Full Paper

Electroanalytical Studies and Simultaneous Determination of Amlodipine Besylate and Atorvastatine Calcium in Binary Mixtures Using First Derivative of the Ratio-Voltammetric Methods

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Abstract
The electrochemical behavior of atorvastatin and amlodipine at a glassy carbon electrode has been studied using different voltammetric techniques. First derivative of the ratio voltammetric methods for determination of amlodipine and atorvastatin in tablets in the presence of the other compound has been described. This technique depends on the measuring of first derivative of the ratio voltammograms of each concentration as a function of the increased concentrations. DP and SW voltammetric methods depend on first derivative of the ratio-voltammetry by measurements of the selected potentials for amlodipine and atorvastatin. The linear response was within the range of $4 \times 10^{-6} - 1 \times 10^{-4}$ M for amlodipine and $2 \times 10^{-6} - 1 \times 10^{-4}$ M for atorvastatin. The proposed methods have been extensively validated.

Keywords: Amlodipine, Atorvastatine, Ratio derivative, Voltammetry, Pharmaceuticals

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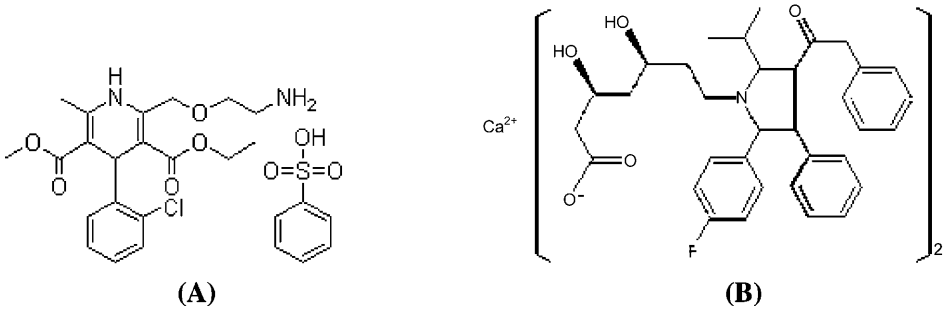
1. Introduction

The amlodipine besylate (AML) is chemically described as 3-ethyl-5-methyl (±)-2-[(2-aminoethoxy)methyl]-4-(*o*-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate, monobenzenesulfonate. AML is a dihydropyridine calcium ion antagonist (or slow-channel blocker) that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. It is a peripheral arterial vasodilator that acts directly on vascular smooth muscle to cause a reduction in peripheral vascular resistance and reduction in blood pressure. Within the physiologic pH range, amlodipine is an ionized compound (pKa 8.6), and its kinetic interaction with the calcium channel receptor is characterized by a gradual rate of association and dissociation with the receptor binding site, resulting in a gradual onset of effect [1–3].

The atorvastatin calcium (ATOR) is chemically described as [R-(R*, R*)]-2-(4-fluorophenyl)-β,δ-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2:1) trihydrate. ATOR is a synthetic lipid-lowering agent. Atorvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. This enzyme catalyzes the conversion of HMG-CoA to mevalonate, an early and rate-limiting step in cholesterol biosynthesis. ATOR is a selective, competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of sterols, including cholesterol. Cholesterol and triglycerides circulate in the bloodstream as part of lipoprotein complexes [1–3].



(A) (B)

Scheme 1. The chemical structures of amlodipine besylate (A) and atorvastatin calcium (B).

Use of AML-ATOR combination tablets may provide a more integrated approach to treatment of cardiovascular risk.

The widespread use of these compounds as binary combination dosage forms and the need for clinical and pharmacological studies require fast and sensitive analytical techniques to assay the presence of the drug in pharmaceutical dosage forms and biological samples. Most of the reported methods were influenced from each other and by interference of endogenous substances and potential loss of the drugs in the re-extraction procedure.

AML and ATOR have been studied and determined simultaneously by very few procedures: Liquid chromatography with UV detection [4–7] and derivative spectrophotometric and partial least square methods [8]. The reported methods were influenced by interference of excipients and potential loss of drugs in the re-extraction procedure and involving lengthy, tedious and time-consuming sample preparation and extraction processes requiring a sophisticated and expensive instrumentation. Electrochemical techniques offer high sensitivity and require no large sample volumes. The advance in experimental electrochemical techniques in the field of drug analysis is because of their simplicity, low cost, and relatively short analysis times no need for derivatization or time-consuming extraction steps when compared with other techniques. Selectivity in voltammetric analysis is due to the fact that different electroactive species undergo oxidation and/or reduction at different electrode potentials, but many analytes might interfere if they have close peak potentials or if they are present as major components in the sample. In old strategies, for resolving overlapping voltammograms were to use chemical reagents for extracting or complexing one component in the mixture, in order to shift or to dispose of it further apart the peak potentials or to mask one component completely. Later on, voltammetric determination of mixtures has been assessed using different techniques such as derivative, derivative ratio and chemometric methods.

The simultaneous determination of two compounds associated is often a difficult task for the analyst and the problem is even more complicate if these compounds are included in a pharmaceutical dosage form where excipients are interfering. Due to their sensitivity, electrochemical techniques, as many others, pose some problems in the analysis of multicomponent mixtures that yield overlapped signals or when the analytical signal is partially distorted. These problems can very often be overcome by using various chemical procedures such as altering the pH of supporting electrolyte, which shift one signal relative to the other, suppress one of them or make the other sharper. This kind of process usually involves time-consuming manipulation of the sample in order to obtain a specific and selective signal. In recent years, ratio derivative methods have been found to be useful in the estimation of drugs from their mixtures especially using spectrophotometric techniques [9–11]. Ratio derivative methods have been demonstrated as being very useful in the resolution of overlapping signals [9–11]. One of the classic analytical problems of multicomponent analysis is that the analyte of interest is often accompanied by other compounds effecting in the same working region. Salinas et al. [9] designed a new spectrophotometric method, which is based on the derivation of the ratio-spectra for resolving binary mixtures. Ratio-spectra derivative spectrophotometric method has been found to be useful in the determination of mixtures with two or more components having overlapping spectra and in eliminating interference from formulation matrix [9–14]. Hence, we have applied the theory of this method to our overlapping differential pulse and square wave voltammograms for removing these binary mixture interferences on their voltammograms. Ratio derivative method involves calculating and plotting one of the mathematical derivatives of a curve, which offers an alternative approach to drug analysis. Although the derivative transformation does not increase the information content of a given voltammogram, this method shows good sensitivity and specificity and permits discrimination in the face of the broad potential interference arising from unexpected effects. This method permits the determination of a component in their binary mixture at the potentials corresponding to a maximum or minimum and also the use of the peak-to peak between consecutive maximum and minimum. The main advantage of derivative of the ratio method may be the option of doing easy measurements in correspondence of peaks so it permits the use of the potential of highest value of analytical signals (maximum or minimum). Moreover, the presence of a lot of maxima and minima is another advantage by the fact that these potentials give an opportunity for the determination of active compounds in the presence of other active compounds and excipients which possibly interfere with the analysis. For applying this method, we calculated all necessary equations and parameters using Microsoft Excel Programme. Direct voltammetric methods are not effective in AML and ATOR binary mixtures because they are subjected to interferences by each other. First derivative of the ratio-voltammetric methods have proved advantageous in eliminating voltammetric interferences. Monitoring of these compounds in their binary mixtures is important for their simultaneous determination in pharmaceutical dosage forms.

The electroanalytical determinations of AML [15, 16] and ATOR [17, 18], as single component, have been studied by voltammetric and polarographic methods. However, to the best of our knowledge, no electroanalytical assay has been performed on AML and ATOR in their binary mixtures using glassy carbon electrode either in bulk form or in pharmaceuticals. Also, there have been no studies published related to the electrochemical oxidation details on the glassy carbon electrode of these compounds in the presence of each other.

