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Electrochemical oxidation of azithromycin and its derivatives

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Abstract

The electrochemical oxidation of azithromycin was investigated in order to elucidate the mechanism for possible oxidative metabolic pathways in humans. Electrochemical studies were carried out by cyclic voltammetry and preparative scale electrolysis at glassy carbon electrodes. It was found that azithromycin undergoes anodic oxidation at one or both amine groups with the rapid follow-up chemistry of intermediate radical cation. Main products of the oxidation were determined by HPLC analysis and were identified as a protonated azithromycin and products obtained by demethylation of the 3′-dimethylamino or macrolactone amino group.

Keywords: Azithromycin; Erythromycin; Anodic oxidation; Cyclic voltammetry; Electrochemical studies

1. Introduction

Azithromycin (**1**) (*N*-methyl-9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A, Sumamed, Zithromax) is a member of the family of 15-membered macrolide antibiotics called azalides. It is obtained by chemical modification of the naturally occurring 14-membered macrolide erythromycin (**2**) from which it differs by the insertion of a methyl-substituted nitrogen into position 9a of the large macrolactone ring [1,2]. This modification chemically distinguishes azalides from macrolides, which have only carbon- and oxygen-containing rings. The insertion of the nitrogen into the ring significantly alters the chemical, microbiologic and pharmacokinetic properties of these drugs. Such modification provides increased acid stability to azalides improving their resistance to the gastric environment [3]. In addition, the formation of the anhydrohemiketal derivative associated with gastrointestinal toxicity observed with erythromycin is prevented [3,4].

The mechanism of action of azithromycin is similar to other macrolides; it binds to the 50S subunit of bacterial ribosomes, resulting in inhibition of natural messenger RNA-directed polypeptide synthesis [5,6]. However, compared with other macrolide antibiotics, azithromycin has enhanced antimicrobial activity, a more favorable adverse reaction profile, and improved pharmacokinetic properties. The in vitro profile of azithromycin is characterized by a broad spectrum of activity against Gram-positive and Gram-negative bacteria [7,8].

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Azithromycin is eliminated slowly from the body, the primary route of excretion being via the bile. More than 50% of azithromycin related material excreted in bile is unchanged azithromycin. The other metabolities are *N*-demethylated derivatives of azithromycin, together with the products of O-demethylation, deconjugation of the cladinose sugar and hydroxylation of the desosamine and/or aglycone rings providing the remainder.

This class of macrolides consists of a large macrolactone ring with the two sugar moieties attached to it: cladinose at the C-3 position and desosamine at the C-5 position (Fig. 1). Almost all macrolides posses a tertiary amine group at the desosamine sugar. In addition, azalides have another amine group as a part of the macrolactone ring. Due to their pK_a values ($pK_{a1} = 8.6$ and $pK_{a2} = 9.5$) azithromycin at physiological pH values is mostly in the protonated form. Both amine groups are easily oxidized and these oxidations form a basis for the analytical determination of macrolides [9,10]. However, although the electrochemical detection of macrolides is the routine analytical method, the mechanisms of oxidation and oxidation products are still unknown.

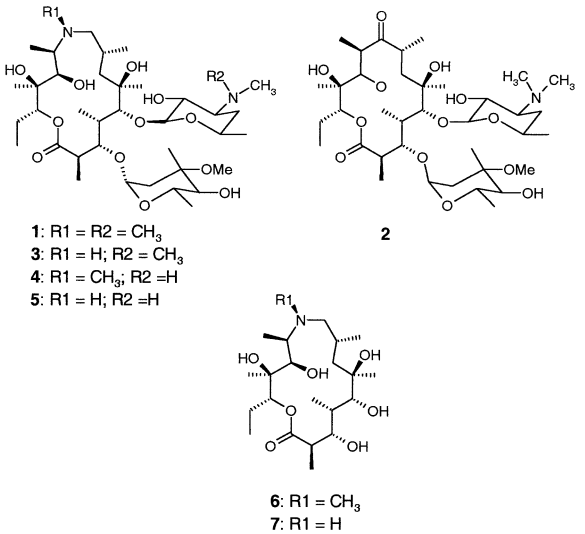


Fig. 1. Chemical structures of azithromycin, erythromycin and derivatives of azithromycin investigated in this work.

