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NOVEL STABILITY- INDICATING METHOD FOR DETERMINATION OF RELATED SUBSTANCES OF CYANOCOBALAMIN INJECTION USING RP- HPLC

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ABSTRACT
The main aim is to develop a simple and sensitive method for determining the cyanocobalamin-related impurities (USP and EP Impurities) in an injection dosage form using the reverse-phase high-performance liquid chromatography (RP-HPLC) method. Chromatographies separation on the Cosmicill Adore pH C18 (octadecylsilane) column of the dimension (150 mm × 3.9 mm, 3 µm) was carried out in the gradient mode with mobile phases; Mobile phase A (0.2 % Trifluroacetic buffer pH-3.0), Mobile phase-B: (95 % Acetonitrile: 5 % Water). The mobile phase was pumped at a flow rate of 1.0 mL min^{-1} and the analyte was monitored with a UV detector at a wavelength of 360 nm. The method was developed and validated under the stress conditions such as acidic, basic, peroxide, thermal, photolytic, and humidity degradation, respectively. The method was validated with respect to specificity, linearity, precision, accuracy, limit of detection, and limit of quantification provided by the ICH guidelines. The results of linear regression analysis of the calibration plot revealed a good linear relationship between response and concentration with a correlation coefficient value of r^2 = 0.9999. The accuracy of known impurities was obtained in the range of 94–105 %. The identification limits and limits of quantification of related compounds of cynocobalamin varied between 0.287 to 0.318 ppm, and 0.950-1.052 ppm respectively. Chromatographic interference was not found during the degradation and excipients were detected from the injection. The proposed method was successfully used to estimate the cyanocobalamin-related impurities in a injection dosage form.

KEYWORDS: cyanocobalamin; related impurities; RP-HPLC; stress conditions; injection dosage form.

INTRODUCTION
Cobalamin, also known as vitamin B_{12} , is a water-soluble micronutrient that is one of the B group vitamins and has a cobalt-centered corrin ring in its molecular structure.[1] It plays a pivotal role in the production of DNA, blood cells, and the repair of neurological functions.[2] Cobalamin has several bioactive forms and many analogues by differences in their upper axial ligand such as hydroxocobalamin, cya- nocobalamin, and methylcobalamin.[3] Among these co- balamins, cyanocobalamin is mainly used in fortified foods and supplements for humans and animals because it is the most stable analogue compared with other cobalamins.[4] Cyanocobalamin has hematopoietic activity apparently identical to that of the antianemia factor in purified liver extract.[5-6] Parenteral (intramuscular) administration of vitamin B_{12} completely reverses the megaloblastic anemia and GI symptoms of vitamin B_{12} deficiency. Cyanocobalamin (International Union of Pure and Applied Chemistry (IUPAC)name: cobalt(3+);[(2R,3S, 4R,5S)-5-(5,6-dimethylbenzimidazol-1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl][(2R)-1-[3[(1R,2R,3R,5Z, 7S,10Z,12S, 13S,15Z,17S,18S,19R)–2,13,18-tris(2-amino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)–3,5,8,8,13,15,18,19-octamethyl-2,7,12,17-tetrahydro-1H-corrin-24-id-3-yl]propanoylamino] propan -2-yl]phosphate; cyanide Cyanocobalamin empirical formula is $C_{62}H_{88}N_{13}O_{14}P.CN.Co$ and it has a molecular weight of 1355.3676 g/mol. İt exists as Dark red crystals or a red, crystalline powder; odourless. It is Soluble in water and ethanol practically insoluble in acetone and ether. The anhydrous form of cyanocobalamin is highly hygroscopic. Cyanocobalamin (vitamin B_{12}) injection is a sterile aqueous solution of cyanocobalamin containing sufficient acetic acid or hydrochloric acid to adjust the pH to about 4 using a suitable buffering agent.[7] The official requirements for the pH of cyanocobalamin injections are between 3.8 and 55[7] and 4.7 to 7.0.[8] Commercial cyanocobalamin parenteral preparations are available either as vitamin B_{12} alone or in combination with B vitamins such as thiamine (B_1) and pyridoxine (B_6). Sterile aqueous solutions of vitamin B_{12} are stable

