Development and Validation of LC-MS/MS Method for the Analysis of P-Toluenesulfonicacid, In Antiviral Drug Acyclovir

Noor Shabana1, K. Dayanan Reddy1*
Research Scholar, Department of Chemistry, P.V.K.N Govt.College, Chittoor, India
Assistant Professor, Department of Chemistry, P.V.K.N Govt.College, Chittoor, India 1*

ABSTRACT: A sensitive and selective liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method was developed and validated for the trace analysis (>1 ppm level) of p-Toluenesulfonicacid, a genotoxic impurity, in acyclovir drug substances. The chromatographic separation was achieved on Symmetry C-18 (150 X 4.6mm, 3.5µm) column using a mobile phase consisting of 5 mM ammonium acetate and methanol (70:30 v/v) at flow rate of 0.4 mL/min. The API-4000 LC/MS/MS was operated on an electrospray in negative mode. The selected ion monitoring (SIM) mode was used during the analytical run and it is able to quantitate up to 1.5 ppm of p-Toluenesulfonicacid. The newly developed method was validated as per achieved on International Conference on Harmonization (ICH) guidelines

KEYWORDS: Acyclovir, p-Toluenesulfonicacid, Method Validation, LC/MS/MS, Trace analysis

I. INTRODUCTION

The chemical name for acyclovir is 2-amino-1,9-dihydro-9- [(2-hydroxyethoxy) methyl]-6H-purine-6-one, (or) 9- [(2-hydroxyethoxy) methyl]- guanine. Its molecular formula is C8H11N5O3, and molecular weight 225.21 g/mol [1]. Acyclovir is a potent anti-viral agent useful in the treatment of Herpes Simplex Virus (HSV) infections. Acyclovir exerts its antiviral activity by competitive inhibition of viral DNA, through selective binding of acyclovir to HSV-thymidine kinase with about 200 fold greater affinity than for mammalian enzyme [2].

Pharmaceutical genotoxic impurities (PGIs) may induce genetic mutations, chromosomal breaks (rearrangements) and they have potential to cause cancer in human [3-4]. Therefore exposure to even low levels of such impurities present in final active pharmaceutical ingredient (API) may be of significant toxicol importance [5]. Hence it is significant for process chemists to avoid such genotoxic impurities in the manufacturing process [6]. However it would be difficult or impossible to eliminate PGIs completely from the synthetic scheme. Therefore it is a great challenge to analytical chemists to develop an appropriate analytical method to quantify the impurity accurately and control their levels in APIs. According to the European Medicines Evaluation Agency (EMEA) and feedback from US Food and Drug Administration (USFDA) the proposed use of a threshold of toxicological concern (TTC), it is accepted that genotoxic impurities will be limited to a daily dose of 1.0-1.5 μg/day [7-8].

Though p-Toluenesulfonicacid is a well known carcinogen, this data would ascertain that the regulatory authorities may be expected to control the levels of p-Toluenesulfonicacid 1.5 ppm in the drug substance. p-Toluenesulfonic acid is used as counter-ions for basic drugs during the synthesis of the drug substance in pharmaceutical industry because of its strong acidic and hydrophilic properties [9-10] as well as the catalyst system is being an organic in character[11]. It has broad application towards, oxidative degradation [12], transesterification of an ester [13], esterification of carboxylic acid [14] and reductive amination of aldehydes and ketones [15] as well as reaction mediator [16]. The utilization of alcohols from synthetic reaction or in the salt formation steps may result in the formation of corresponding alkyl
tosylates, which are potential and known to be genotoxic impurities [17]. A method capable of such a lower level of detection is great challenge for analytical method development for controlling these genotoxic impurities. It was deemed necessary to develop an assay method for simultaneous quantification of p-Toluenesulfonicacid and acyclovir in API by LC-MS/MS with negative mode. This liquid chromatography method was developed and validated for use in bioavailability and bioequivalence studies. Some of the analytical methods have been reported for acyclovir such as fluorescence [18-20] direct UV [21-24] and HPLC–MS [25]. In that numerous HPLC methods have been reported for estimation of acyclovir in pharmaceutical formulations has been reported [26-34]. Among chromatographic techniques; the reversed-phase (RP) HPLC was widely used for the analysis. Present study involves development of LC-MS/MS method using simple mobile phase which was sensitive and rapid for quantification of acyclovir in impurity as well as subsequent validation of developed method according to ICH guide lines [35]. To the best of our knowledge no published method is available for the simultaneous determination of p-Toluenesulfonicacid and acyclovir API using LC-MS/MS. This method provides high degree of precision, accuracy, sensitivity and stability by simple liquid – liquid extraction based on liquid chromatography separation and detection in negative mode by electro-spray tandem mass spectrometry.

