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DESIGN OF HPLC METHOD FOR QUANTIFICATION OF CYANOLOBALAMIN INJECTION

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Abstract: **An accurate and selective HPLC with ultraviolet absorbance detection was developed for the Quantification of vitamin B_{12} (cyanocobalamin) in vitamin B_{12} injections. The method involves an isocratic mode of elution of cyanocobalamin from a reversed phase 150mm, 4.6mm, 5mm hypersil base-deactivated (BDS) C-18 column. Cyanocobalamin was quantitatively analysed using the HPLC method comprising methanol/ phosphate buffer pH7 (30/70 v/v) using ascorbic acid as an internal standard. Separation for quantitative determination was achieved within 6 minutes at a flow rate of 1.4ml/min .The absorbance of the drugs was monitored at 278nm at a detector sensitivity of 0.03.The standard calibration curves were linear with correlation coefficient (R^2) of 0.996. The limit of quantification (LOQ) and detection (LOD) were 0.069ug/ml and 0.0228ug/ml respectively. Four brands of cyanocobalamin injections sampled from some pharmacies and health facilities were analysed with the method and then compared with the standard UV method. The mean percentage recovery of the stated content of four batches of three brands of the injections were 99.00 ± 3.95, 96.05 ± 0.85, 96.00 ± 5.23, 94.36 ±8.73 as against a standard UV method va1ues of 100.33 ± 4.67, 96.97 ± 0.50, 96.22 ± 6.20, 94.47 ± 10.64 respectively. Moreover, within and between run relative standard deviations were 1.682% and 1.656% respectively for cyanocobalamin. These values were all less than the maximum threshold value of 2% for a method to be good as a precise method according to the ICH guidelines. All the validation parameters were assessed statistically and proven to be valid.**

Keywords: **Cyanocobalamin, Chromatograms, Pharmacopoeia, Spectrophotometric, Ascorbic acid.**

1. INTRODUCTION

Vitamin B_{12} is a water-soluble vitamin of the “B complex vitamins which exist in several forms and contains the mineral cobalt; so compounds with vitamin B_{12} activity are collectively called "cobalamins". Methylcobalamin and 5-deoxyadenosylcobalamin are the forms of vitamin B_{12} that are active in human metabolism. Vitamin B_{12} is naturally present in some foods, added to others, available as a dietary supplement and a prescription medication. Vitamin B_{12} plays roles in red blood cell formation, nerve cell maintenance, and methyl donation in DNA synthesis. [Institute of medicine, 1998]. The water-soluble vitamin is constantly excreted from the body system and must therefore be replenished in the diet or else results in its deficiency. Deficiency of vitamin B_{12} affects immunologic and hematologic parameter in the body and can potentially cause severe and irreversible damage, especially to the brain and nervous system. At levels only slightly lower than normal, a range of symptoms such as fatigue, depression, mania, psychosis and poor memory may be experienced. Vitamin B_{12} deficiency affects people of all ages, but children under six years of age, pregnant women, vegetarians, the terminally ill and the aged are the most affected usually due to low levels of vitamin B_{12} in diet or malabsorbtion. [Davis et al., 1973] Vitamin B_{12} status is typically assessed via serum or plasma vitamin B_{12} levels and values below approximately 170–250 pg/ml (120–180 pmol/l) for adults indicate a vitamin B_{12} deficiency. Vitamin B_{12}

deficiency and it's associated symptoms are normally treated by administering vitamin B_{12} capsules, tablets or injections in large dose over a period of time depending upon severity of the deficiency condition. However, the most effective way of treating vitamin B_{12} deficiency and its associated symptoms is by injecting intramuscularly or intravenously due to gastro-intestinal absorption impairment when administered orally.[Carmel, 1992]. The quality of these vitamin B_{12} injections are sometimes questionable due to the upsurge of fake and low quality B_{12} injections on the market and due to high demand for certain brands of the vitamin coupled with the emergence of some dishonest and profit making oriented manufacturers, one is bound to come across imitation or fake formulations on the market. However, there are several standard procedures for assessing the quality of Vitamin B_{12} in formulations. This is to ensure that the various formulations meet the required standard of quality. Some of these methods are microbiological, spectrophotometric methods (UV/visible), electroluminescence, inductive-coupled plasma (ICP) - mass spectrometry (MS) (ICP-MS), atomic absorption spectroscopy, radioimmunoassay (RIA), high-performance liquid chromatography (Hplc), capillary electrophoresis etc. [Glick, 2006]. Determination of the best way of measuring vitamin B_{12} concentration would require critical consideration of the required/desired sensitivity and specificity, the available time, and the process of preparation of the sample as well as cost. The project seeks to design an accurate and validated selective high performance liquid chromatography with ultraviolet absorbance detection (hplc-uv) for the quantification of vitamin B_{12} (cyanocobalamin) in vitamin B_{12} injections using ascorbic acid as internal standard.

2. MATERIALS AND METHOD

Survey:

A survey was carried out in the Kumasi metropolis to determine the different formulations of cyanocobalamin as well as different brands of cyanocobalamin injections sold in the Kumasi metropolis and their country of origin.

Table 3.2(a) Vitamin B12 (cyanocobalamin) formulations sold in the Kumasi metropolis and their country of origin.

