

Electrochemical reduction of 2,5-dimethoxy nitrobenzenes: nitro radical anion generation and biological activity

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Abstract

The electrochemical reduction of four 2,5-dimethoxy nitrobenzene 6-substituted derivatives in aqueous and mixed media by cyclic voltammetry, fast and differential pulse polarography were studied. In aqueous media, pH > 8, it was possible to observe a polarographic peak or wave due to the nitro radical anion formation. This fact is not very common in nitro aromatic compounds where the observation of the nitro radical anion formation peak (or wave) requires the presence of inhibitors or aprotic solvent, besides alkaline pH. In mixed media, all the studied compounds show a well-defined reversible couple, due to the one electron reduction of the nitro group to produce the corresponding nitro radical anion. Cyclic voltammetric studies show that the nitro radical anion generated was relatively stable, although this intermediate specie shows a tendency to undergo further chemical reactions. From these experiments, the kinetic behaviour of the nitro radical anions electrochemically generated was characterized and permitted us to calculate the rate constant k_2 of the second order chemical reaction of the radical anions and their half-life. The electrochemical behaviour is correlated with the in vivo studies of oxygen consumption on *Trypanosoma cruzi* cell suspensions.

Keywords: Cyclic voltammetry; Nitrobenzenes; Nitro radical anion; Oxygen consumption

1. Introduction

The incorporation of a nitro group in organic molecules of therapeutic use is mainly a consequence of its easiness to be reduced in presence of different enzymatic systems (flavine-dependent reductases, cytochrome P450) [1–3]. Thus, in nitroheterocyclic compounds, one of the most extensive families used in medicine, the nitro group reduction constitutes the base for its clinic application. The nitroimidazole drugs are selectively toxic on anaerobic bacteria and protozoans, due to its therapeutic activity is based on the nitro group reduction, accepting electrons at level of the pyruvate metabolism, at potentials that are unable to be generated in aerobic cells. Moreover, nitroimidazole drugs are excellent sensitizer in hypoxic cells of mammalians, adding a cytotoxic effect. These precedents affirm that the mechanism of cytotoxicity in bacteria, protozoans and hypoxic mammalian cells exhibits a common route. It has been observed that under anaerobic conditions or low oxygen tension (hypoxia), the reduction

process involves initially the transfer of 1-electron (nitro radical anion generation) and the subsequent nitroso and hydroxylamine derivative formation, involving 2- and 4-electron reductions, respectively [4–6]. These facts are a good example that illustrates the importance of the nitro group reduction to achieve therapeutic effects.

Actually, it is widely accepted that most of the biological properties of the nitroaromatic compounds depend on the ease with which the nitro group suffers nitroreductive processes in the cell [4,7]. In this sense, the thermodynamic parameters that characterize the ease of reduction are very important to define the type of biological properties of the different nitrocompounds [5]. Such as it has been previously demonstrated by several authors [8–10], the toxic effects exerted by some nitrodrugs on tumoral cells and in microorganisms are highly dependent on the one electron reduction product formation ($R-NO_2^-$). Therefore, the knowledge of the redox characteristics of such species to anticipate cytotoxic effects in biological systems becomes relevant. In this line, Olive [11] found in a series of nitroheterocyclic compounds a direct correlation between the half-wave polarographic potentials and the

nitroreduction rate in different biological systems. As the first step in the reduction process is defined by the reaction $R\text{-NO}_2 + e^- \rightleftharpoons R\text{-NO}_2^-$ involving the couple $R\text{-NO}_2/R\text{-NO}_2^-$, consequently, the reduction potential of this couple would be an appropriate parameter to define the reduction properties of these compounds. Furthermore, correlations between electroaffinities and reduction potentials, obtained by electrochemical techniques or by pulse radiolysis, for both nitroheterocyclic [11–13] and nitroaromatic compounds [14,15] have been reported by several authors.

Chagas's disease is a real and important medical, economic and social problem in Latin America [16]. Trypanosomes, the disease causal agents, are substantially more sensitive to oxidative stress than their biological host. This is consistent with the fact that the most effective drugs up to now, are chemicals that easily produce redox cycling. The knowledge of electrochemical properties is essential for a better understanding of the biological activity and properties of free radicals emerging during biological conversions.

Specifically, in the scope of our investigations tending to find new therapeutic alternatives for the Chagas's disease [17], we have synthesized four 2,5-dimethoxy nitrobenzene derivatives (Fig. 1), and studies about their electrochemical characteristics that permit one to describe their redox path and mainly the feasibility of obtaining stable nitro radical anions have been conducted. The present work is part of a chemical–biological study from substituted nitroaromatic derivatives with the aim to determine the feasibility of producing nitro radical anions. Consequently, if nitro radical anions are produced, they would be aggressive for some cellular systems.

