Asian Journal of Chemistry; Vol. 37, No. 2 (2025), 291-299

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2025.32978

Quality by Design and Green Chemistry Analytical Method: New Combined Methodology for Quantification of Cyanocobalamin and Benzyl Alcohol in Parenteral Formulations

NISHANT GOSWAMI and MUDHULKAR RAJU*

Department of Chemistry, Koneru Lakshmaiah Education Foundation, Bowrampet, Hyderabad-500043, India

*Corresponding author: E-mail: m.raju@klh.edu.in

Received: 2 November 2024; *Accepted*: 18 December 2024; *Published online*: 31 January 2025; AJC-21878

Developing methods for the analysis of hazardous chemicals and complex parenteral formulations is a challenging process. To address these challenges, the integration of green chemistry principles and design of experiments proves highly effective. This study introduces a novel approach that combines green analytical chemistry and design of experiments (DoE) to develop a sensitive, reproducible and stability indicating method by high-pressure liquid chromatography method for quantification of cyanocobalamin and benzyl alcohol in liquid parenteral formulations. A high-pressure liquid chromatography methodology for quantification of cyanocobalamin and benzyl alcohol was established with an analytical quality by design principle. The method employs the central composite response, which optimizes the connection between factors and responses with reduced number of design points and experimental runs, yielding statistically significant results and enhancing analytical quality. Separation of cyanocobalamin and benzyl alcohol was achieved on Zorbax C-8 column (250 * 4.6 mm with 5 µm) using buffer as ammonium formate (pH 2.5) and acetonitrile with a gradient mobile phase at a pump flow of 1.0 mL/min. Detection was performed by ultra violet detector set to a wavelength of 254 nm. The proposed method is validated in line with ICH Q2 (R2) guidelines, evaluating parameters such as linearity, accuracy, precision, system suitability, sensitivity, robustness and solution stability, all within acceptable ranges. The eco-affability and greenness of new developed method were checked by using the Analytical Greenness metric, Green Analytical Procedure Index and National Environmental Method Index, confirming its suitability as an environmentally sustainable analytical approach.

Keywords: Cyanocobalamin, Benzyl alcohol, RP-HPLC, Analytical quality by design, Green analytical chemistry.

INTRODUCTION

The immune system, enzymatic activities, growth, development, repair and maintenance of the body all depend on vitamins, which are vital organic chemical components needed in minute quantities [1,2]. Deficiencies in vitamins can lead to variety of physiological disorders or abnormalities, depending on specific vitamin that lacking in the diets [3]. Examples of such disorders include night blindness and mental retardation [4]. Nicotinamide (vitamin B3) is crucial for carbohydrate and cellular metabolism for instance and DNA healing through its role in nonredox adenosine diphosphate-ribose transfer reactions [5]. Cobalamin (vitamin B12) is a water soluble vitamin that contains cobalt at its core and is also known as cyanocobalamin (CNM), a term used for any cobalamins with similar biological functions [6]. Cobalamin exists in four distinct forms such as methylcobalamin, hydroxocobalamin, cyanocobalamin and 5′-deoxyadenosylcobalamin [7,8]. The essential functions of vitamins in physiological processes are paralleled by the protective role of preservatives, which shield products from microbial degradation.

Preservatives are chemical agents designed to protect food items, pharmaceuticals and cosmetics from deleterious changes induced by microorganisms [9]. Preservative used in appropriate quantity, they prevent the growth of microorganisms at the time of manufacturing and usage phases of therapeutic products [10]. Benzyl alcohol is a water-soluble preservative commonly utilized in injectable pharmaceutical formulations and cosmetic [11].

Though toxic to neonates and infants, the USFDA agency recognizes it safe for adults at concentrations up to 5% [12]. Recent studies investigated the effects of benzyl alcohol as a preservative in bacteriostatic normal saline when administered *via* intra-articular injection. This research particularly relevant in context of MRI-based arthrography, where gadolinium cont-

rast agents are diluted with saline [13]. Limited chromatographic methods have been stated for the quantification of benzyl alcohol in presence of other drugs [14-17].

Recently, the principles of green chemistry have gained significant prioritization and implementation by both public and private industries. These approaches aim to reduce environmental contamination affecting sol, air and water, thereby enhancing the quality of life for humans and wildlife. According to the definition, Green chemistry is the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances [18,19]. The present investigations endeavor to reduce the use of solvents and other contaminants. Typically, tools such as the National Environmental Methods Index (NEMI), Green Analytical Procedure Index (GAPI) and Analytical Eco-Scale, Analytical GREEnness (AGREE), are utilized to evaluate the eco-friendliness of analytical methods. This study specifically employs Analytical Eco-Scale, GAPI and AGREE to assess the greenness of methodology. Limited literature is available that utilizes AGREE and GAPI for such assessments [20-26].

The conventional procedure to change one-method factor-at-one-time method optimization procedure is the time taken and error and trial concept [27-35]. In contrast, the QbD approach offers a systematic method development process that begins by identifying potential risks that could lead to failures. This is followed by determining method critical parameters (CMPs) and optimizing them using the design of experiment (DoE). The QbD approach also facilitates comprehension of both the single impacts and interaction effects of method critical parameters (CMPs). This study objects to optimize a unique analytical method for estimation of cyanocobalamin and benzyl alcohol in formulation pharma products, incorporating both green chemistry and AQbD concept.

