Electrochemical, UV–Visible and EPR Studies on Nitrofurantoin: Nitro Radical Anion Generation and its Interaction with Glutathione

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(Received 12 May 1999; In revised form 8 September 1999)

This paper deals with the reactivity of the nitro radical anion electrochemically generated from nitrofurantoin with glutathione. Cyclic voltammetry (CV) and controlled potential electrolysis were used to generate the nitro radical anion *in situ* and in bulk solution, respectively and cyclic voltammetry, UV–Visible and EPR spectroscopy were used to characterize the electrochemically formed radical and to study its interaction with GSH.

By cyclic voltammetry on a hanging mercury drop electrode, the formation of the nitro radical anion was possible in mixed media (0.015 M aqueous citrate/DMF, 40/60, pH 9) and in aprotic media. A second order decay of the radicals was determined, with a k_2 value of 201 and 111 $M^{-1} s^{-1}$, respectively. Controlled potential electrolysis generated the radical and its detection by cyclic voltammetry, UV–Visible and EPR spectroscopy was possible. When glutathione (GSH) was added to the solution, an unambiguous decay in the signals corresponding to a nitro radical anion were observed and using a spin trapping technique, a thiyl radical was detected.

Electrochemical and spectroscopic data indicated that it is possible to generate the nitro radical anion from nitrofurantoin in solution and that GSH scavenged this reactive species, in contrast with other authors, which previously reported no interaction between them.

Keywords: Nitrofurantoin, nitro radical anion, scavenging, glutathione (GSH), cyclic voltammetry, controlled potential electrolysis, ESR and UV–Visible spectroscopy

INTRODUCTION

Nitrofurantoin (N-(5-nitro-2-furfuryldine)-1-aminohydantoin) is a widely utilized urinary antimicrobial drug which has been associated with pulmonary fibrosis, neuropathy, hepatitis, and hemolytic anemia in patients with glucose-6-phosphate dehydrogenase deficiency.[1] Although the molecular mechanism leading to nitrofurantoin-induced cell toxicity is still uncertain, the antimicrobial activities as well as other clinical toxicities of nitrofurantoin may be due to

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the reductive metabolic activation of the 5-nitro function to the anion radical, nitroso, and hydroxylamine derivatives.[2]

Nitrofurantoin intracellular activation may proceed via one-electron reduction of the nitro group to the nitro anion radical, catalyzed by several intracellular flavoprotein reductases, including enzymes located in the cytosol and microsomal fractions[3] and in the outer mitochondrial membrane.[4] Under aerobic conditions, the nitro radical anion readily auto-oxidizes to the parent compound and concomitant $O_2^{\bullet -}$ formation has been shown upon nitrofurantoin activation by liver and lung microsomes.[5] This process, referred to as redox cycling, can give rise to large amounts of $O_2^{\bullet -}$, which subsequently undergo spontaneous or enzymatic dismutation to produce H_2O_2.

The voltammetric behavior of nitrofurantoin on mercury has been previously reported.[6] Also, Symons *et al.*[7] studied the effect of electrode material on the electrochemical reduction of three nitrofurans, including nitrofurantoin. However, these electrochemical studies did not involve a detailed cyclic voltammetric study about the nitro radical anion and consequently, a quantitative characterization of radical species was not considered. The generation of nitro radical anion from nitrofurantoin by rat hepatocytes[8,9] and lipoamide dehydrogenase[10] and its detection by ESR spectroscopy has been also reported.

On the other hand, some papers concerning the reactivity between the nitro anion radical from nitrofurantoin and glutathione (GSH) have been published.[11,12] In these reports, no detectable reaction of the anion radical metabolite of nitrofurantoin, with reduced GSH was found. Furthermore, it was reported that even a 100 mM GSH concentration did not affect the steady-state concentration of the radical 5-nitrofuran derivatives.[11]

Cyclic voltammetric studies of other nitrocompounds,[13,14] have demonstrated that GSH is capable of reacting with a nitro radical anion electrochemically formed in the mercury surface and moreover, these studies revealed that GSH is a more powerful scavenger than other thiol compounds, like captopril, penicillamine, N-acetylcysteine.

Taking into account the above-mentioned antecedents and the controversial results about the reactivity between the nitro radical anion of nitrofurantoin with GSH, we have examined both the formation and the kinetic characterization of the nitro radical through cyclic voltammetry in this paper. Also, we have used electron spin resonance (ESR) and UV–Visible spectroscopy to show that the drug is reduced electrochemically to its corresponding nitro radical and that this species is scavenged by GSH.

