An Orthogonal Approach for Method development, Validation and Trace Level Quantification of 2,5-Diamino-4,6-dichloro Pyrimidine Impurity in Abacavir Sulfate by LC-MS/MS and GC-MS

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The trace level quantification of 2,5-diamino-4,6-dichloro pyrimidine (DADCP) present in abacavir sulfate by LC-MS/MS and GC-MS has been proposed. LC-MS/MS method was established for 2.5 ppm with respect to test concentration (10 mg/mL), LOQ and LOD were established as 1.3 and 0.4 ppm, respectively. Linearity, method precision, accuracy at LOQ, 100 %, 150 %, solution, mobile phase stability and robustness parameters were studied against the ICH Q2(R1) guideline. Similarly, the GC-MS method was established for 2.5 ppm of 2,5-diamino-4,6-dichloro pyrimidine with respect to test concentration (50 mg/mL), LOQ and LOD were established as 1.1 and 0.4 ppm, respectively. Linearity, method precision, accuracy at LOQ, 100 %, 150 %, solution stability and robustness parameters were studied against the ICH Q2 (R1). No analytical method was found in trace level quantification of 2,5-diamino-4,6-dichloro pyrimidine in abacavir sulfate. The orthogonal method of LC-MS/MS and GC-MS was well robust and can be used for determination of trace level of 2,5-diamino-4,6-dichloro pyrimidine in different pharmaceutical compounds.

Keywords: Quantification, 2,5-Diamino-4,6-dichloro pyrimidine, Abacavir sulfate, LC-MS/MS, GC-MS.

INTRODUCTION

Abacavir (ABC) is an antiretroviral medication used to prevent and treat HIV/AIDS [1]. It is a synthetic carbocyclic nucleoside and is the enantiomer with 1S, 4R absolute configuration on the cyclopentene ring. Vivo, abacavir sulfate dissociates to its free base abacavir [2]. The administration of abacavir at 600 mg once daily and 300 mg twice daily in human immunodeficiency virus-infected subjects [3]. And abacavir prevents to halt the inroads of the human immunodeficiency virus (HIV). Without treatment, HIV gradually undermines the body's immune system, 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 an 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 [4]. DADCP impurity

is the structural alert for the potential impurity in the abacavir sulfate and it needs to be control less than the 2.5 ppm in the final drug substance and drug product. But so far no method was provided for low quantification of DADCP potential impurity.

Many analytical methods were developed to determine the drug substance and drug product of abacavir sulfate and its process and degradation impurities like the UPLC method for determination of abacavir sulfate its related substances and degradation products [5]. HPLC method for assay of abacavir sulfate, determination of abacavir sulfate, its related substances and degradation products are available in literature. Stereo selectivity of abacavir sulfate [6], determination of abacavir in plasma, simultaneous determination with other antiretroviral products, human serum and biological matrices [7-14]. Electrochemical method for determination of abacavir sulfate [15], mass spectrometric method for determination of abacavir sulphate, its related substance and degradation products and its characterization are known [16-18].

Objective of this present work is to quantify the trace level of 2,5-diamino-4,6-dichloro pyrimidine (DADCP) in abacavir sulfate by using LC-MS/MS and GC-MS in the way

of orthogonal method. 2,5-Diamino-4,6-dichloro pyrimidine is the impurity [19,20,22] which is part of synthetic process in abacavir sulphate, no method was available for trace level quantification of 2,5-diamino-4,6-dichloro pyrimidine (DADCP) in abacavir sulphate. LC-MS/MS method and GC-MS methods are specific to all process and degradation impurities.

EXPERIMENTAL

Abacavir sulfate (99.9 % w/w) and 2,5-diamino-4,6-dichloro pyrimidine (DADCP) (98.0 %) were provided by the process research department of Custom Pharmaceutical Services of Dr. Reddy's laboratories limited, Hyderabad, India. HPLC-grade of Acetonitrile Rankem (Mumbai, India) used for the studies. Millipore Milli Q plus (Bangalore, India) purification system was used to prepare high pure water.

