J Fluoresc (2009) 19:673–679
DOI 10.1007/s10895-009-0461-3

ORIGINAL PAPER

Simple and Sensitive Spectrofluorimetric Method for the Determination of Oseltamivir Phosphate in Capsules Through Derivatization with Fluorescamine

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Received: 26 July 2008 / Accepted: 16 January 2009 / Published online: 28 January 2009

Abstract A new, simple and sensitive spectrofluorimetric method has been developed for the determination of oseltamivir phosphate (OSP) in capsules. The method is based on the reaction between oseltamivir and fluorescamine in borate buffer solution of pH 8.50 to give highly fluorescent derivatives that are measured at 483 nm using an excitation wavelength of 381. The different experimental parameters effecting the development and stability of the reaction product were carefully studied and optimized. The fluorescence intensity concentration plot is rectilinear over the range 50–450 ng mL^{-1} with a lower detection limit (LOD) of 1.219 ng mL^{-1} and limit of quantitation (LOQ) of 4.064 ng mL^{-1}. Selectivity was validated by subjecting stock solution of OSP to acidic, basic, oxidative, and thermal degradation. No interference was observed from excipients present in formulations. The developed method was successfully applied to determination of the drug in capsules. The mean % recovery (n=6) was 100.08. The results obtained were in good agreement with those obtained using a reported spectrophotometric method.

Keywords Oseltamivir phosphate · Fluorescamine · Spectrofluorimetry · Capsules

Introduction

Oseltamivir phosphate (OSP) (3R,4R,5S)-4-acetylamino-5-amino-3(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester, phosphate (Fig. 1) is an antiviral drug used in the treatment and prophylaxis of both influenza virus A and influenza virus B. OSP is an ethyl ester pro-drug that is rapidly and extensively metabolized by esterases in the gastrointestinal tract and liver to its active form, oseltamivir carboxylate (OSC) [1–4]. There are some analytical methods in the literature for the analysis of OSP or its active metabolite, OSC in the biological fluids and pharmaceutical preparations. Three high performance liquid chromatography (HPLC) methods with fluorescence in plasma [5] and with UV detection in human serum [6] and in biological materials [7] have been reported for the analysis of OSC. An enzymatic assay based on neuraminidase inhibition has also reported for analysis of OSC [8]. A liquid chromatography/mass spectrometry (LC/MS) with solid phase extraction [2] and LC-tandem MS [9] methods for determination of OSP and OSC in human and animal plasma, urine and saliva have been reported. Three high performance liquid chromatography (HPLC) [10–12] and colorimetric [12] methods have been reported for determination of OSP in pharmaceutical preparations. These methods offered the required sensitivity for the analysis oseltamivir in pharmaceutical dosage forms and biological fluids; however their sophisticated instrumentation and high-analysis cost limited their use for analysis of oseltamivir (OSP). Moreover, these instruments may not available in most quality control laboratories. The reported colorimetric assay for estimation of OSP in capsules [12] is not sensitive enough with the initial determined concentration of analyte (1.50 mg mL^{-1}). The method can not also apply to analysis of drug in biological fluids, and required extraction process. Spectrofluorometry, because of its high sensitivity and selectivity, low cost, and wide availability in most quality control laboratories, is a powerful analytical techniques for the determination of drugs. To the best of our

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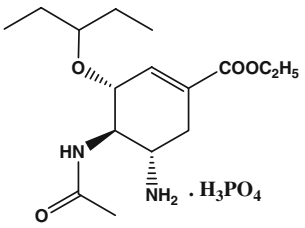
Fig. 1 Structural formula of oseltamivir phosphate



knowledge, there is no reported spectrofluorometric method for the analysis of OST. For these reasons, and growing world demand for this drug, the present study describes the simple, sensitive and economical spectrofluorometric method for the analysis of oseltamivir in its capsules with satisfactory results. The method was based on its reaction with flouerscamine in borate buffer solution of pH=8.50, to form fluorescent product which was measured fluorometrically at 483 nm (λ_{ex} 381 nm). The established method was validated with respect to specificity, linearity, precision, and accuracy. In addition, forced degradation studies were investigated by UV-Vis spectroscopy in order to prove the stability of drug to acidic, basic, oxidative, and thermal degradation.

