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Isocratic liquid chromatography technique for the analysis of cyanocobalamin from beef liver and heart muscle extracts

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Abstract

Reverse-phase high-performance liquid chromatography (RP-HPLC) is among the most widely recommended techniques for analyzing water-soluble vitamins, such as vitamins C and B-complexes. The research study was conducted to detect and quantify cyanocobalamin (vitamin B_{12}) from the beef liver and heart muscle extracts using a validated isocratic RP-HPLC procedure. The analytical column was Luna® Phenomenex 5 µm C_{18} (2) 100 A LC-column (150 × 4.6 mm). The mobile phase consisted of water/ethanol in a ratio of 60:40 (v/v). An enzymatic digestion with 1% potassium cyanide was used for samples (beef liver and heart muscle) extraction. The validated method showed to be linear, R = 0.9977; fast, with a retention time of less than 6.00 min; precise, $\%RSD_r$ of 1.66 to 1.74%; and sensitive, with LOD and LOQ of 0.004 and 0.012 µg/mL, respectively. The detected values of cyanocobalamin from the beef liver (BLV) and heart muscle (HRM) extracts were 52.04 ± 0.13 and 42.04 ± 0.29 µg/mL, respectively. BLV extract indicated a higher level of cyanocobalamin. Hence, the validated isocratic RP-HPLC technique can be recommended to analyze cyanocobalamin and related compounds in research laboratories such as diagnostics, foods, and pharmaceuticals.

Keywords: Cyanocobalamin, ethanol, isocratic, mobile phase, beef liver extract

1. Introduction

Organic compounds required in a minute amount for body growth, proper immune function, and repair are called vitamins [1]. They can provide most nutrients for good health and well-being [2]. Vitamin deficiency-related physiological abnormalities are related to improper amounts in the diet [3]. Cyanocobalamin (Vitamin B_{12}) is a cobalt-centered and water-soluble vitamin [4]. It is predominantly synthesized by microorganisms [5], but it can also occur naturally in animal-based foods and synthetic dietary supplements [6,7]. Some foods containing or rich in cyanocobalamin include milk (dairy), meat, fish (shell), and eggs [8]. The amount of this vitamin reported from the above food substances was 385 µg (milk), 300 µg (meat), 85 µg (fish), and 46 µg (eggs) [9]. Usually, cyanocobalamin is not commonly accessible from plant-based food sources [5].

Cyanocobalamin is crucial in homocysteine balance, an arteriosclerosis risk factor [10]. In addition, it is a vital coenzyme in one-carbon metabolism for many enzymes involved in human growth and development [11]. Furthermore, it is essential for normal brain function, nervous system, and red blood cell synthesis [4]. The suggested daily intake of cyanocobalamin is 1.0 µg; however, because of its low absorption rate in the small intestine, a 3.0 µg level is recommended [11]. In a normal population, cyanocobalamin deficiency rarely manifests. However, individuals with low-rate absorption disorder, infants, and pregnant women can be subjected to its deficiency as a result of their physiologica land/or prescribed dietary needs [12]. Cyanocobalamin deficiency resulted in pernicious anemia, myelin damage, and spongy vacuolation of peripheral and central nervous systems [13]. A dietary supplement can be sufficient for persons at risk of this vitamin deficiency [14].

Cyanocobalamin can exist either in a free form or attached to biomolecules such as a protein. If attached to a biomolecule, it can be unbound by heating at 98 °C for 30 min with potassium cyanide (KCN) solution [15]. Autoclaving at 121 °C in a mixture of KCN and phosphate buffer is another method to unbound cyanocobalamin [16]. Sample digestion with α-amylase and/or pepsin with excess KCN solution is an additional suitable extraction procedure [17]. KCN is usually added to convert all forms of cobalamin into cyanocobalamin [18]. However, to avoid the use of this highly toxic

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compound, sodium metabisulphate and/or sodium nitrite can be considered as suitable alternative compounds [19].

One of the microbiological assays for vitamin B_{12} analysis is the application of *Lactobacillus leishmania* [20]. Some disadvantages of the microbial method are time consumption and low specificity [21,22]. A liquid chromatography technique with a UV-visible detector is the recommended and suitable analytical procedure [22]. Other alternative protocols for cyanocobalamin analysis are polar-graphic and spectrophotometric [23].

