Research Article

Development of Validated Stability Indicating RP-HPLC Method for the Estimation of *levo*-Milnacipran Hydrochloride in Pure and Pharmaceutical Formulations

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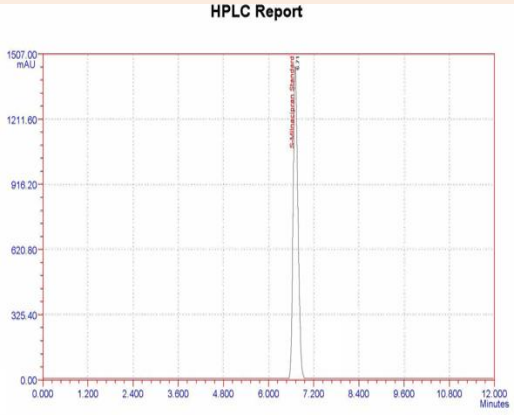
Abstract

A simple, specific, accurate and stability indicating RP-HPLC method was developed and validated for the determination of levo-Milnacipran hydrochloride in pure and tablet dosage form. The chromatographic conditions comprised of a reverse-phase, Kromasil-(C18) column (250mm x 4.6mm, 5µ) with mobile phase consisting of a mixture of Methanol, Water and Acetonitrile in the ratio of 85:5:10(v/v). Flow rate (1.1ml/min). Wavelength (217nm), Retention time (6.7min) and the calibration curve was found linear between 30-210µg/ml. The %recovery was found to be in the range of 99.22-100.22%. The method was validated and effectively separates the drug from its degradation product.



Keywords: (1S, 2R)-Milnacipran, levo-Milnacipran, RP-HPLC, validation, stability indicating method and dosage forms

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Introduction

levo-Milnacipran hydrochloride is an antidepressant. It is mainly used in the treatment of depression [1-3]. levo-Milnacipran is an active enantiomer of milnacipran and therefore has similar effects and pharmacology, acting as a serotonin norepinephrine reuptake inhibitor [2,4,5]. levo-Milnacipran hydrochloride is available in the market mainly in tablet dosage forms with different dosage concentrations. Most commonly it is administered orally [6]. The drug causes a sustained elevation in the synaptic levels of noradrenaline or serotonin or both in the central nervous system thus revealing the depression. Chemical structure of *levo*-Milnacipran hydrochloride is shown in **Figure 1**.

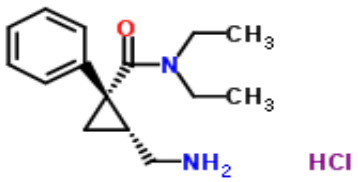

Figure 1 Chemical structure of *levo*-Milnacipran hydrochloride

Common adverse effects include nausea or vomiting, constipation, sweating and erectile dysfunction. Literature survey reveals that very few RP-HPLC methods have been reported for the estimation of *levo*-Milnacipran hydrochloride. In the present study a new RP-HPLC method has been reported for the estimation of *levo*-Milnacipran hydrochloride in pure and tablet dosage form.

Materials and Methods

Chemicals and reagents

levo-Milnacipran hydrochloride was obtained as a gift sample from Micro Labs Limited, Hyderabad, India. All the chemicals used like sodium hydroxide, hydrochloric acid and hydrogen peroxide are of analytical grade and mobile phase solvents of HPLC grade were purchased from MERCK Chem. Ltd., Mumbai, India.

Instrument and chromatographic conditions

In this method quantitative estimation of *levo*-Milnacipran Hydrochloride was done using isocratic RP-HPLC Peak instrument, separation module equipped with LC-7000 UV detector. The Peak HPLC software was used for peak integration along with data acquisition and data processing. The column used for the separation of analytes was Kromasil (C18) column (250mm x 4.6mm, 5µ). Mobile phase consisting of Methanol, Water and Acetonitrile in the ratio of 85:5:10 (% v/v) at a flow rate of 1.1ml/min. It was filtered through 0.45µm nylon filter and sonicated for 5min in ultrasonic bath. Samples were analyzed at 217nm at an injection volume of 20µl.

