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Development and validation of a new spectrophotometric method for the determination of acyclovir

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Abstract
A new spectrophotometric method has been developed for the analysis of acyclovir in bulk and dosage forms. The method is based on the diazo coupling reaction between diazotized acyclovir and *p*-dimethylaminobenzaldehyde (DMAB). Spot tests and thin layer chromatographic analysis confirmed the formation of a greenish-yellow adduct which was stable in the laboratory environment for more than three hours. Critical factors affecting optimal detector response were identified and optimized. The optimal temperature and coupling reaction time were established at 50 ^{o}C and 10 min. The azo adduct was determined at 404 nm where neither diazotized acyclovir nor DMAB has any significant absorptivity. Methanol was found as the best diluting solvent after coupling. The assays of acyclovir were linear over the range 1.81-9.06 µg/mL with a correlation coefficient of 0.9998 and limit of detection of 0.024 µg/mL. The method was accurate (error < 3 %) and precise (RSD < 2.7 %) over three days assessment. There was no interference from commonly used excipients. The method was successfully applied to the determination of acyclovir in tablets and creams with similar accuracy to the official USP spectrophotometric method. The method is rapid, simple and cost-effective and could find application in the in-process quality control of acyclovir.

Keywords: Acyclovir; Diazo coupling; *p*-Dimethylaminobenzaldehyde; Spectrophotometric determination

INTRODUCTION

Acyclovir, chemically 9-[(2-hydroxyethoxy) methyl] guanine, has a molecular weight of 225.21. Acyclovir and its sodium salt are active against herpes simplex viruses (HSV-1 and HSV-2), varicella-zoster infections, and Epstein-Barr virus. Acyclovir is an acyclic nucleoside analogue, and it is incorporated into viral DNA inside an infected cell where it interferes with viral replication (IARC, 2000). Acyclovir is frequently given orally in the management of first and recurrent episodes of muco-cutaneous herpes in selected patients, for the acute treatment of herpes zoster (shingles) and for the treatment of chickenpox in adults and children. Acyclovir is also used topically in the treatment of muco-cutaneous HSV infections, although it is substantially less effective than systemic therapy (Worall, 1996).

Acyclovir in biological fluids and dosage forms has been analyzed by HPLC (Emami *et al*., 2009; Boulieu *et al*., 1997), high performance capillary electrophoresis (Zhang *et al*., 2000) and spectrophotometric and spectrofluorimetric methods (Reddy *et al*., 2011; Basavaiah and Prameela, 2002; El-Din *et al*., 2006; Sultan, 2003; Mustafa *et al*., 2004; Pant *et al*., 2009). These methods

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include a number of spectrophotometric methods have been reported for the determination of acyclovir both in the bulk and in dosage forms. These include the formation of a Schiff base λ_{max} 470 nm between the amino in the acyclovir and aldehyde functional group supplied by ethanolic vanillin in an acidic pH (Reddy *et al*., 2011). The use of Folin-Ciocalteu reagent to generate a blue chromogen that absorbs at 760 nm has been documented (Basavaiah and Prameela, 2002).

Other methods include coupling reaction with 3-methylbenzothiazolin 2-one hydrazone, following oxidation with cerium ammonium sulfate, potassium persulfate to produce a blue adduct λ_{max} 630 nm (El-Din *et al*., 2006) or with ferric chloride as oxidant to produce a green specie λ_{max} 616 nm (Sultan, 2003).

A spectrophotometric method for determination of acyclovir in pure and dosage forms has also been reported. The method is based on complexation reaction with either copper (II) using a borax/ sodium pH 9 hydroxide buffer or cobalt (II) in a non-aqueous medium using 1 % pyridine in methanol. The formed complexes absorbed maximally at 290 and 287 nm respectively (Mustafa *et al*., 2004).

These methods have their relative merits but nearly all of them employ sophisticated equipment not readily available in developing countries, expensive reagents, buffer systems, strongly acidic or non-aqueous media or show poor regression equations.

In this present work, we present a simple, sensitive, accurate, inexpensive analytical method using *p*-dimethylaminobenzaldehyde (DMAB) as a coupling agent for the diazotized drug. This is also the first time the diazotization of acyclovir prior to determination is being reported.

