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Assay method development and validation for simultaneous estimation of duloxetine and methylcobalamin in capsules by RP-HPLC

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ABSTRACT

The main objective of this work is to develop a simple, fast, accurate, precise, rugged and linear Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for simultaneous quantitative estimation of Duloxetine and Methylcobalamin in capsules and validate as per ICH guidelines. The optimized method uses a reverse phase column, Phenomenex Luna C18 (250 X 4.6 mm;5µ), a mobile phase of triethylammonium phosphate buffer (pH 3.5):acetonitrile in the proportion of 40:60 v/v, flow rate of 0.9ml/min and a detection wavelength of 260 nm using a UV detector. The developed method resulted in Duloxetine eluting at 3.48min and Methylcobalamin at 2.19min, having a run time of 5minutes. Duloxetine exhibited linearity in the range 100-300µg/ml, while Methylcobalamin exhibited linearity in the range 5-15µg/ml. % Relative standard deviations of system, intra day and ruggedness were found to be less than 2 for both the drugs. Percentage Mean recoveries were found to be in the range of 90-110, during accuracy studies by absolute method. A simple, fast, accurate, precise, linear and rugged RP-HPLC method was developed for simultaneous quantitative estimation of Duloxetine and Methylcobalamin in capsules and validated as per ICH guidelines. Hence it can be used for the routine analysis of Duloxetine and Methylcobalamin in capsules in various pharmaceutical industries.

Keywords: RP-HPLC, Duloxetine, Methylcobalamin, method development, validation.

INTRODUCTION

Duloxetine hydrochloride (**Figure 1**) is a selective serotonin and norepinephrine reuptake inhibitor (SSNRI). IUPAC name of Duloxetine is ((3S)-N-Methyl-3-naphthalen-1-yloxy-3-thiophen-2-ylpropan-1-amine). This mainly used for the treatment of depression, anxiety and pain associated with diabetic peripheral neuropathy or fibromyalgia [1-4].

Methylcobalamin (**Figure 2**) IUPAC name is Coα-[α-(5,6-dimethylbenz-1H-imidazolyl)]-Coβmethylcobamide. It is used in the treatment of trigeminal neuralgia, megaloplastic anemia, diabetic neuropathy and facial paralysis in Bell's palsy syndrome. The combined dosage forms of these drugs are used for the treatment of neuropathic pain associated with peripheral neuropathy especially diabetic polyneuropathy [1-4].

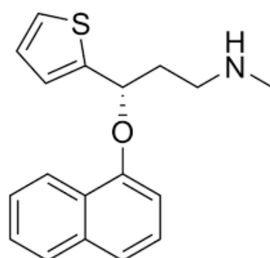


Fig. 1: Structure of Duloxetine

A detailed literature survey reveals that there exists literature on assay methods by using UV spectroscopy [2-4] and UPLC [1], while there is hardly any literature reported on simultaneous quantitative estimation of Duloxetine and Mecobalamin in formulation by RP-HPLC. In addition the combination of these two drugs is not official in US pharmacopeia. Accordingly, we here report a new and a rapid RP-HPLC validated method for the simultaneous quantitative estimation of Duloxetine and Methylcobalamin in capsules using triethylammonium phosphate buffer (pH 3.5) as per ICH guidelines.