Preparation of standard solution

For the preparation of standard solution, accurately weighed working standard of 10mg of *levo*-Milnacipran hydrochloride into a 10ml clean dry volumetric flask, added 10ml of diluent, sonicated for 5min and made up to the final volume with diluent (standard stock-1mg/ml). From the standard stock solution, 1ml was pipetted out into a 10ml volumetric flask and made up to the mark with the diluent.

Preparation of sample solution

Ten tablets were weighed and average weight of each tablet was calculated then the weight equivalent to 10 tablets was transferred into a 10ml volumetric flask, 10ml of diluent was added and sonicated for 10min. Further the volume was made up with diluent and filtered. From the filtered solution 1ml was pipetted out into a 10ml volumetric flask and made up to 10ml with diluent.

Analysis of the marketed formulations

Twenty tablets (FETZIMA 50mg) were weighed accurately and crushed to form fine powder. Accurately weighed quantity of powder equivalent to about 50mg of levo-Milnacipran hydrochloride was dissolved in 50ml of volumetric flask with mobile phase. The flask was sonicated for 10min and then the solution was filtered using nylon membrane and appropriate volumes of the aliquot were transferred into three different 10ml volumetric flasks and then volume was made up to the mark with mobile phase to obtain 150µg/ml of levo-Milnacipran hydrochloride. The chromatographic conditions and peak areas were obtained.

Method development

The HPLC method carried out in this study aimed at developing chromatographic system capable of eluting and resolving levo-Milnacipran hydrochloride and its degradation products with the general requirements for system suitability. Initial trails were done on Zodiac (C18) column (250mm x 4.6mm, 5µ) performing elution with mobile

phase Methanol: Water (90:10 v/v) with gradient elution. This resulted in broad peaks, chromatogram retention time was reported 11.2min which was very longer and therefore can't be considered for analysis of drug samples. Various trails have been carried out in order to develop most suitable method for routine analysis of levo-Milnacipran hydrochloride. Further trails were done on Peak HPLC Kromasil (C18) column (250mm x 4.6mm, 5µ) and the isocratic method was employed for mobile phase with Methanol, Water and Acetonitrile in the ratio (85:5:10 v/v) at flow rate 1.1ml/min. Adding some proportion of Acetonitrile to the mobile phase initially improved the peak shape. However for improving the tailing factor and retention time was further improved by studying the separation of drug samples of different concentrations at different pH values ranging from 7.0 to 3.0. It was found that good resolutions of the peaks were achieved at acidic pH of 4.9.

After many logical trials, chromatographic condition was established such that it could be suitable for separation of drug and it is degradation products also separating impurities during elution from the chromatographic column.

Method Validation

The validation of the method was carried out as per ICH Guidelines [7, 8]. The parameters assessed were linearity, specificity, precision, accuracy, stability, LOD and LOQ.

Linearity and range

The linearity of an analytical procedure is the ability to obtain test sample results that are directly proportional to the concentration of an analyte in the sample. Seven solutions were prepared containing 30, 60, 90, 120, 150, 160 and 210µg/ml of *levo*-Milnacipran hydrochloride concentrations of the test solution. Each solution was injected in to HPLC system. Linearity was evaluated by linear-regression analysis. Corresponding peak area values of different concentrations were determined and graph was plotted between concentration on x-axis and peak area values on y-axis.

Precision

The Precision of the method was studied in terms of intraday and interday precision of sample injections of 150µg/ml concentration. Intraday precision was investigated by injecting drug samples on the same day where as in case of interday precision drug samples were injected on consecutive days into the chromatographic column. The standard deviation and relative standard deviation (coefficient of variation) were reported for each type of precision.

Recovery

The accuracy of the method was determined by recovery, by spiking of standard drug solution to pre analyzed sample at three different levels i.e., at 50, 100, and 150%. The resultant solutions were then re-analyzed by the developed method. At each concentration, sample was injected thrice to check repeatability and from the data it was analyzed and found that method was accurate.

Specificity

The specificity of the method was determined by injecting blank, drug solution and placebo sample solutions into the chromatographic column. The chromatograms were evaluated for analyzing the specificity of the method.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Robustness of the

method was assessed by making variations in mobile phase, wavelength and pH. An appropriate concentration of 150µg/ml of *levo*-Milnacipran hydrochloride was subjected to analysis.

