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Development and Validation of RP-HPLC Method for Determination of Antidepressant Medicine Milnacipran Hydrochloride in Pharmaceutical Formulations

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ABSTRACT

Milnacipran hydrochloride is a racemic mixture of (±)-[1RS, 2SR]-2-(aminomethyl)-N, N-diethyl-1phenylcyclopropanecarboxamide hydrochloride. It is a drug officially approved by several developing and developed countries globally except United States of America as medicament for depression and depressive therapies. Few liquid chromatographic (LC) methods for determination of Milnacipran combined with other antidepressants in human plasma have already been published. Hence, the proposed research article predominantly focuses on method development and validation of antidepressant Milnacipran Hydrochloride by sensitive and precise reverse phase high performance liquid chromatography.

INTRODUCTION

Milnacipran is an antidepressant drug and it is available in some European Countries and in Japan for the treatment of depression. Recently, the Food and Drug Administration (FDA) approved it for the management of fibromyalgia syndrome, characterized by widespread pain and decreased physical function. This agent is unique among clinically available dual-reuptake inhibitors anti-depressants in its preferential blockade of norepinephrine (NE) reuptake over serotonin (SER) reuptake and minimal activity at other receptors or transporters. This profile is in contrast to those of SERNE reuptake inhibitors (SNRI) (e.g., venlafaxine and duloxetine), where SER reuptake is preferentially blocked, or NE-specific agents (noradrenergic reuptake inhibitors) (e.g. Reboxetine) in which the reuptake of NE is blocked. A micellar electrokinetic capillary chromatographic method was developed for separation and determination of antidepressants and their metabolites in biological fluids and LC enantio separation of Milnacipran was investigated on different cellulose-based chiral stationary phases.

However, no simple and sensitive isocratic RP-HPLC method with PDA detection is been reported for the determination of MIL in pharmaceutical formulations. Hence, the presented research work was aimed to develop and validate the simple, specific and sensitive RP-HPLC method for the determination of MIL in pharmaceutical formulation. The present work describes a simple reverse phase LC method for the determination of MILNA in Capsule dosage form. The method was validated according to ICH guidelines

Experimental:

MATERIALS AND METHODS:

Waters 2695 gradient system with autosampler, column oven, and PDA detector (Waters 2998). Data collection and analysis were performed using Empower- version 2 software. Separation was achieved on Inertsil ODS250X4.6mm, 5µ Column. The column was supported with waters symmetry C18, (waters C18, 20 x 3.9 mm, 5 µ) guard column. The peak purity was checked with the photodiode array detector. Sartorius analytical weighing balance - Model BP 211D, Thermo Orion 3 star pH Meter & Elema Fast clean Batch ultrasonicator was used for study.

Reagents and chemicals used

HPLC grade Acetonitrile was purchased from Fisher Scientific Chemicals (United Kingdom, Great Britain). Analytical reagent grade TEA was purchased from Merck Chemicals UK. Pure drug sample of MILNA, % purity 99.95 was obtained as a gift sample from Glenmark Pharmaceuticals Ltd. Mumbai. These samples were used without further purification. Tablet formulations (IXEL, Torrent Pharmaceuticals Ltd., India) containing labeled amount of 50 mg of MIL were procured from local market. Milli-Q Water was collected from a Milli-Q Liocel system (Millipore Ltd. Mumbai).

Chromatographic Conditions

The isocratic elution with Water: ACN: TEA (30: 70: 0.1 v/v/v) pH 7.8 with OPA mobile phase at the flow rate of 1.0 mL min^{-1} was carried out. The runtime was set at 8.0 min and temperature was maintained at 30 ^{0}C. The volume of injection was 20 µL, prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. Detector signal was monitored at a wavelength of 215 nm.

