Available online at **www.joac.info**

ISSN: 2278-1862

Journal of Applicable Chemistry

2019, 8 (5): 2217-2224

(International Peer Reviewed Journal)

Bio-Analytical Method Development and Validation for the Estimation of Oseltamivir in Human Plasma by RP-HPLC

B. Jansi Rani, G. Kiran Kumar* and K. Nagaraju

Sir C.R.R.College of Pharmaceutical Sciences, Santhi Nnagar, Eluru-534007 A. P., **INDIA**

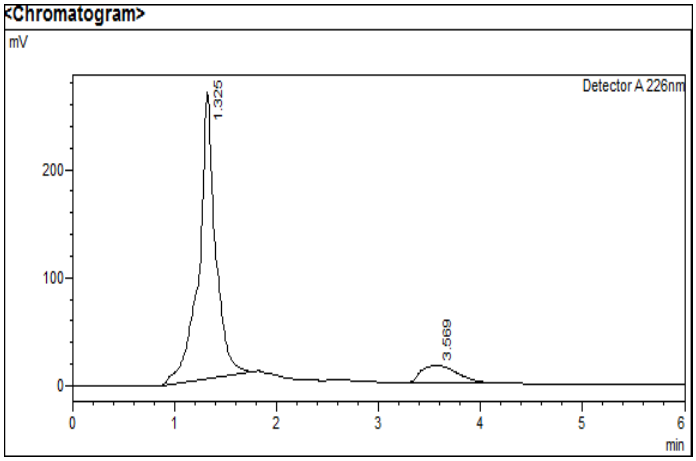
Email: bjr18k@gmail.com, kirru.pharma@gmail.com

Accepted on13th September, 2019

ABSTRACT

A novel, precise, accurate and rapid isocratic reversed phase high performance liquid chromatographic method was developed and validated for the estimation of Oseltamivir in human plasma. The method showed good separation and resolution for Oseltamivir with hypersil ODS (C18) column (4.6 x 250 mm, 5 µm) using Acetonitrile and water (70:30) as mobile phase at a flow rate of 1 mL/min and wavelength of 226 nm. The calibration curves were linear over the concentration ranges of 10-50µg/ml for oseltamivir respectively. All the analytes were separated within 5 min. The proposed method could be applied for the routine laboratory analysis of Oseltamivir in human plasma samples and analysis of Oseltamivir in pharmaceutical formulations.

Graphical Abstract



Typical chromatogram.of *oseltamivir*

Keywords: Oseltamivir, RP-HPLC, Human plasma.

INTRODUCTION

Oseltamivir chemically Ethyl(3R,4R,5S)-4-acetamido-5-amino-3-pentan-3-xyloxycyclohexene-1-carboxylatephosphate, trade name are Tamvir, Tamiflu. Oseltamivir is an inhibitor of the influenza neuramidase enzyme and is used as therapy and prophylaxis against influenza A and B. Oseltamivir has not been associated with clinically apparent liver injury. Oseltamivir phosphate is an ester prodrug of an antiviral enzyme inhibitor which, after absorption, is converted in the liver to oseltamivir carboxylase, the active intermediate. Oseltamivir carboxylase is a potent inhibitor of the enzyme neuro amidase of the influenza virus particle. Inhibition of this enzyme causes a decrease in viral replication. Oseltamivir is active against both influenza A and B virus but has no activity against other common upper respiratory tract viruses. In addition, resistance to oseltamivir can develop rapidly [1]. Oseltamivir was approved for in the United States in 1999 and is commonly used during influenza outbreaks. Oseltamivir is available as capsules of 30, 45 and 75 mg and as an oral suspension (6 mg mL^{-1}) under the brand name of Tamiflu. The recommended oral dose for therapy in adults is 75 mg twice daily for 5 days.

A survey of literature reveals [2-6] that there is no prominent RP-HPLC method developed so far for the estimation of Oseltamivir in human plasma. A very few methods are available for the estimation of above drug in human plasma by HPLC. It is necessary to estimate the bio availability of those drugs by using simple, accurate and specific method. Hence it is proposed to develop a new method for the estimation of the drug in human plasma by RP-HPLC [7, 8].

