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A Stability Indicating U-HPLC Method for Milnacipran in Bulk Drugs and Pharmaceutical Dosage Forms

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ABSTRACT

The objective of the current study was to develop a validated, specific and stability-indicating reverse phase UHPLC method for the quantitative determination of Milnacipran and its related substances. The determination was done for active pharmaceutical ingredient and its pharmaceutical dosage forms in the presence of degradation products, and its process-related impurities. The drug was subjected to stress conditions of hydrolysis (acid and base), oxidation, photolysis and thermal degradation per International Conference on Harmonization (ICH) prescribed stress conditions to show the stability-indicating power of the method. Significant degradation was observed during acid, base, oxidative and neutral stress hydrolysis. The chromatographic conditions were optimized using an impurity-spiked solution and the samples generated from forced degradation studies. In the developed UHPLC method, the resolution between Milnacipran and its process-related impurities was found to be greater than 2.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.999 for Milnacipran and it's all the five impurities. The chromatographic separation was achieved on a C18 stationary phase. The method employed a linear gradient elution and the detection wavelength was set at 220 nm. The mobile phase consists of buffer and acetonitrile delivered at a flow rate of 0.2 $mL \cdot min^{-1}$. Buffer consists a mixture of 10 mM Sodium dihydrogen phosphate monohydrate and 10 mM hexane sulfonate sodium salt, pH adjusted to 2.5 using ortho phosphoric acid. The mobile phase A consists of buffer and acetonitrile (950:50, v/v) and mobile phase B consists of acetonitrile. The stress samples were assayed against a qualified reference standard and the mass balance was found to be close to 99.5%. The developed RP-LC method was validated with respect to linearity, accuracy, precision and robustness.

Keywords: Milnacipran; UHPLC; Forced Degradation; Validation; Stability Indicating

1. Introduction

Milnacipran is an anti-depressant drug, chemically as [2-(aminomethyl)-*N*,*N*-diethyl-1-phenylcyclopropanecarboxamide] hydrochloride and its structural formula is $C_{15}H_{22}N_2O \cdot HCl$. Savella® is generic name for Milnacipran, is a serotonin serotonin-norepinephrine reuptake inhibitor (SNRI) used in the clinical treatment of fibromyalgia. Fibromyalgia syndrome (FMS) is a complex syndrome characterized by chronic widespread muscular pain associated with other symptoms such as fatigue, cognitive dysfunction, sleep disturbance, depression, anxiety, and stiffness. In January 2009, Milnacipran (under the brand name Savella) has been approved only for the treatment of fibromyalgia by the Food and Drug Administration (FDA) in the United States for the management of adult FMS patients. Milnacipran has proven efficacy in managing global FMS symptoms and pain as well as diminishing symptoms of fatigue and cognitive dysfunction without affecting sleep. Due to its antidepressant activity, Milnacipran can be beneficial to FMS patients with coexisting depression. Inhibition of both neurotransmitters works synergistically to treat both FMS as well as depression [1-7].

Few HPLC methods were available in literature for the analysis of Milnacipran includes chiral analysis of Milnacipran by a non-chiral HPLC-circular dichroism [8], a chiral HPLC method for enantionmeric separation of Milnacipran and its FMOC—derivative on cellulose based stationary phases [9], Determination of Milnacipran, a serotonin and noradrenaline reuptake inhibitor, in human plasma using liquid chromatography with spectrofluorimetric detection [10], Determination of Milnacipran in Serum by LC/MS [11], Pharmacokinetics of milnacipran

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in renal impairment using HPLC and GCMS [12], Acute electrophysiological effects of intravenous Milnacipran, a new antidepressant agent using HPLC [13]. GC method also reported in literature for the determination of Milnacipran in human plasma using GC-MS [14]. No LC methods were reported in major pharmacopeia's like USP, EP, JP and BP. M. Srinivasa Rao *et al.* has described an HPLC assay procedure to quantify Milnacipran in Pharmaceutical formulations [15], Peketi, *et al.* developed RP-HPLC method for analysis of Milnacipran in bulk and formulations [16], Priti J. Mehta *et al.* developed Stability-indicating assay for Milnacipran in formulation capsules [17], comparative validation study to assay Milnacipran hydrochloride in capsules by a stability-indicating LC and a second order derivative UV spectroscopic methods [18]. Extensive literature survey reveals there is no rapid stability-indicating LC method for determination of related substances and for quantitative estimation of Milnacipran in bulk drugs. The purpose of the present research work was to develop a suitable, single and rapid stability-indicating U-HPLC method for the determination of Milnacipran and its related substances.

Hence, an attempt has been made to develop an accurate, rapid, specific and reproducible method for the determination of Milnacipran and all the five impurities in bulk drug samples and in pharmaceutical dosage forms along with method validation as per ICH norms. The stability tests were also performed on both drug substances and drug product as per ICH norms [19-22].