MATERIALS AND METHODS

Chemicals

Nitrofurantoin N-(5-nitro-2-furfuryldine)-1-aminohydantoin (Figure 1). (Chile Laboratories, Santiago, Chile). Glutathione (GSH), Dimethylformamide (DMF), spectroscopic grade, anhydrous acetonitrile, for UV spectroscopy, were purchased from Merck. Tetrabutylammonium hexafluorophosphate (TBAHFP) was purchased from Aldrich.

Drug Solutions

Stock solutions of 10 mM nitrofurantoin in dimethylformamide or acetonitrile were prepared and protected from daylight. An aliquot, to obtain

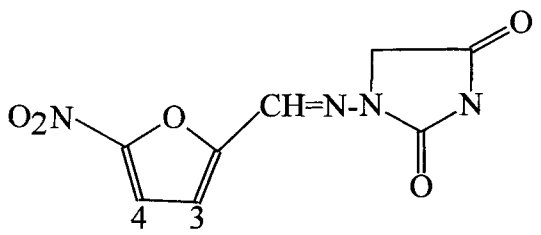

FIGURE 1 Chemical structure of nitrofurantoin.

a final concentration between 0.05 and 1 mM, was taken and diluted in the corresponding media: (a) Mixed media: 15 mM aqueous citrate/DMF, 40/60, pH 9 containing 0.1 M of TBAHFP and 0.3 M of KCl as electrolytes. (b) Aprotic media: Anhydrous acetonitrile containing 0.1 M TBAHFP as supporting electrolyte.

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 for the different mixtures of DMF and aqueous solution, according to a previously reported procedure).[16] These corrections are due to the pH meter being calibrated with aqueous buffers and the pH measurements being made in mixed solvent solutions.

Electrochemical Measurements

Cyclic voltammetry experiments were carried out in an INELECSA assembly PDC 1212, containing a generator/potentiostat 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. A DTK 166 Pentium microcomputer was used for data control, acquisition, and treatment. A routine drug concentration of 1 mM for all the experiments was used. A Metrohm hanging mercury drop electrode (h.m.d.e) with a drop surface of 1.90 mm^2 as the working electrode and a platinum wire as a counter electrode were used in the cyclic voltammetric measurements. All potentials were measured against an Ag/AgCl reference electrode.

Controlled Potential Electrolysis

CPE were carried out on either a platinum coil electrode or a mercury pool at −0.65 V in anhydrous acetonitrile containing 0.1 M TBAHFP as the supporting electrolyte or the optimum mixed media (15 mM aqueous citrate/DMF, 40/60, 0.1 M TBAHFP, 0.3 M KCl, pH 9). Oxygen was removed by pure, dry pre-saturated nitrogen. A three-electrode circuit with Ag/AgCl electrode was used as reference. An INELECSA PDC 210 linked to 486 DTK computer was used to carry out electrolysis of nitrofurantoin.

UV–Visible Studies

A UNICAM UV-3 spectrophotometer was used in order to obtain further information on the mechanism of electrolysis by monitoring, either the progress of the electrolysis or the reactivity of electrolysis products with GSH both in mixed and aprotic media. UV–Visible spectra were recorded in the 220–600 nm range at different intervals. Acquisition and data treatment were carried out with Vision 2.11 software. An electrolytic cell of our own construction based on a 1 cm UV cuvette, with a platinum foil or a stirred pool of mercury as working electrodes were used for the *in situ* generation of the reduction species.

ESR Measurements

The nitro radical anion from nitrofurantoin was generated *in situ* by electrochemical reduction (−0.65 V) at room temperature. A 5 mM solution of nitrofurantoin containing 0.1 M TBAHFP in acetonitrile, was purged with nitrogen for 10 min, reduced and its ESR spectrum was recorded immediately in the microwave band X (9.85 GHz) in a Bruker 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.

GSH was dissolved in 0.1 M tetrabutylammonium hydroxide to obtain final solutions with concentrations varying between 1 and 20 mM. To test the thiyl radical formation, 150 mM 5,5′-dimethyl-1-pyrroline N-oxide (DMPO, Aldrich) was used.

RESULTS

The main goal of this paper was to assess the possible scavenging effect of the nitro radical anion from nitrofurantoin by glutathione. For this purpose electrochemical, UV–Visible and ESR spectroscopic techniques were used.

