Chromatographic and Computational Studies on the Chiral Recognition of Sulfated β-Cyclodextrin on Enantiomeric Separation of Milnacipran

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Highlights:

1. This is the first report of the enantiomeric separation of milnacipran hydrochloride with RP-HPLC using sulfated β-cyclodextrin, as a chiral mobile phase additive.
2. Molecular docking studies for chiral recognition mechanism.
3. A cost-effective method as separation time was limited to 5 minutes with the help of the core-shell column.

ABSTRACT

A new, cost-effective and fast chromatographic method using sulfated β-cyclodextrin (SβCD) as a chiral mobile phase additive (CMPA) was developed and validated for the enantiomeric separation of milnacipran. Milnacipran is an anti-depressant drug. Levo-milnacipran is the active enantiomer with less adverse effects than dextro-milnacipran. Hence, it is imperative to separate the enantiomers of milnacipran. Various parameters affecting enantiomeric resolution, for instance, the effect of type and concentration of cyclodextrins, the effect of pH of the mobile phase, effect of type and concentration of the organic solvent in the mobile phase and effect of type of achiral column, were investigated. We demonstrated successful resolution of enantiomers of milnacipran on reverse-phase HPLC with core-shell Kinetex C_8 column (150x4.6mm, 5μ), using a mobile phase consisting of 18:82 v/v acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate buffer pH 3.0 (adjusted with orthophosphoric acid) containing 10mM SβCD with a flow rate 1.0 ml/minute. The column temperature was ambient and UV detection was carried out at 227 nm with an injection volume of 20μl. This method for enantiomeric separation of milnacipran was validated in accordance with ICH guidelines and successfully applied to the marketed formulation of Levo-milnacipran. Furthermore, molecular docking was used to identify the chiral recognition mechanism. The results of molecular docking corroborated with our experimental findings.

Keywords:

Enantiomeric separation, Reversed phase-HPLC, Chiral mobile phase additive, Milnacipran hydrochloride, Sulfated β-cyclodextrin, Molecular docking.

1. Introduction

Milnacipran is an antidepressant drug that belongs to the class of serotonin and norepinephrine reuptake inhibitors (SNRIs). It shows better selectivity than serotonin for blocking the norepinephrine reuptake [1]. Milnacipran hydrochloride (±)-[1R(S), 2S(R)]-2-(aminomethyl)-N,N-diethyl-1-phenylcyclopropane-1-carboxamide hydrochloride has two chiral centres (**Figure 1**). Levo-milnacipran is the active enantiomer of milnacipran and was approved for the treatment of the major depressive disorder (MDD) by the US Food and Drug Administration (FDA) in 2013. Its efficacy is significantly higher than racemic milnacipran with fewer risks of cardiovascular disturbances and organ/tissue toxicity [2]. Hence, it is imperative to separate enantiomers of milnacipran.
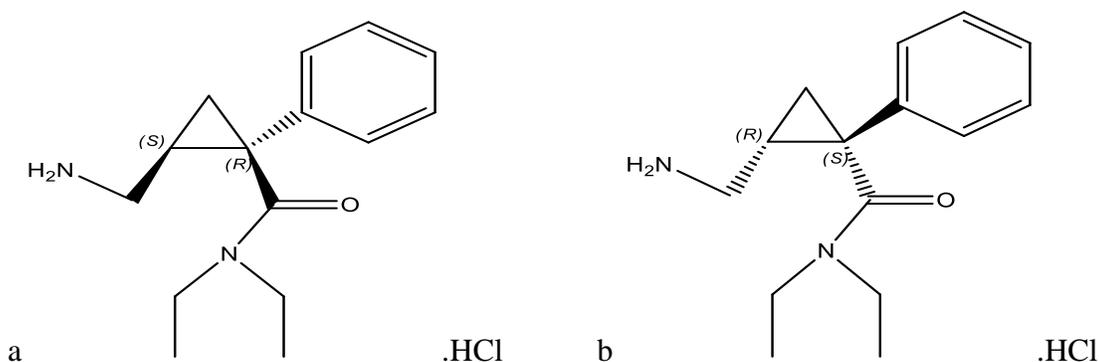

Figure 1: Structures of (a) (1R, 2S) - milnacipran hydrochloride (Dextro) and (b) (1S, 2R) - milnacipran hydrochloride (Levo)

Ever since the Thalidomide disaster in 1960, the US-FDA guidelines made it mandatory to evaluate properties of each enantiomer and racemates separately, and chiral switches for marketing off-patent drugs as a single enantiomer are the driving forces for increased marketing of single enantiomers of chiral drugs. Therefore, it is very important to separate enantiomers and to quantify the drug and chiral impurity. This requires a powerful means of enantiomeric separation and detection. [3, 4] The commonly used technique for chiral separation is High-Performance Liquid Chromatography (HPLC). Direct techniques utilize chiral stationary phases (CSPs) or chiral mobile phase additives (CMPA), and indirect techniques involve chiral derivatization [5]. In the CMPA technique, an enantiomerically pure compound (chiral mobile-phase additive) is added to the HPLC mobile phase. Reversed-phase HPLC is used to separate the enantiomers. This method offers several advantages like low cost of separation attributed to relatively inexpensive achiral stationary phases, absence of any derivatization step and improved flexibility compared to CSPs.

