Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

Der Pharmacia Sinica, 2013, 4(5):46-53

Der Pharmacia Sinica
ISSN: 0976-8688
CODEN (USA): PSHIBD

A simple and validated RP-HPLC method for the estimation of methylcobalamin and Alphalipoic acid in soft gelatine capsule dosage form

Nandini R. Pai and Seema S. Sawant*

Department of Chemistry, D. G. Ruparel College of Arts, Science & Commerce, Senapati Bapat Marg, Mahim (West), Mumbai, Maharashtra, India

---

ABSTRACT

Simple, precise, rapid and selective reverse phase high performance liquid chromatographic (RP-HPLC) methods have been developed and validated for the assay determination of Methylcobalamin 1500 mcg and Alphalipoic acid 300 mg in soft gelatine capsule formulation. Two separate chromatographic conditions were used for estimation of Methylcobalamin[1] & Alphalipoic acid[2]. The methods use Phenomenox Luna (C-18, 250 x 4.6 mm, 5 µm) column and gradient elution for both the methods. The aqueous mobile phase contained 0.02 M phosphate buffer adjusted to pH 3.5 with hexane-1-sulphonic acid, sodium salt as ion pairing reagent and acetonitrile for both the methods. Separation and quantification was achieved by changing the proportion of the system linearly with a time-schedule programme. Detection was carried out in the range of 200 to 600 nm using photodiode array detector and set at 240 nm for alphalipoic acid and at 266 nm for Methylcobalamin and further analysis was carried out using a UV detector. These methods have been validated and found to be applicable in routine analysis for Methylcobalamin & Alphalipoic acid capsule. The precision is exemplified by relative standard deviations of 0.78% for Methylcobalamin and 0.53% for Alphalipoic acid. Good linearity was observed between the concentration of the analytes and peak area with correlation coefficients of 0.99995 and 0.99941 respectively. Mean recoveries obtained during spiking experiments were found to be 101.43 % and 99.43 % respectively

Keywords: Methylcobalamin, Alphalipoic acid, Validation

INTRODUCTION

Methylcobalamin (MCB)[1], (1R, 2R, 4S, 7S)-7-{[(2S)-3-hydroxy-2-phenylpropanol]oxy}-9,9-dimethyl-3-oxa-9-azonia tricycle [3.3.1.02,4] nonane, is a supplement for vitamin, used in treatment of Vitamin B12 deficiency of dietary origin. It is official in Japanese pharmacopoeia[1]. Alphalipoic acid (ALP)[3], (R)-5-(1, 2-dithiolan-3-yl) pentanoic acid, is antioxidant, and used in treatment of diabetes and HIV. It has also been used for cancer, liver ailments, and various other conditions. It is official in United State Pharmacopoeia[2]. Combination of Methylcobalamin and Alphalipoic acid treats both the problems associated with all types of neuropathy i.e., neuralgia (neuronal pain) and neuron degeneration and also is used in management of diabetic neuropathy. Alphalipoic acid is a universal antioxidant which prevents oxidative damage of neurons. Methylcobalamin increases myelin sheath formation thereby regenerates neuron. This has stimulated research on accurate and efficient analytical methods for the determination of vitamins which is problematic because of their instability and the complexity of the matrices in which they are usually analysed. Literature survey reveals some reported methods for the analysis of Methylcobalamin & Alphalipoic acid by ultra-violet (UV)[8], high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC)[5]. Estimation of alaphalipoic acid by UV, HPLC[10] and GC, either individually or in combination with other drugs are also reported. To the best of our knowledge, there are very few analytical methods reported for assay determination of binary mixture containing Methylcobalamin and Alphalipoic acid in soft gelatine capsule[20] formulation. Therefore, an attempt

has been made to develop a simple, accurate, rapid and reproducible gradient HPLC methods for simultaneous determination of Methylcobalamin and Alphalipoic acid in soft gelatine capsule[20] dosage form and validate it, in accordance with ICH guidelines[15]. The proposed methods are very simple, rapid, and validated according to ICH guidelines[15]. In the present work, assay determination of Methylcobalamin and Alphalipoic acid in soft gelatine capsule is proposed using a simple gradient program by setting the wavelength at 240 nm for alphalipoic acid and at 266 nm for methylcobalamin and at different chromatographic conditions