Standard Solutions and Calibrations Graphs

Standard stock solutions of MILNA (100µg mL-1) were prepared separately in mobile phase. To study the linearity range of each component, serial dilutions of MILNA were made from 0.5 - 50 µg mL^{-1}, respectively in mobile phase and injected on to the column. Calibration curves were plotted as concentration of drugs versus peak area response. The system suitability test was performed from six replicate injections of mixed standard solution. A typical chromatogram obtained from a standard solution is shown in Fig. No. 1

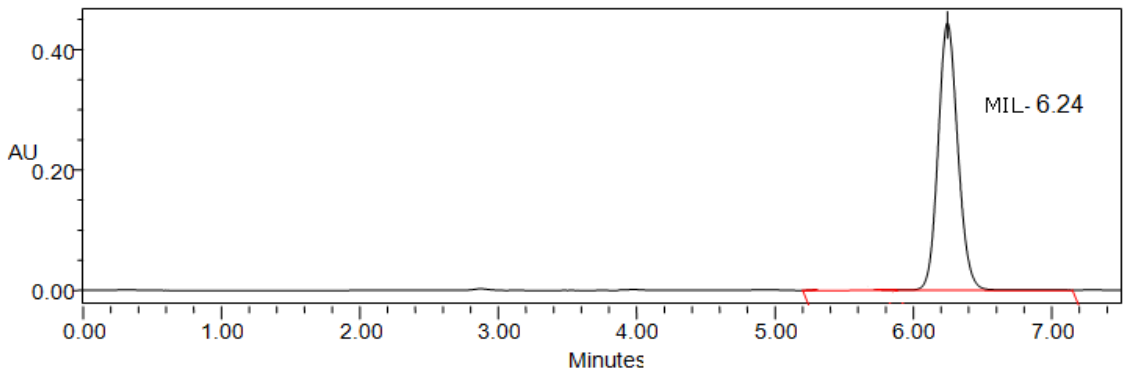

Fig. No. 1 Typical Chromatogram of Milnacipran HCl

Analysis of Tablet Formulation

Twenty tablets were weighed and net contents of each tablet was calculated. Tablet powder equivalent to 50 mg MIL was accurately weighed and transferred to a 100 mL volumetric flask containing about 60 mL of mobile phase, ultrasonicated for 5 min and volume was made up to the mark with the mobile phase. The above solution was centrifuged at 2500 RPM in the research centrifuge for 10 minutes and was filtered through 0.45 µm nylon filter. The first 10 mL of the filtrate was rejected and subsequent filtrate was further diluted with mobile phase to obtain the solution of 20 µg mL^{-1}. The resulting solution was used as sample solution for assay and was analyzed as given under the described chromatographic conditions.

Contents : Milnacipran – 50 mg

Manufacturer : Torrent pharmaceuticals Ltd. India.

METHOD DEVELOPMENT:

METHOD OPTIMIZATION:

A well-defined symmetrical peak was obtained upon measuring the response of eluent under the optimized conditions after thorough experimental trials that can be summarized. Two columns were used for performance investigations, including inertsil C18 (250 mm × 4.6 mm, 5.0 µ) and Kromasil C18 (250 mm × 4.6 mm, 5.0 µ), the First column was the most suitable one since it produced symmetrical peaks with high resolution. The UV detector response of MILNA was studied and the best wavelength was found to be 215 nm showing highest sensitivity.

Mobile phase composition:

Several modifications in the mobile phase composition were performed in order to study the possibilities of changing the selectivity of the chromatographic system. These modifications included the change of the type and ratio of the organic modifier, the pH, the flow rate, the temperature, the concentration of Triethylamine additives etc.

Type of organic modifier:

Initially, the methanol was used as an organic modifier which gives the poor baseline with baseline drift. Hence the response for drug compounds was reduced to improve the peaks shape and peak response, acetonitrile was tried as an organic modifier. The baseline was found good and peak response was improved. The peak shape and peak symmetry were also improved and hence acetonitrile was selected as organic modifier.

Ratio of organic modifier:

The effect of changing the ratio of organic modifier on the selectivity and retention times of the test solutes was investigated using mobile phases containing concentrations of 70-50% Acetonitrile. Table 1 shows that 70% Acetonitrile was the best one giving well symmetrical peak and higher no. of theoretical plates. Ratios less than 70% resulted in peak with very long unacceptable retention times, whereas ratios higher than 70% with decreased peak purity angle.

