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ORIGINAL PAPER

Interaction of Thyroxine with 7 Hydroxycoumarin: A Fluorescence Quenching Study

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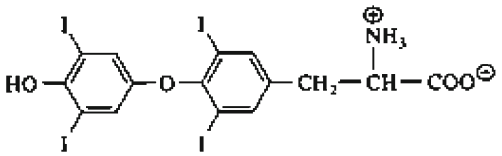
Abstract The interaction between thyroxine hormone and 7 hydroxycoumarin (7HC) was investigated using fluorescence quenching method. The experimental results showed that thyroxine could quench the fluorescence of 7HC by forming the 7HC–thyroxine complex with static quenching. The apparent binding constants (K) between 7HC and thyroxine were determined to be 1.51×10^{4} (297 K) and 9.06×10^{3} (310 K). The binding sites (n) 0.98±0.1. The thermodynamic parameters showed that the interaction between 7HC and thyroxine was driven mainly by hydrogen bonding interactions and van der Waals force. Calibration for thyroxine, based on quenching titration data, was linear in the concentration range 2.0×10^{-8} to 3.0×10^{-7} mol/l. The relative standard deviation was 2.58% for 2.0×10^{-7} mol/l thyroxine (n=4) and the 3σ limit of detection was 3.42×10^{-8} mol/l in cationic surfactant CTAB medium.

Keywords Thyroxine · 7-Hydroxycoumarin · CTAB · Fluorescence quenching · Stern–Volmer

Introduction

Thyroxine (3,5,3′,5′-tetraiodothyronine) is one of the most important hormones of the thyroid gland. It has a vital role in normal growth and development of the body and in the maturation of sexual organs. It is used to make up a hormonal deficiency on a regular maintenance basis and to treat the associated syndrome (myxoedema). It may also be used in the treatment of goitre and thyroid cancer [1]. It has L- and D-forms. The L-form is twice as active physiologically as the racemic product, and the D-form has very little activity [2]. Many means have been developed for thyroxine measurement such as capillary electrophoresis with laser-induced fluorescence [3], bioluminescent immunoassay by use of isotopic ^{135}I (RIA) label [4], time-resolved fluorescence [5], and luminol chemiluminescence [2].



Thyroxine

Coumarins exhibit strong fluorescence in the visible region which makes them suitable for use as colorants, in dye lasers and as nonlinear optical chromophores. They possess distinct biological activity and have been described as agents with potential for anticancer and anticoagulant activity. They can also induce modifications in cell growth, development and intracellular communication mechanisms. The photophysical properties of these compounds depend on the nature and position of a substituent group in the parent molecule and due to a change in the surrounding media [6–8]. 7hydroxycoumarin (7HC, its molecular structure is shown in Fig. 1), also known as umbelliferone, is a major biotransformed product of coumarin family, which is a widely distributed natural product [9–11].

In the present study, a fluorimetric titration was put forward for the interaction between thyroxine and 7HC. The probable mechanism of 7HC fluorescence quenching by thyroxine was studied by means of Stern–Volmer modeling. The experimental results showed that static

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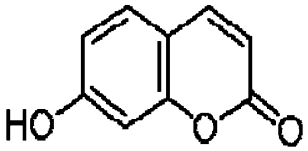
Fig. 1 Molecular structure of 7HC



quenching occurred with a complex formation between 7HC and thyroxine. In addition, the binding properties of 7HC–thyroxine complex were investigated based thermodynamic results.

Experimental

Apparatus

A Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) equipped with a xenon lamp (150 W) and 1.0 cm quartz cell was used to measure the fluorescence spectra and fluorescence intensity. The excitation and emission wavelength for 7HC were set at 334 and 464 nm, respectively, with the excitation and emission slit widths set as 2.5 and 5.0 nm. A Jenway (3040 Ion Analyser) pH meter was used for pH measurements.