MATERIALS AND METHODS

Instrument

The HPLC system was composed of LC 2010 Shimadzu system fitted with Prominence PDA detector with LC Solution software. Analytical column used for these methods is Phenomenox Luna C18 (250 mm x 4.6 mm), 5µm

Reagents and materials

All the reagents were of analytical-reagent grade. De-ionized water (Millipore), HPLC-grade acetonitrile, monobasic potassium phosphate, hexane -1- sulfonic acid, sodium salt AR grade, ortho-phosphoric acid AR grade and sodium hydroxide AR grade were used. Highly purified working standards, Methylcobalamin JP (B12 with purity 92.04%)-Biocon Ltd, and Alphalipoic acid USP (purity 99.72%)-Biocon Ltd, The analysed samples (Methylcobalamin Soft gelatin Capsule of Gelnova Lab.) was a capsule containing 1.5 and 300 mg of Methylcobalamin and Alphalipoic acid per capsule respectively

Preparation of mobile phase

For Assay of Methylcobalamin & alphalipoic acid, the gradient elution system started with an aqueous 0.02 M monobasic potassium phosphate buffer adjusted to pH 3.5 with dilute ortho-phosphoric acid solution and containing 3.76 g of hexane-1-sulphonic acid, sodium salt as gradient system Solvent A and Acetonitrile as Solvent B. Refer Table 1 & 2 for the gradient elution programme for assay of Methylcobalamin & alphalipoic acid respectively

Table 1: Gradient elution programme for the assay of Methylcobalamin

Time	Buffer (Solvent A)	Acetonitrile (Solvent B)	Gradient
0.00	80	20	Linear
13.00	80	20	
13.01	50	50	
23.00	50	50	
23.01	80	20	
32.00	STOP		

Table 2: Gradient elution programme for the assay of Alphalipoic acid

Time	Buffer (Solvent A)	Acetonitrile (Solvent B)	Gradient
0.00	60	40	Linear
10.00	60	40	
10.01	35	65	
16.00	35	65	
16.01	60	40	
28.00	STOP		

Preparation of diluent

Weighed and dissolved 6.8 g Potassium dihydrogen Orthophosphate and 0.9 g of Sodium Hydroxide in 1000 ml water. Adjusted the pH 6.8

Chromatographic conditions

For Assay of Methylcobalamin: A Reverse phase column Phenomenox Luna C-18 was a 250 X 4.6 mm id., 5 µm particle size and end capped. After sample preparation immediately transferred in an amber vial and kept at stable temperature, 20°C. Column oven temperature was set at 40°C. The wavelength was set at 266 nm. The flow rate was 1.0 ml/min and the volume injected was 50 µl

For Assay of Alphalipoic acid: A Reverse phase column Phenomenox Luna C-18 was a 250 X 4.6 mm id., 5 µm particle size and end capped. After sample preparation immediately transferred in an amber vial and kept at stable temperature, 20°C. Column oven temperature was set at 40°C. The wavelength was set at 240 nm. The flow rate was 1.2 ml/min and the volume injected was 10 µl

PROCEDURE

Preparation of standard solutions

Methylcobalamin standard solution: Weighed accurately 20 mg of Methylcobalamin working standard in 100 ml volumetric flask, dissolved in 70 ml of diluent and diluted up to the mark with diluent and mixed. Further diluted 5 ml to 50 ml with diluents

Alphalipoic acid standard solution: Weighed accurately 100 mg of Alphalipoic acid working standard in 200 ml volumetric flask, dissolved in 100 ml of diluent and diluted up to the mark with diluent and mixed

Sample preparation

20 intact capsules were weighed accurately. Transferred the content of the capsules in a petridish and with the help of spatula made a homogenous paste. Cleaned out the capsule shells with a small quantity of n-hexane to remove all the traces of contents. Weighed the empty capsule shells. From the differences in the weight of intact capsules & it's empty capsule shells; determined the average fill weight

Methylcobalamin sample preparation: A portion of the paste equivalent to 1.0 mg of Methylcobalamin transferred into 50 ml amber colour volumetric flask. About 40 ml of diluent was added and sonicated for 20 minutes, with intermittent shaking and the solution was diluted to volume with diluent, mixed well. The sample solution was then filtered through 0.45 µm membrane filter

Alphalipoic acid sample preparation: A portion of the paste equivalent to 100.0 mg of Alphalipoic acid transferred into 200 ml amber colour volumetric flask. About 160 ml of diluent was added and sonicated for 20 minutes, with intermittent shaking and the solution was diluted to volume with diluent, mixed well. The sample solution was then filtered through 0.45 µm membrane filter

