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Quantitative thin-layer chromatography of cobalamin complexes

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Abstract: Quantitative thin-layer chromatography was used to study the hydroxo-, cyano- and dicyanocobalamin transformation equilibria. A new stereoisomer of cyanocobalamin was shown to be formed from dicyanocobalamin on the chromatographic plate. The formation, stability and UV spectrum of this compound (isocyanocobalamin) was studied.

Keywords: *Quantitative thin-layer chromatography; cobalamin complexes; reflectance spectroscopy; cyanocobalamins.*

Introduction

The hydroxocobalamin–cyanocobalamin and cyanocobalamin–dicyanocobalamin equilibria have been studied by several different methods [1–4], which often yield different equilibrium constants [1, 3]. If side-reactions, decomposition or re-transformation of the complexes formed during the chromatographic process do not disturb the equilibria, chromatography can be used to investigate them by separation of the different complexes. Recently, thin-layer chromatography (TLC) has been applied to qualitative studies of organic complexes formed in solution [5–8]; no data have been reported, however, on the use of TLC for quantitative investigations of complex equilibria, or for studying the complex formation of cobalamin. The main aim of the present work was to investigate the equilibria between hydroxo-, cyano- and dicyanocobalamin in the presence of potassium cyanide, using a quantitative thin-layer chromatographic method developed for the separation of cobalamin derivatives [9].

Experimental

TLC separations were performed on precoated 20 × 20 cm Kieselgel 60 chromatoplates (E. Merck, Darmstadt, F.R.G.) using a mixture of acetone–acetonitrile–isopropanol–diethylamine–5% aqueous ammonium hydroxide (30:30:10:3:27, v/v) as eluent [9]. Samples (corresponding to about 2.5 μg of cyanocobalamin) were transferred to TLC plates in 1 cm lines and the chromatogram was developed for up to 15 cm in a

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presaturated chamber protected from light. After the run the plate was dried in an air stream at room temperature and the separated spots were evaluated by densitometry using an Opton KM-3 chromatogram-spectrophotometer (Opton Feintechnik GmbH, Oberkochen, F.R.G.).

All solvents and reagents were of analytical grade and were obtained from Reanal (Budapest, Hungary). The compounds investigated were prepared at Chemical Works of Gedeon Richter Ltd. (Budapest, Hungary) and were considered to be of the highest available quality on the basis of HPLC examination.

Results and Discussion

In aqueous solution the following cobalamin complexes can be formed:

$$MOH + HCN \rightleftharpoons MCN + H_2O \quad (1)$$

$$MH_2O^+ + HCN \rightleftharpoons MCN + H_3O^+ \quad (2)$$

$$MCN + CN^- \rightleftharpoons M(CN)_2^- \quad (3)$$

where MOH = hydroxocobalamin, MH_2O^+ = aquocobalamin, MCN = cyanocobalamin, and $M(CN)_2^-$ = dicyanocobalamin, respectively.

The interaction between hydroxocobalamin and cyanide ions was studied using the TLC method recently published [9]. Cyanide ions were added in increasing amount to aqueous solutions of hydroxocobalamin of constant concentration, and after 2.0 min the cyanide complexes formed were separated on silica gel. A series of typical chromatograms (Fig. 1a, b and c) shows that cyanide ions reacted with hydroxocobalamin to form

