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Research Article

Effect of Ascorbic Acid on the Degradation of Cyanocobalamin and Hydroxocobalamin in Aqueous Solution: A Kinetic Study

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Abstract. The degradation kinetics of 5×10^{-5} M cyanocobalamin (B_{12}) and hydroxocobalamin (B_{12b}) in the presence of ascorbic acid (AH_2) was studied in the pH range of 1.0–8.0. B_{12} is degraded to B_{12b} which undergoes oxidation to corrin ring cleavage products. B_{12b} alone is directly oxidized to the ring cleavage products. B_{12} and B_{12b} in degraded solutions were simultaneously assayed by a two-component spectrometric method at 525 and 550 nm without interference from AH_2. Both degrade by first-order kinetics and the values of the rate constants at pH 1.0–8.0 range from 0.08 to 1.05×10^{-5} s^{-1} and 0.22–7.62×10^{-5} s^{-1}, respectively, in the presence of 0.25×10^{-3} M AH_2. The $t_{1/2}$ values of B_{12} and B_{12b} range from 13.7 to 137.5 h and 2.5–87.5 h, respectively. The second-order rate constants for the interaction of AH_2 with B_{12} and B_{12b} are 0.05–0.28×10^{-2} and 1.10–30.08×10^{-2} M^{-1} s^{-1}, respectively, indicating a greater effect of AH_2 on B_{12b} compared to that of B_{12}. The k_{obs}–pH profiles for both B_{12} and B_{12b} show the highest rates of degradation around pH 5. The degradation of B_{12} and B_{12b} by AH_2 is affected by the catalytic effect of phosphate ions on the oxidation of AH_2 in the pH range 6.0–8.0.

KEY WORDS: ascorbic acid; cyanocobalamin; degradation; hydroxocobalamin; kinetics; two-component spectrometry.

INTRODUCTION

Cyanocobalamin (vitamin B_{12}) (B_{12}) and hydroxocobalamin (vitamin B_{12b}) (B_{12b}) were originally isolated from liver fractions and *Streptomyces griseus* (1,2). These vitamins are complex organometallic cofactors associated with cobalamin-dependent enzymes that are involved in different catalytic functions in bacteria and humans (3). B_{12} deficiency produces profound pathological effects on the blood, nervous system, and other organs. The most prominent effect of B_{12} deficiency is megaloblastic anemia that is caused by the disruption of DNA synthesis (4). B_{12} [α-(5,6-dimethylbezimidazol-1-yl)cobamide cyanide] and B_{12b} [Coα-[α-(5,6-dimethylbezimidazolyl)]-Coβ-hydroxocobamide] (5) are chemically related (Fig. 1). The CN group attached to Co^{3+} in the tetrapyrrolic corrin macrocyclic ring in B_{12} is replaced by an OH group in B_{12b}. B_{12} undergoes photolysis to B_{12b} in aqueous solution which is degraded to irreversible oxidation products (6–11). Similar reactions occur on the chemical degradation of B_{12} and B_{12b} in the presence of ascorbic acid (AH_2) (12–14) and may be expressed as follows:

$$\underset{B_{12}}{[Co^{3+}CN]} \xrightarrow{h\nu/AH_2} \underset{B_{12b}}{[Co^{3+}OH]} + CN^- \quad (1)$$

$$\underset{B_{12b}}{[Co^{3+}OH]} \xrightarrow{O_2} \text{Corrin macrocyclic ring cleavage products} \quad (2)$$

The effect of thiamine (15,16), nicotinamide (17,18), and riboflavin (19–22) on the photolysis of B_{12} and B_{12b} has been investigated. Several studies have been conducted on the incompatibility and interaction of B_{12} and AH_2 that lead to the loss of B_{12} in aqueous solution (12,13,23–27). B_{12b} has been found to be less stable than B_{12} in the presence of AH_2 (28–30) and is destroyed to form unknown oxidation products (9,12,31,32). 3,3-Dimethyl-2,5-dioxopyrrolidine-4-propionamide and 3,3-dimethyl-2,5-dioxopyrrolidine-4-propionic acid have been identified as the oxidation products of B_{12} (33). The chemical (34–36) and photodegradation (21,37) of B_{12} and B_{12b} has been reported to follow first-order kinetics.

The early studies on the stability and degradation of B_{12} and B_{12b} are largely qualitative and no systematic work on the kinetics of degradation of these vitamins in the presence of other vitamins has been carried out. One of the reasons for this approach has been the nonavailability of specific methods for the simultaneous assay of B_{12} and B_{12b} in degraded

