ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 14, Issue 4, 2021

Online - 2455-3891
Print - 0974-2441

Research Article

CHARACTERIZATION OF OSELTAMIVIR PHOSPHATE API AND SIMULTANEOUS QUANTIFICATION AND VALIDATION OF ITS IMPURITIES BY UPLC

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Received: 22 December 2020, Revised and Accepted: 29 January 2021

ABSTRACT

Objective: The purpose of the study is to develop a high sensitive and short runtime method to quantify oseltamivir phosphate impurities (C and D) and characterization of oseltamivir phosphate API.

Methods: The active pharmaceutical ingredient (API) characterization was done using spectroscopic techniques such as mass, infrared spectroscopy (IR), differential scanning calorimetry (DSC), proton nuclear magnetic resonance (H-NMR), phosphorus nuclear magnetic resonance (P-NMR), carbon-13 nuclear magnetic resonance (C13-NMR), and two-dimensional nuclear magnetic resonance (2D-NMR). The impurities (C and D) quantification was done using ACQUITY UPLC BEH C18- 100 mm × 2.1 mm, 1.7 µm column connected to ACQUITY UPLC with PDA detector. The optimized chromatographic conditions were achieved at 0.3 mL/min flow rate using gradient system with 0.1% orthophosphoric acid in water and acetonitrile as mobile phase. Both impurities are measured at λ_{max} 210 nm at 30°C column temperature.

Results: The finalized method has given good peak shape and resolution for impurity-C and impurity-D at Rt = 3.39 and 4.33 min, respectively, and the quantification method is linear and its r2 > 0.999 as a correlation coefficient. The recoveries of impurity-C and impurity-D were found in the range of 100±15% at 0.05, 0.1, and 0.15 and limit of quantitation (LOQ) % concentration levels. The other validation parameters such as specificity, system precision, sensitivity, method precision, ruggedness, robustness, and solution stability were established for this method, and the results are satisfactory as per International Council for Harmonization (ICHQ2).

Conclusion: The characterization data confirm the structure of oseltamivir phosphate active pharmaceutical ingredient (API). The validated method shall be used for regular analysis as well as release analysis in quality control (QC).

Keywords: Oseltamivir phosphate active pharmaceutical ingredient, Impurity-C, Impurity-D, Characterization, Method development and validation, Ultra-performance liquid chromatography.

 DOI: http://dx.doi.org/10.22159/ajpcr.2021v14i4.40595. Journal homepage: https://innovareacademics.in/journals/index.php/ajpcr

INTRODUCTION

Oseltamivir phosphate is a white crystalline solid with the chemical name (3R,4R,5S)-4-acetylamino-5-amino-3(l-ethylpropoxy)-1-cyelohexene-l-carboxylic acid ethyl ester, phosphate. The chemical formula is $C_{16}H_{28}N_2O_4$ (free base) and molecular weight of oseltamivir is 312.4 gr/mol. The chemical formula is $C_{16}H_{31}N_2O_8P$ (salt) and molecular weight of oseltamivir is 410.4 gr/mol for oseltamivir phosphate salt. The structural formula is shown in Fig. 1. Oseltamivir phosphate is an antiviral drug used in the treatment and prophylaxis of both influenza virus A and influenza virus B. It is an ethyl ester prodrug that is rapidly and extensively metabolized by esterase in the gastrointestinal tract and liver to its active form, oseltamivir carboxylate [1]. There are several process impurities/related substances associated with the manufacture of oseltamivir phosphate. Different process-related impurities are formed with various synthetic routes and manufacturing processes. We have taken two impurities for quantification by ultra-performance liquid chromatography (UPLC). Those are impurity-C and impurity-D. The structures and chemical formula are shown in Fig. 2. These two impurities quantification by ultra-performance liquid chromatography (UPLC) methods are never present together and characterization study for oseltamivir phosphate by mass, infrared spectroscopy (IR), differential scanning calorimetry (DSC), proton nuclear magnetic resonance (H-NMR), phosphorus nuclear magnetic resonance (P-NMR), carbon-13 nuclear magnetic resonance (C13-NMR), and two-dimensional nuclear magnetic resonance (2D-NMR).

Literature survey

Few chromatographic methods have been appeared in the literature. Those are development and validation of RP-HPLC method for the determination of oseltamivir phosphate in bulk drug and in dosage forms [2]. A stability indicates LC method for oseltamivir phosphate [3]. Degradation behavior of oseltamivir phosphate under various stress conditions was used stability-indicating HPLC method [4].

A literature search revealed that, nobody has reported characterization of oseltamivir phosphate active pharmaceutical ingredient (API) and quantification of its impurities by ultra-performance liquid chromatography (UPLC). The current work describes a simple and sensitive ultra-performance liquid chromatography (UPLC) method for the simultaneous quantification of two impurities (impurity-C and D) in the oseltamivir phosphate drug substance and characterization of oseltamivir phosphate by mass, infrared spectroscopy (IR), differential scanning calorimetry (DSC), proton nuclear magnetic resonance (H-NMR), phosphorus nuclear magnetic resonance (P-NMR), carbon-13 nuclear magnetic resonance (C13-NMR), and two-dimensional nuclear magnetic resonance (2D-NMR) spectral data.

MATERIALS AND METHODS

Instruments

Mass (Quattro Premier XE Micromass system with MassLynx software), Fourier transform infrared (PerkinElmer Spectrum Two

with Spectrum software), differential scanning calorimetry (DSC Q 2000 with TA Instrument explorer software), and nuclear magnetic resonance (Bruker instrument with TopSpin software) were used for characterization of oseltamivir phosphate. Waters ACQUITY ultra-performance liquid chromatography system with Empower-3 software was used for impurity-C and impurity-D quantification in oseltamivir phosphate active pharmaceutical ingredient and its pharmaceutical dosage forms.