RESULTS AND DISCUSSION

Method development

During method development it became obvious that different work up procedures have to be used for determination of two components due to differences in solubility and stability. So, diluent 0.05 M monobasic potassium phosphate buffer adjusted to pH 6.8 was selected for dissolving both the components. The selectivity of a chromatographic separation is described by the combined affinities that the mobile phase and stationary phase exert on the sample components. A reliable chromatographic assay also requires acceptable resolution, reasonable retention times and good peak symmetry. Different proportions of acetonitrile in the mobile phase exerted a striking change in the 'K' values of Methylcobalamin whereas the peak behaviour of Alphalipoic acid remained unaffected. The profile of a gradient elution system affects the retention of solutes in a similar way to the proportions of the solvent with greater elution strength in a binary mobile phase under isocratic conditions. The gradient conditions were chosen in terms of peak shape, column efficiency, chromatographic analysis time and selectivity. Several gradient programs were tried to separate all the interfering peaks. Elution of both Methylcobalamin & alphalipoic acid from the column was in order of decreasing polarity of the mobile phase used; as the proportion of acetonitrile increased the retention times for both ingredients got decreased and some of them eluted with solvent front. Due to soft gelatine capsules, interference by other excipient's peak were also observed; but by gradient elution method, this problem got nullified. The greater the proportion of phosphate buffer with ion pair reagent, the better peak shapes, and resolution from the other interfering peaks. The peak asymmetry (T) and theoretical plate counts of both the components were very close to the ideal value 2 and not less than 2000 respectively.

Refer Figure 1 & 2 for Methylcobalamin & Alphalipoic acid Standard solution chromatograph respectively.

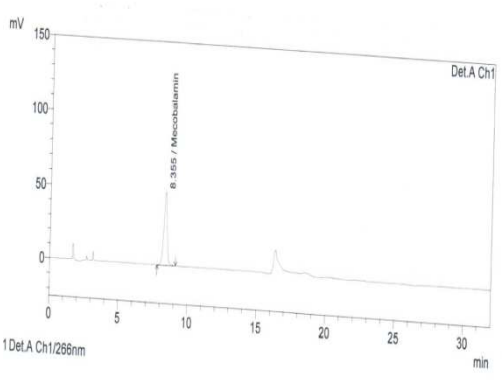

Figure 1: Methylcobalamin standard solution chromatograph

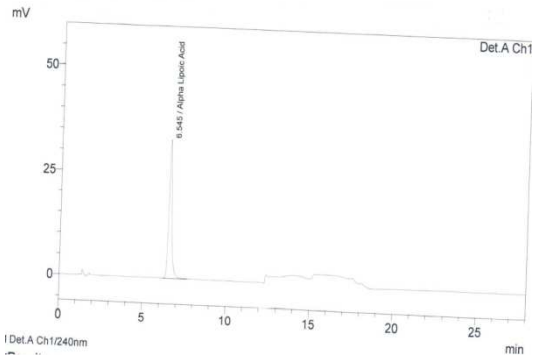

Figure 2: Alphalipoic acid standard solution chromatograph

In order to test the applicability of this procedure to a commercial formulation, 'Methylcobalamin Soft gelatin Capsule of Gelnova Lab. was analysed. The sample peaks were identified by comparing the UV-Vis spectrum of each one with those of the standard reference components.

Refer Figure 3 & 4 for Methylcobalamin & Alphalipoic acid Sample solution chromatograph respectively.