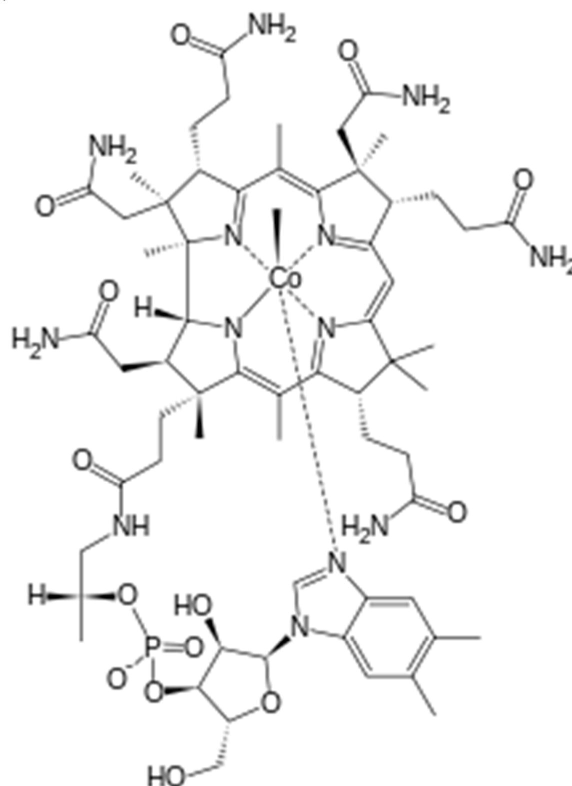


Fig. 2: Structure of Methylcobalamin

MATERIALS AND METHODS

Chemicals and Reagents

Analytically pure sample of Duloxetine and Methylcobalamin with purities greater than 95% were obtained as gift samples from Chandra Labs, Hyderabad, India and tablet formulation [DUZELA M] was procured from Medplus pharmacy, Hyderabad, India with labelled amount 30mg and 1.5mg of Duloxetine and Methylcobalamin respectively. Acetonitrile (HPLC grade) was obtained from Sigma aldrich (Hyderabad, India), water (HPLC grade), Triethylamine (AR grade), ortho phosphoric acid (AR Grade) were obtained from SD Fine chemicals (Hyderabad, India), 0.22 and 0.45µm Nylon membrane filters were obtained from Spincotech Private Limited, Hyderabad, India.

Instrument

HPLC analysis was performed on Shimadzu LC-20AD Prominence Liquid Chromatograph comprising a LC-20AD pump, Shimadzu SPD-20A Prominence UV-VISIBLE detector and a reverse phase C18 column, Phenomenex Luna (250 X 4.6 mm; 5µ). A manually operating Rheodyne injector with 20 µL sample loop was equipped with the HPLC system. The HPLC system was controlled with “Lab solutions lite” software. A double beam UV-visible spectrophotometer (Shimadzu, model UV-1800) having two matched quartz cells with 1 cm light path and loaded with UV probe software (version 2.41) was used for recording of spectra and measuring absorbance. An electronic analytical weighing balance (0.1mg sensitivity, Shimadzu AY 220), digital pH meter (DELUX model 101), a sonicator (sonica, model 2200 MH).

Method

Selection of Wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of Methylcobalamin and Duloxetine. Suitable wavelength selected for simultaneous estimation is 260nm (**Figures 3-4**).

