Selective Fluorimetric Determination of Trace Amounts of Cobalt(II) with the System of Fluorescein-Hydrogen Peroxide-Tiron†

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(Received February 27, 1989)

The decomposition reactions of fluorescein (Fl) or its halo derivatives in the basic region were systematically investigated in the presence of an oxidizing agent such as hydrogen peroxide and/or a metal ion such as cobalt(II) and/or catalytic agent such as tiron as an activator. Moreover, a highly selective spectrophotofluorimetric method for the determination of trace amounts of cobalt(II) was established by measuring the difference in relative fluorescence intensities (ΔF) between Fl-tiron-hydrogen peroxide and Fl-tiron-hydrogen peroxide-cobalt(II) solutions at an emission wavelength of 510 nm, with an excitation wavelength of 490 nm (the calibration graph was linear in the range of 0—6 ng cobalt(II) per 25 cm^3). The applications to the assay of cobalt in cyanocobalamine (vitamin B_{12}) preparations and its eye drops were also investigated, the results were relatively good.

For the spectrophotometric determination of trace amounts of cobalt(II), various kinetic and catalytic reactions between diphenol compounds, such as Pyrogallol Red (PR), phenylfluorone (Phfl), *o*-hydroxyhydroquinonephthalein (Qnph), and cobalt(II), in the presence of an oxidizing agent such as hydrogen peroxide have been investigated.[1-5] In this paper, we investigated the use of fluorescein (Fl) as a fluorescence reagent for the spectrofluorometric determination of trace metal ions; firstly, the fluorescence reactions among Fl or its halo derivatives and an oxidizing agent, such as hydrogen peroxide and/or metal ions such as cobalt(II) were systematically investigated; secondly, a fluorimetric method for the determination of trace amounts of cobalt(II) using the fluorescence reaction (fluorescence-quenching) among trace amounts of cobalt(II), Fl, tiron and hydrogen peroxide in the basic region was proposed and applied to the assay of cyanocobalamine (vitamin B_{12}) preparations.

Experimental

Apparatus and Reagents. Fluorescence-intensity measurements were made on a Shimadzu Model RF-540 spectrofluorophometer using 10-mm silica cells and a xenon arc source. A Hitachi-Horiba model F-8 pH meter, equipped with glass and a calomel combined electrode, was used for all the pH measurements.

Deionized water was used throughout. All reagents were of an analytical reagent grade. A stock solution of cobalt(II) (1.0×10^{-2} mol dm^3) was prepared by dissolving cobalt chloride in water, correcting by chelatometry,[6] and a 5.0×10^{-4} mol dm^{-3} cobalt(II) working solution was prepared by the suitable dilution of the stock solution just before use. Fl, its halo-derivative solution (1.0×10^{-3} mol dm^{-3}), and a 0.3% tiron solution were prepared by dissolving, respectively, Fl, its halo-derivatives[7,8] (highly purified Fl derivatives by recrystallization), and tiron in methanol. A 1.0% hydrogen peroxide solution was prepared by the dilution of a 30% hydrogen peroxide solution (Mitsubishi-Edogawa Chemical Co., Ltd.).

Standard Procedure. To each series of 30-cm^3 Erlenmyer flask, add an aliquot of a solution containing between 0 and 6.0 ng of cobalt(II). Add 2.0 cm^3 of a 1 M sodium hydroxide solution (1M=1 mol dm^{-3}), 1.0 cm^3 of a 1.0% hydrogen peroxide solution, 1.0 cm^3 of a 3.0% tiron solution, and 1.0 cm^3 of a 1.0×10^{-3} mol dm^{-3} Fl solution, and dilute to 10.0 cm^3 volume with water. A reflux condenser is attached, and the mixed solution (Solution A, Fl/cobalt(II)/tiron/hydrogen peroxide solution) is heated for 45 min together with Solution B (Fl/tiron/hydrogen peroxide solution), after with Solutions A and B are diluted to a 25-cm^3 volume with water. Measure the difference in fluorescence intensities (ΔF) between Solutions A and B at the emission wavelength of 510 nm with the excitation wavelength of 490 nm.

