Reactivity of 1,4-Dihydropyridines toward Alkyl, Alkylperoxyl Radicals, and ABTS Radical Cation

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A series of C4-substituted 1,4-dihydropyridines (DHP) with either secondary or tertiary nitrogen in the dihydropyridine ring were synthesized. All of these compounds together with some commercial DHP derivatives were tested for potential scavenger effects toward alkyl, alkylperoxyl radicals, and ABTS radical cation in aqueous media at pH 7.4. Kinetic rate constants were assessed either by UV/vis spectroscopy or GC/MS techniques. Tested compounds reacted faster toward alkylperoxyl radicals and ABTS radical cation than alkyl ones. *N*-Ethyl-substituted DHPs showed the lowest reactivity. Kinetic results were compared with either trolox or nisoldipine. Using deuterium kinetic isotope effect studies, we have proved that the hydrogen of the 1-position of the DHP ring is involved in the proposed mechanism. This fact is mostly noticeable in the case of alkyl radicals. In all cases, the respective pyridine derivative was detected as the main product of the reaction.

Introduction

It is a well-established fact that 1,4-dihydropyridine (DHP) calcium channel antagonists in addition to the well-known inhibition of the voltage-dependent (slow) Ca^{2+} channel exhibit a variety of other biological activities, such as regulation of calmodulin, Ca^{2+}-dependent ATPases, sarcoplasmatic reticulum and mithocondrial Ca^{2+} channels, and inhibition of Na^{+} channels, which also contribute to their pharmacological and clinical effects (*1*, *2*). On the other hand, a relatively new effect of calcium antagonists is related with their possible antioxidant effect (*3*−*8*), which is becoming an important additional property for the treatment of pathologies such as ischemia-reperfusion injury, inflammatory vascular disease, arteriosclerosis, and central nervous system (CNS) trauma and stroke (*9*). Furthermore, Prabha et al (*10*) have suggested a causal relationship between hypertension and free radical damage, as well as between antihypertensive agents and antioxidants. Possible radical scavenging properties, in combination with a lipophilic character and high affinity for the DHP calcium channel receptors, could be the basis for the protective activity in free radical-involved pathologies that appears to accompany many instances of hypertension and also CNS disorders.

The antioxidant effect of the DHP derivatives has been widely studied in biological models through membrane lipoperoxidation. Mak (*6*) has reported the protective effects of DHP against free radical impairment endothelial cell proliferation, using the tetrazolium MTT assay. Recently, our laboratory (*11*) and others authors (*12*, *13*) have reported the DHP antilipoperoxidant activity on membranes using TBARS measurements or the crocin bleaching test. More recently (*14*), the redox properties of lacidipine have been used to support its antioxidant effect. Nevertheless, in such a study, a direct measurement of the reactivity of lacidipine with free radicals was not assessed.

Studies on the reactivity of DHP toward alkyl and alkylperoxyl radical species become relevant, taking into account its participation in the peroxidation processes of membranes. In the state of the art, no systematic studies relating structural changes of DHP and its reactivity toward radical species have been found. We did not find any studies on the possible mechanisms involved in the scavenging activity of the DHP compounds. However, in a recent paper (*15*), it is described that nisoldipine acts scavenging $O_2^{\bullet -}$. The proposed mechanism involved a proton abstraction from the *N*-position of the dihydropyridine ring in the nisoldipine molecule as the first step.

In the present work, a study on the reactivity of nine DHP compounds toward alkyl, alkylperoxyl radicals, and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) radical cation is presented. Also, a detailed discussion based on experimental results relating the chemical structure and the oxidation peak potentials of the DHP compounds is included.

Materials and Methods

Chemicals. All solvents were of HPLC grade, and all reagents were of analytical grade.

Compounds. DHP derivatives (Figure 1, I−VI) were synthesized in our laboratory according to a previously described procedure (*16*). IR, NMR, and elemental analyses, obtaining the following results, analyzed the final products:

(I) 4-Methyl-2,6-dimethyl-3,5-dimethoxycarbonyl-DHP (4-Me-DHP). IR (KBr): ν_{max} 3342, 2950, 1680, 1650, 1435, 1351,

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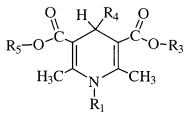
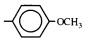
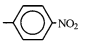
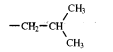
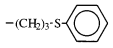


Compounds	R_1	R_3	R_4	R_5
I 4-Me--DHP	−H	$-CH_3$	$-CH_3$	$-CH_3$
II p-MeO-DHP	−H	$-CH_3$		$-CH_3$
III p-Nitro-DHP	−H	$-CH_3$		$-CH_3$
IV N-Et-4-Me	$-CH_2CH_3$	$-CH_3$	$-CH_3$	$-CH_3$
V N-Et-p-MeO	$-CH_2CH_3$	$-CH_3$		$-CH_3$
VI N-Et-p-Nitro	$-CH_2CH_3$	$-CH_3$		$-CH_3$
VII Nisoldipine	−H			$-CH_3$
VIII Nimodipine	−H	$-(CH_2)_2OCH_3$		
IX CDA 50105	−H		$-CH_3$	

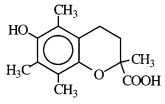
Trolox = 

Figure 1. Chemical structures of the DHP derivatives and trolox.

1226, 1056 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.96 (d, 3H, J = 6.5 Hz, >CH−C**H**$_3$), 2.29 (s, 6H, −C**H**$_3$), 3.73 (s, 6H, −O−C**H**$_3$), 3.83 (q, 1H, J = 6.5 Hz, >C**H**−CH_3), 5.73 (s, 1H, −N**H**−). ^{13}C NMR (75 MHz, $CDCl_3$): (20.35 × 2), 23.20, 29.30, (51.92 × 2), (105.27 × 2), (145.64 × 2), (169.19 × 2). Anal. calcd for $C_{12}H_{17}O_4N$: C, 60.25; H, 7.13; N, 5.86. Found: C, 60.27; H, 7.23; N, 5.87. mp: 147−149 °C.

(II) 4-(4-Methoxyphenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-DHP (*p*-MeO-DHP). IR (KBr): ν_{max} 3349, 2949, 1697, 1650, 1431, 1383, 1251, 1213, 1027 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.33 (s, 6H, −C**H**$_3$), 3.65 (s, 6H, −O−C**H**$_3$), 3.75 (s, 3H, Ar−O−C**H**$_3$), 4.94 (s, 1H, Ar−C**H**<), 5.76 (s, 1H, −N**H**−), 6.75 (d, 2H, J = 8.6 Hz, Ar−**H**), 7.2 (d, 2H, J = 8.6 Hz, Ar−**H**). ^{13}C NMR (75 MHz, $CDCl_3$): (19.51 × 2), 38.31, (50.94 × 2), 55.05, (104.00 × 2), (113.30 × 2), (128.53 × 2),139.86, (143.93 × 2), 157.85, (168.06 × 2). Anal. calcd for $C_{18}H_{21}O_5N$: C, 65.24; H, 6.39; N, 4.23. Found: C, 65.00; H, 6.47; N, 4.36. mp: 181−183 °C.