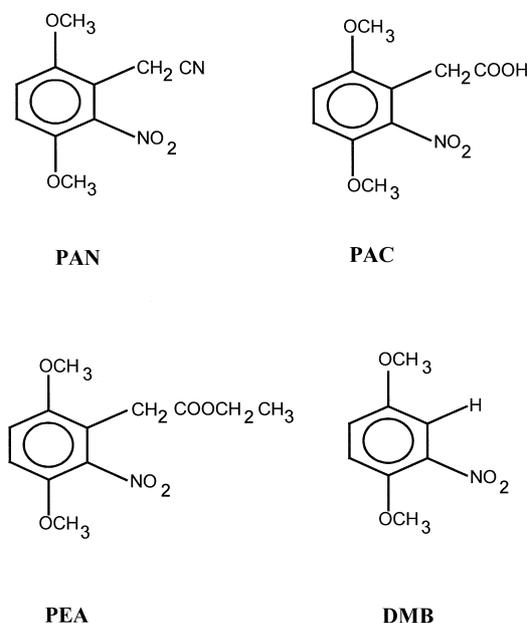


Fig. 1. Chemical structures of the 2,5-dimethoxy nitrobenzene derivatives.

Despite the fact that the electrochemistry of nitroaromatics has been extensively studied [18], no electrochemical studies have been reported for the above synthesized compounds. Furthermore, although nitroaromatics follow a well known generally similar reduction pattern [19], both easiness of formation and the stability of the nitro radical anion are strongly dependent on the molecular structure.

2. Experimental

2.1. Materials and methods

2.1.1. Compounds

All the nitrocompounds were synthesized in our laboratory [20], starting from 2,5-dimethoxy benzaldehyde by following a published procedure [21].

PEA (2,5-dimethoxy nitrobenzene-6-methylenecarboxylic acid ethyl ester). IR (KBr) (cm^{-1}): 3030 (C–H), 1740 (C=O), 1530 (NO_2), 1360 (NO_2). RMN-¹H (CDCl_3) (300 MHz): 1.24 (t, 3H, $J = 8$ Hz, CH_3); 3.63 (s, 2H, Ar- CH_2); 3.80 (s, 6H, OCH_3); 4.16 (q, 2H, $J = 8$ Hz, CH_2); 6.84 (s, 2H, Ar).

PAC (2,5-dimethoxy nitrobenzene-6-methylenecarboxylic acid). IR (KBr) (cm^{-1}): 3600–3200 (COOH), 1700 (C=O), 1520 (NO_2), 1360 (NO_2). RMN-¹H (acetone- d_6) (300 MHz): 3.58 (s, 2H, CH_2); 3.86 (s, 3H, OCH_3); 3.87 (s, 3H, OCH_3); 7.18 (s, 2H, Ar). m.p.: 210–211.5°C.

PAN (2,5-dimethoxy-6-cyanomethyl nitrobenzene). IR (KBr) (cm^{-1}): 2980 (C–H aliph.), 2020 (C \equiv N), 1520 (NO_2), 1360 (NO_2). RMN-¹H (CDCl_3) (300 MHz): 3.66 (s, 2H, CH_2); 3.92 (s, 3H, OCH_3); 3.94 (s, 3H, OCH_3); 7.0 (s, 2H, Ar). m.p.: 148–150°C.

DMB (2,5-dimethoxy nitrobenzene). Prepared by nitration of 1,4-dimethoxybenzene with nitric acid in glacial acetic acid at 0°C. IR (KBr) (cm^{-1}): 3030 (C–H Ar), 1520 (NO_2), 1360 (NO_2), 1247 (OCH_3). RMN-¹H (CDCl_3) (300 MHz): 3.81 (s, 3H, OCH_3 , C-5); 3.92 (s, 3H, OCH_3 , C-2); 7.04 (d, 1H, $J = 9.18$ Hz, 3-H); 7.11 (d, 1H, $J = 9.18$ Hz, $J = 3.05$, 4-H); 7.39 (d, 1H, $J = 3.05$ Hz, 6-H). m.p.: 70–71°C.

All chemicals were of analytical grade and twice-distilled water was used.

2.1.2. Stocks compound solutions

Stock solutions of 1 mM of each compound in DMF (for mixed media) or ethanol (aqueous media) were prepared, to obtain complete dissolution.

2.1.3. Aqueous media

The routine solution concentration for polarography was 0.1 mM in ethanol/0.04 M Britton–Robinson buffer (20/80), adjusting the ionic strength at 0.3 M with KCl. For the C.V. experiments, a 1-mM concentration of each nitrocompound in the same medium was used.

2.1.4. Mixed media

The following optimum composition was used: 0.015 M aqueous trisodium citrate/DMF (40/60), pH 9.0, 0.1 M TBAI and 0.3 M KCl. For the studies conducted at pH 7.4 the same composition as that of the media at pH 9.0 was used. The pH measurements in mixed media were corrected according to a previously reported procedure [22].

