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Fluorimetric Determination of Thyroxine Hormone with Eu(III)-(Pyridine-2,6-Dicarboxylate) Tris Complex

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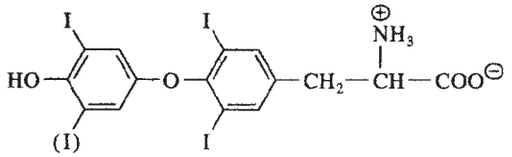
We describe a method for the determination of thyroxine (Thy) using its quenching effect on $Eu(PDA)_3^{3-}$ tris complex fluorescence. The relative fluorescence intensities are measured at fixed $\lambda_{exc} = 282$ nm, $\lambda_{em} = 615$ nm by titrating the metal complex with Thy in piperazine buffer solution at pH 6.5. Data indicated an associative type of reaction of two molecules valid between $0.0 < R < 1.0$, R being the mole ratio of $Eu(PDA)_3^{3-}$ to Thy. Over this ratio and up to ($R \gg 1.0$) collisional quenching of $Eu(PDA)_3^{3-}$ complex ion emission is seen as a result of heavy atom effect, intermolecular energy transfer playing the main role. This is also confirmed by the Stern-Volmer equation. In optimized experimental conditions, the L- form of Thy is determined in a range of 15.5–551.6 μg/ml ($2.0 \times 10^{-5} - 7.1 \times 10^{-4}$ *M*) with relative error of ±1.17%.

KEY WORDS: Thyroxine hormone; Eu(III) chelate; Stern-Volmer Equation; heavy atom effect.

INTRODUCTION

The lanthanides are uniquely fluorescent metals that display emission in aqueous solution [1,2]. Europium(III) and terbium(III) form highly fluorescent chelates with certain organic ligands. The chelates show large Stokes shifts, narrow emission bands, and long fluorescence lifetimes [3–5]. Europium aquo-ions form fluorescent chelates with ligands such as isothiocyanotophenyl-EDTA [6], 4,7-*bis*(chlorosulfophenyl)-1, 10-phenanthroline-2,9-dicarboxylic acid (BCPDA) [7], 2-naphthoyl-trifloroacetone (NTA) [8], and stable mono, bis and tris complexes with pyridine-2, 6-dicarboxylic acid (PDA) [9–11]. The stability constants of $Eu(PDA)_3^{3-}$ complex, expressed as log β_1, β_2, and β_3 values are 8.83, 15.98, and 21.03 respectively [12]. The acidic dissociation constants of the pyridine-2,6-dicarboxylic acid being $pKa_1 = 2.22$, $pKa_2 = 5.29$, all ligands are in anionic dicarboxylate form (dipicolinate anion, PDA^{2-}) before complexation in neutral solutions. The importance of this europium chelate is its enhanced fluorescence at 615 ± 5 nm, the excitation radiation absorbed by the ligand near UV being transfered to the central Eu^{3+} ion by an internal energy transfer process, leading to $^5D_0 \gg {}^7F_2$ emission. This characteristic metal ion emission has a narrow half-bandwidth of ~10 nm and sensitivity to the detailed nature of the ligand environment, termed as hypersensitivite bond [13].

Thyroid hormones have a major role in the regulation of metabolic processes. Stimulation of the thyroid gland by the pituitary hormone TSH (thyroid stimulating hormone) causes the release of thyroxine-T4



(Thy; 3,5,3′,5′-tetraiodothyronine) and triiodothyronine-T3. The iodine content provides hormonal activity of the gland. Thy has L- and D- forms. L- form is twice as physiologically active as the rasemic product, D- form has nearly no activity [14]. Many methods have been developed for Thy measurement, such as

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radioimmunologic assay (RAI) by use of radioactive isotope I^{131} [15], time resolved fluorescence [16], capillary electrophoresis with laser-induced fluorescence [17], and chemiluminescence [18]. In this paper, a method is put forward for the determination of Thy using its quenching effect on the $Eu(PDA)_3^{3-}$ complex fluorescence.

EXPERIMENTAL

Reagents

Pyridine-2,6-dicarboxylic acid (Aldrich Chem.) was used without further purification. A stock solution of the disodium salt was prepared by dissolving an accurately weighed amount of the acid in a solution containing 99% equivalence of sodium hydroxide. The solution was filtered through Millipore filter paper and stored in a polyethylene bottle.