(III) 4-(4-Nitrophenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-DHP (*p*-Nitro-DHP). IR (KBr): ν_{max} 3343, 2948, 1703, 1655, 1518, 1434, 1384, 1347, 1218, 1020 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 2.38 (s, 6H, −C**H**$_3$), 3.66 (s, 6H, −O−C**H**$_3$), 5.12 (s, 1H, Ar−C**H**<), 5.85 (s, 1H, −N**H**−), 7.46 (d, 2H, J = 8.8 Hz, Ar−**H**), 8.12 (d, 2H, J = 8.8 Hz, Ar−**H**). ^{13}C NMR (75 MHz, $CDCl_3$): (20.63 × 2), 40.73, (52.11 × 2), (103.92 × 2), (124.39 × 2), (129.53 × 2), (145.81 × 2), 147.29, 155.64, (168.38 × 2). Anal. calcd for $C_{17}H_{18}O_6N_2$: C, 58.96; H, 5.24; N. 8.09. Found: C, 58.76; H, 5.03; N, 8.25. mp: 165−168 °C.

(IV) 4-Methyl-2,6-dimethyl-3,5-dimethoxycarbonyl-*N*-ethyl-DHP (*N*-Et-4-Me). IR (KBr): ν_{max} 2954, 1696, 1631, 1434, 1389, 1212, 1163, 1056 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 0.88 (d, 3H, J = 6.6 Hz, >CH−C**H**$_3$), 1.16 (t, 3H, J = 7.1 Hz N−CH_2−C**H**$_3$), 2.4 (s, 6H, −C**H**$_3$), 3.7 (q, 2H, J = 7.1 Hz N−C**H**$_2$−CH_3), 3.72 (s, 6H, −OC**H**$_3$), 3.77 (q, 1H, J = 6.6 Hz, >C**H**−CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 15.56, (16.15 × 2), 21.73, 28.12, 39.51, (51.12 × 2), (108.75 × 2), (148.01 × 2), (168.50 × 2). Anal. calcd for $C_{14}H_{21}NO_4$: C, 62.84; H, 7.85; N, 5.23. Found: C, 63.05; H, 7.62; N, 5.13. mp: 78−80 °C.

(V) 4-(4-Methoxyphenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-*N*-ethyl-DHP (*N*-Et-*p*-MeO). IR (KBr): ν_{max} 2948, 1690, 1629, 1433, 1390, 1256, 1213, 1152, 1035 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.06 (t, 3H, J = 5.9 Hz N−CH_2−C**H**$_3$), 2.47 (s, 6H, −C**H**$_3$), 3.67 (q, 2H, J = 5.9 −N−C**H**$_2$$CH_3$), 3.7 (s, 6H, −OC**H**$_3$), 3.75 (s, 3H, Ar−OC**H**$_3$), 5.04 (s, 1H, Ar−C**H**<), 6.75 (d, 2H, J = 8.7 Hz, Ar−**H**), 7.09 (d, 2H, J = 8.7 Hz, Ar−**H**). ^{13}C NMR (75 MHz, $CDCl_3$): 16.06, (16.42 × 2), 37.19, 40.42, (51.26 × 2), 55.17, (107.16 × 2), (113.32 × 2), (127.91 × 2), 138.53, (148.53 × 2), 157.90, (168.61 × 2). Anal. calcd for $C_{20}H_{25}NO_5$: C, 66.77; H, 6.96; N, 3.9. Found: C, 66.85; H, 7.08; N, 3.66. mp: 106−108 °C.

(VI) 4-(4-Nitrophenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-*N*-ethyl-DHP (*N*-Et-*p*-Nitro). IR (KBr): ν_{max} 2945, 1690, 1624, 1511, 1382, 1345, 1252, 1154, 1025 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ 1.04 (t, 3H, J = 7.1 Hz, N−CH_2C**H**$_3$), 2.5 (s, 6H, −C**H**$_3$), 3.72 (s, 6H, −OC**H**$_3$), 3.72 (q, 2H, J = 7.1 Hz, N−C**H**$_2$−CH_3), 5.19 (s, 1H, Ar−C**H**<), 7.34 (d, 2H, J = 8.5 Hz, Ar−**H**) 8.07 (d, 2H, J = 8.5 Hz, Ar−**H**). ^{13}C NMR (75 MHz, $CDCl_3$): 16.06, (16.4 × 2), 38.25, 40.0, (51.44 × 2), (105.70 × 2), (123.29 × 2), (127.74 × 2), (146.42 × 2), 149.32, 153.61, (167.94 × 2). Anal. calcd for $C_{19}H_{22}N_2O_6$: C, 60.9; H, 5.88; N, 7.48. Found: C, 61.10; H, 5.99; N, 7.34. mp: 156−157 °C.

NMR Spectroscopy. The NMR spectra were recorded on a Bruker spectrometer Advance DRX 300.

FT-IR. The IR spectra were recorded on a Bruker spectrometer IFS 55 Equinox. Elemental analyses were performed in Fisons Instrument equipment.

Drugs. Nisoldipine and nimodipine were supplied by Sanitas Laboratories (Santiago, Chile). These drugs were assayed without previous purification. CDA 50105 was supplied by Alter Laboratories (Madrid, Spain). Trolox (6-hidroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) was purchased from Aldrich Chemical Co.

UV/vis. The progress of the reaction with both ABAP (2,2′-azobis (2-amidinopropane)-derived alkyl and alkylperoxyl radicals was followed by UV/vis spectroscopy using an UNICAM UV-3 spectrophotometer. Acquisition and data treatment were carried out with a Vision 2.11 software.