2.1.5. Polarography

A Tacussel[®] assembly operated in d.c. and d.p.p. mode, similar to one previously described was used [23]. Operating conditions: pulse amplitude, 60 mV; potentials scan rate, 5 mV/s; drop time, 1 s; voltage range, 0 to -2000 mV; current range, 1.25 to 5 μ A; temperature, 25°C.

2.1.6. Cyclic voltammetry

Experiments were carried out in an Inelecsa[®] assembly, similar to the one previously described [24].

2.1.7. Electrodes

A Metrohm[®] h.m.d.e. with a drop surface of 1.82 mm² (C.V.) and d.m.e (d.p.p. and d.c.) as the working electrode and a platinum wire as a counter electrode were used. All potentials were measured against an SCE.

All voltammetric measurements were carried out at a constant temperature of 25°C and the solutions were purged with pure nitrogen for 10 min before each voltammetric run. In C.V. experiments, the return-to-forward peak current ratio, I_{p_a}/I_{p_c} , for the reversible electron transfer (the R-NO₂/R-NO₂⁻ couple) was measured, varying the scan rate from 0.1 V s⁻¹ up to 5.0 V s⁻¹.

The experimental I_{p_a}/I_{p_c} ratios were calculated according to Nicholson's procedure, using individual cyclic voltammograms [25]. Furthermore, E_λ was selected to reduce the influence of the second cathodic peak.

2.1.8. Growth of parasites

Trypanosoma cruzi epimastigotes Tulahuen strain were grown at 28°C in a modified Diamond's monophasic

medium [26] with 7.5 μ M hemin instead of blood and 4% fetal calf serum [27].

2.1.9. Cell respiration

Oxygen uptake measurements were carried out with a microprocessor Oximeter WTW[®] model 539 equipped with an oxygen sensor Trioximatic 300. Epimastigotes were suspended in an-80 mM NaCl 0.1 M phosphate buffer pH 7.4 to a final volume of 1.7 ml keeping the temperature constant at 28°C. All tested nitrocompounds were dissolved in dimethylsulfoxide and the DMSO final concentration never exceeded 1%. Controls were carried out using 80 mM NaCl 0.1 M phosphate buffer pH 7.4 and 1% DMSO. Under this condition, the cellular respiration of the parasites was not affected.

3. Results and discussion

3.1. Aqueous media

3.1.1. Polarography

All the tested nitrocompounds were reduced at the d.m.e. in ethanol/0.04 M Britton–Robinson buffer, 20/80 medium. Thus, well-defined waves or peaks depending of d.c. or d.p.p. mode respectively, were obtained. Although all the compounds follow a similar polarographic behaviour, there are some differences depending on the nature of the substituent involved.

The DMB compound produces two cathodic peaks in the 2–12 pH range (Fig. 2). The main peak (I) appears in all the pH range and the second one (II) appears only at acidic pHs. Both peaks are shifted to more cathodic potentials when the pH increases. The peak potential dependence (peak I) with pH is showed in Fig. 3. From this figure we can observe a linear dependence between pHs 2 and 10. A break at pH 6 is observed and beyond pH 10, the peak potential was pH-independent. The limiting current of peak I remains practically constant in all the pH range. This polarographic peak (I) corresponds to the reduction of the nitro group, to generate the corresponding

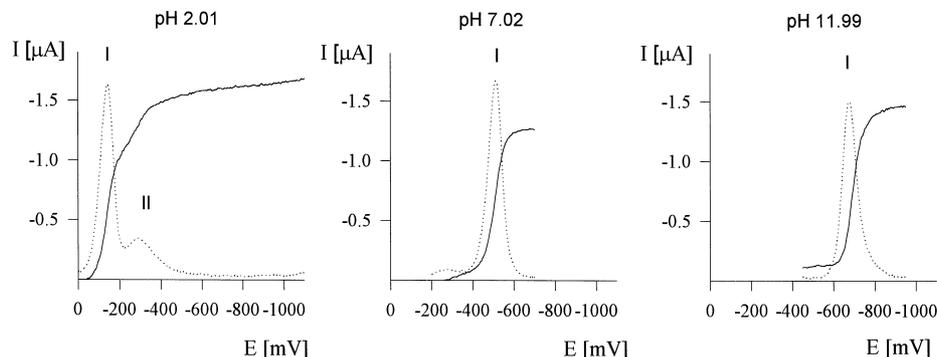


Fig. 2. d.c. and d.p.p. polarograms for DMB at different pH.