Europium perchlorate stock solution, 0.2 *M*, was prepared from Eu_2O_3 (99.9%, BDH). A weighed amount of the oxide was suspended in ~20 ml hot water. The mixture was added slowly with stirring to a dilute solution (~30 ml) containing slightly less than the eqivalent amount of perchloric acid (BDH) to dissolve ca. 98% of the oxide and kept near boiling point. The mixture was left boiling until the reaction was complete (pH > 6). The solution was then filtered to remove unchanged oxide and transfered to a volumetric flask. Europium perchlorate stock solutions were standardized before use, by titrating against 0.01 M EDTA solution using xylenol orange as the indicator at pH 5.8 [19].

$Eu(PDA)_3^{3-}$ complex solutions were prepared by mixing the appropriate volumes of metal perchlorate and pyridine-2,6-dicarboxylate solutions, adjusting pH to 5.8 with dilute NaOH and total ionic strength to 0.5 *M* with 2.0 *M* $NaClO_4$. Final formal concentrations of the metal ion varied from 2.5×10^{-5} to 3.5×10^{-4} *M* in the series studied.

Thyroxine L- and D- forms (Sigma) were used without further purification to prepare stock solution of the hormone.

Measurements

The fluorescence measurements were made on a Jasco Model FP-550 Spectrofluorometer using 10-mm quartz cell or flow cell. The instrument has a xenon lamp, a dual monochromator. Monochromatic readings were taken from the digital display with 0.25 s time constant and with 3-nm bandwidth on excitation side, 5 nm on the emission side. The absorption spectra were recorded by Jasco V-530 UV/Vis Spectrophotometer.

RESULTS AND DISCUSSION

Calibration of $Eu(PDA)_3^{3-}$ Complex Ion Emission

Eu^{3+} aquo ions form stable mono-$[Eu(PDA)(H_2O)_6]^+$, *bis*-$[Eu(PDA)_2(H_2O)_3]^-$, and tris-$Eu(PDA)_3^{3-}$ complexes. Stepwise formation of such complexes led to significant enhancement in $^5D_0 \gg {}^7F_1$ (~590 nm) and $^5D_0 \gg {}^7F_2$ (~615 nm) band intensities [12]. The typical behavior was explained by Eu^{3+} energy acceptor levels being suitable for energy transfer throughout the near UV and visible regions (absorption bands like $^5F_1 \gg {}^5I_6$, $^5H_6$ at 285.4 nm and $^7F_1 \gg {}^7I_7$ at 285 nm) and the overlap of these excitation bands with the broad absorption band of PDA ligands, which acted as energy donors transfering the absorbed energy via a ligands-to-metal ion. The relative fluorescence intensities (F_0) at fixed $\lambda_{exc} = 282$ nm, $\lambda_{em} = 615$ nm showed a linear dependence to its molar concentration ($F_0 = 3.0 \times 10^6 C + 42.95$, $r^2 = 0.9867$). The detection limit (S/N = 2) was $1.7 \times 10^{-7} M$.

Interaction of Thyroxine with $Eu(PDA)_3^{3-}$ Complex

The interaction of the two molecules were investigated using spectral techniques and fluorimetric titrations. A significant interaction was observed in UV visible absorption and emission spectra. Figure 1 shows the hypochromic effects of $Eu(PDA)_3^{3-}$ presence on the 256 nm and 302 nm absorption band of hormone. Data point out the presence of an interaction, pronouncing new formations between the two structures.

Further evidence was also found in emission spectrum of two structures excited at 566 nm or 282 nm. Figure 2 shows typical 615 nm fluorescence of $Eu(PDA)_3^{3-}$ complex,

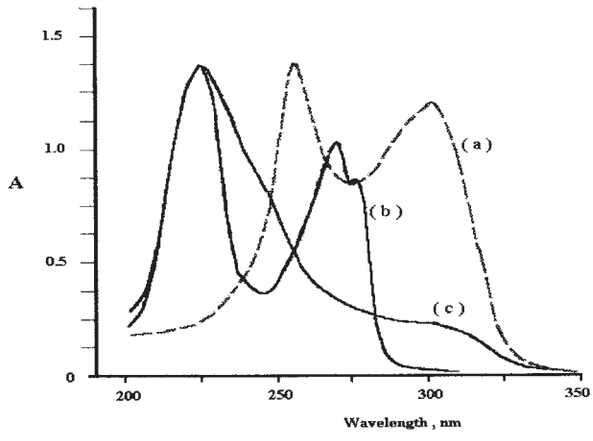
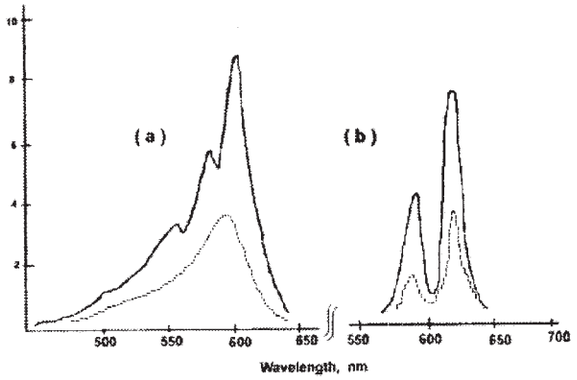