Fig. 3: UV spectrum of standard Duloxetine

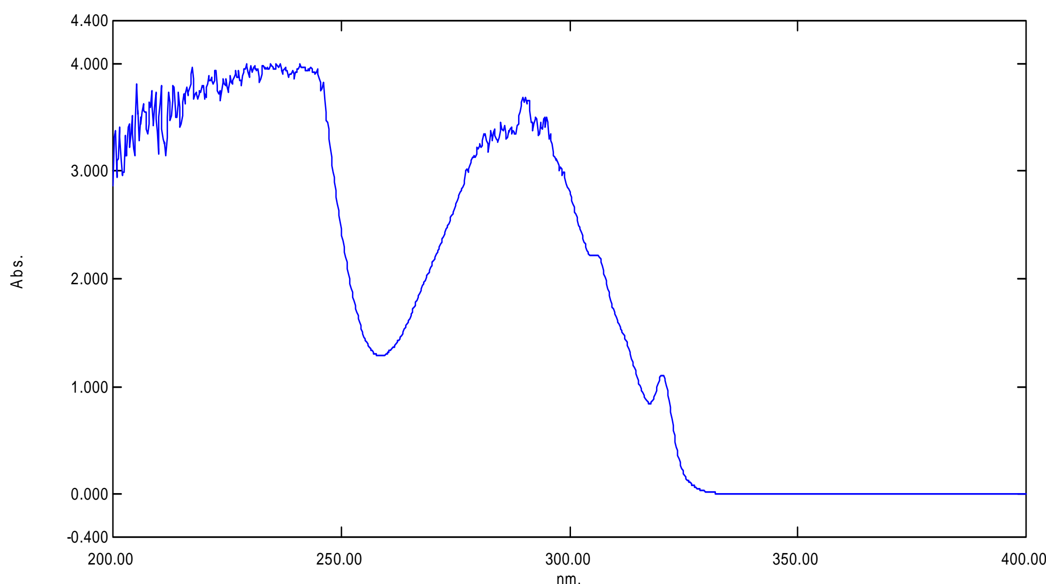
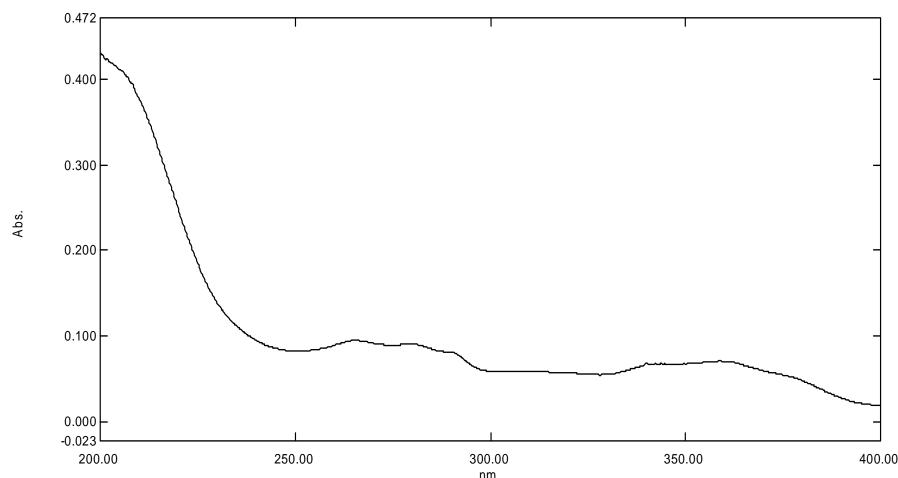


Fig. 4: UV spectrum of standard Methylcobalamin



Chromatographic conditions

The developed method uses a reverse phase C18 column, Phenomenex Luna C18 (250 X 4.6 mm; 5μ), mobile phase of triethylammonium phosphate buffer (pH 3.5):acetonitrile in the proportion of 40:60 v/v. The mobile phase was set at a flow rate of 0.9 ml/min and the volume injected was 20μl for every injection. The detection wavelength was set at 260nm.

Buffer Preparation

The buffer solution was prepared by adding 5 ml of triethylamine to 1000 ml of HPLC grade water and later pH was adjusted to 3.5 using 30% v/v of ortho phosphoric acid in water. The buffer was filtered through 0.45μ filter to remove all fine particles.

Mobile phase Preparation

The mobile phase was prepared by mixing buffer and acetonitrile in the ratio of 40:60 v/v and later it was sonicated for 10 minutes for the removal of air bubbles.

Diluent

Diluent used is the mobile phase itself.

Preparation of mixed standards solution

Weigh accurately 10mg of Methylcobalamin and 200 mg of Duloxetine in 100 ml of volumetric flask (covered with aluminium foil as Mecobalamin is light sensitive) and dissolve in 80ml of mobile phase and make up the volume

with mobile phase. This solution contains 100µg/ml of Methylcobalamin and 2000µg/ml of Duloxetine. 1ml was pipetted out from this stock solution and made up to 10ml using mobile phase, to get 10µg/ml of Methylcobalamin and 200µg/ml of Duloxetine, treated as mixed working standards solution, 100% target concentration.

Preparation of sample solution

10capsules were emptied and total weight was taken. Later total contents were taken into a mortar, crushed and then uniformly mixed. Powder equivalent to 10mg of Mecobalamin and 200mg was transferred to 100ml volumetric flask) (covered with aluminium foil as Mecobalamin is light sensitive containing 70ml of mobile phase. This suspension was subjected to intermittent shaking with sonication for 5 minutes and later made the solution up to 100ml mark using the mobile phase. This solution was filtered using 0.22micron syringe filter. Filtrate working sample solution is equivalent to a concentration of 10µg/ml for Methylcobalamin and 200µg/ml for Duloxetine, concentrations equal to 100% target concentration of mixture of standards.

