PART-[B]

ACHIRAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF PHARMACETUICALS

SECTION-1

Development of Stability Indicating HPLC Method and Validation of Duloxetine Hydrochloride
Introduction

A definition of drug as per FDA is “A substance recognized by an official pharmacopoeia or formulary. The drug product is “the finished dosage form that contains a drug substance, generally but not necessarily in association with other active or inactive ingredients. Method corresponding to the estimation of drug substance or drug product need to be validated before their introduction into routine use [1]. Method validation is the process by which it is established that performance characteristics of the method meet the requirements for the intended analytical applications. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use has developed a text on the validation of analytical procedures [2]. The United States Food and Drug Administration (USFDA) have proposed guidelines on submitting samples and analytical data for methods validation [3, 5]. The United States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation [6].

A dosage form is the physical form in which a drug is produced and dispensed, such as a tablet, capsule or an injection. The solid dosage forms include pill, tablet and a capsule and can be designed to have time based drug release in specific applications. Sustained release dosage forms are designed to release a drug at a predetermined rate in order to maintain a constant drug concentration for a specific period of time with minimum side effects. An enteric coating is a barrier applied to oral medication that controls the location in the digestive system where it is absorbed. Most enteric coatings work by presenting a surface that is stable at the highly acidic pH found in the stomach, but breaks down rapidly at a less acidic (relatively more basic) pH. For example, they will not dissolve in the acidic juices of the stomach (pH ~3), but they will in the alkaline (pH 7-9) environment present in the small intestine. Materials used for enteric coatings include fatty acids, waxes, shellac, plastics, and plant fibers.

Drugs that have an irritant effect on the stomach, such as aspirin, can be coated with a substance that will dissolve only in the small intestine. Likewise, certain drugs are acid-activated, for such types of drugs, enteric coating added to the formulation tends to avoid activation in the mouth and esophagus.
One of the important acid-activated drug is duloxetine, which undergoes many degradation reactions and the most common degradation is acid hydrolysis. Considering these facts the enteric-coated formulation is required to avoid contact with acidic pH of stomach [7]. Site of absorption for duloxetine is the intestine and in order to help this, the formulation of delayed release dosage form found to be a good approach as it is shown good drug levels in the intestine [8, 9].

Therefore it is necessary to bypass the duloxetine through acidic pH of the stomach which can be achieved by formulating delayed release dosage form by using different enteric polymers. In general, protection of the drug from acidic environment is done by coating the drug with enteric polymers by using suspension layering technique in Fluidized bed processor (FBP) with different enteric polymers like HPMCAS (Hydroxy Propyl Methyl Cellulose Acetate Succinate), Acryl EZE and HPMCP (Hydroxy propyl methyl cellulose phthalate).

1.1 Description

Duloxetine hydrochloride, chemically known as (+)-(S)-N-methyl-γ-(1-napthyloxy)-2-thiophenepropylamine hydrochloride is an important anti-depressant drug (Fig. 1). This drug primarily targets major depressive disorder (MDD), generalized anxiety disorder (GAD), pain related to diabetic peripheral neuropathy and in some countries stress urinary incontinence (SUI). Its common brand names are Cymbaltais, Ariclaim, Xeristar, Yentrevem Duzela, Dulane and it is manufactured and marketed by Eli Lilly and Company. Duloxetine has not yet been FDA approved for stress urinary incontinence or for fibromyalgia.

Duloxetine is a white to off white powder. The empirical formula of duloxetine is C_{18}H_{19}NOS with a molecular weight of 297.4 g/mol. The CAS number of duloxetine is 116539-59-4.
1.2 Indication

The major indications are the acute and maintenance treatment of major depressive disorder (MDD), as well as acute management of generalized anxiety disorder. Also used for the management of neuropathic pain associated with diabetic peripheral neuropathy, and fibromyalgia.

1.3 Mechanism of Action

Duloxetine has no significant affinity for dopaminergic, adrenergic, cholinergic, histaminergic, opioid, glutamate, and GABA receptors. Duloxetine is a potent inhibitor of neuronal serotonin and norepinephrine reuptake and a less potent inhibitor of dopamine reuptake. The antidepressant and pain inhibitory actions of duloxetine are believed to be related to its potentiation of serotonergic and noradrenergic activity in the CNS. The mechanism of action of duloxetine in SUI has not been determined, but is thought to be associated with the potentiation of serotonin and norepinephrine activity in the spinal cord, which increases urethral closure forces and thereby reduces involuntary urine loss.
Duloxetine Hydrochloride

1.4 Pharmacodynamics

Duloxetine is in a class of medications called selective serotonin and norepinephrine reuptake inhibitors (SSNRIs) and primarily targets major depressive disorders (MDD) and stress urinary incontinence (SUI). Duloxetine is also used to treat pain and tingling caused by diabetic neuropathy (damage to nerves that can develop in people who have diabetes). Known also as LY248686, it is a potent dual inhibitor of serotonin (5-hydroxytryptamine, 5-HT) and norepinephrine (NE) reuptake, possessing comparable affinities in binding to NE and 5-HT transport sites. Interestingly, its behavior contrasts to most other dual-reuptake inhibitors. Furthermore, duloxetine lacks affinity for monoamine receptors within the central nervous system.

1.5 Pharmacokinetics

Duloxetine has an elimination half-life of about 12 h (range 8 to 17 h) and its pharmacokinetics are dose proportional over the therapeutic range. Steady-state plasma concentrations are typically achieved after 3 days of dosing. Elimination of duloxetine is mainly through hepatic metabolism involving two P450 isozymes, CYP1A2 and CYP2D6.

1.6 Absorption and Distribution

Orally administered duloxetine hydrochloride is well absorbed. There is a median 2 h lag until absorption begins (Tlag), with maximal plasma concentrations (Cmax) of duloxetine occurring 6 h post dose. Food does not affect the Cmax of duloxetine, but delays the time to reach peak concentration from 6 to 10 h and it marginally decreases the extent of absorption (AUC) by about 10%. There is a 3 h delay in absorption and a one third increase in apparent clearance of duloxetine after an evening dose as compared to a morning dose.