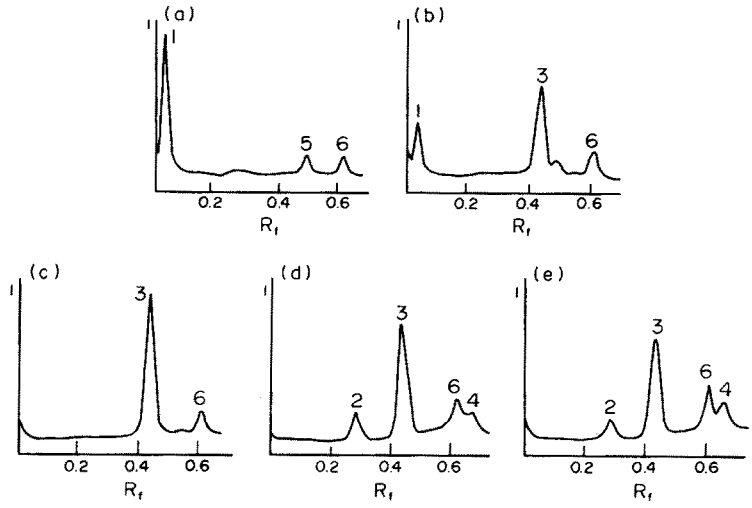

Figure 1
Chromatogram showing the effect of cyanide ions in the cobalamin system. Plate: Kieselgel 60, 20 × 20 cm, line application; eluent: acetone–acetonitrile–isopropanol–diethylamine–5% aqueous NH_4OH (30:30:10:3:27 v/v); evaluation by densitometry at 360 nm in reflectance mode, R_f-values: **1**: hydroxocobalamin (0.03), **2**: isocyanocobalamin (0.29), **3**: cyanocobalamin (0.44), **4**: dicyanocobalamin (0.69), **5**, **6**: unknown (0.50; 0.62). Concentrations: hydroxocobalamin: 1.05×10^{-4} M; sodium acetate: 0.1 M. Potassium cyanide: a, none, b, 6.25×10^{-5} M, c, 1.25×10^{-4} M, d, 6.25×10^{-2} M, e, 1.07×10^{-1} M.

cyanocobalamin; in the presence of excess cyanide, no hydroxocobalamin was detected (Fig. 1c). Increasing the cyanide ion excess produced, in addition to the dicyanocobalamin peak (No. 4), a new peak (No. 2) indicating the formation of a new compound, named hereafter isocyanocobalamin (Fig. 1d and e). The effect of the potassium cyanide concentration on the quantity of isocyanocobalamin produced is shown in Fig. 2. When a solution of cyanocobalamin containing cyanide ions was stored under defined conditions and applied to the TLC plate from time to time, the peak area of isocyanocobalamin remained constant. The quantity of isocyanocobalamin formed markedly depends, however, on the time elapsing between the application of the sample on the layer and the start of the run. Figure 3 indicates that the formation of isocyanocobalamin required more than 4 min between sample application and elution.

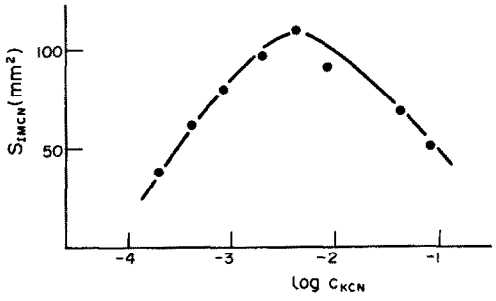

Figure 2
Dependence of isocyanocobalamin peak area S on potassium cyanide concentration. $c_{cyanocobalamin} = 1.43 \times 10^{-4}$ M, other conditions as in Fig. 1.

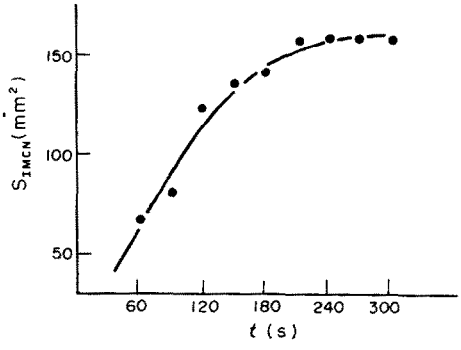

Figure 3
Dependence of isocyanocobalamin formation on the interval between sample application and elution. $c_{cyanocobalamin} = 2.21 \times 10^{-3}$ M, $c_{KCN} = 7.68 \times 10^{-2}$ M, other conditions as in Fig. 1.

To clarify the nature of isocyanocobalamin, the separated spots were dissolved both in distilled water and in 0.01 M aqueous potassium cyanide solution and re-chromatographed. The results are shown in Fig. 4.

Figure 4a shows the initial chromatogram. When isocyanocobalamin is dissolved in water it is converted to cyanocobalamin, dicyanocobalamin and a photochemical decomposition product (Fig. 4b). This last compound has not been identified; it probably does not contain a cyano group. When isocyanocobalamin was dissolved in 0.01 M potassium cyanide the chromatogram obtained (Fig. 4c) was similar to the chromatogram of cyanocobalamin dissolved in potassium cyanide solution (Fig. 4e). Dissolving and re-separating the spot of cyanocobalamin with water as eluent, no isocyanocobalamin was seen in the chromatogram (Fig. 4d); when the same spot was dissolved in potassium cyanide solution, the chromatogram obtained (Fig. 4e) was similar to the