Electrochemical Generation of Nitro Radical Anion from Nitrofurantoin

Cyclic Voltammetry

According to previous studies with different nitro compounds, the optimal conditions to obtain the signal of nitro radical anion were with mixed and/or aprotic media, respectively.[17,18] Therefore, solutions containing 15 mM aqueous citrate/DMF, 40/60, 0.1 M TBAHFP, 0.3 M KCl, pH 9, and 100% of DMF + 0.1 M TBAHFP were prepared and used as mixed and aprotic media. In Figure 2 the electroreduction of nitrofurantoin in mixed media is shown. As can be seen from this figure, two different reduction processes are evident: (a) a first reversible peak (Ic, Ia), which corresponds to the one-electron transfer resulting in the generation of the nitro radical anion ($E_{pc} = -610$ mV; $E_{pa} = -540$ mV) and (b) a second irreversible peak (IIc), which corresponds to the formation of the hydroxylamine derivative involving a three-electron transfer process from the previously formed radical ($E_{pc} = -950$ mV). The characterization of the nitro radical anion of nitrofurantoin was carried out by applying a methodology based upon the Ipa/Ipc ratio, which is possible to obtain only from the cyclic voltammogram of the isolated couple $ArNO_2$/$ArNO_2^{\bullet -}$ (inset, Figure 2).[19–21] In this condition, there is no interference with other electron transfers such as peak IIc.

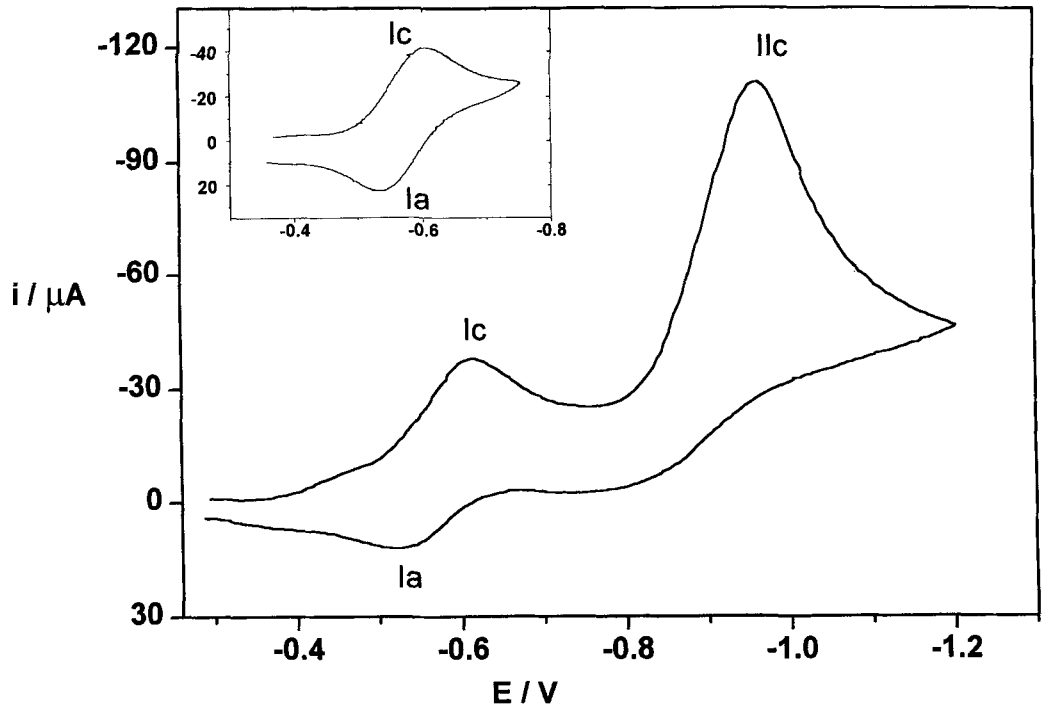

FIGURE 2 Cyclic voltammogram of 5 mM nitrofurantoin solution in mixed media (0.015 M aqueous citrate/DMF, 40/60) at pH 9.0. Sweep rate: 1 V s^{-1}.

From the obtained experimental data in both media, it can be concluded that: (a) the first step in the electroreduction of nitrofurantoin corresponds to an ECi mechanism, i.e., an irreversible chemical reaction following the charge-transfer step occurs; (b) the chemical reaction is of second order, i.e. a disproportionation reaction (dismutation) according to the following equation:

$$2R{-}NO_2^{\bullet -} + 2H^+ \rightarrow R{-}NO_2 + R{-}NO + H_2O$$

(c) There is an increase in the stability of the nitro anion formed from nitrofurantoin with an increase in pH in mixed medium, as illustrated by an increase in the Ipa/Ipc ratio (Table I). Consequently, the lifetime of the $R{-}NO_2^{\bullet -}$ species is increased at more alkaline pH; (d) studies at pH 7.4 to test the appearance of the signal corresponding to the one-electron reduction process were successful after successive sweeps; (e) pH 9 was selected for kinetic characterization due to a best resolution of the signals in this condition; (f) pH-independent values of cathodic peak potentials of the reversible couple were found, proving that no proton transfer precedes the electrode process (Table I); (g) experimental k_2 values in optimal mixed and aprotic media were: $k_2 = 201 \pm 13\,L\,mol^{-1}\,s^{-1}$ and $k_2 = 110 \pm 21\,L\,mol^{-1}\,s^{-1}$, respectively.