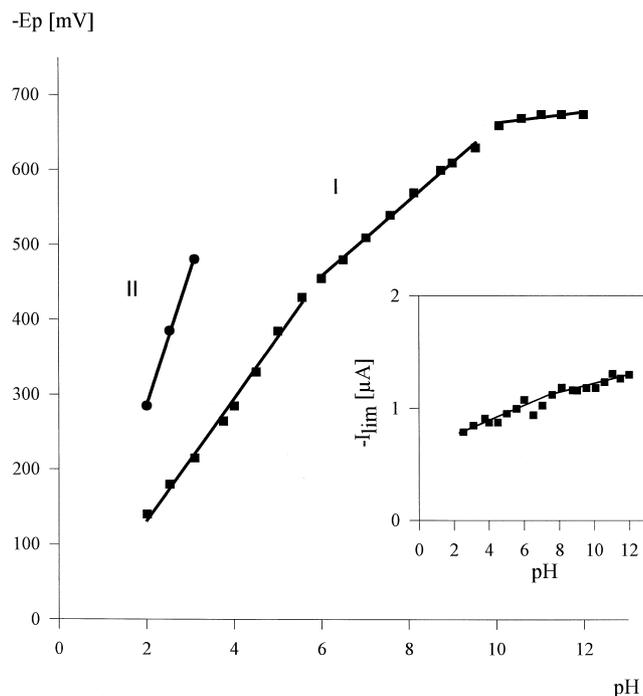


Fig. 3. Peak potential (d.p.p.) and current limit (d.c.) dependence with pH for DMB.

hydroxylamine derivative, according to the well-known general reaction:



The second observed peak (II) in the acidic range is strongly pH-dependent and can be attributed to:



The PAC compound exhibits three cathodic signals (Fig. 4). At acidic pHs, only one peak (peak I) was observed but at $\text{pH} > 8$, two peaks (peak III and peak IV), at more cathodic potentials were observed. According to the limiting current vs. pH behaviour (inset in Fig. 5), we can conclude that the appearance of peak III is related with the vanishing of peak I. Specifically, as can be seen in Fig. 6, as the pH increases, the initial wave I is split in two new

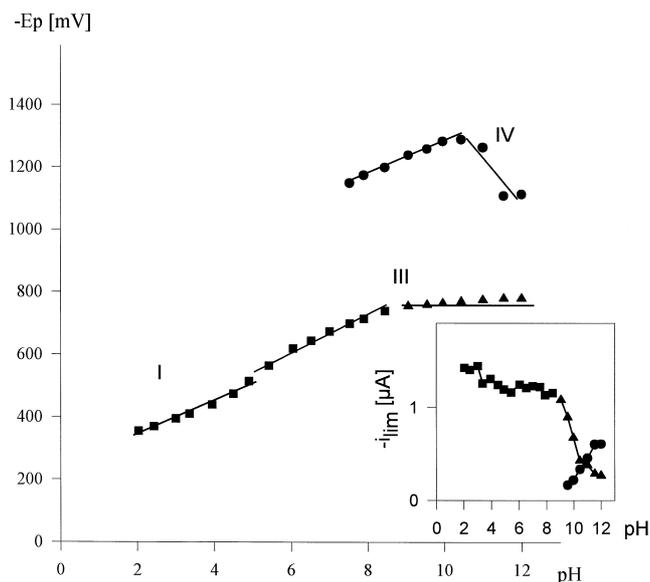


Fig. 5. Peak potential (d.p.p.) and current limit (d.c.) dependence with pH for PAC.

waves: III and IV. Furthermore, at the final stage of the splitting ($\text{pH} 12$), we can observe a 1:3 ratio for waves III and IV, respectively. This fact means that the electrons transferred in waves III and IV are one and three, respectively. The 1-electron transfer was determined by means of the logarithmic analysis of wave III, obtaining slope values about 60 mV. This splitting is a well-known matter in the nitroaromatic's behaviour [28–31] and is due to the following:



The PEA and PAN compounds show a behaviour similar to the above described, which can be summarized in the graphs of Figs. 7 and 8. Moreover, in Table 1 we report a resume of the polarographic pH-behaviour of all the studied compounds. From these results it can be concluded that for all the substituted compounds (PAN, PAC, PEA), it was possible to observe at $\text{pH} > 8$, a polarographic peak or

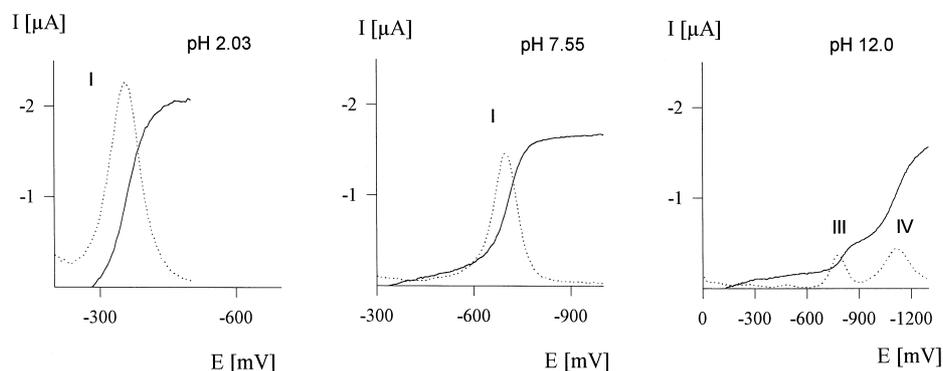


Fig. 4. d.c. and d.p.p. polarograms for PAC at different pH.

