Research Article

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Reactivity of C4-indolyl substituted 1,4-dihydropyridines toward superoxide anion ($O_2^{\bullet-}$) in dimethylsulfoxide

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Reactivity of two new C4-indolyl substituted 1,4-dihydropyridines (1,4-DHPs) toward superoxide anion ($O_2^{\bullet-}$) in dimethylsulfoxide (DMSO) is reported. Reactivity was followed by electrochemical and spectroscopic techniques. Gas chromatography-mass spectrometry (GC–MS) was used to identify the final products of the reaction. C4 indolyl-substituted-1,4-DHPs reacted toward $O_2^{\bullet-}$ at significant rates, according to the calculated kinetic rate constants. Results are compared with 4-phenyl-DHP and the commercial 1,4-DHPs, nimodipine, nisoldipine, and amlodipine. Indolyl-substituted 1,4-DHPs were more reactive than the commercial derivatives. The direct participation of proton of the 1-position of the secondary amine in the quenching of $O_2^{\bullet-}$ was demonstrated. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: C4-indolyl substituted 1,4-dihydropyridines; superoxide radical anion; reactivity cyclic voltammetry; UV–Visible spectroscopy; first-order kinetic rate constants

INTRODUCTION

Interest has recently grown in studying antioxidants, an important class of biologically active compounds essential for maintaining the so-called antioxidant status of the human body. Their main function consists in diminishing the concentrations of reactive oxygen species in the cells of living organisms. Oxidative stress may arise in the human body as a result of the formation of active intermediates of radical nature in the course of oxygen reduction. Reactive oxygen species are the by-products of the most important metabolic reactions involving molecular oxygen. A variety of enzymatic and non-enzymatic biomolecules have been reported to reduce molecular oxygen to superoxide radicals, $O_2^{\bullet-}$.[1–3] This radical can be induced by electron transfer reaction *in vivo* to generate other reactive oxygen species such as hydroxyl free radical and hydrogen peroxide. The $O_2^{\bullet-}$ is reactive and toxic, causing oxidation of bio-macromolecules as well as initiate radical-chain oxidation in tissues. In consequence, compounds maintaining their original pharmacological activities but with another added ability, such as its reactivity toward $O_2^{\bullet-}$, are of major interest.

1,4-DHP analogs of nifedipine, are of major significance in the treatment of a number of cardiovascular diseases, including angina, hypertension, peripheral vascular disorders, and a number of arrhythmic conditions. The synthesis, the properties of these drugs and the mechanisms by which they function have been extensively reviewed during the past years.[4–7]

In the last years our effort has been focused in the synthesis of new C4-substituted-1,4-DHPs[8–11] with strengthened antioxidant properties and the study of the interactions of different redox centers coexisting in the DHP molecule. An example of this is the inclusion of the indole moiety as good choice for a second redox center in a 1,4-DHP molecule. The indole nucleus it has been reported to possess a wide variety of important biological properties such as anti-inflammatory,[12] antibacterial,[13] anticonvulsant,[14] and antioxidant.[15]

Lavilla *et al.*[16] have previously reported the design, synthesis, and pharmacological evaluation of a series of 4-(3-indolyl) DHPs. Also, these authors demonstrated that these derivatives show a similar activity to that of nifedipine on the inhibition of radical oxygen-derived species production. The antioxidant properties of such DHPs was displayed regardless of their substitution pattern.

Several authors[17–19] have recognized that 1,4-DHPs offer an antioxidant protective effect that may contribute to their pharmacological activity. Moreover, a direct reactivity between some commercial 1,4-DHPs and superoxide radical anion based on electrochemical methods was also previously reported by our laboratory.[20,21]

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In this paper, a study on the reactivity of synthesized C4-indolyl substituted 1,4-DHP derivatives with superoxide anion ($O_2^{\bullet}$) in dimethylsulfoxide (DMSO) is reported. To account the reactivity, spectroscopic, electrochemical, and chromatographic techniques were used. Apparent first-order kinetic rate constant values were calculated.

EXPERIMENTAL METHODS

C4-indolyl substituted 1,4-dihydropyridines (Fig. 1)

Ethylacetoacetate (15.0 mmol) and concentrated ammonia hydroxide (10.0 mmol) were added to a mixture of 6 mmol of indole 3-carboxaldehyde or indole 5-carboxyaldehyde in 20 ml of ethyl alcohol.

This mixture was heated under reflux for 30 h under nitrogen atmosphere. The crude solid is filtered and recrystallized in ethyl alcohol/water (50/50). Synthesized compounds had the following characteristics.

2,6-Dimethyl-3,5-dietoxycarbonyl-4-(3-Indolyl)-1,4-dihydropyridine (4-(3-indolyl)-DHP)

Yield: 73%. m.p.:183–184 °C. IR (KBr): δ_{max} 3344.4; 2978.1; 1676.7; 1487.1; 1367.9; 1305.6; 1215.4; 1100.3; 1020.4; 807.2; 744.7. ^{1}H-NMR (300 MHz, DMSO δ_6): δ_{max} 1.18 (*t*, 6H, 2× —CH_2—C**H$_3$**); 2.31 (s, 6H, 2× —C**H$_3$**); 4.03 (q, 4H, 2× —O—C**H$_2$**—CH_3); 5.23 (s, 1H, Ar—C**H**<); 6.975 (m, 3H, *J* = 7,83 Hz, 3× Ar—H); 7.33 (d, 2H, *J* = 7.92 Hz, 2× Ar—H); 7.92 (d, 2H, *J* = 7.92 Hz, 2× Ar—H); 8.89 (s, 1H, DHP-N—H); 10.74 (s, 1H, Indole-N—H). ^{13}C-NMR (75 MHz, DMSO δ_6): (2×)13.70; (2×)17.64: 29.98; (2×)58.25; (2×)101.43; 110.85; 117.54; 118.91; 119.77; 121.31; 122.17; 125.25; 135.54; (2×)143.58; (2×)166.71. Anal. Calcd. for $C_{21}H_{24}N_2O_4$: C 68.46; H 6.57; N 7.60. Found: C 67.89; H 6.71; N 7.61.