Controlled Potential Electrolysis

CPE of nitrofurantoin in mixed and aprotic media to obtain the nitro radical anion in bulk solution (big quantity) were carried out. Thus, electrolysis at an applied potential of −650 mV was made and cyclic voltammetric and spectroscopic (UV–Visible and EPR) curves for detection were recorded.

TABLE I pH dependence of the $R{-}NO_2/R{-}NO_2^{\bullet -}$ couple from nitrofurantoin in the optimal mixed medium. Sweep rate: $1\,V\,s^{-1}$

	pH					
	7.4	8.0	9.0	10	11	12
Ipa/Ipc	0.58	0.80	0.90	0.91	0.94	0.96
$-E_{pc}$ (mV)	0.61	0.60	0.61	0.61	0.60	0.62

(a) Cyclic voltammetry Figure 3 shows the cyclic voltammograms from electrolyzed nitrofurantoin at different intervals of time within 30 min. As the time of electrolysis increases the signals Ic, Ia and IIc decrease, both in long and short sweep modes (Figure 3A and B, respectively).

The evolution of the peak current corresponding to each peak with the electrolysis times are shown in Table II. We can observe that in both conditions, i.e., long and short amplitude, there is an increase in the anodic current value in the first 15 min of electrolysis concomitantly with a decay of the cathodic currents from the beginning of electrolysis. These results indicate that in addition to the disappearance of the nitro compound in the solution (reflected by the drop of the Ic), a generation of the nitro radical anion in a measurable concentration is produced. Also, this generation is supported by the increase of the I_{pa}/I_{pc} ratio of the couple $RNO_2/RNO_2^{\bullet -}$ (Table II). Similar results were obtained in aprotic media and the values of the peak current and current ratio are summarized in Table II.

(b) UV–Visible spectroscopy UV–Visible curves of the time-course of electrolysis were recorded at different intervals in aprotic medium (Figure 4). We can see that the absorption at $\lambda_{max} = 366$ nm decreases during CPE. This absorption is characteristic of a nitroaromatic moiety[24] and therefore its diminution indicates that there was reduction of this group. On the other hand, the increases in the absorption at 292 and 420 nm, indicates that there is a new species in solution. Doing a differential spectrum (inset, Figure 4) it is possible to observe clearly the spectrum of this new species. According to a previous study,[10] similar spectroscopic changes were observed after the reduction of nitrofurantoin by heart lipoamide dehydrogenase and the nitro radical anion was identified in a similar fashion as a new formed species.

