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REACTIVITY OF THE NITRO RADICAL ANION FROM NISOLDIPINE WITH N-ACETYLCYSTEINE: EPR SPECTROSCOPIC AND ELECTROCHEMICAL EVIDENCE

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ABSTRACT

This paper reports the scavenging of the nitro radical anion of nisoldipine by N-acetylcysteine assessed by cyclic voltammetry (CV) and EPR spectroscopic techniques.

Studies by CV on the reactivity of the radical were conducted on the mercury electrode in mixed media at pH 9.0 (0.012 M aqueous citrate buffer/DMF 40/60, 0.1 M TBAI). Interaction rate constant was significantly higher than that of the second order decay rate constant of the radical (k_i = 4.857 [Msec]$^{-1}$ *vs* k_2 = 283 [Msec]$^{-1}$.

EPR spectra recorded *in situ* using DMF/0.1 N NaOH (pH 13) of the nitro radical anion from nisoldipine electrochemically generated was completely inhibited for a 20 mM concentration of N-acetylcysteine.

Key Words: Nitro radical anion, cyclic voltammetry, EPR, nisoldipine, N-acetylcysteine.

RESUMEN

En este trabajo se evalúa la interacción entre el anión radical nitro de nisoldipino y N-acetilcisteína tanto por voltametría cíclica sobre mercurio como por Espectroscopía de Resonancia Paramagnética de Espín Electrónico. Los resultados obtenidos por voltametría cíclica en medio mixto a pH 9.0 (tampón acuoso citrato 0.012 M/DMF, 40/60) muestran que la constante de interacción es significativamente mayor que el decaimiento natural de segundo orden del radical (k_i = 4.857 [Msec]$^{-1}$ *vs* k_2 = 283 [Msec]$^{-1}$.

El espectro de Resonancia Paramagnética de Espín correspondiente al anión radical nitro de nisoldipino obtenido por generación electroquímica es

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inhibido completamente por una concentración 20 mM de N-acetilcisteína, otorgando una prueba directa del efecto atrapador de este tiol sobre este tipo de radicales.

Palabras Claves: Anión radical nitro, voltametría cíclica, RPE, nisoldipino, N-aceilcisteína.

INTRODUCTION

Nisoldipine (± 3-isobutyl-5-methyl 1,4-dihydropyridine-4-(2-nitrophenyl-)pyridine-3,5-dicarboxylate) is a calcium antagonistic dihydropyridine derivative, which has been developed as an antihypertensive and antianginal drug.

1,4-dihydropyridine derivatives are rapidly and extensively metabolized in rat, dog and man[1-4]. There are also no important species differences regarding the metabolic patterns. Most metabolites have been identified. However, some common biotransformation reactions, such as, dehydrogenation of the 1,4-dihydropyridine system, oxidative ester cleavage, oxidative O-demethylation and subsequent oxidation of the resulting primary alcohol to the carboxylic acid has to be considered[3,4]. However, the reduction of the nitro group to yield amino metabolites has been reported as an additional biotransformation route for nisoldipine[5]. This pathway accounts for about 1% and 3% of the dose for this drug. In spite of these data, the significance of the reduction intermediates of nisoldipine has not yet been established.

Electrochemical techniques can detect and quantify the interaction between a reduction product and its target (e.g. DNA or its individual bases) as interaction will result in modifications to the current-voltage response[5-9]. In cyclic voltammetry after traversing the potential region of interest the direction of the scan is reversed, and the electrode reactions of intermediates and products formed on the forward scan can often be detected. Cyclic voltammetry is, therefore, extremely useful for measuring the stability or reactivity of reduction products, as reflected in the return-to-forward peak current ratio.

