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Selective Spectrofluorimetric method for the Determination of Valacyclovir in bulk and tablets through Derivatization with *O*-Phthalaldehyde in presence of 3-Mercaptopropionic Acid

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ABSTRACT: *A new, selective and sensitive spectrofluorimetric method was developed and validated for the determination of valacyclovir via derivatization with o-phthalaldehyde in presence of 3-mercaptopropionic acid in alkaline medium. The resulted derivative has a strong blue fluorescence that was measured at 452 nm after excitation at 336 nm in aqueous solution. The reaction conditions were carefully studied and optimized. Under the optimum conditions, the fluorescence intensity was linear over a concentration range of 0.40 – 2.00 μg/mL (R^2 = 0.9998) with a detection limit of 0.020 μg/mL. The proposed method was fully validated and effectively applied to the analysis of valacyclovir in pure form and tablets. Statistical comparison of the results obtained by the proposed and reference method revealed no significant difference in the performance of the two methods regarding the accuracy and precision respectively. A proposal for the reaction pathway with o-phthalaldehyde was postulated.*

Keywords: *Valacyclovir, O-phthalaldehyde, Derivatization, Spectrofluorimetry.*

I. INTRODUCTION

Valacyclovir (VA) is a nucleoside analogue DNA polymerase inhibitor. It is chemically describes as l-valine-2-[(2-amino-1, 6-dihydro-6-oxo-9-hipurin-9yl) methoxy] ethyl ester (Fig. 1). Valacyclovir is a (l-valyl ester) prodrug of acyclovir. After oral administration, valacyclovir is rapidly absorbed from the gastrointestinal tract and nearly completely converted to acyclovir and L-valine by first-pass intestinal and hepatic metabolism. Acyclovir has demonstrated antiviral activity against herpes simplex virus type 1, 2, and varicella zoster virus both in cell culture and in vivo. VA is converted rapidly into acyclovir after its oral administration via first-pass metabolism. The absolute bioavailability of acyclovir after oral administration of VA reaches 55% which is relatively higher than that of acyclovir (10-20%) [1,2].

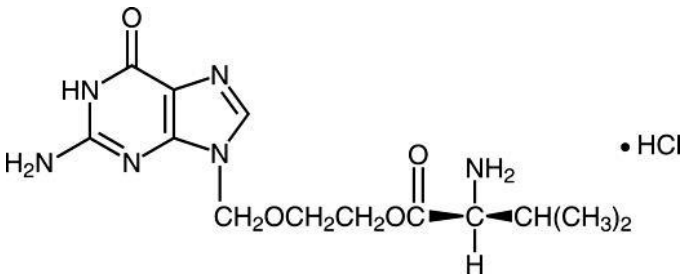


Fig. 1 Valacyclovir hydrochloride structure.

literature review of VA revealed that few analytical methods based on spectrophotometry [3-6], high-performance liquid chromatography (HPLC) with UV detection [7-11], HPLC with MS detection [12-13], and micellar electrokinetic chromatography [14] were reported. Only one spectrofluoremetric method for the determination of VA in tablets and spiked plasma was recorded [15]. Spectroluorimetry has been widely used to estimate pharmaceuticals due to its simplicity, high sensitivity, low cost and less time consumption comparing with other analytical techniques. Therefore, it is still significant to develop a new simple and sensitive spectrofluorimetric method for the determination of such an important drug, valacyclovir.

O-Phthalaldehyde (OPA), in combination with a thiol compound, is known to react with primary amines [16]. The reaction of OPA with amino acids in presence of 2-mercaptoethanol was first reported by Roth in 1971 [17]. It has been used for the determination of many amino acids [17] as well as cholesterol [18]. Several pharmaceutical compounds have been also determined through this approach [19-22]. No attempts have been made for the fluorimetric determination of VA. This paper describes, for the first time, the derivatization of VA with *o*-phthalaldehyde in presence of 3-mercaptopropionic acid. The proposed method is sensitive, accurate, simple and selective. It was applied for the determination of VA in bulk and as well as in pharmaceutical preparations.

II. EXPERIMENTAL

2.1. Apparatus

Fluorescence spectra and measurements were obtained using fluorescence spectrophotometer F-2700 (Hitachi, Japan) equipped with xenon lamp. Excitation and emission wavelengths were set at 336 nm and 452 nm, respectively. The slit widths for excitation and emission monochromators were fixed at 5 nm. All measurements were performed in 1 cm quartz cell at room temperature.