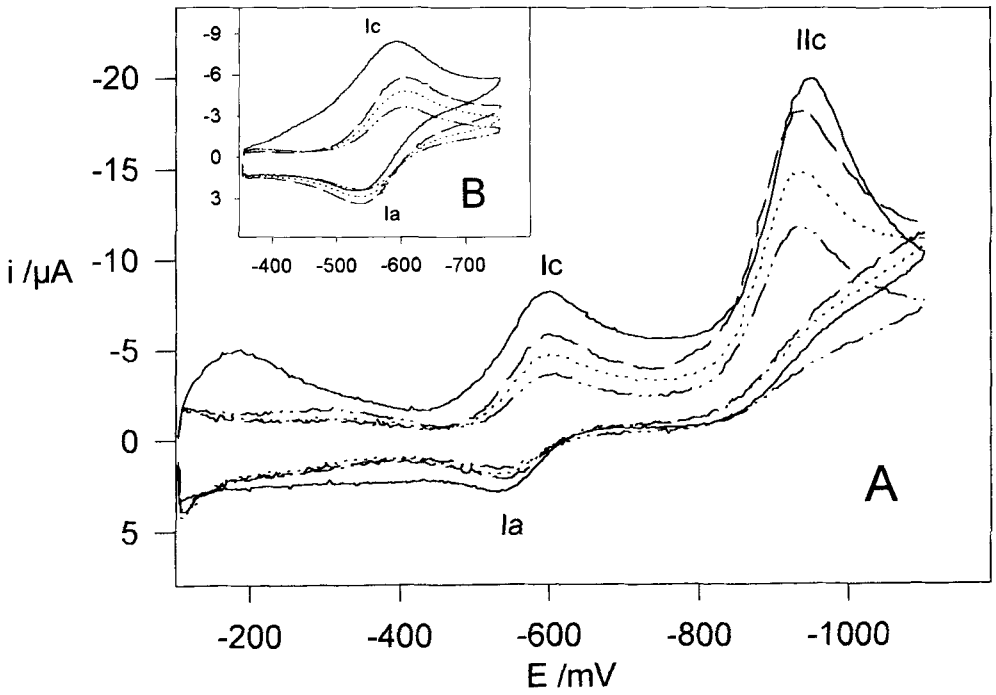

FIGURE 3 Long (A) and short (B) amplitude cyclic voltammograms of 1 mM of nitrofurantoin (in mixed media) electrolyzed at −650 mV at different times: (—) 0, (– – –) 10, (· · · · · ·) 15 and (–· –·) 30 min. Other conditions as in Figure 2.

TABLE II Cyclic voltammetry, at two different potential amplitudes, peak current evolution after CPE in mixed and aprotic media for 1 mM nitrofurantoin concentration

Time (min)	Long sweep			Short sweep					
				Mixed media			Aprotic media		
	Ic (μA)	Ia (μA)	IIc (μA)	Ic (μA)	Ia (μA)	Ipa/Ipc	Ic (μA)	Ia (μA)	Ipa/Ipc
0	8.24	3.38	20.15	8.35	2.46	0.78	10.89	3.28	0.81
15	6.12	3.45	17.89	6.12	3.19	1.02	8.45	4.15	1.06
20	4.12	3.04	14.67	4.63	2.78	1.10	5.12	3.89	1.10
30	3.26	2.81	12.04	3.58	2.52	1.15	3.48	3.51	1.08

To confirm the formation of the nitro radical anion, the variations of the absorption at 420 nm after bubbling of pure oxygen in the mixture of reaction were studied. In Figure 5 we observe a decrease of the absorption with the increase of O_2 bubbling time reaching 20.6% of diminution in 30 min. The decrease of the peak could be ascribed to the reactivity of nitro radical anion (responsible for the absorbance at 420 nm) with the molecular oxygen, according to the following well-known equation:

$$R{-}NO_2^{\bullet -} + O_2 \rightarrow O_2^{\bullet -} + R{-}NO_2$$

(c) EPR The electrochemical reduction of nitrofurantoin to the corresponding nitro radical anion and its detection by EPR was carried out in aprotic media. The spectrum of this radical is shown in Figure 6 (curve 2). After the interpretation of the EPR spectrum by means of a simulation process we were able to determine the

