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Excitation and emission spectra of the reaction between NBD-Cl and VAC (4 µg ml\(^{-1}\))

TLC chromatograms of VAC degradation

Alkali induced degradation

Acid induced degradation

Photolytic degradation

Oxidative degradation

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Abstract

Two stability- indicating methods were developed and validated for determination of valacyclovir HCl (VAC) in the presence of its degradation product, acyclovir. The first was TLC- densitometric method, in which chloroform: methanol: ammonia (50:14:2 v/v/v) was used as mobile phase. Silica gel 60 F254 was used as a stationary phase and the chromatogram was scanned at 253 nm. Using this chromatographic system, VAC can be readily separated from its degradation product and give compact spot at \(R_F\) value of \((0.55 \pm 0.03)\). The peak area concentration plot is rectilinear over the range 20 - 300 ng band\(^{-1}\). The second method represents the first attempt for spectrofluorimetric determination of VAC. The method was based on the reaction between VAC and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in an alkaline medium (pH 8.5) to form highly fluorescent product that was measured at 500 nm after excitation at 465 nm. The fluorescence intensity concentration plot is rectilinear over the range 1-10 µg ml\(^{-1}\). The proposed methods were successfully applied for the determination of VAC in its commercial tablets with average percentage recovery of 100.13 ± 0.33 and 98.50 ± 1.75 for TLC- densitometric and spectrofluorimetric methods, respectively without interference from common excipients. The results of the proposed methods were statistically analyzed and found to be in accordance with those given by a reported method. In addition the proposed methods were extended to stability study of VAC, where the drug was exposed to acidic, alkaline, oxidative and photolytic degradation according to ICH guidelines.

**Keywords:** Valacyclovir HCl, Stability indicating, TLC- densitometric, spectrofluorimetric, acidic and alkaline degradations.

1. Introduction

Valacyclovir hydrochloride (VAC) is L-Valine 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9yl) methoxy] ethyl ester hydrochloride (Fig. 1). After its oral administration,
VAC is rapidly converted into acyclovir which has demonstrated antiviral activity, against herpes simplex virus type I (HSV-1), type 2 (HSV-II) and Varicella zoster virus [1]. The oral bioavailability of acyclovir is higher after administration of VAC relative to acyclovir itself [2-3]. The mechanism of action of acyclovir involves the highly selective inhibition of virus DNA replication, via enhanced uptake in virus-infected cells and phosphorylation by viral thymidine kinase [4-5]. Few analytical methods have been reported for the determination of VAC. These methods include: spectrophotometric [6-8], chromatographic [9-22], capillary electrophoretic [23] and electrochemical methods [24]. To the present time there is no spectrofluorometric method reported for analysis of VAC. Accordingly we here present the first attempt for spectrofluorimetric method in addition to TLC-densitometry for the determination of VAC in pure form and pharmaceutical tablets. TLC-densitometric method is becoming a routine technique for analysis of drugs due to its low operating cost, high sample-throughput, and need for minimum sample clean-up. The major advantage of TLC-densitometric lies in several samples can be run simultaneously using a small quantity of mobile phase thus lowering analysis time and cost per analysis. Unlike HPLC, wherein substantial amounts of mobile phase and time are required for analysis of multiple samples [25]. TLC-densitometric method has the advantages of being sensitive, cheap and one of the simplest chromatographic technique that does not need large precautions as in HPLC technique which should be taken to prevent several problems in HPLC such as plugging of the column and variation of retention time. Both proposed methods of analysis of the cited drug are able to determine the drug in pure form and pharmaceutical tablets without interference from tablet excipients. Furthermore, both methods were developed to be stability-indicating methods that can be used for determination of VAC in presence of its degradation products.

2. Experimental

2.1. Apparatus

Camag TLC scanner 3 S / N 130319 in the absorbance mode utilizing deuterium lamp as a source of radiation with Wincats software. Samples were applied with Camag micro-syringe 100 µl, using a Camag Linomat 5 autosampler, (Camag, Muttenz, Switzerland).
A Perkin Elmer LS 45 luminescence spectrometer (United Kingdom) equipped with 150-watt xenon arc lamp and 1 cm quartz cell. Slit width for both monochromators were set at 10 nm. The spectrometer is connected to a PC loaded with the FL Winlab™ software, Spectronic™ Genesys™ 2PC. Ultraviolet/visible spectrophotometer (Milton Roy Co, USA) with matched 1 cm quartz cell and was connected to PC loaded with Winspec™ application software.

