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Stability Indicating Analytical Method Development and Validation of a RP-HPLC Assay Method for the Quantification of Milnacipran Hydrochloride in Bulk and Its Formulation

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Abstract: *The present study was focused to develop a novel stability-indicating reverse phase high pressure liquid chromatographic (RP-HPLC) assay method with its validation study, to quantify Milnacipran in bulk and its formulation. The chromatographic separation was carried out for the assay determination at a flow rate of 1.0 mL/min on a C_{18} Phenomenex Luna (250 x 4.6 mm x 5µm) column with a mobile phase proportion of water and Methanol in the ratio of 55:45 (% v/v) and was monitored at 254nm. The developed method was validated fulfilling the guidelines of ICH. Linearity of the method was established in between 10- 60µg/ml. The percent relative standard deviation for the precision studies was within 2%. Stability studies are carried for various conditions. The method was simple, precise, accurate, robust and rapid that can be utilized to quantify Milnacipran in bulk and its formulation. The ability to elude the Milnacipran under forced degradation conditions proves the stability indicating nature of the developed method.*

Keywords: *Method development, Milnacipran, Stability indicating*

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I. Introduction

Milnacipran, is a selective norepinephrine and serotonin reuptake inhibitor and inhibits norepinephrine uptake with greater potency than serotonin [1,2]. It is a racemic mixture with the chemical name (±)-[1R(S),2S(R)]-2-(amino methyl)-N,N-diethyl-11phenylcyclopropane carboxamide hydrochloride (Figure-1). Milnacipran can be used as antidepressant and also for the treatment of fibromyalgia [3-5].

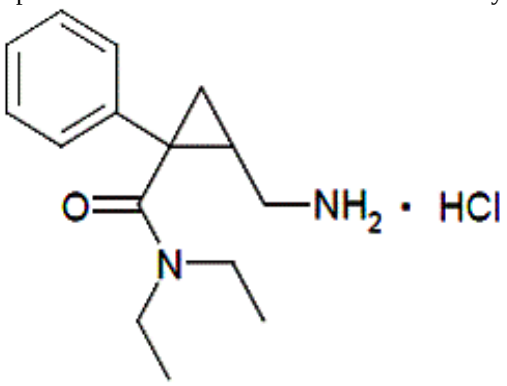

Fig 1: Structure of Milnacipran

A very few literature was reported by non-conventional techniques like colorimetric [6] and by HPTLC [7,8] in dosage forms. Most of the reported HPLC method was related to determination of Milnacipran in dosage forms [9-17] and few other chromatographic methods were reported using special detection technique of LCMS/MS [13-14] for estimation in plasma samples. In the purview of above discussions and based on non-availability of reliable and sensitive stability indicating RP-HPLC method for MIL the present attempt was made to develop Milnacipran by reversed phase HPLC method and to validate as required by International council for harmonization (ICHQ1) guidelines. Thus the developed method could be more appropriate in the quantification of MIL in presence as per stability testing protocol.

II. Materials and Methods

Instrument and chromatographic conditions

A Shimadzu (Kyoto, Japan) UFLC (LC-20AD) chromatographic system, the output signal was monitored and processed using LC solutions as chemstation, a UV/VIS PDA detector (SPD-20AT) and an LC-20AD solvent delivery module was used during the method development and validation. A Phenomenex Luna C18 (250 x 4.6 mm, 5 µm) column was used as stationary phase. Isocratic elution was used with a flow rate of 1.0 ml/min. An injection volume of 20 µl was used for the analysis and validation of MIL. The detection was carried at 254 nm.

Chemicals and reagents

Milnacipran(MIL) working standard was obtained from Perkin pharmaceuticals, India. HPLC grade methanol and AR grade of hydrochloric acid and hydrogen peroxide were purchased form SD fine chem. Ltd, Mumbai. HPLC grade water and AR grade Orthophosphoric acid was purchased from Merck, Mumbai and sodium hydroxide from Molychem, Mumbai.