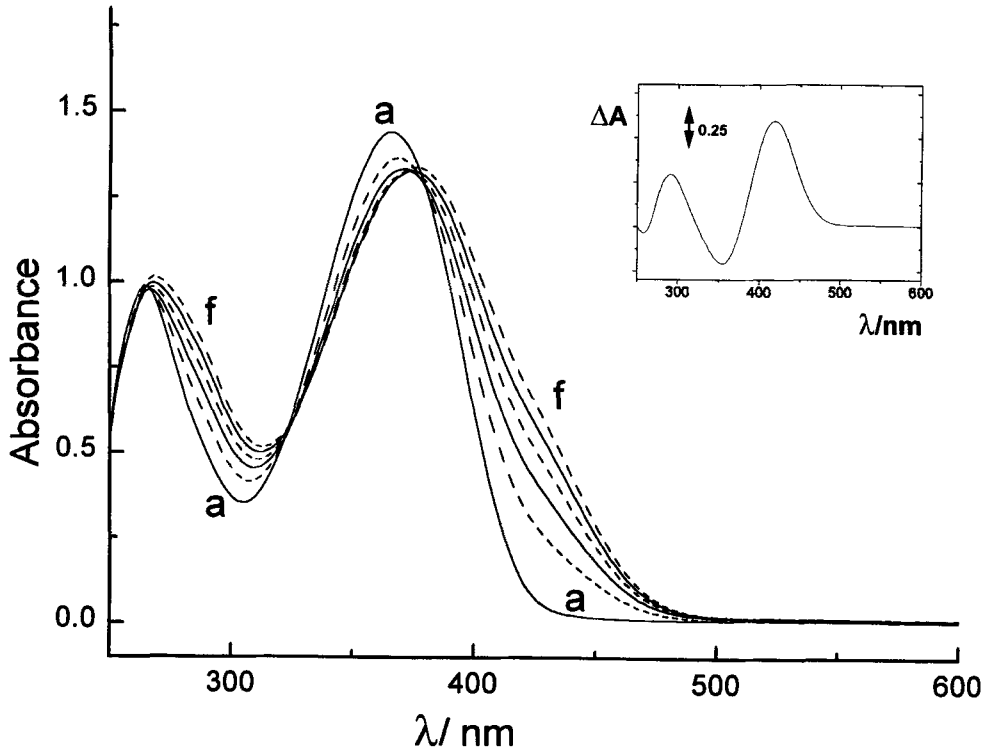

FIGURE 4 Time-course of CPE of 0.05 mM nitrofurantoin solution followed by UV–Vis spectrum. (a–f): 25 min. Inset: Differential spectrum of the nitro radical anion formation from nitrofurantoin.

coupling constants for all magnetic nuclei. In Table III the comparison between our values of the hyperfine constant with the constant values obtained in Ref. [22] after enzymatic reduction of nitrofurantoin are included. As can be seen, no significant differences between the values were observed.

In conclusion, after this voltammetric and spectroscopic experiments we were able to generate the nitro radical anion from nitrofurantoin in solution (*in vitro*) by characterizing its main electrochemical and spectroscopic parameters.

Reactivity of the Nitro Radical Anion Electrochemically Generated from Nitrofurantoin with Glutathione (GSH)

Studies on the reactivity of GSH with the generated radical on microelectrodes were not possible to perform, due to interference of the thiol molecule on the signal of the radical. Thus, only studies with generation of nitro radical anion by electrolysis were conducted.

(a) Cyclic voltammetry By this technique we were able to evaluate the second voltammetric peak produced in the reductive process. In this regard the signal was proportional to the quantity of radical present in the solution, i.e. proportional to the radical produced by electrolysis.

Figure 7 shows the cyclic voltammograms of nitrofurantoin after 15 min in the absence and the presence of different concentrations of GSH. It is possible to observe that the peak IIc decreases concomitantly with the increase in GSH concentration. In the same figure, the evolution of this effect is plotted. The explanation for this change in the peak current may be due to the potential reaction of GSH with the radical lowering of amount of nitrofurantoin available for voltammetric measurements.

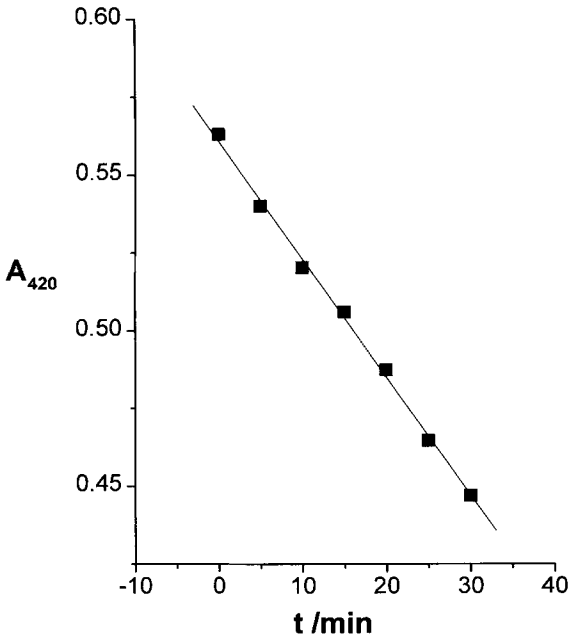

FIGURE 5 Effect of oxygen on the time-course of absorbance variation at 420 nm during CPE of a 0.05 mM nitrofurantoin solution. Oxygen was bubbled after 15 min of the onset of CPE.

(b) UV–Visible spectroscopy In Figure 8, we can observe the effect of GSH on the evolution of the absorption at 420 nm. Curve A represents the increase that occurs during the electrolysis of nitrofurantoin in aprotic media. On the other hand, curve B represents the same evolution when 1 mM GSH was added before the start of electrolysis. Its clear that the quantity of radical produced during the electrolysis is significantly lower in the presence of GSH, since at 25 min of CPE a decrease of 67% in the intensity was observed.

