CHAPTER-2
Development and Validation of Stability Indicating HPLC Methods for Analysis of API and its Pharmaceutical Dosage Form
Section 2.1
Determination of Prasugrel Hydrochloride in Pharmaceutical Dosage Form by validated stability indicating method

![Chemical structure of Prasugrel hydrochloride]

**Fig. 2.1.F-1:** Chemical structure of Prasugrel hydrochloride

### 2.1.1 Drug profile

**Prasugrel hydrochloride**

**Chemical name:** (RS)-5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7- tetrahydrothieno[3,2-c]pyridin-2-yl acetate

**Molecular formula:** $\text{C}_{20}\text{H}_{20}\text{FNO}_3\text{S}$

**Molecular weight:** 373.44

**Description:** Off white powder

**Properties:** Soluble in water

**Therapeutic category:** Platelet inhibitor
2.1.2 Introduction

Chemically Prasugrel hydrochloride is (RS)-5-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno [3,2-c]pyridin-2-yl acetate (Fig. 2.1.F-1) [1]. Prasugrel is thienopyridine derivative, it is a prodrug inactive in body. Prasugrel is rapidly form its metabolite. Efficiency of this metabolite as compared to other thienopyridine is more, very less dependence on enzymes cytochrome P-450. Binding of this metabolite is very specific, which results to inhibit activation of f ADP-mediated platelet. Patients suffering from heart problem showed more potent effect of prasugrel than Clopidogrel. [2-8].

2.1.3 Literature review

Stereo isomers of active metabolite of Prasugrel were analyzed by using LC-MS in human plasma by derivatizing reagent bromomethoxyacetophenone. [9].

Sensitive and rapid and LC-MS method of analysis for estimation of the Prasugrel and its metabolite in plasma. Analysis was carried on C_{18} column. Detection was performed by Mass spectrometer. Another two LC-MS-MS based bio analytical assays were reported to analysis of Prasugrel [11].

Validated HPTLC method for analysis of Prasugrel was also reported [12].

RP- HPLC assay was reported for Prasugrel tablets. Column used a Inertsil C_{18}, (150 × 4.6 mm, 5 µm). Mixture of 0.02 M aqueous KH_{2}PO_{4} solution having pH 3.1 and acetonitrile used as mobile phase [13].

A spectrophotometric validated method for the estimation of Prasugrel was also reported. Methanol is used as a solvent to dissolve the drug and to extract drug product. The E_{max} is observed at 201 nm [14].

Another spectrophotometry assay method for the analysis of Prasugrel in drug product. Prasugrel exhibits maximum absorbance at 254 nm (method A). In method B (D1) is a first derivative method showing minima at 272 nm and method C is an area under curve method (244 to 264 nm) [15].

In the present research work we develop HPLC method for quantification of Prasugrel along with its degradants.
Force degradation study data required to get deep knowledge of degradation path of drug [16-17]. In order to this, stress studies of Prasugrel were performed. Specificity of method was checked by analyzing the stressed samples by using proposed method.

2.1.4 Present work

Chemicals and reagents

Lupin Pharmaceutical Ltd, Mumbai, India, provided Prasugrel reference standard as a gift sample. Acetonitrile (chromatography grade) was procured from Rankem, India. Hydrogen peroxide ammonium acetate, sodium hydroxide and acetic acid procured from Merck. HPLC grade water was used to perform all experiments. Tablet of Prasugrel namely “Prasudoc” tablet were used for analysis.

HPLC instrument and chromatographic parameters

HPLC instrument (Agilent-1100 series) having Chemstation software. Agilent technologies Zorbax XDB C₈ (150 × 4.6 mm, 3.5 µm) HPLC column maintained at 30 °C using column oven. Instrumental setting for Flow rate was 1.0 ml/min. 0.05M ammonium acetate (pH 4.5 with acetic acid) and acetonitrile 40:60, v/v used as mobile phase. Injected 10 µl of injection and detection wavelength was 254 nm. To monitor degradation, the PDA was used in scan mode in UV region. To express specificity, peak purity values were determined the mentioned software.

Standard preparation

10 mg/ml Prasugrel stock was prepared and working level was prepared by diluting this solution. ACN : Water in ratio of 30:70, v/v used as diluent

Sample Preparation

Twenty tablets were taken and determined their average weight and crushed; powder containing 50 mg of Prasugrel transferred to volumetric flask (50 ml). Added 15 ml diluent and 10 minute sonication has been performed with shaking and diluted to mark with diluent. Centrifuged this solution at the rate of 10,000 rpm for 10 minute. 0.45 µm filters were used to filter this solution. Diluted this solution to get concentration 200 µg/ml.
**Stress degradation study**

**Acidic degradation**

Tablet powder containing 20 mg of drug is added to 10 ml flask; 3 ml of 1 N HCl solution is added, and heated this for 3 hrs at 60 °C on water bath. Neutralized this solution by using 1N NaOH and made up the volume with diluent. Prasugrel 200 μg/ml concentration was prepared from this solution.

**Alkali degradation**

Tablet powder containing 20 mg of Prasugrel is weighed and transferred to flask (10 ml), 3 ml diluent. NaOH solution (0.01M) was added and store at ambient temperature for half an hour. Neutralized solution by 0.01N HCl. Prasugrel 200 μg/ml concentration was prepared from this solution.

**Oxidative degradation**

Powder having 20 mg of Prasugrel is dissolved in 3 ml diluent and added 2 ml of 30% hydrogen peroxide. Kept this solution at 60 °C for 1 hr. Volume made up with diluent to 10 ml. Prasugrel 200 μg/ml concentration was prepared from this solution.

**Thermal degradation**

Tablet powder was exposed to heat at 90 °C for 24 hrs. Prasugrel 200 μg/ml concentration was prepared from this solution.

**UV-Short (254 nm) degradation**

Tablet powder was exposed to UV at 254 nm for one day. Prasugrel 200 μg/ml concentration was prepared from this solution.

**UV-Long (366 nm) degradation**

Tablet powder was exposed to UV at 366 nm for one day. Prasugrel 200 μg/ml concentration was prepared from this solution.
2.1.5 Method validation

Specificity

Peak purity determination method was used for the specificity. No interference should be observed at retention time of main peak.

Linearity

To check linearity, standard solutions having concentration in the range of 10 to 200% of the assay level concentration. Each solution was analyzed. Calibration graph was plotted by using concentration versus peak response.

Precision (repeatability)

To study method precision, injected six test preparations (200 μg/ml). Percentage RSD of assay values for six measurements was calculated. Six Sample preparations were analyzed by different analyst on another instrument to carried out intermediate precision. Percentage RSD of six preparations was calculated.

Accuracy (recovery test)

For recovery, known amount of drugs were spiked in placebo at 80, 100 and 120% of working level concentration. Each level was prepared in triplicate.

Robustness

The flow rate robustness was studied by changing ± 0.1 ml/min. Column oven temperature was investigated at ± 2 °C to set value. To study organic strength effect, mobile phase was altered as buffer and acetonitrile 38:62, v/v, 42:58, v/v. Assay of tablet were performed in triplicate. Detector wavelength was studied at ± 254 nm.

Quantification limit and Detection limit

Calibration curve method was employed to establish detection and quantification limit of method.

The LOQ and LOD solutions were injected 6 times. Peak area percentage RSD was determined.
Chapter 2

Stability of analytical solution

Stability of analytical solution was performed upto 24 hrs to observe any significant degradation of analytical solution. Peak response cumulative percentage RSD was determined.

