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Colorimetric Assay of Acyclovir in human serum via Charge-Transfer Complexation with 2,3-Dichloro-5,6-dicyano-p-benzoquinone (DDQ)

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ABSTRACT
Acyclovir, a purine-based nucleoside antiviral agent, is widely utilized in the therapeutic management of Herpes simplex and various other viral infections. This study focuses on the development and validation of a novel, simple, and rapid spectrophotometric method for its quantitative determination. The underlying mechanism involves a charge transfer reaction between Acyclovir and the 2,3-Dichloro-5,6-dicyano-p- benzoquinone (DDQ) reagent system at a controlled pH of 7.0. This reaction yields a stable, red colored product exhibiting maximum absorbance (λmax 420 nm}, which was subsequently used for the quantitative assessment of Acyclovir content in formulations. The method demonstrated excellent linearity, with the calibration curve extending up to a concentration of (1.0-22.0 µg/mL}. The key optical parameters determined were a molar absorptivity 6778.821(l . mole-1. cm-1) and a Sandell's sensitivity of 0.033 (µg/cm2). Precision was found to be 0.461%.
In conclusion, the spectrophotometric reaction produces a highly stable product, and the proposed method is demonstrably cost-effective, while possessing adequate accuracy, precision, and sensitivity. This robust methodology is therefore well-suited for the convenient and reliable quality control determination of Acyclovir in human serum.
Keywords: Serum, colorimetric, acyclovir, DDQ, Charge- transfer.
Received: 03/01/2026 Accepted: 17/02/2026 Published: 31/03/2026

1. Introduction

Acyclovir (9-[(2-hydroxyethoxy)methyl]guanine) is a synthetic purine-based nucleoside analogue that exhibits potent in vitro and in vivo inhibitory activity against various herpes viruses, including Herpes Simplex Virus (HSV), Varicella Zoster Virus (VZV), Epstein-Barr Virus (EBV), Cytomegalovirus (CMV), and Human Herpesvirus 6 (HHV-6) [1-3]. Acyclovir mediates its antiviral effect by functioning as a pseudo-substrate, thereby inhibiting viral DNA polymerase enzyme activity. The phosphorylation of Acyclovir to its active form, acyclovir monophosphate, constitutes the preliminary reaction, which is catalyzed by either viral or cellular thymidine kinase enzymes [4-6]

The British Pharmacopoeia (2005) specifies a UV spectrophotometric method for the quantification of acyclovir. Additionally, numerous unofficial assay methods, employing a variety of techniques and reaction pathways, have been successfully developed for the drug. These methods include: Polarography [7], Radioimmunoassay [8-9], Near-Infrared (NIR) spectroscopy [10], Microcellar electrokinetic chromatography [11] HPLC coupled with UV detection [12-13], HPLC with MS detection [14-15], and HPLC with fluorimetric detection [16-17].

The determination of acyclovir in pharmaceutical products has also been achieved using methods based on the derivatization of the drug with chromogenic reagents [18-20]. Furthermore, a derivative method [21] and a differential spectrophotometric method [22] have also been documented. Spectrophotometric methods remain the most favored for routine analytical procedures due to their simplicity, acceptable sensitivity, and cost-effectiveness. However, some of the aforementioned colorimetric methods present limitations, such as a prolonged reaction time and low selectivity for the analyte. Consequently, the present study aims to develop a simple and accurate colorimetric method for the determination of acyclovir in its dosage form.

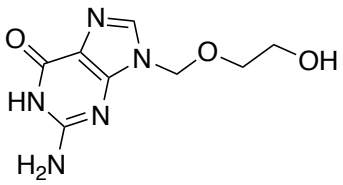

Figure 1: Structure of Acyclovir

2. RESULTS AND DISCUSSION

2.1 Characterization of the Compounds

2.1.1 Physical properties:

In this study, the first charge transfer complex of Acyclovir as an electron donor with DDQ as an electron

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acceptor were prepared by mixing the solution of both the electron donor and the electron receptor in acetonitrile solvent which form complex in a ratio of 1:1, . Table1 representing the complexes with the physical properties of the prepared complex. The validity of the structures has been proven by studying the infrared spectra of charge transfer complexes, the H1NMR and 13C NMR proton NMR spectra as well as the ultraviolet and visible spectra of the complex in the solution

Table1: The physical properties of the prepared complex.