Analysis of water-soluble vitamins with liquid chromatography technique in infant milk [24], okra fruit [25], dietary supplements and ingredients [26], and blood serum [27] have been cited in the existing literature. Recently, cyanocobalamin evaluation was cited using fish species [18], meat brand varieties [5], and plant-based sources such as edible mushroom and laver [28]. However, cyanocobalamin evaluation from the beef liver and heart muscle extracts is lacking. Hence, this research intends to separate, detect, and quantify the amount of cyanocobalamin from the beef liver and heart muscle extracts using a validated isocratic RP-HPLC technique.

2. Experimental

2.1. Chemical reagents and apparatus

The chemical reagents consist of cyanocobalamin (Catalog No. 9868-19-15, Sigma-Aldrich, USA), pepsin (Catalog No. 501784856, Sigma-Aldrich, USA), α-amylase (CAS No. 9000-90-2, Sigma-Aldrich, USA), and HPLC-grade ethanol (Catalog No. AC611050040, Sigma-Aldrich, USA). Other chemical reagents are potassium hydroxide (Catalog No. 109112000, Merck Millipore, Germany), potassium cyanide (Catalog No. 104965, Merck Millipore, Germany), and potassium hydrogen phosphate (Catalog No. 104873, Merck Millopore, Germany). The EMD-Millipore machine (Model No. 13681, Switzerland) was the source of the Milli-Q water. Apparatus includes; Filter papers (Whatman grade 1, 125 mm, Maidstone, England, United Kingdom, Code No. WHAT1001125), 0.22 µm Syringe Filters (Minisart) (Bornstein, Germany, LOT No. 00807103), analytical weighing balance (RADWAG, 220 g × 0.1 mg, Model No. AS/220/C/2, Poland), vortex machine (Model No. S10100A, BioRAD, USA), micropipettes, Eppendorf tubes, and falcon tubes. All chemical reagents used in this research study were of analytical grade.

2.2. Cyanocobalamin extraction from the beef liver and heart muscle

On the 24th of September 2021, fresh beef liver and heart muscle were purchased from Iyama-rama butchery, Grahamstown (Makhanda), Eastern Cape of South Africa. Each sample was washed separately with Milli-Q and stored at -20 °C before analysis. The extraction of cyanocobalamin from each sample was carried out using an enzymatic method, a rarely reported procedure. Each sample was cut up into pieces and homogenized with a warring blender. To each 3.0 g of the homogenate, pepsin (40 mg) and α-amylase (60 mg) were added, followed by the addition of 10 mL of 1% potassium cyanide (KCN) solution. Pepsin and α-amylase enzymes unbound the cyanocobalamin attached to proteins and carbohydrates, respectively [4]. The 1% KCN converts all forms of vitamin B_{12} to cyanocobalamin [4]. Each mixture was vortexed for 10 min, allowed to stand at 37 °C for 24 h, and filtered through Whatman No. 1 filter paper. Each filtrate (extract) was then diluted with a phosphate buffer, pH 5.8 (diluting solution), in a ratio of 5:10 (v/v). Lastly, each diluted extract was further filtered with a 0.22 µm micro-pore filter syringe into their respective HPLC vials.

2.3. Preparation of standard stock and working solutions

The preparation of cyanocobalamin standard stock solution was as reported by [29], with modifications in the working concentration levels. 2 mg of the cyanocobalamin was dissolved in 5 mL of the diluting solution (phosphate buffer, pH 5.8) to produce a stock solution. The preparation of 200 µg/mL working solution was from the stock to generate varying concentration levels of 0.01, 0.1, 0.25, 0.5, 1, 2.5, 15, 20, 30, 50, and 120 µg/mL.

