ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 11, Issue 12, 2018

Online - 2455-3891
Print - 0974-2441

Research Article

STABILITY-INDICATING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ESTIMATION OF METHYLCOBALAMIN, ALPHA-LIPOIC ACID, PYRIDOXINE HCL, AND FOLIC ACID IN BULK AND COMBINED DOSAGE FORM

PADMAJA V[1]*, PRASANTHI M[1], MAYURI P[2]

[1]Department of Pharmaceutical Analysis, Nirmala College of Pharmacy, Atmakuru, Mangalagiri, Guntur, Andhra Pradesh, India, [2]Departmen of Pharmaceutics, Nirmala College of Pharmacy, Atmakuru, Mangalagiri, Guntur, Andhra Pradesh, India. Email: vellaturipadmaja@gmail.com

Received: 16 April 2018, Revised and Accepted: 02 August 2018

ABSTRACT

Objectives: The purpose of the research is to develop a simple, precise, economical, accurate, reproducible, and sensitive method for the estimation of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid drug product by reversed-phase high-performance liquid chromatography (RP-HPLC) method.

Methods: New analytical method was developed for the estimation of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid in drug product by RP-HPLC. The chromatographic separation was achieved on the Inertsil C18, 250 mm × 4.6 mm, 5 µm at ambient temperature. The separation achieved employing a mobile phase consists of buffer (added 5.05 g hexane-1-sulfonic acid is dissolved into 1000 mL of distilled water):acetonitrile in the ratio of 10:90% v/v. The flow rate was 1 mL/min and UV-visible spectrophotometer at 285 nm. The average retention time for methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid was found to be 3.5, 6.7, 8.5, and 9.3, respectively.

Results: The developed method was validated as per ICH guidelines. All validation parameters were within the acceptable ranges. The assay methods were found to be linear from 0 to 2130 µg/mL for methylcobalamin, 0 to 142.5 µg/mL for alpha-lipoic acid, 0–4.54 µg/mL for pyridoxine hydrochloride, and 0–2 µg/mL for folic acid. The correlation coefficient was 0.999 for all drugs, respectively. The mean percentage values for the developed method were found to be within the range of 98–100.6%. The developed method was also found to be robust.

Conclusion: It is concluded that developed method was accurate, precise, linear, reproducible, robust, and sensitive.

Keywords: Alpha-lipoic acid, Folic acid, Methylcobalamin, Pyridoxine hydrochloride and Reversed-phase high-performance liquid chromatography, Validation.

 DOI: http://dx.doi.org/10.22159/ajpcr.2018.v11i12.26715

INTRODUCTION

Methylcobalamin is a cobalamin, a form of Vitamin B_{12}. It differs from cyanocobalamin in that the cyano at the cobalt is replaced with a methyl group [1] and is chemically Coα-[α-(5,6-dimethylbenz-1H-imidazolyl)]-Coβmethyl cob amide. Methylcobalamin is also used in the treatment of peripheral neuropathy, diabetic neuropathy, and as a preliminary treatment for amyotrophic lateral sclerosis [2]. The alpha-lipoic acid is chemically (R)-5-(1,2-dithiolan-3-yl)pentatonic acid. Alpha-lipoic acid is easily absorbed and transported across cell membranes; thus, free radical protection occurs both inside and outside of cells. Pyridoxine, also known as Vitamin B_6, is a form of Vitamin B6 found commonly in food and used as dietary supplement [3]. Pyridoxine responsiveness is associated with two particular missense mutations in the AGT gene which lead to Gly170Arg and Phe152Ile amino acid replacements [4-6]. Folic acid, also known as Vitamin B9, is a form of synthetically produced water-soluble vitamin found in fortified food and supplements. Folate is naturally derived from food, particularly from dark green leafy vegetables [7]. Folate and the biologically active folic acid, which is converted to dihydrofolic acid in the liver, are essential in meeting the requirements of the function of the human body. Folate is used to synthesize, repair, and methylate deoxyribonucleic acid [8]. According to literature review some methods are developed and validated by using different techniques like High performance liquid chromatography [9,10], ultra performance liquid chromatography [11], ultravoilet-visible [12] technique with other combination of drugs. The proposed method aimed to develop and validate a stability-indicating method for the estimation of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid in bulk and combined dosage form by reverse-phase high-performance liquid chromatography (Figs. 1-4).

