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Kinetics of Spontaneous Conversion of Methylcobalamin into Hydroxocobalamin in Aqueous and Methanol Solutions HPLC/MS

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Abstract **Original Research Article**

This work aimed to determine the stability of methylcobalamin in injection solutions under light-protected conditions, identify the products of its spontaneous transformation, and determine the kinetics of these transformations. We have found that methylcobalamin is unstable and transforms into hydroxocobalamin spontaneously at room temperature in aqueous and methanol solutions protected from light. Kinetics, reaction products, and rate constants have been determined. A method for the qualitative and quantitative determination of methylcobalamin and its conversion products is proposed.

Keywords: vitamin B12, methylcobalamin, hydroxocobalamin, kinetics, HPLC/MS.

Abbreviations

K – rate constant
RT – room temperature.
RSD – Relative standard deviation.

INTRODUCTION

Methylcobalamin is one of four members of the B12 family. The light sensitivity of methylcobalamin has long been known. The products of its degradation under the action of light, their UV (Mehmood Y *et al.,* 2015), and MS spectra are described, and found that one of the products of photodegradation is hydroxocobalamin (Chamle A *et al.,* 2019). In works (Campos-Giménez E *et al.,* 2008; Heudi O *et al.,* 2006; Van Wyk J *et al.,* 2010), it is proposed to determine vitamin B12 by HPLC without distinguishing which substance from the B12 family is determined. Other methods for determining B12 are described in the review (Tsiminis G *et al.,* 2017). We know from private correspondence that in many laboratories B12 is determined by the spectrophotometric method (Bruno R *et al.,* 1981) or even colorimetrically (Ahmed F *et al.,* 2003). The method for determining cobalt colorimetrically is more than 200 years old, but it is no worse than the current HPLC for B12, since the total molarity of B12 or cobalt in solution is determined, and it is assumed that these molars are equal. The disadvantage of existing methods is revealed when it is necessary to determine not the total content of vitamin B12, but only one member of the family, namely, methylcobalamin. We used the Agilent 1260 HPLC/DAD/MS instrument to analyze methylcobalamin and developed a new method.

MATERIALS AND METHODS

Chemicals

Water HPLC grade purchased from Agilent. HPLC-grade solvents were used. Reference standards of cyanocobalamin, methylcobalamin, and hydroxocobalamin were from Sigma. A vehicle injection solution containing methylcobalamin, and minor components for solution stabilization, was kindly presented by Infuserve America Inc.

Samples and standards

Solutions of methylcobalamin, hydroxocobalamin, and cyanocobalamin were prepared and stored in glass containers protected from light at RT.

Preparation of standard solutions

An accurately weighed 5 mg of each B12 standard was dissolved in 10 mL of methanol. The

injection solution was diluted 10 times with methanol just before testing. All the solutions were filtered through the 0.45µm cellulose acetate membrane filter.

Instrument

Agilent HPLC/DAD/MS instrument consists of the following components: Diode Array Detector (DAD). The following wavelengths have been established: 222 nm and 350; Normal-phase Column Poroshell Ascentis Si, 250x4.6mm with particles size 5µm; Quaternary pump with the flow: 0.8 ml/min, and high-pressure limit of 600 bar. Single Quadrupole mass selective detector with electrospray ionization and 150 V fragmentor. The gas temperature is 300°C, the capillary voltage is 4000 V, and the nebulizer is 15psi.

Mobile phase (MP): methanol/water 80/20 + 0.1% formic acid; 25°C; flow- 0.8ml/min

Qualitative analysis

Qualitative analysis of the components was carried out using UV and MS spectra specific to each of the components.

Quantitative Analysis

The method for the quantitative determination of methylcobalamin is described below. Quantitative determination of hydroxocobalamin was done using standard according to the calibration curves.

The system's suitability

The system's suitability has been validated according to the (Evaluating System Suitability, 2019). Relative retention times for cyanocobalamin and hydroxocobalamin are 0.6 and 1.0, respectively, which proves the method is suitable.