at pH 4.5-5. However, the presence of reducing agents even in trace quantities can markedly influence potency as a result of irreversible oxidation. Cyanocobalamin is converted to hydroxocobalamin (B_{12b}) at pH 3.5-6.5 on exposure to light. In parenteral solutions, cyanocobalamin is unstable in the presence of thiamine and nicotinamide. Amide cyclization and amide hydrolysis in acidic and basic media also contribute to the overall stability of B12 solutions.[9]

Organic impurities might be produced during the storage and manufacturing of Active Pharmaceutical Ingredient (API) or pharmaceutical products. Its permissible limit is based on drug research or known safety data. If the content of such impurities in the drugs reaches the prescribed level, it may harm the patients and, in some cases, it may also jeopardize their life. These impurities play a vital role in the safety and effectiveness of drugs. As per the regulatory Guidelines such as International Council for Harmonization (ICH), these impurities have to be controlled to specified limits. The most widely used chemical methods for vitamin B_{12} determination are capillary electrophoresis[10,11] and high-performance liquid chromatography (HPLC) with various detection methods such as UV/VIS[12–17], AAS[18] fluorescence,[19,20] or mass spectrometric detectors.[21-26] However, there are limited methods for the determination of cyanocobalamin-related impurities by RP-HPLC. To the best of our knowledge, so far five potential impurities of cyanocobalamin were already identified in the literature is the USP method[27] and two European pharmacopeias with unexplored relative response factor (RRF) . The impurities are listed in Table 1. USP method also has the limitation of poor column life and short mobile phase stability. There is no method available for concurrent estimation of USP impurities and EP impurities along with forced degradation studies.

The objective of the research work reported in this paper is to develop a suitable stability-indicating method to estimate the process impurities of cyanocobalamin (Impurity B–H) in dosage form to prevail over the published research. The method was validated with required parameters such as precision, specificity, linearity, accuracy, LOD, LOQ, and robustness following the ICH guidelines[28] to check whether the developed method meets the regulatory requirements.

Table 1: Related impurities of cyanocobalamin mentioned in listed in USP and EP.

Name of the impurity	IUPAC name	Molecular Formula
50-Carboxy Cyanocobalamin (Cyanocobalamin Impurity B)	Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-cyanocobalamin a,b,c,d,g-pentaamide	$C_{63}H_{87}CoN_{13}O_{15}P$
32-Carboxy Cyanocobalamin (Cyanocobalamin Impurity D)	Cobınıc acıd-acdeg-pentamıde, cyanıde, dıhydrogen phosphate (ester), ınner salt, 3'-ester wıth 5,6-dımethyl-1-.alpha.-d-rıbofuranosyl-1h-benzımıdazole	$C_{63}H_{87}CoN_{13}O_{15}P$
7β,8β-Lactose Cyanocobalamin (Cyanocobalamin Impurity F)	7β, 8β-Lactocyanocobalamin;Cyanocobalamin Impurity F;Cyanocobalamin Impurity 6;7β,8β -Lactocyanocobalamin;Cyanocobalamin 7β,8β-lactone;	$C_{63}H_{85}CoN_{13}O_{15}P$
34-Mythyl Cyanocobalamin (Cyanocobalamin Impurity C)	Cobınamıde,co-(cyano-.kappa.c)-nb-methyl-, dıhydrogen phosphate (ester), ınner salt, 3'-ester wıth (5,6-dımethyl-1-.alpha.-d-rıbofuranosyl-1h-benzımıdazole-.kappa.n3)	$C_{62}H_{90}CoN_{13}O_{14}$
8-Epi Cyanocobalamin (Cyanocobalamin Impurity E)	(8β)-Cobinamide Cyanide 3'-Ester with 5,6-dimethyl-1-α-D-ribofuranosyl-1H-benzimidazole Dihydrogen Phosphate (ester), inner salt, 8-Epicobalamine; 8-epi-Cyanocobalamin •	$C_{63}H_{88}CoN_{14}O_{14}P$
Hydroxy Cyanocobalamin (Cyanocobalamin Impurity H)	Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-hydroxocobamide	$C_{62}H_{89}CoN_{13}O_{15}P$
50-Mythyl Cyanocobalamin (Cyanocobalamin Impurity G)	Coα-[α-(5, 6-dimethylbenzimidazolyl)]-Coβ-cyano-e-N-methylcobamide	$C_{64}H_{90}CoN_{14}O_{14}P$

