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Validated Stability – Indicating Methods for Determination of Oseltamivir Phosphate

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Authors' contributions

This work was carried out in collaboration between all authors. Author NSR managed the analyses of the study, managed the literature searches, wrote and corrected the manuscript. Author OMA designed the study and wrote the protocol. Author NSS collected the literature, performed the statistical analysis and helped in the manuscript preparation. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Two simple and sensitive stability- indicating methods were developed and validated for the quantitative determination of oseltamivir phosphate in presence of its degradation product.
Place and Duration of Study: Analytical Chemistry Department, Faculty of Pharmacy (Girls), Al-Azhar University, between January 2016 and Augest 2016.
Methodology: The first method depends on densitomeric determination of thin layer chromatograms of the drug using a mobile phase of methanol – toluene – ammonia (8: 10: 2, v/v/v). The second method was UPLC method, in which efficient separation was carried out on phenomenex kinetex 2.6 µm C_{18}100 A column using a mobile phase consisting of 85% potassium hydrogen phosphate - methanol (80:20, v/v), adjusted to pH 3.5 with orthophosphoric acid at a flow rate of 1 mL min^{-1} and UV detection at 207 nm.
Results: Beer' law was obeyed in the range of 1-15 µg/spot and 6-14 µg mL^{-1} of the drug using the two procedures, respectively.

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Conclusion: The proposed methods were successfully applied for the determination of oseltamivir phosphate in bulk powder, laboratory prepared mixtures and pharmaceutical dosage form with good accuracy and precision. The methods were validated according to ICH guidelines. The results obtained were compared with those of the reported method and were found to be in good agreement.

Keywords: Oseltamivir phosphate; densitomery; UPLC; pharmaceutical preparation.

1. INTRODUCTION

Oseltamivir phosphate (OSP) is a non-hygroscopic pro-drug of the active metabolite oseltamivir carboxylate, chemically described as (3R, 4R, 5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate (1:1) [1]. It is a neuraminidase inhibitor, serving as a competitive inhibitor of the activity of the viral neuraminidase enzyme upon sialic acid, found on glycoproteins on the surface of normal host cells [2].

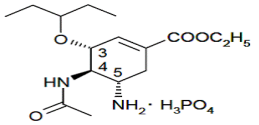


Fig. 1. Chemical structure of oseltamivir phosphate

Several analytical methods were reported for the determination of OSP in pure form and in its pharmaceutical preparations including UV spectrophotometric [3-8], spectrofluorimetric [9,10], capillary zone electrophoresis [11], voltammetry [12] and HPLC [13-18]. The main task of the present work is to develop simple and accurate stability – indicating methods for the selective determination of OSP in presence of its degradation product which can be used for analysis of the drug in raw material and pharmaceutical preparation.

2. MATERIALS AND METHODS

2.1 Instrumentation

- Camag TLC scanner 3, with WINCATS computer software (Switzerland).
- Agilent 1290 Ultra HPLC with binary pump and UV detector (USA).
- Precoated TLC plates, silica gel 60 GF_{254} (20 × 20 cm), (Fluka chemie, Switzerland).
- pH meter combined plus electrode (Adwa model AD1030 pH mv).
- Hamilton 50-µL micro syringe (Germany).
- UV lamp with short wavelength 254 nm, (Desega-Germany).
- Chromatographic tank (25 × 25 × 9 cm).

2.2 Samples

2.2.1 Pure samples

Oseltamivir phosphate; batch no. OP 0010110 was kindly supplied by the Nile Company for Pharmaceuticals and Chemical Industries, Cairo, Egypt. Its purity was found to 99.17%, as referred by the supplier.

2.2.2 Market samples

Taminil-N® capsule; batch no. 0603301, labeled to contain 75 mg OSP, the product of the Nile Company for Pharmaceuticals and Chemical Industries, Cairo, Egypt.