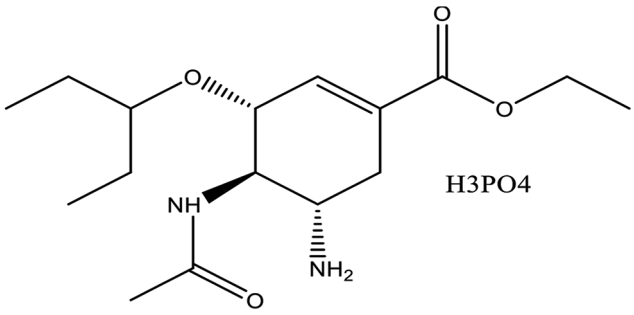

Fig. 1: Oseltamivir phosphate API

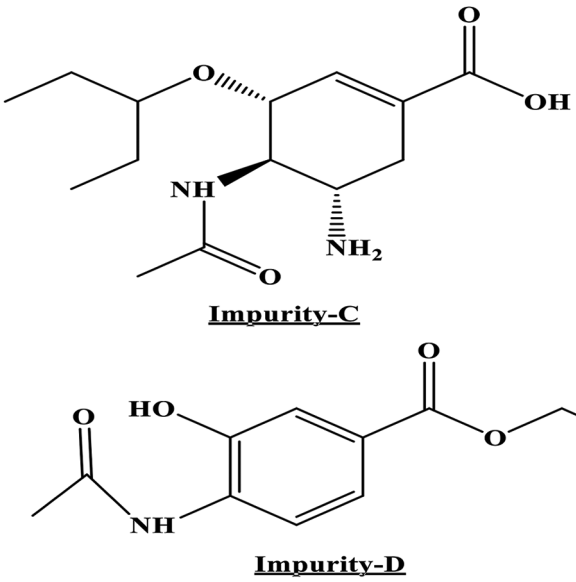

Fig. 2: Chemical structures of impurity-C and impurity-D

MATERIALS AND REAGENTS

Oseltamivir phosphate was kindly supplied by GVK Biosciences. Its purity was reported as 99.56% area. Methanol, impurity-C, and impurity-D were purchased from Sigma-Aldrich. Natflu capsules were purchased from local drugstores. The capsules contain 75.0 mg of oseltamivir phosphate, analytical reagent (AR) grade acetonitrile and methanol. EVOQUA Water Technologies produced ultrapure analytical grade water, orthophosphoric acid from Sigma-Aldrich.

Preparation of buffer solution

Transferred 1.0 mL of orthophosphoric acid in 1000 mL of water, sonicated for 2–3 min, and filtered the solution through a 0.45 µm membrane filter.

Preparation of diluent

Transferred 500 mL of water and 500 mL methanol in 1000 mL mobile phase bottle and mixed well.

METHODS

UPLC system and chromatographic conditions

All separations were performed on Waters UPLC system and operated with Empower-3 software for data acquisition and processing. The analysis was carried out by octadecylsilane column of make ACQUITY UPLC BEH C18 having dimensions 100 mm × 2.1 mm ID, 1.7 µm particle size column. The mobile phase consists of 0.1% orthophosphoric acid buffer as mobile phase-A and acetonitrile as mobile phase-B and has the flow rate as 0.3 mL/min with gradient elution mode of program: Time $_{(min)}$/A (v/v): B (v/v); T $_{0.01}$/100:0, T_6/0:100, T_8/0:100, $T_{8.1}$/100:0, and T_{10}/100:0. The column oven temperature was maintained at 30°C. Samples were monitored and detected at wavelength maxima 210 nm by injecting sample volume 1 µL and data were acquired for 10 min.

Preparation of impurity standard solution (0.1%)

Accurately weighed and transferred 50.15 mg of impurity-C and 50.19 mg of impurity-D into a 50 mL volumetric flask containing 30 mL of diluent, sonicated 1–2 min or until dissolved, and diluted to mark with diluent and mixed well. Transferred 5.0 mL of the above solution into 50 mL of volumetric flask and diluted to volume with diluent and mixed well. Further transferred 1.0 mL of above solution into 100 mL of volumetric flask and diluted to volume with diluent and mixed well.

Preparation of oseltamivir phosphate solution (1.0 mg/mL)

Accurately weighed and transferred 100.25 mg of oseltamivir phosphate into a 100 mL volumetric flask contained 70 mL of diluent, sonicated 1–2 min, and diluted to the mark with diluent and mixed well.

Preparation of oseltamivir phosphate tablet solution

Twenty tablets were taken for formulation analysis and grinded as fine powder. The amount is equivalent to 100 mg and oseltamivir phosphate

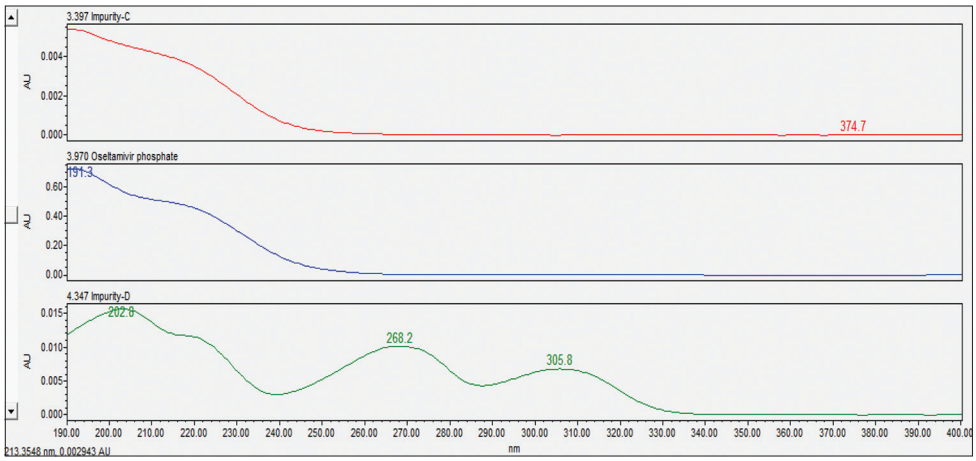

Fig. 3: PDA spectrums of impurity-C, oseltamivir phosphate, and impurity-D

was taken into 100 mL of volumetric flask, sonicated 10 min, and diluted to mark with diluent and mixed well and then filtered through 0.45-micron syringe filter.

