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Analysis of Methylcobalamin and Hydroxocobalamin by HPLC/MS. Method Validation

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Abstract **Original Research Article**

Previously, we found that methylcobalamin spontaneously converts to hydroxocobalamin at room temperature in aqueous and methanol solutions protected from light. The kinetics and rate constants were determined. This work is devoted to the validation of the method for the quantitative determination of methylcobalamin and hydroxocobalamin in pharmaceutical preparations.

Keywords: Vitamin B12, methylcobalamin, validation, hydroxocobalamin, HPLC/MS.

INTRODUCTION

Methylcobalamin is one of four members of the vitamin B12 family. We recently discovered the ability of methylcobalamin to spontaneously convert to hydroxocobalamin in aqueous solutions (Yefimov S, 2022). This discovery made it clear why the chromatograms of methylcobalamin solution show the presence of hydroxocobalamin. The light sensitivity of methylcobalamin has been known for a long time (Mehmood Y *et al.*, 2015; Chamle A *et al.*, 2019), so all manipulations with methylcobalamin and its solutions are carried out in a dark room. Since the process of converting methylcobalamin to hydroxocobalamin in solution is continuous, it is natural to carry out a quantitative analysis of methylcobalamin together with hydroxocobalamin. The work is devoted to the validation of this method.

MATERIALS AND METHODS

Chemicals: Water HPLC grade purchased from Agilent. HPLC-grade solvents were used. Reference standards of 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 and hydroxocobalamin 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); 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: Methanol/water 80/20 + 0.1% formic acid; 25°C; flow- 0.8ml/min.

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 has been validated according to (CDER, 1994; Evaluating System Suitability, 2019; Shabir, 2023; Dr. Deepak, 2013).

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 ($r \geq 0.999$).

The precision/accuracy of the method was determined by the RSD value from the analysis of five samples of the same concentration under the same experimental conditions. The intraday and interday analysis was compared by RSD and recovery.

Limits of Detection (LOD)

LOD characterizes the sensitivity of a method; it is the minimum amount of a substance that can be measured by a given method, whereas the LOQ is the lowest concentration with acceptable linearity, accuracy, and precision. If the equation of the calibration curve is an equation of the first degree (straight line) then LOD is calculated by formula [*]:

[*] LOD = 3.3* σ/a

where the (σ) is the residual standard deviation of the regression line, and (a) is the slope of the line (European Medicines Agency. ICH, 2006). LOQ is 3 times LOD.

A measure of repeatability is the RSD of the mean of five independent tests of the samples of the same concentration.

To prove the specificity of the method, the peak areas of the component in the drug sample and the standard solution of the same concentration were compared. At the same time, the retention time of the component in both chromatograms was almost the same (RSD<1.2%). A minor discrepancy in the magnitude of the peak area indicated the specificity of the method for this component.

The flow rate, column temperature, and mobile phase composition were varied to demonstrate the **robustness** of the method. The tailing factor (T) and the number of theoretical plates (N) were calculated. The results were compared with the acceptable limits.