The present study was undertaken to develop a sensitive and rapid LC/MS/MS method for the determination of p-Toluenesulfonicacid in acyclovir API. Due to its higher selectivity and sensitivity LC/MS/MS has been adopted for quantification of p-Toluenesulfonicacid in acyclovir is a potent anti-viral agent, which is useful in the treatment of Herpes Simplex Virus (HSV) infections.

II. MATERIALS AND METHODS

MATERIALS:
Methanol of HPLC grade was purchased from Merck (Mumbai, India). Analytical grade ammonium acetate, HPLC grade water were purchased from Merck, (Mumbai, India). Water used for the LC-MS/MS analysis was prepared from Milli Q water purification system procured from Millipore (Bangalore, India). Reference substance of p-Toluenesulfonicacid was obtained from Sigma-Aldrich (St. Louis, USA).

Preparation of Stock and Standard Solutions:
Primary stock solutions of p-Toluenesulfonicacid was prepared in 10mg/mL of impurities in 100ml of diluent. Further dilution 0.001mg/mL with diluents further achieved on 0.000015mg/mL with diluent. Diluted final concentration 1.5 ppm to get working solutions for obtaining calibration curve.

METHODS:
Method development and optimization
Optimization of chromatographic conditions was performed, particularly the composition of mobile phase, through several trials to achieve symmetric peak shapes of the analytes peaks, as well as short run time and low cost. Resolution negative mode acyclovir was achieved by using methanol as an organic content in the mobile phase. Separation was attempted using various combinations of methanol and buffer with varying contents of each component on different columns like C18 and C8 of different makes like Hypersil BDS column and Symmetry columns. Finally Symmetry column was found to give the best chromatographic resolution with a flow rate of 0.4 mL/min and total run time of 8 min. The p-Toluenesulfonicacid and acyclovir were eluted at 4.06 and 2.10 min with selected ion monitoring (SIM) mode. The inclusion of 5mM ammonium acetate instead of pure water enhanced the response and improved the reproducibility.

III. INSTRUMENTATION

HPLC operating conditions:
A Shimadzu LC-20 AD Series HPLC system (Shimadzu Corporation, Kyoto, Japan) was used to inject 15μL aliquots of the processed samples on a Symmetry C-18 (150 X 4.6mm, 3.5μm), which was kept at 30±2°C temperature. The mobile phase, a mixture of 5mM ammoniumacetate and Methanol (70:30 v/v) was filtered through a 0.45μm membrane filter (Millipore, USA or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 0.4 mL/min into the mass spectrometer electrospray ionization chamber.
Mass spectrometry operating conditions:
Quantitation was achieved with MS-MS detection using an Applied bio system (AB SCIEX) API-4000 mass spectrometer (Foster City, CA, USA) equipped with Turboionspray™ interface at 400ºC. The MS/MS method consists of negative mode. The ion spray voltage was set at −4500 V. The source parameters viz., the ion source gases GS1, GS2 and Nebulizer gas were set at 35, 30, and 13 psi respectively. The compound parameter viz. the declustering potential (DP) and entrance potential were set at -53 and -10 V. The selected ion monitoring (SIM) mode was used as MS method for quantification of p-Toluenesulfonicacid in acyclovir drug substance. In this method p-Toluenesulfonicacid was monitored with its molecular ion [M-H]⁺ m/z 171.2 (deprotonated) and acyclovir was monitored with its molecular ion [M-H]⁺ m/z 224.2 (deprotonated). The analytical data obtained were processed by Analyst software™ (version 1.5.1).