Cyclodextrins have been widely used as chiral selectors [5,6]. Owing to the internal hydrophobic cavity and external hydrophilic rim, cyclodextrins form inclusion complexes with hydrophobic molecules and enhance the dissolution rate by significantly reducing the non-polar surface area. β-cyclodextrin (βCD), Hydroxypropyl β-cyclodextrin (HβCD), Sulfated β-cyclodextrin (SβCD) were investigated as chiral mobile phase additives for the resolution of milnacipran enantiomers.

The survey of literature revealed that there are chromatographic methods for enantiomeric separation of chiral drugs using β-cyclodextrin as chiral mobile phase additives [7-14]. But there are no reported methods for enantiomeric separation of milnacipran using chiral mobile phase additives. Enantiomers of milnacipran have been separated on chiral columns, hydroxypropyl β-cyclodextrin-based chiral column Astec CYCLOBOND™ I 2000 RSP column in reversed-phase mode with resolution of 1.63[15], acetyl-*β*-cyclodextrin-based chiral column Astec CYCLOBOND™ I 2000 AC in reversed-phase mode with resolution of 1.74[16], Chiralcel OJ in normal-phase mode [17] and by a nonchiral HPLC– circular dichroism detector [18] has been reported.

In view of these facts, efforts were made to develop a cost-effective chiral HPLC method for enantiomeric separation of milnacipran using reverse phase column and β-cyclodextrin and its derivatives as chiral mobile phase additives. Since the method will be developed without using costly chiral stationary phases or costly chiral detector, the cost of the analysis will be significantly reduced. The aim of the present study is to separate and quantify the enantiomers of milnacipran using chiral mobile phase additives and to elucidate the chiral recognition mechanism.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Racemic and Levo-milnacipran hydrochloride was obtained as a gift sample from Arch Pharma Labs, Mumbai, India. All the chemicals used, i.e. sodium dihydrogen orthophosphate dihydrate HPLC grade and orthophosphoric acid AR grade were purchased from S.D. Fine Chem Pvt. Ltd, Mumbai, India. Methanol and acetonitrile HPLC grade were purchased from Merck Chem Ltd., Mumbai, India. Chiral mobile phase additives such as β-cyclodextrin and hydroxypropyl β-cyclodextrin were obtained as gift samples from Signet Chemical Corporation, Mumbai, India and sulfated β- cyclodextrin (degree of substitution 7-11) was purchased from Sigma Aldrich and synthesized in-house (Manuscript under process). Levo-milnacipran hydrochloride capsules

(Fetzima, 120mg) manufactured by Forest Pharmaceuticals, INC. USA were used for assay studies.

2.2 Instrumentation and chromatographic conditions

The HPLC system consists of Jasco 2000 HPLC, intelligent binary HPLC pump, PU-2080 plus, equipped with a 20 µl loop. The detector used was Intelligent UV/Vis detector UV-2075. Data acquisition and processing were performed using BORWIN software. The chromatographic columns used during method development were Kinetex C_8 (150 mm x 4.6 mm I.D., 5µm), Hyperclone C_8 (150 mm x 4.6 mm I.D., 5µm), and HiQsilC_8 (250 mm x 4.6 mm I.D., 5µm). The flow rate of the mobile phase was 1.0 ml/minute and the ambient column temperature was maintained. The eluent was monitored at a wavelength of 227 nm.

2.3 Preparation of the mobile phase

Acetonitrile and 10mM sodium dihydrogen orthophosphate dihydrate buffer pH 3.0 (adjusted with orthophosphoric acid) containing 10mM SβCD were used as mobile phase components. The mobile phase was filtered through a 0.45µm durapore nylon filter and sonicated for 5 minutes before use.

2.4 Preparation of sample solution

Stock solutions of the racemic milnacipran and Levo-milnacipran were prepared by dissolving 20 mg of each drug in 20 ml acetonitrile separately. Working standard solutions were prepared by suitable dilutions of primary stock solution with the mobile phase.

2.5 Preparation of solutions for precision

For precision 15, 30, and 45 ppm concentration solutions of racemic milnacipran were used (7.5, 15, and 22.5 ppm for each enantiomer). 1000 ppm stock solution of racemic milnacipran was used for preparing various concentrations. Aliquots of racemic milnacipran standard stock solution (1000 ppm) of 150, 300, and 450µl were transferred in three different 10 ml volumetric flasks and diluted with the mobile phase to make 15, 30, and 45 ppm respectively. All solutions were pipetted out by 100-1000 µl micropipette.

2.6 Preparation of solutions for accuracy

For accuracy study target concentration for racemic milnacipran was chosen as 30 ppm (15 ppm for each enantiomer). Accuracy test was performed at three concentration level i.e. 50%, 100%, and 150%. For accuracy 7.5, 15, and 22.5 ppm concentration solutions of Levo-milnacipran were used. The volume of racemic milnacipran and Levo-milnacipran required for accuracy study at

different concentration level are mentioned in **Table 1.** Aliquots of racemic milnacipran and Levo-milnacipran standard stock solution (1000 ppm) were transferred in three different 10 ml volumetric flasks and diluted with the mobile phase.