RESULTS AND DISCUSSION

Selection of analytical wavelength

The stock solutions of oseltamivir phosphate and its impurity-C and impurity-D were separately diluted with diluent to get a concentration of 1.0 mg/mL of oseltamivir phosphate API and 0.001 mg/mL of impurity-C and impurity-D, respectively, and were scanned in the wavelength range of 200–400 nm on the ultra-performance liquid chromate (UPLC) system using Photodiode-Array (PDA) detector. Photodiode-Array (PDA) Spectrums of oseltamivir phosphate, impurity-C, and impurity-D are shown in Fig. 3. From the Photodiode-Array (PDA) Spectrums, λ max wavelengths observed are 193 nm for impurity-C, 191.3 nm for oseltamivir phosphate, and 202.9 nm for impurity-D. The best suitable wavelength is 210 nm since it has low baseline noise. Peak purity was passed for oseltamivir phosphate, impurity-C, and impurity-D. From this wavelength study, we have finalized 210 nm for my research work.

Selection of mobile phase

The aim of the study was to separate oseltamivir phosphate and its impurities C and D. Various attempts were made to separate the oseltamivir phosphate and its impurities C and D. Three types of buffers were screened as mobile phase-A. The first one is 0.1% orthophosphoric acid in water, the second one is 10mm ammonium bicarbonate in water, and the third one is 0.1% diethylamine in water. Acetonitrile solvent selected as mobile phase-B. We have done the method screening in the three buffer conditions by ACQUITY UPLC BEH C18 (100 mm × 2.1 mm) 1.7 µm column through gradient elution. In these three buffer conditions, 0.1% orthophosphoric acid in water gives us good peak shape, resolution, and tailing factor then compared to other two buffer conditions. From the above mobile phase screening study, finally, 0.1% orthophosphoric acid in water (mobile phase-A) and acetonitrile (mobile phase-B) were selected as mobile phases most viable for oseltamivir phosphate and its impurities C and D.

Characterization and interpretation of oseltamivir phosphate

Characterization of oseltamivir phosphate is very important for this study. The oseltamivir phosphate API was characterized and confirmed by mass, infrared spectroscopy (IR), differential scanning calorimetry (DSC), proton nuclear magnetic resonance (H-NMR), phosphorus nuclear magnetic resonance (P-NMR), carbon-13 nuclear magnetic resonance (C13-NMR), and two-dimensional nuclear magnetic resonance (2D-NMR).

^{1}H NMR, C^{13} NMR, P-NMR and 2D-NMR (DMSO-d_6)

Observed H-MMR delta values are δ 8.16-8.14 (d, 1H), 6.65 (br s, 1H), 4.28-4.10 (m, 4H), 4.08 (q, 1H), 3.65-3.63 (q, 1H), 3.38-3.35 (br s, 1H), 2.68 (dd, 1H), 2.51-3.49 (m, 1H), 1.87 (br s, 3H), 1.45-1.37 (m, 4H), 1.24-1.21 (t, 3H), and 0.86-0.77 (m, 6H) ppm. C^{13} NMR values