III. Methods

Mobile phase Preparation

The mobile phase consisting of methanol and water in different proportions was tried. Before proceeding for analysis the mobile phase was degassed by use of a sonicator (spincotech pvt ltd.) and filtered through a 0.45 µm HPLC filtration assembly. The diluents used to attain the final concentration consist of a mixture of methanol and water.

Preparation of standard stock solution

25 mg of Milnacipran hydrochloride was accurately weighed and transferred in to a 25 ml volumetric flask. It was then dissolved in 15 ml of mobile phase and then made up to the volume with mobile phase and sonicated for 5 min (Stock solution). From this, a working standard solution of 100 µg/ml of strength was prepared. From the above stock solution by taking suitable aliquots 10- 60µg/ml solutions were prepared.

Preparation of Sample solution (Assay):

A quantity equivalent to 25 mg of drug was weighed and transferred into a 25 ml volumetric flask, 15 ml of diluent was added and sonicated for 10 min, with intermittent shaking and the volume is then made up to the mark with mobile phase and was marked as standard test solution. The resulting solution was thoroughly mixed and filtered through a 0.45 µm membrane filter prior to injection. From this various aliquots were prepared and used for the study.

Method development

A variety of mobile phases were investigated in the development of a stability indicating RP-HPLC method for the analysis of Milnacipran in tablet dosage form. The suitability of mobile phase was decided on the basis of selectivity and sensitivity of the assay, stability studies and separation among degraded products formed during forced degradation studies.

Method validation

The optimized chromatographic conditions were validated by evaluating system suitability, specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness parameters in accordance with the ICH guideline Q2 (R1) [20] Degradation of MIL was performed under neutral, acidic, basic, oxidative, thermal and photolytic stress conditions [21-23].

System Suitability

System suitability parameters were studied to verify the system performance. Six replicate samples containing Milnacipran [40 µg/ml] is analyzed using the developed method. Factors such as theoretical plate count, tailing factor, percent relative standard deviation (%RSD) of peak area and retention time were taken into consideration for testing system suitability.

Linearity

Standard stock solution of the drug was diluted to prepare linearity standard solutions in the concentration range of 10–60 µg/ml. For determination of the limits of detection and quantification based on the standard deviation of the response and slope as per ICH guidelines. The standards were injected and a calibration curve was plotted. Slope, intercept and coefficient of variation (r^2) of the calibration curve were calculated to ascertain linearity of the method.

Precision
Both interday and intraday precision of the proposed method was evaluated by carrying out six independent assays of test sample. Percent relative standard deviation [%RSD] of six assay values obtained was calculated.

Recovery (Accuracy)
Recovery of the method was determined by spiking the marketed sample with 50%, 100% and 150% standard solutions. These mixtures were analyzed by the proposed method. The experiment was performed in triplicate and recovery (%) of spiked drugs was determined.

Limit of detection and limit of quantification
The detection and quantification limits were evaluated from calibration curve plotted in concentration ranges of 10 – 60 µg/ml. The standard drug solutions, for each value of LOD and LOQ concentration were injected 6 times.

Robustness
The robustness was studied by evaluating the effect of small but deliberate variations in the chromatographic conditions. The conditions studied were flow rate (altered by $\pm 0.1 mL min^{-1}$) and temperature ($\pm 5^{o}C$ variation). These chromatographic variations were evaluated for Milnacipran in a system suitability solution with respect to retention time RT and % assay of drugs.