Chromatographic analysis was performed on (Agilent, Germany) apparatus equipped with UV detector, autosampler, and column oven. Chromatographic separation was achieved on CN column (5 µm, 250 mm × 4.6 mm).

2.2. Reagents and solutions

Valacyclovir hydrochloride (Matrix Laboratories Limited, India) standard solution was prepared to contain 0.1 mg/mL of valacyclovir in deionized (DI) water. This solution was freshly prepared at time of study. *O*-phthalaldehyde (OPA) and 3-Mercaptopropionic acid (3MPA) were purchased from Sigma Aldrich, Germany. Solutions of OPA and 3MPA were prepared in methanol at 0.02 M and 0.1 M, respectively, and were kept at refrigerator. The OPA and 3MPA derivatization solution was prepared by mixing suitable volumes of OPA and 3MPA methanolic solutions and diluting it with DI water after adding 2 mL of phosphate buffer solution (pH 11) to get a final concentration of 1×10^{-3} M of both OPA and 3MPA. The mixed reagent solution should be used during one working day only. Phosphate buffer (0.1M) solution was prepared in DI water using disodium hydrogen phosphate and adjusted to pH 11 with 1 M sodium hydroxide. Phosphate buffer solution was kept in refrigerator and used within about 5 days.

Valacyclovir tablets, Valcyclovir (Medical Bahri Company, Damascus, Syria) containing 500 mg and 1000 mg were purchased from local medical stores. All reagents and solvents were of analytical grade.

2.3. Derivatization procedure

Increasing volumes of VA working standard solution were transferred into series of 5 mL volumetric flasks that contain 0.5mL of phosphate buffer pH 11 and 0.3mL of OPA/3MPA derivatization solution. Solutions were mixed gently and allowed to stand at room temperature for 10 minutes. Volumes were made up to mark with DI water and mixed before the fluorescence intensity was measured at 452 nm after excitation at 336 nm against reagent blank that had been treated similarly.

2.4. Determination of 3MPA/OPA stoichiometric relationship

The composition ratio of the derivatization reagent was determined using molar ratio method, in which fixed volumes of VA and OPA solutions correspond to 5.0×10^{-8} and 5.0×10^{-7} moles of VA and OPA, respectively, were added. Increasing volumes of 3MPA solution were then added to each solution and procedure was then completed as described above. The obtained fluorescence intensities were then plotted against 3MPA/OPA molar ratio.

2.5. Determination of OPA/VA stoichiometric relationship

The composition ratio of the derivatization reagent was determined using molar ratio method, in which VA moles were kept fixed at 5.0×10^{-8} and increasing volumes of (OPA/3MPA) reagent were added to each solution and procedure was then completed as described above. Fluorescence intensities were then recorded and plotted against OPA/Drug molar ratio.

2.6. Procedure for pharmaceutical samples

Ten individual tablets were weighed and pulverized carefully. An accurately weighed amount of the powder equivalent to 100 mg of VA was transferred into 100 mL volumetric flask and dissolved in 70 mL of DI water. The content of the flask was sonicated for 20 min then diluted to volume with DI water. A portion of this solution was centrifuged at 5000 rpm for 10 minutes. 0.5 mL of the supernatant was then transferred into 25 mL volumetric flask, and diluted with DI water upto mark. Then suitable volume was transferred into 5 mL volumetric flask and procedure was continued as described under the derivatization procedure.

III. RESULTS AND DISCUSSION

3.1. Fluorescence spectra

Valacyclovir contains primary amino group and can therefore reacts with OPA in presence of 3MPA in alkaline medium to give a strongly fluorescent isoindole derivative. OPA is nonfluorescent itself, and when present in excess does not break down or react to form fluorescent by-products, as shown clearly in figure 2. Under the described experimental conditions, the shiny blue fluorescence was observed at 452 nm after excitation at 336 nm (Fig. 3).