Calibration curve and coefficient of correlation

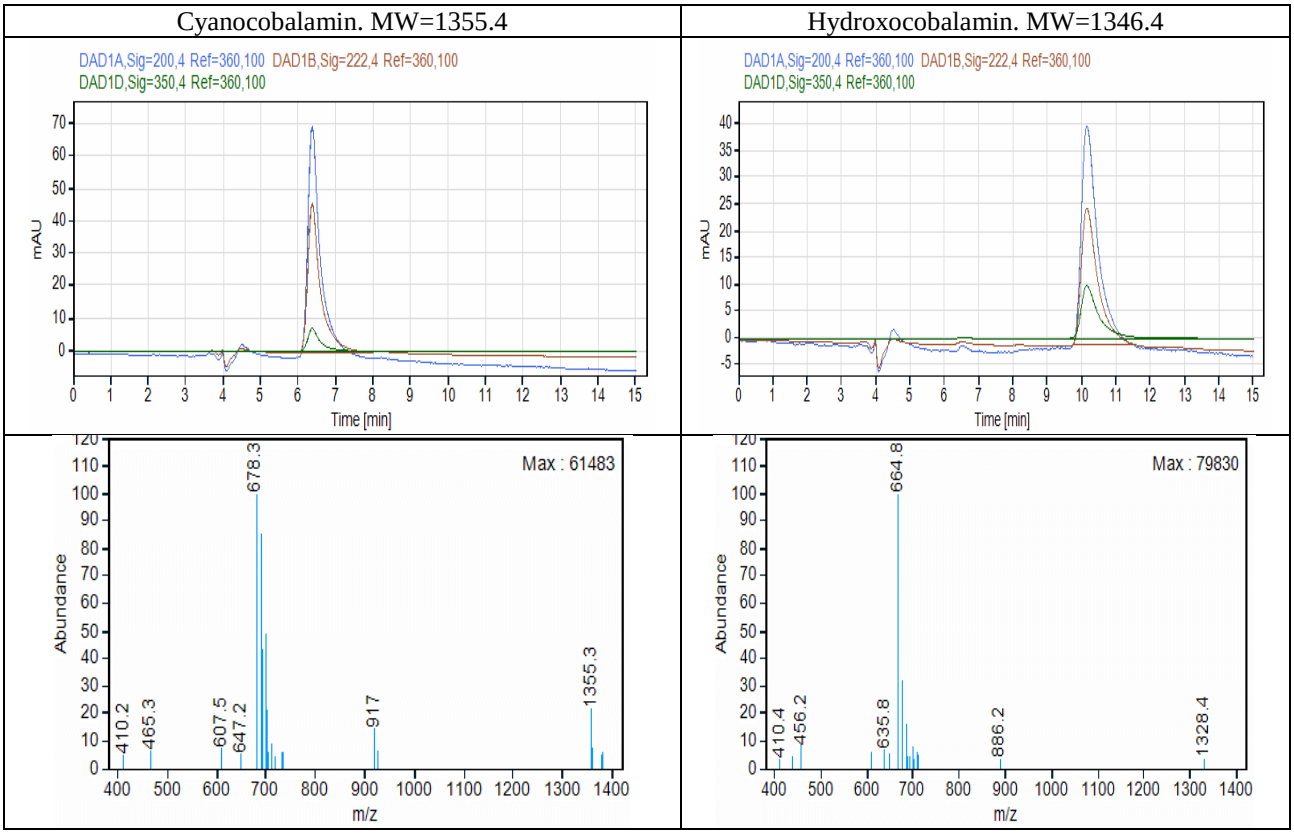
The concentration range of the calibration curve was chosen so that the expected concentration of the component was near the middle. In this range, the calibration curve should be strictly linear ($r \geq 0.999$).

Statistical analysis

Statistical analysis included calculating mean, standard deviation, relative standard deviation (RSD), and correlation coefficient (r). Results $p < 0.05$ were considered statistically significant. The Least-squares regression analysis was used.

RESULTS AND DISCUSSION

Figure 1 shows the chromatograms, MS, and UV spectra of standard solutions of cyanocobalamin and hydroxocobalamin. Cyanocobalamin and hydroxocobalamin are distinguishable both by retention time (6.5 and 10-11 minutes, respectively) and by MS spectra (678.3 and 664.8, respectively). The relative retention time of cyanocobalamin and hydroxocobalamin is 0.6 and 1.0, respectively, which indicates a good separation and adequacy of the chosen method.