RESULTS AND DISCUSSION

Method Development

A Reverse phase HPLC method was developed keeping in mind the system suitability parameters i.e. resolution factor (Rs) between peaks, Peak Asymmetry (A), number of theoretical plates (N), runtime and the cost effectiveness. The optimized method developed resulted in the elution of Methylcobalamin at 2.19min and Duloxetine at 3.48min. **Figures 5 and 6** represent chromatograms of mixture of standard solutions and sample solution respectively. The total run time is 5 minutes. System suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (*RT*), number of theoretical plates (*N*), peak resolution (Rs) and Tailing factor (T) were evaluated for six replicate injections of the standards at working concentration. System suitability parameters for mixture of standard solutions and sample solution are given in Tables 1 and 2. All the parameters were well within the acceptance criteria.

Fig. 5: Typical chromatogram of mixture of standard solutions

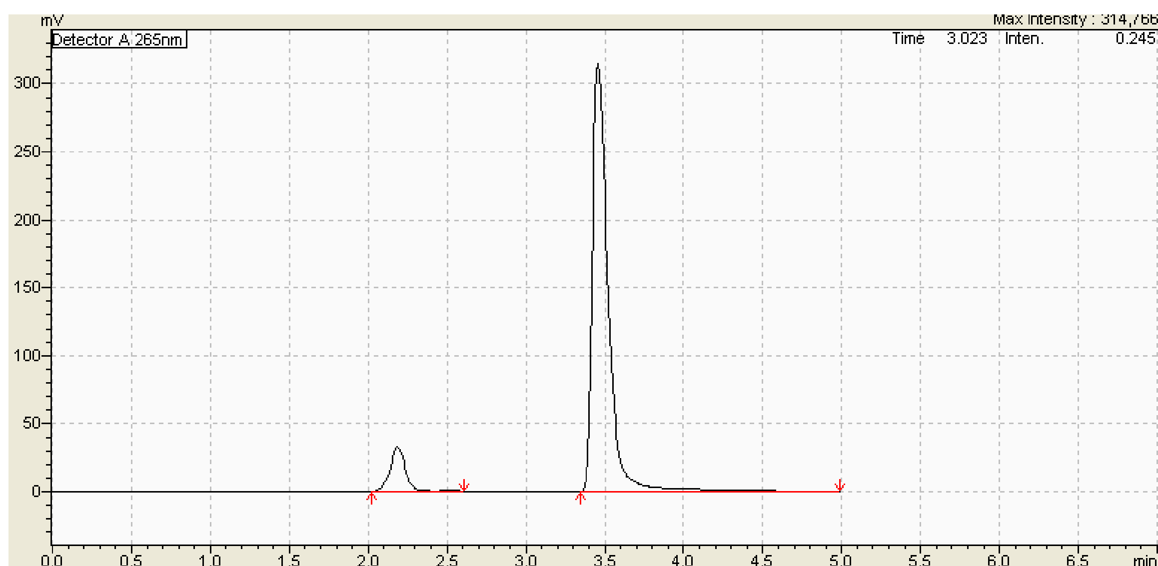


Table 1: System suitability studies results for mixture of standards

Parameters	Acceptance Limits	Methylcobalamin	Duloxetine
Retention time (min)	-	2.194	3.485
Resolution factor (Rs)	Not less Than 2	-	6.939
Number Of Theoretical plates (N)	Not less Than 2000	2204	5601
Tailing factor (T)	Not More Than 2	1.020	1.634