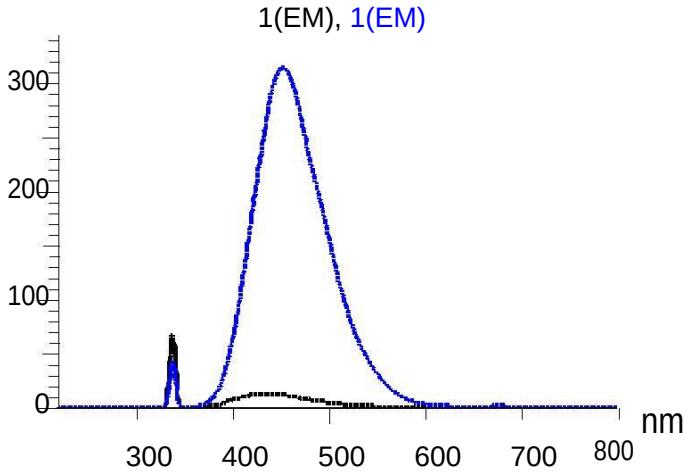


Fig. 2 Emission spectra of VA/OPA derivative (blue) and blank (black) after excitation at λ_{ex}=336 nm.

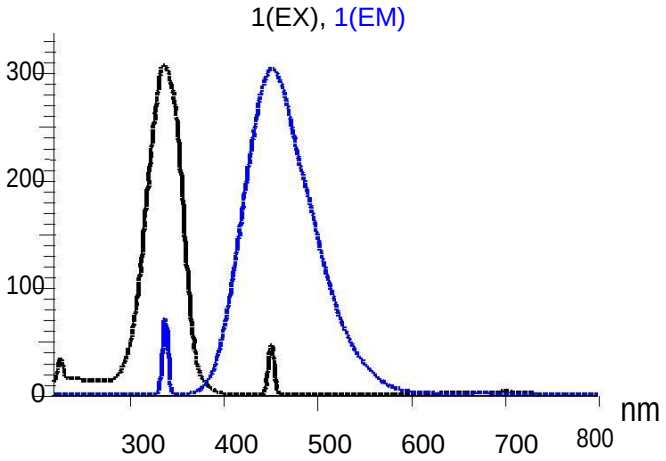


Fig. 3 Excitation (black) and emission (blue) spectra of the VA/OPA derivative (λ_{ex}= 336 nm, λ_{em}= 452 nm).

The fluorescent isoindole product was rapidly formed at room temperature, but subsequently degraded to non-fluorescent derivatives. Fluorescence quantum yield and stability of derivative are usually dependent to the particular amine, excess of OPA, pH of alkaline medium, and the nature of the nucleophile (thiol) used in the cyclization reaction. However, 3MPA seems to be the best thiol compound used along with OPA in derivatization reaction, since it produces the most stable amino acid derivatives with fluorescent yields comparable to other thiols such as 2-mercaptoethanol and N-acetyl cysteine. Thus, 3MPA was chosen for this study.

3.2. Optimization of reaction conditions

3.2.1. Effect of time

The time required for a complete derivatization reaction was the first parameter that has been studied. A high and steady fluorescence intensity was obtained after 5 min and was stable until 45 min (Fig. 4). Thus 10 min was adequate for the derivatization reaction.

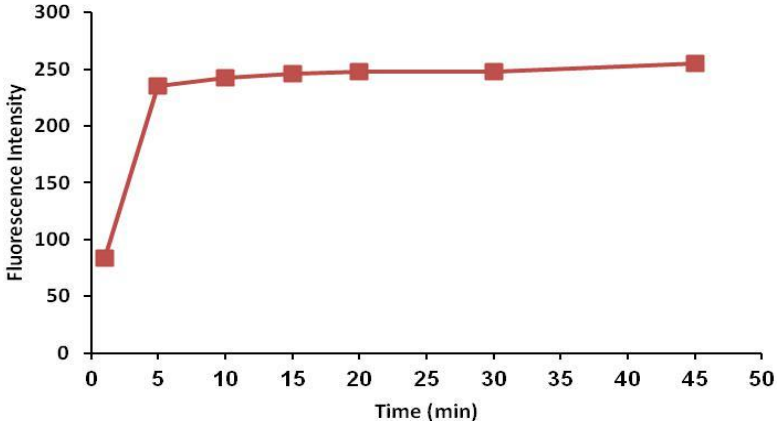


Fig. 4 Effect of the time on the reaction completion of VA (2 μg/mL) with OPA/3MPA.

3.2.2. Effect of pH

To optimize the reaction the pH effect of three different buffers was studied. Borate, bicarbonate, and phosphate buffers (0.1M) were used each at its alkaline working range. Buffers with amino groups such as Tris and Triethylamine were excluded, since they react with OPA. The reaction was carried out at room temperature for 15 min. The highest fluorescence intensity was obtained using phosphate buffer at pH 11 (Fig. 5). A study of needed buffer volume showed that 0.5 mL of phosphate buffer was adequate.