LC-MS/MS: Method development of impurity in abacavir sulfate was done by using Agilent 1100 series LC system with UV detector and connected Agilent MS 6410 QqQ instrument equipped with ESI source and triple quadrupole analyzer (City, CA USA), software used to data analysis is Mass Hunter. MS/MS was done by using nitrogen gas collision gas. By doing DADCP fragmentation, MRM pair was selected.

GC-MS: Methods development of impurity present in abacavir sulfate was done by using Agilent 7890A Gas chromatograph equipped with 5975C mass selective detector. EI source with single quadrupole used to do quantification and selected ion monitoring used for the selection of ions and quantification of impurity. The software used to evaluate the data is Mass Hunter quantification software with 21 CFR part 11 compliance.

Standard solution preparation for LC-MS/MS and GC-MS: In LC-MS/MS, the standard solution were prepared by weighing 10 mg of DADCP and transferred into a 100 mL volumetric flask, dissolved in 10 mL of acetonitrile: water (50:50 % v/v) and made up to the mark with diluent for stock solution-1. Transferred 0.5 mL of stock solution-1 into a 50 mL volumetric flask and made upto the mark with diluent. Transferred 0.33, 0.48, 0.62, 0.95 and 1.25 mL of stock solution-2 into five different 25 mL volumetric flask to prepare 1.3, 1.9, 2.5, 3.8 and 5. 0 ppm of standard solutions with respect to analyte concentration (10 mg/mL). Standard solutions were using for calibration curve method to determine the content of the DADCP in the abacavir sulfate. In GC-MS, the standard solution were prepared by weighing 10 mg of DADCP and transferred in a 100 mL volumetric flask, dissolved by 10 mL of acetonitrile and made up to the mark with diluent for stock solution-1. Transferred 1 mL of stock solution-1 into a 100 mL volumetric flask and made upto the mark with diluent. Transferred 1.5, 2.2, 3.1, 4.5l and 6.0 mL of stock solution-2 into five different 25 mL volumetric flasks to prepare 1.3, 1.9, 2.5, 3.8 and 5.0 ppm of standard solutions with respect to analyte concentration (50 mg/mL). Standard solutions were using for calibration curve method to determine the content of the DADCP in the abacavir sulfate by LC-MS/MS and GC-MS.

The following formula used for calculation of DADCP content.

Content of DADCP in ppm = (Y – C/m) * (weight taken/actual weight), where, Y = area of test sample, C = intercept,

m = slope, Wt. = weight of test sample taken in mg or By using the mass hunter quantitative analysis software and plot the linearity curve by taking concentration on X-axis and area on Y-axis, software will be reported the content of the DADCP present in abacavir sulfate sample.

Sample preparation: Sample preparation was simple and easy procedure for LC-MS/MS and GC-MS. In LC-MS/MS. The sample have been prepared by taking 100 mg of abacavir sulfate sample weighed and transferred into 10 mL volumetric flask and dissolved in 3 mL of 50:50 mixture of acetonitrile and water and made up to the mark with diluent and mix well. This solution was directly used for analysis. Similarly in GC-MS the sample has been prepared by taking 500 mg of abacavir sulfate, sample was weighed and transferred into 10 mL volumetric flask and dissolved in 3 mL of acetonitrile. The sample was not completely soluble, mixed 10 min by using cyclomixer and filtered with 0.45 μ filter and injected into GC-MS system.

Preparation of spiked solution: Spiked solution of DADCP in abacavir sulfate were prepared by taking standard solution at LOQ, 2.5 ppm and 3.8 ppm into separate 10 mL volumetric flasks and added 100 mg of abacavir sulfate solution in method of LC-MS/MS and 500 mg abacavir sulfate in method of GC-MS, dissolved by standard solutions and made upto the mark with standard solutions.