2,6-Dimethyl-3,5-dietoxycarbonyl-4-(5-Indolyl)-1,4-dihydropyridine (4-(5-indolyl)-DHP)

Yield: 61%. m.p.:178–180 °C. IR (KBr): δ_{max} 3351.2; 2978.5; 1657.6; 1481.6; 1371.4; 1303.5; 1208.2; 1100.5; 1018.2; 727.0. ^{1}H-NMR (300 MHz, DMSO δ_6): δ_{max} 1.20 (*t*, 6H, 2× —CH_2—C**H$_3$**); 2.32 (s, 6H, 2× —C**H$_3$**); 4.04 (q, 4H, 2× —O—C**H$_2$**—CH_3); 4.99 (s, 1H, Ar—C**H**<); 6.37 (s, 1H, Ar—H); 7.00 (d, 1H, *J* = 7.92 Hz, 1× Ar—H); 7.25 (m, 3H, *J* = 7,56 Hz, 3× Ar—H); 8.76 (s, 1H, DHP-N—H); 10.94 (s, 1H, Indole-N—H). ^{13}C-NMR (75 MHz, DMSO δ_6): (2×)13.74; (2×)17.96: 48.802; (2×)58.37; (2×)100.20; 102.53; 110.01; 117.72; 120.76; 124.49; 127.07; 134.08; 138.52; (2×)143.88; (2×)166.71. Anal. Calcd for $C_{21}H_{24}N_2O_4$: C 68.46; H 6.57; N 7.60. Found: C 67.00; H 6.85; N 7.38.

2,6-Dimethyl-3,5-diethoxycarbonyl-4-phenyl-1,4-dihydropyridine [4-phenyl-DHP]

2,6-Dimethyl-3,5-diethoxycarbonyl-4-phenyl-1,4-dihydropyridine [4-phenyl-DHP] was synthesized according to a previous paper.[6,7]

Commercial 1,4-dihydropyridine

Amlodipine (99%): (2-[(2-aminoethoxy)methyl]-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester) was purchased from Sigma-Aldrich. Nisoldipine (97.8%): (1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine carboxylic acid methyl 2-methylpropyl ester) was obtained from Laboratorio Chile, Santiago, Chile. Nimodipine: 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid, 2-methoxyethyl 1-methylethyl ester (Laboratorio Saval, Santiago, Chile).

UV–Vis spectrophotometry

UV–Vis spectra were recorded in the 200–1000 nm range by using a diode array detector Agilent spectrophotometer.

Molar absorptivity

This parameter was calculated from 30–200 μM 1,4-DHP solutions in the absence and with an excess of tetrabutylammonium hydroxide (TBA-OH) in DMSO.

Stock superoxide anion solution. It was prepared by weighing 0.071 g (1 mmol) of potassium superoxide (Sigma-Aldrich, USA), dissolving it in DMSO (spectroscopic grade) and sonicating during 0.5–3 h. From the stock solution stored at −40 °C were prepared individual solutions to study the reactivity with selected

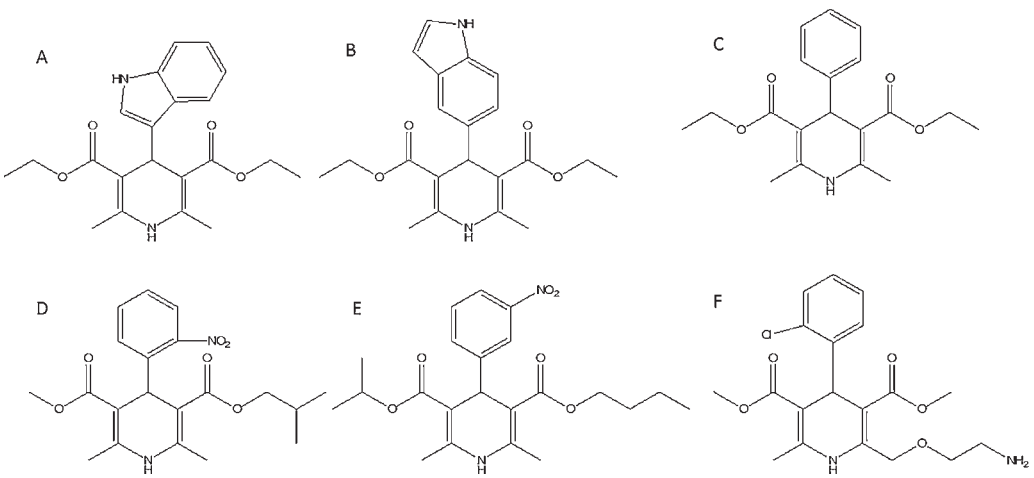

Figure 1. Chemical Structure of derivatives.

1,4-DHPs. Stability studies revealed that the stock solution remains unalterable for at least 2 weeks.

Kinetic experiments were conducted in a pseudo-first order kinetic condition by using a 0.1 mM 1,4-DHP solution and a 20 mM potassium superoxide salt. Measurements were programmed at intervals of 10 min during 5 h.

NMR spectroscopy

The H-NMR spectra were recorded on a Bruker spectrometer advance DRX 300.

Electrochemical experiments

Electrolytic medium was DMSO spectroscopic grade purchased from Merck containing 0.1 M tetrabutylammonium hexafluoro phosphate (TBAHFP, Sigma-Aldrich).

A stationary glassy carbon electrode (0.071 cm^2) was used as working electrode for cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques. The surface of the electrode was polished to a mirror finish with alumina powder (0.3–0.05 μm) before use and after each measurement. Platinum wire was used as auxiliary electrode and all potentials were measured against an Ag/AgCl electrode in saturated KCl. All cyclic voltammograms were carried out at a constant temperature of 25 °C. The return-to-forward peak current ratio, Ipa/Ipc, for the oxygen/superoxide couple was measured for each cyclic voltammogram at 0.1 Vs^{-1} according to the procedure described by Nicholson[22] by using different concentrations of 1,4-DHPs (0–50 mM).