MATERIALS AND METHODS

Chemicals and Reagents

Cyanocobalamin standard was procured from Sigma-Aldrich and its impurities standards were obtained from synzeal labs. Cyanocobalamin injections were purchased from a market, containing 500 µg Cyanocobalamin per 30 mL vial. Analytical grade reagents (such as orthophosphoric acid, trifluoro acetic acid, hydrochloric acid, sodium hydroxide, and hydrogen peroxide) were acquired from Merck's specialties private limited, Mumbai. HPLC-grade solvents (such as water, methanol, and acetonitrile) were acquired from Rankem Private Limited, Mumbai.

Instrumentation

The development and validation activities were accomplished using Water's alliance 2690/2695 high-performance liquid chromatography (HPLC) system

equipped with a low-pressure quaternary gradient pump along with a PDA detector, column oven, auto-sampler, and Waters Empower 3 software. The weighing of the mobile phase reagents, standard, and impurities were measured using the Sartorius E2 analytical balance. The pH of the mobile phase was adjusted using a Metrohm printable pH meter. The mobile phase was filtered through a 0.22 µm filter paper using a glass vacuum filtration apparatus. The dissolved gas and the air bubbles ensnared in the mobile phase were removed using an ultrasonic bath. The samples were centrifuged using Beckman Coulter Life Sciences, Allegra 64R, with a thermostatic cooler system. Thermo Fisher heating oven was used for stress studies.

Experimental Work

Operating Conditions

HPLC method development, validation, and analysis of solutions from forced degradation studies were performed with a Water's alliance 2690/2695 HPLC system with photo diode-array detection. Chromatographic separations were achieved on a 150 mm, 3.9 mm i.d., 3-µm particle size, Cosmicill Adore pH C18 column with a mobile phase gradient prepared from 0.2 % aqueous trifluoroacetic acid and adjusted to pH 3.0 with ortho-phosphoric acid (mobile phase A) and 95:05 (v/v) acetonitrile - Milli Q water (mobile phase B). The gradient program (time (min) /percentage B) was 0.01/15, 45/22, 55/22, 60/15, and 65/15, with a post-run time of 10 min with a constant flow rate of 1.0 $mLmin^{-1}$. The column temperature was maintained at 30 °C and detection was performed at 360 nm. The test concentration was approximately 500 µg mL^{-1} and the injection volume was 20 µL. Data were collected and cyanocobalamin peak purity was checked using Empower 3 software. Calculate the percentage of each impurity in the portion of cyanocobalamin taken:

Percentage of Impurity = (P_{sam}/P_{std}) x (C_{std}/C_{sam}) x (1/F) x 100

P_{sam} = peak area from the sample solution; P_{std} = peak area from the standard solution; C_{std} = concentration of cyanocobalamin in standard solution (mg mL−1), C_{sam} = concentration of cyanocobalamin in the sample solution (mg mL^{-1}) and *F* = relative response factor.

Preparation of Chromatographic Solutions

Preparation of Standard Solution

The standard solution was prepared following the cyanocobalamin standard. An accurately weighed quantity of cyanocobalamin standard was dissolved in 50:50 (v/v) mobile phase A–mobile phase B (diluent) to obtain a solution having a known concentration of about 1000 (µg mL^{-1}).

