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Spectrophotometric and spectrofluorimetric determination of oseltamivir phosphate using 4-chloro-7-nitrobenzo-2-oxa 1,3-diazole

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ABSTRACT

Two simple and sensitive methods were introduced and validated for the determination of oseltamivir phosphate. These methods allowed quantification of oseltamivir phosphate by monitoring its reaction with 4-chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl) in buffered medium (pH 9), either by recording absorbance of the final reaction product at a wavelength of 468 nm or by measuring its fluorescence emission intensity at 535 nm using an excitation wavelength of 480 nm. Different factors affecting the reaction were optimized. Linear relationships with good correlation coefficients (0.9999–0.9997) were found between the absorbance, fluorescence intensity and the concentrations of oseltamivir phosphate in the range of 4–24 and 0.8–12 $\mu\text{g.mL}^{-1}$ for spectrophotometric and spectrofluorimetric methods, respectively. The limits of assays detection were 0.51 and 0.12 $\mu\text{g.mL}^{-1}$ for the first and second method, respectively. Satisfactory precisions of the methods were obtained. The proposed methods were successfully applied to the analysis of oseltamivir phosphate in pure and pharmaceutical dosage forms with good accuracy and the results were compared with those of the pharmacopeial method. The stoichiometry of the reaction was determined and the reaction pathway was postulated.

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KEYWORDS

Swine flu;
Avian flu;
Oseltamivir phosphate;
NBD-Cl;
Spectrophotometry;
Spectrofluorimetry.

INTRODUCTION

Oseltamivir phosphate is an antiviral drug that belongs to a group of medicines called neuraminidase inhibitors (NAIs) used in the treatment and prophylaxis of influenza A (H5N1 subtype commonly known as avian flu and H1N1 subtype commonly known as swine flu) and influenza B^[1,2]. Oseltamivir phosphate has recently become an official drug in the international phar-

macopeia^[3]. Few liquid chromatographic methods have been published for the analysis of oseltamivir phosphate. The determination of oseltamivir carboxylate in pre-clinical plasma samples was reported using HPLC with pre-column fluorescence derivatization^[4]. Oseltamivir phosphate and oseltamivir carboxylate were determined by HPLC-Tandem MS in human and animal plasma and urine after solid phase extraction^[5]. Also HPLC-UV detection methods were published for the estima-

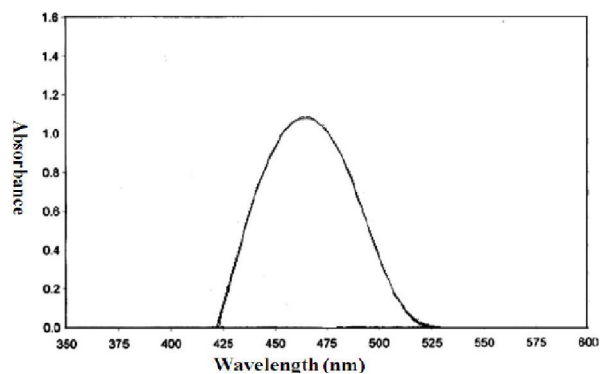


Figure 1 : Absorption spectra of oseltamivir phosphate-NBD complex at 468 nm

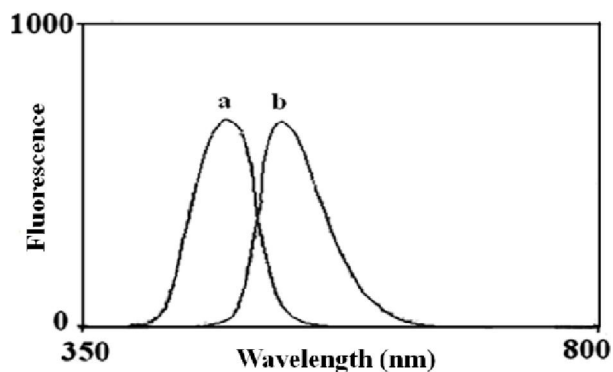


Figure 2 : Excitation (a) and emission (b) spectra of oseltamivir phosphate-NBD complex at 535 nm ($\lambda_{ex} = 480$ nm)

