

1. INTRODUCTION:

Milnacipran is used in the treatment of fibromyalgia and major depressive disorder. Chemically it is [2- (aminomethyl)-N, N-diethyl-1-phenylcyclopropanecarboxamide] hydrochloride. Its structural formula is C₁₅H₂₂NO.HCl.

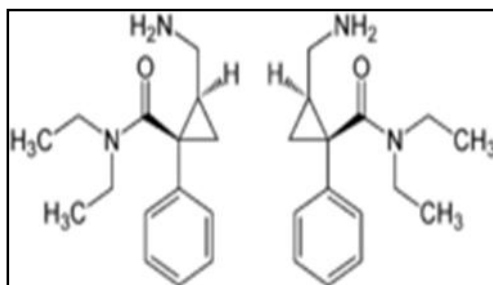


Figure-1: Structure of milnacipran

Milnacipran is a serotonin-norepinephrine reuptake inhibitor (SNRI). Milnacipran has unique pharmacokinetic and pharmacodynamic characteristics that distinguish it from the other marketed serotonin and norepinephrine reuptake inhibitors, venlafaxine, desvenlafaxine, and duloxetine such as equipotent serotonin and norepinephrine reuptake inhibition and a linear dose-concentration trend at therapeutic doses. It is beneficial for fibromyalgia syndrome and as anti-depression[1-7]. Many HPLC methods were described in literature for the chiral analysis of milnacipran by chiral and non-chiral method [8-9]. Few HPLC [10] method with spectrofluorimetric detection technique and LC-MS[11] method available for the determination of milnacipran in human plasma and serum respectively. HPLC and GCMS[12] method available for the , Pharmacokinetics study of milanacipran in renal impairment. Other HPLC, GC-MS, HPLC method also reported for electrophysiological effect of intravenous milnacipran, determination of milnacipran in plasma and in pharmaceutical formulation respectively[13-18].

2. LITERATURE REVIEW:

The literature review shows that various analytical methods were reported for its determination as API, pharmaceutical formulation. Brief details for the same are as under.

R. T. Owen, described efficacy, safety and tolerability profile of milnacipran hydrochloride in fibromyalgia syndrome. Milnacipran is a serotonin-norepinephrine reuptake inhibitor (SNRI), although its norepinephrine reuptake inhibition predominates. It has been marketed in Europe and Japan as an antidepressant for many years, but only

recently has it been investigated as a treatment for fibromyalgia syndrome (FMS). Both open-label and doubleblind, placebo-controlled trials have confirmed its efficacy in FMS, not only on the pain component but also on the fluctuating array of other symptoms such as sleep and cognitive disturbances and fatigue. The phase III trials have employed comprehensive composite endpoints to more accurately capture the many complex domains of FMS. Milnacipran's efficacy was not limited to the short-term, 12-week trials. It also demonstrated durability of response for up to 1 year. Its safety and tolerability were typical of its class, and it was generally well tolerated. Further work will be required to establish its place in FMS therapy by comparing it directly with other agents.

R. M. Gendreau, M. D. Thorn, J. F. Gendreau, *et al.*, described Efficacy of Milnacipran in Patients with Fibromyalgia. Fibromyalgia (FM) is a common musculoskeletal condition characterized by widespread pain, tenderness, and a variety of other somatic symptoms. Current treatments are modestly effective. Arguably, the best studied and most effective compounds are tricyclic antidepressants (TCA). Milnacipran, a nontricyclic compound that inhibits the reuptake of both serotonin and norepinephrine, may provide many of the beneficial effects of TCA with a superior side effect profile. **METHODS:** One hundred twenty-five patients with FM were randomly assigned in a 3:3:2 ratio to receive milnacipran twice daily, milnacipran once daily, or placebo for 3 months in a double-blind dose-escalation trial; 92% of twice-daily and 81% of once-daily participants achieved dose escalation to the target milnacipran dose of 200 mg. **RESULTS:** The primary endpoint was reduction of pain. Both the once- and twice-daily groups showed statistically significant improvements in pain, as well as improvements in global well being, fatigue, and other domains. Response rates for patients receiving milnacipran were equal in patients with and without comorbid depression, but placebo response rates were considerably higher in depressed patients, leading to significantly greater overall efficacy in the nondepressed group. **CONCLUSION:** In this Phase II study, milnacipran led to statistically significant improvements in pain and other symptoms of FM. The effect sizes were equal to those previously found with TCA, and the drug was generally well tolerated.

C. U. Pae, D. M. Marks, M. Shah, C. Han, B. J. Ham, A. A. Patkar and P. S. Masand, described role of milnacipran as an antidepressant. According to them milnacipran is a serotonin and norepinephrine reuptake inhibitor (SNRI) with negligible effects on any presynaptic or postsynaptic receptors. Milnacipran has unique

pharmacokinetic and pharmacodynamic characteristics that distinguish it from the other marketed serotonin and norepinephrine reuptake inhibitors, venlafaxine, desvenlafaxine, and duloxetine such as equipotent serotonin and norepinephrine reuptake inhibition and a linear dose-concentration trend at therapeutic doses. The half-life of milnacipran is approximately 8 hours. In addition, milnacipran does not inhibit the cytochrome P 450 system, indicating minimal propensity for drug-drug interactions. The antidepressant efficacy of milnacipran has been clearly established in a number of randomized, double-blind, placebo-controlled clinical trials, and it has been widely used for treating major depressive disorder. Moreover, evidence suggests that milnacipran is effective and tolerable in the treatment of fibromyalgia and may have usefulness for fatigue and anxiety symptoms. The current paper reviews researches conducted to date that is relevant to the efficacy, tolerability, and mechanism of action of milnacipran in the treatment of depression, fibromyalgia, and other psychiatric syndromes. Future directions of research are also identified.

M. Lecoœur-Lorin, R. Delépée, J. P. Ribet and P. J. Morin, described chiral analysis of milnacipran by a nonchiral HPLC circular dichroism. According to them, the CD detector records both dichroic () and UV () signals at the same wavelength and calculates the anisotropy factor ($g = \Delta\epsilon / \epsilon$), which is linearly related to the e.e. The enantiomeric and chemical composition of a chiral drug can be simultaneously determined on a nonchiral HPLC support. However, the g factor from the CD signal is temperature dependent. Indeed, the temperature has an influence on the stability of the CD signal and the linear regression between g factor and the e.e. of 1*R*,2*S*-enantiomer. So, a decrease in temperature gives rise to an improvement of the above-mentioned linearity correlation. After optimization of chromatographic parameters (porous graphitic carbon-based column, methanol/ phosphate buffer as mobile phase) and selection of CD wavelength, a linear regression of g factor *versus* e.e. of 1*R*,2*S*-enantiomer was obtained at temperature-controlled CD detection and an LOQ of 94% was found. The enantiomeric composition of milnacipran was determined with good accuracy.

A. Patti, S. Pedotti and C. Sanfilippo, described chiral HPLC analysis of milnacipran and Its FMOC-Derivative on cellulose-based stationary phases. The HPLC enantioseparation of the last generation antidepressive drug milnacipran (\pm)-**1** was investigated on different cellulose-based chiral stationary phases (CSPs). On carbamate-type columns, Chiralcel OD and OD-H (\pm)-**1** could be separated with α value about 1.20

but the resolution was quite low because of the tailing of the peaks. Direct determination of (\pm)-**1** with high selectivity and resolution was obtained on Chiralcel OJ in normal phase mode elution. Precolumn derivatization of milnacipran with Fmoc-Cl gave compound (\pm)-**2** which was enantioseparated on all the investigated CSPs and allowed enhanced UV or fluorimetric detection. The Chiralpak IB, that could be considered the immobilized version of Chiralcel OD-H, was found completely ineffective in the chiral recognition of (\pm)-**1** and moderately efficient in the separation of (\pm)-**2**.

C. Puozzo, C. Filaquier and G. J. Zorza, described determination of milnacipran, a serotonin and noradrenaline reuptake inhibitor, in human plasma using liquid chromatography with spectrofluorimetric detection. A sensitive high performance liquid chromatographic during the development method coupled with a fluorimetric detection was set up, validated and then used routinely of the drug. After liquid-liquid extraction, milnacipran and its internal standard were analyzed by reversed-phase liquid chromatography (LC). The drug was derivatized with fluorescamine for fluorescence detection. The identity of the liquid chromatography peaks was controlled using mass spectrometry. The assay linearity was validated up to 1000 ng/ml. The limit of quantification was set at 5 ng/ml. Precision values (relative standard deviations) were lower than 5.4%, whereas the mean accuracy was higher than 95%. The extraction recoveries were higher than 70% for both milnacipran and the internal standard. In clinics, the LC-fluorescence method was routinely used to investigate the pharmacokinetics of milnacipran in patients and proved to be robust and capable of quantifying milnacipran in plasma for at least 36 h (four- to five-fold the elimination half-life).

C. Takeshi, F. Yuji, et al., “Determination of Milnacipran in Serum by LC/MS, developed a method for the determination of milnacipran in serum using a system combining liquid chromatography and mass spectrometry (LC/MS) in which electrospray ionization (ESI) was used. Milnacipran was extracted from serum by liquid-liquid extraction using an Extrelut[®] NT3 column. The chromatographic separations of milnacipran and amoxapine (internal standard) were performed using a XTerra[®] RP 18 (150×2.1mm I.D.) column with a gradient of 0.1% formic acid and 0.1% formic acid in acetonitrile as the mobile phase. The extraction recoveries at concentrations of 10 and 100ng/mL were 85.6 and 83.7%, respectively. The calibration curve was linear in a concentration range from 5 to 300ng/mL and the detection limit was 1ng/mL. The intra-

day and inter-a day assay precision expressed as coefficients of variation (C.V.) were 1.94-2.17% and 5.75-6.20%, respectively. This method proved to be useful in quantifying milnacipran in serum obtained from a patient who took this drug.

C. Puozzo, N. Pozet, D. Deprez, P. Baille, H. L. Ung and P Zech, described pharmacokinetics of Milnacipran in Renal Impairment. The pharmacokinetics of a single 50 mg dose of milnacipran, a new non tricyclic antidepressant drug, were compared in 8 chronic renal failure subjects (Cl_c(reat) between 9 to 84.5 ml.min⁻¹) and in 6 healthy volunteers. Concentrations of unchanged (F2207 racemate and F2695 and F2696, enantiomers) and glucuroconjugated drug (main metabolite) were measured using HPLC and GC-MS. As for drugs mainly eliminated via renal route, the pharmacokinetics of milnacipran were markedly affected by impaired renal function with the elimination half-life of severely impaired subject being approximately three times that of the control group. Milnacipran apparent total clearance and renal clearance were positively correlated with glomerular filtration rate, while non-renal clearance and apparent volume of distribution were unaffected by renal impairment. Plasma concentrations of the glucuroconjugate were gradually increased in plasma, while its total urine excretion remained unchanged. As for the racemate, pharmacokinetics of each enantiomer were modified by renal failure, although, as predictable from its higher renal clearance value, it was more marked for F2696 than for F2695. Considering that modifications were shown to be proportional to the degree of renal impairment and that milnacipran presents low variability, the necessary dose adjustment is therefore easy to predict.