Table 1: Volume of racemic milnacipran and Levo-milnacipran for accuracy studies

Concentration level (For levomilnacipran)	Volume of 100 µg/ml racemic milnacipran (µl)	Volume of 100 µg/ml levomilnacipran (µl)	Final concentration of levomilnacipran (µg/ml)
50% (7.5ppm)	1000	250	7.5
100% (15ppm)	1000	1000	15
150% (22.5ppm)	1000	1750	22.5

2.7 Assay of Levo-milnacipran hydrochloride in the formulation

20 capsules of Levo-milnacipran hydrochloride were weighed and powder equivalent to 25mg Levo-milnacipran hydrochloride was accurately weighed and transferred to a 25ml standard volumetric flask. 20ml of acetonitrile was added to the flask and sonicated for 30 min, and volume was made up using the same solvent. An aliquot of the solution was filtered through a 0.45µm nylon filter and 150µl transferred to a 10ml standard volumetric flask to yield a concentration of 15µg/ml.

2.6 Molecular Docking to estimate complexation stability

The coordinates of βCD were imported from the protein data bank co-crystallized with glycosyltransferase (PDB id 3CGT). The protein around βCD was deleted to obtain the coordinates of βCD. The 3D model of SβCD was constructed by modifying the CH_2OH groups at the 6th position of the glucose moieties of βCD with SO_3^{1-} group to maintain the degree of substitution as 7. The GLIDE module in Schrödinger Suite 2015 was used for docking the drug in the SβCD cavity. The grid for docking was prepared by defining the centre of mass (COM) of the host molecules (considering all atoms) and the dimensions of the inner grid box was fixed at 15 Å x 15 Å x 15 Å and 20 Å x 20 Å x 20 Å for the outer grid box to allow enough sampling space. Prior to docking, drugs were optimized to correct their geometry using the LIGPREP module in Schrödinger Suite 2015 using the OPLS 2005 force field. During docking, the van der Waals radii for the hosts and the guest molecule were scaled to 0.60 Å to soften the potential over the non-polar areas that lie within the grid extents and the partial atomic charges were set to 0.25 (default). The GLIDE Emodel and the standard-precision scoring function were employed to rank the docking solutions.

3. RESULTS AND DISCUSSION

3.1 Chiral HPLC Method development

To achieve enantiomeric separation of milnacipran, factors affecting enantiomeric resolution were investigated, which include the effect of types and concentration of β-cyclodextrins, the effect of pH, effect of organic solvent, and the effect of type of achiral column.

3.1.1 Effect of types and concentration of β-cyclodextrins

The method development was started with the addition of β-CD in the aqueous mobile phase. 10 and 15mM were the concentrations of β-CD used. The saturation solubility of β-CD in water is 15mM, and hence, a higher concentration of β-CD could not be used. Since the limited solubility of β-CD in water was the major limitation. Hp-β-CD was used as CMPA, because it has good solubility in water therefore, it can be used at higher concentrations. 10, 15, and 25mM were the concentration of Hp-β-CD used. Then 10mM S-β-CD was employed as CMPA. The results of trials taken with β-CD, Hp-β-CD, and S-β-CD as CMPA at various concentrations and mobile phase compositions are mentioned in **Table 2.**

Table 2: The results of trials taken with β-CD, Hp-β-CD, and S-β-CD as CMPA

S. N.	CMPA	Mobile phase ACN: Buffer	Retention time (Minutes)	Resolution	Run time (Minutes)
1	10mM β-CD	20:80	19.8	-	25
2	10mM β-CD	10:90	85.08 & 88.06	1.2	100
3	15mM β-CD	10:90	46.76 & 47.87	0.89	52
4	10mM Hp-β-CD	20:80	19.94	-	22
5	10mM Hp-β-CD	10:90	62.06 & 64.47	1.14	70
6	15mM Hp-β-CD	10:90	49.7 & 52	1.24	57
7	25mM Hp-β-CD	10:90	31.8 & 33.4	1.06	38
8	10mM S-β-CD	10:90	38.33 & 48.62	6.41	52
9	10mM S-β-CD	20:80	9.16 & 10.15	2.98	12
10	10mM S-β-CD	30:70	4.4	1.05	6

Mobile phase: Acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate buffer CMPA pH 3.0 adjusted with orthophosphoric acid Column: HiQsil C8 (250x4.6mm, 5μ), Flow rate: 1ml/min, Detection wavelength: 227 nm

From results, one pattern was observed. Whenever the concentration of CMPA was increased, retention times decreased. As the concentration of CMPA increased, the polarity of diastereomeric complex increased and complex will not be retained on the non-polar C8 column, which affects resolution. When β-CD was used, 10mM β-CD gave a resolution of 1.2 in the run-time of 100minutes. When HP-β-CD was used, 15mM HP-β-CD gave a resolution of 1.24 in the run-time

of 57 minutes. Enantiomers of milnacipran were successfully resolved when S-β-CD was used as CMPA. Initially, trials were taken on a fully porous reversed-phase silica column, HiQsil C8 (250x4.6mm, 5μ). The mobile phase composition was found to be important for enantioselectivity. The resolution between enantiomers and retention times increased as the aqueous phase containing CMPA was increased. A good resolution of 2.98 between milnacipran enantiomers was obtained with a mobile phase comprising acetonitrile: buffer (20:80). The run time was 12 minutes. In order to further optimize the method, a core-shell silica column was used.

