World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Available online at: https://wjpsonline.com/
Research Article

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF CYANOCOBALAMIN INJECTION (B12)

Pallam Sowmya[1], Dr. SNVL Sirisha[2]

[1]M. Pharmacy, Department of Pharmaceutical Analysis, Malla Reddy College of Pharmacy, Maisammaguda, Secunderabad, Hyderabad, 500100, Medchal District

[2]Associate Professor, Department of Pharmacognosy, Malla Reddy College of Pharmacy, Maisammaguda, Secunderabad, Hyderabad, 500100, Medchal District

Received: 02-12-2025 / Revised Accepted: 04-12-2025 / Published: 06-12-2025

ABSTRACT:
A precise RP-HPLC method for the pharmaceutical dose estimation of Cyanocobalamin. Chromatogram was run through Sunfire C18 250 x 4.6 mm, 5m. Mobile phase containing 0.01N Kh2po4: Methanol taken in the ratio 60:40 v/v was pumped through column at a flow rate of 1.0ml/min. Temperature was maintained at 30°C. Optimized wavelength selected was 251 nm. Retention time of Cyanocobalamin was found to be 2.461 min. standard %RSD of the Cyanocobalamin were and found to be 0.6. %RSD of Method precision of Cyanocobalamin was found to be 0.4. %Recovery was obtained as 99.74% for Cyanocobalamin. LOD, LOQ values obtained from regression equation of Cyanocobalamin were 0.01, 0.03. Regression equation of Cyanocobalamin is y = 33995x + 858.82.

Key Words: Cyanocobalamin, RP-HPLC, validation, Method Development.

Introduction:

Cyanocobalamin is a synthetic compound of vitamin B12 used to treat vitamin deficiencies.[1] This vitamin is produced naturally by bacteria. Chemically it is a corrinoid a cobalt-containing macrocycle — in which a cyanide group occupies the upper axial ligand position; this makes cyanocobalamin one of the most stable and readily handled cobalamin derivatives for manufacture and formulation [2,3], Biologically, cyanocobalamin is a prodrug: after administration it is converted in tissues to the metabolically active coenzyme forms methylcobalamin and 5-deoxyadenosylcobalamin, which serve as essential cofactors in methionine synthesis and odd-chain fatty acid/amino-acid metabolism respectively. These reactions underpin vitamin B_{12}'s critical roles in DNA synthesis, hematopoiesis and neurologic function.[4,5] Vitamin B12 has many forms, including the cyano-, methyl-, deoxyadenosyl- and hydroxy-cobalamin forms. The cyano form, is the most widely used form in supplements and prescription drugs. [6] Vitamin B12 is quickly absorbed from intramuscular (IM) and subcutaneous (SC) sites of injection; with peak plasma concentrations achieved about 1 hour after IM injection. [7] Clinically, cyanocobalamin is used to prevent and treat vitamin B_{12} deficiency arising from dietary insufficiency, malabsorption (including pernicious anemia), gastric surgery, ileal disease, or long-term use of certain medications. Both parenteral (intramuscular/subcutaneous) and high-dose oral or intranasal preparations are employed depending on etiology and severity; recent studies and guidelines support the efficacy of high-dose oral cyanocobalamin in many patients with pernicious anemia.[8,9]Its Chemically written as cyano[(1R,2R,3R,4R,6Z,8S,11Z,13S,14S,16Z,18S,19S)-8,13,18-tris (2-carbamoylethyl)-3,14,19-tris (carbamoylmethyl)-4-(2-{[(2R)-2-{[(2R,3S,4R,5S)-5-(5,6-dimethyl-1H-1,3-benzodiazol-1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-ylphosphonato]oxy}propyl]carbamoyl}ethyl)-1,4,6,9,9,14,16,19-octamethyl-20,21,22,23-tetraazapentacyclo[15.2.1.1^{2,5}.1^{7,10}.1^{12,15}]tricosa-5(23),6,10(22),11,15(21),16-hexaen-20-yl]cobaltylium.[10]

