A VALIDATED STABILITY-INDICATING RP-HPLC METHOD FOR ABACAVIR SULFATE IN THE PRESENCE OF DEGRADATION PRODUCTS, ITS PROCESS-RELATED IMPURITIES AND IDENTIFICATION OF OXIDATIVE DEGRADANT BY LC-MS/MS

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Stability-Indicating.

ABSTRACT
The objective of the current study was to develop a validated specific LC-MS compatible stability-indicating reverse phase liquid chromatographic method for the quantitative determination of Abacavir sulfate and its related substances. Significant degradation was observed during oxidation, and the major degradant was identified by LC–MS analysis. The chromatographic conditions were developed and optimized using an impurity-spiked solution and the forced degradation samples with a resolution of >2. The chromatographic separation was achieved on YMC Pack Pro C18, 150 mm x 4.6 mm, 3µ particle size column. Using 0.05% TFA in water as mobile phase A and the 0.05% TFA in Acetonitrile as mobile phase B with 1.0mL/min flow rate in gradient mode. The column temperature was maintained at 25°C, detection wavelength was set at 220 nm and the injection volume was 10 µL. Water and Acetonitrile in the ratio 90:10(v/v) was used as a diluent. The developed RP-HPLC method was validated according to ICH guidelines. In this method the LOD and LOQ values for Abacavir and all its related impurities were ranged from 0.0033µg/mL to 0.013µg/mL and 0.010µg/mL to 0.040µg/mL respectively. The percentage recovery for all impurities was ranged from 99 to 102 % w/w. The test solution and mobile phase were observed to be stable up to 48 h after preparation. The validated method produced good results of precision, linearity, accuracy, robustness and ruggedness. The proposed method was found to be suitable precise, sensitive and accurate for the quantitative determination of related impurities in the bulk samples of Abacavir sulfate API.

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INTRODUCTION

Abacavir sulfate is chemically known as ((1S, 4R)-4-(2-Amino-6-(Cyclopropylamine)-9H-purin-9-yl) cyclopent-2-enyl) methanol Hemi sulfate, is a nucleoside reverse transcriptase inhibitor (NRTIs) [1]. It is used as either a 600-mg once-daily or 300-mg twice-daily regimen exclusively in the treatment of human immunodeficiency virus (HIV) infection [2]. Mainly helps to halt the inroads of the human immunodeficiency virus (HIV). Without treatment, HIV gradually undermines the body's immune system by encouraging other infections to take hold until the body succumbs to full-blown acquired immune deficiency syndrome (AIDS). Initially, Abacavir is phosphorylated to its corresponding monophosphate as intracellular reaction. Cytosolic enzymes convert Abacavir monophosphate to carbovir monophosphate (CBV-MP), which is finally phosphorylated to the biologically active moiety, carbovir triphosphate (CBV-TP). CBV-TP inhibits HIV reverse transcriptase by competing with the endogenous substrate dGTP and by chain termination subsequent to incorporation into the growing polynucleotide strand [3]. Side effects of this drug may cause abdominal pain, cough, diarrhea, fatigue, fever or chills, generally ill feeling, headache/migraine, joint pain, mouth ulcers, muscle aches or weakness, nausea, rash, severe blisters in the mouth and eyes, severe peeling skin, shortness of breath, skin tingling or burning, sleep disorders, sore throat, swelling, tiredness, vomiting.

Few HPLC methods have been reported in the literature for determination of abacavir in plasma and therapeutic monitoring and simultaneous determination with other antiretroviral products [4-24], pharmaceutical dosage forms and human serum [25,26], in biological matrices [27-29], using electrochemical determination [30]. Even stability indicating UHPLC method was developed and reported in presence of related substances and degradation impurities [31]. Few articles were found on characterization of degradation impurities [32, 33]. Recently a USP had published a monograph for Abacavir sulfate which proposes RP-HPLC method for impurity analysis [34]. To the best of our knowledge no simple LC-MS compatible RP-HPLC method for the quantitative estimation of Abacavir drug substance in the presence of process-related impurities and degradation products has been reported. The present research work is mainly focused to develop a most accurate single LC-MS compatible stability-indicating HPLC method for the determination of Abacavir and its related substances. The developed LC method was validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness. Forced degradation studies were performed on the drug substance to show the stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines. Developments of such methods are very important to have a good quality product form for Human treatment.