2. Experimental

2.1. Chemicals

Samples of Milnacipran and its related impurities were obtained as gratis sample from Sebondscience Labs (Hyderabad, India) (**Figure 1**). Commercially available 100 mg of Milnacipran tablets (savella®) were purchased from Forest Pharmaceuticals Inc., Cincinnati, USA. HPLC grade acetonitrile, analytical reagent grade Sodium dihydrogen phosphate monohydrate, hexane sulfonate sodium salt and ortho phosphoric acid were purchased from Merck, Darmstadt, Germany. High purity water was prepared by using Millipore Milli-Q plus water purification system. All samples and impurities used in this study were of greater than 99.0% purity.

2.2. Equipment

The U-HPLC system, used for method development, forced degradation studies and method validation was Waters 2695 binary pump plus auto sampler and a 2996 photo diode array detector with Acquity software (Waters Corporation, MA, USA). The output signal was monitored and processed using Empower software on Pentium computer (Digital equipment Co.). Water bath equipped with temperature controller was used to carry out degradation studies for all solution. Photo stability studies were carried out in a photo stability chamber (Mack Pharmatech, Hyderabad, India). Thermal stability studies were performed in a dry air oven (Mack Pharmatech, Hyderabad, India).

2.3. Chromatographic Conditions

The chromatographic column used was Waters BEH column C18 (50 × 2.1) mm with 1.7 µm particles. Buffer consists a mixture of 10 mM Sodium dihydrogen phosphate monohydrate and 10 mM hexane sulfonate sodium salt, pH adjusted to 2.5 using ortho phosphoric acid. The mobile phase A consists buffer and acetonitrile (950:50, v/v) and mobile phase B consists of acetonitrile. The flow rate of the mobile phase was 0.2 $mL \cdot min^{-1}$. The HPLC gradient program was set as: time (min)/% solution B: 0/20, 1.4/20, 7/40, 8.5/40, 8.6/20 and 10/20. The column temperature was maintained at 25˚C and the detection was monitored at a wavelength of 220 nm. The injection volume was 5 µL. Methanol was used as diluent. The concentration is 250 $\mu g \cdot mL^{-1}$ for related substances method and 20 $\mu g \cdot mL^{-1}$ for Assay method.

2.4. Preparation of Solutions

2.4.1. Preparation of Standard Solutions

A stock solution of Milnacipran (2.0 $mg \cdot mL^{-1}$) was prepared by dissolving appropriate amount in the methanol. Working solutions were prepared from above stock solution for related substances determination and assay determination, respectively. A stock solution of impurities (mixture of imp-1, imp-2 imp-3 imp-4 and imp-5) at a concentration of 250 $\mu g \cdot mL^{-1}$ was also prepared in methanol.

2.4.2. Preparation of Sample Solutions

Savella® tablets contain 100 mg of Milnacipran. The inactive ingredients present in Savella® were dibasic calcium phosphate, povidone, carboxymethylcellulose calcium, colloidal silicon dioxide, magnesium stearate, talc, FD&C Red #40 Aluminum Lake, hypromellose, polyethylene glycol and titanium dioxide. Twenty Savella tablets (100 mg) were weighed and the average weight was calculated. The tablets were powdered in a mortar and a sample of the powder equivalent to 25 mg of the active pharmaceutical ingredient (Milnacipran) was transferred to 100 mL volumetric flask. Approximately 75 mL methanol was added and the flask was placed on rotatory shaker for 10 min and sonicated for 10 min to dissolve the material completely. The solution was then diluted to 100 mL and centrifuged at 3000 rpm for 10

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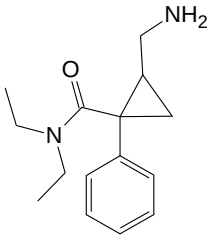
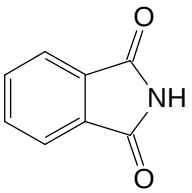
Milnacipran:



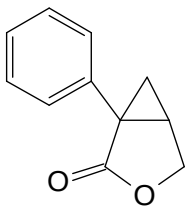
[2-(aminomethyl)-*N*,*N*-diethyl-1-phenylcyclopropanecarboxamide] hydrochloride; Molecular Formula: $C_{15}H_{22}N_2O{\cdot}HCl$; Molecular Weight: 282.81.

Imp-1: Phthalimide



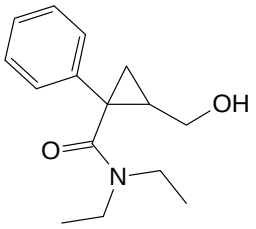
1*H*-isoindole-1,3(2*H*)-dione; Molecular Formula = $C_8H_5NO_2$; Molecular Weight: 147.13.