Forced degradation study
Tablet dosage form was subjected to forced degradation studies. Intentional degradation was carried out by exposing 10mL of reference/test stock solution to 20mL of 0.1 N hydrochloric acid/sodium hydroxide for 24 hours. The solutions were withdrawn in a 10mL volumetric flask, allowed to attain room temperature and then neutralized with acid or base (if necessary). Oxidative degradation of sample solution was conducted by exposing equal volumes of standard/test stock solution and 3.0% hydrogen peroxide solution in a 10mL volumetric flask. The solution was allowed to attain ambient temperature and diluted to mark with water. The photolytic studies, sample in a petriplate was spread as a thin layer (1mm) and exposed to UV light (257 nm) in a photo-stability chamber for 10 days. The method analytical data were collected at a single wavelength of 254 nm. Additional PDA detector data were collected for the peak purity evaluation. An amount of reference sample equivalent to 10 mg of Milnacipran was transferred into 50ml RB flask, added 25 ml of distilled water and kept aside for 24 hours, then filtered the solution through 0.45 micron filter into a 50ml volumetric flask and made up to the mark with diluent. Then about 1.5 ml of filtrate was accurately transferred into 10ml volumetric flask and made up to the mark with mobile phase.

IV. Results and Discussion

The most important aspect in method development in liquid chromatography is achievement of sufficient resolution and reasonable analysis time. The choice of the method depends on factors such as the nature of the drug, the complexity of the sample and the intended use. In this study, the conditions were influenced by solubility, polarity, UV absorption and interference. Prior to chromatographic method development, the detection wavelength was determined by obtaining the UV spectra of solution of Milnacipran. The analyte show maxima absorbance between 254nm with slight variations in absorbance values, and hence the peak intensity. From the spectra obtained, a wavelength detection of 254 nm was chosen in order to achieve a good sensitivity for the determination of all the analytes as well as any other unknown degradation product in the bulk drug.The chromatographic separations of Milnacipran was investigated at 254 nm, using different mobile phases consisting of phosphate and orthophosphoric acid in combination with methanol and acetonitrile on C_{18} column. The elution of the MIL varied substantially with the chromatographic conditions examined. Finally, a mobile phase consisting of Water and Methanol 55:45 v/v on Phenomenex C18 (250x4.6mm, 5µ) offered a good peak shape of the analyte at 35°C. Under these conditions, and using a flow rate of 1.0 ml/min and a run time of 10 min, Milnacipran elutes at about 2.8 min. Figure 2 and 3 shows the chromatograms of Milnacipran in bulk and tablet formulation.