EXPERIMENTAL

Chemicals

All the impurities and the Abacavir standard were of >99% purity and are as follows: Abacavir sulfate (99.7%), imp-A (99.5%), imp-B (99.4%), imp-C (99.7%), imp-D (99.5%), imp-E (99.2%), imp-F (99.1%) and imp-G (99.3%) Figure 1. In addition, HPLC grade acetonitrile was purchased from Qualigens and analytical grade Trifluoroacetic acid was purchased from Acros. Highly pure water was prepared with the Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).
Figure 1: Structures and names of Abacavir and its impurities.

Equipment

The Agilent 1200 series HPLC system with photo diode array used for method development, forced degradation studies and method validation. The output signal was monitored and processed using chemstation software. Cintex digital water bath was used for hydrolysis studies. Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were carried out in a dry air oven (Cintex, Dadar, India).

Chromatographic conditions

A simple LC-MS compatible liquid chromatographic method was developed for the determination of Abacavir and its related substances. Chromatographic separations were achieved on a YMC Pack Pro C18, 150 mm x 4.6 mm, 3µ particle size column having stationary phase of octadecylsilane. The 0.05% TFA in water; was used as mobile phase A and the 0.05% TFA in Acetonitrile; was used as mobile phase B. The flow rate of the mobile phase was 1.0 mL/min with a gradient program of 0/0, 25/20, 35/80, 40/80, 40.1/0.
and 45/0 (time (min)%B). The column temperature was maintained at 25°C and the detection wavelength was set at 220 nm. The injection volume was 10 µL. Water and Acetonitrile in the ratio 90:10(v/v) was used as a diluent.

LC–MS conditions

LC–MS system (Agilent 1100 series liquid chromatography system coupled with a 6410 series triple quardrupole mass spectrometer) was used for the identification of unknown compounds formed during forced degradation. The analysis was performed in positive and negative electrospry ionization modes. The capillary voltage is 4.0 kV. The source and desolvation temperatures were 25°C and 350°C, respectively and the desolvation gas flow was 500Liters/Hour. The same LC conditions were used for LC-MS. The main advantage of this method is that the LC conditions were straight away taken in to LC-MS.

Preparation of standard solutions and sample solutions

The standard solution of Abacavir was prepared at the concentration of 1.0 mg/mL by dissolving appropriate amount of Abacavir hemi sulfate drug substance in diluent. Stock solutions of each impurity were prepared at the concentration of 0.1 mg/mL in the diluent. The sample solutions with required concentrations were prepared using corresponding dilutions to attain the required concentration of impurity and drug substance.

Stress studies/specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [35]. The specificity of the developed LC method for Abacavir was determined in the presence of its impurities (namely imp-A, imp-B, imp-C, imp-D, imp-E, imp-F, imp-G and degradation products) [36]. Forced degradation studies were also performed on Abacavir to provide an indication of the stability-indicating property and specificity of the proposed method. The stress conditions employed for the degradation study included light (carried out as per ICH Q1B), heat (105°C), acid hydrolysis (3 M HCl), base hydrolysis (3 M NaOH) and oxidation (10% H2O2). For thermal studies, the sample exposed for 7 days, whereas the samples were treated up to 48 h for acid, base hydrolysis and 15 hrs for oxidation. The peak purity of the Abacavir stressed samples was checked by using a Agilent 1100 series with photo diode array detector (PDA) and with Agilent LC-MS system. The Summary results of Forced degradation studies are tabulated in Table 1.

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Time</th>
<th>Purity of Analyte after degradation</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstressed sample</td>
<td>--</td>
<td>99.4</td>
<td>--</td>
</tr>
<tr>
<td>Acid hydrolysis (3M HCl)</td>
<td>48 h</td>
<td>99.1</td>
<td>No degradation products formed</td>
</tr>
<tr>
<td>Base hydrolysis (3M NaOH)</td>
<td>48 h</td>
<td>99.2</td>
<td>No degradation products formed</td>
</tr>
<tr>
<td>Oxidation (10% H2 O2)</td>
<td>15 h</td>
<td>94.0</td>
<td>One major degradation product was formed</td>
</tr>
<tr>
<td>Thermal (105°C)</td>
<td>7 days</td>
<td>99.4</td>
<td>No degradation products formed</td>
</tr>
<tr>
<td>Photolytic degradation</td>
<td>10 days</td>
<td>99.4</td>
<td>No degradation products formed</td>
</tr>
</tbody>
</table>

METHOD VALIDATION

The proposed method was validated per ICH guidelines [37].

