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Cyclic voltammetric and EPR spectroscopic studies of benzodiazepines: loprazolam and flunitrazepam

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Abstract

The present paper reports on the electrochemical characterization by cyclic voltammetry of two 1,4-benzodiazepines, loprazolam and flunitrazepam in protic, aprotic and mixed media. In protic media (ethanol + 15 mM citrate buffer pH 8.0) both drugs were irreversibly reduced at HDME involving 4 electrons to give the hydroxylamine derivative. In the optimal mixed media conditions (DMF + 15 mM citrate buffer, 0.1 M TBAI, pH 9.0) and in aprotic media (DMF + 0.1 M TBAI), the one-electron reduction product corresponding to the nitro radical anion was successfully isolated for both drugs. In both electrolytic media (mixed or aprotic media) loprazolam was reduced at less negative potentials compared with flunitrazepam. Radical decay follows second order kinetics. In mixed media the following second order decay constants and half-lives (for a 5 mM concentration) were found: $k_2 = 1573.6 \pm 35.7$ l mol^{-1} s^{-1}, $t_{1/2} = 0.13$ s for flunitrazepam and $k_2 = 455.5 \pm 16.6$ l mol^{-1} s^{-1}, $t_{1/2} = 0.44$ s, for loprazolam. EPR spectra recorded in situ using DMF as an electrolytic solvent showed well-resolved spectra, confirming the reduction of both benzodiazepine derivatives to their corresponding nitro radical anions. The experimental and the simulated hyperfine constant values obtained by INDO calculations are in agreement. The magnitude of such hyperfine splitting constants permits us to conclude that these radical anions are mainly restricted to the benzene ring of the molecules. © 1997 Elsevier Science S.A.

1. Introduction

Benzodiazepines have been on the market for over 30 years and have achieved remarkable clinical success. The introduction of these drugs in clinical practice represented a very significant advance over previous generation of drugs acting on the central nervous system [1].

It is now clear that all of the major centrally mediated actions of benzodiazepines, that is, their anxiolytic, anticonvulsant, muscle relaxant and sedative-anesthetic properties are mediated by benzodiazepine receptors. Moreover, it has also been shown that the benzodiazepine receptor first demonstrated in 1977 is really an A subtype of γ-aminobutyric acid receptor [2].

Discussions on the safety of the benzodiazepines during chronic use have been usefully reviewed [3]. However, systematic studies on the role of the possible formation of toxic species during the biotransformation pathways as a consequence of chronic treatments have not been explored.

The electrochemical reduction and oxidation behaviour of benzodiazepine derivatives has been reviewed extensively by Smyth [4]. Electrochemical studies concerned with the two benzodiazepines under consideration here are rather restricted to papers dealing with its electroanalysis [5–10]. No studies about the cyclic voltammetric behaviour of these drugs leading to a test of possible free radical formation have been found in the literature.

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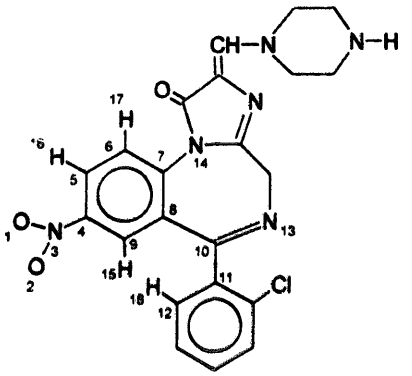
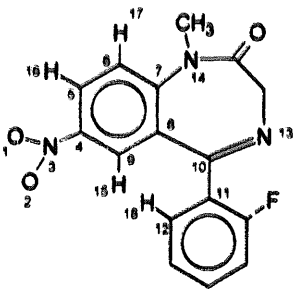
LOPRAZOLAM





FLUNITRAZEPAM

Fig. 1. Chemical structures of the benzodiazepine derivatives.

In this study, we have examined the formation of radical species from two benzodiazepines, loprazolam and flunitrazepam (Fig. 1) in different media using cyclic voltammetry. We have also used electron paramagnetic resonance (EPR) spectroscopy to show that both drugs are reduced electrochemically to their corresponding nitro radical anions.

2. Experimental

2.1. Drugs

2.1.1. Loprazolam

6-(2-chlorophenyl)-2,4-dihydro-2-(4-methylpiperazin-1-yl-methylene)-8-nitroimidazo-[1,2-a]-1,4-benzodiazepin-1-one methanesulphonate monohydrate was obtained from Chile Laboratories, Santiago, Chile.

2.1.2. Flunitrazepam

5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2*H*-1,4-benzodiazepine-2-one was obtained from Hoescht Laboratories, Santiago, Chile.

2.2. Cyclic voltammetry

Experiments were carried out in a INELECSA assembly PDC 1212, containing a generator/potentiostat in conjunction with an A/D converter interface attached to a 12-bit microprocessor and suitable software for totally automatic control of the experiments and data acquisition. An Acer 500$^+$ microcomputer was used for data control, acquisition and treatment.

2.2.1. Electrodes

A Metrohm HMDE, v ith a drop area of 1.92 mm^2 was used as the working electrode and a platinum wire as a counter electrode. All potentials were measured against a SCE.