To our best of knowledge, this study denotes the best attempt to address the (SIM) stability-indicating HPLC method for estimation of cyanocobalamin and benzyl alcohol, despite their formulation in glass vial formulation. This work presents a highly sensitive, reproducible, rapid and simple RP-HPLC method, developed and validated for the estimation of cyanocobalamin and benzyl alcohol in pure for, oral products and parenteral dosage form, in agreement with International Conference on Harmonization (ICH) guidelines [26]. The focus of this study is to target the method development and report single sensitive, simple, robust and precise stability-indicating HPLC methods for the real-time quantification of benzyl alcohol and cyanocobalamin, facilitating their routine quantitative analysis. Moreover, the HPLC method, optimized using the central composite design (CCD) of response surfaces, reduces the number of trials, making the method eco-friendly, time-efficient and cost-effective. The developed method was validated inline with USP Chapter 1225 and ICH Q2(R2) guidelines, assessing specificity, precision, linearity and accuracy.

EXPERIMENTAL

The test samples, predominantly cyanocobalamin injection USP, were formulated at Innovation Plaza (Dr. Reddy's Ltd., Hyderabad, India). Reagents and chemicals utilized in the analysis were of Gradient grade. Acetonitrile was sourced from Rankem, while high pure grade formic acid was sourced from Merck. 30% hydrogen peroxide and hydrochloric acid were obtained from Merck, USA. Milli-Q water from Millipore Technologies (Mumbai, India) was used for all solutions.

Instrumentation and software: Peak separation was achieved on Water HPLC system comprising a Waters 2695 separation module with 2996 photo diode array detector (PDA) with Empower software. The testing utilized a 5 µm particle size, Zorbax C8 (4.6 mm × 250 mm) column. Design-Expert 13 tool, was applied for optimizing the CMPs. This software was established by Lhasa, UK. A balance from Mettler-Toledo was employed for the precise weighing of the sample materials. Exposure of samples to specific degradation conditions was facilitated by employing a dry oven from Thermo Technologies and a photo stability chamber from Thermo Lab Scientific.

HPLC: Prepared solvent A as mobile phase A with the mixing of acetonitrile and buffer in the proportion of 100:900 (v/v). The buffer having pH 2.5 was prepared by dissolving 1.0 g of ammonium formate in 1.0 L mL of HPLC grade (Milli-Q) water and sonicating to dissolve. The pH was adjusted to 2.5 with diluted formic acid solution. An acetonitrile solution, solvent B, was prepared. UV detection studies was performed at 254 nm and a flow rate of 1.0 mL/min. An 10 µL injection volume was chosen for enhanced peak sensitivity. Data acquisition time was set to 45 min. The HPLC system pump operated in gradient mode, following the program: Time (min)/solvent A (v/v): solvent B (v/v): $T_{0.01}$/85:15, T_{03}/85:15, T_{30}/75:25, T_{35}/75:25, T_{40}/85:15, T_{45}/85:15.

Preparation of standard, diluent and sample solutions: The reference standard and test solutions were prepared with a combination of HPLC grade (Milli-Q) water and methanol in a proportion of 80:20 (v/v). Stock solutions (1000 µg/mL) of cyanocobalamin and (15000 µg/mL) of benzyl alcohol were prepared in diluent, while samples were prepared freshly by the appropriate dilution of higher (stock) preparations with the diluent to achieve 200 µg/mL of cyanocobalamin, 3000 µg/mL of benzyl alcohol. These solutions remained stable when stored at 4 ºC, yielding a recovery rate of 98%. A test solution was prepared with the concentration of 200 µg/mL of cyanocobalamin, 3000 µg/mL of benzyl alcohol by pipetting the suitable volume of test solution in suitable volumetric flask. The flask volume filled with diluent and thoroughly mixed.

Stress study of cyanocobalamin (forced degradation study): The purpose of the stress experiments was to elucidate the degradation behaviour, including acidic degradation, oxidation, basic degradation thermal degradation and photolytic degradation of drug ingredients, as well as to separate degradation products from the active peaks [32-34]. Stress studies of cyanocobalamin conducted under various conditions like acid and base experiments achieved by separately mixing 5 mL of 1 molar HCl solution and 1 molar NaOH solution, respectively. Photolytic experiment was performed on test solution charged to photolytic and UV conditions equivalent to 1.2 million lux hr and 200-Watt h m^{-2} at control lab condition, respectively, for 5 days at 254 nm. Oxidative experiment was carried out by

adding 10% H_2O_2 to 25 mL of the prepared sample. Thermal degradation was carried out at 50 ºC for 30 min at water bath.

Analytical quality by design (AQbD) based method scouting: As defined by ICH guideline Q8(R2), QbD is a systematic approach to pharmaceutical development that commences with predefined goals, emphasizing comprehension of product and process and prioritizing process control grounded in scientific principles and quality risk management. Furthermore, the objective is to minimize variations to maintain uniformity and batch failures in product superiority. Implementing an improved quality by design (QbD) methodology in the pharmaceutical product development introduces chances for fast regulatory approval and compliance. This allows manufacturing alterations within predefined parameters to potentially bypass regulatory scrutiny and approval procedures. Method development of the HPLC methodology for quantifying cyanocobalamin and benzyl alcohol embraced AQbD method to reduce inconsistency in the quantification method. The primary objective was to delineate the analytical target profile (ATP) in the development study. The analytical target profile aimed to establish a sensitive, robust, specific, accurate and environmentally sustainable HPLC methodology employing greener chemicals in nature. Numerous initial trials were conducted through an iterative testing to optimize consistent methodology and detecting independent, critical, method parameters and their impacts on dependent variables.

Method validation: The new developed method was validated as per ICH Q2(R2) guidelines, assessing validation parameters like system precision, robustness, specificity, method linearity, accuracy, method precision and stability of sample solution [33-35].

System precision test: The system precision or suitability test ensures the HPLC system is functioning correctly before analysis. This test is performed prior to each sample analysis. System suitability parameters include the %RSD for the peak areas of both cyanocobalamin and benzyl alcohol, the number of theoretical plate counts, the resolution between cyanocobalamin and benzyl alcohol and the tailing factors for both cyanocobalamin and benzyl alcohol peaks.