Precision

The precision of an analytical procedure expresses the closeness of agreement among a series of measurements obtained from multiple samplings of the same homogenous sample under prescribed conditions. The precision of the related substance method was done by injecting six individual preparations of (1mg/mL) Abacavir drug substance spiked with 0.10 % each of imp-B, imp-D, imp-E and 0.15% each of imp-A, imp-C, imp-F and imp-G (each impurity with respect to analyte concentration). The %RSD of the areas for each imp-A, imp-B, imp-C, imp-D, imp-E, imp-F and imp-G were calculated.

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

The detection limit of impurities and drug substance was determined by diluting known concentrations of each related substance and abacavir until the average responses were approximately three times the SD of the responses. The quantitation limit of impurities and drug substance was determined by diluting known concentrations of each related substance and abacavir until the average responses were approximately ten times the SD of the responses. The S/N at these concentrations were also measured and found to be in the range of 3:1 for detection limits and 10:1 for quantitation limit. Precision was carried out at LOQ level by preparing six individual preparations and calculating the %RSD for the areas of Abacavir and its related impurities. Accuracy at LOQ level was also carried out by preparing three recovery solutions of Abacavir with its related impurities at LOQ level.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results, which are directly proportional to the concentration of the analyte in the sample. The linearity test solutions were prepared at six concentration levels
from LOQ to 200% of the specification limit (LOQ, 50%, 75%, 100%, 150% and 200%). The peak area versus concentration data was analyzed with least-squares linear regression. The coefficient of regression, slope and y-intercept of the calibration curves were calculated.

Relative response factor
The relative response factor of each impurity was calculated using slope values from linearity curves by the formula,

\[
RRF = \frac{\text{Slope of the impurity}}{\text{Slope of Abacavir}}
\]

Accuracy
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy test solutions for Abacavir drug substance by standard addition of its related impurities were prepared in triplicate at three concentration levels, 50%, 100% and 150% with respect to specification limit. The percentage recovery of each impurity was calculated.

Robustness
The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To determine the robustness of the developed method, the experimental conditions were altered and the resolution between Abacavir and imp-C was evaluated. The effect of the flow rate on the resolution was studied at 0.8 mL/min and 1.2 mL/min instead of 1.0 mL/min. The effect of wave length on the resolution was studied at 218 nm and 222 nm instead of 220 nm. The effect of the column temperature on the resolution was studied at 20°C and 30°C instead of 25°C. The effect of the Gradient programme on the resolution was studied with gradient programme T/%B: 0/0, 25/18, 35/52, 40/72, 40.01/0 and T/%B: 0/2, 25/22, 35/88, 40/88, 40.01/2 instead of T/%B: 0/0, 25/20, 35/80, 40/80, 40.01/80. In all these four parameters i.e. Flow rate, Wave length, Temperature and gradient programme the theoretical plates, tailing factor and the resolution between analyte and imp-C are calculated.

Ruggedness
The ruggedness of a method was defined as degree of reproducibility of results obtained by analysis of the same sample under variety of normal test conditions such as different laboratories, different analysts, different instruments, different days and different lots of reagents. In this case the study was performed by different analyst on different instrument located at different lab and on different day. Precision studies were carried out for imp-A, imp-B, imp-C, imp-D, imp-E, imp-F and imp-G content in bulk samples at the same concentration levels as in precision. The %RSD of the areas of each imp-A, imp-B, imp-C, imp-D, imp-E, imp-F and imp-G were calculated.

Solution stability and mobile phase stability
The solution stability of Abacavir and its impurities was carried out by leaving a spiked sample solution in a tightly capped volumetric flask at room temperature (25±2°C) for 48 h. The content of imp-A, imp-B, imp-C, imp-D, imp-E, imp-F and imp-G were determined at 12 h intervals up to the 48 hrs period.

The mobile phase stability was also investigated for 48 h by injecting the freshly prepared spiked sample solutions for 48 hrs. The content of imp-A, imp-B, imp-C, imp-D, imp-E, imp-F and imp-G were determined at 12 h intervals up to the 48 hrs period. The prepared mobile phase remained constant during the study period.