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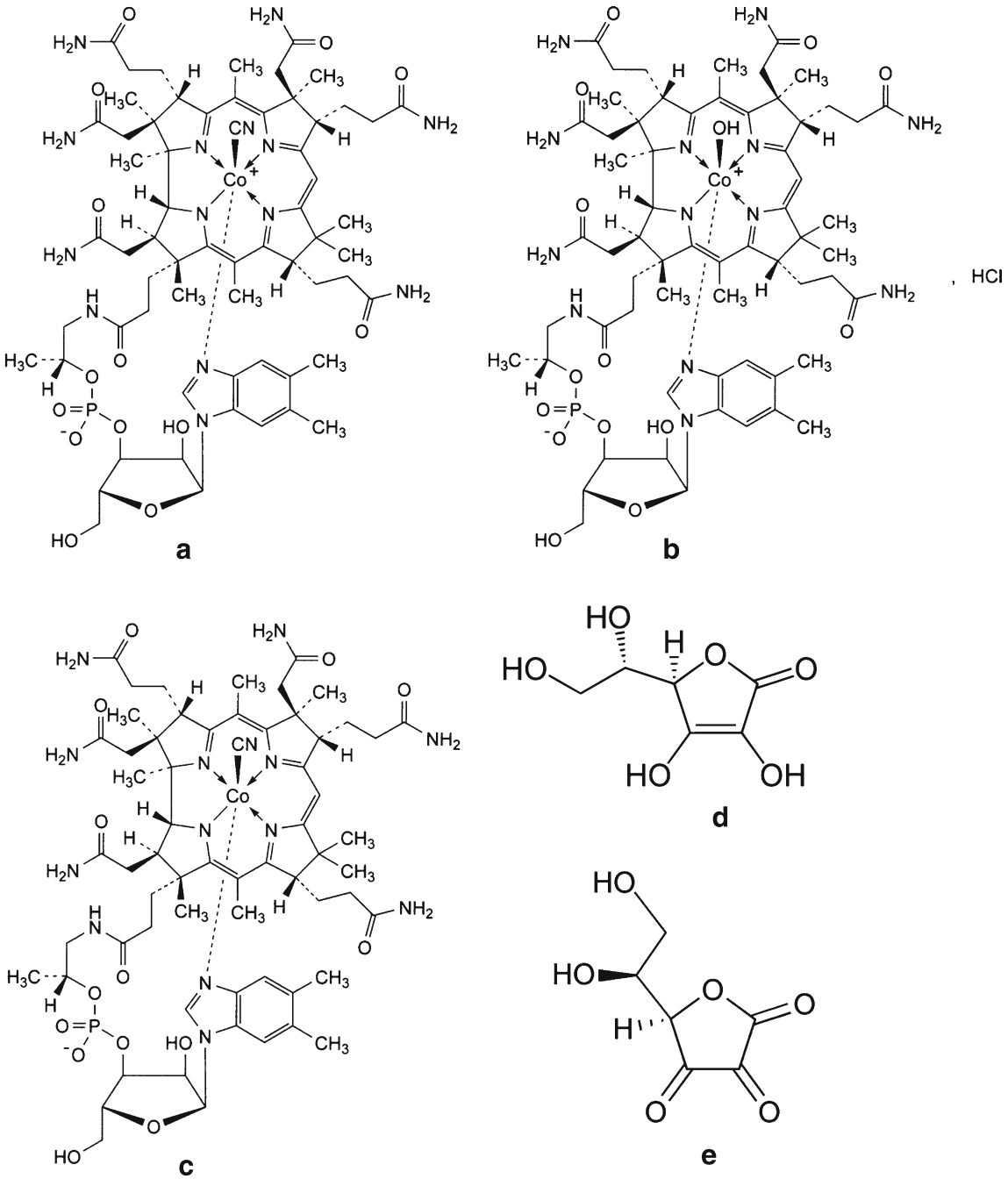

Fig. 1. Chemical structures of **a** cyanocobalamin (B_{12}), **b** hydroxocobalamin (B_{12b}), **c** Cob(II)alamin (B_{12r}), **d** ascorbic acid (AH_2), and **e** dehydroascorbic acid (A)

solutions (10,18,19). In view of the occurrence of vitamins as mixtures in pharmaceutical preparations, there is a need to reinvestigate their interactions and degradation profiles to achieve greater stability and therapeutic efficacy. B_{12} and AH_2 both are components of liquid vitamin B-complex with vitamin C (AH_2) and multivitamin preparations, and B_{12} is liable to degradation in the presence of AH_2 during manufacture, storage, and use. B_{12b} is an intermediate product in the degradation sequence of B_{12} and, therefore, a study of its degradation behavior is of fundamental importance in understanding the stability characteristics of B_{12}. It is necessary to ascertain the role of AH_2 in the degradation of B_{12} and of its degradation product, B_{12b}, together with that of B_{12b} alone. AH_2 is oxidized to dehydroascorbic acid (A) (12,14,38) in these reactions. The present work is based on a kinetic study of the comparison of degradation of B_{12} and B_{12b} in the presence of AH_2 over a wide range of pH (1.0–8.0), that covers the pH range employed for the formulation of vitamin preparations. The study throws light on the interaction of AH_2 with B_{12} and B_{12b} and the extent of formation of oxidation products in these reactions. The magnitude of rate–pH profiles for the degradation of B_{12} and B_{12b} provides an indication of the degree of interaction of AH_2 with B_{12} and B_{12b} as a function of pH. The mode of degradation of B_{12} and B_{12b} in

the presence of AH_2 has also been outlined. The information would be of considerable importance to the chemists and pharmacists in designing vitamin formulations with optimum stability of B_{12} and B_{12b}. The chemical structures of B_{12}, B_{12b}, B_{12r}, AH_2, and A are shown in Fig. 1.

MATERIAL AND METHODS

Materials

B_{12}, B_{12b}, AH_2, and A were obtained from Sigma-Aldrich. All reagents and solvents were of the purest form available from BDH and Merck. The following buffer systems were used throughout the work:

a) For degradation reactions:
KCl / HCl (pH 2.0) and citric acid / Na_2HPO_4 (pH 2.5–8.0); the ionic strength was 0.05 M in each case.
b) For assay:
CH_3COOH/CH_3COONa, 0.2 M (pH 4.0).

Precautions

The degradation, assay, and thin-layer chromatography of B_{12b} solutions were carried out in a dark chamber under subdued light. The solutions were protected from light during preparation and handling.

Degradation of B_{12} and B_{12b}

A 5×10^{-5} M aqueous solution of B_{12} or B_{12b} (100 ml) was prepared at pH 1.0–8.0 using the appropriate buffer, and sufficient amount of AH_2 was added to produce dilutions in the range of $0.25–1.50\times10^{-3}$ M (B_{12}) and $0.05–0.25\times10^{-3}$ M (B_{12b}). The solution was placed in a water bath maintained at 25±1°C in the dark chamber to carry out the degradation. The rate of the reaction was followed by spectrometry.

Thin-layer Chromatography

Thin-layer chromatography (TLC) of the solutions of B_{12} and B_{12b}, degraded in the presence of AH_2, was performed on 250-μm silica gel GF_{254} plates (Merck) using the following solvent systems:

B_{12} and B_{12b}, (a) 1-butanol/acetic acid/0.066 M KH_2PO_4/methanol (36:18:36:10, *v/v*) (39); and (b) methanol/water (95:5, *v/v*) (40). The spots were located visually (red color).