2.1.6 Result and discussion

Chromatographic conditions optimization

In all development trials it was clear that Zorbax XDB C8 column gives a better separation between degradant peaks and Prasugrel as compare to other C18 or CN columns. 0.05 M ammonium acetate adjusted pH with formic acid to 4.5. Mix this buffer with acetonitrile as 40:60, v/v to get final mobile phase. Colum oven maintained at 30 °C and flow rate kept 1.0 ml/Min. Acidic pH of buffer solution reduce the tailing of analyte peak The run time for analysis was less than 10 minute. Prasugrel peak was eluting at 6.9 minute. System suitability data was given in Table 2.1.T-1.

Stress degradation study discussion

Initially during forced degradation experiments, the basic degradation analyte was very vigorous when 0.5N NaOH solution is employed. Table 2.1.T-2 shows degradation percentage in each stress condition, Spectral data and assay of Prasugrel in all tested stress conditions. Fig. 2.1.F-2 to 2.1.F-10 showed the chromatograms of test solution, diluent (blank), base hydrolysis blank, base hydrolysis degradation of tablet, oxidation blank, oxidation degradation of tablet, acid hydrolysis blank, acid hydrolysis degradation of tablet and thermal degradation of tablet respectively. Spectral peak purity of Prasugrel in all stress degradation was shown in Fig. 2.1.F-13 to 2.1.F-18.

Results of method validation

Excipients and diluent did not showed any peak at the Prasugrel retention. UV detector in scan mode of range 200 to 400 nm was used to demonstrate the specificity. Calibration curve were obtained by constructing graph of peak area against the concentration of analyte having equation y = 9.327x + 0.7701. Good precision was observed in method precision and intermediate precision. Percentage
RSD for six determinations was always less than 2.0. Data is shown in table 2.1.T-3. Prasugrel recovery values at 80, 100 and 120% were ranging from 98.9 to 100.6%. Overall recovery mean for nine determinations was 100.0% having overall percentage RSD 1.6. Recovery results are tabulated in table 2.1.T-4.

In robustness experiment assay percentage was within 100 ± 2 proves robustness of method. Table 2.1.T-5 gives the overall robustness data. LOQ of Prasugrel was 0.364 µg/ml having percentage RSD for six injections less than 5.0 and LOD of Prasugrel was 0.11 µg/ml having percentage RSD for six injections less than 10.0.

In solution stability experiment, cumulative percentage Peak area %RSD of Prasugrel was within 0.5. The peak purity and percentage RSD were 999.983, 0.32 respectively.
2.1.7 Figures: Method validation chromatograms of Prasugrel hydrochloride tablet

Fig. 2.1.F-2: Tablet solution chromatogram

Fig. 2.1.F-3: Blank solution chromatogram
Fig. 2.1.F-4: Alkali hydrolysis blank chromatogram

Fig. 2.1.F-5: Alkali hydrolysis degradation of tablet chromatogram
Fig. 2.1.F-6: Oxidative blank chromatogram

Fig. 2.1.F-7: Oxidative degradation of tablet chromatogram
Fig. 2.1.F-8: Acid hydrolysis blank chromatogram

Fig. 2.1.F-9: Acid hydrolysis degradation of tablet chromatogram
Fig. 2.1.F-10: Thermal degradation of tablet chromatogram

Fig. 2.1.F-11: UV short degradation of tablet chromatogram
**Fig. 2.1.F-12:** UV short degradation of tablet chromatogram

**Fig. 2.1.F-13:** Prasugrel peak purity spectra in base hydrolysis degradation
**Fig. 2.1.F-14:** Prasugrel peak purity spectra in oxidation degradation

**Fig. 2.1.F-15:** Prasugrel peak purity spectra in acid hydrolysis degradation
Fig. 2.1.F-16: Prasugrel peak purity spectra in thermal degradation

Fig. 2.1.F-17: Prasugrel peak purity spectra in UV short degradation
Fig. 2.1.F-18: Prasugrel peak purity spectra in UV-long degradation
2.1.8 Tables: Method validation data of Prasugrel hydrochloride tablet

Table 2.1.T-1: System suitability data

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (Min.)</th>
<th>Theoretical Plate Count</th>
<th>USP Tailing</th>
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<tr>
<td>Prasugrel</td>
<td>6.9</td>
<td>9351</td>
<td>1.13</td>
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Table 2.1.T-2: Force degradation study data

<table>
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<tr>
<th>Stress condition</th>
<th>Degradation (%)</th>
<th>Peak purity</th>
<th>Assay (%)</th>
</tr>
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<tr>
<td>Oxidative (30% H₂O₂/60 °C/1 hr)</td>
<td>19.18</td>
<td>999.592</td>
<td>81.23</td>
</tr>
<tr>
<td>Acidic (1N HCl/60 °C/3 hrs)</td>
<td>11.33</td>
<td>999.915</td>
<td>88.38</td>
</tr>
<tr>
<td>Alkali (0.01 NaOH/RT/ 30 min.)</td>
<td>13.38</td>
<td>999.743</td>
<td>86.26</td>
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<tr>
<td>Thermal (90 °C/24 min.)</td>
<td>6.11</td>
<td>999.831</td>
<td>93.13</td>
</tr>
<tr>
<td>UV-short (24 hrs)</td>
<td>No degradation</td>
<td>999.901</td>
<td>99.33</td>
</tr>
<tr>
<td>UV-long (24 hrs)</td>
<td>No degradation</td>
<td>999.925</td>
<td>99.69</td>
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Table 2.1.T-3: Validation Summary

<table>
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<tr>
<th>Experiment</th>
<th>Parameter</th>
<th>Results</th>
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<tr>
<td>Precision (n = 6, % RSD)</td>
<td>System Precision</td>
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<td>Method Precision</td>
<td>0.59</td>
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<tr>
<td></td>
<td>Intermediate Precision</td>
<td>0.86</td>
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<tr>
<td>LOD and LOQ</td>
<td>Limit of detection (µg/ml)</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>Limit of quantification (µg/ml)</td>
<td>0.364</td>
</tr>
<tr>
<td>Linearity</td>
<td>Calibration range (µg/ml)</td>
<td>20 to 400</td>
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<td></td>
<td>Regression equation</td>
<td>y = 9.327x + 0.7701</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>0.999</td>
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### Table 2.1.T-4: Recovery data

<table>
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<tr>
<th>% Level</th>
<th>Spike Amount (mg)</th>
<th>Found Amount (mg)</th>
<th>Mean Recovery % (n=3)</th>
<th>RSD (%)</th>
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<tr>
<td>80</td>
<td>8.13</td>
<td>8.09</td>
<td>99.39</td>
<td>1.71</td>
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<tr>
<td>100</td>
<td>10.19</td>
<td>10.20</td>
<td>100.12</td>
<td>1.22</td>
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<tr>
<td>120</td>
<td>12.35</td>
<td>12.33</td>
<td>99.78</td>
<td>1.66</td>
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### Table 2.1.T-5: Results of robustness study