Fig. 1. (a) 1.00×10^{-3} M Thy solution in alcohol. (b) 3.23×10^{-4} M $Eu(PDA)_3^{3-}$ solution, buffered at pH = 6.5., (c) Thy and $Eu(PDA)_3^{3-}$ solution (1:1 mole ratio) buffered at pH = 6.5.



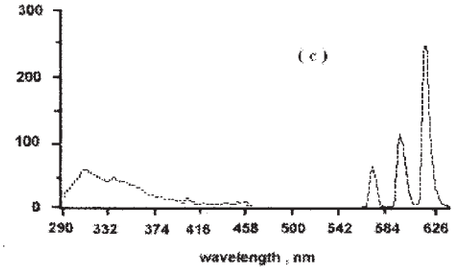


Fig. 2. (a) The excitation spectra at constant λ_{em} = 615 nm; (b) The emission spectra at constant λ_{exc} = 566.8 nm; (c) The emission spectra at constant λ_{exc} = 282 nm of 5.0×10^{-5} *M* $Eu(PDA)_3^{3-}$ tris complex (—), Thy-$Eu(PDA)_3^{3-}$ solution (1:1 mole ratio) (- - -) buffered at pH = 6.5.

576 nm denoting presence of Eu^{3+} ion, 310–350 nm broad emission of the hormone. Figure 3 shows the significant inhibition of complex ion fluorescence in the presence of thyroxine hormone which were carried out by titrating the metal complex with thyroxine at fixed $\lambda_{exc}/\lambda_{em}$ = 282/615 nm and pH 6.5 piperazine buffer solution.

The quenching of $Eu(PDA)_3^{3-}$ fluorescence in the presence of thyroxine hormone can be explained by the following two different approaches:

- There can be an intramolecular energy transfer after (i) an associative reaction by bonding onto the second coordination sphere of $Eu(PDA)_3^{3-}$ or (ii) by displacing one or more PDA^{2-} ligands.
- There can be an intermolecular energy transfer from donor to acceptor molecule, mainly by collision, inhibiting the population of $^5D_0$ energy state of Eu^{3+}, quenching the emission by heavy atom effect.

Although no direct evidence was found in IR spectra, it was aimed to use modified Benesi-Hildebrand

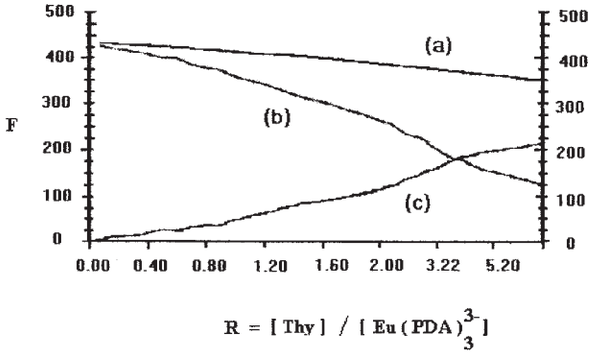


Fig. 3. Fluorimetric titration of $Eu(PDA)_3^{3-}$ with Thy at $\lambda_{exc}/\lambda_{em}$ = 282/615 nm. (a) Dilution of analyte solution, (F_0); (b) titrant (Thy) addition, (F_A); (c) difference, ($\Delta F = F_0 - F_A$).

equations to calculate an apparent binding constant (K_{app}) for above-mentioned reactions (i and ii) [12].

$$(\Delta F)^{-1} = \{\alpha K_{app}[Eu(PDA)_3^{3-}]_0[Thy]\}^{-1} + \{\alpha[Eu(PDA)_3^{3-}]_0\}^{-1} \quad (1)$$

$$K_{app} = \frac{[X]}{[Eu(PDA)_3^{3-}]_{free}\,[Thy]} \quad (2)$$

where ΔF is the decrease in fluorescence intensity of the Eu^{3+}-complex ion in the presence of thyroxine hormone, F_0 is the $[Eu(PDA)_3^{3-}]_0$ initial formal concentration of Eu^{3+}-complex ion, [Thy] is the molar hormone concentration, and α is the proportionality constant that contains all experimental and instrumental parameters at $\lambda_{exc}/\lambda_{em}$ = 282/615 nm.