Reagents

The stock solution of thyroxine hormone (1.0×10^{-4} mol/l) was prepared from L-thyroxine (3-[4-(4-hydroxy-3,5-diiodo phen-oxyl)-3,5-diiodophenyl]-L-alanine, Sigma). The stock solution of 7-hydroxycoumarin (umbelliferone, Fluka; 3.0×10^{-4} mol/l) was prepared by dissolving in hot water. CTAB (cetyl trimethyl ammonium bromide, Sigma) solution (1.0×10^{-2} M) was prepared by dissolving in hot water. Triton100-X (polyoxyethylene 10 iso-octyl phenyl ether, Sigma) and SDS (dodecyl sulfate sodium salt, Merck) were prepared by dissolving in water.

Sodium phosphate buffer solution (PBS, 0.067 mol/l) at pH 7.4 was used for dilution and preparation of solutions. All chemicals were of analytical reagent grade and used without further purification. Each working solution was prepared daily from the stock solution by appropriate dilution.

Procedure

The fluorescence emission intensity of 7HC decreased regularly with the increase of concentration of thyroxine using fluorimetric titration. Different amounts of (0–3.0 ml) 1.0×10^{-4} M thyroxine solution were, respectively, added to 5.0 ml 7HC solution (1.0×10^{-6} M) then diluted to 10.0 ml with PBS (pH 7.4) at room temperature. The final concentration of 7HC was 5.0×10^{-7} M in all working solutions. The fluorescence intensity F, was determined at λ_{ex}=334 nm and λ_{em}=464 nm, and the fluorescence F_0, of the 7-HC without thyroxine was obtained under same conditions.

Results and discussion

Fluorescence quenching

Figure 2 shows the emission spectra of 7HC in the presence of various concentrations of thyroxine. It was observed that the fluorescence intensity of 7HC decreased with the increasing concentration of thyroxine. But there was no significant λ_{em} shift with the addition of thyroxine. These data indicated that thyroxine could interact with 7HC and quench its fluorescence.

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule. Fluorescence quenching can be dynamic, resulting from collisional encounters between the fluorophore and quencher, or static, resulting from the formation of a ground state complex between the fluorophore and quencher. Dynamic and static quenching can be distinguished by their differing dependence on temperature and excited-state lifetime. As in both cases, the fluorescence intensity is related to the concentration of the quencher. Therefore, the quenched fluorophore can serve as an indicator for quenching agent. Fluorescence quenching is described by the Stern–Volmer equation [12]:

$$F_0/F = 1+K_{SV}[Q] = 1 + \tau_0 k_q[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities of 7HC before and after the addition of thyroxine, respectively, k_q the biomolecular quenching rate constant, $[Q]$ concentration of thyroxine as quencher τ_0 is the average lifetime of the excited state of the fluorophore in the absence of quencher.

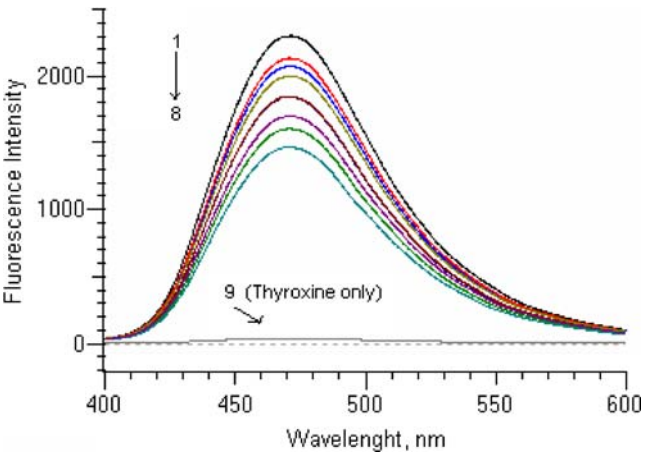


Fig. 2 The quenching effect of thyroxine on 7HC fluorescence. λ_{ex}/λ_{em}=334/464 nm, $C_{7HC}=5.0\times10^{-7}$ mol/l, $C_{thyroxine}$ from 1 to 8: 0, 0.4, 0.6, 0.8, 1.2, 2.0, 2.6, 3.0 ($\times10^{-5}$) mol/l

K_{SV} is the Stern–Volmer constant or dynamic quenching constant.

