Development and Validation of Chiral LC Method for the Enantiomeric Separation of Duloxetine on Amylose Based Stationary Phase

V.P. Rane[1, 2] and **D.B. Shinde**[1,*]

[1]Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad-431004 (MS), India and
[2]Wockhardt Research Centre, Aurangabad-431210 (MS), India

Abstract

A simple, rapid, and robust liquid chromatography method was developed and validated for the enantiomeric separation of duloxetine in bulk drug substance. The enantiomers of duloxetine were resolved on a Chiralpak AD-H (amylose based stationary phase) column using a mobile phase consisting of *n*-hexane–ethanol–diethyl amine (80:20:0.2, v/v/v) at a flow rate of 1.0 mL/min. The resolution between the enantiomers was found to be not less than 2.8 in optimized method. The presence of diethyl amine in the mobile phase played an important role in enhancing chromatographic efficiency and resolution between the enantiomers. The developed method was extensively validated and proved to be robust. The calibration curve for (*R*)-enantiomer showed excellent linearity over the concentration range of 750 ng/mL (LOQ) to 7500 ng/mL. The limit of detection and quantitation for (*R*)-enantiomer were 250 and 750 ng/mL, respectively. The percentage recovery of the (*R*)-enantiomer ranged between 98.3% to 101.05% in bulk drug samples of duloxetine. The proposed method was found to be suitable and accurate for quantitative determination of (*R*)-enantiomer in bulk drug substance.

Introduction

Duloxetine (Figure 1) is chemically [(S)-*N*-methyl-3-(1-napthalenoxy)-3-(2-thienyl)-propanamine potent and balanced dual re-uptake inhibitor of the serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine reuptake being investigated for the treatment of depression and urinary incontinence and diabetic peripheral neuropathic pain (1,2). It is used in treatment for role functioning improvement in generalized anxiety disorder (3). Its pharmacological effects are mainly due to duloxetine while the (R)-enantiomer is considered to be inactive (4). Owing to the pharmacological and toxicological difference between these enantiomers, it is quite important to develop an enantio specific liquid chromatographic method for quality assurance of drugs.

Separation of enantiomers has become very important in analytical chemistry, especially in the pharmaceutical and biological fields because some stereoisomer of racemic drugs have quite different pharmacokinetics and different pharmacological or toxicological effects (5). Recent global advances in new regulatory guidelines for racemic or "pure" pharmaceutical products necessitate development of a rapid, sensitive, and reproducible method for quality control of optical antipodes present in drug substance. Determination of enantiomeric purity, or enantiomeric excess (ee), is of special importance in the control of the purity of chiral synthetic materials and chiral pharmaceuticals. Chiral separation techniques mainly include high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE). The only advantages of CE in chiral separation are the low consumption of both analyte and chiral selector. Moreover, CE has no need for expensive chiral stationary phases because the Chiral selector is simply added to the buffer. The main drawback of CE compared to HPLC is that CE has not shown to be useful as a preparative separation tool. Another advantage of HPLC over CE is the low detection limit, due to much longer path length of the detection cell and the much higher injection volume. Chiral HPLC has been recognized as a useful methodology for the separation of chiral drugs than other techniques.

A through literature survey revealed that a few HPLC methods were reported for determination of duloxetine in bulk drug, pharmaceutical formulation and other biological fluids (6–10). A few CE methods using cyclodextrin as a chiral additive were reported for the determination of duloxetine in bulk drug, human serum, and other biological fluids (11–15). HPLC was also used for determining the duloxetine and its (*R*)-enantiomer

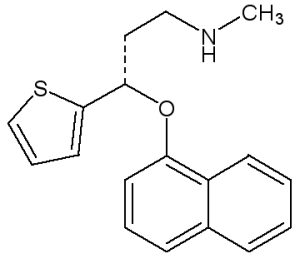

Figure 1. Chemical structure of *S*(+)-duloxetine.

