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# RP-HPLC Method Development and Validation for Simultaneous Estimation of Duloxetin and Methylcobalamine in Combined Dosage Form

**K. Lavanya***, N.V.N. Koteswara Rao, V. Srinivas Rao and K. Chinna Venkanna

*Department of Pharmaceutical Analysis, Lydia College of Pharmacy, Ravulapalem, Andhra Pradesh, India*

## Abstract

A simple, precise, accurate, simultaneous stability indicating RP-HPLC method for the estimation of DLU (Duloxetin) and MCB (Methylcobalamine) in combined dosage form was developed using Intersil-C18 (4.6 x 250mm, 5µm) in an Isocratic mode with mobile phase comprising of Phosphate buffer (pH 4.5) The flow rate was 1 mL/ min and effluent was monitored at 255.0 nm. The retention times were found to be 5.32 min for DLU and 3.59 min for MCB. The assay exhibited a linear dynamic range of 20- 120 µg/mL for DLU and 10- 60 µg/mL for MCB. The calibration curves were linear ($r^2$ = 0.999 for DLU and $r^2$ = 0.999 for MCB) over the entire linear range. Mean % recovery was found to be 99.68 % for DLU and 100.3 % for MCB with % RSD was NMT 2 for both estimations which fully agrees with system suitability which is in good agreement with labeled amount of formulation. The % RSD for Intra- Day & Inter-Day Precision was NMT than 2 for both the drugs. The developed method was validated as per ICH guidelines.

**Keywords:** DLU, MCB, RP-HPLC, Phosphate buffer, Method Validation.

## 1. Introduction

The technique High Performance Liquid Chromatography (HPLC) is so called because of its improved performance over the classical column chromatography. The technique basically involves the use of porous material as a stationary phase and the liquid mobile phase is pumped into the column under high pressure. The development of this technique is attributed to the small particle size of stationary phase. As the particle size is small the resistance to the flow of mobile phase is very high that is the reason why the high pressure is recommended.[1, 18] Analytical method development and validation are key elements of any pharmaceutical development program. HPLC analysis method is developed to identify, quantity or purifying compounds of interest. This technical brief will focus on development and validation activities as applied to drug products. Method validation is the process of proving that an analytical method is acceptable for its intended purpose. The parameters for method validation as defined by ICH (International Conference on Harmonization) guidelines are Accuracy, Precision, Specificity, Limit of Detection, Limit of Quantitation, Linearity, Range, Robustness and Ruggedness[2]. From the literature review [7-16] it has been found that only three analytical methods for the above combination have been reported. Therefore the attempt is made to develop simple, accurate, precise rapid and economical RP-HPLC method for determination of Duloxetin (DLU) and Methylcobalamine (MCB) in combine dosage form.

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].

**Figure 1: Chemical Structure of Duloxetin**

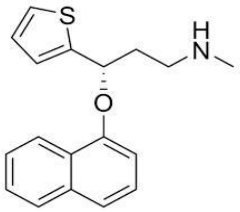
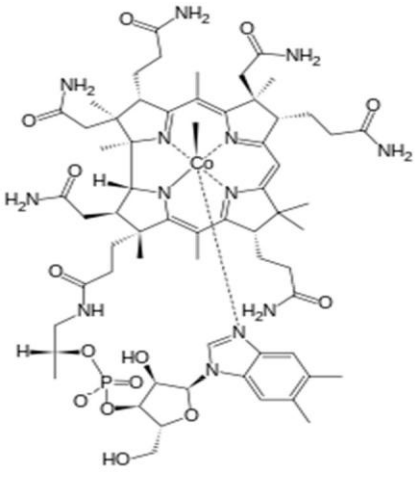



**Figure 2: Chemical Structure of Methyl cobalamine**




**Corresponding Author*:** tejharsha24@gmail.com

## 2. Experimental

### 2.1 Reagents & Chemicals

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, Rajahmundry, 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.

### 2.2 Instruments

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 so lutions 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).

