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Full Paper

A New Electrochemical Method for Simultaneous Determination of Acyclovir and Methotrexate in Pharmaceutical and Human Plasma Samples

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Abstract- Recent clinical and pre-clinical data demonstrate that adjuvant antimicrobial therapy is beneficial in cancer treatment. For the first time, an electrochemical method was proposed for the simultaneous determination of acyclovir (ACV) and methotrexate (MTX) at activated or electropretreated pencil graphite electrode (EPPEG). Their simultaneous determination was achieved by cyclic voltammetry (CV) and adsorptive square wave voltammetry (AdSWV) techniques. The proposed sensor has a wide linear range of 2×10^{-7} to 1.4×10^{-6} M for MTX and 5×10^{-7} to 3×10^{-6} M for ACV. The limits of detection (LOD) values were found 1.13×10^{-8} M and 6.07×10^{-8} M for MTX and ACV, respectively. The proposed method was applied in their pharmaceutical formulations and human plasma. In addition the proposed method could be applied in pharmaceutical laboratories and quality control analysis in the near future.

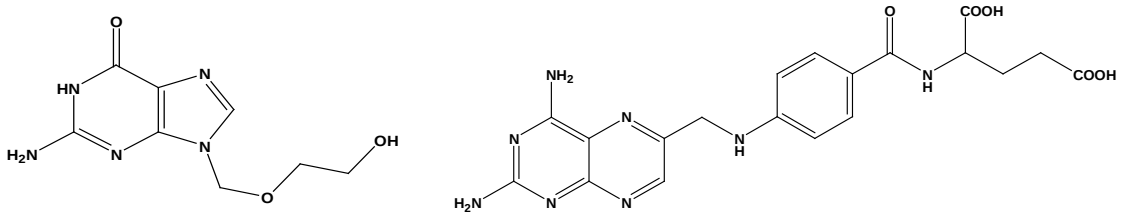
Keywords- Acyclovir, Methotrexate, Adsorptive square wave voltammetry, Electropretreated pencil graphite electrode, Human plasma

1. INTRODUCTION

Antiviral agents is currently used for several types of cancer (gastric, cervical, hematopoietic, liver and brain cancer) associated with virus infections as adjuvant therapy.

However this treatment is effective only in combination with conventional therapies. Many antiviral agents were reported to have antiproliferative and proapoptotic activity in various cancers through inhibition of transcriptional factors, inhibition or activation of human DNA polymerase, suppression of telomerase activity, increases in radiosensitivity and downregulation of angiogenic genes. For example, acyclovir (ACV) (Fig. 1) could be used in cancer therapy is by Treg inhibition in glioblastomas (a highly invasive glioma in the brain). It has been shown that acyclovir has an inhibitory activity on indoleamine 2,3-dioxygenase, and thereby could inhibit Treg function. Given the short survival time of patients with glioblastoma together with relative safety of acyclovir, using this antiviral as an adjunct therapy could have a potential in glioblastoma treatment [1].

Methotrexate (MTX) (Fig. 1) is one of the popular antineoplastic drugs that are used in the treatment of different types of cancers associated with viral infections such as breast cancer, epidermoid cancers of the head and neck, lung cancer, bladder cancer and osteogenic cancer.



Acyclovir (ACV) **Methotrexate (MTX)**

Fig. 1. Chemical structures of the studied drugs

Various analytical methods have been reported for determination of MTX in biological samples and in pharmaceutical preparations. The most commonly used technique is liquid chromatography. It is often combined with solid phase extraction [2-3] and with various types of detection systems, e.g., fluorimetric detector [4], UV spectrophotometric detector [5], mass spectrometer [6] or electrochemical detector [7]. Other methods, which have been successfully used for MTX determination, are ion chromatography with electrochemical detection [8], capillary zone electrophoresis [9] and voltammetry [10-12]. Spectrophotometric methods including color reactions and UV measurements have also been reported [13-14].

There are also several methods were developed for determination of ACV such as spectrophotometry [15-16], high performance liquid chromatography (HPLC) [17-20], capillary electrophoresis (CE) [21-22], radioimmunoassay (RIA) [23-24] and voltammetry

[25-30]. To our knowledge there is no analytical method was developed for simultaneous determination of ACV and MTX.

From the electrochemical point of view, no study has been reported for the electrochemical simultaneous determination of ACV and MTX using pencil graphite electrode (PEG) and application in their pharmaceutical formulations and human plasma. The use of carbonaceous electrodes for electroanalysis has gained popularity in recent years because of their applicability to the determination of substances that undergo oxidation reactions [31-34]. When compared with other carbon-based electrodes, PGEs have the following advantages, such as high electrochemical reactivity, commercial availability, good mechanical rigidity, disposability, low cost and ease of modification [35]. In addition, it was reported that pencil lead electrodes offer a renewable surface which is simpler and faster than polishing procedures, common with solid electrodes, and result in good reproducibility for individual surfaces [35].

Therefore the aim of this study was to demonstrate the usefulness of a pencil graphite electrode in the simultaneous determination of ACV and MTX and its application in their pharmaceutical formulations and human plasma. The proposed method is beneficial in their determination in the biological fluids of cancer patients suffering from viral infections whose are treated with both drugs.

2. EXPERIMENTAL

2.1. Reagent and chemicals

Acyclovir was obtained from Global Napi pharmaceuticals (6^{th} October, Egypt) and Methotrexate was obtained from Pharco Technopharma Egypt (New Borg El Arab City, Alexandria, Egypt). They were used without further purification.

Phosphoric acid (85%), glacial acetic acid, boric acid, citric acid, sodium dihydrogen phosphate, sodium hydroxide, hydrochloric acid and potassium chloride were purchased from (Sigma, Cairo, Egypt). Britton-Robinson (BR) buffer solutions in the pH range 2.1–7 were prepared by mixing of 0.04 M of each acid: phosphoric, acetic and boric acids then the pH was adjusted to (2.1–7.0) with the appropriate amount of 0.2 M sodium hydroxide). Teorell and Stenhagen buffer was prepared by mixing 1 M citric acid, 1 M phosphoric acid, 1M sodium hydroxide and 0.2 M hydrochloric acid with different ratios and phosphate buffer was prepared from sodium dihydrogen phosphate 0.05M and sodium hydroxide 0.05 M [36]. Deionized water was used to prepare all of the solutions.

2.2. Instrumentation

A Princeton VersaSTAT MC (VersaSTAT 3, Model RE-1, Princeton Applied Research, AMETEK, USA) connected to a three-electrode cell was used for the electrochemical

measurements. In all measurements, the reference electrode was an Ag/AgCl electrode, the auxiliary electrode was a platinum wire and electrochemically pretreated PGE was used as working electrode. All experiments were performed at room temperature. The pH values of solutions were adjusted using Hanna pH meter (Hanna Instruments Brazil, São Paulo, SP, Brazil) with a combined electrode (glass-reference electrodes).

2.3. Procedure

2.3.1. Preparation of the standard solutions

A stock solution of ACV and MTX (10^{-3} M) was freshly prepared in deionized water and stored in dark in a refrigerator. The required concentrations were prepared from the stock standard solution by serial dilution with deionized water. All experiments were carried out at the ambient temperature of the laboratory (25±5 °C).

2.3.2. Electrochemical pretreatment and preparation of the working electrode

A pencil lead with a diameter of 0.5 mm (Ultra-Polymer, H) and a total length of 60 mm (Tombow, Japan), and a mechanical pencil Model T 0.5 (Rotring, Germany), which was used as the holder for the pencil lead, were purchased from a local bookstore. Electrical contact to the lead was obtained by wrapping a metallic wire to the metallic part of the holder. For each measurement, its tip was polished on a weighing paper to a smoothed finish and a total of 10 mm of lead was immersed into the solution. The electrochemical pretreatment of the polished PGE surface carried out potentiodynamically by scanning the potential between -0.2 and 2.0 V *vs* Ag/AgCl with a scan rate of 100 mV s^{-1} for 5 cycles in 0.05 M phosphate buffer solution (pH 7.0). Then the electropretreated electrode was washed with deionized water for further use [37].

