Asian Journal of Chemistry; Vol. 34, No. 10 (2022), 2583-2586

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2022.23910

Ultra Trace Level Detection and Quantification of Identified Genotoxic Impurity Ethyl (1R,5R,6R)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate in Oseltamivir Phosphate Drug Substance by Liquid Chromatography-Mass Spectroscopy

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Received: 12 May 2022; *Accepted*: 14 June 2022; *Published online*: 19 September 2022; AJC-20959

A new rapid run time LC-MS method was developed and validated for detection (0.03 ppm) and quantification (0.1 ppm) in ultra-trace level of genotoxic impurity (GTI) ethyl-(1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate in oseltamivir phosphate active pharmaceutical ingredient (API). The method is price effective, time redeemable and capable to confirm the parent and daughter ion masses through mass spectrometry and tandem mass spectrometry for further fragmentation. An isocratic program and YMC Pack Pro C4 reverse phase column (150 mm × 4.6 mm × 3.0 µm) was used to achieve separation between oseltamivir phosphate and ethyl-(1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate impurity. Mobile phase-A used was 0.01 M ammonium acetate in water and mobile phase-B used was acetonitrile in the ration of 40:60 v/v. Diluent was used methanol. The chromatographic conditions were used, injection volume: 20 µL, flow rate: 1.0 mL/min, oven temperature: 50 ºC, auto sampler: 5 °C and run time 8.0 min. The detection and quantification levels found at 0.03 and 0.1 ppm, respectively. Ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate impurity is linear from 0.1 to 15 ppm levels with regression coefficient 0.9994. The recoveries were found in the range of 93.8% to 105.0%.

Keywords: Oseltamivir phosphate, Multiple reaction monitoring, LC-MS/MS, Genotoxic impurity.

INTRODUCTION

Oseltamivir phosphate (Fig. 1a) is an antiviral neuraminidase inhibitor and used for the treatment and prevention of influenza A and B, marketed worldwide under the brand name of Tamiflu [1]. Oseltamivir phosphate have got its antiviral action by inhibiting the activity of the viral neuraminidase enzyme found on the surface of the virus, which prevents budding from the host cell, viral reproduction and infectivity [1]. However, antiviral treatment might be beneficial after 48 h for patients with complex, severe or advanced illness or for hospitalized patients. Oseltamivir phosphate is also approved for the prevention of influenza in adults and children aged one year and older. Efficacy of oseltamivir phosphate for the prevention of influenza has not been established in immune compromised patients.

Basically, ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate (Fig. 1b) was used earlier stage for the synthesis of oseltamivir phosphate. Ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo-[4.1.0]hept-3-ene-3-carboxylate has the positive structure alert for some of the analogs, this was confirmed from MCASE and DERAK data base software. This impurity may be potential in genotoxicity, which may affect chromosomal breaks, mutation in genes and leads to leukemia (cancer) in humans. Therefore, ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate impurity is genotoxic, should be controlled in oseltamivir phosphate active pharmaceutical ingredient with the limit of 10 ppm according to maximum daily dose of 150 mg intake (twice 75 mg) per/day.

Thus, there is a significant toxicological importance to control even at low-level presence of such impurities in finished active pharmaceutical ingredient [2,3]. Thus, it is always challenge to avoid such potential genotoxic impurities during synthesis or

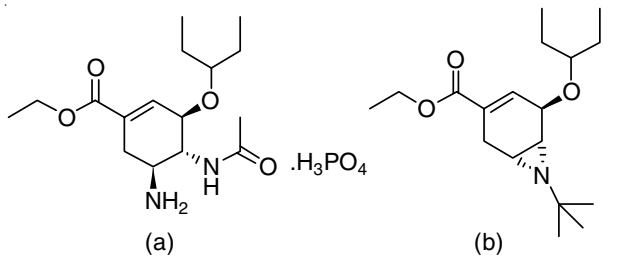

Fig. 1. Structure of oseltamivir phosphate (a) and ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate (b)

manufacturing process. In addition, it is impossible or challenging to control or avoid completely the potential genotoxic impurities during synthesis process. Thus, it is essential and important to develop an analytical method for such a low level for identification and quantification of potential genotoxic impurities, but most of the times it is not possible using regular analytical techniques namely UV, GC and HPLC techniques. Hence, a suitable LC-MS/MS instrument was selected for identification and ultra-trace level quantification of ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate genotoxic impurity due to high sensitivity and selectivity of the instrument.

