Effect of Riboflavin on the Photolysis of Cyanocobolamin in Aqueous Solution

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Abstract: The kinetics of photolysis of cyanocobalamin (CC) by visible radiation in the presence of riboflavin (RF) at pH 2.0-12.0 has been studied. A specific two-component spectrophotometric method has been used for the simultaneous assay of CC and its photoproduct, hydroxocobalamin (HC), at 525 and 550 nm, without any interference from RF. The apparent first-order rate constants for the photolysis of CC in the presence of 1-5 × 10^{-5} M concentrations of RF at pH 2.0-12.0 range from 0.46 (pH 7.0) to 4.22 ×10^{-3} min^{-1} (pH 3.5). The second-order rate constants for the bimolecular interaction of these vitamins are in the range of 8.66 (pH 7.5) to 45.20 M^{-1} min^{-1} (pH 2.0). The rate-pH profile indicates a gradual decrease in rate in the acid pH range followed by the slowest rate and maximum stability around pH 7-8. Thus the cationic form of CC is more susceptible to photolysis compared to the neutral form. The increase in rate in the alkaline region is probably due to the hydrolysis of the amide groups. The kinetic results indicate that RF acts as a sensitizer in the photolysis of CC and in this way promotes the degradation of the molecule. Thus the presence of RF in CC solutions adversely affects the stability of vitamin B_{12}.

Keywords: Cyanocobalamin, riboflavin, photolysis, kinetics, interaction.

INTRODUCTION

Cyanocobalamin (Vitamin B_{12}) is used in the treatment of pernicious anemia [1]. The chemical and biochemical aspects [2, 3], and stability and interactions [4-9] of cyanocobalamin have been reviewed. It is sensitive to light [10-12] and on photodegradation in aqueous solution [4, 13-17] and dietary supplements [18] is converted to hydroxocobalamin and further products [4, 13-17]. Riboflavin [19, 20] and nicotinamide [21] enhance the photodegradation of cyanocobalamin. The present work is based on a kinetic study of the effect of riboflavin on the photodegradation of cyanocobalamin over a wide range of pH using a specific two-component spectrophotometric method [16]. Similar studies of the effect of riboflavin on the photodegradation of folic acid [22] and other compounds [23] have been reported. The information may facilitate the understanding of the interaction and stabilization of cyanocobalamin in pharmaceutical preparations on exposure to light. The chemical structures of riboflavin, cyanocobal-amin and hydroxocobalamin are shown in Fig. (**1**).

MATERIALS AND METHODS

Cyanocobalamin (CC) and hydroxocobalamin (HC) were obtained from Fluka (Switzerland). Riboflavin (RF), lumichrome (LC) and lumiflavin (LF) were obtained from Sigma Chemical Co. (USA). Formylmethylflavin (FMF) and carboxymethylflavin (CMF) were prepared by the methods of Fall and Petering [24] and Fukumachi and Sakurai [25], respectively. Thin- layer chromatography (TLC) was used to confirm the purity of these compounds. All reagents and solvents were analytical grade or of the purest form available from Merck/BDH. The following buffer systems were used throughout. HCl-KCl, pH 2.0; citric acid-Na_2HPO_4, pH 2.5-8.0; $Na_2B_4O_7$-HCl, pH 8.5-9.0; $Na_2B_4O_7$-NaOH, 9.5-10.5; Na_2HPO_4-NaOH, 11.0-12.0; the ionic strength was 0.02 M in each case.

Precautions

The experimental work was carried out in a dark chamber under subdued light. Freshly prepared aqueous solutions of CC and RF were used for each experiment. All the solutions were protected from light before irradiation to avoid any chemical or photochemical effects.

Photolysis

A series of 5×10^{-5} M aqueous solutions of CC containing 1.0-5.0×10^{-5} M RF at the appropriate pH were prepared in 100 ml volumetric flasks (Pyrex) and placed in a water bath maintained at 25 ± 1 ^{o}C in a radiation chamber. The solutions were irradiated with a Philips HPLN 125 W high pressure mercury vapor florescent lamp (emission at 405, 436, 540 and 577 nm), fixed horizontally at a distance of 30 cm from the centre of the flasks. The major emission wavelengths of the irradiation source correspond to the absorption maxima of CC (550 nm) and RF (444 nm) in the visible region [26]. The solution was continuously bubbled with a gentle stream of air. Samples were withdrawn at appropriate intervals for thin-layer chromatography and spectrophotometric assay.