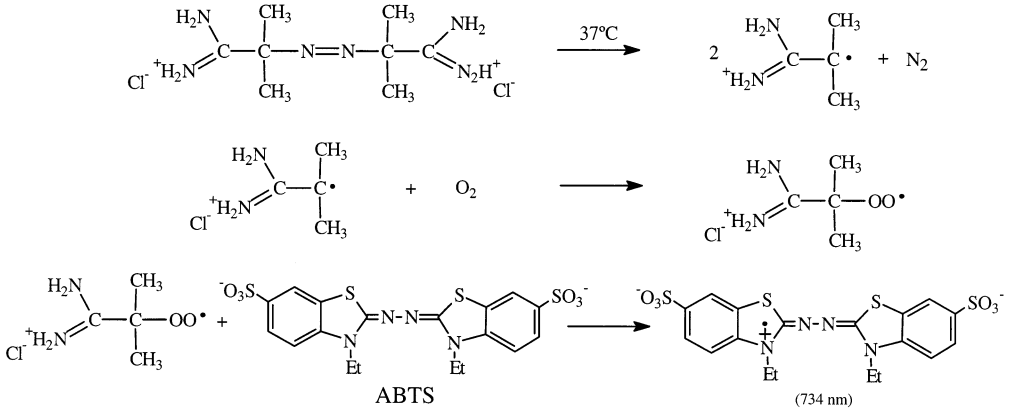
UV/vis spectra were recorded in the 220−750 nm ranges at different intervals. For the DHP compounds, the absorption bands at λ = 330−360 nm were used. Drug concentrations in aqueous buffer (0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4 and 0.04 M Britton−Robinson buffer/DMF (dimethyl formamide) 70/30 pH 7.4 for peroxyl radicals) were determined from the respective calibration curves (10−200 μM). In the case of trolox, the UV band at λ = 300 nm was employed for tracking its concentration. DHP with ABTS radical cation reactivity was studied by following the absorbance at λ = 734 nm.

Reactivity toward Alkyl and Alkylperoxyl Radicals. ABAP dihydrochloride (Aldrich Chemical Co.) was used as radical generator for both types of radicals. Different series of 20 mM ABAP solutions in 0.04 M Britton−Robinson buffer/ethanol 70/30 pH 7.4 (or 0.04 M Britton−Robinson buffer/DMF 70/30 pH 7.4 for alkylperoxyl radicals) were incubated with different solutions of each DHP (20−200 μM) or trolox at 37 °C. Then, samples were obtained at intervals of 10 min for 2 h keeping constant the nitrogen bubble (or oxygen for alkylperoxyl radicals). From these experiments, rate reactions were calculated for each DHP derivative. The kinetic rate constants were calculated as follows:

(1) Alkyl Radicals. Considering that the reaction with these radicals involved less than 10% of the total concentration of DHP present in the media (see Results section) and that the concentration of radicals practically remains constant during the whole experiment, i.e., at least 10 times higher than the reacting concentration of DHP, it is possible to assume that the reaction follows a first-order kinetic. In such a case, it is possible to calculate the kinetic rate constant from the rate of reaction at a certain concentration. On the other hand, in all of the studied concentration ranges (20−200 μM) of 1,4-DHP, the reaction rate exhibited a linear dependence with the concentration.

(2) Alkylperoxyl Radicals. For this type of radical, the reaction rates were calculated by using the kinetic rate constant

Scheme 1. Chemical Generation of Free Radicals[a]



[a] Equation 1, alkyl radicals; eq 2, alkylperoxyl radicals; eq 3, $ABTS^{+\bullet}$ radical cation.

of trolox with peroxyl radicals reported in the literature (i.e., 4.92×10^3 M^{-1} s^{-1} taken from ref *17*). The rate of alkyl or alkylperoxyl radical formation from ABAP is constant at a given temperature (*18*). However, the rate of alkyl or alkylperoxyl radicals formation from this initiator (Scheme 1, eqs 1 and 2) will not be constant as it depends on the concentration of ABAP (rate = k[ABAP]). It appears that over 120 min at 37 °C, only a small amount of the ABAP will decay; therefore, the rate may be considered constant at 37 °C. In neutral aqueous solutions, the half-life of ABAP is about 175 h, and the generation rate of radicals is constant for the first few hours (*19*).

Control solutions containing either DHP or trolox solutions were run in the same conditions as the above mixtures. Time course of the reactivity of synthesized DHP derivatives with the generated alkyl or alkylperoxyl radicals was followed by UV/vis spectroscopy and GC/MS technique.

The relative reactivity toward alkyl or alkylperoxyl radicals was expressed by comparison with either trolox or nisoldipine using the following ratio: k DHP tested/k trolox or k nisoldipine, where k values are the corresponding kinetic rate constants in the presence of free radicals.

Control solutions (in the absence of ABAP-derived radicals) revealed no changes in either their original UV/vis absorption bands or their GC/MS mass fragmentation. Also, a possible photodecomposition of DHP was assessed, but in the time scale of the experiments, this was negligible.

Reactivity toward ABTS Radical Cation. ABTS diammonium salt was purchased from Aldrich Chemical Co.

(1) Bleaching Capacity Assay. A solution of ABAP (2 mM) and ABTS (75 μM) in 0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4 was incubated at 45 °C for 1 h (Scheme 1), a period that generally proved to be adequate to produce an increase in the absorbance at 734 nm near to 0.24 AU (in the absence of ABAP, incubated ABTS did not generated color). By using an extinction coefficient of 1.6×10^4 mol^{-1} L cm^{-1} for ABTS radical cation at 734 nm, we calculated the ABTS radical cation concentration produced in the experiments. The calculated value was 15 μM, indicating that 20% ABTS was oxidized (*20*).

Afterward, the final colored solution was rapidly cooled on ice, nitrogen-bubbled for 5 min, and kept at 4 °C until analysis. When aliquots (2 mL) of the colored solution were placed into a cuvette and kept at 15 °C, they displayed an absorbance (at 734 nm), which remained constant for at least 4 h. The addition of different concentrations of DHP derivatives and trolox to these cuvettes resulted in changes in the AU, which were monitored for 30 min using an UNICAM UV-3 spectrophotometer. From these experiments, rates of reaction were calculated in the linear segment of C_{ABTS}/time plots and the following expression was used to calculate the kinetic rate constants:

$$v = k[\mathrm{DHP}][\mathrm{ABTS}^{\bullet+}]$$

where [DHP] was 5 μM and [$ABTS^{\bullet+}$] was 15 μM. The relative reactivity toward ABTS radical cation was expressed by comparison with either trolox or nisoldipine using the following ratio: k DHP tested/k trolox or k nisoldipine, where k values correspond to the respective kinetic rate constant in the presence of ABTS radical cation.

Voltammetry. Differential pulse voltammetry (DPV) was performed with a BAS CV50 assembly. A glassy carbon stationary electrode was employed as the working electrode. A platinum wire was used as a counter electrode, and all potentials were measured against an Ag/AgCl reference electrode. Measurements of oxidation peak potentials were carried out in 0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4.

