorescence spectra of the CB[7]-PAL complex in the presence of different concentrations of URO are shown in Fig. 2. Fluorescence intensity decreased with increased URO concentration, which is probably due to the competitive inclusion of PAL with URO in the hydrophobic cavity of CB[7].

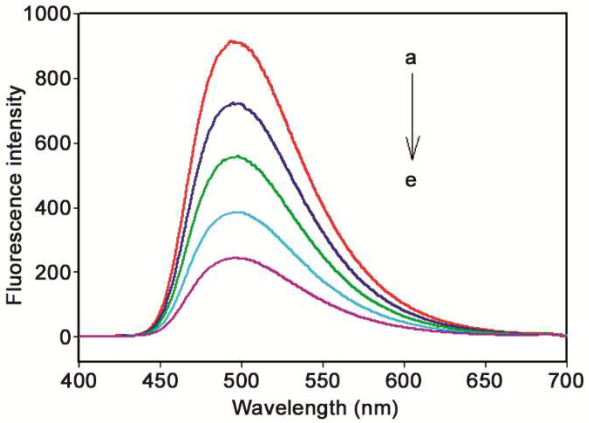

Fig. 2 — Fluorescence spectra of the CB[7]-PAL complex in the presence of URO in aqueous solution with λ_{ex} = 343 nm. [Conc. of URO (μg mL^{-1}): (a) 0; (b) 0.25; (c) 0.50; (d) 0.75; (e) 1.00. $C_{CB[7]} = C_{PAL}$ = 8.0 μ*M*].

The fluorescence enhancement of guest (G) molecules upon the addition of a non-fluorescence host molecule (H) results from the formation of 1:1 host-guest (H-G) complex. The stoichiometry and equilibrium association constant for the inclusion complexes were calculated by using a non-linear version of the Benesi-Hildebrand plot (Eq. 1) [20-22],

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

where F_0 and F are fluorescence intensities of G in the absence and presence of H, respectively. F_∞/F_0 is the fluorescence enhancement when 100% of G has been included and K is the equilibrium association constant for the complexation. The 1:1 complexation 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.

When URO was added into the aqueous solution of the CB[7]-PAL complex, two binding processes coexist in the solution:

$$CB[7] + PAL \Leftrightarrow CB[7] - PAL \quad \ldots (2)$$

$$CB[7] + URO \Leftrightarrow CB[7] - URO \quad \ldots (3)$$

The equilibrium constants are:

$$K_{CB[7]\text{-}PAL} = \frac{[CB[7] - PAL]}{[CB[7]][PAL]} \quad \ldots (4)$$

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

The equilibrium association constant of the system is defined as follows:

$$K = \frac{K_{\text{CB[7]-URO}}}{K_{\text{CB[7]-PAL}}} = \frac{[\text{CB[7]} - \text{URO}][\text{PAL}]}{[\text{CB[7]} - \text{PAL}][\text{URO}]} \quad \ldots (6)$$

Figure 3 shows the enhancement of PAL fluorescence as a function of added CB[7]. The solid line shows the excellent fit to Eq. 1, five such trials were performed, yielding average value of $K_{\text{CB[7]-PAL}} = (1.22 \pm 0.12) \times 10^5\ M^{-1}$.

In the present study, the CB[7]-PAL complex was considered as the host molecule. Fluorescence quenching is given by Eq. 7, as opposed to fluorescence enhancement[23], where C is the concentration of the CB[7]-PAL complex (host). Thus, the equilibrium association constant of the system K (Eq. 6) can be determined according to Eq. 1 because it correlates with the fluorescence intensities of the system:

$$F / F_0 = 1 + (F_\infty / F_0 - 1)\frac{[\text{H}]K}{(C + [\text{H}]K)} \quad \ldots (7)$$

On addition of URO, a dramatic quenching of the fluorescence intensity of the CB[7]-PAL complex is seen (Fig. 4). The solid line shows the excellent fit to Eq. 1, giving the equilibrium association constant of the system $K = (2.02 \pm 0.03)\ M^{-1}$. The association constant value $K_{\text{CB[7]-URO}}$ can be calculated according to Eq. 6 as $K_{\text{CB[7]-URO}} = (2.46 \pm 0.29) \times 10^5\ M^{-1}$. Obviously, $K_{\text{CB[7]-URO}} > K_{\text{CB[7]-PAL}}$. Thus, URO shows stronger binding with CB[7] than with PAL, and PAL molecules can be expelled from the CB[7] cavity by URO molecules. This reduces the fluorescence intensity of the CB[7]-PAL complex because of the complexation between CB[7] and URO.