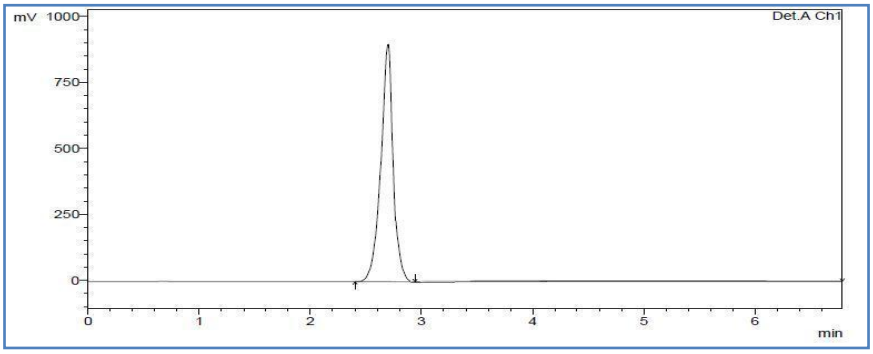

Fig 2: Typical chromatogram of standard

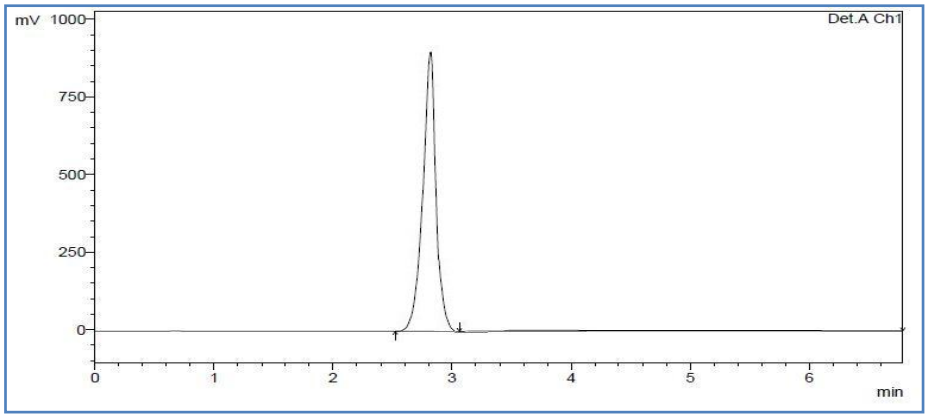

Fig 3: Typical chromatogram of formulation

Method Validation

The method was validated with respect to parameters including linearity, precision, accuracy (recovery), limit of quantitation (LOQ), limit of detection (LOD), selectivity and robustness.

System Suitability

The developed method has produced theoretical plate above 2000 for MIL with tailing factor less than 2. Similarly, the percent relative standard deviation [% RSD] of peak area and retention time was less than 2, which ensure the suitability of the developed method.

Linearity

The calibration curve constructed for MIL was linear over the concentration range of 10- 60µg/ml. Peak areas of MIL was plotted versus its concentration and linear regression analysis performed on the resultant curve. The correlation coefficients of $R^2 = 0.9994$, proves linear regression analysis. Typically, the regression equation for the calibration curve was found to be y = 72914x + 18720. The calibration curve was shown in Figure 4.

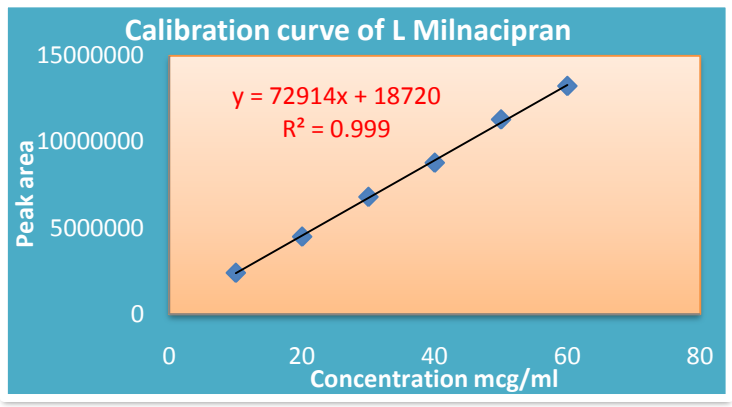

Fig 4: Calibration curve of Milnacipran

Precision

Precision of the assay was investigated with respect to both inter-day and intra-day precision. Inter-day precision was assessed by injecting the same concentrations over 3 consecutive days, and %R.S.D was found to be 0.78 and 1.82 respectively. The result of precision was shown in table 1. The method was successfully applied for the analysis of MIL in tablets for intra-day and inter-day assay respectively.

Table 1: Interday and Intraday precision results

S No	Name	Intra day	Interday
		Peak Area	Peak Area
1	Injection-1	8661987	8939875
2	Injection-2	8705468	8626484
3	Injection-3	8606897	8591678
4	Injection-4	8591928	8419785
5	Injection-5	8791928	8562131
6	Injection-6	8716485	8679862
Mean		8679116	8636636
Std Dev		68225.3	157333
% RSD		0.786086	1.821693

Accuracy

Accuracy of the method was determined by interpolation of replicate (n = 3) peak areas of three accuracy standards (20, 40 and 60µg/ml). In each case, the percent recovery was calculated. The mean % recoveries obtained for three triplicate levels for all three concentration levels was found as 101.44. Results of accuracy were shown in table 2.

Table 2: Results of Accuracy

Spike Level	Peak area (n=3)	Amount spiked (µg/ml)	Amount found (µg/ml)	% Amount recovered
50%	4204548	9.76	9.78	100.25
100%	8591078	39.04	39.99	102.42
150%	12789205	87.86	89.31	101.65

LOQ and LOD

The LOQ and LOD were determined based on signal-to-noise ratios and were determine using an analytical responses of 10 and 3 times the background noise, respectively. The LOD and LOQ of the method were found to be 3.08 and 9.35 µg/ml respectively.