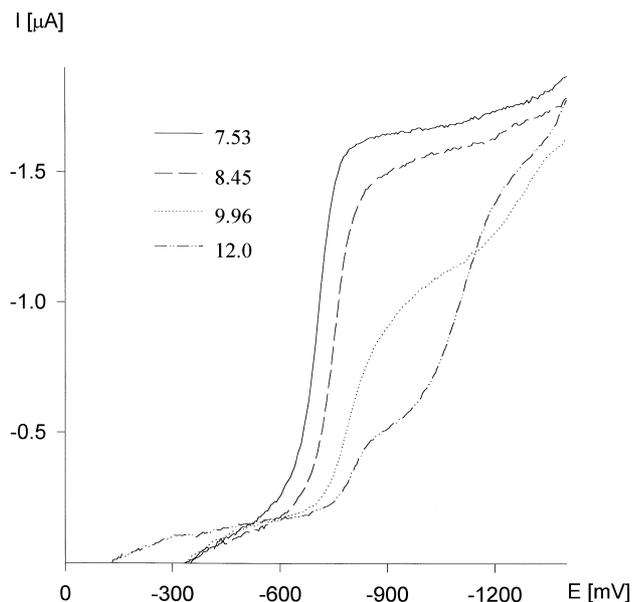


Fig. 6. PAC d.c. polarograms evolution with pH.

wave (peak III) due to the nitro radical anion formation (Eq. (3)). This fact is not very common in nitro aromatic compounds where the observation of the nitro radical anion formation peak (or wave) requires the presence of inhibitors or aprotic solvent, besides alkaline pH. Consequently, we think the formation of the nitro radical anion would be privileged in this type of compounds. On the other hand, DMB compound follows a different pattern with no formation of nitro radical anion in the same conditions.

Furthermore, substitution at position 6 hinders the reduction of the nitro-group as can be concluded from the comparison of the peak potential values. This fact can be

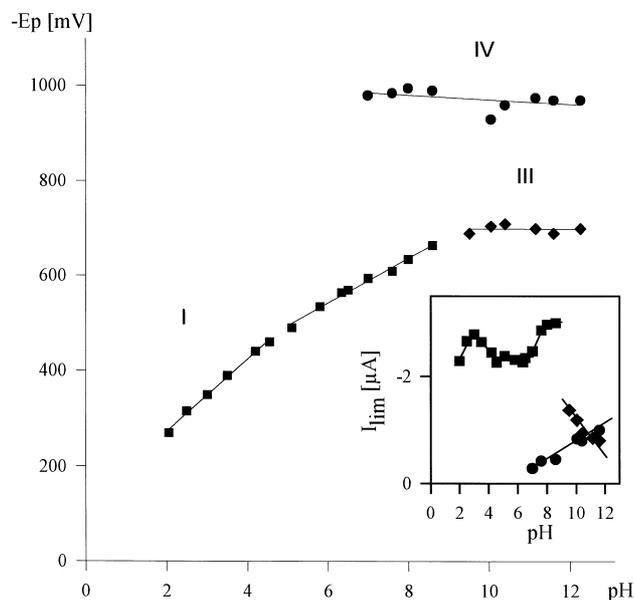


Fig. 7. Peak potential (d.p.p.) and current limit (d.c.) dependence with pH for PEA.

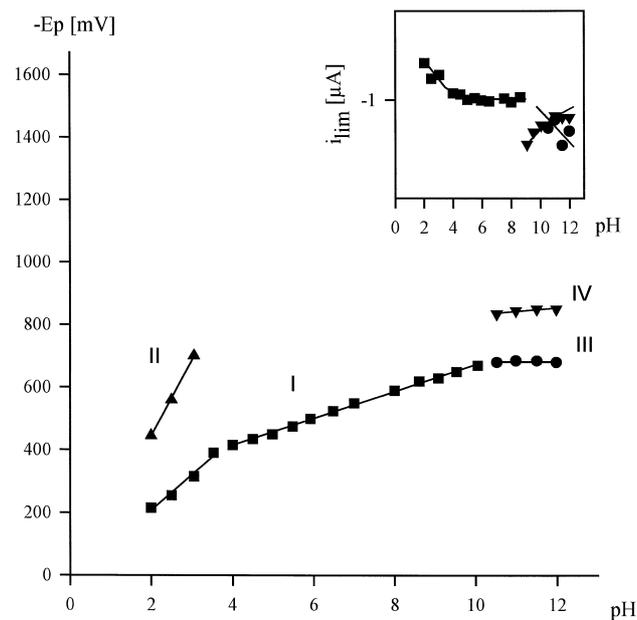


Fig. 8. Peak potential (d.p.p.) and current limit (d.c.) dependence with pH for PAN.