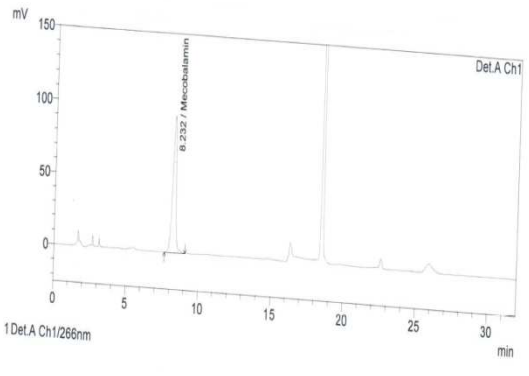

Figure 3: Methylcobalamin sample solution chromatograph

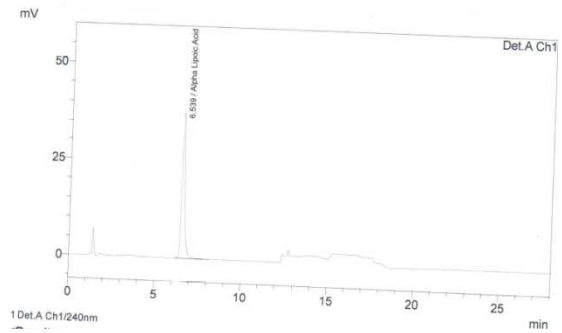

Figure 4: Alphalipoic acid sample solution chromatograph

Method validation

Validation of the analytical method is the process that establishes by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. The preparation and execution should follow a validation protocol. This method has been validated with respect to precision, linear range, accuracy, specificity and robustness

Specificity

The specificity of the present method was described by peak purity. It calculates a peak purity index for each spectral comparison between the reference spectrum and the other peak spectra. Purity angle should be less than the purity threshold for each component and there was no purity flag indicated that the excipients present in the dosage forms did not interfere with the analysis (no noticeable interference from the excipients was observed in the chromatograms). Specificity of the peak purity of Methylcobalamin and Alphalipoic acid were assessed by comparing the retention time of standard and the sample and good correlation was obtained. Both the peaks were found pure. Also there were no peaks when the placebo and blank were injected and no interferences, hence the method is specific. System suitability parameters were determined as tailing factor and theoretical plates for both the peaks

Refer Table 3 for Specificity study observations.

Refer figure 5, 6, 7 & 8 for chromatograph of Methylcobalamin blank, Alphalipoic acid blank, Methylcobalamin placebo, Alphalipoic acid placebo respectively.

Refer figure 9 & 10 for peak purity graph of Methylcobalamin and Alphalipoic acid in sample solution respectively.

Table 3: Specificity study observations

	Methylcobalamin	Alphalipoic acid
Retention Time in minute	8.355	6.545
Tailing Factor (NMT 2.0)	0.973	1.675
Theoretical plates (More than 2000)	7789.55	9755.88
Peak Purity	Peak Purity Index : 1.0000	Peak Purity Index : 1.0000
Blank/Placebo Interference	Not detected	Not detected
% RSD peak area (NMT 2.0 %)	0.05 %	0.12 %