Results and Discussion

Fluorescence-Quenching Reaction between Fluorescein or Its Derivatives and Hydrogen Peroxide, and the Effects of Metal Ions and Oxidizing Agents. Firstly, the decomposition reactions of Fl and its halo derivatives, such as 2,7-dichlorofluorescein (2,7-Cl_2Fl), 3′,4′,5′,6′-tetrachlorofluorescein (3′,4′,5′,6′-Cl_4Fl), 2,4,5,7-tetrachloro-, 2,4,5,7-tetrabromo-, or 2,4,5,7-tetraiodofluorescein (2,4,5,7-Cl_4Fl, 2,4,5,7-Br_4Fl, 2,4,5,7-I_4Fl), in the presence of hydrogen peroxide as an oxidizing agent were systematically investigated by heating at 60 °C under various pH areas. As is shown in Table 1, the decomposition reactions (fluorescence-quenching) were obvious in basic media; all these halo-Fl derivatives except Fl and 3,4,5,6-Cl_4 Fl were easily decomposed[7,8] and then converted to non-fluorescence substances, but these fluorescence intensities were relatively small, unstable and varied widely in value (93%—108%). On the other hand, the fluorescence intensity of 3,4,5,6-Cl_4Fl was very stable, and it was not entirely decomposed by hydrogen peroxide. Fl was slightly decomposed by an oxidation reaction in the presence of hydrogen

† Application of Xanthene Derivatives to Analytical Chemistry. Part LXXVI. Presented at the 37th Annual Meeting of Analytical Chemistry of Japan, Sapporo, October 1988. Part LXXV: I. Mori, Y. Fujita, K. Ikuta, Y. Nakahashi, and M. Ohji, *Fresenius Z. Anal. Chem.*, **334**, 49 (1989).

Table 1. Decomposition Reactions of Fl and Halo-Fl Derivatives at 0.4 M Sodium Hydroxide in the Presence or Absence of 0.025% Hydrogen Peroxide

Fl deriv.	Em max nm	Absence of H_2O_2 R.fl. Int./% 0 min	Absence of H_2O_2 R.fl. Int./% 100 min	Presence of H_2O_2 R.fl. Int./% 0 min	Presence of H_2O_2 R.fl. Int./% 100 min	ΔF %
Fl	510	100.0	99.5	100.0	96.4	3.6
3,4,5,6-Cl_4Fl	530	57.7	57.7	57.7	57.6	0.2
2′,7′-Cl_2Fl	525	45.3	43.8	45.3	24.6	45.7
2′,4′,5′,7′-Cl_4Fl	535	30.1	15.1	30.1	2.5	91.7
2′,4′,5′,7′-Br_4Fl	535	28.3	16.8	28.3	4.8	83.0
2′,4′,5′,7′-I_4Fl	550	22.1	20.9	22.1	12.0	45.7

Fl and halo-Fl: 1.0×10^{-5} mol dm^{-3}; heating at 60 °C; Ex: 365 nm.

Table 2. Inhibition of Fluorescence-Quenching Reactions of Fl-Hydrogen Peroxide Solutions in the Presence of Various Metal Ions in the Presence or Absence of Tiron

Metal ions	Tiron absence R.fl. Int./% mol dm^{-3}	Tiron absence R.fl. Int./% at 510 nm	Tiron absence Ratio of R.fl. Int. at 510 nm	Tiron presence R.fl. Int./% mol dm^{-3}	Tiron presence R.fl. Int./% at 510 nm	Tiron presence Ratio of R.fl. Int. at 510 nm
—	—	31.9	—	—	19.6	—
Co(II)	2.0×10^{-8}	64.5	1.021[a] (20.042[b])	0.2×10^{-8}	99.0	4.051[a] (810.2[b])
Fe(III)	40.0×10^{-8}	119.9	2.760[a] (2.760[b])	8.0×10^{-8}	31.8	0.622 (3.11[b])
Cu(II)	40.0×10^{-8}	117.5	2.682[a] (2.682[b])	40.0×10^{-8}	103.8	4.490 (4.49[b])
Mn(II)	40.0×10^{-8}	73.6	1.307[a] (1.307[b])	20.0×10^{-8}	79.9	3.077 (6.15[b])
Ni(II)	400.0×10^{-8}	145.2	3.533[a] (0.355[b])	20.0×10^{-8}	162.4	7.286 (15.57[b])