Specificity and linearity: Specificity refers to the analytical method denotes its capability to differentiate the analyte(s) from other components within the matrix present in sample. For an HPLC method, this is ensured by the proper separation of the drug peak(s) from additional peaks arising from matrix present in the sample solutions. Specificity was evaluated as per method by injecting of the standard, sample, placebo and blank solutions into the chromatographic system separately. Linearity was determined by generating a calibration curve, plotting the peak areas of the interested components (cyanocobalamin and benzyl alcohol) against their corresponding test concentrations. The correlation coefficient (r^2) and linear equation for both components were calculated to demonstrate a linear relationship.

Robustness: Robustness was evaluated for analytical method by making minor modifications to HPLC parameters, such as changes in buffer pH, HPLC column temperature, flow rate and mobile phase composition. The retention time (RT) and percent RSD of the peak area for both components were maintained with acceptance criteria of $d \leq 2.0\%$. Additionally, the USP tailing factor and USP theoretical plates showed no significant impact. These slight variations indicate that new developed method is found robust in nature for the quantification of both components.

Recovery (accuracy) and precision: The recovery, expressed as % accuracy, indicates the nearness of the measured value to the true value. Accuracy was calculated with the standard addition methodology, wherein the respective standard was spiked into the formulation matrix solution at 50, 100 and 150% of the target concentration. Precision (intra-day repeatability) was performed by evaluating six consecutive preparation of the test solution at the sample concentration. Intermediate precision (inter-day repeatability) was evaluated by performing six preparation of the test solution on another day, using a another HPLC instrument, column and chemist. The %RSD (relative standard deviation) was evaluated for both inter and intra-day precision to assess the consistency of the method.

Stability studies of analytes: The test solution stability of both cyanocobalamin and benzyl alcohol in diluent was evaluated at test concentration levels under various storage conditions. The short-term stability study (accelerated) was also conducted at Lab condition, while the refrigerator (long term) stability study was conducted at 2-8 ºC for 2 days.

Green analytical principles valuation for new proposed method: The primary goal of the method's greenness is the careful selection of chemicals during method development, considering their hazardous impact on health and the environment. A significant environmental concern in analytical testing is the use of solvents. As solvent-free techniques are not always feasible, solvents must be preferred with environmental awareness. Various solvent choice guides designed by pharmaceutical industries offer valuable guidance [20-26]. Another challenge is the use of acids, bases and other supplementary compounds. Due to their differing inherent properties, acids and bases need distinct parameters for greenness valuation. While no specific metrics system exists for analytical chemistry, the GlaxoSmithKline acid and base selection guides provide useful insights for selecting green alternatives [36-40]. The greenness of the new proposed method was evaluated by the GAPI, NEMI and AGREE [41].

RESULTS AND DISCUSSION

Preliminary method development: In this study, initial experiments were led to fix the stationary phase, column dimension and operational column condition like temperature, alongside determining the ideal composition of the mobile phase to meet the green chemistry principle. Based on the nature of the analytes, RP-HPLC was used. Critical parameters, including the pH of buffer used to prepare the mobile phase and composition of the mobile phase A and mobile phase B, were identified as high-risk factors requiring optimization. Initially, Cyano and C18 columns were evaluated to enhance system precision parameters. The Cyano HPLC column exhibited inadequate resolution and high tailing factor for both component peaks. A range of mobile phase composition with altered pH were

explored to assess solubility profiles and identify the superior method conditions. Acetonitrile and methanol, the predominant organic solvents in RP-HPLC, were tested in various compositions to achieve optimal peak resolution, leading to the optimization of a gradient method. Buffer solutions with pH values of 2.2, 2.5 and 3.0 were studied to achieve better separation and peak shape for cyanocobalamin and benzyl alcohol. It was found that a buffer with pH 2.5 provided superior resolution with comparison to other pH values. Firstly, 100% water was used as a method diluent, but the peak symmetry of cyanocobalamin was unsatisfactory. However, addition of 50% acetonitrile to the diluent significantly improved the peak shapes for both analytes.

Analytical method assessment using QbD approach: In AQbD methodology, the analytical target profile (ATP) serves a part analogous to the quality target product profile (QTPP) in product development. The ATP defines the intended characteristics of the critical analytical attributes (CAA) for the method's development and adherence to regulatory requirements. It requires a set of parameters and targeted components that must be measured, including the analytical techniques and concentration series to be applied based on the method's performance criteria. Analytical methodology checking is linked with CMPs, which correspond to critical quality attributes (CQAs) in a cause and effect relationship, influencing the selected critical analytical attributes (CAAs).

Defining analytical target profile: The analytical target profile specifies the performance measures for the analytical methodology, aligning with the QTPP as outlined in the ICH Q8 guidelines.

Defining critical analytical attributes (CAAs): The CAAs are decided by system precision parameters such as resolution, USP plates count and USP tailing factor, to confirm the robustness of the methodology. In this study, the resolution between the peaks is measured as a CAA, its impact on input variables was evaluated using a response surface design. As per ICH Q2(B) advices, the required parameters are: USP plates > 5000, resolution between peaks ≥ 2.0 and USP tailing between 0.8 to 1.5.