Pharmacokinetically, cyanocobalamin is relatively stable (advantageous for storage and formulation), but it is light-sensitive in solution and undergoes biochemical conversion in vivo. Dosing regimens vary by indication: replacement regimens commonly begin with frequent injections to replete stores, followed by maintenance dosing; oral regimens often use microgram-to-milligram doses depending on absorption capacity. [11,12]

Address for Correspondence: Pallam Sowmya, M. Pharmacy, Department of Pharmaceutical Analysis, Malla Reddy College of Pharmacy, Maisammaguda, Secunderabad, Hyderabad, 500100, Medchal District, Telangana, 502313.; **E-Mail:** sowmyapallam3@gmail.com

How to Cite this Article: Pallam Sowmya, Analytical Method Development and Validation for the Estimation of Cyanocobalamin Injection (B12). World J Pharm Sci 2025; 13(04): 219-227; https://doi.org/10.54037/WJPS.2022.100905

Vitamin B12 serves as a cofactor for methionine synthase and L-methylmalonyl-CoA mutase enzymes. Methionine synthase is essential for the synthesis of purines and pyrimidines that form DNA. L-methylmalonyl-CoA mutase converts L-methylmalonyl-CoA to succinyl-CoA in the degradation of propionate. Without vitamin B12, tetrahydrofolate cannot be regenerated from 5-methyltetrahydrofolate, and this can lead to functional folate deficiency.[13]

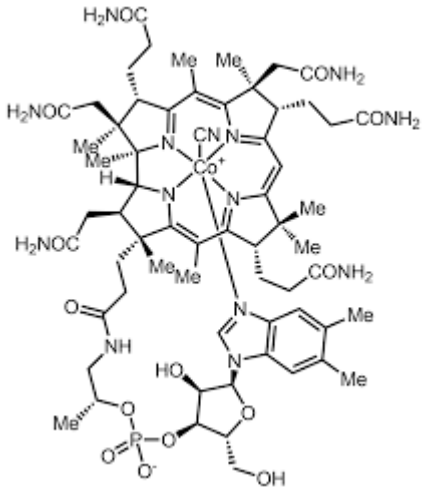

Figure 1: Structure of Cyanocobalamin

High Performance Liquid Chromatography (HPLC) plays a crucial role in the validation of Cyanocobalamin (MK-6482), a novel drug used in the treatment of cancers associated with von Hippel-Lindau (VHL) disease. In the review of literature, more economical methods were observed[14-18], hence a simple, cost-effective stability-indicating simultaneous estimation of Cyanocobalamin by RP-HPLC in pharmaceutical dosage form must be developed and validated as per the guidelines of ICH (Q2 specification).

Materials and Methods:

Cyanocobalamin (API), Cyanocobalamin tablets (VIB 12 1000 mcg Injection), Acetonitrile, Methanol, Ortho Phosphoric Acid, Distilled water. All of the solvents and chemicals were of HPLC quality and obtained from Rankem Chemicals Pvt Ltd.

Instrumentation:

The Method Development and Validation was performed by Waters HPLC Model 2695 equipped with PDA Detector and Empower 3 Software. Analytical weighing Balance, Ultrasonicator, pH Meter, Hot air oven.

Chromatographic Condition:

An Isocratic Elution carried out by using Acetonitrile and 0.1% KH2PO4 Water in 1:1 ratio. Sunfire C18 (4.6 x 250mm, 5µm) column was used to determine the Method at a flow rate of 1ml/min, by maintaining the column Temperature at 30 0C. In addition, with an injection volume of 10µL and the wavelength detected at 251nm.

API Formulation

Preparation of Standard stock solutions and Working Solution: Accurately weighed 5mg of Cyanocobalamin is transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluents and labeled as Standard stock solution. (100µg/ml of Cyanocobalamin) from this 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (10µg/ml of Cyanocobalamin).

Sample Formulation

Preparation of Sample stock solutions and Working Solution: 1 vial of injection consisting of 1000mcg of Cyanocobalamin was transferred into a 10ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (100µg/ml of Cyanocobalamin) from this 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (10µg/ml of Cyanocobalamin)

Method Validation

The established method is validated in accordance with ICH criteria for the purpose of validating analytical methods. The validation metrics were: system appropriateness, accuracy, linearity, limit of detection (LOD), limit of quantification (LOQ), precision, robustness, specificity, and degradation studies.

