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Spectrophotometric determination of acyclovir after its reaction with ninhydrin and ascorbic acid

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ABSTRACT

Acyclovir is a purine-based nucleoside antiviral agent used in the management of Herpes simplex and other viral infections. The present study is aimed at developing and validating a simple and rapid spectrophotometric method for its determination. The mechanism of the proposed method is based on the condensation/coupling reaction between Acyclovir and Ninhydrin-Ascorbic acid at pH 5. A purple colored product with maximum absorption at 540 nm was assayed to quantitatively evaluate the drug content in the formulation. The calibration curve was found to be linear up to 30 µg/ml. Analyte recovery tests carried out by the proposed method gave recovery of between 96.9 – 102.0%. Molar absorptivity and Sandells' sensitivity were determined to be 41,071.43 L mol^{-1} cm^{-1} and 1.84 µg cm^{-2} respectively. The precision was assessed by determining the inter-day and intra-day variation which ranged between 1.45 – 1.63 % and 0.81 – 1.12 % respectively. The results show that the reaction produced a stable product and the proposed method is cost-effective and possesses adequate accuracy, precision and sensitivity. It can therefore be conveniently applied for the determination of acyclovir in dosage forms.

INTRODUCTION

Acyclovir (9-[(2-hydroxyethoxy)methyl] guanine) is a synthetic purine-based nucleoside analogue with potent *in vitro* and *in vivo* inhibitory activity against herpes simplex viruses (HSV), varicella zoster virus (VZV), epstein-Barr virus (EBV), cytomegalovirus (CMV) and human herpes virus 6 (HHV-6) (Corey *et al*, 1982; Keeney *et al*, 1982; Thin, 1988; Wagstaff *et al*, 1994). Acyclovir exerts its antiviral activity by acting as pseudo-substrate and thus inhibits viral DNA polymerase enzyme. Phosphorylation of Acyclovir to the active acyclovir monophosphate occurs via viral or cellular thymidine kinase enzyme constitute the preliminary reaction (O'Brien and Richards, 1989; Gnaan *et al*, 1983; Whitley *et al*, 1982). The British Pharmacopoeia (2005) specifies a UV spectrophotometric method for the determination of acyclovir. A number of unofficial assay methods utilizing a variety of techniques and reaction pathways have also been successfully developed for the drug and some of these include; Polarography (Sheribah *et al*, 2009), Radioimmunoassay (Blum *et al*, 1982; Quin *et al*, 1979), Near IR spectroscopy (Yu and Xiang, 2008), Microcellar electrokinetic chromatography (Yeh *et al*, 2006), HPLC with UV detection (Batterman *et al*, 1998; Pramar *et al*, 1990; Dubhashi and Vavia, 2000; Land and Bye, 1981), HPLC with MS detection (Kourany and Cyr, 1995; Kamel *et al*, 1999), HPLC with flourimetric detection (Testereci *et al*, 1998; Macka *et al*, 1993; Mascher *et al*, 1992; Jankowski *et al*, 1998). Acyclovir determination in pharmaceutical products has also been performed by employing methods based on derivatization of the drug with chromogenic reagents (Basavaiah and Prameela, 2002; El-din *et al*, 2006; Sultan, 2003; Mustafa *et al*, 2004; Chakraborty *et al*, 2011). A derivative (Daabees, 1998) and a differential spectrophotometric method (Mahrous *et al*, 1992) have also been reported. Spectrophotometric methods continue to be the most preferred for routine analytical work due to their simplicity, reasonable level of sensitivity and their low cost. Some of the previously mentioned colorimetric methods have some limitations such as long reaction time and low selectivity for the analyte. The present study therefore aims to develop a simple and accurate colorimetric method for the determination of acyclovir in dosage form.

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EXPERIMENTAL

Materials

Acyclovir tablets were commercial products. The following reagents were obtained from BDH, England: ninhydrin, ascorbic acid, sodium hydroxide, ethanol, citric acid. All the chemicals and reagents used were of analytical grade and were used without further purification. The aqueous solutions were freshly prepared with triple distilled water. A Shimadzu UV-Visible double beam spectrophotometer (Model 1250, Japan) was used for the measurements.

Method

Extraction of Pure Acyclovir from Tablet Formulation

Twenty (20) tablets of acyclovir (label claim: 400 mg each) were finely powdered in a porcelain mortar and extracted with 0.5MolL^{-1} sodium hydroxide (25 mL x 4). This was followed by filtration through Whatman no. 1 filter paper. The filtrate was then evaporated to dryness at reduced temperature. The residue was collected and weighed and its melting point determined to ascertain its purity. This powder was used subsequently as a pure secondary reference sample.