The regulatory agencies US Food and Drug Administration (USFDA), European Medicines Agency (EMA) and International Council for Harmonization [ICHM7 (R1)] had come together and proposed use of a threshold of toxicological concern (TTC) value of 1.0 to 1.5 µg/day intake [4-6], also disclosed about principles and procedures for handling out-of-domain and indeterminate results as part of ICH M7 recommended (Q)SAR analyses [7]. The literature review revealed that so far there is no method reported for the identification and ultra-trace level quantification (0.1 ppm) of ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate in oseltamivir phosphate active pharmaceutical ingredients using LC-MS/MS. Nevertheless, there are few articles published for synthesis, isolation and characterization of process related impurities [8,9], RP-HPLC UV methods reported for the determination of oseltamivir phosphate in both APIs, finished dosage forms and degradation impurities [10-15]. Disclosed about quantification and validation of API and impurities by UPLC [16], organic volatile impurities by GC [17] and reported estimation of related compound-A [18] and estimation of oseltamivir and its metabolite by LC-MS [19,20]. In addition, disclosed about capillary electrophoretic and liquid chromatographic method reported for the determination of oseltamivir phosphate assay in pharmaceutical formulations [21,22]. Also discussed about proactive evaluation of possible genotoxic impurities, regulation and mitigation [23-26]. Thus, there is a superior requirement to develop a rapid, sensitive and selective LC-MS/MS method for the analysis of ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate genotoxic impurity content in oseltamivir phosphate. The developed method was validated as per ICH guidelines [27].

EXPERIMENTAL

Ammonium acetate, methanol and acetonitrile as reagents were of analytical grade and procured from Merck, India). Oseltamivir phosphate and ethyl (1R,5R,6R)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate were received from Cipla Ltd. (R&D), Bangalore, India.

The mass spectrometer system used was an Applied Biosystem Sciex (QTRAP-5500 series and Switzerland). LC was used Agilent HPLC (1260 series, Germany). Additional equipment used such as PCI sonicator (22L500/CC/DTC) and pH meter (Lab India, PICO$^+$, India).

Chromatographic and mass spectrometer parameters: The enhanced chromatographic and typical mass spectrometer parameters are shown in Table-1.

Sample and standard preparation: The concentration of oseltamivir phosphate was fixed at 2 mg/mL based on the mass detector response. The diluent used was methanol. Genotoxic impurity standard solutions were prepared with a concentration of 0.03 ppm (LOD solution), 0.1 ppm (LOQ level), 5.0 ppm (50%), 10.0 ppm (limit level), 12.5 ppm (125%) and 15.0 ppm (150%) with respect to the 2 mg/mL test concentration.

RESULTS AND DISCUSSION

Method development: Preliminarily, the trails were tried with different mobile phase extracts such as acetic acid, formic acid, ammonium formate, ammonium acetate and mixture of

TABLE-1
CHROMATOGRAPHIC AND MASS SPECTROMETER CONDITIONS

Chromatographic parameters		Mass spectrometer parameters		
		Parameter	Oseltamivir	Genotoxic impurity
Mobile phase-A	0.01M ammonium acetate in water	MRM monitoring for *m/z* transition	313.1 > 225.0	310.0 > 184.0
Mobile phase-B	Acetonitrile	Ionization mode	Positive	Positive
Flow rate	1.0 mL/min	Declustering potential (V)	52	40
Column oven temperature	50ºC	Entrance potential (V)	12	14
Injection volume	20 µL	Collision energy (V)	29	20
Sampler cooler temperature	5ºC	Collision exit potential (V)	13	25
Test concentration	2 mg/mL	Ion spray voltage (V)	5500	5500
Mode	Isocratic	Source temperature (ºC)	450	450
Diluent	Methanol	Curtain gas flow (psi)	40	40
Syringe rinse diluent	Methanol	GS1 and GS2	50	50
Run time	8 min	CAD	Medium	Medium

methanol and acetonitrile. In addition, the methods were analyzed with various stationary phase columns, which comprises C8, phenyl, C4, C18 and amino. The final upgraded chromatographic and representative mass spectrometer constraints are shown in Table-1.

Mass confirmation: Ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate impurity mass-to-charge ratio (*m/z*) 310.0 $[M+H]^+$ and oseltamivir mass-to-charge ratio (*m/z*) 313.1 were determined using LC triple quadrupole mass spectrometer. Also, the fragment ion masses is determined for both ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate impurity and oseltamivir using MS/MS further fragmentation technique. The major fragment ion masses of ethyl (1*R*,5*R*,6*R*)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate impurity is 184.0 and oseltamivir is 225.0, respectively.