Preparation of Sample Solution

The pooled sample was prepared by mixing the contents of 3 vials of cyanocobalamin injection formulation (fill volume: 30 mL each). Accurately transferred 30 mL of pooled samples into a 100 mL volumetric flask, made up to mark with diluent, and mixed well to obtain a solution having a known concentration of about 500 µg mL^{-1} equivalent to cyanocobalamin.

Stress Degradation Studies of cyanocobalamin

For the stress degradation study, the solutions were prepared using cyanocobalamin injection. For acid degradation studies into a 10 mL volumetric flask containing 2 mL of cyanocobalamin injection, 1 mL of 1M hydrochloric acid was added and the solution was kept at room temperature for 12 h. The degraded sample was neutralized with 1 mL of 1 M sodium hydroxide, made up to 10 mL mark with diluent and chromatographed. For base degradation, into a 10 mL volumetric flask containing 2 mL of cyanocobalamin injection, 1 mL of 0.5 M sodium hydroxide was added and the solution was kept at room temperature for 1 h. The degraded sample was neutralized with 1 mL of 0.5 M hydrochloric acid, made up to 10 mL mark with diluent and chromatographed. Sımılıarly oxidative degradation was carried out using 5 mL of 3 % v/v of H_2O_2 solution at room temperature for 1 h. For thermal degradation, the cyanocobalamin injection sample is kept in a hot air oven at 80 °C for 7 days. After attaining the room, temperature, sample was prepared as per the test method by taking 2 mL of thermal degradation sample and chromatographed. In photolytic degradation the cyanocobalamin injection sample was kept in the photolytic chamber and exposed to white fluorescent light, 1.2 million Lux hours and UV light 200-watt hours/square meter. After attaining room, temperature the sample was prepared as per the test method by taking 2 mL of photolytic degradation sample and chromatographed. All the above prepared solutions were sonicated, and filtered using a sample filter of 0.22 µm PVDE prior to injection.

Method Validation

Specificity

The specificity of the RP-HPLC method was evaluated to ensure that there is not any interference in the excipients present in the formulations. The specificity was studied by injecting impurities, through degradation products and excipients.

Linearity

Linearity is the ability to obtain test results that are directly proportional to the analyte concentration. Linearity was determined by five different concentrations of cyanocobalamin and its known impurities (LOQ level to 150 % of specification limit), five specified impurities (USP) were taken for the linearity study and the values of their peak area versus concentration were plotted. To calculate the coefficient of correlation, slope, and intercept using linearity curve extrapolation.

Precision

The precision of an analytical method is the degree of agreement amongst individual test results when the method is applied repeatedly to multiple sampling of a

homogeneous sample. The precision of the analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. The system precision and method precision (Repeatability) of the proposed methods was determined by several measurements of known USP impurities. System precision was established by performing six different measurements of the known standard cyanocobalamin and USP impurities at 100 % level. Method precision was determined by six individual preparations of cyanocobalamin spiked with USP Impurities. The percentage RSD of each impurity and total impurities were calculated and recorded in the table.

Accuracy

The accuracy of an analytical method is the nearness between the expected value and the value found. It is obtained by calculating the percent recovery (% R) of the analyte recovered. A study of recovery was performed on LOQ to 200% of the target concentration of each impurity by spiking (n = 3) with 500 µg mL−1 of cyanocobalamin. Spiked samples were extracted and analyzed. Then the % recovery and its relative standard deviation were calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limit of detection (LOD) of an analytical method is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated as an exact value. The limit of quantification (LOQ) of an analytical procedure is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The limit of detection (LOD) and the limit of quantification (LOQ) of the known Impurities and cyanocobalamin were calculated using the signal-to-noise ratio method as per ICH. A typical signal-to-noise ratio is 10:1 for the Limit of Quantification (LOQ) and a signal-to-noise ratio between 3 and 2:1 is generally considered acceptable for estimating the detection limit (LOD). Further, the % RSD of six preparations at LOQ was calculated.