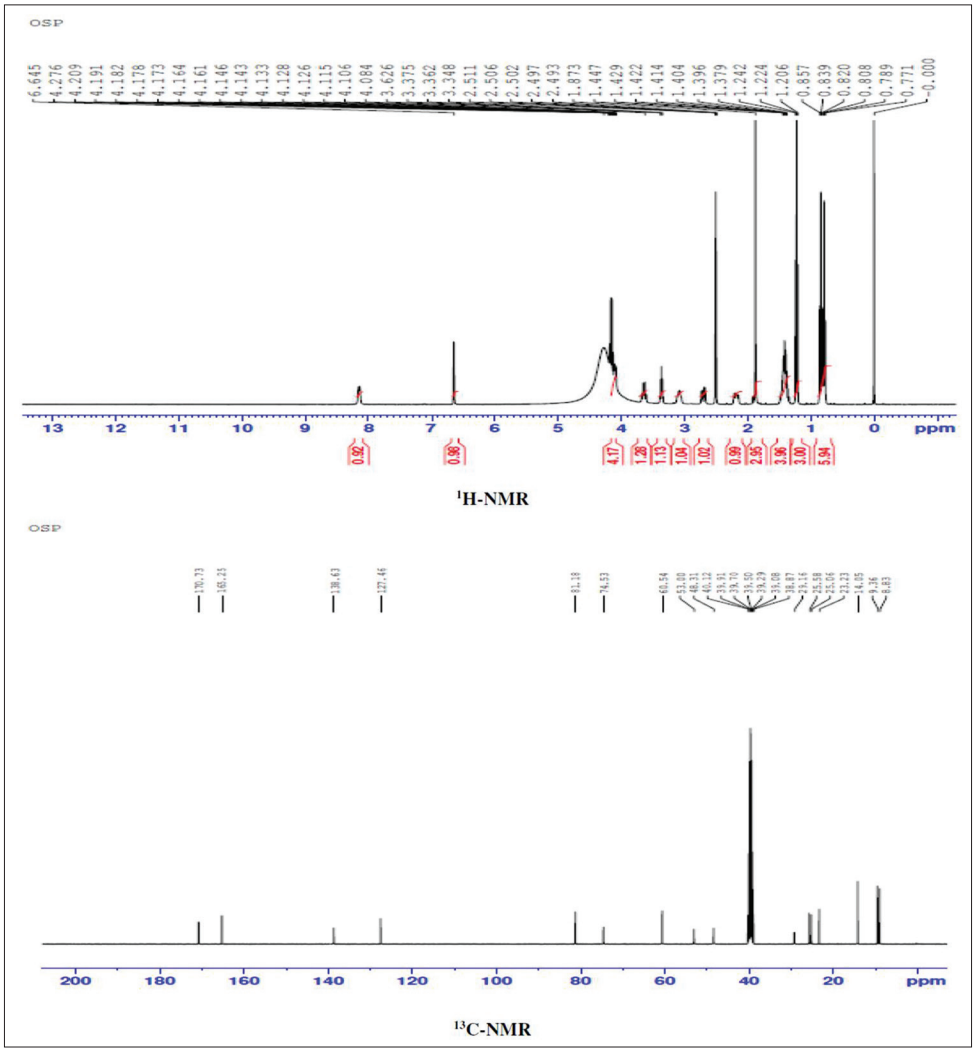

Fig. 4: ^{1}H-NMR and ^{13}C-NMR spectrums of oseltamivir phosphate

are δ 170.73, 165.25, 138.63, 127.46, 81.18, 74.53, 60.54, 53.00, 48.31, 40.12-38.87 (*J* = 125 Hz), 29.16, 25.58-25.06 (*J* = 52 Hz), 23.23, 14.05, and 9.36-8.83(*J* = 53 Hz) ppm. Phosphorus NMR value is δ 0.66 ppm. From these, NMR data support that the proposed structure is oseltamivir phosphate. The typical spectrums are shown in Figs. 4 and 5.

Characterized by Mass, IR, and DSC

Oseltamivir phosphate is confirmed by mass, IR, and DSC. We have observed m/z 313.53(M+1) for oseltamivir and m/z 96.87(M-1) for phosphate. Characteristic IR bands are 1068.44 cm^{-1} for ν (-C-O), 1661.63 cm^{-1} for ν (–C=O), 2938.80cm^{-1} for ν (–C-H), and 3352.29 cm^{-1} for ν (–NH). The melting range is confirmed by DSC, then find 205.64°C (range is 190–206°C). From these, characteristic IR frequencies support that the proposed structure is oseltamivir phosphate. The typical spectrums are shown in Figs. 6 and 7.

Method validation

The finalized method was validated as per the International Council for Harmonization (ICH) Guidelines [5-9]. The following validation parameters were evaluated: Specificity, accuracy, precision, limit of detection, limit of quantification, linearity, solution stability, ruggedness, and robustness.

Specificity

Interference of impurity-C and impurity-D from oseltamivir phosphate peak was ensured as part of specificity. Impurity-C and impurity-D eluted at 3.39 min and 4.33 min while oseltamivir phosphate eluted at 3.96 min, respectively. Based on this, the method has good selectivity by resolving impurities C and D from oseltamivir phosphate API. The specific data and typical chromatograms are as shown in Table 1 and Fig. 8.

System precision and method precision

System precision was evaluated by injecting six replicates, and method precision was evaluated by preparing the six different preparations of impurity standard solution into the chromatographic system as per the test method. The % relative standard deviation (RSD) was calculated for the area of impurity-C and impurity-D. The % relative standard deviation (RSD) of each impurity is not more than 2.0%. For system precision, 0.40% is impurity-C and 0.07% is impurity-D. For method precision, 0.40% is impurity-C and 0.79% is impurity-D. Results are shown in Tables 2 and 3.

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

The LOD and LOQ for the proposed method were determined using calibration standards and calculated using 3.3 σ/s and 10 σ/s formulae, respectively. The limit of detection and limit of quantification are 0.01% and 0.03% for impurity-C. The limit of detection and limit of quantification are 0.004% and 0.01% for impurity-D. The data and typical chromatograms are as shown in Table 4 and Fig. 9.

Linearity

For linearity, stock solution (1 mg/mL) contained impurity-C and impurity-D and further diluted with diluent to get the linearity