Experimental

Material and chemicals

Oseltamivir phosphate was kindly supplied by Roche Pharmaceutical (Istanbul, Turkey). Its purity was reported to be 99.8% by Roche Pharmaceutical. Tamiflu® capsules were purchased from local drugstores. The capsules contain 75 mg of oseltamivir base (98.5 mg oseltamivir phosphate). Fluorescamine was obtained from Sigma (MO, USA). Acetone and all other chemicals and solvents were from Merck (Darmstadt, Germany). All other chemicals and solvents used were analytical grade. Water was purified by aquaMAXTM-ultra, Young lin instrument (Korea) ultrawater purification system.

Apparatus

(a) *Spectrofluorimeter*: A Shimadzu (Kyoto, Japan) spectrofluorimeter Model RF-1501 with xenon lamp and 1 cm quartz cells were used. Exicitation and emission wavelengths were set at 381 nm and 483 nm, respectively.
(b) UV-160 (Shimadzu, Tokyo, Japan) ultraviolet—visible spectrophotometer with 1 cm matched quartz and glass cells was used for spectral measurements.
(c) *pH meter*: A WTW pH 526 digital pH Meter with a combined glass electrode (Wissenschaftlich-technische Werkstatten, Weillheim, Germany) were used for pH measurements.

Solutions

Standard sample and reagent solutions

Stock solution of oseltamivir was prepared by dissolving 5.0 mg of drug (calculated as free base) in 50 mL of water. To prepare standard oseltamivir working solutions, aliquot of 1 mL of the stock solution was diluted to 10.0 mL in a volumetric flask (0.01 mg mL^{-1}). The solutions are stable for 15 days if kept in the refrigerator. The fluorescamine solution was freshly prepared at 1.4 mg/mL in acetone. Aqueous borate buffer (0.1 M, pH=8.5) solution was prepared according to the British Pharmacopoeia [13] using boric acid—potassium chloride.

General procedure and calibration graph

0.05–0.45 mL aliquots of OSP solution were transferred into a series of 10.0 mL volumetric flasks and then the volume was adjusted to about 1 mL with water. To each flask, 0.5 mL of borate buffer solution of pH 8.5 followed by 0.2 mL fluorescamine solution was added and mixed well. The reaction mixture was left for 20 min in the dark, at room temperature (25±5°C) and then completed to mark with distilled water. The fluorescence of the resulting solutions was measured at 483 nm after excitation at 381 nm. Blank experiment was carried out simultaneously. The corrected fluorescence intensity was plotted versus the final drug at six concentrations levels (ng mL^{-1}) to get the calibration graph and the regression equations were derived.

Procedure for assay of capsules

The contents of the entire ten capsules were weighed and their mean mass was determined. An accurately weight of the powder equivalent to 75 mg oseltamivir base was transferred into a 100 mL calibrated flask with about 50 mL of water. The solution was sonicated for 30 min at room temperature and then completed to volume with water and filtered, discarding the first 15.0 mL. Aliquots covering the working concentration range (cited in Table 1) were trasferred into 10 mL volumetric flasks and analyzed as described under "*general procedures and calibration graph*" section. The nominal content of the capsules was determined using the corresponding regression equation of the calibration graph.

Specificity

For the effect of excipients on the assay, used pharmaceutical excipients in formulation of oseltamivir were examined: titanium dioxide, yellow iron oxide, black iron oxide, and red iron oxide, sorbitol, and saccharin sodium. For this, 1.0 μg mL^{-1} excipient solutions was added to 300 ng mL^{-1}

Table 1 Performance data of the proposed method

Parameters	Proposed method
λ_{max}, wavelength (nm)	381/483
Concentration range (ng mL^{-1})[a]	50–450
Limit of detection, LOD (ng mL^{-1})	1.219
Limit of Quantification, LOQ (ng mL^{-1})	4.064
Regression equation Y[b]	
Slope (a)	2.0722
Intercept (b)	17.334
Correlation coefficient (r)	0.9995
$S_{y/x}$	2.302
S_a	0.842
S_b	5.899×10^{-3}

[a] Data obtained from four determination (n=4), [b] Y= aX + b, where Y is the Fluorescence intensity of a 1 cm layer of solution, a is the slope, b is the intercept and X is the concentration of the drug in ng mL^{-1} $S_{y/x}$ Standard deviation of the residuals S_a Standard deviation of the intercept of regression line, S_b Standard deviation of the slope of regression line

of final drug solution and analyzed as described under "*general procedures and calibration graph*" *section*.