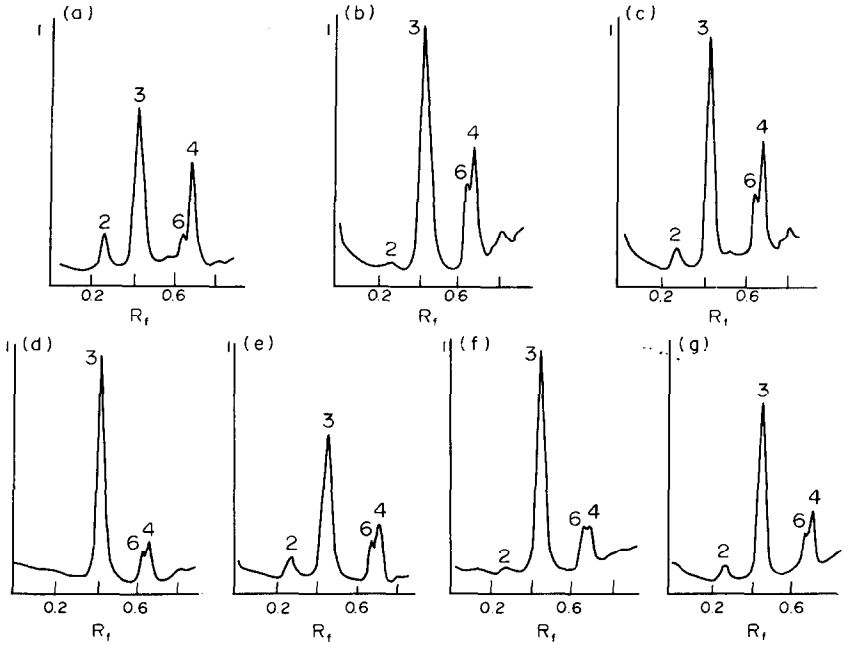

Figure 4
Chromatograms of the eluted spots dissolved in distilled water and in 0.01 M potassium cyanide and rechromatographed. a: original chromatogram, $c_{cyanocobalamin} = 2.21 \times 10^{-3}$ M, $c_{KCN} = 7.68 \times 10^{-2}$ M, b: isocyanocobalamin in water, c: isocyanocobalamin dissolved in 0.01 M potassium cyanide, d: cyanocobalamin in water, e: cyanocobalamin in 0.01 M potassium cyanide, f: dicyanocobalamin in water, g: dicyanocobalamin in 0.01 M potassium cyanide. Other conditions were the same as in Fig. 1.

original one (Fig. 4a). When the dicyanocobalamin spot was dissolved in water most of it was transformed to cyanocobalamin, while dissolving it in potassium cyanide produced detectable amounts of isocyanocobalamin. The relative abundances of each compound formed during these experiments are summarized in Table 1.

Table 1
Rechromatography of TLC components

Component	No. of spot	Solvent	Relative abundance (%)			
			2	3	4	6
Original sample	—	7.68×10^{-2} M KCN	10.2	65.1	18.4	6.3
Isocyanocobalamin	2	water	1.6	64.6	18.8	15.0
Isocyanocobalamin	2	0.01 M KCN	6.3	61.0	24.0	8.7
Cyanocobalamin	3	water	—	82.8	10.8	6.4
Cyanocobalamin	3	0.01 M KCN	7.2	68.2	16.8	7.8
Dicyanocobalamin	4	water	2.9	76.7	9.7	10.7
Dicyanocobalamin	4	0.01 M KCN	5.9	69.2	16.6	8.3

Conditions as in Fig. 4.

These experiments indicate that isocyanocobalamin was formed on the TLC plate when the sample was transferred to it. The dependence of the isocyanocobalamin formation on the cyanide ion concentration suggests that it was formed from dicyanocobalamin. In aqueous solution it was transformed to cyanocobalamin. The UV-visible reflectance spectra of cyano- and isocyanocobalamin were found to be practically identical, showing maxima at 361, 408, 520 and 550 nm, and at 361, 408, 520 and 546 nm respectively. On the basis of all these experiments it is concluded that the new spot appearing on the chromatogram and called isocyanocobalamin was a stereoisomer of cyanocobalamin, the two compounds differing only in the coordination site of cyanide in the complex. Thus isocyanocobalamin is assumed to be formed by the inversion of the ligands bound on the axial coordination sites of the central cobalt atom. This assumption needs further structural investigation. The formation of a stereoisomer of cobalamin has also been observed in the case of Factor $B_{12\ III}$: the latter is similar to cyanocobalamin, the only difference being that it contains 5-hydroxy-benzimidazole instead of 5,6-dimethyl-benzimidazole. Figure 5 shows chromatograms of a 1:1 mixture of cyanocobalamin and Factor $B_{12\ III}$ made in cyanide-free and cyanide-containing solutions. The formation of the isomer discussed is seen in Fig. 5b.