E. Uçaktürk and C. Safak, determined Milnacipran in Human Plasma Using GC-MS. MNP was efficiently derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) before analysis. The role of catalyst, temperature, time, solvent on the trimethylsilylation reaction were evaluated. The proposed method was fully validated by assessment of the following parameters: limits of detection and quantitation, precision, accuracy, linearity, specificity, stability, extraction recovery and robustness/ruggedness. The limit of quantitation (LOQ) was 30 ng mL⁻¹. The calibration curve was linear ($r^2 > 0.9988$) in the range 30–500 ng mL⁻¹. The method was found specific, precise, accurate, selective and reliable according to validation data. This developed method was successfully applied to determine the steady state concentration of MNP in patients.

M. Srinivasa Rao, V. Sivaramakrishna, S. V. M. Vardhan and D. Ramachandran explained validated Rp-Hplc method for the estimation of milnacipran in tablet dosage forms. The analyte was resolved by using a disodium hydrogen phosphate buffer 40mM containing 0.1% triethylamine (adjusted to pH 4.8 by orthophosphoric acid) and Methanol in the ratio of 75:25, v/v). On an isocratic HPLC system (PEAK) consisting UV lamp visible detector, ODS C-18, RP column (150 mm x 4.6 mm i.d., 5 μ m) at a wavelength of 210 nm. The linear dynamic range for Milnacipran was 2.0 μ g/mL – 12.0 μ g/mL. The limit of detection [LOD] and Limit of quantification [LOQ] for Milnacipran was 0.5 μ g/mL and 1.5 μ g/mL respectively.

S. P. Peketi, V. Jagathi et al. described Validated RP-HPLC method for analysis of milnacipran in bulk and formulations. Milnacipran was separated on an Inertsil ODS analytical column with 40:60 (v/v) mixture of methanol and octane sulfonic acid buffer (containing 0.05% w/v octane sulfonic acid with 0.025 % v/v trifluoro acetic acid) as mobile phase at a flow rate of 1.0 ml/min. The effluent was monitored by UV detector at 220 nm. Calibration plots were linear in the range of 10 to 50 μ g mL⁻¹, respectively. The high recovery and low relative standard deviation confirm the suitability of the method for routine quality control determination of milnacipran in capsules.

P. J. Mehta, D. M. Khatri, developed and Validated RP-HPLC Method for determination of milnacipran hydrochloride in pharmaceutical formulations. Where the mobile optimized was phosphate buffer : acetonitrile 72:28 v/v) with C18 column as stationary phase. The flow rate and detection wavelength was 1.0 mL/min and 220 nm respectively. The developed method was validated as per ICH guidelines for specificity, linearity and range, precision, accuracy, robustness, solution stability, limit of quantification and limit of detection and the results of all the validation parameters were well within their acceptance values also the degradation products formed during the different stress condition in specificity studies were separated from milnacipran hydrochloride and also from individual degradation products. The method gave good recovery in the range of 99.1 to 101.0 % of milnacipran hydrochloride when it was applied for its determination in pharmaceutical capsule formulations phase.

C. L. Dias, L. Bajerski et al. described comparative validation study to assay milnacipran hydrochloride in capsules by a stability-indicating LC and a Second Order Derivative UV Spectroscopic Methods. A selective stability-indicating LC method and a second order derivative UV spectroscopic method (UV-D²) were developed to assay

milnacipran in pharmaceutical formulation. The LC method was developed with a Nucleosil C8 analytical column and a mobile phase consisting of acetonitrile, water and triethylamine (at 210 nm). The validation of UV-D² (zero-crossing method) was based on recording the second-derivative spectra for milnacipran hydrochloride at 268.5 nm of its solutions in 0.1 N HCl and the parameters specificity, linearity, precision, and accuracy were evaluated to both methods. The linear dynamic range was 20–100 $\mu\text{g}\cdot\text{mL}^{-1}$ (R^2 0.999). The validation data showed that both methods are reproducible, providing an accurate (98.5% to 101.6%) and precise (RSD 1.0%) quantitation of milnacipran in capsules. The methods proposed showed satisfactory results and were statistically equivalent.

3. AIM OF PRESENT WORK:

The above literature review reveals that there were many methods for the quantitative analysis of milnacipran as a drug substance as well as pharmaceutical dosage form, few methods are there which deals with bionalytical study, spectroscopic study and stability study. The aim of present work is to developed a shortest and optimized method for quantitative analysis of milnacipran. The aim and scope of the proposed work are as under.

- To develop rapid RP-UPLC method for quantification of the drug substance with highest selectivity, precision and accuracy.
- Forced Degradation Study to confirm the stability of the drug substance.
- Perform analytical method validation for the proposed method as per ICH guideline[19].

4. METHOD DEVELOPMENT

4.1 Mobile phase and column selection:

Based on literature review and drug information, acetate and phosphate buffer, TFA were selected. Many experimental trials were performed using selected buffer to obtained best chromatographic condition for assay analysis of milnacipran. During experimental trails different column and organic solvent (i.e. methanol, acetonitrile etc.) used and at last accepted result obtained using following chromatographic condition.

Chromatographic condition:

- Buffer : 0.05% TFA in water
- Mobile phase : Buffer:Acetonitrile(76:24)
- Column : Aquity UPLC BEH C18, (100 x 2.1mm), 1.7 μ m
- Temperature : 30°C
- Flow rate : 0.4 mL/min
- Wavelength : 210 nm
- Injection volume : 1 μ L
- Runtime : 4 minute

5. ANALYTICAL METHOD VALIDATION:

Mobile Phase Preparation:

0.5 mL of Trifluoro acetic acid (TFA) added to 1 Ltr of HPLC grade water. Mix well and sonicate to degass.

Diluent Preparation:

Water: Acetonitrile (50:50) used as a diluents.

Blank Preparation:

Diluent is used as a blank

Standard Preparation:

To prepare a stock solution (500 μ g/mL) for assay analysis, weigh accurately about 50 mg milnacipran reference standard and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50 μ g/mL of milnacipran.

Test Preparation:

To prepare a stock solution (500 μ g/mL) for assay analysis, weigh accurately about 50 mg milnacipran sample and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50 µg/mL of milnacipran.

Procedure:

Inject blank followed by five replicated injection of standard preparation. Asymmetry of first injection of standard should not more than 2.0. Related standard deviation of replicate standard preparation should not more than 2.0 %. If system suitability pass than make duplicate injection of sample preparation.

5.1 Specificity Study:

The evaluation of the specificity of the method was determined against diluent and stress (forced) degradation. The specificity of the method toward the drug was established by means of the interference of the blank preparation and degradation products against drug during the forced degradation study.

Blank Preparation:

Diluent is used as a blank.

Standard Preparation:

To prepare a stock solution (500 µg/mL) for assay, weigh accurately about 50.10 mg milnacipran reference standard and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50.10 µg/mL of milnacipran.

Test Preparation:

To prepare a stock solution (500 µg/mL) for assay, weigh accurately about 50.14 mg milnacipran sample and transfer into 100 mL volumetric flask. Add 70 mL of diluents to dissolve the substance by sonication for one minute and dilute it up to the mark with diluents.

Pipette out 5 mL of above standard stock solution, transfer into 50 mL volumetric flask and dilute it up to the mark with diluent. The concentration obtained is 50.14 µg/mL of milnacipran.

5.1.1 Forced Degradation Study:

Stress study was carried out by application of chemical and physical forced degradation. To perform forced degradation study, the drug content 50 mg in 100mL volumetric flask was employed for acidic, alkaline and oxidant media and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content were allowed to equilibrate to room temperature and diluted with diluent to attain 500 µg/ml concentrations, further 5 mL sample solution diluted to 50mL to attained 50 µg/ml concentration of milnacipran. Pattern of stress (degradation) conditions and preparation for same was described as under:

Acid Degradation:

Acidic degradation study was performed by heating the drug content in 1 N HCl at about 80° C for 1 hour and after cooling to room temperature it was neutralized with 1 N NaOH solution. Further solution was diluted to achieve concentrations 50 µg/ml with diluent.

Alkali Degradation:

Alkaline degradation was performed by heating the drug content in 1 M NaOH at around 80° C for 1 hour and then the mixture was neutralized with 1 M HCl. It was further diluted with diluent to achieve 50 µg/ml concentrations.

Oxidative Degradation Study:

Oxidative degradation study was performed at room temperature for by adding 3% H₂O₂ in the drug content for 30 min then diluted to 50µg/ml with diluent.

Thermal degradation study was performing by keeping powdered drug content at around 80°C for 1 hour. After this it was allowed to come at room temperature.

Photolytic Degradation Study:

Photolytic degradation study was performed by exposing drug content in sun-light for 48 hour, further it diluted to 50 µg/ml using diluent.

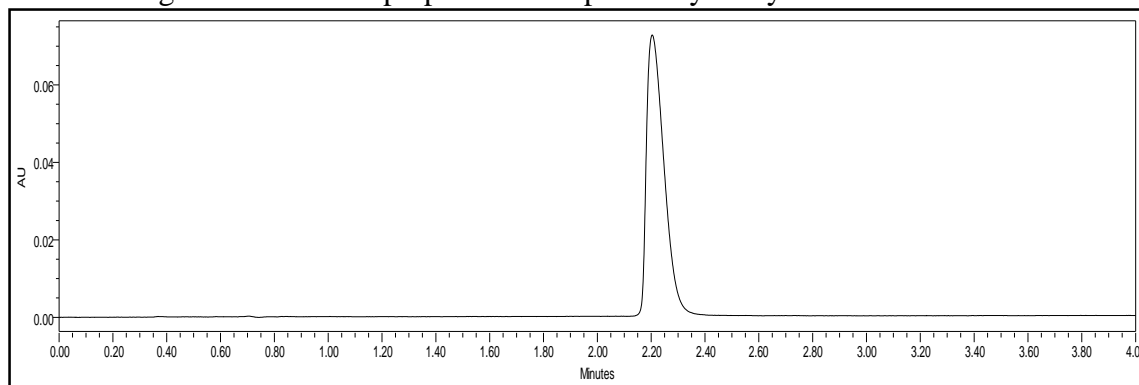
Blank preparation:

Blank preparation was also performed for all degradation to identify the peaks which arise due to blank. Blank was subjected under same all stress condition as that of sample.