2.3.3. Electrochemical measurement

The electrochemical measurement was done using square wave voltammetry by sweeping the electrode potential between 0.1 V and 1.5 V *vs.* Ag/AgCl reference electrode in an electrochemical cell filled with 7 ml Teorell and Stenhagen buffer solution at the optimized pH 2.7 containing 0.1 M KCl.

2.4. Application

2.4.1. Application to dosage form

2.4.1.1. Acyclovir tablets

Ten tablets of Zovirax® (each one contains 400 mg acyclovir) were weighed and powdered in a mortar. A weighed portion of the powder prepared to prepare a solution

equivalent to about 10^{-3} M of acyclovir was transferred into a 100 mL calibrated flask and completed to the volume with deionized water. The contents of the flask were sonicated for 10 min to affect complete dissolution and filtrated. Appropriate solutions were prepared by taking suitable aliquots of the clear filtrate and diluting with deionized water, and the voltammetric procedure was followed.

2.4.1.2. Methotrexate vial

Methotrexate vial (each one contain 50 mg/2 ml) was diluted directly without extraction with deionized form to obtain concentration of 1×10^{-3} M of MTX. Appropriate solutions were prepared by taking suitable aliquots and diluting with deionized water, and then the voltammetric procedure was followed.

2.4.2. Application to human plasma

2.4.2.1. Application to spiked human plasma

Drug-free human plasma samples were obtained from healthy female volunteers. An aliquot volume of plasma (0.5 mL) was added to ACV and MTX solution. Then 1 ml of acetonitrile was added to this mixture as a protein precipitating agent. Samples were vortexed for 3 minutes then centrifuged at 5000 rpm for 15 min. Samples were filtered and the supernatant that contained the drugs mixture was diluted and analyzed according to the voltammetric procedure explained in the procedure of this method.

2.4.2.2. Application to real human plasma samples for voltammetric determination of MTX and ACV simultaneously

The proposed method was applied to real human samples of plasma to confirm its utility. A therapeutic dose of methotrexate (25 mg/ml as a vial for injection) and acyclovir (400 mg/tablet taken orally) was administered to five volunteers. Volunteers participated in the study with a mean age of 50 years (range 48–52 years) and mean body weight of 70 kg (range 60–80 kg). Whole blood samples were collected at 3hrs and 5hrs after dosing, and were transferred to heparinized tubes. The samples were centrifuged at 5000 rpm for 15 min, and the plasma was extracted and stored at −4 °C until analysis.

3. RESULTS AND DISCUSSION

3.1. Effect of electrochemical pretreatment on pencil lead reactivity

The enhancement in electrochemical activity of electropretreated pencil graphite electrode (EPPGE) may be attributed to an increase of oxygen containing groups, such as phenolic, carbonyl and carboxyl groups on electrode surface or to the formation of graphite

oxide films during electrochemical pretreatment of PGE [38-39] when compared with non-treated pencil graphite electrode (NPGE). In the present study, the significant increase in the oxidation peak currents of MTX and ACV at EPPGE confirmed that the electrochemical treatment enhanced the sensitivity of MTX and ACV due to its large specific surface area and special electrical properties, which makes for easier adsorption and provides enough effective reaction sites. The electro-pretreatment of the electrode decreased also the overlap between the peaks of the two drugs and so made their simultaneous determination easier. Fig. 2 shows the square wave voltammograms of 1.0×10^{-5} M MTX and 1.0×10^{-5} M ACV at NPGE, EPPGE before experimental optimization.

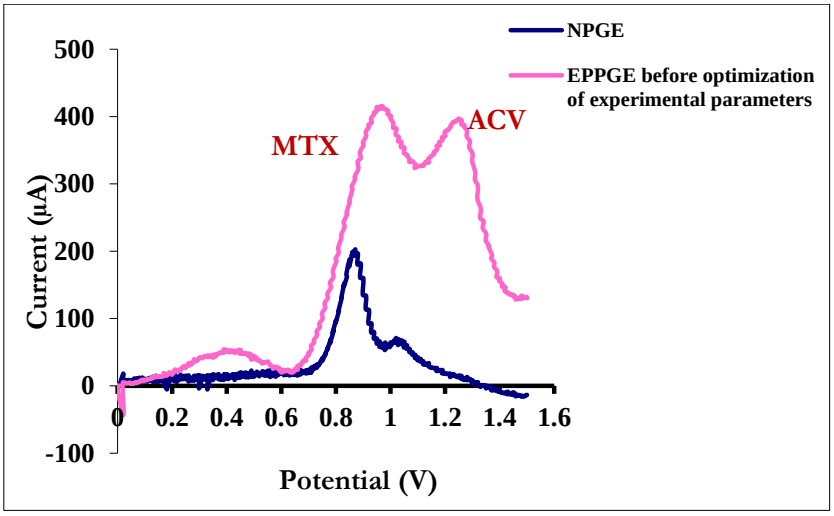


Fig. 2. Square wave voltammogram of 1.0×10^{-5} M ACV and MTX before and after the pretreatment of PGE (i.e at PGE bare and at EPPGE) in Britton Robinson buffer at pH 3.0 before optimization of the experimental conditions

3.2. Voltammetric behaviour of ACV and MTX on EPPGE

In order to elucidate the properties of EPPGE in the determination of ACV and MTX, the electrochemical behaviors of 1.0×10^{-5} M ACV and 1.0×10^{-5} M MTX at EPPGE were studied by cyclic voltammetry and the results are shown in (Fig. 3). Within the potential window from +0.1 V to +1.3 V, under the optimized conditions, the modification of PGE surface by electrochemical pretreatment improves the reactivity of PGE towards the oxidation of ACV and MTX.

It is clear that there is only one anodic peak for each drug when the potential is scanned from 0.1 to 1.3 V. The oxidation process is not accompanied by a reduction wave, which indicated that the oxidation reaction is totally irreversible, and the voltammograms recorded from multicyclic voltammetry showed that the peak current decreased with increasing number of cycles and tended to disappear finally (Fig 4). This fact may be partly attributed to

the poor solubility and of the oxidized product of methotrexate [40] and the adsorption of the oxidized products of ACV and MTX, which accumulated on the electrode surface during the electrochemical process.

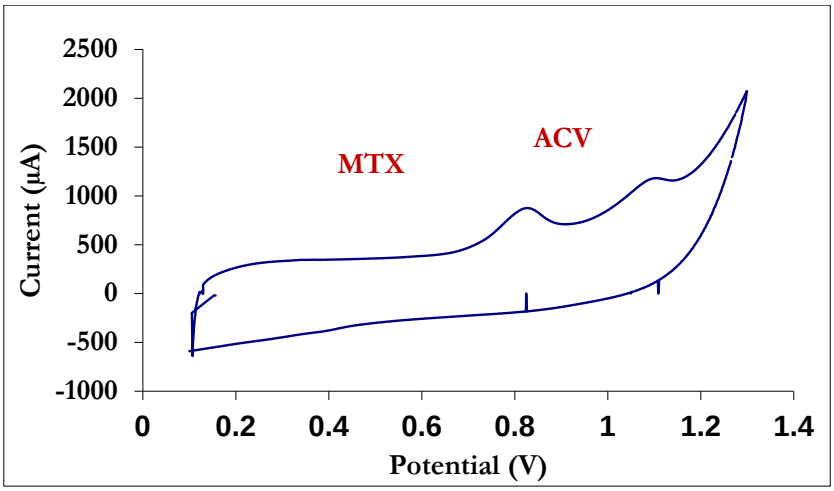


Fig. 3. Cyclic voltammogram for simultaneous determination of 5.0×10^{-5} M of ACV and MTX at EPPGE in Teorell Stenhagen buffer of pH 2.7 at a scan rate of 0.5 Vs^{-1}