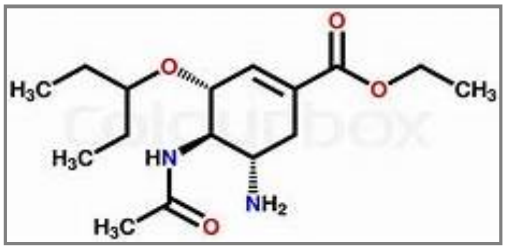


Figure 1. Structure of Oseltamivir.

MATERIALS AND METHODS

Instruments: Systronics PC based double beam spectrophotometer 2202 with 1cm matched quartz cells. SHIMADZU, Model: Prominence Liquid Chromatograph, SPD-20A UV–VIS detector. The output signal was monitored and integrated using Lab solutions software. A Symmetry ODS (C18) (4.6 x 250 mm, 5 m) column was used for separation.

Preparation of Mobile phase: A-Acetonitrile B-Water adjusted to Degas A and B individually in ultrasonic water bath for 5 min. Filter through 0.45 µ filter under vacuum filtration and then, mix A and B in the ratio of 70:30.

Diluent: Acetonitrile and water

Preparation of standard solutions: The stock solutions of Oseltamivir were prepared by dissolving appropriate amount corresponding to 1mg mL^{-1} concentrations in the diluent. All the stock solutions were stored at 2-8°C. The stock solutions of Oseltamivir were further diluted with diluent to give a series of standard mixtures having a final concentration in the range of 10–50 µg mL^{-1} respectively. A standard 1:1 mixture containing 10µg mL^{-1} of Oseltamivir was also prepared in the diluent.

Sample preparation: A simple two step liquid-liquid extraction (LLE) procedure was carried out for the extraction of Oseltamivir from plasma samples. To a series of 500 µL of drug solutions prepared,

200 µL of plasma, acetonitrile were added and mixed for 2 min for de protonation and centrifuged at 5000 rpm for 20 min. The organic layer was separated from this required amount was taken then this solution was injected into HPLC system.

Optimized method parameters are C18 (25X4.6X5) Column, Mobile phase, Acetonitrile:water (70:30 v/v), Flow rate 1 mL min^{-1}, Wavelength 226 nm, Injection volume :20 µL.

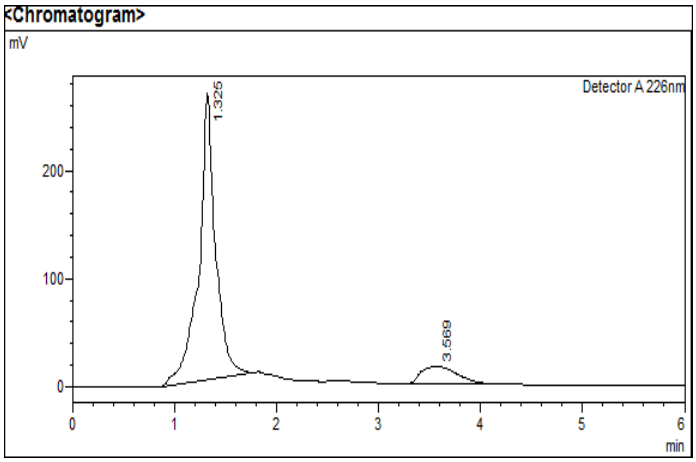


Figure 2. Typical chromatogram.

Validation: Validation of HPLC method was in compliance with recommendations of the ICH guidelines.

System suitability: The system suitability was assessed by six replicate analysis of drug at the concentration of 20 µg mL^{-1} of Oseltamivir. The acceptance criteria is %RSD is not greater than 2 (Table 1).

Table 1. System suitability Data for Oseltamivir

Sample Name	Area	Retention time	Theoretical plates	Tailing factor
Injection 1	1788	3.601	4777	1.581
Injection 2	1901	3.621	3154	1.210
Injection 3	1812	3.709	3077	1.301
Injection 4	1941	3.696	3106	1.413
Injection 5	1907	3.747	3201	1.528
Injection 6	1961	3.681	3150	1.594
% RSD	0.03	0.014	Average :3410	Average :1.437

Selectivity: Selectivity was determined by the analysis of blank and standard samples. The blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification (LLOQ).