AQbD optimization using response surface central composite design: Among the identified factors, pH, flow rate and column temperature identified as high-risk parameter, while the other method parameters were found medium and low-risk. during day-to-day analysis, resolution among cyanocobalamin and benzyl alcohol peaks may vary and is considered a likely risk that requires mitigation through design of experiments (DoE) and the design space approach. The design included 3 factors and 3 center points with block design, resulting in a total of 12 trial experiments. The responses are analyzed and the results are shown in Table-1. The Analysis of Variance ANOVA data (Table-2) evidently indicates that the P-value is less than 0.05, demonstrating statistical significance for responses, as well as resolution among cyanocobalamin and benzyl alcohol peaks. This significance confirms that the model is valid for the given response. Figs. 1 and 2 illustrate the all-factor composite response surface plots, including 2D contours, cube plots and 3D contours, for the analyzed responses. The resolution between the cyanocobalamin and benzyl alcohol peaks, the response of interest, is influenced by both the buffer pH and the column temperature. The projected R^2 of 0.8965 is in realistic agree-

TABLE-1
DESIGN OF EXPERIMENTS (DoE) TRAILS AND HPLC DATA

Standard	Run	Factor 1	Factor 2	Factor 3	Response
		Column temperature (°C)	Flow rate (mL/min)	pH	Resolution between cyanocobalamin and benzyl alcohol
2	4	45	0.8	2.3	4.0
4	10	45	1.2	2.3	5.8
3	11	35	1.2	2.3	10.3
1	12	35	0.8	2.3	8.7
10	5	40	1.0	2.5	9.1
11	7	40	1.0	2.5	8.9
12	8	40	1.0	2.5	7.7
9	9	40	1.0	2.5	7.7
5	1	35	0.8	2.7	10.4
6	2	45	0.8	2.7	5.4
8	3	45	1.2	2.7	7.7
7	6	35	1.2	2.7	13

TABLE-2
ANOVA TABLE FOR RESOLUTION AMONG CYANOCOBALAMIN AND BENZYL ALCOHOL

Response	Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-value	
Resolution between cyanocobalamin and benzyl alcohol	Model	0.2010	3	0.0670	65.48	< 0.0001	Significant
	Column temp.	0.1510	1	0.1510	147.52	< 0.0001	
	Flow rate	0.0295	1	0.0295	28.81	0.0010	
	pH	0.0206	1	0.0206	20.12	0.0028	
	Residual	0.0072	7	0.0010			
	Cor Total	0.2155	11				

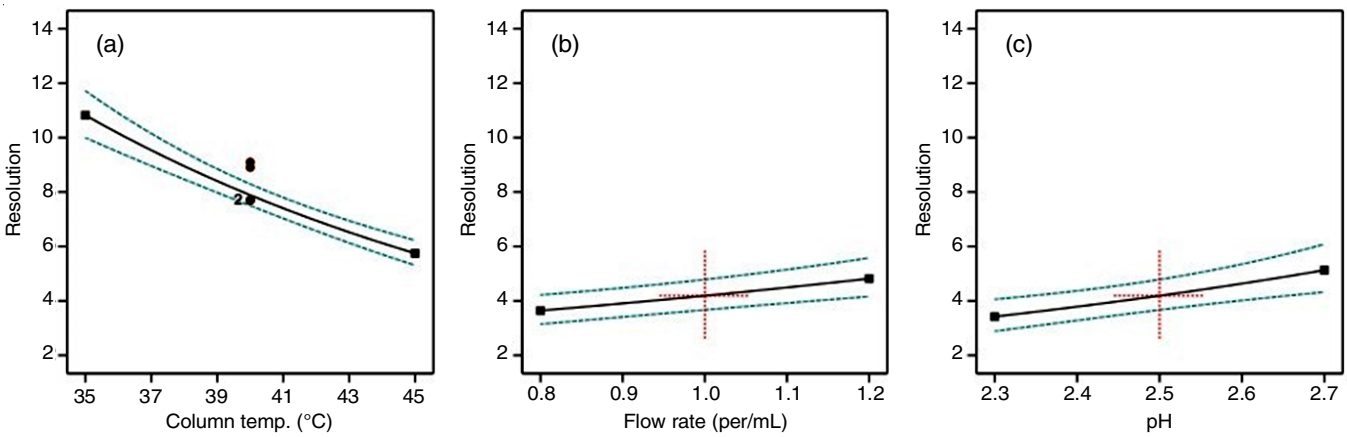

Fig. 1. Plot of resolution between cyanocobalamin and benzyl alcohol (all factor coding)

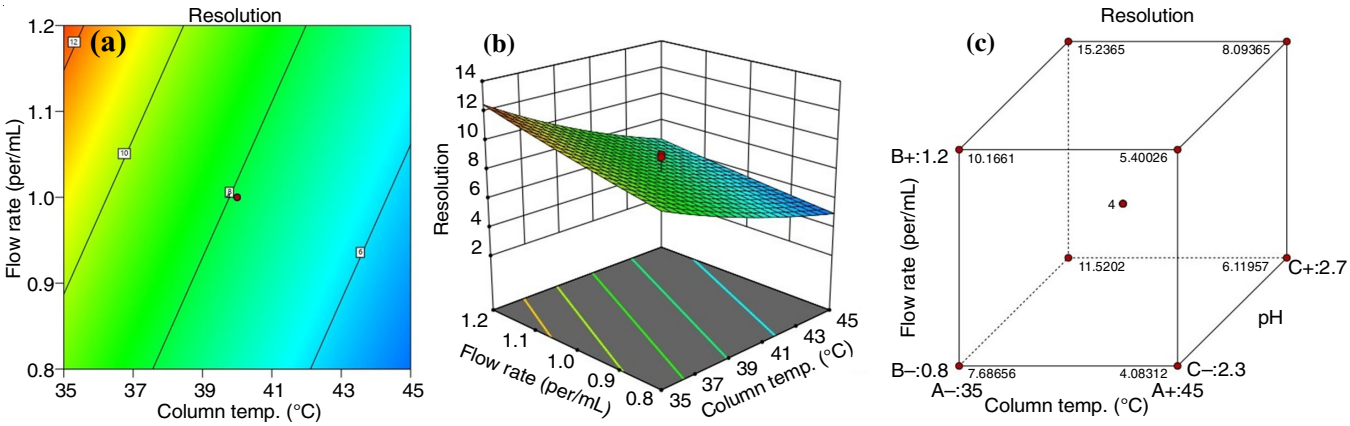

Fig. 2. (a) Contour plot of resolution between cyanocobalamin and benzyl alcohol, (b) 3D plot of resolution between cyanocobalamin and benzyl alcohol, (c) Cube plot of resolution between cyanocobalamin and benzyl alcohol

ment with adjusted R^2 of o.9508, with a discrepancy of not more than 0.2. The adequate precision, which quantifies the s/n ratio, is greater than 4, indicating a robust sign; specifically, it is 24.415, suggesting a reliable signal. Thus, the model is appropriate for exploring the design space.