In the second experiment, drug stock solution subjected to various stress conditions: 1 mL aliquots of OSP solution (0.1 mg mL^{-1}) were transferred into 10.0 mL volumetric flasks and then 1 mL of 1N HCl, 1N NaOH, 30% H_2O_2 was added. The mixture of these solutions was left at room temperature for 1 h and 2 h and at 80°C for 1 h and then all mixture solutions completed to mark with distilled water. Blank experiment was carried out simultaneously. The absorbance of the resulting solutions was measured between 200–800 nm. Each solution was analyzed in triplicate.

Results and discussion

Derivatization of oseltamivir with fluorescamine

Fluorescamine was chosen as a derivatizing agent because it is intrinsically nonfluorescent but reacts rapidly with primary and secondary amines, amino acids, peptides, proteins to yield fluorescent derivative. Excess reagent is rapidly converted to a nonfluorescent product by reaction with water, making fluorescamine useful for determining compounds of solutions with amines groups (Fig. 2) [14, 15]. OSP, which is nonfluorescent compound, has a primary amino group product and was found to react with fluorescamine in solution of pH 8.5, forming fluorescent derivative with λ maximum emission at 483 nm after excitation at 381 nm (Figs. 3, 4, 5). The derivatization reaction between oseltamivir and fluorescamine proceeded in borate buffer at room temperature in 20 min. The spectrofluorimetric properties of the product as well as the different experimental parameters affecting the development of the reaction product and its stability were investigated and optimized. Such factors were changed individually while the others were kept constant. The factors include pH, type of the buffer, concentration of the reagent, reaction time.

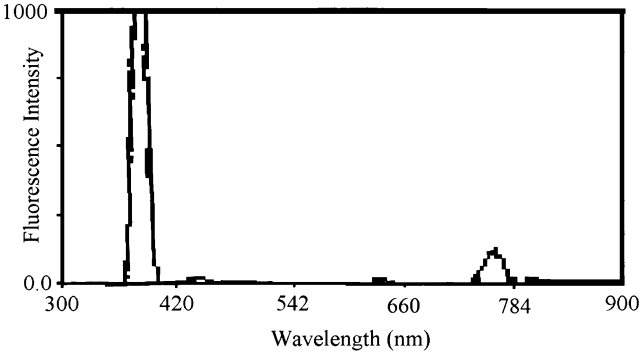


Fig. 3 The emission spectrum of OSP in water (C=300 ng mL^{-1}; λ_{ex}= 381 nm and λ_{em}=483 nm)

Effect of pH

The influence of pH on the fluorescence intensity of the reaction product was studied. Phosphate and borate buffer systems were tested, and higher fluorescence intensities were obtained when the reaction was carried out using

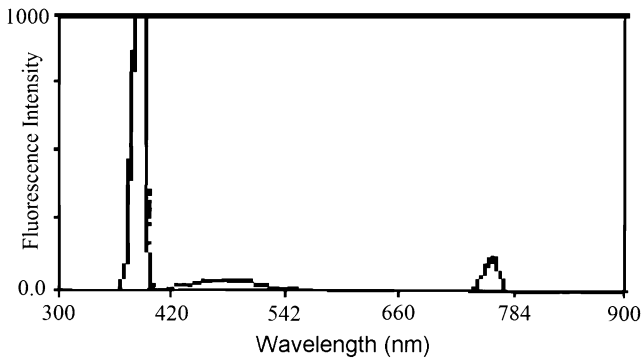


Fig. 2 The emission spectrum of fluorescamine in acetone-water (C= 28 μg mL^{-1}; λ_{ex}=381 nm and λ_{em}=483 nm)