Imp-2: (±)Cis-Lactone



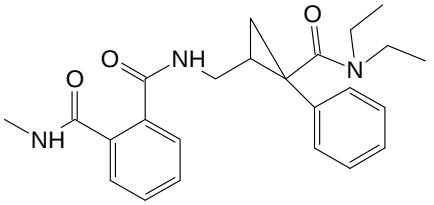
1-phenyl-3-oxabicyclo[3.1.0]hexan-2-one; Molecular Formula: $C_{11}H_{10}O_2$; Molecular Weight: 174.20.

Imp-3: Amide Alcohol



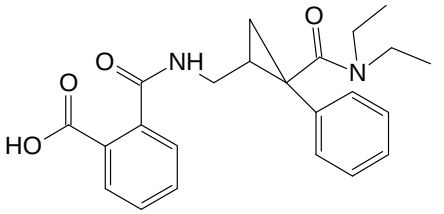
N,*N*-diethyl-2-(hydroxymethyl)-1-phenylcyclopropanecarboxamide; Molecular Formula: $C_{15}H_{21}NO_2$; Molecular Weight: 247.33.

Imp-4: Hydroised imp-1



N1-((2-(diethylcarbamoyl)-2-phenylcyclopropyl) methyl)-N2-methylphthalamide; Molecular Formula: $C_{24}H_{29}N_3O_3$; Molecular Weight: 407.51.

Imp-5: Hydroised imp-2



2-((2-(diethylcarbamoyl)-2-phenylcyclopropyl) methylcarbamoyl)benzoic acid; Molecular Formula: $C_{23}H_{26}N_2O_4$; Molecular Weight: 394.46.

Figure 1. Chemical structures and labels of Milnacipran and its impurities.

min. The supernatant was collected and filtered through a 0.22 µm pore size Nylon 66-membrane filter. The filtrate was used as sample solution.

2.5. Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress testing of the drug substance can help to identify the likely degradation products, which can in turn help to establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used.

The specificity of the Milnacipran in the presence of its impurities namely imp-1, imp-2, imp-3, imp-4, imp-5 and degradation products was determined by developed U-HPLC method. Forced degradation studies were also performed on Milnacipran to provide an indication of the stability indicating property and specificity of the proposed method [19,20]. The stress conditions employed for degradation study includes light (carried out as per ICH Q1B), heat (60˚C), acid hydrolysis (1N HCl), base hydrolysis (0.1 NaOH) and oxidation (5% H_2O_2). For heat and light studies, study period was 10 days where as for acid, base, peroxide and water hydrolysis the test period was 48 h. Peak purity of stressed samples of Milnacipran was checked by using 2996 Photo diode array detector of Waters Corporation, MA, USA.

2.6. Analytical Method Validation

The developed chromatographic method was validated for linearity, precision, accuracy, sensitivity, robustness and system suitability.

2.6.1. Precision

The precision of the related substance method was checked by injecting six individual preparations of (250 $\mu g{\cdot}mL^{-1}$) Milnacipran spiked with 0.15% each imp-1, imp-2, imp-3, imp-4 and imp-5. The %RSD area of each imp-1, imp-2, imp-3, imp-4 and imp-5 was calculated. Precision study was also determined by performing the

same procedures on a different day (interday precision).

The intermediate precision (ruggedness) of the method was also evaluated using different analyst, different column and different instrument in the same laboratory.

Assay method precision was evaluated by carrying out six independent assays of test sample of Milnacipran against qualified reference standard. The %RSD of six assay values obtained was calculated. The intermediate precision of the assay method was evaluated by different analyst and by using different instrument from the same laboratory.

2.6.2. Sensitivity

Sensitivity was determined by establishing the Limit of detection (LOD) and Limit of quantitation (LOQ) for imp-1, imp-2, imp-3, imp-4 and imp-5 estimated at a signal-to-noise ratio of 3:1 and 10:1 respectively, by injecting a series of dilute solutions with known concentration. The precision study was also carried out at the LOQ level by injecting six individual preparations of imp-1, imp-2, imp-3, imp-4 and imp-5, calculated the %RSD for the areas of each impurity.

2.6.3. Linearity and Range

Linearity test solutions for assay method has prepared from stock solution at five concentration levels from 50 to 200% of assay analyte concentration (10, 15, 20, 30 and 40 $\mu g \cdot mL^{-1}$).

A linearity test solution for related substance method was prepared by diluting the impurity stock solution to the required concentrations. The solutions were prepared at seven concentration levels. From LOQ to 200% of the permitted maximum level of the impurity (*i.e.* LOQ, 0.0375%, 0.075%, 0.1125%, 0.15%, 0.225% and 0.3% was subjected to linear regression analysis with the least square method. Calibration equation obtained from regression analysis was used to calculate the corresponding predicted responses. The residuals and sum of the residual squares were calculated from the corresponding predicted responses.

Linearity was checked for three consecutive days in the same concentration range for both assay and related substance method and calculated the %RSD Value of the slope and Y-intercept of the calibration curve. Upper and lower levels of range were also established.