This work aimed to study the detailed voltammetric behavior of AML and ATOR individually and at a glassy carbon electrode in their mixtures using different electrochemical techniques. This study described a fully validated, simple, rapid and more sensitive procedure for the determination of AML and ATOR in binary mixtures and in

pharmaceutical formulations employing first derivative of the ratio-differential pulse and first derivative of the ratio-square wave voltammetric methods at the glassy carbon electrode. As an advantage, the determination procedure did not require sample pretreatment or any time-consuming extraction step prior to the drug assay. In addition, the purposed method can be considered as a stability-indicating and fully validated assay. As a comparison method, first derivative spectrophotometric method has been used for the determination of both drugs in the presence of each other [8].

2. Experimental

2.1. Instrumentation

All voltammetric measurements at a glassy carbon electrode were performed using a BAS 100 W (Bioanalytical System, USA) electrochemical analyzer. A glassy carbon working electrode (BAS; ∅: 3mm diameter), an Ag/AgCl reference electrode (BAS; 3 M KCl) and platinum wire counter electrode (BAS) and a standard one-compartment three-electrode cell of 10mL capacity were used in all experiments. The glassy carbon electrode was polished manually with aqueous slurry of alumina powder (∅: 0.01 μm) on a damp smooth polishing cloth (BAS velvet polishing pad), before each measurement. All measurements were realized at room temperature.

The pH value of the solutions was measured by Model 538 pH meter (WTW, Austria) using a combined electrode (glass electrode-reference electrode) with an accuracy of ± 0.05 pH and calibrated with standard buffers (FIXANAL, Riedel-de Haen, Germany).

DPV conditions were: pulse amplitude, 50 mV; pulse width, 50 ms; scan rate, 20 mV s^{-1} and SWV conditions were: pulse amplitude, 25 mV; frequency, 15 Hz; potential step, 4 mV. All measurements were carried out at ambient temperature of the laboratory (23 – 27 °C).

For the comparison UV-derivative spectrophotometric studies, a Shimadzu 1601 PC double beam spectrophotometer with a fixed slit width (2 nm) was used, coupled an IBM-PC computer running spectrophotometric software Schimadzu UVPC software.

2.2. Reagents and Solutions

AML, ATOR and dosage forms were kindly provided by Pfizer Pharm. Ind.(Istanbul-Turkey). Each film-coated tablet (Caduet) was contained 10 mg AML and 10 mg ATOR and the inactive ingredients calcium carbonate, colloidal silicon dioxide (anhydrous), croscarmellose sodium, hydroxypropyl cellulose, magnesium stearate, microcrystalline cellulose, opadry II white 85F28751 or opadry II blue 85F10919, polysorbate 80, pregelatinized starch, and purified water [1, 3].

All chemicals were of reagent grade quality (Merck, Sigma or Riedel) and they were employed without further purification. The standard stock solutions of AML and ATOR (1×10^{-3} M) were prepared daily by direct dissolution in methanol and kept in the dark in refrigerator. Working solutions under voltammetric investigation were prepared by dilution of the stock solution and contained 20% methanol as constant amount. 0.1 M H_2SO_4, 0.5 M H_2SO_4, 0.2 M Phosphate buffer at pH 2.0 – 8.0, 0.04 M Britton – Robinson buffer at pH 2.00 – 12.0 and 0.2 M Acetate buffer at pH 3.7 – 5.7 were used for supporting electrolyte.

2.3. Procedure

Standard solutions were prepared by dilution of the stock solution with selected supporting electrolyte to give solutions containing AML in the concentration range of 4×10^{-6} to 1×10^{-4} M and ATOR in the concentration range of 2×10^{-6} to 1×10^{-4} M.

The DP and SW voltammograms of the separate and binary mixtures prepared at different concentrations of AML were recorded and stored in the computer. According to the theory of the ratio derivative method [9 – 12], the stored voltammograms of the mixtures were divided, potential-by-potential, by a standard DP or SW voltammograms of ATOR solution (2×10^{-5} M in Britton Robinson buffer at pH 5.0 contained 20% methanol) using Microsoft Excel program. Then, the first derivative of the ratio-voltammograms were calculated and drawn. The values of these first derivative peaks were measured at suitably selected potentials in the range of 400 – 1076 mV for DPV, 500 – 1132 mV for SWV and plotting against the corresponding concentration to obtain the calibration graph.

The similar procedure was followed for the different concentrations of ATOR when AML 2×10^{-5} M in Britton – Robinson buffer at pH 5.0 contained 20% methanol used as a divisor. The stored voltammograms of the mixtures were divided, potential-by-potential, by a standard DP or SW voltammograms of AML solution (2×10^{-5} M in Britton Robinson buffer at pH 5.0 contained 20% methanol) using Microsoft Excel program. Then, the first derivative of the ratio-voltammograms were calculated and drawn. The calibration curve was obtained by plotting the drug concentration against the signal in the first derivative of ratio voltammogram between 400 and 1076 mV for DPV, 500 and 1132 mV for SWV.

2.4. Validation of the Methods

The ruggedness and precision were checked in the same day ($n = 5$) and three different days ($n = 5$) over a week period. Relative standard deviations were calculated to check the ruggedness and precision of the method [19 – 22]. The precision and accuracy of analytical methods are described in a quantitative fashion by the use of relative errors (Bias %). One example of relative error is the accuracy, which

describes the deviation from the expected results. All solutions were kept in the dark and were used within 24 h to avoid decomposition. However, voltammograms of the sample solutions recorded a week after preparation did not show any appreciable change in assay values.

2.5. Pharmaceutical Dosage Forms Assay Procedure

Ten tablets of Caduet (each tablet contains 10 mg AML and 10 mg ATOR) were accurately weighed and finely powdered by pestle in a mortar. An adequate amount of this powder, corresponding to a stock solution of concentration 1×10^{-3} M of each compound was weighed, transferred into a 50 mL-calibrated flask and completed to the volume with methanol. The content of the flask were sonicated for 10 min for complete dissolution. The sample from the clear supernatant liquor was withdrawn and quantitatively diluted with the selected supporting electrolyte and solution contained constant amount methanol as 20%.

This solution was then transferred to a voltammetric cell and DP and SW voltammograms were recorded. For the calculation of compounds amount in pharmaceutical dosage form, the same procedure was used as described Section 2.3. Both of the drug contents in each tablet were determined referring to the related regression equations.

2.6. Recovery Studies

Because other components of the matrix of film coated tablet dosage forms may interfere with the analysis or accurate quantitation of the analyte, potential effects from matrix components must be investigated. If the proposed method is used to measure an analyte in a complex sample matrix (e.g., a pharmaceutical formulation), a standard addition recovery method can be used. Recovery experiments are performed in the presence of the matrix [19–22]. To study the accuracy, reproducibility and to check the interference from the excipients used in the formulations of these techniques, recovery experiments were carried out using the standard addition method. In order to know whether the excipients show any interference with the analysis, known amounts of the pure AML or ATOR were added to the preanalyzed film coated tablet dosage forms.

The mixtures were calculated using the same way described in Section 2.3 by both proposed techniques. The recovery results obtained after five repeated experiments for both techniques.

3. Results and Discussion

3.1. Voltammetric Responses and Optimization of the Solution pH for the Oxidation of AML and ATOR

AML appears to be an electroactive drug. The anodic behavior of AML has been studied using glassy carbon electrode individually [15, 16]. One of these studies is related with adsorptive stripping voltammetry and the other one is related with diffusion controlled process. Also, ATOR is an oxidizable and reducible molecule. The reductive determination of ATOR has been studied using hanging mercury drop electrode [17]. The determination of alone ATOR has been a subject of considerable interest by electrooxidation and a method using glassy carbon electrode [18] has been developed for its determination.

