Study of Forced Degradation Behaviour of a Novel Anti-Platelet Drug of Vorapaxar by Uplc-Ms/Ms

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Abstract: A sensitive, precise, specific, linear and stability indicating isocratic UPLC-MS/MS method was developed for the analysis of vorpaxar as per ICH guidelines Q1A (R2). Various forced degradation studies were conducted to establish an impurity profile for vorpaxar. Degradation products were produced upon exposing vorpaxar to different degradation conditions (acidic, basic, oxidative, photolytic, aqueous and thermal); the chromatographic separation was achieved with 5mM Ammonium Formate Buffer (pH: 4.0): Methanol: Acetonitrile, (40:30:30, % v/v) using the Pursuit XRs-100Å, C18, 4.6 x 50 mm, 10 μm analytical column. The total analysis time was 3.5 min and flow rate was set to 0.2 ml/min. The mass transitions of vorapaxar and vorapaxar-D5 obtained were m/z 591.4/447.2 and 498.6/447.2. The standard curve shows correlation coefficient (r²) greater than 0.999 with a range of 35.0-105.0 pg/ml using linear regression model. The drug showed instability in the solution state (under acidic, alkaline and oxidative stress conditions), but it was relatively stable in the solid-state, at thermal and photo degradation conditions.

Keywords: Vorapaxar; Forced degradation, UPLC-ESI-MS/MS; Stability, Validation.
1. INTRODUCTION

Vorapaxar is a reversible antagonist of the protease-activated receptor-1 (PAR-1) expressed on platelets, but its long half-life makes it effectively irreversible. Vorapaxar inhibits thrombin-induced and thrombin receptor agonist peptide (TRAP)-induced platelet aggregation in in vitro studies. Vorapaxar does not inhibit platelet aggregation induced by adenosine diphosphate (ADP), collagen or a thromboxane mimetic and does not affect coagulation parameters ex vivo. PAR-1 receptors are also expressed in a wide variety of cell types, including endothelial cells, neurons, and smooth muscle cells, but the pharmacodynamics effects of vorapaxar in these cell types have not been assessed. The chemical name of vorapaxar sulfate (Fig 1.0) is ethyl \[\{(1E)-2-[5-(3fluorophenyl)pyridin-2-yl]ethen-1-yl\}-1-methyl-3-oxo dodecahydro naphtho[2,3-c]furan-6-yl\] carbamate sulfate. The empirical formula is C_{29}H_{33}FN_{2}O_{4}·H_{2}SO_{4}, and its molecular weight is 590.7. Vorapaxar sulfate is a white to off-white solid. Vorapaxar sulfate is freely soluble in methanol and slightly soluble in ethanol, acetone, 2-propanol, and acetonitrile.

Fig 1. Chemical Structures of A) Vorapaxar sulfate B) Vorapaxar-D₅

1.1 Review of Literature

From the literature survey, it is evident that very few research articles were available for estimation of Vorapaxarin pre-clinical and clinical studies by LC-MS/MS. Literature survey reveals, a comprehensive LC-MS study of degradation behaviour of Vorapaxar under various ICH prescribed stress conditions has been lacking. So, it was decided to carry out forced decomposition studies according to ICH requirements and develop a selective and validated stability-indicating LCMS method. An integral aim of the study was to identify new degradation products, if any, and to postulate the %degradation of the drug.

2. MATERIALS AND METHODS

Vorapaxar sulphate (ALSACHIM), high purity grade methanol, acetonitrile and water, ammonium formate, formic acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide (3 % w/v) procured from, CDH Chemicals, Delhi, India. The degradant products and their fragments were identified on a QSight® Triple Quadrapole UPLC-MS/MS system (Perkin Elmer). The data processing was accomplished using Simplicity ™ 3Q software.