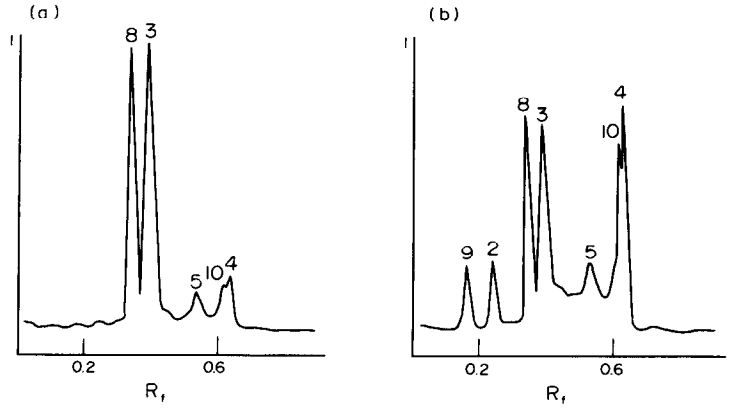

Figure 5
Isomer formation from Factor $B_{12\ III}$: $c_{cyanocobalamin} = 3.69 \times 10^{-4}$ M, $c_{Factor\ B_{12\ III}} = 3.73 \times 10^{-4}$ M. a: chromatogram in the absence of cyanide ions, b: chromatogram in the presence of cyanide ions. R_f-values: **2**: isocyanocobalamin (0.27), **3**: cyanocobalamin (0.41), **4**: dicyanocobalamin (0.65), **8**: Factor $B_{12\ III}$ (0.35), **9**: isomer of Factor $B_{12\ III}$ (0.17), **10**: dicyano derivative of Factor $B_{12\ III}$ (0.63), **5**: unknown (0.53). Other conditions were the same as in Fig. 1.

The complex equilibria in the hydroxo-, cyano-, dicyanocobalamin system are summarized in Fig. 6. The formation constant of dicyanocobalamin is given by

$$K_2 = \frac{[M(CN)_2^-]_s}{[MCN]_s[CN^-]_s} \tag{4}$$

where $[M(CN)_2^-]_s$, [MCN] and $[CN^-]_s$ are the equilibrium concentrations of dicyanocobalamin, cyanocobalamin and cyanide ions, respectively. Exact knowledge of the isomerization reaction is necessary since the concentration of cyanocobalamin measured on the thin-layer plate after separation differs from its concentration in solution:

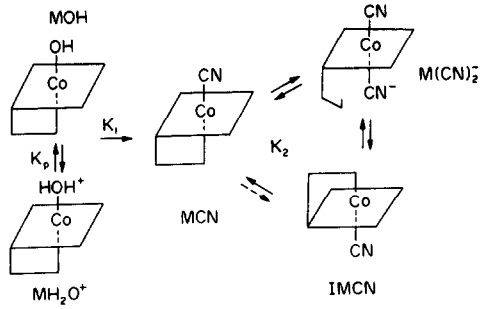

Figure 6
Complex equilibria in the hydroxo-, cyano-, dicyanocobalamin system.

$$[MCN]_m = [MCN]_s + [MCN]_f, \tag{5}$$

where [MCN] is the measured concentration of cyanocobalamin on the layer, $[MCN]_f$ the concentration of cyanocobalamin formed from dicyanocobalamin on the layer, and $[MCN]_s$ the equilibrium concentration of cyanocobalamin in the solution. Decomposition of dicyanocobalamin on the layer results in the formation of cyano- and isocyanocobalamin. The ratio of the cyano- and isocyanocobalamin formed can be calculated from the following formula:

$$k = \frac{[MCN]_f}{[IMCN]_f}, \tag{6}$$

where $[IMCN]_f$ is the concentration of isocyanocobalamin. From equations (5) and (6)

$$[MCN]_m = [MCN]_s + k\,[IMCN]_f. \tag{7}$$

Determining the peak areas of cyanocobalamin and isocyanocobalamin after decomposition times $t_1, t_2 \ldots t_n$ on the TLC plate and plotting them against each other (Fig. 7)