Molecular modeling calculations were optimized at the B3LYP/6-31G(d) level of density functional theory using the Gaussian 03 program[24,25]. The results confirmed the complete inclusion of URO in the hydrophobic cavity of CB[7] (Fig. 5). In the energy-minimized structure, the whole URO molecule is embedded in the CB[7] cavity. The photochemical property of PAL is strongly dependent on its local microenvironment, hence the addition of URO causes PAL to lose its protection in the CB[7] hydrophobic cavity, resulting in decrease of the fluorescence intensity of PAL[26].

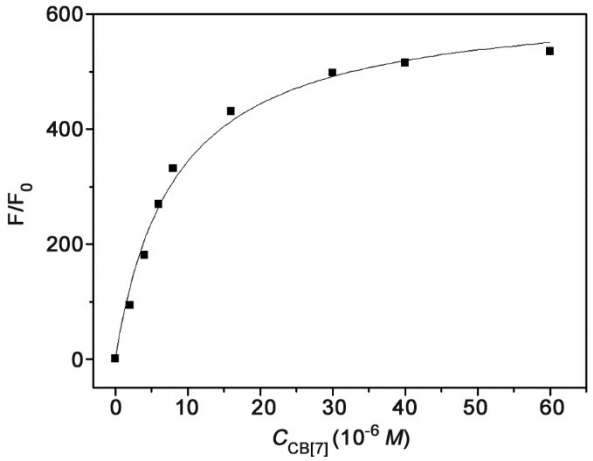

Fig. 3 — Fluorescence enhancement of PAL as a function of added CB[7] in aqueous solution. [$\lambda_{ex}/\lambda_{em}$ = 343/495 nm. The solid line shows the excellent fit to Eq. 1].

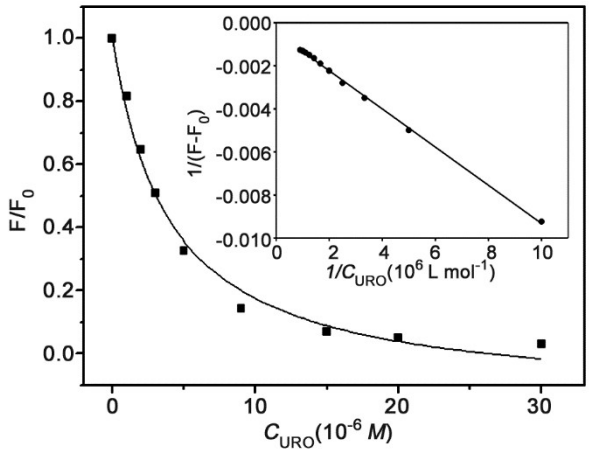

Fig. 4 — Fluorescence suppression of the CB[7]-PAL complex as a function of added URO in aqueous solution. [$\lambda_{ex}/\lambda_{em}$ = 343/495 nm. Solid line shows the excellent fit to Eq. 1. Inset shows the linear double reciprocal plot indicating 1:1 complexation. $C_{\text{CB[7]}} = C_{\text{PAL}} = 8.0\ \mu M$].

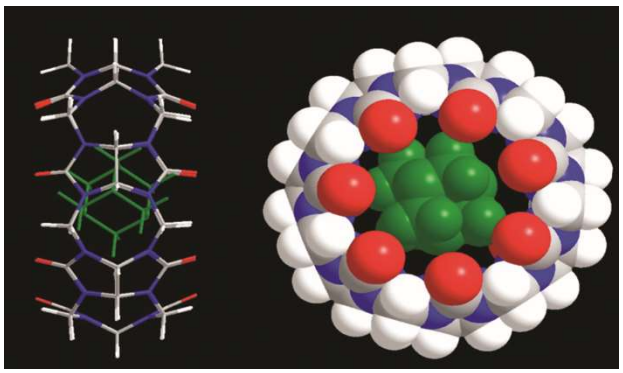

Fig. 5 — Energy-minimized structure of the CB[7]-URO complex in the ground state using balls and tubes for the rendering of atoms. [Color codes: URO, green; CB[7], oxygen, red; nitrogen, blue; carbon, gray; hydrogen, white].