GC/MS. A GC mass selective Hewlett-Packard 5890/5972 Detector (Palo Alto, CA) and Hewlett-Packard 7673 Autosampler were used for the measurements. A Hewlett-Packard Pentium II Data System Laser Jet 4000 printer, controlled instrumentation, and data handling were also used.

(1) Chromatography Column. A Hewlett-Packard Ultra-1 column, 25 m × 0.2 mm i.d. × 0.11 film thickness (Little Falls, Wilmington, DE) was used.

(2) Chromatographic Conditions. Detector temperature, 300 °C; injector temperature, 250 °C; split ratio, 1/10; pressure, 13 psi; purge flow, 40 mL min^{-1}; purge time, 0.5 mL min^{-1}.

(3) Temperature Program. The oven temperature was programmed from 130 to 305 °C (hold for 5 min) at 15 °C min^{-1}; the run time was 16.67 min. Helium was used as the carrier gas with an inlet pressure of 35 kPa. The identification of the samples was based on the analyses of the mass spectra (full scan).

(4) Deuterium Kinetic Isotope Effect (DKIE) Studies. (a) Studies by ^{1}H NMR Spectroscopy. To establish the exchange among the proton of the position 1 with a deuterium atom, 4-Me-DHP was dissolved in DMSO-d_6 carrying out a normal ^{1}H NMR. After this, 20 μL of D_2O was added to the sample in order to observe the possible change in the typical −N**H**− signal.

(b) DKIE Influence on the Kinetic Rate Constants of 4-Me-DHP. Both control solutions (H_2O/DMSO 70/30 in 10 mM phosphate buffer pH 7.4) and deutered solutions (D_2O/DMSO-d_6 70/30 in 10 mM phosphate buffer pH 7.4) containing 100 μM 4-Me-DHP were incubated at 37 °C for 2 h. Then, the same experimental procedure as was previously described in Reactivity toward Alkyl and Alkylperoxyl Radicals in the Experimental Section was followed.

Results and Discussion

Reactivity toward Alkyl and Peroxyl Radicals. The original UV absorption bands between $\lambda = 330-360$ nm corresponding to the DHP compounds were followed to assess reactivity after its addition to an aqueous media containing either alkyl or peroxyl radicals. These absorption bands decreased with time in parallel with the

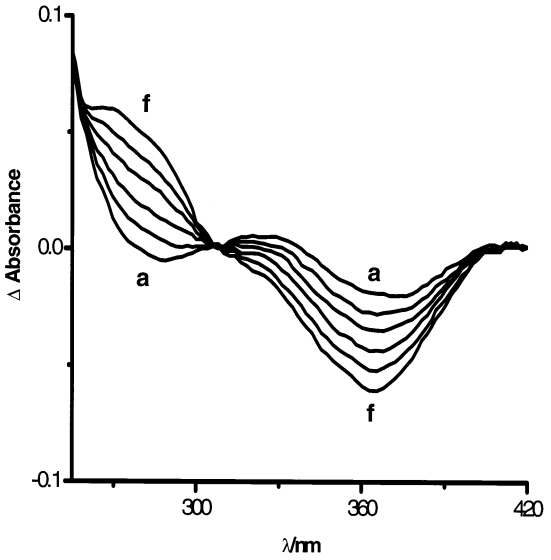


Figure 2. UV/vis differential spectra corresponding to reaction between 100 μM *p*-MeO-DHP solution and 20 mM ABAP-derived alkyl radicals solution in 0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4; a−f, 120 min.

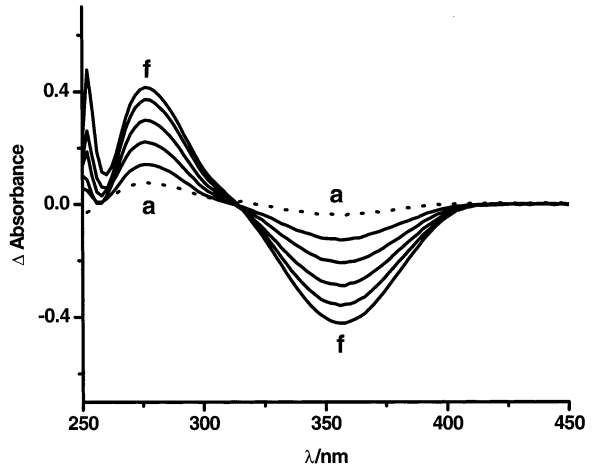


Figure 3. UV/vis differential spectra corresponding to reaction between 100 μM *p*-MeO-DHP solution and 20 mM ABAP-derived alkylperoxyl radicals solution in 0.04 M Britton−Robinson buffer/DMF 70/30 at pH 7.4; a−f, 120 min.

apparition of a new band at 270 nm (Figures 2 and 3). This latter band might correspond to the oxidized derivative, i.e., the related pyridine derivative, which is consistent with previous observations (*21*). To compare the reactivity, kinetic rate constant values (*k*) were calculated as was shown in the Experimental Section for tested DHP derivatives and *k* values of the reference compounds (trolox or nisoldipine) were used (Tables 1 and 2). Clearly, DHP derivatives reacted with alkyl and peroxyl radicals at different degrees, the latter being the most reactive species, which is supported by the respective *k* values.

Thus, comparing *k* values for 4-substituted DHP with *N*-ethyl-DHP, the former values were significantly bigger. However, trolox was more reactive than all studied DHP derivatives for both radicals. In the case of the alkyl radicals, as can be seen from Table 1, trolox was 11.4-fold more reactive than *p*-MeO-DHP (the less reactive 4-substituted DHP) and 24-fold more reactive than *N*-ethyl *p*-MeO-DHP (the less reactive *N*-ethyl-substituted DHP).

Table 1. Kinetic Rate Constants of DHP with ABAP-Derived Alkyl Radicals in 0.04 M Britton−Robinson Buffer/Ethanol 70/30 at pH 7.4 and 37 °C

compd	$k \times 10^5$ (s^{-1})[a]	rel. nisoldipine[b]	rel. trolox[c]
4-Me-DHP	1.55 ± 0.06	1.5	0.15
p-MeO-DHP	0.88 ± 0.04	0.8	0.09
p-nitro-DHP	1.31 ± 0.07	1.3	0.13
N-Et-4-Me	0.53 ± 0.03	0.5	0.05
N-Et-*p*-MeO	0.42 ± 0.02	0.4	0.04
N-Et-*p*-nitro	0.53 ± 0.03	0.5	0.05
nisoldipine	1.06 ± 0.05	1	0.1
nimodipine	0.27 ± 0.04	0.26	0.03
CDA 50105	0.53 ± 0.05	0.5	0.05
trolox	10.1 ± 0.2	9.5	1

[a] Kinetic rate constant calculated assuming a first-order kinetic for the reaction between DHP derivatives and alkyl radicals. [b] Ratio between kinetic rate constants of the tested DHP derivatives/kinetic rate constant of nisoldipine in the presence of alkyl radicals. [c] Ratio between kinetic rate constants of the tested DHP derivatives/kinetic rate constant of trolox in the presence of alkyl radicals.