2.4. Instrumentation and chromatographic condition

The HPLC system (Shimadzu Corporation, Kyoto, Japan.) consists of a connecter (LC-20AD), 20 MPa pump (LC-2AB), auto-sampler (SIL-2A), and diode array detector (SPDA-M20A). The PDA detector wavelength ranges from 190 to 800 nm. The control of the system, data acquisition, and evaluation were carried out by "LC Lab Solution" software. The chromatographic separation was done with a 5µm C18 (2) 100A Luna® column (150 × 4.6 mm (Phenomenex, USA), maintained at room temperature (25 ± 5 °C). The mobile phase delivery was isocratic, made up of water/ethanol in a 60:40 (v/v) ratio. The flow rate was 0.5 mL/min with an injection volume of 20 µL for a 10 minute run. The detected UV-visible absorbance was at 270 nm wavelength [30]. The analysis of each working standard solution and sample extract was conducted in duplicate.

3. Results and discussion

3.1. Linearity

The linearity was carried out with a regression curve under 11 points. The regression curve was detected as linear, which indicates a high correlation between the varying working standard concentration levels and mean peak areas. The obtained regression curve value

Table 1. Characteristics of the validated isocratic RP-HPLC procedure

Characteristic variables	Cyanocobalamin (Vitamin B_{12})
Calibration range, µg/mL	0.01 to 120
Correlation coefficient, R	0.9977
Linear regression equation	y = 18949x - 1411.4
LOD [1], µg/mL	0.004
LOQ [2], µg/mL	0.012
Detection wavelength, nm	270

[1]LOD, limit of detection; [2]LOQ, limit of quantification

'R' was 0.9977 (Table 1). Fig. 1 shows the cyanocobalamin standard curve, generated over concentration levels of 0.01 to 120 µg/mL.

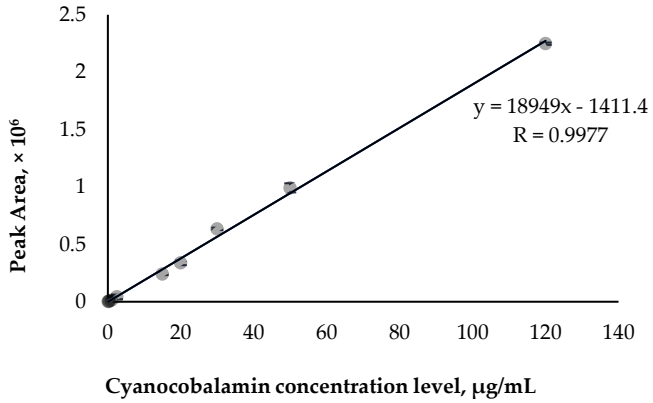

Figure 1. Cyanocobalamin standard curve. The standard curve was generated over concentration levels between 0.01 to 120 µg/mL with pure cyanocobalamin (HPLC grade) prepared in phosphate buffer, pH 5.8. The analysis was carried out in duplicate on the same day.

3.2. Sensitivity

The limit of detection (LOD) was calculated based on the 3.3-fold differences of the analyte signal and baseline noise. LOD implies a compound concentration that produces a signal-to-noise ratio of above 3.0. Whereas the limit of quantification (LOQ) was determined based on 10-fold variations. LOQ means a compound concentration equal to 10 times the value of the signal-to-noise ratio.

$$LOD = 3.3 \times \frac{(Sy)}{m} \quad (1)$$

$$LOQ = 10 \times \frac{(Sy)}{m} \quad (2)$$

Table 2. Precision and recovery accuracy of the validated isocratic RP-HPLC procedure

Intra-day			Inter-day	
Content, µg/mL	Precision (RSD_r[1]), % (n = 5)	Recovery accuracy, % (n = 5)	Precision (RSD_r[1]), % (n = 5)	Recovery accuracy, % (n = 5)
7.5	1.74	97.05 ± 0. 64	1.72	99.87 ± 0.32
15	1. 66	91.10 ± 0.17	1. 68	89.42 ± 0.63

[1]RSD_r, relative standard deviation of repeatability; n = number of repeat in duplicate

The 'SY' denotes the standard deviation of the y-intercept and 'm' implies the slope of the linear regression curve. Both LOD and LOQ were determined as per the International Conference of Harmonization (ICH) guidelines. As depicted in Table 1, the LOD and LOQ values were obtained as 0.004 and 0.012 µg/mL respectively. Hence, these values indicated the high sensitivity of the validated analytical procedure.