AH_2 and A, (c) acetic acid/acetone/methanol/benzene (5:5:20:70, *v/v*) (41); and (d) ethanol/10% acetic acid (90:10, *v/v*) (42). The spots were located under UV (254 nm) excitation (AH_2) using a Uvitech lamp (Cambridge, UK), or by spraying with a 3% aqueous phenylhydrazine solution (orange red color) (A).

Spectral Determination

All spectral determinations on B_{12} and B_{12b} and the degraded solutions were carried out on a Shimadzu UV-1601 recording spectrophotometer using quartz cells of 10-mm path length.

Spectrometric Assay

The assay of B_{12} and its degradation product, B_{12b}, in degraded solutions in the presence of AH_2 was carried out at by a two-component spectrometric method at 525 and 550 nm (pH 4.0, acetate buffer) (10). The degradation products of B_{12b} formed by the breakdown of the corrin ring may absorb in the UV region and do not interfere with the assay method. The method was validated in the presence of the highest concentration of AH_2 to ensure their accuracy, reproducibility, and specificity under the experimental conditions used.

RESULTS AND DISCUSSION

Degradation Products of B_{12}, B_{12b}, and AH_2

TLC has been applied to detect the products formed on the degradation of B_{12} and B_{12b} in the presence of AH_2. The experimental conditions for the degradation of these vitamins (5×10^{-5} M) at pH 1.0–8.0 involved AH_2 concentrations of $0.25–1.5\times10^{-3}$ M (B_{12}) and $0.05–0.25\times10^{-3}$ M (B_{12b}). TLC of the degraded solutions of B_{12} (R_f 0.46 and 0.42 in solvent systems (a) and (b), respectively) showed the presence of B_{12b} only at all pH values (R_f 0.26 and 0.05 in solvent systems (a) and (b), respectively) whereas no degradation product has been detected in B_{12b} solutions in the pH range studied.

B_{12} is known to be converted to B_{12b} by reducing agents including AH_2 (12–14). B_{12b} is destroyed more rapidly by AH_2 than B_{12}, with a loss of color to indicate the release of cobalt from the molecule (24) and formation of the corrin ring cleavage oxidation products (9,13,32). These products could not be identified by TLC in this study. The oxidation products of B_{12} have been identified as 3,3-dimethyl-2,5-dioxopyrrolidine-4-propionamide and 3,3-dimethyl-2,5-dioxopyrrolidine-4-propionic acid (33). In the acid medium, the breakdown products of B_{12} include mixtures of carboxylic acid, γ-lactone and 1-amino-2-propanol (9,13), which do not absorb in the visible region. An R_f 0.22 and 0.73 in solvent systems (c) and (d), respectively, has been detected for A as an oxidation product of AH_2 (R_f 0.30 and 0.49 in (c) and (d), respectively) in the degraded solutions of B_{12} and B_{12b}.

Spectral Characteristics and Color Changes of B_{12} and B_{12b} in Degraded Solutions

B_{12} and B_{12b} exhibit absorption maxima at 278, 361, and 550 nm and 274, 351, and 525 nm, respectively, in water (5,43). AH_2 absorbs at 265 nm at pH 6.4 (44) and does not interfere in the visible region. The absorption spectra of a B_{12b} solution (pH 5.0) stored in the presence of 1×10^{-3} M AH_2 in the dark are shown in Fig. 2. A slight increase in absorbance in the 450–500 nm region after about 90 min gives an indication of the presence of the reduced form of B_{12b} (Cob(II)alamin, B_{12r}), absorbing at 474 nm (45). After about 180 min, the spectrum of the colorless solution showed negligible absorption in the visible region as a result of the oxidation of corrin ring (33).

A B_{12} solution (pH 5.0) stored in the dark in the presence of AH_2 became slightly lighter in color and gradually turned reddish brown. This was followed by a change in color to yellow, and ultimately, the solution became colorless. These changes were more rapid in the degraded solution of B_{12b}. The color changes involved in the degradation of B_{12} have

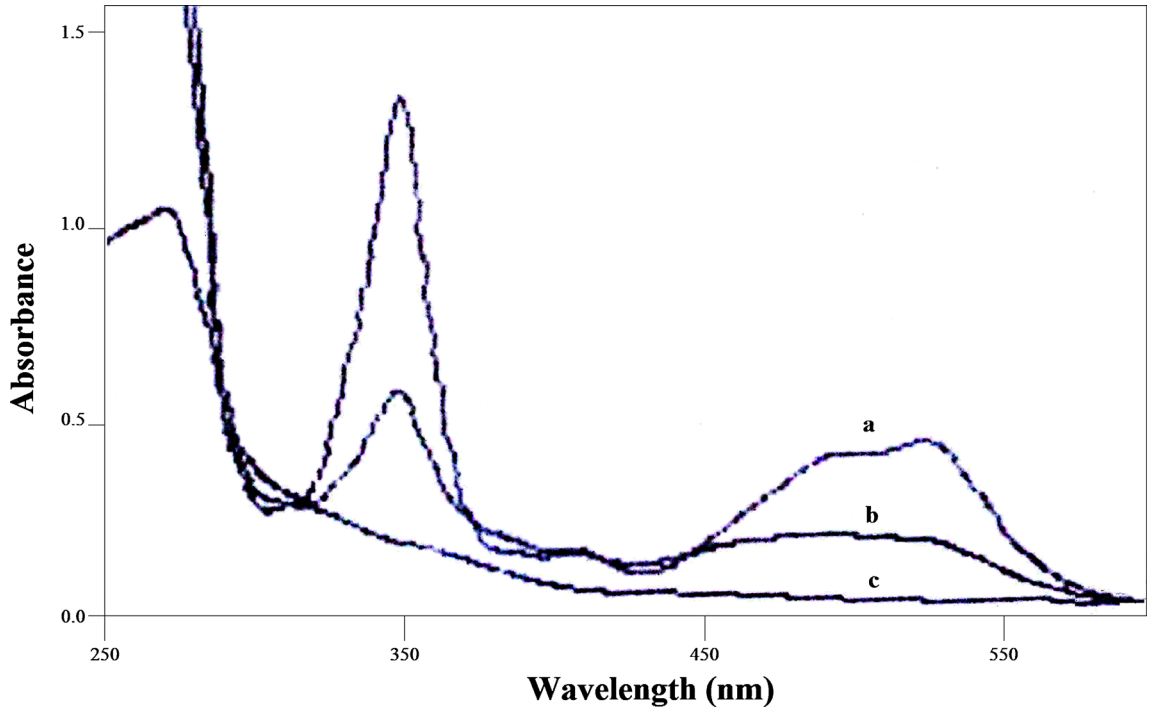

Fig. 2. Absorption spectra of hydroxocobalamin solution (5×10^{-5} M) stored in the presence of ascorbic acid (1.5×10^{-3} M). **a** 0 h, red, **b** 1.5 h, brownish red, **c** 3 h, colorless