explained considering the steric hindrance caused by a bulky group in *o*-position (position 6) preventing attainment of coplanarity of the nitro group and the aromatic ring. The distortion of the coplanar arrangement decreases the resonance interaction between the electroactive group and the aromatic ring, and the observed shift towards negative potentials corresponds to this decrease [32]. For the PAC compound the notoriously more negative value can be explained by the additional effect of the hydrogen bond between the carboxylic hydrogen and the oxygen of the nitro group. This bonding requires an extra energy for reduction. At $\text{pH} > \text{pK}$, the carboxylic group would be ionized and the more negative reduction potential would result from a diminished electrophilic character of the molecule due to the ionized carboxylate group.

3.2. Mixed media

3.2.1. Polarography

For the purpose of obtaining better stabilization of the above obtained nitro radical anion, we changed the media by the introduction of an aprotic solvent such as DMF. A similar polarographic behaviour as that in aqueous media is observed in mixed media for all the nitrocompounds studied (Fig. 9). The main differences are a better separation obtained between the peaks (or waves) corresponding to reactions III and IV and more negative reduction potentials (by 200–400 mV). Furthermore, the peak corresponding to Eq. (2) does not appear which means that the arylhydroxylamines are not protonated at pH 2 in these media.

According to the obtained results, the mixed media protonation of the nitro radical anion occurs at higher pHs

Table 1
Overall redox mechanisms in protic media at different pH

Compound	E_p (pH 7) [mV]	pH range	Slope [mV/pH]	Electrode reaction involved
DMB	-510	2-6	81.9	I
		6-9.5	50.8	I
		10-12	independent	I
		2-3.5	178.7	II
PAN	-560	2-3.5	113.6	I
		4-10	43.1	I
		2-3.5	297	II
		10-12	independent	III
PEA	-580	10-12	independent	IV
		2-7	58.8	I
		7-12	independent	III
		7-12	independent	IV
PAC	-680	2-7.5	65.5	I
		7.5-12	independent	III
		7.5-12	independent	IV

than the aqueous media. In order to obtain a more adequate time scale for the experiments and better conditions for the study of the nitro radical anions generated from the studied compounds, we carried out cyclic voltammetric experiments.

3.2.2. Cyclic voltammetry

Employing the cyclic voltammetric technique (C.V.) in mixed media (DMF/aqueous buffer) at the d.m.e., it was possible to observe the reversible couple due to the monoelectronic reduction of the nitro group to the corresponding radical anion for all the compounds studied (Fig. 10).

From the ΔE_p value of about 60 mV, the monoelectronic character of the couple was confirmed. In Fig. 11, the evolution of the first redox system with pH for PAC is presented ($\nu = 1 \text{ V s}^{-1}$). As the pH increases, the I_{p_a}/I_{p_c} ratio increases to reach one at pH 9.0 which is interpreted as showing, that the chemical reactions following the electrons transfer becomes slow as the pH increases and that the protons are involved. The observation of this couple for other nitrocompounds with pharmacological importance has been previously reported [33,34].

From the C.V. studies, the kinetic behaviour of the nitro radical anions electrochemically generated can be charac-