All cyclic voltammetry was carried out at a constant temperature of 25°C and the solutions were purged with pure nitrogen for 10 min prior to the voltammetric runs.

The return-to-forward peak current ratio, I_{pa}/I_{pc}, for the reversible first electron transfer (the $R\text{-}NO_2/R\text{-}NO_2^{\cdot}$-couple) was measured, varying the scan rate from 0.1 V s^{-1} up to 5.0 V s^{-1}.

2.3. UV–Vis spectroscopy

Spectra were recorded with an UV-3 Unicam® spectrophotometer. Data analysis was carried out with Vision® software. All measurements were performed using dimethylformamide as the solvent and 0.1 M TBAI.

2.4. Methods

The experimental I_{pa}/I_{pc} ratios were calculated according to Nicholson and Shain's procedure [11], using individual cyclic voltammograms. The potential E_A was selected to minimize the influence of subsequent cathodic peaks.

Kinetic reaction orders for the nitro radical anion were quantitatively assayed for first and second-order coupled reactions according to previously published theoretical studies [12–14].

2.5. Protic media

This medium was prepared with ethanol + 15 mM citrate buffer, 20/70, pH 8.0 and KCl to adjust the ionic strength to 0.3 M.

2.5.1. Mixed media

In order to obtain the optimal mixed media, different supporting electrolytes such as KCl, LiCl and tetra-butylammonium iodide (TBAI) were tested. Likewise, citrate, camphor and hexamethylphosphotriamide (HMPA) were tested as surface-active substances. The concentrations of these substances varied between 0.01 M and 0.1 M. Percentages of DMF varied from 20% to 80% (v/v). From these experiments, the following optimum composition was selected: 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 medium at pH 9.0 was used.

2.5.2. Aprotic media

0.1 M TBAI in dimethylformamide was used for voltammetric measurements in this medium.

2.6. pH in mixed media

pH measurements were corrected according to the following expression [15]: $pH^* - B = \log U_H^0$, where pH^* equals $-\log a_H$ in the mixed solvent, B is the pH meter reading and the term $\log U_H^0$ is the correction factor for the glass electrode, calculated from different mixtures of DMF plus aqueous solvent, according to a previously reported procedure [16].

2.7. Drug solutions

Stock solutions of 10 mM of the 1,4-benzodiazepine derivatives either in DMF or ethanol were prepared. Drug concentrations of 5.0 mM were used normally.

2.7.1. Saturated ethanol solution

This saturated solution was prepared by adding 0.24 g NaOH to 30 ml absolute ethanol (0.2 M).

2.8. EPR measurements

The nitro radical anions from benzodiazepine derivatives were generated in situ by electrochemical reduction at room temperature. A 5 mM solution of each benzodiazepine derivative containing 0.1 M tetrabutyl ammonium perchlorate in DMF was degassed with nitrogen for 10 min and immediately its EPR spectrum was recorded in the microwave band X (9.85 GHz) in a Brucker ECS 106 spectrometer, using a rectangular mode cavity with a 50 kHz field modulation. Hyperfine splitting constants were estimated to be accurate within 0.05 G.

2.8.1. Theoretical calculations

Full geometry optimization of the benzodiazepines in spin-paired and free radical forms was carried out by AM1 methods [17]. INDO calculations were done employing the open shell UHF option.

3. Results and discussion

The main goal of this paper was to establish the formation of the nitro radical anion from the two benzodiazepines, which have in common the presence of a nitro group in the molecule (Fig. 1).

Firstly, we have characterized the redox behaviour of both drugs by cyclic voltammetry on the mercury electrode in different electrolytic media.

3.1. Protic media

Cyclic voltammograms of flunitrazepam and loprazolam showed that both drugs were irreversibly reduced in this medium, with no indications of return peaks, irrespective of the sweep rate.

In the case of flunitrazepam, a first signal appeared which corresponded to the reduction of the nitro group via 4 electrons to give the hydroxylamine derivative. At more negative potentials, a second signal corresponding to the reduction of the azomethyne group was observed [4]. Finally, an oxidation peak appeared which corresponded to the subsequent oxidation of the hydroxylamine derivative formed in the negative sweep. In the second sweep a cathodic peak appeared, which corresponded to the reduction of the nitroso derivative.

Since loprazolam has the same reductive groups as flunitrazepam ($-NO_2$; $-C=N-$, Fig. 1), a similar redox pattern was observed. Now, the reductive processes appeared at less negative potential values, i.e., loprazolam was more easily reduced.

