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ORIGINAL ARTICLE

Validated spectrophotometric methods for the evaluation of oseltamivir counterfeit pharmaceutical capsules

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Abstract Four rapid, reliable and economical spectrophotometric methods have been established for the quantitative determination of Oseltamivir phosphate (OST) without the interference of ascorbic acid (ASC) found in some of its counterfeit capsules. The first method involves the use of derivative spectrophotometry with the zero-crossing technique where OST was easily determined using its ^{1}D ($\Delta\lambda = 3$) at 219 nm. The second method is based on a first-order derivative ratio spectrophotometry (^{1}DD, $\Delta\lambda = 5$) where 218 nm was selected for its quantification, while the third method applies a more advanced spectrophotometric method based on the ratio difference spectrophotometry (RD) in which the difference in absorbance ratio was measured between 217 and 210 nm. In the fourth method, difference spectrophotometric method (ΔA) is applied by subtracting absorbance at 252 from that at 263 nm where the difference in absorbance was zero for ASC. The proposed methods were validated for linearity, accuracy, precision and selectivity. Synthetic mixtures of different proportions and commercial capsules were assayed by the proposed methods and the results revealed good accuracy and repeatability of the developed methods.

1. Introduction

OST (Ethyl (3R, 4R, 5S)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1 cyclohexene-1-carboxylate phosphate; Fig. 1a) is a prodrug of Oseltamivir carboxylate, an inhibitor of the enzyme neuraminidase, which has a role in the infectivity and replication of influenza A and B viruses. It is used orally as capsules or suspension for the treatment and post-exposure prophylaxis of influenza A and B.[1]

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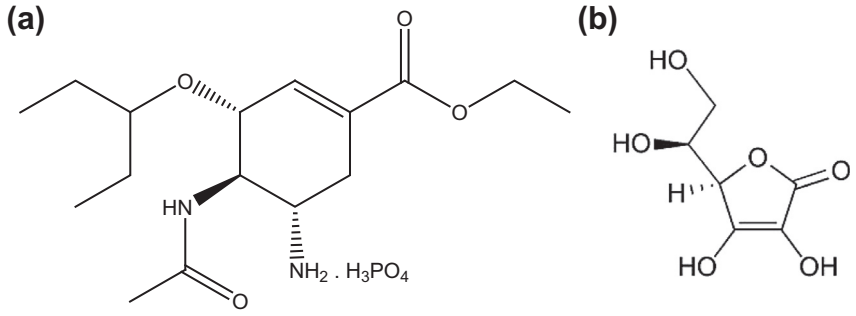

Figure 1 Chemical structure of OST (a) and ASC (b).

OST recently became official in the USP 2011[2] which describes the HPLC method for its assay in bulk powder as well as in capsules. Some spectrophotometric methods[3–5] were reported for the assay of OST. HPLC methods were described for the analysis of OST both in bulk and in commercial pharmaceuticals.[6–8] Also a selective HPLC method was applied to evaluate potentially counterfeit OST capsules.[9] Few HPLC methods were developed for the assay of OST in biological fluids using liquid chromatography coupled to tandem mass spectrometry.[10,11] Other methods were applied to its quantification in its capsules like spectrofluorimetry[12] and capillary electrophoresis.[13] A new HPTLC method was recently developed for the evaluation of such OST counterfeited pharmaceutical formulations.[14]

The specter of an avian influenza pandemic has given OST much notoriety, and anticipation of the potential public health threat has prompted a demand for its pharmaceutical products. Consequently, criminal elements have already produced counterfeit OST preparations and specific cases of the seizure were observed in the United Kingdom of 5000 counterfeit packets of the OST products.[15] Also, ASC (Fig. 1b) was reported widely in counterfeit OST products which lacked the active ingredient. These products had been purchased through the internet and easily have gone unnoticed in the developing countries, where insufficient analytical resources hamper the ability to monitor and preserve drug quality.[16] This work describes the application of derivative, derivative ratio, ratio difference and difference spectrophotometry for the quantitative estimation of OST in the presence of ASC. The proposed methods do not require solving equations or working with additional sophisticated software. In addition, the methods are direct, inexpensive and do not need separation, specific detectors or pretreatment steps enabling its application in quality control analysis of OST in its capsules.