MLW type thermostatically controlled water bath (Memmert GmbH, Schwabach, Germany) was used for heating purposes.

2.2. Material and chemicals

VAC was obtained as a gift from (Hikma Pharma, Cairo, Egypt). Valtovir® tablets (Hikma Pharma, Cairo, EGYPT) labeled to contain 1110 mg VAC per tablet was purchased from local market. Acyclovir was obtained as a gift from GlaxoSmithKline (Cairo, Egypt). 4-chloro-7 nitrobenzo-2-oxa-1,3-diazole NBD-Cl (Sigma Chemical Co., St. Louis, USA) was freshly prepared as 0.1 % w/v in methanol. Chloroform, hydrochloric acid, ammonia solution, hydrogen peroxide (30 %v/v), sodium hydroxide, boric acid and methanol were purchased from El-Nasr Chemical Co. (Cairo, Egypt). All chemicals and reagents used were of analytical grade.

Aluminum plate precoated with silica gel 60 F254, (20 × 10 cm) with 250 µm thickness) (Merck, Darmstadt, Germany).

2.3. Standard solution preparation

An accurately weighed amount (10 mg) of VAC was quantitatively transferred into a 100-ml calibrated flask, dissolved in 20 ml methanol, and then completed to volume with the same solvent to obtain a stock solution of 0.1 mg ml⁻¹. The stock solution was further diluted with methanol to obtain working solutions in the concentration range of 5-75 µg ml⁻¹ and 10-100 µg ml⁻¹ for TLC- densitometric and spectrofluorimetric methods, respectively

2.4. Preparation of sample solutions

An accurately weighed amount of finely powdered twenty tablets equivalent to 25 mg of VAC was transferred into a 50-ml volumetric flask. The powder was dissolved in 25 ml methanol, sonicated for 15 min, diluted to the mark with methanol and mixed
well. The resulting solution was centrifuged at 3000 rpm for 5 min. Two ml of this solution was diluted to 10 ml with methanol.

2.5. General Analytical procedure

2.5.1. For TLC- densitometric method

TLC- densitometric method was performed in aluminum plates (10 x 20 cm) coated with 0.2 mm layers of silica gel 60 F_{254}. Standard or sample solutions were applied as 6.0 mm at bands at the concentration range of 20 - 300 ng band^{-1} to the plate at 10 mm from the bottom and 10 mm from the side edges of the plate. The band was dried with the aid of an online nitrogen gas. Linear ascending development with chloroform: methanol: ammonia (50:14:2 v/v/v) as a mobile phase was performed in a Camag twin-trough glass chamber previously saturated with mobile phase for 15 min at room temperature. After development, the plates were dried in a current of hot air using a hair-dryer. Densitometric scanning in reflectance mode at 253 nm was performed at 20 mm/s scanning speed and the slit dimension was kept at 6.0 × 0.30 µm. The TLC chromatograms were manipulated by WINCATS software.

2.5.2. For spectrofluorimetric method

One milliliter of the working standard or sample solution in the concentration range of 10 - 100 µg ml^{-1} was transferred into 10 ml test tubes, then 1.0 ml of borate buffer solution (pH 8.5) and 1.2 ml of NBD-Cl reagent (0.1% w/v) were added. The reaction was allowed to proceed at 70 °C in a water bath for 20 min. After cooling the test tubes in ice bath, 0.2 ml of conc. hydrochloric acid was added at the wall of the test tube and mixed well. Finally the contents of the test tube were quantitatively transferred into 10-ml calibrated flasks and completed to the mark with methanol. The fluorescence intensity of the resulting solutions was measured at 500 nm (λ_{ex}, 465 nm). A blank experiment was performed similarly omitting the drug.