Protic Media

$$ArNO_2 + 4e^- + 4H^+ \xrightarrow{I} ArNHOH + H_2O$$

$$ArNHOH \underset{IIa}{\overset{IIc}{\rightleftharpoons}} ArNO + 2e^- + 2H^+$$

$$R_1{-}C{=}N{-}R_2 + 2e^- + 2H^+ \xrightarrow{III} R_1{-}CH{-}NH{-}R_2$$

Mixed Media

$$ArNO_2 + e^- \underset{IVa}{\overset{IVc}{\rightleftharpoons}} ArNO_2^{\cdot -}$$

$$ArNO_2^{\cdot -} + 3e^- + 4H^+ \xrightarrow{V} ArNHOH + H_2O$$

$$R_1{-}C{=}N{-}R_2 + 2e^- + 2H^+ \xrightarrow{III} R_1{-}CH{-}NH{-}R_2$$

Aprotic Media

$$ArNO_2 + e^- \underset{IVa}{\overset{IVc}{\rightleftharpoons}} ArNO_2^{\cdot -}$$

$$ArNO_2^{\cdot -} + 3e^- + 4\,Bu_3N^{\cdot +} \xrightarrow{VI} ArNHO^{\cdot} + 3\,C_4H_8 + 3\,Bu_3N + H_2O$$

$$ArNHO^{\cdot} \xrightarrow{VII} ArNHO^- + e^-$$

$$R_1{-}C{=}N{-}R_2 + 2e^- + 2H^+ \xrightarrow{III} R_1{-}CH{-}NH{-}R_2$$

Scheme 1. Overall nitroreduction mechanisms for loprazolam and flunitrazepam in different electrolytic media.

Table 1
Cyclic voltammetric parameters for the reduction of flunitrazepam and loprazolam in different electrolytic media

	Protic media, pH 8.0				
	$-E_p^{I}$/mV	$-E_p^{IIc}$/mV	$-E_p^{IIa}$/mV	$-E_p^{III}$/mV	
Flunitrazepam	880	317	289	1400	
Loprazolam	710	330	250	1250	
	Mixed media, pH 9.0				
	$-E_p^{IVc}$/mV	$-E_p^{IVa}$/mV	$-E_p^{V}$/mV	$-E_p^{III}$/mV	
Flunitrazepam	820	730	1170	1745	
Loprazolam	780	725	1140	1703	
	Aprotic media				
	$-E_p^{IVc}$/mV	$-E_p^{IVa}$/mV	$-E_p^{VI}$/mV	$-E_p^{VII}$/mV	$-E_p^{III}$/mV
Flunitrazepam	1005	939	1920	–	2217
Loprazolam	1000	926	1829	1176	2122

E_p values are given versus SCE.

The reduction mechanism and the corresponding peak potential values are summarized in Scheme 1 and Table 1. These results are consistent with early investigations of nitrobenzodiazepines in protic solvents at all pH values [18].

3.2. Mixed media

The addition of DMF to an aqueous citrate buffered solution (Fig. 2) of either loprazolam or flunitrazepam at pH 9.0 enables the presence of three different reduction process: (a) a first reversible peak, which corresponds to the one-electron transfer processes resulting in the generation of the nitro radical anion; (b) a second irreversible peak, which corresponds to the formation of the hydroxylamine derivative involving a three-electron transfer process and (c) a third irreversible peak, which corresponds to the reduction of the azomethine group and involves a two-electron transfer process. The results in this electrolytic medium are summarized in Scheme 1 and Table 1.

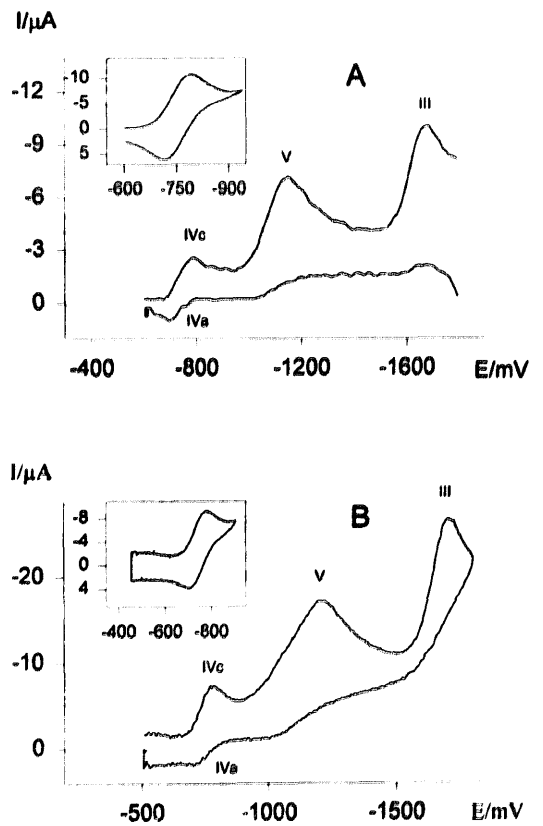


Fig. 2. Cyclic voltammograms in the optimal mixed media: (A) loprazolam (B) flunitrazepam. Inserts: Isolated cyclic voltammogram corresponding to the one-electron reduction product. Sweep rate: 1 V s^{-1}.