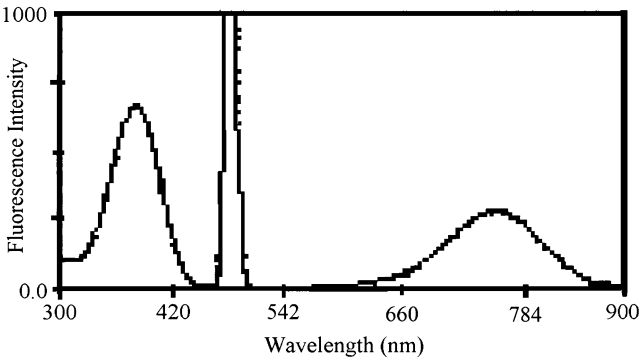


Fig. 4 Excitation spectrum of the reaction product of OSP (300 ng mL^{-1}) with fluorescamine (λ_{ex}=381 nm)

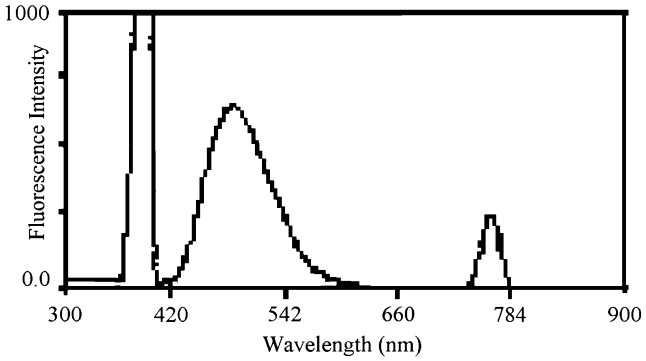


Fig. 5 Emission spectrum of the reaction product of OSP (300 ng mL^{-1}) with fluorescamine (λ_{ex}=381 nm, λ_{em}=483 nm)

borate buffer system (Fig. 6). The pH was varied over the range of 7.5–9.5 using borate buffers, and the maximum fluorescence intensity was obtained at pH 8.5 as shown in Fig. 7.

Effect of the concentration of fluorescamine

The amount of reagents on the intensity of the fluorescence development was examined by measuring the fluorescence intensity of the solutions containing a fixed concentration of OSP and varied amounts of the respective reagent. It was found that 64-fold molar excess of fluorescamine were sufficient for the maximum fluorescence (Fig. 8).

Effect of reaction time and temperature

Different time intervals were examined to ascertain the time after which the solution attains its highest fluorescence intensity. It was found that after 20 min, the reaction reaches the highest fluorescence intensity (Fig. 9). The influence of temperature on development of fluorescence was studied in the range from 30–60°C and at room temperature (25±5°C). It was found that the rate of reaction was not increased with the increasing temperature, and

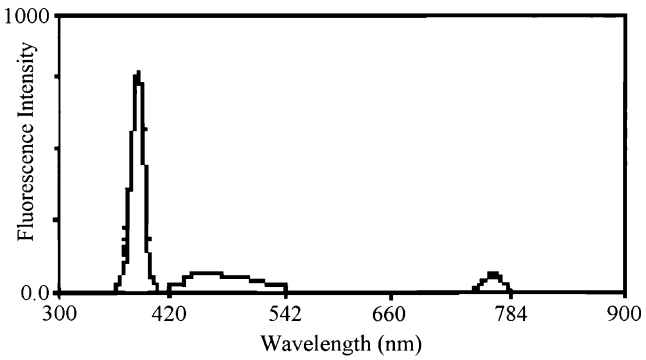


Fig. 6 Emission spectrum of fluorescamine—borate buffer system ((λ_{ex}=381 nm, λ_{em}=483 nm)

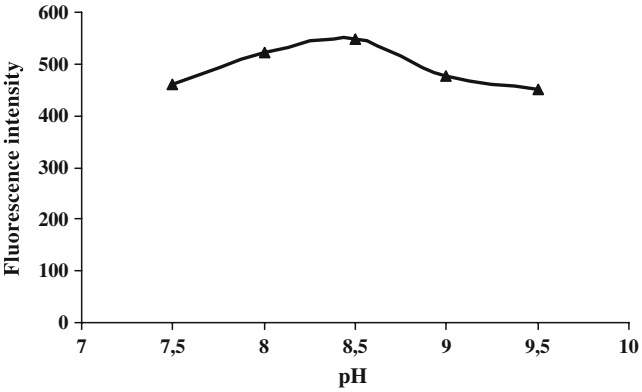


Fig. 7 Effect of the pH on the development of the reaction product of oseltamivir (250 ng mL^{-1}) with fluorescamine

readily occurred at room temperature and remains stable for about 6 h.