Linearity: For all methods, 5-point calibration curve were prepared on single day. The results obtained were used to calculate the equation of the line by using linear regression by the least square method (Table 2).

Procedure for calibration curve: The extracted plasma samples, 10–50 µg mL^{-1} of Oseltamivir were injected each time into the column and the corresponding chromatograms were obtained. From these chromatograms retention times and the area under the curve of the drug to that of the reference standard for each dilution was calculated. The linearity range was found to be from 10-50 µg mL^{-1}.

The regression equation of the curve was computed. Correlation coefficient was found to be 0.997 and regression equation was found to be Y=10047x.

Acceptance Criteria: The plot of concentration versus peak area for each drug should be linear with a correlation coefficient (R^2) not more than 1.

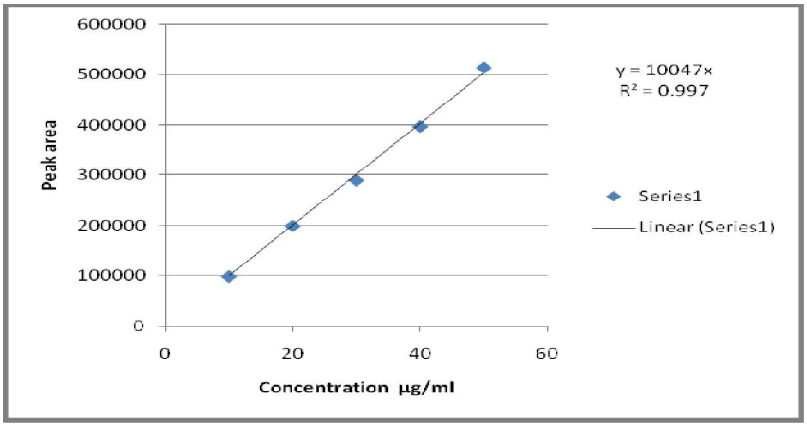


Figure 3. Calibration curve for Oseltamivir.

Table 2. Linearity Data for Oseltamivir

S.No	Linearity Level	Concentration (µg mL^{-1})	Area
1	I	10	97783
2	II	20	198873
3	III	30	289745
4	IV	40	397142
5	V	50	514506
Correlation Coefficient			0.997

Accuracy at LLOQ: The analyte response at the LLOQ should be at least five times the response compared to blank response. Analyte peak (response) should be identifiable, discrete, and reproducible, and the back-calculated concentration should have precision that does not exceed 20% of the CV and accuracy within 20% of the nominal concentration.

Accuracy at LLOQ was carried at three level 50%,100%,150% by using 1,2,3 µg mL^{-1} and mean % recovery was found98% and results are given in table 3.

Table 3. Accuracy at LLOQ-data for Oseltamivir

Sample ID		Concentration (µg mL^{-1})		%Recovery	Statistical Analysis
		Amount added (µg)	Amount found (µg)		
50 %	1	1	0.98	98	**Mean** = 92.3
	2	1	0.88	88	
	3	1	0.91	91	
100 %	1	2	2.01	100.5	**Mean** = 101.3
	2	2	1.998	99.9	
	3	2	2.07	103.5	
150 %	1	3	3.00	100	**Mean** = 99.74
	2	3	2.996	99.86	
	3	3	2.981	99.36	**97.78%**

Precision: Precision is carried out in the following levels .a) Precision at LLOQ b) Inter-day precision c) Intra-day precision (Table 4).

The repeatability of the method was examined at single intermediate levelby injecting the solution consisting of Oseltamivir in to the HPLC system for two consecutive days (intra-day and inter-day) respectively. The results were given in following table 4. Acceptance criteria of Relative Standard Deviation should not be more than 2 %.

Table- 4. Precision data for Oseltamivir

Injection	Precision of LLOQ	Inter-day Precision	Intra-day Precision
	Area	Area	Area
Injection-1	24377	2822	2812
Injection-2	21081	2812	2781
Injection-3	20154	2761	2862
Injection-4	20601	2873	2753
Injection-5	21366	27l3	2891
Injection-6	23170	2879	2849
Average	21718	2810	2824
Standard Deviation	1635	48.32	47.55
%RSD	0.753	1.74	1.68

The accuracy of an analytical method: It is the closeness of the test results to the true value. It was determined by application of the analytical procedure to recovery studies, where known amount of standard S and test samples (50%, 100%, and 150%) were spiked. From this, percentage recovery values were found to be 100. Acceptance criteria of Mean recovery should be in between 98-102% (Table 5).