2.2.3 Degraded sample

Degraded OSP was laboratory prepared by refluxing 10 mg of pure drug with 25 ml 2N NaOH for 75 minutes at 100°C. The solution was cooled, neutralized with 4N HCl to pH 7 and evaporated till dryness under vacuum. Residue was extracted three times, each with 25mL methanol then filtered into a 100 mL volumetric flask and diluted to the volume with methanol. The obtained solution was claimed to contain degradate derived from 0.1 mg mL^{-1} intact drug.

2.3 Chemicals and Reagents

All reagents used were of analytical grade, solvents were of spectroscopic grade, and water used throughout the procedure was freshly distilled.

- Methanol and potassium hydrogen phosphate; HPLC grade (Sigma-Aldrich, Merck).
- Orthophosphoric acid (Fisher, UK)
- Sodium hydroxide, hydrochloric acid, toluene and ammonia (El-Nasr Co., Egypt).

2.4 Standard Solution

Stock solution of the drug (1 mg mL^{-1}) was prepared by dissolving 100 mg of OSP in 100 mL methanol.

2.5 Procedures

2.5.1 Densitometric method

2.5.1.1 Chromatographic conditions

Analysis was performed on precoated (20 × 20 cm) TLC aluminum silica gel 60 GF_{254} plates. Samples were applied to the plates using Hamilton micro syringe (50 μL). Plates were spotted2 cm apart from each other and 1.5 cm apart from the bottom edge. The chromatographic tank was pre-saturated with the mobile phase for 20 minutes, and then developed by ascending chromatography using methanol - toluene - conc. ammonia (8: 10: 2 v/v/v) as a mobile phase. The plates were air dried, detected under UV- lamp 254 nm and scanned under the following conditions:

- Silt dimension: 6.0 × 0.3 µm.
- Wavelength: 220 nm.
- Scanning speed: 20 mm/s.
- Data resolution: 100 nm / step.
- Measurement mode: absorption.
- Result output: chromatogram and area under the peak.

2.5.1.2 Construction of calibration curve

Into a series of 10 - mL volumetric flasks, aliquots of standard OSP solution (1 mg mL^{-1}) equivalent to (0.01-0.15 mg) OSP were diluted to volume with methanol. 5μL were spotted on a TLC plate following the above mentioned specific chromatographic conditions and scanned at 220 nm. Calibration curve was constructed by plotting area under the peak versus the corresponding drug concentrations in μg / spot.

2.5.1.3 Assay of laboratory prepared mixtures

Aliquots of intact OSP solution containing (1.5 – 13.5 mg mL^{-1}) were introduced into a series of 10- mL volumetric flasks containing (13.5 – 1.5 mg mL^{-1}) of the degraded OSP and then diluted to the volume with methanol. The same procedure under "2.5.1.2.Construction of calibration curve" was applied and the peak areas of the obtained chromatograms were measured then the concentration of the drug was calculated from its corresponding regression parameters.

2.5.2 UPLC method

2.5.2.1 Chromatographic conditions

At ambient temperature, isocratic separation was carried out on phenomenex Kinetex 2.6 µ m C_{18} 100 A, column (75 mm x 4.6 mm) using a mobile phase composed of 85% potassium hydrogen phosphate - methanol (80:20 v/v), pH adjusted at 3.5 with orthophosphoric acid. The mobile phase was pumped at flow rate 1 mL min^{-1}, the injection volume was 20 µL and the detection was carried out at 207 nm.

2.5.2.2 Construction of calibration curve

Aliquots of standard drug solution (0.1mg mL^{-1}) containing 6-14 µg mL^{-1} OSP were transferred into a series of 10- mL volumetric flasks and diluted to the volume with methanol. 20 µL of each solution were injected into the UPLC system six times for each concentration and chromatographed under the above mentioned conditions. Calibration curve was obtained by plotting the peak area against concentration of the drug in µg mL^{-1}

2.5.2.3 Assay of laboratory prepared mixtures

Different aliquots of intact OSP containing (1.2 - 10.8 µg mL^{-1}) were transferred into a series of 10- mL volumetric flasks containing degradate (10.8 - 1.2 µg mL^{-1}). 20µL of each solution were injected into the UPLC column and the corresponding chromatograms were recorded at 207 nm. The intact drug concentrations were calculated from the corresponding regression equation.