* Author to whom correspondence should be addressed: email shinde_devanand@yahoo.co.in.

using hydroxypropyl-beta-cyclodextrin as chiral selector with resolution of isomers 1.3, analysis time about 60 min, and a vancomycin chiral stationary phase (Chirobiotic V) with resolution of isomers 1.7, analysis time approximately 20 min (16)

Polysaccharide-based stationary phases are quite popular with wide recognition for direct resolution of enantiomers. To the best of our knowledge, there were no validated LC methods for the determination of the enantiomeric purity of duloxetine in bulk drugs. The disadvantage of previously reported methods is longer analysis time, shorter resolution, and could not be used for preparative HPLC separation. In the present investigation, we report the development and validation of a normal-phase LC method using polysaccharide amylose based stationary phase (Chiralpak AD-H) column for determination of enantiomeric purity of duloxetine in bulk drugs. We developed a rapid method with short analysis time and better resolution, enantioselectivity, and robust method. The main advantages of developed method are the method is useful for preparative HPLC separation and routine analysis in quality control labs due to short run time and better resolution. The developed method was validated with respective linearity, accuracy, precision, limit of detection (LOD) and quantitation (LOQ), and robustness.

Experimental

Chemicals

Samples of (*R*)-enantiomer and duloxetine (free base) were obtained from Cipla pharmaceutical limited (Mumbai, India). HPLC grade *n*-hexane and diethyl amine were purchased from Qualigens Fine chemicals (Mumbai, India). The HPLC grade ethanol was purchased from Merck Ltd. (Mumbai, India).

Equipment

HPLC system used was an Agilent Technology (1100 series, Germany) system equipped with auto sampler, quaternary pump, degasser, and a UV Detector. The out put signal was monitored and processed using Agilent Chemstation software.

Sample preparation

The stock solution of the (*R*)-enantiomer and duloxetine (5.0 mg/mL) was prepared by dissolving an appropriate amount of substance in methanol. For quantitation of (*R*)-enantiomer in duloxetine, a solution of 1.0 mg/mL concentration was used.

Chromatographic condition

The chromatographic column used was 250 × 4.6 mm ChiralPak AD-H (Daicel Chemical Industries, Ltd., Tokyo, Japan) packed with 5 µm particles. The mobile phase was *n*-hexane–ethanol–diethyl amine (80:20:0.2, v/v/v). The flow rate of the mobile phase was 1.0 mL/min. The column temperature was maintained at 30°C, and the eluent was monitored at a wavelength of 254 nm. The injection volume was 10 µL. Protein based chiral stationary phase Chiral AGP (Chrom Tech Ltd., Cheshire, UK) and cellulose based chiral stationary phase Chiralcel OJ-H (Diacel Chemical Industries, Ltd., Tokyo, Japan) were employed during the method development.

Method Validation

System suitability

The system suitability was determined by injecting a racemic mixture containing equal quantity of (*R*)-enantiomer and duloxetine. Because the enantiomers form a critical band pair in the chromatogram, the qualification criteria was resolution between the two enantiomers, shown to be not less than 2.8 and tailing factor should not exceed 1.5. The separation factor (α) was calculated as the ratio of retention factors, $\alpha = k_2/k_1$. The resolution factor (R_s) was calculated as $R_s = 2(t_2 - t_1)/(w_1 + w_2)$ where, t_1, t_2 refer to the retention time of the first and second enantiomers; w_1 and w_2 are the peak widths for the first and second eluting enantiomers, respectively.

Precision

Method reproducibility was determined by measuring repeatability and intermediate precision (between-day precision) of retention times and peak areas for each enantiomer.

In order to determine the repeatability of the method, replicate injections ($n = 6$) of a 1.0 mg/mL solution containing duloxetine spiked with (*R*)-enantiomer (0.5%) was carried out. The intermediate precision was also evaluated over three days by performing six successive injections each day.

Linearity of (*R*)-enantiomer

Linearity was assessed by preparing six calibration sample solutions of (*R*)-enantiomer covering from 750 ng/mL (LOQ) to 7500 ng/mL (750, 1500, 3000, 5000, 6000, and 7500 ng/mL), prepared in mobile phase from (*R*)-enantiomer stock solution.

The regression curve was obtained by plotting peak area versus concentration, using the least squares method. Linearity was checked for three consecutive days in the same concentration range from the same stock solution. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve was calculated.