AML and ATOR appear to be electroactive drugs and there are no reports in scientific literature about the simultaneous determination of AML and ATOR using selective and sensitive electroanalytical techniques. To demonstrate the usefulness of a solid electrode for the simultaneous determination of AML and ATOR, which may offer advantages for the use of such electrodes as sensors, the electrochemical behavior of AML and ATOR on a glassy carbon electrode was investigated in this research. AML and ATOR was subjected to a voltammetric study in DPV and SWV modes, and to cyclic and linear sweep voltammetric study with the aim of characterizing their electrochemical oxidation behavior. Also, concerning the detailed electro-oxidative behavior and possible oxidation mechanism of AML and ATOR at solid electrodes such as glassy carbon electrode will be discussed. AML and ATOR were electrochemically oxidized in a broad pH range using a glassy carbon disc electrode, producing rather complex signals at high anodic potential. The peak currents and peak potentials were determined in supporting electrolytes containing 20% methanol (v/v) to maintain solubility. Therefore, several measurements with different electrochemical techniques were performed using various supporting electrolytes and buffers in order to obtain such information. The cyclic, linear sweep, DPV and SWV voltammetric behavior of 6×10^{-5} M AML and 6×10^{-5} M ATOR individually and their synthetic mixtures at the same concentrations were examined with varying pH over a wide range of values from acidic (0.5 M H_2SO_4) to alkaline (pH 12.00) in acid solutions and different buffer aqueous media. All anodic responses will be discussed in this study.

As a first step, AML and ATOR were subjected to a cyclic and linear sweep voltammetric studies with the aim of the detailed characterizing its electrochemical oxidation behavior, on glassy carbon electrodes and to a sensitive and selective simultaneous voltammetric studies in DPV and SWV modes.

All voltammograms from CV, LSV, DPV and SWV methods, AML exhibited one distinct and well defined anodic peak at different potential values in different supporting electrolyte compositions and at all pH values between pH 0.3 and 12.00 (Figs. 1a and b). However, using all voltammetric techniques, ATOR gave different response than AML. Cyclic voltammetric measurements showed an irreversible nature of the oxidation process for both compounds in all pH values. The scanning was started at 0.0 V in the positive direction in 0.5 M H_2SO_4 and at pH 5.0 Britton – Robinson buffer, the anodic oxidation of AML did not occur until about +0.92 V and +0.81 V, respectively, on

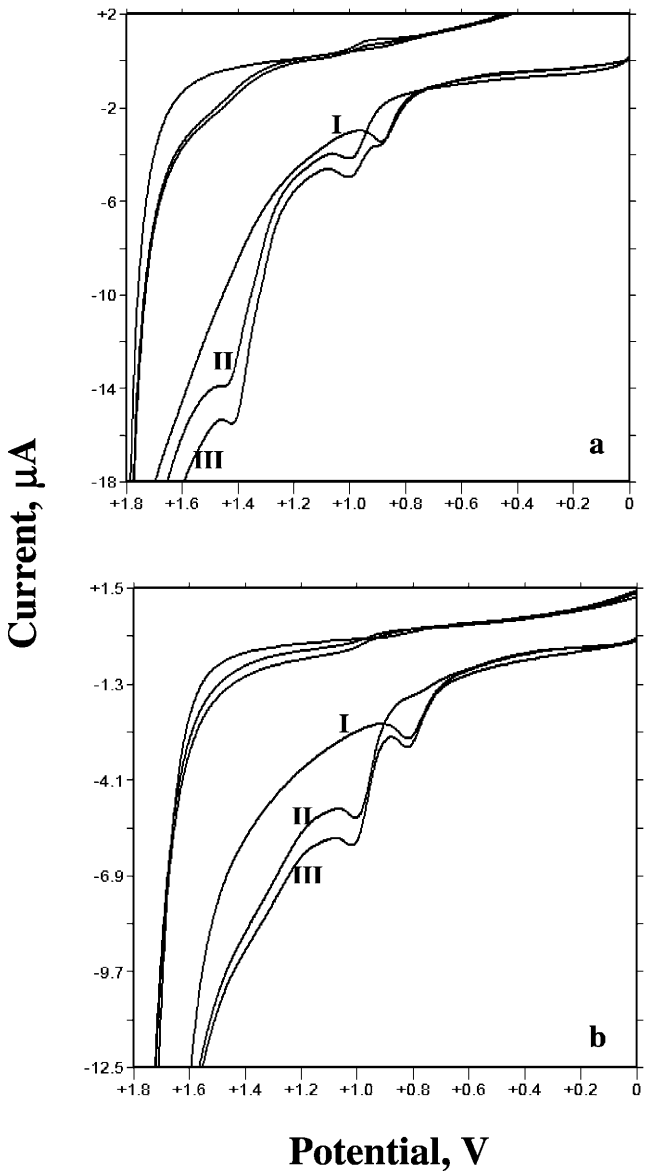


Fig. 1. Cyclic voltammograms obtained for a glassy carbon electrode (a) in 0.5 M H_2SO_4; (b) in pH 5.00 Britton–Robinson buffer containing 6×10^{-5} M AML (I); ATOR (II); their binary mixture (III). Scan rate: 100 mV s^{-1}.

glassy carbon electrode (Figs. 1a and b). By reversing at +1.80 V, no reduction signal corresponding to the anodic response was observed on the cathodic branch in all media. The AML peak decreased to the second or higher cycles. This phenomenon may be partly attributed to the consumption of adsorbed AML on the electrode surface.

Cyclic and linear sweep voltammograms of ATOR showed one distinct and well defined anodic peak and one additional ill-defined anodic wave at different potentials depending on pH values and supporting electrolyte compositions (Fig. 1a). Figure 1a shows a well-defined oxidation peak and an ill-defined wave which are observed at about +0.995 V and +1.44 V for ATOR and a sharp peak at about +0.89 V for AML with glassy carbon electrode in 0.5 M H_2SO_4 supporting electrolyte using by cyclic voltammetry. Successive recording of the voltammetric signal resulted in a decrease of the wave for ATOR and AML, suggesting significant deleterious adsorption processes. However, for ATOR second ill-defined wave became a peak (at high potential) on the repetitive scanning (not shown).

The electrochemical oxidation of AML and ATOR in Britton–Robinson buffer at pH 5.0 was explored next. As shown in Figure 1b, glassy carbon electrode exhibits an extensive oxidation wave at about +0.82 V for AML and +1.02 V for ATOR. Figure 1b shows the voltammograms given by each of the individual and mixture of AML and ATOR at their studied concentrations. A considerable overlap of the peaks is clearly evident in Figures 1a and b in the studied pHs and media. According to Figure 1 (curve III), their binary mixtures were affected between each compound current.

Next, the responses of pH on the voltammetric waves were explored for both compounds. Over the pH range of 0.3 to 12.0, a linear variation of peak potentials (E_p) and peak currents with pH were observed for AML and ATOR, as shown in Figures 2a, b and 2 c, d, respectively.

The DPV data for both compounds were similar to those obtained by cyclic and SWV. For the DPV response in all working media, the relationship between the peak potential and pH can be expressed by the following equations:

For AML:

$E_p = 1026.5 - 55.74$ pH $\quad r = 0.996$ (between pH 2.0 and 9.0)

For ATOR:

$E_p = 1046.8 - 23.54$ pH $\quad r = 0.997$ (between pH 4.0 and 12.0)

For AML and ATOR, potential values remain pH-independent at lower pH values than 2.0 and 4.0, respectively. After pH 2.0 for AML and pH 4.0 for ATOR, the potentials shift to less positive values with increasing pH (Figs. 2a and c). It seems that the electroactive grouping responsible for the oxidation process is in acid-base equilibrium with pKa of about 9.0 for AML and 4.0 for ATOR. At pH < pKa the conjugate base predominates in the supporting electrolyte. When pH > pKa, the conjugate base must be formed by a rapid dissociation of the protonated form.