2.5.3 Application to pharmaceutical preparation

The contents of five Taminile-N® capsules were evacuated carefully, mixed and weighed. An accurately weighed quantity of the powder equivalent to 100 mg of OSP was dissolved into a 100- mL volumetric flask, diluted to volume with methanol and filtered. The obtained solution labeled to contain 1 mg mL^{-1} of the drug was analyzed by densitometric method. 10 mL of the obtained solution (1 mg mL^{-1}) was diluted to 100 mL with methanol, the obtain solution labeled to contain (0.1 mg mL^{-1}) of the drug to be analyzed

by the proposed UPLC method. The drug concentrations were calculated from the appropriate regression parameters.

3. RESULTS AND DISCUSSION

Two chromatographic methods were developed for the selective determination of OSP in presence of its degradation product; UPLC and TLC methods.

3.1 Degradation of OSP

Previous study [18] was done to produce incomplete degradation of OSP based on exposure to acidic, basic, oxidative and thermal stress conditions and the stressed samples were not confirmed by IR or mass as the present study. In our study complete degradation of OSP was performed upon heating for 75 minutes with 2N NaOH under reflux, leading to hydrolysis of ester and amide linkage to produce 4,5- diamino-3- (pentan-3-yloxy) cyclohex-1-enecarboxylic acid; as shown in Scheme 1.

The prepared alkaline degradate residue was confirmed by IR and Mass spectra; The IR spectrum of the intact drug exhibits characteristic peak of (NH_2 stretching) at 3349 cm^{-1}, Peak of (C-H aliphatic) at 2970 cm^{-1}, characteristic peak of (C=O stretching) of ester at 1720 cm^{-1}, strong peak of (C=O stretching) of amide at 1659 cm^{-1} and characteristic peak of (C-O stretching) of ether at 1069 cm^{-1}. While in the degradate; the absence of (C=O) peak of ester at 1720 cm^{-1} and appearance of broad peak at 3427 cm^{-1} of (OH) stretching of carboxylic acid, indicates hydrolysis of ester bond. Also disappearance of (C=O) characteristic peak at 1659 cm^{-1} indicate hydrolysis of amide bond, and presence of characteristic peak of (C-O) at 1063 and peak of (C-H aliphatic) at 2969 cm^{-1} indicate presence of ether linkage.

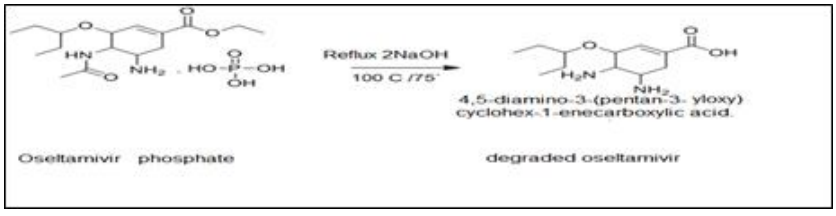
EI mass showed molecular ion peak at *m/z* = 410 with high intensity (10.10%) for the intact drug and showed molecular ion peak at *m/z* = 242 with high intensity (20.00%). In the degradate this indicate decreasing in molecular ion peak equal 168 unit. This means loss of $C_2H_5^*$, CH_3CO^* radicals of ester and acetamide moieties and also absence of $H_3PO_4^-$group respectively.

3.2 Densitometric Method

Quantitative TLC scanning was developed for the densitometric determination of intact OSP in presence of its degradation product. The difference in R_f values between the intact drug and its corresponding degradate was the basis of the determination. Best separation was achieved using a mobile phase of methanol – toluene – ammonia (8: 10: 2, v/v/v), having R_f 0.84 for intact OSP and 0.46 for its degradate. The separated spots of the drug were scanned densitometrically at 220 nm without any interference from its degradate.