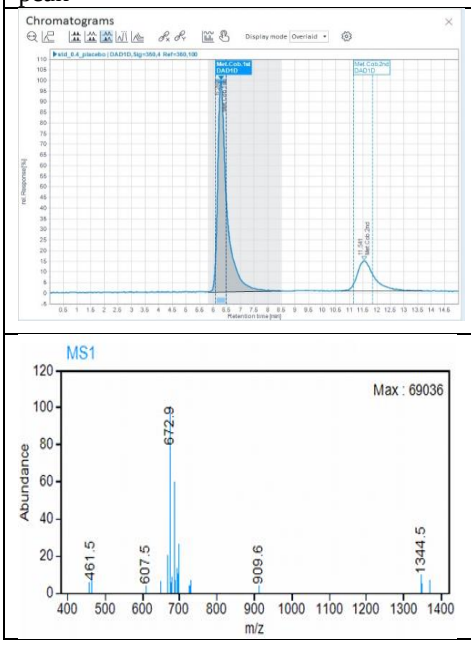
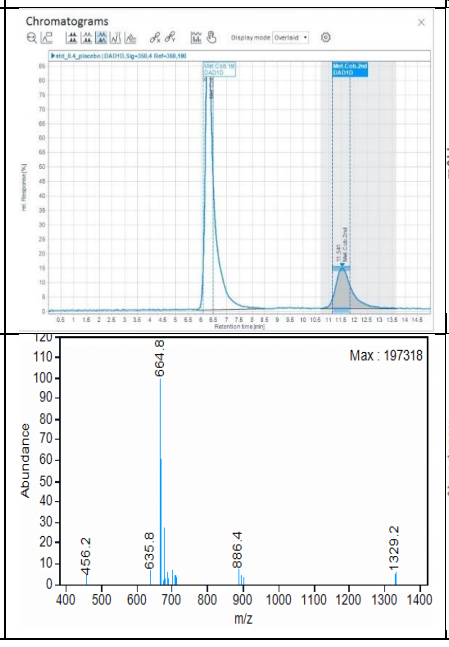
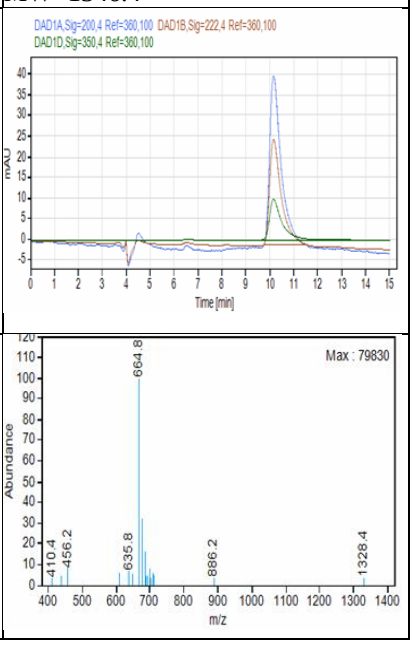
Statistical Analysis

Statistical analysis included calculating mean, standard deviation (S.D.), relative standard deviation (RSD), and correlation coefficient (r). Results $p < 0.05$ were considered statistically significant. The Least-squares regression analysis was used. In most cases, the OpenLAB CDS program performed the calculation automatically.

RESULTS AND DISCUSSION

Qualitative Analysis

A freshly prepared standard methanol solution of methylcobalamin (MW=1344.4) gives two peaks on the chromatogram Figure 1. The relatively larger, leading peak 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.

Methylcobalamin (MW=1344.4) front peak	Methylcobalamin second peak	Hydroxocobalamin standard. MW=1346.4
		

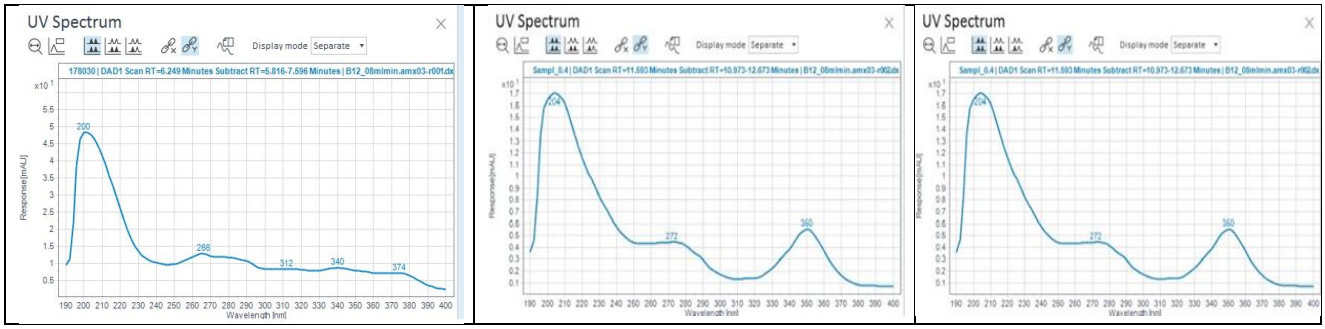


Figure 1: Chromatograms, MS spectra, and UV spectra of the standard methanol solutions of methylcobalamin and hydroxocobalamin

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, but the sum molarity (methylcobalamin + hydroxocobalamin) is changed not significant Figure 2.