Method validation: The specificity was carried out by injecting LOQ solution of oseltamivir and genotoxic impurity with respect to test sample. The retention time of oseltamivir and genotoxic impurity was observed at 1.52 and 6.81 min respectively (Fig. 1). No interference was found at the retention time of API.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated from S/N (signal to noise) ratio. The LOD and LOQ of GTI was originated with the concentration of 0.03 ppm and 0.1 ppm and S/N ratio was more than 3 and 10, respectively. The developed method was carried out linearity and concluded a concentration of five levels 0.1-15 ppm (LOQ, 50%, 100%, 125% and 150%). The X-axis was employed concentration and Y-axis was peak area used for calibration curve. The intercept, correlation coefficient and slope values was obtained over regression analysis and the data is shown in Table-2. For method precision, six separate solutions were prepared by spiking with the impurities limit level with respect to test concentration. Intermediate precision was carried out by different instrument, different day and different column. For all the overhead quantifications were calculated %RSD and observed below 5. The standard addition method was used for accuracy study. Consequently, accuracy was determined at LOQ, 50%, 100% and 150.0%. The recovery was found in the range of 93.8% to 105.0%. The developed method robustness was performed by making trivial and deliberate changes in experimental environments and mobile phase changed 0.1 units *i.e.* 1.0 to 1.1 mL/min, 1 to 0.9 mL/min for flow rate and temperature was changed by 2 ºC *i.e.* 48 ºC and 52 ºC. The % RSD values from robustness study and method precision were calculated and found to be below 10 and confirmed that the method was robust. Genotoxic impurity was quantitatively spiked at limit level concentration and stored at 5 ºC for solution stability. The standard solutions and spiked solutions were injected initially at different intervals. The sample solution and standard solution were stable up to 22 h at 5 ºC. Validation results were plotted and the values are represented in Table-2.

TABLE-2
THE SUMMARY OF ANALYTICAL VALIDATION RESULTS

Parameter	Results
LOD (0.03 ppm) (s/n)	3.9
LOQ (0.1 ppm) (s/n)	11.6
Linearity range (ppm)	0.1-15
Correlation coefficient	0.9994
Slope	884115
Intercept	35653
System precision (%RSD)	1.2
Method precision (%RSD)	0.88
Intermediate precision (%RSD)	1.46
% Recovery at LOQ (0.3 ppm)	93.8-101.7
% Recovery at 50% (5 ppm)	97.7-103.6
% Recovery at 100% (10 ppm)	96.3-103.5
% Recovery at 150% (15 ppm)	98.9-105.0

Conclusion

A validated rapid run time LC-MS method for the detection (LOD 0.03 ppm) and quantification (LOQ 0.1 ppm) of gentoxic impurity in oseltamivir phosphate is developed. The method is simple, cost-effective, precise, accurate, linear and robust with the lesser run time and sensitive and selective for the ultra-

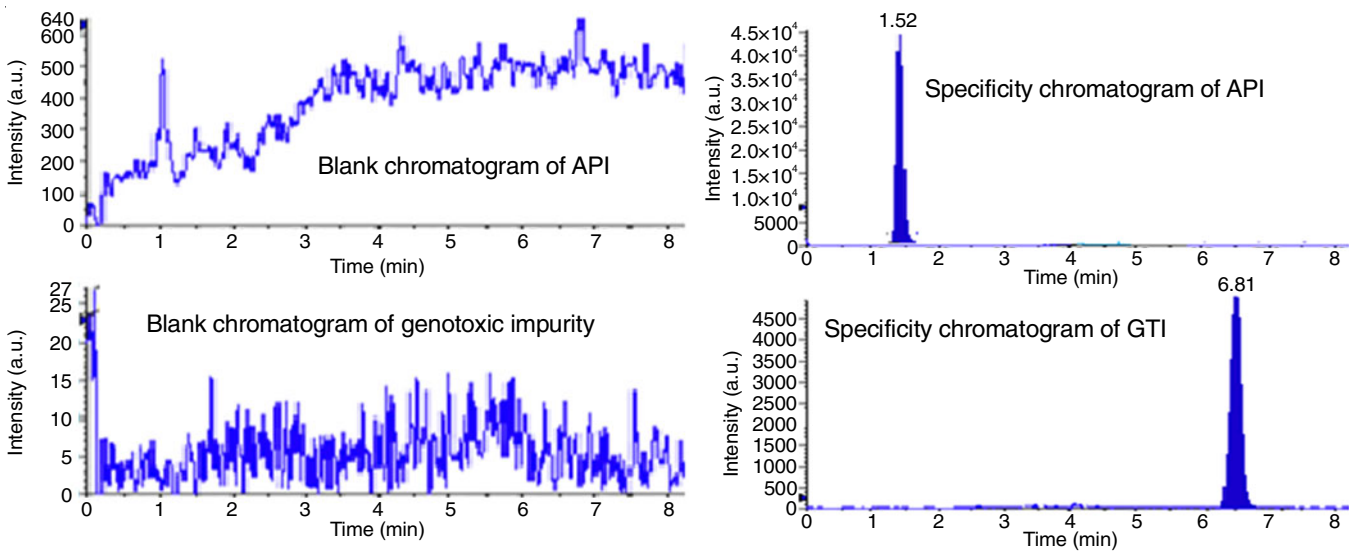

Fig. 1. Specificity chromatogram of blank, API and genotoxic impurity

trace level. More batches were analyzed using validated method for ethyl (1R,5R,6R)-7-(*tert*-butyl)-5-(pentan-3-yloxy)-7-azabicyclo[4.1.0]hept-3-ene-3-carboxylate genotoxic impurity as method applicability and impurity content was not detected. The developed method can be used for quality control, routine and stability studies in the pharmaceutical industry.

ACKNOWLEDGEMENTS

The authors are thankful to the Management of Cipla Ltd., Bangalore, India for provision to carry out the research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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