MATERIALS AND METHODS

Equipment

The chromatographic technique was performed on a Waters, 2695 separation module, Empower, Version 2.0, reversed-phase C18 column (Inertsil, 250 mm × 4.6 mm, 5 µm) as stationary phase, with Sartorius analytical balance and vacuum microfiltration unit with 0.45 µ membrane filter.

Materials

Pharmaceutically pure samples of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid were obtained from ICON Laboratories, Vijayawada, India.

High-performance liquid chromatography grade methanol and acetonitrile were procured from on laboratories, Vijayawada, India.

Selection of wavelength (for detection)

In setting up the conditions for the development of assay method, the choice of detection wavelength was based on the scanned absorption spectrum for methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid. The UV spectrum of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid was obtained

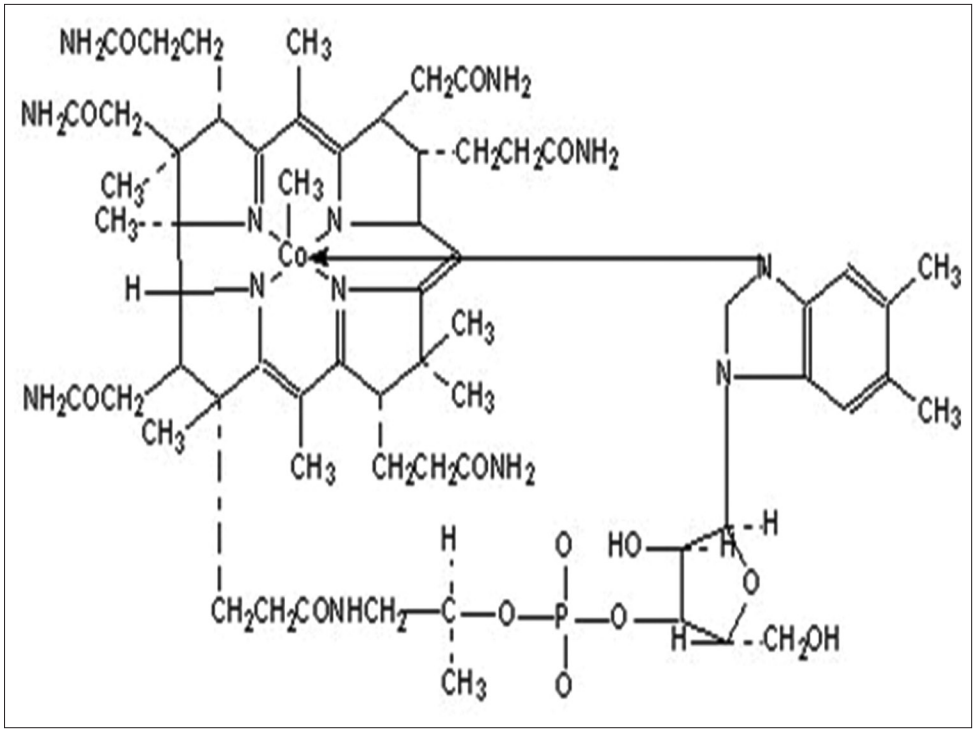

Fig. 1: Chemical structure of methylcobalamin

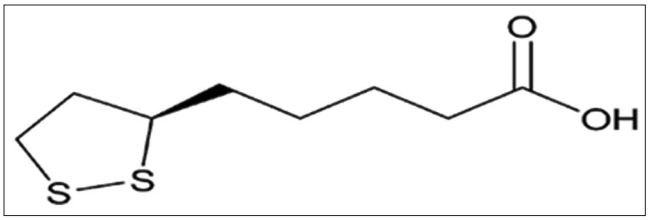

Fig. 2: Chemical structure of alpha-lipoic acid

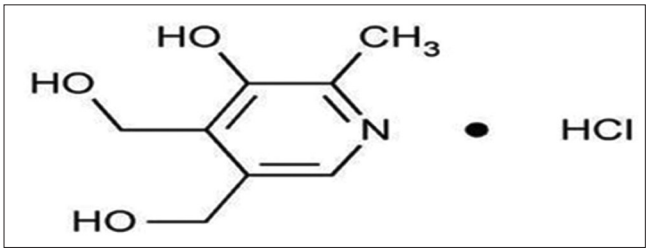

Fig. 3: Chemical structure of pyridoxine hydrochloride

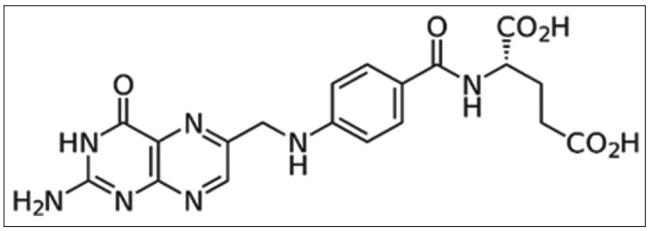

Fig. 4: Chemical structure of folic acid