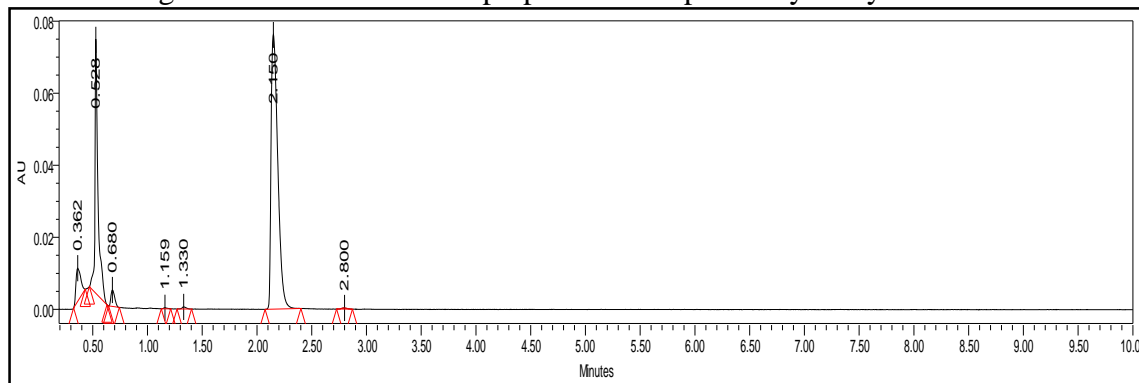
Table-1:Chromatographic sequence for Specificity study

No.	Description	Injection Replicate	Remarks
1	Blank	1	As such
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	Acidic Forced Degradation
5	Blank preparation of acidic stress	1	
6	Test preparation of acidic stress	2	
7	Bracketing Standard	1	Alkali Forced Degradation
8	Blank preparation of alkali stress	1	
9	Test preparation of alkali stress	2	
10	Bracketing Standard	1	Oxidative Forced Degradation
11	Blank preparation of oxidative stress	1	
12	Test preparation of oxidative stress	2	
13	Bracketing Standard	1	Thermal Forced Degradation
14	Blank preparation of thermal stress	1	
15	Test preparation of thermal stress	2	
16	Bracketing Standard	1	Thermal Forced Degradation
17	Blank preparation of photolytic stress	1	
18	Test preparation of photolytic stress	2	
19	Bracketing Standard	1	

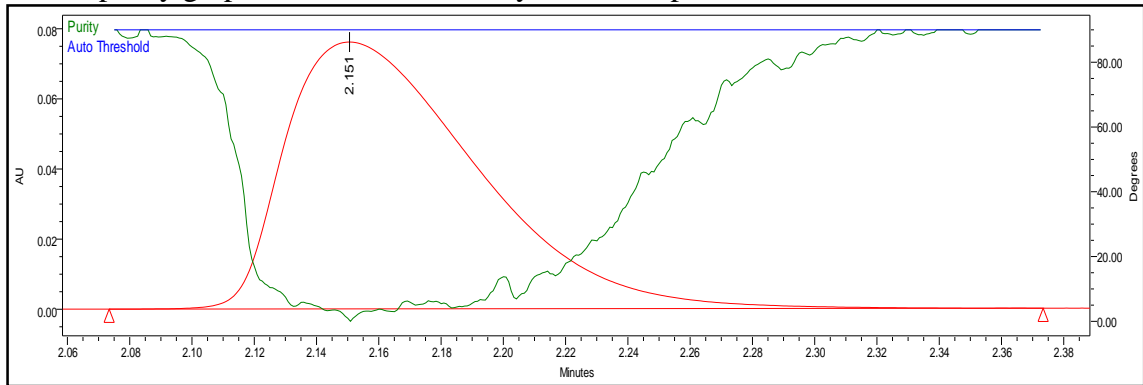
Chromatogram of standard preparation of specificity study:



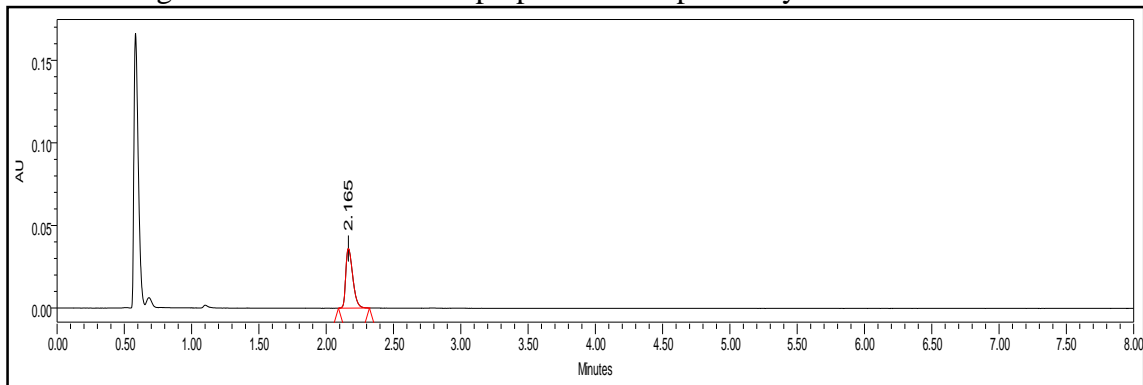
Chromatogram of acidic stress test preparation of specificity study:



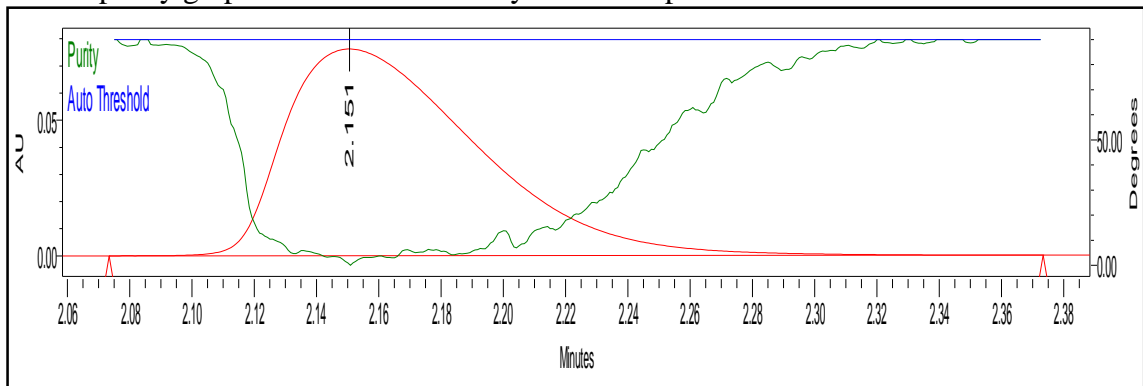
Peak purity graph of acidic stress study of milnacipran:



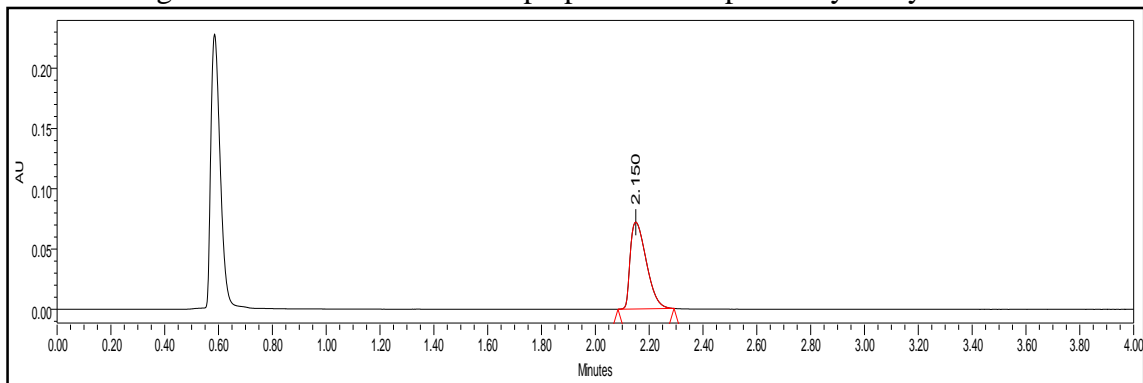
Chromatogram of alkali stress test preparation of specificity stud :



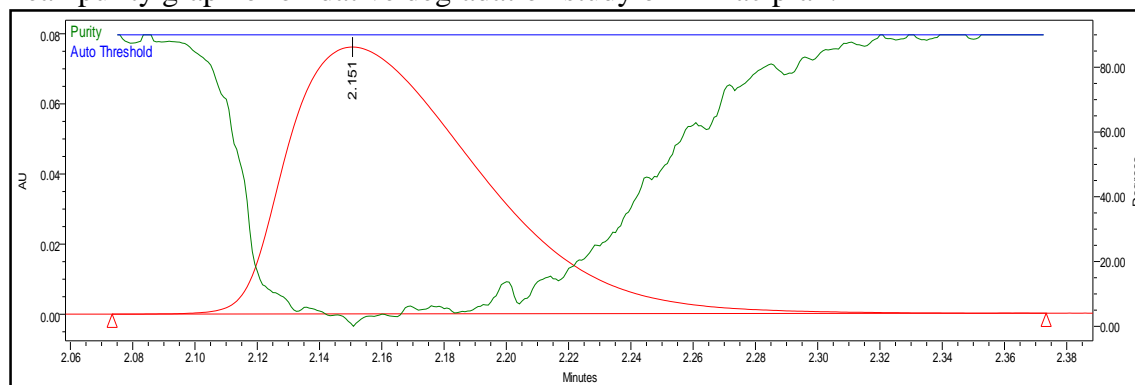
Peak purity graph of alkali stress study of milnacipran:



Chromatogram of oxidative stress test preparation of specificity study :



Peak purity graph of oxidative degradation study of milnacipran:



Observations:

- (1) Any interference was not observed from blank to the peak of interest, in addition to this peak purity was also within the acceptance criteria proved by the photo diode detector.
- (2) From the above chromatogram it can be conclude that there is no interference of any degradation product to the peak of interest and impurity has been generated by each stress condition.

Table-2: Degradation result of stress condition

Degradation Condition	Time	Peak Purity		% Degradation
		Purity Threshold	Purity Angle	
Acidic	1 Hrs at 80°C	12.4	9.5	5.4%
Basic	1Hrs at 80°C	18.6	9.7	62%
Oxidative	1Hrs at 80°C	15.7	10.6	8%
Thermal	1Hrs at 80°C	7.2	2.3	0.2%
Photolytic	48 hrs	19.4	4.9	0.1%

5.2 Linearity and Range:

The linearity plot was prepared with 8 concentration levels (20, 30, 40, 50, 60, 70 and 80 µg/ml of Milnacipran). These concentration levels were respectively corresponding to 40, 60, 80, 100, 120, 140 and 160% of standard solution concentration. The peak areas vs. concentration data were evaluated by linear regression analysis.