The reduction of a homocyclic or heterocyclic nitrocompound is complex. Under appropriate conditions the nitro group can accept a single electron to yield the radical anion, which is not stable under alkaline conditions. Two- and four-electron addition form the nitroso and hydroxylamine derivatives, respectively. A further two-electron addition produces the amino, being the result of a total six-electron reduction. The electrochemistry of 4-(nitrophenyl) substituted, 1,4-dihydropyridines has been extensively studied in the last few years[10-13]. Thus, our laboratory has been devoted to kinetically characterize the nitro radical anions electrochemically generated both in mixed media and aprotic media[14-17].

Thiol-containing compounds are important in many biochemical and pharmacological reactions: disulfide bonds are important in deciding the total structure of proteins, and in many drugs the thiol group is an important reactive center that determines both their effects and their side-effects. Aminothiols, such as glutathione can protect biological systems from the cellular damage caused by ionizing radiation and free radicals. Thiols can also protect against the action of most chemotherapeutic agents, including the anaerobic cytotoxicity of nitro aromatic compounds due to oxygen reactive species formation (redox cycling), such as, superoxide anion or hydroxyl radical[18,19]. Moreover, N-acetylcysteine has been employed as cytoprotective agent in damage produced by radiations[20] and paracetamol[21].

Recently, we have proved the *in vitro* reactivity of the one-electron reduction product from several nitroaryl 1,4-dihydropyridines with some relevant biological targets, such as, glutathione, cysteamine and the nuclei acid bases, adenine and uracil[17].

Considering the above described data, in this paper we assessed the feasibility of the interaction between the nitro radical anion from nisoldipine and a relevant thiol such as N-acetylcysteine by both EPR spectroscopy and cyclic voltammetry.

MATERIALS AND METHODS

Drug

Nisoldipine was obtained from Sanitas Laboratories, Santiago, Chile.

Dimethylformamide (DMF), spectroscopic grade, tetrabutylammonium iodide (TBAI), were purchased from Merck and tetrabutylammonium perchlorate was purchased from Fluka.

N-acetylcysteine was purchased from Sigma, St. Louis, USA.

Cyclic voltammetry

Experiments were carried out in an INELECSA assembly PDC 1212, containing 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 486 SX microcomputer was used for data control, acquisition, and treatment.

Electrodes

A Metrohm h.m.d.e. with a drop surface of 1.80 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 voltammograms were carried out at a constant temperature of 25°C and the solutions were purged with pure nitrogen for 10 minutes before the voltammetric runs.

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

Methods

The experimental I_{pa}/I_{pc} ratios were calculated according to Nicholson's procedure, using individual cyclic voltammograms[22]. Furthermore, E_λ was selected to reduce the influence of the second cathodic peak. Fifteen runs with E_λ varying between -1195 and -1185 mV *versus* SCE did not show a significative variation in I_{pa}/I_{pc} values (Coefficient of Variation = 2.2%).

Kinetic reaction orders for the nitro radical anion was quantitatively assayed for first and second-order coupled reactions according to some previous studies[23,24].

To ensure that changes in the voltammetric parameters (I_{pa}/I_{pc} ratio, E_{pc}, etc.) of the drug by the addition of N-acetylcysteine was due to reaction between electrochemically generated radical, and not due to an electrode adsorption phenomenon, 100 µl of cyclohexanol was added to mixed media[8,17].

To quantitatively estimate the interaction rate constant (k_i) for the reaction between the nitro radical anion generated from nisoldipine and N-acetylcysteine, a method previously developed in our laboratory was used[15,16].

Mixed media

To obtain the optimal mixed media conditions, different supporting electrolyte as KCl, LiCl tetrabutyl ammonium iodide (TBAI) and tetrabutyl ammonium perchlorate were tested. Similarly, citrate, camphor and hexamethylphosphotriamide (HMPA) were tested as active-surface substances. The concentrations of these type of substances varied between 0.01 M and 0.1 M. Percentages of DMF varied from 40% 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 MTBAI and 0.3 M KCl. For the studies conducted at pH 7.4, the same composition than that of the media at pH 9.0 was used.

