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CHROMATOGRAPHIC STUDY OF PHOTOLYSIS OF AQUEOUS CYANOCOBALAMIN SOLUTION IN PRESENCE OF VITAMINS B AND C

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ABSTRACT

Aqueous cyanocobalamin solutions (pH 1-7) have been photolysed in the presence of individual B (thiamine HCl, riboflavin, nicotinamide and pyridoxine HCl) and C (ascorbic acid) vitamins with visible light. The degraded solutions were subjected to thin-layer chromatography using several solvent systems and the R_f values of the vitamins and their photoproducts were determined. The major photoproducts have been identified by comparison of their R_f values with those of the authentic compounds. Cyanocobalamin leads to the formation of hydroxocobalamin. Thiamine HCl gives rise to 4-methyl-5-(ß-hydroxyethyl) thiazole and 2-methyl-4-amino-5-hydroxymethyl-pyrimidine in trace amounts whereas riboflavin degrades extensively to formylmethylflavin and lumichrome, and to a smaller extent to lumiflavin and carboxymethylflavin. Ascorbic acid is oxidized to dehydroascorbic acid. Nicotinamide and pyridoxine HCl do not undergo any degradation. The extent of degradation depends upon the pH.

INTRODUCTION

Aqueous solutions of cyanocobalamin (vitamin B_{12}) are degraded on exposure to light (Veer *et al.*, 1950; Baxter *et al*., 1953; Bayer, 1964; Pratt, 1964, 1972; Vogler *et al*., 1976; Ahmad, 2001; Ahmad and Hussain, 1993a; Ahmad *et al*., 1992, Ansari, 2002) or on interaction with other vitamins/reducing agents (Blitz *et al*., 1954, 1956; Feller and Macek, 1955; Macek, 1960; Ahmad and Hussain, 1993b; Ismail, 1995). The main photoproduct of this reaction, hydroxocobalamin (vitamin B_{12b}), has been identified by chromatographic methods (Kirchbaum, 1981; Ahmad *et al*., 1992; 2003). In the presence of other B or C vitamins such as thiamine hydrochloride, riboflavin, nicotinamide, pyridoxine hydrochloride and ascorbic acid, the photolysis of cyanocobalamin is accelerated and the individual B or C also tend to degrade (Patel and Soni, 1964; Ahmad, 2001; Ahmad and Hussain, 1992; Ahmad *et al*., 2003; Ansari, 2002). The object of present study is to identify the degradation products of B and C vitamins in cyanocobalamin solutions, photolysed under specified conditions, by thin-layer chromatography and to observe the effect of pH on product formation.

MATERIALS AND METHODS

Vitamins: Thiamine hydrochloride (B_1), riboflavin (B_2), nicotinamide (B_3), pyridoxine hydrochloride (B_6), cyanocobalamin (B_{12}), hydroxocobalamin (B_{12b}) and ascorbic acid (C) were obtained from Sigma Chemical Co.

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Vitamin photoproducts: 4-Methyl-5-(ß-hydroxyethyl) thiazole, lumichrome, lumiflavin and dehydroascorbic acid were obtained from Sigma Chemical Co. 2-Methyl-4-amino-5-hydroxymethylpyrimidine, formylmethylflavin and carboxymethylflavin were synthesized by the methods of Neal (1968), Fall and Petering (1956) and Fuchumachi and Sakurai (1954), respectively.

All reagents and solvents used were of analytical grade or of the highest purity available from BDH/Merck.

Photolysis

Aqueous cyanocobalamin solutions (5×10^{-5} M) containing the individual B (5×10^{-4} M) and C (5×10^{-3} M) vitamins in the pH range 1.0-7.0 (100 ml) were irradiated with Philips HPLN 125 W high pressure mercury vapour fluorescent lamp (emission at 405, 436 and 545 nm) fixed horizontally at a distance of 30 cm. The intensity of the lamp, determined by ferrioxalate actinometry was $1.14 \pm 0.10 \times 10^{17}$ quanta S^{-1}.

Thin-layer Chromatography

The photolysed solutions of cyanocobalamin containing other B and C vitamins were subjected to thin-layer chromatography (TLC) using the following adsorbents and solvent systems.

Adsorbents

A_1: Silica gel G.
A_2: Silica gel G_{254}.
A_3: Whatman CC 41 cellulose powder.

Solvent systems

S_1: 1-Butanol-acetic acid-0.066 M KH_2PO_4 –methanol (36:18:36:10,v/v) (Cima and Mantovan, 1962).
S_2: Methanol-water (95:5, v/v) (Covello and Schettino, 1964).
S_3: Pyridine-2-butanol-water (66:17:17, v/v) (Johnson and Goodwin, 1963).
S_4: Chloroform-methanol-ammonia solution (30%) (70:30:1, v/v) (Hameed, 1973).
S_5: 1-Butanol-acetic acid-water (40:10:50, v/v organic phase) (Ahmad *et al*., 1980).
S_6: 1-Butanol-1-propanol-acetic acid-water (50:30:2:18, v/v) (Ahmad *et al*., 1980).
S_7: Distilled water (Bollinger, 1965).
S_8: Acetic acid-acetone-methanol-benzene (5:5:20:70, v/v) (Ganshirt and Malzacher, 1960; Ishikawa and Katsue, 1964).
S_9: Ethanol-10% acetic acid (90:10, v/v) (Bolliger, 1965).

The spots were detected visually, under UV light (254/365 nm) or on spraying with a location reagent (3% aqueous phenylhydrazine solution).