The increase in the slope between pH 2.0 and 9.0 indicated the presence of an antecedent acid-base equilibrium with pKa of about 9.0 which is supposed to correspond to the pKa value of AML. AML pKa value was reported as 8.6 in the literature [23]. The slope of this equation was 55.74 mV/pH. According to the obtained slope value of this Equation, 2 electrons and 2 protons are involved in the rate-determining steps. This closeness of the slope to the expected theoretical value of 59 mV/pH indicates that the number of proton and electron involved in the oxidation of AML is equal [24, 25].

The increase in the slope at pH >4.0 indicated the presence of an antecedent acid-base equilibrium with pKa

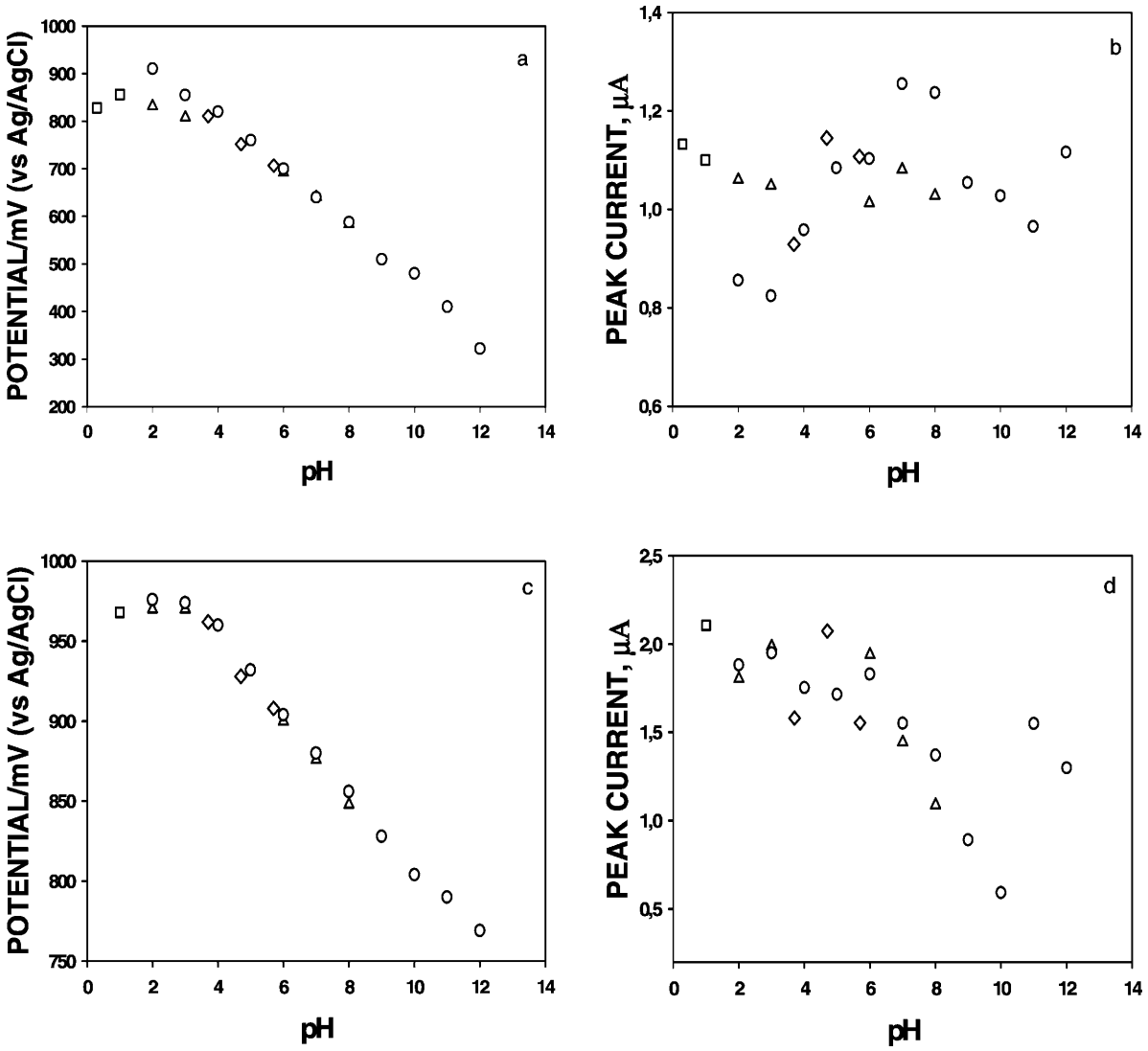


Fig. 2. Effect of pH on AML peak potential (a) and peak current (b); on ATOR peak potential (c) and peak current (d); AML and ATOR concentration 6×10^{-5} M with constant amount methanol (20%). (□) H_2SO_4; (◇) acetate (0.2 M); (△) phosphate (0.2 M); (○) Britton – Robinson buffer (0.04 M).

of about 4.0 which is supposed to correspond to the pKa value of ATOR [26]. As the slope of this equation are found that 23.54 mV/pH. This value is close to the theoretical value of 30 mV/pH that involve 2 electrons and a proton transfer in the rate-determining step [24, 25, 27].

The experimental results showed that shapes of the curves, maximum peak currents and resolution of the peaks were better in acidic pH values for ATOR (Fig. 2d and Fig. 3a, b). The main peak of ATOR that appeared at less positive potential was the best developed and became sharper in Britton – Robinson buffer at pH 5.0. Also for AML the good response was obtained in Britton – Robinson buffer at pH 5.0 (Fig. 1b). For the simultaneous determination of both compounds, Britton – Robinson buffer at pH 5.0 with constant amount of methanol (20%) was selected for the working solution. The first peak and second wave shift to less positive potentials and close to each other as the pH increases so much so that it virtually overlapped above pH 7.0. The second wave increases up to pH 8.0, above which it exhibits a shoulder at less positive potentials. At pH > 8.0, the peak looks like split shape (Fig. 3c and d). After pH 6.0, both electrochemical responses of ATOR were shifted and close to each other and become a shoulder depending on the pH value.

Scan rate studies were carried out to assess at both compounds, under diffusion or adsorption control. Using the concentration of 6.0×10^{-5} M AML and 6.0×10^{-5} M ATOR in Britton – Robinson buffer at pH 5.0, the voltammetric peak currents were observed as the scan rate over the range of 5 – 1000 mV s^{-1} for AML and the range of 5 –