separately by scanning the sample over the wavelength range of 200–400 nm against blank as methanol. After thorough examination of the spectra, the wavelength 260 nm was selected for further analysis. The overlay spectrum for methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid is shown in Fig. 5.

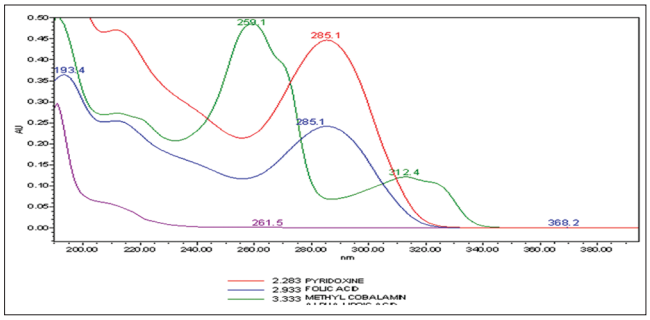

Fig. 5: UV spectrum of methylcobalamin, alpha-lipoic acid, pyridoxine HCL, and folic acid (285 nm)

Chromatographic conditions

The sample separation was achieved on a C18 (Inertsil, 250 mm × 4.6 mm, 5 µm) column, aided by mobile phase mixture of buffer: acetonitrile (10:90%v/v). The flow rate was 1 mL/min and ultraviolet detector at 285 nm, injection volume is 10 µL and maintained at ambient temperature.

Preparation of mobile phase

Buffer preparation: An accurate amount of 5.05 g hexane-1-sulfonic acid is dissolved into 1000-mL distilled water and adjusted to pH - 2.5 with OPA. Then it was filtered through 0.45-µ membrane filter.

Mobile phase: Then, 10 volumes of buffer and 90 volumes of acetonitrile were added and sonicated for 10 min.

Preparation of standard stock solution

Weighed accurately about 1500 mg methylcobalamin, 100 mg alpha-lipoic acid, 3 mg of pyridoxine, and 1.5 mg folic acid and transferred into 100-mL volumetric flask, added 70 mL of diluent, sonicated to dissolve, and diluted up to volume with diluents to give a primarily stock solution containing 150 µg/mL of methylcobalamin, 10 µg/mL of alpha-lipoic acid, 0.3 µg/mL of pyridoxine hydrochloride, and 15 µg/mL of folic acid.