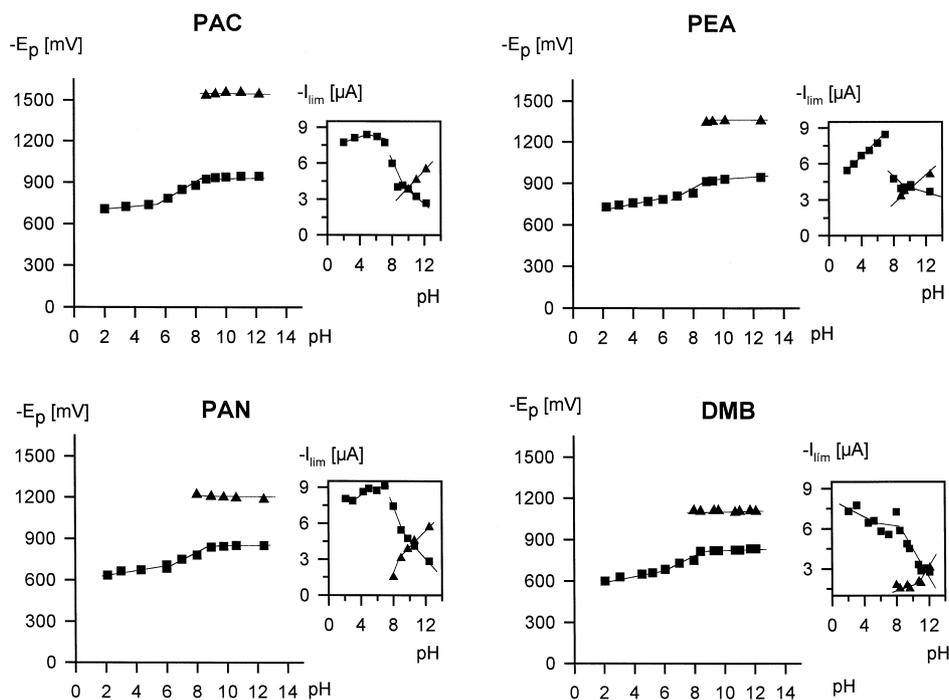


Fig. 9. Peak potential (d.p.p.) and current limit (d.c.) dependence with pH for the studied nitrocompounds, mixed media: DMF/aqueous citrate: (A) PAC, (B) PEA, (C) PAN, (D) DMB.

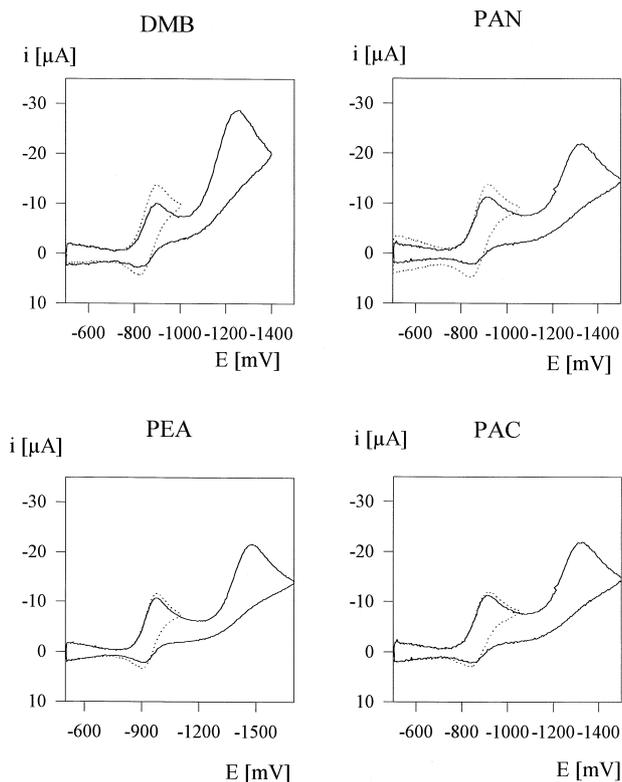


Fig. 10. Cyclic voltammograms for the nitrocompounds at different voltage ranges, sweep rate 1 V s^{-1} , DMF/aqueous citrate (60/40), pH 9.0.

terized. Taking the current ratio (I_{p_a}/I_{p_c}) as an experimental follow-up parameter of the reaction and applying the criteria developed by Nicholson and Shain [35], we can obtain some conclusions about the mechanisms involved.

The results show (Fig. 12) that as the scan rate increased, the current ratio (I_{p_a}/I_{p_c}) increased towards unity,

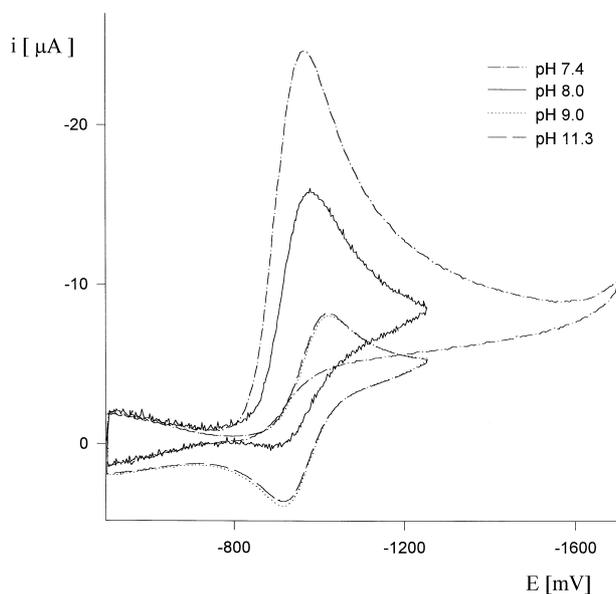


Fig. 11. Isolated couple at different pH for PAC.