<table>
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<tr>
<th>Parameter</th>
<th>Set Value</th>
<th>Assay % (n=3)</th>
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<tbody>
<tr>
<td>Flow rate</td>
<td>0.9 ml/min.</td>
<td>99.56</td>
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<td></td>
<td>1.1 ml/min.</td>
<td>99.18</td>
</tr>
<tr>
<td>Organic modifier</td>
<td>58%</td>
<td>99.32</td>
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<tr>
<td></td>
<td>62%</td>
<td>98.97</td>
</tr>
<tr>
<td>Column oven temperature</td>
<td>28 °C</td>
<td>98.81</td>
</tr>
<tr>
<td></td>
<td>32 °C</td>
<td>99.23</td>
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<tr>
<td>Detector Wavelength</td>
<td>250 nm</td>
<td>99.88</td>
</tr>
<tr>
<td></td>
<td>258 nm</td>
<td>99.75</td>
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Section 2.2
Stress Degradation Studies of Dronedarone in Pharmaceutical Dosage Form by a Validated Stability Indicating LC Method

![Chemical structure of Dronedarone hydrochloride](image)

**Fig. 2.2.F-1:** Chemical structure of Dronedarone hydrochloride

### 2.2.1 Drug profile

**Dronedarone hydrochloride**

**Chemical name:** N-{2-buty1-3-[4-(3-dibutylaminopropoxy)benzoyl]benzofuran-5-yl} methanesulfonamide

**Molecular formula:** C_{31}H_{44}N_{2}O_{5}S

**Molecular weight:** 556.73

**Description:** Off white powder

**Properties:** The HCl salt is soluble in dichloromethane and methanol, Water insoluble

**Therapeutic category:** Anti-arrhythmic agent
2.2.2 Introduction

Dronedarone HCl is chemically N-{2-butyl-3-[4-(3-di-
butylaminopropoxy) benzoyl] benzofuran-5-yl} methane sulfo-
namide (Fig. 2.2.F-1) [21]. Dronedarone is belonging to the
benzofuran class of anti-arrhythmic compounds including
amiodarone, mainly used for cardiac arrhythmias (ir-
regular heart beat). For patients requiring drug therapy or
electroshock therapy to retain their normal rhythms,
Dronedarone is a useful option [22-23]. Dronedarone is
indicated for the reduction of cardiovascular morbidity and
mortality in atrial fibrillation or atrial flutter suffering
patient [24-26].

Electrophysiological properties of Dronedarone are same as
amiodarone. The main advantage of dronedarone is it doesn’t
contain iodine; therefore there is no possibility of iodine
related side effect [27]. Method of preparation of Dronedarone
is reported in US patent [28].

2.2.3 Literature survey

Bio analytical LC method for the analysis of Dronedarone and
debutyl Dronedarone was reported. Reported method is
sensitive for determination of Dronedarone, Amiodarone in
plasma [29].

Dronedarone pharmacokinetics was studied by using LCMS
was reported. This method is useful for determination of
Dronedarone and debutyl Dronedarone. [30].

ICH guideline on stability testing of new drug substance
contains that stress degradation study to be determine the
stability of drugs [16, 31]. Stress studies of drug are required
for method development and validation.

2.2.4 Present work

Chemicals and reagents

Standard Dronedarone were obtained from Aldrich (India),
ammonium acetate and acetonitrile (HPLC grade) were procured
from Merck Chemicals. Hydrogen peroxide, sodium hydroxide
and hydrochloric acid were obtained from
Qualigen. The drug product of Dronedarone (Multaq tablet 400 mg) was purchased from the market. During all validation experiments

**Chromatographic setup**

Agilent-1100 series HPLC was used. Analysis was performed by Agilent chemstation. Software, column was Zorbax XDB C₈ (150 × 4.6 mm, 3.5 um,) was used. Column temperature was 30 °C. Instrumental setting for flow was set at rate of 1.0 ml/min. Filtered acetonitrile and ammonium acetate 50mM (60:40; v/v) is used as mobile phase. Injected 10 µl injection and UV detector was kept at 220 nm. Peak purity was examined by using PDA detector.

**Standard solution**

Stock solution (1000 µg/ml) was prepared in 50 ml volumetric flask. Further diluted this stock, to get desired concentration. Diluent was water and acetonitrile as 50:50, v/v.

**Preparation of tablet solution**

Average weights of 20 tablets were calculated. Tablet powder containing 50 mg of Dronedarone were weighed and transferred into flask (50 ml), added diluent sonicated to dissolve and make up with diluent and solution was centrifuge at for 10 minute at 10,000 rpm. Diluted this solution to get solution having Dronedarone concentration 200 µg/ml

**Stress degradation study**

Degradation study was design as per the ICH guideline [18]. Forced degradation was studied under acid, alkali, oxidative, photolytic thermolysis, conditions.

**Acidic degradation**

Weighed tablet powder containing 20 mg of Dronedarone in and transfer to volumetric flask (10ml). 3 ml of each diluent and 1N HCl solution was added. The mixture was heated at 60 °C by using water bath for 3 hrs, neutralized with 1M sodium hydroxide and make up to mark with same solvent and Dronedarone solution having concentration 200 µg/ml was prepared from this solution.
**Base degradation**

Weighed tablet powder containing 20 mg of Dronedarone in and transfer to volumetric flask (10ml). Added diluent to dissolve the drug and added 2 ml of 0.1N NaOH solution. Keep the mixture at 60 °C for half an hour on water bath. 0.1N HCl solution is used to neutralize the solution. Make up the volume up to mark with diluent. Dilute this solution to working level concentration.

**Oxidative degradation**

Weighed tablet powder containing 20 mg of Dronedarone in and transfer to volumetric flask (10ml). Added diluent to dissolve and 2 ml of 30% hydrogen peroxide solution, heated the mixture at 60 °C for 1 hr on water bath. Allow it to cool and the make up with diluent. Dilute this solution to working level concentration.

**Thermal degradation**

Dronedarone tablet powder was exposed to 90 °C for 24 hrs. Prepare the solution having 200 μg/ml of Dronedarone.

**Photolytic degradation**

Expose the tablet powder to 1.2 million lux hours of light in 24 hrs. Prepare the solution having 200 μg/ml of Dronedarone.

**UV-Short degradation**

Tablet powder was kept in UV chamber to 254 nm for one day. Solution having concentration 200 μg/ml of Dronedarone was prepared from exposed sample.

**UV-Long degradation**

Tablet powder was kept in UV chamber to 366 nm for one day. Solution having concentration 200 μg/ml of Dronedarone was prepared from exposed sample.
2.2.5 Method validation

Specificity

Peak purity determination method was used for the specificity. No interference should be observed at retention time of Dronedarone.

Linearity

To check linearity, standard solutions having concentration in the range 10 to 150% of the standard concentration (200 µg/ml) was prepared. Each solution were analyzed in triplicate.

Method precision (repeatability)

For method precision study, injected tablet sample preparation six times and determined concentration of drug.

Intermediate precision (reproducibility)

To evaluate reproducibility of method performed assay of tablet six times by another chemists. Determined mean of the six measurements.

Accuracy (recovery test)

For recovery, known amount of drug were spiked in placebo at 80, 100 and 120% of working level concentration. Each level was prepared in triplicate.

Robustness

Ability of any analytical method to remain unchanged due to small deliberated changes in parameters is termed as robustness. To confirm this, analytical methods were purposely altered. Robustness was measured by evaluating assay system suitability parameters and resolution between main peak and oxidation degraded product at RRT 0.87 in all altered conditions.

Mobile phase flow was at the rate of 1.0 ml/min. Changed it ±0.1 ml/min. Column oven temperature impact was studied at ± 2 °C while other parameters were kept constant. Organic composition robustness was studied in ratio of acetonitrile and aqueous solution of 50 mM ammonium acetate (62:38, v/v and 58:42, v/v). UV detector ruggedness was studied at 220 ± 4 nm and 216 nm. At all conditions, the assay was performed in triplicate.
**Quantification limit and Detection limit**

The detection limit and quantitation limit was determined by plotting calibration graph in range of detection to 150% of standard solution concentration.