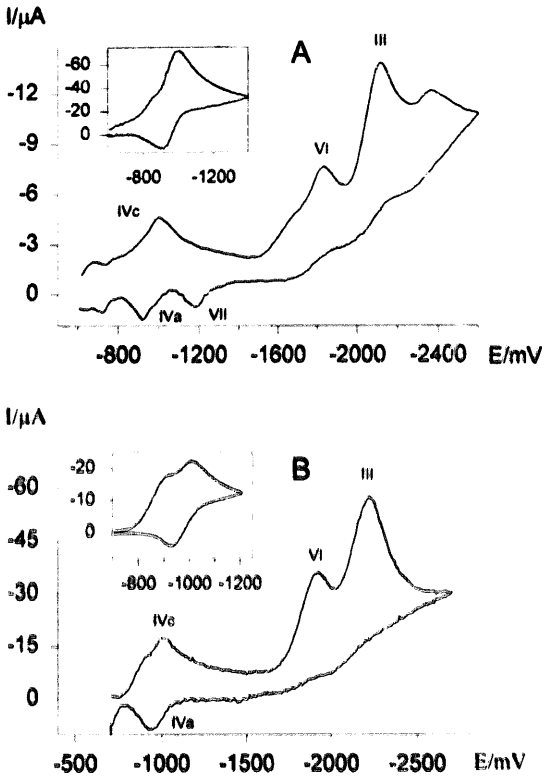


Fig. 3. Cyclic voltammograms in aprotic media: (A) loprazolam (B) flunitrazepam. Inserts: Isolated cyclic voltammogram corresponding to the one-electron reduction product. Sweep rate: 1 V s^{-1}.

In conclusion, in this electrolytic medium the isolation and the kinetic characterization of the nitro radical anions electrochemically generated from both flunitrazepam and loprazolam was established successfully.

3.3. Aprotic media

The cyclic voltammetric behaviour in this medium for loprazolam is shown in Fig. 3. As can be seen in this figure, this electrolytic medium enables the presence of three different reduction processes to be established. Since our main interest is focused on the formation of the nitro radical anion from these two benzodiazepine derivatives, we have determined the optimal electrochemical conditions (i.e., range potential, switching potential, sweep rate) to characterize such species. However, under optimal selected conditions for the isolation of reversible couples, the appearance of pre-peaks was observed at a more positive potential than the one corresponding to the couples for both drugs (Inserts Fig. 3). It is noteworthy that these pre-peaks disappeared completely when a NaOH-saturated ethanol solution was added. This type of

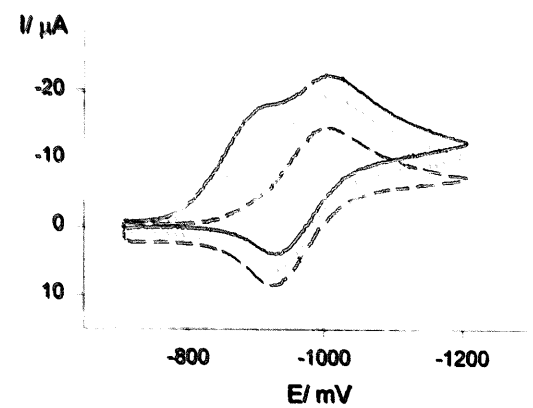


Fig. 4. Cyclic voltammograms of 1 mM flunitrazepam in DMF + 0.1 M TBAI. Effect of the addition of NaOH saturated ethanol. (—) 0, (.....) 0.1 ml, (— — —) 0.5 ml. Sweep rate: 1 V s^{-1}.

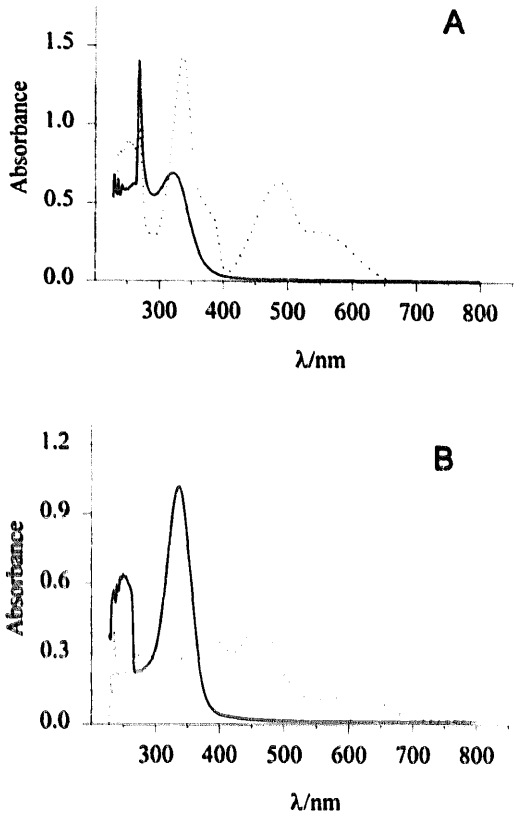
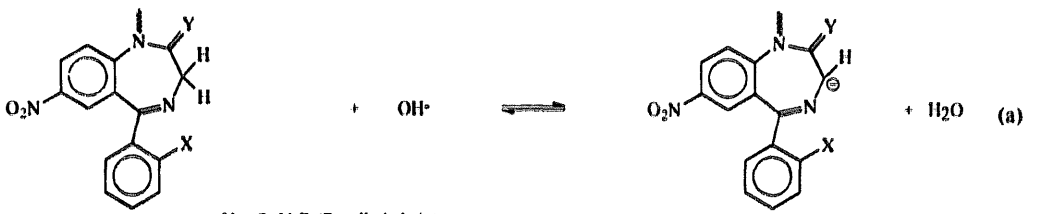