1.7 Metabolism

The major biotransformation pathways for duloxetine involve oxidation of the naphthyl ring followed by conjugation and further oxidation. Both CYP1A2 and CYP2D6 catalyze the oxidation of the naphthyl ring in vitro. Metabolites found in plasma
include 4-hydroxy duloxetine glucuronide and 5-hydroxy, 6-methoxy duloxetine sulfate. Many additional metabolites have been identified in urine, some representing only minor pathways of elimination. Most (about 70%) of the duloxetine dose appears in the urine as metabolites of duloxetine; about 20% is excreted in the feces.

1.8 Toxicity

Symptoms of overdose include tremors, convulsions, reduced activity, intermittent tremors, and rigidity.

1.9 Side Effects

The major side effects are with heart, like hot flush, palpitations, heart attack, hypertensive crisis, abnormal heart rhythm. Duloxetine hydrochloride has side effects on central nervous system, like drowsiness, headache, dizziness, sleeplessness, fatigue, decreased appetite, lack of energy, agitation, tremor, decreased sexual activities, tingling, abnormal dreams, anxiety, migraine, elevated body temperature, sleep disorder, yawning, partial loss of sensitivity, lethargy, vertigo, aggression, anger, restless leg syndrome, seizures upon treatment discontinuation. Apart from these, duloxetine hydrochloride has side effects on skin, eye, ENT, gastrointestinal, genitourinary, hypersensitivity, metabolic and musculoskeletal.

[2] Literature Overview

A through literature review, it has been observed that numerous HPLC methods were reported for estimation of duloxetine hydrochloride as a drug substance, or in biological samples and pharmaceutical dosage forms. Analytical methods for the determination of the impurities were also reported.

N.V.V.S.S. Raman, K.A. Harikrishna, A.V.S.S. Prasad, K. Ratnakar Reddy, and K. Ramkrishna have reported stability indicating reverse-phase HPLC method for determination of duloxetine hydrochloride in the presence of process and degradation impurities. They reported separation of all impurities using a C18 column and potassium
dihydrogen orthophosphate in the mobile phase. The run time of the method is 55 min [10).

**P. R. M. Reddy, J. Sreeramulu, Petla Y.N., and A. R. Reddy** have developed a reverse-phase HPLC method and found satisfactory results with validation exercises. This method used a mixture of ion pairing reagent and phosphate buffer in the mobile phase [11].

**Dantu Durga Rao, Shakil S. Sait, A. Malleswara Reddy, Dinesh Chalkole, Y. Ramakoti Reddy and K. Mukkanti** have reported Analysis of duloxetine hydrochloride and Its related compounds in pharmaceutical dosage Forms and in vitro dissolution studies by stability indicating UPLC. A reproducible gradient method has developed using acetonitrile and an inorganic buffer in the mobile phase [12].

### [3] Aim of Present Study

Duloxetine undergoes many degradation reactions and the most common degradation is acid hydrolysis, considering these facts the enteric-coated formulation is required to avoid contact with acidic pH of the stomach. Site of absorption for duloxetine is the intestine and in order to help this, the formulation of delayed release dosage form found to be a good approach as it is shown good drug levels in the intestine. The common challenging with sustained release formulation during method development and validation are extended sample preparation and low recovery. It has become very important to have a precise and accurate method for accurate estimation of duloxetine hydrochloride in a sustained release formulation.

The major objective of the present work was to develop and validate the stability indicating HPLC method as per International Conference on Harmonisation (ICH) for the
estimation of duloxetine hydrochloride the delayed release enteric-coated formulation. The method should have the application in accurate determining of the duloxetine in pharmaceutical formulation to support routine analysis of quality control laboratories. The present work deals with the estimation of duloxetine hydrochloride in presence of related substance. The developed method validated in terms of specificity, linearity, accuracy, precision, stability in analytical solution, robustness and ruggedness.

Another important objective of this work was to use volatile mobile phase for method development. This helps in easy method transfer to LCMS as well as also reduce the down time of hardware due to good miscibility with organic solvents [13].

[4] Experimental

4.1 Chemicals and Drugs

Duloxetine hydrochloride was obtained from Tatva Chintan Pharma Chem Pvt. Ltd. Delayed release enteric-coated duloxetine hydrochloride formulation was purchased from local market. HPLC grade water, acetonitrile, methanol, acetic acid and acetic acid were purchased from Merck.

4.2 High Performance Liquid Chromatography

The method development and validation was performed on an Agilent 1200 HPLC system consist of a quaternary pump, column oven, photo diode array detector and an auto injector. C18 columns were used for method development. The HPLC system was controlled and analytical data were processed using Agilent ChemStation software (Version B.04).

4.3 Chromatographic Conditions

An Agilent ZORBAX StableBond C-18 column (250 × 4.6 mm, 5 µm particle size,) was used as a stationary phase. The buffer contains the 5mM ammonium acetate
solution and the pH adjusted to 4.0 with dilute acetic acid. The buffer used a solvent A and acetonitrile used a solvent B, and a gradient mixture of solvent A & B was used as mobile phase. The gradient program is mentioned in Table 1.

<table>
<thead>
<tr>
<th>Time</th>
<th>% A (Aqueous Phase)</th>
<th>% B (Organic Phase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>85</td>
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<tr>
<td>9</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 1: Gradient table for duloxetine hydrochloride

The flow rate was 1.2 mL/min and the injection volume was 5 µL. The column oven temperature was maintained at 35 °C. Chromatography monitored at a wavelength at 230 nm. Analytical data were processed using Agilent ChemStation software (Version B.04.03).

4.4 Diluent Preparation

Buffer and acetonitrile in the ratio of 50:50 v/v was used as diluent.

4.5 Preparation of Stock Solutions

Standard stock solution (500 µg/mL) of duloxetine hydrochloride was prepared by dissolving appropriate amount of the standard in diluent and this stock solution used for preparing remaining working solutions. The resulted solution was filtered through a 0.45-µm membrane filter.