Detection limit = $3.3\sigma/S$

Quantitation limit = $10\sigma/S$

Where $\sigma$ = peak area standard deviation

$S$ = calibration curve slope.

Both solutions were injected 6 times. Peak area percentage RSD was determined.

**Solution stability**

Solution stability of analytical solution was examined for 24 hrs. Cumulative percentage RSD of peak area was evaluated. Analyte peak was examined for peak purity at each interval.

**2.2.6 Result and discussion**

**Chromatographic conditions optimization**

Our objective of method development was method should be specific for analyte peak and the tailing factor should be less than 2. Dronedarone and its degradants were separated on Zorbax XDB C$_8$ (150 × 4.6 mm, 3.5 µm) column. Composition of mobile phase was optimized to achieve good chromatographic condition. Zorbax XDB C$_8$ column showed good results than other C$_{18}$ or CN columns. System suitability data is shown in table 2.2.T-1.

Development studies confirmed that the acetonitrile and ammonium acetate 50mM (60:40, v/v) is an optimal condition as mobile phase. Peak shape Dronedarone has less tailing. All possible degradants were well resolved from main peak. Dronedarone peak retention is about 8.7 minute.

**Result of stress degradation study**

Target was kept 10 to 30% degradation of analyte in all attempted conditions [17, 32]. This is not achieved in photolytic degradation after using harsher conditions. The drug product showed degradation in thermal acid hydrolysis, base hydrolysis and oxidation condition. Table 2.2.T-2 indicates the forced degradation
study data of Dronedarone. Peak purity values were obtained from spectral analysis. These values in the range of 999 to 1000 for tablet solution confirms the specificity of proposed method. Fig. 2.2.F-2 to 2.2.F-4 shows the chromatograms of Dronedarone tablet, diluent and placebo solution. Fig. 2.2.F-5 to 2.2.F-11 showed the chromatograms of acid hydrolysis blank, acid hydrolysis degradation of tablet, base hydrolysis blank, base hydrolysis degradation of tablet, oxidation blank, oxidation degradation of tablet and thermal degradation of tablet respectively. Fig. 2.2.F-14 to 2.2.F-17 showed the peak purity spectra of Dronedarone in acidic condition, basic condition, oxidation and thermal condition respectively.

**Result of method validation**

There was no any interference at analyte peak retention. The peak purity values for Dronedarone peak were more than 999 at 220 nm, indicating that the peaks of Dronedarone were pure and no specific interference from formulation excipients and degradants.

In linearity, peak response was found linear over the concentration. Observed calibration curve equation was

\[ y = 12.967x + 1.3752. \]

In precision study, the percentage RSD for six preparations was 0.23 and 0.39 respectively and mean of assay percentage was 99.61 and 99.91 respectively. Table 2.2.T-3 showed the precision data.

In robustness study assay percentage of Dronedarone at all deliberated conditions was found to be within the range of 98.21 to 99.60. Data is tabulated in table 2.2.T-5.

Observed LOD value for Dronedarone is 0.126 µg/ml and observed LOD value for Dronedarone is 0.318 µg/ml. The percentage RSD of six replicate measurements of LOQ and LOD solutions were less than 10.

In solution stability experiment, cumulative percentage RSD of peak area was within 0.39 after 24 hrs. The peak purity 999.906, indicates the solution have the stability of 24 hrs at room temperature.
2.2.7 Figures: Method validation chromatograms of Dronedarone HCl tablet

Fig. 2.2.F-2: Tablet solution chromatogram

Fig. 2.2.F-3: Diluent chromatogram
**Fig. 2.2.F-4:** Placebo solution chromatogram

**Fig. 2.2.F-5:** Acid hydrolysis blank chromatogram
Fig. 2.2.F-6: Acid hydrolysis degradation of tablet chromatogram

Fig. 2.2.F-7: Alkali hydrolysis blank chromatogram
Fig. 2.2.F-8: Alkali hydrolysis degradation of tablet chromatogram

Fig. 2.2.F-9: Oxidative degradation of tablet chromatogram
Fig. 2.2.F-10: Oxidative degradation of tablet chromatogram

Fig. 2.2.F-11: Thermal degradation of tablet chromatogram
Fig. 2.2.F-12: UV short degradation of tablet chromatogram

Fig. 2.2.F-13: UV long degradation of tablet chromatogram
Fig. 2.2.F-14: Dronedarone peak purity spectra in acid hydrolysis.

Fig. 2.2.F-15: Dronedarone peak purity spectra in alkali hydrolysis.
**Fig. 2.2.F-16:** Dronedarone peak purity spectra in oxidative degradation.

**Fig. 2.2.F-17:** Dronedarone peak purity spectra in thermal degradation.
Fig. 2.2.F-18: Dronedarone peak purity spectra in UV short degradation.

Fig. 2.2.F-19: Dronedarone peak purity spectra in UV long degradation.
2.2.8 Tables: Method validation data of Dronedarone hydrochloride tablet

Table 2.2.T-1: System suitability data

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (Min.)</th>
<th>Theoretical Plate Count</th>
<th>USP Tailing</th>
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<td>Dronedarone</td>
<td>8.5</td>
<td>6577</td>
<td>1.09</td>
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Table 2.2.T-2: Results of stress study

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<tr>
<th>Condition</th>
<th>Degradation (%)</th>
<th>Spectral Peak purity</th>
<th>Assay (%)</th>
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<td>Acidic (1N HCl/60 °C/3 hrs)</td>
<td>12.33</td>
<td>999.905</td>
<td>88.18</td>
</tr>
<tr>
<td>Alkali (0.1 NaOH/60 °C/30 min.)</td>
<td>21.98</td>
<td>999.719</td>
<td>77.26</td>
</tr>
<tr>
<td>Oxidative (30% H₂O₂/60 °C/1 hr)</td>
<td>26.18</td>
<td>999.902</td>
<td>73.53</td>
</tr>
<tr>
<td>Thermal (90 °C/24 hrs)</td>
<td>8.52</td>
<td>999.369</td>
<td>92.12</td>
</tr>
<tr>
<td>UV-254 nm (24 hrs)</td>
<td>No degradation</td>
<td>999.631</td>
<td>99.52</td>
</tr>
<tr>
<td>UV-366 nm (24 hrs)</td>
<td>No degradation</td>
<td>999.801</td>
<td>99.33</td>
</tr>
</tbody>
</table>
Table 2.2.T-3: Validation summary

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>System Precision</td>
<td>0.33</td>
</tr>
<tr>
<td>(n = 6, % RSD)</td>
<td>Method Precision</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Intermediate Precision</td>
<td>0.39</td>
</tr>
<tr>
<td>LOD and LOQ</td>
<td>Limit of detection (µg/ml)</td>
<td>0.126</td>
</tr>
<tr>
<td></td>
<td>Limit of quantification (µg/ml)</td>
<td>0.318</td>
</tr>
<tr>
<td>Linearity</td>
<td>Calibration range (µg/ml)</td>
<td>20 to 300</td>
</tr>
<tr>
<td></td>
<td>Regression equation</td>
<td>y = 12.967x + 1.3752</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Table 2.2.T-4: Recovery data