Ruggedness

The ruggedness of the method was determined by changing the analyst performing the analysis on the instrument. The method was validated by performing the analysis by different chemists, in order to check the repeatability and to minimize the human errors.

Limit of Detection and Limit of Quantification

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected. LOD was calculated using the formulae, LOD=S/N where Average Baseline Noise obtained from Blank was named as (S), Signal obtained from LOD solution (0.25% of target assay concentration) was named (N).
The Quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the formulae, LOQ= S/N, where S was Average Baseline Noise obtained from Blank and N was Signal Obtained from LOD solution (0.75% of target assay concentration).

Forced degradation studies

The Forced degradation for *levo*-Milnacipran hydrochloride was studied by injecting dilute solutions of known concentrations treated with different forced degradation conditions. The typical forced degradation conditions used include acidic, basic, thermal, hydrolytic and photolytic degradation (in the range of ICH conditions). The drug samples were injected into HPLC column [9] and degradation studies have been carried out.

Photolytic degradation

To demonstrate the degradation in presence of light, *levo*-Milnacipran hydrochloride was kept in open petri dish under UV light and normal light. Drug sample was checked after exposed to 48 hours under UV and normal light. Sample solutions were prepared and injected into the chromatographic column. Further, chromatograms were compared with initial values and degradants were estimated in chromatograms.

Thermal degradation

To study the thermal degradation, drug samples were kept in Petri dish and placed in oven at temperature ranging from 40°C to 80°C for 48 hours. After 48 hours of exposure to high temperatures sample solutions were prepared and injected into the column. Degradedness in chromatogram were evaluated and compared to initial values.

Hydrolytic degradation under acidic condition

In order to study the degradation under acidic conditions, acid hydrolyzed samples at zero hours and 48 hours were prepared. Acid hydrolyzed sample solution was prepared by adding 300mg of drug sample in 20mL of 0.1N hydrochloric Acid. After keeping it for 48 hours 5ml of acid hydrolyzed sample was transferred in to 25ml volumetric flask and 5ml of sodium hydroxide was added for neutralization and volume was made upto the mark with diluent. The above prepared solution was injected into the chromatographic column after system suitability solution was injected and degradants in chromatogram were evaluated and compared with standard values.

Alkali degradation

To study the alkali degradation drug sample was weighed accurately and 300 mg of drug sample was added to 20ml of 0.1N Sodium hydroxide. It was kept for 48 hours and 5ml of alkali hydrolyzed sample was transferred to 25ml of

volumetric flask and 5ml of 0.1N hydrochloric acid was added for neutralization and made up to the mark with diluents. The above prepared solution was injected into the column after system suitability solution has been injected and then degradants in the chromatograms were evaluated and compared with initial values.

Hydrogen Peroxide

To study degradation of drug on oxidation, samples were prepared at zero hours and after 48 hours. 300mg of drug sample was mixed with 20ml of 3% hydrogen peroxide. After 48 hours 5ml of oxidized sample was transferred into 25ml volumetric flask and made upto the mark with diluents. The prepared oxidized sample was injected into the column after system suitability solution has been injected and degradants in chromatogram were compared with initial values.

Aqueous

300mg of drug sample was added to 20ml of aqueous solution. After 48 hours, 5ml of sample solution was added to 25ml volumetric flask and made up with diluent. The above solutions were injected once after system suitability solution has been injected and evaluated the degradants in chromatogram and compared with initial values (without aqueous solution).

Results and Discussions

The present study was carried out to develop a sensitive, précised and accurate stability indicating RP-HPLC method for the estimation of levo-Milnacipran hydrochloride in pure as well in pharmaceutical dosage form. In order to develop the method under isocratic conditions, mobile phase- Methanol, Water and Acetonitrile (85:5:10% v/v) were taken, pH was maintained at 4.9 and flow rate was adjusted to 1.1 ml/min. The mobile phase was freshly prepared and filtered through a 0.45μm membrane filter and degassed by an ultrasonic bath. The injections were carried out through a 20.0µl loop. The analytes were detected and quantified by UV detection at a wavelength of 217nm. The retention time observed was 6.71min which allows a rapid determination of the drug. In **Figure 2**, a typical standard chromatogram has been shown. In addition, system suitability test was performed by measurement of general characteristics such as peak asymmetry, number of theoretical plates and RSD (%) of peak area observed for a standard solution. The different parameters such as linearity, precision, specificity, robustness, ruggedness and recovery were tested and satisfactory results have been reported compared to other methods. The present method is good for analysis of different drug samples illustrated by results obtained from the analysis.