Robustness

The method was found to be robust, as small but deliberate changes in the method parameters have no damaging effect on the method performance. The results of robustness were shown in table 3. As expected, the retention time of the analytes altered with increasing mobile phase flow rate and vice versa. Although flow rate and temperature has no adverse effect on the method since standard and samples would generally be analyzed concurrently and at the same flow rate and temperature for routine quality control analysis.

Table 3: Results of Robustness

Robust Parameters	Variation	System suitability Results	
		USP Plate Count	% RSD
Flow rate	0.9 $mLmin^{-1}$	2956	1.64
	1.0 $mLmin^{-1}$	3104	0.98
	1.1 $mLmin^{-1}$	2861	1.01
Temperature	30 ^{o}C	2865	1.46
	35 ^{o}C	3045	0.73
	40 ^{o}C	2968	1.48

Application of validated method for assay of MIL in pharmaceutical dosage form

Developed method was successfully implemented in the assay of MIL in pharmaceutical dosage form. Assay was found to be 99.88%. The method validation results were satisfactory as per ICH Q2R1 guidelines. The peak areas were found to be linear over the concentration range 10- 60µg/ml with a correlation coefficient of 0.999. Method specificity can be proved using "Peak purity" parameter in LC solution software of HPLC. The unaffected assay of the drug in the blend confirms the non-interference by any excipients. Intra-day and inter-day precision were less than 2%. Percent recovery in accuracy study was within the limit of 98 to 102%. The results of validation were summarised in table 4.

Table 4: Summary of validation parameters of the developed method

S.No.	Validation parameters	MIL
1	Linearity	10- 60µg/ml
	Regression equation	y = 72914x + 18720
	Correlation coefficient	0.999
2	Precision	
	Intraday precision	0.78
	Interday precision	1.82
3	Accuracy	101.44
4	LOD [µg/ml]	3.08
5	LOQ [µg/ml]	9.35
6	Robustness	Robust

Forced degradation studies

MIL was found to degrade significantly with peroxide and very mild degradation was observed in acid, base, photolytic, thermal and hydrolytic stress conditions. Figure 5 and 6 shows the chromatograms of degradation studies. Photodiode array detector was employed to check and ensure the homogeneity and purity of MIL peak in all the stressed sample solutions. Assay studies were carried out for stress samples against MIL qualified working standard. The results are presented in Table 5. The purity and assay of MIL was unaffected by the presence of its degradation products and thus confirms the stability-indicating power of the developed method.

Table 5: Results of forced degradation studies

Degradation condition	% Assay of MIL	% Degradation
Acid degradation	96.8	3.2
Base degradation	95.4	4.6
Peroxide degradation	89.9	10.1
photolytic degradation	94.6	5.4
Thermal degradation	93.9	6.1
Hydrolytic studies	94.6	5.4