Quantification

Under the described experimental conditions, a calibration graph for proposed method was constructed. Table 1 summarizes the value for Beer's law limit, regression equation, correlation coefficient, limit of detection (LOD), and limit of quantitation (LOQ) for method. For evaluation of linearity, determination of OSP was done at six concentration levels, and each concentration was analyzed for four times. The data given in Table 1 indicated that linear relationship was found between the fluorescence intensity at λ_{max} and the concentration of the drug in the range 50–450 ng mL^{-1}. The correlation coefficient was 0.9995 indicating good linearity. LOQ was determined by establishing the lowest concentration that can be measured according to ICH guidelines [16]. LOD was determined by establishing the minimum level at which the analyte can be

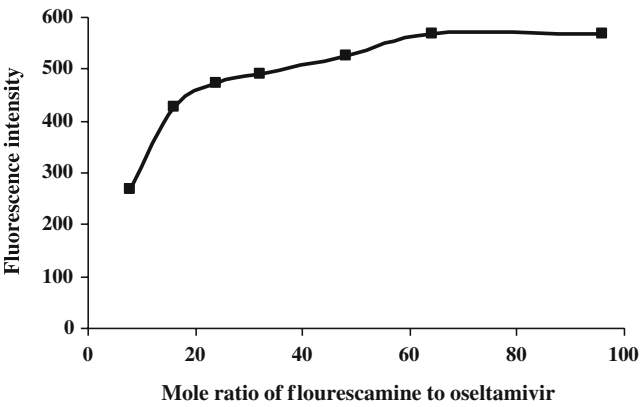


Fig. 8 Effect of amount of fluorescamine on the fluorescence intensity of the reaction product of oseltamivir (250 ng mL^{-1}) at pH 8.5

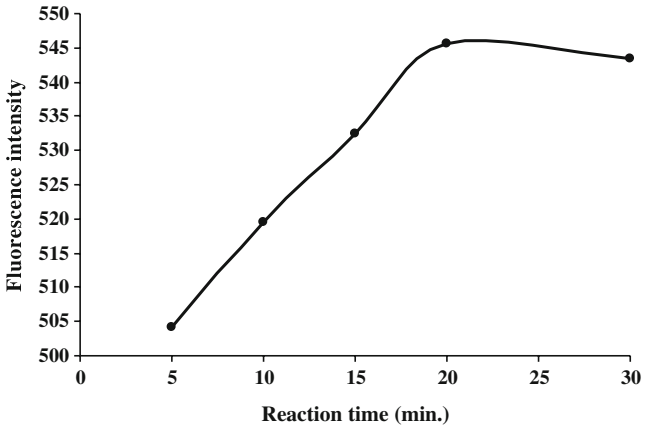


Fig. 9 Effect of reaction time of on the fluorescence intensity of the reaction product of oseltamivir (250 ng mL^{-1}) with fluorescamine at pH 8.5

reliably detected (Table 1). LOQ and LOD were calculated according to the following equation.

$$LOQ = 10\sigma/s$$

$$LOD = 3\sigma/s$$

Where, σ is the standard deviation of the intercept of regression line; s is the slope of the calibration curve. LOD and LOQ values of proposed method were calculated to be 1.219 ng mL^{-1} and 4.064 ng mL^{-1}, respectively.

Accuracy and precision

The interday and intraday precisions were examined by analysis of OSP with the concentration of 150.0 ng mL^{-1}, 250.0 ng mL^{-1}, and 350.0 ng mL^{-1} (each n=6) for seven consecutive days. The reagent solutions were prepared freshly and analyzed as described under "*general procedures and calibration graphs*" section. The analytical results (Table 2) obtained from these investigations revealed that the standard deviations (SD) values for intraday and interday precisions were 0.57–1.69 and 0.47–2.48, respectively. The relative standard deviation (R.S.D, %) values for intraday and interday precisions were 0.23–0.60 and 0.31–0.69, respectively. The recoveries (%) were 99.43–101.20 and 99.43–102.82, respectively. The repeatability and reproducibility were fairly good, as indicated by low values of R.S.D.