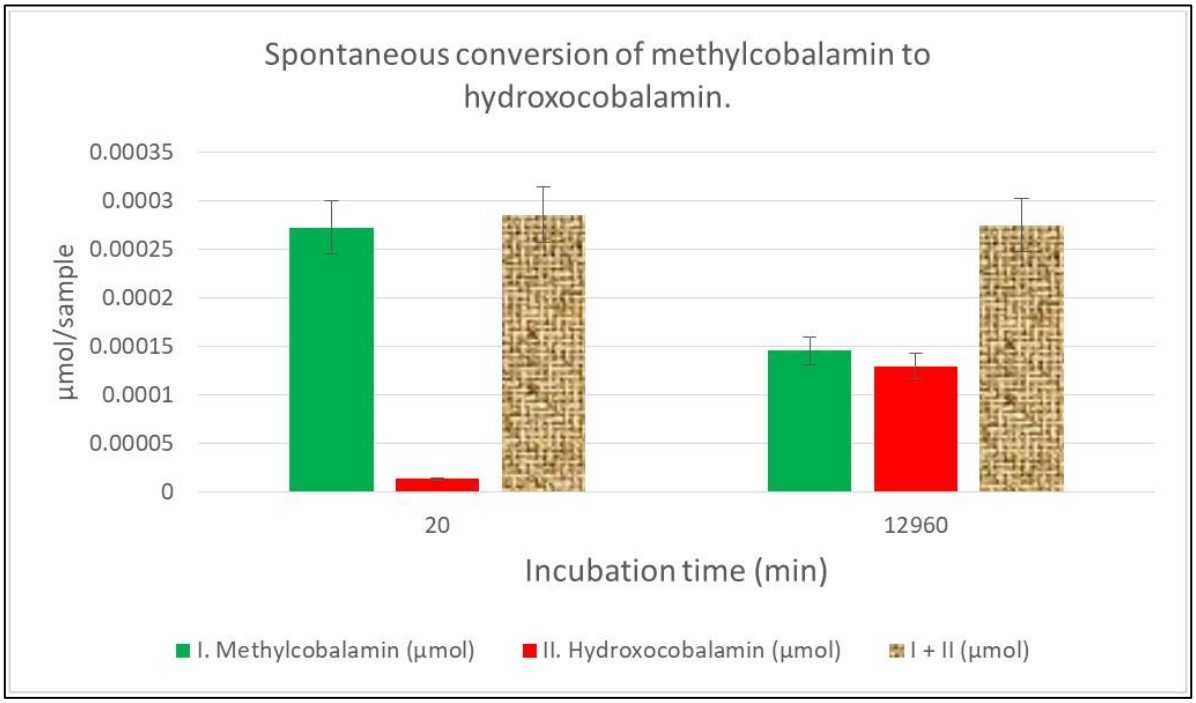


Figure 2: A standard methanolic solution of methylcobalamin 2.97 mM was incubated at room temperature in an amber glass vial. The amount of methylcobalamin and hydroxocobalamin in the solution was measured at 20 and 12960 minutes after preparation

Quantitative Analysis

The method for the quantitative analysis of methylcobalamin in aqueous and methanol solutions is described in detail in our previous work (Yefimov S. 2022). Here we briefly outline the main stages of this analysis. The conversion reaction of methylcobalamin proceeds according to the scheme:

(1) A ---> B ---> c, d, e.

where "A" is methylcobalamin, "B" is hydroxocobalamin, and "c", "d", and "e" are the breakdown products. The validity of our assumption is confirmed experimentally. According to this scheme, the amount (or peak area) of methylcobalamin in the sample decreases exponentially:

(2) A = A0 * exp (-K * t),

where "A0" is the amount of methylcobalamin at the time t=0, "K" is the reaction rate constant, and "t" is time.