EXPERIMENTAL

Equipment. A Lambda 25 digital UV/VIS spectrometer (Perkin Elmer model) with 1 cm path length and matched quartz cells was used, thermostated water bath, vortex mixer.

Materials and Reagents. All reagents and chemicals used were of analytical grade and include acyclovir CRS, sulphuric acid, sodium nitrite, sulphamic acid. The drugs used for this study were obtained from pharmaceutical stores in Ibadan, Nigeria. They include Acyclovir brands, Virest® 200 mg tablets and Virest® 5 % cream.

Procedures. Standard solution of pure reference acyclovir (2×10^{-3} M) was prepared in 0.1 M HCl. A 0.03% DMAB solution was prepared in a solution of optimized 0.0625 M sulphuric acid solution.

Analytical procedure. A 5 mL of the iced-cold 2×10^{-3} M acyclovir solution was transferred into a 25 mL beaker, 1.5 mL iced 0.1 M freshly prepared sodium nitrite solution was added and stirred for 5 min in an ice bath. Thereafter, 0.2 mL of 0.2 M sulphamic acid solution was added and stirred for another 1 minute. Freshly prepared diazotized solution was used on each day throughout this study. Sample solutions for spot test and TLC were prepared by mixing 0.5 mL of the diazotized drug and 0.5 mL of the DMAB solution. Color change was noted immediately and after 20 min both at room temperature and at 70 ^{0}C.

Optimization of reaction conditions. The analytical wavelength was selected by recording the spectra of the diazotized drug, DMAB and the reaction product between the diazotized acyclovir and DMAB. The spectra were overlaid on one another and the analytical wavelength selected by inspection. For the development of maximum colour intensity, the volume and concentration of the reagents for diazotization were optimized. The optimization of temperature and time was

done using the method of steepest ascent (Karnes and March, 1993). This was carried out at 30, 50, 60, 70 and 80 °C, after 5 and 20 minutes each. Ten test tubes each containing 0.5 mL of 2 x 10^{-3} M of diazotized acyclovir was used. 0.5 mL DMAB solution was then added to each of the test tubes and vortex mixed. At the end of the various reaction times and temperatures, the reaction was stopped by cooling in ice and the reaction mixture was made up to 5 mL final volume with methanol. The absorbance reading of each of the mixtures was taken at 404 nm with methanol as blank solvent. Optimization of the time required for coupling to take place at the selected temperature (50 °C) was done at 0, 2, 5, 10, 20 and 30 minutes. The reaction mixtures were made up to 5 mL at these times with methanol, and the absorbance readings were taken at 404 nm with methanol as blank. All of these determinations were done in duplicate.

Stoichiometric ratio determination. Job's method of continuous variation (Rose, 1964) was used to determine the optimal stoichiometric ratios at which the diazotized drug will combine with DMAB. Increasing volumes (from 0-1.0 mL) of diazotized solution of acyclovir were made up to 1.0 mL with the DMAB solution. The reaction was then maintained at 50 °C for 10 min. At the end of this interval, the reaction was stopped by cooling in ice and the reaction volume made up to 5 mL with methanol. The absorbance readings were taken at 404 nm using methanol as blank. All procedures were carried out in duplicate.

Validation. Calibration line was generated from the 3-day average of curves using concentrations of the diazotized drug equivalent to 0, 1.812, 2.71, 3.62, 4.53, 7.24 and 9.06 µg/mL. 0.5 mL DMAB solution was added to each of the test tubes and each of the reaction mixture was incubated at 50 ^{0}C for 10 min. Thereafter, the reaction was stopped and the required volume of methanol was added to each test tube to make the final reaction mixture of 5 mL. The absorbance readings were taken at 404 nm. Model recoveries and repeatability of the new methods were carried out on three successive days as stipulated by the USP (USP, 1999). Intra- and inter-day accuracy and precision of the new method were assessed from the results of replicate analyses on the pure drug solution. The mean values and relative standard deviation values for replicate analysis at three different concentration levels were calculated. The limit of detection (*LOD*) and limit of quantification (*LOQ*) were calculated according to the current ICH guidelines (ICH, 2011) as the ratio of 3.3 and 10 standard deviation of the blank (n = 6), respectively divided by the slope of the calibration line.