<table>
<thead>
<tr>
<th>% Level</th>
<th>Spike Amount (mg)</th>
<th>Found Amount (mg)</th>
<th>Mean Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>320.23</td>
<td>316.02</td>
<td>99.19</td>
<td>1.22</td>
</tr>
<tr>
<td>100</td>
<td>400.53</td>
<td>398.29</td>
<td>99.53</td>
<td>1.10</td>
</tr>
<tr>
<td>120</td>
<td>480.45</td>
<td>479.11</td>
<td>99.88</td>
<td>1.44</td>
</tr>
</tbody>
</table>
Table 2.2.T-5: Robustness study data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set Value</th>
<th>Assay % (n=3)</th>
<th>Resolution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (±10%)</td>
<td>0.9 ml/min.</td>
<td>99.22</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1.1 ml/min.</td>
<td>99.59</td>
<td>2.8</td>
</tr>
<tr>
<td>Organic modifier (±2%)</td>
<td>58%</td>
<td>99.53</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>62%</td>
<td>98.99</td>
<td>2.7</td>
</tr>
<tr>
<td>Column oven temperature (±2 °C)</td>
<td>28 °C</td>
<td>98.21</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>32 °C</td>
<td>99.33</td>
<td>2.8</td>
</tr>
<tr>
<td>Detector Wavelength (±4 nm)</td>
<td>216 nm</td>
<td>99.51</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>224 nm</td>
<td>99.60</td>
<td>2.9</td>
</tr>
</tbody>
</table>

*Resolution between the oxidized degraded product at RRT 0.87 and analyte peak.
Section 2.3
Determination of Guanfacine Hydrochloride in Pharmaceutical Dosage Form by validated stability indicating method

2.3.1 Drug profile
Guanfacine hydrochloride

**Chemical name:** N-(diaminomethylidene)-2-(2,6-dichlorophenyl) acetamide

**Molecular formula:** C9H9Cl2N3O

**Molecular weight:** 246.09

**Description:** Off white powder

**Properties:**
- In acetone: slightly soluble.
- In water and acetone: Sparingly soluble.

**Therapeutic category:** Antihypertensive
2.3.2 Introduction

Guanfacine hydrochloride (Fig. 2.3.F.-1) is chemically N-(diaminomethylidene)-2-(2,6-dichlorophenyl) acetamide [33]. It comes in cardiovascular category and used for lowering blood pressure [35-36]. Drug acts on the central nervous system $\alpha-2a$ nor epinephrine auto receptors, results in reduced peripheral sympathetic outflow and reducing peripheral sympathetic tone [37]. Guanfacine also used for attention deficit hyperactivity disorder treatment, rebound, as well as induce sleep [38].

2.3.3 Literature review

A literature survey reveals common spectrofluorometric assay method was reported for Guanethidine sulphate, Ganoxan sulphate, Guanfacine hydrochloride, and Guanochloro sulphate in tablets, spiked human serum and urine sample [39].

Quantitative determination of Guanfacine in urine by validated GC-MS method was reported. protriptyline is used as an internal standard (IS). Solvent extraction technique was employed for extraction of Guanfacine and IS [40].

Scintillation proximity assay method was also reported for Guanfacine hydrochloride [41].

Spectrophotometric analysis of guanafacine was also reported [42].

No HPLC method which is stability indicating for quantification of Guanfacine hydrochloride was reported.

2.3.4 Present work

Chemicals

Guanfacine hydrochloride standard was supplied by Mylan pharmaceutical (Hyderabad, India) and tablet “Tenex” (2.0 mg) were purchased from market. Acetonitrile (HPLC grade) obtained from Rankem. Hydrogen peroxide, sodium hydroxide and hydrochloric acid were purchased from Qualigens Ltd. Double distilled water was used for all study preparations.
**HPLC Instrumentation conditions**

Agilent Technology HPLC 1100 series, were used Agilent Chemstation software was used to monitor the signal output. The column used was C$_{18}$ (25 cm × 0.46 cm, 5 µm), Apollo technology, Japan. Column temperature was maintained at 30 °C using column oven. Instrumental setting of mobile phase was kept 1.0 ml/min. Acetonitrile and buffer in ratio of 35:65, v/v. The buffer used contains 0.05 M ammonium acetate. 5 μl of injection volume was injected and monitored at 220 nm wavelength.

**Standard solution**

Guanfacine hydrochloride stock solution (3.0 mg/ml) was prepared. (water:ACN in ratio 70:30, v/v) and diluting upto 25 ml with diluent. Further dilute this stock solution to get standard solution by transferring 10 ml standard stock solution to dilute to 100 ml with same solvent. This solution contains 300 µg/ml of Guanfacine hydrochloride.

**Sample preparation of dosage form**

Twenty tablets of mean weight were crushed in mortar. Powdered containing 30 mg of Guanfacine hydrochloride is transferred to volumetric flask (100 ml). The drug powder was extracted in diluent and was sonicated for 25 minute and diluted to 100 ml.

**Procedure for forced degradation**

**Acidic degradation**

Drug product powdered to 15 mg of Guanfacine hydrochloride was taken into flask (50 ml), added 5 ml diluent to dissolve. 1.0 N HCl solution was added and kept aside at room temperature for about 3 hrs, allow attending room temperature and volume made with diluent.

**Alkali degradation**

Drug product powdered to 15 mg of Guanfacine hydrochloride was weighed and transferred in volumetric flask (50 ml), added 5 ml diluent to dissolve. 0.1 N NaOH was added (5 ml) and heated at 60 °C under reflux for 1 hr. allows attending room temperature and volume made with diluent.
**Oxidative degradation**

Drug product powdered to 15 mg of Guanfacine hydrochloride was transferred in volumetric flask (50 ml), added 5 ml diluent to dissolve. To it 5 ml of 30% H₂O₂ solution was added and heated at 60°C under reflux for 1 hr and volume made up with diluent.

**Thermal degradation**

75 mg of Guanfacine hydrochloride was kept at 80 °C for 24 hrs. Dilution was performed to get the 300 µg/ml concentration of Guanfacine.

**UV degradation (Short)**

75 mg of compound was kept at UV short (254 nm) light for 48 hrs. Dilution was performed to get the 300 µg/ml concentration of Guanfacine.

**UV degradation (Long)**

75 mg of compound was kept at UV long (366 nm) light for 48 hrs. Dilution was performed to get the 300 µg/ml concentration of Guanfacine.

**2.3.5 Validation of Method**

**Precision (Repeatability)**

For method precision, six replicate assays by the proposed method were done. The percentage RSD of six measurements was determined. Repeatability was performed by another chemist.

**Accuracy**

For recovery, known amount of drugs were spiked in placebo at 80, 100 and 120% of working level concentration. Each level was prepared in triplicate.

**Linearity**

Six different concentrations levels tested from 30 µg/ml to 450 µg/ml. Injected each level in triplicate. Plot the calibration graph for response over the concentration range of analyte.
Detection limit and Quantitation limit

Signal/noise ratio test procedure was employed for prediction of LOD and LOQ for Guanfacine hydrochloride. Predicted LOQ and LOD solutions were injected 6 times. Peak area percentage RSD was determined.

Robustness

To determine the robustness, chromatographic parameters were intentionally changed. The system suitability parameters such as resolution between the peaks of Guanfacine hydrochloride and adjacent alkali degradation product and percentage assay were evaluated. Flow rate robustness was verified by changing it ±0.2 unit. Mobile phase robustness was studied by varying organic composition in mobile phase by ±10%. Column oven temperature impact was studied by checking resolution at ±5 °C of set value. The wavelength robustness was checked at 216 and 224 nm.