The exponent (2) is linearized by taking a logarithm:

(3) ln(A)=ln(A0) -K*t

Figure 3 shows how the peak area of methylcobalamin 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 linear regression by the Least Squares method. The desired initial peak area of methylcobalamin is calculated by the formula: **A0=exp(ln(A0))**. The slope of the regression line corresponds to the reaction rate constant

($K=0.00051(min^{-1})$), and the point of intersection of the line with the y-axis corresponds to the ln(A0) then A0 are 587.7 (red) and 288.8 (green).

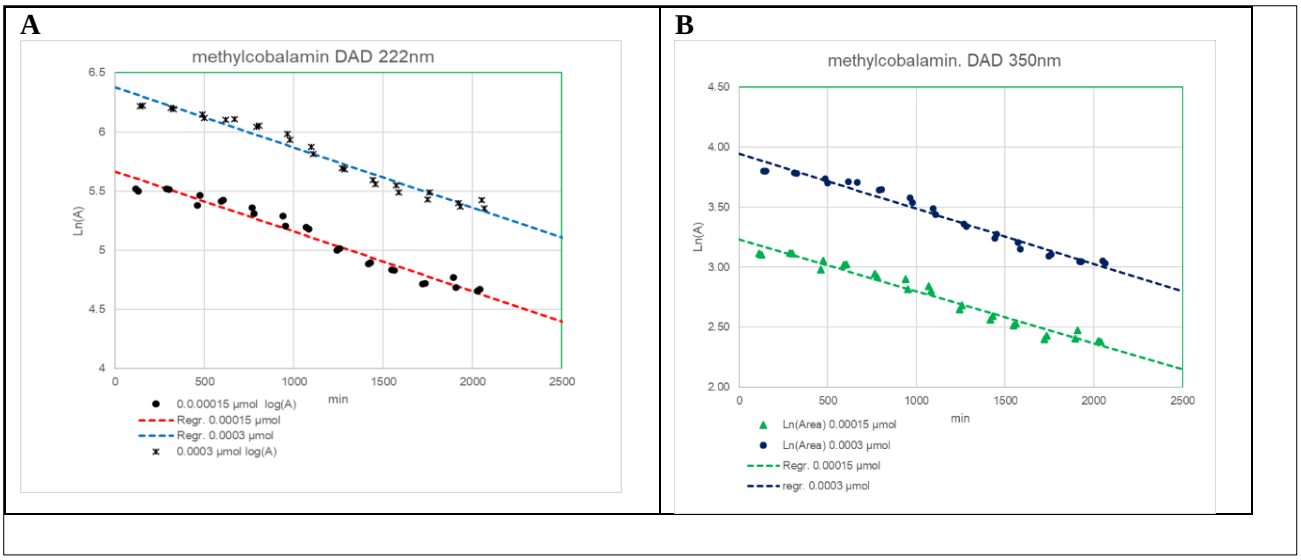


Figure 3 A, B: The time dependencies of the methylcobalamin transformation as a logarithm of peak area. The dashed lines are the theoretical model, icons are the experimental data. Panel A corresponds to DAD 222nm, and panel B corresponds to DAD 350nm

Having the area of the chromatogram peak at the initial time (A0) and the amount of methylcobalamin in the sample at the initial time (M0 (µmol)), we can calculate the value of the molar absorption by formula 4.

(4) E=M0/A0 (µmol/area).

This value is the intrinsic property of the species and is constant for the given instrument geometry and certain conditions: wavelength, temperature, and mobile phase composition. The molar absorption 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) at any time of incubation by formula 5.

(5) M=E*A (µmol).

Hydroxocobalamin is stable enough to be quantified using a standard calibration curve. 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 per injection (Table 2.). Now, having the "E" values for methylcobalamin, and the calibration curve for hydroxocobalamin, we can determine the amount of methylcobalamin and hydroxocobalamin in a sample.

Method Validation

System suitability (Table 1) the standard solution of methylcobalamin and hydroxocobalamin was tested five times. The results were averaged, and the RSD was calculated automatically using the OpenLAB CDS software. The acceptable limit is in line with the recommendations (Dr. Deepak, 2013; Bose, 2014; Shabir A, 2023).