2. Experimental

2.1. Instruments

UV/Visible spectrophotometric measurements were carried out using a Shimadzu Model1800 ultraviolet–visible spectrophotometer.

2.2. Materials and reagents

OST was obtained from The Nile Company for Pharmaceuticals and Chemical Industries (Cairo, Egypt) while ASC was obtained from El-Borg (Alexandria, Egypt). Taminil-N capsules were purchased locally. Analytical-grade methanol was from S.D. Fine Chemicals (Mumbai, India).

2.3. Standard solutions

Stock standard solutions of 1000 μg mL^{-1} OST or ASC were prepared in methanol. The standard solutions were appropriately diluted to prepare the working standard solutions using methanol.

2.4. Construction of calibration curves

For ^1D method, aliquots from the working standard solution of OST, within the concentration range listed in Table 1, were accurately transferred into a set of 10-mL volumetric flasks. Dilution was made to volume with methanol and the absorption spectra for each solution were recorded against methanol as a blank. The ^1D amplitudes were measured at 219 nm (zero-crossing of ASC) and plotted against the concentrations of OST where a linear relationship is obtained.

Furthermore for the ^1DD method, the absorption spectra were divided (amplitude by amplitude at each wavelength) by the spectrum of a 5-μg mL^{-1} solution of ASC. The values of ^1DD amplitudes were measured at 218 nm and were found proportional to the concentrations of OST.

In the RD method, the difference between the amplitudes of the ratio spectra at 220 and 217 or 210 were plotted versus the corresponding concentrations and the regression equations were computed.

Moreover, in the ΔA method, OST was determined by plotting the difference in absorbance values at 236 and 252 nm (the difference is zero for ASC) against its corresponding concentration.

2.5. Assay of capsules

The contents of ten capsules of Taminil-N were carefully evacuated, mixed and weighed. An accurately weighed amount of the powder equivalent to 50 mg OST was transferred to a 50-mL volumetric flask. A volume of 30 mL of methanol was added and the flask was sonicated for 30 min, and then completed to volume with methanol followed by filtration. Then, the procedures were carried out on the filtrate as mentioned under construction of calibration curves.

Table 1 Regression and statistical parameters for the determination of OST using the proposed methods.

Parameters	^{1}D	^{1}DD	RD	ΔA
Linearity range (μg mL^{-1})	10.00–50.00	10.00–50.00	10.00–50.00	10.00–50.00
LOQ (μg mL^{-1})	10.00	10.00	10.00	10.00
LOD (μg mL^{-1})	3.12	3.82	4.15	4.35
Intercept (a)	-4.00×10^{-4}	-5.00×10^{-2}	-0.23	-1.86×10^{-2}
Slope (b)	2.40×10^{-3}	4.28×10^{-2}	0.14	9.67×10^{-3}
Correlation coefficient (r)	0.9990	0.9990	0.9990	0.9991
S_a	1.84×10^{-3}	3.60×10^{-2}	0.12	8.14×10^{-3}
S_b	5.45×10^{-5}	9.10×10^{-4}	2.86×10^{-3}	2.01×10^{-4}
$S_{y/x}$	1.75×10^{-3}	3.44×10^{-2}	0.10	7.31×10^{-3}
a/S_a	0.22	1.39	1.92	2.29
S_b^2	2.97×10^{-9}	8.28×10^{-7}	8.18×10^{-6}	4.04×10^{-8}
S_b%	2.27	2.13	2.04	2.08
F	1357.04	1547.24	1524.27	1659.66
Significance F	4.40×10^{-5}	3.62×10^{-5}	3.70×10^{-5}	3.25×10^{-5}

S_a is standard deviation of intercept, S_b is standard deviation of slope, and $S_{y/x}$ is standard deviation of residuals.