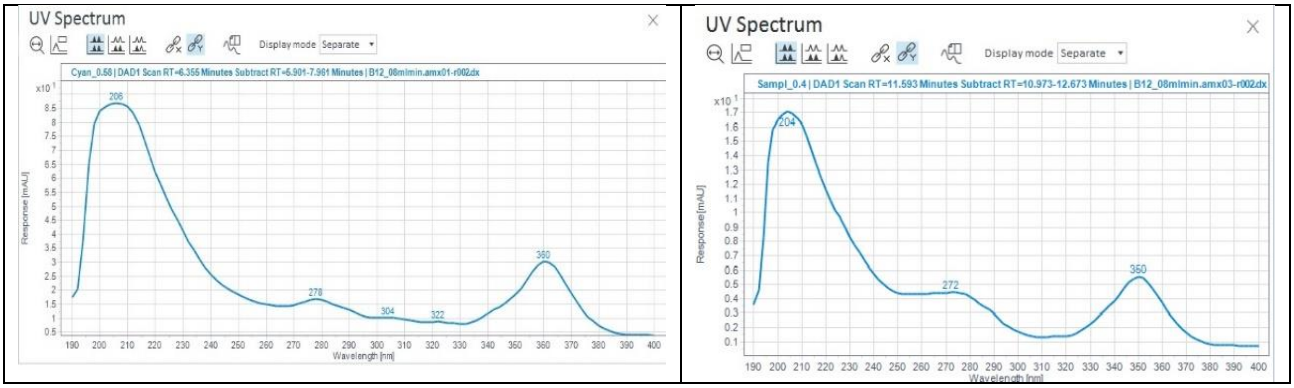


Figure 1: Chromatograms, MS spectra, and UV spectra of the standard methanol solutions of cyanocobalamin (0.5g/l) and hydroxocobalamin (0.2g/l) are presented

A freshly prepared standard methanol solution of methylcobalamin (MW=1344.4) gives two peaks on the chromatogram Figure 2. The relatively larger, leading peak probably belongs to methylcobalamin since the main signal of the mass spectrum - 672.9 corresponds to the doubly charged ion [methylcobalamin + H] ++, and a weak signal of the singly charged positive ion 1344.5 is also observed. The second peak obviously belongs to hydroxocobalamin, with a full agreement in terms of retention time and spectra.