RESULTS AND DISCUSSION

MS/MS conditions: Initial MS/MS method development was started with direct mass in the LC-MS with single quadrupole. The mass of DADCP was observed in positive mode of ionization in ESI as 179 [M+H]+ with dichloro pattern after subtracting background of the system in scan mode *i.e.*, 100 a.m.u. to 1600 a.m.u [Fig. 1(A)]. The base peak mass is molecular ion of DADCP. 179 [M+H]+ is the precursor ion for the MS/MS study. The collision energy at 10 eV product ion of 179 [M+H]+ was 143.10 *m/z* stable fragment [Fig. 1(B)]. But still 179 [M+H]+ was not vanished at 35 eV also and at 50 eV product ion was the 89 which was stable ion and the 179 [M+H]+ was vanished [Fig. 1(C)]. Similarly the abacavir sulfate was injected into Q1, the 287 [M+H]+ mass observed [Fig. 1(D)]. No such fragments found as like DADCP in abacavir sulfate. Fragmentor for all this study was 135, temperature was 350 °C, gas flow was 10 L/min and the capillary voltage for the study was 4000 volts, EMV played critical role in low level detection of DADCP at 300 volts of EMV, it was detected.

LC conditions: Initial LC method development was started with Zorbax C18, 150 mm.4.6 mm and 3.5 μm size columns the separation of DADCP and abacavir were closely eluted. For better separation and peak sharpness YMC pack pro C18 150 mm × 4.6 mm and 3.0 μm column was used, in order to get the short method for quantification. The less particle size column was taken and both DADCP and abacavir are polar should be separated. The ratio of organic and aqueous also having effect on the separation of DADCP and abacavir sulfate. The mobile phase chosen for this study was 0.1 % formic acid in water and acetonitrile, initial study was studied with different ratio of water and acetonitrile, at 50 % of 0.1 % formic acid in water and acetonitrile with isocratic elution the separation of
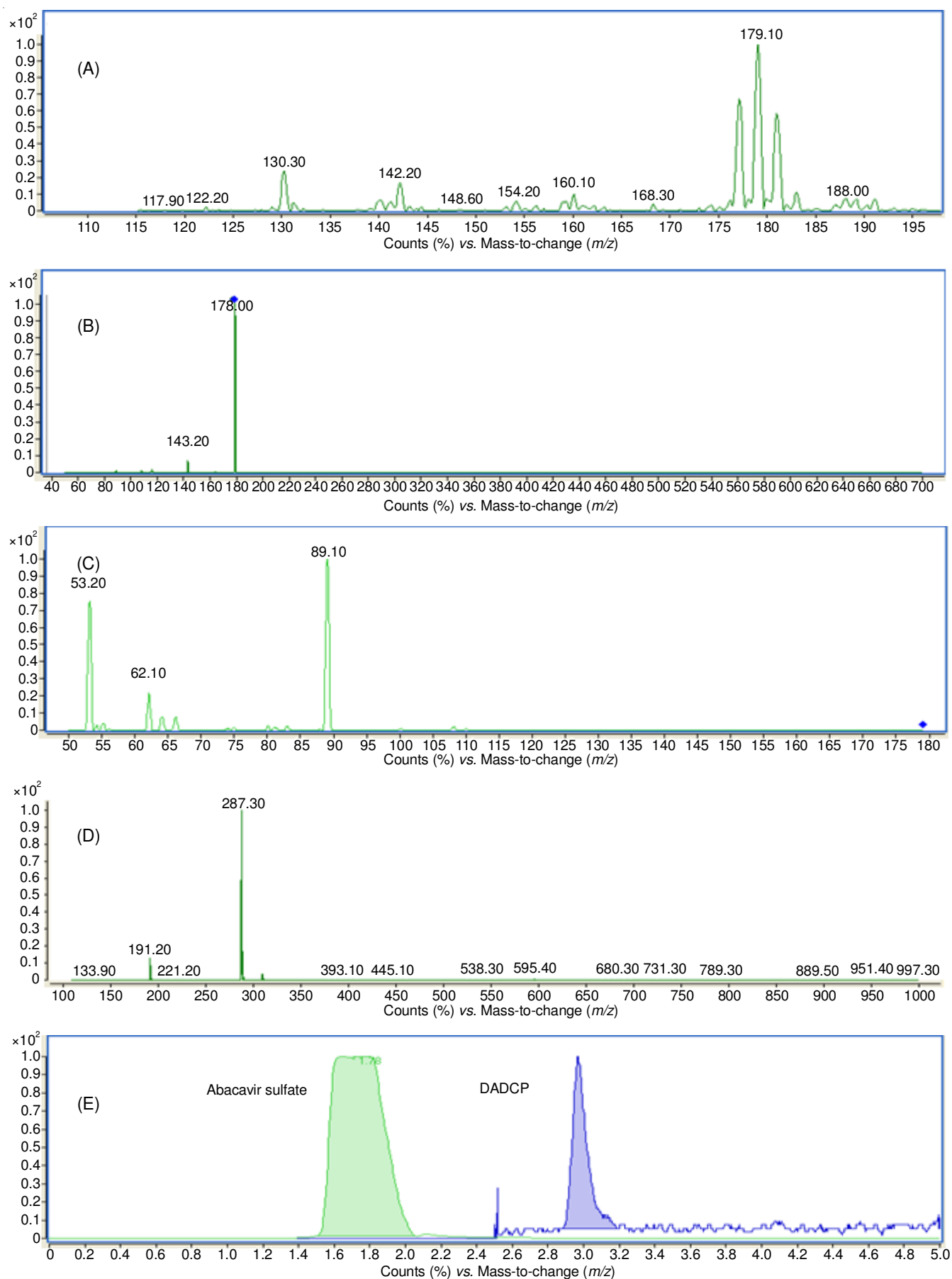
Fig. 1. (A)ESI mass spectrum of DADCP and MS/MS spectra of *m/z* 179 [M+H]⁺ ion at (B) MS/MS spectra of *m/z* 179 [M+H]⁺ ion at 10 eV (C) 50 eV for DADCP (D) ESI mass spectrum of abacavir sulfate (E) VWD chromatogram of abacavir sulfate and TIC of DADCP