CV and DPV were performed with a BAS-CV 100 assembly and the routine 1,4-DHP concentration was 1 mM. For measurements in oxygen media, O_2 gas was bubbled directly into the cell in order to obtain solutions 1.0 mM O_2 in DMSO, and during the measurement, O_2 gas was flushed over the cell solution using an apparatus consisting of two flow-meters (Cole Palmer 316SS) for oxygen and nitrogen, respectively, equipped with needle valves.

Controlled-potential electrolysis (CPE)

Studies on exhaustive electrolysis were carried out at constant electrode potential (1100 mV) on glassy carbon mesh electrode during 2 h continuously stirred in a 30 ml cell capacity at 1 mM solutions. A three-electrode circuit with a reference electrode Ag/AgCl and platinum wire as counter electrode were used. A BAS-CV 100 assembly was also used to electrolyze the 1,4-DHPs solutions.

GC–MS

A gas chromatograph/mass selective detector (5890/5972) combination (Hewlett-Packard, Palo Alto, CA, USA) and a Hewlett-Packard 7673 autosampler were used for the analyses. The *m/z* range monitored was 45–550 with a scan rate of 1 scan/s; the normal energy electron was set at 70 eV. A Hewlett-Packard Ultra-1 column, 25 cm, −0.2 mm i.d., −0.11 mm film thickness (Little Falls, Wilmington, DE, USA), was used.

HPLC

This technique was used to analyze 1,4-DHP solutions and products generated after the reaction with superoxide anion and the electrolysis (CPE).

Equipment and operation conditions

HPLC measurement were carried out by using a Waters assembly equipped with a model 600 controller pump and a model 996 photodiode array (PDA) detector. The acquisition and treatment of data were made by means of the Millennium version 2.1 software. As chromatographic column a Kromasil C-18 column (4.6 × 150 mm^2) was used. As precolumn a C-18 Bondapak precolumn (30 × 4.6 mm^2) was employed. The injector was a 20 μl Rheodyne valve. An aliquot of the electrolyzed solution and the result solutions of 5 h of reaction toward superoxide radical anion of each 1,4-DHPs was taken and a 20 μl volume of these solutions were injected into the chromatographic system. A mixture of acetonitrile/0.05 M phosphate buffer (55/45) at pH 4.3 was used as mobile phase. The PDA detector operated at 250 nm (product) and 360 nm (1,4-DHPs) for quantification. The flow of mobile phase was maintained at 1 ml/min and a helium bubbling of 30 ml/min was applied to remove dissolved gases.

RESULTS AND DISCUSSION

Reactivity of C4-substituted 1,4-dihydropyridines with superoxide radical anion assessed by voltammetric techniques

Cyclic voltammetry

In a previous paper[23] the optimal conditions for generating and studying the superoxide redox couple were reported. Moreover, this couple was used to reveal an interaction between superoxide with a commercial 1,4-DHP. In the present paper, we have used such methodology to assess the reactivity of synthesized 1,4-DHPs with superoxide. Thus, we have examined the cyclic voltammetric response of $O_2/O_2^{\bullet}$ redox couple on a glassy carbon electrode in the absence and in the presence of 1,4-DHP derivatives as shown in Fig. 2. In this Figure the cyclic

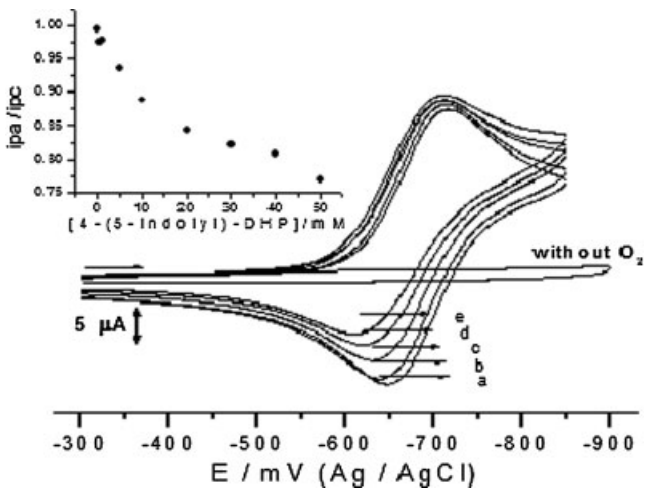

Figure 2. Cyclic voltammograms corresponding to the O_2/O_2:redox couple: (A) O_2 saturated solution; (B) +1 mM; (C) +10 mM; (D) +30 mM; and (E) +50 mM of 4-(5-indolyl)-DHP. Sweep rate: 0.1 Vs^{-1}. Electrolytic media: DMSO + 0.1 M de TBAHFP. Insert: Ipa/Ipc ratio of $O_2/O_2^{\bullet}$ redox couple in the presence of different concentrations of 4-(3-indolyl)-DHP

voltammetric response at four concentrations of 4-(5-indolyl)-DHP in oxygenated DMSO + 0.1 M TBAHFP solutions is displayed. As can be seen, after the addition of 4-(5-indolyl)-DHP solution, the oxidation peak current of $O_2^{\bullet-}$ (anodic current (Ipa), oxygen regeneration) decreases whereas the reduction current (cathodic current (Ipc), $O_2^{\bullet-}$ formation) increases. On the other hand, in the insert of Fig. 2 the effect of increasing concentrations of 4-(5-indolyl)-DHP derivative on the Ipa/Ipc ratio is displayed. These data support that 4-(5-indolyl)-DHP reacts with $O_2^{\bullet-}$, that is, it scavenges $O_2^{\bullet-}$ in DMSO, in a concentration-dependent way. Considering that the direct interaction between the *in situ* generated superoxide and the tested compounds is connected with the decrease of Ipa/Ipc ratio, we have used this ratio as a reactivity criterion. Thus, we have extended the study to other molecules with comparative purposes. Figure 3 shows the variation of Ipa/Ipc ratio as a function of the concentration of the synthesized 1,4-DHPs and commercial 1,4-DHPs (nisoldipine, amlodipine, and nisoldipine). In quantitative terms, 4-(5-indolyl)-DHP produced a decrease of 20% on the current ratio at the same concentration compared with the commercial ones.