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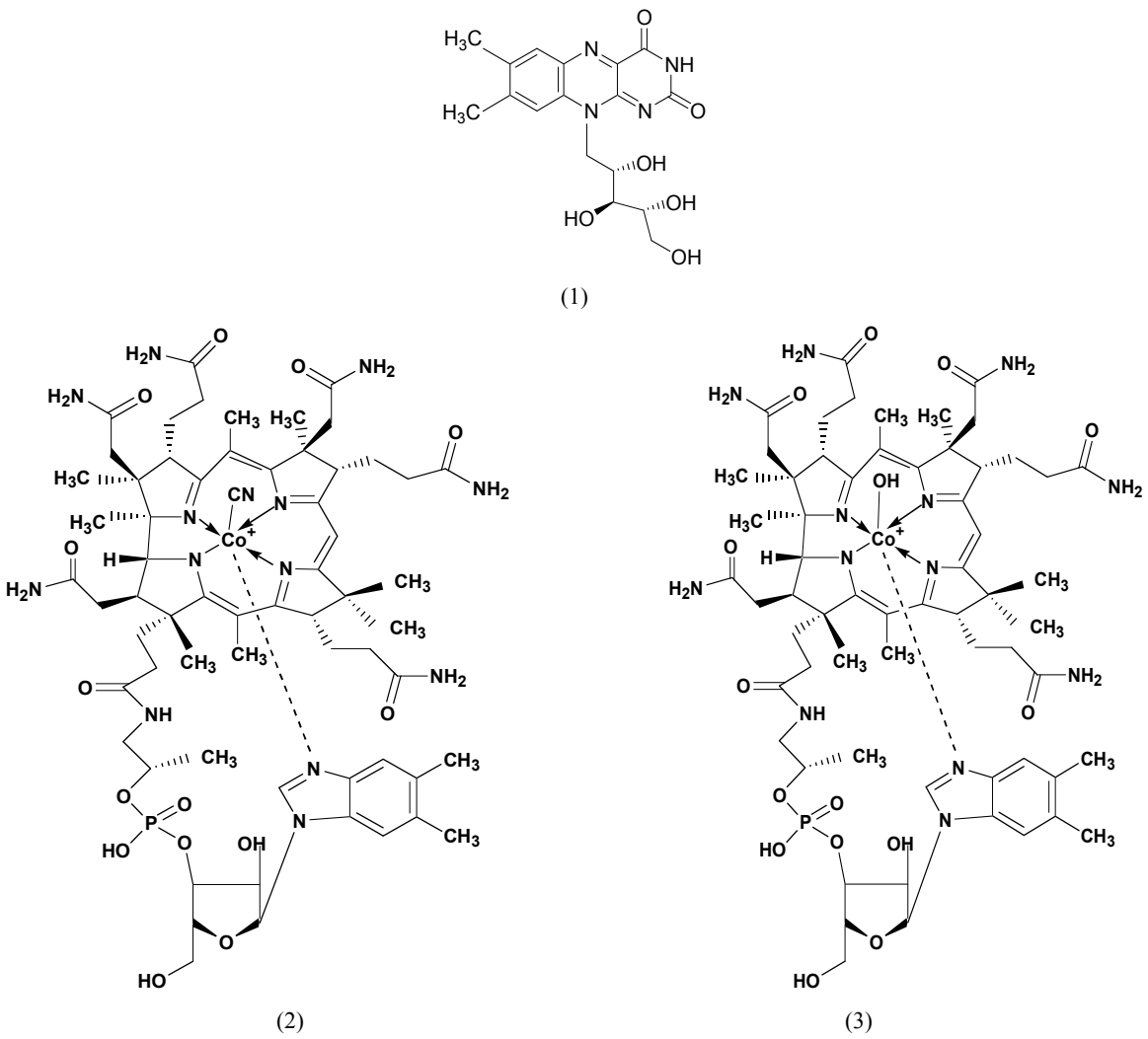

(1) (2) (3)

Fig. (1). Chemical structures of riboflavin (1), cyanocobalamin (2) and hydroxocobalamin (3).