DADCP and abacavir were well separated [Fig. 1(E)]. The temperature of column oven was studied but no variation it the retention time and response, by change of flow, the peak of DADCP coming close to the abacavir sulfate hence the flow was optimized to 0.7 min. Finally ambient temperature was chosen for the final quantifications.

GC-MS conditions: GC-MS method development trails have been started with solubility of abacavir sulfate drug substance, solubility with DMSO, DMA were more compare to low boilers *e.g.* methanol, acetonitrile, dichloro methane, *etc*. With respect to 50 mg/mL, the solubility of abacavir sulfate was not there in low boilers and but at lower analyte solubility, the required level response is not sufficient to show the LOQ and LOD as well as the recovery. 50 mg/mL concentration was chosen to start the development of the DADCP in abacavir sulfate. The column stationary phase have started with non-polar to mid polar such as DB-1, HP-5 and DB-1701, DADCP is mid polar hence the retention time and the peak shape was good in DB-1701 column, split ratio, injection volume, oven temperature program, injector temperature and auxiliary temperature were optimized. The mass conditions were initiated with scan mode; the ionized, abundant mass was selected for selected ion monitoring [Fig. 2(A) & (B)].

Optimization of LC-MS/MS and GC-MS method

LC-MS/MS: LC and mass conditions were optimized, the liquid chromatography optimized conditions were run time with 5.0 min, mobile phase containing 0.1 % formic acid in 50:50 v/v % acetonitrile: water, flow was 0.8 mL/min, load of injection was 10 µL, column was YMC Pack pro C18 150 mm × 4.6 mm, 3.0 µm, column temperature was 25 °C, concentration of analyte for response of impurity was 10 mg/mL. MS/MS spectrometry condition were optimized and MS/MS optimized condition were capillary voltage as 4000 volts, source temperature was 300 temperature, gas flow 10L/h and fragmentor 135 [Fig. 2(C)].

GC-MS: Gas chromatography and mass spectrometric conditions were optimized to reach the 2.5 ppm as specification with respect to API concentration 50 mg/mL. The simple gas chromatographic conditions were optimized with DB-1701 column as with 30 m × 250 µm × 0.25 µm and injection volume as 2 µL, Helium flow was 1.2 mL/min, split ratio was 5:1, injector temperature was 250 °C, auxiliary temperature was 265 °C, oven temperature program was 150 °C for 0 min hold then 5 °C/min ramp to 250 °C for 2 min hold, the total run time was 22 min according to oven program. The optimized EI-MS conditions were selected, mass for the selected ion monitoring was 142.9, 177.9, 179.9, the mass selected mass abundant mass gave enough response to quantify the impurity of its limit (2.5 ppm), the solvent delay was 6.8 min, gain factor was 1.0, EMV was 1541, timed events off was 9.0 min, resolution was low mode, the tune file was auto tune and the Column#2 used to connect mass and gas chromatography with 1 m × 180 µm × 0 µm because no stationary for this column, this is working like as interface between gas chromatography and mass spectrometry [Fig. 2(D)].

Validation

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix as per ICHQ2 (R1) [21], *etc*. Specificity in mass spectrometric methods, the impurity is main focused component and the impurity should be specified from the other impurities and active pharmaceutical ingredients. The specificity study was done by spiking the DADCP in abacavir sulfate and injected in LC-MS as well as in GC-MS; no other mass number was interfered with the DADCP. In the LC-MS method of abacavir sulfate peak was eluted before the DADCP hence the abacavir sulfate eluted run time kept in the waste by mass parameter, but in GC-MS the peak was not eluted because the boiling point of abacavir sulfate was more than 250 °C, it is not enter the gas chromatography by injector. Hence, both methods were specifying with active pharmaceutical ingredients and other impurities.

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 (amount) of analyte in the sample. Mass detectors are near linear detector hence the range of solutions were kept from 1.3-5.0 µg/mL for both LC-MS/MS and GC-MS methods and the correlation coefficient were found to be 0.997 and 0.998, respectively (Table-1).

<table>
<tr><td colspan="3">TABLE-1
LC-MS/MS AND GC-MS VALIDATION
RESULTS OF DADCP IN ABACAVIR SULFATE</td></tr>
<tr><td>Parameter</td><td>LC-MS/MS</td><td>GC-MS</td></tr>
<tr><td>LOQ in ppm w.r.t. analyte concentration</td><td>1.3</td><td>1.1</td></tr>
<tr><td>LOD in ppm w.r.t. analyte concentration</td><td>0.4</td><td>0.4</td></tr>
<tr><td>Slope (m)</td><td>112.4</td><td>3448</td></tr>
<tr><td>Intercept (c)</td><td>38.1</td><td>731</td></tr>
<tr><td>Correlation coefficient(r)</td><td>0.997</td><td>0.998</td></tr>
<tr><td>Method precision (% RSD)</td><td>2.8</td><td>6.3</td></tr>
<tr><td>LOQ precision (% RSD)</td><td>4.3</td><td>4.4</td></tr>
<tr><td>Intermediate precision (% RSD)</td><td>1.3</td><td>3.3</td></tr>
<tr><td>Accuracy at 1.3 ppm (% Recovery)</td><td>100.1</td><td>109.5</td></tr>
<tr><td>Accuracy at 2.5 ppm (% Recovery)</td><td>95.2</td><td>107.2</td></tr>
<tr><td>Accuracy at 3.8 ppm (% Recovery)</td><td>101.4</td><td>112.7</td></tr>
<tr><td>Solution stability and mobile phase difference with respect to initial (% variation)</td><td>0.4</td><td>0.1</td></tr>
<tr><td colspan="3">w.r.t. = with respect to test sample</td></tr>
</table>

Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Method precision and intermediate precision were established and method precision results were meeting the ICH Q2 R1 guidelines and % RSD of the DADCP in abacavir sulfate was found to be 2.8 % at 2.5 ppm by LC-MS/MS and 6.3 % at 2.5 ppm by GC-MS. The intermediate precision was done by different day, different column, different analyst and different lots of chemicals. The % RSD were found to be 1.3 and 3.0 by LC-MS and GC-MS, respectively (Table-1).

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. Accuracy of DADCP impurity in abacavir sulfate at 2.5 ppm and 3.8 ppm were studied. The % recovery at 2.5 ppm was found to be 95.2 and
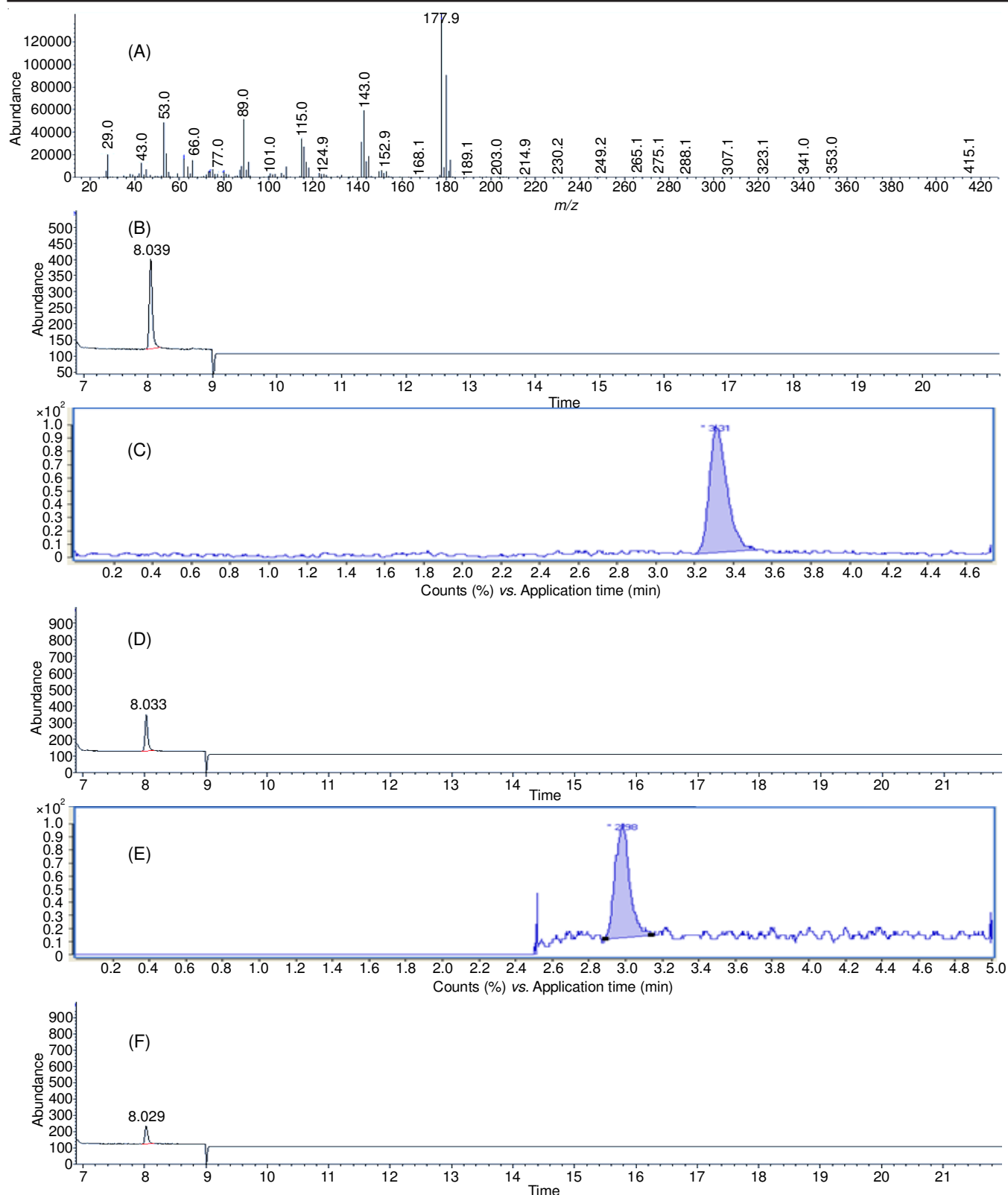
Fig. 2. (A) Mass spectrum of DADCP by GC-MS (B) GC-MS Total ion chromatogram (C) 2.5 ppm Total ion chromatogram of LC-MS/MS (D) 2.5 ppm Total ion chromatogram of GC-MS (E) LOQ Total ion chromatogram of LC-MS/MS (F) LOQ Total ion chromatogram of GC-MS