Consistent with our results, other authors have reported that the hydrogen at the *N*-position of indole compounds[24] also acts as a proton donor to $O_2^{\bullet-}$.

These results substantiate that both indole and DHP moieties would contribute to the reactivity toward superoxide anion. Also, no significant differences in the reactivity between indolyl-substituted 1,4-DHPs were found. In all the cases, the results also support that superoxide radical anion reacted with the derivatives in a concentration-dependent trend.

Differential pulse voltammetry (DPV)

In these experiments the voltammetric response of 1,4-DHPs on a glassy carbon electrode both in the absence and in the presence of a 1.5 mM potassium superoxide salt solution was observed. C4-indolyl-substituted 1,4-DHPs exhibited two anodic voltammetric signals. As can be seen in Fig. 4A, 4-(5-indolyl)-DHP derivative exhibited a peak I at 992 mV which correspond to the oxidation of the DHP ring. The second peak II due to the oxidation of the indole moiety appeared at 1288 mV. After the addition of the superoxide, peak I completely disappeared (Fig. 4B), but a new peak III at approximately 600 mV appeared. This peak III would correspond to the electrochemical oxidation of the DHP anion ($R—N^-$) generated by the action of superoxide radical anion, which abstracted the proton of the 1-position. On the other hand, peak II suffered a little shift toward more anodic potential values, that is, 1300 mV (Fig. 4B). Current intensity of this peak II did not change under these experimental conditions. Similar experiments but using an organic base such as, TBA-OH instead of superoxide anion were performed. Results revealed that changes affected the intensity of the anodic signal of the DHP ring and the appearance of the signal corresponding to the DHP anion.

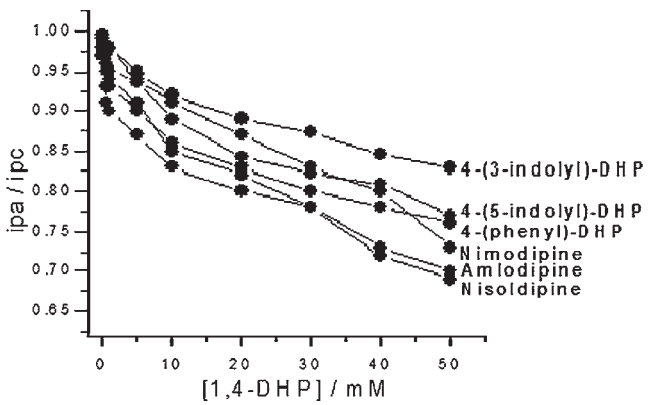

Figure 3. Ipa/Ipc ratio of $O_2/O_2^{\bullet-}$ redox couple in the presence of different concentrations of: 4-(3-indolyl)-DHP, 4-(5-indolyl)-DHP, 4-phenyl-DHP, nimodipine, nisoldipine, and amlodipine. Sweep rate: 0.1 Vs^{-1}. Electrolytic media: DMSO + 0.1 M de TBAHFP

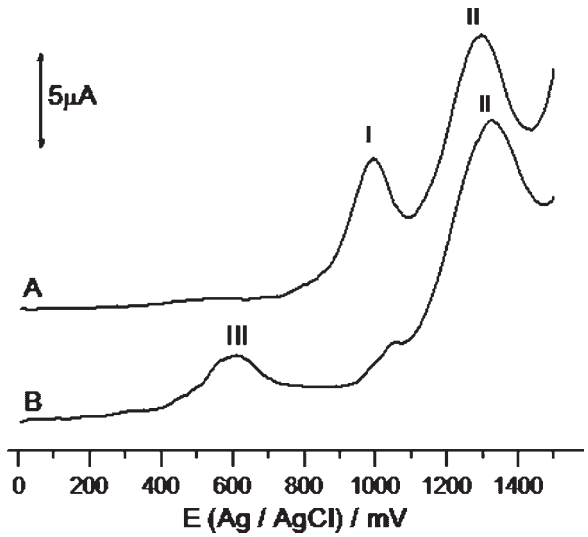

Figure 4. Differential pulse voltammograms of: (A) 1 mM 4-(5-indolyl)-DHP (B) 1 mM 4-(5-indolyl)-DHP + 1.5 mM de KO_2. DMSO + 0.1 M TBAHFP

In conclusion from different voltammetric experiments the interaction between the 1,4-DHP derivatives and superoxide anion has been documented. CV and DPV experiments required quite different conditions and therefore this could explain the apparent discrepancy in terms of the concentrations at which the effects were achieved. Thus, in the CV experiments, the $O_2^{\bullet-}$ is electrochemically generated *in situ* on the electrode surface from oxygen-saturated solutions, therefore only the DHP molecules reaching the electrode surface will be able to modify the anodic current corresponding to the superoxide anion. This could explain the high concentration required to observe changes in the current. In the VPD experiments, the superoxide anion (KO_2) is added to the solution together with the 1,4-DHP derivative. Accordingly, DHP-anion is the species that reaches the electrode surface and it is subsequently oxidized to pyridine. Consequently, a lower concentration is required to observe changes in the voltammograms.

