Development and Validation of an HPLC Method for Quantification of Thienopyridine Analogues by RRLC method

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ABSTRACT

A simple, sensitive, and specific reversed phase liquid chromatographic method was developed and validated for quantification of related substances of clopidogrel bisulfate.

Chromatographic separation was performed on an Hypersil column (C18, 100x2.1 mm, 1.9μ) via gradient elution with mobile phase consisting with mobile phase A(Phosphate buffer with pH 2.6 and Acetonitrile(20:80%v/v)) and Mobile phase B( water and acetonitrile(15:85%v/v))

The method was validated pertaining to linearity, sensitivity, precision, accuracy, limit of quantification and limit of detection.

Keywords: Clopidogrel bisulfate, Linearity, Precision, Accuracy and Rapid resolution liquid chromatography (RRLC).

INTRODUCTION

Various international guidelines provide substantiation based recommendations of coprescribing antiplatelets drugs and statins in secondary prevention of cardiovascular events in patients with atherothrombosis (acute coronary syndromes (ACS), cerebrovascular disease, and peripheral arterial disease (PAD))\(^1\). Thienopyridine class of molecules involves clopidogrel hydrogen sulfate (fig-1), and its analogues. It remains the oral thienopyridine agent most prescribed in the world with a loading dose of 300 or 600 mg and maintenance dose of 75 mg \(^2\). Clopidogrel bisulfate is a prodrug converted about 15% in the liver by cytochrome P450 enzymes in a two-step process to the thiolic active metabolite\(^3\) that irreversibly blocks...
the P2Y12 receptor by disulfide bonding. The first step involves cytochrome P450-dependent monooxygenation\(^4\) to 2-oxo-clopidogrel and the second cytochrome P450-dependent oxidative opening\(^4\) of the thiolactone ring to an intermediate sulfenic acid metabolite subsequently reduced to the active thiolic metabolite. Few other methods have been reported for the estimation of clopidogrel by spectrophotometric\(^5,6\). Thin layer chromatography\(^7\), High-performance thin-layer chromatography \(^8\), High-performance liquid chromatography\(^9,10\), Liquid chromatographic -potentiometric detection method\(^11\) and Gas chromatography-Mass spectrometry\(^12\). Clopidogrel was determined by LC in the presence of its impurities using a chiral column and its impurities were determined at low levels\(^13\). The major objective of the present work is to develop a single method for the separation of chiral and non chiral impurities of clopidogrel bisulfate in drug substance with shorter runtime. The accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) of the method were determined in accordance with ICH guidelines\(^14,15\).

![Structure of Thienopyridine compound (Clopidogrel bisulfate)](image)

**Fig-1: Structure of Thienopyridine compound (Clopidogrel bisulfate)**

**MATERIALS AND METHODS**

**Chromatographic conditions**

The method was developed by using Hypersil C18 (100x2.1 mm, 1.9μ) column. Mobile phase A (Buffer and Acetonitrile 90:10 v/v) and Mobile phase B (water and acetonitrile, 20:80 v/v) with gradient elution programming. The mobile phase was filtered through a nylon membrane (pore size 0.45μm) filter. The flow rate of the mobile phase was 0.4ml/min with total runtime 14 min. The column temperature was maintained at 35 °C and the wavelength was monitored at 210 nm. The injection volume was 3μL.

**Equipment**

The Agilent 1290 infinity system equipped with Binary pump with maximum pressure 1200bar, Auto sampler micro cell) and data handling system (chemstation software) and UV detector, Cintex digital water bath, Semi Analytical balance, pH meter and Filtration unit and 0.22μ membrane filters.

**Impurity Chemical Names**

**Imp-A** : [Thieno[3,2-c]-4,5,6,7-tetrahydro pyridine]

**Imp-B** : [Methyl-o-chlorophenyl]-4,5-dihydro thieno [2,3-c]pyridine-6(7H)-acetate]
Imp-C : [Thieno[3,2-c]-4,5-dihydro pyridine]
Imp-D : Methyl-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate

Mobile Phase preparation

Buffer: Take 1000ml of milli-Q water, filter through 0.22μ membrane filter, sonicate the water for 10-15min, after add 100 μl of trifluoro acetic acid mix thoroughly and adjust pH to 2.8 with diluted H₃PO₄ solution.

Mobile phase A: Buffer and Acetonitrile in the ratio 90:10 mix thoroughly
Mobile phase B: Water and Acetonitrile in the ratio of 20:80 mix thoroughly and degass through 0.22μ filter. Preparation of IMP-B: Take 10.0mg of imp-B in to 10ml of volumetric flask dissolved and diluted mark with diluent.

System suitability preparation

Weigh accurately 10.0 mg of the standard into a 10 ml volumetric flask and dissolved. To this add Transfer 10μL of above solution and make up to the mark with same solution.

Standard preparation

Weigh about 10.0 mg of working standard into a 10 ml volumetric flask, dilute to volume with diluent and sonicate to dissolve.

Test preparation

Weigh about 10.0 mg of sample into a 10 ml volumetric flask, dilute to volume with diluent and sonicate to dissolve.

Method validation

The proposed method was validated as per ICH guidelines.

Table-1: System suitability

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Result</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Resolution between the peak due to clopidogrel bisulfate and Impurity-B</td>
<td>2.51</td>
<td>Not less than 1.5</td>
</tr>
</tbody>
</table>

Fig-2: Impurity blend chromatogram
RESULTS AND DISCUSSION

Limit of detection

It is the lowest concentration of analyte that can be detected but cannot be quantified. Prepared dilutions of impurity and analyte in different concentrations and injected them into the chromatographic system till the signal to noise ratio is between 2 and 3.