107.2 % in LC-MS/MS and GC-MS, respectively. The % recovery at 3.8 ppm was 101.4 and 112.7 % in LC-MS/MS and GC-MS, respectively (Table-1).

Limit of quantitation, limit of detection, precision and accuracy at limit of quantitation: The quantitation limit of

an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices. The LOQ and LOD were establishment

by using STYEX method. The linearity solution were kept for the establishment for LOQ and LOD from 1.3 ppm to 5.0 ppm.

The LOQ values were found to be 1.3 ppm and 1.1 ppm in LC-MS/MS and GC-MS, respectively (Table-1). LOQ precision was studied and the precision limits were within the limits as per ICH Q2 R1. The %RSD of LOQ precision of DADCP in abacavir sulfate by LC-MS and GC-MS were 4.3 and 4.4 %, respectively, The LOD values were three times less than the LOQ values 0.4 ppm was LOD in the LC-MS/MS and GC-MS [Fig. 2(E) & (F)].

Solution stability and mobile phase stability: Solution stability and mobile phase stability were studied for the DADCP in presence of the abacavir sulfate. The DADCP as 2.5 ppm was spiked in abacavir sulfate and injected into LC-MS/MS with interval of 12 h and obtained 100 % recovery of content of DADCP in the abacavir sulfate and with same mobile phase 100 % recovery of content of DADCP in the abacavir sulfate, similarly 100 % recovery of DADCP content in the abacavir sulfate by GC-MS in acetonitrile diluent.

Robustness: The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. In LC-MS/MS. the flow, temperature, mobile phase variation have been done and linear robust parameters applied to the method, its robust with different temperature and linear robust with flow, variation in mobile phase concentration and MRM pair. In GC-MS, the method was robust by change in flow and column with different equivalents.

Conclusion

An orthogonal approach for LC-MS/MS and GC-MS quantitative method was developed for quantitation of DADCP impurity in abacavir sulfate at 2.5 ppm level. Both liquids and gas chromatography with mass detection were used to detect the trace level quantification and method was specific towards the process impurities and volatile solvents, so far no orthogonal method was used for the determination of the DADCP impurity in abacavir sulfate active pharmaceutical ingredient and other pharmaceutical compounds. Availability of any one of these two instruments in GMP lab, the DADCP can be analyzed. All validation parameters such as system suitability, linearity, LOQ, LOD, method precision, intermediate precision, accuracy, solution stability, mobile phase stability and robustness met the predefined acceptance criteria as per ICH Q2 (R1).

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