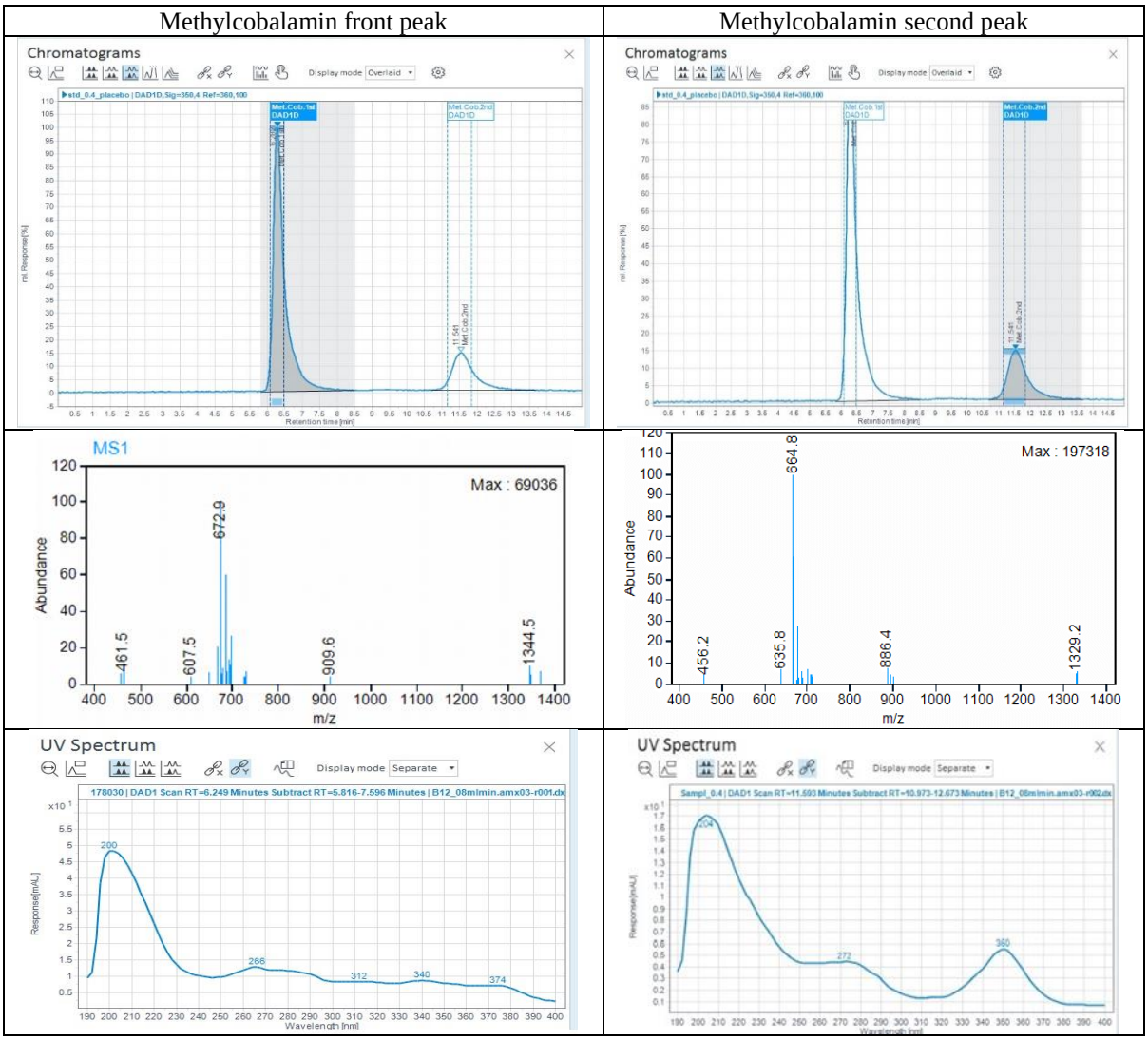


Figure 2: Chromatograms, MS spectra, and UV spectra of the standard methanol solutions of methylcobalamin (0.4g/l) are presented

During the aging of the standard solution, the ratio of the magnitudes of the two peaks continuously changes, namely, the methylcobalamin peak decreases and the hydroxocobalamin peak increases Figure 3.

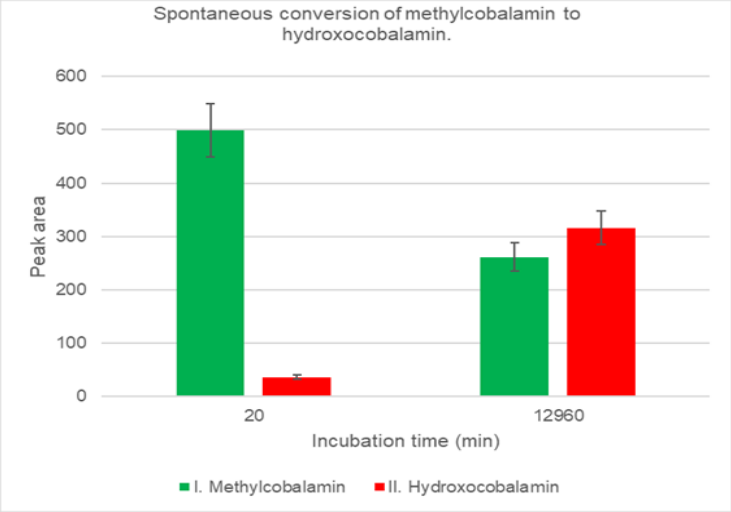


Figure 3: A standard methanolic solution of methylcobalamin 2.97 mM was incubated at room temperature in a glass container protected from light. The injection volume was 0.1µL. RSD<5%

Quantitative Analysis

Determining the content of methylcobalamin in samples using a calibration curve is not possible because the standard is constantly changing. We quantified methylcobalamin by molar absorbance at 222nm and 350nm wavelengths. The molar absorbance was found from the kinetic curves and the proposed reaction scheme. The conversion reaction of methylcobalamin presumably proceeds according to the scheme:

A + X ---> B --> c, d, e ……. **(1)**

Where "A" is methylcobalamin, "B" is hydroxocobalamin, "X" is an unknown component of the system, presumably air oxygen, and "c", "d", and "e" are the breakdown products of B12.