Stability of Solution

The stability of cyanocobalamin and its known Impurities solution is determined. The working standard solution of cyanocobalamin and its known Impurities to be tested was kept on the bench at a temperature of 25 °C and analyzed in a chromatographic time interval of 48 h.

RESULTS AND DISCUSSION

Method Development

The objective of method development was to separate cyanocobalamin and its process-related impurities with a shorter run time to increase the throughput at quality control laboratories, good resolution, and peak shapes. Initial method development trials were conducted on different stationary phases like C8, C18, Phenyl, and Cyano along with the optimization of other chromatographic conditions like detection of wavelength, type and quantity of organic/inorganic buffer, pH of the mobile phase, thermostat and column oven temperature. Every time system suitability criteria were evaluated during the trial runs to ensure the strength of the method. The resolution between impurities was critical and thus considered as the system suitability criteria. Satisfactory resolution and good peak shape of the analyst was observed on Cosmicill Adore pH C18 (3.9 mm × 150 mm, 3 µ) HPLC column at flow rate 1.0 mL·min^{-1}, λ 360 nm, column oven temperature 30˚C and injection at room temperature and the mobile phase was 0.2 % aqueous trifluoroacetic acid and adjusted to pH 3.0 with ortho-phosphoric acid, (mobile phase A) and 95:05 (v/v) acetonitrile- milli Q water (mobile phase B). The gradient program (time (min)/percentage B) was 0.01/15, 45/22, 55/22, 60/15 and 65/15. The standard solution was injected in six replicates and the average of the injections was used for calculation. The first injection is considered for the system suitability of the standard solution with parameters such as tailing factor is about 1.15, USP plate count about 8530 and the % RSD of the six injections about 0.23. The spike solution containing all the impurities spiked at known concentrations was injected to check the specificity of the method. The optimized chromatogram was shown in Figure 1 and chromatographic conditions were shown in Table 2. From this, the retention time, the relative retention time for cyanocobalamin and the known impurities were obtained. The minimum linearity studies provided the relative response factor values for the impurities obtained which are tabulated as shown in Table 3. The cyanocobalamin-related impurities labeled in the chromatograms are as follows: 50-Carboxy Cyanocobalamin, 32-Carboxy Cyanocobalamin, 7β,8β-Lactose Cyanocobalamin, 34-Mythyl Cyanocobalamin, 8-Epi Cyanocobalamin, Hydroxy Cyanocobalamin (Ph.Eur Impurity-H) (Process) and 50-Mythyl Cyanocobalamin (Ph.Eur Impurity-H) (Process).

Table 2: Chromatographic Conditions.

Column	Cosmicill Adore pH 150 X 3.9 mm, 3µm		
Mobile phase A	0.2 % aqueous TFA and adjusted to pH 3.0 with OPA		
Mobile phase B	95:05 (v/v) acetonitrile- milli Q water		
Gradient Program	t (min)	mobile phase A	mobile phase B
	0.01	85	15
	45	78	22
	55	78	22
	60	85	15
	65	85	15

Flow rate	1.0ml/min
Injection Volume	20µL
Column Temperature	35°C
Detection	UV at 360nm