Table 5. Accuracy of Oseltamivir

Sample ID	Concentration (µg mL^{-1})		%Recovery	Statistical Analysis
	Amount added (µg)	Amount found (µg)		
LQC	10	12.02	120.2	**Mean** = 108.3
LQC	10	10.36	103.6	
LQC	10	10.12	101.2	
MQC	30	30.17	100.5	**Mean** = 95.07
MQC	30	28.38	94.61	
MQC	30	27.03	90.12	
HQC	50	48.18	96.36	**Mean** = 96.99
HQC	50	46.82	93.65	
HQC	50	50.48	100.97	99.8%

Recovery: Recovery were performed by comparing the analytical results for extracted samples at three concentrations (LQC, MQC, and HQC) with unextracted standards that represent 100% recovery (Table 6).

Table 6. Recovery data for Oseltamivir

Sample ID	Concentration (µg mL^{-1})		%Recovery	Statistical Analysis
	Amount added (µg)	Amount found (µg)		
LQC	10	10.12	101.2	**Mean** =100.45%
MQC	30	30.17	100.5	
HQC	50	46.82	99.65	

Stability Studies:

Freeze and Thaw Stability: Analyte stability was determined after three freeze and thaw cycles. The results are given in table 7.

Table 7. Freeze and Thaw Stability data

Name of the drug	Amount added	Amount found (µg)	% change
Oseltamivir	LQC (10 µg)	10.12	1.2
		10.08	0.8
		10.82	8.2
	HQC (50 µg)	50.21	0.42
		50.33	0.66
		50.61	1.22

Short-Term temperature stability: Three aliquots of LQC and HQC were thawed at room temperature and kept at this temperature for 22 h and results are given in the table 8.

Table 8. Short-term temperature stability data

Name of the drug	Amount added	Amount found	% change
Oseltamivir	LQC (10 µg)	10.07	0.7
		10.41	0.41
		10.16	0.16
	HQC (50 µg)	49.51	0.49
		51.02	1.02
		50.42	0.42

Long-Term stability: Long-term stability was determined by storing three aliquots of LQC and HQC under the same conditions as the study samples for 22 days. The results were given in the table 9.

Table 9. Long-term temperature stability data

Name of the drug	Amount added	Amount found (µg)	% change
Oseltamivir	LQC (10 µg)	10.04	0.4
		10.71	7.1
		10.09	0.9
	HQC (50 µg)	49.05	1.9
		50.52	1.04
		50.64	1.28

Stock solution stability: The stability of stock solutions of drug was evaluated at room temperature for 6 h. Results are given in the table 10.

Acceptance criteria: Stability sample results should be within 15% of nominal concentrations.

Table 10. Stock solution stability data

Name of the drug	Amount added	Amount found	% change
Oseltamivir	1000µg	1901 µg	19.9 %

RESULTS AND DISCUSSION

To ascertain the maximum wavelength, λ_{max} of the drugs, the drug solutions of Oseltamivir were scanned between the wavelength ranges of 200-400 nm. The absorption maximum was found to be 226nm where most appropriate chromatogram with good peak area, Plate count and less tailing factor were observed. The study is carried out at 226 nm.

Ethanol, Methanol and Methanol-Ethanol in different ratios were tried for protein precipitation but complete protein precipitation was achieved with absolute Acetonitrile at least three times the volume of plasma. Methylene chloride, Ethyl acetate, chloroform, n-hexane and diethyl ether were evaluated either alone or in different ratios for the extraction of all analytes from plasma. Recovery of

Oseltamivir was better when extracted with Acetonitrile. So, best results in terms of recoveries were obtained with a simple LLE procedure involving extraction with Acetonitrile. Organic layer was separated.

Feasibility of different solvent systems such as methanol-water and acetonitrile–water in different compositions, pumped at different flow rates (in the range of 0.5-1.2 mL min^{-1}) were evaluated. Best results were obtained using Acetonitrile–water in the ratio of 70:30 at a flow rate of 1 mL min^{-1}.