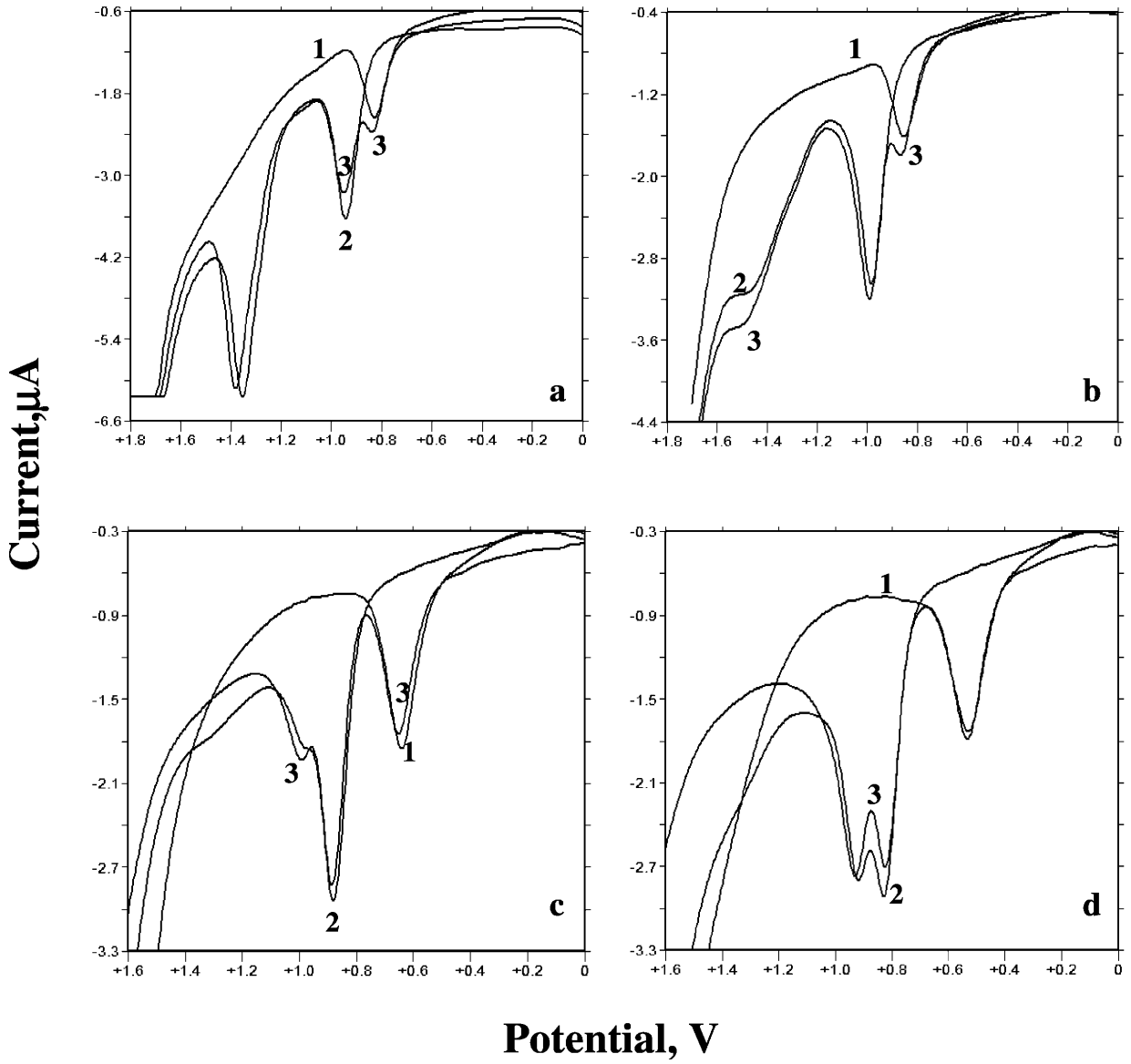


Fig. 3. DPV curves obtained for a glassy carbon electrode in 0.5 M H_2SO_4 (a); in pH 3.0 Britton – Robinson buffer (b); in pH 7.0 Britton – Robinson buffer (c) and in pH 9.0 Britton – Robinson buffer (d) containing 6×10^{-5} M AML (1); ATOR (2); their binary mixture (3).

750 mV s^{-1} for ATOR. The linear responses for AML and ATOR were observed with the square root of the scan rate as follows

I_p (μA) = 0.13 $v^{1/2}$ (mV s^{-1}) – 0.10 $r = 0.997$ for AML

I_p (μA) = 0.23 $v^{1/2}$ (mV s^{-1}) – 0.34 $r = 0.994$ for ATOR

A plot of the logarithm of the peak current versus the logarithm of the scan rate for AML and ATOR gave a straight line with a slope of 0.53 and 0.55, respectively, close to the theoretical value of 0.5, which is expected for an ideal reaction of solution species [28]. Such dependence indicated that the oxidation of AML and ATOR was indeed diffusion controlled. It is expected for an ideal reaction of solution species [28].

The equations obtained were:

$\log I_p$ (μA) = 0.53 $\log v$ (mV s^{-1}) – 0.997 $r = 0.997$ for AML

$\log I_p$ (μA) = 0.55 $\log v$ (mV s^{-1}) – 0.82 $r = 0.998$ for ATOR

Tafel analysis of voltammograms from the oxidation of 6×10^{-5} M AML and 6×10^{-5} M ATOR (at a scan rate of 5 mV s^{-1}) was conducted in Britton – Robinson buffer at pH 5.0. The αn values of the anodic reaction corresponding to the voltammetric oxidation peak was obtained using Tafel plot (log I vs. E_p). The value of 0.36 and 0.32 were obtained in Britton – Robinson buffer at pH 5.0 for AML and ATOR, respectively. The exchange current densities are obtained as

$I_o = 2.24 \times 10^{-11}$ μA/cm^2 for AML and 3.02×10^{-11} μA/cm^2 for ATOR. These values together with the absence of cathodic response in cyclic voltammetry (Fig. 1) confirmed the irreversibility of the oxidation processes of both compounds on glassy carbon electrode.

Voltammetric methods, especially cyclic voltammetry are most suitable for investigating the redox behavior of the new pharmaceutical compounds which can give insights into its metabolic fate [29–31]. Cyclic voltammetric curves from the redox properties of active compounds might have profound effects on the understanding of the redox mechanism related to the activity of the AML and ATOR compounds. The anodic oxidative behavior of ATOR has already been discussed as detail [18]. There are two main groupings present in the structure of ATOR, which might undergo electrooxidation: the pyrrole ring and phenylcarbamoyl moiety. Our results confirm that the electroactive center corresponding to the first and second anodic peak was the heterocyclic amine (pyrrole ring) and phenylcarbamoyl group, respectively.

AML was oxidized at glassy carbon electrode in all media and pH values, producing only one anodic peak. It may be attributed to the oxidation 1,4 dihyropyridine ring, as can be observed from the same group members [32, 33] to give the corresponding pyridine derivative in a 2 electron and 2 proton oxidation process. The proton bounded N position of the pyridine group is responsible for the oxidation mechanism. In aqueous media, AML undergoes a simple anodic process that includes two-electrons and two- protons and produces the corresponding pyridine derivative with the concomitant release of protons.

3.2. Simultaneous Electroanalytical Determination of AML and ATOR

The aim of this work was to develop a rapid, simple, selective and sensitive simultaneous determination of AML and ATOR in their binary mixtures and dosage forms. Various electrolytes, such as sulfuric acid, Britton–Robinson, acetate and phosphate buffer were examined. In order to develop a voltammetric procedure for determination of the drug, we selected the DPV and SWV techniques, since the peaks were sharper and better-defined at lower concentration of AML and ATOR than those obtained by cyclic and linear sweep voltammetry with a lower background current, resulting in improved resolution. DPV and SWV are effective and rapid electroanalytical techniques with well-established advantages, including good discrimination against background currents and low detection limits [24, 25]. pH is one of the variables that influence voltammograms most strongly and hence the resolution of mixtures as it affects different analytes in different forms. The best results with respect to signal enhancement and peak shape accompanied by sharper response was obtained with Britton–Robinson buffer at pH 5.0 with a constant amount of methanol (20%) for both compounds. These supporting electrolytes were chosen for the subsequent experiments