(c) EPR When the nitro radical anion was formed by CPE, GSH was added to the solution. By the analysis of the spectrum we try to establish the scavenger ability of the thiol in this system. In Figure 6 (curves 2 and 3) the scavenging effect of GSH on the nitro radical anion is shown. As illustrated in this figure, the presence of GSH decreased the intensity of the signal until it completely disappeared. Furthermore, to assess the type of the interaction, spin trapping

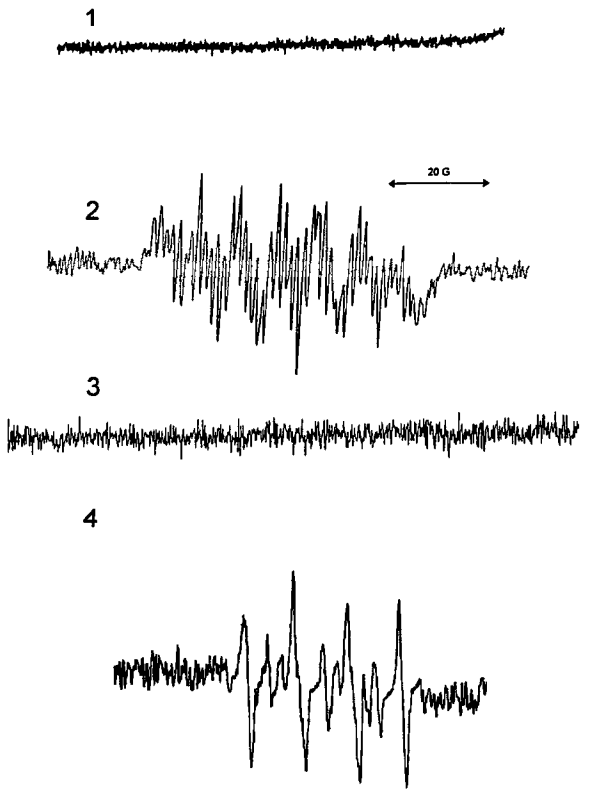

FIGURE 6 Experimental ESR spectra of: (1) 0.1 M TBAHFP in acetonitrile, (2) nitro anion radical from nitrofurantoin at 1 mM drug concentration, (3) nitro anion radical from nitrofurantoin +20 mM GSH, (4) DMPO–GS adduct obtained after electrolysis of 5 mM nitrofurantoin in the presence of 20 mM GSH and 150 mM DMPO. Note that the receiver gain in 4 is five-fold higher than the 1–3 spectra.

TABLE III Experimental hyperfine constant values for the nitro radical anion electrochemically generated from nitrofurantoin. Comparison with constant values obtained from Ref. [4]

	a_{NO_2} (G)	a_{H4} (G)	a_{H3} (G)	$a_{H\alpha}$ (G)	$a_{CH=N}$ (G)	a_{-N-N-} (G)
This paper	9.1	4.8	2.4	0.86	3.1	0.8
*Ref. [4]	10.75	5.67	1.65	0.73	2.23	0.7

**Tritrichomonas foetus* hydrogenosomal and cystolic enzyme reduction.

studies using DMPO were conducted. For these experiments two types of variations were used: (a) the addition of DMPO was done at the same time as GSH, (b) DMPO was added immediately

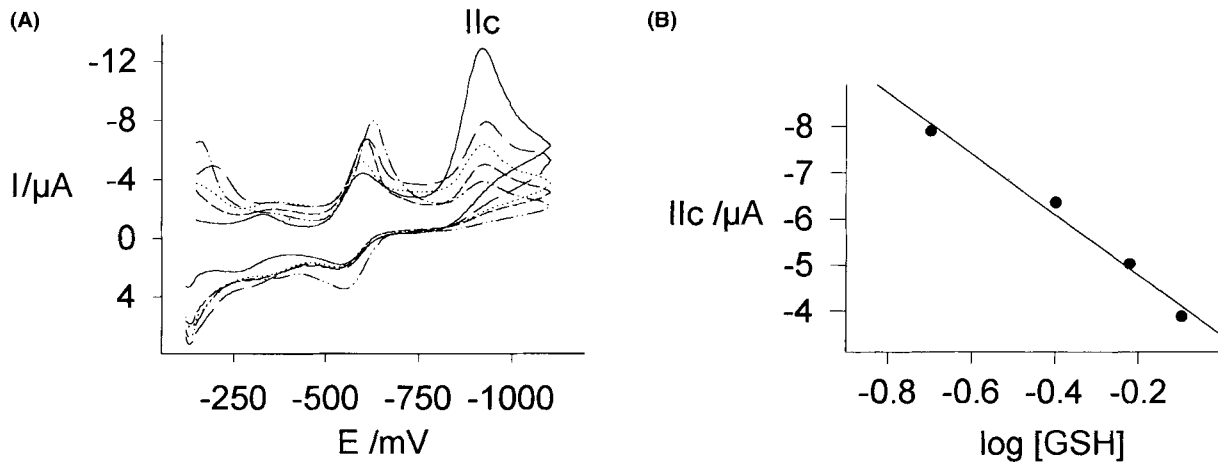

FIGURE 7 Cyclic voltammograms of 1 mM of nitrofurantoin (in mixed media) electrolyzed 15 min at −650 mV in the presence of different GSH concentrations: (——) 0, (– – –) 0.2, (· · · · · ·) 0.4, (– – –) 0.6 and (–· –· –) 0.8 mM. Other conditions are the same as in Figure 2.