Comp.	Formula	Color	MP	Yield
DDQ	$C_6Cl_2(CN)_2O_2$	Yollow	210-215ºC	------
ACV	$C_8H_{11}N_5O_3$	White	256.5ºC	------
ACV-DDQ	$C_{16}H_{11}N_7O_5Cl_2$	Dark red	>300^{o}C	58%

MP: Melting point

2.1.2 Infrared spectra:

The study of infrared spectra in this study was based on the diagnosis of the active aggregates of receptor ((C=O, C≡N, C=C , N-H, O-H, C=N) and the changes in the shapes and locations of these beams when forming the charge transport complexes. The infrared spectra of the charge transport complexes showed slight differences in the amplitude oscillation sites of the effective aggregates compared to their primary components, Figures (2) represent the infrared spectra of the CV.DDQ complex. By comparing the infrared spectra of the CV.DDQ charge transfer complex with its primary components acyclovir and DDQ), the following was observed: There was no change in the amplitude frequency of the O-H group, while the complex recorded a slight change of about 10 cm-1 in the frequency of the N-H group the primary amine, which can be attributed to the occurrence of some kind of interference between the components of these complex. The components of these complexes, as well as the spatial factors that play an important role in determining the nature of the relationship between the giver and the receiver. A displacement in the frequency of the cyanide group from 2229cm-1 in free DDQ to 2213cm-1 in the CV.DDQ charge transfer complex is also observed.

Table (2) Amplitude vibration positions of effective total in IR Spectra of CV.DDQ complex

Table 2: Amplitude vibration positions of effective total in IR Spectra of CV.DDQ complex

Comp.	ν_{OH}	ν_{NH}	ν_{CO}	ν_{CN}	$\nu_{CN,\ C=C}$	ν_{CH}	$\nu_{C\text{-}Cl}$
AC	3436	3285 3179	1702	--	1625 1538	2921 2854	-
DDq	--	--	1678	2229	1554	--	802
CV-DDQ	3436	3295 3179	1698 1647	2213	1600 539	2936 2811	812

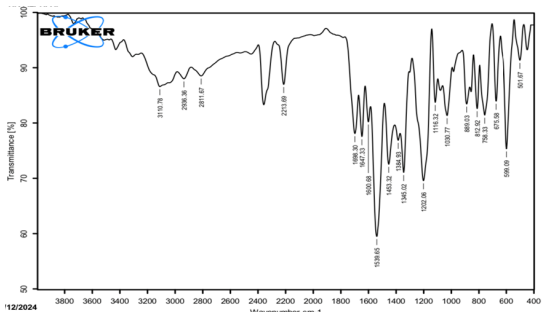

Figure 2: Infrared spectrum of the CV.DDQ charge transport complex

2.1.3 HNMR:

In general, the HNMR spectra of the cyclovir complex with DDQ showed the following signals: a single signal at the chemical displacement between (10.65) ppm and the proton of the NH group, a single signal at the chemical displacement of 7.82 ppm due to the H1 proton, the protons of the NH2 group showed a single signal at the chemical displacement 6.52 ppm, the protons of the H2,3 group showed a single signal at the chemical displacement 5.35 ppm, and the first two triple signals were observed at the chemical displacement 3.47 ppm. The second at (3.46) ppm is due to the protons of the methylation groups H 6 , 7 and H 4,5 respectively, while the proton of the OH group showed a single signal at the region (4.7) ppm, these protons belong to cyclovir, and this is consistent with what is stated in the chemistry literature while DDQ did not show any displacement because it does not contain protons.

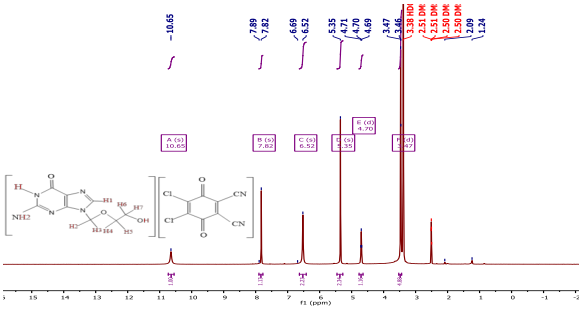

Figure (3) ^{1}HNMR of CV.DDQ

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2.1.4 ^{13}C NMR:

THE 13C NMR spectra of the charge transport complexes was recorded in the DMSO-d6 solvent and their data are summarized in Table (5-3) and Figures (5-3) and (6-3) represent the 13C NMR spectra of the charge transport complexes of cyclovir and brassetmol, respectively. In general, the 13C NMR spectra of the prepared complexes showed all the signals from the carbon atoms of DDQ receptor and the donor of acyclovir, confirming the validity of the proposed structures of the prepared complex. The ^{13}C NMR spectra of the cyclovir complex showed the following signals: The appearance of signals at chemical displacement ppm 60.32, 71.42 and 73.62 are due to methylated carbon atoms C13, C12 and C10 respectively.