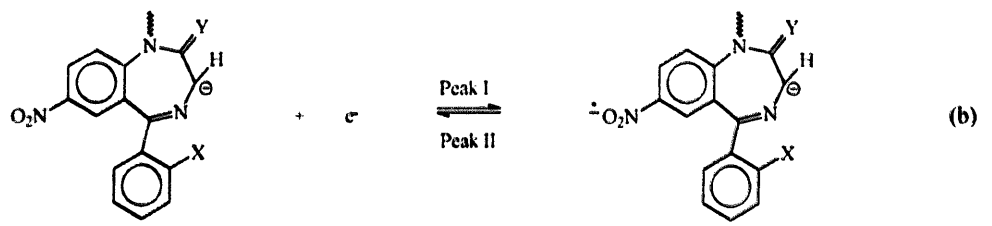
Fig. 5. UV–Vis absorption spectra of 0.1 M solutions in DMF+0.1 M TBAI of: (A) flunitrazepam and (B) loprazolam. (—) in the absence of NaOH (.....) in the presence of 0.5 ml NaOH solution.

behaviour is illustrated in Fig. 4 for flunitrazepam. The gradual disappearance of the pre-peak is directly related to the quantity of base added. Therefore, it can be concluded that this behaviour can be attributed to an equilibrium between the un-ionized (I) and ionized (II) form of the drugs according to the following equation:



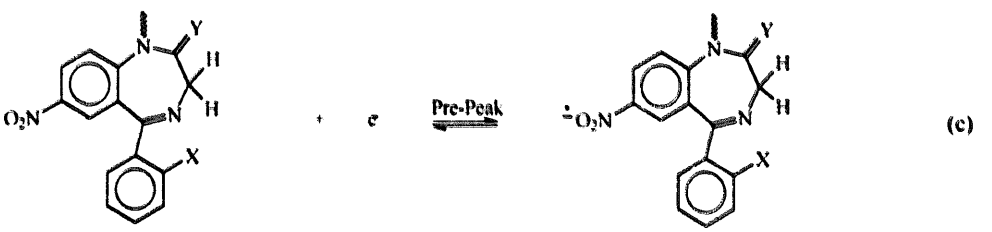
For both drugs the existence of these equilibria was followed by using cyclic voltammetry and UV–Vis spectroscopy. The evolution of the UV–Vis absorption spectra of the drugs is shown in Fig. 5. As can be seen in this figure, the spectral patterns indicate the transitions of the un-ionized forms of the benzodiazepines to their ionized forms. Thus, in the absence of NaOH addition, both derivatives exhibited two major UV–Vis bands (flunitrazepam: $\lambda_1 = 268$ nm, $\lambda_2 = 320$ nm; loprazolam: $\lambda_1 = 250$ nm, $\lambda_2 = 338$ nm). But, in the presence of an excess of NaOH, the spectra changed dramatically. The UV–Vis bands of flunitrazepam were shifted to the visible region ($\lambda_1 = 332$ nm, $\lambda_2 = 488$ nm), with an increase in band intensity. In contrast, the UV–Vis spectrum of loprazolam now exhibited three major bands ($\lambda_1 = 378$ nm, $\lambda_2 = 466$ nm, $\lambda_3 = 642$ nm), with a decrease in band intensity compared with the original one. From the above experimental facts, we can conclude that when a strong base is added to the aprotic media, equilibrium is displaced to the anionic form of the drugs

(II), these species being responsible for the single signal observed by cyclic voltammetry according to the following equation:

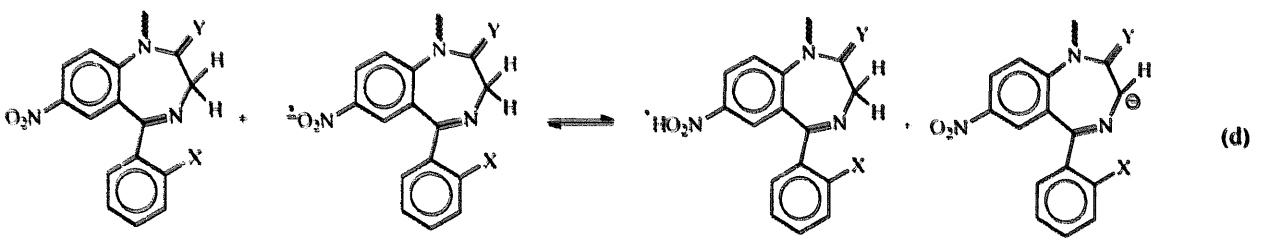


II III

Since the anionic form (II) of the drug failed to occur in the solutions when base was not added, it may be assumed that radical anions formed (IV) at the first voltammetric peak potential act as negatively charged species capable of deprotonating the parent molecule (I). This would finally result in neutral radicals (V) and benzodiazepine anions (II), which are reduced at a more negative potential than the neutral species (I), the reduction of these species being responsible for the production of the above mentioned pre-peaks. This phenomenon can be described by the following equations:



I IV



I IV V II

In conclusion, when the base is not present in the solution of the drugs in the aprotic medium, the reversible couples with pre-peaks are due to a combination of equations (c), (d) and (b). Consequently, to avoid pre-peaks we must always work by adding base to the medium in order to have only the reduction of the ionized form of the drugs; hence further studies in this medium will involve the anionic forms of the benzodiazepines (II). Similar behaviour was previously observed for a series of nitroaryl-1,4-dihydropyridine derivatives in this laboratory [19].