2.6. Procedures for stability indicating assay:

Alkali induced degradation: Into 10-mL test tube, 1 ml of VAC solution (1 mg ml^{-1}) was transferred and 1 ml of 0.05 M NaOH was added. The solution was left at room temperature for 2 hours. After the specified time, the solution was neutralized to pH 7 using1 ml of 0.05 M HCl solution and completed to the volume with methanol.
Acid induced degradation: Into 10-ml volumetric flask, 5 ml of VAC solution (1 mg ml$^{-1}$) was transferred and refluxed with 5 ml of 1 M HCl for 2 hours. After the specified time, the solution was neutralized to pH 7 using 1 M NaOH solution. The solution was transferred quantitatively into 50-ml volumetric flask and completed to the volume with methanol.

Oxidative induced degradation with hydrogen peroxide was carried out by transferring 1.0 ml of VAC solution working solution (1 mg ml$^{-1}$) into 10 ml volumetric flask then 1 ml of hydrogen peroxide (30.0 % v/v) was added. The solution was kept at room temperature for a period of 2 hours. The solution was heated in boiling water bath for 10 minutes to completely remove the excess of hydrogen peroxide. The volume was completed to 10 ml with methanol.

The photochemical stability of the drug was studied by exposing the stock solution (0.1 mg ml$^{-1}$) to direct artificial day light for five days.

For TLC- densitometric method 6 ml of the above solution was diluted to 10 ml with methanol in volumetric flask then 4 µl was applied to TLC plate and the plate was developed and analyzed as described in "general analytical procedures". For spectrofluorimetric, 1.0 ml of the above solution was used in the "general analytical procedures".

3. Results and discussion

3.1. Method optimization for the TLC-densitometric measurements.

TLC procedure was optimized with a view to develop a stability-indicating assay method. The pure forms of drug and its degradation product, acyclovir were spotted on TLC plates and run in different solvent systems. Initially, chloroform-methanol in varying ratios was tried. Chloroform–methanol (5:1.4 v/v) as a mobile phase gave good resolution with RF value of 0.56 ± 0.03 for VAC and 0.3 ± 0.03 for acyclovir unfortunately with tailing peaks. Addition of 0.2 ml ammonia to the above mobile phase gave compact spots for both drug and acyclovir. The final mobile phase consisted of chloroform: methanol: ammonia (5:1.4:0.2 v/v/v) which gave a sharp and symmetrical peaks. To obtain a well-defined spots the chamber was saturated with the mobile phase for 15 min at room temperature prior to the analysis.

3.2. Method optimization for the spectrofluorimetric measurements.
VAC contains a primary amino group that could react with NBD-Cl through a condensation reaction. This reaction was investigated in this study to develop the first spectrofluorimetric method for determination of VAC. The reaction product was colored yellow and exhibited highest fluorescence intensity at $\lambda_{em}$ 500 nm after excitation at $\lambda_{ex}$ 465 nm, (Fig. 2). The experimental parameters affecting the development and stability of the reaction product were investigated and optimized. Each parameter was changed individually while the others were kept constant. These parameters include; pH, volume of the buffer, concentration of NBD-Cl, reaction time, temperature and diluting solvent.

**Effect of pH**

The effect of the solution pH on the reaction product formation was studied in the pH range of 7 - 10. The fluorescence intensity of the reaction product was highly dependent on pH. The highest intensity was obtained in the pH range of (8.3 - 8.8). Higher or lower solution pH resulted in a decrease on the fluorescence intensity. Therefore the optimum pH was 8.5 using borate buffer solutions (Fig. 3).

**Effect of the Volume of the buffer:**

Several volumes (0 - 3 ml) of borate buffer of pH 8.5 were used for general assay procedure. The maximum fluorescence intensity was obtained when the buffer volume was in the range of 0.8 - 1.5 ml. Lower or higher volume showed marked decrease in the fluorescence intensity, Therefore the selected volume of borate buffer was 1 ml.

**Effect of the volume of NBD-Cl solution:**

Different volumes of NBD-Cl (0.1 % w/v) were used in performing the general analytical procedure. Increasing NBD-Cl volume produced a gradual increase in the fluorescence intensity until reaching a steady state at 1.0 - 1.5 ml of NBD-Cl. Further increase in the volume resulted in slight decrease in the fluorescence intensity. So, 1.2 ml NBD-Cl was chosen for subsequent works (Fig. 4).