Cyanocobalamin formulation	Country of Origin
Tablet	UK , Ghana , Nigeria
Capsule	UK
Injection	China, Lichensteine

Table 3.2 (b) Brands of vitamin B_{12} injections sold in the Kumasi metropolis

Brand	Country of Origin	Batch no	Strength (%)	Man. Date	Exp. Date
Cyanamin-12	China	0910119	0.005	10/2009	10/2012
Binox-12	Lichensteine	1550351	0.005	02/2010	02/2013
BenaR	China	100602	0.005	06/2010	06/2013

3. DEVELOPMENT OF HPLC METHOD OF ANALYSIS

Method development strategy:

Cyanocobalamin and ascorbic acid were evaluated for their physicochemical properties as well as their interaction in the injection. Upon critical examination on the physicochemical properties of these drugs, the choice of mode of HPLC was RPLC. A number of mobile phase combinations as well as column types were considered, with the prime aim of achieving better resolution for drugs for quantitative purposes. Mobile phase combinations included methanol/water, methanol/phosphate buffer in various combinations and at different pH-values.

Preparation of phosphate buffers:

50ml of monobasic potassium phosphate was poured into a 200ml volumetric flask and the specified volume of 0.2M sodium hydroxide added and then made up to the mark with distilled water according to the table shown below.

Table 3.6.2 Volume of 0.20M NaOH required for preparing various PH

PH	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.8	8.0
0.2M NaOH / ml	3.6	5.6	8.1	11.6	16.4	22.4	29.1	34.7	44.5	46.1

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Preparation of standard solutions of reference cyanocobalamin:

0.05g cyanocobalamin was dissolved in mobile phase. Standard solutions were prepared by further dilution with the mobile phase to the required concentrations. Concentration ranging from 0.03% to 0.005% were prepared and used for the calibration curve.

Selection of column:

A number of RPLC columns exist for the simultaneous separation of acidic, basic and neutral drugs using isocratic mode of elution. Amongst these columns, ODS C-18, 250mm, 4.6mm; C8, 250mm, 4.6mm; Hypersil BDS C8, 150mm, 4.6mm. Hypersil BDS C-18, 150mm, 4.6mm was chosen for the method design due to the fact that cyanocobalamin and ascorbic acid are more polar and therefore easily eluted from the coloumn

The effect of mobile phase pH on the retention time on reverse phase HPLC columns:

In reverse phase chromatography, solvent strength increases with increase in the organic portion of the mobile phase. Solvent strength can be controlled by adjustment of the pH of the mobile phase. For ionisable acidic or basic drugs, the pH of the mobile phase affects their elution rates in RPLC. Using the effect of pH on partition coefficient of a base and by knowing the pH of the mobile phase and the pKa of the analyte, it is possible to predict approximately the retention time of the analyte upon change in pH, whilst all other parameters of the HPLC system remains fixed. This is achievable when a first chromatogram is obtained. In the case of accuracy in the calculations, pH of the mobile phase was used, because this was the actual pH environment in which the drug was being analysed, and also ensuring that the pH was set in the pH range of 2 to 8 units because of the tendency of extremes of pH to dissolve silica gel.

Selection of pH of mobile phase:

Various proportions of mobile phase of (methanol /water/phosphate buffer) were prepared and a specified concentration of cyanocobalamin and Ascorbic acid added and then injected into the HPLC system. The pH of the aqueous phase ranging from 4 to 8 was adjusted upwards with the phosphate buffer solutions prepared. The pH of the aqueous portion as well as that of the mobile phase was monitored and peaks obtained after injection into the HPLC system were accessed with the one with the best separation and resolution.

Selection of UV detection wavelength and detector sensitivity:

A suitable wavelength of maximum absorption was obtained for the analysis by scanning with UV spectrophotometer of solution containing the sample dissolved in the mobile phase using the mobile phase in the reference cell. With careful investigation of the UV spectrum of the sample, wavelength of maximum absorption of 278nm was most suitable for both cyanocobalamin and ascorbic acid. After varying the sensitivity ranging from 0.001 to 0.1, 0.030 was most suitable for the detection.

Internal Standard:

Ascorbic acid was chosen as the internal standard for the method design. Mobile phase pH and compositions were varied until there was a resolution between the test drugs and the proposed internal standard. Ascorbic acid worked best for cyanocobalamin owing to the fact that it gave the best resolution with it under the established conditions without interfering with others and also meets all the requirements for the selection of an internal standard. In the method design, a constant amount (0.04mg) of the ascorbic acid was used.

HPLC Pump flow rate:

Flow rates were carefully chosen in order to ensure pumps reproducibility of results. Upon several adjustments, 1.40mL/min (column back pressure; 111-115bar) was chosen for cyanocobalamin analysis.

Chart recorder speed:

The speed of the recorder was of great concern because it helped in the elucidation of resolved peaks and shapes of peaks. The appropriate speed employed was 5mm/min at attenuation 0.

Method performance:

2mg of pure cyanocobalamin powder was dissolved in 100ml of the mobile phase and then subjected to the conditions established for the method design and 0.04mg of ascorbic acid added and then analysed. In the determination of limit of quantitation (LOQ) and limit of detection (LOD), six replicate solutions were analysed differently for the reference solutions of cyanocobalamin. Resultant peaks were then analysed for their mean peak area ratios (MPAR), mean peak areas (MPA) and concentrations. Mean and standard deviation values were calculated and then deduced LOQ and LOD values for cyanocobalamin using specific equations. The relative standard deviation (RSD %) as a fractional error expressed as a percentage was deduced for the analysis. RSD% value of less than 2% is achievable for HPLC method for analysis. Intermediate precision was assessed with the developed method in three different days after the development phase was over and standard deviations and RSD% values compared.