RESULTS AND DISCUSSION

Cyanocobalamin is a component of liquid vitamin preparations and is used in µg quantities. Aqueous solutions of cyanocobalamin are liable to degradation on exposure to light and the degradation is accelerated by some B and C vitamins (Ahmad, 2001; Ansari, 2002; Ahmad *et al.*, 2003). In the present work cyanocobalamin solutions were photolysed at pH 1-7 in the presence of

individual B/C vitamins and the degraded solutions were subjected to TLC using a number of solvent systems developed by various workers. In addition to these several other solvent systems have also been reported for the separation and identification of water-soluble vitamins (Bollinger and Konig, 1969; Katsui, 1972; Moffat, 1986). The R_f values of the vitamins and the photoproducts detected in this study are reported in Table 1. The individual vitamins and their photoproducts were identified by comparison of their R_f values and colour of spot fluorescence with those of the authentic compounds in specific solvent systems given under thin-layer chromatography.

Hydroxocobalamin (solvent systems S_1 and S_2) was found to be the only photoproduct of cyanocobalamin at pH 1-7 alone, or in the presence of individual B/C vitamins. The relative intensity of the TLC spots appears to depend upon the rate of reaction and the added vitamin and decreases with an increase in pH. Hydroxocobalamin is susceptible to further degradation and may give rise to irreversible oxidation products (Macek, 1960; Bonnett, 1963; Connors *et al.*, 1986), however, no other degradation products of this reaction were detected by TLC using the present solvent systems. This suggests that hydroxocobalamin is the only primary photoproduct of cyanocobalamin in aqueous solutions and its formation is influenced by vitamins of B/C group.

Thiamine hydrochloride (solvent systems S_3 - S_4) showed trace amounts of 4-methyl-5-(β-hydroxyethyl)thiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine at pH 4-7, the intensity of their spots increased with an increase in pH indicating that their rate of formation is pH dependent. Riboflavin (solvent systems S_5 - S_6) showed extensive degradation with an increase in pH. The photoproducts detected in these reactions included formylmethylflavin and lumichrome (major) at pH 2-4 and lumiflavin and carboxymethylflavin (minor) at pH 7. Two unknown products (yellow fluorescence) were also detected at pH 7 (Table 1).

Ascorbic acid (solvent systems S_8 - S_9) was extensively degraded to dehydroascorbic acid (pH 4-7) probably by chemical and photochemical oxidation. It was detected by spraying with aqueous phenylhydrazine solution. The intensity of the spots increased with an increase in pH. Nicotinamide and pyridoxinehydrochloride (solvent systems S_7 - S_8) did not show any photoproducts under the present conditions. The photoproducts of B and C vitamins detected in this study are in agreement with those reported for thiamine HCl (Vaid, 1998), riboflavin (Fasihullah, 1988; Ahmad and Rapson, 1990) and ascorbic acid (Shaikh, 1997).

One of the problems in the TLC study of vitamins and their photoproducts is that no single solvent system can separate all these compounds in degraded solutions and, therefore, several solvent systems have to be used to achieve the separation of individual vitamins and their photoproducts. Similarly, different combinations of adsorbents and solvent system (Table 1) give better resolution of certain vitamins and their photoproducts. Since most of the vitamins and their photoproducts are either fluorescent (365 nm) or quench fluorescence (254 nm), the sensitivity of detection is very high and even trace amounts of the photoproducts can be detected. In certain cases (e.g. photolysed solutions of cyanocobalamin and riboflavin), some minor unknown products of riboflavin were also detected, the R_f values of these compounds in solvent system S_5 and S_6 are reported in Table-1.

It may be concluded from the present study that there is a considerable possibility of mutual interactions between B and C vitamins, in aqueous solution on exposure to light, giving rise to a number of photoproducts as described above. The formation of the photoproducts of B and C vitamins increases with pH except that of cyanocobalamin and, therefore, a pH around 4 may be

more suitable to achieve a relatively greater photostability of all B and C vitamins including cyanocobalamin in pharmaceutical preparations.

Table 1
R_f values of B and C vitamins and their photoproducts

Adsorbent	A_1	A_1	A_2	A_2	A_3	A_3	A_1	A_1	A_2	A_2	Detection**	
Solvent system	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_8	S_9	Colour	Flour 254/ 365 nm
Cyanoco-bal-amin	0.46	0.42									red	
*Hydroxo-cobalamin	0.25	0.05									red	
Thiamine HCl			0.03	0.00								dark
*4-Methyl-5-(β-hydroxy-ethyl) thiazole			0.70	0.58								dark
*2-methyl-4-amino-5-hydroxyme-thylpyri-midine			0.29	0.32								dark
Riboflavin					0.47	0.27						yellow
*Formyl-methylflavin					0.70	0.70						yellow
*Lumi-chrome					0.66	0.63						blue
*Lumiflavin					0.53	0.41						yellow
*Carboxy-methylflavin					0.38	0.20						yellow
*Unknown					0.53	0.50						yellow
*Unknown					0.50	0.22						yellow
Nicotina-mide							0.48	0.44				dark
Pyridoxine HCl							0.52	0.11				dark
Ascorbic acid									0.30	0.49		dark
*Dehydro-ascorbic acid									0.22	0.73	Orange red	

*Photoproducts of B and C vitamins.
**Colour: visual (red) or on spraying with a location reagent (orange red); fluorescence under UV light (254nm, dark; 365 nm, yellow/blue).

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