Anodic oxidation of amines in aqueous and non-aqueous solutions has been extensively studied [11–13]. Tertiary amines were found to undergo dealkylation to the corresponding secondary amines and aldehydes but in many cases the large amount of original amines remained as protonated amine salt.

The aim of this work was to investigate the mechanism of the electrochemical oxidation of azithromycin and its derivatives and to identify the products obtained. Elucidation of the mechanism of the electrochemical oxidation would provide a deeper insight into the chemistry and electrochemistry of azalides. What is more, the identification of the obtained products would enable their comparison with the azithromycin metabolites eliminated from the body.

2. Materials and methods

Azithromycin (**1**) was obtained in-house as dihydrate, 9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A (**3**), 3′-*N*-demetyl-*N*-methyl-9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A (**4**), 3′-*N*-demetyl-9a-aza-9-deoxo-9-dihydro-9a-homoerythromycin A (**5**), *N*-methyl-9a-aza-9-deoxo-9-dihydro-9a-homoerythronolide (**6**), 9a-aza-9-deoxo-9-dihydro-9a-homoerythronolide (**7**) were synthesized in-house from azithromycin as starting compound according to the procedures described previously [1] and with purity higher then 95% as determined by HPLC analysis. Erythromycin (**2**) was purchased from Aldrich.

2.1. Cyclic voltammetry

Cyclic voltammetry experiments have been carried out at a glassy carbon electrode ($A = 3.1\ mm^2$) with the EG&G Princeton Applied Research (PAR) potentiostat, Model 283. The reference electrode was Ag/AgCl electrode (PAR) and Pt wire served as a counter electrode.

The concentration of macrolides varied between 1 and 5 mM in methanol solution of 0.1 M $LiClO_4$ supporting electrolyte or in aqueous solution of phosphoric buffer (0.1 M) of different pH values. The working electrode potential was scanned

between 0 and 1 V versus Ag/AgCl with scan rates between 0.01 and 10 V/s.

The working electrode was polished intensively with aluminium oxide on a polishing cloth and ultrasonically degreased in methanol prior to each electrochemical measurement.

2.2. Constant potential electrolysis

Constant potential electrolyses have been carried out in the solution of 0.1 M $LiClO_4$ in methanol at the large glassy carbon electrode ($A = 6$ cm^2). The concentration of macrolides was 1 mg/ml in a conventional 20 ml, two-compartment H-type electrolysis vessel. The electrolyses were carried out at the potential of the corresponding current peak as determined from cyclic voltammetry experiments. The electrolysis was performed until completion i.e. until registered current dropped to background level. The current versus time was registered and the charge necessary to drive the reaction to completion was calculated by the integration of I–t curve.

2.3. HPLC analysis

HPLC analysis was performed with a Hewlett-Packard liquid chromatograph series 1100. A reversed-phase Chromegabond γRP-1 alumina analytical column, 150×4.6 mm I.D., 5 μm particle size, (ES Industries, Marlton, NY, USA) with two guard column (50×4.6 mm I.D., 5 μm particle size, ES Industries) provided the separation.

The mixture of phosphate buffer/acetonitrile (80:20, v/v) was used as a mobile phase. The final pH was adjusted to 10.6 with 1.0 M KOH. The flow rate was 1.0 ml/min. The samples were automatically injected with a HP1100 autosampler.

The detection of the column effluent was performed with Bioanalytical Systems (BAS, West Lafayette, IN, USA) LC-4C amperometric detector equipped with glassy carbon electrode, set at the oxidation potential of 0.82 V relative to Ag/AgCl reference electrode. The resulting current was monitored as an amplifier output in mV s.

The interface and HPLC system were controlled by PC and HP CHEMSTATION software.