been ascribed to the formation of the reduced form of B_{12} (B_{12r}) (red to reddish brown), reaction intermediates (brown to yellow) and corrin ring oxidation products (yellow to colorless) (24). In the presence of air B_{12r} is slowly oxidized to B_{12b} (32): similar rapid color changes have been observed in B_{12b} solutions, degraded in the presence of AH_2. These changes indicate that B_{12} undergoes degradation to B_{12r} which may further degrade to colorless oxidation products by the cleavage of the corrin ring or be oxidized to B_{12b} which could also be transformed to the colorless oxidation products in the presence of AH_2 (32).

Assay of B_{12} and B_{12b} in Degraded Solutions

The absorption maxima of B_{12} and B_{12b} occur at 361 and 550 nm and 351 and 525 nm, respectively, in aqueous solution (43). The British Pharmacopoeia (5) method for the assay of these vitamins is based on the measurement of their absorbance at 361 and 351 nm, respectively. However, due to overlapping of the absorption bands in this region and in the 525 and 550 nm region, the assay of the individual vitamins is not possible. In order to overcome this problem, a two-component spectrometric method was developed and applied to study the kinetics of photolysis of B_{12} alone (11,19), and also in the presence of nicotinamide (18) and riboflavin (19,20) and the degradation of B_{12} in parenteral solutions (31). Since B_{12b} is also a product of photolysis of B_{12}, both B_{12} and B_{12b} can be conveniently assayed by this method in B_{12} solutions degraded in the presence of AH_2. AH_2 absorbs at 265 nm (pH 6.4) (44) and does not interfere in the simultaneous assay of B_{12} and B_{12b} by this method. Since the ultimate degradation of B_{12} and B_{12b} results in the cleavage of corrin ring to form the oxidation products, which may absorb in the UV region, the set of wavelength 525 and 550 nm in the visible region has been preferred for the two-component assay of B_{12} and B_{12b} in degraded solutions. B_{12b} has directly been assayed at 525 nm to avoid any interference from degradation products. The assay method has been validated in the presence of AH_2 and has a precision of ±2% (Table I). The results of the assay of B_{12} and B_{12b} in a typical degradation reaction carried out at pH 5.0 are given in Table II. The increasing loss of molar balance for B_{12} and B_{12b}, with time, is due to the formation of the oxidation products (OP) and is an indication of the destructive effect of AH_2 on both B_{12} and B_{12b}. The gradual decrease in the OP/B_{12b} ratios suggests that B_{12b} may be produced by more than one pathway. This has been discussed under the section on the mode of degradation of B_{12}. Control solution of B_{12} and B_{12b} kept in the dark in the absence of AH_2 did not show any degradation during the period of the reactions.

Kinetics of Degradation of B_{12} and B_{12b}

B_{12} is known to degrade to B_{12b} and B_{12b} to irreversible oxidation products in aqueous solution in the presence of AH_2. Both reactions are affected by AH_2 and the degradation of B_{12b} alone is much faster than B_{12} in the presence of AH_2 as discussed in the above sections. These reactions have been studied at pH 1.0–8.0 to assess the degradation behavior of B_{12} and B_{12b}. Therefore, the assay data on the degradation of B_{12} and B_{12b} were subjected to kinetic treatment, and it was found that these vitamins degrade by an apparent first-order kinetics in the presence of AH_2. This is in agreement with the previous observations that the chemical degradation of B_{12} and B_{12b} follows first-order kinetics (34–36). The apparent first-order rate constants (k_{obs}) for the degradation of B_{12} and B_{12b} are

Table I. Validation Data (n=5) for Cyanocobalamin (B_{12}) and Hydroxocobalamin (B_{12b})

	B_{12}	B_{12b}
Absorption maxima	550	525
Molar absorptivity (M^{-1} cm^{-1})	8.70×10^3	8.64×10^3
Linearity range ($M\times10^{-5}$)	1.0–5.0	1.0–5.0
Correlation coefficient	0.9998	0.9998
Slope	8700	8640
Intercept	−0.0018	−0.0090
SE (±) of slope	0.0031	0.0040
SE of intercept	0.0032	0.0030
SD of intercept	0.0073	0.0068
Recovery range (%)	98.79–101.03	98.96–101.85
Accuracy (%)±SD	100.03±0.865	100.19±1.121
% RSD	0.865	1.119
LOD ($M\times10^{-5}$)	0.276	0.260
LOQ ($M\times10^{-5}$)	0.835	0.787

LOD limit of detection, *LOQ* limit of quantification

given in Table III and IV, respectively. The values of k_{obs} indicate that the degradation of B_{12} and B_{12b} is promoted by AH_2, and the rate increases with an increase in AH_2 concentration throughout the pH range employed. In order to correlate the values of k_{obs} with AH_2 concentrations, these values were plotted against the respective AH_2 concentrations for the two vitamins and the second-order rate constants (k_2) for the interaction of AH_2 with B_{12} and B_{12b} were determined from the slopes of the straight lines (correlation coefficients 0.996–0.999) (Table V). A comparison of the values of k_2 for B_{12} and B_{12b} under the reaction conditions employed shows that the rate of interaction of AH_2 with B_{12b} is about 20 (pH 1.0) to 100 (pH 5.0) times faster than that of B_{12}. Earlier studies have shown that the degradation of B_{12b} is faster than B_{12} in the presence of AH_2 (28,46), but no comparison of the kinetic data in these reactions has been made.