The linear relationship in Eq. (1) between $F_0/\Delta F$ versus 1.0/[Thy], as given in Fig. 4, the intersect/slope gave directly the apparent binding constant (K_{app}). Calculated K_{app} values (Table I) for different R-values definitely pointed out associative reaction between Thy and $Eu(PDA)_3^{3-}$, the binding rate and stoichiometry of which change $0.0 < R < 0.5$ and $0.5 < R < 1.0$. The structural changes seem to be true up to 1:1 mole ratio, the intramolecular interaction causing quenching of the 615-nm fluorescence.

On the other hand, there may be intermolecular energy transfer between two molecules termed as *collisional* or *resonance*, both causing quenching of donor luminescence yet sensitizing (sometimes quenching) acceptor emission. Thus the experimental data must obey the Stern-Volmer equation given below.

$$\frac{F_0}{F_A} = \frac{k_1 + k_2 + k_q[A]}{k_1 + k_2} = 1.0 + k_q\tau[A] \quad (3)$$

Where *F* is the measured fluorescence intensity of $Eu(PDA)_3^{3-}$ with or without A; *A* is the molar concentration of acceptor molecule = [Thy] for this case, *k* is the

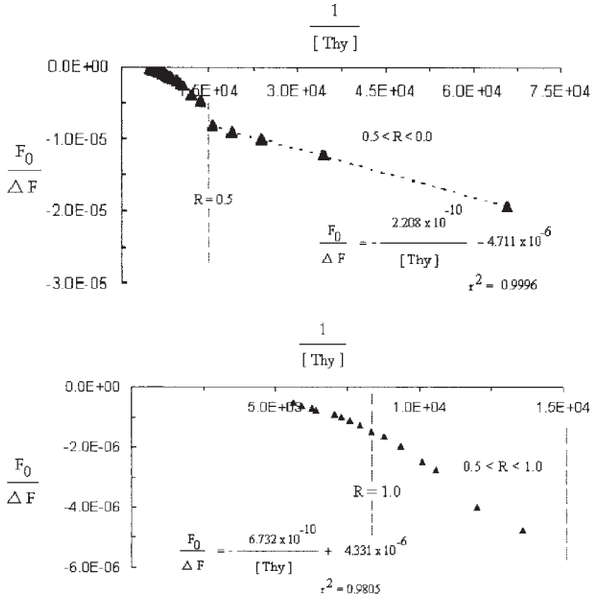


Fig. 4. The linear regression of $F_0/\Delta F$ and 1.0/;[Thy] for different R values.

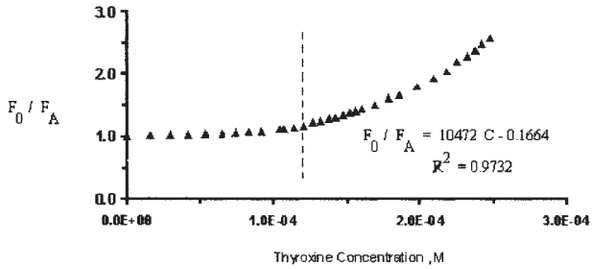


Fig. 5. The linear regression of $F_0/\Delta F$ and [Thy] for different R values.

specific rate constants, s^{-1}. k_1 is the fluorescence of $Eu(PDA)_3^{3-}$ at $\lambda_{exc}/\lambda_{em}$ = 282/615 nm; k_2 is the thermal deactivation of excited $Eu(PDA)_3^{3-}$ molecule; k_q is the quenching of $Eu(PDA)_3^{3-}$ fluorescence when A is present in aqueous media, usually written as $\cong k_3$, for energy transfer; and τ is the measured lifetime of excited $Eu(PDA)_3^{3-}$ molecule $(1/k_1 + k_2)$ when no A is present, s.

Under conditions of steady illumination and no irreversible photochemical reactions, the Stern-Volmer equation gave a linear relationship between F_0/F_A and [Thy], intersects at value 1.0, and a slope of $k_q\tau$.