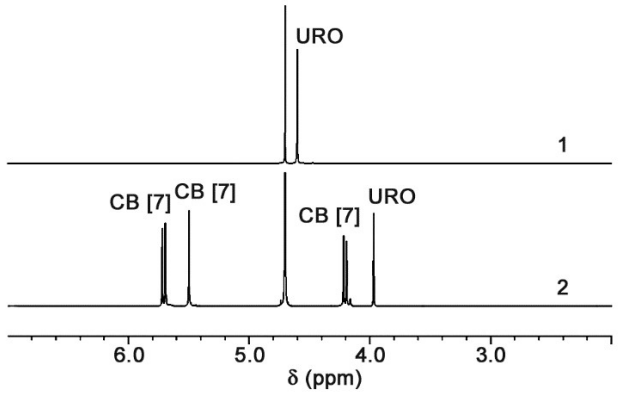

Fig. 6 — ^{1}H NMR spectra (600 MHz) of URO (1), the CB[7]-URO complex (2).

Table 1 — Effect of interferents on estimation of URO (tolerance error ±5.0%)

Interferents	Tolerance ratio
Starch, glucose, sucrose, lactose, sorbitol, mannitol, boracic acid, hexane diacid	3000
Methyl cellulose	2000
Gelatin, glycine	1500
Sodium hydroxymethyl cellulose,	1000
Sodium carboxymethyl cellulose	500
NH_4^+, Na^+, K^+	100
Mg^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}	50
Alanine, cysteine, cystine, phenylalanine, valine	0.2

Table 2 — Comparison with other spectral methods for the determination of URO

Technique	Sample	Linear range (μg mL^{-1})	Minimum working level (μg mL^{-1})	Ref.
Spectrophotometry	Urine	0.042 – 0.46	0.014	11
	Tabellae	1 – 12	0.33	12
	Tabellae	2 – 35	0.35	13
Spectrofluorimetry	Tabellae	0.60 – 40	0.12	13
Proposed method	Food sample	0.004 – 1.26	0.0013	This work

Table 3 — Determination of URO in dried beancurd sticks (n = 4)

Samples	Amount added (μg/g)	Amount found (μg/g)	RSD [a] (%)	Recovery (%)
1	0.10	0.088	7.4	88.00
2	0.30	0.26	6.5	86.67
3	0.60	0.62	5.8	103.33
4	1.00	0.95	4.4	95.00

[a]Average of four determination.

The formation of the CB[7]-PAL complex was confirmed by ^{1}H NMR spectroscopy[27]. The interaction of URO with CB[7] was also studied with ^{1}H NMR. Figure 6 shows the ^{1}H NMR spectra of URO as a function of CB[7]. In the presence of CB[7], URO signals significant upfield shift after the complex formation (Fig. 6b). This behavior is characteristic of the URO molecule encapsulated in the CB[7] cavity[28,29]. The results are consistent with the foregoing discussion.

The effect of interference by some foreign species was studied for the determination of 1.00 μg mL^{-1} of URO. With a relative error of less than ±5%, the tolerance limits for the foreign species are shown in Table 1. The results indicate that most of the foreign species do not affect the determination of URO under these conditions. Thus, this method had high selectivity.

Under the optimum experimental conditions, a linear relationship between ΔF and the concentration of URO was obtained in the range of 0.004–1.26 μg mL^{-1}. The linear regression equation obtained was $\Delta F = 560.12C$ (μg mL^{-1}) + 20.289 with correlation coefficient of 0.999 and a detection limit of 0.0013 μg mL^{-1}. The proposed method proves to have higher sensitivity than any other spectral method for the determination of URO reported in the literatures, as presented in Table 2.

The proposed method was applied to the determination of URO in soy foods. The accuracy, RSD of the method was assessed by the determination of four replicate recoveries at each value. The recoveries were in the range of 88.00–103.33%. The results are presented in Table 3. As can be seen, the precision and accuracy were satisfactory. Thus, the present method can be applied for the detection of URO in soy foods.

In summary, a new fluorescent probe method for the determination of URO with high sensitivity and selectivity has been developed. The general concept used in this approach is based on the competition between URO and PAL for occupancy of the CB[7] cavity, resulting in ΔF of the CB[7]-PAL supramolecular complex. The detection limit is as sensitive as 0.0013 μg mL^{-1}. In addition, the proposed method was successfully applied to determine URO in food samples. The results indicate a potential future in food analysis to guarantee safety, quality and authenticity.

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