Standard solution preparation:

Weigh accurately 50.07mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 500.7 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.07 µg/ml of Milnacipran.

Linearity Standard Solution Preparation:***Stock Solution:***

Weigh accurately 50.12mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.2 µg/ml of Milnacipran.

Linearity Level 1 (40%):

2 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 20.05 µg/ml of Milnacipran.

Linearity Level 2 (60%):

3 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 30.07 µg/ml of Milnacipran.

Linearity Level 3 (80%):

4 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 40.10 µg/ml of Milnacipran.

Linearity Level 4 (100%):

5 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 50.12 µg/ml of Milnacipran.

Linearity Level 5 (120%):

6 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 60.14 µg/ml of Milnacipran.

Linearity Level 6 (140%):

7 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 70.17 µg/ml of Milnacipran.

Linearity Level 7 (160%):

8 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 80.19 µg/ml of Milnacipran.

Table 3: Sequence of Linearity and range study

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Linearity level-1 (40%)	2
4	Linearity level-2 (60%)	2
5	Linearity level-3 (80%)	2
6	Linearity level-4 (100%)	2
7	Linearity level-5 (120%)	2
8	Linearity level-6 (140%)	2
9	Linearity level-7 (160%)	2
10	Bracketing Standard	1

Linearity Level 8 (180%):

9 ml of stock solution was pipette out and transferred into 50 ml volumetric flask. The solution was diluted to volume with diluent. The concentration obtained is 90.22 µg/ml of Milnacipran.

For each linearity level, the solution was injected in duplicate. Linearity was evaluated by linear regression analysis.

Table 4(A): Summary of Linearity and range study

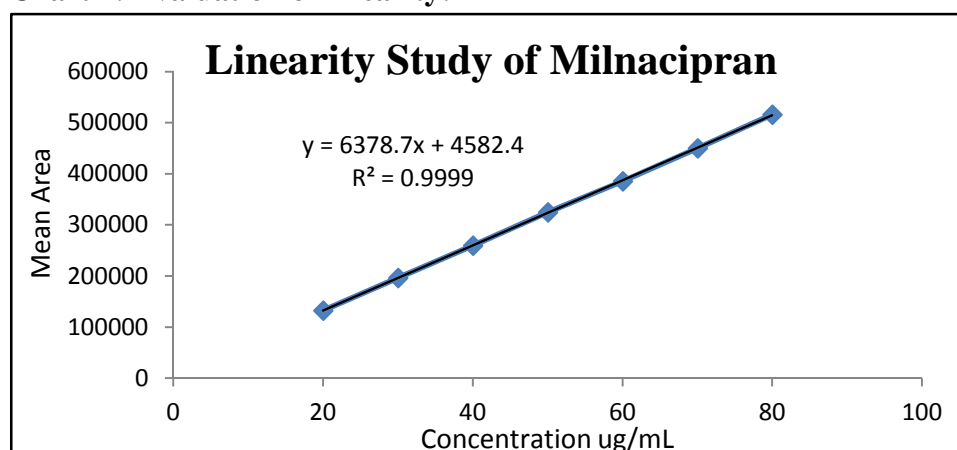
Observation			
Data for Standard Preparation			
Replicate	Area	Standard Weight	50.07
1	323631	Standard Potency	99.32
2	324810		
3	325029		
4	325188		
5	325833		
Average	324898.2		
Stdev	804.44		
%RSD	0.25		
Data for Leniarity Level Preparation			
Linearity Level	Replicate	Area	Mean Area
Level-1 (40%)	1	131755	132034
	2	132313	
Level-2 (60%)	1	196785	196260
	2	195735	
Level-3 (80%)	1	260529	259783
	2	259037	
Level-4 (100%)	1	324143	324521
	2	324899	
Level-5 (120%)	1	384971	385689
	2	386407	
Level-6 (140%)	1	451027	450268
	2	449509	
Level-7 (160%)	1	516488	516074
	2	515660	

Table-4 (B): Summary of Linearity and Range study:

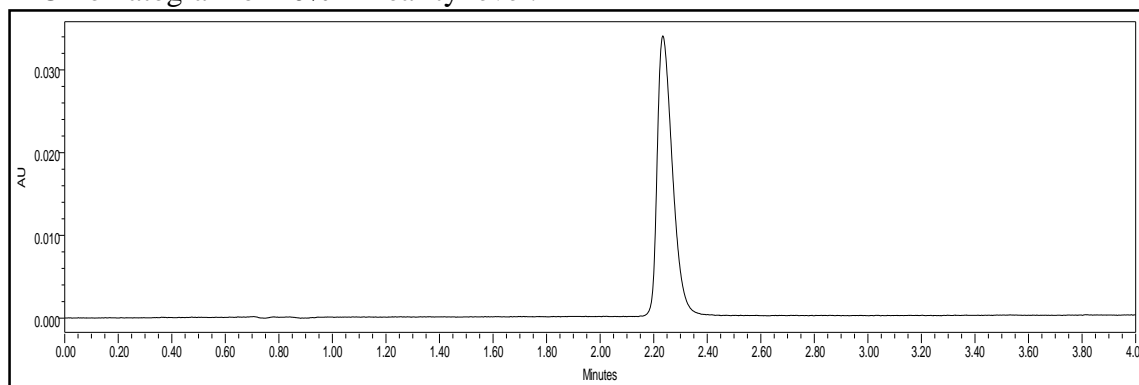
Linearity Level	% of Level	Concentration ($\mu\text{g/ml}$)	Mean Area
1	40	20.0	132034
2	60	30.0	196260
3	80	40.0	259783
4	100	50.0	324521
5	120	60.0	385689
6	140	70.0	450268
7	160	80.0	516074
Correlation Co-efficient			0.999
Slope			3189
Intercept			4582

Table-4(C): Summary of Linearity and Range study:

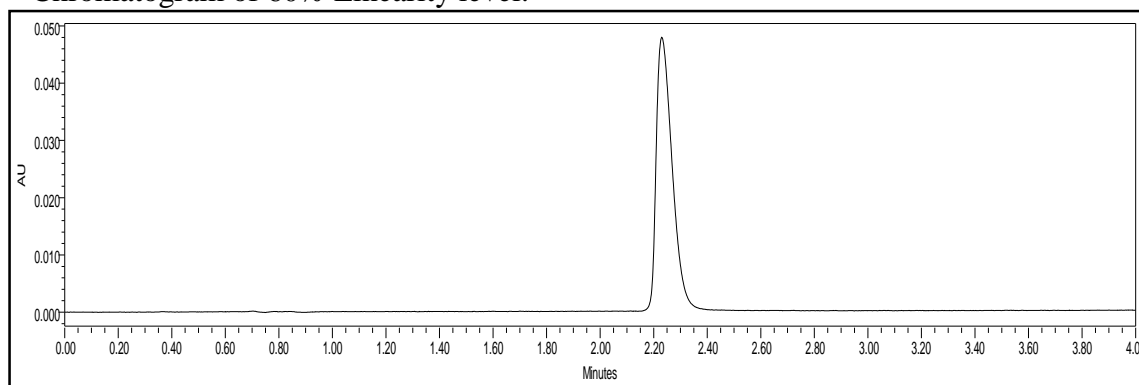
Linearity Level	% of Level	Concentration ($\mu\text{g/ml}$)	Mean Area
1	40	20	132034
2	60	30	196260
3	80	40	259783
4	100	50	324521
5	120	60	385689
6	140	70	450268
7	160	80	516074
Correlation Co-efficient			0.999
Slope			6378
Intercept			4582

Chart 1: Evaluation of linearity:

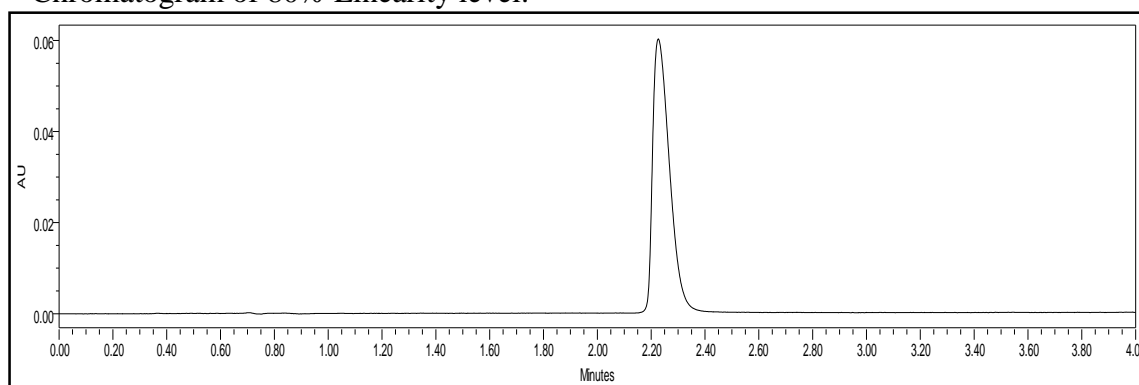
Chromatogram of 40% Linearity level:



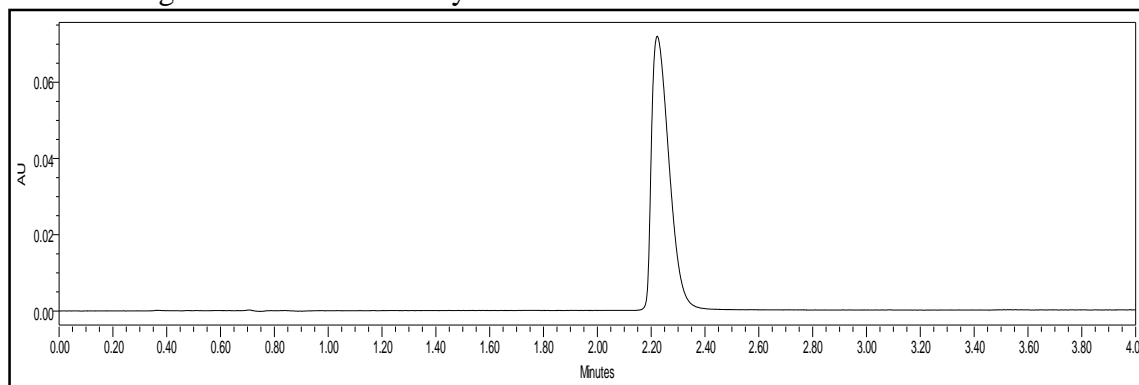
Chromatogram of 60% Linearity level:



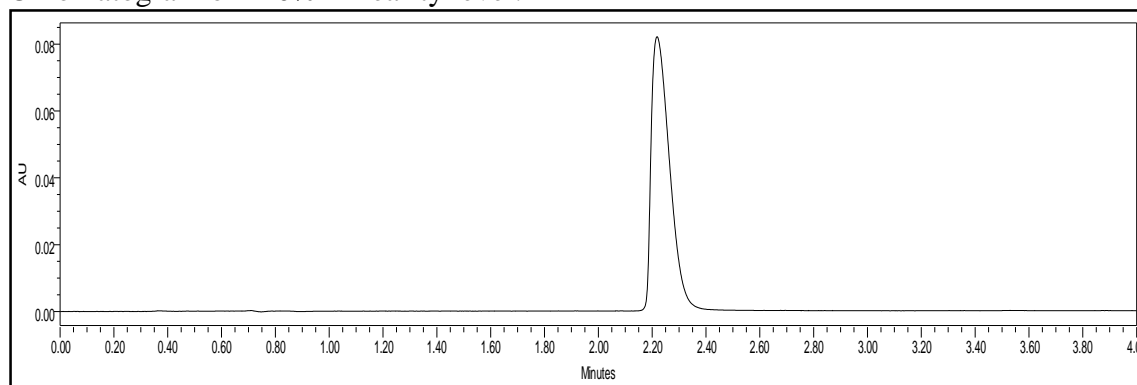
Chromatogram of 80% Linearity level:



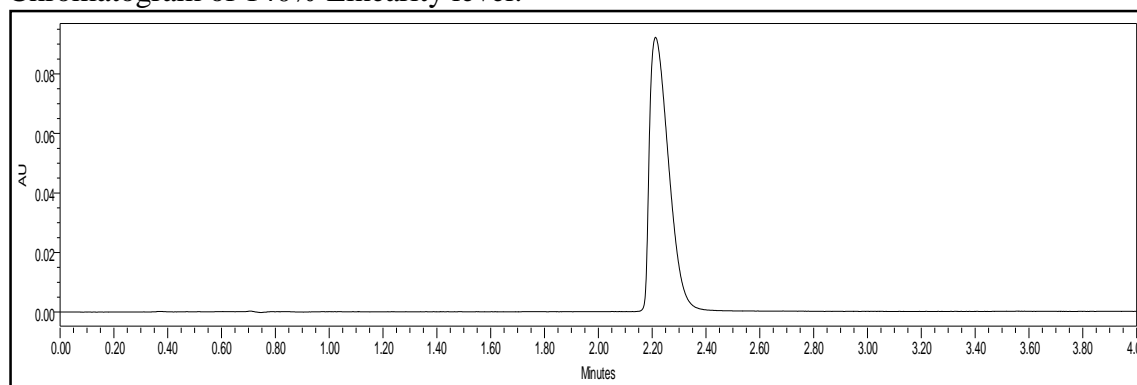
Chromatogram of 100% Linearity level:



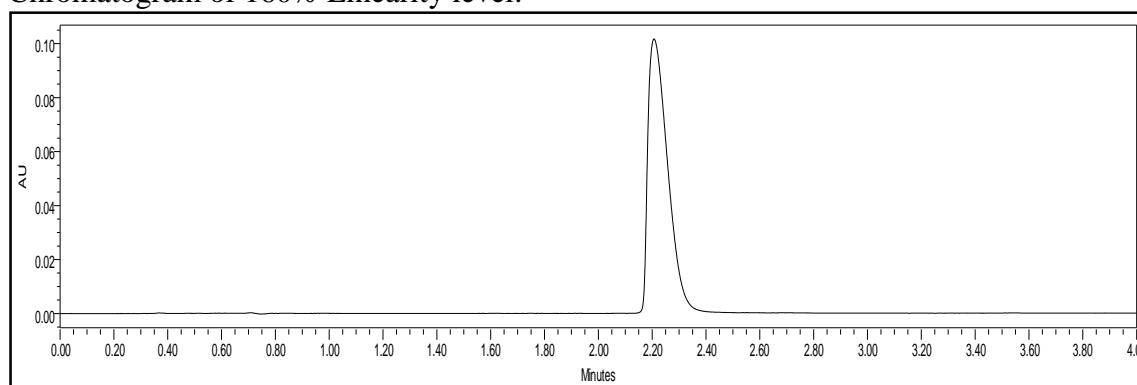
Chromatogram of 120% Linearity level:



Chromatogram of 140% Linearity level:



Chromatogram of 160% Linearity level:



Correlation coefficient of the linearity study was found to $R^2 = 0.999$ with linear regression equation $y = 6378x + 4582$, which proves the method is highly linear over the working range 20 – 80 $\mu\text{g/ml}$.

5.3 Limit of Detection and Limit of Quantitation:

LOD is the lowest amount of the drug content which can be **detected** by the proposed method while **LOQ** is the lowest amount which can be **quantified** by the method. The guideline suggest minimum signal to noise ratio (S/N) more than 3.3 for LOD and more

than 10 for LOQ. On the basis of linearity data theoretically it can be also calculated by the given formula,

$$\text{LOD} = 3.3 \sigma / S$$

$$\text{LOQ} = 10 \sigma / S$$

Where σ = Residual Standard Deviation of regression line and S = Slope of regression line.

LOQ value is precised by six replicate injections and checked for linear response with respect to other linearity levels by extended linearity curve. For LOD and LOQ study, blank, standard preparation, LOD preparation and LOQ preparation was prepared as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Weigh accurately 50.12mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.2 $\mu\text{g/ml}$ of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.12 $\mu\text{g/ml}$ of Milnacipran.

LOD and LOQ solution:

Solution-A:

Weigh accurately 50.02mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 500.2 $\mu\text{g/ml}$ of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.02 $\mu\text{g/ml}$ of Milnacipran.

Pipette out 5 mL of this solution and transfer into 50 mL volumetric flask and dilute it up to the mark with diluents. The concentration obtained is 5.0 µg/ml of Milnacipran. This solution is designated as Solution-A.

LOD preparation:

5 ml of above Solution-A was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.5 µg/ml of Milnacipran. 7 ml of this solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.07 µg/ml of Milnacipran

LOQ preparation:

2 ml of above Solution-A was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 0.2 µg/ml of Milnacipran.

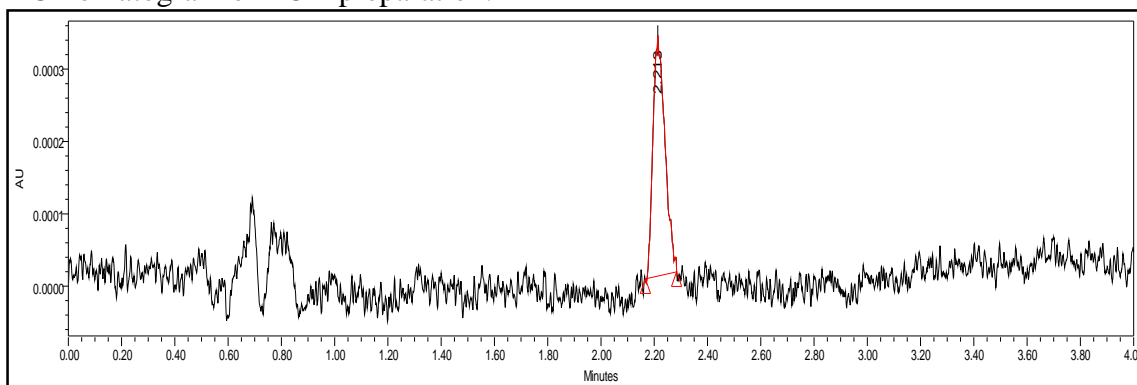
Table 5: Sequence of LOD and LOQ study:

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Blank	1
4	LOD	2
5	LOQ	6
6	Bracketing Standard	1

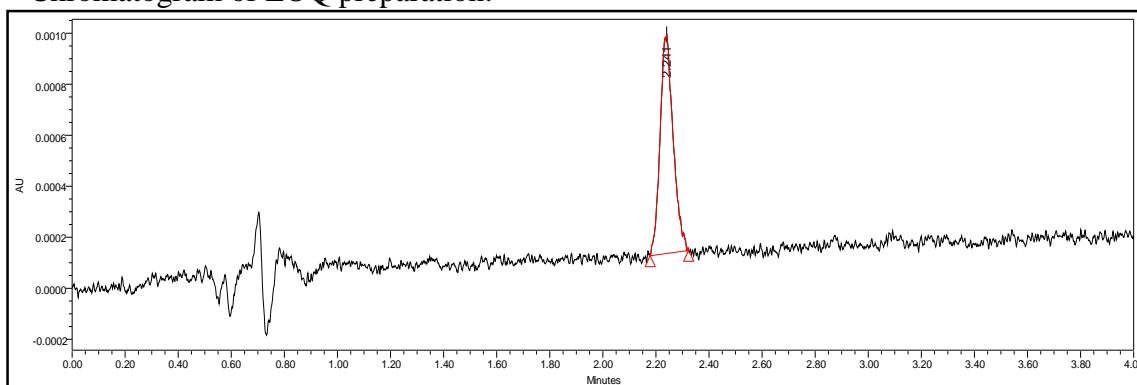
Table-6: Summary of LOQ study:

Observation			
<i>Data for Standard Preparation</i>		<i>Data for LOQ Preparation</i>	
Replicate	Area	Replicate	Area
1	1601287	1	2837
2	1617662	2	2934
3	1612469	3	2841
4	1609947	4	2908
5	1615378	5	2947
Average	1611348.6	6	3017
Stdev	6335.43	Average	2914
%RSD	0.39	STDEV	68.40
		%RSD	2.35

Chromatogram of LOD preparation:



Chromatogram of LOQ preparation:



LOQ of the analytical method can be evaluated by establishing linearity up to the LOQ value. Hence, the linearity study is extended to the LOQ value,

Chart 2: LOD and LOQ study of Milnacipran

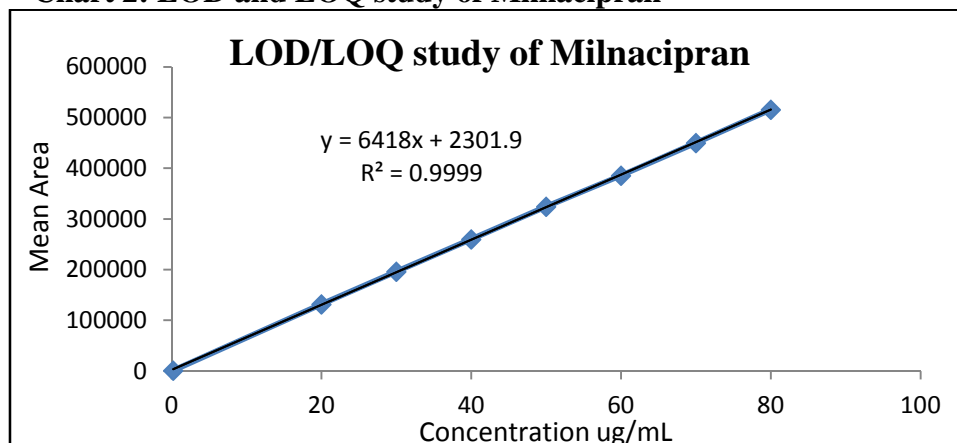


Table-7: Summary of LOQ study by evaluating linearity:

Linearity Level	% of Level	Concentration ($\mu\text{g/ml}$)	Mean Area
1	LOQ	0.20	1376
2	40	20	132034
3	60	30	196260
4	80	40	259783
5	100	50	324521
7	120	60	385689
8	140	70	450268
9	160	80	516074
Correlation Co-efficient			0.999
Slope			6418
Intercept			2301

All the results of LOD and LOQ data were within the acceptance criteria, hence it can be concluded that the LOD and LOQ of the method was $0.07 \mu\text{g/ml}$ and $0.20 \mu\text{g/ml}$ respectively which correspond to 0.14% and 0.4% of working concentration.