4. RESULTS AND DISCUSSION

4.4.0 CHROMATOGRAMS AND HPLC CONDITION FOR ANALYSIS

4.4.1 Chromatograms of cyanocobalamin and ascorbic acid in methanol/phosphate buffer (30/70 $^{v}/_{v}$)

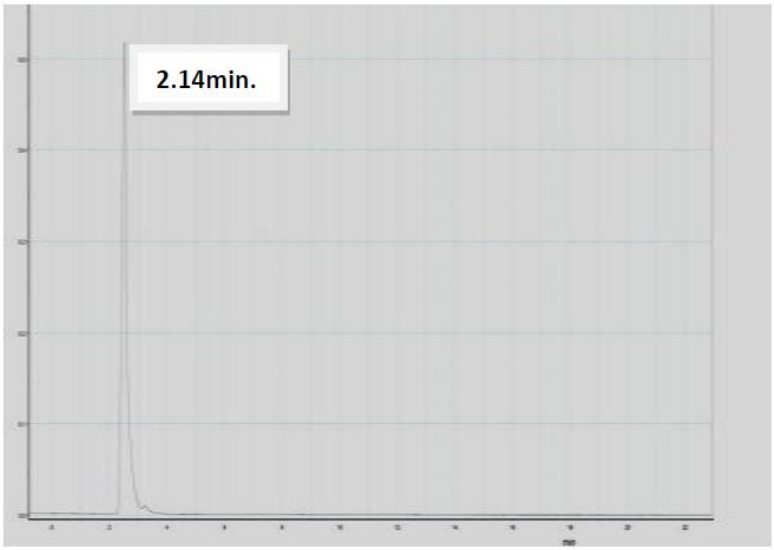

Fig. 4.4.1.1 Chromatogram of ascorbic acid

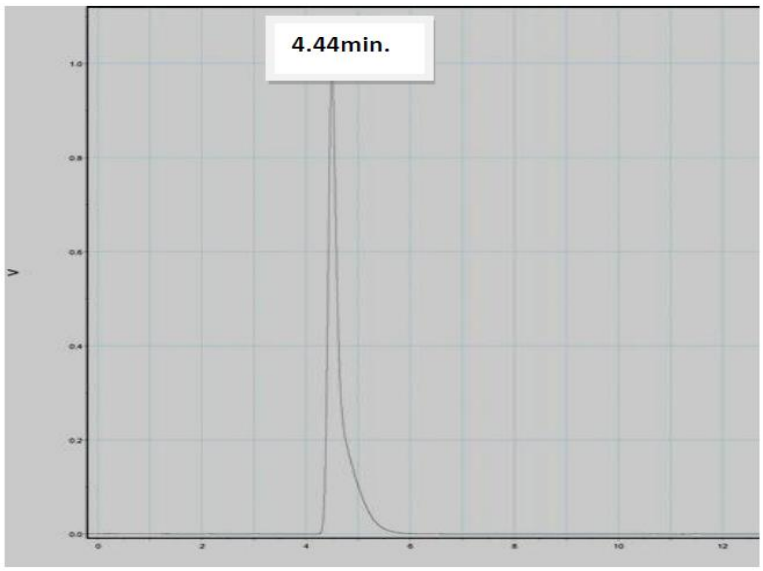

Fig.4.4.1.2 Chromatogram of cyanocobalamin

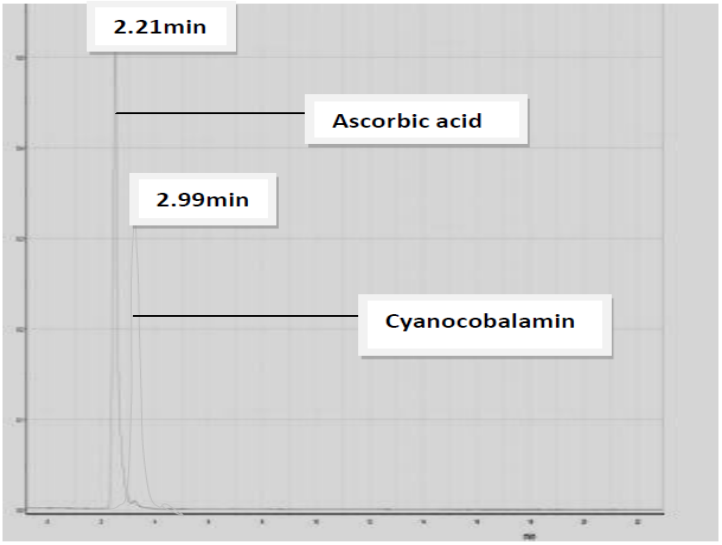

Fig 4.4.1.3 Chromatogram of cyanocobalamin and ascorbic acid at pH 6

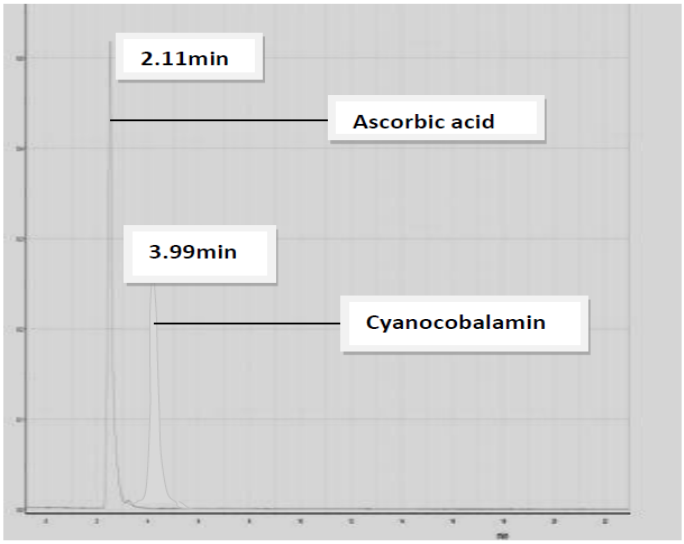

Fig 4.4.1.4 Chromatogram of cyanocobalamin and ascorbic acid at pH 6.5

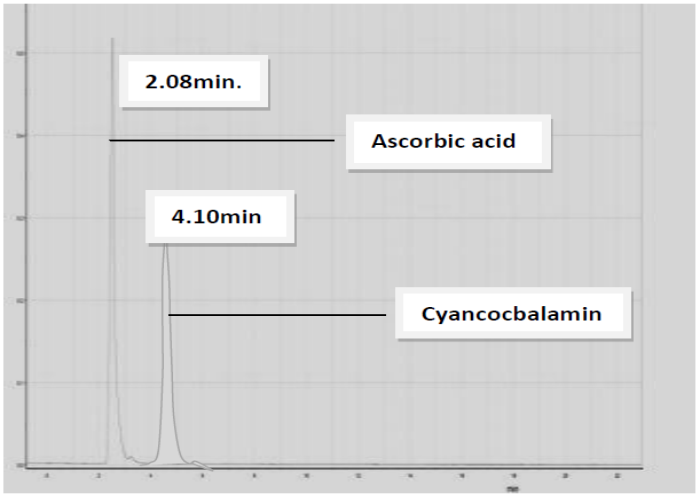

Fig 4.4.1.5 Chromatogram of cyanocobalamin and ascorbic acid at pH 6.8

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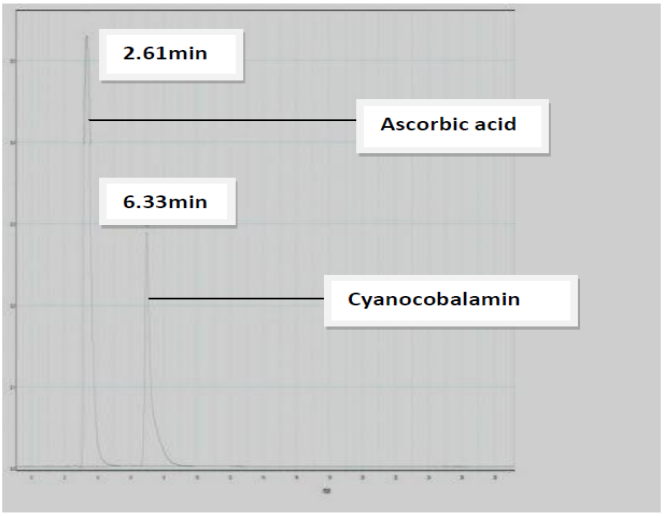

Fig 4.4.1.6 Chromatogram of cyanocobalamin and ascorbic acid at pH 7

Table 4.4.1 Data for chromatograms showing their retention times and peak areas

Sample mixture	pH of mobile phase	Peaks	Peak name	Retention time / min.
Cyanocobalamin/Ascorbic acid	6.0	1	Ascorbic acid	2.21
		2	Cyanocobalamin	2.99
Cyanocobalamin/Ascorbic acid	6.5	1	Ascorbic acid	2.11
		2	Cyanocobalamin	3.99
Cyanocobalamin/Ascorbic acid	6.8	1	Ascorbic acid	2.08
		2	Cyanocobalamin	4.10
Cyanocobalamin/Ascorbic acid	7.0	1	Ascorbic acid	2.61
		2	Cyanocobalamin	6.33

4.4.2 Hplc condition for assay of commercial samples:

Table 4.4.2.1 Hplc condition for analysis

Parameter	Condition
Mobile phase	Phosphate buffer PH7/Methanol (70/30 $^{v}/_{v}$)
Stationary phase	150mm, 4.6mm, 5µm Hypersil base-deactivated C-18 Coloumn
Wavelenght of analysis	278nm
Sensitivity	0.03
Flow rate:	1.40ml/min.