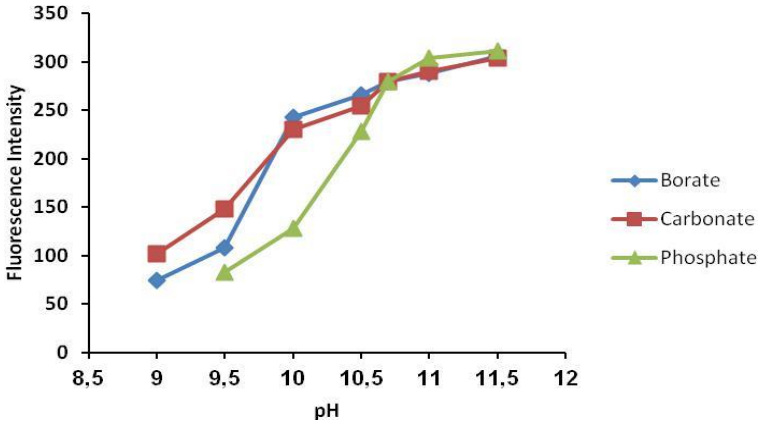


Fig. 5 Effect of the pH on the reaction completion of VA (2 μg/mL) with OPA/3MPA.

3.2.3. Effect of temperature with time

In this study, the reaction between VA and OPA/3MPA was performed using pH 11 phosphate buffer at different temperatures (25° C, 30° C, 40° C, 50° C and 60° C) at seven time intervals (5, 10, 15, 20, 30, 45, and 60 minutes). A steady and maximum fluorescence was noticed at room temperature (25 ± 2°C) within 5 minutes and continued to be stable until one hour. An identical profile was obtained at 30° C with a slight decrease in the fluorescence after 30 min. High temperatures up to 60 °C has affected the reaction negatively, which can be due to the instability of the isoindole compound (Fig. 6). As a result, the reaction was performed at room temperature and 10 min was adequate for a complete reaction.

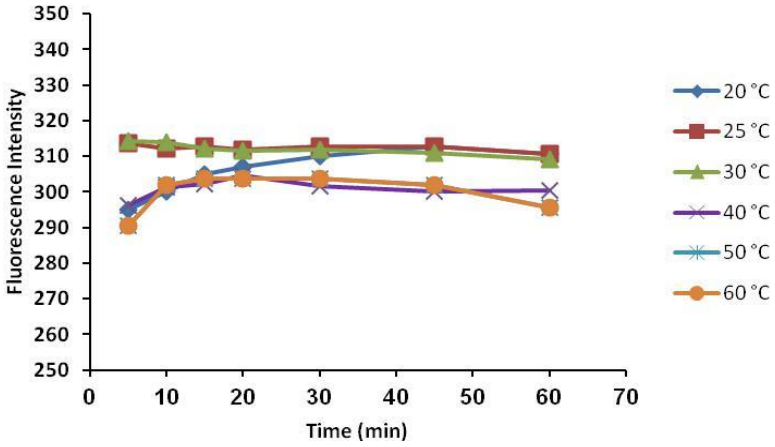


Fig. 6 Effect of the temperature and time on the reaction completion of VA (2 μg/mL) with OPA/3MPA.

3.2.4. Effect of OPA reagent volume

The influence of the volume of reagent solution was examined by addition of increasing volumes of OPA/3MPA (1.0×10^{-3} M) derivatization solution. Volumes were ranged from 0.1 – 0.6 mL. High fluorescence intensities were attained when more than 0.2 mL of the reagent solution was added. Thus a fixed volume of 0.3 mL was used in the optimal procedure. (Fig. 7).