Drug solution

Stock solutions of 10 mM of nisoldipine in DMF were prepared and protected from daylight to avoid photodecomposition. A routine drug concentration of 5.0 mM for all the experiments was used. 50 mM stock solutions of N-acetylcysteine in citrate buffer pH 9.0 were prepared to obtain solutions with concentrations ranging from 0.1 mM to 5 mM.

EPR measurements

The nitro radical anion from nisoldipine was generated *in situ* by electrochemical reduction at room temperature. A 5 mM solution of nisoldipine containing 0.1 M tetrabutyl ammonium perchlorate in DMF/aqueous 0.1 N NaOH pH 13, 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 Hz field modulation. Hyperfine splitting constants were estimated to be accurate within 0.05 G.

RESULTS

The main goal of this paper was to assess the reactivity of the nitro radical anion from nisoldipine with N-acetylcysteine, using cyclic voltammetry and EPR spectroscopy.

In previous works[9-12] has been proved that the addition of an aprotic solvent to an aqueous buffer enables the isolation of the reversible couple corresponding to the one-electron reduction, i.e. the nitro radical anion.

Thus, we have studied the experimental conditions to obtain an optimal mixed media to stabilize the radical and characterize its reactivity with N-acetylcysteine. Results of these studies showed that a mixed media containing 15 mM aqueous citrate solution/DMF, 40/60, 0.1 M TBAI, 0.3 M KCl and pH 9.0 permitted the isolation and stabilization of the nitro radical anion of nisoldipine (Figure 1). We have also assayed this mixed media at pH 7.4 (closer to a physiological condition) to test the reactivity of the radical with N-acetylcysteine. However, at this pH the couple was evidenced, but exhibited a poor resolution. Therefore, we selected the above described conditions to study the reactivity.

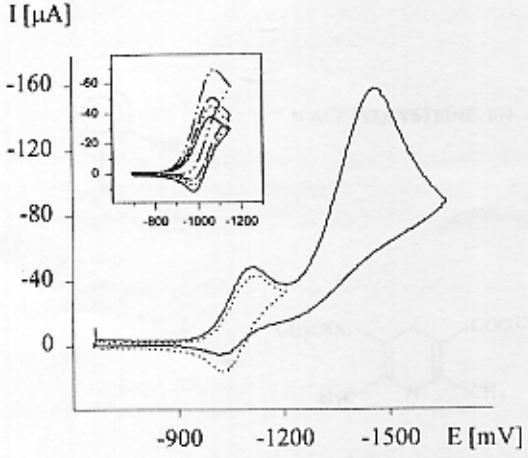


FIG. 1. Cyclic voltammograms (CV) of nisoldipine in mixed media at pH 9.0. Solid line: extended sweep. Dashed line: short sweep. Sweep rate: 1 Vs^{-1}. Inset: Effect of N-acetylcysteine on the isolated Ar-NO_2/Ar-$NO_2^{\bullet-}$ couple: (——) 0 (.....) 1 (——) 2 mM (~.~) 5 mM.

Furthermore, the stability of the Ar-NO_2 species was assessed 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 behavior for an irreversible chemical reaction following a charge-transfer step, i.e. the EC process[25]. Furthermore, the cathodic peak potential depends on nisoldipine concentrations and sweep rates, with a $dE_{pc}/d\log c$ and $dE_{pc}/d\log v$ values varying between 20 and 22 mV. These values agree 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.

Also, to check the order of the following chemical reaction, I_{pa}/I_{pc} ratio dependence on concentration of the nisoldipine was studied. The theory of cyclic voltammetry for a second-order reaction initiated electrochemically has been studied exhaustively by Olmstead[25]. In our experiments we have found that an increase in the nisoldipine concentration, keeping both DMF percentage and scan rate constant, resulted in a decreased I_{pa}/I_{pc} value according to the predicted by Olmstead[25]. From the above experimental data it can be concluded that the chemical reaction was of second order, i.e. a disproportionation reaction (dismutation).