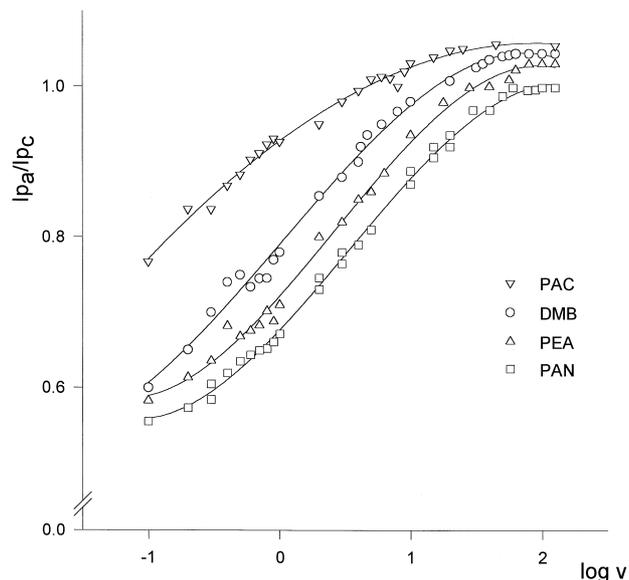
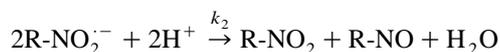
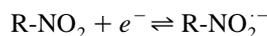


Fig. 12. I_{p_a}/I_{p_c} vs. $\log v$ for all the nitrocompounds.

a typical behaviour for an irreversible chemical reaction following a charge-transfer step, i.e., an EC process [36]. Furthermore, our results clearly reveal a dependence of the I_{p_a}/I_{p_c} ratio on the concentration of the nitro derivatives, suggesting a second order reaction for the chemical step according to the following mechanism:



The theory of cyclic voltammetry for a second order reaction initiated electrochemically was exhaustively studied by Olmstead et al. [37]. According to this theory, we

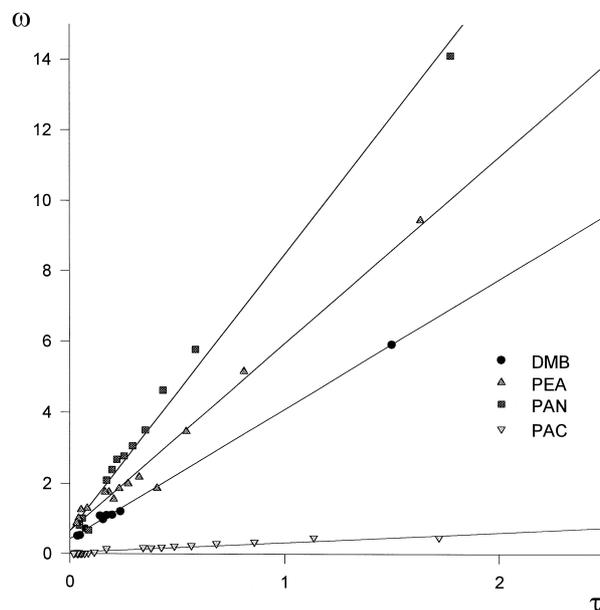


Fig. 13. ω vs. τ for all the nitrocompound derivatives.

Table 2

Second order decay constant (k_2) and half life ($t_{1/2}$) for the nitro radical anions, calculated at pH 9.0 in mixed media (DMF/aqueous citrate)

Compound	Decay constant K_2 [l/mol × s]	Half life $t_{1/2}$ [s]
DMB	3679 ($r = 0.9992$)	0.27
PEA	5299 ($r = 0.9905$)	0.19
PAN	7838 ($r = 0.9925$)	0.13
PAC	279 ($r = 0.9937$)	3.58

have obtained linear relationships (Fig. 13) between the kinetic parameters ω and τ . This is an indication that the nitro radical anion is consumed in a second order chemical reaction. Furthermore, the above theory permitted us to calculate the rate constant k_2 of the second order chemical reaction. Table 2 exhibits the rate constant k_2 for the second order reaction of the radical anions and their half-life. From the results of Table 2 it is possible to observe that the nitro radical anion formed by PAC compound is much more kinetically stable than the other nitro compounds.

To test the possibility of in vivo free radical formation from this type of nitroderivatives, we have used a cell system containing epimastigotes of *T. cruzi* Tulahuen strain because of its well-known capability of producing redox cycling with some nitrocompounds such as nifurtimox and benznidazole [38,39].

From these studies we selected DMB and PAC because of their extreme cathodic peak potential values. Results showed that both derivatives produced an increase in the oxygen consumption at 50 μ M concentration, the increase being more important with DMB (40.0% \pm 2.0%) than with PAC (12.8% \pm 2.5%). The above results can be ascribed to in vivo nitro radical anion formation, with the subsequent redox recycling that involves reductive oxygen species formation (i.e., O_2^- , OH^\cdot). According to the electrochemical data: E_{pc} of -510 mV and -680 mV for DMB and PAC, respectively (Table 1), the DMB compound that possesses the more positive reduction potential, produces the main increase in the oxygen consumption on the strain of *T. cruzi* epimastigotes. The PAC compound, with more negative reduction potential, produces a minor increase in oxygen consumption.

Acknowledgements

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