Three reduction process were then established in the optimal experimental conditions for this medium: (a) a first reversible peak, corresponding to the one-electron transfer process resulting in the generation of the nitro radical anion; (b) a second irreversible cathodic peak corresponding to the reduction of the nitro radical anions to form the protonated nitroso dianion. This peak involves the participation of the supporting electrolyte (Bu_4N^+), where the electrogenerated base may generate butene (Hoffman elimination) from Bu_4N^+ [20]. Addition of a saturated ethanol solution of NaOH also acts as a suitable proton donor because the EtOH can protonate any dianion and (c) a third irreversible peak, corresponding to the reduction of the azomethine group involving a 2-electron transfer process. However, in the case of loprazolam an additional anodic signal was observed, which corresponds to the oxidation of the protonated nitroso dianion. These overall reduction processes are summarized in Scheme 1 and Table 1.

Table 2
Dependence of the current ratio of loprazolam and flunitrazepam with DMF

DMF/%	I_{pa}/I_{pc}	
	Loprazolam	Flunitrazepam
20	0.68	0.66
30	0.77	0.73
40	0.80	0.79
50	0.87	0.84
60	0.88	0.89
70	0.88	0.89
80	0.88	0.89

3.4. Kinetic characterization of the nitro radical anions from loprazolam and flunitrazepam

3.4.1. Mixed media

Cyclic voltammograms for both benzodiazepines in the optimal mixed media (DMF + 0.015 M citrate buffer 60/40 + 0.3 M KCl + 0.1 M TBAI, pH 9.0) also exhibited a well-defined signal (Inserts Fig. 3). Chemical reversibility of this first reduction step, as determined by the I_{pc}/I_{pa} ratio in the cyclic voltammogram, increases with the addition of DMF, reaching limiting values at approximately 60% DMF for both drugs (Table 2).

From the above results it is clear that the one-electron transfer process resulting in the generation of the nitro radical anion becomes apparent by the addition of DMF, this trend being the same for the two benzodiazepines under study.

On the other hand, we have studied the stability of the $R\text{-}NO_2^{\cdot-}$ species by changing the electrochemical conditions, i.e., the scan rate, the switching potential, and keeping the chemical conditions of the solution constant. Results show that as the scan rate increased, the I_{pa}/I_{pc} increased towards unity, typical behaviour for an irreversible chemical reaction following a charge-transfer step, i.e., the EC process [14]. To check the order of the following chemical reaction, the I_{pa}/I_{pc} ratio dependence on the concentration of the benzodiazepine derivatives was evaluated. The theory of cyclic voltammetry for a second-order reaction initiated electrochemically has been studied exhaustively by Olmstead et al. [14]. In our experiments we have found that an increase in the nitrobenzodiazepine concentration, keeping both DMF percentage and scan rate

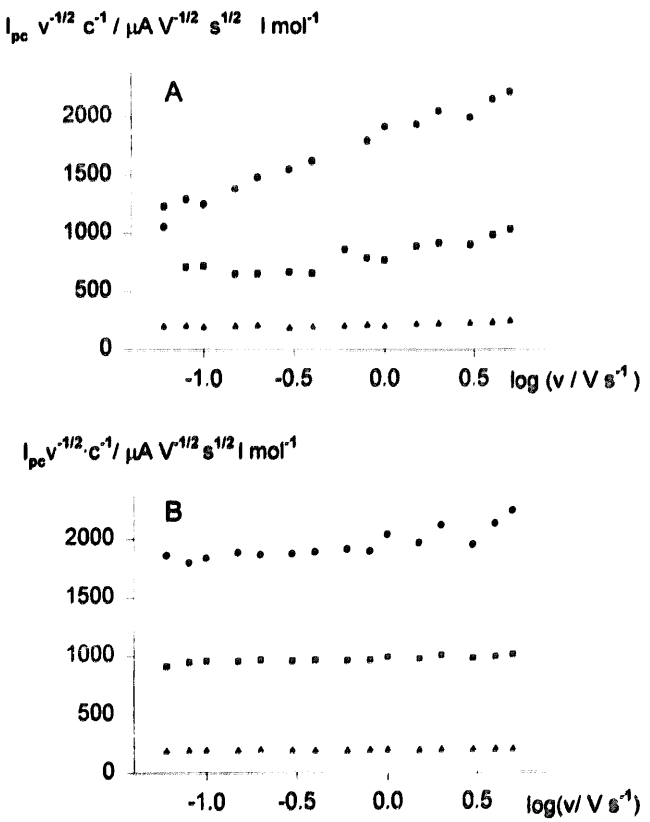


Fig. 6. Dependence of the current function (ψ) with the logarithm of the sweep rate. (A) Flunitrazepam (●) 0.5 mM, (■) 1 mM, (▲) 5.0 mM. (B) Loprazolam (●) 0.5 mM, (■) 1 mM, (▲) 5.0 mM.