**Effect of heating time and temperature**

The general analytical procedure was carried at different temperature or for different heating period. It was found that either increasing the temperature of the reaction mixture or heating for longer time produced a corresponding increase in the
fluorescence intensity of the reaction product. However, it was noticed that heating at a relatively low temperature (70 °C) for a longer period (20 min) was better than heating at a higher temperature for a shorter period of time, regarding the reproducibility of the fluorescence intensities.

**Effect of diluting solvent:**

In order to select the most appropriate solvent for dilution, different solvents were tested including; methanol, ethanol, acetonitrile, and acetone. Methanol was found to be a best diluting solvent as it gave highest fluorescence intensity, and therefore was selected as diluting solvent. Unfortunately the high reading obtained with methanol was accompanied with the presence of high fluorescence background. This was attributed to the hydrolysis of NBD-Cl to the corresponding hydroxy derivative, namely, 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) [26]. The fluorescence of NBD-OH could be quenched by decreasing the pH of the reaction medium to less than one [27]. Therefore acidification of the reaction mixture prior to dilution and measurement of the relative fluorescence intensity was necessary to effectively decrease the background fluorescence. In the same time, the fluorescence of the reaction product was not affected by acidification, thus the sensitivity was ultimately enhanced. The suitable amount of hydrochloric acid required for acidification was found to be 0.2 ml of the concentrated acid.

**3.3. Stoichiometry and mechanism of the reaction**

Job’s method of continuous variation [28] was employed for investigating the stoichiometry of the reaction. Equimolar solutions of both the drug and NBD-Cl reagent (1 X 10^{-3} M) were prepared. Then portions of mixture of master solutions of the drug and reagent were made up comprising different complementary proportions (0.2: 2.8, 0.4: 2.6, 0.5: 2.5…………2.6: 0.4, 2.8: 0.2). Results suggested that VAC reacts with NBD-Cl in the ratio 1:1 (Fig. 5). Based on this ratio the suggested mechanism of the condensation reaction with NBD-Cl was presented in (Fig. 6).

It should be noted that, the degradation product, acyclovir could not undergo the condensation reaction with NBD-Cl and this can be inferred from the absence of any fluorescence property due to the reaction. This may be attributed to tautomerism of the amino group in the guanine base making the proton of amino group acidic therefore no condensation reaction could occur with NBD-Cl.
3.4. Method validation

The proposed methods have been validated according to ICH guidelines [29]. The investigated parameters include: linearity and range, sensitivity, precision, accuracy, recovery and robustness.

The International Conference on Harmonization (ICH) guideline entitled ‘stability testing of new drug substances and products’ requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [30]. An ideal stability-indicating method is one that quantifies the drug and resolves its degradation products.

**Linearity and range:**

The proposed methods showed a good linear correlation in concentration range of 20 - 300 ng band\(^{-1}\) \((r^2 = 0.9992)\) for densitometric method and 1 - 10 µg ml\(^{-1}\) \((r^2 = 0.9991)\) for spectrofluorimetric method. The high value of correlation coefficient assured the validity of the proposed methods (table 1).

**Accuracy:**

Standard addition method was applied to examine of the accuracy of the proposed methods. Known amounts of pure drug were added to a previously analyzed sample solution. The mixtures were re-analyzed by the proposed procedures in six replicates and the percentage recovery was calculated. The calculated high percentage average recovery and low relative standard deviation indicate the suitable accuracy of the method (Table 2).

**Precision:**

The interday and intraday precisions [31] were examined by analyzing six replicates of VAC solutions at three concentration levels (80, 120 and 300 ng band\(^{-1}\) for TLC-densitometric and 3, 4 and 6 µg ml\(^{-1}\) for spectrofluorimetric method) in seven successive days. The low value of RSD (less than 1.28 %) indicates fairly the good, repeatability and reproducibility of the proposed method (Table 3).