Fig. 6: Typical chromatogram of sample solution

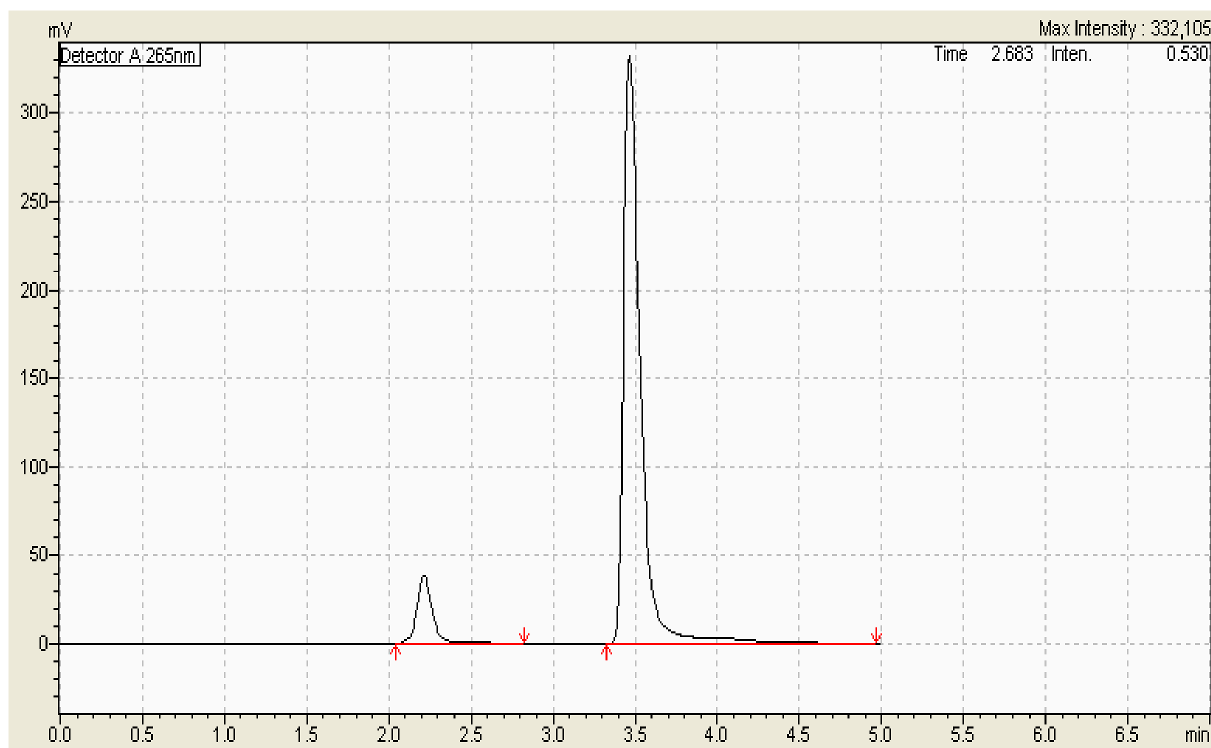


Table 2: System suitability studies results for sample

Parameters	Acceptance Limits	Methylcobalamin	Duloxetine
Retention time (min)	-	2.21	3.465
Resolution factor (Rs)	Not less Than 2	-	6.943
Number Of Theoretical plates (N)	Not less Than 2000	2654	5323
Tailing factor (T)	Not More Than 2	1.147	1.718

In order to test the applicability of the developed method to a commercial formulation, 'DUZELA M' formulation was chromatographed at working concentration and it is shown in **Figure 6**. The sample peaks were identified by comparing the relative retention times with the standard solutions (**Figures 5-6**). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample. Integration of separated peak area was done and each drug concentration was determined by using the peak area concentration relationship obtained in the standardization step. The protocol affords reproducible quantification of the two drugs with error less than 10%, which is the standard level in any pharmaceutical quality control.

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. HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines [5] for validation of analytical procedures. The method was validated for the parameters like specificity, linearity, accuracy, system precision, intra-day precision and Ruggedness.

Precision

System precision

Six replicate injections of the mixture of standards solution at working concentration showed % RSD (Relative Standard Deviation) less than 2 concerning peak area for both the drugs, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in **Table 3**.

Method precision

Method precision was determined by performing assay of sample under the test of repeatability (Intra day precision) at working concentrations.

Repeatability (Intra day precision)
Six consecutive injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning % assay for both the drugs which indicate that the method developed is method precise by the test of repeatability and hence can be understood that the method gives consistently reproducible results (**Table 4**).