3.3 UPLC Method

To optimize UPLC parameters such as peak shape, peak symmetry and run time, many trials were taken. Mixture of commonly used solvents like water, methanol and acetonitrile with or without buffers in different concentrations at different pH values were tested as mobile phases. Good resolution with good peak shape and purity were obtained on phenomenex kinetex 2.6 µm C_{18}100 A column using mobile phase consisting 85% potassium hydrogen phosphate - methanol adjusted to pH 3.5 with orthophosphoric acid in composition of (80:20 v/v). Different flow rates (0.8 -1.4 mL min^{-1}) at different wavelengths (200 – 400) were tried; optimal flow rate was found to be 1 mL min^{-1} with detector responses at 207 nm. The peak of intact OSP was well resolved from the peak of its degradate and clearly developed at retention time of 4.252 for intact OSP and 2.111 for its degradate respectively; Fig. 3.



Scheme 1. Suggested degradation pathway of OSP

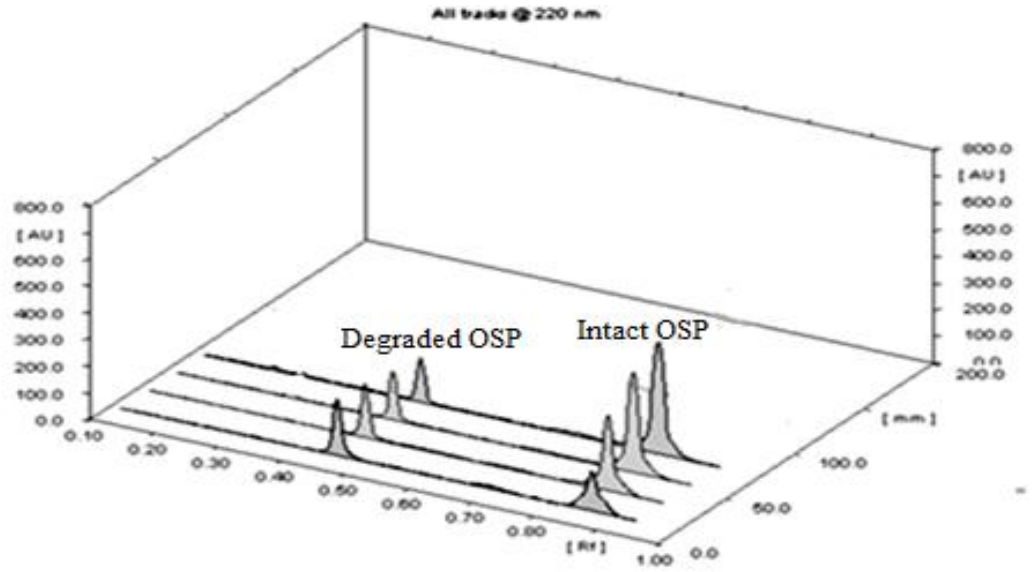


Fig. 2. Densitometric chromatogram of OSP and its alkaline degradate at 220 nm

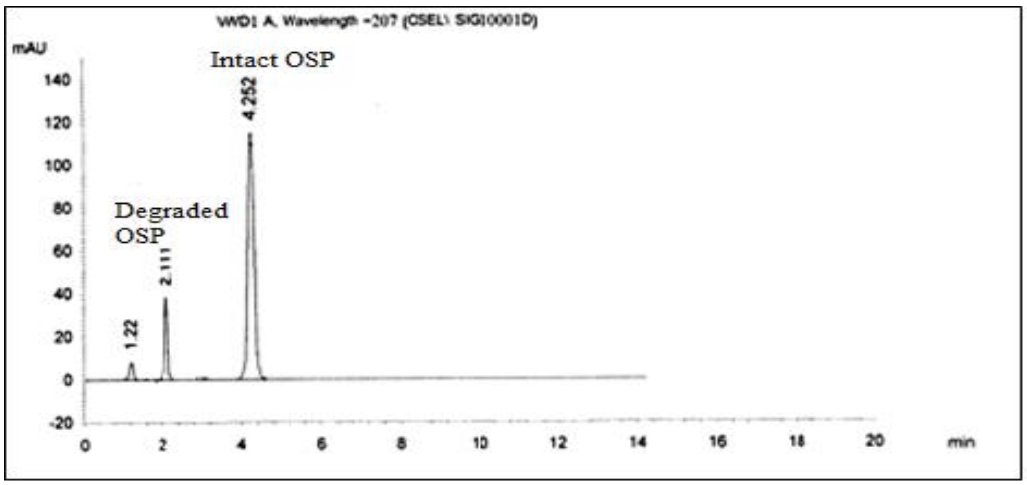


Fig. 3. UPLC chromatogram of mixture of intact OSP and degraded OSP (14 µgmL^{-1}) at 207 nm

3.4 Methods Validation

The methods were validated as per ICH guidelines [19].

3.4.1 Linearity

A good linear relationship was obtained in TLC method between peak areas of the separated spots and the corresponding drug concentration over the range of 1-15 µg/spot. While linearity between peak area and corresponding drug concentration in UPLC method was obtained over the range of 6-14 µg mL^{-1}; Table 1.

3.4.2 Accuracy and precision

Accuracy (R %) of the proposed methods was demonstrated by analyzing different concentrations covering the points in the calibration range. The average percentage recovery was found to be 100.32 and 99.18% using the two proposed methods, respectively; Table 1. Intraday and interday precision (RSD%) of the proposed methods were determined using three different concentrations with-in linearity range each in triplicate, within the same day for intraday analysis and three different days for interday analysis. RSD % values for the two methods were found to be less than 2%

indicating good precision of the developed methods; Table 2.

3.4.3 LOD and LOQ

LOD and LOQ were determined according to ICH using the standard deviation of multiple blank samples and the slope of the calibration curve; Table 1.

3.4.4 Selectivity