2.1 Preparation of standard stock solution

Standard stock solution of vorapaxar (1.0 mg/mL) and vorapaxar-D₅ (1.0 mg/mL) were prepared by accurately weighing about 10 mg and transferring into 10 mL volumetric flask and dissolved in acidified methanol. All stock solutions were stored in refrigerated conditions (2-8°C) until analysis.

2.2 Preparation of Acidified Methanol

1.0 mL of formic acid was transferred into 1000.0 mL of methanol (HPLC grade) which was filtered through a 0.45µ membrane disc filter and sonicated to degas.

2.3 Preparation of 5mM Ammonium Formate Buffer (pH: 4.0)

About 0.315g of ammonium formate was weighed, and transferred to a 1000.0 mL volumetric flask and made up to volume (1000.0mL) with ultra-pure water. Finally, the pH of the solution was adjusted to pH: 4.0 with formic acid and filtered through a 0.45µ membrane disc filter and sonicated for degassing.

2.4 Preparation of mobile Phase

The mobile phase used was 5mM ammonium formate buffer (pH: 4.0): methanol: acetonitrile, (40:30:30 % v/v) and the mobile phase was filtered through a 0.45µ membrane filter and sonicated before use.

2.5 Preparation of Internal standard spiking solution

The Vorapaxar-D₅ (internal standard) spiking solution (50.00 pg/mL) was prepared from standard stock solution of Vorapaxar-D₅ (1000.00 µg/mL) in mobile phase (5mM ammonium formate buffer (pH: 4.0): methanol: acetonitrile, (40:30:30, % v/v)). Internal standard spiking solution (vorapaxar-D₅) was stored in refrigerated conditions (2-8°C) until analysis.

2.6 Preparation of standard solutions

Standard solutions of different concentrations of vorapaxar were prepared from vorapaxar stock solution (1000µg/mL) in mobile phase 5mM ammonium formate buffer (pH: 4.0): methanol: acetonitrile, (40:30:30, % v/v)). To each aqueous standard solution, 100 µL of 50.00 pg/mL of vorapaxar-D₅.
was added and vortexed for 5 min and injected into the UPLC-ESI-MS/MS for analysis.

2.7 Method Development

Various mass spectrometric and chromatographic parameters were optimised for estimation of vorapaxar by LC-MS/MS.

2.8 Selection of internal standard

For selection of internal standard; carbidopa, levodopa, pramipexole, ropinirole were tried with optimized mobile phase and column conditions. Finally Vorapaxar-D$_5$ was selected as IS (internal standard) due to its compatibility with analyte chromatographic conditions. The peak elution times for the Vorapaxar and Vorapaxar-D$_5$ were found at 1.62 and 0.56 min.

2.9 Optimization of Mass spectroscopic Parameters

The standard concentrations of vorapaxar and Vorapaxar-D$_5$ (Fig. 2) were prepared in acidified methanol (50.00 pg/mL) and injected with a flow rate of 5µL/min in positive ion mode mass spectrometer. High intense positive ions were obtained for Vorapaxar (591.4/447.2) and Vorapaxar-D$_5$ (498.6/447.2) after optimization of mass parameters.

Fig 2. Parent ion mass spectra (Q1) and (Q3) of Vorapaxar & Vorapaxar-D$_5$

2.10 Optimised Chromatographic conditions

The chromatographic separation was achieved with 5mM ammonium formate buffer (pH: 4.0): methanol: acetonitrile, (40:30:30 % v/v) using the Pursuit XRs-100Å, C$_{18}$, 4.6 x 50 mm, 10 µm as analytical column. The chromatograms of blank (Mobile phase) and standard samples (LOQ and ULOQ) were shown in Fig.3-5. This was followed by method validation.
Fig 3. Blank chromatogram (mobile Phase)

Fig 4. Standard chromatogram of LOQ sample (50% Linearity level).
Method Validation

2.11 System suitability

Six replicate injections of aqueous standard 100% level (70.0 pg/mL) along with internal standard (50.0 pg/mL) were injected into UPLC-MS/MS and %RSD was calculated.