NMR studies

NMR spectra of 1,4-DHPs in d_6-DMSO in the absence and in the presence of KO_2 were recorded. In Fig. 5A, the extended NMR spectrum corresponding to 4-(3-indolyl)-DHP derivative is shown. It can be observed the signal at 8.8 ppm accounting for the proton of the N1-position of the secondary amine of DHP ring and the signal at 10.7 ppm corresponding to the proton of the indole moiety. These signals disappeared in the presence of an excess of KO_2 (Fig. 5B). On the other hand, the signal at 5.2 ppm

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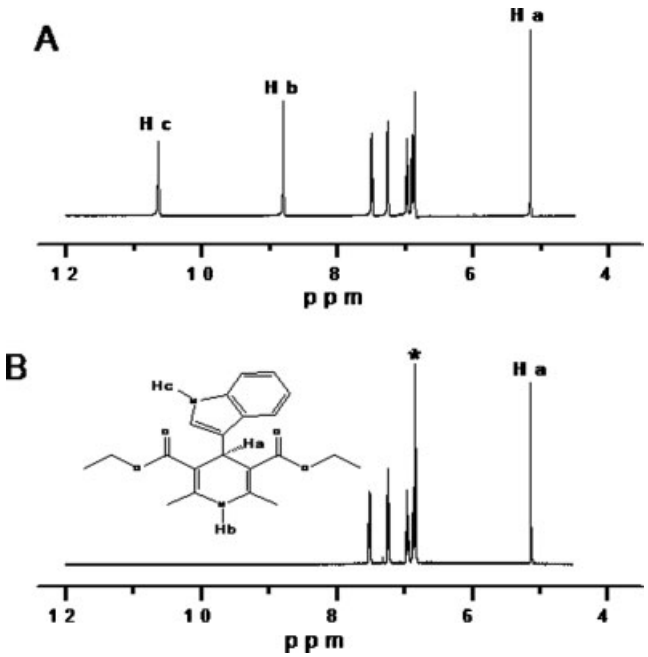

Figure 5. Extended ^{1}HNMR spectrum in d_6DMSO of 5 mM 4-(3-indolyl)-DHP: (A) Absence KO_2 (B) 20 mM KO_2

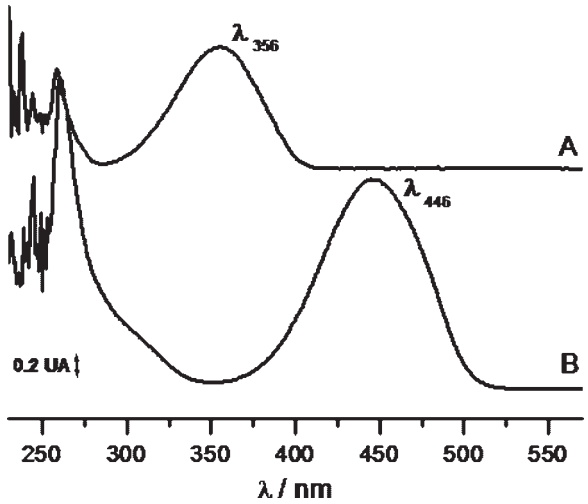

Figure 6. UV–Vis spectra of (A) 0.1 mM 4-(5-indolyl)-DHP; and (B) 0.1 mM 4-(5-indolyl)-DHP + 20 mM KO_2 solution in DMSO

corresponding to the proton of C-4 position of the DHP ring remains unaltered. So, we can conclude that the superoxide acts as a base abstracting the protons at 1-positions of both DHP ring and the indole moiety. This was fully proved by ^{1}H-NMR spectra where the signals corresponding to the N—H group protons disappeared and a new signal corresponding to the formation of the HO_2 moiety appeared at 6.8 ppm in all of the spectra of the 1,4-DHP derivatives. p*K*a values of 1,4-DHPs and indole are 19.0[25] and 21.0,[26] respectively. In consequence, the first step of the interaction between superoxide and the derivatives correspond to the abstraction of both protons at 1-position on the DHP ring and indole in the experimental conditions here used.

Reactivity of C4-indolyl substituted 1,4-DHP toward superoxide anion assessed by UV–Visible spectroscopy

Indolyl-substituted 1,4-DHPs exhibited a main UV–Vis band in the zone at $\lambda = 360$ nm, which in the presence of superoxide anion potassium salt disappeared together with the appearance of a new intense UV–Vis band close to 450 nm (Fig. 6). This latter band is due to UV–Vis. absorption of the anionic species generated by the addition of the superoxide anion. To test the above results, we also use TBA-OH as a base. Results of these experiments were similar.

Considering the above results, anion molar absorptivities (ε_{Anion}) corresponding to all 1,4-DHP derivatives were determined in the region of $\lambda = 450$ nm by using an excess of TBA-OH. Then, ε_{Anion} values were used to calculate the DHP anion concentration involved in the reaction through the calibration curve method as is described in Section "Experimental Method." Results from these experiments are summarized in Table 1. As expected molar absorptivities corresponding to the DHP anions were significantly higher than that of parent derivatives.

Now, kinetic studies were carried out under a pseudo first-order kinetic (20 mM potassium superoxide and 0.1 mM 1,4-DHP solutions in DMSO at 37 °C). Time-course of the reaction revealed that the intensity of the UV–Vis band corresponding to the DHP anion ($\lambda = 440$–450 nm) significantly decreased. This effect is illustrated for 4-(5-indolyl)-DHP in Fig. 7A. UV–Vis spectra exhibited two isosbestic points at $\lambda = 279$ and 396 nm, respectively. This characteristic supports that there are two main species during the reaction.[27] As can be seen in Fig. 7B for 4-(5-indolyl)-DHP, the absorption at 446 nm clearly decreased while the absorption at 308 nm increased. Analyses of the reaction products by gas chromatography-mass spectrometry (GC–MS) support the oxidation of the 1,4-DHP ring. Fragments with *m/z* 366, *m/z* 275, *m/z* 245, and *m/z* 321 containing the pyridine ring sustain the formation of this derivative as the final product of the reaction (Fig. 8). This main product of the reaction between superoxide and 1.4-DHPs absorbs in the region of 310 nm.[6] In this zone, superoxide also exhibits a wide absorption band close to 300 nm, which interferes with the absorption of pyridine derivative.