Method selectivity. To demonstrate the selectivity of the method in the presence of commonly utilized pharmaceutical excipients and additives such as starch, lactose, magnesium stearate, talc, gelatin and their mixtures, recovery studies of diazotized acyclovir (4.53 µg mL^{-1}) from the matrices containing these excipients were carried out. Four replicates were determined in each instance.

Assay of dosage forms. A brand of acyclovir tablet and a brand of acyclovir cream were analyzed. For the tablets, weight uniformity test was carried out on 20 tablets and the content of the active ingredient determined by the new method. An amount of the powdered drug equivalent to 0.045 g of acyclovir was weighed into 100 mL volumetric flask, about 60 mL of 0.1 M HCl added and then shaken thoroughly for 20 min. The solution was made up to volume with 0.1 M HCl and then filtered. The filtrate was used as stock solution for subsequent diazotization as described above. From the filtrate, a 0.05 mL aliquot of the diazotized acyclovir stock solution (equivalent to 4.53 µg/mL acyclovir) was then transferred into test tubes and 0.5

mL of 0.03 % DMAB was added to each of the tubes. The reaction was allowed to proceed for 10 min at 50 ^{0}C and then made up to 5 mL with methanol. The absorbance reading was taken at 404 nm using methanol as blank solvent. The official UV spectrophotometric method was adopted for acyclovir tablets at 255 nm (BP, 2010a). Six replicate determinations were carried out using both methods. For the cream, a quantity of the well-mixed cream equivalent to 45.3 mg of acyclovir was added to 50 mL 0.1 M HCl and then partitioned with 50 mL ethyl acetate. The clear lower aqueous layer was collected after separation and the organic layer washed with 20 mL 0.1 M HCl. The combined washings and aqueous layer was then diluted to 100 mL with 0.1 M HCl, mixed well and filtered. The filtrate was used as stock solution for subsequent diazotization and analysis as previously described. The official UV spectrophotometric method of analysis for acyclovir cream at 255 nm was also carried out (BP, 2010b). Six replicate determinations were carried out using both methods.

RESULTS AND DISCUSSION

The proposed method is based on the coupling reaction between the diazotized acyclovir and DMAB. A persistent yellowish-green was formed which was quite distinct from the colorless appearance of the diazotized acyclovir and DMAB solution. Since the dimethylamino moiety in DMAB has a moderate activating influence on the aromatic skeleton, *ortho* substitution of an incoming electrophile might be possible. This reaction will be further aided by the presence of the aldehyde group which is *meta*-directing. Thus the 3- and 5-positions on the DMAB molecule are equivalent and will be available for electrophilic attack. The proposed coupling reaction pattern between diazotized acyclovir and DMAB is presented in Scheme 1. This reaction was previously optimized for the determination of reduced and diazotized nitroimidazoles, which represented the first application of DMAB as a coupling component in spectrophotometric determination, as well as for diazotized cephalosporins [Adegoke and Umoh, 2009; Adegoke and Quadri, 2012].

Evidence for the formation of new chemical species by diazotization of acyclovir and coupling with DMAB was established by TLC analysis. The R_f value showed the adduct was more non-polar than the diazotized acyclovir or DMAB as shown in Table 1. In all the three mobile phase systems adopted, the adduct was obtained as a single spot justifying only one product formed and the R_f values were distinct from that of the diazotized acyclovir and DMAB. The colour of the adduct was stable for more than three hours as judged by the constant absorbance readings over this period.

Method development. Critical variables required for optimal colour development were the amount of acids used for preparing both acyclovir and DMAB solutions as well as the concentration of sodium nitrite and sulphamic acid used in the diazotization steps. For the development of maximum colour intensity of the adduct, 0.1 M HCl, 0.1 M sodium nitrite and 0.2 M sulphamic acid were found to be necessary for the diazotization process. An excess of the sulphamic acid led to drastic reduction in the intensity of the colour of the reaction product.

Selection of analytical wavelength. The UV-VIS absorption spectra of the acyclovir, diazotized acyclovir, DMAB and the azo adduct formed between them are presented in Figure 1. The spectra show the formation of a new chromogen distinct from the starting materials. The diazotized acyclovir showed peaks at 215, 250 nm while the azo adduct absorbed maximally at 245, 350 nm with a shoulder at 404 nm.