4.6 Preparation of Sample Solutions

For delayed release enteric-coated formulation sample, 5 capsules (20 mg of duloxetine label claim) were opened and the enteric-coated granules were finely
grounded using agate mortar and pestle. The grounded material, which was equivalent to 100 mg of duloxetine, was transferred a 100 mL volumetric flask containing 90mL of methanol. To extract the duloxetine hydrochloride from placebo matrix, the resulted solution was sonicated for 30 min with intermediate shaking. The final volume was made up with methanol. The resulted solution was filtered through a 0.45 µm membrane filter. This solution corresponds to analyte concentration of 1 mg/mL, and further dilutions were prepared in diluent.

4.7 Method Validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use as well as results from method validation can be used to judge the quality, reliability and consistency of analytical results.

4.7.1 Specificity

Specificity is the ability of the method to measure accurately analyze response in the presence of its potential impurities. The selectivity of this method was indicated by checking the peak purity measurement and the absence of any endogenous interference at retention times of duloxetine peak. The absence of interfering peak was evaluated by injecting a blank consisting of diluent and placebo. Force degradation studies were carried out under different stress conditions to understand the degradation behavior duloxetine hydrochloride. The assay and peak purity of duloxetine hydrochloride were taken under consideration to evaluate the results. The stress conditions were as follows:

A. Acidic Stress Condition

An acidic stress study was carried by dissolving the drug at 500 µg/mL concentration in 0.1M hydrochloride under and kept for 60 min in water bath at 50°C.
B. Alkaline Stress Condition

An alkaline stress study was carried by dissolving the drug at 500 µg/mL concentration in 0.1M NaOH solution and kept for 60 min in water bath at 50°C.

C. Oxidative Stress Condition

The oxidative stress study was carried by dissolving the drug at 500 µg/mL concentrations in 30 % v/v hydrogen peroxide solution and kept for 60 min in water bath at 50°C.

D. Photo Degradation

Solid drug substance was exposed to ultra violet light of 254 nm for 10 days.

E. Thermal Degradation

Solid drug substance was exposed to dry heat at 60°C for 10 days.

4.7.2 Precision

Precision is the closeness of agreement between measurements from multiple sampling of a homogeneous sample under recommended conditions. The precision of the method was checked by analyzing six replicate samples of duloxetine hydrochloride (at analyte concentration, i. e. 200.00 µg/mL). Relative standard deviation (%RSD) of retention time and peak were calculated for duloxetine hydrochloride. The intermediate precision was determined over 3 days by performing six successive injections on each day.

4.7.3 Linearity

Linearity corresponds to the capacity of the method to supply results directly proportional to the concentration of the substance being determined within a certain interval of concentration. Detector response linearity was assessed by preparing 5 calibration sample solutions covering from 100 to 400 µg/mL (100, 150, 200, 250, 400
µg/mL) Regression curve was obtained by plotting peak area versus concentration, using the least squares method.

4.7.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the value found. The accuracy of this method was carried out at three concentrations (100, 200, 300 µg/mL) level by spiking placebo with known concentration of duloxetine hydrochloride. Accuracy was evaluated in triplicate at each concentration level.

4.7.5 Ruggedness

To determine the ruggedness, the recovery experiments carried out duloxetine hydrochloride in formulation samples were again carried out in laboratory B using a different instrument.

4.7.6 Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. To evaluate the robustness of the developed method, the experimental conditions were deliberately altered and the peak purity and system suitability parameters (i.e. theoretical plates and symmetry factor) were monitored. The following parameters were changed to establish the robustness of the method.

(a) Flow Rate Variation:

The flow rate of the mobile phase was changed to 1.0 mL/min and 1.3 mL/min from 1.2 mL/min.

(b) Effect of pH:

The effect of the mobile phase pH was checked by varying the pH value to 3.5 and 4.5 from actual value of 4.0

(c) Column Oven Temperature Variation:

The effect of column temperature on results was studied at 31.5 and 38.5°C instead of 35°C
4.7.7 Solution stability

The sample was analyzed for 24 h at room temperature, i.e., at 25°C. Relative standard deviation (RSD) of the assay for duloxetine hydrochloride was observed during the study.

[5] Result and Discussion

5.1 Method Development

Duloxetine hydrochloride, chemically known as (+)-(S)-N-methyl-γ-(1-napthyloxy)-2-thiophenepropylamine hydrochloride. The standard solution of 200 µg/mL concentration was used for the method development and optimization. To determine the $\lambda_{\text{max}}$, the standard solution was screened between 200 to 400 nm using a UV diode array detector and we got $\lambda_{\text{max}}$ at 230 and 29 nm (Fig. 2).

![Figure 2: UV spectra of Duloxetine hydrochloride](image-url)
In order to achieve the best sensitivity, the $\lambda_{\text{max}}$, with higher intensity (i.e. 230 nm) has selected for the further study. Duloxetine hydrochloride has logP of 4.72 and has a pKa values, 9.7. Duloxetine is basic nature and it is soluble in water and methanol.

### 5.1.1 Development and Optimization of Chromatographic Conditions

Numerous stability indicating HPLC methods were reported for the estimation of duloxetine HCl and related products and these methods used inorganic salts in the mobile phase. Such methods can not be transferred to LC-MS due to non-volatile mobile phase system.

The volatile mobile phase will help in easy method transfer to LC-MS and reduce unnecessary instrument downtime due to good miscibility with organic solvents. One of the published report describes solubility of various aqueous buffers like ammonium acetate at pH 5.0, ammonium phosphate at pH 3.0 and pH 7.0, and potassium phosphate at pH 3.0 and pH 7.0 with various organic solvents (methanol, acetonitrile and tetrahydrofuran). The outcome of this study shown that the ammonium acetate at pH 5.0 was the most soluble buffer and potassium phosphate at pH 7.0 was the least soluble buffer [14].