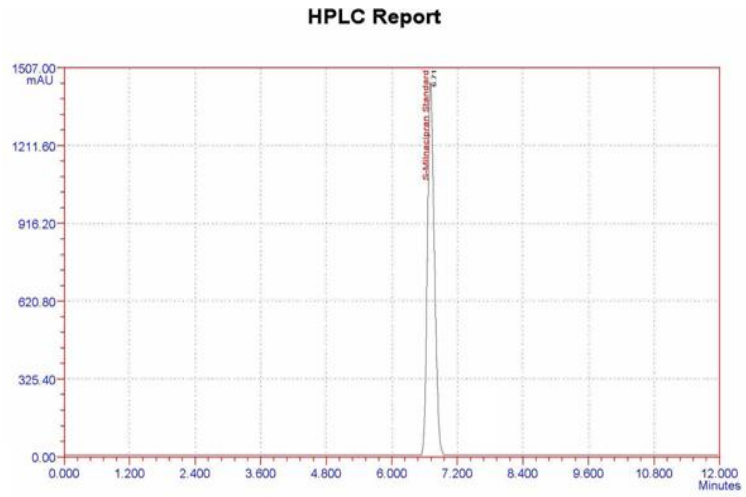

Figure 2 Standard chromatogram of levo-Milnacipran hydrochloride

Linearity

The calibration curve showed good linearity in the range of 30-210µg/ml, for *levo*-Milnacipran hydrochloride (API) with correlation coefficient (r^2) of 0.999. A typical calibration curve has the regression equation of y = 9667.0x + 11606. Results are given in Table 1 and Figure 3.

Table 1 Table showing values of concentration vs. area

S. No	Concentration (µg/ml)	Area
1	30	322442
2	60	582048
3	90	854069
4	120	1219602
5	150	1455480
6	180	1734523
7	210	2044926
	Slope	9611
	Intercept	19897
	CC	0.998

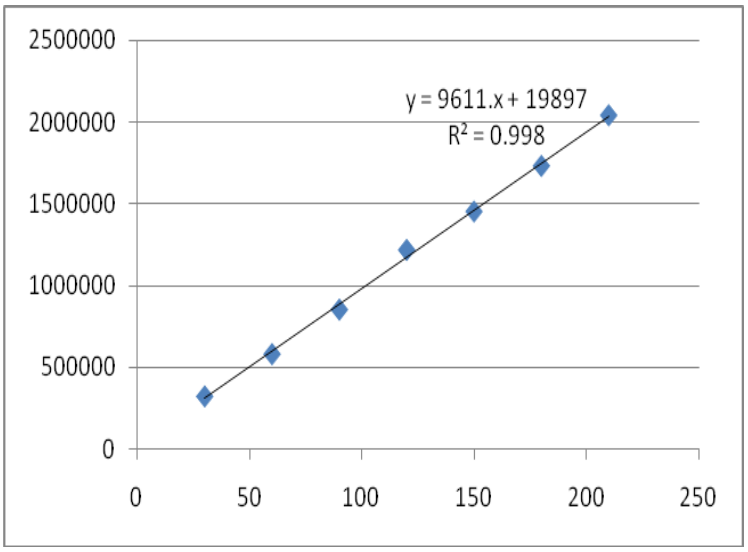

Figure 3 Calibration curve of *levo*-Milnacipran hydrochloride

Specificity

The drug solution when introduced into the column elutes at retention time 6.71min forming sharp peak, it was noted that no other peak was reported in the chromatogram depicting the fact that either blank or placebo solution doesn't consist of drug sample and also drug was unaffected by presence of other excipients and it forms sharp peak at same retention time repeatedly. Thus the developed method is specific for levo-Milnacipran hydrochloride.

Precision

Intraday precision was carried out using test samples prepared and analyzed on the same day. Interday precision was assessed by analysis of the same solutions on consecutive days. The % RSD was found to be 0.46 for intraday precision and 1.33 for interday precision. The low % RSD values below 2 indicate that the method is precise. The results are given in **Table 2** and **Table 3**.