Table 1: The R_f values for the thin layer chromatographic analysis

Mobile phase	Diazotized acyclovir	Adduct	DMAB
Ethyl acetate/Methanol (8:2)	0.025	0.625	0.56
Ethyl acetate/Methanol (9:1)	0	0.69	0.5
Ethyl acetate/Hexane (5:5)	0	0.75	0.625

Table 2: Analytical and Validation parameters for the assay of acyclovir

Parameter	Value
Beer's law limit (µg/mL)	1.812 - 9.06
Limit of detection (µg/mL)	0.024
Limit of quantification (µg/mL)	0.071
Molar absorptivity (L $mol^{-1}cm^{-1}$)	$1.1x10^4$
Sandell's sensitivity (µg cm^{-2} per 0.001 A)	0.018
Intercept ± 95 % CI	0.04 + 0.0045
Slope ± 95 % CI	0.0574+0.0032
Correlation coefficient, r	0.9998
Coefficient of determination, r^2	0.9996

Table 3a: Intra-day accuracy and precision

Amount added (µg/mL)	Amount found (µg/mL)	Mean recovery (%)*	RSD (%)	Relative error (%)
3.624	3.637	100.35±0.06	1.84	0.35
5.436	5.458	100.41±0.07	0.31	2.56
7.248	7.305	100.79±0.12	1.64	0.77

**n=4*

Table 3b: Inter-day accuracy and precision

Amount added (µg/mL)	Amount found (µg/mL)	Mean recovery (%)*	RSD (%)	Relative error (%)
3.624	3.623	99.97±0.09	2.67	0.03
5.436	5.514	101.43±0.13	2.47	1.43
7.248	7.244	99.94±0.14	1.94	0.06

**n=12*

Table 4: Interference studies of commonly used excipients

Drug Concentration	Recovery (%) *					
	Starch	Lactose	Talc	Magnesium stearate	Gelatin	Mixture of all excipients
4.53 µg/mL	97.1±0.2	97.8±0.3	98.3±0.3	106.2±0.1	106.7±0.2	106.7±0.3

** Recovery (%) = mean ± SD, n=4*

Table 5: Comparative determination of acyclovir in dosage forms

Drug Formulation (label claim)	New method			Official Method		Statistics (p values)	
	% recovery ± SD	95 % CI (of 4.5µg/mL)	RSD	% recovery ± SD	RSD	F-test	t-test
Virest Tab (200 mg)	99.45±0.6	4.48±0.48	0.61	99.65±0.23	0.23	0.08	0.69
Virest cream (5 %)	104.53±0.64	4.7±0.51	0.61	104.25±0.23	0.22	0.04	0.36

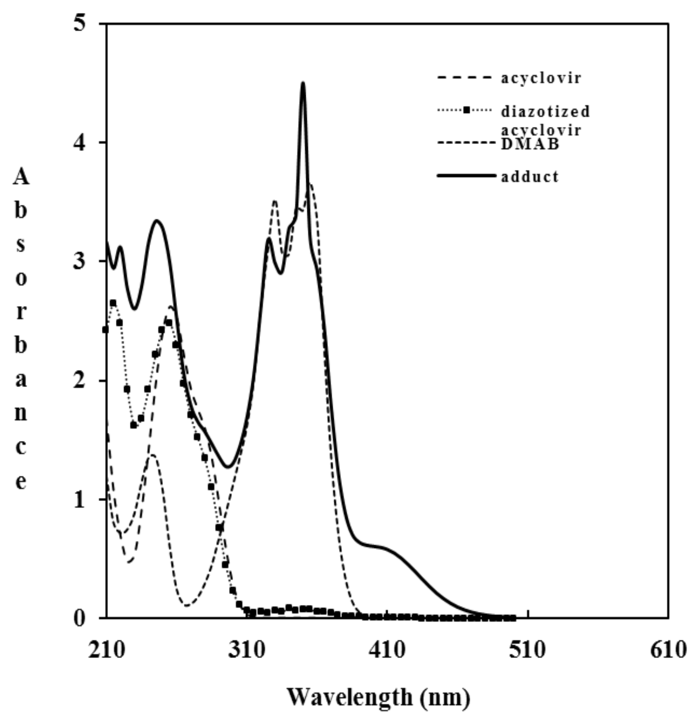


Fig 1: Overlaid Absorption spectra of acyclovir, diazotized acyclovir, DMAB and the azo adduct formed.