The selectivity of the proposed methods was assured by applying the proposed methods to laboratory prepared mixture of the intact drug together with its degradation product. The proposed procedures were adopted for the selective determination of intact OSP in presence of up to 90 % of its corresponding degradate, as shown in Table 2.

3.4.5 Robustness

The robustness of the proposed densitometric method was assessed by studying the influence of deliberate variation in the mobile phase contents ratio. It was observed that no significant difference in R_f value upon variation in ammonia volume from 0.01- 0.09 mL. The RSD% did not exceed 1.82%. Also robustness was checked by studying the effect of different sources of methanol; it was found that using methanol (Sigma – Aldrich, Germany, El-Nasr Co., Egypt) gave RSD % did not exceed 0.29% whereas peak area remains acceptable throughout the assay, which proved the robustness of the method. While the robustness of the proposed UPLC method was assured by study the influence of deliberate variation in the mobile phase contents ratio by ± 1% and variation of the pH of the mobile phase by ± 0.1 units indicate that RSD% did not exceed 0.738 which proved the robustness of the method. The required parameters for the system suitability test were capacity factor (k`), number of theoretical plates (N), resolution (R), selectivity (α) and tailing factor (T). It was found that, the deliberate variations did not affect the system suitability parameters, confirming robustness of the method; Table 3.

The stability of standard solution was determined. Two solutions of the drug were prepared, one of them was kept at room temperature while the other was kept in refrigerator and they were analyzed against freshly prepared standard. It was found that, OSP solution was stable for 2 days either kept at room temperature or in refrigerator.

The proposed methods were used for determination of OSP in its pharmaceutical preparation where no interference from excipients and additives were observed. The results presented in Table 4 revealed mean percentage recoveries of 100.25 ± 0.82 and 100.79 ± 1.23% for the two proposed methods; respectively. The recovery of the proposed methods was also validated by applying the standard addition technique.

Statistical comparison between results obtained by applying the proposed procedures and those obtained by applying the reported method [4] for OSP showed less calculated t and F values than the tabulated ones revealing no significant difference in accuracy and precision, at 95% confidence limit [20]; Table 5.