### 2.3 Preparation of Standard Solutions

#### 2.3.1 Preparation of phosphate buffer 4.5:

Weighed 6.8 grams of $KH_2PO_4$ was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 4.6 with ortho phosphoric acid.

#### 2.3.2 Preparation of mobile phase:

A mixture of pH 4.6 Phosphate buffer 300 mL (30%), 700 mL of ACN (70%) are taken and degassed in ultrasonic water bath for 5 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration.

#### 2.3.3 Standard Stock Solution (A):

200mg of Duloxetine HCl working standard was accurately weighed and transferred into a 100ml clean dry volumetric flask and about 2ml of DMF is added. Then it is sonicated to dissolve it completely and made volume upto the mark with the diluant. Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted upto the mark with diluant.(10µg)

#### 2.3.4 Standard Stock Solution (B)

Accurately weighed quantity of MCB (10.0 mg) was transferred to 100 mL volumetric flask and dissolved in mobile phase. The volume was made up to mark with mobile phase to get final concentration of 200 µg/mL. The resultant solution was then sonicated for 10.0 min in ultrasonicator. ). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted upto the mark with diluant.(20µg)

### 2.4 Optimization of Mobile Phase and Chromatographic Conditions

#### 2.4.1 Procedure:

The chromatographic conditions were set as per the optimized parameters. The mobile phase was allowed to equilibrate with stationary phase as was indicated by a steady baseline. Solution (C) was injected in the Rheodyne injector (20.0 µl) and the respective chromatograms were recorded. Various mobile phases were tried by permutations and combinations and also by varying column, flow rate, column temperature and type of buffers with varying pH and solvents. The various mobile phases tried are as follows.

- **Trial 1** Water : Methanol (40: 60)
- **Trial 2** Buffer : Methanol (50: 50)
- **Trial 3** Buffer : Methanol (40: 60)
- **Trial 4** Buffer : Acetonitrile (40:60)
- **Trail 5** Buffer : Acetonitrile (30:70)

Above mentioned mobile phases were tried. The mobile phase containing Phosphate buffer: Acetonitrile (30: 70) at pH 4.5, injection volume- 20.0 µL flow rate of 1 mL/min was selected, due to its high resolving power, sensitivity and suitability, for the determination of DLU and MCB. The chromatogram is shown in **Figure.** Hence the following optimized chromatographic parameters were selected to carry out further experimentation.

| | |
|---|---|
| Column | Inertsil C18 (4.6 x 250mm, 5µm) |
| Mobile phase | Phosphate buffer: Acetonitrile (30:70) |
| pH | 4.5 (Using PDA) |
| Flow Rate | 1 mL/min |
| Wave length | 255.0 nm |
| Injection volume | 20.0 µL |
| Column temperature | Ambient |
| Run time | 10.0 min |

### 2.5 System Suitability Studies

System suitability is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be carried out. It is performed to ensure that the system is operating properly and read to deliver results with acceptable accuracy and precision. The tests were performed by collecting data from five replicate injections of standard solutions.

**Procedure:**

The chromatographic conditions were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady baseline. Five replicate injections of mixed working standard solution (C) were injected in to the system, the chromatograms were recorded for both the drugs and the results are shown in **Table 1 & 2.**