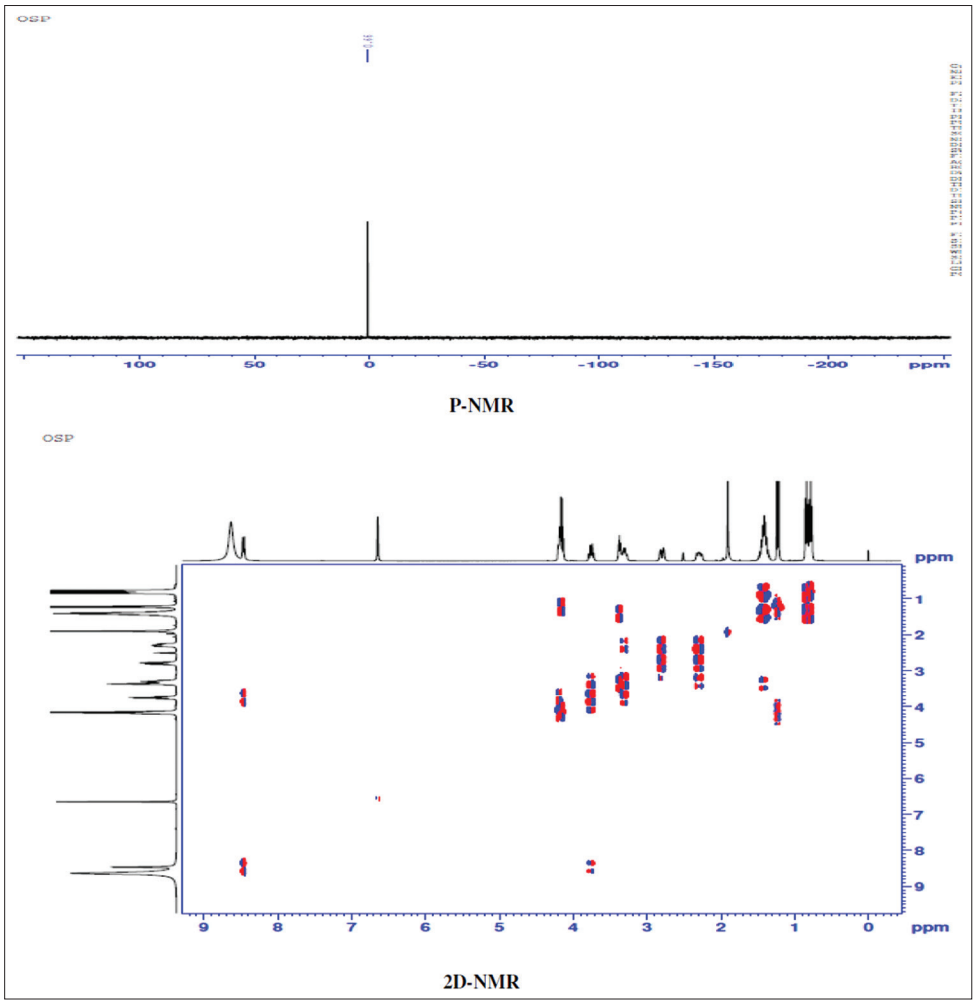

Fig. 5: P-NMR and 2D-NMR spectrums of oseltamivir phosphate

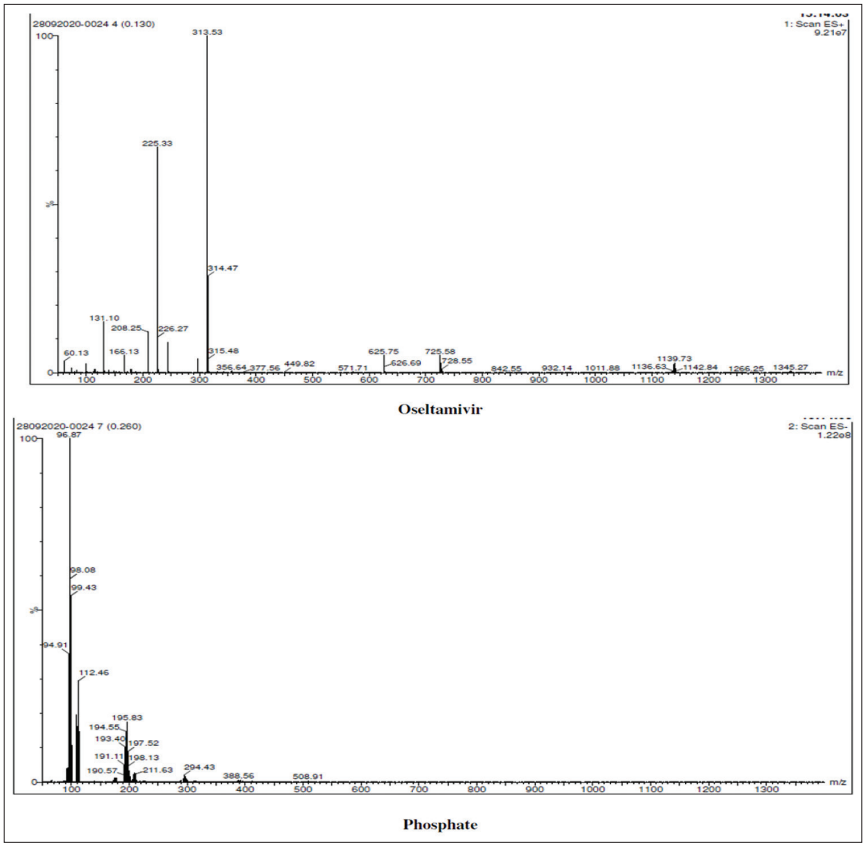

Fig. 6: Mass spectrums of oseltamivir phosphate

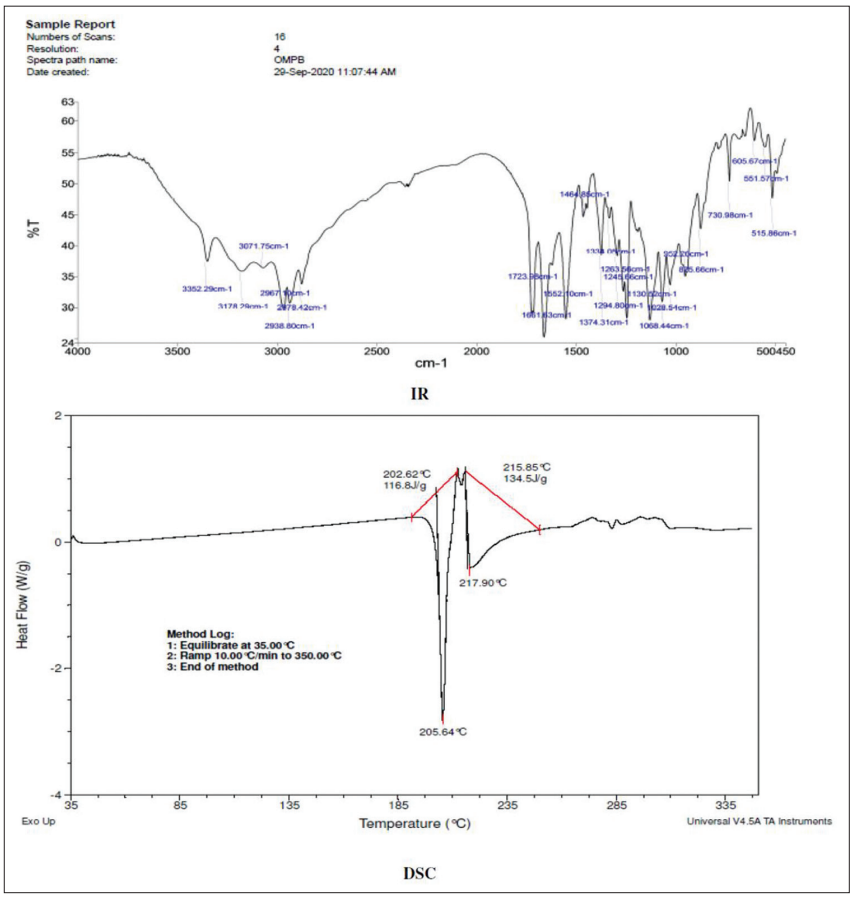

Fig.7: IR and DSC spectrums of oseltamivir phosphate

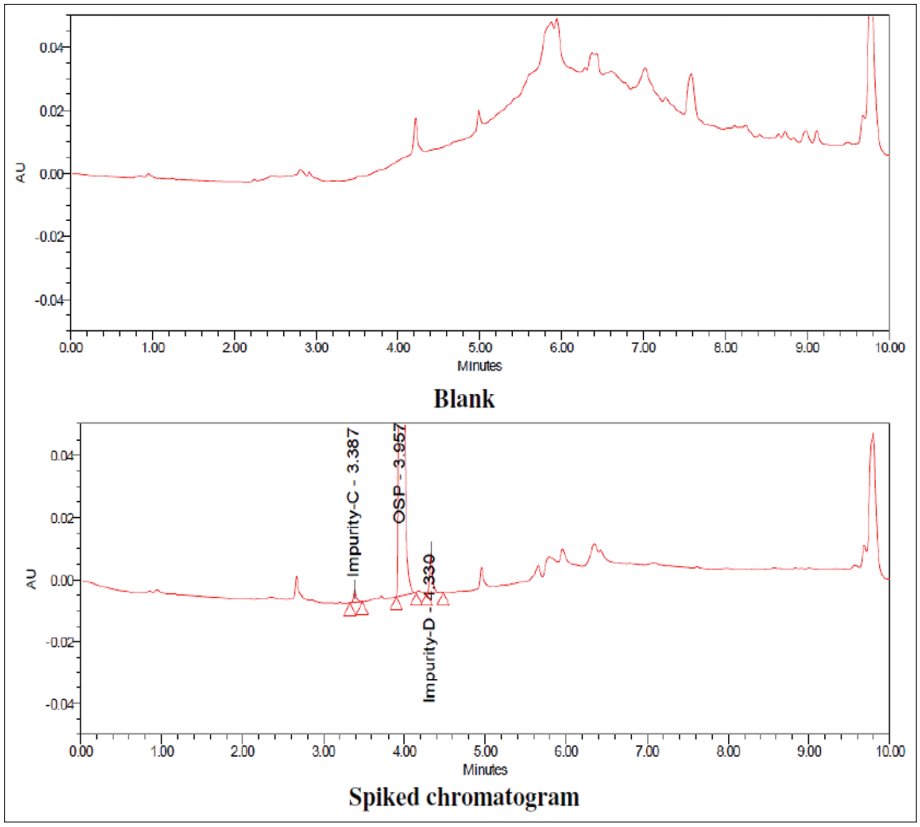

Fig. 8: Typical chromatograms for specificity

Table 1: Specificity data for impurity-C and impurity-D

S.No.	SST parameters	Impurity-C	Impurity-D	Oseltamivir phosphate API
1	Specification concentration (*W.R.S)	0.1%	0.1%	*NA
2	Specification concentration (*W.R.S)	0.001 mg/mL	0.001 mg/mL	1.0
3	Retention time (min)	3.39	4.33	3.96
4	Relative Retention time (min)	0.86	1.09	1.00
5	*USP Tailing	1.33	1.36	1.41
6	*USP Resolution	*NA	8.32	5.32
7	*USP Plate count	50972	75171	45818

*W.R.S: With respect to sample, NA: Not Applicable, USP: United States Pharmacopeia

Table 2: System precision data for impurity-C and impurity-D

No. of injections	Area of impurity-C	Area of impurity-D
Injection-1	4859	16513
Injection-2	4835	16522
Injection-3	4810	16542