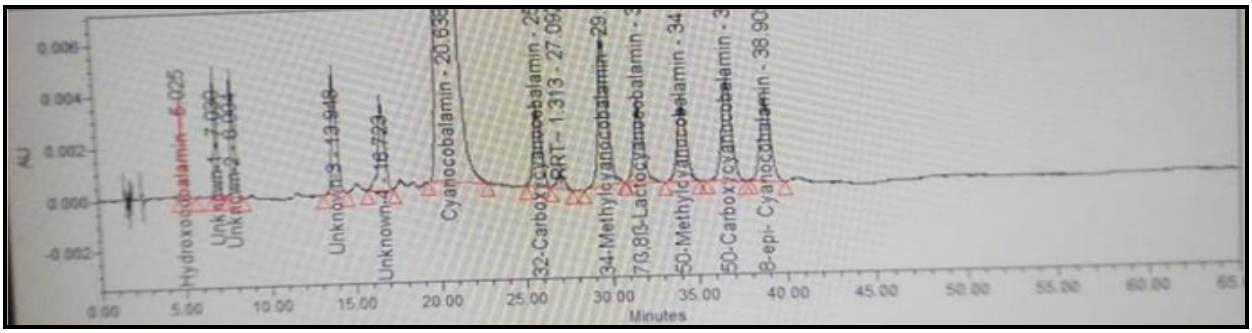

Fig. 1: Optimized chromatogram from spiked solution.

Table 3: Relative retention time and factor data.

Analyte	RRT[a]	RRF[b]
Hydroxy Cyanocobalamin	0.24	1.00
Cyanocobalamin	1.00	NA
32-Carboxy Cyanocobalamin	1.24	1.06
34-Methyl Cyanocobalamin	1.43	0.95
7β,8β- Lactose Cyanocobalamin	1.53	0.93
50-Methyl Cyanocobalamin	1.65	1.00
50-Carboxy Cyanocobalamin	1.76	1.10
8-Epi Cyanocobalamin	1.88	1.12

[a]RRT, relative retention time
[b]RRF, relative response factor

Stress Studies of cyanocobalamin

Stress studies were performed on cyanocobalamin injection to provide a stability-indicating property and also the specificity of the developed method. The degradation was performed on the cyanocobalamin injection intentionally by various conditions which are mentioned in the experiment part to evaluate that the proposed chromatographic method is suitable to separate the main moiety from its impurities. Also, the peak purity checked through the photodiode array detector gets passed, and the determined purity angle was lower than the calculated purity threshold which is a part of the development activity. As shown in starting from acid, base, peroxide, thermal, photolytic, and humidity are represented and the percentage of degradation for each stress condition is recorded in Table 4.

Table 4. Forced degradation data.

S.NO	Sample name	Degradation condition	Degradation	Assay (%)	Mass balance
1	Control sample	NA	NA	103.50	NA
2	Thermal sample	80 ºC for 7 days	6.678	94.12	97.4
3	Base hydrolysis	0.5N NaoH ; 1mL- 60 min at room temperature	5.650	93.28	95.6
4	Acid Hydrolysis	1N HCl ; 1mL- 120 min at room temperature	6.755	96.80	100.1
5	Peroxide Degradation	3 % peroxide 60 min at room temperature	3.575	98.40	98.5
6	Photolytic Condition	1 Cycle	5.896	94.65	97.1

Method Validation

Specificity

The study is performed by spiking the individual impurities at a known concentration with cyanocobalamin and checking their separation. The impurities were found to be separated from each other and also from cyanocobalamin and also the peak purity of the injection was checked. Further, the individual injection of the impurities was injected at the same concentration as injected above to confirm the retention time.

Linearity

A series of solutions of cyanocobalamin and its known impurities were injected from LOQ to 150 % as discussed, and the obtained peak area versus concentration was plotted to provide the linearity graph from which the slope, intercept and correlation coefficient values are obtained. These values are tabulated in Fig. 2 and Table 5.