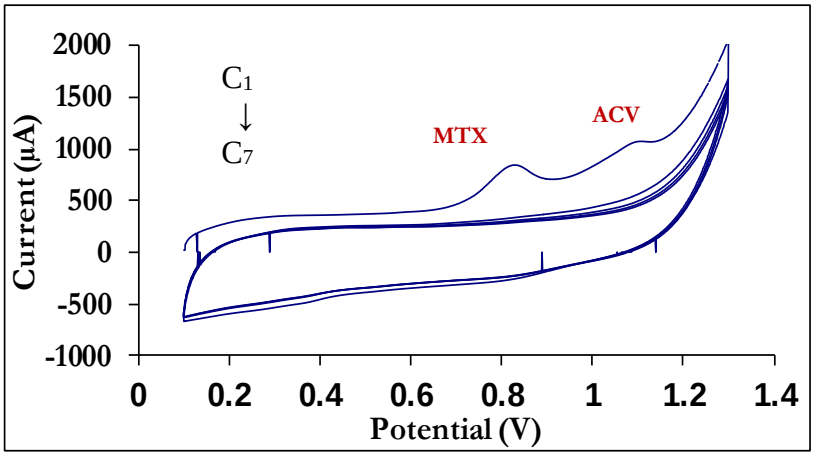


Fig. 4. Successive cyclic voltammograms of $5.0\times l0^{-5}$ M ACV and MTX in Teorell Stenhagen buffer (pH 2.7) at EPPGE surface, t_{acc}=60 s., scan rate 100 mVs^{-1}

3.3. Effect of scan rate

The influence of scan rate (υ) on the oxidation peak is investigated in the range from 50 to 1000 mV s^{-1}. It was found that the increase in the peak current (I) of ACV or MTX oxidation is linear to the scan rate (υ) (Fig. 5 a,b) according to the following equation:

I_p (µA) = 0.806 + 614.73 υ (V s^{-1}) (r=0.9989) for ACV

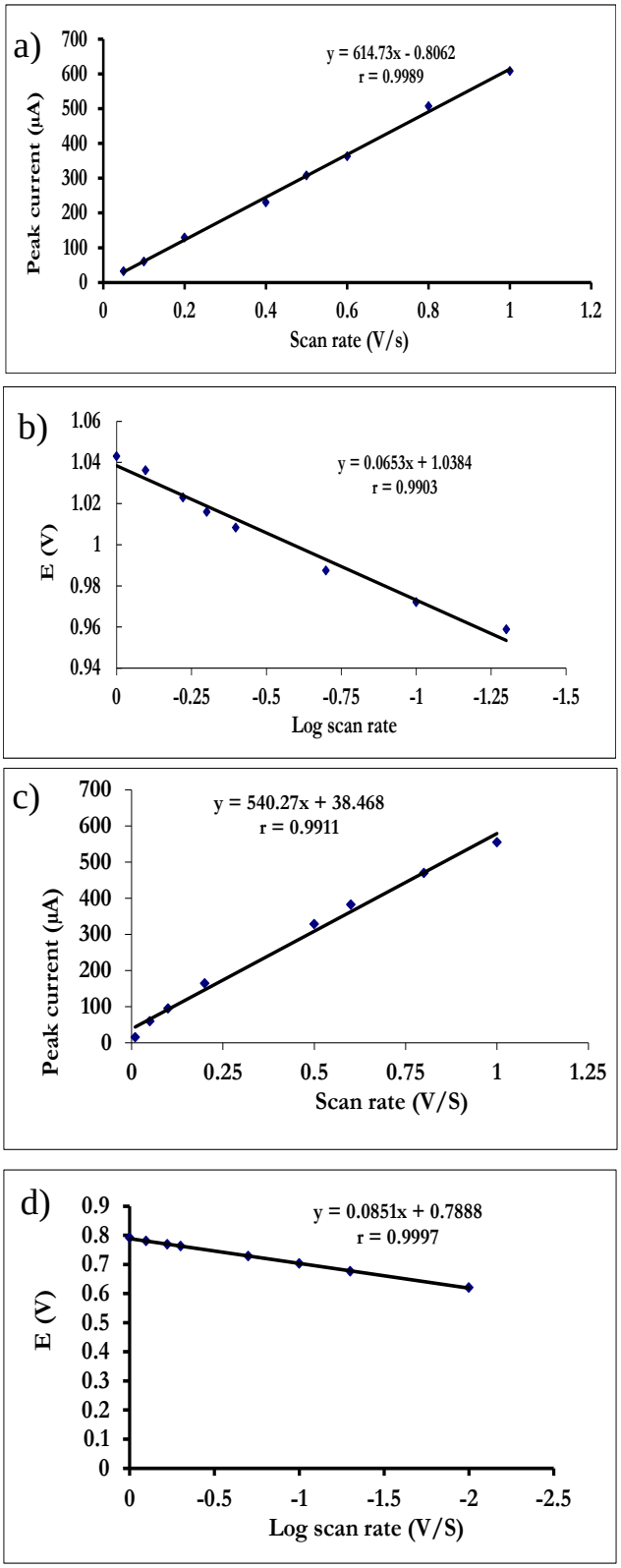


Fig. 5 a) Dependence of ACV oxidation peak current (µA) on scan rate (υ V S^{-1}); b) Dependance of ACV oxidation peak potential (E_p) on log scan rate (υ/ V S^{-1}); c) Dependence of MTX oxidation peak current (µA) on scan rate (υ V s^{-1}); d) Dependance of MTX oxidation peak potential (E_p) on log scan rate (υ/ V s^{-1})

I_p (μA)=38.47 + 540.27 υ (V s^{-1}) (r=0.9911) for MTX

The plot of the peak potential E_p versus log of scan rate (log υ) (Fig. 5 c,d) was found according to the linear regression equation:

E_p(V)=1.0384+0.0653 log υ (V s^{-1}) (r=0.9903) for ACV
E_p(V)=0.7888+0.0851 log υ (V.s^{-1}) (r=0.9997) for MTX

For the irreversible electrode process, the relationship between the oxidation peak potential and scan rate can be used to calculate (αn) by the following equation [41]:

Slope=2.303 RT/ (1-α**)** nF

where T is the temperature (298 K), α is the transfer coefficient, n the number of electrons transferred in the rate determining step, υ is the scan rate and F is the Faraday constant (96.480 C mol^{-1}), R is the universal gas constant (8.314 J mol^{-1} K^{-1}). For ACV the slope is 0.07 approximately, and αn was calculated to be 0.84, while for MTX the slope is 0.09 approximately, and αn was calculated to be 0.66. Generally, α is assumed to be 0.5 in totally irreversible electrode process. Therefore, the value of n=1.68 (≈2) was obtained for the oxidation peak of ACV and n=1.32 (≈1) for the oxidation peak of MTX. Therefore it is beneficial in calculation of (n) number of electrons transferred in the rate determining step of the reaction as will be discussed in reaction mechanism later.

3.4. Electrode mechanism

As shown in Fig. 2 the activation of PGE surface improved the sensitivity of the method. It demonstrated the square wave voltammograms recorded for ACV and MTX at PGE bare and EPPGE, two overlapped peaks for the two studied drugs were observed at PGE bare, but at EPPGE the overlap between peaks disappeared because they appeared at 1.2 V for ACV and 0.88 V for MTX with enhancement of the currents of their peaks. As shown also in Fig. 3 no peaks were observed in the cathodic sweep indicating that the oxidation of ACV and MTX is an irreversible process. At EPPGE ACV is adsorbed and oxidized onto the electrode surface. The electrochemical behavior of guanine, the parent base of ACV, at carbon electrodes was studied before by many workers [26,42-44]; therefore we may assume that the oxidation process is located on the guanine moiety in the molecule. On the basis of the results obtained from cyclic voltammetric study, it can be concluded that the electrochemical oxidation of ACV involves *two electrons and two protons irreversible process* to the formation of *8-oxoacyclovir*, which is analogous to the initial oxidation product of guanine [26,42-44] as summarized in Fig. 6. MTX is adsorbed also and oxidized onto the electrode surface. The amino group at the C_2 position on the pyrimidine ring is electroactive and the possible mechanism of MTX oxidation [40] is illustrated in Fig 7. On the basis of the results obtained

from cyclic voltammetric study, it can be concluded that the electrochemical oxidation of MTX involves a one electron one proton irreversible process.