Injection-4	4834	16512
Injection-5	4862	16533
Injection-6	4847	16532
Mean±SD	4841±19.22	16526±12.01
%RSD	0.40	0.07

Table 3: Method precision data for impurity-C and impurity-D

No. of injections	Area of impurity-C	Area of impurity-D
Preparation-1	4788	16516
Preparation-2	4839	16554
Preparation-3	4813	16530
Preparation-4	4793	16607
Preparation-5	4823	16539
Preparation-6	4806	16240
Mean±SD	4810.33±19.01	16498±130.08
%RSD	0.40	0.79

Table 4: LOD and LOQ data for impurity-C and impurity-D

Impurities	LOD Con. (%)	LOQ Con. (%)	LOD area	LOQ area
Impurity-C	0.01 (0.0001 mg/mL)	0.03 (0.0003 mg/mL)	1803	3739
Impurity-D	0.004 (0.00004 mg/mL)	0.01 (0.0001 mg/mL)	716	2760

concentrations such as limit of quantification (LOQ), 0.05, 0.0075, 0.1, 0.125, 0.15, 0.2, 0.25, and 0.3%. Moreover, these solutions were injected (n=2) into the ultra-performance liquid chromatography (UPLC) system. The obtained peak areas of each component were recorded. The correlation coefficient obtained for impurity-C and D, 1.000 and 1.000. The linearity data and correlation graphs are shown in Table 5 and Fig. 10.