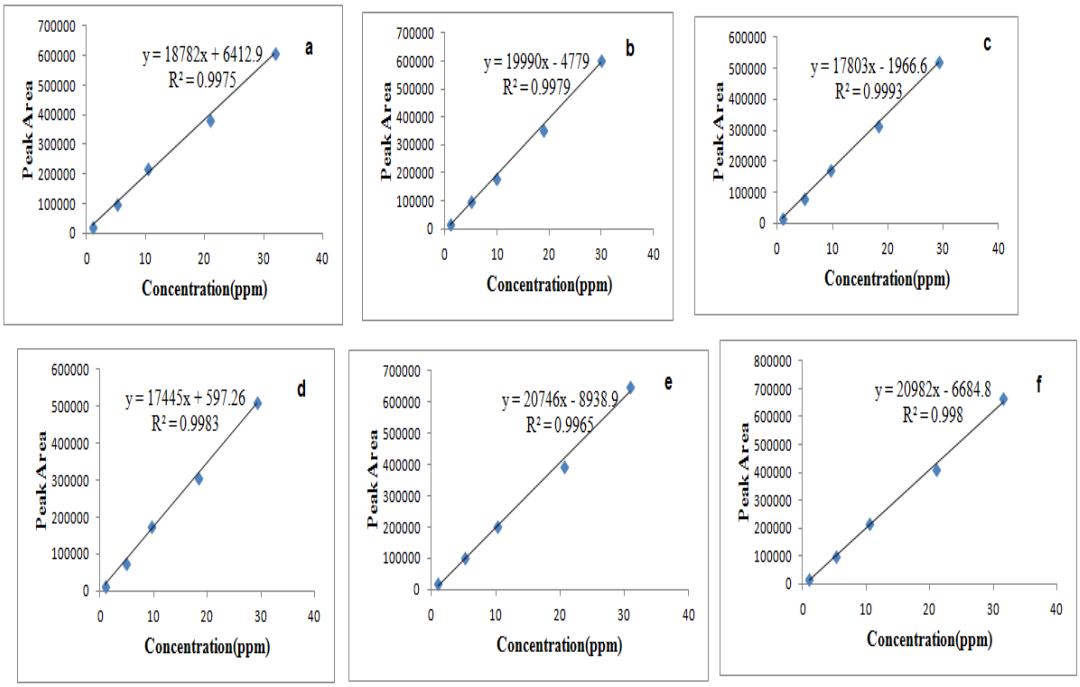

Fig. 2: Calibration plot of a) cyanocobalamin b) 32-carboxycyanocobalamin c) 34-Mythyl Cyanocobalamin d) 7β,8β- Lactose Cyanocobalamin e) 50-Carboxy Cyanocobalamin f) 8-Epi Cyanocobalamin

Table 5: Calibration curves, LODs, and LOQs.

Analyte	Linearity Range(ppm)	Regression equation	R^2	LOD(ppm)	LOQ(ppm)
Cyanocobalamin	1.024-31.782	Y=18782X+6412.9	0.9975	0.310	1.024
32-Carboxy Cyanocobalamin	0.981-29.855	Y=19990X-4779	0.9979	0.297	0.981
34-Methyl Cyanocobalamin	0.950-29.115	Y=17803X-1966.6	0.9984	0.287	0.950
7β,8β- Lactose Cyanocobalamin	0.965-19.188	Y=17445X +597.26	0.9983	0.292	0.965
50-Carboxy Cyanocobalamin	1.03-30.90	Y=20746X -8938.9	0.9965	0.312	1.03
8-Epi Cyanocobalamin	1.052-31.560	Y=20982X +6684.8	0.9980	0.318	1.052

Precision

We have studied two precision parameters, namely the system precision which tells about the % RSD of the six replicate standard solutions injected which is represented in Table 6. The % RSD values are obtained for the Impurities through method precision where six individual spiked sample preparations were done and the values were within 2.0 %. The % RSD value of the combined precision was less than 5.0 % and the respective values are shown in Table 6 and Table 7.

Table 6: System Precision.

S.NO	32-Carboxy	34-methyl	7β,8β- Lactose	50-Carboxy	8 epi	Total impurities
1	1.113	1.873	1.321	1.482	1.285	7.074
2	1.111	1.841	1.343	1.487	1.322	7.104
3	1.182	1.843	1.389	1.587	1.271	7.272
4	1.176	1.885	1.341	1.515	1.272	7.189
5	1.092	1.945	1.381	1.510	1.265	7.193
6	1.131	1.998	1.366	1.584	1.312	7.391
Mean	1.134	1.897	1.356	1.527	1.287	7.203
SD	0.036	0.062	0.026	0.046	0.023	0.115
% RSD	3.18	3.28	1.92	3.01	1.79	1.59

Table 7: Method Precision.