Preparation of buffer solution (pH 5)

4.95g of citric acid and 2.0g of sodium hydroxide were dissolved in 100 mL distilled water and its pH was then adjusted to 5.0 with sodium hydroxide (Nagaraja *et al*, 2011).

Preparation of 1% w/v ninhydrin Solution

1.0 g of ninhydrin was weighed and dissolved in some ethanol in a 100 mL volumetric flask and the volume made up to mark (Arayne *et al*, 2008).

Preparation of 0.1% w/v ascorbic acid solution

0.1 g of ascorbic acid was weighed and dissolved in some distilled water in a 100 mL volumetric flask and the volume made up to mark (Subbayamma and Rambabu, 2008).

Preparation of acyclovir stock solution.

100 mg of the pure drug was dissolved in 100 mL of 0.5 MolL^{-1} NaOH in a volumetric flask to form the stock solution (1 mg / mL).

Construction of calibration curve and its validation

From the stock solution of acyclovir (1mg / mL), 1.0 – 3.0 mL were quantitatively transferred to separate 10 mL calibrated screw-cap test-tubes. 4.0 mL of the buffer solution (pH 5.0), 1.0 mL of 1%w/v ninhydrin solution and 0.5 ml of 0.1%w/v ascorbic acid solution were added and shaken for three minutes. The volume in each tube was adjusted to 9.0 mL with distilled water and kept in a boiling water bath for 15 minutes; the tubes were then removed and chilled in ice water. The solution in each tube was finally made up to 10.0 mL with distilled water. The blank solution was prepared in a similar manner but without the drug. The absorption maximum was determined using the spectrophotometer. The limits of detection and quantification were estimated according to IUPAC rules (LOD = B + 3σ/S and LOQ = B + 10σ/S where B is the value of sample blank solution, σ is the standard deviation of 10 replicates of the blank, and S slope of calibration curve). Quadruplicate preparations were made for each volume and their absorbances read with the spectrophotometer. The plot of absorption against concentration was obtained from the mean of five replicate determinations. The curve was validated by methods previously reported (Onah and Odeani, 2002). Molar absorptivity, Sandells' sensitivity, regression equations and standard deviations were also determined. The analytical technique was optimized by a method reported earlier (Onah and Ajima, 2011).

To study the accuracy of the proposed method, analyte recovery tests were carried out using the standard addition method. For these, known quantities of pure acyclovir were mixed with definite amounts of pre-analyzed formulation and the mixture was analyzed as previously described. The total amount of the drug was then determined and the amount of the added drug recovered was calculated by difference. Precision of the method was assessed by evaluating the inter-day and intra-day variation which were determined by replicate analysis (n = 5) of calibration standards at three different concentration levels, five times per day on five consecutive days. The stability of the product formed from the reaction was also evaluated over a period of days. As part of the preliminary method optimization studies, the reaction was carried out at several pH values ranging between 2 -11 to determine the effect of pH on absorbance of the product formed and to establish the optimal pH for the analytical procedure. Maximum absorbance and stability of the product formed was obtained at pH of 5 and this was used subsequently for the analysis.

Determination of actual content of Acyclovir in different tablet formulations using the proposed method

Five different brands of acyclovir tablet formulation were assayed using the developed method. For each brand, the contents of 20 tablets were weighed, ground into a fine powder. An accurately weighed portion of the powder equivalent to 100 mg acyclovir was transferred into a 100 mL volumetric flask. 25 mL of distilled water was added and after some minutes of mechanical shaking, the suspension was made up to mark with the solvent. After filtration, suitable amounts of the filtrate were then taken and the same procedure was applied as described for the calibration curve. The content of each label claim was verified by comparing the concentrations obtained from the validated curves with the actual concentrations of the drug taken. Standard deviations were also calculated for each brand.

RESULTS

Acyclovir was found to react with ninhydrin and ascorbic acid to form a purple colored product which absorbs maximally at 540 nm (Fig 1). The purple colored solution was stable beyond 24

hours. The limit of detection (LOD) and limit of quantitation (LOQ) (calculated using LOD = 3σ/slope of calibration curve and LOQ = 10σ/slope of calibration curve) were determined to be 0.3 µg / mL and 0.8 µg / mL respectively.

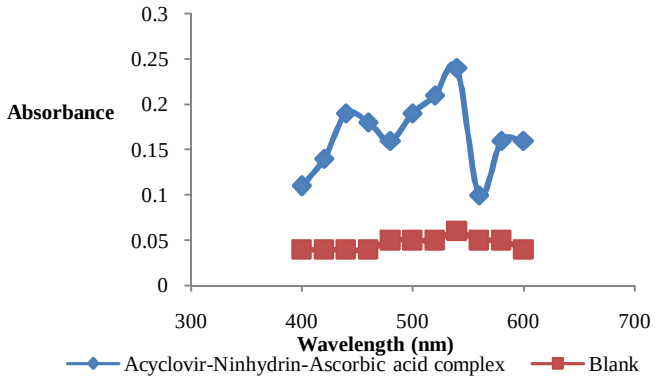

Fig. 1: Absorption spectrum for acyclovir-ninhydrin-ascorbic acid condensation product.