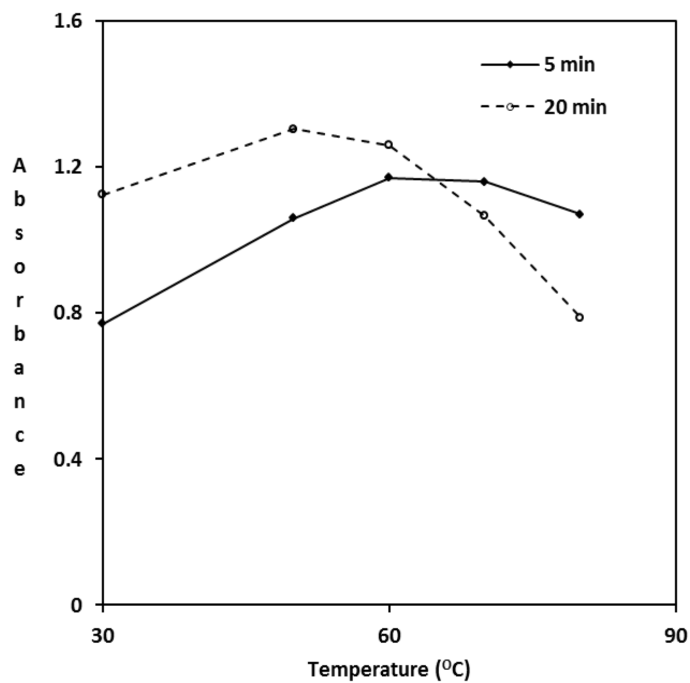


Fig 2: Optimization of coupling temperature

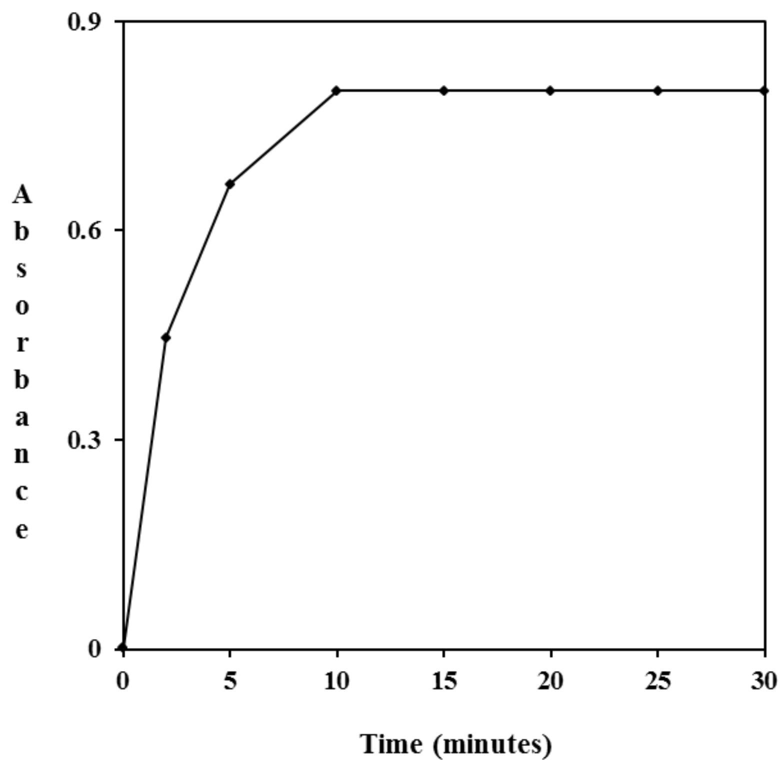


Fig. 3: Optimization of coupling reaction at 50 ^{o}C

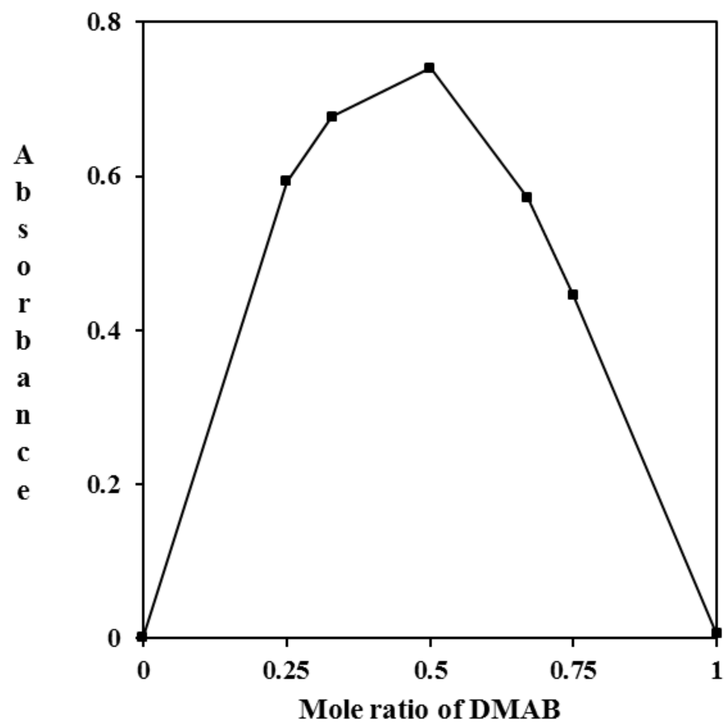
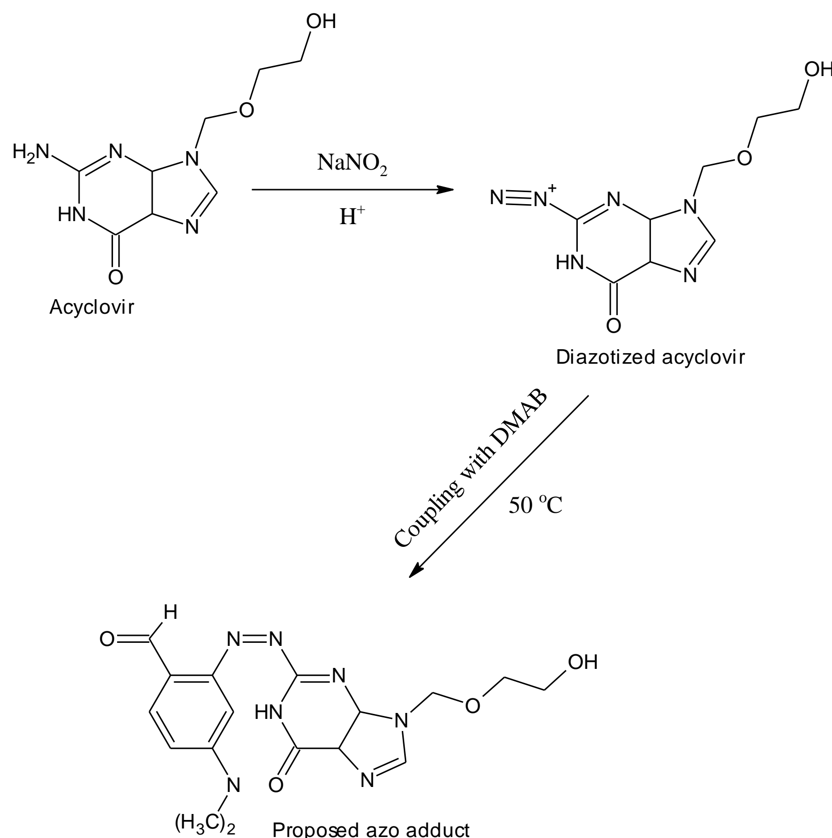


Fig. 4: Stoichiometric ratio of diazotized acyclovir and DMAB



Scheme 1: Proposed coupling pattern between diazotized acyclovir and DMAB

The analytical wavelength was selected as 404 nm where both the diazotized acyclovir and DMAB had extremely low absoptivities. The wavelength also gave optimal detector response. Critical looks at the azo adduct presented in Scheme 1 showed that the extent of chromophoric elongation produced by the diazo coupling of acyclovir with DMAB is minimal. This might be unconnected with the lack of extensive chromophore in acyclovir. However, the chromophoric elongation produced is sufficient to permit the determination of the compound at the visible region of the spectrum.