One always can avoid precipitation of the buffer salt by using a very low concentration of buffer salt (10 mM) in the aqueous portion of the eluent. However, the resulting low buffer capacity can lead to slow equilibration, irreproducible retention, and poor peak shapes. Furthermore, both retention and selectivity are functions of the salt concentration of ionizable solutes [15-18]. Thus, knowing the relationship between the buffer’s solubility limit and volume fraction of organic co-solvent is useful in reverse phase HPLC.

The maximum buffer capacity of this weak acid–base pair buffer is achieved at the half titration point, where the concentration of the acid is equal to the concentration of its corresponding conjugate base, or in other words, when the pH of the solution is equal to the $pK'_a$ of the buffer compounds. This fact is important because the buffer capacity sharply decreases when the solution pH move away from the buffer $pK'_a$. 

When the pH is one unit below or above the pKₐ, the buffer capacity is reduced to 1/3 of its maximum value, and it is only about 4% when pH equals to pKₐ ± 2. As shown in Table 2, the mobile phase buffer pH need to be within the ± 1 unit of buffering reagent’s pKₐ value [19].

<table>
<thead>
<tr>
<th>Buffering Agent</th>
<th>pKa</th>
<th>Useful pH Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td>3.13, 4.76, 6.40</td>
<td>2.1 - 7.4</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>4.8</td>
<td>3.8 – 5.8</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>7.2</td>
<td>6.2 – 8.2</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.2</td>
<td>8.2-10.2</td>
</tr>
<tr>
<td>Borate</td>
<td>9.24</td>
<td>8.25 – 10.25</td>
</tr>
</tbody>
</table>

*Table 2: Properties of common buffering reagents*

It is necessary to know the pKₐ of the analytes before buffer pH can be chosen. A buffer 2 pH unit above or below pKₐ is recommended for good peak shape. From the below Henderson-Hesselback equation, i.e. pH = pKₐ + log ([A⁻]/[HA]), it can be determined that 99% of an analyte is in a single form if the solution pH is 2 units above or below the analyte’s pKₐ. Good peak shape is possible when an analyte is in a single form. Duloxetine hydrochloride has a pKₐ values, 9.7 and with respect to Henderson-Hesselback equation, the mobile phase pH need to be outside the range of 7.7 to 11.7 pH.

Considering these all information, we preferred to develop the HPLC method using 10mM ammonium acetate buffer as a mobile phase. The pH was studied over a pH range of 2.5 to 6.5 and buffer at pH 4.0 was adopted, because it was giving the best system suitability results. The chosen mobile phase has below characteristics to give robust and sensitive method;

1. The mobile phase pH is lower than 2 units of duloxetine’s pKₐ. This will help to keep analyte in a single form i.e. the ionic form and results in sharper peak shape.
The mobile phase pH is within the ±1 unit of acetate buffer’s pKa i.e. 4.8. This will provide the buffering effect and results in to the robustness method.

The ammonium acetate buffer at pH 4.0 has a more solubility in organic phase compare to other inorganic buffers, this will help to avoid the buffer precipitation in HPLC and reduce the breakdown.

Important cosolvents for reverse phase mobile phase are acetonitrile, methanol and tetrahydrofuran. In general the most of the buffers are more soluble in methanol than acetonitrile and on the other hand the acetonitrile is more preferred cosolvent in reverse phase chromatography due to its low U.V. cutoff and lower viscosity than methanol. For method development, acetonitrile was considered as a cosolvent in mobile phase.

Among the different makes of C18 columns, the better system suitability results were achieved on ZORBAX StableBond C18 (250 x 4.6 mm, 5 µm) column. The special ZORBAX silica support is designed to reduce or eliminate strong adsorption of basic compounds. The densely covered, stericallyprotected, diisobutyl n-octadecylsilane stationary phase is chemically stable and gives longer column life. As a result, Agilent ZORBAX SB-C18 is a stable, reversed-phase packing that can be used for basic, neutral, or acidic samples.

Various combinations of aqueous and organic phase have been tried to achieve optimum chromatographic resolution of duloxetine hydrochloride and related substances. The system suitability results with optimized gradient (Table 3) were satisfactory.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow Rate (mL/min)</th>
<th>% of Acq. Ammonium Acetate Buffer, (10mM) 4.0pH</th>
<th>% Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>1.2</td>
<td>25</td>
<td>75</td>
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<td>7</td>
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<td>9</td>
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</tr>
<tr>
<td>12</td>
<td>1.2</td>
<td>70</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3: Optimized Gradient Table
The flow rate was 1.2 mL/min and the injection volume was 5 µL. The column oven temperature was maintained at 35 °C. Chromatography monitored at a wavelength of 230 nm. The typical chromatogram of duloxetine hydrochloride in final method is shown in Fig.3.

![Typical chromatogram of duloxetine in final method](image)

**Figure 3:** Typical chromatogram of duloxetine in final method

### 5.2 Results of Method Validation

#### 5.2.1 Results of System suitability

The system suitability results are summarized in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>RT</th>
<th>T</th>
<th>N</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deuloxetine</td>
<td>4.7</td>
<td>1.2</td>
<td>33883</td>
<td>0.73</td>
</tr>
</tbody>
</table>

(RT: Retention time in min, T: USP tailing factor, N: number of theoretical plates, S: Symmetry)

**Table 4: System suitability results.**
5.2.2 Results of Specificity

Peak purity of the principle peak was checked in the impurities spiked solution and stressed solution using the photo diode array detector. Peak purity was passed for all the solution at the threshold level 999.9. Representative peak purity report is shown in Fig. 4.

![Peak purity report of duloxetine](image)

**Figure 4:** Peak purity report of duloxetine

The peak purity result corresponding the duloxetine is summarized in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>Purity Factor</th>
<th>Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duloxetine</td>
<td>999.993</td>
<td>999.900</td>
</tr>
</tbody>
</table>

**Table 5:** System suitability results.

As can be seen in overlay of duloxetine and blank chromatogram (Fig. 5), blank chromatograms is free from any interference at RTs duloxetine, i.e. 4.7 min. The blank sample is consisted of diluent and placebo corresponding to delayed release formulation.