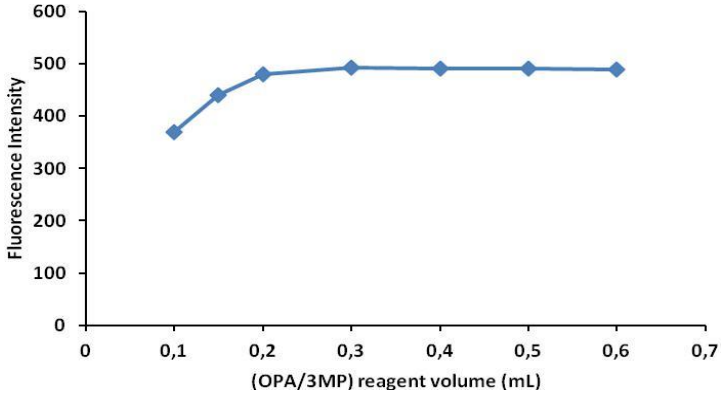


Fig. 7 Effect of the volume added of OPA/3MPA solution on the derivatization reaction of VA (3.5 μg/mL)

3.2.5. Effect of solvent

Effect of diluting solvent was also investigated. Six different solvents (methanol, ethanol, isopropanol, acetone, acetonitrile, and dimethylsulfoxide) have been tested and compared to water as diluting solvent. A relatively lower and less stable fluorescence intensity was observed in methanol and acetonitrile in comparison

with water, with an emission peak shifted to 440 nm. However, cloudy solutions were generated with other solvents.

3.2.6. Stoichiometric relationship of the derivatization reagent

The stoichiometrirc relationship between OPA and 3MPA during the derivatization reaction with VA was determined by molar ratio method. In this method VA and OPA moles were kept at a fixed concentration so that the OPA moles were 10 times greater that VA moles to insure a complete reaction of drug with reagent at any OPA/3MPA ratio.

As can be seen from Figure 8, OPA/3MPA ratio was found to be 2:1, that means each two molecules of OPA need one molecule of 3MPA during the derivatization reaction with VA. But, a slight increase in the fluorescence intensity was observed at the ratio of 1:1 compared with 2:1 (OPA/3MPA). Thus, to insure a higher sensitivity, a ratio of 1:1 has been reserved during our study.

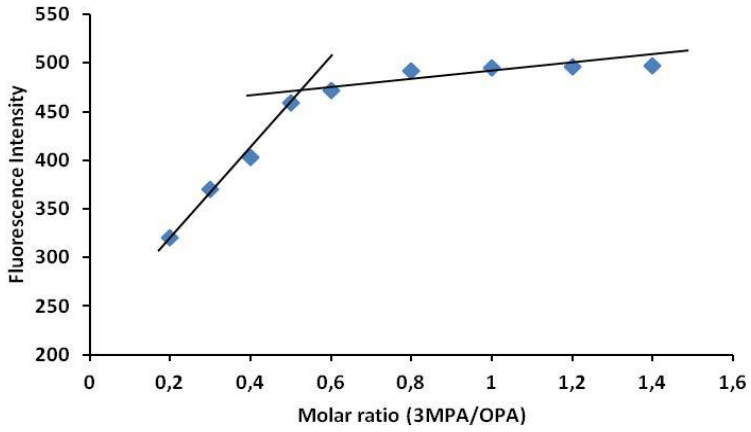


Fig. 8 Molar ratio plot for the stoichiometric relationship between OPA and 3MPA.

3.2.7. Mechanism of the reaction with *o*-pthalaldehyde

The stoichiometry of the reaction between VA and *o*-pthalaldehyde was studied adopting the molar ratio method (Fig. 9). It is obvious that, the reaction proceeds at two ratios of VA/OPA correspond to 1:1 and 1:2, confirming that one molecule of the drug condenses with one or two molecules of *o*-phthalaldehyde, since it contains two amine groups in its structure. A schematic explanation of the reaction pathway is given in Scheme 1.