3. Results and discussion

OST is used for the treatment and post-exposure prophylaxis of influenza A and B. Criminal elements have already produced counterfeit OST preparations. ASC was reported in the WHO as a main adulterating agent used widely in counterfeit OST products which lacked the active ingredient. These products had been purchased through the internet and easily have gone unnoticed in the developing countries, where insufficient analytical resources hamper the ability to preserve drug quality.[16] Hence it is very important to develop analytical methods which are not only accurate, precise, and rapid but also simple and economic for the determination of the studied drug in its pharmaceutical dosage form and this is the main task of the developed spectrophotometric methods. The UV-spectrophotometric methods have the advantages of saving time and cost when compared to the chromatographic techniques, also they do not require solving equations or working with additional sophisticated software enabling their application in pharmaceutical industry.

This work concerns with the development and validation of four selective spectrophotometric methods, ^{1}D, ^{1}DD, RD and ΔA methods for the determination of the suggested drug (OST) in the presence of adulterating agent (ASC).

3.1. ^{1}D method

Fig. 1a shows a considerable overlapping between the absorption spectra of OST and ASC in methanolic solution, so direct spectrophotometry cannot be applied. Derivative spectrophotometry is very useful in the resolution of signal overlap or interference.[17] The application of the ^{1}D method showed appropriate resolution between OST and ASC spectra. Thus, OST could be determined in presence of its counterfeit ingredient (ASC) by measuring ^{1}D amplitudes at 219 nm which corresponds to zero-crossing point of ASC as shown in Fig. 2b.

3.2. ^{1}DD method

The main disadvantages of the zero-crossing method are the risk of small drifts in the working wavelengths and the fact that the working wavelengths generally do not produce corresponding peaks in the derivative spectrum.[18] A correct choice of divisor

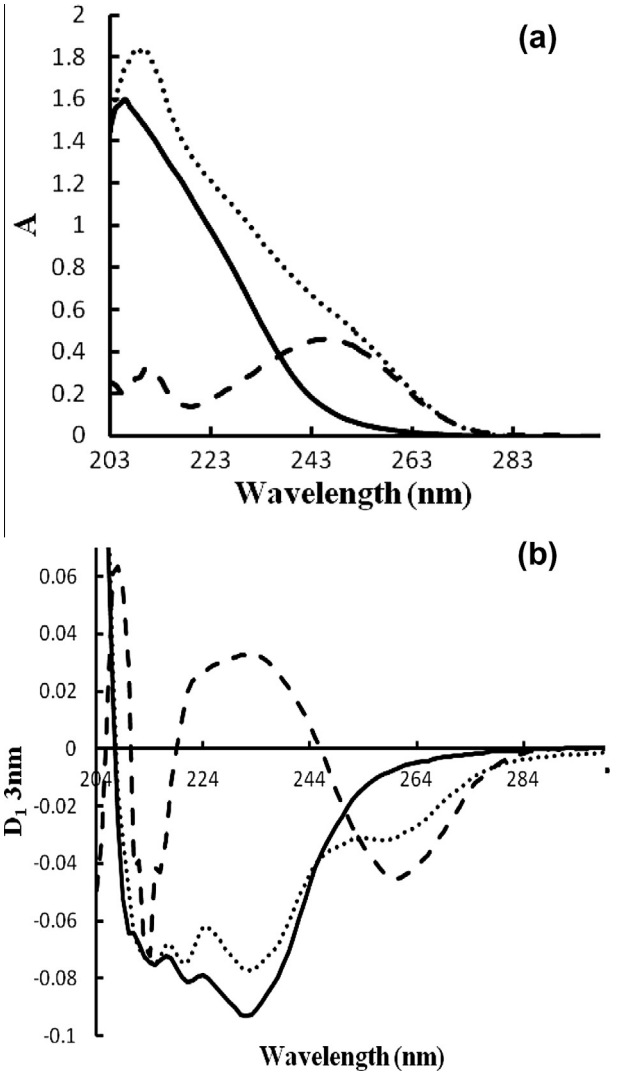

Figure 2 The absorption (a) and the first derivative (b) spectra of 35 μg mL^{-1} OST (—), 5 μg mL^{-1} ASC (—) and their mixture (. . ..) in methanol.

concentration is fundamental. If the concentration of the divisor is increased or decreased, the resulting values of the derivative ratio are proportionally decreased or increased with the consequent variation in both the sensitivity and the range of linearity. The absorption spectra of standard OST solutions were divided (amplitude by amplitude at each wavelength) by the spectrum of ASC of concentration 5 μg mL^{-1} where ratio spectra were obtained (Fig. 3a). Then, the first derivative of the ratio spectra was calculated as shown in Fig. 3b. From these figures, the ^{1}DD amplitudes were measured at 218 nm.