Table 1: System suitability parameters for methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid

Parameters	Methylcobalamin	Alpha-lipoic acid	Pyridoxine hydrochloride	Folic acid	Acceptance criteria
Retention time (min)	13.505	14.399	3.544	6.732	±10
Theoretical plates	43409	19674	117890	3951	>2000
Tailing factor	1.22	1.89	1.61	1.81	<2
RSD	0.777	0.100	0.047	0.047	<2

RSD: Relative standard deviation

Table 2: Method precision values for methylcobalamin

Rt (min)	Area	USP plate	USP tailing
13.505	5962	52654	1.21
13.512	5973	51764	1.27
13.505	5984	45180	1.57
13.512	5945	52104	1.27
13.505	5898	53121	1.21
13.512	5979	51792	1.26
Mean: 5957			
%RSD: 0.534			

Rt: Retention time, %RSD: Percentage relative standard deviation

Table 3: Method precision values for alpha-lipoic acid

Rt (min)	Area	USP plate	USP tailing
14.399	218860	19674	1.89
14.409	218860	19470	1.88
14.399	218652	19685	1.89
14.409	217920	19518	1.87
14.399	218845	19677	1.89
14.409	218367	19496	1.89
Mean: 218584			
%RSD: 0.173			

Rt: Retention time, %RSD: Percentage relative standard deviation

Table 4: Method precision values for pyridoxine hydrochloride

Rt (min)	Area	USP plate	USP tailing
3.544	117890	6272	1.33
3.547	117967	6014	1.33
3.544	117478	6274	1.42
3.547	117785	6018	1.36
3.544	117769	6275	1.33
3.547	117967	6014	1.33
Mean: 117809			
%RSD: 0.156			

Rt: Retention time, %RSD: Percentage relative standard deviation

Sample solution

Accurately weighed 10 tablets were crushed to powdered form and then five tablets equivalent of sample were taken into a 250-mL volumetric flask. To this, 200 mL of diluent was added, sonicated to dissolve, and diluted to volume diluent and further diluted to 5–100 mL with the diluents, which was filtered through 0.45-µ Nylon syringe filter.

Method validation

System suitability

The typical values for evaluating system suitability of a chromatographic procedure are relative standard deviation (RSD) <2%, tailing factor <2%, and theoretical plates >2000. The retention time, peak area, theoretical plates, and tailing factor were evaluated for the system.

Linearity

Linearity was studied by analyzing five standard solutions covering the range from 0 to 2130 µg/mL for methylcobalamin, 0–142.5 µg/mL for alpha-lipoic acid, 0–4.54 µg/mL for pyridoxine hydrochloride, and 0–2 µg/mL for folic acid. A calibration curve with concentration versus peak area was plotted by injecting the above-prepared solutions.

Table 5: Method precision values for folic acid

Rt (min)	Area	USP plate	USP tailing
6.732	158056	3954	1.81
6.738	158808	3944	1.84
6.732	157872	3957	1.80
6.738	157920	3957	1.84
6.732	158224	3951	1.81
6.738	158808	3944	1.84
	Mean: 158281		
	%RSD: 0.269		

Rt: Retention time, %RSD: Percentage relative standard deviation

Table 6: LOD and LOQ values calculated from calibration curve

Parameters	LOD=3.3 (SD/S) (µg/mL)	LOQ=10 (SD/S) (µg/mL)
Methylcobalamin	40.631	123.12
Alpha-lipoic acid	4.55	13.815
Pyridoxine Hcl	0.087	0.264
Folic acid	0.0447	0.135

LOD: Limit of detection, LOQ: Limit of quantitation, SD: Standard deviation, S: lope

Accuracy

The accuracy of the method was determined by calculating the recoveries of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid by analyzing solutions containing approximately 50%, 100%, and 150% of the working strength of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid.