Recovery

To study the accuracy of the proposed methods, the standard addition method was applied. For this, known amounts of pure drug were added to a known amount of capsule solution, and the mixtures were analyzed by the proposed procedures in the six replicates. From the amount drug found, the percentage recovery was calculated from: Recovery %= $[(C_t - C_u)/ C_a] \times 100$, where C_t is the total concentration of the analyte found; C_u, concentration of the analyte presents the formulation; C_a, concentration of the pure analyte added to the formulation. The results were reproducible with low S.D., R.S.D. (%) and with high percentage average recovery (%) values as shown in Table 3.

Table 2 Interday and intraday assay of oseltamivir base by the proposed method (n=6)

Proposed method	Concentration (ng mL^{-1})		Recovery (%)	R.S.D. (%)
	Taken	Found ± S.D.		
Interday	150.0	152.03±0.47	101.35	0.31
	250.0	248.58±0.90	99.43	0.36
	350.0	359.87±2.48	102.82	0.69
Intraday	150.0	151.80±0.91	101.20	0.60
	250.0	248.97±0.57	99.59	0.23
	350.0	348.03±1.69	99.43	0.50

Specificity

The influence of used capsules excipients on proposed method were examined as described in experimental section and no interference was observed from these excipients. The specificity and stability were also investigated by accelerated stress testing. Oseltamivir solutions were subjected to degradation under alkaline, acid and oxidative conditions for 1 h, 2 h at room temperature and at 80°C for 1 h. The absorbance spectra of drug solutions after subjected to different stress conditions were shown in Figs. 10, 11, and 12. There was significant degradation of OSP by basic hydrolysis in both waiting and heating conditions. The moderately degradation in acidic condition within 2 h at room temperature was formed while no meaningful changes were observed when it was subjected to thermal stress at 80°C for 1 h. After waiting or heating the drug solutions in hydrogen peroxide conditions, the most severe degradation was observed. No degradation was observed in neutral conditions. A literature survey revealed that the drug stability has been investigated in presence of

Table 3 Determination of oseltamivir base in capsules by standard addition method (n=6)

Concentration (ng mL^{-1})			Recovery (%)	R.S.D.(%)
Taken	Added	Found ± S.D.		
75.0	50.0	127.23±1.37	101.78	0.48
150.0	50.0	200.15±2.13	100.08	0.49
150.0	100.0	254.99±1.27	101.97	0.22

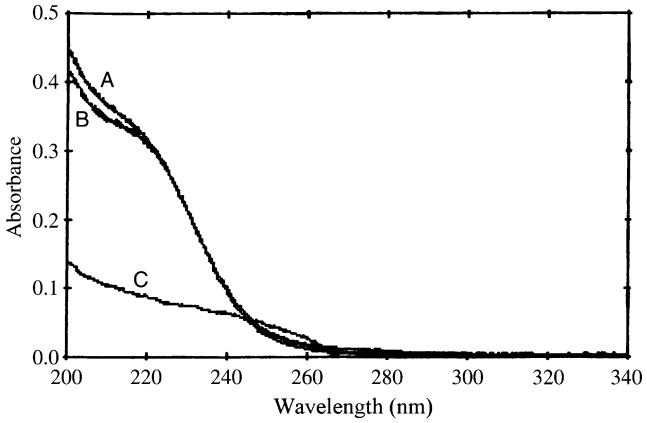


Fig. 10 Absorption spectra of OSP solutions **a** before heating, **b** subjected to thermal stress at 80°C for 1 h, and **c** subjected to hydrogen peroxide oxidation at 80°C for 1 h. Concentration of drug was 10.0 μg mL^{-1}