3.3. RD method

The most striking feature of this method is its simplicity, rapidity and accuracy. This is a newly developed method having the ability for solving severely overlapped spectra without prior separation meanwhile it does not require any sophisticated apparatus or computer programs.

The utility of the RD method is to calculate the unknown concentration of the component of interest present in a mixture together with an unwanted interfering one.

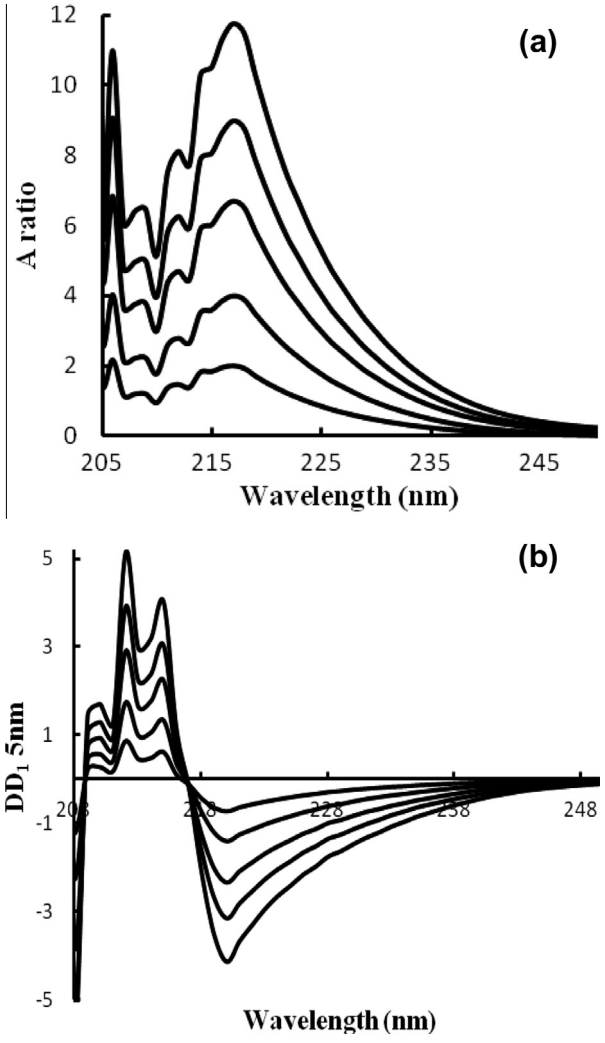

Figure 3 The absorption ratio spectra (a) and ^{1}DD spectra (b) of OST (10, 20, 30, 40 and 50 μg mL^{-1}) using 5 μg mL^{-1} ASC as divisor.

For the determination of concentration of the component of interest by this method, the only requirement is the contribution of the two components at the two selected wavelengths where the ratio spectrum of interfering component shows the same amplitudes (constant) whereas the component of interest shows significant difference in these two amplitude values at these two wavelengths with concentration. Ratio spectra were obtained as mentioned in the ^{1}DD method (Section 3.2) and then amplitudes at 217 and 210 nm were selected and subtracted (Fig. 3a).

3.4. ΔA method

The developed dual wavelength method provides a simple method for the selective determination of OST using its zero order spectra. The principle of this method is that the absorbance difference at two points on the spectra is directly proportional to the component of interest, independent of the interfering component. The pre-requisite for this method is the selection of two wavelengths where the interfering component shows the same absorbance value while the component of interest shows significant difference in absorbance. Selection of the suitable wavelengths plays an important role; hence different wavelengths were tried. Using the absorbance values at 236 and 252 nm (where ASC has the same absorbance values as shown in Fig. 2a) gave the best selectivity when used for the determination of OST.

4. Validation

ICH guidelines[19] for method validation were followed for the developed methods.