Table 1. Regression analysis and validation parameters for the determination of OSP by the proposed methods

Parameter	Densitometric method	UPLC method
λ_{max} (nm).	220	207
Linearity range	1-15 (μg/spot)	6-14 (μgmL^{-1})
Regression equation		
slop± SD(S_Y)	699.5209±6.71	8.7134 ± 0.044
Intercept ± SD(S_X)	536.3395± 61.02	-0.69087 ± 0.46
SD of residual(S_{YX})	258.26	0.242
Correlation coefficient (r^2)	0.99981	0.99996
Accuracy (Mean± SD%)	100.32 ± 0.57	99.18 ± 0.34
Precision (RSD %)		
Intraday	1.76	1.63
Interday	1.02	0.54
LOD	0.098 (μg/spot)	0.150 (μgmL^{-1})
LOQ	0.37 (μg/spot)	0.501 (μgmL^{-1})

Table 2. Determination of OSP in laboratory prepared mixtures with its degradation product by the proposed methods

Densitometric method			UPLC method		
Intact (µg/spot)	Degraded (µg/spot)	Recovery % of Intact	Intact (µg mL^{-1})	Degraded (µg mL^{-1})	Recovery % of Intact
13.5	1.5	97.03	10.8	1.2	101.31
11.5	3.5	98.26	9.6	2.4	101.32
9.5	5.5	98.33	8.4	3.6	100.95
7.5	7.5	97.33	7.2	4.8	102.04
5.5	9.5	101.80	6	6	101.66
3.5	11.5	97.17	4.8	7.2	101.66
1.5	13.5	101.33	3.6	8.4	101.19
			2.4	9.6	101.33
			1.2	10.8	102.25
Mean% ± SD		98.75±1.92			101.52±0.41

Table 3. Robustness results for the determination of OSP by the proposed UPLC method

Mobile phase ratio Buffer : MeOH	K`	N	R	α	T
81/19	1.29	4586	4.75	3.42	0.85
80/20	1.23	4670	4.50	3.04	0.89
79/21	1.19	4815	4.45	3.41	0.91
pH					
3.6	1.34	4512	4.26	3.12	0.83
3.5	1.23	4670	4.39	3.22	0.89
3.4	1.17	4389	4.28	3.22	0.85

Table 4. Application of standard addition technique for the determination of OSP by the proposed methods

Densitometric method				UPLC method			
Recovery ± SD%	Standard addition			Recovery ± SD%	Standard addition		
	Claimed taken (µg/spot)	Pure added (µg/spot)	Recovery % of pure added		Claimed taken (µgmL^{-1})	Pure added (µgmL^{-1})	Recovery % of pure added
	6	2	99.80	100.79±1.23	6	6	98.81
100.25±0.82	6	4	99.59		6	7	97.92
	6	6	99.92		6	8	97.01
	6	8	99.79				
Mean ± SD%			99.92 ± 0.13				97.91 ± 0.90

Table 5. Results obtained by the proposed method compared with reported method for determination of OSP in pharmaceutical dosage form

Parameters	Taminil-N® capsule		
	Densitometric method	UPLC method	Reported method
Linearity range	1-15 (µg/spot)	6-14 (µgmL^{-1})	20-70 (µgmL^{-1})
N	4	6	4
Mean %	100.25	100.79	100.38
SD	0.829	1.23	1.67
Variance	0.672	1.51	2.78
t-	0.354 (2.45)	1.40 (2.31)	---
F-	4.13(9.12)	1.84 (9.01)	---

-The values in parenthesis are the theoretical t- and F- at P = 0.05.

-Reported method [4] for determination of OSP depends on measuring the UV absorbance in methanol at 221.4 nm.

4. CONCLUSION

The proposed study describes densitometric and UPLC methods for estimation of oseltamivir phosphate in presence of its degradation product. The methods were validated and found to be simple, accurate, precise and selective. The two methods proved their ability to be used for stability indication of the drug. Therefore, they can be conveniently adopted for estimation, stability studies and routine quality control analysis of oseltamivir phosphate.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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