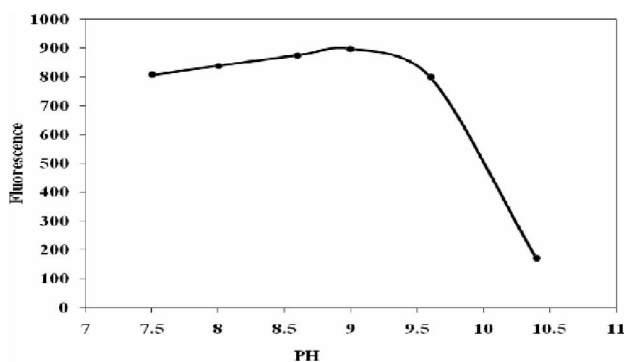


Figure 3 : Effect of pH of borate buffer on the fluorescence of oseltamivir phosphate ($12 \mu\text{g.ml}^{-1}$)-NBD complex

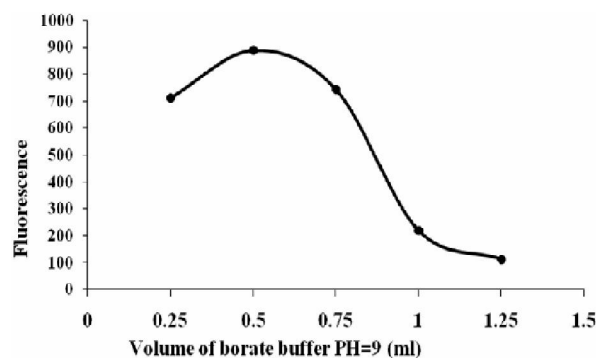


Figure 4 : Effect of volume of borate buffer on the fluorescence of oseltamivir phosphate ($12 \mu\text{g.ml}^{-1}$)-NBD complex

tion of oseltamivir phosphate in tamiflu® capsules^[6,7].

These methods offered the required sensitivity and selectivity for the analysis of oseltamivir phosphate; however their sophisticated instrumentation and high-analysis cost limited their use in quality control laboratories for analysis of oseltamivir phosphate in its pharmaceutical dosage forms. Moreover, these instruments are not available in most quality control laboratories specially, third world countries. Since avian flu became an important issue worldwide and the presence of tamiflu® capsules in the third world markets occurred, the necessity of the presence of simple, accurate and cheap methods for the determination of oseltamivir phosphate in tamiflu® capsules in quality control laboratories of the third world countries arose. In general, spectrophotometry and spectrofluorimetry are considered ones of the most convenient analytical techniques, because of their inherent simplicity, low cost, and wide availability in most quality control laboratories. To the best of our knowledge, there were no reported spectrophotometry or spectrofluorimetry methods on oseltamivir phosphate being an aliphatic compound with no conjugation. For

these reasons, the aim of our study was to present two simple, sensitive and economical methods for the analysis of oseltamivir phosphate in its pure and pharmaceutical dosage forms. These methods were based on measuring of the absorbance and fluorescence intensity of the condensation reaction product of the drug with 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) reagent in basic buffered medium.

EXPERIMENTAL

Apparatus

UV/VIS spectrophotometer (UNICAM UV 300) thermospectronic was used for spectrophotometric determinations. RF-1501 Spectrofluorimeter (Shimadzu, Japan) with 1-cm matched quartz cells was used for all spectrofluorimetric determinations. Digital pH meter (Hanna 8417). Thermostatically controlled water bath, (Memmert, Germany).

Chemicals and reagents

Oseltamivir phosphate (F. Hoffmann-La Roche Ltd,

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Basel, Switzerland) was used. Its purity was $98.99\% \pm 0.66$ as assayed by the official HPLC method^[3]. Daily freshly prepared 0.1 % (w/v) 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl; sigma) in methanol. Borate buffer pH 9 (prepared according to B.P). Methanol (labscan, ltd, Dublin, Ireland). All solvents and other chemicals used were of analytical grade.

Market samples

Tamiflu[®] capsules (F. Hoffmann-La Roche Ltd, Basel, Switzerland) are labeled to contain 75 mg oseltamivir equivalent to 98.5 mg oseltamivir phosphate per capsule.