Second-order constant for the decay of the Ar-$NO_2^{\bullet}$ species was assessed from single cyclic voltammograms of nisoldipine, according to Olmstead's procedure[25] from the following relationship:

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

Confirming the second order character of the following chemical reaction, plot of kinetic parameter, ω, *vs* time constant, τ, was linear, with average correlation coefficients not lower than 0.97 for the drug. As expected, the second order rate constant decreased and half-life increased as DMF percentages increased. Experimental k_2 value in the optimal mixed media was: $k_2 = 283 \pm 16.6$ l mol^{-1} s^{-1} and the calculated half-life, $t_{1/2} = 0.70$ minutes for 5 mM concentration of nisoldipine.

The characteristic linear dependence between ω versus τ for a second order chemical reaction of the radical was maintained when N-acetylcysteine was added to the reaction medium. However, concomitantly with the increase in its concentration, an increase in the slope of this plot was observed (Figure 2). This effect is due to the contribution of two different simultaneous competitive decay pathways, i.e. natural decay of the radical and its reaction with N-acetylcysteine (see Scheme).

Addition of N-acetylcysteine to nisoldipine solution decreased the I_{pa}/I_{pc} value as compared with the control (without N-acetylcysteine, Inset. Figure 1). The decrease in the current ratio was of 15% at nisoldipine: N-acetylcysteine ratio of 5:1.

The interaction rate constant (calculated according to the procedure described in ref. 16) in the optimal experimental conditions was $k_i = 4.857$ $[Msec]^{-1}$. This value was significantly higher than the corresponding to the natural decay of the radical ($k_2 = 283$ $[Msec]^{-1}$).

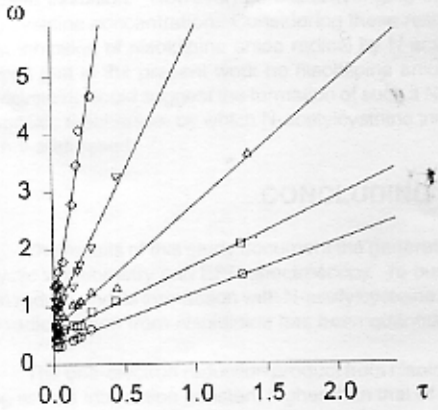
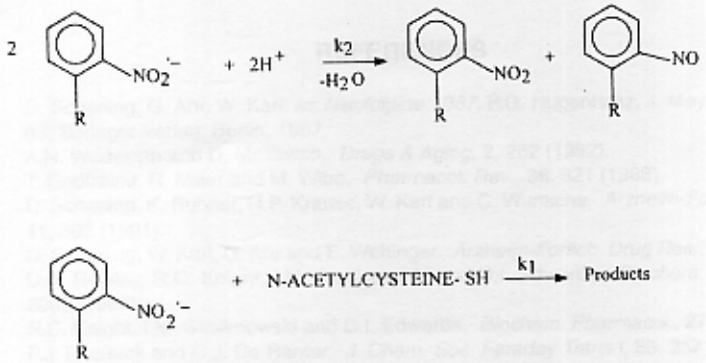
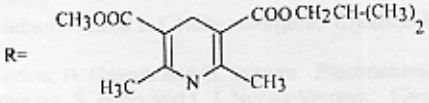


FIG. 2. Dependence of ω versus τ. (○) No additions. Addition of N-acetylcysteine: (□) 0.2 mM (Δ) 0.5 mM (▽) 1.0 mM (◇) 5 mM.





Scheme. Parallel reactions involved in the decay of the nisoldipine nitro radical anion when N-acetylcysteine is added.

EPR studies

The *in situ* generation leading to radical species was carried out in DMF/0.1 N aqueous NaOH pH 13 by applying the potential corresponding to the first one-electron reversible reduction process, just found in the cyclic voltammetric experiments.