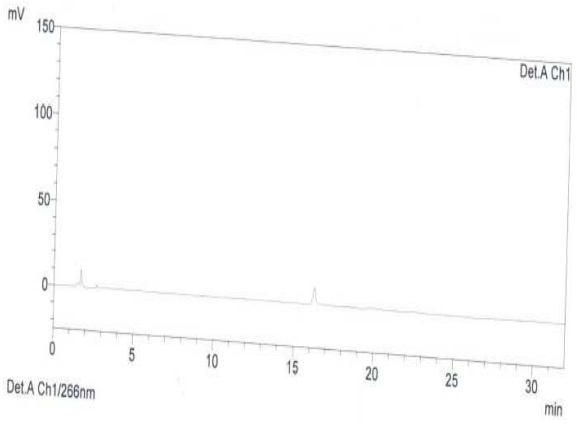

Figure 5: Methylcobalamin Blank Chromatograph

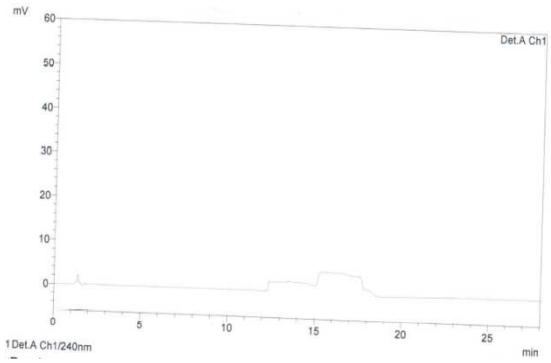

Figure 6: Alphalipoic acid Blank Chromatograph

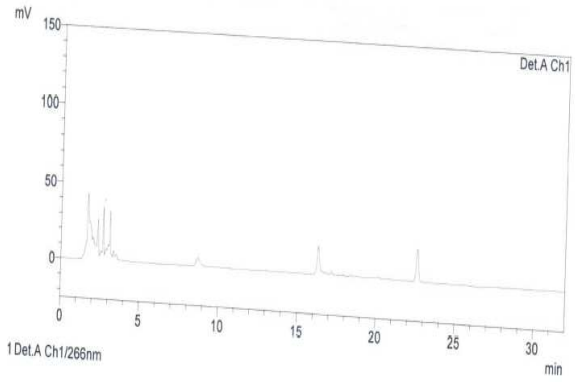

Figure 7: Methylcobalamin Placebo Chromatograph

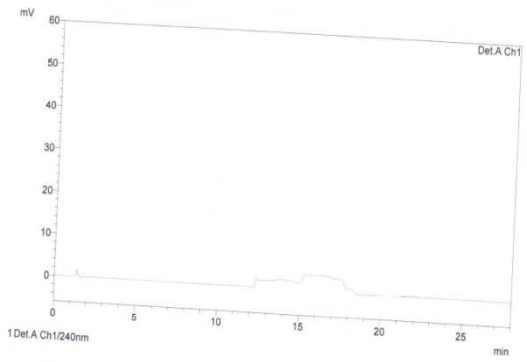

Figure 8: Alphalipoic acid Placebo Chromatograph

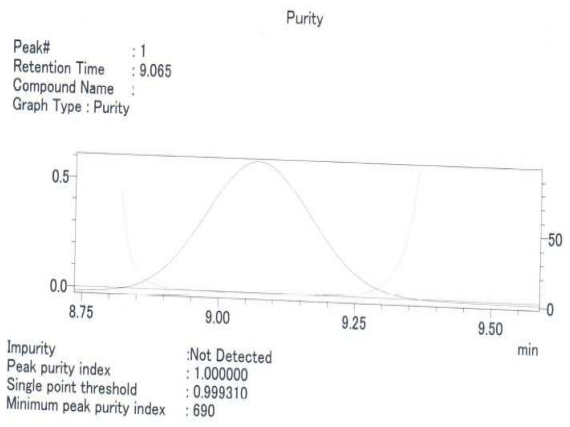

Figure 9: Peak purity graph of Methylcobalamin

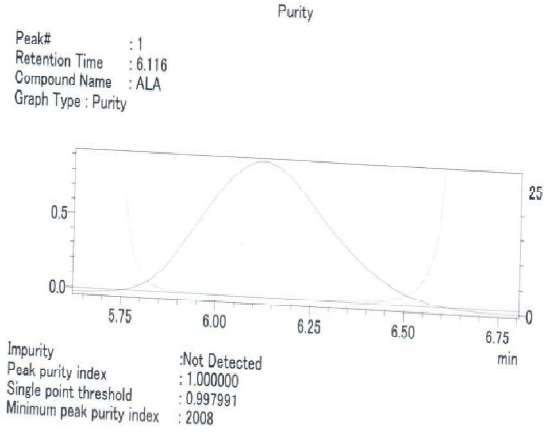

Figure 10: Peak purity graph of Alphalipoic acid

Linearity & range

Linearity was obtained in the range of standard concentration of both contents of capsule. A series of six solutions at low and high concentration levels from 75 to 200% of the nominal concentration levels for Methylcobalamin and a series of five solutions at low and high concentration levels from 80 to 120% of the nominal concentration levels for Alphalipoic acid were prepared, each solution was injected three times and the regression was calculated by the method of least-squares. Peak areas were calculated and the results interpolated on the calibration graph for both contents. It could be shown that the correlation is linear over a wide range.

Refer Table 4 for Linearity & range study observations.

Refer Figure 11 & 12 for linearity graph of Methylcobalamin and Alphalipoic acid respectively.

Table 4: Linearity & Range study observations

	Methylcobalamin	Alphalipoic acid
Concentration range	15 - 40 µg/ml	400 - 600 µg/ml
Correlation coefficient	0.99995	0.99941
Slope	8674.92	4072.12
Y – Intercept	-2498.98	10136.83
R-square	0.99990	0.99882