Method validation: In accordance with ICH Q2(R2) guidelines, the new developed methodology was validated across various parameters, including system precision, linearity, specificity, recovery, precision, robustness and stability under specified storage conditions.

System suitability test: The system precision parameter indicated that the number of theoretical plates exceeded 5000, a benchmark considered acceptable for the analysis. Parameters like USP tailing factor and %RSD of components were within acceptable limits (Table-3). These findings confirmed that the HPLC methodology is suitable and that the data found well within the acceptable limits.

Specificity: A PDA HPLC system was used to confirm the peak purity and validated the proposed methodology for specificity. All degradation peaks were well-separated from cyanocobalamin and benzyl alcohol peaks and purity angles remained within threshold values (Table-4), indicating absence of interferences. To evaluate specificity, 10 µL solutions of standard, stressed sample, placebo and blank were separately injected into the chromatographic system. No interfering peaks were observed other than those of the target analytes (Table-4). This confirms that developed methodology is specific for determination of cyanocobalamin and benzyl alcohol.

Linearity: The linearity was established through calibration curves showing a linear relationship between mean peak areas and analyte (cyanocobalamin and benzyl alcohol) concentrations (Table-3).

Robustness: The robustness was assessed by slight variations in HPLC parameters, including the buffer pH, HPLC column temperature, mobile phase composition and flow rate. The percent RSD of the area and retention time within the acceptable limit of not more than 2.0%. Additionally, the USP tailing and the USP theoretical plates showed no significant changes. These results indicate that the developed methodology is robust for the determination of both components.

Recovery (accuracy): Recovery was determined, which reflects the nearness to the actual value. The % recovery ranged from 95% to 105% for cyanocobalamin and benzyl alcohol across for all test concentrations (Table-3). These results fall within the acceptable limits, confirming that the developed methodology is accurate.

Precision: The precision of the methodology was evaluated by calculating the % RSD of % recovery for intra-day and another day (intermittent) precision by preparing the sample solution as per the methodology. The data demonstrated that

TABLE-3
METHOD VALIDATION DATA FOR PROPOSED ANALYTICAL METHOD

Parameter name	Cyanocobalamin	Benzyl alcohol
System suitability		
USP tailing (less than 2.0)	1.1	1.2
USP plate (more than 5000)	18846	6996
RSD percent (n = 6, less than 2.0)	0.3	0.5
Specificity		
Diluent interference (no peak should be at analyte RT)	No peak	No peak
Placebo interference (no peak should be at analyte RT)	No peak	No peak
Peak purity	Passed	Passed
Linearity		
Range (µg/mL)	47.2-378	751.3-4507.8
Slope	11604	798
Intercept	24907	36260
Correlation coefficient > 0.999	0.9999	0.9999
Recovery (n = 6 avg percentage)		
50% avg ± SD	100.8 ± 0.5	99.5 ± 0.5
100% avg ± SD	99.1 ± 1.1	98.9 ± 0.9
150% avg ± SD	100.3 ± 0.9	99.6 ± 0.3
Precision		
Inter-day, n = 6 %RSD less than 2.0	0.3	0.4
Intermediate precision		
Intra-day, n = 6 %RSD less than 2.0	0.2	0.5
Ruggedness, n = 12 %RSD less than 2.0	0.4	0.6
Solution stability		
Bench top (0 and 24 h % difference should be less than 2.0)	1.8	0.7
2?8 °C (initial and 24 h % difference should be less than 2.0)	1.6	0.8
Bench top (initial and 48 h % difference less than 2.0)	0.3	0.7
2?8 °C (0 and 48 h % difference < 2.0)	0.5	0.8

TABLE-4
DEGRADATION SUMMARY RESULTS AT DIFFERENT STRESS CONDITIONS AND PEAK PURITY FOR CYANOCOBALAMIN

Degradation type and conditions	Degradation (%)	Assay (%)	Purity index	Single point threshold	Peak purity
Bare cyanocobalamin	0.56	98.9	1.000	0.999	Passes
Acidic degradation (1 M HCl, refluxed at 60 °C for 30 min)	2.0	97.7	1.000	0.998	Passes
Basic degradation (1 M NaOH, refluxed at 60 °C for 30 min)	6.3	92.1	1.000	0.996	Passes
Photolytic degradation (1200 KLX Visible light and 200 Watt h m^{-2} UV light exposure)	5.94	93.1	1.0000	0.999	Passes
Oxidative degradation (5 mL 10%H_2O_2 at RT)	0.98	97.7	1.000	0.999	Passes
Thermal degradation at 50 °C for 30 min	1.5	97.5	1.000	0.999	Passes

proposed methodology is precise and reproducibility, the observed % RSD is below 2.0% (Table-3 and Fig. 3). These findings suggest that the method is repeatable and reliable.

Stability studies of analytical solutions: The solution stability of both components (cyanocobalamin and benzyl alcohol) in the diluent was evaluated at test concentration levels at room temperature and at 2-8 ºC over a period of 2 days. No degradation of either analyte was observed under these conditions (Table-3). These findings confirmed that the test samples were stable for 2 days at room temperature and that their standard solutions maintained stability for 2 days at 2-8 ºC (refrigerated).