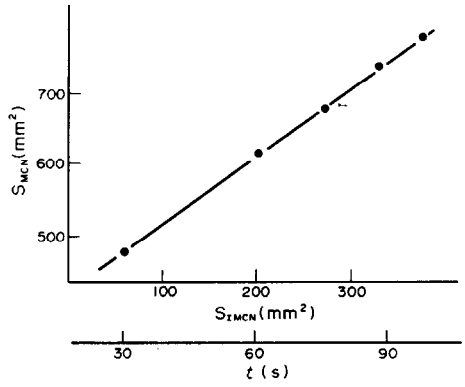

Figure 7
Dependence of cyanocobalamin and isocyanocobalamin concentration on the time between sample application and TLC elution $c_{cyanocobalamin} = 9.14 \times 10^{-4}$ M, $c_{KCN} = 8.70 \times 10^{-3}$ M, pH = 10.05 samples in 0.1 M sodium acetate detection wavelength: 360 nm.

reveals a linear relationship giving the slope k and the intercept $[MCN]_s$ ($y = 0.93\ x + 424$). The value of k depends on the total concentrations of cyanocobalamin and cyanide ions and also on the pH of the solution. When k-values determined in solutions of constant total cyanocobalamin and cyanide concentration, are plotted against pH, a linear relationship is obtained in the pH range 8.9–10.1 (Fig. 8) ($k = -3.01$ pH $+31.42$).

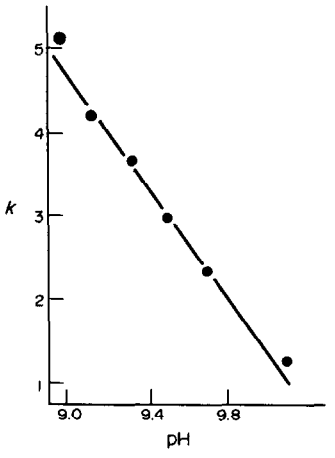

Figure 8
Dependence of the cyanocobalamin:isocyanocobalamin ratio on pH. $c_{cyanocobalamin} = 2.94 \times 10^{-4}$ M, $c_{KCN} = 6.70 \times 10^{-3}$ M dissolved in 0.3 M sodium hydrogen carbonate–sodium carbonate buffer solution.

To calculate the formation constant of dicyanocobalamin, k values have been determined for every solution used in the equilibrium measurements. The equilibrium concentrations of dicyanocobalamin, cyanocobalamin and cyanide ions ($[M(CN)_2^-]$; $[MCN]_s$; $[CN^-]_s$) can be expressed as follows:

$$[M(CN)_2^-]_s = [M] - [MCN]_s \tag{8}$$

$$[MCN]_s = [MCN]_m - k\,[MCN]_f \tag{9}$$

$$[CN^-]_s = \frac{C - 2[M(CN)_2^-]_s - [MCN]_s}{K_D^{-1}\,[H^+] + 1} \tag{10}$$

where [M] = the total concentration of cyanocobalamin; C = [HCN] + $[CN^-]$ + 2 $[M(CN_2^-]_s$ + $[MCN]_s$, the total concentrations of cyanide ions; $K_D = 1.39 \times 10^{-9}$, the dissociation constant of hydrogen cyanide; $[IMCN]_f$ is the measured concentration of isocyanocobalamin, $[MCN]_m$ is the measured concentration of cyanocobalamin, and k the ratio of cyano- and isocyanocobalamin formed on the layer. The formation constants determined at different pH values, and the corresponding k-values, are shown in Table 2.

Table 2
Formation constants of dicyanocobalamin determined by quantitative TLC.

pH	log K_2*	k	log K_2‡
9.20	3.48 ± 0.14†	2.00	4.10 ± 0.16†
9.50	4.02 ± 0.06†	1.26	4.08 ± 0.05†
10.05	4.80 ± 0.13†	1.00	4.11 ± 0.11†

0.1 M sodium acetate buffers used throughout.
* Without taking into account isocyanocobalamin formation.
† Calculated from seven experiments; mean ± standard deviation.
‡ Taking into account isocyanocobalamin formation.

Table 3
Equilibrium constants determined by quantitative TLC

Reaction	log K		Ref.
	A	B	
$MOH + HCN \rightleftharpoons MCN + H_2O$	> 10	6.45–6.85	1
		> 12	3
$MCN + CN^- \rightleftharpoons M(CN)_2^-$	4.10	4	2
		4	3
		3.8	4

A = Present results.
B = Literature data.

Notwithstanding the possible formation of isocyanocobalamin on the plate, TLC was found suitable for the determination of the formation constant of dicyanocobalamin. The results obtained by the quantitative TLC method are compared with previous values in Table 3. This comparison indicates the applicability of TLC to the study of this type of coordination equilibrium.

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