Standard solutions

(1) Stock standard solution

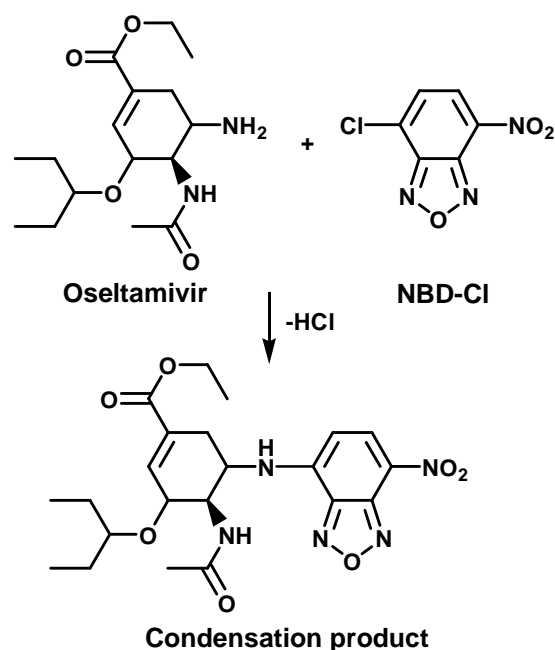
An accurately weighed 20 mg of oseltamivir phosphate was transferred into 50-mL volumetric flask, 30 mL of methanol were added to dissolve and the volume was completed to the mark with the same solvent. Aliquot equivalent to 8 mg of oseltamivir phosphate was transferred from the previous solution into 100-mL volumetric flask and the volume was completed to the mark with methanol to obtain a working standard solution of $80 \mu\text{g} \cdot \text{mL}^{-1}$.

(2) Capsules solution

The content of 3 capsules (Tamiflu[®]) were accurately weighed and finely powdered. A quantity of the mixed powder equivalent to 8 mg of oseltamivir phosphate was transferred into a 100-mL volumetric flask, extracted with 40 ml methanol, sonicated for 15 min., completed to the volume with the same solvent, shaken well for 10 min. and filtered.

Analytical procedure

Aliquots equivalent to 40-240 μg (for spectrophotometric method) and 8-120 μg (for spectrofluorimetric method) of oseltamivir phosphate working standard solution or capsules solution were transferred into a series of test tubes, 0.5 ml of borate buffer pH 9 and 1 ml of NBD-Cl solution (0.1 % w/v in methanol) were added to each test tube. The reaction was allowed to proceed in a boiling water bath for 20 minutes. Then the test tubes were cooled and 0.5 ml of 0.5 M HCl was added and mixed well. The contents were quanti-



Scheme 1 : The suggested scheme of the reaction mechanism between oseltamivir phosphates with NBD-Cl

tatively transferred into 10-ml volumetric flasks and the volume was completed to the mark with methanol. The absorbance and fluorescence intensity of the resulting solutions were measured at 468 nm (for spectrophotometric method) and 535 nm ($\lambda_{\text{ex}} = 480 \text{ nm}$) for spectrofluorimetric method, against blank solutions treated similarly.

RESULTS AND DISCUSSION

7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) reagent is an activated halide derivative that reacts with primary and secondary amines and gives a colored and/or fluorescent derivative^[8,9]. Oseltamivir phosphate contains a primary aliphatic amino group that is found to react with NBD-Cl in alkaline medium. This condensation reaction hasn't been reported yet for oseltamivir. Therefore, this reaction is investigated in the present study. Under the recommended conditions, the derivatized oseltamivir product is found to have absorbance at 468 nm and exhibits highest fluorescence intensity at $\lambda_{\text{em}} 535 \text{ nm}$ when excited at $\lambda_{\text{ex}} 480 \text{ nm}$. (Figure 1 & 2) show the spectral characteristics of the derivatized Oseltamivir phosphate with NBD-Cl reagent.

Optimization of the reaction conditions

Different experimental parameters affecting the

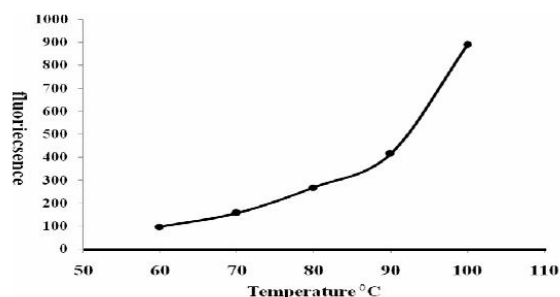


Figure 5 : Effect of temperature on the fluorescence of oseltamivir phosphate ($12 \mu\text{g.ml}^{-1}$)-NBD complex