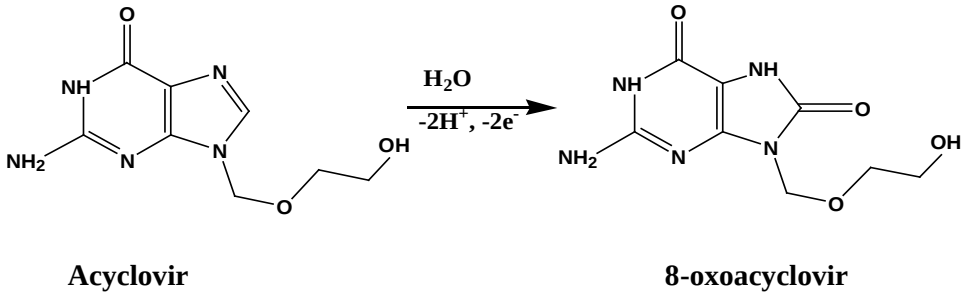


Fig. 6. Suggested electrode reaction mechanism of ACV

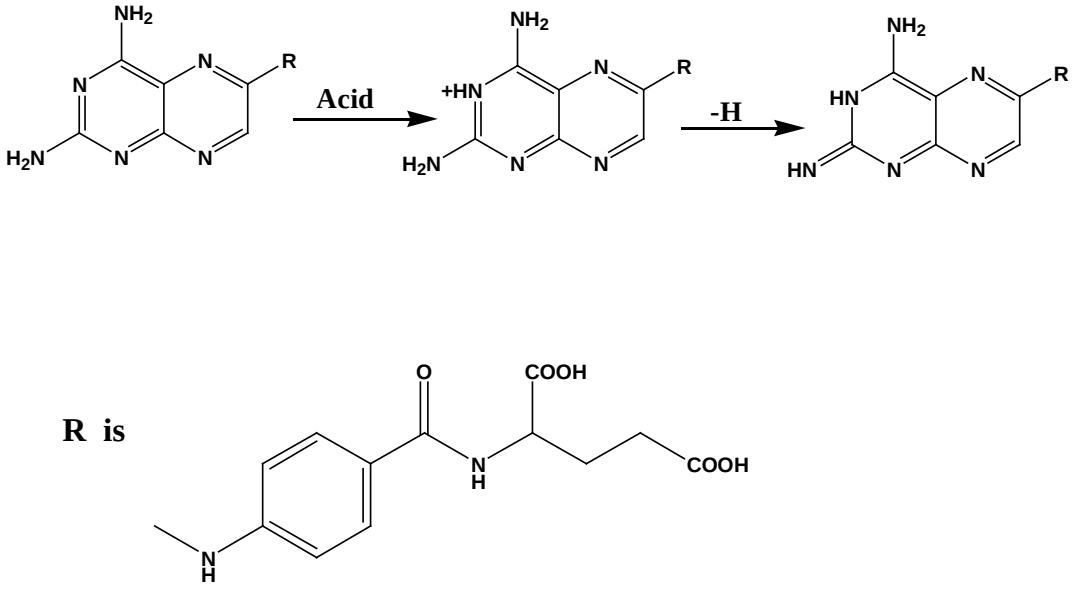


Fig. 7. Oxidation mechanism of MTX

3.5. Optimization of the experimental parameters

The resulting oxidation peak currents of MTX and ACV, using adsorptive square wave stripping voltammetry (AdSWV) technique at the working EPPGE, were characterized with respect to the pH, supporting electrolyte composition, accumulation potential, frequency, pulse amplitude, step height and accumulation time.

3.5.1 Effect of pH and buffer type

The effect of the electrolyte solution pH on the oxidation of MTX and ACV at the EPPGE surface was investigated. The Britton-Robinson buffer solution was used in the pH

range from 2.0 to 10.0 and their oxidation peak appeared only from 2.0 to 6.0 pH. The current peak, (*Ip*), versus pH plot shows that their oxidation peak current is maximized at pH 2.7 as shown in Fig. 8.

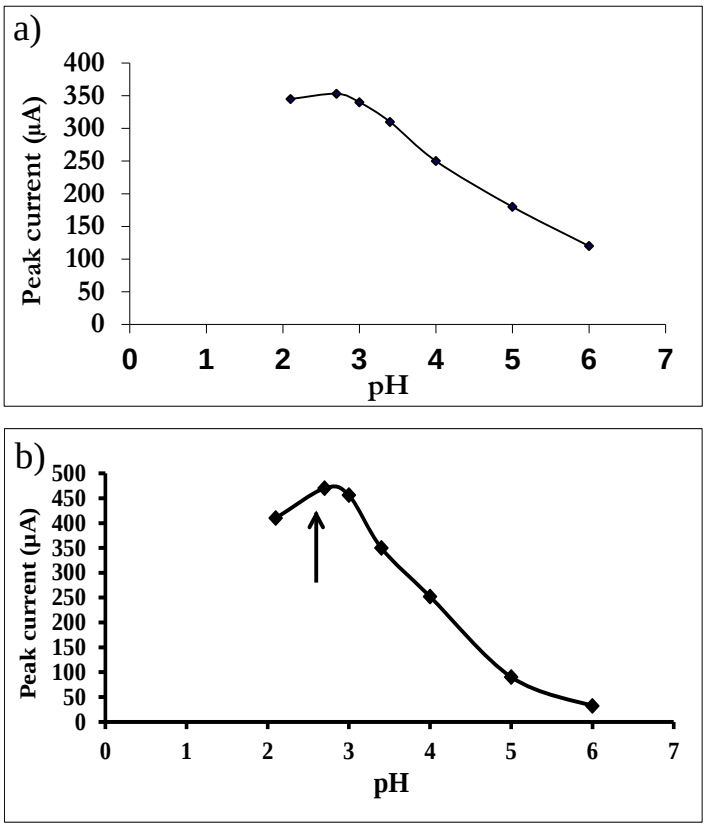


Fig. 8. Dependence of peak current of MTX (a) and ACV (b) oxidation on buffer pH in Britton Robinson buffer (MTX and ACV concentrations are 1.0×10^{-5} M for both)

The pH also affects MTX and ACV oxidation peak potential, (*Ep*). The anodic peak of MTX shifted toward more positive potentials with increasing the solution pH. While the anodic peak of ACV shifted toward less positive potentials with increasing the solution pH (as shown in Fig. 9). The plot of *Ep* versus pH was linear with slope of 0.033 and -0.052 V between pH 1.0 and 6.0 for MTX and ACV, respectively. These results suggest the importance of an acid medium for simultaneous determination of MTX and ACV because decreasing the pH allowed sufficient separation of the corresponding peaks of both drugs. This study leads us to select a solution pH of 2.7 as the convenient value for simultaneous voltammetric determination of MTX and ACV.

The effects of the other different buffer solutions, such as acetate buffer, Clark-Lubs buffer, Teorell Stenhagen buffer on MTX and ACV peak, were also tested. The Teorell Stenhagen buffer solution (pH=2.7) showed higher sensitivity so it was used throughout the further study.