The forced degradation of the azathioprine under all the stress condition was carried out as a part of specificity study. The result and observation of the forced degradation study are discussed below.
**Figure 5:** Overlay of duloxetine and blank chromatograms

**A. Acidic Stress Conditions:**

**Figure 6:** Chromatogram of Acid hydrolysis
An acidic stress study was carried in 0.1M hydrochloric acid and kept for 60 min in water bath at 50°C. There was major degradation in acidic condition and major degradation product of 17% area was appeared a RT of 6.4 min. The degradation product is well separated and RRT is 1.36 with respect to main peak. The Chromatogram of acid hydrolysis is presented in Fig. 6.

**B. Alkaline Stress Condition**

An alkaline stress study was carried in 0.1M NaOH solution and kept for 60 min in a water bath at 50°C, there was no degradation observed. The purity and chromatographic profile of duloxetine remain unchanged (Fig. 7)

![Figure 7: Chromatogram of Base hydrolysis](image)

**C. Oxidative Stress Condition**

The oxidative stress study was carried by dissolving the drug at 500 µg/mL concentrations in 30 % v/v hydrogen peroxide solution and kept for 60 min in water bath at 50°C.
D  Photo Degradation

Solid drug substance was exposed to ultraviolet light of 254 nm for 10 days and there was no degradation found observed (Fig. 9)

E  Thermal Degradation

During photo degradation, the solid drug substance was exposed to dry heat at 60°C for 10 days. The purity and chromatographic profile of duloxetine remain unchanged (Fig. 10)
The peak purity was monitored during the force degradation study, and peak corresponding to duloxetine hydrochloride was found pure in all tested conditions. The results corresponding to the peak purity are summarized in Table 6.

<table>
<thead>
<tr>
<th>Stress Conditions</th>
<th>Purity Factor</th>
<th>Purity Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>999.993</td>
<td>999.900</td>
</tr>
<tr>
<td>Alkaline</td>
<td>999.972</td>
<td>999.900</td>
</tr>
<tr>
<td>Oxidative</td>
<td>999.976</td>
<td>999.900</td>
</tr>
<tr>
<td>Photo</td>
<td>999.987</td>
<td>999.900</td>
</tr>
<tr>
<td>Thermal</td>
<td>999.996</td>
<td>999.900</td>
</tr>
</tbody>
</table>

Table 6: Peak purity results of force degradation study
5.2.3 Results of Precision

The precision was determined for duloxetine hydrochloride at concentration of 200 ug/mL. The relative standard deviation of retention time and area was calculated for six replicates injection. The percentage relative standard deviation of duloxetine hydrochloride area repeatability for system precision and method precision were found to 0.38 and 0.97 respectively. The result of precision of retention time and area is presented in Table 7.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Duloxetine</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT</td>
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<td>4.693</td>
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</tr>
<tr>
<td>MP-4</td>
<td>4.687</td>
<td>3053</td>
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<tr>
<td>MP-5</td>
<td>4.695</td>
<td>3114</td>
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<tr>
<td>MP-6</td>
<td>4.703</td>
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<tr>
<td>Average</td>
<td>4.695</td>
<td>3069</td>
<td></td>
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<tr>
<td>SD</td>
<td>0.006</td>
<td>29.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% RSD</td>
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<td>0.97</td>
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<table>
<thead>
<tr>
<th>S.N.</th>
<th>Duloxetine</th>
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<tr>
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<td>RT</td>
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<tr>
<td>SP-1</td>
<td>4.694</td>
<td>3052</td>
<td></td>
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<td>SP-2</td>
<td>4.692</td>
<td>3034</td>
<td></td>
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<td>SP-3</td>
<td>4.693</td>
<td>3063</td>
<td></td>
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</tr>
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<td>SP-6</td>
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<td>SD</td>
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<tr>
<td>% RSD</td>
<td>0.044</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Results of precision study

The intermediate precision was determined over 3 different days by performing six successive injections on each day in another laboratory by performing six successive injections. In intermediate precision study, results showed that %RSD values were satisfactory. The results for intermediate method precision are summarized in Table 8.
### Table 8: Results of intermediate precision study

<table>
<thead>
<tr>
<th></th>
<th>Day-I</th>
<th></th>
<th>Day-II</th>
<th></th>
<th>Day-III</th>
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<tbody>
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<td>SN</td>
<td>RT</td>
<td>Area</td>
<td>SN</td>
<td>RT</td>
<td>Area</td>
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</tr>
<tr>
<td>1</td>
<td>4.698</td>
<td>3087</td>
<td>4.603</td>
<td>3183</td>
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<td>2</td>
<td>4.692</td>
<td>3098</td>
<td>4.612</td>
<td>3173</td>
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<tr>
<td>3</td>
<td>4.688</td>
<td>3077</td>
<td>4.603</td>
<td>3191</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.689</td>
<td>3083</td>
<td>4.622</td>
<td>3134</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.681</td>
<td>3089</td>
<td>4.629</td>
<td>3128</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.699</td>
<td>3091</td>
<td>4.682</td>
<td>3118</td>
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<td></td>
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<tr>
<td>Average</td>
<td>4.690</td>
<td>3088</td>
<td>Average</td>
<td>4.625</td>
<td>3155</td>
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</tr>
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<td>SD</td>
<td>0.006</td>
<td>7.148</td>
<td>SD</td>
<td>0.03</td>
<td>31.44</td>
<td></td>
</tr>
<tr>
<td>% RSD</td>
<td>0.125</td>
<td>0.232</td>
<td>% RSD</td>
<td>0.642</td>
<td>0.997</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 5.2.4 Results of Linearity

The calibration curves constructed for duloxetine hydrochloride was linear over the concentration range from 100 to 400 µg/mL. The regression was found to be linear all over the concentration range and correlation coefficients values was 0.9997. The results
show that good correlation existed between the peak area and concentration duloxetine hydrochloride (Fig 11).