Green analytical metric tools: The greenness of the developed methodology was assessed using AGREE, GAPI and NEMI tools. The methodology involves a total run time of 45 min to analyze two components with a flow of 1.0 mL/min. Each sample preparation consumes less than 20 mL of acetonitrile and 20 mL of methanol, causing in a total solvent usage of under 50 mL per sample analysis. The green assessment results, summarized in Table-5, demonstrate that the method scores well on the AGREE tool, with a score of 0.78, indicating it is greener compared to previous methods. Table-5 also includes the results of the GAPI tool evaluation, which assesses the test obtaining, methodology, test preparation, reagents/solvents used, technique and quantification. Fig. 4 illustrate the greenness of the new method.

Conclusion

A joint approach of analytical quality-by-design (AQbD) and green analytical chemistry principles was utilized to develop a new robust methodology for the separation of two analytes in a parenteral formulation. The greenness of the methodology was assessed by metric tools such as NEMI, AGREE and GAPI. The method was deemed excellent due to the use of environmental friendly solvents used in diluent and in mobile phase.

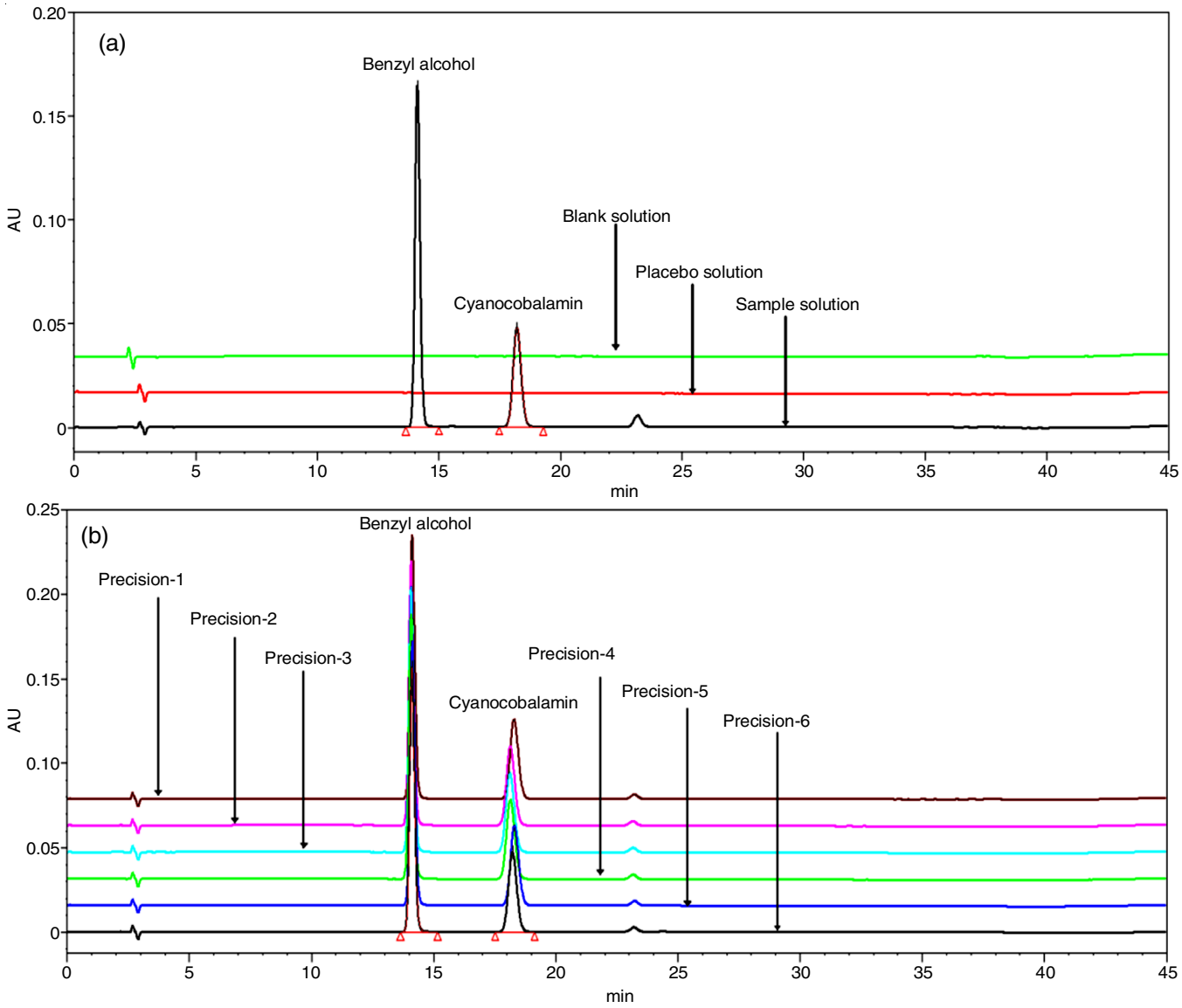

Fig. 3. (a) Overlay RP-HPLC chromatogram of the blank, placebo and sample and (b) Overlay RP-HPLC chromatogram of individual samples subjected to various stress conditions

TABLE-5
ECO SCALE FOR EVALUATING THE ANALYTICAL METHODOLOGY

S. No.	Green analytical chemistry principles	Proposed sample procedure
1	As such analytical techniques should be used to avoid sample preparation	Off-line
2	Minimum sample size should be used for testing	1 g
3	If possible, measurement should be performed on-time	At-line
4	Simple step analytical processes and operations should be use to reduce energy and minimize the consumption of reagents	No steps in test preparation
5	Automatic and miniaturized technique should be use	Semi-automatic and miniaturized
6	Derivatization/extraction should not be use	No derivatization
7	Generation of a big quantity of solvent waste should be avoided and appropriate management of waste should be available	1 g
8	Multi-analyte methods are recommended *versus* single analyte method	2 Analytes determined with single run;
9	The energy should be less during analysis: Select the most energy-saving technique method, or the closest equivalent	High performance
	Otherwise, asses the power consumption of a single analysis in kWh	0.15
10	bio sources reagents should be selected.	Bio-based reagent used
11	Toxic reagents should be avoided	No toxic reagents or solvents used
12	Operator's safety should be taken care	The dangers not avoided ware: (a) Bioaccumulative, (b) Flammable, (c) Explosive