Figure 5 clearly points out that there was a significant change in the Stern-Volmer plot around R = 1.0. Over this ratio and up to R~6.9 the slope was sharper (found to be ~10472), denoting an increased quenching effect of the hormone molecule. If we assume a new structural formation at equimolar concentrations (1.00×10^{-4} *M* of $Eu(PDA)_3^{3-}$ and Thy), replotting of the data after R = 1.00 gave a Stern-Volmer line with an intersect of 1.00 and a slope of ~7932.

The results from the experimental data are summarized below. Intramolecular energy transfer data indicated an associative reaction between two molecules forming a new structure in two consequent steps:

$$0 < R < 0.5 \qquad K_{app} = 2.13 \times 10^4 \quad (r^2 = 0.9996)$$

$$0.5 < R < 1.0 \quad K_{app} = 6.43 \times 10^3 \quad (r^2 = 0.9805)$$

followed by collisional quenching of the complex emission with excess Thy (R >> 1.0). *Heavy atom effect* played the main role, inhibiting the observed emissive intensity at 615 nm.

$$1.0 < R < 6.9 \qquad \text{Stern-Volmer} \qquad (r^2 = 0.9732)$$

Determination of Thyroxine

Stock solutions of 5.15×10^{-4} *M* thyroxine (D- and L- forms) were prepared from pure crystalline solids by dissolving 0.010 g in 25.0 ml ethanol with a single drop of concentrated HCl. A series of volumes; 0.10 – 1.00 ml were taken and diluted up to 1.00 ml with piperazine buffer.

Table I. Calculated Kapp Values

R–mole ratio	K_{app} (M^{-1})	pK_{app}	Equation of Linear regression	r^2	α
$0.0 < R < 0.5$	$2.13 (\pm 0.21) \times 10^4$	4.33 ± 0.04	Fig. 4 (a)	0.9996	2.12×10^5
$0.5 < R < 1.0$	$6.43 (\pm 0.15) \times 10^3$	3.81 ± 0.01	Fig. 4 (b)	0.9805	2.31×10^5
$1.0 < R < 6.0$	Cannot be calculated	—	—	—	

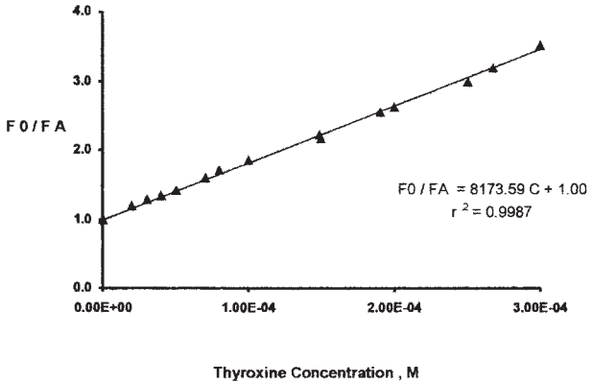


Fig. 6. The linear regression between F_0/F_A versus Thy concentration.

Two milliliter 2.85×10^{-5} *M* $Eu(PDA)_3^{3-}$ stock solutions were added over 1.00 ml of thyroxine solutions prepared above (coded as sample) or over 1.00 ml of buffer solutions (coded as control). To use the Stern-Volmer type of linear regression, the hormone concentrations must be above 1.9×10^{-5} *M* selected as final R = 1.00 value. The final 3.00-ml solution in the quartz cell was read for its relative emission intensity. The relative standard deviations (%RSD) in the optimized experimental conditions were less than ±1%.

There were no differences in the emission intensities found for D- or L- forms of hormone; thus the Stern-Volmer type of linear calibration graph (Fig. 6) was given for only L-thyroxine. Thus, for the above calibration the final $Eu(PDA)_3^{3-}$ concentration was so choosen (1.9×10^{-5} *M*) that F_0 is 100 ± 1, and calculation of hormone concentration was straight forward and linear within 15.5 μg/ml (2.0×10^{-5} *M*) up to 551.6 μg/ml (7.10×10^{-4} *M*). The accuracy of the proposed method was tested for five thyroxine samples (Table II).

Table II. Accuracy of L-Thyroxine Determination Using $Eu(PDA)_3^{3-}$ Fluorescence

F_{Thy}	Calculated concentration μg / ml	True value concentration μg / ml	Absolute error xi-xt μg / ml	Relative error (xi-xt) /xt %
75	31.75	32.00	−0.25	−0.78
58	68.97	68.00	0.97	1.43
42	131.52	130.00	1.52	1.17
38	155.39	156.00	−0.61	−0.39
28	244.90	244.00	0.90	0.37

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