Thin-Layer Chromatography (TLC)

TLC of the photolysed solutions of CC was carried out to detect the photoproducts of CC and RF by using the following systems:

CC: 250-µm silica gel GF_{254} plates using the solvent systems: (A) 1-butanol-acetic acid-0.066 M potassium dihydrogen phosphate-methanol (36:18:36:10, v/v) [27]; and (B) methanol-water (95:5, v/v) [28]. The spots were located visually (red colour) or under UV-light.

RF: 250-µm cellulose plates (Whatman CC 41) using the solvent systems: (C) 1-butanol-acetic acid-water (40:10:50, v/v, organic phase); and (D) 1-butanol-1-propanol-acetic acid-water (50:30:2:18, v/v) [29]. The compounds were detected by their characteristic florescence emission under UV (365 nm) excitation.

Spectral Measurements

All spectral measurements on RF, CC and their photolysed solutions were carried out on a Shimadzu UV-240 recording spectrophotometer using silica cells of 10 mm path length.

Light Intensity Measurements

The intensity of the Philips HPLN 125 W high pressure mercury vapor fluorescent lamp was determined by potassium ferrioxalate actinometry [30] as 1.17 $\pm 0.10\times10^{17}$quanta s^{-1}.

Assay Method

The assay of CC and its photoproduct, HC, in degraded solutions was carried out using a two-component spectrophotometric method of Ahmad *et al*. [16] by the measurement of absorbance at 525 and 550 nm (pH 4.0, acetate buffer). The method was validated in the presence of RF to ensure its accuracy and specificity. The reproducibility of the method was determined by the analysis of synthetic mixtures of CC and HC in the presence of the highest concentration of RF (5×10^{-5}M) used in the reactions.

RESULTS AND DISCUSSION

Photoproducts of CC and RF

TLC of the solutions of CC photolysed in the presence of RF using solvents systems (A) and (B) showed HC as the only products of its photolysis at all pH values. This product is formed by the cleavage of Co^{3+} - CN bond [31] as follows:

$$\underset{\text{CC}}{[Co^{3+} - CN]} \xrightarrow{h\nu} \underset{\text{HC}}{[Co^{3+} OH] + CN^-}$$

The photolysed solutions of CC were also subjected to TLC using the solvent systems (C) and (D), and FMF, LC and CMF (acidic solutions) and FMF, LC, LF and CMF (neutral and alkaline solutions) were detected as the photoproducts of RF. All the photoproducts were identified by comparison of the R_f values and colour/ fluorescence of the spots with those of the authentic compounds (Table **1**).

Assay of CC and HC in Photolysed Solutions

CC and HC exhibit absorption maxima at 361 and 550nm, and 351 and 525 nm, respectively, in aqueous solution [26]. A two-component spectrophotometric method has been reported for the assay of CC and HC at 351 and 361 or 525 and 550 nm (pH 4.0) in photolysed solutions. RF absorbs at 223, 267, 374 and 444 nm [10] and would interfere with the assay of CC and HC at 351 and 361 nm. It has negligible absorbance beyond 500 nm and, therefore, to compensate for any absorbance contribution at 525 nm, an equivalent amount of RF was used as a blank. The degradation products of RF do not absorb in the region of analytical wave lengths. The method has been validated in the presence of RF and the results of the assay of synthetic mixtures of CC and HC in the concentration range likely to occur in the photolysed solution are given in Table **2**. The values of RSD are within ± 3% which show that the method is accurate and reliable for the assay of CC and HC in photolysed solutions in the presence of RF. The results of the assay of CC and HC in a photolysis reaction carried out at pH 4.0 are given in Table **3**. The data show decreasing concentrations of CC and increasing concentrations of HC, with time, giving a constant molar balance.