Table 2. Kinetic Rate Constants of DHP with ABAP-Derived Alkylperoxyl Radicals in 0.04 M Britton−Robinson Buffer/DMF 70/30 at pH 7.4 and 37 °C

compd	$k \times 10^{-3}$ ($M^{-1}\,s^{-1}$)[a]	rel. nisoldipine[b]	rel. trolox[c]
4-Me-DHP	3.35 ± 0.08	3.5	0.68
p-MeO-DHP	2.6 ± 0.05	2.7	0.53
p-nitro-DHP	1.02 ± 0.02	1.1	0.21
N-Et-4-Me	1.84 ± 0.02	1.9	0.37
N-Et-*p*-MeO	1.45 ± 0.04	1.5	0.29
N-Et-*p*-nitro	0.34 ± 0.02	0.4	0.07
nisoldipine	0.96 ± 0.05	1	0.20
nimodipine	1.84 ± 0.07	1.9	0.37
CDA 50105	2.94 ± 0.07	3.1	0.60
trolox	4.92	5.1	1

[a] Kinetic rate constants were calculated by using the rate constant for the reaction between trolox and alkylperoxyl radicals ($k = 4.92 \times 10^3\ M^{-1}\,s^{-1}$, taken from ref *17*). [b] Ratio between kinetic rate constant of the tested DHP derivatives/kinetic rate constant of nisoldipine in the presence of alkylperoxyl radicals. [c] Ratio between kinetic rate constant of the tested DHP derivatives/kinetic rate constant of trolox in the presence of alkylperoxyl radicals.

Although DHP derivatives were significantly reactive toward peroxyl radicals (Table 2), trolox continued to be the most active compound but at a lesser magnitude, i.e., 4.8-fold more reactive than *p*-nitro-DHP (the less reactive 4-substituted DHP) and 14.5-fold than *N*-ethyl- *p*-nitro-DHP (the less reactive *N*-ethyl-substituted DHP).

The 4-methyl-substituted DHP derivatives were the most reactive compounds (the highest *k* value). When DHP derivatives were compared with well-known antioxidant DHP drugs, such as nisoldipine, a comparable activity was found (Tables 1 and 2).

The time course of the absorbance corresponding to the formed pyridine derivative and the parent DHP derivatives with both alkyl (A) and peroxyl radicals (B) is presented in Figure 4. From Figure 4A,B, it can be concluded that after 4 h of reaction between either alkyl or peroxyl radicals and DHP derivatives no changes were observed in both the parent DHP and the absorbances of the pyridine derivative. These results support that under these experimental conditions there is no formation of side products interfering with the reaction.

Dependence of reaction rate with concentration of DHP derivatives showed a linear behavior in all of the ranges assayed (20−200 μM) for the alkyl radicals. This linear dependence and the fact that half-life was concentration-independent support the fact that the kinetics involved

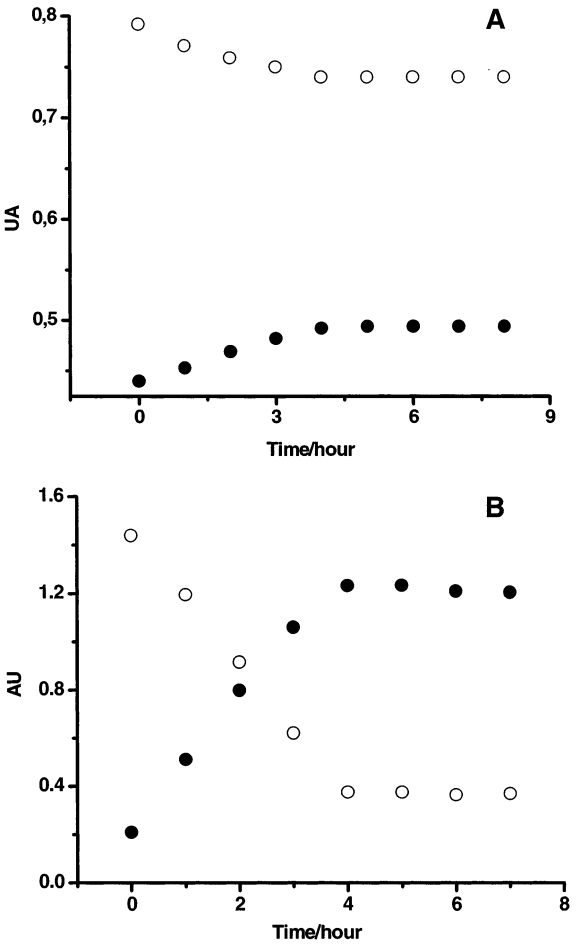


Figure 4. (A) Time course of band at 354 nm (○) and band at 276 nm (●) for reaction between 4-Me-DHP at 100 μM solution and 20 mM ABAP-derived alkyl radicals solution in 0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4 and 37 °C. (B) Time course of band at 276 nm (●), band at 354 nm (○) for reaction between 4-Me-DHP at 200 μM solution and 20 mM ABAP-derived alkylperoxyl radicals solution in 0.04 M Britton−Robinson buffer/DMF 70/30 at pH 7.4 and 37 °C.

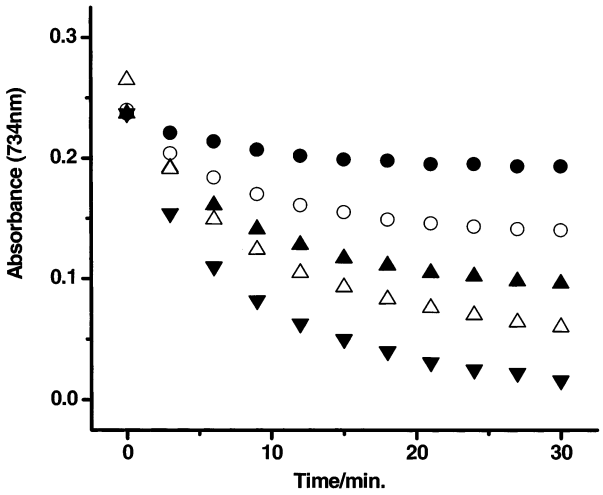


Figure 5. Time course of the absorbance at 734 nm of 15 μM ABTS radical cation solution in the presence of different concentrations of 4-Me-DHP in 0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4 and 15 °C. (●) 1, (○) 2.5, (▲) 5, (△) 7.5, and (▼) 10 μM.