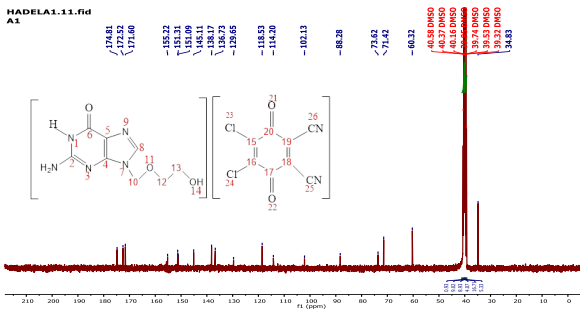

Figure 4: ^{1}3CNMR of CV.DDQ

2.1.5 UV. Visible:

Spectrophotometric procedures are popular for their sensitivity in the assay of drugs and hence, charge-transfer complex formation has received considerable attention for the quantitative determination of many pharmaceutical compounds . Acyclovir react with DDQ to give red color charge-transfer complex, which exhibits absorption maxima at 420 nm against its reagent blank, Figure5. The some bands may be attributed to the formation of DDQ radical anion, which probably resulted from the dissociation of the donor-acceptor complex in relatively high polar solvents like acetonitrile . Therefore, in order to avoid the maximum interference from the reagent blank, the absorbance measurements were carried out at 420 nm in the subsequent work.

2.1.6 Optimization of experimental variables [23].

2.1.6. 1 Univariable Method:

The experimental variables affecting the development and stabilities of charge-transfer complex formation

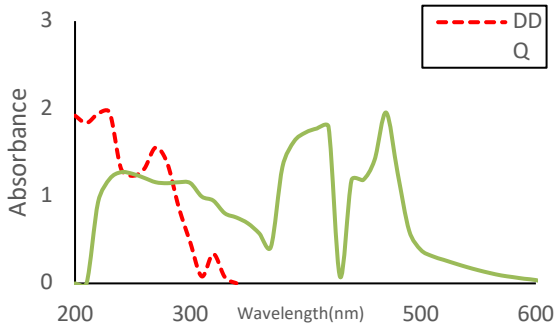

Figure 5: Absorption spectra for the reaction product of (10-3M) Acyclovir against (10-3M) DDQ reagent blank solution.

were achieved through a number of preliminary experiments. Such factors include reagent volume, reaction time, pH and temperature. For this reason, a variable was modified while maintaining the other variables at their constant values, then by maintaining that variable at its optimized value, another was modified; all variables were optimized via this method. Table 3 shows the optimal conditions obtained in the present study.

Table 3 : The optimal conditions obtained in the present study.

Experimental Conditions	The Value
λ_{max} (nm)	420
Amount (ml) of 4.4×10^{-4} M AC	1ml
Amount (ml) of 4.4×10^{-4}M DDQ	1ml
Time (min.)	10min
Temperature (Cº)	40ºC
Medium , PH	7

2.1.6. 2 Calibration graph:

Employing the optimum experimental conditions, a linear calibration graph for the determination of acyclovir, by charge-transfer complex formation with DDQ, was obtained, Figure 6, which shows that Beer's law was obey in the concentration range of (1.0-22.0) µg.mL-1, with a correlation coefficient (R2= 0.9992) and detection limit of 0.60 µg.mL-1.

2.1.6. 3 Spectral Characteristics of the Proposed Method:

Under optimum experimental conditions of the proposed method, the regression plot showed linear dependence of absorbance signals on the concentrations of the studied drug in the range given. The regression

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equations, correlation coefficients, molar absorptivities, detection limits and sandell sensitivity in addition to other parameters are given in Table 4.