Recovery of (*R*)-enantiomer in bulk sample

The study was carried out in triplicate at 4000, 5000, and 6000 ng/mL of the duloxetine target analyte concentration. The recovery of (*R*)-enantiomer was calculated from the slope, and Y-intercept of the calibration curve obtained was ensured by determining recovery of the spiked amount of (*R*)-enantiomer in duloxetine.

LOD and LOQ of (*R*)-enantiomer

LOD and LOQ of (*R*)-enantiomer were achieved by injecting a series of dilute solutions of (*R*)-enantiomer (17).

The precision of the developed enantioselective method for (*R*)-enantiomer at LOQ was checked by analyzing six test solutions prepared at the LOQ level and calculating the percentage relative standard deviation of area.

Robustness

To determine robustness of the method, experimental conditions were purposely altered, and chromatographic resolution between enantiomers was evaluated.

The flow rate of the mobile phase was 1.0 mL/min. To study

the effect of the flow rate on the resolution of enantiomers, it was changed 0.2 units from 0.8 to 1.2 mL/min, while the other mobile phase components were held constant, as stated in "Chromatographic condition" section. The effect of change in percent of ethanol on resolution was studied by varying from ± 1% and the effect of percent of diethyl amine on resolution was studied by varying from ±0.1%, while the other mobile phase components were held constant, as stated in "Chromatographic condition" section. The effect of column temperature on resolution was studied at 25°C and 35°C instead of 30°C while other mobile phase components were held constant, as stated in chromatographic condition section.

Solution stability and mobile phase stability

Stability of duloxetine in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on laboratory bench for 2 days. Content of (*R*)-enantiomer was checked at 6 h intervals up to the study period.

Mobile phase stability was carried out by evaluating the content of (*R*)-enantiomer in duloxetine; sample solutions were prepared freshly at 6 h intervals for 2 days. The same mobile phase was used during the study period.

Results and Discussion

Optimization of chromatographic conditions

The objective of this study was to separate the enantiomers of duloxetine and accurately quantify the (*R*)-enantiomer. To develop the suitable chiral HPLC method for the separation of the enantiomers of duloxetine, different mobile phases and stationary phases were employed. For this, different chiral columns were used, namely: Chiralcel OJ-H, Chiral-AGP, and Chiralpak AD-H. The enantiomeric separation for duloxetine was not achieved by using either Chiralcel OJ-H using mobile phase (hexane–ethanol, 80:20) or Chiral-AGP using mobile phase 10mM ammonium acetate pH 5.5 by acetic acid–ethanol (65:35). There was an indication of separation on Chiralpak AD-H column using the mobile phase consisting of *n*-hexane–ethanol (50:50, v/v) but the peak shape was broad. For further improvement in resolution, peak shape, and column efficiency, the peak modifier diethyl amine was used. Better separation was achieved on the Chiralpak AD-H column (resolution between enantiomers was found to be > 2.8) using the mobile phase *n*-hexane–ethanol–diethyl amine (80:20:0.2, v/v/v), which produces better resolution and chromatographic analysis time less than 7 min.

The mechanism of chiral separation methods is the interaction of chiral stationary phase (CSP) with analytes to form short-lived, transient diastereomeric complexes. The complexes are formed as a result of hydrogen bonding, dipole–dipole interactions, pi bonding, electrostatic interactions, and inclusion complexation (18,19). As discussed earlier in method development, enantiomers of duloxetine could not be separated on Chiral AGP and Chiralcel OJ-H (*tris*-4-methyl benzoate ester derivative of cellulose). Chiral stationary phase (CSP) that gave the best resolution was Chiralpak AD-H (3,5-*tris*-dimethylphenylcarbamate derivative of amylose coated on silica gel). The separation of duloxetine enantiomers on Chiralpak AD-H was due to the interaction between the polar group of analytes and the polar carbamate group on the CSP. The carbamate group on the CSP interacts with the NH group of analytes through hydrogen bonding, the oxygen atom of duloxetine form dipole–dipole interaction between CSP. The π–π interaction occurred between phenylcarbamate and duloxetine aromatic ring, steric fit, which are stabilized by insertion of aromatic portion of duloxetine in to chiral grooves (asymmetric centers). Amylose forms a helical structure and posses more defined grooves (asymmetric centers) making it different than cellulose derivatives. These polysaccharides contain a large number of chiraly-active sites and thus a relatively high probability of interaction with the solute, leading to separation of the two enantiomers. Peak tailing may results from the silanol effect. Small amounts of diethyl amine basic modifier in the mobile phase, when analytes contains amino basic functions, will reduce peak tailing by masking the residual silanol group of the chiral stationary phase.