Table II. Degradation of Cyanocobalamin (B_{12}) in the Presence of Ascorbic acid (AH_2) at pH 5.0

Time (h)	B_{12} ($M\times10^5$)	B_{12b} ($M\times10^5$)	$B_{12}+B_{12b}$ ($M\times10^5$)	OP[a] ($M\times10^5$)	OP/B_{12b}
0	5.00	–	5.00	–	–
2	4.68	0.20	4.88	0.12	0.60
4	4.25	0.47	4.72	0.28	0.60
6	3.85	0.73	4.58	0.42	0.58
8	3.54	0.95	4.49	0.51	0.53
10	3.25	1.21	4.46	0.54	0.45
12	2.96	1.47	4.43	0.57	0.39
14	2.70	1.69	4.39	0.61	0.36
16	2.46	1.90	4.36	0.64	0.34
20	2.13	2.19	4.32	0.68	0.31
24	1.77	2.52	4.29	0.71	0.28
28	1.47	2.79	4.46	0.74	0.26
32	1.24	2.98	4.22	0.76	0.25

Experimental conditions were as follows: initial concentration of B_{12}, 5×10^{-5} M; concentration of AH_2, 1.5×10^{-3} M; degradation time, 32 h; temperature, 25±1°C; storage, in dark

[a] Oxidation products (OP) of B_{12}=[initial concentration of B_{12}−(molar concentration of B_{12}+molar concentration of B_{12b} at various time intervals)]

Effect of pH

The pH has a significant effect on the degradation of both B_{12} and B_{12b} in the presence of AH_2. The rate–pH profiles give an indication of the interactions between drugs and the rate at which different ionic/non-ionic species undergo degradation as a function of pH. These profiles are helpful in the prediction of the pH of maximum stability of drug substances for the formulation of liquid preparations (38,47). The k_2 *versus* pH profiles for the degradation of B_{12} and B_{12b} in the presence of AH_2 are shown in Figs. 3 and 4, respectively. Both profiles are bell-shaped with maxima around pH 5.0. Such profiles imply acid/base dissociation in the reactants. This is possible in the case of AH_2 (pK_a, 4.17) (44) whose monoanion AH^- is the reactive species and its concentration would determine the rate of interaction between AH_2 and B_{12} or B_{12b}. As the reaction of AH_2 species (AH_2 and AH^-) with B_{12} and B_{12b} proceeds, the formation of AH^- anions is increased with pH and hence the rate of interaction between AH_2 and B_{12} or B_{12b}, reaching a maximum around pH 5.0. A decrease in pH from 5.0 to 1.0 leads to an increase in the nonionic species of AH_2 and thus the resultant decrease in the rate of interaction. Moreover, the protonated form of B_{12} (pK_a 3.3) (48) may be less susceptible to degradation than the neutral form. An increase in pH tends to destroy the AH^- anions by oxidation (49), resulting in a gradual decrease in the rate of interaction between AH_2 and B_{12}/B_{12b}. A similar rate–pH profile for the degradation of B_{12b} in the presence of formate anions (pH_{max} 5.5) has been reported (50), indicating a similarity of the reaction between ascorbate and formate anions as reductants of B_{12} and B_{12b}, respectively.

Effect of Buffer

The buffer salts normally used to maintain the pH of pharmaceutical preparations may lead to the degradation of drug substances by acid-base catalysis (38,47). Phosphate salts are commonly used as buffers in the pH range 6.0–8.0 and have been found to catalyze the degradation of drugs including phenytoin (51), codeine (52), ciclosidomine (53), spironolactone (54), gonadorelin (55), riboflavin (56–59), and formylmethylflavin (60). Since the degradation of B_{12} in this study has also been carried out in phosphate buffer (pH 6.0–8.0), it was considered necessary to evaluate the catalytic effect of phosphate species on the degradation of B_{12} in 0.02–0.10 M phosphate buffer in the presence of 1.5×10^{-3} M AH_2. The graphs of k_{obs} *versus* phosphate concentration are linear with a negative slope (R^2=0.997–0.999) and the second-order rate constants (k_2) for the degradation of B_{12} in the presence of phosphate species at pH 6.0, 7.0, and 8.0 obtained from the slopes of the plots are 1.04, 0.40, and 0.21×10^{-4} M^{-1} s^{-1}, respectively. The values of apparent first-order rate constants, k_o, at zero buffer concentration at pH 6.0, 7.0, and 8.0 obtained from y-intercepts of the second-order plots are 1.74, 0.63, and 0.26×10^{-5} s^{-1}, indicating the inhibitory effect of phosphate species on the reaction. The decrease in the values of k_2, with pH, suggests that the monovalent phosphate species ($H_2PO_4^-$) (pK_a 7.21) are more effective than the divalent phosphate species (HPO_4^{2-}) in causing the catalytic degradation of AH_2 which would in turn have a lower effect on the degradation of B_{12}.

Table III. Apparent First-Order Rate Constants (k_{obs}) for the Degradation of Cyanocobalamin (B_{12}) in the Presence of Ascorbic acid (AH_2) at pH 1.0–8.0