System suitability parameters:

The working standard solution was injected six times into the HPLC system, and the chromatographic analysis was carried out according to the created and optimized parameters. The system appropriateness parameters were determined by computing the percentage RSD of retention times, theoretical plates, and peak areas from six replicate injections.

Specificity: Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So this method was said to be specific.

Precision:

The technique and system precision have been proven by producing a standard solution at a known concentration and administering six repetitive injections to ensure the proposed approach's consistency. Intermediate accuracy was

also achieved by creating six functioning sample solutions and injecting each solution six times. The area was measured, and the mean, standard deviation, and percentage of RSD were computed. The results were satisfactory, falling below the 2% limit.

Linearity:

To test the drug's linearity, serial dilutions from 2.5 to 15 µg/ml were prepared. A graph was used to show the link between peak area response and medication concentration. It was found to be linear at the specified drug concentration.

Accuracy:

Accuracy was performed in triplicate for various concentrations of Cyanocobalamin equivalent to 50%, 100% and 150% of the standard amount were injected into the HPLC system per the test procedure.

Sensitivity:

Limit of detection and Limit of Quantification

LOD and LOQ were estimated using ICH recommendations based on the average slope and standard deviation of the calibration curve.

Based on the response's standard deviation and calibration curve's slope, the LOD and LOQ can be estimated. The formulae given below can be used to calculate LOD and LOQ:

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

where S is calibration curve of the slope and σ is the response of the standard deviation.

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there was no recognized change in the result and are within range as per ICH Guidelines.

Assay:

The assay and % purity was calculated for brand VIB 12 1000 mcg Injection with label claim 40g. The observed value was compared with that of standard value without interference from the excipients used in the tablet dosage form

Degradation Studies

These investigations are carried out under various stress situations to describe the stability of the pure pharmacological material and are useful in establishing the best storage settings. These research cover base, peroxide, acid, neutral hydrolysis, photo, and heat degradation.

Oxidation:

In a flask, 1 mL of Cyanocobalamin stock solution and 1 mL of 20% hydrogen peroxide (H2O2) were added. The solutions were heated to 60 degrees Celsius for 30 minutes. The HPLC investigation involved diluting the resulting solution to 10µg/ml, injecting 10µl into the system, and recording chromatograms to test sample stability..

Acid Degradation Studies:

In a flask, 1 mL of Cyanocobalamin stock solution and 1 mL of 2N HCl were added. The solutions were heated to 60 degrees Celsius for 30 minutes. The HPLC investigation involved diluting the resulting solution to 10µg/ml, injecting 10µl into the system, and recording chromatograms to test sample stability.

Alkali Degradation Studies:

In a flask, 1 mL of Cyanocobalamin stock solution and 1 mL of 2N NaOH were added. The solutions were heated to 60 degrees Celsius for 30 minutes. The HPLC investigation involved diluting the resulting solution to 10µg/ml, injecting 10µl into the system, and recording chromatograms to test sample stability.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105°C for 6h to study dry heat degradation. For HPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 100µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to obtain 10µg/ml solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

RESULT AND DISCUSSION

Table 1 Optimized Condition

Parameter	*Condition*
Mobile phase	*Acetonitrile: 0.01KH_2PO_4 (50:50 v/v)*
Flow rate	*1 ml/min*
Column	*Sunfire C18 (4.6 x 250mm, 5µm)*
Detector wave length	*251nm*
Column temperature	*30°C*
Injection volume	*10µL*
Diluent	*Water and Acetonitrile in the ratio 50:50*