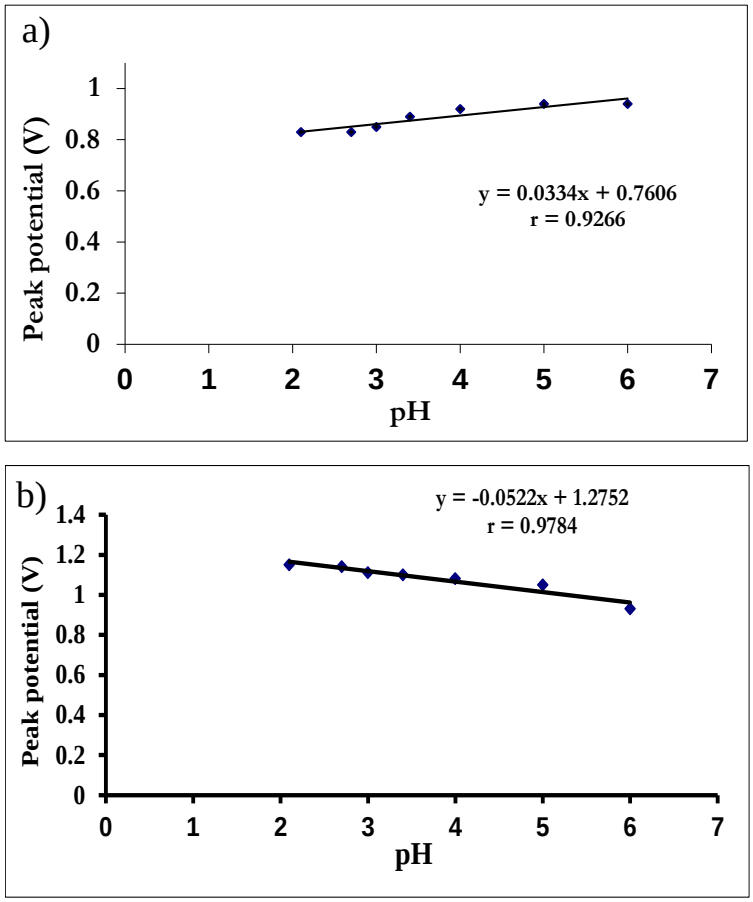


Fig. 9. Dependence of peak potential of MTX (a) and ACV (b) oxidation on buffer pH in Britton Robinson buffer (MTX and ACV concentrations are 1.0×10^{-5} M for both)

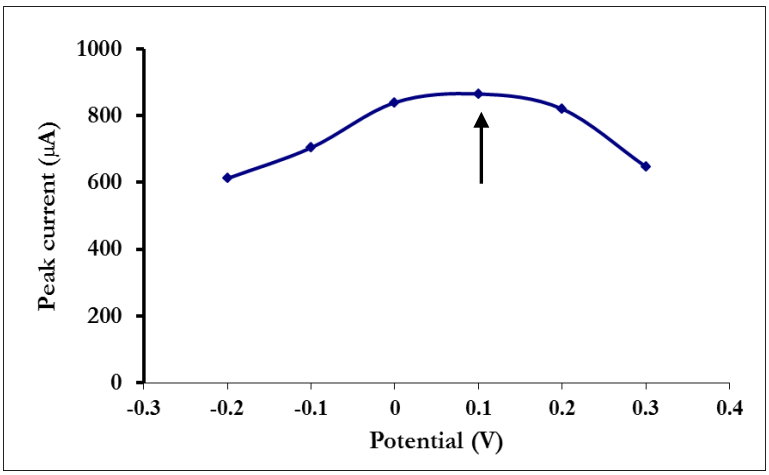


Fig. 10. Dependence of the oxidation peak current of ACV (1.0×10^{-5} M) on initial (accumulation) potential (E_{acc}) on the activated PGE in Teorell Stenhagen buffer pH 2.7 containing 0.1 M KCl

3.5.2. Effect of supporting electrolyte

Several electrolytes were tested including KCl, $NaClO_4$ and KNO_3 and the results showed that the best one is KCl. The supporting electrolyte concentration is also effective and so it was optimized. By varying the KCl concentration from 0.01 M to 0.3 M, it was found that 0.1 M KCl in the solution gave the best results so it was chosen as the optimum one. The presence of KCl increases the ability of the analyte to adsorb on the electrode surface [45]. Therefore the optimum conditions for studying the square-wave adsorptive stripping voltammetry of MTX and ACV involve Teorell Stenhagen buffer at pH 2.7 containing 0.1 M KCl.

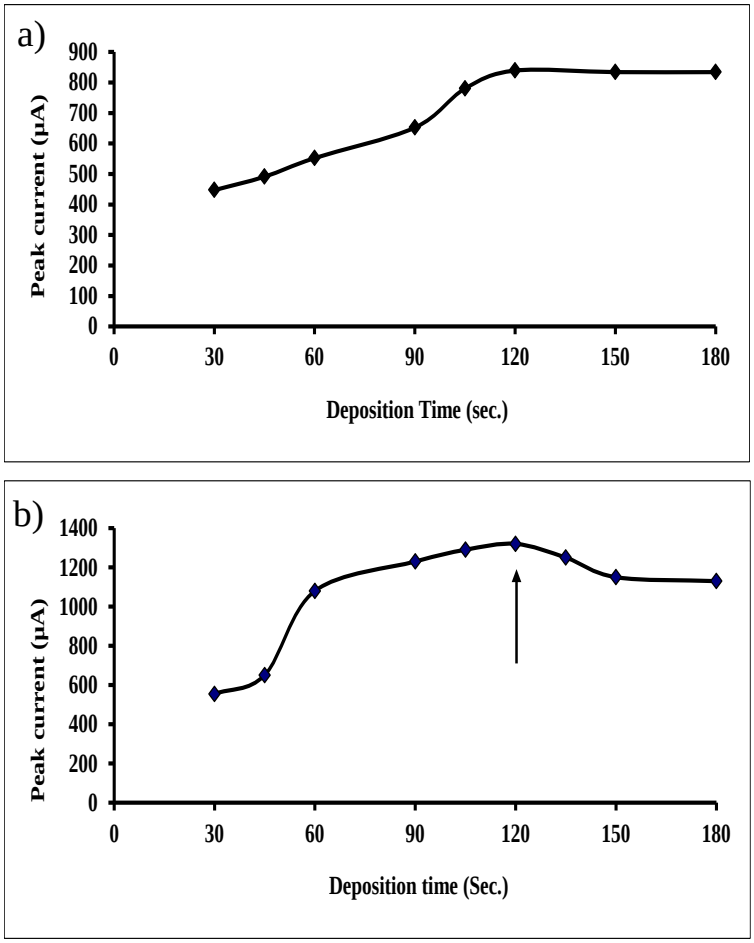


Fig. 11. Dependence of peak current of MTX (a) and ACV (b) oxidation (1.0×10^{-5} M for both) on the deposition time onto the EPPGE surface in Teorell Stenhagen buffer pH 2.7 containing 0.1 M KCl

3.5.3. Effect of accumulation potential and time

It is important to fix the accumulation (deposition) potential (E_{acc}) and time (t_{acc}) when adsorption studies were undertaken. Bearing this in mind, the effect of E_{acc} and t_{acc} has been

studied by AdSWV method. The influences of the E_{acc} on the oxidation of 1.0×10^{-5} M ACV were studied as a model. When the accumulation potential changed from - 0.1 to +0.4V, the oxidation peak current of ACV increased upto +0.2 V then decreases again as shown in Fig. 10. Therefore, E_{acc} of +0.2V was chosen as the optimum one for the rest of the study.
According to the accumulation time, it significantly affects the oxidation peak current of MTX and ACV. The peak currents of 1.0×10^{-5} M of MTX and ACV increased greatly up to 120 sec. then decreased slowly as shown in Fig. 11. This may be attributed to the saturation of the EPPGE surface with the adsorbed layer of the oxidation products of MTX and ACV.

3.5.4. Effect of square wave voltammetric parameters

Final optimization of the analytical signal centered upon varying the square-wave parameters such as the frequency, step height and pulse amplitude.

3.5.4.1. Effect of frequency

The effect of frequency was studied in the range 10–250 Hz. A nearly linear relationship was obtained between the peak current and frequency of the signal up to 200 Hz. This may be attributed to the increase in the effective scan rate but at higher values of frequency, the peak heights decreased. Hence the frequency of 200 Hz was chosen for entire analysis.