Robustness

Robustness is the measure of method remains unaffected by small deliberate changes in method parameters such as flow rate and detection of wavelength on assay of the analyte of interest. Here, the detection wavelength varied ±2 nm and flow rate was varied ±0.1 mL/min.

Solution stability

The solution stability of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid in diluents was determined by storing sample solution in a tightly capped volumetric flask at room temperature for 24h. The amount of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid was measured at different time intervals such as 12 and 24 h, and the results obtained were compared methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid freshly prepared solutions.

RESULTS AND DISCUSSION

Selection of wavelength (for detection)

System suitability

The system suitability of the method was checked by repeated preparations for 160 µg/mL for methylcobalamin, 120 µg/mL for alpha-lipoic acid, 80 µg/mL for pyridoxine hydrochloride, and 40 µg/mL for folic acid. The typical values for evaluating system suitability of a chromatographic procedure are RSD <2%, tailing factor <2%, and theoretical plates >2000. The retention time, peak area, theoretical plates, and tailing factor were evaluated for the system suitability data of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid are shown in Table 1.

Table 7: Accuracy results of methylcobalamin by HPLC

Accuracy	Conc. (ppm)	Area	Amount added	Amount recovered	% recovery	Results
50	50	4994	0.367	0.37	100.8	Mean: 0.28
50	50	5111	0.378	0.38	100.5	S.D: 0.280
50	50	5111	0.379	0.38	100.3	%RSD: 100
100	100	10447	0.781	0.78	99.9	Mean: 0.07
100	100	10107	0.75	0.75	100.0	S.D: 0.07
100	100	10447	0.78	0.78	100.0	%RSD: 0.070
150	150	15482	1.15	1.15	100.9	Mean: 100.3
150	150	15482	1.15	1.15	100.0	S.D: 0.51
150	150	15146	1.13	1.13	100.0	%RSD: 0.500
					Mean: 100.2	
					%RSD: 0.25	

Ppm: Parts per million, SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 8: Accuracy results of alpha-lipoic acid by HPLC

Accuracy	Conc. (ppm)	Area	Amount added	Amount recovered	% recovery	Results
50	50	110448	250.7	252.94	100.9	Mean: 100.8
50	50	110448	251.4	252.94	100.6	S.D: 0.15
50	50	110448	250.8	252.94	100.9	%RSD: 0.150
100	100	220245	501.5	504.4	100.6	Mean: 100.5
100	100	220064	502.8	503.98	100.2	S.D: 00.25
100	100	220064	500.4	503.98	100.7	%RSD: 0.250
150	150	331868	759.6	760.03	100.1	Mean: 100.4
150	150	330680	752.2	757.31	100.7	S.D: 0.33
150	150	330680	753.1	757.31	100.6	%RSD: 0.330
					Mean: 100.6	
					%RSD: 0.21	

Ppm: Parts per million, SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 9: Accuracy results of pyridoxine hydrochloride by HPLC

Accuracy	Conc. (ppm)	Area	Amount added	Amount recovered	% recovery	Results
50	50	80094	3.79	3.79	100.0	Mean: 100.1
50	50	80094	3.78	3.79	100.3	S.D: 0.41
50	50	80094	3.76	3.79	100.8	%RSD: 0.400
100	100	162301	7.68	7.68	100.0	Mean: 100
100	100	162301	7.69	7.68	99.9	S.D: 0.13
100	100	162301	7.67	7.68	100.1	%RSD: 0.130
150	150	246824	11.59	11.68	100.8	Mean: 100.7
150	150	248096	11.68	11.74	100.5	S.D: 0.15
150	150	248096	11.65	11.74	100.8	%RSD: 0.150
					Mean: 100.4	
					%RSD: 0.35	

Ppm: Parts per million, SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 10: Accuracy results of folic acid by HPLC