Core-shell silica column:

Core-shell silica columns have solid core covered with a porous layer or shell of a silica adsorbent. The solid core plus the porous shell provides columns with speed and efficiency with low operating back pressure and it also reduces band broadening thus improving resolution. The porous shell provides less band broadening by reducing the dispersion of the solute molecules within a packed bed owing to a lower pore volume available for longitudinal diffusion (B term in the van Deemter equation-Eq.1). A shorter diffusion path length because of solid core, diminishes the contribution of the C term to band broadening due to the fast mass transfer. The contribution to A term (column packing regularity) is significantly reduced, because of a rougher surface of core-shell particles [19]. In equation 1, H is height equivalent to the theoretical plate, A is eddy diffusion, B is longitudinal diffusion, C is mass transfer and μ is linear velocity

$$H=A+B/\mu+C\mu\ldots\ldots\ldots\ (Eq.1)$$

Two achiral HPLC columns were investigated for enantiomeric resolution. One was a column with fully porous silica particles (HiQsil C8, 250x4.6mm, 5μm) and another was a column with core-shell silica particles (Kinetex C8, 150x4.6mm, 5μm).

From the comparison between two achiral columns, huge disparity was observed in the retention times and resolution. The probable parameters were the change in the length of the column and core-shell particles. Hence, to identify the cause, further studies were conducted on another fully porous silica column i.e. Hyperclone C_8 150mmx4.6mm, 5μm. The same chromatographic conditions were applied for enantiomeric separation of milnacipran hydrochloride on Hyperclone C_8 Column. The results obtained are shown in **Table 3** and **Figure 2**.
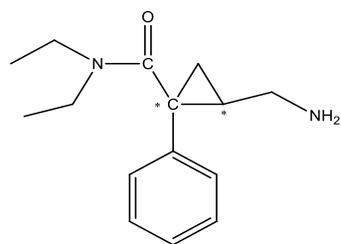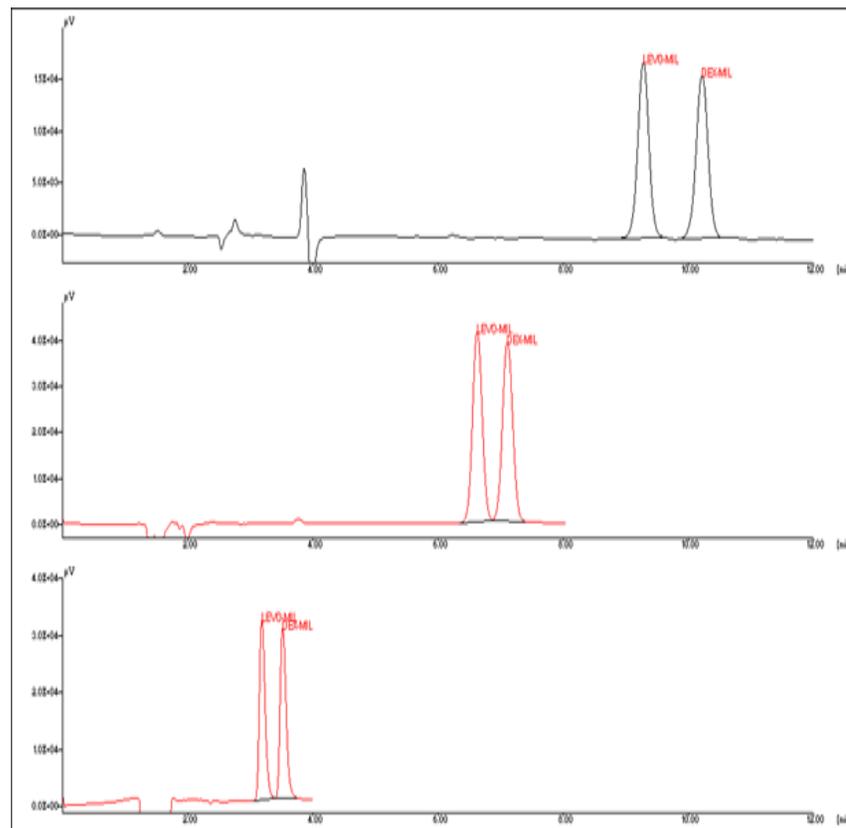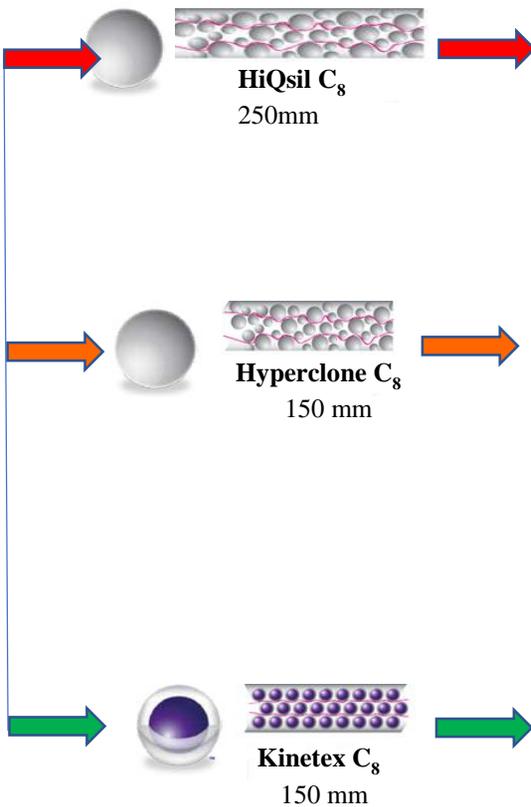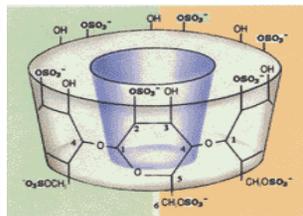
Figure 2: Comparison of chromatographic performances of three achiral columns