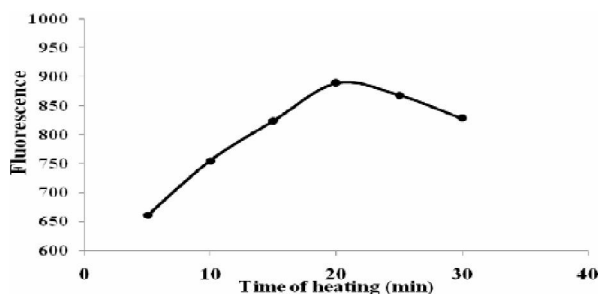


Figure 6 : Effect of heating time on the fluorescence of oseltamivir phosphate ($12 \mu\text{g.ml}^{-1}$)-NBD complex

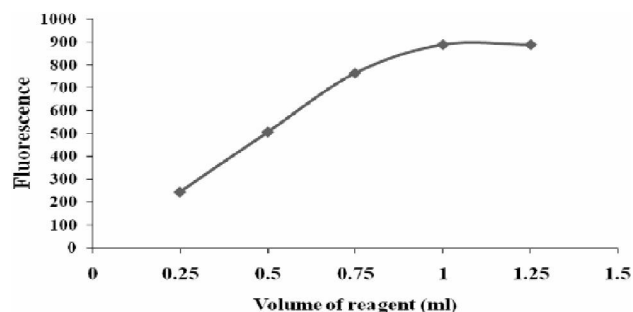
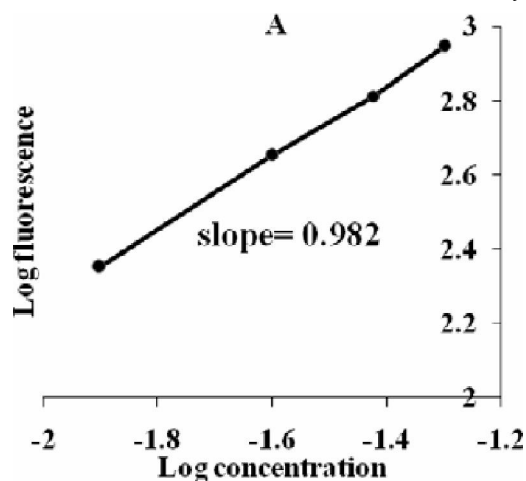


Figure 7 : Effect of volume of NBD-Cl 0.1 % w/v in methanol on the fluorescence of oseltamivir phosphate ($12 \mu\text{g.ml}^{-1}$)-NBD complex

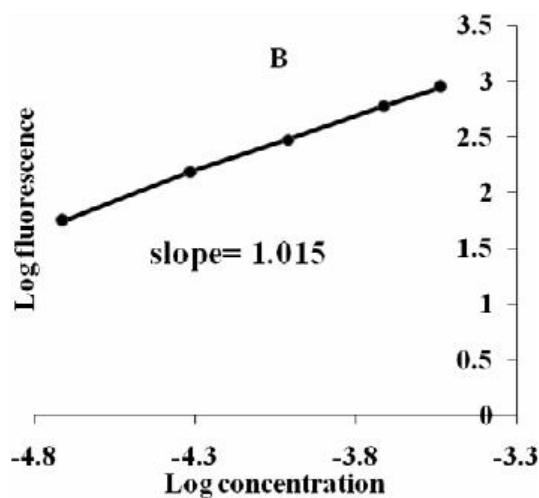
reaction development and its stability were carefully studied and optimized. Investigating the effect of pH indicated that the color and fluorescence intensity was pH dependent and the optimum pH was found to be 9 by using borate buffer (Figure 3).

At higher pH values, the background fluorescence of the reagent increased resulting in a net decrease in fluorescence of the drug solutions. The effect of the volume of borate buffer was studied and it was found that 0.5 ml was sufficient to get the highest fluorescence intensity, (Figure 4).

Regarding the influence of temperature on the reaction between oseltamivir phosphate and NBD-Cl



(A) Variable NBD concentrations and constant oseltamivir phosphate concentration



(B) Variable oseltamivir phosphate concentrations and constant NBD concentration

Figure 8 : Stoichiometry of the reaction between oseltamivir phosphate and NBD adopting limiting logarithmic method

reagent, the range of 25-100°C with constant heating time was used; the results showed that increasing the temperature to 100°C accelerated the reaction, (Figure 5). Also the influence of time was studied in order to optimize the assay conditions. The results showed that the maximum intensity was attained after 20 min and that longer reaction time decreased the fluorescence intensity, (Figure 6). Therefore, the experiments were carried out at 100°C for 20 min all over the work.