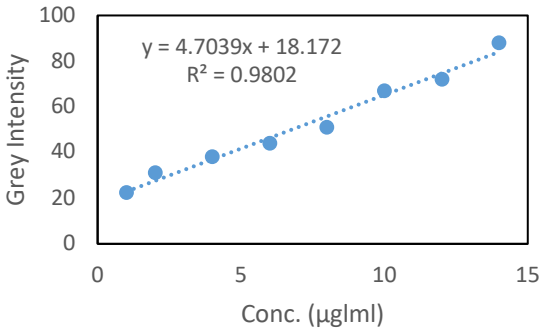
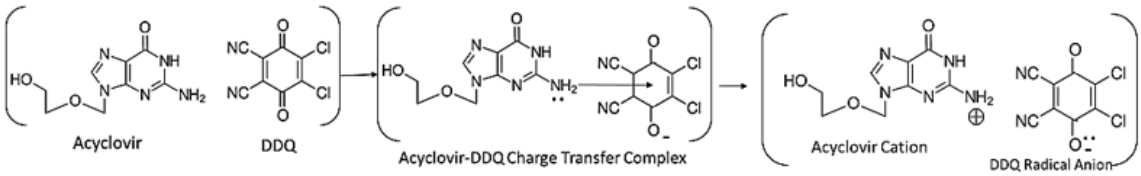


Figure 6: Calibration graph of Acyclovir under optimum experimental conditions.

in the intensity of the orange color was observed linearly when the concentrations of the drug compound ACV

Table (4): Spectral characteristics and statistical data of the regression equation for determination of Acyclovir via charge transfer formation.

Parameters	Value
Color	red
Medium	pH 7
λ_{max}, nm	420
Beers law range (µg/ml)	1.0-22.0
LOD (µg/ml)	0.60
LOQ (µg/ml)	2.009
Ɛ (l . mole^{-1}. cm^{-1})	6778.821
Sandells sensitivity (µg/cm^2)	0.033
Regression equation : Y=bX+a	y=4.7039x+18.172
Intercept (a)	18.172
Slope (b)	4.7039
Determination coefficient (R^2)	0.9802
RSD%	0.461



Scheme (1): Structure of the formed charge transfer complex

The structure of the formed charge transfer complex can be represented as in Scheme 1. The mechanism of the reaction depends on the formation of an original donor-acceptor (DA) complex through the interaction between one of the nitrogen atoms of amine moieties in the acyclovir (as n-electron donor) and DDQ (as π-acceptor). Then, the dissociation of DA-complex may be promoted by the solvent, especially that with high ionizing power such as acetonitrile, where complete electron transfer from the donor to the acceptor moiety takes place. This is followed by formation of the DDQ radical anions as a predominant chromogen.

Estimation of CV in Serum Using µ-chip:

The implementation of the microchip was examined using it with a sample of pharmaceuticals to designate the ACV drug complex. The validity of the method was studied under optimal conditions, the color intensity of the complex produced by the combination of the ACV compound with the DDQ reagent was monitored (Fig. 7 and when drawing the intensity values against the concentrations of the drug compound ACV, an increase were increased at the µg/ml range 1.0 -22.0 µg/ml then the intensity is almost fixed, and Figure 6 shows the calibration curve calculations derived from the intensity of the images after they have been analyzed using ImageJ software. The intensity increased linearly with the concentration of the drug compound ACV. To determine the concentration of the drug compound ACV in a sample of pharmaceuticals, concentrations ranging from (1.0-14.0) µg/ml were identified for the drug compound ACV. Our device further simplifies these processes, using integrated chemicals for chromatography and a smartphone for quantitative analysis. To demonstrate the potential of a 3D printed microfluidic device for field measurements, a sample of pharmaceuticals was analyzed using a new 3D printed microfluidic device pre-filled with A CV reagents and an image of the device used to detect the.

The implementation of the microchip was examined using it with a serum sample to determine the ACV compound. The validity of the method was studied under optimal conditions, where the color intensity of

the resulting complex was monitored from the combination of the ACV compound with the DDQ

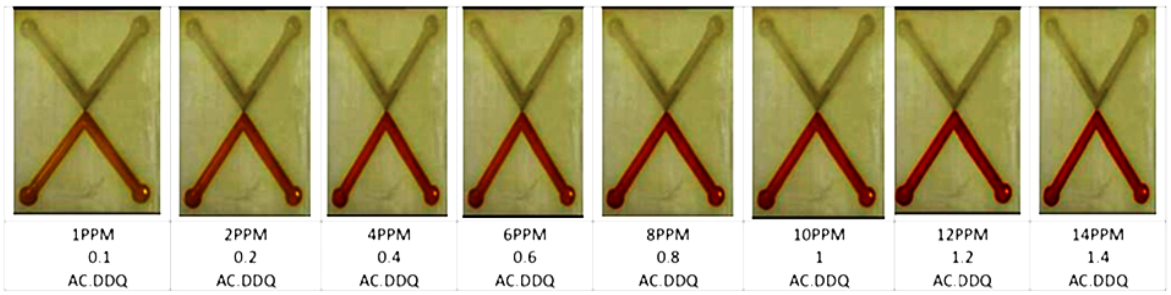


Figure (7): Images for the chromatography of the ACV drug compound in the microchip device at different concentrations

reagent, and the serum sample was analyzed using the new 3D printed microfluidic device pre-filled with ACV compound reagents. Table (5) shows the calculated concentrations of the ACV compound in the serum sample using the calibration curve shown in the Figure (7).