2.12 Selectivity and Specificity

Ten batches of plasma were examined, out of which six were free from plasma interference. The biological matrix interference-free plasma samples were used for evaluating the selectivity and specificity experiment.

2.13 Linearity and Range

The linearity of calibration curve for vorapaxar was assessed at 50 % to 150 % of the target concentration at different levels in the range of 35.0 pg/mL to 105.0 pg/mL in aqueous standards.

2.14 Precision

Method repeatability was assessed by quality control standards of 45 n.g/mL (LQC), 70 n.g/mL (MQC) and 95 n.g/mL (HQC) standards in six replicates on the same day (Intra-day) and five different days (Inter-days).

2.15 Limit of detection (LOD) and limit of quantification (LOQ)

Six lower limit of quantification standards were used to calculate the signal to noise ratio (S/N) of LOQ and LOD of analyte.

2.16 Robustness

Robustness was carried out by varying the method parameters like flow rate (±5%) and Column temperature (±5%) was made to evaluate the impact on the system suitability parameters of the developed method. Six replicate injections of aqueous standard solutions of 100% level (70.0 pg/mL) along with internal standard was injected into UPLC-MS/MS and %RSD was calculated. Results are presented in Table 3. No significant changes were observed in studied system suitability parameters when deliberate variations are made in the chromatographic conditions which mean that the proposed method is robust.

2.17 Stability

Stability of Vorapaxar & Vorapaxar-D$_5$ in aqueous standards was using two replicates of 70.0 pg/mL and 50.00 pg/mL at ambient and refrigerated conditions with different time intervals.

2.18 Solution Stability

The storage conditions of samples need to maintain the integrity of a drug. For this reason, stability studies play an important role in analytical method development. A Vorapaxar at a concentration of 70 pg/mL solution and Vorapaxar-D$_5$ (IS) solution at 50 pg/mL of were prepared from fresh stock solutions. A portion of the freshly prepared standard solutions (Vorapaxar & Vorapaxar-D$_5$) were kept at ambient temperature ($25^\circ$C) for 24 hours and then analyzed.

Fig 5. Standard chromatogram of ULOQ sample (150% Linearity level)
by the proposed method. A second portion of the freshly prepared standard solutions (Vorapaxar & Vorapaxar-D5) were stored at refrigerated temperature (between 2°C and 8°C) for 24 hours and then analyzed. The results were compared with those obtained from samples analyzed at initial moment (0.0 hours).

2.19 Filter validation (Filter Interference)

Vorapaxar (VP) at concentration of 70.00 pg/mL solution and Vorapaxar-D5 (VPD5) (IS) solution at 50.00 pg/mL were prepared from fresh stock solutions. Some portion of Vorapaxar (VP) and Vorapaxar-D5 (VPD5) standard solutions (70.00 pg/mL and 50.00 pg/mL) was filtered through three different filters namely 0.45µm PVDF filter, 0.45µm PTFE and 0.45µm Nylon filter and some portion was centrifuged and injected into the UPLC-MS/MS system.

2.20 Stress degradation studies

2.20.1 Preparation of Acid induced degradation sample

About 10 mg of vorapaxar was taken in a 10 ml volumetric flask and dissolved in 25 ml methanol and diluted with 0.1 N HCl. The solution was set aside for 72 h at ambient temperature. After degradation, solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

2.21 Preparation of alkali induced degradation product

About 10 mg of vorapaxar was taken in a 10 ml volumetric flask and dissolved in 10 ml methanol and diluted with 0.1 N NaOH. The solution was set aside for 72 h at ambient temperature. After degradation, solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

2.22 Preparation of hydrogen peroxide induced degradation product

About 10 mg of vorapaxar was taken in a 10 ml volumetric flask and dissolved in 10 ml methanol and diluted with 3% v/v H2O2. The solution was set aside for 72 h at ambient temperature. After degradation, solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