Levo-Mil=Levo-milnacipran , Dex-Mil= Dextro-milnacipran
Chromatographic conditions: Mobile phase: Acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate buffer 10mMS-β-CD pH 3.0 adjusted with orthophosphoric acid (20:80), Flow rate: 1ml/min, Detection wavelength: 227 nm

Table 3: Comparison of chromatographic performances of three achiral columns

S. N.	Achiral Column	Retention time (Minutes)	Resolution	Run time (Minutes)
1	HiQsil C_8 Column (250mmx4.6mm, 5μ)	9.1 & 10.1	2.98	12
2	Hyperclone C_8 Column (150mmx4.6mm, 5μ)	6.7 & 7.1	1.76	8
3	Kinetex C_8 Column (150mmx4.6mm, 5μ)	3.2 & 3.5	2.48	4

Mobile phase: Acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate buffer 10mM S-β-CD pH 3.0 adjusted with orthophosphoric acid (20:80), Flow rate: 1ml/min, Detection wavelength: 227 nm

From these results, it was observed that the variations in retention times and resolution were not only due to change in length of the column but also due to the core-shell particles i.e. Kinetex C_8 column. In the case of Hyperclone C8, the resolution between enantiomers was not good though retention times were higher than Kinetex C8. This can be attributed to peak width since it is inversely proportional to resolution (Eq.2). In Hyperclone C8, peaks are wide hence reducing resolution and in kinetex C8 peaks are sharp hence good resolution is obtained.

$$Rs = 2(t2-t1)/ (W1+W2) \ldots\ldots\ldots\ldots \text{(Eq.2)}$$

It is beneficial to use a core-shell column because all the system suitability parameters were satisfied with less analysis time. The consumption of the chiral selector (Sulfated-β-CD) was less when the core-shell column was used. Hence, the method with the core-shell column can be considered cost-effective. Therefore, it was decided to develop the method on the Kinetex C8 column.

3.1.2 Optimization of concentration of sulfated β-cyclodextrin:

The concentration of SβCD had a prominent effect on retention time and ultimately on the resolution of milnacipran enantiomers. 8 and 10mM concentrations of SβCD were investigated. At 8mM concentration, we observed an increased retention time with a decrease resolution. On the other hand, at 10mM concentration of SβCD, the resolution was better with relatively less retention time seen. This can be attributed to enhanced host-guest complex formation between SβCD and milnacipran enantiomers due to higher SβCD concentration. The polar diastereomeric complex formed was not retained on the non-polar stationary phase. Therefore, 10mM of SβCD was considered optimum. The Mobile phase was acetonitrile: 10mM sodium dihydrogen phosphate buffer 10mM S-β-CD pH 3.0 adjusted with orthophosphoric acid (20:80).

3.1.3 Optimization of pH of the mobile phase

The pH of the mobile phase is one of the important parameters governing the resolution of enantiomers. It was observed that the best separation was obtained when the pH of the mobile phase was kept 3. Although, the retention times were nearly the same for pH 3, 4 and 6., the resolution was best (Rs=2.48) with pH 3. This was because, at pH 4 and pH 6, wider peaks were observed and hence resolution was decreased. The result obtained with different pH of the mobile phase is shown in **Figure 3**.
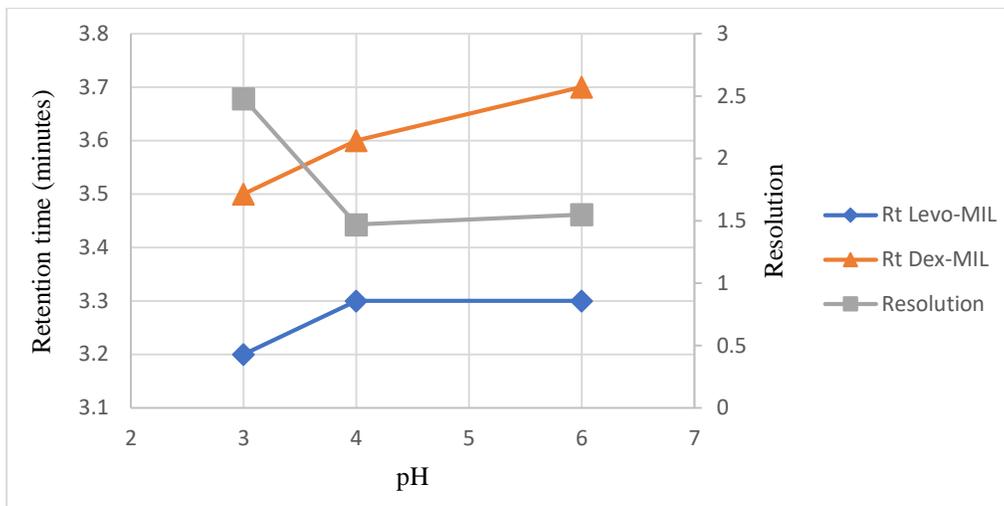
Figure 3: Effect of pH on retention times and resolution
Chromatographic conditions: Mobile phase: Acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate buffer 10mM S-β-CD pH (3, 4, 6) adjusted with orthophosphoric acid (20:80), Column: Kinetex C8 (150x4.6mm, 5μ), Flow rate: 1ml/min, Detection wavelength: 227 nm