3.3. Precision and recovery accuracy

The method precision was determined with two (2) varying standard concentration values of 7.5 and 15 µg/mL. These concentration values were not part of the regression curve points, analyzed in duplicate. Finally, the intra-day and inter-day precision was represented as a percentage relative standard deviation of repeatability ($\%RSD_r$).

$$\%RSD = \frac{Mean\ recovered\ standart\ deviation}{Mean\ recovered\ concentration\ level} \times 100 \quad (3)$$

The intra-day and inter-day precision of repeatability were analyzed within the first day and between two (2) days, respectively. The analysis was conducted in one laboratory, by a single analyst. The intra-day and inter-day precision values are presented in Table 2. In addition, the accuracy recovery (intra-day and inter-day) was calculated using similar standard concentration values used for the intra-day and inter-day precision of repeatability evaluation. (Table 2). Hence, the low $\%RSD_r$ values of ≤ 2.5% as suggested by AOAC [31] and sufficient recovery values indicated the high precision and accuracy of the validated isocratic RP-HPLC technique. Notwithstanding, a higher $\%RSD_r$ values, ≤ 8% was recently adopted by AOAC, which as well indicated a high precision and accuracy of a liquid chromatography analytical procedure [32].

3.4. Accuracy spike

The accuracy-spike recovery was determined by spiking each extract of the beef liver and heart muscle with a known standard (reference standard) concentration value of 35 µg/mL in a 50:50 (v/v) ratio. The accuracy spike was calculated using the relation below.

$$\%MR = \frac{(P - Q)}{R} \times 100 \quad (4)$$

P, Q, R, and % MR represent peak areas of spiked sample extract, un-spike (unspike) sample extract, reference standard, and percentage mean recovery (accuracy spike), respectively.

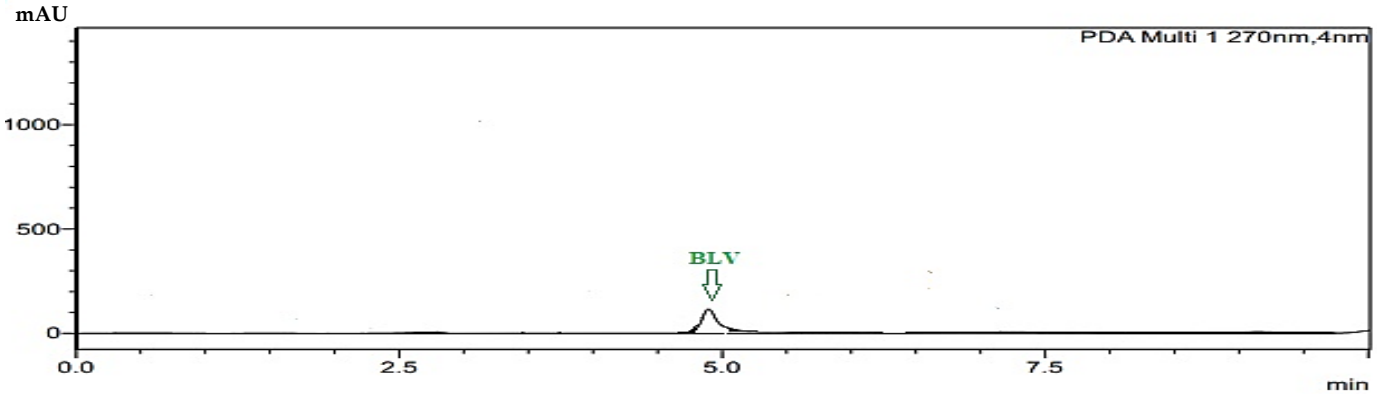

Figure 2. A chromatogram for the beef liver (BLV) extract, determined with a UV-visible detector using an isocratic gradient of 60% water/40% ethanol. The flow rate, injection volume, and detection wavelength were 0.5 mL/min, 20 µL, and 270 nm, respectively.

Table 3 shows each sample extract accuracy spike and amounts of cyanocobalamin obtained. Fig. 2 and Fig. 3 reveal chromatograms of the beef liver (BLV) un–spike (unspike) and heart muscle (HRM) un–spike (unspike) extracts, respectively. Additionally, chromatograms of the beef liver spiked (BLVS) extract, heart muscle spiked (HRMS) extract, and reference standard (RSTD) for the accuracy spike were provided as supplementary materials.