![Figure 11: Linearity results for Duloxetine](image)

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1539</td>
</tr>
<tr>
<td>150</td>
<td>2241</td>
</tr>
<tr>
<td>200</td>
<td>3063</td>
</tr>
<tr>
<td>250</td>
<td>3872</td>
</tr>
<tr>
<td>400</td>
<td>6083</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
</tr>
<tr>
<td>Slope</td>
</tr>
<tr>
<td>$r^2$</td>
</tr>
</tbody>
</table>

5.2.5 Results of Recovery Study in Formulation

The recovery study was carried out in triplicate by spiking placebo with three concentrations (100, 200 and 300 µg/mL) of duloxetine hydrochloride standards and
assaying for the chromatographic method. The recovery results from both laboratories are summarized in Table 9.

<table>
<thead>
<tr>
<th>% Level</th>
<th>Added (µg)</th>
<th>Recovered (µg)</th>
<th>% Recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>100</td>
<td>98</td>
<td>98.0</td>
<td>99.3</td>
</tr>
<tr>
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<td>199</td>
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<td></td>
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<tr>
<td>150</td>
<td>300</td>
<td>284</td>
<td>94.7</td>
<td>95.1</td>
</tr>
<tr>
<td>150</td>
<td>300</td>
<td>279</td>
<td>93.0</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>300</td>
<td>293</td>
<td>97.7</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>102</td>
<td>102.0</td>
<td>99.7</td>
</tr>
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<td>281</td>
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<td>95.3</td>
</tr>
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<td>300</td>
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<tr>
<td>150</td>
<td>300</td>
<td>287</td>
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</tr>
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</table>

Table 9: Recovery result of Duloxetine

5.2.6 Results of Solution Stability

The sample was analyzed for 24 h at room temperature, i.e., at 25°C. Relative standard deviation (RSD) of the assay for duloxetine hydrochloride was observed during the study. The duloxetine hydrochloride sample injected at various time intervals, like 0, 3, 6, 9, 12, 15, 18 and 24 h. The % RSD of assay corresponding to duloxetine hydrochloride was remain less than 2% during the solution stability study. The data are presented in Table 10, it can be seen from the data that sample solution and mobile phase are stable for 24h at room temperature, i.e., at 25°C.
5.2.7 Results of Robustness

To evaluate the robustness of the developed method, the experimental conditions were deliberately altered and the peak purity and system suitability parameters (i.e. theoretical plates and symmetry factor) were monitored. The results were satisfactory for all deliberately changed chromatographic conditions and this confirmed the robustness of the method. The results are summarized in Table 10.

<table>
<thead>
<tr>
<th>Flow Rate (mL/min)</th>
<th>Parameters</th>
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<th>Purity Threshold</th>
<th>Theoretical Plates</th>
<th>Symmetry Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>999.993</td>
<td>999.900</td>
<td>33709</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>999.989</td>
<td>999.900</td>
<td>33462</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>999.994</td>
<td>999.900</td>
<td>33921</td>
<td>0.76</td>
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</table>

<table>
<thead>
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<th>Symmetry Factor</th>
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</thead>
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<tr>
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<td>999.900</td>
<td>33891</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>999.995</td>
<td>999.900</td>
<td>33456</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>999.998</td>
<td>999.900</td>
<td>33712</td>
<td>0.70</td>
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<table>
<thead>
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<td>Parameters</td>
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</tr>
<tr>
<td>35.0</td>
</tr>
<tr>
<td>38.5</td>
</tr>
</tbody>
</table>

**Table 10**: Results of robustness study
5.2.8 Results of Ruggedness

To check the method ruggedness, the recovery experiments corresponding to carried out duloxetine hydrochloride were again carried out in laboratory B using a different instrument. The results are satisfactory and confirm ruggedness of the method (Table 9).

[6] Conclusion

A simple, suitable, precise and fast HPLC method has been developed and validated for the determination of duloxetine hydrochloride in enteric-coated extended release formulation. There was a systematic approach in the selection of mobile phase and HPLC column for method development.

The developed method was compatible with LC-MS detection due to the volatile mobile phase system. The method was completely validated with respect to specificity, system suitability, linearity, accuracy, precision, robustness, ruggedness and solution stability. The result of validation showed satisfactory data for all the method validation parameters tested.

The develop method can be used for the accurate estimation of duloxetine hydrochloride in pharmaceutical formulation. The less run time and simple mobile phase make this method more suitable to support routine analysis in quality control labs.
References


### List of Research Article

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<td>DEVELOPMENT AND APPLICATION OF A VALIDATED CHIRAL HPLC METHOD FOR ENANTIOSELECTIVE ESTIMATION OF ZOPICLONE IN PHARMACEUTICAL</td>
<td>Journal of Analytical Chemistry</td>
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<td>Development and Application of a Validated Chiral LCMS Method for Direct Enantioselective Estimation of Pregabalin in Pharmaceutical Formulation.</td>
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Development and validation of a stability indicating method for the enantioselective estimation of omeprazole enantiomers in the enteric-coated formulations by high-performance liquid chromatography

Samir Vyas, Ajay Patel, Kartik D. Ladva¹, H. S. Joshi², Atul H. Bapodra³

ABSTRACT

Omeprazole is widely prescribed in the form of enteric-coated formulations, due to the rapid degradation of the drug in the acidic condition of the stomach. In the current article, we are reporting the development and complete validation of a stability indicating chiral high-performance liquid chromatography (HPLC) method for the enantioselective analysis of omeprazole in the enteric-coated formulations. A precise and sensitive enantiomeric separation of omeprazole was obtained on Chiralcel OD-H analytical column (250mm × 4.6 mm, 5µm particle size) using normal phase chromatography. The analysis was performed under UV detection at 301nm wavelength. During method development, the addition of methanol to the mobile phase helped in getting the sharp peaks. The developed method showed linear response over a wide concentration range of 0.39-800µg/ml and the regression coefficients value ($r^2$) was obtained more than 0.999 for (S-) and (R)-omeprazole. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for (R)-omeprazole were found to be 0.39 and 0.78 µg/ml, respectively for 5 µl injection volume. The percentage recovery of (R)-omeprazole ranged from 93.5 to 104 in spiked formulation samples and omeprazole sample solution and mobile phase were found to be stable for at least 24 h at room temperature. The proposed method was found to be suitable and accurate for the quantitative determination of undesired enantiomer in the enteric-coated omeprazole formulations.