Solution stability

Stability of the Guanfacine hydrochloride bulk drug solution and tablet assay solutions were measured at time intervals upto 48 hrs. Standard solution stability was checked by the area percent and spectral purity data comparison of analyte. Assay of tablet sample solution was determined at both intervals.

2.3.6 Result and discussion

Chromatographic conditions optimization

The primary goal is the development of HPLC method to separate Guanfacine hydrochloride and degradation products. The separation of was achieved on Apollo C<sub>18</sub> column. The Guanfacine retention time is about 7.0 min which reduced the total run time. System suitability data of method is shown table 2.3.T-1.

Forced degradation study

Singh and Bakshi [17] suggest degradation of 20 to 80% for developing the stability indicating method. Optimized conditions were used to get degradation in range of 10 to 30%. In basic conditions, analyte undergoes the degradation for the formation of both polar and non polar impurities. The percentage alkali degradation was found to be 42.1 with analyte peak having the peak purity 999.325.
In oxidative degradation analyte undergoes degradation for the formation of both polar and non-polar impurities within short time period. The percentage degradation was found to be 59.2 with analyte peak having the peak purity 999.293.

In thermal degradation analyte undergoes degradation for the formation of polar impurities. The thermal degradation percentage was found to be 8.9 with analyte peak having the peak purity 999.655.

The peak purity data proves that analyte peak is pure and no co-elution of another peak. Degradation study data is tabulated in Table 2.3.T-2.

Spectral analysis report gives peak purity values for analyte peaks, which demonstrate specificity. Fig. 2.3.F-2 shows the chromatogram of Guanfacine hydrochloride tablet solution. Fig. 2.3.F-3 and 2.3.F-4 shows chromatogram of diluent and Guanfacine hydrochloride standard solution respectively. Fig. 2.3.F-5 and 2.3.F-6 shows chromatograms of alkali hydrolysis blank and alkali hydrolysis degradation of tablet respectively. Fig. 2.3.F-7 and 2.3.F-8 shows the chromatogram of oxidative blank and oxidative degradation of tablet respectively. Fig. 2.3.F-9 shows chromatograms of thermal degradation of tablet. Peak purity values were greater than 0.999 for main peak which confirms the specificity of method.

**Results of method validation**

Guanfacine peak purity values in all stress condition were greater than 999 at 220 nm, which confirms the specificity of method. Percentages RSD of six measurements of method precision were 0.36. Percentage RSD of six measurements of intermediate precision was 0.42. Precision study result proves that method is rugged (percentage RSD values was less than 2%). Data is given in Table 2.3.T-3. The recovery values for Guanfacine at 80, 100 and 120% were ranging from 98.66 to 100.31%. The mean of all individual nine determinations was 99.97% having overall percentage RSD 1.8. Recovery data were shown in Table 2.3.T-4. The calibration graph was plotted and observed linear in the studied concentrations having correlation coefficient value 0.999 having equation $y = 13.917x + 1.8702$. The LOD for Guanfacine hydrochloride was 0.011 and LOQ was 0.038 µg/ml, having percentage RSD of 4.9 and 3.5% respectively. Assay percentage at all deliberate conditions of robustness studies are within 98.21 to 99.59, which prove the method is robust. The resolution between analyte peak and adjacent alkali degradants was
observed 2.5 to 3.3 in all conditions. Robustness results are tabulated in table 2.3.T-5.

The results of solution stability indicate that the Guanfacine solution was stable for 48 hrs at bench top with peak purity value 999.721.
2.3.7 Figures: Method validation chromatograms of Guanfacine hydrochloride tablet.

**Fig. 2.3.F-2:** Tablet sample solution chromatogram

**Fig. 2.3.F-3:** Diluent chromatogram
Fig. 2.3.F-4: standard solution chromatogram.

Fig. 2.3.F-5: Blank alkali hydrolysis chromatogram
Fig. 2.3.F-6: Alkali hydrolysis degradation of tablet chromatogram

Fig. 2.3.F-7: Blank for oxidative degradation
Fig. 2.3.F-8: Oxidative degradation of tablet

Fig. 2.3.F-9: Thermal degradation of tablet chromatogram
**Fig. 2.3.F-10:** Blank for acidic degradation

**Fig. 2.3.F-11:** Acidic degradation of tablet chromatogram
2.3.8 Tables: Method validation data of Guanfacine hydrochloride tablets

Table 2.3.T-1: System suitability data

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (Min.)</th>
<th>Theoretical Plate Count</th>
<th>USP Tailing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanfacine</td>
<td>7.2</td>
<td>11291</td>
<td>1.36</td>
</tr>
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</table>

Table 2.3.T-2: Result of stress study

<table>
<thead>
<tr>
<th>Condition</th>
<th>Degradation (%)</th>
<th>Spectral Peak purity</th>
<th>Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic (1N HCl/RT/3 hrs)</td>
<td>No degradation</td>
<td>999.756</td>
<td>99.17</td>
</tr>
<tr>
<td>Alkali (0.1 NaOH/60 °C/ 1 hr)</td>
<td>42.1</td>
<td>999.325</td>
<td>57.22</td>
</tr>
<tr>
<td>Oxidative (30% H₂O₂/60 °C/1 hr)</td>
<td>59.2</td>
<td>999.293</td>
<td>41.11</td>
</tr>
<tr>
<td>Thermal (80 °C/24 hrs)</td>
<td>8.9</td>
<td>999.655</td>
<td>90.85</td>
</tr>
<tr>
<td>UV-short (48 hrs)</td>
<td>No degradation</td>
<td>999.788</td>
<td>99.12</td>
</tr>
<tr>
<td>UV-long (48 hrs)</td>
<td>No degradation</td>
<td>999.771</td>
<td>99.03</td>
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Table 2.3.T-3: Validation summary

<table>
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<tr>
<th>Experiment</th>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>System Precision</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Method Precision</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Intermediate Precision</td>
<td>0.42</td>
</tr>
<tr>
<td>LOD and LOQ</td>
<td>Limit of detection (µg/ml)</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Limit of quantification (µg/ml)</td>
<td>0.038</td>
</tr>
<tr>
<td>Linearity</td>
<td>Calibration range (µg/ml)</td>
<td>30 to 450</td>
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<tr>
<td></td>
<td>Regression equation</td>
<td>( y = 13.917x + 1.8702 )</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Table 2.3.T-4: Results of recovery experiment

<table>
<thead>
<tr>
<th>% Level</th>
<th>Spike Amount (µg/ml)</th>
<th>Found Amount (µg/ml)</th>
<th>Mean Recovery % (n=3)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>241.2</td>
<td>239.2</td>
<td>99.2</td>
<td>1.61</td>
</tr>
<tr>
<td>100</td>
<td>302.3</td>
<td>303.7</td>
<td>100.5</td>
<td>1.45</td>
</tr>
<tr>
<td>120</td>
<td>362.7</td>
<td>362.7</td>
<td>100.2</td>
<td>1.36</td>
</tr>
</tbody>
</table>
Table 2.3.T-5: Data of robustness