Effect of pH:

The effect of changing the pH of the mobile phase on the selectivity and retention times of the test solutes was investigated using mobile phases of pH ranging from 5.0-8.0. The pH 7.8 with OPA was the most appropriate one giving well-resolved peaks and highest no. of theoretical plates. At pH values > 5.0 produced peak broadening in the mobile phase.

Effect of Flow rate:

The effect of flow rate on the formation and separation of peaks was studied by varying the flow rate from 0.8-1.2 a flow rate of 1.0 ml/min was optional for good shape and symmetry of peaks in a reasonable time.

Effect of Temperature:

The effect of temperature on the formation, separation and resolution was studied by varying the temperature from 20 - 35^{o}C; we found that at lower temperatures the peaks are not well resolved, whereas at temperature above 28^{o}C the peaks shows good symmetry and purity.

Table No. 1: System suitability parameters with peak purity data

System Suitability Parameter	Milnacipran Hydrochloride
Retention Time	6.24
Theoretical plates[a] (T.P.)	5764
Peak Tailing[a]	1.12
K prime	2.49
% R.S.D. (T.P.)	0.78
PA and PT	0.117(0.739)

[a]USP - NF 29 section 621, Page.No. 2135.

METHOD VALIDATION

METHOD VALIDATION PARAMETER

The developed method was validated in terms of system suitability, specificity, linearity and range, precision, accuracy, limit of detection, limit of quantification, solution stability and robustness as per USP and ICH guideline

System suitability

The system suitability test was performed to ensure that the complete testing system was suitable for the intended application and it was performed by injecting the five replicate injections of standard preparation (20 µg mL-1). The parameters measured were retention time, theoretical plates, and asymmetry and peak area of MIL.

Linearity:

The stock solution was prepared by dissolving accurately weighed 50 mg in 50 ml of mobile phase to obtain a final concentration of 1.0 mg/ml. From this stock solution, Standards within concentration range 0.1-50 µg/mL were freshly prepared in mobile phase prior to analysis. Six replicates per concentration were injected and Chromatograms were recorded. Respective calibration curves were plotted of Area against concentration of each drug.

Accuracy

Accuracy of the method was calculated by recovery studies at three levels by standard addition method. The proposed method when used for extraction and subsequent estimation

of Milnacipran from pharmaceutical dosage form after spiking with additional drug afforded recovery of 98–102%.

Precision:-

System Precision:

The system precision is a measure of the method variability that can be expected for a given analyst performing the analysis and was determined by performing six replicate analyses of the same working solution. System precision was determined with the Capsule sample.

Precision for repeatability {Intraday and Interday}:

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The intra-day precision of the developed LC method was determined by preparing the tablet samples of the same batch in nine determinations with three concentrations and three replicate each. The R.S.D. of the assay results, expressed as a percentage of the label claim, was used to evaluate the method precision. The inter-day precision was also determined by assaying the capsule in triplicate per day for consecutive 3 days.

Range

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in the pharmaceutical product, to give accurate, precise and linear results.

Limit of Detection and Limit of Quantification

LOD is the lowest concentration of an analyte that can reliably be differentiated from background levels. LOQ of an individual analytical procedure is the lowest amount of analyte that can be quantitatively determined with suitable precision and accuracy. LOD and LOQ were calculated from standard deviation of the response and the slope of the three linearity curves using the formula 3.3 α/S for LOD and 10 α/S for LOQ where α is standard deviation of response and S is mean of slope of three calibration curves. The LOQ was verified by injecting six replicates at its concentration at the LOQ level of MIL.

Specificity:-

The specificity defined as the ability of method to measure the analyte accurately and specifically in the presence of components present in the sample matrix was determined by analysis of chromatograms of drug-free and drug-added placebo formulation. In peak purity analysis with photodiode detector, purity angle should be less than purity threshold for both the analytes.

Robustness of the method: -

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. The standard solution is injected in five replicates and sample solution of 100% concentration is prepared and injected in triplicate for every condition and % R.S.D. of assay was calculated for each condition.