Hypersil ODS C18 (4.6, 250 mm, 5 µ) column was used throughout the optimization and method development process.

Using the optimized chromatographic conditions mentioned, resolved sharp peaks corresponding to Oseltamivir were obtained at R_T of 3.6 min respectively. The proposed RP-HPLC assay for the estimation of Oseltamivir was validated for selectivity, linearity, precision, accuracy, recovery robustness, LOD, LOQ and stability. The results were given in the table 11.

Table 11. Results of the optimized method

S.No	Parameter	Specifications	Inference Oseltamivir
1	Specificity	No interference	No interference
2	Resolution	NLT 2	
3	Tailing factor	NMT 2	1.450
4	Number of Theoretical plates	NLT 2000	7949
	Precision		1.68
5	Intraday (n=6)	RSD NMT 2.0%	1.74
	Inter ay (n=6)		
6	Linearity range	Correlation coefficient NLT 0.999	0.997
7	Accuracy–Mean Recovery (%)	Range 98 –102 %	99.8 %
8	Recovery (%) from plasma		98.45 %
9	Freeze and Thaw Stability	Within 15% of nominal concentrations	LQC 0.8, HQC 1.22
	Short term Temperature Stability		LQC 0.7, HQC 0.98
	Long term Temperature Stability		LQC 0.9, HQC 1.9

APPLICATION

The proposed method could be applied for the routine laboratory analysis of Oseltamivir in human plasma samples and analysis of Oseltamivir in pharmaceutical formulations.

CONCLUSION

An RP-HPLC method is developed and validated as per ICH guidelines for simultaneous estimation of Oseltamivir in human plasma. In present study an attempt has been made to estimate drugs and their active metabolites simultaneously in order to estimate the amount converted into metabolite and remained as such. The mobile phase was selected after trying various combinations of polar solvents. The proportion of solventsandvariationofbufferswasfoundtobequitecriticalasslightvariationin it adversely affected the resolution of peaks. Considering all the fact the following parameter was finally fixed for this method.

The proposed method was found to be rapid, accurate, precise, specific, robust, rugged and economical. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good. This method offers an advantage than reported methods that the drug can

be estimated. Thus the method is not time consuming and can be used in clinical studies for the analysis of bioavailability and bioequivalence.

ACKNOWLEDGEMENTS

Authors are very much thankful for the management of Sir C.R Reddy Educational Institutions for providing eminent facilities.

REFERENCES

[1]. R. Agrawal, P. V. Rewatkar, G. R. Kokil, A. Verma, A. Kalra, Oseltamivir: a first line defense against swine flu, *Medicinal Chemistry*, **2010**, 6(4), 247–251.
[2]. J. Joseph-Charle , method development and validation for the determination of Oseltamivir in bulk 168
[3]. Balasubramanian Narasimhan, Khan Abida, Kona Srinivas, Method development and validation for the determination of Oseltamivir in bulk drug and dosage form by using RP-HPLC, *International Journal of Pharma tech Research*, **2010**, 2(1), 363-366.
[4]. N. Lindegardh, W. Hanpithakpong, Y. Wattanagoon, P. Singhasivanon, N. J. White, N. P. J. Day, Method development and validation for the determination of Oseltamivir in bulk drug by using RP-HPLC, *Pharmaceutical analysis Acta*, **2015**,
[5]. S. M. Malipatil, Kishwar Jahan, S. K. Patil, Method development and validation for the determination of Oseltamivir in bulk drug and dosage form by using RP-HPLC, *Indo-Global Journal of Pharmaceutical Sciences*, **2011**, 1(1), 57-62.
[6]. Agrawal, Oseltamivir, a first line defense against swine flu, *Medicinal Chemistry*, **2010**, 6(4), 247–251.
[7]. Nagaraju Kavuri, T. Sunitha, Estimation of Atorvastatin, Fenofibrate and Fenofibric acid in H. plasma, Lambert academic publishing, **2015**, 6, 85-90.
[8]. Guidance for Industry Bi analytical Mehod Validation U.S Department of Health and Human Services Food and Drug Admnistration, Center for Drug Evaluation and Research (CDER),Center for Veterinary Medicine, Biopharmaceutics, September **2013**.