Table 1: System suitability

Test parameters	Methilcobalamin (St.)			Hydroxocobalamin (St.)			Aceptable limit
	Mean	±S.D.	%RSD	Mean	±S.D.	%RSD	
Peak area (counts*min.)	N/A	N/A	N/A	27.89	3	0.11	**RSD ≤2**
Retention time (min)	6.56	0.01	0.1	8.75	0.08	0.01	**RSD ≤2**
Theoretical plates (N)	30309.00	56.57	0.3	22391	50	0.00	**>2000**
Tailing factor (T)	1.10	0.014	1.1	1.24	0.01	0.01	**≤2**
Relative retention	1.38			1.38			≥1
Resolution (USP)	5.69			5.69			≥2

Linearity, Range, and Limit of Detection

The working range corresponds to the range of the linear section of the calibration curve (Tables 2 and 3). The linearity is more than satisfactory; the correlation coefficient is almost equal to one. The limit of detection is satisfactory for testing pharmaceutical products (Tables 2 and 3). We state the LOD in micrograms, which is complete information on the sensitivity of the method. The typical injection volume is 1 microliter.

Table 2: Hydroxocobalamin DAD 350nm, Linearity, Range, LOD

Hydroxocobalamin. 350nm.			
X (μmol/inj)	Y (peak area)	Y (linear)	ΔY
6.2E-05	53.586	52.248	1.338
0.00016	138.371	136.470	1.901
0.00031	271.938	276.840	4.902
0.00062	556.187	557.580	1.393
0.00094	840.611	838.321	2.290
a	899808.3		
b	-3.9		
c	N/A		
r	0.99995		
Mean ΔY (n=xx)	2.365		
S.D. ΔY (n=x)	1.471		
LOD (μmol/inj)	**5.395E-06**		
"X"- the content of hydroxocobalamin in the sample; "Y" - the peak area; "Y calc."- the calculated peak area; "ΔY" - the residues; "a" - the slope of the regression line;"a","b", "c" - the parameters of regression curve; "r" - the correlation coefficient; "S.D. ΔY" - the residual standard deviation of the regression curve.			

To determine the LOD of methylcobalamin, we cannot use the dependence of the peak area on the amount of analyte introduced into the sample, since this amount is constantly changing. Therefore, the amount of the analyte is determined by the formula (5) based on the peak area and the predetermined molar absorbance: **M=E*A**.

Table 3: Methylcobalamin DAD 350nm, Linearity, Range, LOD

Methylcobalamin. 350nm.			
M=E*Y (μmol/inj)	Y (peak area)	Y (linear)	ΔY
7.9E-05	13.434	52.248	1.338
0.00018	31.108	136.470	1.901
0.00025	43.208	276.840	4.902
0.00029	49.469	557.580	1.393
0.00036	61.428	838.321	2.290
a	169491.53		
b	-1.421E-14		
c	N/A		
r	1		
Mean ΔY (n=xx)	2.365		
S.D. ΔY (n=x)	1.471		
LOD (μmol/inj)	**2.864E-05**		
"M"- the content of metylcobalamin in the sample; "Y" - the peak area; E-molar absorption; "Y calc."- the calculated peak area; "ΔY" - the residues; "a" - the slope of the regression line;"a","b", "c" - the parameters of regression curve; "r" - the correlation coefficient; "S.D. ΔY" - the residual standard deviation of the regression curve.			

Accuracy/recovery and precision (Table 4) samples containing three different starting concentrations of methylcobalamin were measured five times, and the mean value of sum moles of methylcobalamin and hydroxocobalamin was measured. The recovery was determined based on the calibration curve (hydroxocobalamin) and molar absorption (methylcobalamin). The data confirm the accuracy, reproducibility, and precision of the method. Interday analysis (check the next day) shows no significant degradation.

Table 4: The recovery was calculated as the ratio of the sum of moles of methylcobalamin and hydroxocobalamin in solution to the number of moles of methylcobalamin introduced into the solution multiplied by 100

Methyl cobalamin (µmol/inj)	Methylcobalamin + Hydroxocobalamin Mean recovery (µmol/inj)	±S.D.	RSD (%)	Recovery (%)
2.60E-04	2.60E-04	0.0001	0.2	100
3.64E-04	3.61E-04	0.0007	0.7	99
4.17E-04	4.12E-04	0.0004	0.3	99
5.21E-04	5.14E-04	0.0040	0.8	99
Recovery data are the mean of five independent determinations (n=5). *Bottom line corresponds to analysis after 48 hours.				