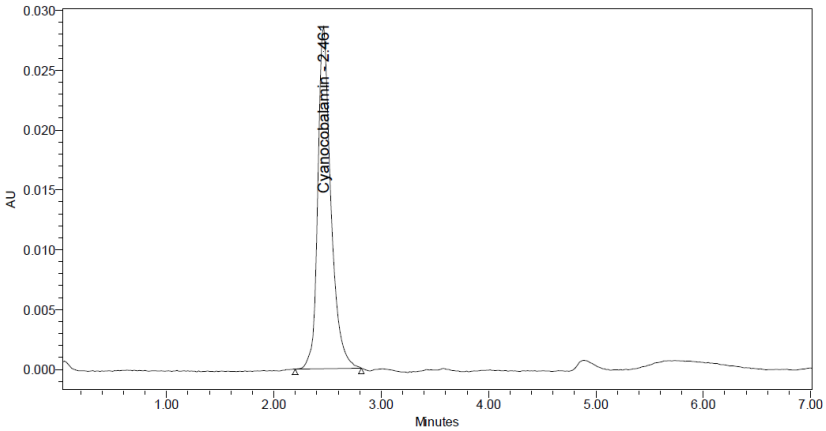

Figure 2: Optimized Chromatogram

System Suitability:

Table 2: System Suitability

S no		*Cyanocobalamin*		
Injection	*RT*	*area*	*Plate Count*	*Tailing*
Injection-1	*2.455*	*338583*	*2594*	*1.21*
Injection-2	*2.458*	*339591*	*2588*	*1.21*
Injection-3	*2.458*	*339075*	*2575*	*1.20*
Injection-4	*2.458*	*342872*	*2587*	*1.19*
Injection-5	*2.461*	*340026*	*2602*	*1.19*
Injection-6	*2.461*	*343948*	*2587*	*1.20*
Mean		*340683*		
Std ev		*2194.2*		
RSD		*0.6*		

Standard solution was injected six times, and their corresponding chromatograms were obtained. The theoretical plate count exceeded 2,000, the USP tailing was under 2, and the percent RSD was under 2%, according to observations. All of the requirements for system suitability were met and fall within acceptable ranges.

Linearity:

Six concentrations ranging from 2.5 to 15 µg/ml were prepared and linearity was estimated in a duplicate manner. The linearity equation for Cyanocobalamin was y = 33995x + 858.82. For the alibration curve over the concentration range, the data have shown a good correlation.

Table 3: Linearity Data

Concentration (ppm)	**Peak area*
0	*0*
5	*606877*
10	*1211958*
15	*1823212*
20	*2430736*
25	*3044340*
30	*3603248*
y :	*33995x + 858.82*
R^2	*0.9999*
Slope	*33995*
Intercept	*858.82*

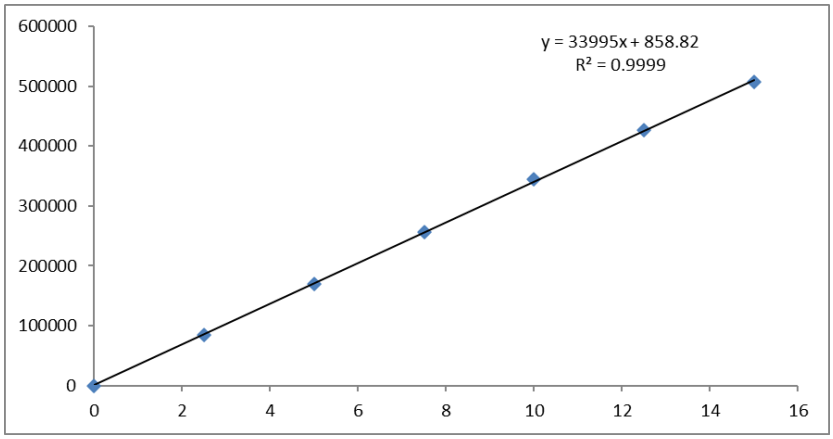

Figure 3: Calibration Curve of Cyanocobalamin

Accuracy:

Three doses were given at each level, and the mean % recovery was calculated. Cyanocobalamin's recovery rate was observed to be between 99% and 100.%, which is within the acceptable ranges

Table 4: Accuracy Data

Level	*Amount Spike (µg/mL)*	*Amount recovered (µg/mL)*	*% Recovery*	*Avg %*	*Mean %Recovery*
		5.00	*99.91*		
50%	***5***	*4.97*	*99.32*	*99.64*	
		4.98	*99.69*		
		9.96	*99.56*		
100%	***10***	*9.96*	*99.61*	*99.62*	***99.74%***
		9.97	*99.69*		
		14.99	*99.92*		
150%	***15***	*15.00*	*99.99*	*99.96*	
		15.00	*99.98*		