	$k_{obs}\times10^5$ [s^{-1}]±SD[a]					
pH	0.25[b]	0.50[b]	0.75[b]	1.00[b]	1.25[b]	1.50[b]
1.0	0.08±0.005	0.10±0.006	0.11±0.006	0.12±0.005	0.13±0.007	0.14±0.009
1.5	0.10±0.006	0.12±0.005	0.13±0.007	0.15±0.008	0.16±0.009	0.18±0.008
2.0	0.11±0.006	0.14±0.008	0.18±0.009	0.21±0.009	0.23±0.008	0.26±0.008
2.5	0.30±0.01	0.35±0.02	0.38±0.02	0.41±0.02	0.45±0.03	0.50±0.01
3.0	0.49±0.03	0.53±0.03	0.58±0.04	0.63±0.04	0.67±0.03	0.72±0.03
3.5	0.77±0.04	0.82±0.04	0.88±0.05	0.94±0.04	0.99±0.04	1.05±0.04
4.0	0.99±0.05	1.06±0.05	1.12±0.05	1.19±0.06	1.26±0.07	1.32±0.04
4.5	1.02±0.06	1.08±0.06	1.14±0.06	1.21±0.05	1.28±0.05	1.36±0.07
5.0	1.05±0.05	1.12±0.05	1.18±0.05	1.26±0.06	1.33±0.08	1.40±0.08
5.5	0.95±0.05	1.04±0.05	1.11±0.07	1.19±0.06	1.27±0.05	1.33±0.06
6.0	0.91±0.04	0.97±0.04	1.03±0.05	1.10±0.04	1.17±0.06	1.22±0.05
6.5	0.49±0.02	0.53±0.02	0.56±0.03	0.60±0.03	0.64±0.03	0.68±0.03
7.0	0.29±0.02	0.31±0.02	0.34±0.02	0.37±0.02	0.40±0.02	0.43±0.02
7.5	0.16±0.01	0.18±0.01	0.19±0.01	0.20±0.01	0.21±0.01	0.23±0.01
8.0	0.11±0.01	0.12±0.01	0.13±0.01	0.14±0.01	0.15±0.01	0.16±0.01

Experimental conditions were as follows: initial concentration of B_{12}, 5×10^{-5} M; concentration of AH_2, 0.25–1.50$\times10^{-3}$ M; degradation time, 15 h; temperature, 25±1°C; storage, in dark
[a] $n=3$
[b] AH_2 concentration ($M\times10^3$)

In the present work, the degradation studies on B_{12} and B_{12b} have been carried out in the presence of AH_2 and the values of pH_{max} obtained are around pH 5.0 (Figs. 3 and 4). Since the phosphate species present in the buffer system (pH 6.0–8.0) are also catalytic to ascorbate monoanion (AH^-) in the pH range 5.85–7.22 (61), there is a possibility that the buffer species may catalyze the oxidation of AH^- anions thereby inhibiting their effect on the degradation of B_{12} and B_{12b}. This has in fact been confirmed by conducting the B_{12} degradation reactions in phosphate buffer. It is evident that the rates of B_{12} and B_{12b} degradation increase with a decrease in buffer concentration due to the presence of a relatively greater amount of AH^- anions which would interact with these vitamins. Phosphate species tend to destroy AH^- anions above pH 5.0, and their rate of oxidation is minimum at pH 5.6 (62) which is slightly above the pH_{max} for B_{12} and B_{12b}. The greater stability and reactivity of AH_2 at pH 5.0 compared to that of pH 7.0 is due to its lower redox potential at pH 5.0 ($E°=-0.117$ V) than that at pH 7.0 ($E°=+0.058$ V) (63). The decrease in the rate of degradation of these vitamins above pH 5.0 (Figs. 3 and 4) is probably due to a gradual decrease in the availability of AH^- anions for interaction with B_{12} and B_{12b}.

Table IV. Apparent First-Order Rate Constants (k_{obs}) for the Degradation of Hydroxocobalamin (B_{12b}) in the Presence of Ascorbic acid (AH_2) at pH 1.0–8.0

	$k_{obs}\times10^5$ [s^{-1}]±SD[a]				
pH	0.05[b]	0.10[b]	0.15[b]	0.20[b]	0.25[b]
1.0	0.04±0.002	0.08±0.004	0.13±0.006	0.17±0.008	0.22±0.01
1.5	0.06±0.003	0.15±0.005	0.23±0.010	0.28±0.142	0.34±0.02
2.0	0.11±0.01	0.28±0.01	0.41±0.02	0.50±0.03	0.64±0.03
2.5	0.17±0.01	0.47±0.02	0.68±0.03	0.80±0.03	1.04±0.04
3.0	0.42±0.03	0.89±0.04	1.40±0.06	1.82±0.08	2.30±0.09
3.5	0.85±0.03	1.76±0.09	2.64±0.10	3.55±0.14	4.47±0.20
4.0	1.24±0.05	2.63±0.14	4.05±0.16	5.37±0.25	6.65±0.28
4.5	1.45±0.06	2.82±0.11	4.25±0.18	5.65±0.23	7.04±0.32
5.0	1.55±0.06	3.04±0.12	4.65±0.19	6.02±0.29	7.62±0.36
5.5	1.42±0.06	2.75±0.14	4.15±0.17	5.48±0.25	6.80±0.37
6.0	0.80±0.03	1.69±0.07	2.54±0.10	3.40±0.16	4.20±0.22
6.5	0.65±0.03	1.30±0.05	2.05±0.12	2.72±0.12	3.45±0.14
7.0	0.52±0.03	1.12±0.05	1.65±0.07	2.24±0.09	2.76±0.13
7.5	0.42±0.02	0.77±0.04	1.21±0.05	1.55±0.06	2.02±0.08
8.0	0.35±0.02	0.63±0.03	0.99±0.05	1.28±0.06	1.60±0.08

Experimental conditions were as follows: initial concentration of B_{12b}, 5×10^{-5} M; concentration of AH_2, 0.05–0.25$\times10^{-3}$ M; degradation time, 15 h (pH 1–2), 3 h (pH 2.5), 1 h (pH 3.0–6.5), 3 h (pH 7.0–8.0); temperature, 25±1°C; storage, in dark
[a] $n=3$
[b] AH_2 concentration ($M\times10^3$)