The Stern–Volmer quenching plots from the fluorescence titration data under two different temperature were investigated (Fig. 3). The results showed that the Stern–Volmer plots were both linear with the slopes decreasing with increasing temperature. The dynamic quenching constants, K_{SV} for the interaction between 7HC and thyroxine were found from slopes of curves. The corresponding Stern–Volmer quenching constants are shown in Table 1. Because the fluorescence lifetime of the coumarin derivatives is 10^{-9} s in literature [12–14], the quenching rate constants k_q calculated from $K_{SV}=\tau_0 k_q$. The order of magnitude of k_q was 10^{13} in the present work.

According to the literature [15] for dynamic quenching the upper limit of k_q expected for a diffusion-controlled bimolecular quenching rate constant of various quenchers with biomolecules is 10^{10} l/mol s. Considering that in our experiment the rate constant of 7HC quenching procedure initiated by thyroxine is much grater than 10^{10} l/mol s. It can be concluded that the quenching is not initiated by dynamic quenching, but probably by static quenching from the formation a complex between of 7HC and thyroxine and due to the presence of heavy atom in such a complex the intersystem-crossing rate is enhanced and thus the fluorescence quantum yield is decreased. The K_{SV} decreased with increased temperature, it showed that there is less quenching at high temperature, which suggests the 7HC-thyroxine complex is less stable at high temperature.

Binding constant and binding sites

For the static quenching interaction, if it is assumed that there are similar and independent binding sites in the biomolecule, the binding constant (K) and the number sites (n) can be got from the double logarithm regression curve of $\log(F_0-F)/F$ versus $\log[Q]$ based on the following equation [15],

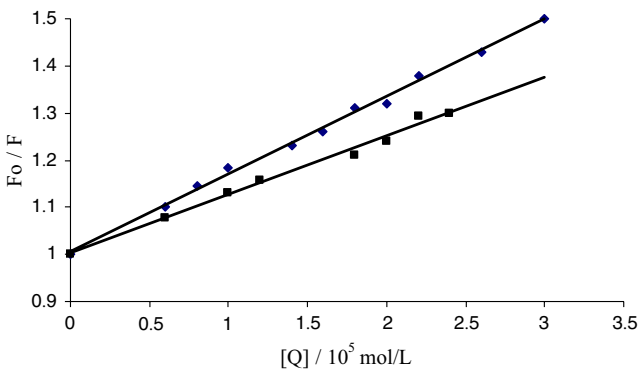


Fig. 3 Stern–Volmer curves for the binding of 7HC and thyroxine at 297 (*filled diamond*) and 310 K (*filled square*). $\lambda_{ex}/\lambda_{em}$

Table 1 Stern–Volmer quenching constant of the system of 7HC–thyroxine at different temperatures

T (K)	Linear regression equation	K_{SV} (l/mol)	R
297	$F_0/F = 1.005 + 1.648 \times 10^4[Q]$	1.648×10^4	0.9963
310	$F_0/F = 1.002 + 1.243 \times 10^4[Q]$	1.243×10^4	0.9907

$$\log(F_0 - F)/F = \log K + n\log[Q] \quad (2)$$

where K is the binding constant of 7HC with thyroxine and number sites, n can be determined by the slope of double logarithm regression curve based on the equation. Figure 4 shows the double logarithm curve and Table 2 gives the corresponding calculated results. It was found that the binding constant decreased with the increasing of temperature, resulting in a reduction of the stability of the 7HC–thyroxine complex. The values of binding sites were almost equal to unity indicating that there was one independent class of binding site on thyroxine for 7HC.