2.23 Thermal (Dry heat) degradation product

10mg Vorapaxar was taken on Petri dishes (10 cm in diameter) and spread as a thin layer of 1 mm, and exposed to 80°C for 72 hrs. After degradation, the degradation sample was dissolved in methanol and diluted with mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

2.24 Photolytic Degradation Product

About 10 mg of vorapaxar, was taken in a 10 ml volumetric flask and dissolved in 10 ml methanol. The solution was exposed to UV radiation (254 nm) at 1.2 million lux-hours for 72 h (using the photo stability chamber Thermolab 400G, New Delhi, and India). After degradation, solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

2.25 Neutral Degradation Studies

About 10 mg of vorapaxar, was taken in a 10 ml volumetric flask and dissolved with methanol and diluted to 10 mL with water. The solution was set aside for 72 h at ambient temperature. After degradation, solution was diluted with the mobile phase to get the final concentration 100 ng/mL and injected into UPLC-MS/MS system.

3. RESULTS AND DISCUSSION

3.1 Method development

The mass parameters of analyte and internal standard optimized and showed the abundant intensity of ions. The UPLC-MS/MS method described here satisfies the requirement of routine analyses since it has a short run time. The MS optimization was performed by direct injection of Vorapaxar and Vorapaxar–D5 into the mass spectrometer. The mass parameters were optimized to obtain better ionization of Vorapaxar and Vorapaxar–D5 molecules. Initially, mass parameters were also tuned in atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) ion sources, but inadequate response was observed in APCI ion source. The pure drug of Vorapaxar and Vorapaxar-D5 were prepared in methanol and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters.

3.2 Method validation

3.2.1 System suitability

The relative standard deviation of retention time and peak area response of 6 consecutive injections was observed as <1.0%, indicating excellent injection repeatability.

3.2.2 Selectivity and Specificity

No significant response was observed at retention times of Vorapaxar and Vorapaxar–D5 in mobile phase samples. It can be concluded that the method is specific for estimation of Vorapaxar in the presence of matrices. The chromatograms of blank samples (mobile phase) Vorapaxar Standards samples are shown in Fig.3-5.

3.2.3 Linearity

Linearity was plotted as a peak area ratio (Vorapaxar peak area / Vorapaxar–D5 peak area) on the y-axis against Vorapaxar concentration (pg/ml) on the x-axis. The correlation coefficient for Vorapaxar over the concentration range of 35.0 to 105.0 pg/mL was 0.999 (Tab-1 and Fig-6). The regression equation for Vorapaxar was y = 0.00079x - 0.00065. Linearity was found to be quite satisfactory and reproducible.
**Table 1. Calibration curve details of Vorapaxar (VP)**

<table>
<thead>
<tr>
<th>Linearity Level (%)</th>
<th>Nominal Conc. (pg/mL)</th>
<th>Vorapaxar Mean Peak Area (n=3)</th>
<th>Vorapaxar-D5 Mean Peak Area (n=3)</th>
<th>Mean Peak Area Ratio (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>35.00</td>
<td>23261</td>
<td>863472</td>
<td>0.027</td>
</tr>
<tr>
<td>65</td>
<td>45.00</td>
<td>30239</td>
<td>862241</td>
<td>0.035</td>
</tr>
<tr>
<td>80</td>
<td>55.00</td>
<td>37217</td>
<td>861010</td>
<td>0.043</td>
</tr>
<tr>
<td>100</td>
<td>70.00</td>
<td>46521</td>
<td>865779</td>
<td>0.054</td>
</tr>
<tr>
<td>115</td>
<td>80.00</td>
<td>53499</td>
<td>863548</td>
<td>0.062</td>
</tr>
<tr>
<td>135</td>
<td>95.00</td>
<td>62803</td>
<td>861317</td>
<td>0.073</td>
</tr>
<tr>
<td>150</td>
<td>105.00</td>
<td>69782</td>
<td>864086</td>
<td>0.081</td>
</tr>
</tbody>
</table>

Correlation coefficient: 0.99908  
Y-Intercept: -0.000651  
Slope: 0.00079  
Standard Error: 0.00089

**Fig 6. Calibration curve for Vorapaxar (VP)**

### 3.3 Precision & Accuracy

The precision of the proposed method was evaluated at three different concentration levels and precision and accuracy results were found to be 0.39 to 0.59 and 100.20 to 100.91% for intraday precision and 0.11 to 1.28 and 98.76 to 101.03 for interday precision (Table 2).