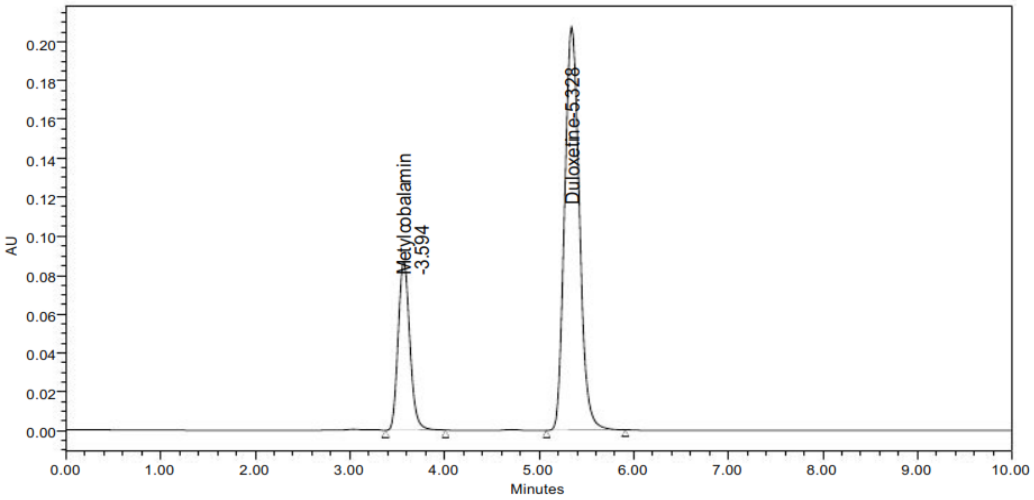



**Fig. 3: Typical chromatogram of mixture of standard solutions**

**2.6 Analysis of Marketed formulation**

**2.6.1 Preparation of Sample Solutions**

Twenty tablets were weighed accurately and crushed to fine powder. Each tablet contains 30 mg of Duloxetine and 1.5 mg of MCB. A quantity of powder equivalent to 200 mg of DLU and 10mg of MCB was weighed and dissolved in 25 mL of the mobile phase in a 100 mL volumetric flask. The volume was made up to give a concentration of 2000 µg/mL of DLU and 100 µg/mL of MCB. The solution was filtered through 0.45 µ nylon membrane filter. From this filtrate, different dilutions ranging from 20-120 µg/mL of DLU & 10-60µg/mL of MCB were prepared in 10 mL volumetric flasks with the mobile phase. 20 µL of each of these solutions were injected 5 times and the chromatograms were recorded. The amount of Duloxetine and Methylcobalamine present in each tablet formulation was calculated by comparing the peak area of the tablet solution with that of standard using the given formula:

Equal volume (20.0 µL) of standard and sample solution was injected separately after equilibrium of stationary phase. The chromatograms were recorded and the response i.e. peak area of major peaks were measured. The amount of drug in a Tablet was calculated using following formula

$$\%\ \text{Assay} = \frac{\text{sample Avg. Peak Area}}{\text{Standard Avg. Peak Area}} \text{x} \frac{\text{Wt. of Drug (mg)}}{\text{Dilution of Standard}} \text{x} \frac{\text{Dilution of Tablet Solution}}{\text{Wt. of Sample}} \text{x} \frac{\%\ \text{Purity Avg. Wt.}}{\text{Label Claim}} \text{x100}$$

**Table 1: System suitability test results**

| Sr. No | Parameters | DUL | MCB |
|---|---|---|---|
| 1 | Peak area (mV*min) | 2273955 | 754695 |
| 2 | No. of theoretical plates | 5081 | 4011 |
| 3 | Retention time (min) | 5.183 | 3.562 |
| 4 | Asymmetry | 1.14 | 1.15 |

## 3. Results and Discussion

The chromatographic conditions were optimised to develop RP-HPLC method for simultaneous determination of Duloxetin and Methylcobalamine with adequate resolution and rapid analysis time.

**3.1 Method Validation:**

The analytical method was developed and validated according to ICH guidelines. Analytical variable parameters such as linearity, precision, accuracy, specificity and system suitability were tested using the optimized chromatographic conditions and instruments.

**3.1.1 Linearity:**

Mixed standard stock solution was suitably diluted with the mobile phase to obtain the concentrations ranging from 20-120 µg/mL of DUL & 10-60 µg/mL of MCB.. The solutions were filtered through 0.45 µ nylon membrane filter paper and 20 µL of each of the solutions were injected and the chromatograms were recorded. A good linear relationship ($R^2$= 0.999 for Duloxetine and $R^2$= 0.998 for Methylcobalamine was observed between the concentrations of the drugs and their corresponding peak areas. The results of linearity studies are shown in Table 2.