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set Value</th>
<th>Assay % (n=3)</th>
<th>Resolution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>0.8 ml/min.</td>
<td>99.22</td>
<td>2.9</td>
</tr>
<tr>
<td>(± 20%)</td>
<td>1.2 ml/min.</td>
<td>99.59</td>
<td>2.7</td>
</tr>
<tr>
<td>Organic modifier</td>
<td>31.5%</td>
<td>98.99</td>
<td>3.3</td>
</tr>
<tr>
<td>(± 10% )</td>
<td>38.5%</td>
<td>99.53</td>
<td>2.5</td>
</tr>
<tr>
<td>Column oven temperature</td>
<td>25 °C</td>
<td>98.21</td>
<td>2.9</td>
</tr>
<tr>
<td>(±5 °C)</td>
<td>35 °C</td>
<td>99.33</td>
<td>2.8</td>
</tr>
<tr>
<td>Detector Wavelength</td>
<td>216 nm</td>
<td>99.51</td>
<td>2.7</td>
</tr>
<tr>
<td>(±4 nm)</td>
<td>224 nm</td>
<td>99.60</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*Resolution between analyte peak and adjacent alkali degradant.
Section 2.4  
**Determination of Epinastine Hydrochloride in Pharmaceutical Dosage Form by validated stability indicating method**

![Epinastine Hydrochloride Chemical Structure](image)

*Fig. 2.4.F-1: Epinastine hydrochloride chemical structure.*

### 2.4.1 Drug profile

**Epinastine hydrochloride**

**Chemical name:** (RS)-3-amino-9,13b-dihydro-1H-dibenz (c,f)imidazo (1,5-a) azepine

**Molecular formula:** \( C_{16}H_{15}N_{3} \)

**Molecular weight:** 249.311

**Description:** Off white powder

**Properties:** Sparingly soluble in alcohol, water and slightly soluble in acetone

**Therapeutic category:** Antiallergic
2.4.2 Introduction

Chemically Epinastine is known as RS-3-amino-9,13b-dihydro-1H-dibenz(c,f)imidazo(1,5-a)azepine (Fig. 2.4.F-1) [43]. It is one of the most potent inducers of pruritus. Variety of antihistamines showed anti-allergic and anti-inflammatory activity in skin. Therefore these agents are frequently used for the treatment of cutaneous pruritic diseases such as atopic dermatitis in which mast cells and released histamine play significant roles. Epinastine hydrochloride exhibits potent inhibitory action on not only the H1-receptor, but also on inflammatory mediator release from mast cells, following its systemic administration. In addition to antihistaminic effect, several studies have been demonstrated and found Epinastine hydrochloride exerting a variety of unique pharmacological mode of action [44-48].

2.4.3 Literature survey

Quantitative determination of Epinastine in plasma of rat by using HPLC was reported [49].

Determination of Epinastine in human serum was reported by using CE. [50].

HPLC and UV derivative spectrophotometric analysis of epinastine in solid oral dosage was also reported. RP_{18} HPLC column with basic mobile phase was used to perform the HPLC analysis [51].

A chiral method for enantiomeric separation of Epinastine was reported by using column a Chiralcel OD-R [52].

Auto titrimetry determination of Epinastine was reported [53].

2.4.4 Present work

**Material and reagents**

Epinastine hydrochloride was procured from Lupin pharmaceutical Ltd. (India) and formulated tablet was procured from local market. Hydrochloric acid (HCl) and trifluoroacetic acid (TFA) was procured from Merck Chemicals. Hydrogen peroxide (H_{2}O_{2}), Acetonitrile and sodium hydroxide (NaOH) was obtained from Rankem.
**Instrumental conditions**

Agilent-1100 HPLC system was used for analysis. Analyte separation was achieved by using YMC ODS A (YMC Co. Ltd, 25 cm × 0.46 cm, 5 um). Analytical column was maintained at 30 °C using column oven. Acetonitrile and buffer mixed at ratio of 35: 65, v/v used as eluent and at rate flow was 1.0 ml/min. Buffer used was 0.05% v/v solution in water. UV detector was used at 220 nm to quantify Epinastine. Injection volume was kept 10 µl. Agilent chemstation software was used to process the chromatograms.

**Standard stock solution**

Accurately weighed and transferred about 75 mg Epinastine into a volumetric flask (50 ml) and dissolved with diluent. Diluent was composed of acetonitrile and water 70:30; v/v. From stock solution transferred 5 ml to flask (100 ml) and make up with diluent to get standard solution.

**Preparation of sample**

Tablet powder containing 75 mg of Epinastine was taken into 50 ml flask. Added 20 ml of diluent to dissolve by means of sonication and make up with diluent. Centrifuge this solution, From stock solution transferred 5 ml to flask (100 ml) and make up with diluent.

**Stress degradation study**

**Acidic degradation**

Accurately weighed epinastine (75 mg) transferred into flask (50 ml), dissolved and make up with diluent. Added 1.0 N. HCl solution (10 ml), and heated at 60 °C for 3 hrs on water bath, Adjusted pH 7 with 1.0 N NaOH solution and make up the volume with diluent upto 50 ml, Further taken 5 ml of this stock solution in to volumetric flask (100 ml) and make up the volume with diluent.

**Alkali degradation**

Accurately weighed epinastine (75 mg) transferred into flask (50 ml), dissolved and make up with diluent. Added 0.1 N. NaOH solution (10 ml), and heated at 60 °C for 3 hrs on water bath, Adjusted pH 7 with 0.1N HCl solution and
make up with same solvent up to 50 ml, Further taken 5 ml of this stock solution in to volumetric flask (100 ml) and make up the volume with diluent.

**Oxidative degradation**

Accurately weighed epinastine (75 mg) transferred into flask (50 ml), dissolved and make up with diluent. Added 30% H$_2$O$_2$ solution (10 ml), and heated at 60 °C for 3 hrs on water bath, make up the volume with diluent upto 50 ml, Further taken 5 ml of this stock solution in to volumetric flask (100 ml) and make up the volume with diluent.

**Thermal degradation**

Epinastine was exposed at 80°C for 48 hrs. Then the solution of concentration 150 µg/ml was prepared from exposed sample.

**Thermal degradation**

Epinastine drug powder was exposed to heat at 80°C for two days. Prepared solution containing the concentration to the assay level.

**UV-Short (254 nm) degradation**

Powder of drug product exposed to 254 nm in UV chamber for two day. The solution was prepared in diluent to achieve assay level concentration.

**UV-Long (366 nm) degradation**

Powder of drug product exposed to 366 nm in UV chamber for two day. The solution was prepared in diluent to achieve assay level concentration.

Allow it to cool and the make up with diluent. Dilute this solution to working level concentration.

**2.4.5 Method Validation**

**Precision**

Six assay measurements of tablets were performed in method precision study. Same experiment was repeated by another chemist to evaluate the intermediate precision of method.
Accuracy (Recovery test)

For recovery, known amount of drugs were spiked in placebo at 80, 100 and 120% of working level concentration. Each level was prepared in triplicate.

Linearity

Seven different concentration solution ranging from LOQ to 200% of the assay concentration were prepared and analyzed in triplicate.

Detection limit and Quantification limit

Calibration curve was constructed between analyte concentration and response of samples to determining the detection limit and quantification limit.

Detection limit and Quantification limit were estimated by using below formulae:

Quantification limit = Cq x Styx / b
Detection limit = Cd x Styx / b

Where, Styx is residual variance due to regression; Cd/Cq is coefficient for LOD/LOQ; b is slope.

Standard solution at LOQ and LOD level was prepared and determined the precision of peak area.

Robustness

The flow rate of the method for mobile phase was altered ±0.1 ml/min and determined the resolution between analyte peak and its alkali degradant. Organic composition robustness was studied at ± 5%. Column oven temperature was changed to ±5 °C to set value.