When 2-propanol was used as an organic modifier, the enan-

Table I. System Suitability Report*

Enantiomers	R_t (min)	α	Rs	N	T
(*R*)-enantiomer	5.20	1.13	2.89	6769	1.2
(*S*)-enantiomer	5.9	–	–	8636	1.15

* *n* = 3, R_t = retention time, α = enantioselectivity, Rs = USP resolution, *N* = number of theoretical plates (USP tangent method); and T = USP tailing factor.

Table II. Validation Results of the Developed Liquid Chromatographic Method

Validation parameter	Results
Repeatability (n = 6, % RSD)	
Retention time (*R*-enantiomer)	0.6
Retention time (*S*-enantiomer)	0.8
Peak area (*R*-enantiomer)	1.3
Peak area (*S*-enantiomer)	0.8
Intermediate precision (n = 9, % RSD)	
Retention time (*R*-enantiomer)	0.7
Retention time (*S*-enantiomer)	0.6
Peak area (*R*-enantiomer)	1.4
Peak area (*S*-enantiomer)	1.2
LOD-LOQ (R-enantiomer)	
LOD (ng/mL)	250
LOQ (ng/mL)	750
Precision at LOQ (%RSD)	1.9
Linearity (R-enantiomer)	
Calibration range (ng/mL)	750-7500
Calibration points	6
Correlation coefficient	0.999
Slope (%RSD)	1.5
Intercept (%RSD)	4.5

tiomers were not separated on the Chiralpak AD-H column. But excellent separation was obtained when ethanol was used as organic modifier.

A representative chromatogram of duloxetine enantiomers is shown in Figure 2A, showing an excellent resolution (R_s = 2.89) between two enantiomers, and symmetric peak shape with tailing at 1.25 was obtained. In the optimized method, the typical retention time of the (*R*)-enantiomer and duloxetine were about 5.2 and 5.9 min, respectively. The system suitability test results of the chiral liquid chromatographic method on Chiralpak AD-H are presented in Table I.

Validation results of the method

In the precision study, the percentage relative standard deviation (RSD) was less than 0.6% and 0.8% for the retention times of the (*R*)-enantiomer and duloxetine, respectively. Peak area (RSD) 0.8% for duloxetine and 1.3% for (*R*)-enantiomer (Table II). In the intermediate precision study, the results showed that RSD values were in the same order of magnitude as those obtained for repeatability (Table II).

The LOD and LOQ concentration were estimated to be 250 and 750 ng/mL for (*R*)-enantiomer, when the signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for (*R*)-enantiomer at limit of quantification was less than 1.9% RSD (Table II).

The described method was linear in the range of 750–7500 ng/mL for (*R*)-enantiomer in duloxetine. The calibration curve was drawn by plotting the peak area of (*R*)-enantimer verses its corresponding concentration with a correlation coefficient of 0.999. The equation of the calibration curve for (*R*)-enantiomer was $Y = 18967x - 9710.7$. Linearity was checked for (*R*)-enantiomer over the same concentration range for three consecutive days. The percentage relative standard deviation of the slope and Y-intercept of the calibration curve were 1.5 and 4.5, respectively (Table II).

The bulk sample did not show the presence of the (*R*)-enantiomer; therefore, standard addition and recovery experiments were conducted to determine the accuracy of the present method for the quantitation of the (*R*)-enantiomer in bulk drug samples.

The recovery and standard addition experiments were conducted for (*R*)-enantiomer in bulk samples in triplicate at 4000, 5000, and 6000 ng/mL of the analyte concentration. Recovery was calculated from slope and Y-intercept of the calibration curve, obtained in the linearity study and percentage recovery ranged from 98.3% to 101.05% (Table III).

An HPLC chromatogram of spiked (*R*)-enantiomer at 0.5% level in duloxetine sample was shown in Figure 2B.