KEY WORDS: Enantiomeric purity; method development, normal phase chiral HPLC; omeprazole; pharmaceutical formulations, validation

Omeprazole is one of the most important Proton Pump Inhibitors (PPIs). PPIs are a group of drugs whose main action is pronounced and long-lasting reduction of gastric acid production. Omeprazole has a stereogenic center at the sulphur atom, and it exists as the two optically active forms, (S)-(-) and (R)-(+) omeprazole [Figure 1].[1] Omeprazole was first approved as a racemic mixture, but the (S)-enantiomer was later introduced to the market. The major difference is that (S)-omeprazole is metabolized more slowly and reproducibly than the (R)-omeprazole and racemic omeprazole, because of the stereoselective metabolism by human cytochrome P450 enzyme.[2] Therefore lower doses of (S)-omeprazole can be used to produce equivalent acid suppression than omeprazole doses. Omeprazole is unstable in acidic environment.[3] In aqueous media, the degradation rate proceeds with a half-life of less than 10 min at pH values lower than 4.3.[4] Omeprazole is thus formulated as enteric-coated granules encapsulated in a gelatin shell or as enteric-coated tablets.[5,6]

One study reported the comparison of these two techniques, high-performance liquid chromatography (HPLC) and capillary electrophoresis for the enantioselective analysis of omeprazole.[7]
The major objective of this present work is to develop a stability indicating method and perform the systematic validation as per the International Conference of Harmonization (ICH) guidelines. The same method was used to estimate the (R)-omeprazole in the enteric-coated pharmaceutical formulations.

Chemicals and drugs

HPLC grade solvents were used as mobile phase, which is manufactured by Merck and procured from commercial source. Bulk drug samples and enteric-coated capsule formulation of omeprazole, (R) and (S)-omeprazole were obtained from local market.

Chromatographic conditions

The chiral analysis was performed on a Shimadzu LC-2010 HPLC system consist of a quaternary pump, a column oven, a photo diode array detector and an auto injector. Enantiomeric separation achieved at 40°C column oven temperature using Chiralcel OD-H column (250 × 4.6 mm, 5 μm particle size, Daicel make). The mobile phase consisted of 85% of n-hexane, 8% of methanol and 7% a mixture of isopropyl alcohol and ethanol (85:15, v/v). Mobile phase was chosen as the diluent to achieve clean blank chromatogram without any interference. Flow rate was 0.75 ml/min and injection volume was 5 μl. Data analysis performed at a wavelength of 301 nm.

Sample preparation

The standard stock solutions of (R)- and (S)-omeprazole were prepared by dissolving appropriate amount of the standard samples in mobile phase. A stock solution concentration was fixed at 800 μg/ml. For formulation sample, 8 capsules (10 mg of (S)-omeprazole label claim) were opened and the enteric-coated granules were finely ground using agate mortar and pestle. The ground material, which was equivalent to 80 mg of (S)-omeprazole was extracted in to methanol in a 50-ml volumetric flask by ultrasonication. The resultant mixture was filtered through a 0.45-μm membrane filter. This solution corresponds to analyte concentration of 1600 μg/ml, and further dilutions were prepared in diluent.

Method validation

Specificity

Specificity of this method was indicated by the absence of any endogenous interference at retention times of enantiomeric peaks. The absence of interfering peak was evaluated by injecting a blank sample consisting of diluent and placebo.

Stability indicating method

The drug was subjected to forced degradation under acidic (1M hydrochloric acid, 5 ml), basic (1M sodium hydroxide, 5 ml), and oxidative (3% hydrogen peroxide, 5 ml) stress conditions.

Precision

The precision of the method was checked by analyzing nine replicate samples of (S)-omeprazole (at analyte concentration, i.e., 800.00 μg/ml) spiked with 0.1% (0.8 μg/ml) of (R)-omeprazole on different days and RSD of area under the peaks was calculated.

Linearity of omeprazole enantiomers

Linearity corresponds to the capacity of the method to supply results directly proportional to the concentration of the substance being determined within a certain interval of concentration. Detector response linearity was assessed by preparing 12 calibration sample solutions covering from 0.39 to 800 μg/ml (0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.0, 100.0, 200.0, 400.0, and 800.00 μg/ml), Regression curve was obtained by plotting peak area versus concentration, using the least squares method. Duplicate injections given for each concentration level.

Sensibility (LLOD and LLOQ)

LLOD and LLOQ were achieved by giving six injections of lowest three concentration levels, prepared for linearity study. The signal to noise ratio and RSD of the area is considered to evaluate LLOD and LLOQ.

Recovery study of (R)-omeprazole in formulation

The standard addition and recovery experiments were conducted to determine accuracy of the present method. The study was carried out in triplicate by spiking placebo with
three concentrations (0.12, 0.15 and 0.18%) of standard (R)-omeprazole and assaying for the chromatographic method. The recovery for (R)-omeprazole was calculated from the slope and Y-intercept of the calibration curve, drawn in the concentration range of 0.39-500 μg/ml.

Ruggedness

To determine the ruggedness, the recovery experiments carried out for (R)-omeprazole in formulation samples were again carried out in laboratory B using a different instrument.

Robustness

For the HPLC method, the robustness was determined by the analysis of the samples under a variety of conditions making small changes in the percentage of methanol in mobile phase (7 and 9%, v/v), in the flow rate (0.7 and 0.8 ml/min), in the column temperature (35 and 45°C), and changing the wavelength (299 and 303 nm). The change in chromatographic resolution between enantiomers was evaluated for the study.