2.6.4. Accuracy

The accuracy of the assay method was evaluated in triplicate at five concentration levels, *i.e.* 10, 15, 20, 30 and 40 $\mu g \cdot mL^{-1}$ in bulk drugs and pharmaceutical dosage forms. At each concentration, three sets were prepared and injected in triplicate. The percentage of recovery was calculated at each level.

The accuracy of the related substance method was evaluated in triplicate at 0.075%, 0.125%, 0.15%, 0.225% and 0.3% of the analyte concentration (250 $\mu g \cdot mL^{-1}$). The percentage of recoveries for imp-1, imp-2, imp-3, imp-4 and imp-5 were calculated.

2.6.5. Robustness

To determine the robustness of the developed method, experimental conditions were deliberately changed and the resolution (R_s) between Milnacipran, imp-1, imp-2, imp-3, imp-4 and imp-5 were evaluated. The flow rate of the mobile phase was 0.2 $mL \cdot min^{-1}$. To study the effect of flow rate on the developed method, 0.02 units of flow was changed (*i.e.* 0.18 and 0.22 $mL \cdot min^{-1}$). The effect of column temperature on the developed method was studied at 20˚C and 30˚C instead of 25˚C. The effect of pH on resolution of impurities was studied by varying ± 0.1 pH units (*i.e.* buffer pH altered from 2.5 to 2.4 and 2.6). In the all above varied conditions, the components of the mobile phase were held constant.

2.6.6. Solution Stability and Mobile Phase Stability

The solution stability of Milnacipran in the assay method was carried out by leaving the test solutions of samples in tightly capped volumetric flasks at room temperature for 48 h. The same sample solutions were assayed at 6 h intervals up to the study period against freshly prepared standard solution. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 6 h intervals up to 48 h. Mobile phase prepared was kept constant during the study period. The %RSD of assay of Milnacipran was calculated for the study period during mobile phase and solution stability experiments.

The solution stability of Milnacipran and its related impurities were carried out by leaving both spiked sample and unspiked sample solution in tightly capped volumetric flask at room temperature for 48 h. Content of imp-1, imp-2, imp-3, imp-4 and imp-5 was determined at every 6 h interval, up to the study period.

Mobile phase stability was also carried out for 48 h by injecting the freshly prepared sample solutions, for every 6 h interval. Content of imp-1, imp-2, imp-3, imp-4 and imp-5 was checked in the test solutions. Mobile phase prepared was kept constant during the study period.

3. Results and Discussion

3.1. Method Development and Optimization

The HPLC method carried out in this study aimed at developing chromatographic system capable of eluting and resolving Milnacipran and its degradation products and that complies with the general requirements for system suitability. Initial trials were done on LC by Waters Symmetry C18 column (150 mm × 4.6 mm i.d., particle size 5

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μm) and the gradient method for mobile phase, water: methanol was (time (min)/% solution B): 0/20, 5/20, 30/70, 45/70, 45.1/20, 52/20 at flow rate 1.0 mL·min^{-1}. Longer retention time and poor peak shape of Milnacipran was problem with the above method. Replacing the organic modifier with acetonitrile improved the peak shape but high retention time and tailing of Milnacipran were still to be improved. Studied the separation and peak shape by varying pH from 7.2 to 2.5, and obtained better separations at acidic pH but the asymmetry of the Milnacipran peak was very high and the imp-2 is eluting closely to the Milnacipran peak . Different columns such as Inertsil ODS 3V, Luna C18 3 um and different buffers such as potassium dihydrogen phosphate, triethyl amine, Trifluoroacetic acid were also tried with different gradient methods to achieve the best chromatographic separation. The use of ion pair agent in acidic pH as buffer along with acetonitrile improved the peak shape of Milnacipran and obtained good resolution between all the impurities and Milnacipran. But the long retention times were still unavoidable. Transferred the condition on to U-HPLC with BEH C18 column (50 × 2.1) mm, 1.7 μm and the % of acetonitrile played a key role in the retention times and resolution between impurities.

After many logical trials, chromatographic condition was established such that which could be suitable for separation of drug-degradation products and drug-five known impurities.

Using the optimized conditions Milnacipran and its known impurities were well separated with a resolution of greater than 2. The system suitability results are given in **Table 1**.

Table 1. System suitability report.

Compound	USP Resolution(R_s)	USP Tailing factor (T)	No of theoretical plates USP tangent method (N)
imp-1	-	1.2	8041
Milnacipran	27.4	1.1	41921
imp-2	11.3	1.0	22308
imp-3	2.2	1.1	47689
imp-4	2.7	1.0	70254
imp-5	10.5	1.0	92618

3.2. Results of Forced Degradation Studies

The drug was exposed to 1N HCl at 70˚C for 5 h. Milnacipran has shown significant sensitivity towards the treatment of 1N HCl. The drug gradually undergone degradation with time in 1N HCl and prominent degradation was observed (~15%).