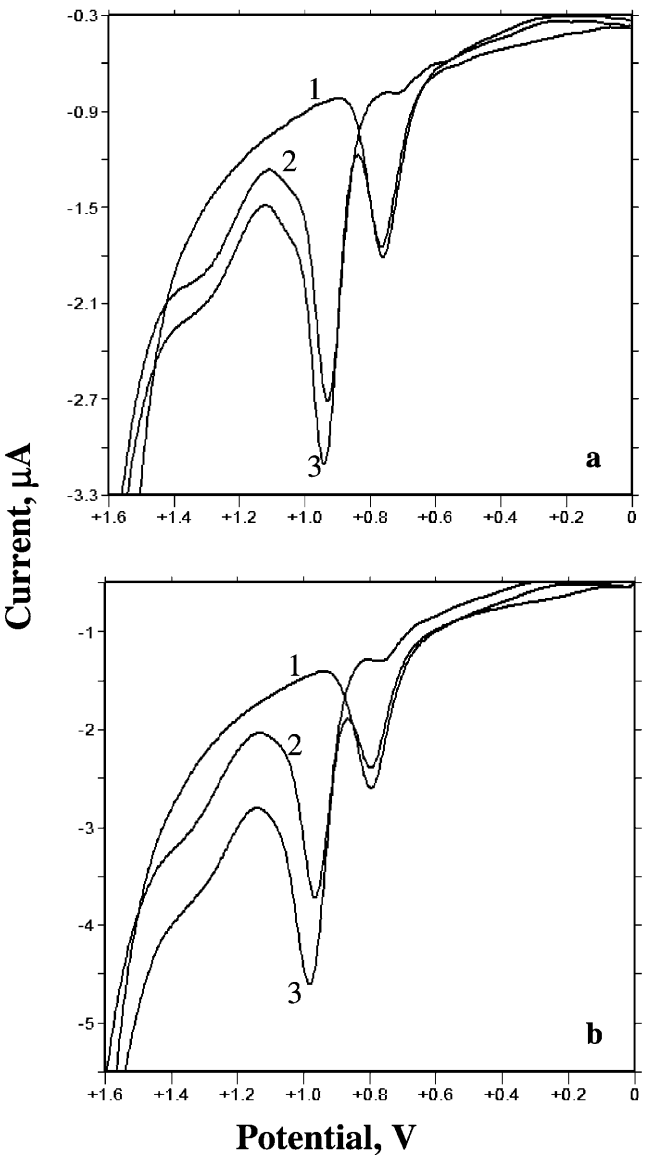


Fig. 4. DPV (a) and SWV (b) curves obtained in pH 5.00 Britton–Robinson buffer, containing 6×10^{-5} M AML (1); ATOR (2) and their binary mixture (3).

(Fig. 4). The main DPV and SWV peaks of ATOR that appeared at less positive potential was the best developed and became sharper in this supporting electrolyte. For this reason, Britton–Robinson buffer at pH 5.0 was chosen as the working medium. Also for AML, the good response was obtained in Britton–Robinson buffer at pH 5.0 (Fig. 4a and b). For the simultaneous determination of both compounds, Britton–Robinson buffer at pH 5.0 with constant amount of methanol (20%) was selected for the working solution. After pH 6.0, (Fig. 3c) both electrochemical responses of ATOR were to close up to each other and become a shoulder depending on the pH value. Also after pH 9, this situation was obtained as a split peak (Fig. 3d).

In Figure 4, the zero order DP and SW voltammograms of AML and ATOR between 0.0 and + 1.60 V potential ranges are shown. It can be easily seen that the DP and SW

voltammograms of AML and ATOR are overlapped and as a result, the determination of these two compounds was not possible for reliable direct current measurements. Ratio derivative voltammetry can be suitable to obviate this problem. The main advantage of the ratio derivative voltammetric methods like ratio derivative spectrophotometric methods [9–13] may be the option of doing measurements in correspondence of peaks, hence a potential greater sensitivity and accuracy. While the main disadvantages of the zero crossing method in derivative voltammetry for resolving of the binary mixtures of components with overlapped voltammograms are the risk of small drifts of the working potentials and circumstance that the working potentials generally do not fall in correspondence of peaks of the derivative voltammograms.

The ratio derivative method permits the use of the different concentrations as the divisor to obtain the different calibration graphs. An accurate choice of divisor standard concentration is fundamental for several reasons. Hence we tested the methods with various divisor concentrations. We carried out preliminary investigations, to select the standard solution as divisor at an appropriate concentration of AML and ATOR in the range $4 \times 10^{-6} - 1 \times 10^{-4}$ M and $2 \times 10^{-6} - 1 \times 10^{-4}$ M, respectively. We randomly selected the divisor concentration within the linearity ranges. The results of all tests are not shown for the sake of brevity and because these do not add to the scientific value of the work. A concentration of 2×10^{-5} M of AML and 2×10^{-5} M ATOR as divisor gave best results in term of signal-to-noise ratio and highest correlation coefficient values, being and indication of the quality of fitting of the data to the straight line.

The ratio voltammograms and the first derivative of these ratio voltammograms of different AML standards at increasing concentrations in Britton–Robinson buffer at pH 5 (with 20% constant amount methanol) obtained by dividing each of these voltammograms with the voltammograms of the standard solution of ATOR 2×10^{-5} M are shown in Figure 5. In Figure 5a and b were shown the ratio-voltammograms of AML/ATOR with DPV and SWV, respectively. In Figure 5c and d were shown the correspond-

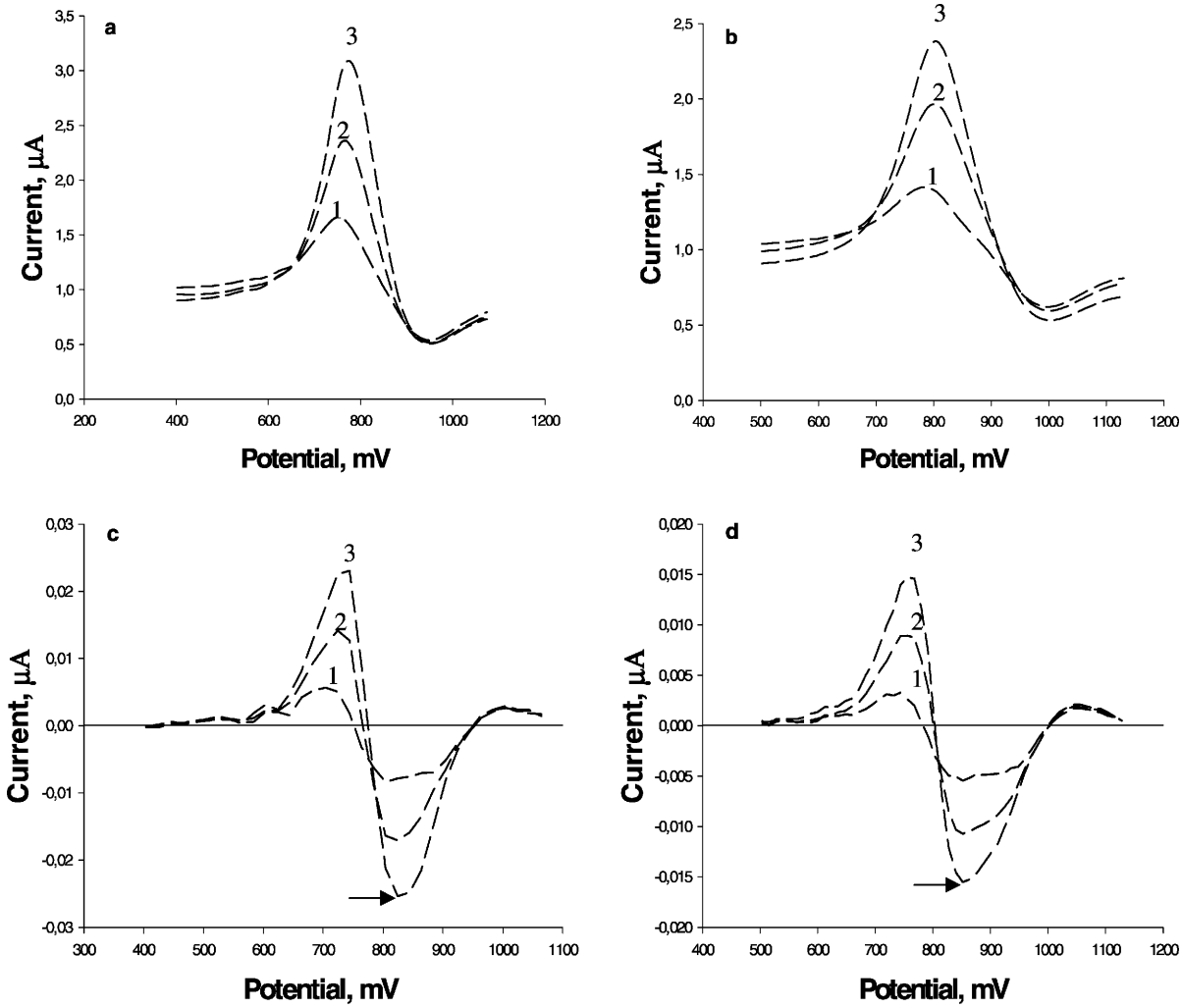


Fig. 5. (a;b) Ratio-voltammograms and (c;d) first derivative of the ratio-voltammograms of AML of (1) 2×10^{-5} M; (2) 6×10^{-5} M and (3) $1 \times 10^{-}4$ M using a 2×10^{-5} M ATOR as divisor. The arrows indicate the working potential. [a, c show DPV results and b, d show SWV results].