We assume that the concentration of component X in the solution is constant, then its concentration is included in the reaction rate constant. In this case, the reaction scheme is simplified (1a):

A ---> B ---> c, d, e. ……. **(1a)**

The validity of our assumption is confirmed experimentally.

According to this scheme (1a), the amount (or peak area) of methylcobalamin in the sample decreases exponentially:

A = A0 * exp (-K * t) ……. **(2)**

Where "A0" is the amount of methylcobalamin at the time t=0, i.e., at the moment of mixing the components of the solution, "K" is the reaction rate constant, and "t" is time.

Figure 4 shows how the peak area of methylcobalamin in a 0.00297M solution changes over time. The protected from light standard solution was incubated at RT the initial amount of methylcobalamin was 0.0003 µmol and 0.00015 µmol per injection. The icons are the experimental data, the dashed lines are the exponent (2) where K=0.00051(min^{-1}) and A0 are 587.7 (red) and 288.8 (green).

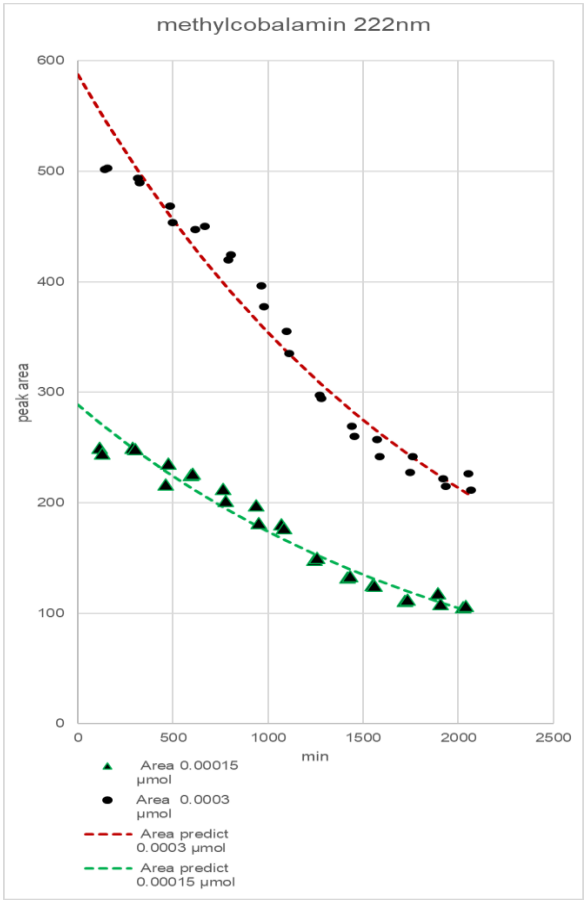


Figure 4: The time dependencies of the methylcobalamin degradation. The dashed lines are the theoretical model

The exponent (2) is linearized by taking a logarithm, formula 3.

ln(A)=ln(A0) -K*t ……. **(3)**

Now we can apply the Least Squares method to determine the slope, which corresponds to the reaction rate constant (K), and the point of intersection of the line with the y-axis, this point corresponds to the ln(A0) Figure 5 A, B. The desired initial peak area of methylcobalamin is calculated by the formula**:** A0=exp(ln(A0)).