3.1.4 Optimization of the organic solvent

The effect of concentrations of methanol on retention times and resolution were studied. Instead of 20 % acetonitrile, 20% methanol was used as an organic solvent, but retention times were quite high with high resolution. On using 30% Methanol, retention times decreased with a satisfactory resolution. But due to high column back-pressure and increased retention times with methanol as compared to acetonitrile, acetonitrile was selected as an organic solvent. With 20% acetonitrile in mobile resolution between enantiomers was 2.48. In order to increase resolution, 18% acetonitrile was tried, which increased retention times and resolution (2.96).

Final Developed HPLC Method:

The final optimized mobile phase was acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate buffer, pH 3.0 adjusted with orthophosphoric acid with 10mM Sulfated

β cyclodextrin as chiral mobile phase additive. The order of elution was determined by injecting a single enantiomer. The Levo-milnacipran eluted early and Dextro-milnacipran eluted later. The chromatogram of the optimized method is shown in **Figure 4**. The system suitability parameters for the final developed method are mentioned in **Table 4**.
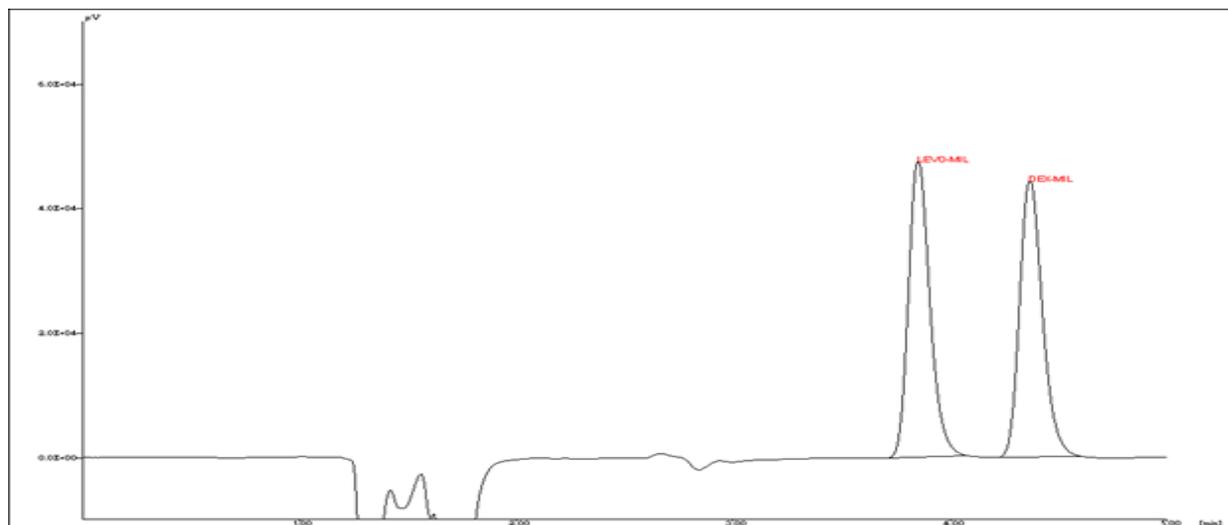
Levo-Mil = Levo-milnacipran , Dex-Mil = Dextro-milnacipran
Chromatographic conditions: 1) Mobile Phase: Acetonitrile: 10mM sodium dihydrogen orthophosphate dihydrate Buffer containing 10mM sulfated β-CD, pH 3.0, (18:82 v/v); 2) Column: Kinetex C_8, 150 mm x 4.6 mm, 5μ. 3) Column temperature: Ambient 4) Flow rate: 1ml/minute; 5) Detection wavelength: 227 nm
Figure 4: Enantiomeric separation of racemic milnacipran hydrochloride

Table 4: System suitability parameters

Enantiomers	Retention times (minutes)	Resolution	Number of theoretical plates	Symmetry Factor
Levo -Mil	3.85	-	8365.27	1.25
Dex -Mil	4.36	2.96	9330.82	1.26

Levo-Mil=Levo-milnacipran , Dex-Mil= Dextro-milnacipran

3.2 Method Validation:

After optimization of the parameters affecting the enantiomeric resolution of milnacipran, the method was validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, accuracy, and robustness as per ICH guidelines on both the columns.

3.2.1 Specificity:

Specificity study was conducted for the interference of any other peak at the retention time of the enantiomers peak in blank solution. Diluent was injected as a blank solution. Both peaks were well separated and there was no interference from blank. All system suitability parameters were as per norms. The system suitability parameters are shown in **Table 6**.