Analytical Solution Stability:

To demonstrate the stability of standard working solutions and of tablet sample solutions during analysis, both solutions were analyzed over a period of 12 h while being stored at room temperature and for 24 h when refrigerated at 4 ^{0}C.

METHOD VALIDATION

The proposed method was validated according to the ICH guidelines with respect to linearity, specificity, accuracy, precision, LOD & LOQ and robustness. System suitability was established by injecting standard solution and results were given in (Table 8.1). The chromatograms were checked for the appearance of any extra peaks. No chromatographic interference from the tablet excipients was found. Peak purity was performed by using PDA detector where Peak Threshold is greater than Peak Angle.

Table. No. 2 System suitability parameters

System Suitability Parameter	Milnacipran Hydrochloride
Peak area	1108545
Retention Time	6.24
Theoretical plates[a] (T.P.)	5764
Peak Tailing[a]	1.12
USP Resolution[a]	-----
K prime	2.49
% R.S.D. (T.P.)	0.78
Purity Angle	0.117
Purity Threshold	0.437

[a]USP-NF 29 section 621, pp. 21

Linearity

The linearity of the developed method was determined in triplicate at different concentrations ranging from 0.1-50 µg mL-1. The regression analysis equation was y = 120781.66x+8122.88 and correlation coefficient (r) was 0.9992, showing good linearity. The results confirmed the linearity of the standard curves over the range studied and the excellent reproducibility of the assay method (Reference Table No.3)

Table No. 3: Linearity of Milnacipran (n = 6)

Standard Concentrations	0.1 µg/ml	1 µg/ml	10.0 µg/ml	20.0 µg/ml	30.0 µg/ml	40.0 µg/ml	50 µg/ml
Replicates	**Peak Area**						
1	12512	125543	1203567	2369465	3664534	4715466	6099463
2	12535	125456	1212145	2364985	3665768	4727565	6099564
3	12646	124566	1195467	2438596	3662183	4722635	6087546
4	12597	124967	1206478	2465556	3662348	4737353	6087489
5	12789	125145	1203435	2368976	3663434	4725252	6077896
6	12578	125213	1206578	2369767	3663024	4727242	6099756
Mean	12609	125148.3	1204611	2396224.2	3663549	4725918	6091952
SD	99.75	354.0077	5480.90	44128.581	1377.78	7151.09	9079.23
% RSD	0.79	0.28	0.45	1.84	0.37608	0.15	0.14

Formulation Analysis

The assay for the marketed tablets was established with present chromatographic condition developed. The average drug content was found to be 99.42 % of the labeled claim. No interfering peaks were found in chromatogram, as indicated by Peak Purity test. The results are given in Table No. 4

Table No. 4: Analysis of commercial Formulation

Sr. No.	Quantity claimed (mg/tab)	Quantity found (mg/tab)	% Label claim Determined
1.0	50.00	49.48	98.96
2.0	50.00	49.45	98.9
3.0	50.00	49.23	98.46
4.0	50.00	49.55	99.1
5.0	50.00	49.6	99.2
6.0	50.00	49.33	99.32
	Mean	49.44	98.99
	SD	0.138275	0.301662
	% RSD	0.279683	0.30474

Precision

Precision studies were carried out by repeating the analysis of the samples six times and the results shows that the mean assay value and %RSD are well within the acceptance criteria for the precision study. Similarly, the results of intermediate precision study (intraday, interday and different analyst) were also determined for

Mean assay value and %RSD. The results of precision and intermediate precision are shown in Table No. 5

Table No. 5: System precision of Milnacipran HCl (n=6)

Sample	% Assay
1	**99.4**
2	**99.0**
3	**98.7**
4	**99.1**
5	**99.1**
6	**99.4**
Mean	**99.12**
SD	**0.264**
%RSD	**0.262**

Accuracy

The accuracy was evaluated by the recovery of a known amount of MIL in synthetic mixture prepared by mixing MIL to placebo, to obtain concentrations of 80-120% of the labeled claim. The accuracy was calculated as the percentage of the drug from that percentage recovery values were calculated. The results were shown in table No. 6