3. Results and discussion

3.1. Cyclic voltammetry

The comparison of the cyclic voltammograms of di-amine macrolides **1**, **3** and **4** in methanolic solution is given in Fig. 2a, while the cyclic voltammograms for mono-amine macrolides **2**, **6** and **7** are shown in Fig. 2b. All macrolides investigated show one irreversible oxidative current peak. The irreversibility of the electrode process indicates that the electrode reaction is accompanied by a fast follow-up homogeneous chemical reaction giving a product, which is not electroactive under the experimental conditions studied. The homogeneous reaction is so fast that even at the highest scan rates employed ($\nu =$ 10 V/s) there was no sign of a reductive wave in the reverse scan.

Current peak heights were not linear with either the scan rate or with the square root of the scan rate indicating mixed diffusion/adsorption control of the electrooxidation. Since current peaks for di-amine compounds (Fig. 2a) are approximately twice as high as current peaks for mono-amine macrolides (Fig. 2b), it is obvious that current waves from Fig. 2 represent amine oxidation processes and that current peaks at Fig. 2a is a composite of two oxidation processes, macrolactone amino and desosaminil amino group oxidation. No separate peaks for the oxidation of these two amino groups have been observed, thus the oxidation potentials of both amine groups lay within 0.030 V from each other.

Another current peak appears on the voltammograms for almost all macrolides investigated at the potentials more positive than the main current peak. In some cases (e.g. for **1**, **2** and **3**) the third peak can be distinguished at even more positive potentials. The ratio of the current post-peaks to the main current peak is independent of the scan rate in the range of 5 mV/s–10 V/s. This might be explained by an reaction scheme in which a fast homogenous chemical reaction is interposed be-

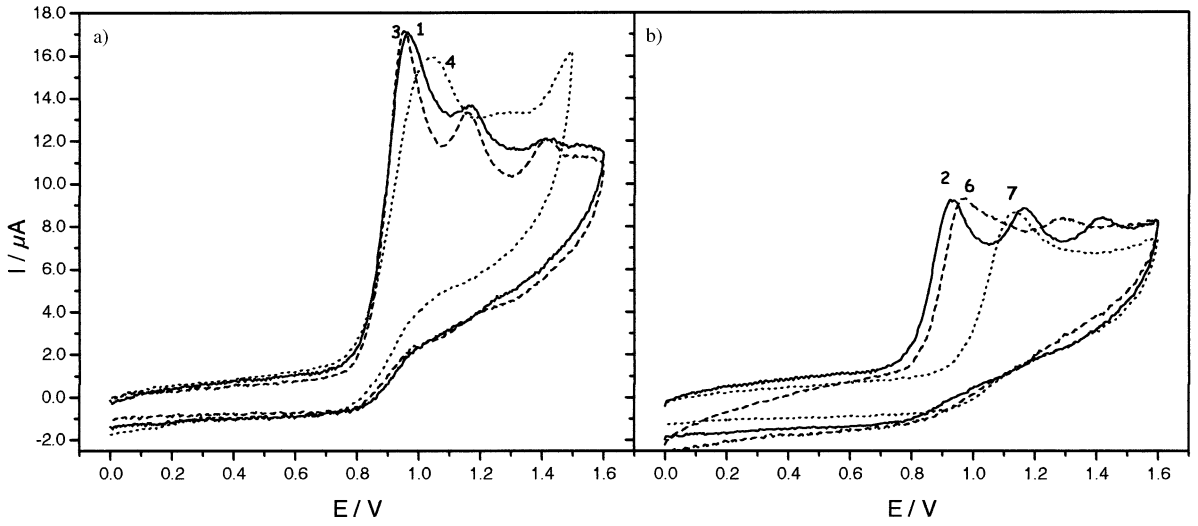


Fig. 2. Cyclic voltammograms of (a) di-amine macrolides **1**, **3** and **4**, (b) mono-amine macrolides **2**, **6** and **7** at GC electrode in 0.1 M $LiClO_4$/methanol solution, $\nu = 100$ mV/s, $c = 2$ mM.

tween two electron transfer steps (ECE mechanism). In that case, the second and third current peaks represent the electrochemical oxidation of the products obtained by homogenous chemical reaction following the first charge transfer. The interposed homogenous chemical reaction was so fast that it could not be detected in the time scale of the cyclic voltammetry experiments.