DISCUSSION
Method development and optimization
Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-G are potential impurities of abacavir drug substance. The main objective of the chromatographic method was to separate imp-A, imp-B, imp-C, imp-D, imp-E, imp-F, imp-G and the generated degradation products from the analyte peak during stress studies. Imp-C was co-eluted with analyte peak by using different stationary phases, such as C8, cyano and phenyl with various mobile phases, such as phosphate and acetate buffers, and organic modifiers, including acetonitrile and methanol, in the mobile phase. Selection of column has played a critical role in achieving the separation of imp-C from analyte peak. Initially the method development is started by using sodium dihydrogen orthophosphate buffer with a pH value of 3.0 as mobile phase A and acetonitrile as mobile phase B with a gradient programme T/%B: 0/2, 20/12, 30/60, 35/60, 35.01/2 post run: 5 min at a flow rate of 1.0 mL/min on a 250 mm x 4.6 mm ID column and 5µ particle size C8 stationary phase. When an impurity-spiked solution was injected, for the analyte peak system suitability parameters are very poor, the resolution between the imp-C and analyte was very poor. Imp-C was almost co-eluted with the analyte hence the peak purity of analyte also failed. To improve the peak shape we replaced the mobile phase A with 10 mM Ammonium acetate buffer and injected the impurity-spiked solution. Peak shape was slightly improved but the imp-C was still having less resolution from the analyte peak. Next trial was taken with 0.05% TFA in water as mobile phase A and 0.05% Acetonitrile as mobile phase B and injected impurity-spiked solution. Peak shape was improved but the imp-C was still having less resolution from analyte peak. C8 stationary phase was not successful in achieving a favorable resolution of imp-C and Analyte peak and the coelution of other process impurities. Next trails had performed
on cyan and phenyl columns but results are not good, so we tried on C18 stationary phase with same TFA conditions and different gradient programs to get more resolution of imp-C from Analyte and to avoid co-elution of all process related impurities and degradant impurities.

Chromatographic separation was successfully achieved on C18 stationary phase, YMC Pack Pro C18, 150*4.6 mm, 3.0µm column, by using 0.05% TFA in water as mobile phase A and 0.05% TFA in Acetonitrile as mobile phase B with a gradient programme T/%B: 0/0, 25/20, 35/80, 40/80, 40.01/0, post run: 5 min. The column temperature was maintained at 25°C and detection was monitored at wave length 220 nm. The injection volume is 10µL. In the optimized conditions, the impurities namely imp-A, imp-B, imp-C, imp-D, imp-E, imp-F, imp-G and drug substance Abacavir were well separated with a resolution more than 3.5 and all degradant impurities are having resolution more than 2.0 from analyte peak. The method was specific for Abacavir from its pot.

RESULTS

Method Validation

Precision

The %RSD for the areas of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-G were well within 1.41%, conforming good precision of the method. The %RSD values are reported in Table 3.

Table 3: Regression and precision data.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Abacavir</th>
<th>Imp-A</th>
<th>Imp-B</th>
<th>Imp-C</th>
<th>Imp-D</th>
<th>Imp-E</th>
<th>Imp-F</th>
<th>Imp-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (µg/mL)</td>
<td>0.009</td>
<td>0.013</td>
<td>0.01</td>
<td>0.005</td>
<td>0.009</td>
<td>0.01</td>
<td>0.013</td>
<td>0.005</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>0.026</td>
<td>0.038</td>
<td>0.029</td>
<td>0.015</td>
<td>0.028</td>
<td>0.029</td>
<td>0.040</td>
<td>0.014</td>
</tr>
<tr>
<td>Regression equation (y)</td>
<td>374.78</td>
<td>127.43</td>
<td>396.88</td>
<td>329.81</td>
<td>367.95</td>
<td>471.37</td>
<td>315.15</td>
<td>318.56</td>
</tr>
<tr>
<td>Slope (m)</td>
<td>1.30</td>
<td>-2.03</td>
<td>1.34</td>
<td>-4.21</td>
<td>-0.94</td>
<td>-0.48</td>
<td>0.64</td>
<td>-4.21</td>
</tr>
<tr>
<td>Intercept (C)</td>
<td>0.9999</td>
<td>0.9993</td>
<td>0.9996</td>
<td>0.9994</td>
<td>0.9996</td>
<td>0.9993</td>
<td>0.9996</td>
<td>0.9993</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.41</td>
<td>0.77</td>
<td>1.1</td>
<td>0.82</td>
<td>0.93</td>
<td>1.5</td>
<td>1.2</td>
<td>0.99</td>
</tr>
<tr>
<td>Precision at LOQ level(%RSD)</td>
<td>0.85</td>
<td>1.24</td>
<td>1.32</td>
<td>0.27</td>
<td>1.25</td>
<td>1.13</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>Precision (%RSD)</td>
<td>4.21</td>
<td>3.67</td>
<td>3.21</td>
<td>2.27</td>
<td>1.25</td>
<td>1.13</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td>Ruggedness(%RSD)</td>
<td>1.00</td>
<td>0.34</td>
<td>1.06</td>
<td>0.88</td>
<td>0.98</td>
<td>1.26</td>
<td>0.84</td>
<td>0.85</td>
</tr>
<tr>
<td>Relative response factor</td>
<td>9.01</td>
<td>5.06</td>
<td>4.52</td>
<td>3.52</td>
<td>2.52</td>
<td>1.52</td>
<td>1.02</td>
<td>0.52</td>
</tr>
</tbody>
</table>