The chromatographic resolution of the duloxetine and (*R*)-enantiomer peaks was used to evaluate the method robustness under modified conditions. The resolution between duloxetine and (*R*)-enantiomer was greater than 2.5 and enantioselectivity (α) was better under all separation conditions tested, demonstrating sufficient robustness. As a flow rate of mobile phase and column temperature is increased, the resolution decreased to (2.68) and enantioselectivity was not affected (1.13). As the percentage of ethanol in mobile phase increased the resolution was decreased (2.7) and selectivity also deceased to (1.15), while as the percentage of diethyl amine in the mobile phase increased improved resolution (3.0) and selectivity (1.12) was observed.

The % RSD of duloxetine content during solution stability and mobile phase stability experiments was within 1.5%. Hence, duloxetine sample solution and mobile phase were stable for at least 48 h.

Table III. Recovery Results of (*R*)-Enantiomer in Bulk Sample

Amount spiked (ng)	Amount found	Recovery (%)	% RSD
4000	3950	98.75	1.5
5000	5050	01.05	2.1
6000	5850	97.50	1.8

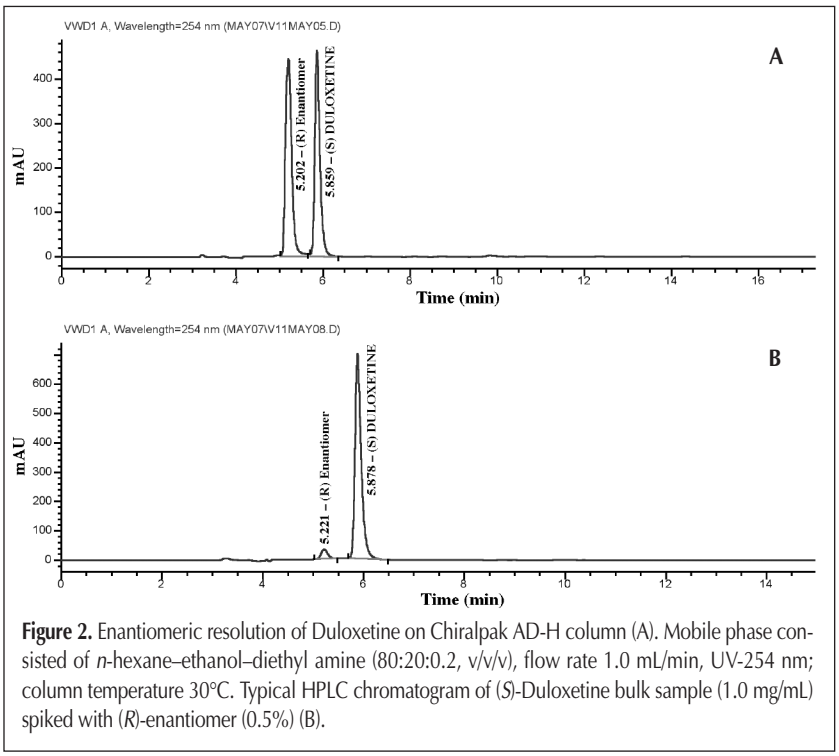

Figure 2. Enantiomeric resolution of Duloxetine on Chiralpak AD-H column (A). Mobile phase consisted of *n*-hexane–ethanol–diethyl amine (80:20:0.2, v/v/v), flow rate 1.0 mL/min, UV-254 nm; column temperature 30°C. Typical HPLC chromatogram of (*S*)-Duloxetine bulk sample (1.0 mg/mL) spiked with (*R*)-enantiomer (0.5%) (B).

Conclusion

A simple, rapid, and accurate chiral HPLC method has been developed and validated for the enantiomeric separation of duloxetine. Chiralpak AD-H (amylose-based chiral stationary phase) was found to be selective for the enantiomers of the drug. Baseline separation with resolution greater than 2.8 is achieved between the two enantiomers within 7 min. The effect of organic modifiers and temperature on resolution and

retention of enantiomers have been evaluated. The method was completely validated with respect to accuracy, precision, linearity, LOD, LOQ, and robustness as per ICH guidelines. The developed method can be conveniently used by the quality control department for the quantitative determination of chiral impurity (*R*-enantiomer) in the bulk drug substance.

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