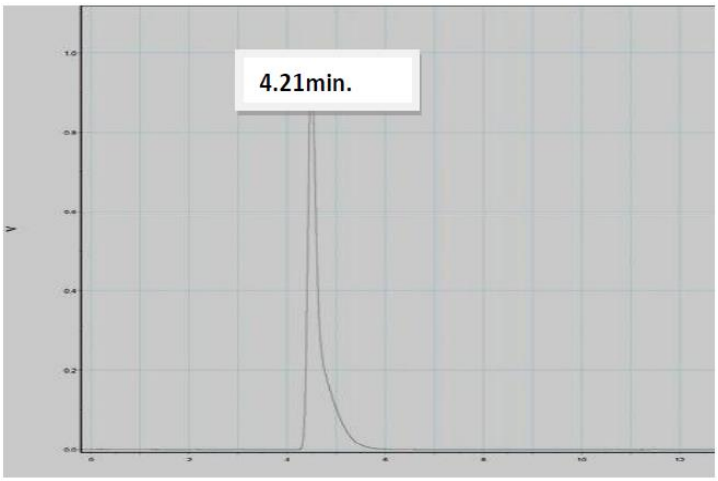

Fig. 4.4.2.1 Chromatogram of cyanocobalamin

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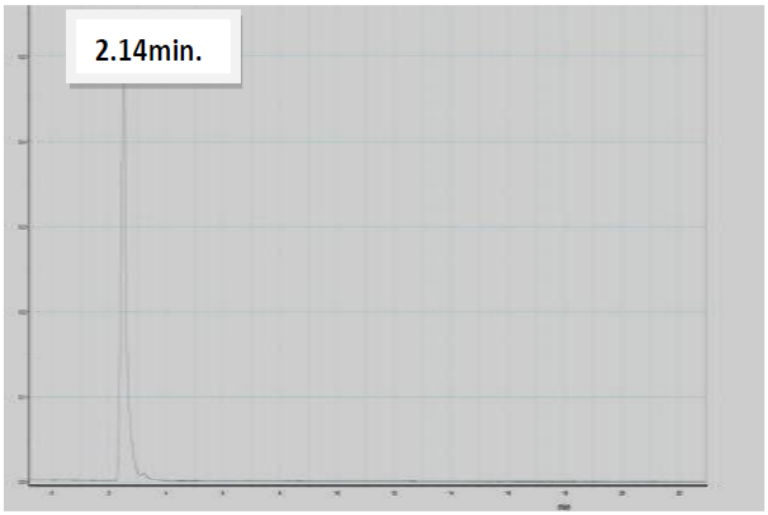

Fig.4.4.2.2 Chromatogram of ascorbic acid

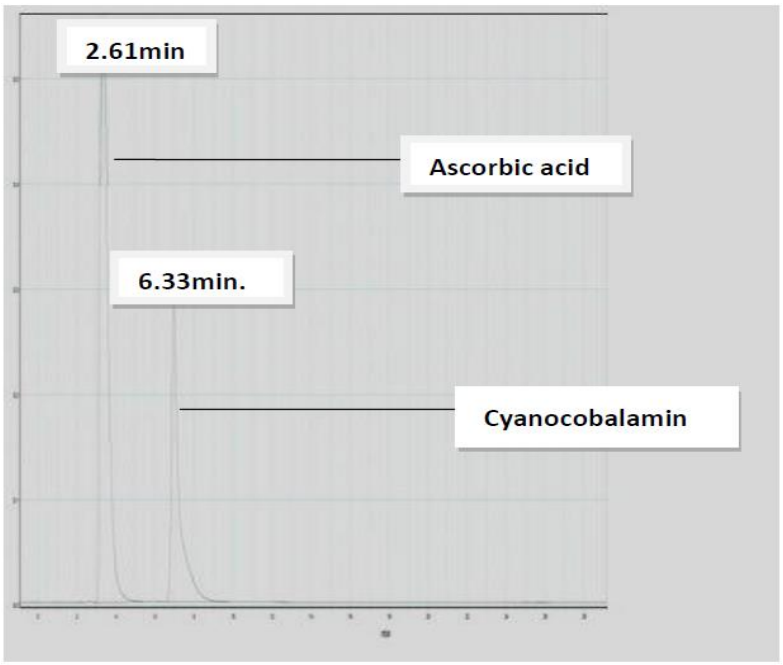

Fig. 4.4.2.3 Chromatogram of cyanocobalamin and ascorbic acid

Table 4.4.2.2 Hplc data for calibration graph

Conc./%	Peak area (mm^2)				MPA-B_{12}	MPA-IS	MPAR
	Cyanocobalamin		Internal standard				
	No of determinations		No of determinations				
	1	2	1	2			
0.005	3.09	3.09	7.47	7.47	3.09	7.47	0.41
0.010	5.16	5.63	7.33	7.52	5.40	7.43	0.73
0.015	7.65	7.55	7.24	7.12	7.62	7.37	1.03
0.020	10.49	10.49	7.41	7.41	10.49	7.41	1.42
0.025	12.47	12.20	7.49	7.51	12.30	7.50	1.64
0.030	15.52	15.52	7.68	7.68	15.52	7.68	2.02

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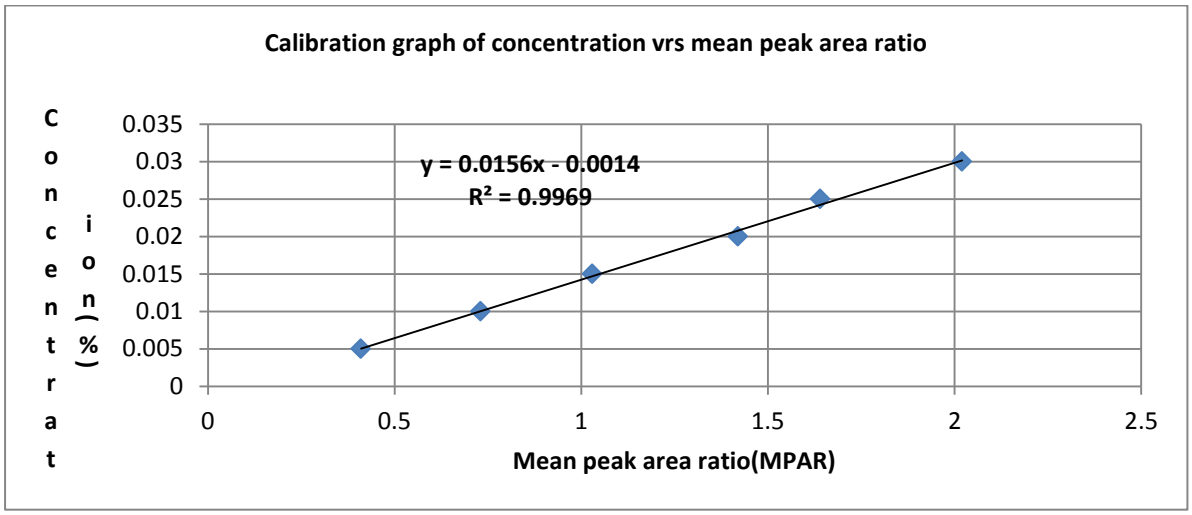