5.4 Precision Study:

Precision study was established by evaluating method precision and intermediate precision study. Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated.

Intermediate precision of the analytical method was determined by performing method precision on another day by another analyst using different make of raw materials under same experimental condition. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation and 95% confidence interval for the same was calculated. Overall assay value of method precision and intermediate precision was compared and % difference and overall % relative standard deviation was calculated.

For method precision, blank, standard preparation and six sets of test preparations was prepared as per method as under:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Weigh accurately 50.04mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 500.4 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.04 µg/ml of Milnacipran.

Test preparation:

Weigh accurately 50.12mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.2 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.04 µg/ml of Milnacipran.

The same procedure was used for preparing the six Test preparation Sets. Same approach was applied for the intermediate precision study on the second day with different analyst.

Table-8: Sequence of precision study:

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Test preparation (Set-1)	2
4	Test preparation (Set-2)	2
5	Test preparation (Set-3)	2
6	Test preparation (Set-4)	2
7	Test preparation (Set-5)	2
8	Test preparation (Set-6)	2
9	Bracketing Standard	1

Table-9:Data for method precision

Data for Standard preparation					
Replicate	Area		Standard Weight	50.04	
1	324816		Standard Potency	99.32	
2	323957				
3	325022				
4	325235				
5	324563				
Average	324719				
Stdev	493.07				
%RSD	0.15				
Data for Test preparation					
Set No.	Replicate	Area	Mean Area	Wt. of Sample	% Assay
1	1	325861	325635	50.12	99.44
	2	325409			
2	1	326022	326208	50.14	99.58
	2	326394			
3	1	324917	324693	50.09	99.21
	2	324469			
4	1	324561	324738	50.07	99.27
	2	324914			
5	1	327917	327597	50.11	100.06
	2	327276			
6	1	328885	325304	50.1	99.38
	2	321722			

% Assay calculation is as under:

$$\% \text{ Assay} = \frac{A_T}{A_S} \times \frac{W_1}{100} \times \frac{5}{50} \times \frac{100}{W_2} \times \frac{50}{5} \times P$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

Table-10: Summary of Intermediate precision study

Observation					
Data for Standard preparation					
Replicate	Area		Standard Weight	50.15	
1	326714		Standard Potency	99.32	
2	325721				
3	326408				
4	326317				
5	325596				
Average	326151				
Stdev	475.26				
%RSD	0.15				
Data for Test preparation					
Set No.	Replicate	Area	Mean Area	Wt. of Sample	% Assay
1	1	327521	327307	50.07	99.61
	2	327093			
2	1	330118	331204	50.18	101.02
	2	332289			
3	1	325371	324559	50.01	99.33
	2	323747			
4	1	327802	326096	49.98	99.86
	2	324389			
5	1	325821	325912	50.09	99.59
	2	326002			
6	1	328184	328074	50.27	99.89
	2	327963			

% Assay calculation is as under:

$$\% \text{ Assay} = \frac{A_T}{A_S} \times \frac{W_1}{100} \times \frac{5}{50} \times \frac{100}{W_2} \times \frac{50}{5} \times P$$

Where,

A_T = Average Area of Test Preparation.

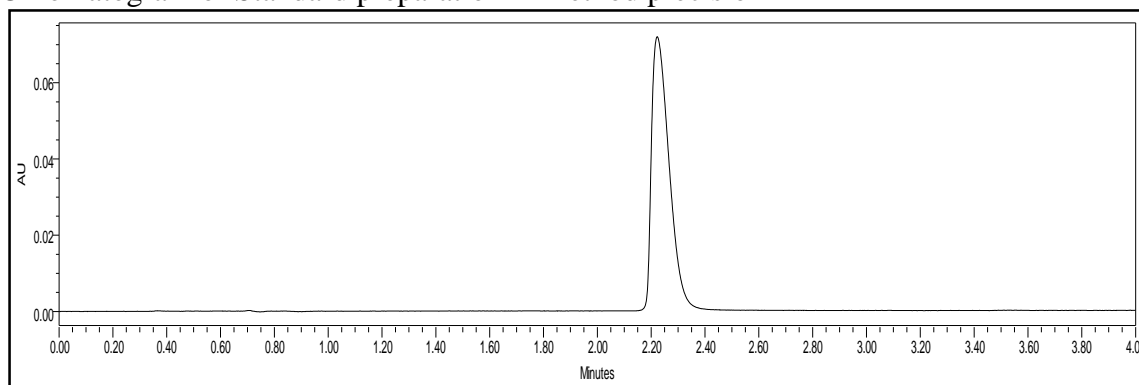
A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

Chromatogram of Standard preparation in method precision



Chromatogram of standard solution in intermediate precision

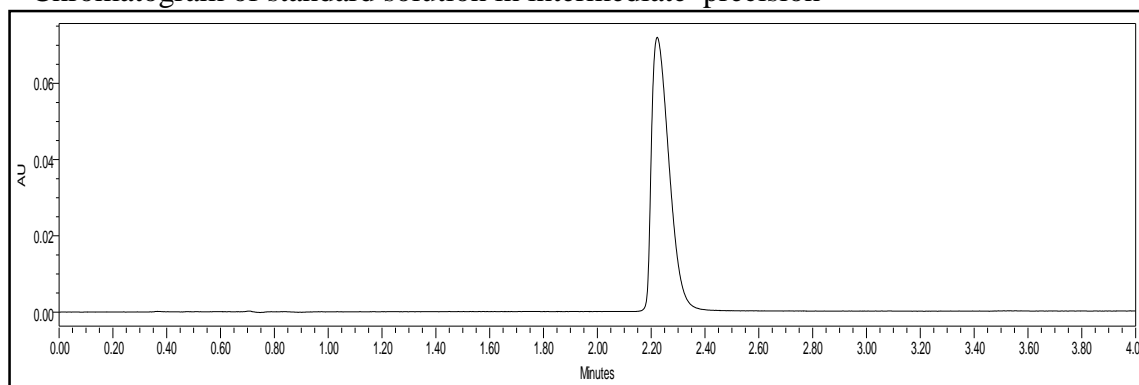


Table-11: Summary of precision study

Summary of precision study:				
Study	%Assay	Mean Assay %	Std. Dev.	%RSD
Method Precision	99.44	99.49	0.31	0.31
	99.58			
	99.21			
	99.27			
	100.06			
	99.38			
Intermediate Precision	99.61	99.88	0.59	0.59
	101.02			
	99.33			
	99.86			
	99.59			
	99.89			
Overall	99.69			
	0.50			
	0.50			

Overall the data for the precision study suggest % Assay value for each Test Preparation is between 98 – 102% which is under the acceptance criteria while % RSD of all results are less than 2%. Hence from all the observation it can conclude that the proposed method is highly precise.

5.5 Accuracy Study:

This Experiment can be performed by the recovery test. Recovery of the method was evaluated at 3 different concentration levels (Generally corresponding to 50, 100 and 150% of test solution concentration) by addition of known amounts of standard to placebo preparation. For each concentration level, 3 sets were prepared and injected in duplicate.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Weigh accurately 50.10mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.0 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.10 µg/ml of Milnacipran.

Sample preparations for accuracy levels are as under:

Accuracy level 1 (50%):

Test stock solution:

Weigh accurately 25.01 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 250.1 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 25.01 µg/ml of Milnacipran.

The same procedure was applied for preparing the three sets.

Accuracy level 2 (100 %):

Test stock solution:

Weigh accurately 50.18 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.8 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.18 µg/ml of Milnacipran.

The same procedure was applied for preparing the three sets.

Accuracy level 3 (150 %):

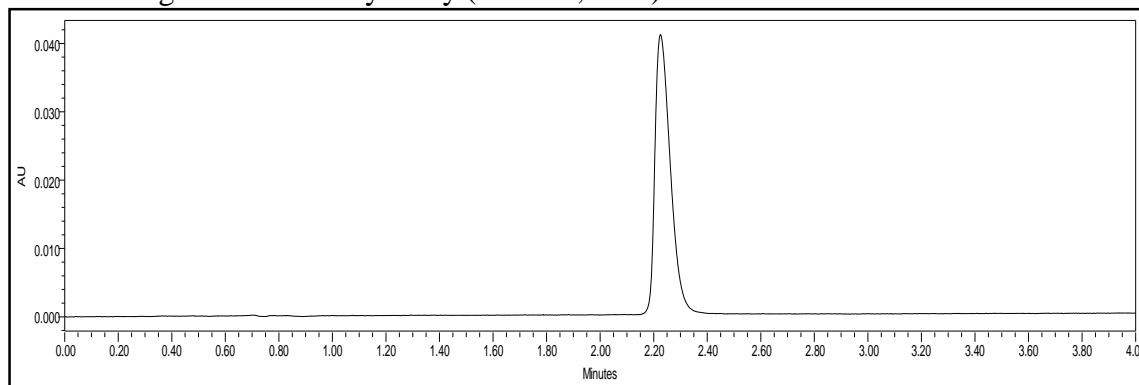
Test stock solution:

Weigh accurately 75.07 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 750.7 µg/ml of Milnacipran.