IV. RESULTS AND DISCUSSION

METHOD VALIDATION

Specificity and selectivity
Specificity is the ability of the method to assess unequivocally the analyte response in presence of components that may be expected to be present in the sample. Acyclovir and p-Toluenesulfonicacid compounds solutions were prepared individually at a concentration of about 0.01 mg/mL in the diluents and a solution of acyclovir spiked with p-Toluenesulfonicacid were also prepared. Specificity was established by injecting acyclovir spiked with its impurity where in no interference was observed. Blank and specificity chromatograms are shown in Fig.1(a), (b).

Fig.1. (a) Specificity chromatogram of p-Toluenesulfonicacid
Robustness
The robustness of the developed method was studied with slight and deliberate changes in experimental conditions. The effect of changes in flow rate of mobile phase (-2% to +2%) while the amounts of the other mobile phase components were held constant, column oven temperature (-2°C to +2°C). i.e at 28°C and 32°C buffer units was studied.

Determination of LOD and LOQ:
The LOD and LOQ, as a measure of method sensitivity, were calculated from S/N (signal to noise) ratios. To determine LOD and LOQ values for a p-Toluene sulfonic acid concentration were reduced sequentially such that they yield S/N ratio as 3.2 and 10.1 respectively. The determined LOD and LOQ chromatograms were shown in Fig.2(a),(b). Data generated from six injections of (without API) containing 1.5 ppm of each p-Toluene sulfonic acid with respect to an API sample concentration 10 mg/mL. The LOQ of 1.5 ppm is typical for the p-Toluene sulfonic acid, with a LOD approximately three times less than LOQ. In addition, the relative efficiency of SIM modes in sensitivity improvement was also evaluated.

Fig.1. (b) Blank chromatogram of p-Toluenesulfonicacid

Fig.2. (a) LOD chromatogram of p-Toluenesulfonicacid
Recovery studies:
The recovery studies by the standard addition method were performed to evaluate accuracy and specificity, accordingly the accuracy of the method was determined in triplicate at LOQ level in bulk drug sample. The recoveries were calculated. Excellent recovery values of p-Toluenesulfonicacid 98.56 - 99.35 percentage were obtained. At such a low levels these recoveries and %RSD is <1.0 was satisfactory. Sample and accuracy at LOQ chromatograms are shown in Fig.3(a),(b), and the relative standard deviation, %RSD were calculated from the average of triplicate analysis, which were shown in Table1. Further, the stability of p-Toluenesulfonicacid was found as 48 hr and the stability of this impurity at different time intervals is presented in Table 2.

Fig.2. (b) LOQ chromatogram of p-Toluenesulfonicacid

Fig.3. (a) Sample chromatogram of p-Toluenesulfonicacid
Fig. 3. (b) Spiked chromatogram of p-Toluenesulfonic acid

Table 1
Accuracy/recovery of p-Toluenesulfonic acid

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound Name</th>
<th>Sample Area</th>
<th>Level</th>
<th>Standard Area</th>
<th>Spiked area</th>
<th>Theoretical concentration</th>
<th>Measured concentration</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>p-Toluene sulfonicacid</td>
<td>LOQ</td>
<td>0</td>
<td>206377</td>
<td>203415</td>
<td>1.5</td>
<td>1.4785</td>
<td>98.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50%</td>
<td>0</td>
<td>614510</td>
<td>609978</td>
<td>4.5</td>
<td>4.4668</td>
<td>99.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100%</td>
<td>0</td>
<td>1245120</td>
<td>1234100</td>
<td>9</td>
<td>8.9203</td>
<td>99.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>150%</td>
<td>0</td>
<td>1842122</td>
<td>1830124</td>
<td>13.5</td>
<td>13.4121</td>
<td>99.35</td>
</tr>
</tbody>
</table>