LOQ-precision

Six replicates of impurity-C and D at limit of quantification (LOQ) concentration are injected into the ultra-performance liquid chromatography (UPLC) system and then calculated the % relative standard deviation and it is not more than 2.0%. The % relative

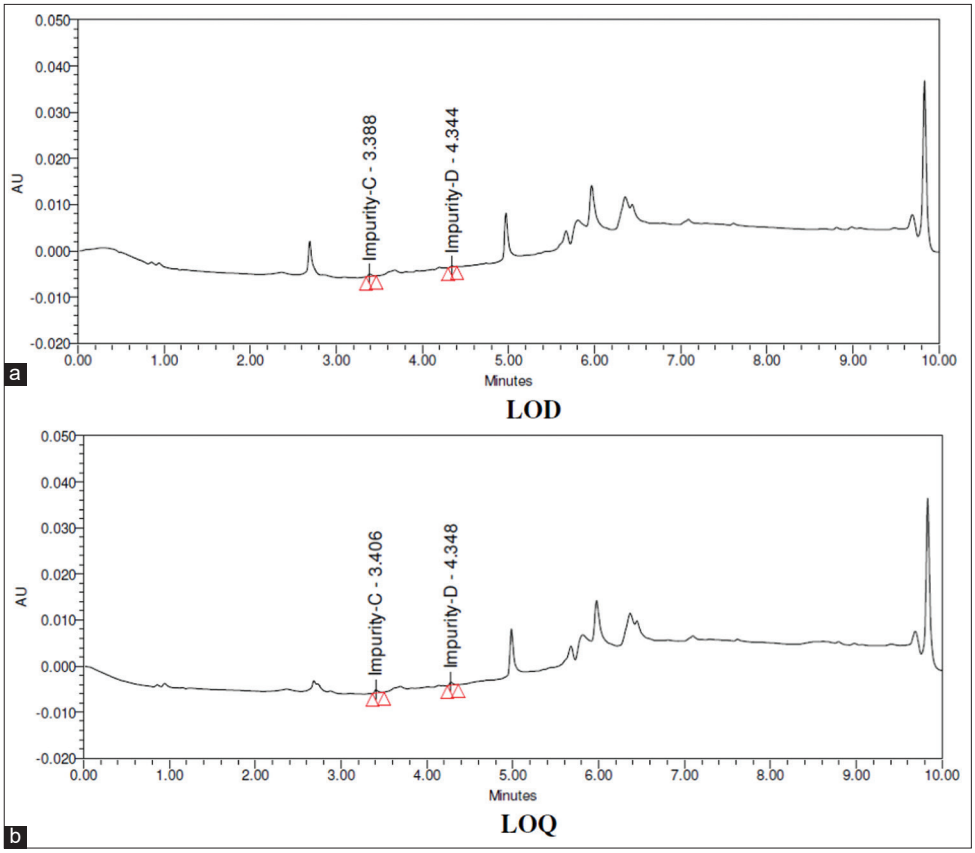

Fig. 9: (a) LOD and (b) LOQ chromatograms of impurity-c and impurity-D

Table 5: Linearity data with LOQ

Area of impurity-C			Area of impurity-D		
Con. (%)	**Injection-1 and 2**	**Average area**	**Con. (%)**	**Injection-1 and 2**	**Average area**
0.03	2013	1997	0.01	1783	1798
	1980			1812	
0.05	2795	2779	0.05	8523	8349
	2763			8175	
0.075	4116	4153	0.075	12494	12580
	4190			12666	
0.10	4830	4826	0.10	16445	16457
	4822			16469	
0.125	5870	5856	0.125	20141	20211
	5841			20280	
0.15	6900	6899	0.15	24546	24663
	6897			24779	
0.2	8920	8859	0.2	32706	32498
	8798			32290	
0.25	10916	10910	0.25	40750	40802
	10903			40853	
0.3	13052	13034	0.3	49152	49146
	13015			49139	
Correlation (r^2)		1.000	Correlation (r^2)		1.000

standard deviation is 1.05 for impurity-C and 1.28 for impurity-D. Results are summarized in Table 6.

Recovery

The recovery was estimated at 0.05, 0.1, 0.15, and limit of quantification (LOQ) levels. The recovery was found within the range of 100 ± 15. The obtained recoveries at 0.05, 0.1, 0.15, and 0.03 concentrations are 107.00, 96.84, 94.58, and 90.95% for impurity-C, respectively. The obtained recoveries at 0.05, 0.1, 0.15, and 0.01 concentrations are 102.64, 98.52, 98.50, and 105.18% for impurity-D, respectively. The results are indicated in Table 7.

Ruggedness and robustness

The ruggedness of the method was evaluated by injecting the standard organic volatile impurities in six replicates with different analysts on different days. Robustness means to perform by making small variations in the ultra-performance liquid chromatography (UPLC) method parameters. The changed parameters are column flow and column temperature. The % relative standard deviation (RSD) for six organic volatile impurities is not more than 2.0% for ruggedness and robustness. The results are summarized as shown in Tables 8 and 9.

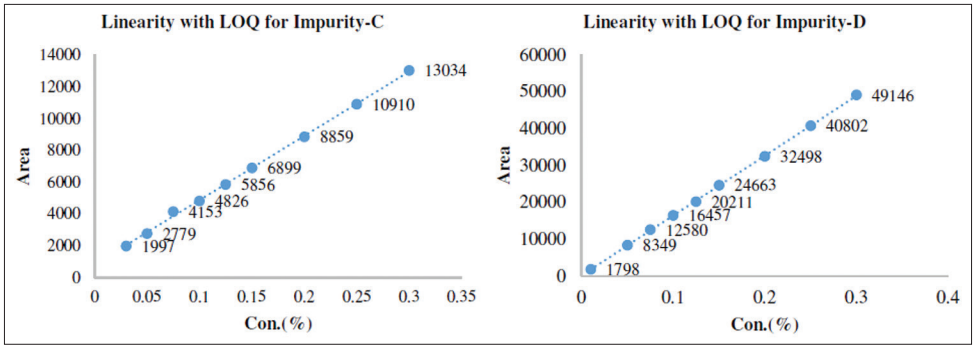

Fig. 10: Correlation graphs of impurity-C and D

Table 6: LOQ-precision data

No. of injections	Area of impurity-C (0.03%)	Area of impurity-D (0.01%)
Injection-1	2013	1783
Injection-2	1980	1812
Injection-3	2011	1815
Injection-4	2000	1800
Injection-5	1962	1853
Injection-6	1975	1813
Mean±SD	1990±20.88	1813±23.12
%RSD	1.05	1.28