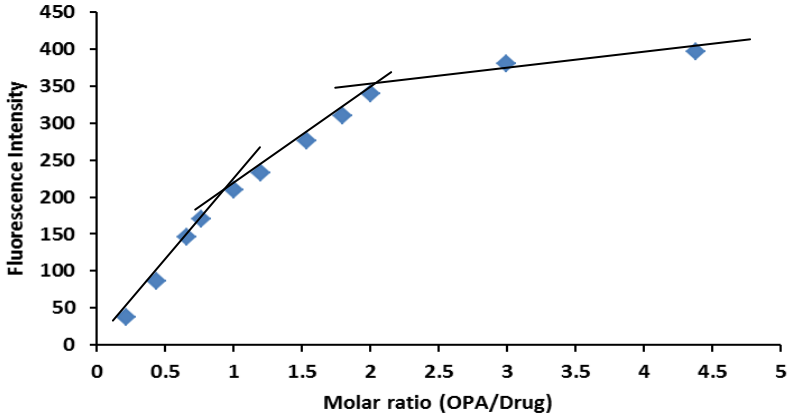
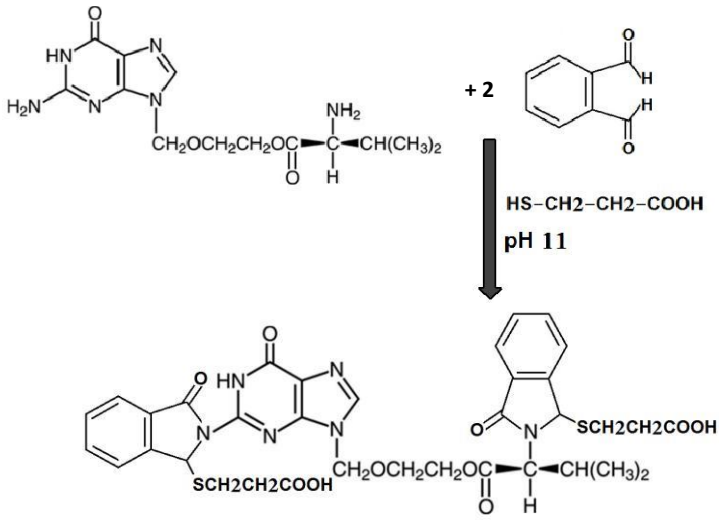


Fig. 9 Molar ratio plot for the stoichiometric relationship between VA and OPA.



Scheme 1 Proposed reaction pathway between VA and OPA in presence of 3MPA.

3.3. Validation of the proposed method

3.3.1. Linearity

Under the optimum experimental conditions, standard calibration curve was constructed at nine concentration levels (n=5). The correlation coefficient was 0.9998 indicating very good linearity, over the concentration range of 0.4 – 2.0 μg/mL. The intercept, slope, limit of detection (LOD), and limit of quantitation (LOQ) are summarized in Table 2. LOD and LOQ values were calculated as $3.3S_b$/m and $10S_b$/m, respectively. Where S_b is the standard deviation of intercept of regression line and m is the slope of the calibration curve [23, 24] (Table 1).

Table 1. Statistics and analytical parameters of VA determination.

Parameter	Result
$\lambda_{ex}/\lambda_{em}$ (nm)	336/452
Linear range (μg/mL)	0.4 – 2.0
Slope	149.1
Standard deviation in the slope	0.704
Intercept	0.6061
Standard deviation in the intercept	0.919
Correlation coefficient	0.9998
Limit of detection (μg/mL)	0.020
Limit of quantification (μg/mL)	0.061

3.3.2. Selectivity

The effects of some common excipients used in pharmaceutical forms were examined by analyzing solutions containing suggested amounts of each excipient. Frequently encountered excipients or additives were studied such as lactose, microcrystalline cellulose (Avicel), soluble starch, polyvinylpyrrolidone (PVP k30), talc, and magnesium stearate. None of the studied excipients has given any fluorescent product. So the proposed method is suitable for analysis of valacyclovir in its dosage forms and application in quality control laboratories.

3.3.3. Precision

The repeatability of proposed method was estimated by measuring five replicate samples of each concentration of valacyclovir prepared in one laboratory on the same day. The precision expressed as the relative standard deviation (RSD%) ranged from 0.54% to 2.27% for the smallest concentration, indicating good precision (Table 2).

Table 2. Precision and accuracy for determination of VA in pure form using proposed method.

Valacyclovir (μg/mL)		SD	RSD%	Recovery %	*t*-test[b]
Taken	Found[a]	(μg/mL)			
0.400	0.402 ± 0.009	0.008	1.99	100.50	0.55
0.600	0.605 ± 0.011	0.009	1.48	100.83	1.24
0.800	0.802 ± 0.011	0.009	1.12	100.25	0.49
1.000	0.995 ± 0.009	0.008	0.80	99.50	1.39
1.200	1.198 ± 0.009	0.008	0.66	99.83	0.55
1.400	1.391 ± 0.011	0.009	0.64	99.36	2.23
1.600	1.602 ± 0.012	0.010	0.62	100.12	0.44
1.800	1.806 ± 0.015	0.012	0.66	100.33	1.11
2.000	2.011 ± 0.013	0.011	0.54	100.55	2.23

[a] Average of five determinations ± Confidence limit.
[b] The tabulated *t*- value at 95% confidence limit for 4 degrees of freedom (n =5) is 2.78.