Selectivity (Specificity) assay

The results of the analysis of the standard solution and the test solution with the same concentration of the test component were compared. The presence of other ingredients does not affect the parameters of the chromatograms. The relative standard deviation of the compared peak areas does not exceed 1.5% (Table 5). Thus, the method is specific to each of the tested components.

Table 5: Specificity

Test parameters	Metylcobalamin 0.00025 µmol/inj standard solution (0.1% Formic acid in H2O/Methanol 80/20)			Metylcobalamin 0.00025 µmol/inj solution, containing benzyl alcohol, phosphate monobasic, NaCl.			Hydroxocobalamin 0.0003 µmol/inj standard solution (0.1% Formic acid in H2O/Methanol 80/20)			Hydroxocobalaamin 0.0003 µmol/inj solution, containing benzyl alcohol, phosphate monobasic, NaCl.		
	Mean	±S.D.	%RSD	Mean	±S.D.	%RSD	Mean	±S.D.	%RSD	Mean	±S.D.	%RSD
Retention time (min)	6.60	0.10	1.5	6.59	0.01	0.3	8.8	0.10	1.1	8.80	0.07	0.8
Theoretical plates (N)	30309	50	12	30522	52	11	22391	65	0.3	22401	61	0.3
Tailing factor (T)	1.10	0.001	0.1	1.05	0.001	0.1	1.24	0.002	0.2	1.15	0.001	0.1

Robustness (Table 6) as part of establishing the robustness of the method, the chromatographic parameters (T and N) of each of the two components were determined with a change in flow rate, column temperature, and composition of the mobile phase. These parameters changed insignificantly and were within acceptable limits. Thus, the method is robust.

Table 6: Robustness

Parameter	Metylcobalamin 0.00029 µmol/inj								Hydroxocobalamin 0.00012 µmol/inj							
	T	% RSD	N	% RSD	R time	% RSD	Resolution (USP)	% RSD	T	% RSD	N	% RSD	R tme	% RSD	Resolution (USP)	% RSD
Flow rate 0.8 mL/min	1.1	0.1	30309	12	6.6	0.5	5.7	0.40	1.24	0.1	22391	12	8.75	0.5	5.7	0.4
Flow rate 0.7 mL/min	1.2	0.1	30311	6	7.5	0.5	6.4	0.3	1.23	0.1	22411	15	10	0.5	6.3	0.5
Temperature 25°C	1.1	0.1	30309	12	6.6	0.5	5.7	0.40	1.24	0.1	22391	12	8.75	0.5	5.7	0.4
Temperature 26°C	1.26	0.1	30298	15	6.5	0.6	5.6	0.6	1.31	0.2	22377	13	8.71	0.6	5.7	0.6
Mobile phase composition																
Formic acid 0.1%	1.1	0.1	30309	12	6.6	0.5	5.7	0.40	1.24	0.1	22391	12	8.75	0.5	5.7	0.4
Formic acid 0.2%	1.1	0.1	30356	14	6.6	0.5	5.7	0.40	1.21	0.1	22401	12	8.71	0.5	5.7	0.4

CONCLUSION

The method for the quantitative analysis of methylcobalamin and hydroxocobalamin in injection solutions has been validated. The method has been tested for sensitivity, accuracy, reproducibility, specificity, and robustness. Due to the continuous transformation of methylcobalamin into hydroxocobalamin, the method has two features:

1. The amount of methylcobalamin in the sample at a given time is determined not by a calibration curve, but by peak area and molar absorbance. Molar absorption is predetermined from the kinetic curve.
2. The total number of moles of methylcobalamin and hydroxocobalamin in solution remains approximately constant, while the amount of methylcobalamin decreases.

Abbreviations

K – Rate constant
RT – Room temperature.
RSD – Relative standard deviation
T - The tailing factor
N - Number of theoretical plates
r - Correlation coefficient
R time – Retention time.

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