The peak potentials of compounds **4** and **7** are shifted to the more positive potentials in comparison to the oxidation potentials of corresponding tertiary amines. This behavior is expected for secondary amines due to the stronger electron-withdrawing effect of hydrogen atom than that of methyl group. Furthermore, the peak potential of **7** coincides with the potentials of the second current peak of **6**. This suggests that electrooxidation of **6** might result in **7** and that the fast homogenous follow-up reaction of anodic oxidation process is in fact demethylation reaction yielding corresponding secondary amine.

The cyclic voltammograms of investigated macrolides in aqueous solutions show one irreversible current peak. The potential of current peak and current peak height are pH dependent (Fig. 3). The peak potential shifts toward more positive potentials with a decrease of pH indicating the participation of proton in the oxidation reaction.

At the same time, as the pH of the solution approaches the pK_a of azithromycin, the current peak height starts to decrease with a decrease of pH, completely disappearing at $pH < 5$. It is obvious that investigated macrolides are electroactive only in their non-protonated forms and that the first step of electrooxidation is the removal of an electron from lone-pair electrons of the amino nitrogens.

3.2. Constant potential electrolysis

Constant potential electrolysis was carried out by applying a potential pulse from the potential where no reaction occurs to the potential of the corresponding current peak. The solution was electrolyzed until the completion of reaction and current as a function of time was registered.

When electrolysis was carried out in methanolic solutions, the resulting current exponentially decayed to the background level. The number of electrons consumed in the oxidation process was calculated from the current–time curves (Table 1).

However, when electrolysis was carried out in aqueous medium at $pH > pK_a$ of investigated macrolides, the current did not exponentially decay but rather remained constant even on prolonged electrolysis.

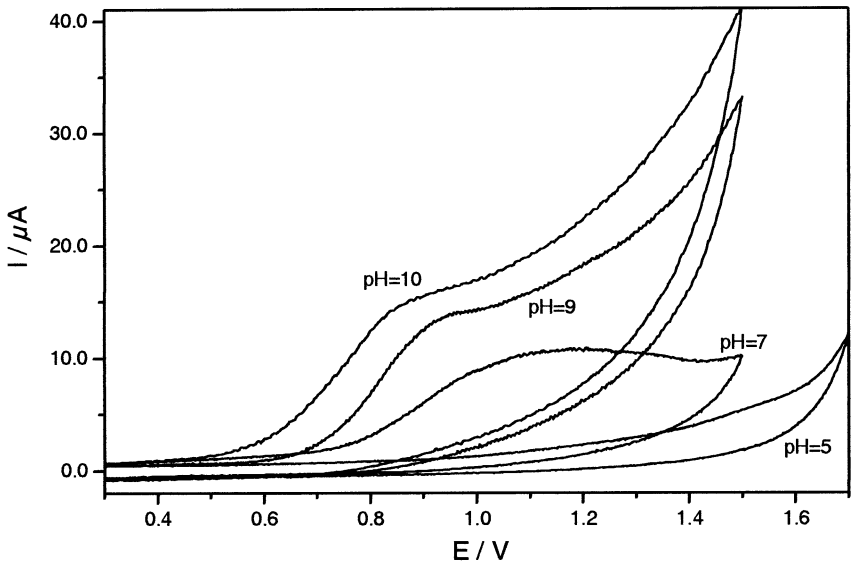


Fig. 3. Cyclic voltammograms of azithromycin at GC electrode in Britton–Robinson buffer, ν = 100 mV/s, c = 2 mM.