3.3.4. Accuracy

The proposed method was applied on the available commercial tablets at three different concentration levels (80%, 100%, and 120%) and recoveries are mentioned in Table 3. However, the method's accuracy is judged by (1) determining the average amount of VA in pure form at several levels, and using a significance test to compare it with actual amount u [23]:

$$t = \frac{|\bar{X} - \mu|}{SD}\sqrt{n}$$

As shown in table 2, the calculated *t*-value is less than tabulated *t*(0.05,4) value (2.78), and thus there is no significant differences between the taken and found concentration at 95% confidence level. Accuracy was indicated as well by analyzing the recoveries of known different amounts of VA (Table 2) which varied from 99.36 to 100.83%. (2) comparing the results obtained from the presently proposed method, that has been applied on commercial tablets, with those obtained from a reference method such as HPLC [7]. The resulted values were statistically compared with each other (Table 4) using *t*- and *F*-tests. t_{exp} was calculated using the following equation [23]:

$$t_{exp} = \frac{|\bar{X}_A - \bar{X}_B|}{\sqrt{(S_A^2/n_A) + (S_B^2/n_B)}}$$

Where $\bar{X}_A$ and $\bar{X}_B$ are VA mean values in each pharmaceutical product using the proposed and reference methods, respectively. S and n are the standard deviation and the number of replicate trials conducted on samples, respectively. With respect to *t*- and *F*-tests, no significant differences were found between the calculated values of both the proposed and the reported methods at 95% confidence level.

Table 3. Application of the proposed method to the determination of VA in tablets.

Tablets	Labeled amount of VA	Amount taken (μg/mL)	Amount found[a] (μg/mL)	Recovery %
Valcyclovir	500 mg	0.800	0.811	101.36
		1.000	1.009	100.91
		1.200	1.210	100.80
Mean found%				**101.02**
RSD%				**0.29**
Valcyclovir	1000 mg	0.800	0.797	99.57
		1.000	1.005	100.51
		1.200	1.196	99.67
Mean found%				**99.91**
RSD%				**0.51**

[a] Average of three determinations

Table 4. Precision and accuracy for determination of VA in tablets.

Tablets	Labeled amount of VA	Average VA found (mg/tablet) ± SD [a] (Recovery%) [b]		*t*- and *F*- test [c]
		Proposed method	Reference method [d]	
Valcyclovir	500 mg	504.13 ± 2.76 (100.82)	505.6 ± 1.62 (101.12)	0.94, 2.90
	1000 mg	1002.10 ± 6.10 (100.21)	1006.06 ± 3.49 (100.60)	1.16, 3.04

[a] Average and standard deviation of five determinations for the proposed method, and three determinations for the reference method.
[b] Recoveries were calculated considering the labeled amount reported by the manufacturer.
[c] the tabulated *t* value at 95% confidence limit for 4 degrees of freedom (n =5) is 2.78 and the tabulated *F* value at 95% confidence limit for (4, 2) degrees of freedom for the proposed and reference methods, respectively, is 6.944.
[d] HPLC [7].

3.3.5. Robustness

Robustness was examined by evaluating the influence of small variations in the experimental conditions such as volume of reagent (±0.05mL), volume of buffer solution (±0.05mL) and reaction time (±5 min). These minor changes that may happen during the analysis did not have any significant effect on fluorescence intensity of the reaction product.

3.4. Application to Tablets

The proposed method was successfully applied to the analysis of available commercial tablets (Valcyclovir® Tablets) labeled to contain 500 and 1000 mg of valacyclovir. The mean recovery values were 100.82 and 100.21 for valcyclovir 500 mg and 1000 mg, respectively, which were identical to the recoveries recorded by the reference method (HPLC) as revealed by *t*- and *F*-test (Table 4).

IV. CONCLUSION

New, simple, and rapid spectrofluorimetric method for the determination of VA has been successfully developed and validated. The method involved the formation of a fluorescent isoindole derivative resulted from the reaction of VA with OPA in presence of 3MPA. The proposed method was specific, precise and accurate with a comparable low detection limit value of 0.020 μg/mL. The method was effectively applied for determining VA in pure form and in tablets without any interference with the excipients. Therefore, the developed method can be suitable for routine analysis of VA in quality control laboratories.

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