As can be seen in Figure 3, the hyperfine pattern corresponding to the nitroanion of nisoldipine appears completely resolved into 35 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 H_3 and H_5, one triplet due to two equivalent hydrogens, H_4 and H_6 and one doublet due to hydrogen of the dihydropyridine ring. The hyperfine constant values were obtained by an EPR simulation program and are listed in Table I.

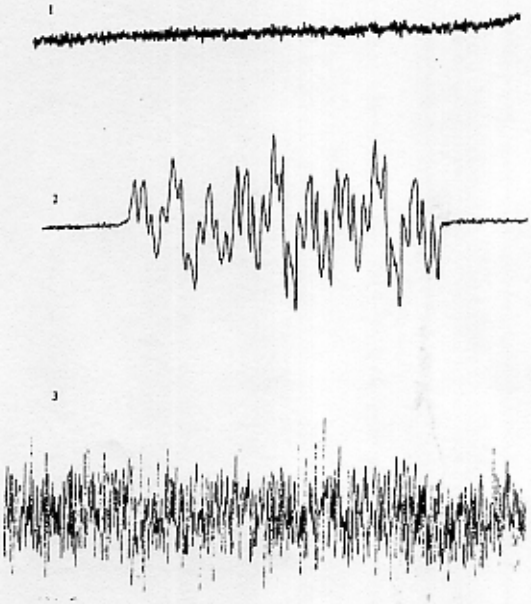


FIG. 3. EPR spectrum of nisoldipine anion radical electrochemically generated. Line 1 is the control (DMF/0.1 N NaOH, 0.1 M tetrabutylammonium perchlorate. Line 2 is an EPR spectrum of nisoldipine anion radical produced in a system of 1 mM nisoldipine and 0.1 N NaOH, final pH 13. Line 3 illustrates the complete inhibition of free radical generation by 20 mM N-acetylcysteine, final pH 13.

TABLE I. Experimental hyperfine splitting constants for the nitro radical anion from nisoldipine.

a^{*}_{NO2}	a_{H5}	a_{H3}	a_{H4}	A_{H6}	$A_{H\text{-}DHP}$
10.87	3.59	2.83	1.09	1.09	0.65

*a values are expressed in Gauss.

As can be seen from Figure 3, the addition of 20 mM N-acetylcysteine to nisoldipine nitro anion radical generated by electrochemical reduction resulted in a decrease of the peak intensity until a complete inhibition. However, partial scavenging effect becomes to be evident from a 10 mM N-acetylcysteine concentration. Considering these results, it can be possible to offer a direct evidence for an inhibition of nisoldipine anion radical by N-acetylcysteine under our experimental conditions. The fact that in the present work no nisoldipine anion radical was detected following addition of N-acetylcysteine could suggest the formation of such a N-acetylcysteine/nisoldipine conjugate. However, the specific mechanism by which N-acetylcysteine inhibits the generation of nisoldipine anion radical was not addressed.

CONCLUDING REMARKS

The results of this study document the generation of nitro radical anion from nisoldipine directly by cyclic voltammetry and EPR spectroscopy. To our knowledge is the first EPR study of nisoldipine anion radical and its interaction with N-acetylcysteine. On the other hand, a significant reactivity of the nitro radical anion from nisoldipine has been quantitatively demonstrated by cyclic voltammetry.

The one-electron reduction product from nisoldipine significantly reacted with N-acetylcysteine, being its rate interaction constant higher than that of the natural decay of the radical. These findings enhance our understanding of nisoldipine metabolism and will be useful to support a chemical basis for the use of N-acetylcysteine as free-radical scavenger of this type of species. Moreover, these data could be extended to substantiate a possible new therapeutic applications, i.e. to prevent the appearance of potential cytotoxicity phenomena caused for reduction intermediates of nisoldipine such as, the Ar-$NO_2^{\bullet-}$ species.

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