Fig.4.4.2.4 Calibration graph of concentration against mean peak area ratio (MPAR)

Table 4.4.2.3 Data for hplc assay of cyanocobalamin injections

Brand	Ampoule No	Peak areas No of determinations				MPAR	Conc.	%Conc. ($^{v}/_{v}$)
		Cyanocobalamin		Ascorbic acid				
		1	2	1	2			
Bena^{R} 1	1	2.76	2.74	7.30	7.10	0.382	0.00475	95.00
	2	3.01	3.00	7.22	7.20	0.418	0.00529	104.80
	3	2.77	2.75	6.99	7.14	0.391	0.00486	97.20
Bena^{R} 2	1	2.75	2.74	7.09	7.05	0.388	0.00482	96.30
	2	2.72	2.70	7.08	7.04	0.384	0.00476	95.10
	3	2.79	2.76	7.16	7.12	0.389	0.00484	96.74
Cyanamin-12	1	2.99	2.97	7.37	7.31	0.407	0.00510	102.00
	2	2.78	2.74	7.35	7.21	0.379	0.00468	93.60
	3	2.71	2.69	7.317	7.09	0.375	0.00462	92.40
Binox-12	1	2.55	2.55	7.30	7.24	0.351	0.00427	85.34
	2	3.03	3.04	7.45	7.39	0.409	0.00514	102.77
	3	2.80	2.82	7.40	7.28	0.383	0.00475	94.97

Table 4.4.2.4 Data for comparison of hplc method to standard BP(UV) method

Sample	Ampoule	Method of of analysis (%)			
		Hplc method	Comment	UV method	Comment
Bena^{R} 1	1	95.00	Passed	97.20	Passed
	2	104.80	Passed	105.7	Passed
	3	97.20	Passed	98.10	Passed
Bena^{R} 2	1	96.30	Passed	97.20	Passed
	2	95.10	Passed	96.40	Passed
	3	96.74	Passed	97.32	Passed
Cyanamin -12	1	102.00	Passed	103.40	Passed
	2	93.60	Failed	92.15	Failed
	3	92.40	Failed	93.10	Failed
Binox -12	1	85.34	Failed	83.44	Failed
	2	102.77	Passed	104.67	Passed
	3	94.97	Failed	95.30	Passed

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Table 4.4.2.5 Data for mean % content of brands of injections

Sample	Method of analysis			
	Mean % content (Hplc)	Comment	Mean % content (UV)	Comment
Bena 1	99.00 ± 3.95	Passed	100.33 ± 4.67	Passed
Bena 2	96.05 ± 0.85	Passed	96.97 ± 0.50	Passed
Cyanamin-12	96.00 ± 5.23	Passed	96.22 ± 6.20	Passed
Binox-12	94.36 ± 8.73	Failed	94.47 ± 10.64	Failed

4.5.0 METHOD PERFORMANCE ASSESSMENT

4.5.1 Within run precision or repeatability:

Table 4.5.1 Data for repeated measurement of cyanocobalamin

No of runs	Peak area (mm^2)				MPAR	Mean concentration
	Cyanocobalamin		Internal standard			
	1	2	1	2		
1	10.02	10.32	7.32	7.30	1.391245	0.019869
2	10.64	10.54	7.61	7.56	1.396177	0.019943
3	10.14	10.24	7.15	7.20	1.420206	0.020203
4	11.13	10.24	7.63	7.60	1.403152	0.020047
5	10.39	10.55	7.25	7.22	1.447132	0.020707
6	10.24	10.60	7.52	7.50	1.387483	0.019812

4.5.2 Between run precision (Intermediate precision):

Table 4.5.2 Data for between run precision (Intermediate precision)

No of run	1st day		2nd day		3rdday	
	MPAR	Mean conc.	MPAR	Mean conc.	MPAR	Mean Conc.
1	1.391245	0.019869	1.458716	0.020881	1.458571	0.020879
2	1.396177	0.019943	1.433103	0.020497	1.469761	0.021046
3	1.420206	0.020203	1.365563	0.019483	1.419944	0.021046
4	1.403152	0.020047	1.499293	0.021489	1.478992	0.021185
5	1.447132	0.020707	1.479167	0.021188	1.50000	0.021500
6	1.387483	0.019812	1.470994	0.021265	1.49786	0.021468

Table 4.5.3.1 Data for test for accuracy of the method (% recovery)

Concentration prepared	Concentration recovered	% Concentration recovered
0.0200	0.019869	99.35
0.0200	0.019943	99.97
0.0200	0.020203	100.10
0.0200	0.020047	100.24
0.0200	0.020707	103.54
0.0200	0.019812	99.06

Table 4.5.3.2 Data for test of precision of the method (Student t-test)

Days	Statistical parameter					
	Mean concentration	Standard deviation	RSD (%)	t_r	t_o	Comment
1st	0.02011	0.000338	1.682	0.133	3.365	$t_r < t_o$ hence precise
2nd	0.02087	0.000756	1.707	0.470	3.365	$t_r < t_o$ hence precise
3rd	0.02089	0.000420	1.580	0.865	3.365	$t_r < t_o$ hence precise

t_o is the theoretical student t value for (N-1) degrees of freedom at 98.0% confidence interval

t_r is the experimental t value for N = 6

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Table 4.5.3.3 Data for test for robustness of the method (F-Test)