Solution stability

The sample was analyzed for 24h at room temperature, i.e., at 25°C. Resolution and composition of omeprazole enantiomers were observed during the study period.

Results and Discussion

Method development and optimization

Among the chiral columns evaluated, Chiralcel OJ-R and Chiralcel OJ-H did not show potential results for enantiomeric separation of omeprazole. The Chiralcel OD-H column was found to be suitable for better resolution of omeprazole enantiomers. The solutes can bind to the carbamate groups on the chiral stationary phase forming transient diastereomers through hydrogen bonding using the C=O and NH groups and also through dipole–dipole interaction using the C=O moiety. Omeprazole has NH functional group and this could well be contributing to the interactions with the carbamate groups on CSP, resulting in separation.\[11\]

During method development the methanol was chosen as a polar organic modifier of the mobile phase, because the methanol has proven good organic modifier for resolution of omeprazole enantiomers.\[12,13\] The percentage of methanol between 0 and 10% had strong effect on separation and sharpness of the enantiomeric peaks corresponding to omeprazole. The increase in certain percentage of methanol content in mobile phase drastic increased the resolution, and number of theoretical plates of the two enantiomers, but decreased the resolution with the higher percentage of methanol [Table 1]. In order to obtain sharp peaks without compromising on the resolution, the 8% (v/v) of methanol content chosen in mobile phase.

In the final method, the typical retention times of (S)-omeprazole and (R)-omeprazole were about 14 and 15 min, respectively [Figure 2]. A chromatogram of spiked (R)-omeprazole at 0.1% of concentration level in (S)-omeprazole formulation sample is shown in Figure 3.

Validation

Specificity

The blank chromatogram was clean without any interference from diluent and placebo of the capsules.

Stability indicating method

The drug shows 30%, 15% and 21% degradation under acidic, basic and oxidative conditions, respectively. The purity factor is within the threshold limit for forced degradation and formulation samples, hence the developed method was found to be stability indicating and results are free from any interference.

Precision and sensibility

The determined precision, intraday precision, LLOD and LLOQ values are reported in Table 2.

Recovery

The recovery experiments were conducted to determine the accuracy of the present method for the quantification of (R)-omeprazole in formulation samples. (R)-omeprazole was spiked to the extracted (S)-omeprazole sample (500 μg/ml) in triplicate at 0.12, 0.15 and 0.18% of target analyte concentration. Recovery was calculated from the slope and Y-intercept of the calibration curve obtained in linearity study. The same recovery experiments were also conducted using a different system in laboratory B at the same concentration levels tested in laboratory A and results were well in agreement. This confirms the ruggedness of the method. The results are summarized in Table 3.

Linearity

The calibration curve constructed for omeprazole was linear over the wide concentration range of 0.39-800 μg/ml. The each solution was injected in duplicate and RSD of area under the peak was < 2% across the study. The regression was found to be linear over the concentration range and correlation coefficient was greater than 0.999 for both enantiomers.
Robustness

The chromatographic resolution of the (S)- and (R)-omeprazole enantiomers peaks was remain more than 1.5 under all modified conditions, which demonstrate the sufficient robustness of the method.

Solution stability

No significant change was observed in resolution and composition of omeprazole enantiomers during the solution stability study for 24h at room temperature, i.e., at 25°C.

Application

The accuracy data proved that the developed method can be used for the quantitative determination of undesired enantiomer of omeprazole in the enteric-coated pharmaceutical formulations.

Conclusions

This is the first report to describe the stability indicating chiral HPLC method for enantioselective analysis of omeprazole enantiomers. In this study we found the importance of methanol as polar organic modifier in normal phase chiral chromatography, which improved the peak shape of omeprazole enantiomers. The method was completely validated and shown satisfactory data
for all the method validation parameters tested. The method was sensitive (LLOQ=0.78 μg/ml) and linear over the thousand fold concentration ranges. This method can be use for routine analysis in quality control laboratories.

References


Source of Support: Nil, Conflict of Interest: None declared.
FORMULAS

Mean:

\[
\text{Mean} = \frac{\text{Sum of all Values}}{\text{Number of Values}}
\]

Standard Deviation (SD):

\[
\text{SD} = \sqrt{\frac{\sum_{i=1}^{n} (y_i - \bar{y})^2}{(n - 1)}}
\]

Where mean (M) is

\[
\bar{y} = \frac{\sum_{i=1}^{n} y_i}{n}
\]

Relative Standard Deviation (RSD) [%]:

\[
\text{RSD} = \frac{\text{Standard Deviation} \times 100}{\text{Mean}}
\]

Recovery (%):

\[
\text{Recovery} = \frac{\text{Obtained (Recovered) value} \times 100}{\text{True value (Added)}}
\]

Quantitation from Calibration Curve:

\[
x = \frac{y - \text{Intercept}}{\text{Slope}}
\]

Limit of Detection: \( = \frac{3.3 \times \text{SD}}{S} \)

Limit of Quantification = \( \frac{10 \times \text{SD}}{S} \)

Where;
- **SD** is standard deviation
- **S** is the standard deviation of the slope
LIST OF SYMBOLS AND ABBREVIATIONS

conc : Concentration
h : Hour
ppm : Parts Per Million
g : gram
mg : Milligram
min : Minute
mL : Milliliter
N : Normality
nm : Nanometer
No : Number
UV : Ultraviolet
µg : Microgram
µL : Microliter
m : Micrometer
% : Percentage
° C : Degree Centigrade
r : Correlation Coefficient
≥ : Greater Than or Equal to
≤ : Less Than or Equal to
$ : Dollar
AU : Absorbance Unit
mAU : Milli Absorbance Unit
6-MP : 6-Mercaptopurine
ACN : Acetonitrile
API : Active Pharmaceutical Ingredient
Avg : Average
CEC : Capillary Electrophoresis Chromatography
cGMP : Current Good Manufacturing Practice
UV  : Ultra Violet
WHO : World Health Organization