The plots of absorbance versus drug concentration were linear up to 30 µg / mL. Optimization of the various concentrations during the experimentations did not show any significant differences in standard deviations ($p \geq 0.01$) which suggests that the method produced consistent results. Several replicate determinations were carried out from which regression equations, standard deviations in slope and intercept were calculated. The regression equation shown in Equation 1 satisfied all the conditions investigated.

$A_{abs} = 0.35x - 0.062$ (regression coefficient = 0.993)…… (1)

where A_{abs} = absorbance of analyte, x = concentration of analyte in the final mixture in moles.

Table 1: Results of recovery study using Standard addition method.

S/n	Brand	Endogenous Value (µg/ml)	Pure Acyclovir added (µg/ml)	Amount Found (µg/ml)	% Recovery ± SD*
1.	Hepirax®	10	5	14.91	99.4 ± 1.16
			10	19.38	96.9 ± 0.92
			15	24.80	99.2 ± 1.63
2.	Vivorax®	10	5	15.18	101.2 ± 1.79
			10	20.14	100.7 ± 0.88
			15	25.58	102.3 ± 1.11
3.	Zovirax®	10	5	14.91	99.4 ± 2.07
			10	20.14	100.7 ± 1.63
			15	25.50	102.0 ± 1.54
4.	Lovir®	10	5	15.02	100.1 ± 1.24
			10	19.86	99.3 ± 1.43
			15	24.78	99.1 ± 1.95
5.	Virest®	10	5	14.95	99.7 ± 2.10
			10	19.98	99.9 ± 1.66
			15	24.95	99.8 ± 1.38

*Mean value of three determinations.

The results of the analyte recovery tests conducted by this method are shown in Table 1. Recovery values were found to range between 96.9-102.0%. The relative standard deviations of the slope and intercept were calculated to be 0.0412% and 0.0158% respectively. Molar absorptivity and Sandells' sensitivity were similarly calculated to be 41,071.43 L mol^{-1} cm^{-1} and 1.84 µg cm^{-2} respectively. The inter-day variation ranged between 1.45 – 1.63 % and the intra-day variation ranged from 0.81 – 1.12 % for the method. The condensation product formed from the reaction was found to remain stable beyond 24 hours.

Table 2: Summary of validation parameters obtained for the method.

Parameter	Value
Regression Equation	Y = 0.35x – 0.062
Correlation co-efficient (r^2)	0.9930
Range	10 – 30 mcg/ml
Limit of detection (LOD)	0.3 mcg/ml
Limit of quantitation (LOQ)	0.8 mcg/ml
λ_{max}	540 nm
Molar absorptivity	41,071.43 L/mol/cm
Sandells sensitivity	1.84mcg/cm^2
Inter-day precision	1.45 – 1.63 %
Intra-day precision	0.81 – 1.12 %

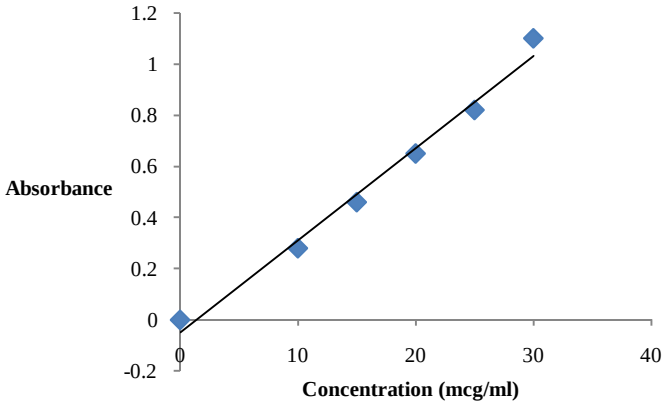

Fig. 2: Beer Lamberts plot of acyclovir-ninhydrin-ascorbic acid condensation product.