3.5.4.2 Effect of step height

The influence of step potential was investigated between 1 and 20 mV. The peak heights of the studied drugs increased up to 10 mV because the effective scan rate was increased, but at higher values of step potential peak shape was distorted. So 10 mV was chosen as the optimum step potential in the entire analysis.

3.5.4.3. Effect of pulse amplitude

The analytical signal was dependent on the pulse amplitude even if this parameter seems to be less important than the frequency. Pulse amplitude was examined in the range from 5 to 40 mV. Peak heights of the studied drugs increased upon increase of the pulse amplitude up to 25 mV and at higher values the peak shape was distorted. Thus 25 mV was chosen as the optimum pulse amplitude for all subsequent work.

Fig. 12 shows the square wave voltammograms of a solution containing mixture of MTX and ACV of same concentrations (1×10^{-5} M) at the EPPGE after optimization of all experimental parameters. As can be seen in this figure, two well defined oxidation peaks of MTX and ACV at about 0.79 and 1.14 V, respectively, observed. The peak potential separation of more than 300 mV permits the simultaneous voltammetric measurement of two components of the solution. This is an important role that the EPPGE plays.

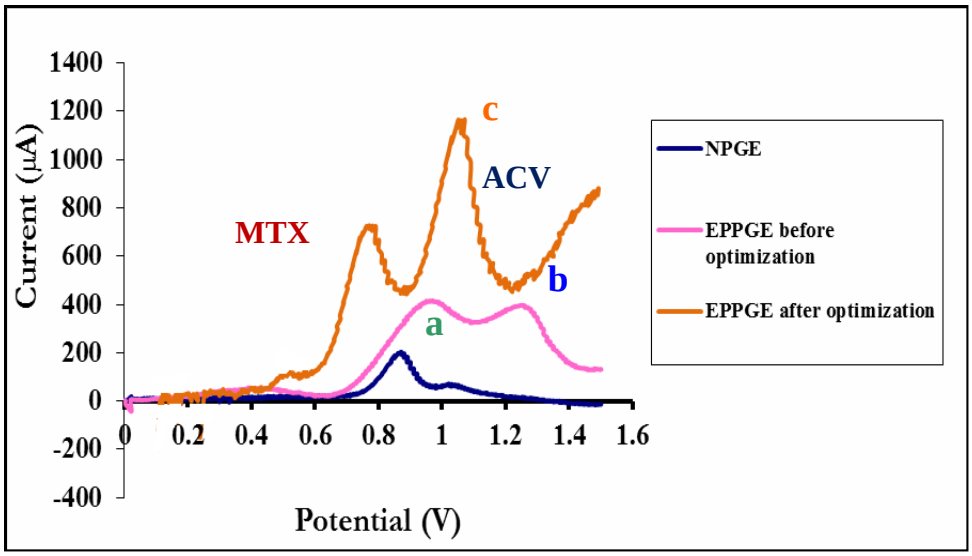


Fig. 12. Square wave voltammograms of 1.0×10^{-5} M MTX and ACV at (a) NPGE, b) EPPGE before optimization and (c) EPPGE after experimental optimization in Teorell Stenhagen buffer at pH 2.7 containing 0.1 M KCl. *PGE pretreatment conditions*: 0.05 M phosphate buffer (pH 7): Scanning potential range: -0.2 to 2.0 V *vs* Ag/AgCl, scan rate: 100 mV s^{-1}, scan number: 5 cycles

3.6. VALIDATION OF THE PROPOSED METHOD

The method was validated according to International Conference on Harmonization (ICH) guidelines [46] for sensitivity, precision, accuracy and recovery.

3.6.1. Linearity

The applicability of the proposed adsorptive square-wave stripping voltammetric (AdSWV) procedures as an analytical method for the determination of MTX and ACV was examined by measuring the stripping peak current as a function of concentration of the bulk drug for at least three times under the optimized operational parameters. The results showed linear relation in the ranges of 2.0×10^{-7} to 1.4×10^{-6} M and 5.0×10^{-7} to 3.0×10^{-6} M for MTX and ACV, respectively.

The linear regression equations are expressed as:

$Ip\ (\mu A)=(3.55\times10^{8})\ C_{MTX}\ (M)+57.24$ for MTX

$Ip\ (\mu A)=(2.17\times10^{8})\ C_{ACV}\ (M)+161.36$ for ACV

Where I_p is the peak current in µA, the regression plots showed that there is a linear dependence of the current intensity on the concentration of the studied drugs over the range

as given in Table 1, Fig. 13 and 14. A SWV of MTX and ACV simultaneously determined onto the EPPGE surface in Teorell Stenhagen buffer solution (pH 2.7) is shown in Fig. 15.

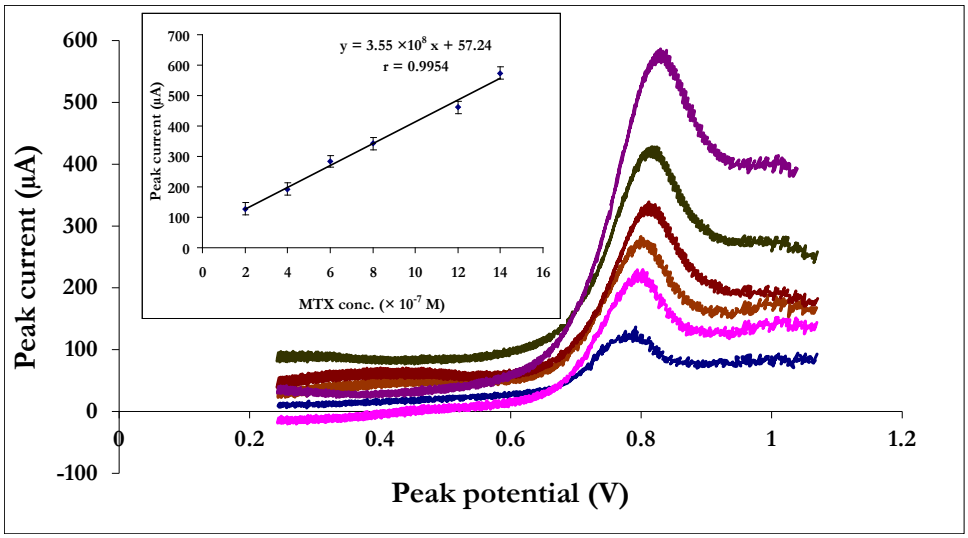


Fig. 13. Square wave voltammograms of different concentrations of MTX on the activated PGE surface 0.2, 0.4, 0.6, 0.8, 1.2 and 1.4 µM of MTX. Inset: calibration curves of the corresponding concentrations of MTX

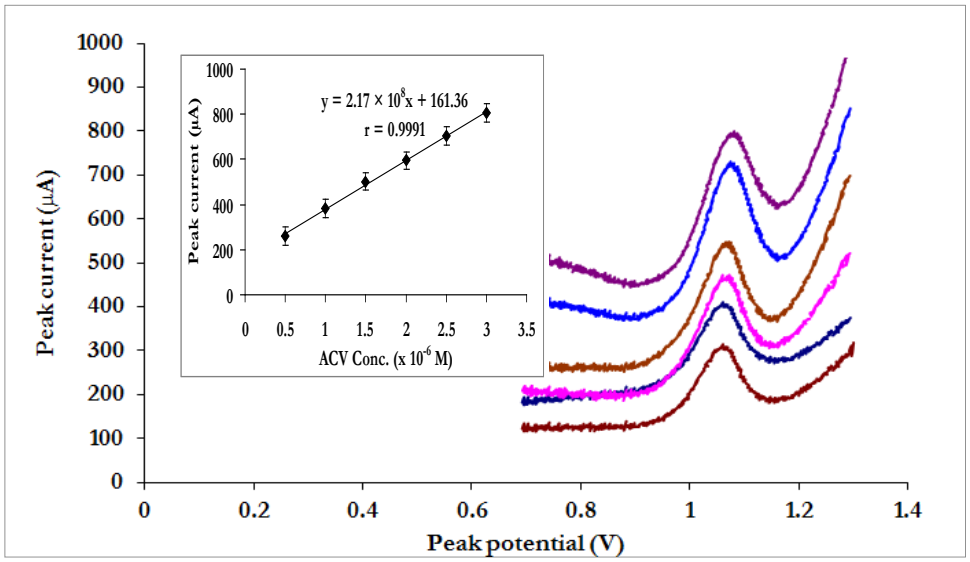


Fig. 14. Square wave voltammograms of different concentrations of ACV on the activated PGE surface 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM of ACV. Inset: calibration curves of the corresponding concentrations of ACV