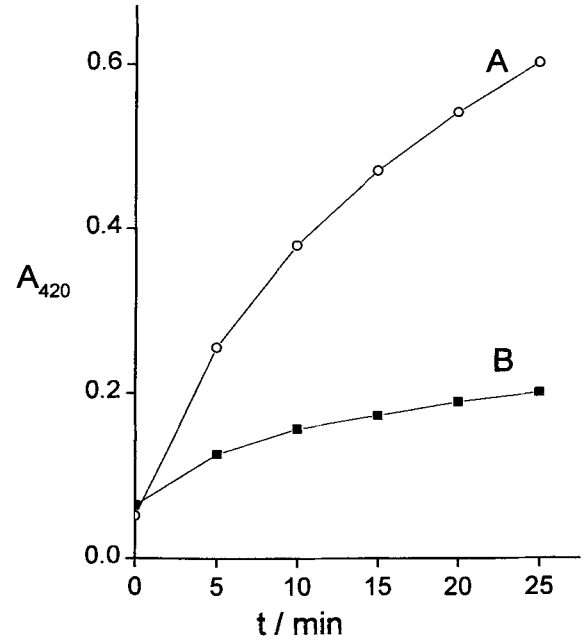

FIGURE 8 Time-course of absorbance variation at 420 nm: (○) CPE of a 0.05 mM nitrofurantoin solution in acetonitrile, (■) CPE in the presence of 1 mM GSH.

after the electrolysis. In both cases, after about 10 min of the addition, a typical ESR spectrum of DMPO appeared (Figure 6, curve 4). This spectrum had a constant value of 13.91 G, the typical value obtained when the trapping of $GS^{\bullet}$ radical by DMPO occurs.[23] Also, in this spectrum additional lines are observed. Control experiments were conducted in order to substantiate this observation. Results from these experiments indicated that the additional lines were due to the nitro radical anion–DMPO adduct formed in parallel with the GS–DMPO. However, these results clearly demonstrate that GSH scavenged the nitro radical anion from nitrofurantoin under anaerobic conditions.

Thus, the present results support the view that under oxygen-depleted conditions, the scavenging by GSH of a reactive drug metabolite (i.e. nitro radical) would appear to be the most likely mechanism of GSH depletion. Since, in the absence of oxygen, superoxide anion or hydrogen peroxide are not formed.

DISCUSSION

In the present study we have investigated the electrochemical reduction of nitrofurantoin to the corresponding nitro radical anion, which was kinetically characterized in both mixed and aprotic media: $k_{2,\,\mathrm{mixed}} = 201 \pm 13\ \mathrm{L\,mol^{-1}\,s^{-1}}$ and

$k_{2,\,\text{aprotic}} = 110 \pm 21\ \text{L mol}^{-1}\ \text{s}^{-1}$, respectively. This radical also was characterized by UV–Visible and EPR spectroscopy.

Furthermore, we have unambiguously demonstrated that in anaerobic conditions, GSH scavenged the nitro radical anion electrochemically generated from nitrofurantoin. These conclusions were reached after considering the following experimental facts: (a) the decrease of peak current, corresponding to the second signal (IIc) in the cyclic voltammograms after 15 min of electrolysis; the absorption at 420 nm by UV–Visible and the EPR signal intensity of the nitro radical after the addition of different GSH concentrations. Furthermore, the EPR spectrum completely disappeared at a 20 mM GSH concentration. (b) The appearance of the signal corresponding to the adduct formation between thiyl radical ($GS^{\bullet}$) and DMPO.

Finally, these findings clearly support that the nitro radical anion formation from nitrofurantoin is scavenged by GSH and could support the view that under hypoxic or anaerobic conditions, it is not possible to discard the reactivity of this thiol molecule with this type of species and perhaps, partially explain the significant loss of GSH under the above experimental conditions. Furthermore, the present results provide a chemical support to employ other different thiol compounds than GSH to treat a possible intoxication exerted by nitrofurantoin, i.e. N-acetylcysteine.

Acknowledgments

This work was supported by grants from FONDECYT (Project 8970023) and D.I.D. (U. de Chile). Also, the collaboration of CEPEDEQ is also acknowledged.

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