Accuracy	Conc. (ppm)	Area	Amount added	Amount recovered	% recovery	Results
50	50	80094	3.79	3.79	100.0	Mean: 100.4
50	50	80094	3.78	3.79	100.3	S.D: 0.41
50	50	80094	3.76	3.79	100.8	%RSD: 0.400
100	100	162301	7.68	7.68	100.0	Mean: 100.0
100	100	162301	7.69	7.68	99.9	S.D: 0.13
100	100	162301	7.67	7.68	100.1	%RSD: 0.130
150	150	246824	11.59	11.68	100.8	Mean: 100.7
150	150	248096	11.68	11.74	100.5	S.D: 0.15
150	150	248096	11.65	11.74	100.8	%RSD: 0.150
					Mean: 100.4	
					%RSD: 0.35	

Ppm: Parts per million, SD: Standard deviation, %RSD: Percentage relative standard deviation

Table 11: Robustness data for methylcobalamin, alpha-lipoic acid, pyridoxine HCL, and folic acid

Drug	Parameter	Retention time	Area
Methylcobalamin	Decreased flow rate (0.8 mL)	13.506	100178
	Increased flow rate (1.2 mL)	13.496	96114
	Wavelength (283 nm)	13.506	104399
	Wavelength (287 nm)	13.506	92044
Alpha-lipoic acid	Decreased flow rate (0.8 mL)	14.404	221198
	Increased flow rate (1.2 mL)	14.395	215464
	Wavelength (283 nm)	14.404	176205
	Wavelength (287 nm)	14.399	258132
Pyridoxine hydrochloride	Decreased flow rate (0.8 mL)	3.548	118836
	Increased flow rate (1.2 mL)	3.545	117704
	Wavelength (283 nm)	3.548	360499
	Wavelength (287 nm)	3.550	23881
Folic acid	Decreased flow rate (0.8 mL)	3.548	118836
	Increased flow rate (1.2 mL)	3.545	117704
	Wavelength (283 nm)	13.506	104399
	Wavelength (287 nm)	13.505	92044

nm: Nanometer

Table 12: Solution stability of methylcobalamin

Stability (h)	Rt	Area	USP plate count	USP tailing	% assay
Initial	13.506	99220	47146	1.13	100
6	13.506	99587	47182	1.17	100.5
12	13.506	99220	47146	1.13	100.6
18	13.506	99220	47146	1.13	100.4
24	13.506	99733	47077	1.14	100.6

H: Hours, Rt: Retention time, % assay: Percentage assay

Table 13: Solution stability of alpha-lipoic acid

Stability (h)	Rt	Area	USP plate count	USP tailing	% assay
Initial	14.404	217168	20032	1.91	100.0
6	14.404	217168	20032	1.91	100.3
12	14.404	216158	20092	1.89	100.3
18	14.404	217690	20002	1.93	101.1
24	14.404	217168	20032	1.91	100.3

H: Hours, Rt: Retention time, % assay: Percentage assay

Table 14: Solution stability of pyridoxine HCL

Stability (h)	Rt	Area	USP plate count	USP tailing	% assay
Initial	3.548	117699	5824	1.34	100.8
6	3.548	117669	5837	1.32	100.1
12	3.548	117849	5827	1.24	100.4
18	3.548	117391	5834	1.26	100.2
24	3.548	115679	5874	1.30	100.0

H: Hours, Rt: Retention time, % assay: Percentage assay

Table 15: Solution stability of folic acid

Stability (h)	Rt	Area	USP plate count	USP tailing	% assay
Initial	6.739	158182	3842	1.86	100.2
6	6.739	157546	3855	1.83	100.3
12	6.739	158278	3842	1.85	100.7
18	6.739	158030	3846	1.84	100.4
24	6.739	158535	3837	1.85	100.6

H: Hours, Rt: Retention time, % assay: Percentage assay

Linearity

Linearity was studied by analyzing six concentrations ranging from 0 to 2130 µg/mL for methylcobalamin, 0 to 142 µg/mL for alpha-lipoic acid, 0 to 4 µg/mL for pyridoxine hydrochloride, and 0 to 2 µg/mL for folic acid. A calibration curve with concentration versus peak area was plotted by injecting the above-prepared stock solutions. Correlation coefficient values for methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid are 0.999, respectively. The linear regression data for the calibration plot indicate a good linear relationship between peak area and concentration. The linearity data for all drugs are shown in Figs. 6-9.