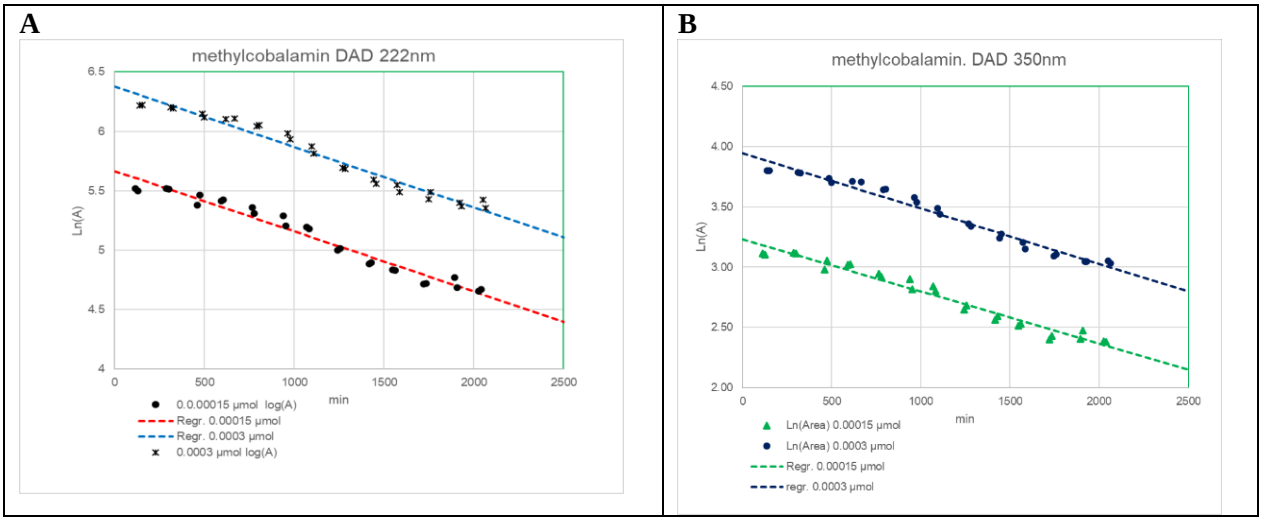


Figure 5 A, B: The time dependencies of the methylcobalamin degradation as a logarithm of peak area. The dashed lines are the theoretical model. Column A corresponds to DAD 222nm, and column B corresponds to DAD 350nm

Now we know the area of the chromatogram peak at the initial time (A0) and we know the amount of methylcobalamin in the sample at the initial time (M0 (µmol)), this is the amount that we added to the solution. Considering that the thickness of the optical cell (path length) is constant for a given instrument, we can calculate the value of the molar optical density using formula 4.

E=M0/A0 (µmol/area) ……. **(4)**

This value will be constant for certain measurement conditions: wavelength, temperature, and mobile phase composition. The molar optical densities at wavelengths of 222 and 350 nm are, respectively, E_{222}=5.1E-7(µmol/area) and E_{350}=5.9E-6(µmol/area). Here, "area" means the area of the corresponding peak in the chromatogram. Having the value of "E", we can determine the amount of methylcobalamin M (µmol) in a sample by the peak area (A) on a chromatogram at any time of incubation by formula 5:

M=E*A ……. **(5)**

Hydroxocobalamin is stable enough to be quantified using a standard. The dependence of the peak area at 222 and 350 nm on the amount of hydroxocobalamin is linear (r=0.9999) at least in the range from 6E-05 to 1E-03 micromoles. E_{222} and E_{350} are defined as 4.4E-07 and 1.13E-06 µmol/area, respectively. Now, having the "E" values, you can determine the amount of methylcobalamin and hydroxocobalamin in commercial solutions. The analysis data are shown in Table 1.

Table 1: Three injection solutions with declared methylcobalamin concentrations of 20, 1.0, and 20 g/L were analyzed by HPLC/DAD/MS at a DAD wavelength of 222 nm. RSD<5%.

Declared methylcobalamin concentration (g/L)	Sample injection amount (µmol)	Detected methylcobalamin (µmol)	Methylcobalamin recovery (%)	Detected hydroxocobalamin (µmol)	Total B12 recovery (%)
20.0	1.49E-03	1.32E-03	81	2.17E-04	96
1.0	1.49E-03	5.43E-04	37	1.24E-03	120
20.0	7.44E-04	6.38E-04	79	1.23E-04	96

As seen from the table, the content of methylcobalamin in the solutions is out of range and, therefore, the solution must be destroyed. But if the customer would ask to determine the total content of vitamin B12 in the solution, then for solutions of 20g/L the results are within the acceptable limit. Thus, the change of the name transfers the product from being destroyed into a quite suitable one.

CONCLUSION

It was established in the work that methylcobalamin in aqueous and methanol solutions is

converted into hydroxocobalamin and this transformation is not caused by light. A method for the qualitative analysis of methylcobalamin and hydroxocobalamin by retention time, UV, and MS spectra is substantiated. A method for the quantitative determination of methylcobalamin has been developed and tested, the validation of which is our next task. The main drawback of quantitative analysis, which must be overcome, is the long time for kinetic measurements.

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