Precision:

Table 5: Precision Data

S. No	*Day 1*	*Day 2*
Injection-1	*341144*	*339052*
Injection-2	*338471*	*341555*
Injection-3	*338412*	*338488*
Injection-4	*339103*	*334825*
Injection-5	*341568*	*338867*
Injection-6	*339279*	*339594*
Mean	*339663*	*338730*
S.D	*1361.6*	*2198.1*
%RSD	*0.4*	*0.6*

Sensitivity:

Table 6: LOD and LOQ Data

Molecule	*LOD*	*LOQ*
Cyanocobalamin	*0.01µg/ml*	*0.03 µg/ml*

Robustness:

Table 7: Robustness data

Parameter	*Optimized condition and %RSD*		*Used condition*	*Obtained %RSD*
Flow rate (±0.1ml/min)	*1ml/min*		*0.9ml/min*	*0.3*
			1.1 ml/min	*0.4*
MP Composition (5%v/v)	*40:60*	*0.6%*	*35:65*	*0.5*
			45:55	*0.1*
Column Temp (±3^{0}c)	*30^{0}c*		*27 ^{0}C*	*0.5*
			33 ^{0}C	*0.3*

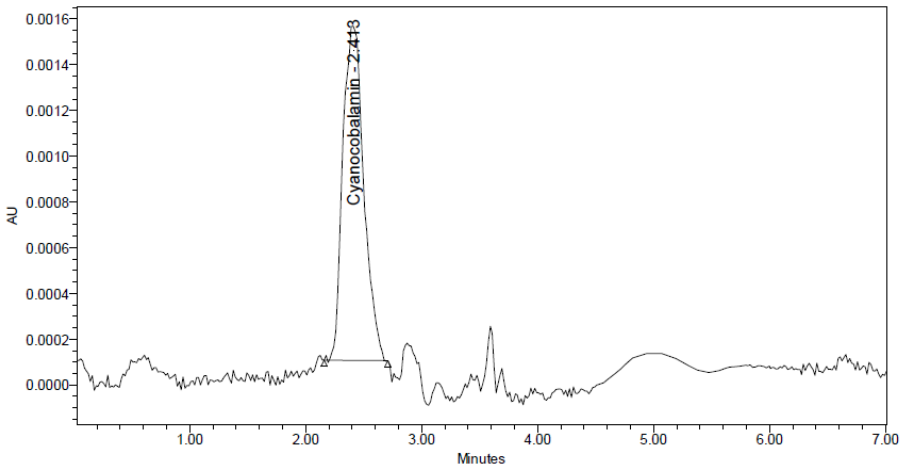

Figure 4: LOD Chromotogram

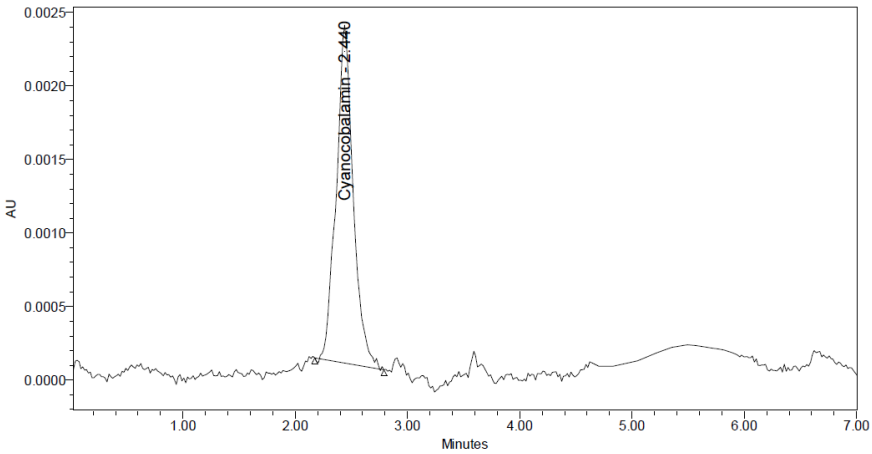

Figure 5: LOQ Chromotogram

Assay

Table 8: % Assay Purity Data

Formulation	*Label claim(mg)*	*% Assay**
VIB 12	*Cyanocobalamin 1000 mcg*	*99.50 %w/w*