Table V. Second-Order Rate Constants (k_2) for the Interaction of Ascorbic acid (AH_2) with Cyanocobalamin (B_{12}) and Hydroxocobalamin (B_{12b}) at pH 1.0–8.0

	k_2 [M^{-1} s^{-1}]±SD[a]	
pH	$B_{12}\times10^2$	$B_{12b}\times10^2$
1.0	0.05±0.002	1.10±0.06
1.5	0.06±0.003	1.36±0.05
2.0	0.11±0.004	2.56±0.11
2.5	0.15±0.008	4.20±0.19
3.0	0.19±0.007	9.20±0.51
3.5	0.22±0.009	17.88±0.72
4.0	0.25±0.012	26.60±0.99
4.5	0.27±0.014	29.02±0.99
5.0	0.28±0.013	30.08±1.20
5.5	0.27±0.015	27.20±1.09
6.0	0.24±0.009	16.81±0.67
6.5	0.16±0.008	13.80±0.65
7.0	0.10±0.006	11.04±0.49
7.5	0.06±0.002	8.08±0.34
8.0	0.05±0.002	6.40±0.29

Experimental conditions were as follows: initial concentration of B_{12} and B_{12b}, 5×10^{-5} M; concentration of AH_2, $0.25–1.5\times10^{-3}$ M (B_{12}) and $0.05–0.25\times10^{-3}$ M (B_{12b}); degradation time, 1–15 h; temperature, 25±1°C; storage, in dark

[a] $n=3$

Distribution of Degradation Products of B_{12}

The present study has shown that the degradation of B_{12} in the presence of AH_2 leads to the formation of B_{12b} and the oxidation products (OP) at pH 1.0–8.0. The product distribution at 50% degradation of B_{12} along with the $t_{1/2}$ values of B_{12} and B_{12b} are given in Table VI. A comparison of the molar concentrations of B_{12b} and OP at various pH values shows that the ratios of OP to B_{12b} decrease with pH from 1.81 (pH 1.0) to 0.10 (pH 8.0). This could be due to the simultaneous oxidation of AH_2/AH^- species with an increase in pH and thus being less effective in causing the oxidation of B_{12b}. The greater stability of B_{12} at pH 1.0–2.0 (97–99% protonated form) (pK_a 3.5) (48) indicates that the protonated form of B_{12} (B_{12} H^+) is resistant to degradation. The values of OP at pH 1.0–2.0 may include certain amount of B_{12r} which would also be more stable to oxidation in this pH range. The relatively high values of OP and the greater stability of B_{12b} at pH 1.0–2.0 suggest that these may have a contribution from B_{12r}. The $t_{1/2}$ values for B_{12} and B_{12b} in the pH range indicate that B_{12} is more stable to degradation than B_{12b} as reported in the earlier studies (28,32,46).

Mode of Degradation

It is well known that B_{12} is incompatible with reducing agents such as AH_2 and is degraded in its presence at room temperature. B_{12b} is very unstable in the presence of AH_2 and is destroyed by the release of Co atom with subsequent disappearance of the color (12,24,28,29). These changes result in the cleavage of the corrin ring to form the oxidation products (9,12,28). The degradation of B_{12} by H_2O_2 (64) and HOCl (65) also results in the discoloration of the solutions and the formation of the oxidation products. In the light of the previous studies and on the basis of the present findings, schemes for the mode of degradation of B_{12} and B_{12} in the presence of AH_2 are presented.

Degradation of B_{12}

The degradation of B_{12} by AH_2 in the pH range 1–8 proceeds slowly with AH_2 species (pK_{a1}, 4.17) (44) up to around pH 3 (3) and then with an enhancement in the rate up to pH 5 due to the participation of the AH^- anions (4). This is followed by a decline in the rate of reaction due to the loss of AH^- anions. This process leads to the formation of reduced B_{12} (B_{12r}) by one electron transfer depending upon the pH and the redox potential of the B_{12}/B_{12r} system (E° pH 7.0= −0.04 V) (66). B_{12r} is directly oxidized to form B_{12b} by the replacement of CN^- by an OH^- group (5). Alternatively, it could undergo cleavage of the corrin ring to produce colorless oxidation products (6). The (5) and (6) reactions may occur simultaneously under the conditions employed. B_{12r} can also disproportionate to B_{12s} (Co(I)alamin) and B_{12b} (7) (13). Thus, B_{12b} could be generated by more than one pathway ((5) and (7)), whereas OP are formed through B_{12b} only. This would result in a gradual decrease in the OP/B_{12b} ratios

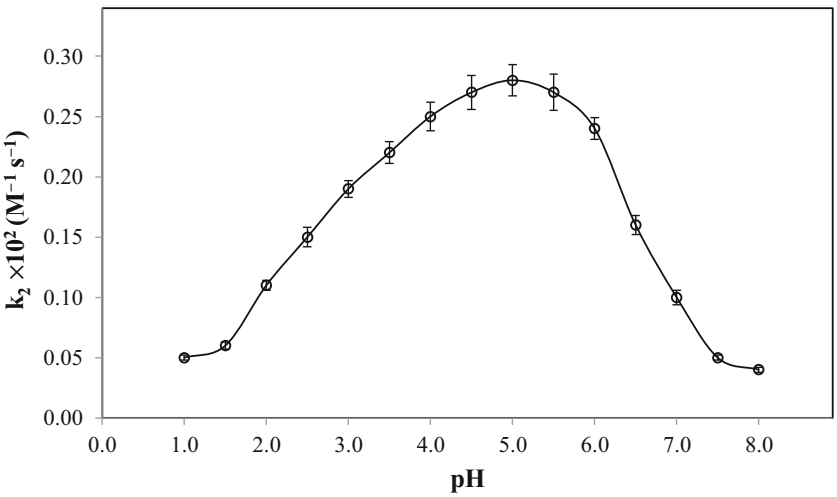

Fig. 3. k_2–pH profile for the degradation of cyanocobalamin (B_{12}) at pH 1–8

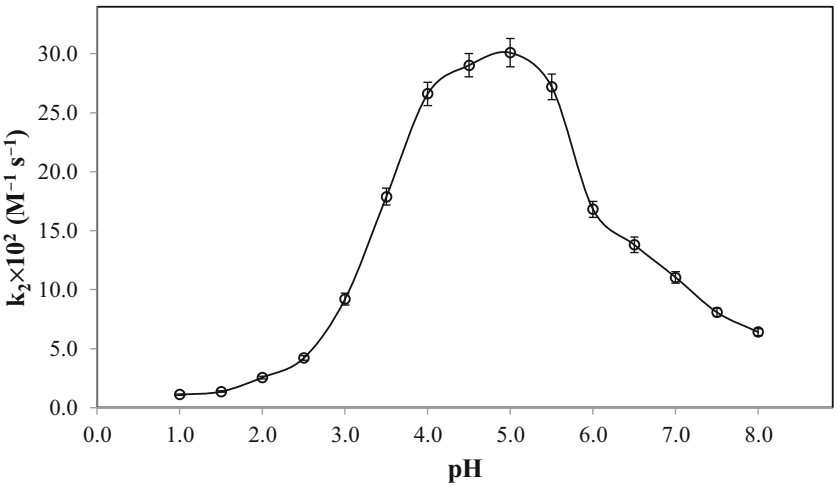

Fig. 4. k_2–pH profile for the degradation of hydroxocobalamin (B_{12b}) at pH 1–8