**Table 2: Linearity study data for DLU and MCB**

| S.No. | Concentration of DUL | Peak Area of DUL | Concentration of MCB | Peak Areak of MCB |
|---|---|---|---|---|
| 1 | 20 ppm | 561876 | 10 ppm | 187098 |
| 2 | 40 ppm | 1116970 | 20 ppm | 370710 |
| 3 | 60 ppm | 1685203 | 30 ppm | 560397 |
| 4 | 80 ppm | 2237066 | 40 ppm | 744043 |
| 5 | 100 ppm | 2800383 | 50 ppm | 934181 |
| 6 | 120 ppm | 3371108 | 60 ppm | 1123745 |
| Correlation Coefficient 0.999 | | | | |

**Fig 4: Calibration curve of DUL**

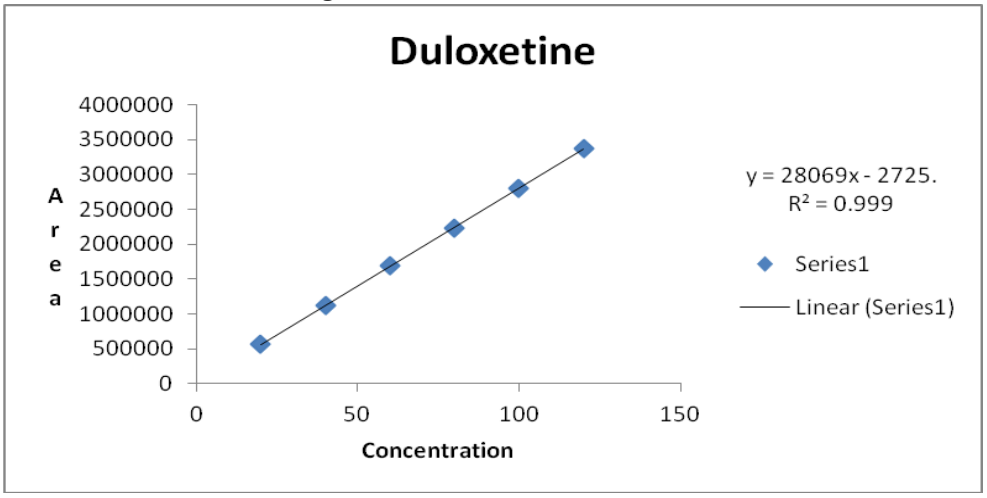
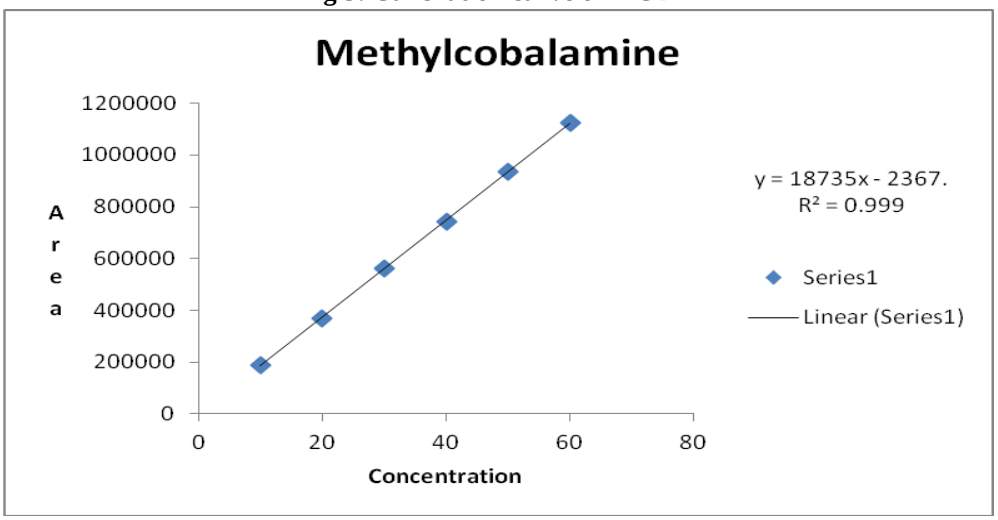



**Fig 5: Calibration curve of MCB**




**3.1.2 Accuracy:**

The accuracy studies were performed on 50%, 100 % and 150 % of the analytical method target concentrations of DUL and MCB. Standard and sample preparations were injected into HPLC system and three determinants for each concentration level were obtained.