Optimization studies. The effect of temperature allowed for coupling reaction to take place as a function of time at levels of 30, 50, 60, 70 and 80 °C is presented in Figure 2. At 5 min reaction time, the absorbance increased gradually and peaked at 60 °C. Thereafter a decline was observed at 70 °C and 80 °C. This is most likely due to thermal decomposition of the azo adduct formed. At 20 min however, the peak absorptivity was obtained at 50 °C. This optimal coupling reaction temperature was thereafter investigated for the time required to attain the maximum absorptivity. The result is presented in Figure 3. The optimal coupling temperature and time were established as 50 °C and 10 min respectively. Thereafter, the measurement of the absorbance of the coloured adduct was found to be independent of time as the absorbance was stable for more than three hours. In order to select the most appropriate dilution solvent after coupling reaction, different solvents were investigated. With methanol as dilution solvent, higher absorbance values, better regression equation and better coefficient of determination were

obtained and this was utilized for further studies. The suitability of methanol as the diluting solvent may be due to its ability to mop up excess water in the medium which may promote hydrolytic cleavage of the azo adduct formed between acyclovir and DMAB. For the stoichiometric ratio determination, optimal detector response was obtained when the diazotized drug combined with DMAB in a 1:1 ratio. Figure 4 presents the results obtained for the stoichiometric ratio determination. The 1:1 ratio corroborates the TLC result (which gave a single spot for the azo adduct in the three mobile systems adopted) and also justifies the proposed structure of the azo adduct presented in Scheme 1.

Method validation. In terms of linearity and sensitivity, the new method produced excellent correlation for the regression of absorbance of the complex on the concentration of acyclovir (R^2 =0.9996). The calculated apparent molar absorptivity is 1.1 x 10^4 L Mol^{-1} cm^{-1}. The Sandell's sensitivity obtained is 0.018 μg cm^{-2}. The LOD and LOQ, estimated from the ICH guidelines are 0.024 μg/mL and 0.071 μg/mL respectively. The various analytical and validation parameters are presented in Table 2. A cursory look at the various parameters obtained for the validation of the new spectrophotometric method for acyclovir reveal the highly sensitive nature of this new DMAB method and makes it stand at par with previously described colorimetric method for acyclovir. The reproducibility of the new method was determined by performing replicate determinations. The percentage relative standard deviation (% RSD) was used to estimate the intra-day and inter-day variations at three different acyclovir concentrations. Accuracy was determined as the difference between the estimated and the reference values. The model recoveries and repeatability of the method are shown in Tables 3a and 3b. The results show excellent intermediate precision (% RSD ≤ 2.67) and accuracy (% RE ≤ 2.56).

Method selectivity. The recovery of acyclovir in the presence of commonly utilized excipients is presented in Table 4. In all cases of the excipients studied, the recovery of acyclovir was greater than 97 % with very high precision. This thus implies the ability of the method to analyze acyclovir when present in the matrix of these excipients.

Assay of dosage forms. The suitability of the new method described in this report for the assay of acyclovir tablets and cream was examined and the results obtained were statistically compared with the official methods using *F*-ratio test, *t*-test, confidence limits and ANOVA. The results are presented in Table 5. The recovery of acyclovir from the dosage forms was in the range of 99.45 to 104.53% when compared with the official spectrophotometric method. Some clearly recognizable advantages of the spectrophotometric method described in this report are simplicity, accuracy and lack of interference from commonly utilized excipients. The method also adopted common laboratory reagents and so it applicability in different laboratory settings will be possible.

CONCLUSIONS

A new spectrophotometric method was developed and validated for the determination of acyclovir using *p*-DMAB as a coupling component for the diazotized drug. The method adopted for the determination of acyclovir is simple, accurate, sensitive, and less time-consuming and free from extreme experimental conditions such as heating at very high temperatures. It was successfully adopted for the analysis of acyclovir in dosage forms. The method could find application as a rapid method for the in-process quality control of acyclovir in bulk and dosage forms.

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