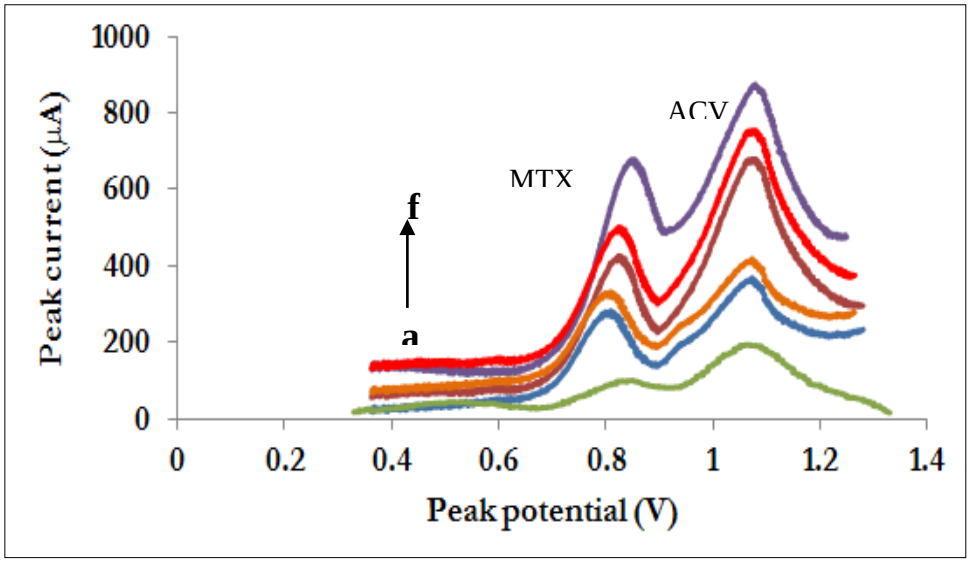


Fig. 15. Square wave voltammograms of EPPGE in Teorell Stenhagen buffer solution (pH 2.7) containing different concentrations of MTX and ACV. a–f corresponding to mixed solutions of 0.2+0.5, 0.4+1.0, 0.6+1.5, 0.8+2.0, 1.2+2.5, and 1.4+3.0 µM of MTX and ACV, respectively

Table 1. Analytical parameters for simultaneous voltammetric determination of MTX and ACV

Parameter	**MTX**	**ACV**
Linearity range (M)	2×10^{-7} to 1.4×10^{-6}	5×10^{-7} to 3×10^{-6}
Slope (µA M^{-1})	3.55×10^{8}	2.17×10^{8}
Intercept (µA)	57.24	161.36
Correlation coefficient (r)	0.9954	0.9991
% RSD of slope*	0.71	0.77
% RSD of intercept*	2.12	2.47
LOD (M)	1.13×10^{-8}	6.07×10^{-8}
LOQ (M)	3.42×10^{-8}	1.84×10^{-7}

***** n=5 where n is no. of experiments

3.6.2. Detection and quantitation limits

The limits of detection were calculated by the equation: (LOD=3SD/b) while the quantitation limits were estimated by equation: (LOQ=10SD/b) where (SD) is the standard deviation of intercept and (b) is the slope of the regression line. The calculated detection limits were 1.13×10^{-8} M and 6.07×10^{-8} M for MTX and ACV, respectively, and the

quantitation limits were 3.42×10^{-8} M and 1.84×10^{-7} M for MTX and ACV, respectively, as given in Table 1.

3.6.3. Precision/ Repeatability

For method precision, the intra- and inter-day variations for the determination of MTX and ACV were carried out at three different concentration levels covering the low, medium and higher ranges of their calibration curve. The precision and repeatability of the developed method for the studied drugs were determined in five replicate analyses for each concentration level. The results confirmed both the good precision of the proposed procedure and stability of the drugs' solutions. These results were indicated in Table 2 that proved that the method is precise and confident.

Table 2. Intra- and inter-day precision of the proposed method for simultaneous determination of MTX and ACV

Drug	**Drug Conc. ($\times10^{-7}$ M)**	**Intra-day precision**		**Inter-day precision**	
		%Recovery±SD*	**%RSD**	**%Recovery±SD***	**%RSD**
MTX	***2.0***	99.9±2.39	2.13	97.1±2.35	1.34
	6.0	101.2±2.69	1.23	98.2±3.23	2.34
	12.0	97.8±0.52	2.03	102.2±1.45	1.87
ACV	***10.0***	97.9±2.33	2.43	99.3±2.39	3.14
	20.0	98.2±2.38	1.26	100.2±1.39	2.89
	30.0	102.8±1.32	2.01	102.8±1.55	2.15

* Average of five determinations

Table 3. Robustness study for the voltammetric determination of MTX and ACV

Parameter	**MTX Recovery (%)±SD[a]**	**ACV Recovery (%)±SD[a]**
No variation[b]	99.8±2.09	98.4±2.45
pH		
2.6	98.3±2.78	101.6±2.43
2.8	101.4±1.32	101.1±1.89
Accumulation time		
110 sec.	97.8±1.23	99.5±1.56
130 sec.	101.3±2.35	102.4±1.86
Frequency		
190 Hz	99.7±1.56	101.2±1.61
210 Hz	100.4±2.54	100.5±2.05

[a] Average of five determinations
[b] Following the general assay procedure conditions

3.7. Interference

The tolerance limit was defined as the maximum concentration of the interfering substances that caused an error less than ±5.0% for the simultaneous determination of MTX and ACV. The experimental results show that 1:1, 1:10 and 1:100 of glucose, starch, sucrose, and ascorbic acid did not interfere with MTX and ACV oxidation peak, except ascorbic acid may affect MTX oxidation peak (by increase in the peak current) only in high concentration levels (more than 100 times). Because they have different electroactive group with MTX and ACV, their oxidation potentials are far from that of the studied drugs and thus they don't affect the oxidation peak current of MTX and ACV [25,47].

3.8. Analytical applications

3.8.1. Application to dosage forms

3.8.1.1. Application to methotrexate vial

The accuracy of the developed method was evaluated by quantifying MTX in its vials. The nominal content of the vial was calculated from the corresponding regression equations of previously plotted calibration plots. The mean results for the determination of MTX using the proposed method were found (48.6 mg MTX/2 ml in each vial) which is very close to the declared value of 50 mg MTX/2 ml. The obtained results showed that the proposed method could be applied with great success to MTX assay in tablets without any interference (Table 4).

Table 4. Comparative and recovery studies for the studied drugs from their dosage forms by the proposed method and reported method

Parameter	**MTX**	**ACV**
Labeled claim	Methotrexate vial (50 mg MTX / 2 ml)	Zovirax® (400 mg ACV per tablet)
Amount found	48.6 mg/2ml	397.2 mg ACV per tablet
% Recovery	97.2	99.3
% RSD*	3.23	2.89
% Bias*	-0.7	-2.8
Student t_{test}	$t_{calc.}$= 0.86	$t_{calc.}$=0.98
F_{test}	$F_{calc.}$= 2.31	$F_{calc.}$= 2.76

* Average of five determinations.
At 95% confidence level: t_{tab}=2.3, F_{tab}=6.39

3.8.2 Application to acyclovir tablets

The accuracy of the developed method was evaluated by quantifying ACV in its tablets. The nominal content of the tablet amount was calculated from the corresponding regression equations of previously plotted calibration plots. The mean results for the determination of ACV using the proposed method were found (397.2 mg ACV/tablet) which is very close to the declared value of 400 mg ACV / tablet. The obtained results showed that the proposed method could be applied with great success to ACV assay in tablets without any interference (Table 4).