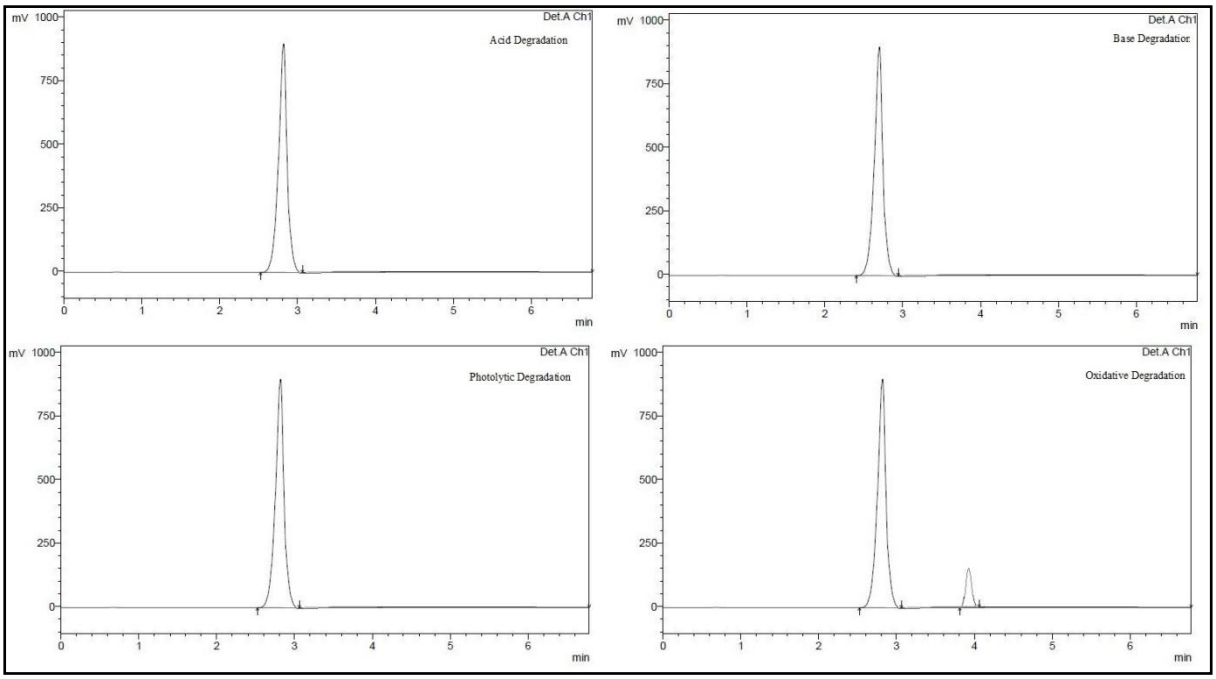

Fig 5: Typical chromatograms of MIL forced degradation samples of Acid, Base, Peroxide and Photolytic studies

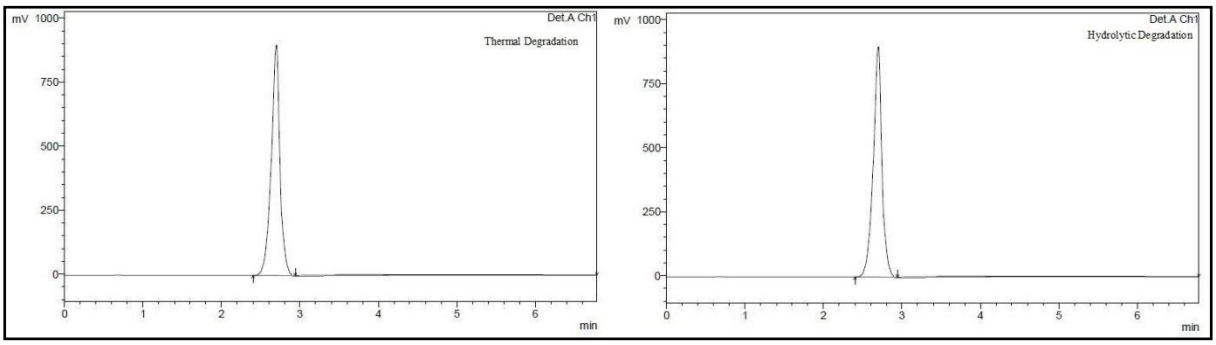

Fig 6: Typical chromatograms of MIL forced degradation samples of thermal and hydrolytic studies

V. Conclusion

An isocratic liquid chromatographic method has been described and validated for qualitative and quantitative determination of MIL in bulk and its formulation. The method shown acceptable assay precision (<2% RSD) and mean %Recovery obtained at 50-150% of the target analytical concentration was within limit. In addition to its high sensitivity and robustness, the proposed RP-HPLC method proved reliable for stability indicating analysis of MIL. Hence the proposed method can be used for routine quality control analysis of dosage forms and stability samples.

Acknowledgement

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