Linearity range is from LOQ to150% with respect to 1.0 mg/mL of Abacavir for impurities.

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

The determined limit of detection, limit of quantification and precision at LOQ values for Abacavir and its related impurities are reported in Table 3. Accuracy at LOQ values for Abacavir and its related impurities are reported in Table 4.

Linearity

Linear calibration plot for Abacavir and its related impurities were obtained over the calibration ranges tested, i.e. LOQ to 0.20% for Abacavir, Imp-B, Imp-D, Imp-E and LOQ to 0.30% for Imp-A, Imp-C, Imp-F and Imp-G. The correlation coefficient for Abacavir and its related impurities obtained was greater than 0.9993 and reported in Table 3. The above result shows that an excellent
correlation existed between the peak area and the concentration of Abacavir, Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-G.

**Relative response factor**
The relative response factors for all Abacavir related impurities were calculated and reported in Table 3.

**Accuracy**
The percentage recovery of impurities in Abacavir samples varied from 97.8 to 100.4%. The LC chromatogram of spiked sample at 0.10% level of Imp-B, Imp-D, Imp-E and at 0.15% level of Imp-A, Imp-C, Imp-F, Imp-G is shown in Fig. 2. The % recovery values for Abacavir and its related impurities are presented in Table 4.

Table 4: Evaluation of accuracy.

<table>
<thead>
<tr>
<th>Amount spiked</th>
<th>% Recovery&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Abacavir</th>
<th>Imp-A</th>
<th>Imp-B</th>
<th>Imp-C</th>
<th>Imp-D</th>
<th>Imp-E</th>
<th>Imp-F</th>
<th>Imp-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ</td>
<td>98.2 ± 0.77</td>
<td>97.9 ± 0.18</td>
<td>98.9 ± 0.32</td>
<td>99.2 ± 0.47</td>
<td>98.3 ± 0.91</td>
<td>99.7 ± 0.42</td>
<td>97.8 ± 0.12</td>
<td>99.1 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>99.3 ± 0.42</td>
<td>98.3 ± 0.27</td>
<td>99.4 ± 0.81</td>
<td>100.1 ± 0.07</td>
<td>99.7 ± 0.17</td>
<td>98.7 ± 0.49</td>
<td>99.2 ± 0.09</td>
<td>99.4 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>100%</td>
<td>100.4 ± 0.43</td>
<td>97.2 ± 0.33</td>
<td>98.4 ± 0.19</td>
<td>100.0 ± 0.21</td>
<td>98.8 ± 0.95</td>
<td>99.2 ± 0.87</td>
<td>98.5 ± 0.96</td>
<td>98.4 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td>100.3 ± 0.05</td>
<td>99.2 ± 0.13</td>
<td>98.9 ± 0.16</td>
<td>99.6 ± 0.45</td>
<td>99.1 ± 0.02</td>
<td>99.3 ± 0.29</td>
<td>99.7 ± 0.19</td>
<td>98.9 ± 0.82</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Amount of seven impurities spiked with respect to specification level (0.10% for Imp-B, Imp-D, Imp-E and 0.15% for Imp-A, Imp-C, Imp-F, Imp-G) individually to 1.0 mg/ml of Abacavir.<n
<sup>b</sup> Mean ± %RSD for three determinations.

**Robustness**
In all the deliberate varied chromatographic conditions (flow rate, wave length, column temperature and Gradient), all analytes were adequately resolved and elution orders remained unchanged. The resolution between critical pair, i.e. for Abacavir and imp-C was greater than 3.1 and tailing factor for Abacavir was less than 1.1. In all these conditions theoretical plates for Abacavir, tailing factor for Abacavir and the resolution between Abacavir and Imp-C are reported in Table 5.