Tiron absence—Fl: 4.0×10^{-5} mol dm^{-3}; H_2O_2: 0.025%; NaOH: 0.08 M;
Tiron presence—Fl: 4.0×10^{-5} mol dm^{-3}; H_2O_2: 0.04%; NaOH: 0.08 M; Tiron: 0.14%; Ex: 490 nm; at heating for 45 min on a reflux.
a) Rate of R. fl. Int. of {(Fl-H_2O_2-metal ion)−(Fl-H_2O_2)}/(Fl-H_2O_2). b) Rate of inhibition on fluorescence quenching under correction of coexisting metal-ion concentration—40.0×10^{-8} mol dm^{-3}.

peroxide, and its fluorescence reaction could easily be reproduced (97.5%—102.4%). Thus, because Fl has an appropriate stability and decomposed property for an oxidation-destruction reaction using hydrogen peroxide, and because the subsequent purification of Fl[7,8] was very easy, Fl was used as a fluorescence reagent for further investigations.

Secondly, the enhancement effect of such metal-ions as cobalt(II), iron(III), manganese(II), and copper(II) by the fluorescence-quenching reaction on the Fl used was systematically investigated. As is shown in Table 2, the difference in fluorescence intensity (ΔF) between Solution A (Fl/hydrogen peroxide/metal ions solution) and Solution B (Fl/hydrogen peroxide solution) was largest and showes most clearly in the presence of trace amounts of cobalt(II). That is, the amounts of ΔF in the presence of cobalt(II) was about 60-times that of ΔF in the presence of nickel(II) (ratio of inhibition of fluorescence-quenching; rates of relative fluorescence intensity on {(Fl-hydrogen peroxide-metal ion)−(Fl-hydrogen peroxide)}/(Fl-hydrogen peroxide), using a correction of the coexisting metal-ion concentration (ratio of fl.Int.), 20.042 for cobalt(II) vs. 0.355 for nickel(II)), the fluorescence-decomposition reaction of Fl was inhibited in proportion to the coexisting cobalt(II) concentrations.

Thirdly, the effect of the oxidizing agent was investigated in the oxidation and decomposition reactions of Fl; sodium hypochlorite, hydrogen peroxide, sodium nitrite, potassium iodate, chloramine B, potassium peroxodisulfate, etc. were used as oxidizing agents. The use of 1.0—2.0 cm^3 of a 1.0% hydrogen peroxide solution as an oxidating agent gave a large and reproducible ΔF. Although the quenching reaction was influenced by the amounts of hydrogen peroxide, further investigation was carried by using Fl and, finally, 0.04% hydrogen peroxide.

Effect of Activator. In this decomposition reaction of Fl, the fluorescence intensity of Fl-hydrogen peroxide-cobalt(II) system remained in the basic medium was significantly enhanced by the coexistence of a biphenol organic reagent, such as tiron, pyrocatechol, or gallic acid, as is shown in Table 3. In measuring the difference in fluorescence intensities the best results were produced using tiron as an activator; the optimum tiron concentration was 0.1—0.3% in the final solution. The inhibition phenomena of fluorescence-quenching (=difference in fluorescence intensities between Solution A (Fl/hydrogen peroxide/tiron/cobalt(II) solution) and Solution B (Fl/hydrogen peroxide/tiron solution)) were larger and more obvious than in the absence of a tiron-quenching ratio (difference in relative fluorescence intensities between Solutions A and B against the