4.1. Accuracy and precision

Solutions containing three different concentrations of OST, within the linearity range, were analyzed in five replicates

Table 2 Evaluation of the precision and accuracy of the proposed methods for the determination of OST.

Methods	Added concentration[a]	Found ± RSD%[b]	E_r%[c]
^{1}D method	20.00	100.30 ± 1.52	0.30
	30.00	100.91 ± 0.93	0.91
	40.00	101.18 ± 1.27	1.18
Mean		100.80 ± 1.24	0.80
^{1}DD method	20.00	98.64 ± 1.50	−1.36
	30.00	99.09 ± 1.14	−0.91
	40.00	100.97 ± 0.83	0.97
Mean		99.57 ± 1.16	−0.43
RD method	20.00	101.40 ± 0.87	1.40
	30.00	101.92 ± 1.06	1.92
	40.00	98.88 ± 0.90	−1.12
Mean		100.73 ± 0.94	0.73
Δ*A* method	20	98.70 ± 1.58	−1.30
	30	101.06 ± 0.61	1.06
	40	99.27 ± 1.80	−0.73
Mean		99.68 ± 1.33	−0.32

[a] Final concentration in g mL^{-1}.
[b] Mean recovery ± relative standard deviation for five determinations.
[c] Percentage relative error.

(Table 2). Satisfactory recoveries, small relative errors (E_r%), with small relative standard deviations (RSD%) were obtained, which indicated the high accuracy and precision of both methods.

4.2. Selectivity

The selectivity of the proposed methods was checked by analyzing synthetic mixtures containing different ratios of both OST and ASC, where good percentage recoveries were obtained indicating that ASC did not interfere with OST (Table 3).

4.3. Limit of detection and limit of quantitation

LOD and LOQ were calculated using the formulae given by Miller[20] where the limit of detection, LOD = 3 S/b and the limit of quantitation, LOQ = 10 S/b, where S is the standard deviation of replicate blank responses (under the same conditions as for sample analysis) and b is the sensitivity, namely the slope of the calibration graph. Using the proposed methods, LOD and LOQ for each method were calculated and are presented in Table 1.

4.4. Linearity

The linearity of the proposed methods was evaluated by analyzing series of different concentrations of OST. According to ICH, at least five concentrations must be used. Under the experimental conditions described, the graphs obtained by plotting ^{1}D, ^{1}DD, RD and ΔA values at the specified wavelengths versus concentration (in the ranges stated in Table 1) show linear relationships. The slopes, intercepts and correlation coefficients obtained by the linear least squares regression treatment of the results are also given. An important statistical parameter for indicating the random error in the estimated values of y is the standard error of the estimate, or the standard deviation about regression, or the standard deviation of the residuals, $S_{y/x}$. The smaller the standard error of the estimate the closer the points are to the straight line. Standard deviation of intercept (S_a) and slope (S_b) is also presented for each compound using the proposed methods of measurements. The high values of the correlation coefficients with negligible intercepts together with the high F-values indicate the good linearity of the calibration graphs. The linearity was further evaluated by calculation of the percentage relative SD of the slope (S_b%). Also, the small degree of scatter of the experimental data point around the line of regressions could be confirmed by the small values of the variances around the slopes S_b.[2] For more confirmation, the Student's t-test was performed to determine whether the experimental intercept (a) of the above-mentioned regression lines was not significantly different from the null hypothesis. The calculated values of t (a/S_a) do not exceed the 95% criterion of t = 2.31 for 5 samples. So the intercepts are not significantly different from zero in the proposed methods. Thus, the hypothesis that (a) is of negligible value is confirmed.[20,21]

For equal degrees of freedom, increase in F-values means increase in the mean of squares due to regression and decrease in the mean of squares due to residuals. The greater the mean of squares due to regression, the more the steepness of the regression line is. The smaller the mean of squares due to residuals, the less the scatter of the experimental points around the regression line is. Consequently, regression lines with high F-values (low significance F) are much better than those with lower ones. Good regression lines show high values for both (r) and (F) values.[21]

5. Assay of capsules

The proposed methods were applied to the determination of OST in its commercial capsules. Satisfactory results were

Table 3 Evaluation of the proposed methods for the determination of OST in its laboratory-prepared mixtures with ASC.