Table 3
pH dependence of the $R\text{-}NO_2/R\text{-}NO_2^-$ couples from loprazolam and flunitrazepam in the optimal mixed media

pH	Loprazolam		Flunitrazepam	
	$-E_{pc}$ /mV	I_{pa}/I_{pc}	$-E_{pc}$ /mV	I_{pa}/I_{pc}
8.0	780	0.88	820	0.89
9.0	782	0.90	819	0.91
10.0	784	0.94	822	0.93
11.0	781	0.96	819	0.95
12.0	783	0.97	820	0.98

Scan rate: 1.0 V s^{-1}.

constant, resulted in an increased I_{pa}/I_{pc} value. However, according to the theory proposed by Olmstead et al. [14], this ratio should decrease because the second-order reaction is favoured over the electrochemical reaction of the radical. Our results show the opposite variation, but this effect is only apparent. It has been observed that the current function of the cathodic peak ($\psi = I_{pc}/v^{1/2}\, c_0$) is a function of benzodiazepine concentration and scan rate (Fig. 6). This type of behaviour indicates weak adsorption of the reactant [21]. The relative influence of the adsorption phenomenon is higher at low concentrations than at high concentrations, and the I_{pa}/I_{pc} ratio shows a relative increase with increasing benzodiazepine concentration. Experimental variations suggest that these effects mask the actual dependence on concentration for a second-order reaction. At a given scan rate the adsorption effects can be corrected qualitatively by taking into account the value of ψ at each concentration. The corrected values for the cathodic peak currents can be obtained as $(I_{pc})_{corr} = I_{pc}/\psi$. Then, if these values are normalized with respect to the maximum values, the $(I_{pa}/I_{pc})_{corr}$ ratio decreases as the concentrations increase, as is accounted for by the theoretical predictions for the above-mentioned mechanism. The variation of I_{pa}/I_{pc} resembles theoretical predictions for an E_rC_i second-order reaction (i.e., a decrease in this ratio as c_0 increases). Considering the above results we decided to select a concentration of 5 mM for the studies in order to minimize adsorption effects.

Second-order constants were assessed from single cyclic voltammograms for each drug, according to Olmstead's procedure [14] from the following relationship:

$$\log \omega = \log(k_2 c_0 \tau)$$

Confirming the second order character of the following chemical reaction, plots of kinetic parameter, ω, vs. time constant, τ, were linear, with average correlation coefficients not lower than 0.98 for the two derivatives.

Furthermore, the cathodic peak potentials depend on benzodiazepine concentrations and sweep rates, and values for

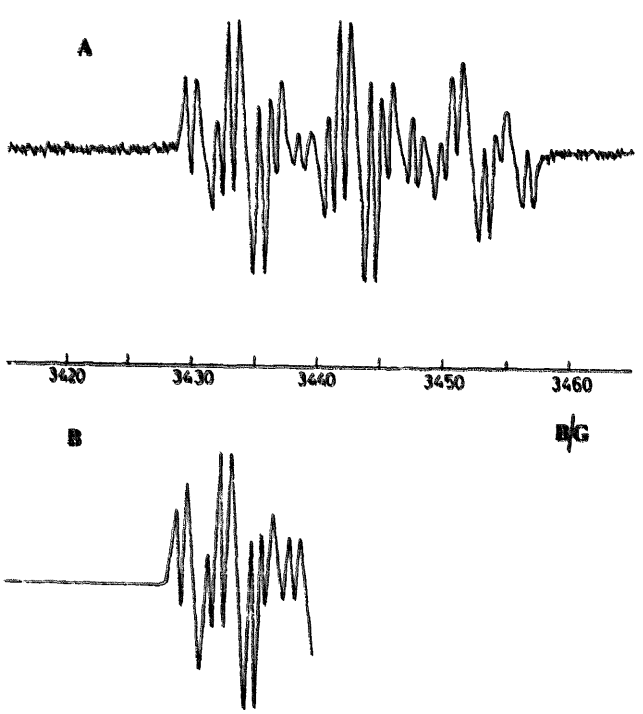


Fig. 7. (A) Experimental EPR spectrum of the radical anion of loprazolam in DMF. (B) Computer simulated EPR spectrum of loprazolam.

$dE_{pc}/d\log c$ and for $dE_{pc}/d\log v$ between 20 and 22 mV, are respectively obtained for the two benzodiazepines. These values are in agreement with the theoretical value of 19.5 mV for an EC_i process where the chemical step follows second order kinetics. The fact that the current ratio does not reach a value of unity can be ascribed to competition with a small amount of heterogeneous protonation.