Table 1
Number of electrons, z, obtained from controlled potential coulometry for the macrolides investigated

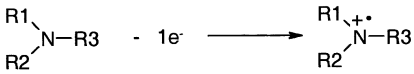
Macrolide	z
1	2.5±0.1
2	2.1±0.1
3	2.0±0.1
4	2.1±0.1
5	1.9±0.1
6	1.2±0.1
7	1.0±0.1

The course of the electrochemical oxidations was followed by cyclic voltammetry of electrolyzed solutions while the solutions at the end of electrolysis were analyzed by HPLC electrochemical detection. The end of electrolysis was characterized by complete disappearance of the anodic current wave in the registered cyclic voltammograms. However, one or more new products together with the starting macrolide were detected in the HPLC analysis (Fig. 4). The presence of a substantial quantity of the starting compound at the end of electrolysis indicates that its recovery took place during the HPLC determination.

In the case of azithromycin electrooxidation approximately 70% of the starting compound was recovered after the end of electrolysis (Table 2). Three other chromatographic peaks, B, C and D, assigned to the compounds **3**, **4** and **5**, respectively, were also detected (Fig. 4). Table 2 summarizes the products and the yields obtained from the control potential electrolysis in methanolic solutions for all macrolides investigated.

3.3. Mechanism of electro-oxidation

From the results obtained it was found that azithromycin and its derivatives undergo oxidative degradation and that the first step in the oxidation process is the removal of the electron from one of the nitrogen atoms to form aminium cation radical:



(1)

where R3 = H or CH_3.

The fact that the investigated macrolides are not oxidizable in acidic solutions, the dependence of current wave height on the number of amine groups in the molecule, together with the observed

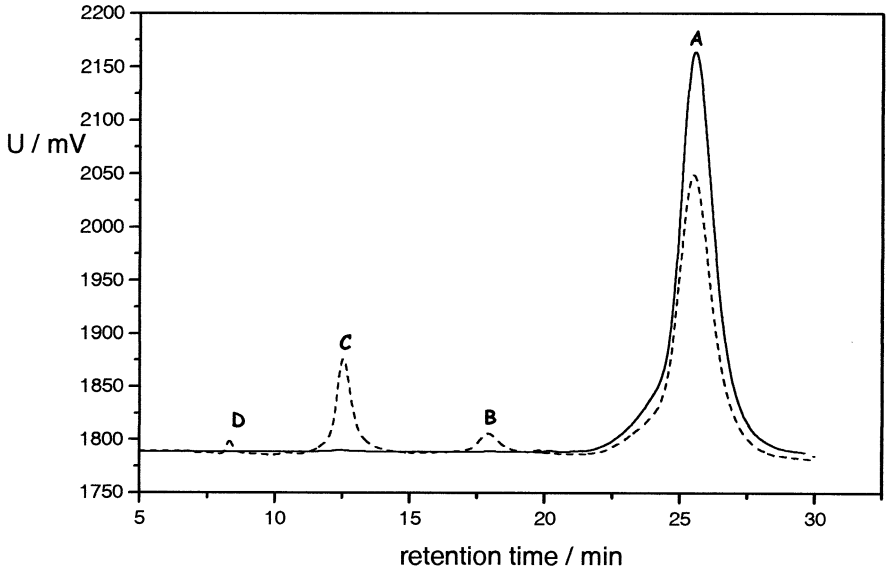


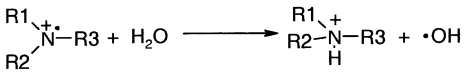
Fig. 4. Electrochemical detection chromatograms of azithromycin before (——) and after (---------) exhaustive electrolysis at GC electrode.

Table 2
Yields of products obtained by HPLC analysis and expressed as area% from chromatograms

Macrolide	Recovered (%)	B (%)	C (%)	D (%)
1	67.5	8.4	18.1	3.0
2	75.1	–	–	–
3	80.4	–	–	15.2
4	91.1	–	–	7.3
5	98.4	–	–	–
6	89.3	–	–	–
7	99.0	–	–	–

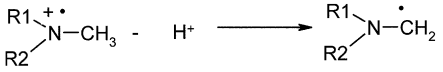
anodic shift of the oxidation potentials of **4** and **7** support the above consideration.