Table 3. Each sample accuracy-spike and cyanocobalamin detected value

Sample extract	Accuracy-spike, % n = 2	Cyanocobalamin value, µg/mL n = 2
Beef liver	109.29 ± 0.11	52.04 ± 0.13
Heart muscle	100.89 ± 0.19	42.04 ± 0.29

n = number of repeat in duplicate.

A naturally occurring vitamin B_{12} exists in varying conformational structures. Thus, this vitamin requires transformation into a single form uch as cyanocobalamin. The potassium cyanide (KCN) solution was used during each sample extraction to convert all forms of vitamin B_{12} to cyanocobalamin [4]. Notwithstanding, the addition of KCN solution may produce a problem of this compound toxicity. During the method optimization, each sample extract with and without the addition of KCN solution was analyzed. An extract with the addition of KCN solution yielded an adequate amount of cyanocobalamin. The mobile phase for the analytical procedure was selected after several evaluations with ethanol, methanol, acetonitrile, orthophosphoric acid, water, and buffer solution. Better separation and sensitivity were achieved with this mobile phase consisting of water/ethanol in a 60:40 (v/v) ratio. The examined flow rates were 0.5, 1.0, and 2 mL/min. A 0.5 mL/min flow rate produced an optimal signal-to-noise with minimum separation time. Both 270 nm and 360 nm wavelengths were tested. A 270 nm wavelength yielded a better resolution of peaks. However, a 360 nm wavelength was recommended by the AOAC official method [33]. The linearity, limit of detection (LOD), limit of quantification (LOQ), recovery accuracy, precision, and sensitivity analysis were detected within the valid ranges. The validation of this analytical procedure complied with the International Conference of Harmonization (ICH) [34].

The detected amounts of cyanocobalamin in the sample extracts of this study were similar to the reported values from multivitamin tablets [7]. However, the obtained levels were greater than the cited values from meat products [5], dietary ingredients/supplements [26], infant formula [33], and poly-vitaminated premixes [35]. The United States National Academy of Science (US-NAS) recommended an intake level of vitamin B_{12} between 0.40–2.80 µg/day for all ages.

Notwithstanding, a higher amount may be required by older persons [36] and individuals with low absorption rate disorders [12].

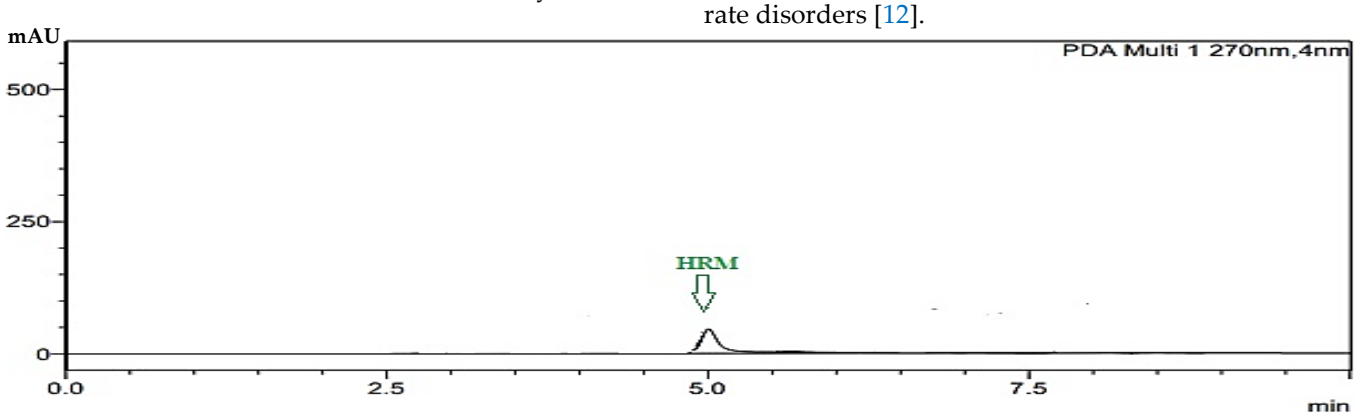

Figure 3. A chromatogram for the heart muscle (HRM) extract, determined with a UV-visible detector using an isocratic gradient of 60% water/40% ethanol. The flow rate, injection volume, and detection wavelength were 0.5 mL/min, 20 µL, and 270 nm, respectively.