Table 3. Effects of Activators on Fluorescence-Quenching Reaction

Activators	Procedure A R.fl. Int./% 510 nm		Procedure A Ratio of R.fl. Int.[a] at 510 nm	Procedure B Ratio of R.fl. Int.[a] at 510 nm
	Fl-H_2O_2	Fl-H_2O_2-Co(II)		
—	16.8	34.8	1.071	0.102
Tiron	17.1	61.8	2.614	4.051
Pyrogallol	16.0	49.5	2.094	0.25
Pyrocatechol	25.4	50.9	1.004	0.10
2,3-dihydroxy benzoic acid	23.7	49.6	1.093	0.10
Luminol	17.4	35.3	1.029	0.10
Resorcinol	17.9	34.7	0.939	0.10
Phenol	16.3	38.2	1.344	0.10
Glycerol	19.4	32.9	0.696	0.10
Gallic acid	30.0	65.4	1.180	0.10
Phenylalanine	22.1	39.8	0.801	0.10
Norepinephrine	23.0	64.5	1.804	0.10

Procedure A—Co(II) taken: 8.0×10^{-9} mol dm^{-3}; activator: 4.0×10^{-4} mol dm^{-3}; hydrogen peroxide: 0.03%; NaOH: 0.08 M; Fl: 4.0×10^{-5} mol dm^{-3}. Procedure B—Co(II) taken: 2.0×10^{-9} mol dm^{-3}; activator: 0.14%; hydrogen peroxide: 0.04%; NaOH: 0.08 M; Fl: 4.0×10^{-5} mol dm^{-3}; Ex: 490 nm; at heating on reflux for 45 min.

a) Rate of R.fl. Int. on {(Fl-H_2O_2-Co(II)-activator)—(Fl-H_2O_2-activator)}/(Fl-H_2O_2-activator).

relative fluorescence intensity of Solution B); they were 4.051 (in the presence of tiron) and 1.021 (without tiron), while the rates of fl.Int. were 810.2 (in the presence of tiron) and 20.042 (without tiron) for 40.0×10^{-8} mol dm^{-3} cobalt(II)). Thus, the fluorescence-quenching among Fl, hydrogen peroxide, and cobalt(II) in the presence of tiron gave the largest value. That is, the apparent fluorescence-quenching reaction in the presence of cobalt(II) and tiron was about 40-times that in the absence of tiron, and about 300-times that with copper(II) alone; rates of fl.Int.: 810.2 for cobalt(II), 4.490 for copper(II). The maximum and almost costant value was obtained by using a final tiron concentration of 0.2%—0.1%; for the analytical procedure, a final tiron concentration of 0.14% was used.

Fluorometry of Cobalt(II). Fluorescence Spectra. Figure 1 shows the excitation spectrum of the Fl solution and the emission spectra of Fl-hydrogen peroxide and Fl-hydrogen peroxide-cobalt(II) solutions after heating reactions at reflux for 45 min on basic media. The excitation spectrum of the Fl-hydrogen peroxide solution showed an excitation band with a maximum at 490 nm when an emission wavelength at 510 nm was used and an emission band at 510 nm when an excitation wavelength at 490 nm was used. Also, ΔF at 510 nm was proportional to the concentration of trace cobalt(II).

Effect of Concentration of Alkali. The quenching phenomena were recognized over a wide basical range; a maximum and almost constant ΔF value was obtained in a basic medium of a 0.02—0.5 M NaOH final solution under the optima excitation and emission wavelengths (Ex 490 nm, Em 510 nm). Although the elution of metal-ions from the glassware was presumed, a final concentration of 0.08 M NaOH was selected for the recommended procedure for the assay of cobalt(II).