The percentage recoveries of Duloxetine and Methylcobalamine were calculated using standard at the same concentration at each concentration level. The results are presented in Table 3& 4.

**Table 3: 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 | | |

**Table 4: Results of Accuracy studies for Methylcobalamin**

| 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 | | |

**3.1.3 Precision:**

**System Precision**:

System precision of the proposed method was checked by injecting five replicate preparations of the standard drug solutions of Duloxetine (60µg/ml) and Methylcobalamine (30µg/ml). The corresponding peak areas were measured and % RSD calculated as exhibited in Table 5.

**Table 5: System precision results of Duloxetine and Methylcobalamin**

| SI. No. | RT | Area | RT | Area |
|---|---|---|---|---|
| 1 | 5.351 | 2249906 | 3.576 | 750376 |
| 2 | 5.332 | 2249724 | 3.561 | 750874 |
| 3 | 5.326 | 2249452 | 3.597 | 751087 |
| 4 | 5.370 | 2249197 | 3.532 | 751477 |
| 5 | 5.337 | 2247457 | 3.542 | 751720 |
| 6 | 5.319 | 2247088 | 3.539 | 751365 |
| Mean | | 2248804 | | 751149.8 |
| Std.dev | | 1216.077 | | 481.0977 |
| %RSD | | 0.054077 | | 0.064048 |

**3.1.4 Specificity:**

The specificity of the proposed method was determined to check whether there is any interference due to presence of excipients, impurities or other components with the retention time of analytical peaks. The HPLC chromatograms were recorded for the drug-matrix (mixture of the drug and excipient) which showed almost no interfering peaks within retention time ranges indicating that the method is quite specific.

**3.1.5 Robustness:**

Robustness of the developed analytical method was tested by evaluating the affect of small variations in analytical method parameters such as change in flow rate of 1.2 mL/min by ±0.2 mL/min and change in wavelength by ±2 nm. The results are shown in Table 6.

**Table 6: Results of Robustness data**

| Parameter | Duloxetin | | Methylcobalamine | |
|---|---|---|---|---|
| | Theoretical plates | Tailing Factor | Theoretical plates | Tailing Factor |
| **Flow rate (0.8ml/min)** | 6118 | 1.14 | 4422 | 1.16 |
| **Flow rate(1ml/min)** | 5533 | 1.12 | 4019 | 1.13 |
| **Flow rate (1.2ml/min)** | 3593 | 1.16 | 3593 | 1.16 |
| **Organic composition (10% less)** | 5688 | 1.16 | 4004 | 1.15 |
| **Organic composition (Actual)** | 5533 | 1.12 | 4019 | 1.13 |
| **Organic composition (10% more)** | 5351 | 1.16 | 3814 | 1.18 |