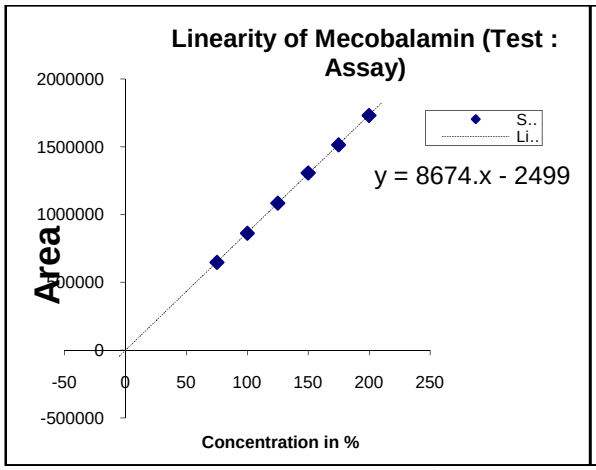

Figure 11: Linearity graph of Methylcobalamin

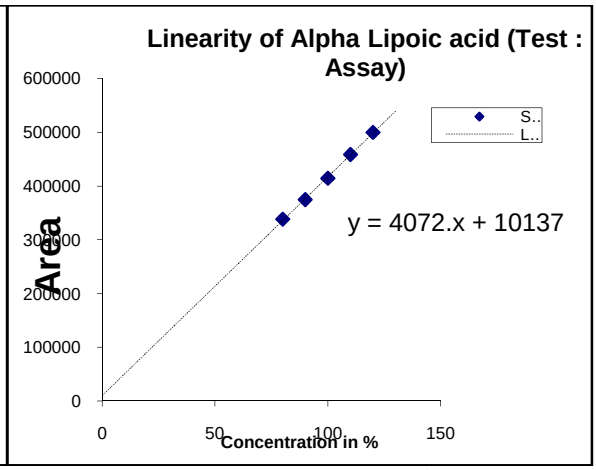

Figure 12: Linearity graph of Alphalipoic acid

Accuracy (recovery studies)

To check the degree of accuracy of the method, recovery studies were performed in triplet by standard addition method at 80%, 100%, and 120% of specification limit for alphalipoic acid (500 µg/ml) and at 75%, 100% & 125% of specification limit for Methylcobalamin (20 µg/ml) . Known amounts of standard solutions of methylcobalamin & alphalipoic acid was mixed with the placebo paste to prepare the different concentrations and were subjected to the proposed HPLC method. The % recovery was found to be within the limits of the acceptance criteria of 98% - 102%.

Refer Table 5 for results of recovery studies.

Table 5: Results of recovery studies

Methylcobalamin		Alphalipoic acid	
Level	% Recovery	Level	% Recovery
75 %	101.26	80 %	100.61
100 %	101.25	100 %	98.59
125 %	101.79	120 %	99.10
Mean	**101.43**	**99.76**	**99.43**
% RSD	**0.31**	**0.81**	**1.06**

Precision

Precision was carried out for Inter and Intraday analysis for soft gelatine capsule dosage form. Precision was evaluated by carrying out six independent sample preparations of a single lot of formulation. The sample preparation for soft gelatine capsule dosage form was carried out in same manner as described above. Relative standard

deviation (% RSD) was found to be less than 2% for methylcobalamin & alphalipoic acid, which proves that the method is precise.

Refer Table 6 for precision study observations.

Table 6: Precision study observations

	Sr. No.	Assay in %			
		Methylcobalamin		Alphalipoic acid	
		Method Precision	Intermediate Precision	Method Precision	Intermediate Precision
	1	167.55	165.17	104.34	104.40
	2	164.18	165.09	104.08	104.42
	3	164.85	165.65	103.24	103.25
	4	167.12	165.74	103.73	103.68
	5	166.12	163.92	103.05	103.75
	6	166.02	163.28	104.30	103.96
	Mean	165.97	164.81	103.79	103.91
	SD	1.29	0.99	0.55	0.45
	RSD	0.78	0.60	0.53	0.43
Method Precision - Intermediate Precision :	Mean	165.39		103.85	
	SD	1.25		0.48	
	RSD	0.76		0.46	

Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The variations may be variation in column oven temperature by ± 5°C, variation in mobile phase pH by ± 0.1 units. The observed results were within the limit.

Refer Table 7 for the results of Robustness parameter.