Within run variance		Between run variance		F	F_o	Comments
$\overline{X_w}$	$\sum (X_i - \overline{X_w})^2$	Y	$\sum (\overline{X_{ii}} - Y)^2$			
0.02011	6.48×10^{-7}	0.02062	3.955×10^{-7}	27.46	49.432	F < Fo hence precise

$$F = \frac{[(\sum n (\overline{X_{ii}} - Y)^2/(K-1))}{[\sum (X_i - \overline{X_w})^2/(N-K))]}$$

Where $\overline{X_w}$ is the mean concentration for day I result as indicated in Table 4.6.3 coloumn 2

N = 18, n = 6, Y = 0.02062, K = 3

$\overline{X_{ii}}$ is the mean concentration of the mean concentration for each day,

X_i is the concentrations obtained for day 1 as indicated in table 4.6.2 coloumn 3,

Where F_o is the F value at 98.0% confidence interval for 2 against 15.F is the experimental value and since F is less than F_o, the null hypothesis is not rejected and therefore the experimental concentrations obtained do not differ significantly from each other even though they were carried out on three different days. The method is therefore robust.

4.5.4 Determination of limit of quantitation (LOQ) and limit of detection (LOD):

From the calibration graph from fig.4.5.2

$Y = 0.015\,\overline{X_i} - 0.001$

Where $\overline{X_i}$ is the Mean peak area ratio (MPAR), Y is the concentration

Table 4.5.4 Data for LOD and LOQ determination

Y	$\overline{X_e}$	$\overline{X_i}$	$(X_e - \overline{X_i})$	$(X_e - \overline{X_i})^2$
0.005	0.414	0.410	0.004	1.6×10^{-5}
0.010	0.727	0.730	-0.003	9.0×10^{-6}
0.015	1.034	1.030	0.004	0.000016
0.020	1.417	1.420	-0.003	9.0×10^{-6}
0.025	1.640	1.640	0	0
0.030	2.021	2.020	0.001	1.0×10^{-6}
				$\sum(\overline{X_e} - \overline{Y_i})^2 = 5.10\times10^{-5}$

$$SD = \left[\frac{\sum(\overline{X_e} - \overline{Y_i})2}{(N-1)}\right]^2$$

Where SD is the residual standard deviation

X_e is the estimated mean peak area ratio from the calibration graph

Y_i is experimental mean peak area ratio from table 4.5.2(b) last column

N is the number of concentration prepared

$SD = \left[\frac{\sum(5.10\times10-5)2}{(6-1)}\right]^2 = 1.040\times10^{-10}$

According to the ICH guidelines for method validation, $LOQ = \left[\frac{10SD}{s}\right]$

Where S is the slope from the calibration in fig.4.5.2

But S = 0.015, Therefore, $LOQ = \left[\frac{10 \times 1.04 \times10-10}{0.015}\right] = 6.90 \times 10^{-8}\,\% = 0.069$ug/ml

Similarly, $LOQ = \left[\frac{3.3SD}{S}\right] = \left[\frac{3.3 \times 1.04\times10-10}{0.015}\right] = 2.20\times10^{-8}\,\% = 0.0228$ug/ml

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5.1.3 HPLC method design:

During the method development, a number of factors were considered to arrive at the mobile phase that could resolve both the target drug and the chosen internal standard at a reasonable time for quantitation. Some of these factors include the mobile phase composition, chemistry of the drug (e.g pKa, solubility, spectroscopic properties and it's interactions with other compounds and surfaces). The design of an hplc method of analysis is normally carried out by considering the sample matrix (e.g biological, pharmaceutical etc.), the mobile phase in which the analyte is soluble and most stable, stationary phase which will elute the drug faster as well as resolving it from other drugs in the sample matrix, the method of detection of the sample (e.g UV, refractive properties, conductive properties) and flow rate of the mobile phase etc.

A reverse phase hplc coloumn was chosen because cyanocobalamin has a lot of polar groups on its structure and therefore does not retain much longer on the column. UV detection was chosen for the method because of the presence of extensive chromophoric system in cyanacobalamin. The choice of mobile phase was important for successful liquid chromatography. In this work, the organic phase was initially changed while the aqueous medium was fixed. Various mobile phase compositions comprising of water/methanol at (90/10, 80/20, 70/30, 60/40, 50/50) $^{v}/_{v}$ employed. In each composition, a quantity of the cyanocobalamin and a constant amount of internal standard (0.04mg) were added. The composition was injected whilst varying the conditions (e.g wavelength, sensitivity and the flow rate). Mobile phase composition of 70/30$^{v}/_{v}$ was found most suitable because it gave the best resolution; however, the separation of the drug and the internal standard was very poor. A buffer was therefore employed to generate ionic species which could have different affinities for the coloumn and hence separation of the drug and internal standard being achieved. The organic phase composition was then fixed whilst the aqueous phase (conducting species) was varied. That is, pH was increased thereby decreasing amount of phosphate buffer in solution and ionic strength. Mobile phase composition was fixed at 70/30 $^{v}/_{v}$ phosphate buffer/methanol whilst varying pH of the aqueous medium, it could be observed that in acidic mobile phase of pH 4 cyanocobalamin tends to ionise and shows little retention, thereby eluting in the void. This behaviour of cyanocobalamin stems from the fact that it is a basic ionic drug and also posses other functional groups for ion transfer. With pH values towards neutral medium (pH 6 – 7) of the mobile phase, cyanocobalamin retains better thus resolving from solvent front.