Table 2
Solution stability data of p-Toluenesulfonic acid in diluent

<table>
<thead>
<tr>
<th>S.No</th>
<th>Compound Name</th>
<th>Injection time(hr)</th>
<th>Sample area</th>
<th>Standard area</th>
<th>Spiked area</th>
<th>Theoretical concentration</th>
<th>Measured concentration</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>p-Toluene sulfonicacid</td>
<td>0</td>
<td>0</td>
<td>206377</td>
<td>203415</td>
<td>1.5</td>
<td>1.4785</td>
<td>98.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0</td>
<td>201300</td>
<td>200450</td>
<td>1.5</td>
<td>1.4937</td>
<td>99.58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0</td>
<td>224578</td>
<td>224150</td>
<td>1.5</td>
<td>1.4971</td>
<td>99.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>0</td>
<td>240512</td>
<td>238941</td>
<td>1.5</td>
<td>1.4902</td>
<td>99.35</td>
</tr>
</tbody>
</table>
Linearity and range:
The linearity test for the method was performed according to the guidelines laid by ICH. This method was evaluated at six different concentrations of analytes with in the range of 1.5 – 13.5 ng/mL. These standard solutions were prepared by suitable dilution of stock solution with mobile phase. The linearity of the plot was evaluated using least squares linear regression analysis by selected ion monitoring (SIM). The linearity of p-Toluenesulfonicacid was satisfactorily established with a six point calibration curve between LOQ to 150% of analyte concentrations (40%, 60%, 80%, 100%, 120% and 150%). The calibration curve was produced by plotting the average of triplicate of p-Toluenesulfonicacid injections against the concentrations expressed in percentage. The slope, intercept and correlation coefficient values were derived from linear least-square regression analysis and the data were presented in Table 2. It reveals that good correlation existed between the peak areas concentration of p-Toluenesulfonicacid. Repeatability was checked by calculating the relative standard deviation (%RSD) of six determinations by injecting six freshly prepared solutions containing and 1.5 ppm of p-Toluenesulfonicacid on the same day. The low %RSD values confirm the good precision of the developed method. Table 3.

Table 3
Linearity plot of p-Toluenesulfonicacid the concentration range of 1.5- 13.5 ppm level.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Peak Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>208241</td>
</tr>
<tr>
<td>3.6</td>
<td>475713</td>
</tr>
<tr>
<td>5.4</td>
<td>745610</td>
</tr>
<tr>
<td>7.2</td>
<td>984563</td>
</tr>
<tr>
<td>9</td>
<td>1245121</td>
</tr>
<tr>
<td>10.8</td>
<td>1478541</td>
</tr>
<tr>
<td>13.5</td>
<td>1804510</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.9995</td>
</tr>
<tr>
<td>Slope</td>
<td>134850.1</td>
</tr>
<tr>
<td>Intercept</td>
<td>9277.882</td>
</tr>
</tbody>
</table>
The present development study is based on validation of a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantification of p-Toluenesulfonic acid in APIs. It has been established that it is highly sensitive with a limit of detection (LOD) of 0.5 ppm Trace level ammonium acetate is added to the mobile phase to enhance ionization and detection. Selected sample solvents were assessed for the effect on standard stability with and without presence of API. As a systematic approach, it is very important to utilize the comprehensive chromatographic knowledge gained throughout the lifecycle of the development of a drug candidate based on continuous understanding of the API manufacturing process. The method which is able to quantify them at ppm level is developed and validated. We can conclude that the developed method could be very useful for monitoring of p-Toluenesulfonic acid in acyclovir in its pure and tablet form.

V. CONCLUSION

The present development study is based on validation of a highly sensitive, specific, reproducible and high-throughput LC-MS/MS method to quantification of p-Toluenesulfonic acid in APIs. It has been established that it is highly sensitive with a limit of detection (LOD) of 0.5 ppm Trace level ammonium acetate is added to the mobile phase to enhance ionization and detection. Selected sample solvents were assessed for the effect on standard stability with and without presence of API. As a systematic approach, it is very important to utilize the comprehensive chromatographic knowledge gained throughout the lifecycle of the development of a drug candidate based on continuous understanding of the API manufacturing process. The method which is able to quantify them at ppm level is developed and validated. We can conclude that the developed method could be very useful for monitoring of p-Toluenesulfonic acid in acyclovir in its pure and tablet form.

REFERENCES

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 4, Issue 3, March 2015