S.NO	32-Carboxy	34-methyl	7β,8β- Lactose	50-Carboxy	8 epi	Total impurities
1	1.118	1.94	1.323	1.475	1.241	7.097
2	1.097	1.955	1.328	1.531	1.317	7.228
3	1.131	1.956	1.33	1.525	1.268	7.210
4	1.125	1.998	1.343	1.551	1.271	7.288
5	1.119	1.976	1.350	1.542	1.254	7.241
6	1.127	2.001	1.367	1.583	1.343	7.421
Mean	1.120	1.971	1.340	1.535	1.282	7.248
SD	0.012	0.025	0.017	0.036	0.039	0.128
% RSD	1.077	1.262	1.236	2.318	3.066	8.959

Accuracy

The study was performed to find the recovery of the impurities spiking it with cyanocobalamin from LOQ, 50, 100, 150, and 200 % of target concentration and it has provided satisfactory results. The percentage of recovery for each impurity was calculated, and it was found to be around 90.0% to 110.0 % the % RSD for each impurity at each level ranged from 0.2 to 4.1%, and the corresponding results are tabulated in Table 8.

Table 8: Accuracy data of related impurities.

Analyte	Mean % Recovery at LOQ level & %RSD	Mean % Recovery at 100% level & %RSD	Mean % Recovery at 150% level & %RSD
32-Carboxy Cyanocobalamin	99.35 & 1.625	99.6 & 1.077	98.1 & 1.086
34-Mythyl Cyanocobalamin	95.65 & 2.560	98.9 & 1.262	99.6 & 1.031
7β,8β-Lactose Cyanocobalamin	96.98 & 3.870	99.6 & 1.236	99.8 & 1.120
50-Methyl Cyanocobalamin	94.80 & 2.860	99.6 & 2.318	99.8 & 1.340
8-Epi Cyanocobalamin	102.3 & 2.145	100.8 & 3.066	99.2 & 1.540

Limit of Detection and Quantification (LOD and LOQ)

The linearity study was used to determine the limit of detection (LOD) and limit of quantification (LOQ) for the impurities using the signal-to-noise ratio concept. Further, the precision studies performed at the LOQ level were found to provide good results, and the % RSD calculated for each impurity was found to be less than 3%. The obtained values for the LOD, and LOQ, are tabulated in Table 5.

Stability

The standard solution containing cyanocobalamin was very stable which is understood from the similarity factor value which is about 1.0 for the initial injection and the solution kept on the benchtop for 48 h (Table 9). Next is the stability of the impurities studied by injecting the spike solution in which the difference is less than 1 % between the initial one and the one injected after 48 h. Finally, the stability of the mobile phases was checked by injecting the system suitability solutions and the spike solution which showed that the system suitability parameters provided good repeatability even after 3 days. The only thing is that a decrease was observed in the plate count each day. Then on studying the RRTs of the impurities from the spike solution it was found that the RRT values varied by ± 0.01 units as per the study.

Table 9: Solution stability of the standard solution.

Time in hours	Similarity factor for Cyanocobalamin
Initial	Not applicable
After 48 h	1.00

CONCLUSION

A simple RP-HPLC method for quantification of known related impurities in the cyanocobalamin injectable formulation was successfully developed and validated. The proposed methodology is specific, precise, linear, accurate, and stable, for the quantification of related impurities of cyanocobalamin injection. Moreover, the developed method has the lowest detectable and quantification capability. The degradation impurities formed during the stress study are well resolved, indicating the stability indicating nature of the method. Based on the above advantages, the proposed method could be useful for monitoring possible degradation impurities of cyanocobalamin in its injectable formulation in quality control departments of manufacturing units.

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