Table 7: Recovery data for impurity-C and impurity-D

Accuracy Con. (%)	Area of Imp-C+Sample	Imp-C area in Sample	Imp-C area in 0.1% Standard
Accuracy at 0.05	4519	1929	4841
Accuracy at 0.1	6617		
Accuracy at 0.15	8797		Imp-C area in LOQ Standard
Accuracy at 0.03 (LOQ)	3739		1990
% Recovery at 0.05	107.00		
% Recovery at 0.1	96.84		
% Recovery at 0.15	94.58		
% Recovery at 0.03 (LOQ)	90.95		
Accuracy Con. (%)	**Area of Imp-D+Sample**	**Imp-D area in Sample**	**Imp-D area in 0.1% Standard**
Accuracy at 0.05	9335	853	16527
Accuracy at 0.1	17135		
Accuracy at 0.15	25272		Imp-D area in LOQ standard
Accuracy at 0.01 (LOQ)	2760		1813
% Recovery at 0.05	102.64		
% Recovery at 0.1	98.52		
% Recovery at 0.15	98.50		
% Recovery at 0.01 (LOQ)	105.18		

Table 8: Ruggedness data for impurity-C and impurity-D

Different Days and analysts		%RSD for impurity-C	%RSD for impurity-D
Day-1	Analyst-1 (*n=6)	0.45	1.17
	Analyst-2 (n=6)	0.41	1.39
	Analyst-1 and 2 (n=12)	0.41	1.26
Day-2	Analyst-1 (n=6)	0.84	0.89
	Analyst-2 (n=6)	0.61	0.53
	Analyst-1 and 2 (n=12)	0.41	1.39
Analyst-1	Day-1 and 2 (n=12)	0.64	1.29
Analyst-2	Day-1 and 2 (n=12)	0.54	1.04

*n=6 (number of injections)

Table 9: Robustness data for impurity-C and impurity-D

Name of impurity	Flow rate (mL/min)		Column temperature (°C)	
	0.25 mL/min (%RSD)	0.35 mL/min (%RSD)	25°C (%RSD)	35°C (%RSD)
Impurity-C	1.05	0.90	1.25	1.07
Impurity-D	0.32	0.44	0.90	0.64

Table 10: Oseltamivir phosphate pharmaceutical analysis

Name of API	API label claim (*mg)	Impurity-C (%)	Impurity-D (%)
Oseltamivir Phosphate	75	Not detected	Not detected

*mg: milligram; API: Active pharmaceutical ingredients

Pharmaceutical analysis

The prepared oseltamivir phosphate **(**oseltamivir 75 mg) tablet solution (250 mg/mL) was injected. From these oseltamivir 75 mg tablet analyses, impurity-C and D found to be within the specified limits (i.e., not detected). The results are shown in Table 10.

Solution stability

The impurity-C and impurity-D standard solutions were prepared in selected diluent at specified concentration on the 1st day and kept at room temperature. These standard solutions were injected at initial, 1, 2, 6, 12, 24, 48, and 72 h. Moreover, the solution stability was checked for impurity C and D at each interval, and the obtained solution stability data is 100 ± 2%. Based on these data, impurity-C and D standard solution was stable up to 72 h. The corresponding data are presented in Table 11.

Table 11: Solution stability data for impurity-C and D

Impurity-C		
Time interval	**Area of impurity-C (*n=1)**	**% Solution stability**
Initial h	4919	*NA
After 1 h	4903	99.67
After 2 h	4892	99.45
After 6 h	4859	98.78
After 12 h	4858	98.76
After 24 h	4847	98.54
After 48 h	4845	98.50
After 72 h	4838	98.35
Impurity-D		
Time interval	**Area of impurity-D (*n=1)**	**% Solution stability**
Initial h	16643	*NA
After 1 h	16580	99.62
After 2 h	16532	99.33
After 6 h	16513	99.22
After 12 h	16503	99.16
After 24 h	16471	98.97
After 48 h	16468	98.95
After 72 h	16443	98.80

*n=1 (number of injections); h: hours, NA: Not applicable

CONCLUSION

The reported characterization data (mass, infrared spectroscopy (IR), differential scanning calorimetry (DSC), proton nuclear magnetic resonance (H-NMR), phosphorus nuclear magnetic resonance (P-NMR), carbon-13 nuclear magnetic resonance (C13-NMR), and two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopic technics were used to identify and confirm the structure of oseltamivir phosphate active pharmaceutical ingredient (API). Using ultra-performance liquid chromatography (UPLC) instrument, a short runtime method was developed and validated by an extensive experiment for the quantification of impurity-C and impurity-D in oseltamivir phosphate active pharmaceutical ingredient (API) and its finished drug product. The ultra-performance liquid chromatography (UPLC) method was optimized and found to be economical, reproducible, accurate, linear, precise, and robust. The developed and validated method was used for the separation, identification, and quantitation of these impurity-C and impurity-D in oseltamivir phosphate active pharmaceutical ingredient (API) and its pharmaceutical substances. According to the characterization and quantitative results, the method could be applied to the quality control of pharmaceutical preparations.

ACKNOWLEDGMENT

The authors thank Dr. P Shyamala and Dr. R Murali Krishna (Physical Chemistry Department, Andhra University, Visakhapatnam, Andhra Pradesh, India) and Dr.KMV Narayana Rao and Dr. M. Durga babu (GVK Biosciences Pvt. Ltd, Hyderabad, Telangana, India) for their encouragement, valuable inputs, and cooperation, while carrying out this research work. The authors are also grateful to GVK Biosciences Pvt. Ltd, Hyderabad, Telangana, India, for providing facilities to carry out this research work.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally. Dr. M Durga babu was involved in data collection, reviewing, and editing of the manuscript.

CONFLICTS OF INTERESTS

The author declares no conflicts of interest.

AUTHORS FUNDING

Self-sufficient.

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