3.2.1. Degradation in Basic Solution

The drug was exposed to 0.1N NaOH at 70˚C for 5 h. Milnacipran has shown significant sensitivity towards the treatment of 0.1N NaOH. The drug gradually undergone degradation with time in 0.1N NaOH and prominent degradation was observed (~25%).

3.2.2. Oxidative Conditions

The drug was exposed to 5% hydrogen peroxide at 70˚C for 15 hours and it was degraded up to (~14%). Milnacipran has shown significant sensitivity towards the treatment of 5% hydrogen peroxide and the drug gradually undergone prominent degradation (~10%).

Milnacipran was stable under forced photo and thermal degradation. DP-2 was common degradation product found in all degradation. Similarly, DP-1 for acid and base degradation; DP-3 for base and neutral degradation was common degradation products. It was labeled by logical matching of all chromatograms for retention time and wavelength for those peaks (See **Figure 2**).

From the degradation studies, Peak purity test results derived from PDA detector, confirmed that the Milnacipran peak was homogeneous and pure in all the analyzed stress samples. The mass balance of stressed samples was close to 99.5%. No degradants were observed after 10 min in the extended runtime of 20 min of all the Milnacipran samples. The developed U-HPLC method was found to be specific in the presence of imp-1, imp-2, imp-3, imp-4, imp-5 and its degradation products confirm the stability indicating power of the developed method.

3.3. Method Validation

3.3.1. Precision

The %RSD of assay of Milnacipran during assay method precision study and intermediate precision study was 0.3 and the %RSD of area of imp-1, imp-2, imp-3, imp-4 and imp-5 in related substance method precision study were within 2.0. Confirming the good precision of the developed analytical method.

3.3.2. Sensitivity

The limit of detection of imp-1, imp-2, imp-3, imp-4 and imp-5 were 0.001%, 0.009%, 0.007%, 0.006% and 0.008% (of analyte concentration, *i.e*., 250 μg·mL^{-1}) respectively for 5 μL injection volume. Under the same conditions, the LOQ were 0.003, 0.027, 0.021, 0.018 and 0.023% (of analyte concentration, *i.e*. 250 μg·mL^{-1}) respectively.

The precision at LOQ concentration for imp-1, imp-2, imp-3, imp-4 and imp-5 were below 2%.

3.3.3. Linearity and Range

Linear calibration plot for assay method was obtained over the calibration ranges tested, *i.e*. 10 - 40 μg·mL^{-1} and the correlation coefficient obtained was greater than

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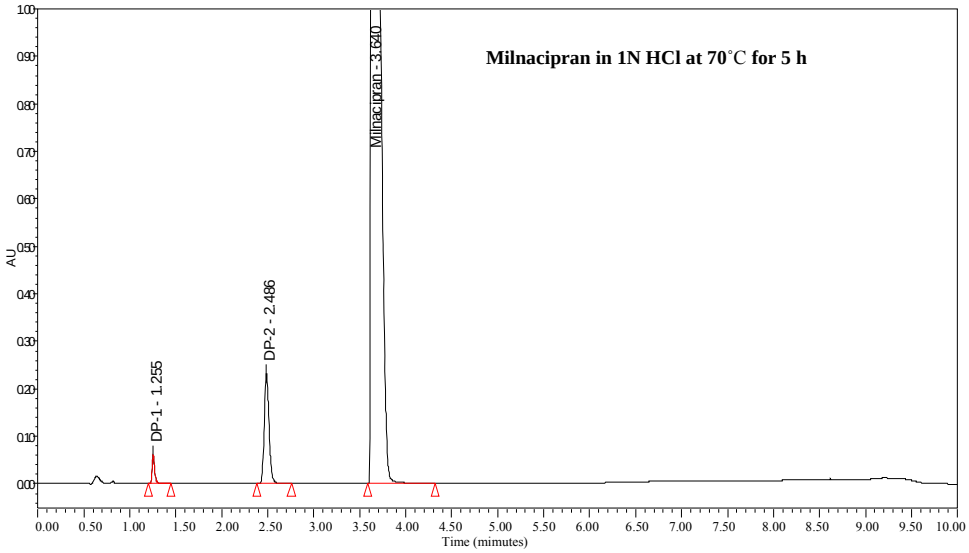
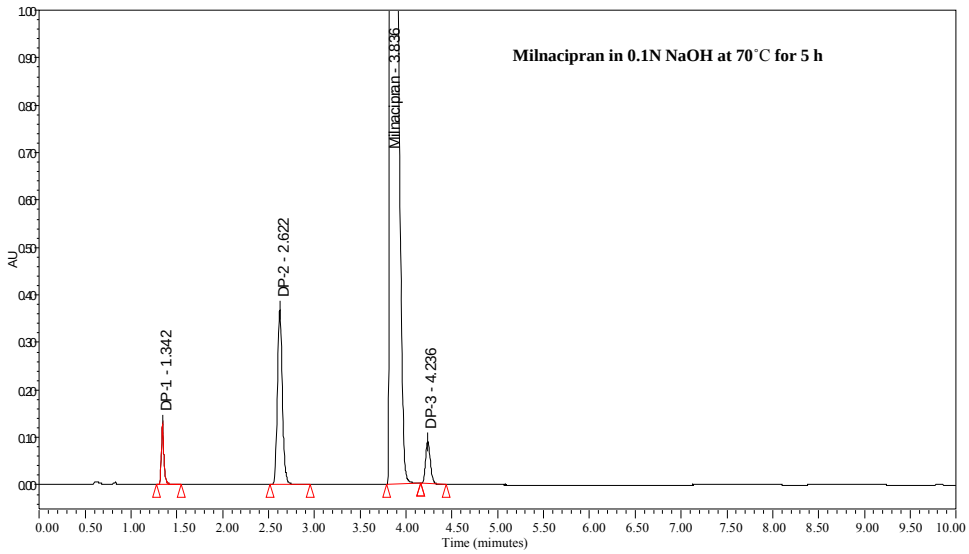