Table 3: System precision results of Duloxetine and Methylcobalamin

n	Mecobalamin	Duloxetine
1	230550	2179758
2	231057	2172126
3	233111	2206233
4	224039	2189605
5	227008	2223782
Average	229153	2194301
Std dev	3605.43	20837.37
% RSD	**1.57**	**0.94**

Linearity
Standard solutions of Duloxetine and Methylcobalamin at different concentrations were prepared. Calibration curves (**Figures 7 and 8**) were constructed by plotting the percentage concentration level versus corresponding peak area for both the drugs. The results show an excellent correlation between peak areas and concentration within the concentration range of 5-15µg/ml for Methylcobalamin and 100-300µg/ml for Duloxetine (**Tables 5 and 6**). The correlation coefficients were greater than 0.995 for both the drugs, which meet the method validation acceptance criteria and hence the method is said to be linear for both the drugs.

Table 4: Intra day precision results of Duloxetine and Methylcobalamin

n	%ASSAY	
	Mecobalamin	Duloxetine
1	108.78	106.67
2	107.81	104.03
3	105.55	105.75
4	103.78	105.03
5	108.3	107.76
6	106.46	102.62
Average	106.78	105.31
Std dev	1.89	1.84
% RSD	**1.77**	**1.75**

Table 5: Calibration data for Duloxetine

% Level	Concentration (µg/ml)	Peak area
50	100	1081456
75	150	1648954
100	200	2168970
125	250	2654643
150	300	3357126
Regression equation		y=11114.06x-40581.8
Regression coefficient		0.996

Table 6: Calibration data for Methylcobalamin

% Level	Concentration (µg/ml)	Peak area
50	5	129022
75	7.5	193836
100	10	259419
125	12.5	316328
150	15	394128
Regression equation		y=26108.16x-2535
Regression coefficient		0.998