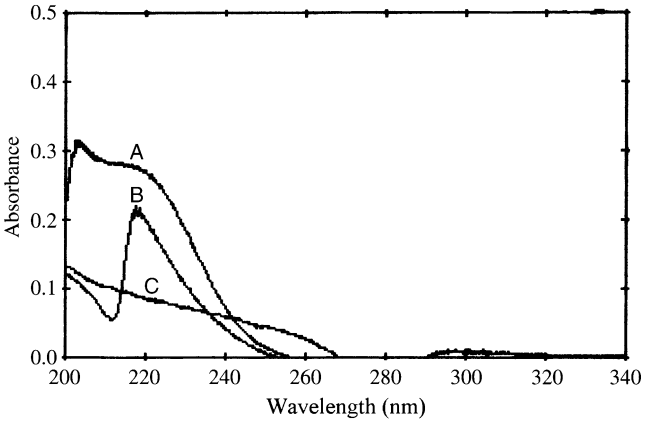


Fig. 12 Absorption spectra of OSP solutions (at room temperature for 2 h) subjected to **a** acidic hydrolysis, **b** alkaline hydrolysis and **c** hydrogen peroxide oxidation. Concentration of drug was 10.0 μg mL^{-1}

degradation products (base, acid and hydrogen peroxide) by HPLC very recently [17] and similar degradation results were observed when the degradation test determined by UV-Vis spectrophotometer in this study. According to literature survey, this is the first time the degradation of oseltamivir under different temperature and force condition were determined by ultraviole absorption spectrophotometry.

Application to capsules

The proposed method was successfully applied to the determination of OSP in capsules. As it shown in Table 4, there is a satisfactory agreement between the results of the proposed and reference method [12] (colorimetric method based on the formation ion pair between drug and Congo red) as well as the labeled amounts on the pharmaceutical product. The mean recovery value was 100.08% for proposed method. Its standard deviation value was found to be 0.84 (Table 4). Using Student's *t*-test and the varience ratio *F*-test at 95% confidence level, revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively.

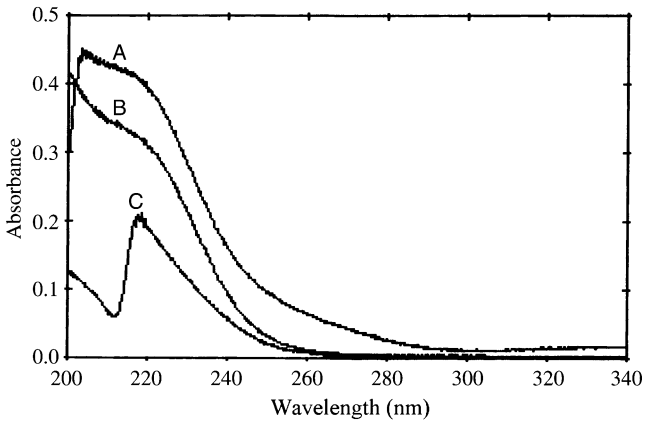


Fig. 11 Absorption spectra of OSP solutions (at 80°C for 1 h) subjected to **a** acidic hydrolysis, **b** thermal stress, **c** alkaline hydrolysis. Concentration of drug was 10.0 μg mL^{-1}

Conclusion

The proposed method has the advantage of being simple, sensitive, selective, accurate, and suitable for routine analysis of oseltamivir in pure form and in its capsules forms without any interference from the excipients. Also, the advantages of the proposed method in comparison to the reported methods are less toxic organic compound and solvent used. Furthermore, the developed method required no extraction step and drug can be measured directly in aqueous solution and stable for about 6 h. Since the proposed method allowed us to the assay of the drug at very low detectable value (1.219 ng mL^{-1}), it can be extended to chromatographic separation by high performance liquid chromatography using a fluorescence detector. But, the

Table 4 Statistical evaluations of the results obtained by the proposed and comparison methods for the assay of OST in capsules (each capsules contains 75 mg of oseltamivir base)

Statistical values	Proposed methods	Reference method [12]
Mean (mg)[a]	75.06	76.69
Recovery (%)[b]± S.D.	100.08±0.84	102.25±1.26
R.S.D. (%)[b]	1.12	1.64
Confidence limits	75.05±0.57	
t-calculated [b]	1.68	
F-calculated [b]	0.44	

Theoretical values at 95% confidence limit; t=2.23 and F=5.05
[a] Six independent analyses, [b] Each values is the average of six determination

developed method may not be applicable for the analysis of OSP in biological fluids since the other chemicals with primary or secondary amines group in the maintained matrix will interfere with the assay.

Acknowledge This work was supported by Research Fund of the Istanbul University. Project number: 478/27122005.

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