(a)



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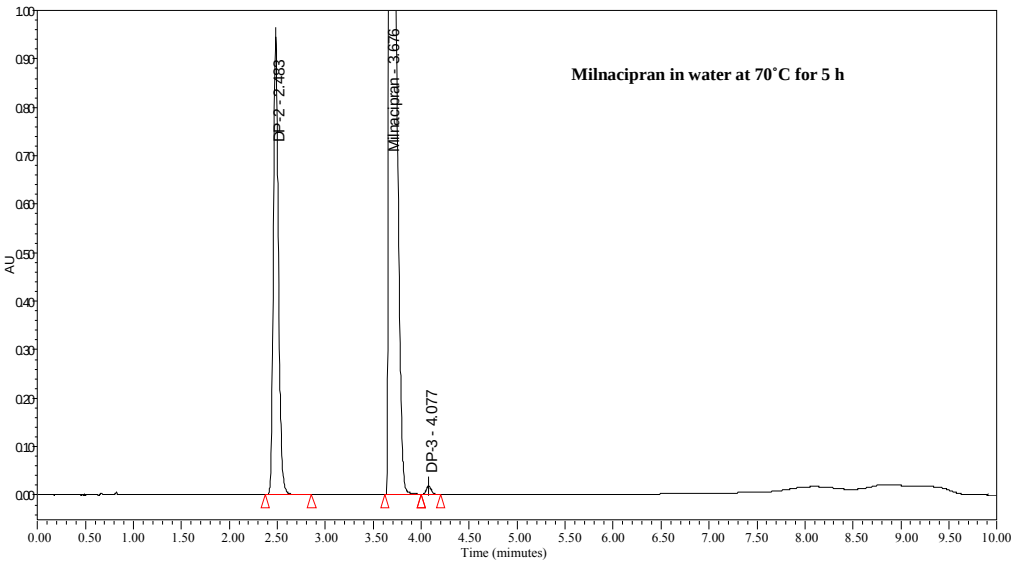

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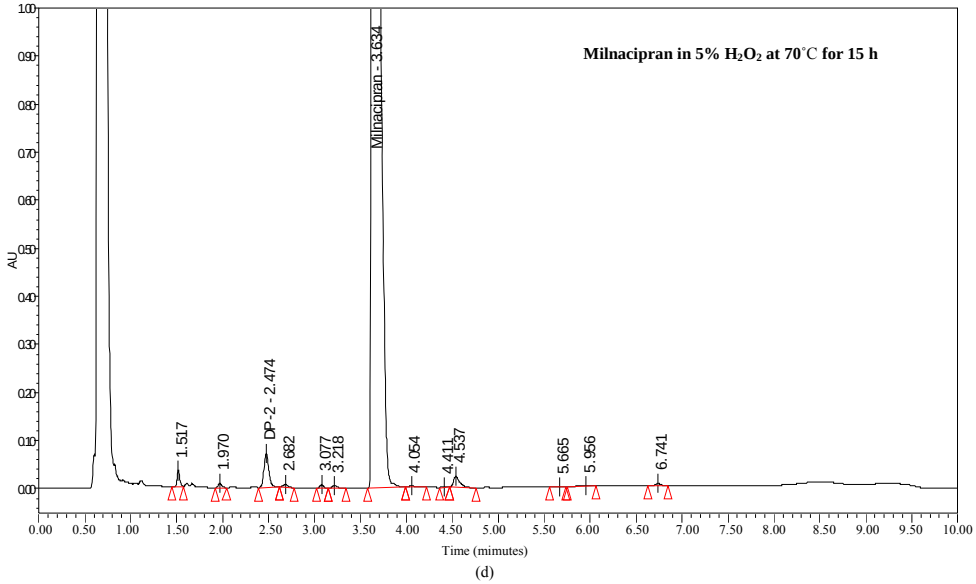

Figure 2. Typical chromatogram of stressed Milnacipran samples.