ing first derivative of the ratio voltammograms of Figure 5a and b, respectively. For calibration graph, the potentials were selected which exhibited the best linear response to the analyte concentration, i.e., in the first derivative mode 0.84 V and 0.86 V for AML, with DPV and SWV methods, respectively.

For determining the other component, ATOR, an analogous procedure was followed. Figure 6 show the ratio spectra of different standard solutions of ATOR in Britton – Robinson buffer at pH 5 (Figs. 6a and b) and their first derivatives of the ratio voltammograms (Figs. 6c and d), using the standard DP or SW voltammograms of a 2×10^{-5} M of ATOR solution as the divisor. As seen in Figure 6c and d, there obtain more than one maxima and minima and it was found that the maximum at 0.90 V for DPV and at 0.94 V for SWV is suitable for the assay of ATOR in binary mixture with AML for both voltammetric methods.

Once the optimum working conditions has been established, calibration graphs were obtained at 0.84 V with DPV and 0.86 V with SWV (for AML) and 0.90 V with DPV and 0.94 V with SWV (for ATOR), and showed that the proposed method is applicable over the ranges $4 \times 10^{-6} - 1 \times 10^{-4}$ M for AML and $2 \times 10^{-6} - 1 \times 10^{-4}$ M for ATOR. The characteristic parameters of the regression equations are summarized in Table 1. The calibration graphs of each drug at selected potentials were achieved by plotting the values of the first derivative of the ratio voltammetric response of AML/ATOR and ATOR/AML, with variable concentrations of AML and ATOR. The proposed method is applicable over the ranges $4 \times 10^{-6} - 1 \times 10^{-4}$ M for AML and $2 \times 10^{-6} - 1 \times 10^{-4}$ M for ATOR. The linearity ranges, limits of detection (*LOD*), limit of quantification (*LOQ*), repeatability, reproducibility, precision, recovery, bias % and selectivity were evaluated for both methods. The characteristic parameters and necessary statistical data of the regression equations are compiled in Table 1. The *LOD* and *LOQ* values were calculated using the following equations [19 – 22].

$$LOD = 3.3 \times SD/m$$

$$LOQ = 10 \times SD/m$$

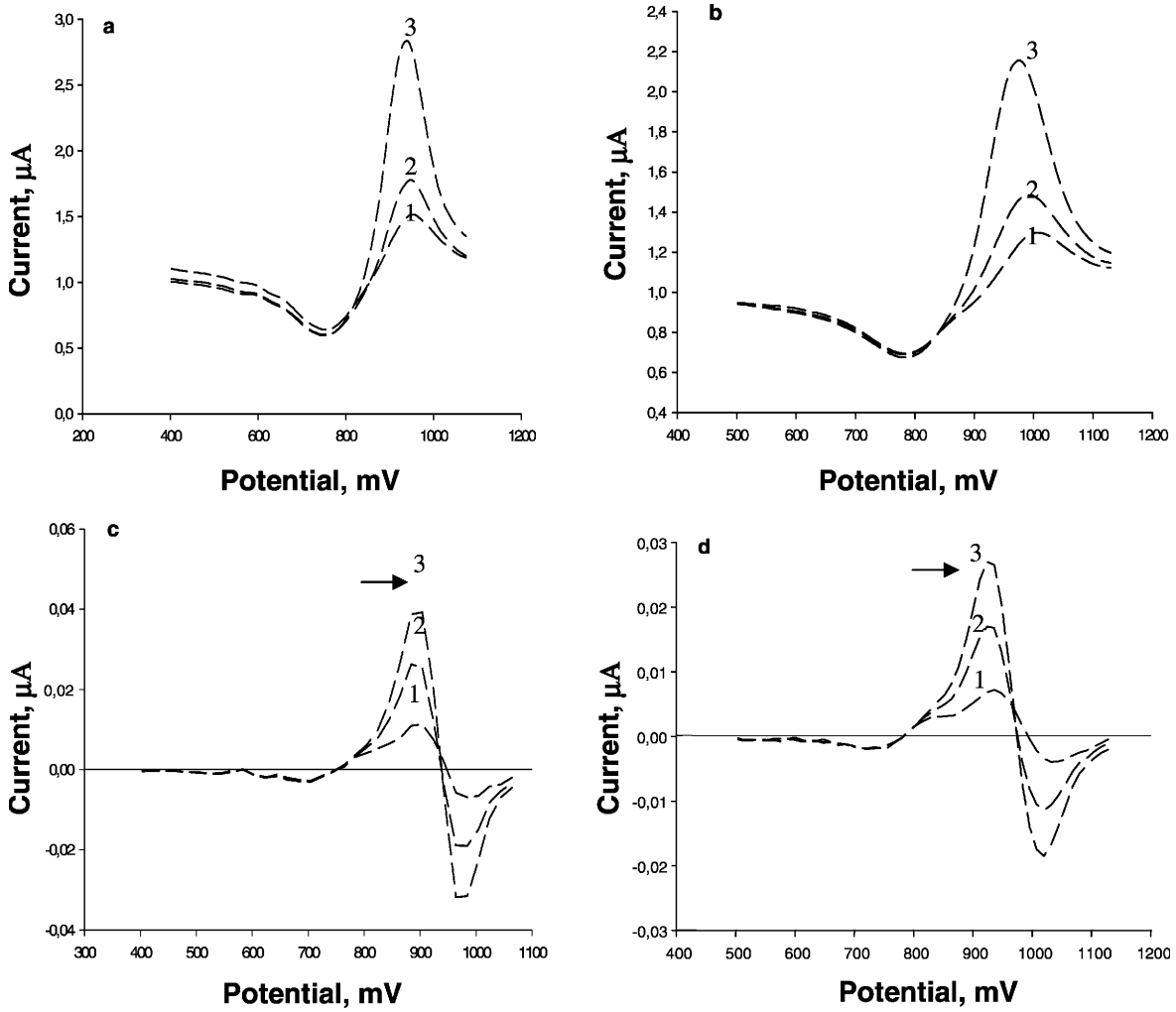


Fig. 6. (a;b) Ratio-voltammograms and (c;d) first derivative of the ratio-voltammograms of ATOR of (1) 2×10^{-5} M; (2) 6×10^{-5} M and (3) 1×10^{-4} M using a 2×10^{-5} M AML as divisor. The arrows indicate the working potential. [a, c show DPV results and b, d show SWV results].

Table 1. Statistical data for the calibration graphs of AML and ATOR by the first derivative of the ratio DPV and SWV techniques. Each value is obtained from five experiments.