Thermodynamic parameters and nature of the binding forces

Considering the dependence of binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. Hence, the thermodynamic parameters depend on the temperatures and

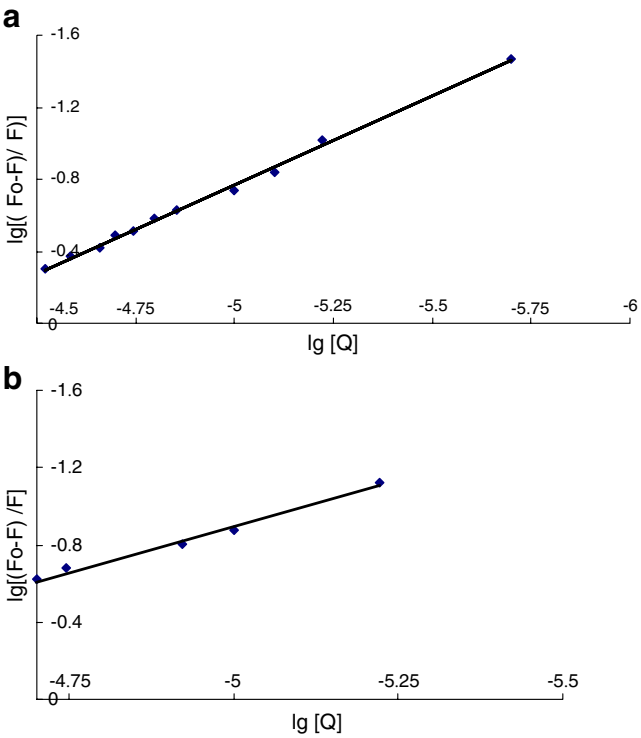


Fig. 4 Double-log plot of thyroxine quenching effect on 7HC fluorescence at 297 (**a**) and 310 K (**b**)

Table 2 Binding parameters for the system of 7HC–thyroxine

T (K)	K, binding constant (l/mol)	n, binding site	R
297	1.51×10^4	0.99	0.9970
310	9.06×10^3	0.97	0.9904

were analyzed to characterize the acting forces between 7HC and thyroxine. The acting forces between a small molecule and biomolecule include hydrogen bond, van der Waals force, electrostatic force, hydrophobic interaction force, and so on. The thermodynamic parameters were calculated using the following three equations. If the temperature does not vary significantly, the entalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta G = -RT\ln K \tag{3}$$

where R is the gas constant, T is the experimental temperature and K is the binding constants at corresponding T. Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from the following equations:

$$\ln K_2/K_1 = \Delta H/R[1/T_1 - 1/T_2] \tag{4}$$

where K_1 and K_2 are the binding constant at the experiment temperatures T_1 and T_2, respectively.

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

The thermodynamic parameters for the interaction of 7HC with thyroxine are shown in Table 3. The negative value of ΔG reveals that the interaction process is spontaneous. An important source of negative contribution to ΔH and ΔS will arise if a hydrogen bond is formed [16]. The negative ΔH and ΔS values for the interaction of 7HC and thyroxine indicate that the binding is mainly enthalpy driven and entropy is unfavorable for it, and that the hydrogen bonding and van der Waals forces played major role in the interaction.

Effect of surfactant and calibration graph

The effects of surfactants on the fluorescence of 7HC–thyroxine complex were studied using the three surfactants which have anionic, cationic and nonionic properties. Each surfactant concentration were above critical micelle concentration (cmc). Surfactant molecules are useful for supersensitive determination of trace amounts of a component due to their solubilization, raising super sensitivity and they are extremely important because they can serve as structural and functional model for complex bio-aggregates [17, 18]. In Fig. 5, more quenching was observed when cationic surfactant, CTAB was added to 7HC–thyroxine complex solution. The containing bromide chemical structure of CTAB probably caused a *heavy atom quenching effect*, in which the fluorescence intensity of 7HC is decreased. The fluorescence intensities were slightly increased with addition of neutral surfactant Triton X-100 and anionic surfactant SDS.