Table 3. Kinetic Rate Constants of DHP Derivatives with ABTS Radical Cation in 0.04 M Britton−Robinson Buffer/Ethanol 70/30 at pH 7.4 and 15 °C

compd	k (M^{-1} s^{-1})[a]	rel. nisoldipine[b]	rel. trolox × 10^2[c]
4-Me-DHP	201 ± 3	3.9	5
p-MeO-DHP	131 ± 2	2.6	3.2
p-nitro-DHP	92 ± 1	1.8	2.2
N-Et-4-Me	15 ± 1	0.3	0.37
N-Et-*p*-MeO	50 ± 2	1	1.2
N-Et-*p*-nitro	22 ± 1	0.4	0.55
nisoldipine	51 ± 2	1	1.25
nimodipine	43 ± 2	0.8	1.05
CDA-50105	291 ± 3	5.7	7.1
trolox	4100 ± 10	80	100

[a] Kinetic rate constant calculated from the $ABTS^{\bullet+}$ concentration/time plots. [b] Ratio between kinetic rate constant of the tested DHP derivatives/kinetic rate constant of nisoldipine. [c] Ratio between kinetic rate constant of the tested DHP derivatives/kinetic rate constant of trolox.

under these experimental conditions corresponds to first-order kinetics. Furthermore, the calculated percentage of DHP derivatives reacting with the radicals was low (less than 10%) and a practically constant concentration of the alkyl radicals under these conditions can be assumed.

In the case of peroxyl radicals, for some of the most reactive compounds, the rate exhibited a narrower linear dependence (20−120 μM). However, for the resting DHP derivatives, a linear dependence between rate and concentration in all of the studied ranges (20−200 μM) was observed.

Reactivity toward ABTS Radical Cation. ABTS radical cation was generated by oxidation of ABTS with radical alkylperoxyl. All of the tested DHP compounds scavenged the ABTS radical cation at different degrees. When different DHP concentrations were added to an ABTS radical cation solution, a diminution of the absorbance at 734 nm corresponding to the radical cation was observed. In Figure 5, the time course of the absorbance at 734 nm in the presence of different DHP concentrations is shown. This result reveals a concentration-dependent effect, i.e., the bleaching ability of DHP depends on the concentration of the DHP. To assess the reactivity of the DHP, the slopes of the absorbance at the 734 nm time course were used. In Table 3, kinetic rate constants of the different tested compounds toward ABTS radical cation are shown. Both 4-Me-substituted DHPs (CDA-50105; 4-Me-DHP) reacted faster than the other tested DHP derivatives. Conversely, the *N*-ethyl-substituted DHPs were the slowest compounds. However, a comparable reactivity of all tested DHP with two commercial ones assayed, i.e., nisoldipine and nimodipine (Table 3), was found. Furthermore, trolox became 14-fold faster than the most rapid DHP compound, i.e., CDA 50105 (Table 3).

In general terms, by relating the reactivity toward free radicals and the nature of C-4 substituents of DHP compounds, conclusions can be summarized as follows: (i) DHP with electron-donating substituents (4-Me-DHP, *p*-MeO-DHP, and CDA 50105) shows the highest kinetic rate constants toward ABTS radical cation; (ii) *p*-nitro-DHP, a compound with an electron-withdrawing substituent, shows a lower kinetic rate constant; and (iii) *N*-alkyl-DHP compounds show kinetic rate constants lower than the −NH−DHP (Figure 6).

Reduction of ABTS radical cation by DHP derivatives occurs by electron transfer. In the present study, this reaction showed a trend in which the *N*-ethyl-substituted

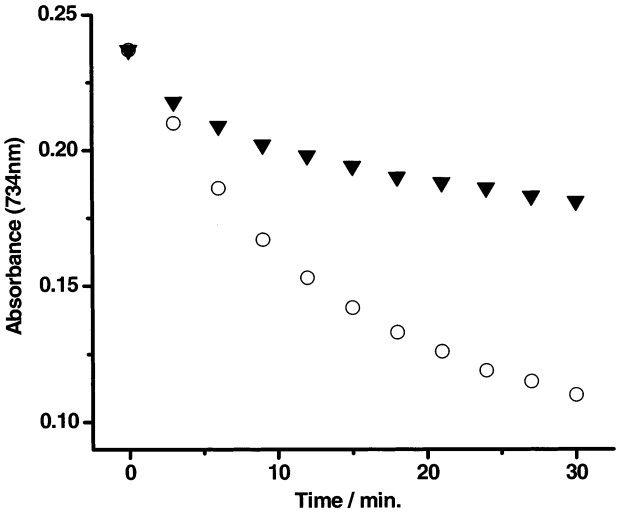


Figure 6. Time course of the absorbance at 734 nm of 15 μM ABTS radical cation solution in the presence of at 5 μM solution of (○) *p*-MeO-DHP and (▼) *N*-Et-*p*-MeO in 0.04 M Britton−Robinson buffer/ethanol 70/30 at pH 7.4 and 15 °C.

Table 4. Peak Potential Values of DHP Derivatives at Different pH Values

	E_p (mV) (DPV)[a]			
compd	pH 2	pH 4	pH 7.4	pH 11
4-Me-DHP	786	764	640	394
p-MeO-DHP	805	775	655	422
p-nitro-DHP	870	850	722	466
N-Et-4-Me	753	748	738	729
N-Et-*p*-MeO	768	767	760	762
N-Et-*p*-nitro	820	815	824	

[a] Values determined by DPV in Britton−Robinson buffer 0.04 M/ethanol 70/30 with 0.1 M KCl.

DHP reacted slower than the parent DHP derivatives (Table 3). This is in opposition with the well-known fact that alkylation makes amines more electron rich, and hence, should increase the energy of the highest occupied molecular orbital thereby making its oxidation easier. This apparent contradiction could be explained based on studies on the dependence of electrooxidation potentials of parent DHP with pH. These experiments (Table 4 and ref *22*) revealed that voltammetric oxidation peak potentials are pH-dependent, i.e., potential values in the acidic zone were higher than the values in the alkaline zone. However, oxidation peak potentials corresponding to *N*-ethyl-DHP derivatives were pH-independent in the whole studied range (pH 1−12). Furthermore, oxidation of *N*-ethyl-DHP derivatives between pH 2−4 was easier than that of the parent DHP compounds. However, at pH 7.4 in which the experiments with ABTS were conducted, the parent DHPs were oxidized easier than *N*-ethyl-DHP. These findings could be explained based on acid−base equilibrium. Thus, at acidic pH (2−4), DHP derivatives would be predominantly in their protonated form (H*N*-DHP). Under these experimental conditions, the oxidation potential values of the parent DHP are higher (more anodic values) than those of *N*-ethyl-DHP (smaller peak potential values). This would agree with the expectations for *N*-alkyl-DHP derivatives. However, at pH 7.4, the parent DHP predominantly would be in its anionic form (−*N*-DHP); such species are oxidized easier than the *N*-ethyl-DHP. This supports that at pH 7.4, the parent DHP has a smaller peak potential value and therefore the reaction of ABTS radical cation with *N*-ethyl-DHP derivatives was slower than with parent DHP (Table 3).