as observed in the present study (Table II). These reactions are presented in Scheme 1:

$$\underset{B_{12}}{[Co^{3+}CN]} + AH_2 \xrightarrow{+e^-} \underset{B_{12r}}{[Co^{2+}CN]}[H^+] + AH^- \quad (3)$$

$$\underset{B_{12}}{[Co^{3+}CN]} + AH^- \xrightarrow{+e^-} \underset{B_{12r}}{[Co^{2+}CN]}[H^+] + A^{2-} \quad (4)$$

$$\underset{B_{12r}}{[Co^{2+}CN]} \xrightarrow{O_2,OH^-} \underset{B_{12b}}{[Co^{3+}OH]} + CN^- \quad (5)$$

$$\underset{B_{12r}}{[Co^{2+}CN]} \xrightarrow{O_2} \underset{OP}{\text{Corrin ring cleavage oxidation products}} \quad (6)$$

$$2\underset{B_{12r}}{[Co^{2+}CN]} \rightarrow \underset{B_{12b}}{[Co^{3+}OH]} + \underset{B_{12s}}{[Co^{+}OH]} + CN^- \quad (7)$$

Degradation of B_{12b}

The degradation of B_{12b} by AH_2/AH^- species (8) follows the same pattern as observed for B_{12}, resulting in the formation of the reduced B_{12b} (B_{12br}) (9). Similar to the reactions of B_{12} given in Scheme 1, B_{12br} may be oxidized to B_{12b} (10) or may undergo cleavage of the corrin ring to yield the oxidation products (11). The formation of the oxidation products may depend on the pH and the rate of interaction of AH^- anions with the B_{12b}. The degradation reactions of B_{12b} are presented in Scheme 2:

$$\underset{B_{12b}}{[Co^{3+}OH]} + AH_2 \xrightarrow{+e^-} \underset{B_{12br}}{[Co^{2+}OH_2]} + AH^- \quad (8)$$

$$\underset{B_{12b}}{[Co^{3+}OH]} + AH^- \xrightarrow{+e^-} \underset{B_{12br}}{[Co^{2+}OH_2]} + A^{2-} \quad (9)$$

$$\underset{B_{12br}}{[Co^{2+}OH]} \xrightarrow{O_2} \underset{B_{12b}}{[Co^{3+}OH]} \quad (10)$$

$$\underset{B_{12br}}{[Co^{2+}OH]} \xrightarrow{O_2} \underset{OP}{\text{Corrin ring cleavage oxidation products}} \quad (11)$$

CONCLUSION

Cyanocobalamin (B_{12}) and hydroxocobalamin (B_{12b}) both degrade in the dark by first-order kinetics in the presence of ascorbic acid (AH_2) at pH 1.0–8.0. A comparison of the

Table VI. Product distribution at 50% degradation ($t_{1/2}$) of cyanocobalamin (B_{12}) solutions at pH 1.0–8.0

pH	$t_{1/2}$ (h)	B_{12} ($M \times 10^{5)}$	B_{12b} ($M \times 10^5$)	OP[a] ($M \times 10^{5)}$	OP/B_{12b}
1.0	137.50 (87.50)[b]	2.50	0.89	1.61	1.81
2.0	74.03 (30.08)[b]	2.50	1.26	1.24	0.98
3.0	26.74 (8.37)[b]	2.50	2.05	0.45	0.22
4.0	14.58 (2.89)[b]	2.50	2.09	0.41	0.19
5.0	13.75 (2.53)[b]	2.50	2.14	0.36	0.17
6.0	15.78 (4.58)[b]	2.50	2.20	0.30	0.14
7.0	44.77 (6.97)[b]	2.50	2.24	0.26	0.12
8.0	120.31 (12.03)[b]	2.50	2.29	0.22	0.10

Experimental conditions were as follows: initial concentration of B_{12} and B_{12b}, 5×10^{-5} M; concentration of AH_2, 1.50×10^{-3} M (B_{12}), 0.025×10^{-3} M (B_{12b}); degradation time, 15 h; temperature, 25±1°C; storage, in dark

[a] Oxidation products (OP) of B_{12}=[initial molar concentration of B_{12}−(molar concentration of B_{12}+molar concentration of B_{12b} at 50% degradation)]

[b] The values in parenthesis are $t_{1/2}$ of B_{12b}

kinetic data shows that the degradation of B_{12b} is much faster than that of B_{12}. The maximum rate of degradation of B_{12} and B_{12b} occurs at pH 5.0, indicating the highest rate of interaction of AH_2 with B_{12} and B_{12b} at that pH. The protonated forms of B_{12} and B_{12b} are less susceptible to degradation as evident from the values of the rate constants at pH 1–3. The enhancement in the rate of reaction in the pH range 1–5 is due to gradual ionization of AH_2 to form the ascorbate monoanion (AH^-) and the reduction in the rate above pH 5 is a result of the loss of AH^- anions by oxidation. The degradation of B_{12} and B_{12b} involves the reduction of Co^{3+} to Co^{2+} in the corrin ring by AH_2, followed by the oxidation to B_{12b} and/or the ring cleavage products. These reactions would depend on the pH, concentration of AH_2 and the redox potential of the Co^{3+}/Co^{2+} system.

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