**Sensitivity**

Sensitivity of the proposed method was evaluated by calculating limit of detection (LOD) and limit of quantification (LOQ) using the formula; "x = n σ / S", where x is LOD or LOQ, n is a numerical value equal to 3.3 or 10 for LOD or LOQ, respectively.
and $\sigma$ is the standard deviation of intercept and $S$ is the slope of calibration graph [30-32]. The calculated LOD and LOQ values for TLC- densitometric were 6.4 and 19.5 ng band$^{-1}$, respectively and for spectrofluorimetric method were 0.25 and 0.75 $\mu$g ml$^{-1}$ respectively. The low values of these parameters indicate the high sensitivity of the proposed method (table 1).

**Robustness**

The robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variation in method parameters and provides an indication of its reliability in routine analysis.

Robustness of TLC- densitometric method was performed by introducing small changes in the mobile phase composition, saturation time and wavelength, then examining their effects on the obtained results. Mobile phases having different composition of chloroform: methanol: ammonia (4.9:1.3:0.1 and 5.1:1.5:0.3, v/v/v) and different wavelengths (248 and 258 nm). Robustness for spectrofluorimetric method was performed by making small changes in general analytical procedure, such as NBD-Cl volume ($\pm$ 0.2 ml), solution pH ($\pm$ 0.2 unit), and volume of the buffer ($\pm$ 0.5 ml). The obtained results from small variation in any of these parameters did not significantly affect the results of both methods. Therefore the procedures of the proposed methods are considered robust.

**3.5. Stability-indicating studies**

VAC was exposed to different stress conditions including acid, alkali, oxidation and photodegradations. After applying the degradation procedures, the proposed methods were applied for determination of the extent of degradation of the cited drug. Alkaline and Acidic induced hydrolysis resulted in the appearance of an additional peak at $R_F$ value of 0.3 $\pm$ 0.03 (Fig. 7 a & b). The new peak appears before the parent drug peak which gives an indication that the degradation product is more hydrophilic and contains polar group. The degradant was identified as acyclovir through comparison between $R_F$ of the degradant and $R_F$ of the standard authentic acyclovir. About 45 % of the drug was degraded in case of acidic degradation while 52 % of the drug was degraded in case of alkaline degradation. On the other hand, the exposure to oxidation with 30 % v/v hydrogen peroxide and photodegradation with direct sun light for five days did not produce any significant effect on the drug chromatograms.
and only the peak of VAC was observed at R\textsubscript{f} = 0.55 (Fig. 7 c & d). This gives an indication about the stability of VAC against oxidation and photodegradation but its liability of for basic and acidic hydrolysis.

3.6. Application to tablets

The proposed methods were applied for determination of VAC in the commercially available tablets. The mean recoveries values were 100.13 ± 0.33 and 98.5 ± 1.75 for TLC- densitometric and spectrofluorimetric methods, respectively. The results of the proposed methods were statistically compared with that of the reference method [8] regarding t- and F- tests at 95 % confidence level. As shown in table 4, there is no significant difference between the results of both proposed and reported method, as the calculated values is less than the tabulated one. This indicated that the proposed methods have good accuracy and precision.

4. Conclusion

The proposed TLC-densitometric and spectrofluorimetric methods are accurate, sensitive and selective methods. They allow the determination of VAC in pure form and in the presence of its degradation product, as well as in pharmaceutical dosage form. So, it can be considered as a stability-indicating one. The satisfactory sensitivity and simplicity make the methods suitable for routine analysis of VAC in quality control laboratories.

References


23. Al Azzam, K. M.; Saad, B.; Makahleah, A.; Aboul-Enein, H. Y.; Elbashir, A. A. Assay and stability-indicating micellar electrokinetic chromatography method for the