The aminium radical cation of azithromycin is a very reactive species and rapidly reacts with the environment to form stable products. Controlled-potential electrolysis in non-aqueous media revealed that there are two pathways for the follow-up chemistry of the aminium radical cation of azithromycin. First, the radical cation abstracts hydrogen atom from the solvent or residual water resulting in azithromycin salt formation (reaction 2) in the overall one-electron process. Bearing a positive charge, the protonated molecule of azithromycin inhibits further electrochemical oxidation.

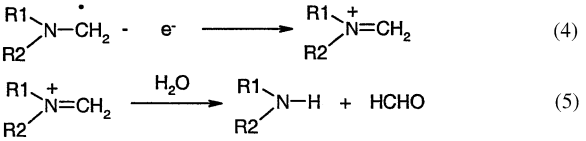


(2)

In the case of **1**, **3** and **4**, the HPLC analysis of the reaction mixture (Table 2) indicates that one or both amine groups undergo a demethylation reaction in parallel to reaction 2 resulting in the corresponding secondary amines. The demethylation reaction proceeds via an overall two-electron ECE reaction scheme (reaction 3–5). It is believed that the rate determining steps in this mechanism are the removal of the α-proton and the formation of an enamine as an intermediate (reaction 3–4) [14]. According to the proposed mechanism, the liberation of the methyl group would be mainly governed by the acidity of α-protons and their statistical abundance.



(3)



(4)

(5)

The distribution of the products in the anodic oxidation of azithromycin and its derivatives depends on the relative rates of the two pathways. From the results presented in Table 2 it is obvious that the main products of the anodic oxidation of the investigated macrolides are their protonated salts and that hydrogen atom abstraction is the primary route of the reaction of the aminium radical cation intermediate. In the case of **1**, **3**, **4** and **5** both amine groups are simultaneously oxidized leading to the corresponding double protonated salts. Competitive demethylation reactions take place on both amine groups as well. However, the removal of the methyl group from desosamine sugar seems to be the favored process in the case of the investigated macrolides. This is in agreement with the hypothesis that the acidity and number of α-protons influence the demethylation reaction rate, since the order of acidity of α-protons based on the inductive effect decreases with an increase in the attached alkyl chain length. Furthermore, the two methyl groups attached to desosamine amine group make the liberation of desosamine methyl group more likely than the liberation of the methyl group from the macrolactone.

The high yields of the recovered secondary amines **4**, **5**, and **7** after the electrolysis indicate that the oxidation of secondary amines is a one-electron process leading to the corresponding salt formation. The number of electrons consumed in oxidation reaction (Table 1) are in agreement with the product yields (Table 2) confirming the proposed mechanism.

The above mechanism explains why the current remains constant at constant potential electrolysis of azithromycin and its derivatives in aqueous media at $pH > pK_a$ of amine groups. Hydrogen abstraction reactions also take place in aqueous media but, at high pH, the macrolide salt is automatically deprotonated regenerating the starting compound, which is available again for oxidation. The net reaction in this case is indirect electrolysis of water and the current in the preparative scale electrolysis remains more or less constant throughout the electrolysis.

4. Conclusions

Cyclic voltammetry and preparative scale electrolysis show that azithromycin and its derivatives undergo oxidative degradation. The anodic oxidation is an irreversible process and the first step is considered to be the removal of electron from one or both amino groups resulting in the intermediate aminum radical cation. The main products of rapid follow-up chemistry of the radical cation were identified as the corresponding protonated compounds together with the various ratio of products obtained by demethylation of 3′-dimethylamino or macrolactone amino group.

In human beings, more than 50% of azithromycin is excreted unchanged via bile while metabolites comprise mostly demethylated derivatives. Further studies are needed to investigate whether the possible metabolic pathway of azithromycin in humans includes the oxidative degradation steps described above.

Acknowledgements

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