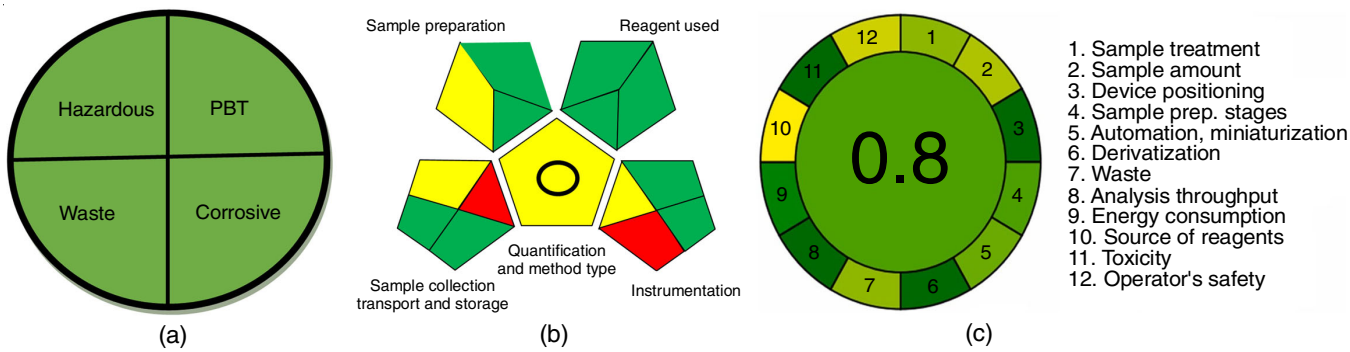

Fig. 4. Representation of (a) NEMI, (b) GAPI and (c) AGREE pictogram for proposed methodology

The AQbD methodology developed, which evaluated 2 responses with a central composite response surface involving 3 factors. It was evaluated that the buffer pH and column temperature significantly influence the resolution among cyanocobalamin and benzyl alcohol. The proposed methodology was validated for recovery, specificity, robustness and precision according to ICH Q2 procedures. The stress study (forced degradation) behaviour of cyanocobalamin under several stress conditions was studied following ICH Q1A and Q1B guidelines. This work presents a simple, robust, rugged and validated methodology for quantifying cyanocobalamin and benzyl alcohol in greener solvents. The method adheres to green analytical principles and AQbD, making it suitable for routine and quality control stability sample analysis.

ACKNOWLEDGEMENTS

The authors thank Dr. Reddy's Laboratories Ltd. (DRL), for analytical and other kinds of support. The authors are also grateful to Intellectual Property Management Department of DRL for technical support. The manuscript has been assigned PUB00696-24 registration number.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. S. Mitra, S. Paul, S. Roy, H. Sutradhar, T.B. Emran, M.U. Khandaker, F. Nainu, M. Almalki, P. Wilairatana and M.S. Mubarak, *Molecules*, **27**, 555 (2022); https://doi.org/10.3390/molecules27020555
2. E. Tourkochristou, C. Triantos and A. Mouzaki, *Front. Immunol.*, **12**, 665968 (2021); https://doi.org/10.3389/fimmu.2021.665968
3. I. Ortigues-Marty, D. Micol, S. Prache, D. Dozias and C.L. Girard, *Reprod. Nutr. Dev.*, **45**, 453 (2005); https://doi.org/10.1051/rnd:2005038
4. W. Elfalleh, N. Nasri, N. Marzougui, I. Thabti, A. M'rabet, Y. Yahya, B. Lachiheb, F. Guasmi and A. Ferchichi, *Int. J. Food Sci. Nutr.*, **60(S2)**, 197 (2009); https://doi.org/10.1080/09637480903067037
5. J.B. Kirkland, *Curr. Pharm. Design*, **15**, 3 (2009); https://doi.org/10.2174/138161209787185823
6. D. Guggisberg, M.C. Risse and R. Hadorn, *Meat Sci.*, **90**, 279 (2012); https://doi.org/10.1016/j.meatsci.2011.07.009
7. L. Ibrahim and A. Usman, *Turk. J. Chem.*, **46**, 320 (2021); https://doi.org/10.55730/1300-0527.3309
8. J.T. Polaski, S.M. Webster, J.E. Johnson Jr. and R.T. Batey, *J. Biol. Chem.*, **292**, 11650 (2017); https://doi.org/10.1074/jbc.M117.787176
9. •. T. Rakuša, R. Roškar, N. Hickey and S. Geremia, *Molecules*, **28**, 240 (2023); https://doi.org/10.3390/molecules28010240
10. D.E. Sherbiny and M.E.K. Wahba, *J. Taibah Univ. Sci.*, **14**, 294 (2019); https://doi.org/10.1080/16583655.2020.1736768
11. L. Stroppel, T. Schultz-Fademrecht, M. Cebulla, R.J. Marhöfer, P.M. Selzer, M. Blech and P. Garidel, *Pharmaceutics*, **15**, 563 (2023); https://doi.org/doi:10.3390/pharmaceutics15020563
12. M. Kester and K.D. Karpa and K.E. Vrana, Elsevier's Integrated Review Pharmacology Book, Elsevier, edn. 2, pp. 41-78 (2012).
13. K.M. Khan, A.R. Khan, S. Perveen, Zia-Ullah, N. Ambreen and W. Voelter, *Open Anal. Chem. J.*, **2**, 74 (2008); https://doi.org/10.2174/1874065000802010074
14. B.J. Floor, A.E. Klein, N. Muhammad and D. Ross, *J. Pharm. Sci.*, **74**, 197 (1985); https://doi.org/10.1002/jps.2600740219
15. G.C. Wood, M.R. Iyer, A.M. Geller, A.M. Fleischner and B.B. Sheth, *J. Liq. Chromatogr. Relat. Technol.*, **21**, 2183 (1998); https://doi.org/10.1080/10826079808006617
16. M.M. Baker, D.S. El-Kafrawy, M.M. Abdel-Khalek and T.S. Belal, *Chromatographia*, **83**, 191 (2020); https://doi.org/10.1007/s10337-019-03832-8
17. ICH Q2(R2) guideline on validation of analytical procedures - 2022.
18. P. Anastas and N. Eghbali, *Chem. Soc. Rev.*, **39**, 301 (2010); https://doi.org/10.1039/B918763B
19. S.K. Muchakayala, N.K. Katari, T. Dongala, V.M. Marisetti, G. Vyas and R.V.K. Vegesna, *J. Iran. Chem. Soc.*, **19**, 1397 (2022); https://doi.org/10.1007/s13738-021-02388-5
20. L.P. Kowtharapu, N.K. Katari, S. Ch, C.A. Sandoval, S.K. Muchakayala and N. Konduru, *Biomed. Chromatogr.*, **36**, e5359 (2022); https://doi.org/10.1002/bmc.5359
21. J. Plotka-Wasylka, *Talanta*, **181**, 204 (2018); https://doi.org/10.1016/j.talanta.2018.01.013
22. D. Mohamed and M.M. Fouad, *Microchem. J.*, **157**, 104873 (2020); https://doi.org/10.1016/j.microc.2020.104873
23. A. Galuszka, Z.M. Migaszewski, P. Konieczka and J. Namieœnik, *Trends Analyt. Chem.*, **37**, 61 (2012); https://doi.org/10.1016/j.trac.2012.03.013
24. L.P. Kowtharapu, N.K. Katari, C.A. Sandoval, S.K. Muchakayala and V.K. Rekulapally, *J. Sep. Sci.*, **45**, 1711 (2022); https://doi.org/10.1002/jssc.202100979
25. M. Sajid and J. Plotka-Wasylka, *Talanta*, **238**, 123046 (2022); https://doi.org/10.1016/j.talanta.2021.123046
26. F. Pena-Pereira, W. Wojnowski and M. Tobiszewski, *Anal. Chem.*, **92**, 10076 (2020); https://doi.org/10.1021/acs.analchem.0c01887
27. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human use ICH Harmonised Guideline, Analytical Procedure Development Q14 (2022).
28. S.K. Muchakayala, K. Pavithra, N.K. Katari, V.M. Marisetti, T. Dongala and R.V.K. Vegesna, *Anal. Methods*, **13**, 3705 (2021); https://doi.org/10.1039/D1AY01096D