Table 3 The thermodynamic parameters of 7HC–thyroxine binding procedure

T (K)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (J/molK)
297	−30.08	−23.76	−21.28
310	−30.08	−23.49	−21.28

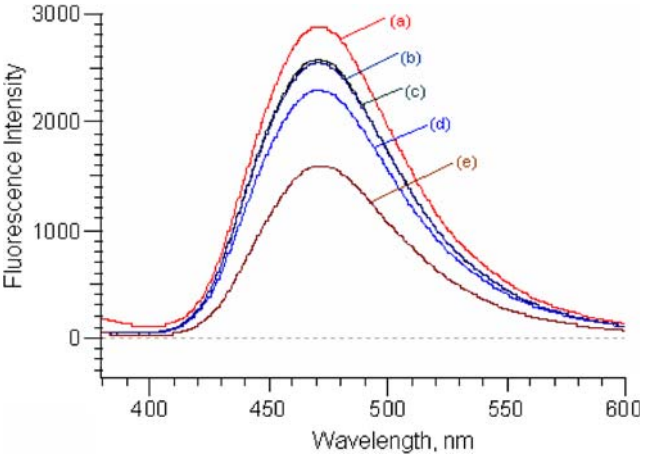


Fig. 5 7HC–thyroxine fluorescence spectra in presence of surfactants. **a** 7HC–thyroxine, 1.0×10^{-3} mol/l Triton X-100; **b** 7HC–thyroxine, 1.0×10^{-2} mol/l SDS; **c** 7HC; **d** 7HC–thyroxine; **e** 7HC–thyroxine, 1.0×10^{-3} mol/l CTAB

Figure 6 shows the calibration graph of thyroxine using of 1.0×10^{-3} mol/l CTAB medium. There was a linear relationship between F_0/F and thyroxine concentration in the range 2.0×10^{-8}–3.0×10^{-7} mol/l. The linear equation was $F_0/F = 0.9943 + 0.0449\times10^7$ [Q] and the correlation coefficient (R^2) was 0.994. The relative standard deviation (RSD) was 2.58% for 2.0×10^{-7} mol/l thyroxine (n=4). The limit of detection (LOD) for thyroxine was

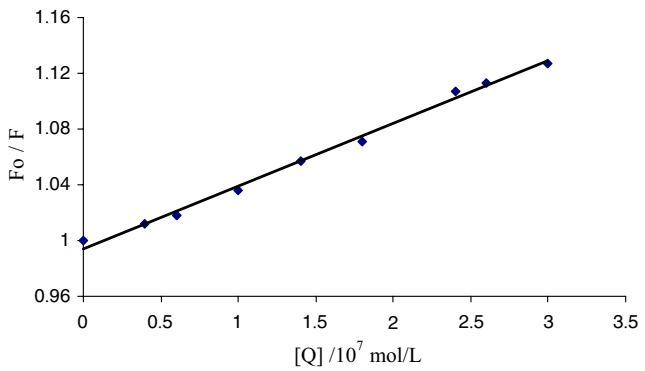


Fig. 6 Stern–Volmer plot of 7HC–thyroxine titration in presence of CTAB

defined as the concentration at which the signal was equal to the three times standard deviation (3σ) of blank solution. The blank signals (F_0) were taken to be the fluorescence intensities at 464 nm in the absence of thyroxine. According to a 3σ, LOD was calculated to be 3.42×10^{-8} mol/l thyroxine. K_{SV} was found to be 4.49×10^{5} l/mol from slope of curve [19]. As a result of these data, CTAB provides more sensitive, reproducible and stable micellar medium for the determination of thyroxine with 7HC.

Conclusion

In this paper, the interaction of thyroxine hormone with 7HC has been studied by fluorescence quenching method. The experimental results indicated that 7HC can interact with thyroxine through hydrogen bond and van der Waals force, and that the probable mechanism of 7HC fluorescence quenching by thyroxine is static quenching. The apparent binding constants (K) between 7HC and thyroxine were determined to be 1.51×10^{4} (297 K) and 9.06×10^{3} (310 K), and the binding site values (n) were 0.98 ± 0.1, respectively. From calibrations for thyroxine, greater sensitivity was achieved with use of the cationic surfactant CTAB and limit of detection (3σ) of thyroxine was 3.42×10^{-8} mol/l. Because of sensitive, practical and simple, the method can be suggested to determine thyroxine in serum and pharmaceutical samples.

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