Degradation studies:

Table 9: Force Degradation Studies

S.No	*% Degradation*	*Peak area*	*Stress Conditions*	*Peak Purity*
1	*5.82*	*321502*	*Acid*	*Passes*
2	*5.61*	*322231*	*Base*	*Passes*
3	*2.96*	*331245*	*Oxidation*	*Passes*
4	*2.18*	*333938*	*Thermal*	*Passes*
5	*1.66*	*335698*	*Photolytic*	*Passes*
6	*0.34*	*340219*	*Hydrolytic*	*Passes*

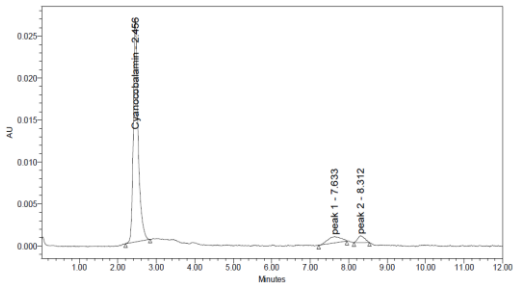

Figure 6: Acid chromatogram of Cyanocobalamin

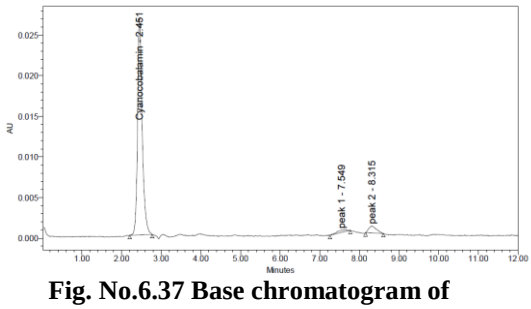

Fig. No.6.37 Base chromatogram of Cyanocobalamin

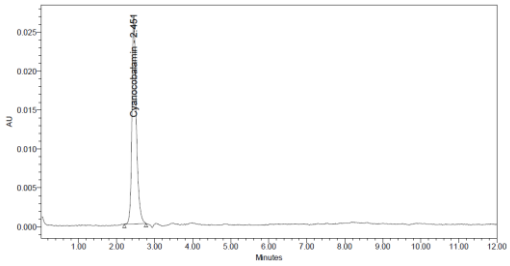

Fig. No.6.38 Peroxide chromatogram of Cyanocobalamin

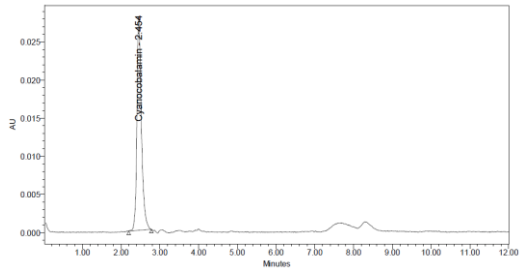

Fig. No. 6.39 Thermal chromatogram of Cyanocobalamin

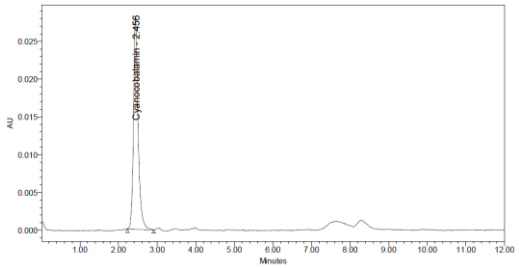

Fig.No. 6.40 UV chromatogram of Cyanocobalamin

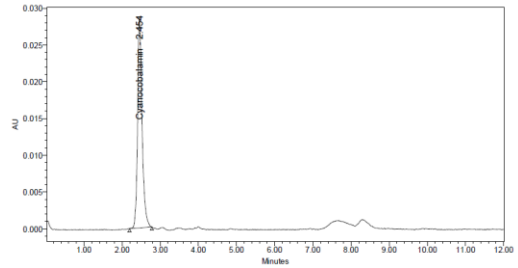

Fig.No.6.41 Water chromatogram of Cyanocobalamin

CONCLUSION:

The developed HPLC method for the estimation of Cyanocobalamin proved to be simple, precise, accurate, and highly reproducible. The chromatographic conditions were successfully optimized to achieve clear separation of Cyanocobalamin with a distinct retention time and excellent peak resolution. System suitability parameters, including theoretical plates, tailing factor, and %RSD, were found to be within acceptable limits, demonstrating the robustness and reliability of the method. Linearity studies confirmed a strong correlation between peak area and concentration across the selected analytical range, while recovery and precision results supported the accuracy and repeatability of this technique. Overall, the validated HPLC method is suitable for routine quantitative analysis of Cyanocobalamin in bulk and pharmaceutical formulations and can also be effectively applied in quality control laboratories for regular monitoring

ACKNOWLEDGEMENT:

The authors are thankful to, Department of Pharmaceutical Analysis, Malla reddy college of Pharmacy, Affiliated to Osmania University, India and Spectrum Pharma Research Solutions, Hyderabad, Telangana, India.

REFERENCES:

1. *StatPearls: Cyanocobalamin*
2. *PubChem — Cyanocobalamin (Vitamin B12).*
3. *Fang H, Kang J, Zhang D: Microbial production of vitamin B12: a review and future perspectives. Microb Cell Fact. 2017 Jan 30;16(1):15*
4. *NIH: Vitamin B12*
5. *Pubchem: Cobalamin*
6. *O'Leary F, Samman S: Vitamin B12 in health and disease. Nutrients. 2010 Mar;2(3):299-316. doi: 10.3390/nu2030299. Epub 2010 Mar 5.*
7. *Cyanocobalamin injection, monograph.*
8. *Valentin Lacombe et al., Oral vitamin B12 supplementation in pernicious anemia: a prospective cohort study, Am J Clin Nutr. 2024 Jul;120(1):217-224*
9. *Ralph Carmel, How I treat cobalamin (vitamin B12) deficiency, Blood. 2008 Jul 7;112(6):2214–2221*
10. *Drugbank: Cyanocobalamin*
11. *ScienceDirect topic entry — Cyanocobalamin: properties and stability overview.*

12. *FDA clinical/pharmacology review: Cyanocobalamin regulatory/clinical pharmacology documentation.*
13. *The metabolic processes of folic acid and Vitamin B12 deficiency*
14. *Ruchi Singh,Harvinder Popli & Meghraj Suryawanshi, Development and validation of an HPLC analytical method for the simultaneous estimation of vitamin B12 and folic acid, ESSENTIAL CHEM2025, VOL. 2, NO. 1, 1–9*
15. *Labaran Ibrahim, Aminu Usman (2022), A simple and rapid RP-HPLC method for the assessment of cobalamin (vitamin B12) in tilapia and snoek fishes, Turkish Journal of Chemistry, (2022) 46: 320-329.*
16. *Wonjong Lee, Yoon-Bok Lee, Moon Haeng Huh, Jae Kwon Choi, Determination of the Chemical Stability of Cyanocobalamin in Medical Food by a Validated Immunoaffinity Column-Linked HPLC Method, Journal of Food QualityVolume 2022, Article ID 1619936, 8 pages*
17. *Xiao Qiu et al. (2019), Determination of active vitamin B12 (cobalamin) in dietary supplements and ingredients by reversed-phase liquid chromatography: Single-laboratory validation, Food Chemistry, Volume 298, 15 November 2019, 125010.*
18. *Vigbedor Bright Yaw, Nathaniel Nii Adu Okine, Adosraku Remmel K, Samuel Osafo Acquah, Design of HPLC Method for Quantification of Cyanolobalamin Injection, International Journal of Novel Research in Life Sciences Vol. 2, Issue 6, pp: (26-39), Month: November-December 2015.*