Table 4. Current isocratic RP-HPLC analytical procedure in comparison with previously reported methods

	Parameters	Values obtained
1.	Regression value, R	0.9977
	Detection limit, µg/mL	0.00033
	Quantification limit, µg/mL	0.00100
	Precision, %	0.16 – 0.74
	Recovery accuracy, %	89.42 – 99.87
	Retention time, min	4.88 – 5.04
	Detection wavelength, nm	270
	Level detected, µg/mL	52.04 ± 0.13; 42.04 ± 0.29
	Sample extracts	Beef liver and heart muscle
	Reference	[*]
2.	Regression value, R	0.9910
	Detection limit, µg/mL	0.0625
	Quantification limit, µg/mL	0.1250
	Precision, %	0.40 – 4.10
	Recovery accuracy, %	80.40 – 108.50
	Retention time, min	8.53
	Detection wavelength, nm	350
	Concentration reported, µg/mL	45.86
	Test sample	Multivitamin tablets
	Reference	[7]
3.	Regression value, R	0.9930
	Detection limit, µg/mL	0.1600
	Quantification limit, µg/mL	0.5200
	Precision, %	1.41 – 4.64
	Percentage recovery, %	96.00 – 101.10
	Retention time, min	8.70
	UV-visible wavelength, nm	328
	Reported amount, µg/mL	1.80 – 2.69
	Test sample	Dietary ingredients/supplements
	Reference	[26]
4.	Regression value R	Nr
	Detection limit, µg/mL	Nr
	Quantification limit, µg/mL	Nr
	Precision, %	Nr
	Percentage recovery, %	Nr
	Retention time, min	12.35 – 12. 50
	UV-visible wavelength, nm	361
	Reported amount, µg/mL	0.24 – 0.39
	Test sample	Infant formula
	Reference	[33]
5.	Regression value, R	0.9950
	Detection limit, µg/mL	0.2000
	Quantification limit, µg/mL	0.3800
	Precision, %	Nr
	Percentage recovery, %	Nr
	Retention time, min	16.00
	UV-visible wavelength, nm	210
	Reported concentration, µg/mL	Nr (less than LOD)
	Test sample	Poly-vitaminated premixes
	Reference	[35]
6.	Regression value. R^2	0.9940
	Detection limit, µg/mL	Nr
	Quantification limit, µg/mL	0.007
	Precision, %	1.50 –7.26
	Percentage recovery, %	79. 61 –88.80
	Retention time, min	7.17
	UV-visible wavelength, nm	361
	Reported Level, µg/mL	3.85 – 8.78
	Test sample	Meat product
	Reference	[5]

[*], the current validated isocratic liquid chromatography technique; Nr, not reported

Table 5. Various analytes retention times of the validated isocratic liquid chromatography procedure

Analytes	Retention time, min
RSTD	4.90 ± 0.39
BLV	4.91 ± 0.05
BLVS	4.91 ± 0.09
HRM	5.01 ± 0.01
HRMS	4.91 ± 0.09

RSTD, reference standard; BLV, beef liver un–spike extract; BLVS, beef liver spiked extract, HRM, heart muscle un–spike; HRMS, heart muscle spiked.

From the result of this study, the beef liver and heart muscles can be considered as good dietary sources of cyanocobalamin.

Table 5 represents the retention times of the reference standard (RSTD) for the accuracy spike, beef liver (BLV) un spike, beef liver spiked (BLVS), heart muscle (HRM) unspike, and heart muscle spiked (HRMS) extracts. The consistency of the current validated analytical procedure in comparison to the previously reported methods is presented in Table 4. The validated isocratic liquid chromatography technique of this research is indicated to be more rapid (retention time), precise (%RSD), and sensitive (LOD and LOQ).

4. Conclusions

The validated isocratic RP-HPLC method was linear, rapid, precise, and sensitive. Also, the separation, detection, and quantification of cyanocobalamin from each sample extract were satisfactory. Hence, this analytical method can be significantly valuable in research laboratories for quality control purposes. In addition, the data obtained can be useful for nutritional labeling, education, and food-based dietary guidelines.

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