**Table 2. Precision and accuracy of Vorapaxar (VP) at three different concentrations**

<table>
<thead>
<tr>
<th>Concentration (pg/ml)</th>
<th>Within-run (Intra-day)</th>
<th>Between-run (Inter-Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Concentration measured (n=10;pg/ml;mean±S.D)</td>
<td>%CV</td>
</tr>
<tr>
<td>45.00</td>
<td>45.41±0.27</td>
<td>0.59</td>
</tr>
<tr>
<td>70.00</td>
<td>70.14±0.33</td>
<td>0.47</td>
</tr>
<tr>
<td>95.00</td>
<td>95.35±0.37</td>
<td>0.39</td>
</tr>
</tbody>
</table>

### 3.4 LOD and LOQ

The LOD and LOQ levels were calculated and found to be 3.71 and 11.24 pg/mL respectively.

### 3.5 Robustness

No significant changes were observed in studied system suitability parameters when deliberate variations are made in the chromatographic conditions which mean that the proposed method is robust (Tab 3).

**Table 3. Robustness of Vorapaxar**

<table>
<thead>
<tr>
<th>Validation Sample</th>
<th>Flow Rate (± 5%)</th>
<th>Column Temp (± 5%)</th>
<th>PH (± 2%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorapaxar</td>
<td>1.04</td>
<td>1.09</td>
<td>1.51</td>
</tr>
</tbody>
</table>

(70.0 pg/mL)
3.6 Solution Stability

The solution stability was assessed by considering stock solution stability for Vorapaxar and Vorapaxar-D5 (Tab-4) and their stability is shown under the studied conditions (ambient and refrigerated conditions), since in all conditions, the %difference values were smaller than 2%.

<table>
<thead>
<tr>
<th>Stability Sample</th>
<th>Ambient temperature</th>
<th>Refrigerated Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%Difference at 0.0Hours</td>
<td>%Difference at 48.0Hours</td>
</tr>
<tr>
<td>Vorapaxar (70.00pg/mL)</td>
<td>0.00</td>
<td>-0.4865</td>
</tr>
<tr>
<td>Vorapaxar-D5 (50.00pg/mL)</td>
<td>0.00</td>
<td>0.5501</td>
</tr>
</tbody>
</table>

3.7 Filter validation (Filter Interference)

The % difference values for Vorapaxar and Vorapaxar-D5 of different filter materials was found to be 0.21 to 0.50% and 0.52 to 0.99 (Tab-5) and no significant interference was observed.

<table>
<thead>
<tr>
<th>Validation Sample</th>
<th>% Difference 0.45µm Nylon</th>
<th>% Difference 0.45µm PVDF</th>
<th>% Difference 0.45µm PTFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorapaxar (70.00pg/mL)</td>
<td>0.26</td>
<td>1.25</td>
<td>1.37</td>
</tr>
<tr>
<td>Vorapaxar-D5 (50.00pg/mL)</td>
<td>0.278</td>
<td>0.315</td>
<td>0.681</td>
</tr>
</tbody>
</table>