Table 7: Results of robustness studies

	Methylcobalamin		Alphalipoic acid	
Parameter	% Assay	% RSD	% Assay	% RSD
Column oven change from 40°C to 35°C	164.89	0.75	103.60	0.49
Column oven change from 40°C to 45°C	165.14	0.73	104.53	0.55
Mobile phase pH change from 3.5 to 3.4	164.37	0.80	102.47	0.68
Mobile phase pH change from 3.5 to 3.6	164.44	0.79	104.72	0.59

Stability of sample solution

During solution stability experiments, RSD for the Methylcobalamin & Alphalipoic acid content were found 0.47% and 0.23% respectively for soft gelatine capsule dosage form which were within 2% RSD. Results of the solution stability experiments confirmed that standard solutions and solutions in the diluent were stable for upto 12 hour during the analysis

CONCLUSION

The developed separate gradient & chromatographic HPLC methods are simple, accurate and precise for the assay determination of Methylcobalamin & Alphalipoic acid from soft gelatine capsule formulation. In the methods for determination of Methylcobalamin and Alphalipoic acid levels in soft gelatine capsule, use of Phenomenox Luna C18 column and a gradient elution mode was applied. Isocratic conditions could not be used mainly because the chromatographic peaks were not well resolved. The methods are simple, specific and rapid with a high degree of accuracy and precision and applied successfully to the routine analysis of Methylcobalamin and Alphalipoic acid in soft gelatine capsule formulation. Thus, the described method is suitable for routine analysis and quality control of pharmaceutical preparations containing these drugs in combination. The simplicity of the procedure should make it highly desirable for quality control of multi-component products in the pharmaceutical and health food industries

REFERENCES

[1] The Ministry of Health and Welfare Ministerial Notification; Japanese Pharmacopoeia, official monograph for part I. 15th ed.; **2006**, pp.590
[2] United State Pharmacopeia, USP36, NF31, **2013**, pp.1517

[3] Goti PP, Savsani JJ, Patel PB., *Inventi Rapid: Pharm Anal Qual Assur*. **2012**, 537(12)
[4] Goti PP, Patel PB., *Journal of pharmacy research*; **2013**, 6; 609 -614
[5] Baheti KG, Galande VR., *Int J Res Pharm Biomed Sci*.; **2011**, 2(3):1199
[6] Sengamalam R, Ravindran M, Gunjan M, Meena S., *J Pharm Res*.; **2011**, 4(2):449
[7] Sharma MC, Sharma AD., *Drug Invent Today*.; 2011, 3(6):95
[8] Ganesan M, Solairaj P, Rajesh SC., *Int J Pharm Pharm Sci.;* **2012**, 4(3):559
[9] Goti PP, Savsani JJ, Patel PB., *Inventi Rapid: Pharm Anal Qual Assur*.; **2012**, 525(12)
[10] Chattopadhyaya I, Akhtar J., *Inventi Rapid: Pharm Anal Qual Assur*.; **2012**, 335(12)
[11] Goti PP, Savsani JJ, Patel PB., *Int J Pharm Pharm Sci*.; **2012**,4(5):33
[12] Raja M, Swathi M, David B, Swathi A., *Int J Pharm Res Dev*.; **2012**,4(6):102
[13] Poongothai S, Ilavarasan R, Karrunakaran C., *Int J PharmPharm Sci*.; **2010**, 2(4):131
[14] Saravanan.J, Shajan A, Joshi NH, Varatharajan R and Valliappan K., *International journal of Chemical & Pharmaceutical Sciences*, **2010**,Dec.; Vol.1 (2)
[15] ICH Q2B, March **1996**
[16] Nandini R. Pai and Deeptaunshu Atul Pusalkar *Der Pharmacia Sinica*, **2012**, 3 (5):526-535
[17] Nandini R. Pai and Deeptaunshu Atul Pusalkar *Der Pharmacia Sinica*, **2012**, 3 (5):526-535
[18] Nandini R. Pai and Swapnali Suhas Patil *Der Pharmacia Sinica*, **2013**, 4(2):76-84
[19] Amir Khan, *Der Chemica Sinica*, **2011**, 2 (2):211-222
[20] Srikanth Inturi, *Der Pharmacia Sinica*, **2011**, 2(5):223-234
[21] Amir Khan, *Der Pharmacia Sinica*, **2012**, 3(4):443-456
[22] Abdul Althaf. S, *Der Pharmacia Sinica*, **2011**, 2 (3):1-19
[23] Naresh Adepu, *Der Pharmacia Sinica*, **2010**, 1 (1): 116-123
[24] Ediga Sasikiran Goud, *Der Pharmacia Sinica*, **2013**, 4(2):33-46
[25] Mohd. Nayeemullah Khan, *Der Pharmacia Sinica*, **2012**, 3(2):177-184