It was necessary to acidify the reaction mixture to about pH 2.0 before carrying out the measurements in order to quench the emission of the reagent blank as

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TABLE 1 : Results of assay validation obtained by applying the proposed methods

Parameters	Spectrophotometric method	Fluorimetric method
Accuracy		
Mean \pm RSD	99.41 \pm 0.338	98.58 \pm 0.453
Precision		
Intraday	0.292	0.526
Interday	0.321	0.709
L.O.D.	0.510	0.125
L.O.Q.	1.545	0.377
Linearity		
Slope	0.0586	7.3054
S.E. of Slope	0.0003	0.7766
Intercept	-0.0609	2.5233
S.E. of Intercept	0.0053	5.2517
Correlation coefficient	0.9999	0.9997
Range (μ g/ml)	4-24	0.8-12

NBD-Cl is hydrolyzed in alkaline medium to give NBD-OH which has excitation and emission maxima at 462 and 532 nm, respectively. Therefore, 0.5 ml of 0.5 M hydrochloric acid solution was added to the reaction mixture before measurements. Several diluting solvents were tested, methanol was found to be the best diluting solvent, and therefore it was selected.

With respect to the influence of the concentration of NBD-Cl, different volumes of 1 mg/ml solution of the reagent was used. It was found that increasing the volume of the reagent increased the fluorescence intensity up to 1 ml, after which no more increase in fluorescence was obtained; therefore, 1 ml reagent solution was added, (Figure 7).

Stoichiometry of the reaction

The stoichiometry of the reaction was investigated by adopting the limiting logarithmic method^[10] where two straight lines were obtained. The values of the slopes of the two lines were 0.982 and 1.015 (Figure 8) proving that the molar ratio of NBD-Cl: oseltamivir was 1:1, and the reaction pathway was proposed as in (Scheme 1). The reaction mechanism can be explained by the formation of a complex which is produced through a nucleophilic substitution reaction type. One molecule of NBD-Cl condenses with one molecule of the drug through its primary aliphatic amino group.

TABLE 2 : Determination of oseltamivir phosphate in drug substance and pharmaceutical product using the proposed methods

Item	Mean \pm R.S.D.		
	Spectrophotometric method	Fluorimetric method	HPLC method
Drug substance	99.41 \pm 0.338	98.58 \pm 0.453	98.99 \pm 0.663
t (2.31) ^a	1.276	1.156	-
F(6.39) ^a	3.812	2.163	-
Tamiflu® capsule			
batch No 36066	99.14 \pm 0.635	98.71 \pm 0.393	98.50 \pm 1.033
t ^a	1.122 (2.26)	0.391 (2.36)	-
F ^a	2.615 (9.01)	6.884 (9.12)	-
Standard addition ^b	100.01 \pm 0.625	98.85 \pm 0.455	-

^aTheoretical values for t and F at $p=0.05$. ^bAverage of three different determinations

Validation of proposed methods

Upon applying the optimum reaction conditions, the calibration curves were constructed in the concentration range of 4-24 μ g/ml for the spectrophotometric method and 0.8-12 μ g/ml for the spectrofluorimetric method where acceptable intercepts and very good correlation coefficients were obtained. The accuracy of the proposed methods was demonstrated using six replicate measurements. Also interday and intraday precisions were estimated from three replicates of three concentration levels repeated over three days. The assays gave satisfactory results as the relative standard deviations were less than 2. The limit of detection and limit of quantitation were calculated for both measurements. The results of validation were presented in (TABLE 1).

Analysis of pharmaceutical dosage form

Tamiflu® capsules were analysed by the proposed methods as well as the official method^[3] and the obtained results were statistically compared with each other. Also statistical comparison between the results obtained by applying the suggested methods and official method for the determination of oseltamivir in bulk powder was calculated (TABLE 2). With respect to t- and F-tests there were no significant differences found between the calculated and theoretical values of the proposed method and the official one at 95% confidence level. This indicated similar accuracy and precision in the analysis of oseltamivir in bulk powder and capsules.

CONCLUSION

This study presented a validated spectrophotometric and spectrofluorimetric analysis of oseltamivir phosphate in raw material and Tamiflu® capsules. The proposed methods are simple, accurate and precise. They were developed via complexation with NBD-Cl. The proposed methods are of great value in quality control laboratories in developing countries owing to their improved simplicity, sensitivity, low cost and their independence on expensive instruments or critical analytical reagents.

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