Kinetics of Photolysis

The kinetics of photolysis of CC in the presence of RF has been studied in the pH range of 2.0-12.0. The apparent first-order rate constants (k_{obs}) for the photolysis reactions at various concentrations of RF are reported in Table **4**. These rate constants were determined by linear regression analysis and the values of correlation coefficients were obtained in the range of 0.995-0.999. The results show that RF promotes the degradation of CC on exposure to light and the rate increases with an increase in RF concentration throughout the pH range. The second-order rate constants (k') for the bimolecular interaction of CC and RF and the values of (k_0) (k_{obs} in the absence of RF) are reported in Table **5**. The k' values range from 8.66 (pH7.5) to 45.20 M^{-1} min^{-1} (pH 2.0) indicating a greater interaction in the acid medium. The values of k_0 are about one-half of the values of k_{obs} for CC at the highest concentration of RF (5×10^{-5} M) indicating that the rate of photolysis is enhanced in the presence of RF. The UV and visible absorption spectra of RF and CC exist in the 200-600 nm region. The 278 and 361 nm absorption maxima of CC overlap the 267 and 444 nm absorption maxima of

Table 1. R_f Values of CC, RF and Photoproducts

Solvent Systems	A	B	C	D	Colour of Spot	Fluorescence λ_{ex} 365 nm
Cyanocobalamin	0.46	0.42			Red	
Hydroxocobalamin	0.25	0.05			Red	
Riboflavin			0.47	0.27		Yellow green
Formylmethylflavin			0.20	0.70		Yellow green
Lumichrome			0.66	0.63		Sky blue
Lumiflavin			0.53	0.41		Yellow green
Carboxymethyflavin			0.28	0.20		Yellow green

Table 2. Spectrophotometric Assay of CC and HC in Synthetic Mixtures in the Presence of RF[a]

CC				HC			
Added ($M\times10^5$)	Found ($M\times10^5$)	Recovery (%)	RSD (%)	Added ($M\times10^5$)	Found ($M\times10^5$)	Recovery (%)	RSD (%)
4.50	4.46	99.10	1.1	0.50	0.51	102.0	1.9
4.00	4.05	101.2	1.5	1.00	0.98	98.0	0.8
3.50	3.48	99.4	0.9	1.50	1.52	101.3	1.6
3.00	3.05	101.7	2.1	2.00	2.03	101.5	0.8
2.50	2.53	101.2	1.6	2.50	2.46	98.4	1.4
2.00	1.95	97.5	2.4	3.00	3.05	101.7	2.0
1.50	1.52	101.3	1.5	3.50	3.42	97.8	1.6
1.00	0.96	96.0	2.1	4.00	4.02	100.5	1.2

[a]Values expressed as a mean of three to five determinations.

RF. Therefore, there is strong possibility of mutual interaction and energy transfer from RF in the excited state to CC resulting in its degradation. Such an energy transfer between molecules has been suggested by Moore [32]. The spectral variations during the photolysis of CC in the presence of RF have been studied by Ansari *et al*. [33].

Table 3. Photolysis of a 5×10^{-5} M Solution of CC in the Presence of 5×10^{-5} M RF at pH 4.0

Time (min)	CC ($M\times10^5$)	HC ($M\times10^5$)	Total ($M\times10^5$)
0	5.00	-	5.00
60	4.03	0.95	4.98
120	3.38	1.64	5.02
180	2.75	2.21	4.96
240	2.28	2.70	4.98
300	1.99	2.97	4.96

Rate-pH Profile

The *k′*- pH profile for the photolysis of CC in the presence of RF is shown in Fig. (**2**). It represents a broad V shaped curve involving different species of the molecule (B_{12}) that undergo photodegradation. The value of k_{obs} for the degradation of CC is the sum of the rate constants for specific acid-catalyzed, water-catalyzed and specific based-catalyzed reactions occurring in different regions.

$$k_{obs} = k_{H^+}[B_{12}H^+] + k_{H_2O}[B_{12}] + k_{OH^-}[B_{12}] \quad (1)$$

where k_{H^+}, k_{H2O} and k_{OH^-} are the rate constants for the hydrogen ion-catalyzed, water-catalyzed, hydroxide ion-catalyzed reactions, respectively.

The gradual decrease in the rate in the pH range 2-6 is the acid-catalyzed photolysis of protonated CC (pKa 3.3) [34], followed by a small plateau of water-catalyzed reaction (6.5-8.5) in which the rate is almost independent of pH. In the pH range of 9-12 the increase in rate is due to the hydrolysis of amide groups [8]. The maximum stability of CC in light occurs on the degradation of the neutral molecule around pH 7-8. A somewhat similar profile for the photolysis of CC alone has been observed by Ahmed *et al*. [16]. This type of profiles has also been reported for the specific acid-base catalyzed degradation of acetaminophen, cefotaxime, indomethacin, methicillin, and phenethicillin [8].