Table 5: Robustness values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Actual value</th>
<th>Changed value</th>
<th>Theoretical plates of Abacavir</th>
<th>Tailing factor of Abacavir</th>
<th>Resolution between Abacavir and Imp-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
<td>0.8 ml/min</td>
<td>240,628</td>
<td>1.1</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.2 ml/min</td>
<td>251,325</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Wave length</td>
<td>220 nm</td>
<td>218 nm</td>
<td>230,041</td>
<td>1.0</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>222 nm</td>
<td>241,398</td>
<td>1.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Temperature</td>
<td>25°C</td>
<td>20°C</td>
<td>229,549</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C</td>
<td>254,624</td>
<td>1.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Gradient</td>
<td>0/0, 25/20, 35/80, 40/80</td>
<td>0/0, 25/18, 35/72, 40/72</td>
<td>228,471</td>
<td>1.1</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0/2, 25/22, 35/88, 40/88</td>
<td>249,569</td>
<td>1.0</td>
<td>3.3</td>
</tr>
</tbody>
</table>

**Ruggedness**
The %RSD for the areas of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F and Imp-G were well within 1.25% and are reported in Table 3. The results obtained by second analyst were well in agreement with the results obtained by the first analyst, conforming good reproducibility of the method.

**Solution stability and mobile phase stability**
The solution stability and mobile phase stability experiments data confirms, no variation was observed in the study period and it indicates that spiked sample solution prepared in diluent was stable up to 48 h at room temperature (25±2°C) with respect to specified method.
Forced degradation studies

Degradation was not observed in Abacavir stressed samples subjected to acid, base, light and heat. Major degradation of the drug substance was detected under oxidative degradation, leading to the formation of one major unknown degradation product at 0.88 RRT Figure 2 along with some known impurities. Peak purity test results derived from the PDA detector and LC-MS, confirmed that the Abacavir peak and the degraded peaks were homogeneous and pure in all of the analyzed stress samples, confirming the stability-indicating power of the developed method.
Identification of major degradation product (RRT 0.88) formed in oxidative degradation

A LC–MS study was carried to determine the m/z value of the major degradation product formed under oxidative degradation using an Agilent 1100 series liquid chromatography system coupled with a 6410 series triple quadrupole mass spectrometer. The same LC conditions were used in LC-MS. The m/z value obtained for the degradation product resolving at 0.88 RRT in ESI positive mode was 303.3 (M+H) and corresponds to a molecular weight of 302.3. The impurity was isolated using preparative HPLC, and its structure was confirmed by characterization through FTIR, and $^1$H/$^{13}$C NMR spectral analysis. The strongest band in the IR spectrum is observed at 1231 cm$^{-1}$ together with adjacent absorptions at 1238 and 1250 cm$^{-1}$ these are assigned to the N-O stretch, because this vibration is accompanied by a large change in dipole moment and polarizability. The blue shifted very strong IR band at 1258 cm$^{-1}$ supports both the assignment to $\nu$ (N-O) and existence of CH…O-N hydrogen bonding, $^1$H/$^{13}$C NMR spectral data also supporting to the proposed structure of Figure 3.

**Imp at RRT 0.88:**

![Structure of Impurity formed in Oxidative degradation.](image)

**CONCLUSION**

In this paper, a sensitive, specific, accurate, validated and well-defined stability-indicating LC method for the determination of Abacavir in the presence of degradation products and its process related impurities was described. The behavior of Abacavir under various stress studies were studied, the degradant in oxidative degradation was identified by LC–MS and presented.
All of the degradation products and process impurities were well separated from the drug substance and drug product demonstrates the stability-indicating power of the method. The method is stability indicating and can be used for routine analysis of production samples and to check the stability of Abacavir samples. The further evaluation can be performed to study the product development matrix interference in dosage forms with respect to Abacavir and its Process and Degradation impurities.

REFERENCES
3. Huff, J. R.; New drugs- Reports of new drugs recently approved by the FDA; Bioorganic & Medicinal Chemistry. 1999, 7: 2667-2669
34. USP monograph on Abacavir Sulfate; the United States Pharmacopoeia. USP 35: 2021-2022.
35. ICH Q1A (R2), Stability Testing of New Drug Substances and Products, 2000.
37. ICH Q2 (R1), Validation of Analytical Procedures: Text and Methodology, November 2005.