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Concentration in mg/mL w.r.t test</th>
<th>% impurity w.r.t test</th>
<th>S/N Ratio</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impurity –A</td>
<td>0.00008</td>
<td>0.008</td>
<td>2</td>
<td>The signal to noise ratio should be in between 2.0 and 3.0</td>
</tr>
<tr>
<td>Impurity –B</td>
<td>0.00007</td>
<td>0.007</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Impurity –C</td>
<td>0.00006</td>
<td>0.006</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Impurity –D</td>
<td>0.00007</td>
<td>0.007</td>
<td>2.8</td>
<td></td>
</tr>
</tbody>
</table>

Fig-3: LOD chromatogram

Limit of quantification

It is the lowest concentration of analyte that can be quantified with an acceptable precision and accuracy. Prepared a series of dilutions of impurities by different concentrations and injected them into the chromatographic system till the signal to noise ratio is between 9.4 and 10.5

<table>
<thead>
<tr>
<th>Impurity</th>
<th>Concentration in mg/mL w.r.t test</th>
<th>% impurity w.r.t test</th>
<th>S/N Ratio</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Impurity –A</td>
<td>0.0003</td>
<td>0.03</td>
<td>9.4</td>
<td>The signal to noise ratio should be in between 9.4 and 10.5</td>
</tr>
<tr>
<td>Impurity –B</td>
<td>0.0002</td>
<td>0.02</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>Impurity –C</td>
<td>0.0002</td>
<td>0.02</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>Impurity –D</td>
<td>0.0002</td>
<td>0.02</td>
<td>10.5</td>
<td></td>
</tr>
</tbody>
</table>
Precision at Limit of Quantification

Six individual impurity solutions at the limit of quantification level were prepared. Injected each solution once and calculated the % RSD for the area of each impurity.

Accuracy at Limit of quantification

Accuracy is measurement of exactness of analytical method which is determined by adding the known amount of impurity to sample at limit of quantification level. The accuracy is calculated in terms of % recovery of impurity. Triplicate test solutions containing impurities at limit of quantification level were prepared. The test sample solution in twice and prepared the impurities at LOQ level in triplicate were prepared. Injected each solution once in to chromatographic system as per the test method and calculated the % recovery of the impurities.

Table-4: Accuracy at limit of quantification level results (% Recovery)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Imp –A</th>
<th>Imp –B</th>
<th>Imp –C</th>
<th>Imp –D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1041.7</td>
<td>92.3</td>
<td>109.1</td>
<td>96.2</td>
</tr>
<tr>
<td>2</td>
<td>93.6</td>
<td>93.5</td>
<td>109.3</td>
<td>95.1</td>
</tr>
<tr>
<td>3</td>
<td>108.6</td>
<td>98.2</td>
<td>97.1</td>
<td>95.5</td>
</tr>
<tr>
<td>Average</td>
<td>102.3</td>
<td>94.7</td>
<td>105.2</td>
<td>95.6</td>
</tr>
</tbody>
</table>

Acceptance criteria: The %RSD of impurities should be in between 80 and 120.

Table-5: Results of Precision at Limit of Quantification level

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Imp –A</th>
<th>Imp –B</th>
<th>Imp –C</th>
<th>Imp –D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.81314</td>
<td>2.47419</td>
<td>2.18537</td>
<td>3.51808</td>
</tr>
<tr>
<td>2</td>
<td>1.73220</td>
<td>2.38513</td>
<td>2.08893</td>
<td>3.52319</td>
</tr>
<tr>
<td>3</td>
<td>1.80089</td>
<td>2.40155</td>
<td>2.09532</td>
<td>3.43820</td>
</tr>
<tr>
<td>4</td>
<td>1.77929</td>
<td>2.41674</td>
<td>2.05633</td>
<td>3.47072</td>
</tr>
<tr>
<td>5</td>
<td>2.00732</td>
<td>2.47917</td>
<td>2.17713</td>
<td>3.54318</td>
</tr>
<tr>
<td>6</td>
<td>1.79172</td>
<td>2.6639</td>
<td>2.07301</td>
<td>3.67392</td>
</tr>
<tr>
<td>Avg.</td>
<td>1.82076</td>
<td>2.47552</td>
<td>2.11268</td>
<td>3.52797</td>
</tr>
<tr>
<td>Std.</td>
<td>0.10</td>
<td>0.11</td>
<td>0.05</td>
<td>0.08</td>
</tr>
<tr>
<td>%RSD</td>
<td>5.2</td>
<td>4.6</td>
<td>2.6</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Acceptance criteria: %RSD of individual impurities area should not be more than 10.
Linearity

Linearity was demonstrated by injecting impurities at limit of quantification level, 25%, 50%, 75%, 100%, 125% and 150% with respect to the specification level. Graph was plotted the calibration curve by taking concentration on X-axis and peak area on Y-axis. Calculated the correlation coefficient and % y-intercept at 100% specification level.

Fig-5: Linearity graphs of Impurity-A, B, C and D

Range

Range is defined as the range of concentration in which method is linear, precise and accurate. The data generated in precision, linearity and accuracy should be considered in establishment of range.

CONCLUSION

The established analytical technique was rapid, accurate, selective and précised. Hence the method is used for the analysis of Clopidogrel and its related substances are validated as per ICH guidelines. The percentage RSD for all parameters was found within the acceptance limit, which indicates that validity of the method in fair agreement. The results of linearity, precision, accuracy and specificity, proved to be within the limits.
ACKNOWLEDGEMENTS

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REFERENCES