3.2.2 Linearity

The working standard solutions for linearity determination were prepared by diluting the stock solution of racemic milnacipran to the required concentrations in the mobile phase. These solutions were injected three times and peak areas were recorded. A linearity curve was plotted between peak areas versus analyte concentration for both the enantiomers. After linearity was plotted, the slope, y-intercepts, correlation coefficient and regression coefficient (r^2) was determined. The linearity equation for Levo-Mil and Dex-Mil is $y = 25453x + 82775$ and $y = 25355x + 85118$ respectively. Good correlation coefficient (r^2) = 0.9991 was obtained for both the enantiomers.

3.2.3 Precision

The precision of the method was performed by repeatability and intermediate precision studies. The repeatability of the method was determined by making 3 injections at three different concentration level (15, 30 and 45 µg/ml) of working standard solutions of racemic milnacipran hydrochloride and %RSD of peak area was calculated for each of the enantiomers. The intermediate precision of the method was performed by intra-day precision, inter-day precision, and different instrument. For intermediate precision, 6 determinations of working standard solution of racemic milnacipran were carried out and %RSD of peak area was calculated for each of the enantiomers. % RSD of replicate injections in repeatability and intermediate precision was found to be less than 2% for both enantiomers.

3.2.4 Accuracy

The accuracy of the method was assessed by conducting recovery studies. Recovery studies for Levo-milnacipran were conducted at three concentration levels i.e. at 50% (7.5 µg/ml), 100% (15 µg/ml) and 150% (22.5 µg/ml) of target concentration. Each concentration level was analyzed in triplicate and % recovery was calculated. The % recovery for both enantiomers was found to be from **99-101%.** The results are shown in **Table 5**.

Table 5: Recovery results

Actual concentration of Levo-milnacipran (µg/ml)	Recovered concentration (µg/ml), Mean ± SD	% Recovery Mean ± SD
7.5	7.52 ± 0.19	99.05 ± 0.53
15	15.2 ± 0.14	101.53 ± 0.95
22.5	22.4 ± 0.17	99.68 ± 0.76

3.2.5 Limit of detection and quantification

The LOD and LOQ were determined by injecting a series of dilute solutions. LOD and LOQ were calculated on the basis of the response and slope of the regression equation. They were calculated from the formula $3.3\sigma/s$ and $10\sigma/s$ respectively where 'σ' is the standard deviation of the y-intercept of the regression line and 'S' slope of the calibration curve. The limit of detection and limit of quantification was found to be **0.125** μg/ml & **0.5** μg/ml respectively for both enantiomers.

3.2.6 Robustness

To determine the robustness of the method, deliberate changes were made in the experimental conditions of the developed method. The resolution between two enantiomers and system suitability parameters were evaluated. The variables evaluated in the study were flow rate (±0.1 ml/min.), pH of mobile phase (±0.2), Mobile phase composition (±2 Units) and variation in detection wavelength (±2 nm). The robustness was determined from triplicate injections of racemic milnacipran hydrochloride. In all cases, the resolution was greater than 2.0, %RSD of peak area was less than 2 and the tailing factor was less than 1.5 for both the enantiomers.

3.3 Application of the method

The validated HPLC method was used for the quantitative analysis of Levo-milnacipran hydrochloride in pharmaceutical formulation (Fetzima). The assay chromatogram of capsule formulation is shown in **Figure 5**. The assay of Levo-milnacipran hydrochloride in the marketed formulation is presented in **Table 6**.

Table 6: Assay of Levo-milnacipran hydrochloride in pharmaceutical formulation

| Formulation	Label Claim (mg)	Amount estimated (mg) mean		% Assay	
		Levo- Mil	Dex- Mil	Levo- Mil	Dex-Mil
Fetzima	120	120.4	-	100.3	-
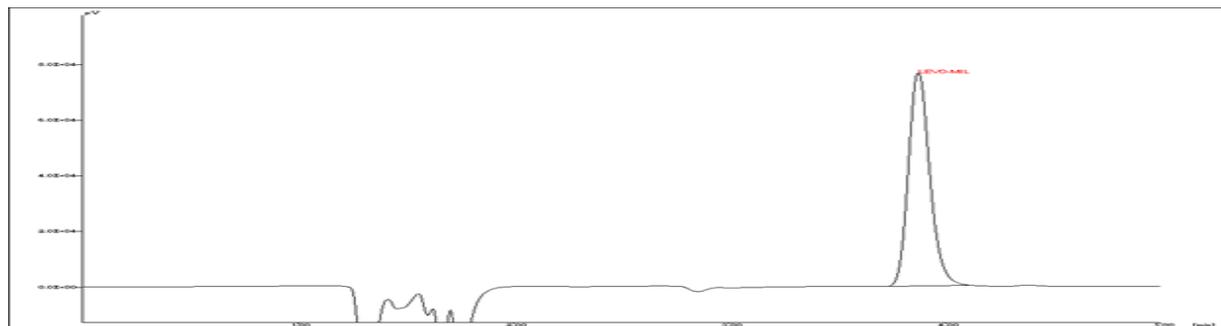
Figure 5: **Assay of Levo-milnacipran formulation**