Table 5. DKIE for the Reaction between 4-Me-DHP Derivative and Alkyl, Alkylperoxyl Radicals, and ABTS Radical Cation

radical	kinetic rate constant in H_2O (k_{H_2O})	kinetic rate constant in D_2O (k_{D_2O})	k_{H_2O}/k_{D_2O}
alkyl radical	1.57×10^{-5} s^{-1}	0.75×10^{-5} s^{-1}	2.1
alkylperoxyl radical	3.3×10^{3} M^{-1} s^{-1}	2.82×10^{3} M^{-1} s^{-1}	1.2
ABTS radical cation	204 M^{-1} s^{-1}	225 M^{-1} s^{-1}	0.91

These results could indicate that the kinetic rate constants toward alkyl, alkylperoxyl, and ABTS radical cation depend on the nature of the substituents in the C-4 position and on the presence of the secondary amine group in the dihydropyridine ring, i.e., the presence of the hydrogen in 1-position.

(1) DKIE Studies. To prove the feasibility of participation of the hydrogen of the 1-position, we have carried out some DKIE studies. For this purpose, the most reactive derivative, the 4-Me-DHP derivative, was selected. As it is noticed from NMR experiments, the addition of a small quantity of D_2O to a solution of 4-Me-DHP makes the −NH− signal at 8.7 ppm disappear, thus demonstrating that the proton of the amine group was isotopically interchanged with deuterium. Detailed descriptions of the NMR signals experimentally obtained are as follows.

(a) Normal Spectra Corresponding to 4-Me-DHP. ^{1}H NMR (300 MHz, DMSO-d_6): δ 0.82 (d, 3H, J = 6.4 Hz, >CH−C**H**$_3$), 2.2 (s, 6H, −C**H**$_3$), 3.6 (s, 6H, −O−C**H**$_3$), 3.7 (q, 1H, J = 6.4 Hz, >C**H**−CH$_3$), 8.7 (s, 1H, −N**H**−).

(b) Spectra of 4-Me-DHP with Addition of Deuterium Oxide. ^{1}H NMR (300 MHz, DMSO-d_6 + D_2O): δ 0.82 (d, 3H, J = 6.4 Hz, >CH−C**H**$_3$), 2.2 (s, 6H, −C**H**$_3$), 3.6 (s, 6H, −O−C**H**$_3$), 3.7 (q, 1H, J = 6.4 Hz, >C**H**−CH$_3$).

DKIE studies are summarized in Table 5. As can be seen, the ratio k_{H_2O}/k_{D_2O} for the alkyl radical is approximately 2.1, thus demonstrating that the proposed mechanism based on the abstraction of the amine hydrogen in the scavenging effect appears supported (*23*, *24*).

In the remaining cases (alkylperoxyl radicals and ABTS radical cation), there is not a significant difference between them and the ratio k_{H_2O}/k_{D_2O} close to 1.0, indicating that the hydrogen abstraction could not be involved in the mechanisms, but further studies must be conducted to clarify this point, mainly concerning the possibility of complex mechanisms involving both types of radicals.

Reactivity Assessed by the GC/MS Technique. To identify the products obtained after the reaction between DHP derivatives and the different free radicals, the GC/MS technique was used. In Table 6, the chromatographic data corresponding to the DHP compounds are shown. Some conclusions on these studies can be summarized as follows: (i) The GC/MS procedure used to characterize the parent DHP and its subsequent reactivity with radicals did not require a derivatization process. (ii) The DHP after the reaction with alkyl, alkylperoxyl, or ABTS radical cation suffered a dehydrogenation process yielding the pyridine derivative metabolites. This conclusion is supported by the respective retention times and mass fragmentation pattern for each compound like displayed in Table 6. Noteworthy, in the scavenging effect of the radicals by DHP, an electron transfer reaction is involved.

Table 6. GC/MS Characteristics of Parent DHP and Their Corresponding Oxidized Derivatives Obtained by Reaction with Alkyl, Alkylperoxyl Radicals, and ABTS Radical Cation

parent drugs				oxidized derivatives			
derivative	M^+	Pb	R_t (min)	derivative	M^+	Pb	R_t (min)
4-Me-DHP	239	224	8.84	4-Me-pyridine	237	206	7.4
p-MeO-DHP	331	224	10.45	*p*-MeO-pyridine	329	266	8.69
p-nitro-DHP	346	224	11.81	*p*-nitro-pyridine	344	327	9.54
nisoldipine	388	371	11.62	nisoldipine	386	284	10.19
nimodipine	418	296	12.9	nimodipine	416	58	11.5
CDA 50105	403	222	7.2	CDA 50105	401	234	6.07

(c) The retention times of the pyridine derivatives were lower than the related parent compounds. This result could be explained on the basis of the different affinity for the stationary phase, considering that aromatic pyridine derivatives have no hydrogen in the *N*-position or in the 4-position, both of them present in the DHP moiety. (d) The mass fragmentation pattern of the aromatized derivatives shows different peak bases depending on C-4 substitution on the DHP rings. (e) The tested DHP derivatives scavenged both alkyl and alkylperoxyl radicals, forming as a final product the pyridine derivative.

The time course of the reaction between *N*-ethyl-DHP derivatives with free radicals assessed by GC/MS technique did not reveal any new signal. The lack of a new signal observed by this technique could be explained based on the previous knowledge that the electrochemical oxidation of *N*-substituted-DHP gives rise to a pyridinium salt as a final product (*25*). This product did not show any signals by GC/MS, probably because the ionization fountain of the chromatograph could repel this positive ion, avoiding its detection.