Stability of analytical solution

Standard solutions and sample solution stability were verified upto 72 hrs by analyzing these solutions against fresh standard solution.

2.4.6 Result and discussion

Chromatographic conditions optimization

To achieve the resolution between degradants and analyte employed various phases and different mixture of 0.05% v/v trifluoroacetic acid as buffer with ACN.
Chapter 2

Chromatographic resolution between degradatn peaks and Epinastine was successfully achieved on YMC ODS A column and mobile phase used was the Acetonitrile and 0.05% v/v TFA in ratio 35:65, v/v. Column temperature was kept 30 °C.

In all development trials it was clear that C₁₈ stationary phase column gives a better separation between degradant peaks and Epinastine peak as compare to other C₁₈ or CN columns. Trifluoroacetic acid 0.05%v/v and acetonitrile as 40:60, v/v used as mobile phase. Column oven maintained at 30 °C and flow rate kept 1.0 ml/Min. Acidic pH of buffer solution reduce the tailing of analyte peak Epinastine peak was eluting at 5.4 minute. System suitability data was given in Table 2.4.T-1.

**Stress degradation study**

The Epinastine peak with tailing less than 2.0, well separated from other stress degradant was observed. Retention time for Epinastine peak is about 5.4 minute.

Conditions employed for stress degradations are to get degrade the Epinastine 10 to 30%. In photolytic and acid condition, degradation was not observed even employed the harsh condition degradation [17]. Epinastine was undergoing fast and significant degradation in base hydrolysis, oxidation and thermal conditions. Stress degradation study data is tabulated in Table 2.4.T-2. peak purity values were analyzed from raw report of PDA and values more than 990 in all stress degradation conditions proves specificity of method. Fig.2.4.F-2 to Fig.2.4.F-3 showed the chromatograms of diluent and Epinastine tablet solution. Figure Fig.2.4.F-4 to 2.4.F-8 shows the chromatograms of base hydrolysis blank, base hydrolysis degradation of tablet, oxidation blank, oxidation degradation of tablet and thermal degradation of tablet respectively.

**Results of method validation**

Average of six assay measurements in method precision were 99.69 with percentage RSD 0.15. Average of six assay measurements in method precision were 99.52 having percentage RSD 0.19. Table 2.4.T-3 represents precision data of method.
Recovery for Epinastine was 99.57 to 100.25 having precision of nine measurements was 0.9. Results are tabulated in table 2.4.T-4.

The calibration curve equation of linearity for epinastine $y = 5.2675x - 4.5055$. The correlation value is 0.999. LOQ of Epinastine was 0.18 µg/ml having percentage RSD for six injections less than 5.0. LOD of Epinastine was 0.05 µg/ml having percentage RSD for six injections less than 10.0.

In robustness study Epinastine peak and alkali degradation peak resolution was always greater than 3.0 in all altered conditions. The data is given in table 2.5.T-5. In solution stability experiments, standard solution and test solution found stable upto 72 hours.
2.4.7 Figures: Method validation chromatograms of Epinastine hydrochloride tablet

Fig. 2.4.F-2: diluent chromatogram

Fig. 2.4.F-3: Chromatogram of tablet solution
Fig. 2.4.F-4: Alkali hydrolysis blank chromatogram

Fig. 2.4.F-5: Alkali hydrolysis degradation of tablet chromatogram
Fig. 2.4.F-6: Oxidative degradation blank chromatogram

Fig. 2.4.F-7: Oxidative degradation of tablet chromatogram
Fig. 2.4.F-8: Thermal degradation of tablet chromatogram

Fig. 2.4.F-9: Acidic degradation blank chromatogram
Fig. 2.4.F-10: Acidic degradation of tablet chromatogram
2.4.8 Tables: Method validation data of Epinastine hydrochloride tablet

Table 2.4.T-1: System suitability data

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (Min.)</th>
<th>Theoretical Plate Count</th>
<th>USP Tailing</th>
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<tbody>
<tr>
<td>Epinastine</td>
<td>5.4</td>
<td>7895</td>
<td>1.33</td>
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</table>

Table 2.4.T-2: Results of Stress degradation

<table>
<thead>
<tr>
<th>Condition employed</th>
<th>Degradation (%)</th>
<th>Peak purity</th>
<th>Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic (1N HCl/60 °C/3 hrs)</td>
<td>No degradation</td>
<td>999.936</td>
<td>99.18</td>
</tr>
<tr>
<td>Alkali (0.1 NaOH/60 °C/3 hrs)</td>
<td>27.88</td>
<td>999.706</td>
<td>73.26</td>
</tr>
<tr>
<td>Oxidative (30% H₂O₂/60 °C/3 hrs)</td>
<td>18.12</td>
<td>999.532</td>
<td>81.23</td>
</tr>
<tr>
<td>Thermal (80 °C/48 hrs)</td>
<td>17.20</td>
<td>999.379</td>
<td>82.52</td>
</tr>
<tr>
<td>UV-short (48 hrs)</td>
<td>No degradation</td>
<td>999.609</td>
<td>99.89</td>
</tr>
<tr>
<td>UV-long (48 hrs)</td>
<td>No degradation</td>
<td>999.977</td>
<td>100.32</td>
</tr>
</tbody>
</table>
### Table 2.4.T-3: Validation summary

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision (n = 6, % R.S.D.)</td>
<td>System Precision</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Method Precision</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>Intermediate Precision</td>
<td>0.19</td>
</tr>
<tr>
<td>LOD and LOQ</td>
<td>Limit of detection (µg/ml)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>Limit of quantification (µg/ml)</td>
<td>0.18</td>
</tr>
<tr>
<td>Linearity</td>
<td>Calibration range (µg/ml)</td>
<td>0.18 to 150</td>
</tr>
<tr>
<td></td>
<td>Regression equation</td>
<td>y = 5.2675x – 4.5055</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>0.999</td>
</tr>
</tbody>
</table>

### Table 2.4.T-4: Results of recovery experiment

<table>
<thead>
<tr>
<th>% Level</th>
<th>Spike Amount (mg)</th>
<th>Found Amount (mg)</th>
<th>Mean Recovery %</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>16.00</td>
<td>15.88</td>
<td>99.57</td>
<td>0.82</td>
</tr>
<tr>
<td>100</td>
<td>20.00</td>
<td>20.05</td>
<td>100.25</td>
<td>0.68</td>
</tr>
<tr>
<td>120</td>
<td>24.00</td>
<td>23.92</td>
<td>99.87</td>
<td>1.29</td>
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</tbody>
</table>
Table 2.4.T-5: data robustness study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set Value</th>
<th>Assay % (n=3)</th>
<th>Resolution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (± 10%)</td>
<td>0.9 ml/min.</td>
<td>99.38</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>1.0 ml/min.</td>
<td>99.69</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>1.1 ml/min.</td>
<td>99.66</td>
<td>4.2</td>
</tr>
<tr>
<td>Organic modifier (± 5%)</td>
<td>33.0%</td>
<td>100.04</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>30.0%</td>
<td>99.69</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>40.0%</td>
<td>99.62</td>
<td>3.3</td>
</tr>
<tr>
<td>Column oven temperature (±5 °C)</td>
<td>25 °C</td>
<td>99.89</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>99.69</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>35 °C</td>
<td>99.53</td>
<td>4.4</td>
</tr>
</tbody>
</table>

*Resolution in analyte peak and RRT 1.2 base degradants peak.
References