Table No. 6: Results of the recovery analysis of Milnacipran hydrochloride

Level (%)	Amount of drug added in (mg)	Amount of drug found in (mg)	Mean Recovery (%)	Statistical Analysis	
S_1 : 80 %	40.1	39.9	99.50	Mean	99.00%
S_2 : 80 %	40.3	40.0	99.25	SD	0.6557
S_3 : 80 %	40.4	39.7	98.26	% RSD	0.6623
S_4 : 100 %	50.1	50.2	100.19	Mean	99.99%
S_5 : 100 %	49.8	49.6	99.59	SD	0.3044
S_6 : 100 %	50.3	50.2	99.80	% RSD	0.3044
S_7 : 120 %	60.2	60.5	100.49	Mean	99.88%
S_8 : 120 %	60.3	60.0	99.50	SD	0.5314
S_9 : 120 %	60.0	59.8	99.66	% RSD	0.5320

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ of Milnacipran HCl were determined by calibration curve method. LOD value was found to be 0.03 µg mL-1 and LOQ was 0.09 µg mL-1. The LOQ was verified by performing six replicate analysis at its concentration, the %RSD of precision at LOQ was 1.31.

Specificity: -

The specificity of the HPLC method is illustrated in Fig. No. 1 where complete separation of Milnacipran hydrochloride was noticed in presence of Capsule excipients. In addition, there was no any interference at the retention time of Milnacipran hydrochloride in the chromatogram of placebo solution. In peak purity analysis with photodiode detector, purity angle was less than purity threshold for both the analytes. This shows that the peak of analytes was pure and excipients in the formulation does not interfere the analytes.

Solution Stability

Stability as described in method development under experimental section was studied. Result of short-term, long-term and the autosampler stability of the Milnacipran hydrochloride solution were calculated from nominal concentrations and found concentration. Results of the stability studies were within the acceptable limit (98–102%) and the retention time, peak area of the Milnacipran hydrochloride remained almost unchanged (% R.S.D. less than 2.0) indicating that no significant degradation within the indicated period, which was sufficient to complete the whole analytical process.

Robustness

Robustness of the method was determined by making slight changes in the chromatographic conditions. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed, and System suitability parameters were found to be within acceptable limits. Results were shown in table 7 indicating that the test method was robust for all variable conditions.

Table No. 7. Result of robustness study

Parameter (Limit)	Level	System Suitability Parameters (SD) n=3				% Assay, % RSD, n=3
		t_R	N	Rs	K	
Flow rate ml/min (± 0.1 mL)	0.8 (-)	5.46	8607	---	4.46	99.59, 0.46
	1.0(+)	6.24	9443	---	5.24	100.01, 0.57
% of Organic (± 2%)	68(-)	5.23	8611	---	4.42	99.66, 0.52
	72(+)	6.14	9441	---	5.21	99.88, 0.60
pH of Mobile Phase (±mL)	7.6(-)	5.12	8603	---	4.47	99.48, 0.78
	8.0(+)	6.26	9442	---	5.32	100.02, 0.55
Separation column	Column I[a]	5.18	8613	---	4.48	99.65, 0.77
	ColumnII[b]	6.19	9440	---	5.15	99.74, 0.58
Measurement Wavelength (± 0.2 nm)	214.8(-)	5.48	8518	---	4.36	99.59, 0.65
	215.2(+)	6.45	9438	---	5.17	99.83, 0.95
Column Temp. (± 2ºC)	28(-)	5.10	8604	---	4.45	100.01, 0.76
	32(+)	6.22	9438	---	5.19	99.58, 0.98

CONCLUSION

In proposed study, sensitive isocratic RP-HPLC method has been developed for determination of MIL. The developed method was validated and was found to be simple, sensitive, accurate and precise. The method was successfully used for determination of MIL in its pharmaceutical formulations. As the method separates the

Drug from its degradation products as well as all the degradation products from each other therefore the method can be used for routine quality control analysis of MIL in industries for batch release.

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