Table 2 Table showing results of intraday precision

Sample (150µg/ml)	**Area**
1	1448390
2	1447815
3	1464755
4	1455647
5	1448326
6	1456094
RSD	**0.46**

Table 3 Table showing results of interday precision

Sample (150µg/ml)	**Area**
1	1463393
2	1483652
3	1465694
4	1483669
5	1440816
6	1439819
RSD	**1.33**

Robustness

Small deliberate changes in chromatographic conditions such as change in mobile phase ratio (± 10ml), change in pH (±1) and detection wavelength of (± 2nm) were studied to determine the robustness of the method. The results were in favor of (% RSD < 2%) the developed RP-HPLC method for the analysis of levo-Milnacipran hydrochloride. The results are given in **Table 4**.

Table 4 Table showing results of robustness

S.No	Parameter	Change	Area	% of Change
1	Standard	……..	1455480	……
2	Mobile phase	Methanol: Water: Acetonitrile 80:5:15	1461570	0.42
3		90:5:5	1466733	0.77
4	pH	4.8	1463854	0.57
5		5.0	1442523	0.89
6	Wavelength	215nm	1473067	1.21
7		219nm	1457347	0.13

Ruggedness

The RSD value 1.49 illustrates that the method is suitable to analyze different drugs as values are in order of repeatability depicting the precision of the method. Inspite of changing the analyst the peak area values were reported in repeated manner thus showing the efficiency of the method and so can be used to estimate various other drug samples using this method. The results are given in **Table 5**.

Table 5 Table showing results of ruggedness

Sample (µg/ml)	Area
1	1474128
2	1487654
3	1423314
4	1458219
5	1465479
6	1454869
RSD	**1.49**

Recovery

At each concentration, sample was injected thrice to check repeatability and from the RSD values it was analyzed that the method was accurate as % recovery values found to be in the range of 99.22 % to 100.22% at three different concentrations 90, 120 and 150µg/ml. The results are given in **Table 6**.

Table 6 Table showing results of recovery

% Recovery	Recovery					
	Target Conc. (µg/ml)	Spiked Conc. (µg/ml)	Final Conc. (µg/ml)	Conc. Obtained	% of Recovery	RSD
50%	60	30	90	89.30261	99.22	0.52
	60	30	90	89.52875	99.48	
	60	30	90	88.62967	98.48	
100%	60	60	120	118.4349	98.69	1.60
	60	60	120	122.1127	101.76	
	60	60	120	119.335	99.44	
150%	60	90	150	152.1755	101.45	0.92
	60	90	150	149.4567	99.638	
	60	90	150	150.3288	100.22	

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD was found to be 0.25µg/ml and the LOQ was found to be 0.8µg/ml estimated by using the standard formulas. The low values of LOD and LOQ illustrate that the developed method is sensitive, accurate and precise as it can be detected and quantify the drug samples with very low concentration.

Stability indicating studies

RP-HPLC study of samples obtained on stress testing of *levo*-Milnacipran hydrochloride under different conditions using mixture of Methanol, Water and Acetonitrile in the ratio 85:5:10 (v/v) as a mobile solvent system suggested the following degradation behavior.

It was noticed that under acid hydrolyzed conditions in presence of 0.1N HCl two other peaks in the chromatogram were observed. Similarly, effect of different stress conditions were evaluated and tabulated in **Table 7**.

Table 7 Table showing results of stress degradation

Condition after 48 hours	Observation on *levo*-Milnacipran hydrochloride
3% Peroxide	Degraded in to Three compounds.
0.1 N Basic	Degraded in to one compound
0.1 N Acidic	Degraded in to two compounds
Sun light	Degraded in to two compounds
UV light	Degraded in to three compounds
Aqueous (HPLC)	Degraded in to two compounds
Thermal (thermal)	Standard peak was spited into three peaks

Conclusion

The RP-LC method developed for quantitative determination of levo-Milnacipran hydrochloride in both pure and tablet drugs dosage forms was accurate, precise and specific. The method was completely validated showing satisfactory results for all the validation parameters tested in the analysis. The developed method is stability indicating and can be used for the routine analysis of production samples and also to check the stability of levo-Milnacipran hydrochloride drug samples.

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