## 4. Application of the method to commercial formulations

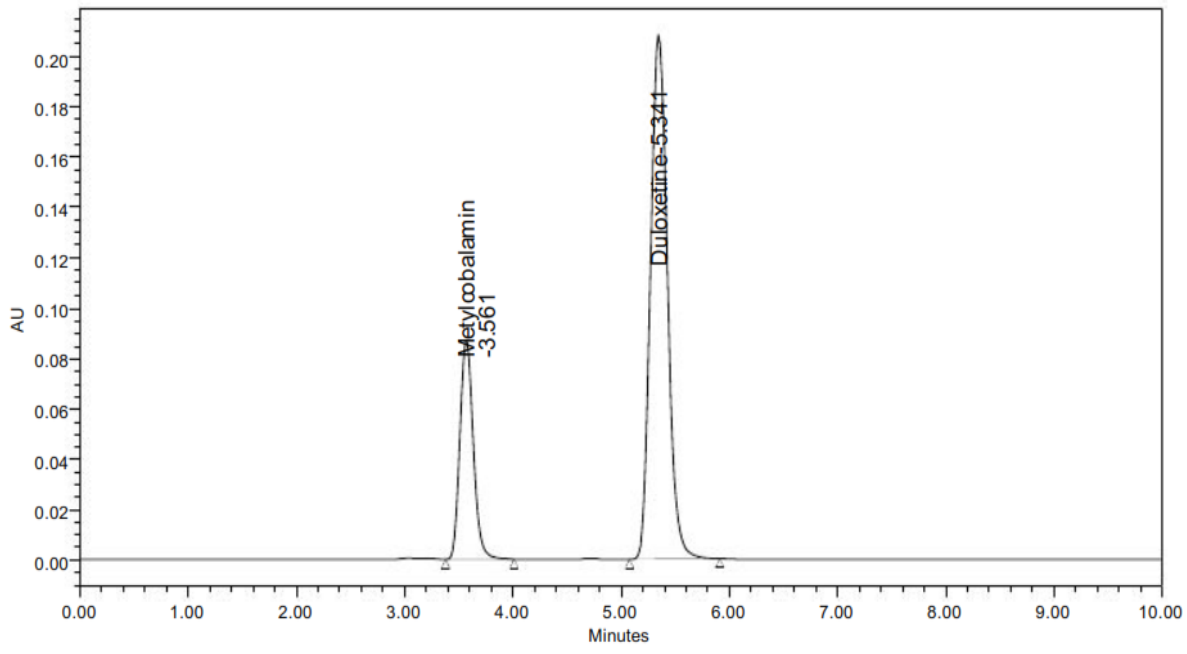
Twenty tablets were weighed accurately and crushed to fine powder. Each tablet contains 30 mg of Duloxetine and 1.5 mg of MCB. A quantity of powder equivalent to 200 mg of DLU and 10mg of MCB was weighed and dissolved in 25 mL of the mobile phase in a 100 mL volumetric flask. The volume was made up to give a concentration of 2000 µg/mL of DLU and 100 µg/mL of MCB. The solution was filtered through 0.45 µ nylon membrane filter. From this filtrate, different dilutions ranging from 20-120 µg/mL of DLU & 10-60µg/mL of MCB were prepared in 10 mL volumetric flasks with the mobile phase. 20 µL of each of these solutions were injected 5 times and the chromatograms were recorded. The amount of Duloxetine and Methylcobalamine present in each tablet formulation was calculated by comparing the peak area of the tablet solution with that of standard using the given formula.

$$\%\ \text{Assay} = \frac{\text{sample Avg. Peak Area}}{\text{Standard Avg. Peak Area}} \times \frac{\text{Wt. of Drug (mg)}}{\text{Dilution of Standard}} \times \frac{\text{Dilution of Tablet Solution}}{\text{Wt. of Sample}} \times \frac{\%\ \text{Purity Avg. Wt.}}{\text{Label Claim}} \times 100$$

**Table7: Results of Marketed Formulation Analysis**

| S. No. | Weight of std | | Weight of Sample (mg) | Peak area of | | Peak area of | | % Label | |
|---|---|---|---|---|---|---|---|---|---|
| | DUL | MCB | | DUL | MCB | DUL | MCB | DUL | MCB |
| 1 | 200 | 10 | 312.20 | 2255315 | 752297 | 2255474 | 752347 | 99.98 | 99.99 |
| 2 | | | 312.12 | | | 2255489 | 752115 | 99.99 | 100.02 |
| 3 | | | 312.35 | | | 2255290 | 752367 | 100.00 | 99.99 |
| | | | | | | | Mean | 99.99 | 100.00 |
| | | | | | | | S.D. | 0.01 | 0.02 |
| | | | | | | | %RSD | 0.01 | 0.02 |

**Fig 6: Typical HPLC Chromatogram corresponding to marketed formulation of DUL and MCB**



## 5. Conclusion

The proposed RP-HPLC method is simple, sensitive, reproducible, less time consuming and is applicable for analysis of Duloxetine and Methylcobalamine in bulk and in tablet dosage forms. The method was duly validated by evaluation of required parameters

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