Table (5) Concentration of ACV µg.ml-1 in serum sample using µ-Chip

Sample	Conc. of CV (µg.ml-1)	RSD%
Serum	5.40	5.24

3. EXPERIMENTAL

UV visible Spectrophotometer (Biotech UV 9200,UK) was used with 1cm quartz cuvettes to record the UV spectra of Acyclovir (ACY). A digital weighing balance (BL2105 Sartorius-Romania) was used for all preparations. pH meter (AD-1030 Adwa-Romania) was also used. Fourier transform infrared (FT-IR) spectra of the compound and the as-prepared compound were recorded in the wavenumber range of 400-4000 cm-1 using an ALPHA II instrument from Bruker, Germany. Mass spectra of the as-prepared compound were recorded using an Agilent Technologies 5975C 110 instruments. Proton nuclear magnetic resonance (NMR) spectra of the as-prepared compound were recorded using an Ascend instrument at 400 MHz from Bruker, using dimethyl sulfoxide-6d as the solvent.

All chemicals, solvents and reagents used in this work were of analytical reagent or pharmaceutical grade and all solutions were prepared fresh daily. Double distilled water was used throughout the investigation.

The reference samples of Acyclovir and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were obtained from FOB, China. Sodium Hydroxide pellets extra pure AR was purchased from FOB, China. HCL was purchased from BDH company. A 50 mg of ACV was accurately weighed and transferred into 50 ml standard volumetric flask. To that 2 ml of 1 M NaOH was added & sonicated for 10 min. After 10 min volume was made up to the mark with the solvent to get 10-2 M . That solution was used as a stock solution. A 10-2 M DDQ solution, was prepared by dissolving 0.05 g of the DDQ in 2 mL of DMF and then the solution was diluted to a final volume 50 mL with acetonitrile. Working solutions were freshly prepared by subsequent dilutions. This solution is prepared daily using red- glass volumetric flask because it is a light sensitive reagent.

4. General Recommended Procedure

Measured volumes of the standard stock solution of the drug containing appropriate amount of acyclovir were transferred into 10 mL calibrated flasks, 1 ml of 10-2 M DDQ solution was added to each, and then diluted to volume with acetonitrile. Absorbance measurements of resulting solutions were done at the wavelength of maximum absorption (420 nm) against reagent blank which prepared by the same manner, but without addition of Acyclovir.

5. Analysis of Acyclovir in serum by µ-chip;

5.1 Blood serum sample treatment:

Blood serum samples were prepared by centrifuge at 3000 rpm for whole heparin-treated blood samples

collected from patients at Al-Jumhouri Hospital Epidemiological Department , prepared plasma samples were stored at 20°C before analysis, and blood serum samples were dissolved at 20°C for approximately 10 minutes. The 0.5 mL volume of the sample is then transferred to a vial and treated with a 0.1 mL solution of 20% perchloric acid V/V . It was then shaken for 20 seconds and centrifugalized for 10 minutes at 10000 rpm. It was filtered for serum , transferred to a 10 mL volumetric vial and completed volume to the label with acetonitrile

5.2 Determination of acyclovir in the blood serum sample:

Volumes (1, 2, 4, 6, 8, 10, 12, 14) of acyclovir solutions were added separately to two series of 10 ml volumetric vials to obtain concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4) µg/ml for a cyclovir, the above solution were injected after adjusting the pH=7 acid function in channel A, and DDQ was injected into channel B. Left to blend for ten minutes, use your smartphone to take pictures of the colors of the solutions formed and processed in the ImageJ. program. The treated serum was injected after regulating the pH=7 acid function into channel A, the reagent was injected into channel B, and left to mix for ten minutes, use a smart phone to take pictures of the colors of the solutions formed and processed in the ImageJ program.

6. Conclusions

The utility of DDQ reagent for the spectrophotometric determination of acyclovir was established. The method based charge-transfer complex formation between the cited drug and DDQ as a chromogenic reagent. The proposed method was found to be accurate, simple and sensitive. It was satisfactorily applied to the determination of acyclovir in human serum.

Acknowledgement

Authors like to thank university of Basrah for the support

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