The influence of pH on the anion radical reaction rates was also examined (Table 3). These results show an increase in the stability of nitroanions formed from the nitrobenzodiazepines with an increase in pH. Studies at pH 7.4 (similar to a physiological condition) to test the appearance of the signal corresponding to the one-electron reduction process were successful. However, we selected pH 9.0 for kinetic characterization due to a better resolution of the signals. On the other hand, we have found pH-independent values of cathodic peak potentials of the reversible couples, proving that no proton transfer precedes the electrode process (Table 3). Therefore, the sequence involved must be electron, proton, etc. These results disagree with the mechanism proposed previously for nitrobenzene in aqueous DMF media [22], which involved a mechanism with a proton-transfer step preceding the transfer of the first electron. But they are in agreement with the results reported by Kastening [23]. It should be noted that our studies involving pH measurements in mixed media were properly corrected, according to a previously published methodology [15,16] (see Section 2).

The absence of protons in the media favoured the stability of the nitroanions from the benzodiazepines. As expected, second order rate constants decreased and half-lives increased as DMF percentages increased. Experimental k_2 values in optimal mixed media conditions were: k_2 (loprazolam) = 455.5 ± 16.6 l mol^{-1} s^{-1} and k_2 (flunitrazepam) = 1573.6 ± 35.7 l mol^{-1} s^{-1}.

3.4.2. Aprotic media

From the single isolated couples corresponding to nitro radical anion formation in this media, i.e., DMF + supporting electrolyte + drops of a NaOH saturated ethanolic solution for both drugs, the nitro radical anions were characterized kinetically.

For loprazolam the current ratio increased as the scan rate was increased, but the ratio was different from unity, indicating the presence of an EC_i type of mechanism [14]. The experimental k_2 value for loprazolam was 120.3 ± 9.8 l mol^{-1} s^{-1}. However, in the case of flunitrazepam at any sweep rate, a maximum value of $I_{pa}/I_{pc} = 1$ was obtained, indicating that under experimental conditions the couple corresponding to its nitroanion was completely reversible.

3.5. EPR studies

The in situ electrochemical reductions leading to radical species were carried out in DMF by applying the potential corresponding to the first one-electron reversible reduction process for the benzodiazepine derivatives, just described in the cyclic voltammetric experiments. Interpretation of EPR spectra by means of a simulation process which led us to the determination of coupling constants for all magnetic nuclei is also included. A typical experimental EPR spectrum for loprazolam is shown in Fig. 7A. Loprazolam exhibits a hyperfine pattern completely resolved into 27 lines. The spectra were simulated in terms of one triplet due to the nitrogen nucleus of the nitro group, two doublets due to the non equivalent hydrogens 15 and 16 (Fig. 7B), two doublets due to the hydrogens 17 and 18 and one triplet due to the nitrogen N 14, which presents a small hyperfine constant. The hyperfine constant values are listed in Table 4. The flunitrazepam EPR spectrum exhibits a hyperfine pattern similar to that of loprazolam. This spectrum was simulated in terms of one triplet due to the nitrogen nucleus of the nitro group, two doublets due to the non equivalent hydrogens 15 and 16, and two doublets due to the hydrogens 17 and 18.

3.5.1. Theoretical calculations

In order to rationalize the experimental results obtained through the EPR spectra, AM1 calculations of both electron-paired and radical anion forms were carried out, with all the internal coordinates completely optimized. Results provided by AM1 calculations show that the nitro group lies in the ring plane for both molecules, either in neutral or in the radical anion

Table 4
Experimental and calculated INDO hyperfine splitting constants for the nitro radical anions from the benzodiazepine derivatives

Molecule	a_{NO2}/G	a_{H16}/G	a_{H15}/G	a_{H17}/G	a_{H18}/G	a_{N14}/G
Loprazolam						
Experimental	8.91	3.36	3.47	0.86	0.76	0.20
INDO	8.89	2.70	3.01	1.35	0.90	0.17
Flunitrazepam						
Experimental	8.87	3.24	3.46	1.04	0.85	–
INDO	8.84	2.44	2.78	1.20	0.98	–

forms. The most stable structures of both nitro radical forms showed that the condensed heterocyclic ring forms a dihedral angle of 20° referred to the nitrobenzene plane. These results are in agreement with experimental data, indicating that the unpaired electron is preferably delocalized towards N 14. Examination of MO coefficients indicated that the SOMOs of the nitro radical forms have antibonding p^z characteristics and are localized mainly on the nitro group. However, the unpaired electron was localized partially on the benzene ring. In order to obtain the theoretical hyperfine constants, INDO calculations were performed using the geometries obtained using AM1 calculations. In Table 4, both experimental and calculated hyperfine constant values are reported. Comparison of these results show good agreement with hyperfine constant assignments.

4. Concluding remarks

In the present study we have investigated the electrochemical reduction of two benzodiazepine derivatives in different media and for the first time we have provided experimental evidence that both derivatives are reduced to nitro anion free radicals capable of generating toxic reactive species. These findings could support the appearance of cytotoxicity phenomena in mammalian cells, considering that these drugs are used over long treatment periods. Furthermore, the cyclic voltammetric technique has proved to be a very useful tool to characterize the kinetic behaviour of the nitro radical anions produced from benzodiazepines in mixed and aprotic media.

Formation of radical species has been confirmed by EPR studies. Moreover, both experimental and computer simulated hyperfine splitting constant values are in agreement. Finally, the magnitude of the hyperfine splitting constants permits us to conclude that these radical anions are mainly restricted to the benzene ring of the molecules.

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