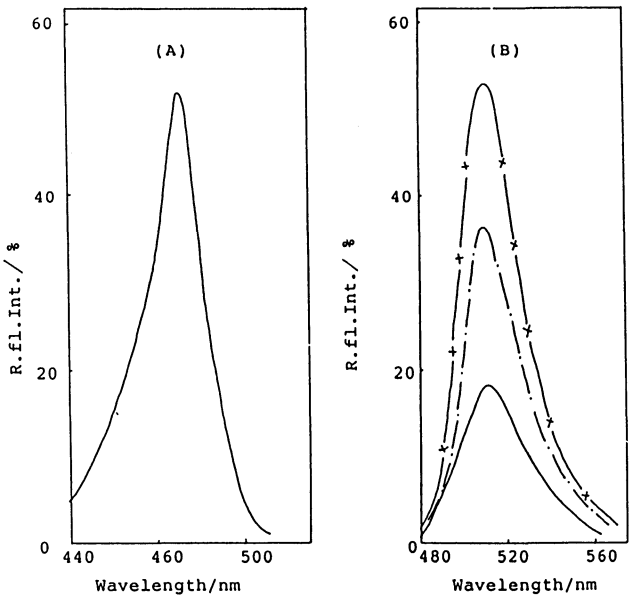

Fig. 1. Excitation spectrum of Fl solution, and emission spectra of Fl-hydrogen peroxide solution in the presence or absence of cobalt(II) at basical media in the presence of tiron.
(A) Excitation spectrum; NaOH: 0.08 M; Fl: 4.0×10^{-5} M; Em 510 nm. (B) Emission spectra; NaOH: 0.08 M; Fl: 4.0×10^{-5} M; hydrogen peroxide: 0.04%; at reflux heating for 45 min; Ex 490 nm;
———: {Fl-H_2O_2-tiron}soln;
— - —: {Fl-H_2O_2-Co(II)-tiron} soln (Co(II) 5.0×10^{-10} M);
—X—: {Fl-H_2O_2-Co(II)-tiron} soln (Co(II) 1.0×10^{-9}M.

Effect of Fl Concentration. As the fluorescence-quenching is influenced by the purity of Fl, previously highly purified Fl was used. The effect of the Fl concentration was then considered. The best result was produced by using 1.0—1.5 cm^3 of a 1.0×10^{-3} mol dm^{-3} Fl (highly purified by recrystallization from Fl acetyl compound) methanol solution per 25 cm^3; a final solution of 4.0×10^{-5} mol dm^{-3} Fl was used for further investigations.

Effects of Temperature and Time. The effect of the temperature was studied at 50 °C, 60 °C, 80 °C, and 100 °C. An inhibition of the fluorescence-quenching was observed when the temperature was increased; the maximum quenching was obtained at reflux on a hotplate. The maximum quenching phenomenon was obtained by heating at reflux for 30 min or less, the fluorescence intensity was thereafter kept constant for longer than 60 min. Therefore, a standing time of 45 min using reflux heating was selected for the standard procedure. Although the coexistence effect of surfactants (nonionic, anionic, and cationic) was investigated, no enhancement effect at all was observed.

Calibration Graph. The calibration graph of cobalt(II) was linear over the range from 0 to 6 ng cobalt(II)/25 cm^3. For 3 ng of cobalt(II) in 25 cm^3, the coefficient of variation, as estimated from 5 replicates, was 2.5%.

Effect of Diverse Ions. The influences of diverse ions on the determination of 2.9 ng of cobalt(II) upon the use of the fluorescence reaction of a Fl-hydrogen peroxide-tiron-cobalt(II) system were also examined. The tolerance limit was taken as the amount that caused about a ±3% error in the fluorescence intensity. The coexistence of numerous metal-ions besides iron(III), manganese(II), copper(II), and nickel(II) did not interfere at all. That is, the coexistence of iron(III) gave a positive error when present in a 400-fold excess over cobalt(II), and the coexistence of copper(II), manganese(II), or nickel(II) also gave a positive error when present in a 50-fold excess over cobalt(II). On the other hand, the coexistence of almost any anion did not interfere, though the cyanide ion gave a negative error when present in a 40-fold excess over cobalt(II). The results are given in Table 4.

Mechanism of Fluorescence-Quenching Reaction. Although further investigation is necessary, the fluorescence-quenching reaction among Fl, hydrogen peroxide, cobalt(II), and tiron was estimated. Firstly, the oxidation and decompostion reaction of Fl by hydrogen peroxide was catalytically enhanced by the presence of small amounts of cobalt(II). Secondly, by the coexistence of tiron as an activator, the total amounts of hydrogen peroxide as an oxidation agent decreased following a reaction between tiron and hydrogen peroxide, or one of tiron, hydrogen peroxide, and cobalt(II). Then the R.fl.Int. of the Fl-hydrogen peroxide-tiron-cobalt(II) solution was found to be proportional to the amounts of cobalt(II); the total hydrogen peroxide concentration was less than the reference solution (Fl/hydrogen peroxide/tiron solution). Thus, the fluorescence-quenching reaction was recognized.