Photochemical Interaction of CC and RF

The basic reactions in the photolysis of CC have been explained in the above section. The photolysis of CC in the presence of RF involves photochemical interaction of the two molecules (given by the second-order rate constant, *k′*). The *k′*-pH profile (Fig. **2**) represents photodegradation of CC in the presence of RF. In this reaction RF appears to play the role of a photosensitizer as observed for such reactions [23,32] and this can be expressed by the following equations.

$$^0RF \xrightarrow{h\nu} {}^1RF \quad (2)$$

$$^1RF \xrightarrow{isc} {}^3RF \quad (3)$$

$$[^0(Co^{3+}CN)\ldots{}^3(RF)] \xrightarrow{\text{interaction}} {}^3(Co^{3+}CN) + {}^0RF \quad (4)$$

$$^3(Co^{3+}CN) \xrightarrow{H_2O} (Co^{3+}OH_2) + CN^- \quad (5)$$

Table 4. First-Order Rate Constants (k_{obs}) for the Photolysis of CC in the Presence of RF ($1.0\text{-}5.0\times10^{-5}$ M)

pH	$k_{obs}\times10^3$ min^{-1}				
	1.0[a]	2.0[a]	3.0[a]	4.0[a]	5.0[a]
2.0	2.26 (0.998)	2.75 (0.996)	3.15 (0.997)	3.59 (0.998)	4.10 (0.995)
2.5	2.25 (0.996)	2.65 (0.997)	3.01 (0.995)	3.50 (0.997)	3.79 (0.998)
3.0	2.48 (0.997)	2.88 (0.995)	3.22 (0.997)	3.57 (0.995)	3.88 (0.998)
3.5	3.02 (0.997)	3.28 (0.995)	3.62 (0.996)	3.95 (0.998)	4.22 (0.996)
4.0	2.16 (0.998)	2.45 (0.995)	2.71 (0.997)	2.92 (0.996)	3.18 (0.999)
4.5	3.31 (0.996)	3.50 (0.996)	3.76 (0.997)	3.93 (0.995)	4.14 (0.996)
5.0	3.52 (0.996)	3.69 (0.997)	3.91 (0.997)	4.06 (0.996)	4.21 (0.998)
5.5	3.48 (0.996)	3.67 (0.997)	3.79 (0.998)	3.99 (0.997)	4.15 (0.996)
6.0	1.59 (0.995)	1.70 (0.995)	1.85 (0.997)	1.97 (0.996)	2.10 (0.998)
6.5	0.58 (0.995)	0.70 (0.997)	0.83 (0.996)	0.92 (0.995)	1.05 (0.997)
7.0	0.46 (0.995)	0.56 (0.996)	0.66 (0.995)	0.73 (0.996)	0.84 (0.997)
7.5	0.77 (0.995)	0.86 (0.997)	0.92 (0.997)	1.02 (0.995)	1.12 (0.996)
8.0	0.73 (0.995)	0.81 (0.996)	0.90 (0.997)	0.97 (0.996)	1.08 (0.995)
8.5	0.87 (0.995)	0.94 (0.996)	1.04 (0.999)	1.12 (0.995)	1.21 (0.996)
9.0	0.58 (0.997)	0.68 (0.998)	0.81 (0.995)	0.92 (0.996)	1.02 (0.995)
9.5	0.77 (0.999)	0.84 (0.996)	1.04 (0.995)	1.15 (0.997)	1.27 (0.996)
10.0	1.43 (0.995)	1.55 (0.996)	1.68 (0.997)	1.84 (0.996)	1.99 (0.998)
10.5	1.53 (0.995)	1.70 (0.998)	1.84 (0.997)	1.99 (0.995)	2.20 (0.996)
11.0	0.65 (0.996)	0.81 (0.997)	1.02 (0.995)	1.27 (0.998)	1.39 (0.995)
11.5	1.52 (0.996)	1.76 (0.995)	1.92 (0.999)	2.14 (0.995)	2.38 (0.997)
12.0	1.54 (0.996)	1.73 (0.995)	1.96 (0.995)	2.17 (0.996)	2.43 (0.997)

[a] RF concentrations ($M\times10^5$). The values in parenthesis are correlation coefficients.