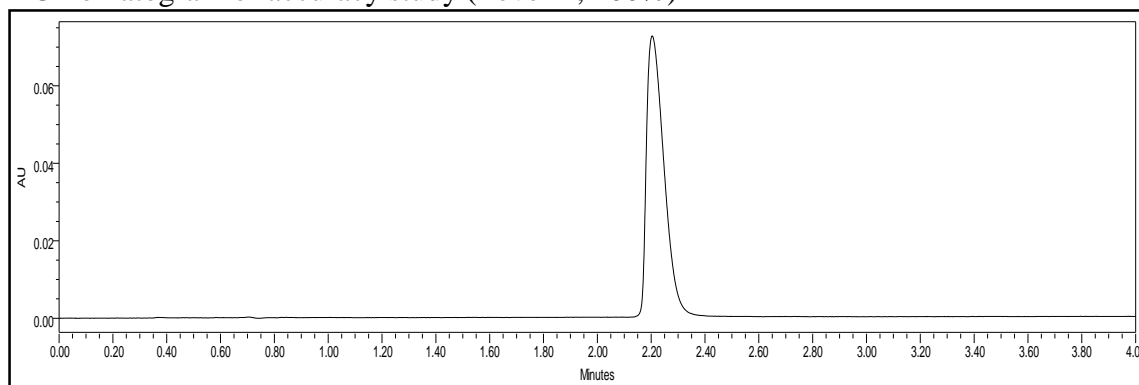
5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 75.07 µg/ml of Milnacipran.

The same procedure was applied for preparing the three sets.

Chromatogram of accuracy study (Level-1, 50%):



Chromatogram of accuracy study (Level-2, 100%)



Chromatogram of accuracy study (Level-3, 150%)

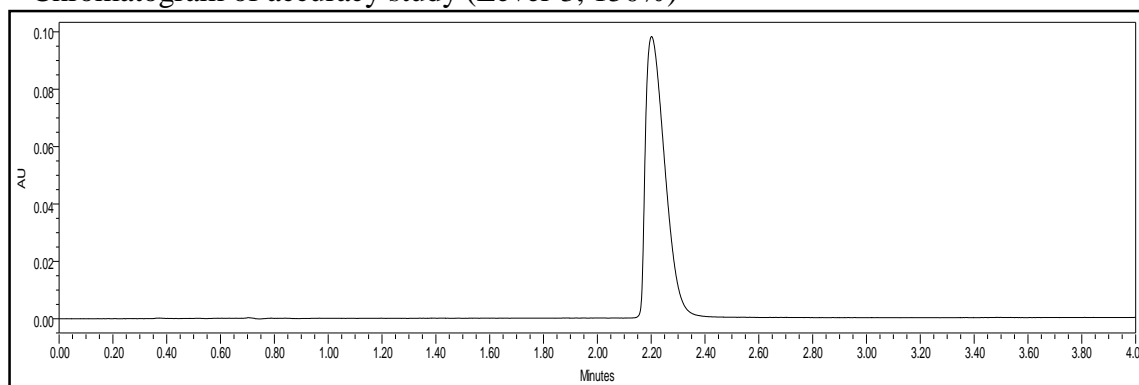


Table-12: Sequence of Accuracy Study

No.	Description	Injection Replicate
1	Blank	1
2	Standard Preparation	5
3	Accuracy level-1 preparation: Set-1	2
4	Accuracy level-1 preparation: Set-2	2
5	Accuracy level-1 preparation: Set-3	2
6	Accuracy level-2 preparation: Set-1	2
7	Accuracy level-2 preparation: Set-2	2
8	Accuracy level-2 preparation: Set-3	2
9	Accuracy level-3 preparation: Set-1	2
10	Accuracy level-3 preparation: Set-2	2
11	Accuracy level-3 preparation: Set-3	2
12	Bracketing Standare	1

Table-13(A): Summary of accuracy study

Observation				
Data for Standard preparation				
Replicate	Area		Standard Weight	50.1
1	324475		Standard Potency	99.32
2	324186		Standard Conc.	50.1
3	324756			
4	329664			
5	321285			
Average	324873			
Stdev	3019.58			
%RSD	0.93			
Data for Test preparation				
Accuracy Level	Set No	Replicate	Area	Mean Area
1(50%)	1	1	161961	161859
		2	161757	
	2	1	162009	162113
		2	162217	
	3	1	161736	162545
		2	163354	
2(100%)	1	1	324856	324693
		2	324529	
	2	1	324204	324484
		2	324763	
	3	1	325284	325153
		2	325021	
3(150%)	1	1	484847	485842
		2	486837	
	2	1	485021	485573
		2	486125	
	3	1	484563	488715
		2	492867	

From the all data (Table-13A and 13B) it has been proven that the % recovery is within the limit of 98 to 102 % this is in the limit of acceptance criteria and % RSD value of % recovery of replicate set is below 2 % .Hence this suggest that proposed method is highly accurate.

Table-13 (B): Summary of accuracy study

Accuracy (Recovery) Study							
Accuracy Level	Set No	Amount added (µg/ml)	Amount Found (µg/ml)	Recovery (%)	Average recovery	Std Dev.	% RSD
I (50%)	1	25.01	24.96	99.80	99.85	0.16	0.16
	2	25.07	25.00	99.72			
	3	25.06	25.07	100.03			
II (100%)	1	50.18	50.07	99.79	99.86	0.13	0.13
	2	50.15	50.04	99.78			
	3	50.14	50.14	100.01			
II (150%)	1	75.07	74.92	99.81	99.95	0.33	0.33
	2	75.09	74.88	99.72			
	3	75.12	75.37	100.33			

Calculation formulas for recovery study are as under:

$$\text{Amount added } (\mu\text{g/ml}) = \frac{\text{Wt. taken}}{\text{Volume 1}} \times \frac{\text{Volume 2}}{\text{Volume 3}} \times 1000$$

Where, Volume = Dilution given for preparing the solution.

$$\text{Amount found } (\mu\text{g/ml}) = \frac{\text{Mean area of test preparation}}{\text{Average area of standard preparation}} \times \text{standard conc.}$$

5.6 Robustness Study:

Robustness of the method was evaluated by assaying test solutions under slight but deliberate changes in analytical conditions, such as change in flow rate, change in proportions of Buffer-Acetonitrile (78:22 and 74:26,v/v), Change in flowrate and change in column-lot.

5.6.1 Change in flow rate:

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Weigh accurately 50.14 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.4 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.14 µg/ml of Milnacipran.

Test preparation:

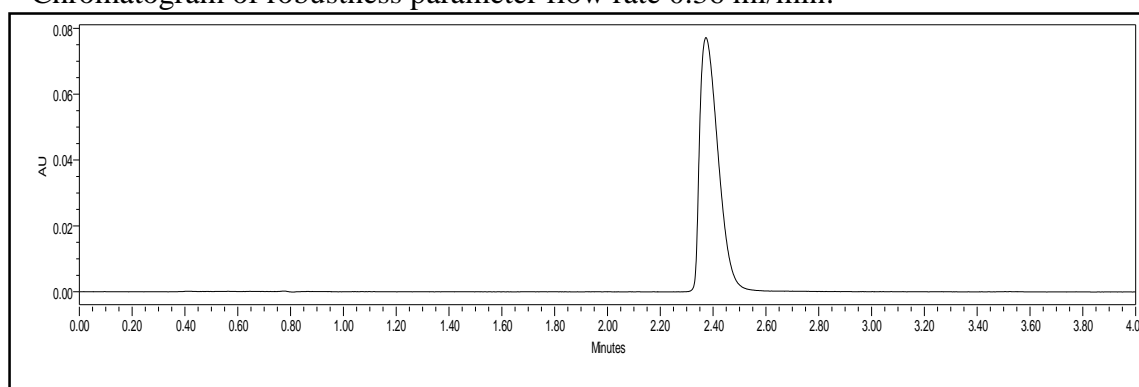
Weigh accurately 50.11 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.1 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.11 µg/ml of Milnacipran.

Table-14: Sequence for flow rate robustness study

No.	Description	Injection Replicate	Chromatographic Parameter
1	Blank	1	Flow rate: 0.36 mL/min
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	
5	Blank	1	Flow rate: 0.44 mL/min
6	Standard Preparation	5	
7	Test Preparation	2	
8	Bracketing Standard	1	

Chromatogram of robustness parameter flow rate 0.36 ml/min:



Chromatogram of robustness parameter flow rate 0.44 ml/min:

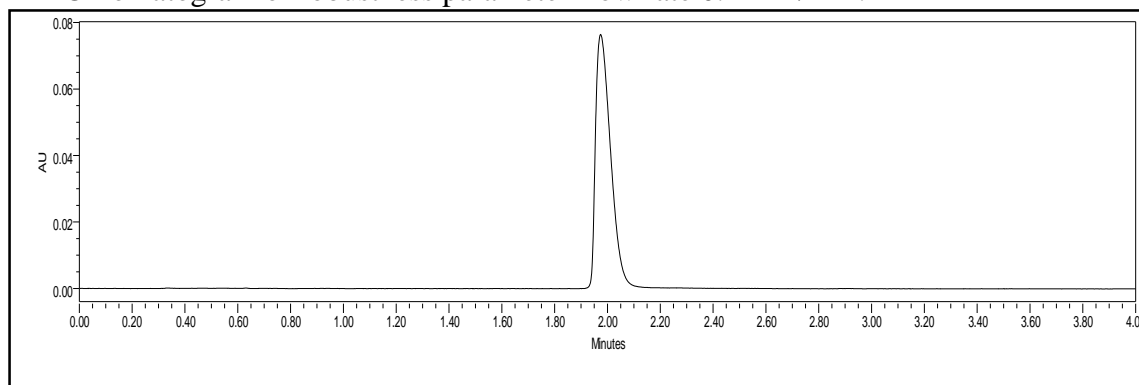


Table-15: Summary for flow change parameter of robustness study.

At 0.36 mL/min flow rate		At 0.44 mL/min flow rate	
<i>Data for standard preparation</i>		<i>Data for standard preparation</i>	
Replicate	Area	Replicate	Area
1	350624	1	288233
2	351186	2	288632
3	350881	3	287967
4	351867	4	287524
5	351852	5	287991
Mean	351282	Mean	288069
Std.dev.	563.49	Std.dev.	405.39
%RSD	0.16	%RSD	0.14
<i>Data for Test preparation</i>		<i>Data for Test preparation</i>	
Replicate	Area	Replicate	Area
1	350705	1	288136
2	350091	2	287614
Mean	350398	Mean	287875
Standard wt. (mg)	50.14	Standard wt. (mg)	50.14
Test wt. (mg)	50.11	Test wt. (mg)	50.11
%Assay	98.97	%Assay	99.19

The calculation formula for the determination of assay is,

$$\% \text{ Assay} = \frac{A_T}{A_S} \times \frac{W_1}{100} \times \frac{5}{50} \times \frac{100}{W_2} \times \frac{50}{5} \times P$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

5.6.2 Change in mobile phase composition:

In this experiment the test samples were analyzed at the mobile phase proportion of (Buffer:Acetonitrile) 78:22 and 74:26 v/v each and the results were observed in terms of assay value.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Refer standard preparation under flow rate parameter of robustness study

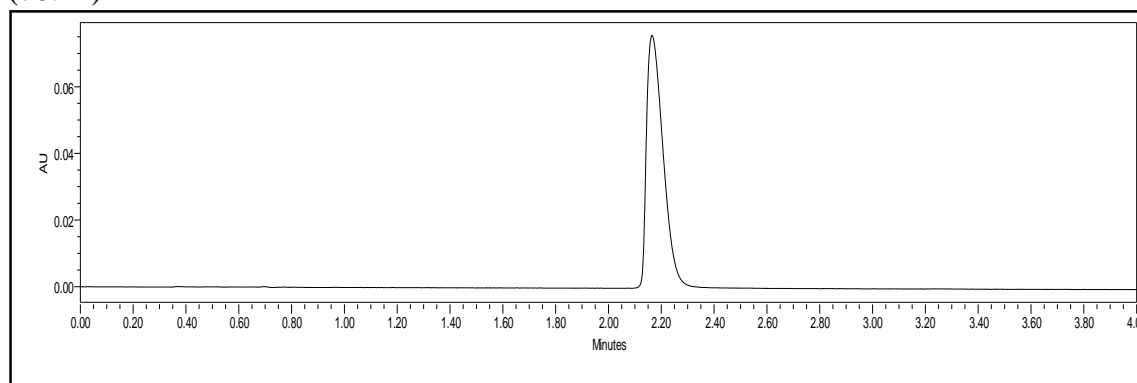
Test preparation:

Refer test preparation under flow rate parameter of robustness study.