Synthetic mixture number	Nominal value (μg mL^{-1})		%Recovery			
	OST	ASC	^{1}D	^{1}DD	RD	ΔA
1	10.00	5.00	98.41	97.73	101.62	97.86
2	10.00	10.00	99.00	99.26	99.89	98.67
3	20.00	10.00	97.91	99.52	101.91	100.29
Mean			98.44	98.84	101.14	98.94
SD			0.55	0.97	1.09	1.24
RSD%			0.56	98.14	1.08	1.25

Table 4 Statistical comparison for the determination of OST in its pharmaceutical formulations using the proposed methods.

Pharmaceutical preparation	Found% ± SD[a]			
	^{1}D	^{1}DD	RD	ΔA
Taminil-N capsules[b]	99.00 ± 1.73	101.00 ± 2.65	100.57 ± 3.21	98.51 ± 2.44
t[c]	1.41	0.23	1.14	
F[c]	2.35	1.47	1.73	

[a] Average of five determinations.
[b] Labeled to contain 75 mg OST per capsule (The Nile Co. For Pharmaceuticals & Chemical Industries – Batch No. 10027).
[c] Theoretical value of T and F at p = 0.05 is 2.31 and 6.39, respectively.

Table 5 Comparison between the proposed and reported spectrophotometric methods.

Method	Reported method[3]	Reported method[4]			Reported method[5]		Reported method[12]	Proposed methods			
		Method A	Method B	Method C	Method I	Method II		^{1}D	^{1}DD	RD	ΔA
λ (nm)	λ_{max} 2085	λ_{max} 530	λ_{max} 545	λ_{max} 512	λ_{max} 520	λ_{max} 590	λ_{ex} 381 λ_{em} 483	λ 219	λ 218	217–210	252–263
Linearity range (μg mL^{-1})	4–24	1–7	5–50	1–15	1.5–4.5	6–18	50–450*	10–50	10–50	10–50	10–50
Procedure		The need of waiting time of 10 min for the reaction to be completed	The formed colored product is stable only for 30 min	The need of waiting time of 20 min with heating at 70 °C for the reaction to be completed	– The formed ion-pair complex with OST was extracted with ethyl acetate. (The ion-pair extraction technique suffers from difficulties & inaccuracies arising from incomplete extraction or emulsion formation.)		– A buffer is required. (The pH adjustment may vary due to pH-meter calibration or personal variation which affects the method ruggedness)	– No use of toxic or expensive solvents – No care is needed regarding the pH adjustment – No need for extraction steps – The proposed methods are direct and not time consuming – The proposed methods determine directly the concentration of OST in presence of the ASC without any interference with minimal cost and time. On the other hand, the reported methods determine OST only			
					– The reported methods require the use of a buffer (The pH adjustment may vary due to pH-meter calibration or personal variation which affects the method ruggedness.)		– The need of pretreatment step with fluorescamine to give a fluorescent derivative				
		The used reagents (p-dimethyl amino cinnamaldehyde in method A, 4-aminophenazone in method B or ferric chloride and 1,10-phenanthroline in method C) add extra cost			– The used reagents (congo red in I or bromochlorophenol blue in II) add extra cost		*Concentration in ng mL^{-1}				

obtained and were in good agreement with the label claims (Table 4). Excellent percentage recoveries and SD suggested that there is no interference from excipients, which are present in OST capsules. Statistical analysis of the results obtained by the proposed methods was performed using Student's *t*-test and the variance ratio *F*-test (Table 4). The calculated values did not exceed the theoretical ones, indicating no significant difference between the performance of the compared methods regarding accuracy and precision.

6. Conclusion

The proposed spectrophotometric methods have the advantages of saving time, cost and environmental protection without sacrificing accuracy when compared to the reported chromatographic techniques, also they do not require solving equations or working with additional sophisticated software enabling their application in pharmaceutical industry. Table 5 shows the comparison between proposed and reported spectrophotometric methods. They can be considered selective and simple enough to be applied in the quality-control analysis of the drug without interference of the commonly encountered capsules additives or ASC that is present in many of its counterfeit pharmaceutical products.

7. Conflict of interest

We have no conflict of interest to declare.

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