3.8 Identification of major degradation products formed under stress conditions by UPLC-MS/MS

The fragmentation for the degradants was also carried out for vorapaxar using product ion scan by UPLCMS/MS. In these stress studies, a total of three degradation products (D1–D3) were observed for vorapaxar, among these products three degradation products, D1 (acidic), D2 (basic), and D3 (peroxide). Under oxidative degradation, the percentage degradation of the drug was 77.32% and complete degradation occurred for acidic and basic hydrolysis after 72 h. Under acidic hydrolysis, vorapaxar was degraded after 72 h with the formation of molecular ion. There was no degradation observed under neutral hydrolysis up to 72 h. The degradation products of acid and base hydrolysis were analyzed by UPLC–MS-MS, the total ion chromatograms of both degradates was showing molecular ions at m/z 449.22 and 445.19 indicating the presence of the degradation product in both cases. The respective acid and base degradation of m/z values of degradants and their fragmentation ions are represented in Figure-7 and 8. The oxidation stress conditions were studied using 3% v/v H₂O₂ up to 72 hr at room temperature. It was found that, 3% v/v H₂O₂ was effective in oxidizing the drug even after 72 hr. The oxidation degradant product has a molecular ion at m/z 493.25 and its fragmentation ions are shown in Figure-9. No neutral, thermal and photo degradation was observed for the solution and solid form of drug after exposure to the water, 80°C, UV light up to 72 h, respectively. It was confirmed that the drug was stable both in solid as well as in solution forms under neutral, photolytic and thermal stress conditions. The detail of stress degradation conditions applied and optimized is given in Table 6.

![Fragmentation mass spectrum of degradation product formed in acid Hydrolysis of vorapaxar.](image-url)
Pharmaceutical analysis

Fig 8. Fragmentation mass spectrum of degradation product formed in base Hydrolysis of vorapaxar.

Fig 9. Fragmentation mass spectrum of degradation product formed in oxidative (peroxide) degradation of vorapaxar.

Table 6. % Degradation behaviour of vorapaxar

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Strength of stress</th>
<th>Temperature</th>
<th>Duration</th>
<th>%Degradation</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Hydrolysis</td>
<td>0.1M HCl</td>
<td>Ambient</td>
<td>72 h</td>
<td>100.00</td>
<td>Complete degradation</td>
</tr>
<tr>
<td>Basic Hydrolysis</td>
<td>0.1M NaOH</td>
<td>Ambient</td>
<td>72 h</td>
<td>100.00</td>
<td>Complete degradation</td>
</tr>
<tr>
<td>Oxidative/Peroxide</td>
<td>3% v/v H2O2</td>
<td>Ambient</td>
<td>72 h</td>
<td>77.32</td>
<td>Degradation observed</td>
</tr>
<tr>
<td>Thermal</td>
<td>-</td>
<td>80ºC</td>
<td>72 h</td>
<td>0.00</td>
<td>Degradation not observed</td>
</tr>
<tr>
<td>Photolytic Studies (UV-Light)</td>
<td>UV lamp (254 nm) at 1.2 million lux-hours</td>
<td>Ambient</td>
<td>72 h</td>
<td>0.00</td>
<td>Degradation not observed</td>
</tr>
<tr>
<td>Neutral(Water)</td>
<td>Water</td>
<td>Ambient</td>
<td>72 h</td>
<td>0.00</td>
<td>Degradation not observed</td>
</tr>
</tbody>
</table>

4. CONCLUSION

An alternative UPLC-ESI-MS/MS method for quantification of Vorapaxar has been successfully developed and validated. The method exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, robustness and stability in various matrices. The method is sensitive enough for quantitative detection of the analyte in pharmaceutical preparations. This method may be useful in further investigation and characterization of other process-related impurities and helps in confirming the identity of degradation products formed. The possibility of further research for this developed method is to synthesize and develop reference standards and monitor their presence in the stability samples from the identified degradation products.

5. ACKNOWLEDGEMENTS

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6. AUTHORS CONTRIBUTION STATEMENT

Dr.Ch.Balasekhara reddy and Dr.P.Srinivasa babu conceived of the presented idea. T.Mohana Rao developed the theory and performed the computations. Dr.Ch.Balasekhara reddy and Dr.P.Srinivasa babu verified the analytical methods. Dr.Ch.Balasekhara reddy encouraged T.Mohana Rao to investigate and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.
8. REFERENCES