Table 1. Regression equation and validation parameters for the proposed TLC-densitometric and spectrofluorimetric methods for determination of VAC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>TLC- densitometric method</th>
<th>Spectrofluorimetric method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity Range</td>
<td>20-300 (ng band$^{-1}$)</td>
<td>1-10 (µg ml$^{-1}$)</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9996</td>
<td>0.9996</td>
</tr>
<tr>
<td>Determination coefficient (r$^2$)</td>
<td>0.9992</td>
<td>0.9991</td>
</tr>
<tr>
<td>Intercept ($a$) ± SD</td>
<td>249.75 ± 24.31</td>
<td>104.03 ± 1.67</td>
</tr>
<tr>
<td>Confidence limit of intercept $b$</td>
<td>192.25</td>
<td>99.74</td>
</tr>
<tr>
<td>Slope ($b$) ± SD</td>
<td>12.48 ± 0.14</td>
<td>22.2 ± 0.29</td>
</tr>
<tr>
<td>Confidence limit of slope $b$</td>
<td>12.16</td>
<td>21.45</td>
</tr>
<tr>
<td>LOD</td>
<td>6.42 (ng band$^{-1}$)</td>
<td>0.25 (µg ml$^{-1}$)</td>
</tr>
<tr>
<td>LOQ</td>
<td>19.47 (ng band$^{-1}$)</td>
<td>0.75 (µg ml$^{-1}$)</td>
</tr>
</tbody>
</table>

(a) Number of determination is 9 for TLC- densitometric method and 7 for spectrofluorimetric method.

(b) at 95% confidence limit.

Table 2. Standard addition method for the recovery studies

<table>
<thead>
<tr>
<th>TLC- densitometric method</th>
<th>Spectrofluorimetric method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (ng band$^{-1}$)</td>
<td>% Recovery$^a$ (± SD)</td>
</tr>
<tr>
<td>Taken</td>
<td>% added</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>50 %</td>
</tr>
<tr>
<td>80</td>
<td>100 %</td>
</tr>
<tr>
<td>80</td>
<td>150 %</td>
</tr>
</tbody>
</table>

a; the value is the average of six determinations.
Table 3. The Intra- and inter-day precision for the determination of VAC by the proposed Methods.

<table>
<thead>
<tr>
<th></th>
<th>Intra-day precision</th>
<th></th>
<th>Inter-day precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount a</td>
<td>% Recovery b ± % RSD</td>
<td>Amount a</td>
</tr>
<tr>
<td>TLC- densitometric method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>99.89 ± 0.33</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>98.60 ± 0.52</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>99.45 ± 0.49</td>
<td>0.30</td>
</tr>
<tr>
<td>Spectrofluorimetric method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>99.07 ± 1.28</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>101.2 ± 0.26</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>100.8 ± 1.11</td>
<td>6</td>
</tr>
</tbody>
</table>

a The amount is ng band$^{-1}$ for TLC-method and µg ml$^{-1}$ for the spectrofluorimetric method.

b the value is the average of six determinations.

Table 4. Analysis of containing 1110 mg of VAC using the proposed and reference methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>% Recovery ± SD</th>
<th>t-value</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proposed method</td>
<td>Reference method</td>
<td></td>
</tr>
<tr>
<td>TLC- densitometry</td>
<td>100.13 ± 0.33</td>
<td>99.44 ± 0.62</td>
<td>1.68</td>
</tr>
<tr>
<td>Spectrofluorimetry</td>
<td>98.5 ± 1.75</td>
<td>99.44 ± 0.62</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Average of five determinations.

a Tabulated values for t and F. are 2.306 and 6.338 respectively.
Fig. 1. Chemical structure of valacyclovir HCl and Acyclovir

Valacyclovir HCl

Acyclovir

Fig. 2. Excitation and emission spectra of the reaction between NBD-Cl and VAC (4 µg ml⁻¹)

Fig. 3: Effect of the pH on the condensation reaction of VAC (4 µg ml⁻¹) and NBD-Cl.
Fig. 4: Effect of the volume of NBD-Cl reagent on the fluorescence intensity of the reaction with VAC (4 µg ml⁻¹).

Fig. 5: The Stoichiometry of the reaction between NBD-Cl and VAC
**Fig. 6.** The suggested mechanism of the condensation reaction of VAC with NBD-Cl.

Fluorescent valacyclovir-NBD
Fig. 7: TLC chromatograms of VAC degradation

Fig. 7a. Alkali induced degradation

Fig. 7b. Acid induced degradation
Fig. 7c. Photolytic degradation

Fig. 7d. Oxidative degradation