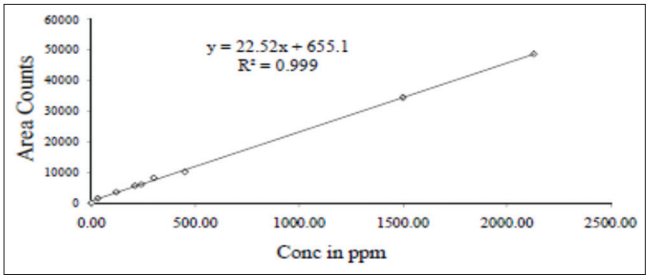

Fig. 6: Graph representing calibration curve of methylcobalamin. Error bars represent standard deviation of the mean (±standard deviation)

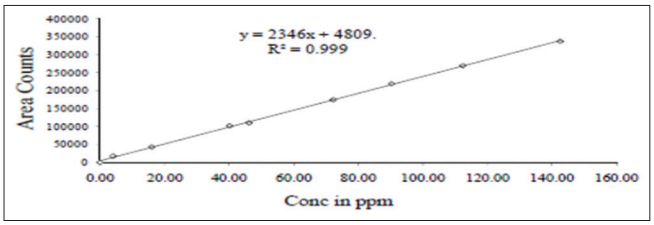

Fig. 7: Graph representing calibration curve of alpha-lipoic acid. Error bars represent standard deviation of the mean (±standard deviation)

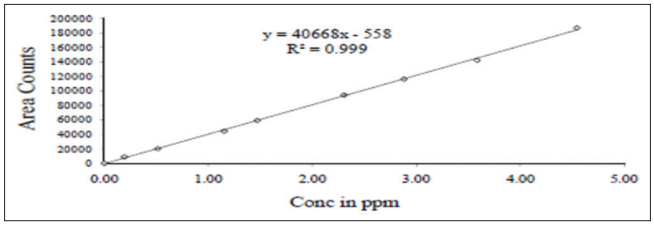

Fig. 8: Graph representing calibration curve of pyridoxine hydrochloride. Error bars represent standard deviation of the mean (±standard deviation)

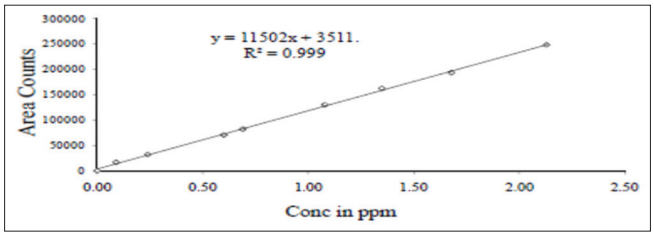

Fig. 9: Graph representing calibration curve of folic acid. Error bars represent standard deviation of the mean (±standard deviation)

Precision

The precision of the method was checked by repeated preparations. The measurement of peak areas of repeated solutions (n=6) for 160 µg/mL of methylcobalamin, 120 µg/mL of alpha-lipoic acid, 80 µg/mL of pyridoxine hydrochloride, and 40 µg/mL of folic acid. The study was expressed as RSD of set of results. The precision of the method (%RSD) was found to be <1% showing a good repeatability. The values of percentage RSD for all drugs are shown in Tables 2-5.

LOD and LOQ

The LOD and LOQ were separately determined based on standard deviation of the Y-intercept and slope of the calibration curve. The LOD of the proposed method was found to be 40.631 µg/mL, 4.55 µg/mL, 0.087 µg/mL, and 0.0447 µg/mL and LOQ was found to be 123.12 µg/mL for methylcobalamin, 13.815 µg/mL for alpha-lipoic acid, 0.264 µg/mL for pyridoxine hydrochloride, and 0.135 µg/mL for folic acid. The results are shown in Table 6.