0.999. The result shows an excellent correlation existed between the peak area and concentration of the analyte.

Linear calibration plot for related substance method was obtained over the calibration ranges tested, *i.e*. LOQ to 0.3% for imp-1, imp-2, imp-3, imp-4 and imp-5. The correlation coefficient obtained was greater than 0.999 for all five impurities. The result shows an excellent correlation existed between the peak area and concentration of imp-1, imp-2, imp-3, imp-4 and imp-5.

The best-fit linear equation obtained was y = 7.3514x + 36.8876. At all concentration levels, standard deviation of peak area was significantly low and RSD was below 1.0%. Analysis of residuals indicated that residuals were scattered within ±2% with respect to 100% concentration response. Linearity was checked for related substances over the same concentration ranges on three consecutive days the %RSD of the slopes and Y-intercept of the calibration plots were with in 2.3 and 5.0 respectively. The range of the method was found from LOQ to 0.3% of the analyte concentration (250 $\mu g \cdot mL^{-1}$).

3.3.4. Accuracy

The percentage recovery of Milnacipran in bulk drug samples ranged from 98.3% - 101.1% and in pharmaceutical dosage forms ranged from 100.2% - 103.1% (**Table 2**). The percentage recovery of imp-1, imp-2, imp-3, imp-4 and imp-5 in bulk drug samples ranged from 99.7% to 102.1% (**Table 3**). HPLC chromatograms of spiked sample with all five impurities in Milnacipran bulk drug sample are shown in **Figure 3**.

3.3.5. Robustness

Close observation of analysis results for deliberately changed chromatographic conditions (flow rate, pH and column temperature) revealed that the resolution between closely eluting impurities, namely imp-1, imp-2, imp-3, imp-4 and imp-5 was always greater than 2.0, illustrating the robustness of the method.

3.3.6. Solution Stability and Mobile Phase Stability

The %RSD of assay of Milnacipran during solution stability and mobile phase stability experiments was within 1.0. No significant changes were observed in the content of imp-1, imp-2, imp-3, imp-4 and imp-5 during solution stability and mobile phase stability experiments. The solution stability and mobile phase stability experiments data confirms that sample solutions and mobile phase used during assay and related substance determination were stable up to the study period of 48 h.

3.3.7. Assay Analysis

Analysis was performed for different batches of Milnacipran in both bulk drug samples (n = 3) ranged from 99.95% - 99.96% and dosage forms (n = 3) ranged from 100.9% - 103.1%.

4. Conclusion

The RP-LC method developed for quantitative and related substance determination of Milnacipran in both bulk drugs and pharmaceutical dosage forms are precise, accurate and specific. The method was completely validated showing satisfactory data for all the method validation parameters tested. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of Milnacipran samples.

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Table 2. Results of accuracy study for bulk drugs and pharmaceutical dosage forms.

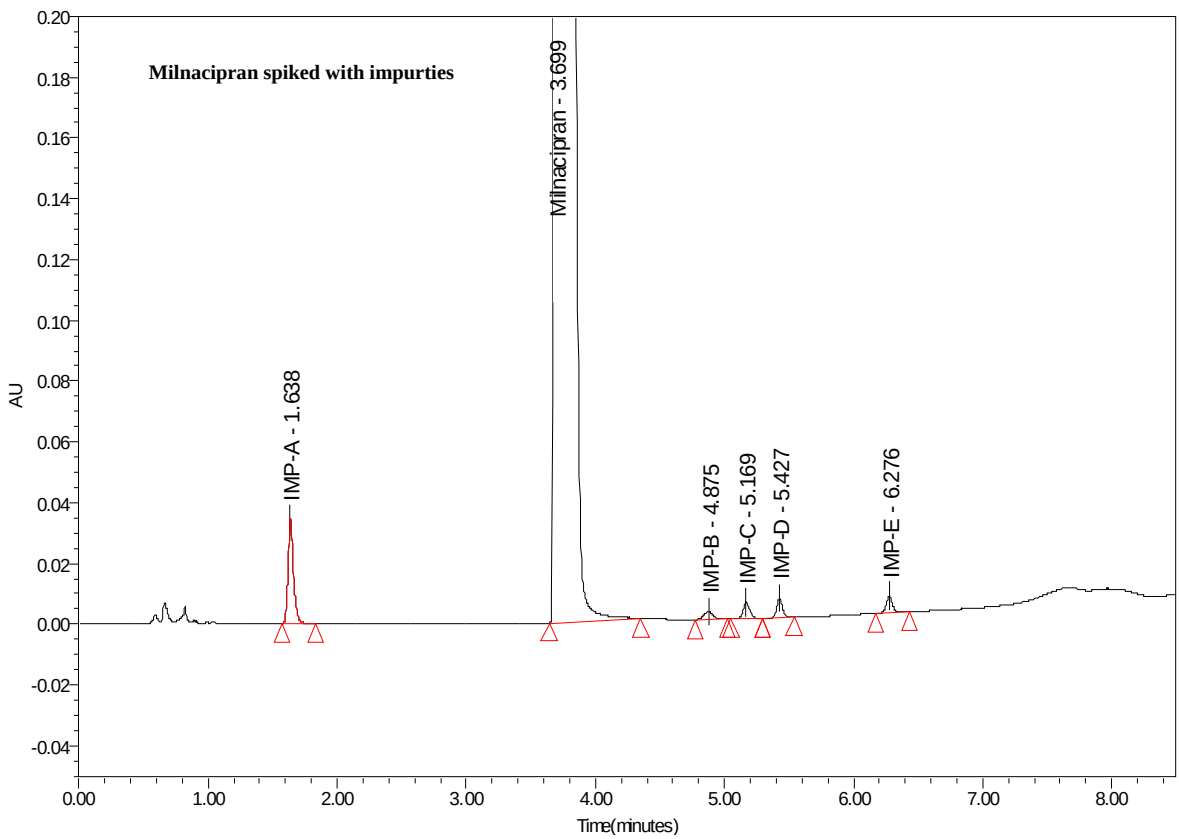
Added (µg) (n = 3)	%Recovery for Bulk drugs	%RSD for Bulk drugs	%Recovery for Pharmaceutical dosage forms	%RSD for Pharmaceutical dosage forms
10	99.8	0.7	100.8	0.4
15	101.1	0.8	102.8	0.2
20	98.3	0.2	103.1	0.5
30	99.4	0.3	101.0	0.6
40	99.1	0.2	100.2	0.2