The ground state RF (^{0}RF) is promoted to the excited singlet state (^{1}RF) by the absorption of a quantum of light (2) and may be converted to the excited triplet state (^{3}RF) by intersystem crossing (isc) (3). The ground state CC [^{0}(Co^{3+} CN)] may react with ^{3}RF to form a transient excited state complex (exciplex) as suggested by Sunshine [34] and Moore [32]. This complex may lead to the formation of the excited triplet state of CC [^{3}(Co^{3+} CN)] and ground state RF (^{0}RF) (4). ^{3}(Co^{3+} CN) is degraded to HC (Co^{3+} OH_2) and CN^- in the presence of water (5). Thus, the role of RF in the photolysis of CC is to enhance the reaction and this is evident from the fact that an increase in RF concentration results in an increase in the rate of the reaction (Table **4**). Since RF (pKa_1 1.9, pKa_2 10.2) [35] also exists in different ionized states in the pH range 2.0-12.0, the reaction between CC and RF and the rate of photolysis would depend on the ionized states of the two molecules and their susceptibility to photochemical interaction in a particular pH range. This could be observed from the shape of the *k*'- pH profile for the photolysis reactions of CC.

Table 5. First-Order Rate Constants (k_0) for the Photolysis of CC in the Absence of RF and Second-Order Rate Constant for the Photolysis of CC (*k*') in the Presence of RF[a]

pH	$k_0 \times 10^3$ (min^{-1})	*k*' (M^{-1} min^{-1})	Correlation Coefficient
2.0	1.81	45.20	0.998
2.5	1.86	39.30	0.995
3.0	2.16	34.90	0.998
3.5	2.70	30.69	0.997
4.0	1.93	25.11	0.997
4.5	3.10	20.90	0.996
5.0	3.35	17.71	0.995
5.5	3.32	16.60	0.996
6.0	1.46	12.89	0.998
6.5	0.47	11.62	0.997
7.0	0.37	9.31	0.998
7.5	0.68	8.68	0.996
8.0	0.64	8.66	0.995
8.5	0.77	8.74	0.996
9.0	0.46	11.29	0.998
9.5	0.62	13.07	0.999
10.0	1.27	14.17	0.998
10.5	1.37	16.28	0.996
11.0	0.45	19.42	0.997
11.5	1.32	20.91	0.996
12.0	1.30	22.14	0.997

[a]The values of rate constants are relative and depend on specific experimental conditions including the light intensity.

Pharmaceutical Implications

Vitamins are often formulated in combination in B complex and multivitamin preparations. These preparations contain both RF and CC which may interact in light leading to the loss of the later vitamin. The photochemical interaction of these vitamin is minimum in the pH range around 7-8 and, therefore, this range is suitable for imparting optimum stability to CC in liquid vitamin preparations. A consideration of the stability of other vitamins in these preparations, in addition to RF and CC, would help to achieve a better formulation with prolonged shelf-life.

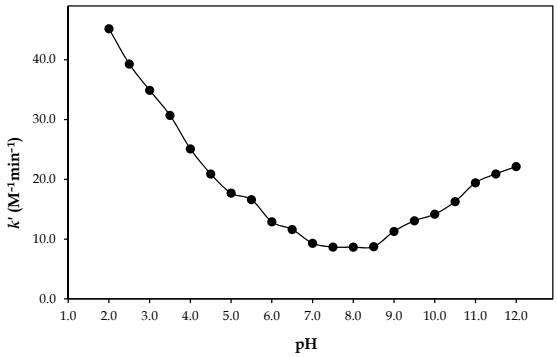

Fig. (2). *k'*-pH profile for the photolysis of cyanocobalamin in the presence of riboflavin.

CONCLUSION

The photolysis of cyanocobalamin in aqueous solution is promoted by riboflavin. This may probably result from their mutual interaction in the excited state followed by energy transfer from riboflavin to cyanocobalamin and its enhanced degradation. The photolysis of cyanocobalamin in aqueous solution is affected by the ionization of the molecule and is greater in the acidic medium compared to that of the neutral solution. The rate of photolysis of cyanocobalamin in the presence of the highest concentration of riboflavin at pH 7.0 is more than double compared to that observed in its absence. The higher stability of cyanocobalamin around pH 7.0 suggests that this region is suitable for maintaining the pH of vitamin preparations containing cyanocobalamin and riboflavin.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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