Accuracy

The accuracy of the method was determined by calculating the recoveries of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid by analyzing solutions containing approximately 50%, 100%, and 150% of the working strength of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid are shown in Tables 7-10.

Robustness

Robustness is the measure of a method remains unaffected by small, deliberate changes in method parameters such as flow rate and detector wavelength on assay of the analyte of interest. Here, the detection wavelength varied ±2 nm and flow rate was varied ±0.1 mL/min. The results are shown in Table 11.

Solution stability

The amount of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid was measured at different time intervals such as 12 and 24 h, and the results obtained were compared methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid freshly prepared solutions. The results are shown in Tables 12-15.

CONCLUSION

From the experimental results and parameters, it was concluded that this newly developed method for the simultaneous estimation of methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid was found to be simple, precise, and accurate. The high resolution and shorter retention time make this method more acceptable and cost-effective, and it can be effectively applied for routine analysis in research institutions, quality control department, and approved testing laboratories.

CONFLICTS OF INTEREST

All authors declare that they have no conflicts of interest.

REFERENCES

1. McDowell LR. Vitamins in Animal and Human Nutrition. Available from: https://www.Booksgoogle.com. [Last retrieved on 2018 Jan 28].
2. Eisai Submits New Drug Application for Mecobalamin Ultra-High Dose Preparation as Treatment for Amyotrophic Lateral Sclerosis In Japan (PDF). Available from: https://www.Eisai.com. [Last retrieved on 2018 Jan 28].
3. World Health Organization. WHO Model Formulary 2008 (PDF). World Health Organization; 2009. p. 496.Available from: https://www.kidney-international.org/article/S0085-2538(15)50104-X/pdf. [Last retrieved on 2016 Dec 08].
4. van Woerden CS, Groothoff JW, Wijburg FA, Annink C, Wanders RJ, Waterham HR, *et al.* Clinical implications of mutation analysis in primary hyperoxaluria Type 1. Kidney Int 2004;66:746-52.
5. Monico CG, Olson JB, Milliner DS. Implications of genotype and enzyme phenotype in pyridoxine response of patients with Type I primary hyperoxaluria. Am J Nephrol 2005;25:183-8.
6. Monico CG, Rossetti S, Olson JB, Milliner DS. Pyridoxine effect in Type I primary hyperoxaluria is associated with the most common mutant allele. Kidney Int 2005;67:1704-9.
7. Dietary supplement fact sheet. Folate. Health Information. Office of Dietary Supplements, US: National Institutes of Health; 2014. Available from: https://www.ods.od.nih.gov/factsheets/Folate-HealthProfessional.
8. Weinstein SJ, Hartman TJ, Stolzenberg-Solomon R, Pietinen P, Barrett MJ, Taylor PR, *et al.* Null association between prostate cancer and serum folate, Vitamin B(6), Vitamin B(12), and homocysteine. Cancer Epidemiol Biomarkers Prev 2003;12:1271-2.
9. Nawaz M. A new validated stability indicating RP-HPLC method for simultaneous estimation of pyridoxine hydrochloride and meclizine hydrochloride in pharmaceutical solid dosage forms. Chromatogr Res Int 2013;2013 Article ID: 747060. pages:7.
10. Shah R, Shah R. Stability indicating RP-HPLC method for simultaneous estimation of dosulepin hydrochloride and methylcobalamin in tablet dosage form. Int J App pharm 2017;9:69-75.
11. Available from: https://www.innovareacademics.in/journals/index.php/ijpps/article/view/3369.
12. Available from: https://www.ijprd.com/ENT%20AND%20VALIDATION%20OF%20ANALYTICAL.