Oxidation of DHPs and Its Reactivity with Free Radicals. The electrooxidation process of DHPs is an ECEC type of mechanism, i.e., the sequence: e, H+, e, H+ (*26*−*28*). The electrochemical oxidation of the DHP compounds generates as the final product the pyridine derivative (*28*). As in the case of the electrochemical oxidation, the reaction of DHP with alkyl, alkylperoxyl, and ABTS radical cation also generated the corresponding pyridine derivative. Consequently, we have obtained a direct correlation between the percentages of pyridine formed (analyzed by GC/MS) with the calculated kinetic rate constants. This phenomenon is illustrated in Figure 7 for the reaction of three C-4-substituted DHPs with alkylperoxyl radicals. These results strongly support the assumption that the reactivity involves an oxidation process of the DHP by the presence of free radicals, i.e., an electron transfer reaction.

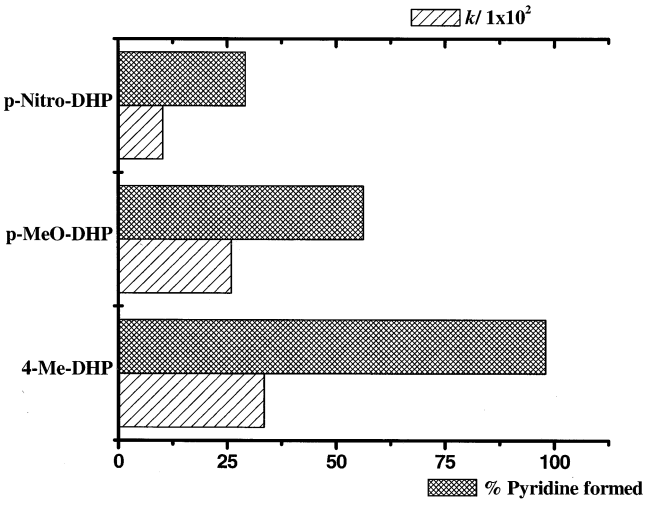


Figure 7. Relationship between kinetic rate constant and percentage of pyridine formed for the reaction between 100 μM DHP solutions and 20 mM ABAP-derived alkylperoxyl radicals solution after 2 h, in 0.04 M Britton−Robinson buffer/DMF 70/30 at pH 7.4.

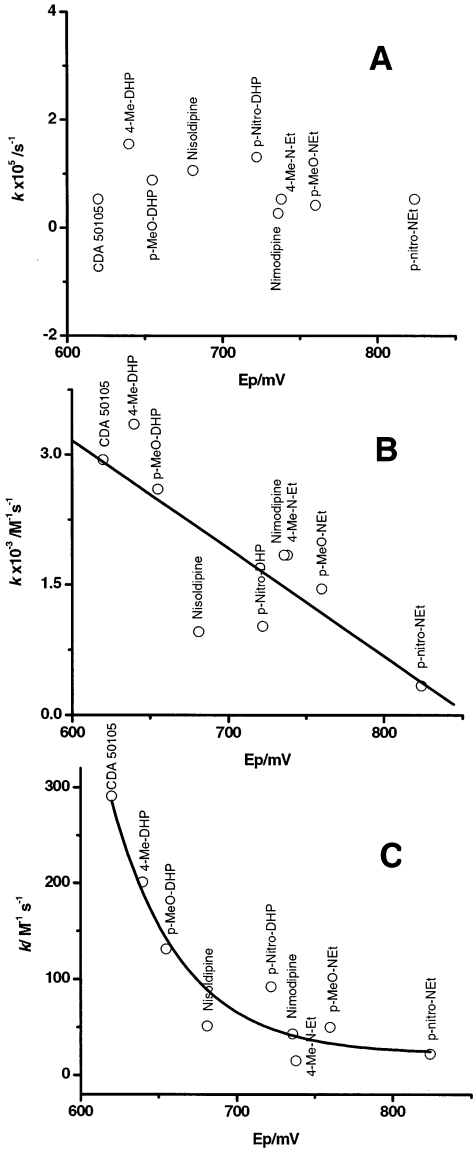


Figure 8. Relationship between kinetic rate constants (*k*) and the oxidation peak potentials of the DHP derivatives. (A) ABAP-derived alkyl radicals; (B) ABAP-derived alkylperoxyl radicals; and (C) ABTS radical cation.

(1) Correlations between Kinetic Rate Constants and Oxidation Potential Values. To further study the mechanism of reactivity of the DHP derivatives, the electrochemical oxidation of the DHPs was studied in the same experimental conditions used for the reactivity studies. Under these conditions, all compounds exhibited only a single oxidation peak. In Figure 8, the relationship between the kinetic rate constants and the oxidation peak potentials is displayed. From these studies, it can be concluded that (i) kinetic rate constants of tested DHP for the reaction with alkyl radicals were independent from the oxidation peak potential values (Figure 8A).

From these results, it can be assumed that the electron transfer is not the determining step. (ii) Kinetic rate constants for the reaction between DHP compounds and alkylperoxyl radicals exhibited a fairly good linear correlation with oxidation peak potential (kinetic rate constant = 10 616 − 12.42 E_p (mV); c.c. = 0.82). Thus, the compounds that are more easily oxidized reacted more rapidly with this radical (Figure 8B). (iii) The kinetic rate constant toward the ABTS radical cation exhibited a dual behavior type, i.e., four of the tested compounds, CDA50105, 4-Me-DHP, *p*-MeO-DHP, and nisoldipine, showed a good linear correlation with oxidation peak potentials (kinetic rate constant = 2732.4 − 3.95 E_p (mV); c.c. = 0.995, Figure 8C). However, the resting five studied compounds did not show a direct relationship with the oxidation peak potential values (Figure 8C).

Conclusions

In the present paper, we have demonstrated the direct participation of the DHP derivatives in the quenching of radical species. In general terms, DHP reacted faster toward alkylperoxyl radicals and ABTS radical cation than alkyl radicals. *N*-ethyl-DHP compounds reacted slowest toward all of the tested radicals. After the reaction between the DHP and the different free radicals, the pyridine derivative was formed as the final product. This fact was assessed using both UV/vis spectroscopy and GC/MS techniques. Kinetic rate constants of tested DHP with alkyl radicals showed independence with the oxidation peak potential values. The kinetic rate constant of tested DHP toward alkylperoxyl showed a direct relationship with the oxidation peak potential values, i.e., compounds reacting faster were most easily oxidized. The kinetic rate constants for some tested DHPs (CDA50105, 4-Me-DHP, *p*-MeO-DHP, and nisoldipine) toward ABTS radical cation exhibited a good linear correlation with the potential peak values (however, other compounds showed independence). DKIE studies clearly demonstrated that the reaction between 4-Me-DHP with alkyl radicals involves the abstraction of the hydrogen in the 1-position of the dihydropyridine ring. For the resting radicals studied, an isotopic effect was not apparent.

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