n =3, Number of determinations.

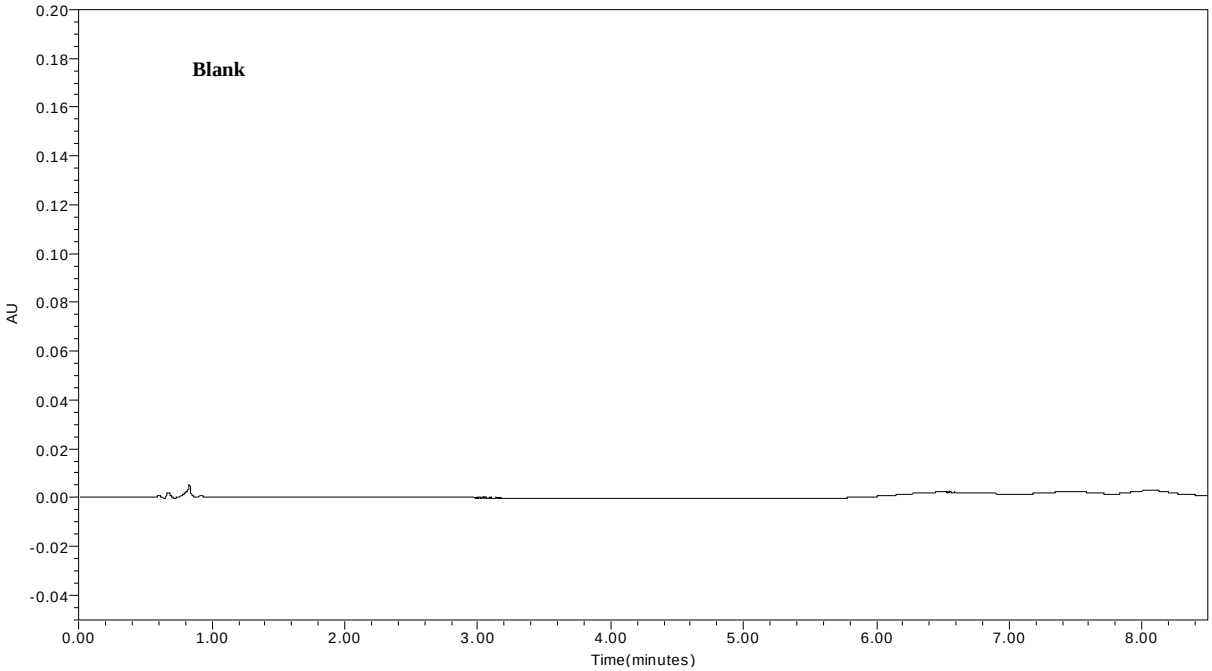
Table 3. Results of accuracy study for impurities.

No. of Accuracy level (n = 3)	%imp-1	%imp-2	%imp-3	%imp-4	%imp-5
Accuracy at 50%	101.5	100.1	99.8	100.1	100.1
Accuracy at 75%	100.8	101.1	101.0	101.5	100.8
Accuracy at 100% level	100.3	100.8	100.1	100.5	101.2
Accuracy at 150% level	101.7	99.7	101.5	99.9	99.8
Accuracy at 200% level	102.1	101.5	100.1	101.7	101.4

n = 3, Number of determinations.



(a)



(b)

Figure 3. Typical chromatogram of Milnacipran spiked with impurities at 0.15% specification level.

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