Taking into account the above-summarized experimental facts, we decide the use of a methodology which was based in the absorptivity changes in the region $\lambda = 440$–450 nm to determine the apparent kinetic rate constants for the reaction between 1,4-DHPs and superoxide.

Table 1. Molar absorptivities corresponding to parent 1,4-DHP derivatives and their anionic species

	Molar absorptivity coefficients/ $M^{-1}\,cm^{-1}$	
Derivative	$\lambda = 350$–360 nm	$\lambda = 445$–460 nm
4-(3-indolyl)-DHP	10 161 ± 16	16 674 ± 22
4-(5-indolyl)-DHP	8 966 ± 20	15 968 ± 28
4-phenyl-DHP	6 900 ± 14	12 625 ± 16
Amlodipine	5 992 ± 8	11 243 ± 10
Nisoldipine	3 332 ± 5	9 976 ± 10
Nimodipine	6 612 ± 9	9 983 ± 9

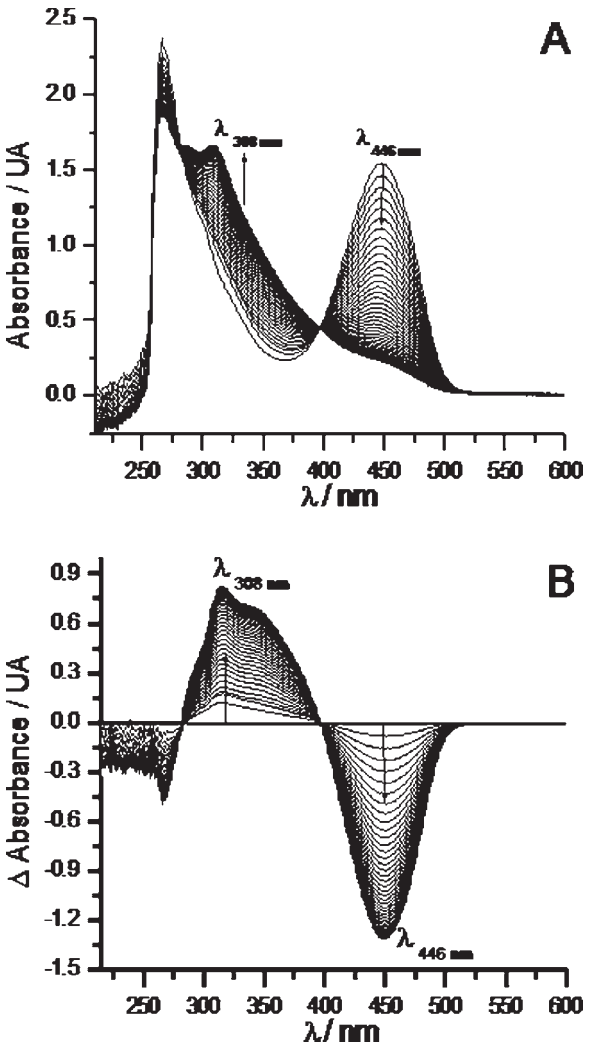

Figure 7. (A) Time-course of the reaction between 0.1 mM 4-(5-indolyl)-DHP and 20 mM KO_2 solutions in DMSO at 37 °C followed by UV–Vis spectroscopy. (B) Differential UV–Vis spectra

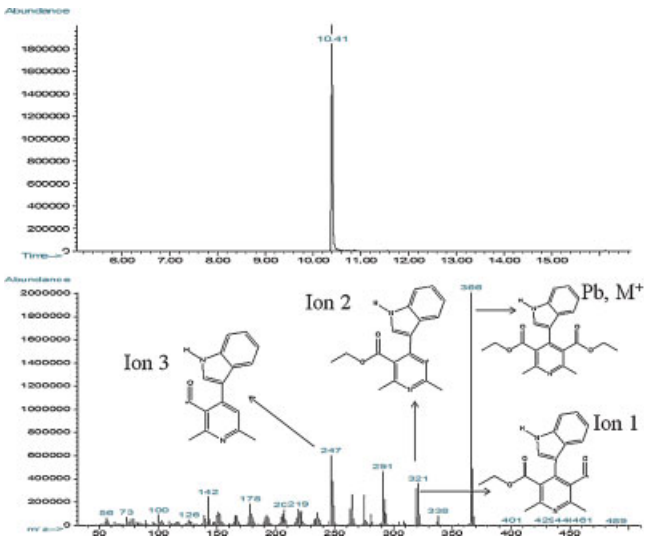

Figure 8. Ion chromatogram and mass spectrum of 0.1 mM 4-(3-indolyl)-DHP in the presence 20 mM KO_2 solution after 5 h reaction

In this reaction, the $O_2^{\bullet}$ acts as a base as well as an oxidant. When we add $O_2^{\bullet}$ to DHP solution a first fast acid–base equilibrium is established, leaving small quantity of DHP in its free form. When we add a strong base such as TBA-OH to a DHP solution in at least equimolar quantitites we convert DHP to DHP^-. However, if $O_2^{\bullet}$ is added to this solution, no conversion to pyridine occurred. This experiment demonstrates that the anionic form of DHP does not react with $O_2^{\bullet}$ in an oxidative process that would lead to pyridine. Therefore, it is the DHP moiety that experiments the oxidation process. Therefore, the following kinetic treatment is proposed for the overall reaction when a large amount of $O_2^{\bullet}$ (at least 200-fold greater than (DHP)) is added.

From the following expressions is possible to determine the apparent kinetic rate constants:

$$\text{acid} - \text{base equilibrium}: \quad \text{DHP} - \text{DHP}$$

$$\text{oxidative process}: \quad \text{DHP} + O_2^{\bullet} \xrightarrow{k_3} \text{pyridine}$$

$$\frac{d[\text{pyiridine}]}{dt} = k_3\,[\text{DHP}]^n[O_2^{\bullet}]^m$$

the $[O_2^{\bullet}]$ is in very large excess over (DHP) then we may consider it as nearly constant.