3.4 Molecular docking to estimate complexation stability

An attempt was made to understand the binding of enantiomers with chiral selectors by employing molecular docking. The details, at atomistic levels, about the interactions between SβCD and each enantiomer of milnacipran hydrochloride were further sought using molecular docking. The GLIDE Emodel energy was used as the yardstick to rank order the complexes. It must be noted that the enantiomer with the best Emodel score is expected to elute before the one with a lower Emodel score. Here, we observed that Levo-milnacipran with Emodel energy -42.3 kcal/mol forms a better complex with SβCD than Dextro-milnacipran with Emodel energy -38.9 kcal/mol. Hence docking studies suggest that Levo-milnacipran will be eluted before Dextro-milnacipran. Our docking studies corroborate with the experimental observations. The inclusion complexes of SβCD with Dextro-milnacipran and Levo-milnacipran are depicted in **Figure 6.**
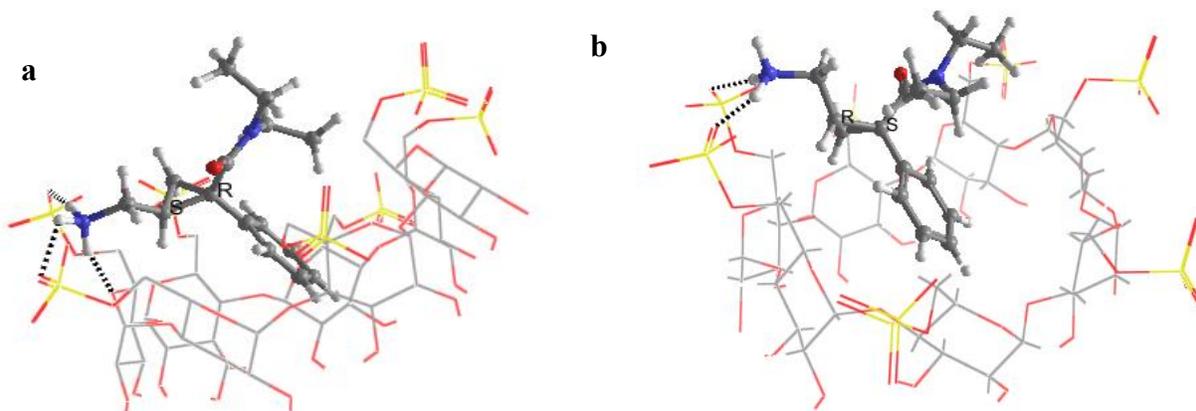

Figure 6: Docked structure of S-β-CD with (a) Dextro-milnacipran and (b) Levo-milnacipran

3.5 Comparison with reported methods:

As mentioned earlier, there are methods reported for the separation of milnacipran enantiomers by chiral columns. JiaYan LE et al [15] have reported enantio-separation of milnacipran by HPLC method with hydroxypropyl β-cyclodextrin-based chiral stationary column Astec CYCLOBOND™ I 2000 RSP column (250 mm × 4. 6 mm, 5μm) the mobile phase was acetonitrile-0. 1% triethylamine acetate (pH 6. 0) (5:95, V/V) and the resolution was 1. 63. Yifeng, C et al [16], have reported enantio-separation of milnacipran by HPLC method with β-cyclodextrin-based chiral column Astec CYCLOBOND™ I 2000 AC (25 cm×4.6 mm, 5μm), the mobile phase was acetonitrile-0.1% (v/v) pH 5.0 triethylamine acetate (TEAA) (5:95, v/v) with a flow rate of 0.4 mL/min and the detection wavelength was 220 nm. The injection volume was

10μL and the column temperature was 25°C. The value of the resolution (R_s) was 1.74 with a run-time of 10minutes. The method reported in this paper is the RP-HPLC method with sulfated β-cyclodextrin as CMPA on achiral core-shell column gave a resolution of 2.96 in the run-time of 5 minutes, indicating that this developed method is superior to the reported chiral separation methods for milnacipran. Hence CMPA technique should be evaluated alongwith CSP technique.

Conclusion:

A cost-effective, validated, simple, fast RP-HPLC method with a good resolution was developed, using sulfated β-cyclodextrin for chiral separation of milnacipran enantiomers. The simplicity of the method is attributed to the fact that there was no need for any derivatization. Furthermore, it excludes the use of various expensive chiral stationary phases and instead makes use of easily available RP-HPLC C_8 columns. Commercially available cyclodextrin bonded chiral columns have preloaded unadjustable amount of cyclodextrin. As shown in this paper, the concentration of cyclodextrin in the mobile phase is the most important parameter governing enantiomeric resolution. If sufficient cyclodextrin is not present on the column, there will not be complete formation of transient diastereomeric inclusion complex and subsequently, satisfactory resolution may not be obtained. The key step in achieving good chiral separation is the formation of stable inclusion complex between milnacipran hydrochloride and SβCD. It was observed that inclusion complex formation stabilized by hydrogen bonding is a major force responsible for the chiral separation, in this case. Out of two achiral columns investigated, the column packed with core-shell particles outperformed the fully porous column. With the help of the core-shell column, there was not only a reduction in analysis time but also a reduction in consumption of costly chiral selector, hence making separation quick and economic.

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