To indicate the accuracy of the proposed AdSWV method, the results of ACV tablets were also compared with another reported method [49] using both student t- and F-tests as shown in Table 4. At 95% of the confidence level, the values calculated from experiments were less than those of critical t and F ones indicating that there were no statistically significant differences between the performances of both methods.

3.8.3. Standard Addition Method

The standard addition method was carried out for the accuracy studies. In this study, a solution of 2.0×10^{-7} M of MTX vial was subjected to additions of 2.0×10^{-7} M, 4.0×10^{-7} M and 6.0×10^{-7} M of standard solution, SWV curves were recorded before and after each addition. Also a solution of 5.0×10^{-7} M of ACV tablets was subjected to additions of 5.0×10^{-7} M, 1.0×10^{-6} M and 1.5×10^{-6} M of standard solution, SWV curves were recorded before and after each addition. Corresponding calculations were performed as shown in (Table 5).

Table 5. Standard addition method for the assay of Zovirax tablets (400 mg ACV/tablet) and Methotrexate vial (50 mg MTX/2 ml)

Parameter	**MTX**			**ACV**		
Added ($\times10^{-7}$ M)	2.0	4.0	6.0	5.0	10.0	15.0
Found ($\times10^{-7}$ M)	1.97	4.06	5.93	5.04	10.36	14.88
%Recovery*	98.5	101.5	98.3	100.8	103.6	99.2
%RSD*	2.97	2.43	2.32	3.41	2.63	2.65
%Bias*	-1.5	1.5	-1.2	0.8	3.6	-0.8

* n=5, where n is no. of experiments

3.8.2. Application to biological fluids

3.8.2.1. In-vitro application to spiked human plasma

To assess the performance of the proposed method for the analysis of MTX and ACV in complex matrices, its utility was investigated by determining MTX and ACV in plasma

samples. To do this, the drug-free plasma samples obtained from healthy volunteer were spiked with known amounts of standard MTX and ACV. Then protein precipitation was done by acetonitrile. After addition of acetonitrile to plasma samples, mixtures were then vortexed for 3 min and centrifuged at 5000 rpm for 15 min. Supernatants were taken and filtered through 0.45 µm syringe filter, then the voltammetric procedure under the experimental conditions was carried out. Quantifications were performed by means of standard addition method and results were shown in Table 6.

Table 6. Results of the recovery analysis of ACV and MTX in spiked human plasma sample obtained using the activated EPPGE electrode

Drug	**Standard added (M)**	**Found (M)**	**(%) Recovery±(%) RSD***	**(%) Bias**
MTX	4×10^{-7}	4.11×10^{-7}	102.8±1.42	2.75
	6×10^{-7}	6.18×10^{-7}	103.0±1.03	3.00
	8×10^{-7}	8.04×10^{-7}	100.5±1.14	0.50
ACV	1.0×10^{-6}	0.96×10^{-6}	96.0±2.45	-4.00
	1.5×10^{-6}	1.55×10^{-6}	103.3±2.33	3.33
	2.0×10^{-6}	1.94×10^{-6}	97.0±1.53	-3.00

* n=5, where n is no. of experiments.

N.B. Amount of MTX and ACV sample spiked to each plasma sample equal 2.00×10^{-7} M for MTX and 5×10^{-7} M for ACV

Table 7. Results of the studied drugs in human plasma samples obtained from volunteers after their administration

Drug	**Dose (mg)**	**Time after taking dose (hrs)**	**Average concentration of the drug (µM)±SD**	**% RSD**
MTX	25(vial)	3 5	0.45±0.019 0.27 ±0.008	4.30 2.93
ACV	400(tab.)	3 5	1.34±0.049 0.76±0.016	3.69 2.08

3.8.2.2. In-vivo application to human plasma

In addition to spiked human plasma, the present method was applied to real samples of plasma to confirm its utility. The results of the levels of the two studied drugs in the actual

cases are calculated from the linear regression equation of the studied drugs mentioned before in linearity study and these results are summarized in Table 7.

3.9. COMPARISON OF THE SENSITIVITY OF THE METHOD WITH PREVIOUSLY REPORTED METHODS

Table 8 compared the proposed method with other reported methods [12,50-52] for determination of MTX. Table 9 compares the proposed method with the other reported methods [22,26,53-54] for determination of ACV.

Table 8. A comparison of the proposed method with several reported methods for single determination of MTX

Method	Reaction conditions	Sample preparation	Linearity Range (M)	LOD (M)	Ref.
UV-Vis spectrophotometry	Need about 20 min. for completeness of the reaction	Tablets and plasma samples extracted by protein precipitation	1.1×10^{-6}-6.6×10^{5}	2.86×10^{-7}	[50]
HPLC/UV	Need about 12 min. for separation	Human plasma samples extracted by solid phase extraction	25×10^{-9}-5×10^{-6}	3×10^{-9}	[51]
CZE	Require not less than 9 min. for separation	Human urine extracted by solid phase extraction	2.2×10^{-6}-1.32×10^{-5}	7.7×10^{-7}	[52][52]
DPV	Boron - Doped Diamond Electrode used as working electrode is not easily available	Tablets and spiked human urine. Urine was used without any pretreatment	5×10^{-8}-2×10^{-5}	1×10^{-8}	[12]
SWV	Electropretreated PGE easily obtained and fabricated. Time of analysis is not more than 3 min.	Injections and human plasma samples.	2×10^{-7} - 1.4×10^{-6}	1.13×10^{-8}	This study

UV-Vis: Ultraviolet-visible
CZE: Capillary Zone Electrophoresis
DPV: Differential Pulse Voltammetry
SWV: Square Wave Voltammetry

Table 9. Comparison of the proposed method with reported methods for the determination of ACV

	Reaction conditions	**Applications**	**Linearity Range (M)**	**LOD (M)**	**Ref.**
Spectrofluorimetry	Need 80 °C and 45 min. for complete reaction	Pure and pharmaceutical dosage forms	2.22×10^{-7}-5.33×10^{-6}	8.88×10^{-8}	[53]
UHPLC-HESI-MS/MS	Gradient eleution that consume large amounts of solvents	Human plasma prepared using protein precipitation	4.44×10^{-9}-8.88×10^{-6}	2.22×10^{-9}	[54]
HPCE	Need 15 min. for complete measurement.	Tablets and creams	2.22×10^{-6}-8.88×10^{-5}	6.66×10^{-6}	[22]
Differential pulse voltammetry	Need 30-35 min. for electrode preparation each time.	Pharmaceutical dosage forms and spiked human serum and urine prepared by protein precipitation	9.0×10^{-8}- 6×10^{-6}	1.48×10^{-8}	[26]
Square wave voltammetry	Need 3-5 min for electrode preparation and measurement	Dosage forms and human plasma samples	$5\times10^{-7}$to 3×10^{-6}	6.07×10^{-8}	This work

UHPLC-HESI-MS/MS: Ultra-high-performance liquid chromatography - heated electrospray ionization - tandem mass spectrometry
HPCE: High Performance Capillary Electrophoresis

4. CONCLUSION

In this work, an electropretrated pencil graphite electrode has been successfully developed for the simultaneous electrocatalytic oxidation of MTX and ACV. This is beneficial in patients with cancer associated with viral infections. Based on the study, influence of several parameters like potential scan rate, pH and concentration were investigated. A suitable electrochemical oxidation mechanism for MTX and ACV was proposed. The modified electrode has been used to determine the studied drugs in their dosage forms and plasma samples. The proposed method offered the advantages of sensitivity, accuracy and time saving as well as simplicity of reagents and apparatus.

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