Therefore, $d[\text{pyiridine}]/dt = k'[\text{DHP}]$, where $k' = k_3\ [O_2^{\bullet}]^m$ assuming first-order condition.

However, if the DHP concentration is low, the steady-state approximation will be useful.

$$\frac{d[\text{DHP}]}{dt} = k_1\,[\text{DHP}^-] - k_2\,[\text{DHP}] - k'\,[\text{DHP}] = 0$$

therefore

$$[\text{DHP}] = \frac{k_1[\text{DHP}^-]}{(k_2 + k')}$$

and

$$\frac{-d[\text{DHP}^-]}{dt} = \frac{d[\text{pyridine}]}{dt} = \frac{-k'\,k_1\,[\text{DHP}^-]}{(k_2 + k')}$$

$$k'' = \frac{k'\,k_1}{(k_2 + k')}$$

$$\frac{-d[\text{DHP}^-]}{dt} = -k''\,[\text{DHP}^-]$$

$$\frac{d[\text{DHP}^-]}{[\text{DHP}^-]} = k''t$$

Integrating:

$$\ln[\text{DHP}^-] = -k''t + \ln[\text{DHP}^-]^0$$

As can we seen in Fig. 9, a plot of ln $[DHP^-]$ *versus* time is linear with a slope equal to k''. The linearity of the plot ($r = 0.9999$) supports the original assumption of pseudo first-order kinetic for the reaction. Also, from equation $\ln[\text{DHP}^-] = -k''\,t + \ln[\text{DHP}^-]_0$ the apparent pseudo first-order kinetic rate constants, k'' can be calculated.

Kinetic experiments

In Fig. 9, a typical linear plot between ln $[DHP^-]$ *versus* time for 4-(5-indolyl)-DHP is shown. All the derivatives exhibited a linear dependence. Calculated apparent kinetic rate constant values are

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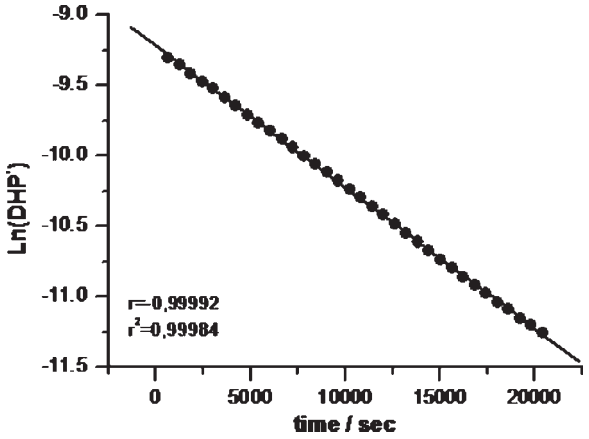
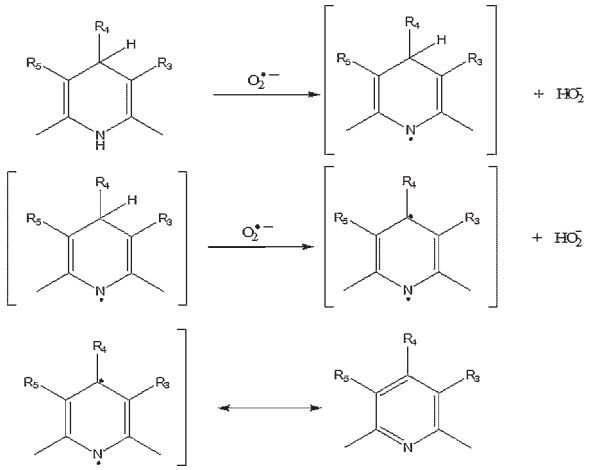


Figure 9. Typical plot of ln [DHP$^-$] *versus* time for the reaction between 0.1 mM 4-(5-indolyl)-DHP and 20 mM KO_2 solutions



Scheme 1. Proposed mechanism of reaction between C4-indolyl 1,4-dihydropyridines with superoxide anion

presented in Table 2. As can be seen C4-indolyl-substituted 1,4-DHPs were the most reactive derivatives. Thus, 4-(3-indolyl)-DHP exhibited a kinetic rate constant that is 16.1- and 15-fold higher than those commercial 1,4-DHPs, nisoldipine, and amlodipine, respectively. Also, indolyl-substituted 1,4-DHPs were more reactive than the 4-phenyl-DHP derivative. From these results it can be concluded that the inclusion of an indole moiety substituting C-4 position of the DHP ring produced a significant increase in the reactivity of the 1,4-DHP. The bigger reactivity of 4-(3-indolyl)-DHP derivative may be is due to the instability of the radical generated at the DHP ring (dihydropyridyl radical) compared with the radical formed in the 4-(5-indolyl)-DHP.

In the following Scheme 1 we propose a logical mechanism for the oxidation of 1,4-DHP free form to pyridine, which was identified by GC–MS as the product of the reaction. Also in previous paper,[8] we have identified a C-centered radical (dihydropyiridyl radical) by electron spin resonance (ESR) after exhaustive oxidation by means of controlled-potential electrolysis (CPE). This radical was trapped with *N-tert*-butylamine-α-phenylnitrone (PBN) as intermediate of the electrochemical oxidation.

Table 2. Apparent first-order kinetic rate constant values for the reaction between C-4 substituted 1,4-DHP derivatives and superoxide anion (KO_2) and their relationship

	Pseudo first-order kinetic rate constant Kpy/s^{-1}	
Derivative	k''[a] (s^{-1}) $\times 10^{-4}$	Ratio[b]
4-(3-indolyl)-DHP	2.25 ± 0.04	16.1
4-(5-indolyl)-DHP	1.79 ± 0.06	12.8
4-phenyl-DHP	0.38 ± 0.03	2.7
Amlodipine	0.15 ± 0.02	3.6
Nisoldipine	0.14 ± 0.01	1.0
Nimodipine	0.03 ± 0.01	0.2

[a] Calculated apparent first-order kinetic rate constants.
[b] Ratio kinetic rate constant derivative/kinetic rate constant nisoldipine.