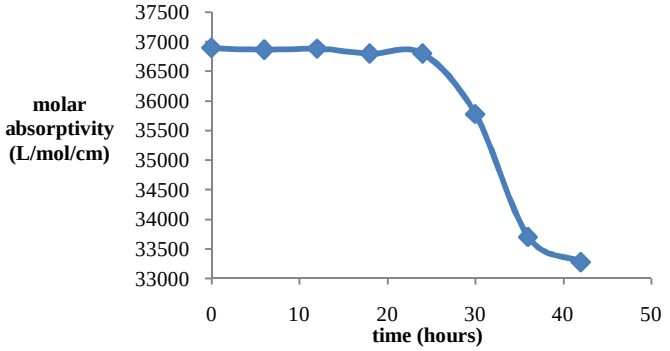

Fig. 3: Plot of molar absorptivity of the condensation product versus time.

DISCUSSION

The proposed method represents an analytical application for ninhydrin determination. Acyclovir possesses a primary amino group as part of the guanine residue which is capable of undergoing condensation and coupling reaction with ninhydrin-ascorbic acid (Figure 4) under acidic conditions created by the use of the citric acid buffer (pH 5) to produce a purple-coloured chromophore (Ruhemann's purple) that absorbs maximally at

540nm. This represents a bathochromic shift of the λmax for the drug, as it is established from literature that acyclovir absorbs only in the UV region with a λmax of 255 nm (BP, 2005). Formation of the purple color serves as evidence of the reaction between acyclovir and the chromogenic reagents. Moore and Stein (1948) had previously shown that for ninhydrin reaction with primary amines, the pH must be maintained at 5 for optimum stability of the colored product.

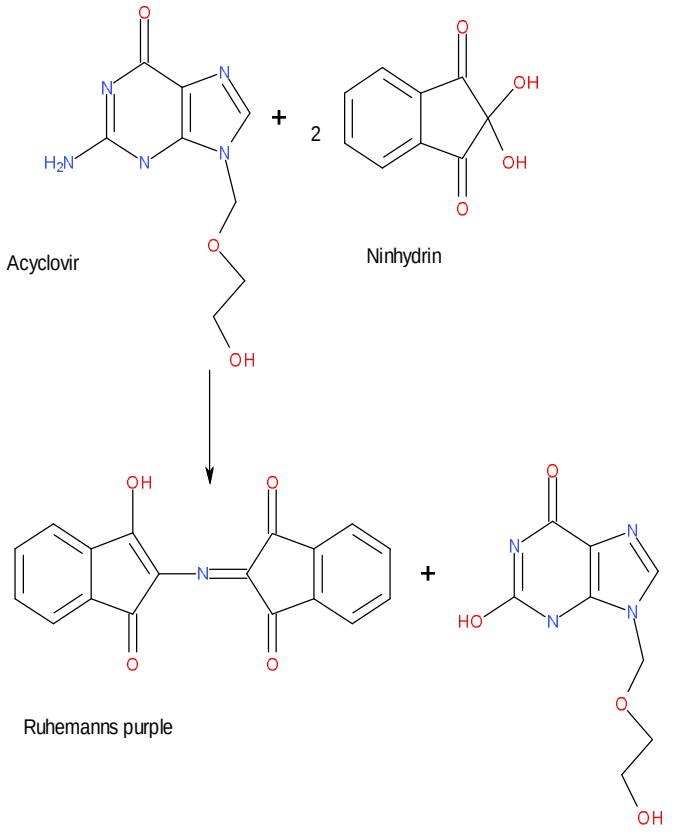

Fig. 4: Proposed reaction pathway between acyclovir and ninhydrin-ascorbic acid.

The stability of the product formed under the conditions mentioned above was therefore investigated. It was found that full color development occurred spontaneously at room temperature (25 ± 1 °C) and the values of absorbances of the product formed were found to remain unchanged after standing for 24 hours (figure 3) at room temperature indicating its stability for that period of time. The observation that a single equation (Equation 1) could be derived to satisfy the different reaction conditions reveals that the reaction produced stable products. The absorption spectrum for the acyclovir-ninhydrin complex showed two absorption maxima at 440nm and at 540nm as shown on figure 1 and this is as a result of the introduction of additional chromophores in conjugation with the existing chromophores sequel to the reaction.

The peak at 540nm was chosen for the spectro-photometric measurements since it was more prominent indicating a higher intensity of absorption. Also, the high molar absorbtivity and relatively low Sandell's sensitivity provides evidence as to the accuracy and sensitivity of the technique. The high values of correlation coefficients obtained for regression equations demonstrate the good linearity of the method. As regards analytical applications, the proposed method was successfully applied for the quantitative determination of Acyclovir in five tablet formulations. The result obtained was in good agreement with the labeled claim.

CONCLUSION

The proposed method has been demonstrated to be quite simple, sensitive and reproducible and is thus suitable for the determination of Acyclovir alone and also in pharmaceutical dosage form. The method can thus be adapted for the quality control of Acyclovir in hospitals and laboratories in less endowed settings where more sophisticated analytical equipment such as HPLC is not readily available.

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