Fig.7: Linearity graph of Duloxetine

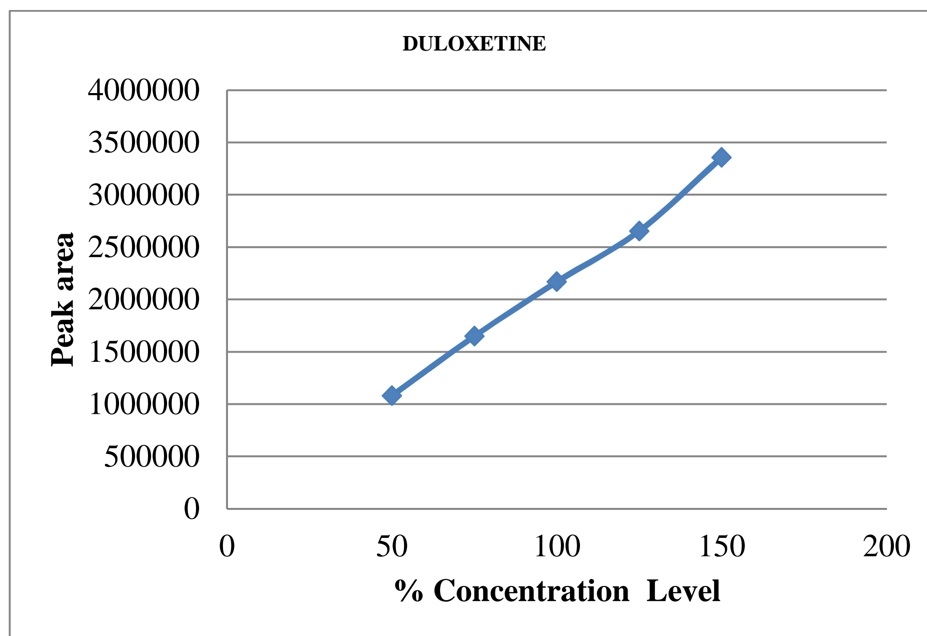


Fig.8: Linearity graph of Methylcobalamin

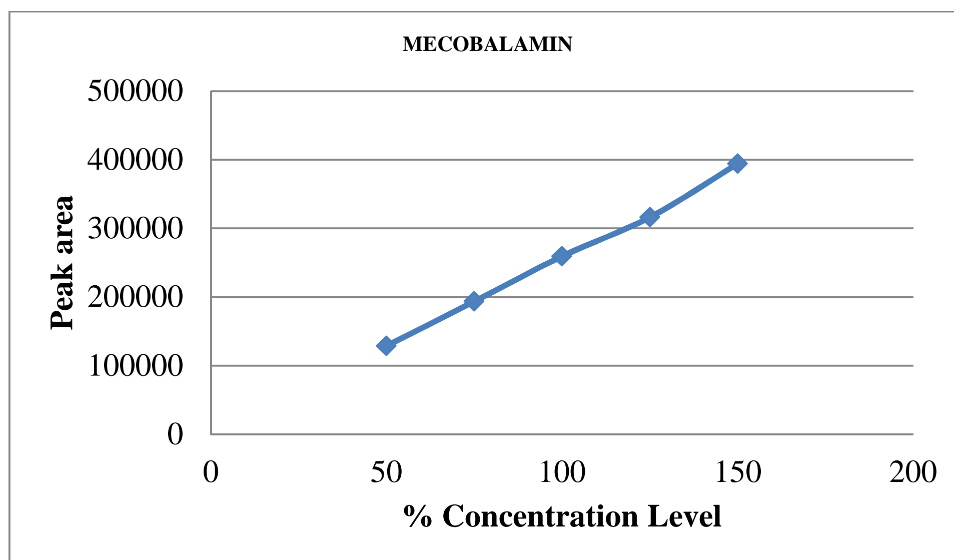


Table 7: Results of Accuracy studies for Duloxetine

LEVEL (%)	% Recovery	% Mean Recovery	%RSD
50	100.072	101.56	0.99
50	102.04		
50	101.35		
100	99.24	100.12	1.27
100	99.55		
100	101.59		
150	104.87	103.81	0.92
150	103.01		
150	103.56		

Accuracy
Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of both the drugs at three different levels (50-150%). At each level, three determinations were performed. Percent mean recovery is calculated as shown in **Tables 7 and 8**. The accepted limits of mean recovery are 90% -110% by

absolute method and all observed data were within the required range, which indicates good recovery values and hence the accuracy of the method developed.

Table 8: Results of Accuracy studies for Mecobalamin

LEVEL (%)	% Recovery	% Mean Recovery	% RSD
50	106.99	105.65	1.47
50	103.94		
50	106.03		
100	95.41	96.46	1.10
100	96.44		
100	97.54		
150	107.58	106.98	0.98
150	107.58		
150	105.76		

Ruggedness

Ruggedness was evaluated by performing assay of the formulations on different day by different analyst by injecting six consecutive injections of the sample at working concentration from the same homogeneous mixture of capsules. This study showed % RSD less than 2 concerning % assay for both the drugs which indicate that the method developed is rugged and hence can be understood that the method gives reproducible results irrespective of day and analyst (**Table 9**).

Table 9: Ruggedness results of Duloxetine and Methylcobalamin

n	Mecobalamin	Duloxetine
1	95.34	105.85
2	93.46	110.85
3	93.43	107.91
4	92.13	108.19
5	95.39	105.95
6	94.16	108.43
Average	93.98	107.86
Std dev	1.25	1.84
% RSD	1.33	1.71

Specificity

Figures 5-6 for mixture of standards drug solutions and sample solution chromatogram reveal that the peaks obtained in the standards solution and sample solution at working concentrations are only because of the drugs as blank had no peak at the retention time of Methylcobalamin and Duloxetine.. Accordingly it can be concluded that, the method developed is said to be specific for the analytes of interest.

CONCLUSION

A reverse phase HPLC isocratic method developed has been validated as per ICH guidelines in terms of specificity, accuracy, system precision, intra day precision, linearity and ruggedness for simultaneous quantitative estimation of Duloxetine and Methylcobalamin in DUZELA M capsules. The developed method resulted in Duloxetine eluting at 3.48min and Methylcobalamin at 2.19min. Duloxetine exhibited linearity in the range 100-300µg/ml, while Methylcobalamin exhibited linearity in the range 5-15µg/ml. % Relative standard deviations of system, intra day and ruggedness were found to be less than 2 for both the drugs. Percentage Mean recoveries were found to be in the range of 90-110, during accuracy studies by absolute method.

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