Table 4. Effects of Foreign Ions

Foreign ion	Added			Rate of R.fl. Int.[a)]	Recovery /%
	as	μg/25 cm^3	Mole ratio		
—	—	—	—	4.051	—
Sn(IV)	Sulphate	1.19	200	4.059	100.2
V(V)	Vanadate	2.55	1000	4.060	100.2
Mo(VI)	Molybdate	4.80	1000	4.052	100.0
Ti(IV)	Sulfate	2.38	1000	4.058	100.2
Al(III)	Nitrate	1.35	1000	4.080	100.7
Th(IV)	Nitrate	11.60	1000	4.063	100.3
Bi(III)	Nitrate	10.45	1000	4.072	100.5
Fe(III)	Sulfate	2.33	800	5.192	128.2
		1.17	400	4.059	100.2
Mn(II)	Chloride	0.27	100	5.224	129.0
		0.14	50	4.082	100.7
Cu(II)	Nitrate	0.32	100	4.581	113.1
Ni(II)	Nitrate	0.29	100	5.807	143.3
NO_3^-	Sodium	3.10	1000	4.050	100.0
CN^-	Potassium	0.13	100	3.621	89.4
S^{2-}	Sodium	1.60	1000	4.031	99.5
F^-	Sodium	0.94	1000	4.043	99.8
Citrate	Sodium	7.40	1000	4.042	99.8
Tartrate	Sodium	9.35	1000	4.044	99.8

Co(II) taken: 2.0×10^{-9} mol dm^{-3} (2.9 ng/25 cm^3); hydrogen peroxide: 0.04%; NaOH: 0.08 M; tiron: 0.14%; Fl: 4.0×10^{-5} mol dm^{-3}; Ex 490 nm; Em: 510 nm;
a) Rate of R.fl. Int. on {(Fl-H_2O_2-tiron-Co(II))−(Fl-H_2O_2-tiron)}/(Fl-H_2O_2-tiron).

Table 5. Results of Determinations of Cobalt in Pharmaceutical Preparations

Sample	Calculated /%	Cobalt found/%			Recovery[a] of proposed
		Proposed	ICP-AES	JP-XI[b]	
Cyanocobalamine No. 1	4.34	4.29	4.37	—	96.8
No. 2	4.34	4.34		4.27	—
Drop eye	0.000043	0.000042	0.000042	0.000054	95.2
Sample[c]	0.0043	0.0043	0.0042		97.5

a) Average recoveries from five determinations; cobalt(II) taken—2.9 ng/25 cm^3. b) Japanese Pharmacopoeia XI. c) Prepared sample.

Application. To verify the usefulness of the recommended procedure, the proposed method was applied to the determination of cobalt in cyanocobalamine (vitamin B_{12}) and also in some eye-drop and synthetic pharmaceutical preparations containing vitamin B_{12}. As is shown in Table 5, which gives the recovery data for 3—4 replicates of each sample, the results were relatively good. By using a reflux heating procedure on a hotplate, the proposed method does not require a preliminary decomposition procedure, and it is very simple, rapid, and selective.

In conclusion, the proposed method for the spectrophotometric determination of cobalt(II) based on the fluorescence-quenching of Fl by coexisting hydrogen peroxide, tiron, and cobalt(II) is simpler, more sensitive, and more reproducible than previous reported methods.[2,4,5,9-11] It works by means of a reaction between such organic reagents as Pyrogallol Red, Pyrocatechol Violet, and tiron and an oxidizing agent. The proposed method is very selective and excellent in its effect on foreign ions. Moreover, for the assay of cobalt containing organo compounds such as cyanocobalamine, the proposed method does not require any particular preparatory procedures; organo compounds are decomposed by standard procedures and their fluorescence is quenched well.

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