At pH 6.8 cyanocobalamin was partially ionised, retained and well resolved from solvent front and ascorbic acid (internal standard). Further variation of pH of the medium towards neutral medium (pH 7) was more suitable for the elution of peaks.The various pH values of mobile phase employed revealed that at pH7 there was substantial elution and resolution of peaks than the others Figure 4.4.1(1-6). This is due to the fact that at pH 7 cyanocobalamin is fully unionized and undegradable into other B_{12} forms. It has been shown from table 4.3.1 that at pH 7 cyanocobalamin has the highest concentration in mobile phase. pH 7 was therefore most suitable for establishing the mobile phase condition for the method design. Upon varying the various parameters (wavelenght of absorption, pH of mobile phase, detector sensitivity and flow rate), the final condition for analysis was obtained. The conditions for the analysis consisted of a reverse stationary phase base-deactivated C-18 material with a mobile phase comprising of phosphate buffer pH 7/mehtanol (70/30 $^{v}/_{v}$). The cyanocobalamin and ascorbic acid were detected at 278nm with detector sensitivity of 0.03% using a flow rate of 1.40ml/min. The retention time was 4.9 ±1.5 minutes for cyanocobalamin and the mode of elution was isocratic. (Table 4.4.2.1). Chromatograms obtained from the analysis of cyanocobalamin with ascorbic acid as internal standard were evaluated for their concentrations using peak areas to obtain the mean peak area ratios (MPAR) as shown in table 4.4.2.2. Peak areas were used based on the fact that peaks were symmetrically shaped and narrow. The data obtained from the conditions for the analysis was then used to prepare a calibration graph of mean peak area ratio against the concentration ranging from 0.005 to 0.03$^{v}/_{v}$ % as indicated in figure 4.4.2.

5.1.4 Method performance assessment:

A designed method for drug analysis must meet all the requirements specified by the ICH guidelines. The method must be specific for the analyte, accurate, reproducible or repeatable precise, linear with respect to varying parameters (e.g peak area ratio against concentration etc.), specified range, limit of detection and limit of quantitation, robust etc. Reproducibility of results were assessed by repeated runs of the samples several times and at different days and data analysed statistically and were found to be reproducible. (Tables 4.5.1 and 4.5.2). Accuracy of results were also assessed by comparing experimental concentrations to expected concentration by computing percentage recovery against expected concentration and were found to be very accurate as indicated in table 4.5.3.1

Between run precision and intermediate precision were carried out to access the methods performance in the same laboratory. Reapeated measurements were compared and then tested for their precision using the student t- test and were all found to be precise (table 4.5.3.2). The standard deviations as well as relative standard deviations were compared, also nature of peaks were compared. Cyanocobalamin analysis produced relative standard deviations of 1.68%, 1.71% and 1.58% respectively for first and second and third occasions. These statistical parameters obtained on the three different occasions were all less than 2% as the ICH guidelines upper limit. (Table 4.5.3)

The linearity of the method was assessed by preparing a calibration graph of concentrations ranging from 0.03 to 0.005 % against mean peak area ratios with a linear correlation coefficient (R^2) of 0.996. (Figure 4.4.2). In the assessment of sensitivity of the method, the LOD and LOQ were calculated and cyanocobalamin produced LOQ of 0.069ug/ml and LOD of 0.0228ug/ml.This parameter explains the sensitivity of the method and was found to be very sensitive for the detection and quantification of such small concentrations. Robustness of the method was also assessed by comparing experimental values of two other days and their variations compared using analysis of variance (ANOVA) and were all found to be insignificant (table 4.5.3.3) and hence the various values obtained for other days do not differ significantly from each other. The designed method was applied for the assay of cyanocobalamin in the injection and then compared with standard UV method. When the cyanocobalamin was analysed using the newly developed hplc method, it produced percentage content ranging from 94% to 105% for the injections , with most of the ampoules meeting the requirements of the BP percentage of the stated content requirement of not less than 95% and not more than 105% of the labeled amount . (Table 4.4.2 (1-5)). The percentage content obtained from the two methods for most of the ampoules were found not to be significantly different from each other.

6. CONCLUSION

Hplc with ultraviolet detection method for the analysis of cyanocobalamin in injections formulation has been developed. This consisted of phosphate buffer pH 7/mehtanol (70/30 $^{v}/_{v}$) for the determination of cyanocobalamin using ascorbic acid as an internal standard. The retention time was 4.9 ±1.5 minutes for cyanocobalamin and the mode of elution was isocratic with experiment conducted at room temperature.The stationary phase was a base-deactivated C-18 column with a mobile phase flow rate of 1.40ml/min. Cyanocobalamin detection and analysis worked best with a mobile phase composition of phosphate buffer pH 7/methanol (70/30) $^{v}/_{v}$ at 278nm with detector sensitivity of 0.03%. The method was found to be specific for the cyanocobalamin, accurate and sensitive with LOQ and LOD found to be 0.069ug/ml and 0.0228ug/ml respectively. Moreover, the method was precise, robust, less time consuming for assessing the quantity of cyanocobalamin in the injection. The maximum elution time on the coloumn was found to be six minutes after injection. The mean percentage recovery of the stated content of four batches of three brands of the injections categories were 99.00 ± 3.95, 96.05 ± 0.85, 96.00 ± 5.23, 94.36 ± 8.73 as against a standard UV method of 100.33 ± 4.67, 96.97 ± 0.50, 96.22 ± 6.20, 94.47 ± 10.64 respectively.

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