29. I. Radic, M. Runje and S. Babic, *J. Pharm. Biomed. Anal.*, **201**, 114091 (2021); https://doi.org/10.1016/j.jpba.2021.114091
30. L.P. Kowtharapu, N.K. Katari, C.A. Sandoval, S.K. Muchakayala and V.K. Rekulapally, *J. AOAC Int.*, **105**, 1247 (2022); https://doi.org/10.1093/jaoacint/qsac072
31. R. Deidda, S. Orlandini, P. Hubert and C. Hubert, *J. Pharm. Biomed. Anal.*, **161**, 110 (2018); https://doi.org/10.1016/j.jpba.2018.07.050
32. P. Jackson, P. Borman, C. Campa, M. Chatfield, M. Godfrey, P. Hamilton, W. Hoyer, F. Norelli, R. Orr and T. Schofield, *Anal. Chem.*, **91**, 2577 (2019); https://doi.org/10.1021/acs.analchem.8b04596
33. A.H. Schmidt and I. Molnár, *J. Pharm. Biomed. Anal.*, **78-79**, 65 (2013); https://doi.org/10.1016/j.jpba.2013.01.032
34. H.K. Chanduluru and A. Sugumaran, *RSC Adv.*, **11**, 27820 (2021); https://doi.org/10.1039/D1RA04843K
35. V.M. Marisetti, P. Katakam, V.K. Siddhani and N.K. Katari, *Biomed. Chromatogr.*, **35**, e5194 (2021); https://doi.org/10.1002/bmc.5194
36. K. Alfonsi, J. Colberg, P.J. Dunn, T. Fevig, S. Jennings, T.A. Johnson, H.P. Kleine, C. Knight, M.A. Nagy, D.A. Perry and M. Stefaniak, *Green Chem.*, **10**, 31 (2008); https://doi.org/10.1039/B711717E
37. D. Prat, A. Wells, J. Hayler, H. Sneddon, C.R. McElroy, S. Abou-Shehada and P.J. Dunn, *Green Chem.*, **18**, 288 (2016); https://doi.org/10.1039/C5GC01008J
38. M. Tobiszewski, S. Tsakovski, V. Simeonov, J. Namieœnik and F. Pena-Pereira, *Green Chem.*, **17**, 4773 (2015); https://doi.org/10.1039/C5GC01615K
39. C. Capello, U. Fischer and K. Hungerbühler, *Green Chem.*, **9**, 927 (2007); https://doi.org/10.1039/b617536h
40. M. Tobiszewski and J. Namieœnik, *Ecotoxicol. Environ. Saf.*, **120**, 169 (2015); https://doi.org/10.1016/j.ecoenv.2015.05.043
41. A. Galuszka, Z. Migaszewski and J. Namiesnik, *Trends Analyt. Chem.*, **50**, 78 (2013); https://doi.org/10.1016/j.trac.2013.04.010