HPLC

Time-course of the reaction between 1,4-DHP derivatives and KO_2 was also followed by HPLC technique. As can be seen in the Fig. 10, the parent 4-(5-indolyl)-DHP at 0.1 mM concentration exhibited a single chromatographic signal (Fig. 10a) with a retention time of 5.48 min in the experimental conditions. The addition of the free radical KO_2, at a 20 mM concentration produced the appearance of a new signal at 5.83 min, which corresponds to the pyridine formation. Time-course of reaction was followed during 5 h with 1 hour intervals (Fig. 10a–f). Oxidation of 4-(5-indolyl)-DHP by superoxide to pyridine was finished at 5 h (Fig. 10f). To compare the chromatographic characteristics of the oxidized products, reaction solutions with KO_2 and those electrolyzed at 1100 mV, were analyzed. Analysis by HPLC of these solutions revealed similar chromatographic characteristics (Table 3). As can be seen from this Table,

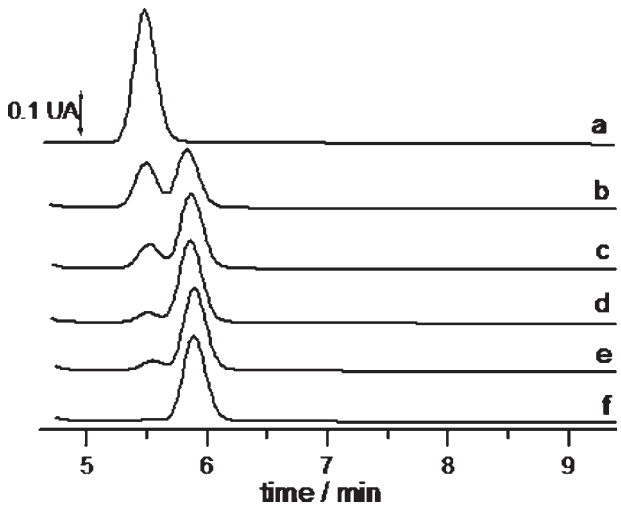


Figure 10. Time-course of the reaction between 0.1 mM 4-(5-indolyl)-DHP and 20 mM KO_2 in DMSO at 37 °C, followed by HPLC with diode array detector ($\lambda = 250$ nm). Parent 4-(5-indolyl)-DHP. Reaction mixture at: (a) $t = 0$, (b) $t = 1$ hour, (c) $t = 2$ hours, (d) 3 hours, (e) 4 hours, (f) 5 hours.

Table 3. Retention times (Rt) of oxidized products obtained by controlled-potential electrolysis (CPE) and the reaction of 1,4-dihydropyridines with superoxide anion obtained from HPLC with diode array detector ($\lambda = 250$ nm)

Derivative	Rt 1,4-DHP[a]/min	Rt reaction solution[b]/min	Rt electrolyzed derivative[c]/min
4-(3-indolyl)-DHP	5.9	6.2	6.2 (Eapp = 1100 mV)
4-(5-indolyl)-DHP	5.5	5.9	5.9 (Eapp = 1100 mV)
4-phenyl-DHP	8.5	9.6	9.5 (Eapp = 1200 Mv)

[a] Average of three independent measurements of parent 1,4-DHP.
[b] Products of the reaction with superoxide radical anion of the derivatives after 5 h at 37 °C.
[c] Controlled-potential electrolysis (CPE) in DMSO + 0.1 M de TBAHFP.

the chromatographic signals and retention times support that products are quite similar, corresponding to the pyridine derivative.

DHP-anion was not possible its identification by HPLC technique because the pH of the mobile phase was pH 4.3. Kinetic experiments were carried out in DMSO (UV–Vis), and aliquots of such reaction solutions were injected into the chromatograph. In consequence, the DHP-anion returns to its protonated-DHP form due to the acidity of the mobile phase, precluding its determination.

GC/MS

To identify the final products after the reaction between superoxide and the 1,4-DHP derivatives, a GC–MS method was used. Figure 8 shows typical extracted ion chromatograms and mass spectra corresponding to a solution resulting after 5 h reaction between an initial 0.1 mM 4-(3-indolyl)-DHP) solution and 20 mM superoxide in DMSO at 37 °C. The chromatogram shows a peak at 10.4 min corresponding to the pyridine derivative. The other 1,4-DHP derivatives exhibited similar characteristics, varying only the product's abundance. In all the compounds, the retention times of the oxidized derivatives were lower than those of the parent derivatives, and the mass spectral fragmentation pattern were different from that of the original derivatives.[28] We can conclude that the only product of the reaction was the pyridine derivative. Analyses of electrolyzed solutions of 1,4-DHPs at 1100 mV by this technique revealed the similarity of the chromatographic characteristics of these products with those obtained by the reaction with the free radical. Average retention time of the oxidized derivatives was 10.4 min and *m/z* of 366 was the most abundant fragment, supporting that both types of products are the same, corresponding to the pyridine derivative.

CONCLUSIONS

1. C4-indolyl substituted 1,4-DHPs significantly reacted with superoxide either electrochemically generated or as the potassium salt.
2. Apparent first-order kinetic rate constant values of synthesized compounds were higher than that of commercial 1,4-DHPs, nifedipine, nisoldipine, and amlodipine.
3. Extended NMR spectra demonstrated an acid–base equilibrium reaction involving the protons at 1-position of both heterocycles.
4. The anionic form of DHP does not react with $O_2^{\bullet}$ to generate pyridine.
5. Analyses of reaction solutions by GC–MS permit us the identification of the pyridine derivative as the final product.
6. Results from voltammetric techniques (CV and DPV) were consistent with the spectroscopic ones, supporting the formation of the DHP anion (R—N^-) and the consumption of the parent 1,4-DHPs in the reaction.

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