	AML		ATOR	
	DPV	OSW	DPV	OSW
Measured potential (V)	0.84	0.86	0.90	0.94
Linearity range (M)	4×10^{-6}–1×10^{-4}	4×10^{-6}–1×10^{-4}	2×10^{-6}–1×10^{-4}	2×10^{-6}–1×10^{-4}
Slope	−225.64	−152.39	382.24	250.56
Intercept	-2.2×10^{-3}	-0.8×10^{-3}	3.9×10^{-3}	2.0×10^{-3}
Correlation coefficient	0.998	0.998	0.999	0.999
SE of slope	6.04	2.22	7.10	4.06
SE of intercept	3.00×10^{-4}	1.10×10^{-4}	3.35×10^{-4}	1.91×10^{-4}
LOD (M)	8.01×10^{-7} (±0.22)	8.53×10^{-7} (±0.13)	5.95×10^{-7} (±0.11)	4.70×10^{-7} (±0.08)
LOQ (M)	2.67×10^{-6}	2.84×10^{-6}	1.98×10^{-6}	1.57×10^{-6}
Repeatability of peak current (*RSD* %)*	1.30	1.74	1.21	1.00
Reproducibility of peak current (*RSD*%)*	1.90	1.84	1.80	2.41

The low values of SE of slope and intercept are greater than 0.999 correlation coefficient in the selected supporting electrolyte confirmed that the precision of the proposed methods. Repeatability and reproducibility results were characterized by *RSD* % and by the difference between theoretical and measured concentrations. There was no significant difference for the assay, which was tested within day (repeatability) and between days (reproducibility).

In order to demonstrate the validity and applicability of the proposed methods, recovery studies were performed by analyzing in synthetic mixtures of AML and ATOR, which reproduced different composition ratios. Satisfactory results were found for different compositions of synthetic mixtures, between 2×10^{-5} and 1×10^{-4} M. Each concentration was analyzed three times under the same conditions. The mean recovery and their *RSD*% values were as 98.35 and 2.22% for AML and 100.26 and 0.83%, for ATOR, respectively, according to DPV technique. Using SWV technique, the mean recoveries and their *RSD*% values were obtained as 99.14 and 2.16% for AML and 100.20 and 0.37% for ATOR between the same concentration ranges, respectively. The selected potentials for DPV and SWV methods were used for the successful determination of AML and ATOR in their synthetic mixtures and capsule dosage forms.

3.3 Analysis of AML and ATOR in Pharmaceutical Dosage Form

When working on synthetic mixture, results encourage the use of the proposed methods described for the simultaneous determination of AML and ATOR in commercial tablet dosage forms. The proposed first derivative of the ratio DPV and SWV methods could be used for the simultaneous determination of AML and ATOR in the presence of each other and without prior separation of the excipients. Each film-coated Caduet tablet contains 10 mg AML and 10 mg ATOR and the inactive ingredients calcium carbonate, colloidal silicon dioxide (anhydrous), croscarmellose sodium, hydroxypropyl cellulose, magnesium stearate, microcrystalline cellulose, opadry II white 85F28751 or opadry II blue 85F10919, polysorbate 80, pregelatinized starch, and purified water [1, 3]. The adequacy of the developed methods was evaluated by quantifying AML and ATOR in commercial pharmaceutical dosage form. Pretreatment was not required for samples nor time-consuming extraction or evaporation steps prior to the analysis. The samples were used after adequate dilutions. The utility of all of the proposed methods was verified by means of replicate estimations of pharmaceutical preparations and results obtained were evaluated by statistically. Table 2, shows the results obtained in the analysis of tablets for proposed first derivative of the ratio voltammetric methods. Results obtained from proposed methods of the analysis of both drugs in tablets indicate that the proposed techniques can be used for simultaneous quantitation and routine quality control analysis of this binary mixture in pharmaceuticals.

A comparison with an official reference determination method has not been possible in any pharmacopoeias, because so far no other procedure for the quantitation of AML and ATOR from pharmaceutical formulations has been reported. For this reason, proposed methods were compared with the literature method which is related with the first derivative spectrophotometric method [8]. Table 2 compares the results of the analysis of AML and ATOR between proposed and literature methods. The amounts of AML and ATOR are fairly close to the labeled amounts for all techniques. However, the proposed method is sensitive, selective and more precise than the first derivative spectrophotometric assay. The results obtained from the two methods were statistically compared with each other at the 95% confidence level with the aid of *t*- and *F*-tests. The *F*- and student *t*-tests were carried out on the data and statistically examined the validity of the obtained results by first derivative spectrophotometric and first derivative of the ratio methods. According to the student's t and variance ratio *F*-test, the calculated t and F values were less than the theoretical values in either test at the 95% confidence level. This indicates that there are no significant differences between the performances of the proposed and spectrophotometric methods with regards to accuracy and precision (Table 2).

Table 2. Results of the assay and the recovery analysis of AML and ATOR in tablet dosage forms using by the first derivative of the ratio DPV and SWV techniques.

	AML			ATOR		
	DPV	OSW	Spectrophotometric method [8]	DPV	OSW	Spectrophotometric method [8]
Labeled claim (mg per tablets)	10.00	10.00	10.00	10.00	10.00	10.00
Amount found (mg) [a]	10.13	10.08	10.14	10.14	10.02	10.19
RSD %	1.87	2.30	2.22	2.19	0.95	2.51
Bias %	−1.30	−0.8	−1.40	−1.40	−0.20	−1.90
Calculated *t*	0.92	0.69	t_{th}: 2.31	0.75	0.19	t_{th}: 2.31
Calculated *F*	0.75	0.96	F_{th}: 2.60	0.79	0.08	F_{th}: 2.60
Added (mg)	3.00	3.00	–	3.00	3.00	–
Found [a]	3.03	3.01	–	3.01	3.02	–
Recovery %	100.92	100.46	–	100.28	100.67	–
Bias %	−0.92	−0.46	–	−0.28	−0.67	–
RSD % of Recovery	1.69	1.79	–	2.86	1.31	–

[a] Mean value of the five determinations.
th: theoretical.

The accuracy of the proposed methods was determined by its recovery during standard addition experiments. The recovery of the procedure was carried out by spiking the already analyzed samples of tablets with the known amounts of standard solutions of AML and ATOR. The results of the recovery analysis for the proposed techniques are tabulated in Table 2. It is concluded that the proposed methods are sufficiently accurate and precise in order to be applied to pharmaceutical dosage forms. High percentage recovery data shows also that the proposed methods are free from the interferences of the excipients used in the formulations.

4. Conclusions

The electrochemical behavior of AML and ATOR on glassy carbon electrode was established and studied as alone and in the presence of each other. AML and ATOR are irreversibly oxidized at high positive potentials in different supporting electrolytes.

The proposed first derivative of ratio DP and SW voltammetric techniques can be used for simultaneous determination of AML and ATOR in film-coated tablet dosage forms. The results obtained show the above described methods are useful not only for simultaneous determination of AML and ATOR in conventional electrolytes, but also in more complex matrices such as dosage forms. The principal advantages of first derivative of ratio DP and SW voltammetric techniques over the other techniques are that they may be applied directly to the analysis of pharmaceutical dosage forms without the need for separation or complex sample preparation, since there was no interference from the excipients. The proposed first derivative of ratio DP and SW voltammetric methods enable the quantitation or mixtures of AML and ATOR with good accuracy and precision, either in laboratory made samples or in pharmaceutical dosage forms.

The advantages of the proposed first derivative of the ratio voltammetric methods over the published methods, is the possibility of performing measurements in correspondence of peaks, hence a potentially greater sensitivity and accuracy. Other advantages of the proposed assay are easy measurement on the separate peaks, higher values of analytical signals and no need to work only at zero-crossing point in comparison with the derivative methods.

The proposed methods are suitable for quality control laboratories, where economy and time are essential. High percentage recovery shows that the methods are free from the interferences of the commonly used excipients and additives in the formulations of drugs.

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