Table-16: Sequence for change in composition robustness study

No.	Description	Injection Replicate	Chromatographic Parameter
1	Blank	1	Buffer:Acetonitrile (78:22)
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	
5	Blank	1	Buffer:Acetonitrile (74:26)
6	Standard Preparation	5	
7	Test Preparation	2	
8	Bracketing Standard	1	

Chromatogram of robustness parameter Mobilephae composition Buffer:Acetonitrile (78:22)



Chromatogram of robustness parameter Mobilephae composition Buffer:Acetonitrile (74:26)

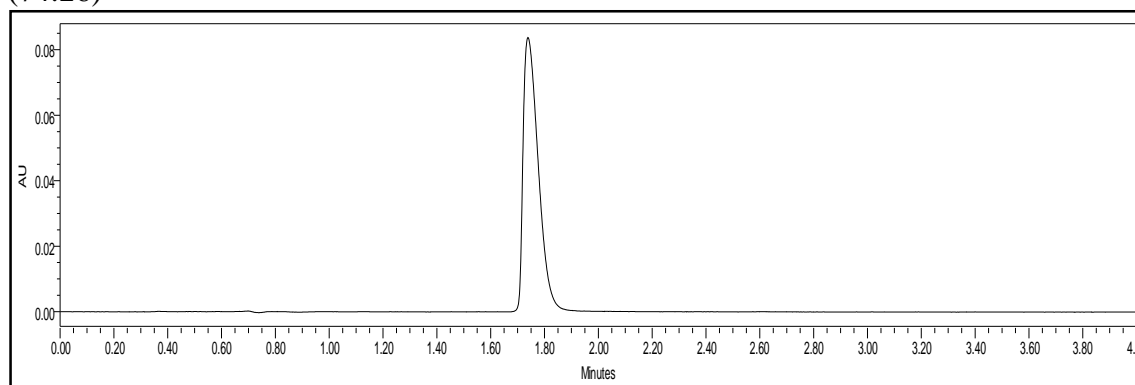


Table-17: Summary of Mobile phase composition change parameter of robustness study

Buffer:Acetonitrile (78:22)		Buffer:Acetonitrile (74:26)	
<i>Data for standard preparation</i>		<i>Data for standard preparation</i>	
Replicate	Area	Replicate	Area
1	322594	1	326268
2	321927	2	326481
3	322246	3	325812
4	321829	4	325623
5	322081	5	326546
Mean	322135	Mean	326146
Std.dev.	301.17	Std.dev.	409.94
%RSD	0.09	%RSD	0.13
<i>Data for Test preparation</i>		<i>Data for Test preparation</i>	
Replicate	Area	Replicate	Area
1	322249	1	326589
2	322563	2	326105
Mean	322406	Mean	326347
Standard wt. (mg)	50.14	Standard wt. (mg)	50.14
Test wt. (mg)	50.11	Test wt. (mg)	50.11
%Assay	99.48	%Assay	99.44

The calculation formula for the determination of assay is,

$$\% \text{ Assay} = \frac{A_T}{A_S} \times \frac{W_1}{100} \times \frac{5}{50} \times \frac{100}{W_2} \times \frac{50}{5} \times P$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

5.6.3 Robust Parameter: Change column lot:

Column used in analytical method was changed to different lot. Sample was assayed by changing the lot of column.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Refer standard preparation under flow rate parameter of robustness study

Test preparation:

Refer standard preparation under flow rate parameter of robustness study

Table-18: Sequence of Column change parameter of robustness study

No.	Description	Injection Replicate	Chromatographic Parameter
1	Blank	1	Change column lot
2	Standard Preparation	5	
3	Test Preparation	2	
4	Bracketing Standard	1	

Chromatogram of robustness parameter column change:

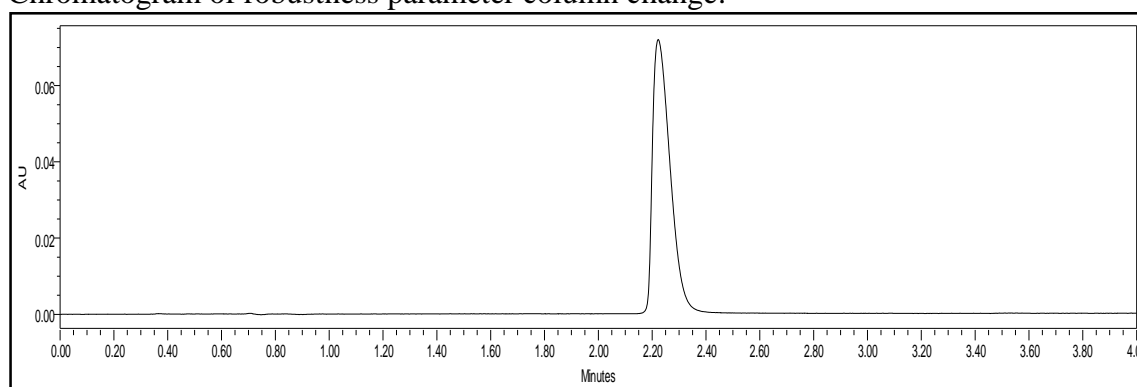


Table-19: Summary for column change parameter of robustness study

Column lot change	
<i>Data for standard preparation</i>	
Replicate	Area
1	324862
2	324252
3	324563
4	325012
5	325146
Mean	324767
Std.dev.	360.39
%RSD	0.11
<i>Data for Test preparation</i>	
Replicate	Area
1	324529
2	324761
Mean	324645
Standard wt. (mg)	50.14
Test wt. (mg)	50.11
% Assay	99.34

The calculation formula for the determination of assay is,

$$\% \text{ Assay} = \frac{A_T}{A_S} \times \frac{W_1}{100} \times \frac{5}{50} \times \frac{100}{W_2} \times \frac{50}{5} \times P$$

Where,

A_T = Average Area of Test Preparation.

A_S = Average Area of Standard Preparation.

W_1 = Weight of Working Standard (mg).

W_2 = Weight of Test Sample (mg).

P = Potency of Working Standard (%).

Table-20: Summary of robustness study

Summary of Robustness Study				
Robust Condition	% Assay	Retention time (min.)	System Suitability	
			Theoretical Plates	Asymmetry
Flow Change 0.36 ml/min	99.14	2.37	5274	1.21
Flow Change 0.44 ml/min	99.36	1.92	5750	1.18
MP Proportion Change A:B= 78:22	99.18	2.34	5359	1.21
MP Proportion Change A:B= 74:26	98.96	1.73	5084	1.18
Column Lot Change	99.88	2.21	5118	1.20

The data and the chromatogram given above suggest that there is no considerable influence of the change in flow rate, mobile phase composition and column lot change on the result of the analysis by this method or on chromatographic suitability of this method. Hence, it can be concluded from this experiment that the method is highly robust.

5.7 Solution stability study:

Solution stability period for the solutions of standard preparation and test preparation was evaluated. The solutions were stored at 5° C and ambient temperature without protection against light and tested at interval of 6, 12 and 24 hrs. The responses for the aged solution were evaluated using a freshly prepared standard solution.

Blank preparation:

Diluent was used as blank.

Standard preparation:

Stock solution: Weigh accurately 50.17 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 501.7 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.17 µg/ml of Milnacipran.

Test preparation:

Stock solution: Weigh accurately 50.20 mg of milnacipran standard and transferred into 100 ml volumetric flask. Added 70ml of diluent into the volumetric flask, dissolved by sonication and dilute up to the mark with diluent. The concentration obtained was 502.0 µg/ml of Milnacipran.

5 ml of above standard stock solution was pipette out and transferred into 50 ml volumetric flask followed by diluted to volume with diluent. The concentration obtained is 50.20 µg/ml of Milnacipran.

Table-21: Summary of solution stability:

Time intervals	Absolute difference in assay for standard solution %		Absolute difference in assay for sample solution %	
	At 5°C	At room temperature	At 5°C	At room temperature
After 6 hours	0.10	0.19	0.12	0.17
After 12 hours	0.33	0.40	0.34	0.5
After 24 hours	0.66	0.84	0.59	0.80

Conclusion:

Solution stability time period for standard solution is 24 hours at 5°C and room temperature. Solution stability time period for test solution is 24 hours at 5°C and at room temperature.

5.8 System Suitability Study:

A system suitability test for the chromatographic system was performed before each validation experiment. Five replicate injections of standard preparation were injected and asymmetry, theoretical plate and % RSD of peak area were determined for same. Only after the system suitability results were in acceptance criteria the experiments were precede further.

The Theoretical plates should be more than 5000, Asymmetry should be less than 2.0 and % RSD should be less than 2.0. As the data suggest the system suitability was within the criteria in each validation experiment. Hence the system was found suitable to perform the validation experiment which confirms the reliability of the data generated during the method validation.

Table-22: Summary of System suitability:

Summary of System Suitability Test			
Experiment Name	Theoretical Plates	Asymmetry	% RSD
Specificity	5462	1.11	0.18
Linearity and Range	5584	1.17	0.25
LOD and LOQ	5963	1.08	0.39
Method Precision	5217	1.18	0.31
Int. Precision	5328	1.19	0.59
Accuracy	5821	1.17	0.93
Robustness	5239	1.21	0.16
Solution Stability	5415	1.15	0.07

6. CONCLUSION

The surveillance and outcome obtained from each validation experiment including specificity, linearity and range, LOD and LOQ, precision, accuracy, robustness, solution stability and system suitability lies well inside the acceptance criteria. Since, all the results are with-in the limit, the developed Analytical method is considered as validated and suitable for anticipated use.

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